

Impact of Sulfur and Vitamin C on the Allergenicity of Mal d 2 from Apple (*Malus domestica*)

Gorji Marzban,[†] Tamar Kinaciyan,[§] Fatemeh Maghuly,[†] Richard Brunner,^{||} Clemens Gruber,[‡] Rainer Hahn,[†] Erika Jensen-Jarolim,^{||} and Margit Laimer^{*,†}

[†]Plant Biotechnology Unit and Down-Stream Processing Group, Department of Biotechnology, and [‡]Department of Chemistry, Vienna Institute of BioTechnology (VIBT), BOKU (University of Natural Resources and Life Sciences), Vienna 1190, Austria

[§]Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, and ^{||}Institute of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna 1090, Austria

ABSTRACT: Mal d 2 is a minor allergen from apple which shows a high conformational stability due to its eight conserved disulfide bridges. Chemical reduction of disulfide bridges and linearization of Mal d 2 lead to enhanced IgE reactivity in vitro and indicate a higher potential for allergenicity. Since food preservatives such as sulfur and vitamin C are reducing and denaturing agents, their influence on Mal d 2 allergenicity was verified by simulated food processing conditions. The immunoreactivity of purified Mal d 2 was investigated after different treatments in vitro and in vivo using IgG/IgE Western blotting, mediator-releasing cell assay, and skin prick and oral smear tests. The conformational changes of Mal d 2 upon addition of 1% and 5% vitamin C were also monitored by attenuated total reflectance Fourier transform infrared spectroscopy. The results show no positive skin and oral smear test reactivity to native, heated, or vitamin C-treated purified Mal d 2. Furthermore, the results confirm that sulfur in combination with heat treatment can influence the structural integrity and thus the allergenicity of Mal d 2, while vitamin C is too weak as a reducing agent to change allergenicity.

KEYWORDS: thaumatin-like proteins, pathogenesis-related proteins, food safety, IgE reactivity, sulfurization, oral allergy syndrome

INTRODUCTION

Thaumatin-like proteins (TLPs) are members of the pathogenesis-related protein 5 family and constitute a homogeneous family of plant proteins expressed in response to different biotic and abiotic stresses.^{1–3} The TLP from apple, Mal d 2, is considered a minor allergen associated with IgE-mediated oral allergy syndrome (OAS), although reports on the significance of Mal d 2 as an allergen are geographically distinct and contradictory.^{4,5} While about 75% of sera from apple-allergic patients in the United States recognized a chemically reduced Mal d 2 band at about 31 kDa, only 15% in Spain and Italy and 5% in Austria and The Netherlands were positively reactive in IgE Western blots.^{6,7}

The structural stability of Mal d 2 is related to 16 cysteine residues which hold tightly the tridimensional conformation of the molecule. Its extreme resistance to low pH, heat denaturation, and proteolysis by gastric fluids led to the assumption that this protein might be able to trigger severe symptoms.⁸ However, this has not been confirmed by clinicians yet.^{1,5,8} The demographic distribution of the prevalence of IgE reactivity to apples in Europe demonstrated a significantly lower rate of sensitization to Mal d 2 compared to the major allergens Mal d 1 and Mal d 3.⁷

Previous studies of the molecular stability of Mal d 2 demonstrated two major features after chemical reduction and denaturation: (a) altered migration in SDS–PAGE (the 23 kDa band appears as a 31 kDa band) and (b) an enhanced IgE reactivity.^{8,9} Altered migration of TLPs in SDS–PAGE after chemical reduction of disulfide bridges was described in apple, cherry, and kiwi.^{9–11} In vitro studies showed that native and heat-denatured Mal d 2 has a much lower affinity for patients'

IgE's than the chemically reduced form.^{8,9} The chemical reduction dissociates the disulfide bridges and destroys the tertiary structure, so the Mal d 2 molecules linearize to a variable extent.^{8,9}

