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Conformational changes of Mal d 2, a thaumatin-like apple allergen, induced by food processing

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ABSTRACT

Mal d 2, a thaumatin-like protein from apple was previously described to react to almost 75% of the apple allergic patient sera. Based on the molecular structure of this protein, the present study focused on the conformational stability of Mal d 2 in relation to *in vitro* IgE-binding under different physico-chemical conditions and proteolysis. The structural integrity of Mal d 2 was monitored using SDS–PAGE, Western blotting using polyclonal antibodies and human sera, fluorescence spectrometry and circular dichroism. Results confirmed the stability of Mal d 2. However, Mal d 2 was reactive to human serum IgEs mainly after reduction of disulphide bridges fixing the α-helical domain II. Contrary to previous assumptions, the current findings suggest that the allergenic epitopes of Mal d 2 are hidden inside the protein structure and none of the rigorous conditions applied in industrial juice processing or digestive proteolysis enhance or reduce the binding to IgE molecules.

1. Introduction

Fruit allergy is an adverse reaction to the consumption of fruit or fruit-based products, mediated by an IgE-response and manifested through pathophysiological response to food (Hoffmann-Sommergruber, 2000). Allergic reactions to fruits in fact are caused by distinct epitopes, which bind IgE-antibodies and initiate a series of clinical symptoms varying from respiratory, cutaneous and gastrointestinal disturbances to systemic anaphylaxis (Fernandez-Rivas et al., 2006). Different fruit manufacturing conditions may induce alterations of immuno-reactive epitopes on allergenic proteins. Therefore, attempts to reduce or deactivate fruit allergenicity through technological processing cause variable results (Soler-Rivas & Wichers, 2001). Processing was shown to destroy existing epitopes on a protein and generate new ones (formation of neoallergens) as a result of conformational changes (Sathe, Teuber, & Roux, 2005).

Principally, allergenic epitopes are divided in two main classes, namely (i) conformational epitopes are expected to be more susceptible to processing-induced changes and (ii) linear epitopes, likely to be more resistant to physical treatments and to be deactivated only by hydrolysis (Restani et al., 2004).

The most frequently observed adverse reactions to fruits are those induced by apple consumption (Ortolani, Ispano, Pastorello, Bigi, & Ansaloni, 1988). Interestingly, apple accounts for nearly 60% of the world fruit production. A 31 kDa apple protein has been reported to react with almost 75% of the apple allergic patient sera (Hsieh, Moos, & Lin, 1995). The N-terminal sequencing of this protein, called Mal d 2, revealed approximately 50% identity to the superfamily of thaumatin-like proteins (TLPs) belonging to the group 5 of pathogenesis-related proteins (PRs). TLPs contain 16 conserved cysteine residues that form eight disulphide bonds essential for protein folding and possibly for allergenicity. Although TLPs are generally assumed to resist hydrolysis by proteases, pH and heat-induced denaturation (Breiteneder, 2004), they perform different extent of structural stability (Gavrovic-Jankulovic et al., 2002). Immuno tissue printing of apple fruit has shown the equal distribution of Mal d 2 in peel and pulp (Marzban et al., 2005). Therefore removal of the peel will not prevent the exposure to the allergen.

Heat treatment is used generally to clear apple juice and to conserve food products by pasteurisation. Further pressed apples are

Abbreviations: DIECA, diethyldithiocarbamic acid; EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol; ESI-Q-TOF-MS, electrospray ionisation quadropole-time of flight mass spectrometry; TLP, thaumatin-like proteins; PRs, pathogenesis-related proteins; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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treated with enzymes to remove suspended solid materials and proteins (Wal, 2003). In hot processing the apple juice is subjected to a flash heating at temperatures between 82 and 85 °C, which cause the coagulation of suspended particles, interfering with juice filtration. The juice is then rapidly cooled and filtered or centrifuged (Bates, Morris, & Crandall, 2001). Pasteurisation, which involves heating the juice to a given temperature and defined period of time, followed by rapid cooling to reduce the microbial content, is the most frequently applied method for preserving apple juice (Wal, 2003). Food processing, particularly thermal processing, was observed to reduce the allergenicity of some foods, and therefore it was even proposed as contribution to a better management of the allergenic risk of foods (Wal, 2003). A decrease or increase of allergenicity can be caused by protein unfolding, misfolding or aggregation as well as by chemical modifications occurring during food processing by addition of sugar (e.g. Maillard reaction). For fruit manufacturer it is important to move toward a knowledge-based management of allergen risk assessment during fruit manufacturing (Sancho et al., 2005).

The current study describes the effects of pH, heat, reducing environment and proteolytic hydrolysis on the physico-chemical integrity and IgE-binding properties of the apple allergen Mal d 2 within a matrix or as purified protein thereby providing the basis for a scientific decision tree.

2. Materials and methods

2.1. Patient serum pool

Patient sera were obtained from five patients allergic to apple with known clinical history and anamnesis. All sera were tested by Western blotting for reactivity to purified Mal d 2 and subsequently pooled.

2.2. Protein extraction from apples

Apples of the cultivar "Topaz", purchased at a local market, were used for the preparation of an extract and for purification of Mal d 2. Protein extraction was performed as previously described (Björksten, Halmepuro, Hannuksela, & Lahti, 1980). Approximately 160 g of apple pulp was shock frozen and homogenised with extraction buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, 0.27 mM ethylene diamine tetraacetic acid (EDTA), 13.3 mM diethyldithiocarbamic acid (DIECA), 2% PVPP, pH 7) in a ratio of 1:2 (w/v). The suspension was stirred for 30 min at 4 °C, filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA), centrifuged at 28.000 g (Beckman Coulter, Avanti™ J-25, JLA-10500, Paolo Alto, CA, USA) for 30 min at 4 °C. The supernatant was immediately frozen at −80 °C. The determination of the total protein content was performed using the BCA protein assay kit (PIERCE, Rockford, IL, USA) according to manufacturer's instructions.

2.3. Purification of Mal d 2

"Topaz" apple (1 kg) was homogenised with carbonate extraction buffer (20 mM Na_2CO_3, 13.3 mM diethyldithiocarbamic acid (DIECA), 1 mM ethylene diamine tetraacetic acid (EDTA), 2 mg/ml PVP, pH 9.0) and stirred for 30 min at 4 °C. pH 9.0 was chosen, since it reduces the viscosity by the aggregation of pectin molecules. To remove debris, the extract was filtered through two layers of Calbiochem Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged at 28.000 g (Beckman Coulter, Avanti™ J-25, JLA-10500, Paolo Alto, CA, USA) for 30 min at 4 °C. The supernatant was lyophilised and stored at −20 °C. Further purification was performed on an AEKTA 100 explorer chromatography system (GE Healthcare, Uppsala, Sweden). The lyophilisate was dissolved in carbonate extraction buffer in a ratio of 1:10 (w/v), centrifuged for 30 min at 4000 g and 4 °C (Beckman Coulter, Allegra R21, S4180 rotor, Paolo Alto, CA, USA) and buffer-exchanged into equilibration buffer (10 mM Tris, 2 mg/ml PVP, pH 8). One hundred and fifty millilitres of the obtained sample were loaded onto an anion exchange column Source 30Q HR 10/10 with column dimensions of 10 mm i.d., 100 mm length and a bed volume of about 8 ml (GE Healthcare, Upsala, Sweden) previously equilibrated with equilibration buffer. Elution was carried out with a linear gradient from 0 to 1 M NaCl in equilibration buffer and the fractions were collected for further analyses. Fractions containing Mal d 2 were subjected to gel filtration on a Superdex 75 column (GE Healthcare, Upsala, Sweden), equilibrated with PBS. For the determination of purity, purified protein was subjected to offline ESI-Q-TOF-MS on a Q-TOF Ultima Global (Waters Micromass, Milford, UK). Spectra were deconvoluted using MaxEnt 1 function of Mass-Lynx 4.0 SP4.

2.4. Conditions to test stability

In order to simulate oxidizing/reducing conditions, purified Mal d 2 was reduced using DTT in a final concentration of 50 mM. A portion of the reduced sample was subsequently treated with iodacetamide in a final concentration of 100 mM in order to block the cysteine residues. The agents were removed by overnight dialysis against water (Dialyse Filter, 0.05 μm, Millipore, Billerica, MA, USA).

Effects of freezing/thawing cycles were determined by subjecting apple extract to three cycles of shock freezing in liquid nitrogen and slow thawing at RT. Samples were taken after every cycle and analysed by SDS–PAGE and Western blotting. Samples of apple extract were stored for one week at RT, for one month at 4 and −20 °C and for 3 months at −80 °C. All samples were analysed in duplicates by SDS–PAGE and Western blotting.

In order to evaluate the thermo-stability, both the fruit extract and the purified protein at pH 3.5 or pH 7 were heated for either 30 s, or 20 min to 60, 80 and 100 °C. The corresponding pH of the fruit extract was adjusted, whereas the purified protein was diluted in the appropriate buffers to a final concentration of 0.25 μg/μl. An acidic pH of 3.5 and a neutral pH of 7.0 were chosen to simulate the commercial apple juice acidity. The temperatures of 60, 85, 100 °C and a combination of 75 and 85 °C were chosen to simulate different thermal processes which are applied in the fruit industries for pasteurisation. Additionally, sucrose was added to samples containing purified Mal d 2 to a final concentration of 15% (w/v).

For proteolytic digestion, pepsin (Sigma, St. Louis, MO, USA) was used for the preparation of a simulated gastric fluid (SGF): 84 mM HCl, 35 mM NaCl, pH 2 and 4000 U of pepsin in 1.52 ml (Thomas et al., 2004). Purified Mal d 2 or apple extract were added in a ratio of 10 U pepsin activity/μg of test protein. Samples were incubated at 37 °C and aliquots of 200 μl were taken after 1, 3, 6 h and overnight incubation. The reaction was stopped by adding 70 μl of 200 mM $NaHCO_3$, pH 11. Samples were analysed directly or stored at −20 °C.

Intestinal digestion was simulated by a mixture of trypsin (Sigma, St. Louis, MO, USA) and chymotrypsin (Sigma, St. Louis, MO, USA). The pH of the apple extract was adjusted to pH 8.3, whereas the purified Mal d 2 was diluted in a buffer containing 65 mM Tris–HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.3 to a final concentration of 0.5 μg/μL (Sen et al., 2002). Protein samples were mixed with enzymes in a ratio of 34.5 U trypsin activity and 0.44 U chymotrypsin activity/mg protein and incubated at 37 °C according to Moreno et al. (2004). Aliquots were taken after 1, 3, 6 h and overnight incubation. The reaction was immediately

quenched with SDS sample buffer (see below) and samples were directly analysed or stored at −20 °C.

2.5. Production of polyclonal antibodies against Mal d 2

Purified Mal d 2 was used to raise polyclonal antibodies in rabbits by immunisation with 500 μg native protein and Freund's Adjuvans.

2.6. SDS–PAGE and Western blotting

Samples were diluted in sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.005% (v/v) bromphenol blue) in a ratio 1:2 (v/v) and either denatured for 1 h at RT or boiled for 10 min at 90 °C. When reducing conditions were required, DTT was added to the mixture to a final concentration of 50 mM. Samples were loaded on 4–20% gradient gels and electrophoresis was carried out on the Novex System (Invitrogen, Carlsbad, CA, USA). Gels were either stained with Coomassie brilliant blue or silver, or electroblotted onto nitrocellulose membranes (BioRad, Hercules, CA on the XCell II Blot Module (Invitrogen) using a buffer containing 50 mM $Na_2B_4O_7 \cdot 10H_2O$, 0.1% (w/v) SDS, 20% (v/v) methanol. After overnight blocking with 3% skim milk powder, polyclonal antibodies were diluted in a ratio of 1:1000 (v/v) in dilution buffer (1% skim milk, 0.1% Tween 20 in PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and added to the membranes. The immuno-reaction was detected using goat anti-rabbit IgG conjugated with alkaline phosphatase diluted in dilution buffer in a ratio of 1:2000 (v/v) (Sigma, St. Louis, MO, USA), followed by bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium NBT (Sigma, St. Louis, MO, USA) in alkaline phosphate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris).

2.7. IgE-Western blotting

A pool of sera from five adult patients (one male and 4 females in the age range of 40–60 years) reacting to Mal d 2 was used. After electroblotting, membranes were blocked with 3% BSA for 2 h at 37 °C and incubated over night at 4 °C with the serum pool in a ratio 1:10 (v/v). Goat anti-human IgE conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) (1:20000, v/v) was used for the detection of the immuno-reaction. Reacting protein bands were visualised by ECL (GE Healthcare, Upsala, Sweden) and chemiluminescence reaction using the Lumi-Imager TM apparatus (Boehringer Mannheim/Roche, Penzberg, Germany).

2.8. Fluorescence spectroscopy

Intrinsic steady-state tryptophan fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer from VARIAN with a Peltier-controlled cell holder (Varian Ltd., Victoria, Australia). Measurements were carried out using a protein concentration of 2 μM in 10 mM phosphate buffer, pH 7. Path length: 10 mm; excitation wavelength: 295 nm; emission range: 300–450 nm, excitation and emission slits: 5 nm; scan rate: 60 nm min^{-1}; PMT voltage: 700 V. Initial measurements were carried out at 25 °C. Finally, emission spectra were recorded at stepwise (5°) increased temperatures (25–87 °C). Spectra were also collected during stepwise (5 °C) cooling of the sample. All experiments were performed under non-reducing conditions.

2.9. Electronic circular dichroism spectrometry

Far-UV electronic circular dichroism (CD) spectra were performed on PiStar-180 spectropolarimeter (Applied Photophysics, Leatherhead, UK) equipped with a thermostated cell holder. Measurements were carried out using a protein concentration of 1 μM in 10 mM phosphate buffer, pH 7. Path length: 2 mm; scan range: 200–250 nm; step size: 0.5 nm; spectral bandwidth: 5 nm; scan time: 390 s. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell. The instrument was flushed with nitrogen with a flow rate of 5 l min^{-1}. Initial measurements were carried out at 25 °C. Finally, CD spectra were recorded stepwise with increased temperatures (25–88 °C). Spectra were also collected during stepwise cooling of the sample. All experiments were performed both under non-reducing and reducing conditions. In the latter case, the protein was treated with DDT in a final concentration of 10 mM.

3. Results

3.1. Impact of denaturation on the immuno-reactivity of Mal d 2

The influence of rigorous sample treatments like heat and reduction of disulphide bridges in presence of detergents has been studied using SDS–PAGE and Western blotting. To our knowledge such conditions are only used in part during the fruit industrial processes but they can reveal the structural integrity of any given protein. Therefore, apple extracts were incubated under different conditions like 1 h at RT, heat denatured, reduced for 10 min at 90 °C and subsequently analysed by SDS–PAGE and silver staining (Fig. 1A), Western blotting with a polyclonal rabbit antiserum against Mal d 2 (Fig. 1B) and a human serum pool (Fig. 1C). Mal d 2, when incubated for 1 h at RT, yielded only a faint band at 23 kD, while thermally denatured and reduced Mal d 2 yielded a clearly visible band after silver staining.

Reducing conditions caused a significant shift of the Mal d 2 mobility in the gel, which appeared as a 31 kD protein band, while untreated and denatured Mal d 2 showed a MW of 23 kD which is equal to the calculated value (Fig. 1A and B). Polyclonal antibodies recognised reduced, denatured Mal d 2 and even dimers at a molecular weight of about 40 kD (Fig. 1B) to the same extent. After 1 h of incubation at RT, however, only a weak immuno-reaction was visible on the membrane (Fig. 1B). Interestingly, unreduced Mal d 2 demonstrated a very low binding to IgEs of patient sera (Fig. 1C), while, the Mal d 2 reactivity was enhanced in the presence of reducing agent, DTT (Fig. 1C). As a consequence, all samples were reduced prior to IgE-Western blotting for better detection.

In order to investigate the influence of reducing conditions on the Mal d 2 *in vitro* immuno-reactivity, purified protein was studied as a (i) reduced, (ii) reduced and re-oxidised and (iii) reduced, alkylated and by the removal of DTT re-oxidised protein (Fig. 1D–F). Denaturing SDS–PAGE revealed that both the reduced and alkylated proteins migrated as double bands, if they were re-oxidised, with the alkylated protein having a slightly higher molecular weight (Fig. 1D). This behaviour indicates a partial refolding, even though the disulfide bridges were blocked. The pAbs against Mal d 2 recognised all forms of Mal d 2 (Fig. 1E). However, serum IgEs showed a higher affinity to the reduced form of Mal d 2. Removal of the reducing agent, even after alkylation of thiol-residues, resulted in a reduction of the *in vitro* immuno-reactivity (Fig. 1F).

3.2. Influence of cold storage, thermal processing, sugar and pH on the stability of Mal d 2

The influence of low temperatures was studied by applying three cycles of rapid freezing and slow thawing. Mal d 2 showed no structural disintegration or fragmentation. The IgG and IgE-reactivity was not altered after repeated freezing (data not shown). After 1 week of incubation at RT, Mal d 2 was better stained by silver and IgG binding resulted in a more intensive band, suggesting a

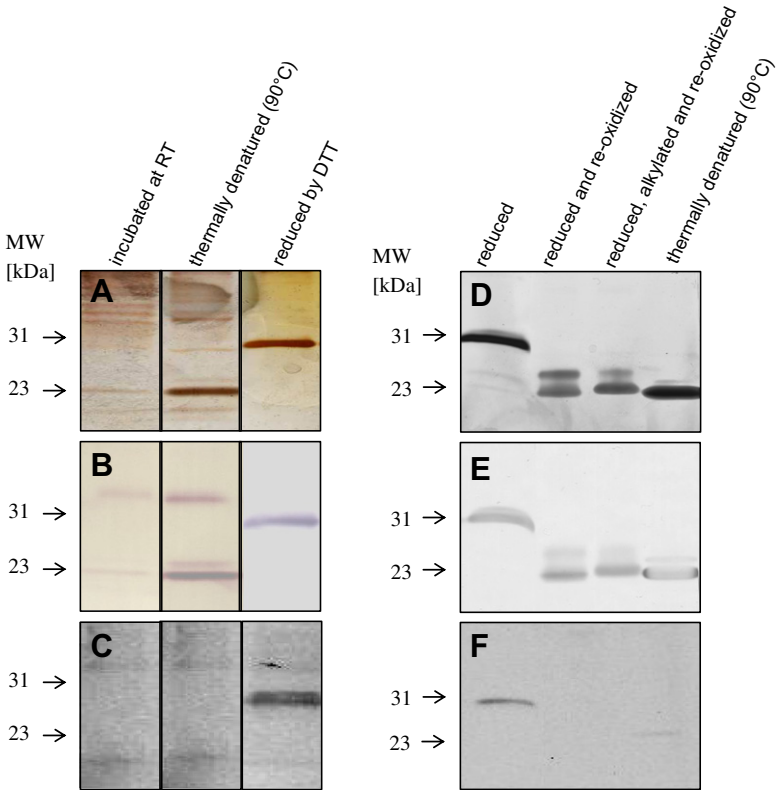

Fig. 1. Influence of physico-chemical treatments on apple extract and purified Mal d 2. (A) silver stained gel; (B) Western blot using anti-Mal d 2 pAbs; (C) Western blot using a human serum pool. Mal d 2, a 23 kDa protein, migrates as a 31 kDa band under reducing conditions. An increased reactivity of pAb was detected after thermal denaturation and reduction of Mal d 2. The band at approximately 40 kDa reacting with the pAbs is probably a dimers of Mal d 2. The slight band at about 17 kDa belongs to Mal d 1, the major allergen from apple. The samples were separated by SDS–PAGE and (D) silver stained and (E) Western blotted with a pAb directed to Mal d 2 or (F) Western blotted with a human serum pool.

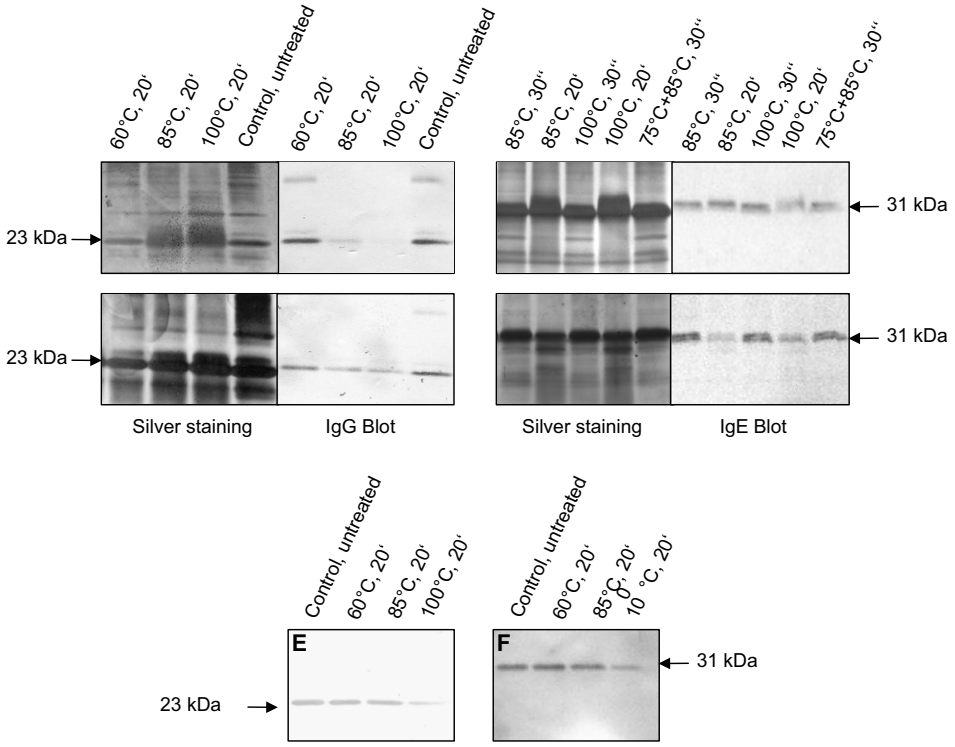

Fig. 2. Influence of heat treatment and pH and sugar on Mal d 2 in the apple extract. Apple extract was heated at different temperature and pH (A,B: pH 7 and C,D: pH 3.5) and subjected to SDS–PAGE and Western blotting. (A,C) denaturing conditions for Western blotting with pAbs directed to Mal d 2. (B,D) reducing conditions for IgE-Western blotting using a human serum pool. Apple extract was heated at different temperatures in the presence of 15% sugar and analysed by SDS–PAGE and Western blotting. (E) Western blot under denaturing conditions for the incubation with anti-Mal d 2 pAbs. (F) Western blot under reducing conditions for the incubation with a human serum pool.

protein denaturation or unfolding at RT (data not shown). Incubation at 4 and −20 °C during one month of storage and at −80 °C for three months showed no alteration of Mal d 2 *in vitro* immunoreactivity.

At pH 7, a treatment of 20 min at higher temperatures (⩾85 °C) caused considerable alterations of Mal d 2 in the apple extract, visible as diffuse bands in the silver stained gel and reduced IgG reactivity (Fig. 2A). However, the IgE-reactivity remained intact, though only a diffuse band was visible after a treatment at 100 °C (Fig. 2B). Under the same conditions, a heat treatment of 30 s did not cause any significant changes in the Mal d 2 immuno-reactivity or molecular fragmentation (data not shown). At pH 3.5 Mal d 2 was more resistant to thermal treatment as there were no significant changes visible in denaturing SDS–PAGE and Western blotting with pAbs even after incubation for 20 min at 100 °C (Fig. 2C). However, the IgE-reactivity decreased after long-term treatment with high temperatures (Fig. 2D). Interestingly, none of the tested treatments was able to elicit an increase of the IgE-reactivity under non-reducing conditions (data not shown).

The influence of high temperatures on purified Mal d 2 was further investigated by heating the protein in the absence and presence of 15% sugar (Fig. 2E and F) for 20 min at 60, 85 or 100 °C. The addition of sugar implicated a slight decrease in both IgE- and IgG immuno-reactivity after 20 min at 100 °C (Fig. 2E and F). These results indicated that temperatures below 100 °C had minimal influence on Mal d 2 structural integrity.

3.3. Conformational changes and thermal unfolding of Mal d 2

The phenomenon of the conformational stability of reduced versus native Mal d 2 was investigated by circular dichroism as independent methodology. The effect of thermal unfolding and refolding was studied by fluorescence spectroscopy and CD spectrometry. Fig. 3A shows the intrinsic tryptophan (Trp) fluorescence

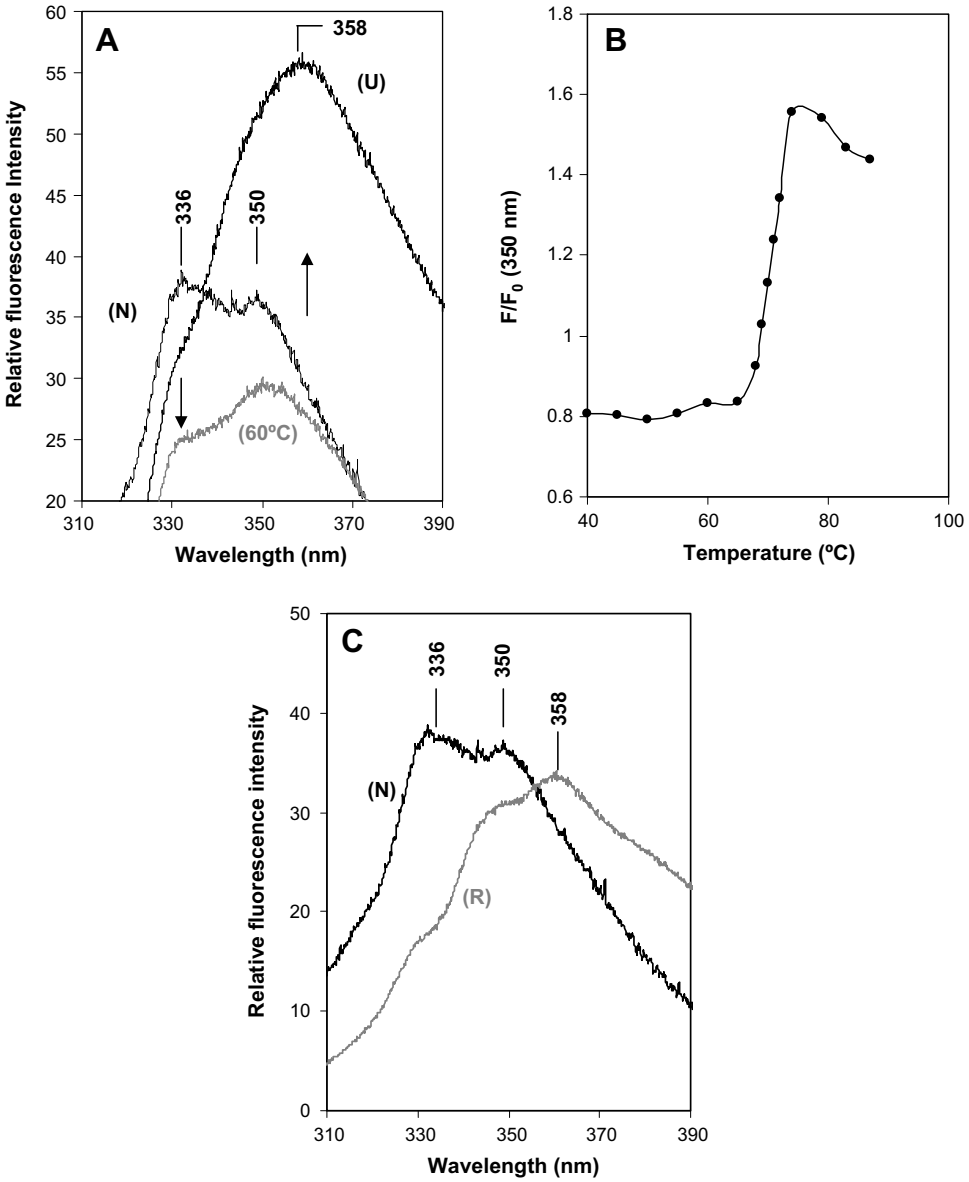

Fig. 3. Thermal unfolding of Mal d 2 followed by fluorescence spectroscopy. Excitation: 295 nm. Emission range: 310–390 nm. Protein concentration 2 μM (10 mM phosphate buffer, pH 7). (A) Emission maxima of native protein at 25 °C (N), at 60 °C, and of unfolded protein at 87 °C (U). (B) Changes in relative tryptophan emission at 350 nm during temperature increase with F being the actual emission intensity at actual temperature and F_0 being the emission intensity at 25 °C. (C) Comparison of emission spectra of native Mal d 2 (N) and refolded (temperature denatured) protein (R).

of Mal d 2 at 25 °C. Principally, the three Trps in Mal d 2 exhibited a low emission intensity, most probably due to energy transfer and quenching by neighbouring amino acids. The two emission maxima at 336 and 350 nm clearly revealed that the Trps in native Mal d 2 are located in at least two different environments, one in a non-polar (336 nm) and one in a polar environment (350 nm). Within the temperature range 25–60 °C only minor structural changes occurred as is obvious from the absence of spectral shifts of emission maxima. Only the relative intensities of peaks at 336 and 350 nm were altered, with the Trp in the more hydrophobic environment in native Mal d 2 being more exposed at 60 °C. Further rise of temperature (60–90 °C) led to increase of emission intensities due to enhanced mobility of residues and thus decreased efficiency in energy transfer. The overall structure collapsed and one red-shifted emission maximum of increased intensity at 358 nm was observed. Fig. 3B clearly indicates that thermal unfolding of Mal d 2 follows a two-state transition. The T_m value was calculated to be 70.5 °C.

Thermal unfolding of Mal d 2 was not reversible (Fig. 3C). Slowly decreasing temperature and analysis of the fluorescence features at 25 °C showed a spectrum dissimilar to both native and completely unfolded Mal d 2 (Fig. 3C). The presence of a maximum at 358 nm and two shoulders at 350 and 336 nm suggests that all three Trps were apparently located in different environments (still mostly polar) after refolding. Aggregation was not observed during the whole heating–cooling cycle.

Fig. 4A shows the far-UV-CD spectrum of native Mal d 2 at 25 °C with a maximum at 210 nm and a shoulder at 215 nm, representing a structure with dominating β-sheets (210 nm) and some contribution from α-helices and random coil-like structures (loops). At 88 °C the obtained CD spectrum had a broad maximum around 208 nm suggesting the loss of secondary structure elements (Fig. 4A). The relative changes in ellipticity at 210, 215 and 222 nm during the whole heating and cooling cycle are shown in Fig. 4B. Between 25 °C and 65 °C both the β-sheets and α-helical structures were stable. Further increasing the temperature led to a typical two-state transition with a calculated T_m value of 74 °C. Upon slowly cooling below the melting point the secondary structures were formed again and refolded Mal d 2 exhibited CD features similar but not identical to its native state (Fig. 4A and B).

The corresponding spectra and changes in ellipticity obtained upon thermal un- and refolding of reduced Mal d 2 are demonstrated in Fig. 5A. The important role of disulphide bridges in stabilisation of the native state is obvious from inspection of both the far-UV spectrum at 25 °C and the transition upon thermal unfolding. The reduced protein at 25 °C exhibited only about half of residual ellipticities between 200 and 250 nm compared to native Mal d 2 and had its maximum at 215 nm indicating dominating β-sheet and loss of α-helical structure. Above 55 °C a two-state transition occurred with a T_m value of 61 °C. The spectrum of the obtained (only partially unfolded) state (maximum around 217 nm) was completely different to that obtained under non-reducing conditions (Fig. 5A and B) and suggested the presence of residual β-sheet structure. Further increase of temperature above 75 °C induced the formation of a random coil-like structure (data not shown).

3.4. Influence of proteolytic digestion on the stability of Mal d 2

To simulate the proteolytic resistance of Mal d 2 in the digestive tract, three gastric and gut enzymes, i.e. pepsin, trypsin and chymotrypsin, were used to digest both an apple extract and purified Mal d 2. Pepsin digestion was performed as separate experiments whereas trypsin and chymotrypsin were applied in combination. Samples of the digested apple extract were taken at different times and analysed by Western blotting with pAbs directed to Mal d 2 and Mal d 1. Mal d 1, the major allergen of apple is a labile protein and was digested within the first hour, whereas Mal d 2 showed no degradation, even after overnight incubation with both enzyme mixtures (Fig. 6A and B). Similarly, the reaction of patients IgE to Mal d 2 was not altered by enzymatic proteolysis (Fig. 6C and D). Additionally the IgE-antibodies used strongly cross react with pepsin (Fig. 6C). During pepsin digestion protein fragments recognised by human sera are probably caused by the degradation of the enzyme itself. Nevertheless, the IgE immuno-reactivity to Mal d 2 was clearly recognisable even after overnight enzymatic digestion.

4. Discussion

Due to their compact structure, Mal d 2 molecules were predicted to be extremely stable and therefore involved in the induction of severe food allergies (Breiteneder, 2004). To our knowledge, the impact of conformational stability of apple Mal d 2 has not

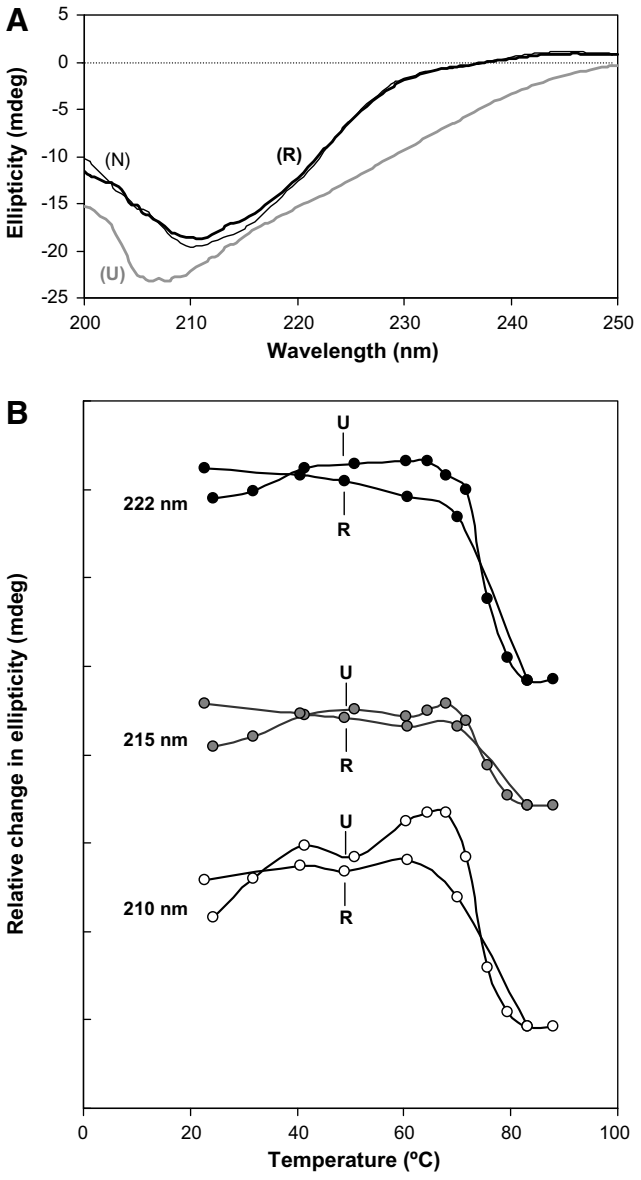

Fig. 4. Thermal unfolding of Mal d 2 followed by electronic circular dichroism spectroscopy. Protein concentration 1 μM (10 mM phosphate buffer). (A) Far-UV-CD spectra of native protein at 25 °C (N), thermally unfolded protein at 88 °C (U) and refolded protein at 25 °C (R). (B) Relative changes in ellipticities at 210, 217 and 222 nm during the whole heating (U) and cooling cycle (R).

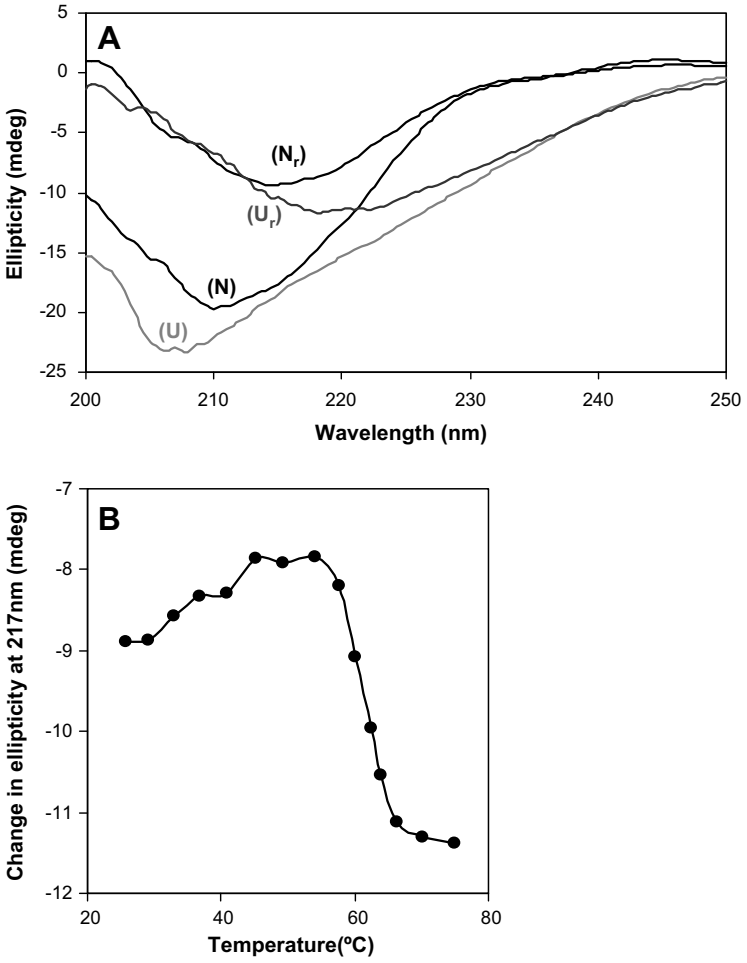

Fig. 5. Thermal unfolding of DTT-reduced Mal d 2 followed by electronic circular dichroism spectroscopy. Protein concentration 1 μM (10 mM phosphate buffer). (A) Spectra of reduced protein at 25 °C (Nr) and at 75 °C (Ur, grey). For comparison spectra of non-reduced native (N) and thermally unfolded (U, grey) Mal d 2 is shown. (B) Change in ellipticity at 217 nm upon during thermal unfolding of reduced protein.

been studied so far in relation to its IgE-reactivity. In the current study, the structural integrity of Mal d 2 in apple extract and as purified protein in relation to its IgE-reactivity was investigated under different physico-chemical conditions, similar to those applied in fruit juice processing. So far, the molecular structure of fruit-specific TLPs from banana, maize and tobacco were studied (Barre et al., 2000; Batalia, Monzingo, Ernst, Roberts, & Robertus, 1996; Koiwa et al., 1997). In a pollen-related TLP from mountain cedar, Jun a 3, structurally conserved IgE-binding epitopes were identified and predicted not only in different allergenic TLPs from banana, cherry and grape, but also in thaumatin from *Thaumatococcos daniellii* (Leone et al., 2006). Based on the sequence homology and the known structure of other TLPs from banana, it is reasonable to assume that Mal d 2 is composed of three domains: (a) the N-terminal domain I corresponding to a β-sandwich built up by 10 antiparallel strands (β1–β10), (b) (domain II) containing one extended and three short α-helices, and (c) a C-terminal domain III consisting of a hairpin segment of two short strands (Leone et al., 2006). The far-UV spectrum of native Mal d 2 reflects these secondary structures.

Interestingly, preliminary experiments have shown two main characteristics for Mal d 2: (1) the altered electrophoretic mobility in the gel, if the disulphide bridges are reduced and (2) the higher IgE-reactivity after reduction. Mal d 2 was identified and characterised as a 23 kDa protein (Krebitz et al., 2003) although in most studies Mal d 2 migrated as a 31 kDa protein in SDS–PAGE (Hsieh et al., 1995; (Krebitz et al., 2003; Oh, Song, Shin, & Chung, 2000). This anomaly in the electrophoretic mobility of Mal d 2 was attributed to treatments with reducing agents (Herndl et al., 2007). The molecular weight of Mal d 2 appeared as increased under reducing conditions in SDS–PAGE (Herndl et al., 2007). The altered migration in SDS–PAGE was previously reported also for the homologous Pru av 2 from cherry (Fuchs et al., 2006; Inschlag et al., 1998) and Act c 2 from kiwi (Gavrovic-Jankulovic et al., 2002), however, the correlation between the reducing milieu and the conformational changes remained unclear.

Momentary conformational changes of Mal d 2 conferred different immunological reactivity. Previously, a significant increase in the IgE-binding under reducing conditions was observed, while none of patient sera tested reacted with the unreduced Mal d 2 (Herndl et al., 2007). Refolding experiments indicated the ability of Mal d 2 to reverse its structure after denaturation or disintegration of disulfide bridges. It was shown that refolding of the Mal d 2 after removal of the reducing agent resulted in reduced IgE-reactivity and nearly a complete reversal of electrophoretic mobility resembling that of the purified protein.

Comparing the CD spectra of the native and reduced Mal d 2, the important role of disulphide bridges in stabilizing the overall structure became evident (Barre et al., 2000). Upon its reduction by DTT, the compactness of the protein was diminished and domain II seemed to have lost part of its α-helical structure. A transition to an intermediate, that fully lost its α-helical structures but

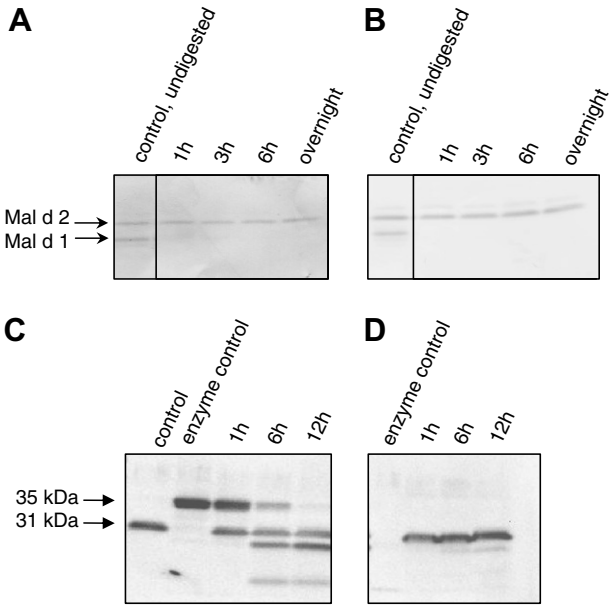

Fig. 6. Resistance of Mal d 1 and Mal d 2 to enzymatic digestion in apple extract. The sample was digested by different gastrointestinal enzymes (A) pepsin; (B) a mixture of trypsin and chymotrypsin and analysed by Western blotting with anti-Mal d 2 and anti-Mal d 1 pAbs. While Mal d 2 resisted the digestion until the end of the experiment, Mal d 1 was digested within 1 h. The Influence of enzymatic digestion on the immunoreactivity of purified Mal d 2 was studied by (C) pepsin and (D) a mixture of trypsin and chymotrypsin. Samples taken at different times were analysed by Western blotting under reducing conditions and visualised with a pool of human sera. Protein control: purified Mal d 2 without enzymes. Enzyme control: enzyme without Mal d 2. Mal d 2 could still be detected by the patients IgE after overnight enzymatic proteolysis.

retained β-strands, occurred at an apparent T_m value of 61 °C. Results obtained suggest a minor role of disulfide bridges for the β-sandwich-like structure of domain I. By contrast, the presence of DTT decreases significantly the structural integrity of domain II and leads to unfolding of its α-helical structure at temperatures, where the structure of domain I was still intact. These findings indicate that the IgE-binding epitopes most probably are localised within the α-helical structure of domain II and become accessible only after the reduction of disulfide bonds.

The heat stability of purified Mal d 2 was studied by fluorescence spectrophotometry and circular dichroism. The three Trps all associated with the N-terminal domain I, facilitates the interpretation of the observed fluorescence spectra. The presence of two emission maxima is fully compatible with two Trps (located within β2 and β5) being buried in the hydrophobic core of the β-sandwich (emission maximum at 336 nm) and one Trp (located in the loop between β4 and β5) being exposed to solvents (emission maximum at 350 nm).

Mal d 2 is relatively stable at high temperatures. Thermal unfolding follows a two step transition with T_m values calculated to be 70.5 °C (Trp fluorescence of domain I) and 74 °C (far-UV-CD of whole protein). At temperatures higher than 80 °C a random coil-like structure was formed. The thermal stability is well reflected by the presence of eight conserved disulfide bridges in Mal d 2 that are formed between the domains I & II and I & III as well as within domain I and domain II (Leone et al., 2006). Upon refolding of thermally unfolded Mal d 2 the overall secondary structure contents was recovered on the whole though the tertiary structure of at least domain I could not be fully restored.

The results obtained by SDS–PAGE and Western blotting essentially led to the same conclusions as measurements by circular dichroism and fluorescence spectroscopy. Short time heating (30 s) or extended heating (20 min) at 60 °C did not cause any detectable alterations. However, it revealed the importance of pH for the immune-reactivity of Mal d 2. The diffuse bands in the silver stained gel and the loss of IgG-reactivity after long-term treatment (20 min) and higher temperatures (⩾85 °C, pH 7) indicated structural changes. On the other hand, no changes are visible in the silver stained gel and the IgG blot at pH 3.5 under the same conditions. In both cases, the IgE-reactivity under reducing conditions remains largely intact, though a slight decrease is observable at higher temperatures. Results obtained demonstrate that even after harsh heat treatment or conditions similar to apple juice manufacturing the IgE-epitopes are still intact, but only accessible under reducing conditions

Purified Mal d 2 resisted enzymatic digestion for over 24 h with pepsin, trypsin and chymotrypsin and could bind to IgEs without alterations. Several *in vitro* gastrointestinal digestions were performed to asses the protein stability in presence of digestive enzymes (Asero et al., 2000; Astwood, Leach, & Fuchs, 1996; Besler, Steinhart, & Paschke, 2001; Thomas et al., 2004). Mal d 2 resisted pepsin digestion in the same extent as described for Mal d 3, a lipid transfer protein from apple (Akkerdaas et al., 2004). By contrast, the homologous TLP of kiwi was degraded within 1 min by the simulated gastric fluid (Polovic et al., 2007). The stability of the tested proteins can not be compared due to different protocols (Polovic et al., 2007). The stability of Mal d 1 was also analysed to test its in vitro proteolytic digestibility (data not shown). Mal d 1 could be only detected with a polyclonal antibody (anti-Mal d 1) only after half a minute in the pepsin digestion and 1 min in the chymotrypsin–trypsin digestion. These data demonstrate the protease lability of Mal d 1 compared to the extreme stability of Mal d 2.

Despite previous reports on the importance of Mal d 2 as a second major apple allergen, a recent population study showed that the incidence of allergy to Mal d 2 compared to other apple allergens is low (Fernandez-Rivas et al., 2006). Apple Mal d 2 showed enhanced IgE-reactivity only after breakage of disulphide bridges. Removal of reducing agent led to a partial re-naturation and immediate loss of IgE-binding capacity *in vitro*. The refolding studies support the hypothesis that predicted IgE-binding epitopes of Mal d 2 may be hidden inside the molecule and therefore, not easily accessible for the IgE-molecules. In conclusion, industrial practice in manufacturing of apple products or digestive enzymes cannot lead to a destruction of the molecular stability of Mal d 2. Further, the ability of the protein to refold nearly to its natural conformation prevents the exposure of binding structures hidden within this protein. In fact, the correlation of these data and the corresponding clinical symptoms are still under investigation and crucial for the risk assessment of different types of apple products.

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