In industrial food manufacturing, apple-derived foods and juices are routinely treated by heat, enzymes, or antioxidants to prevent browning processes. Among food preservation techniques, sulfurization and hot and cold drying processes are the most frequently used conservation methods. Sulfur treatment, carried out by addition of sulfur dioxide or water-soluble sulfite salts (e.g., Na₂S₂O₃), reduces protein disulfide bridges and causes enzyme deactivation by rigorous denaturation of protein structures and preserves dried fruits.¹² In particular, sulfur treatment preserves the texture and color by inhibiting the enzymatic and nonenzymatic browning reaction.¹³ In fact, EU rules (directive 2000/13/EC) keep residual sulfur concentrations strictly low (at a maximum of 0.045%) in plant-derived food due to carcinogenicity and other health risks of sulfur.¹⁴

Vitamin C is added at concentrations up to 0.1% to fruit products as an antioxidant to avoid nonenzymatic browning reactions as well as oxidation.¹⁵ Addition of both vitamin C and sulfur is performed routinely in an acidic milieu, which maximizes the effectiveness of both treatments and inhibits the browning activity of polyphenol oxidases.¹⁵ Vitamin C is the strongest natural reducing agent¹⁶ and is routinely used in juice

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and dried fruit production to enhance color stability. The presence of vitamin C in apple products has implications on disulfide bridges and may lead to increased IgE reactivity or even enhanced allergenicity for sensitized individuals.

The present study focused on the influence of heat treatment and vitamin C or sulfur addition to purified Mal d 2. The conformational stability of Mal d 2 in the presence of vitamin C was investigated using IgE Western blot, mass spectrometry (MS), and attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Furthermore, the characterization of the *in vivo* reactivity to vitamin C-treated Mal d 2 was performed using patients with clinically confirmed allergy to apples.

MATERIALS AND METHODS

Chemicals. Trichloroacetic acid (TCA), acetone, sodium bicarbonate (Na_2CO_3), diethyldithiocarbamic acid (DIECA), sodium chloride (NaCl), potassium chloride (KCl), disodium phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium hydrogen carbonate (NaHCO_3), sodium hydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), hydrochloric acid (HCl), glycerol, sodium borate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), magnesium chloride (Mg_2Cl_2), sodium disulfite ($\text{Na}_2\text{S}_2\text{O}_5$), acetic acid, 2-mercaptoethanol, calcium chloride (CaCl_2), ammonia (NH_4HCO_3), D-glucose, and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). Urea, poly(vinylpyrrolidone) K25, Tris base, sodium dodecyl sulfate (SDS), goat antirabbit IgG conjugated with alkaline phosphatase, goat antihuman IgE antibodies conjugated with horseradish peroxidase, bromochloroindolyl phosphate (BCIP), nitro blue tetrazolium (NBT), bovine serum albumin (BSA), L-glutamine, sodium pyruvate, 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES), citric acid, dithiothreitol (DTT), 4-methylumbelliferyl N-acetyl- β -D-glucosaminide, polyvinyl polypyrrolidone (PVPP), and Triton X-100 were obtained from Sigma-Aldrich (Steinheim, Germany). Skim milk and iodoacetamide were obtained from Fluka (Steinheim, Germany). Formic acid (FA) was obtained from Roth (Karlsruhe, Germany). The Micro BCA protein assay kit was purchased from Pierce (Rockford, IL). Acetonitrile (ACN) and vitamin C were purchased from BDH Prolabo (Leuven, Belgium). The ECL-Advanced Western blot kit was obtained from GE Healthcare (Buckinghamshire, U.K.). RPMI medium was obtained from Invitrogen (Carlsbad, CA). Trypsin and GluC were purchased from Roche (Basel, Switzerland).

Apple Material. Seven different apple breeding lines and cultivars were harvested from an experimental field with drip irrigation near the Adriatic seaside at cultivar-specific dates and stored at 4 °C before extraction: G198, Braeburn, Topaz, Fuji, Golden Delicious, Granny Smith and Pilot, and Pink Lady. Protein extraction was performed as previously described.¹⁷ Five apples per cultivar were peeled (5 mm) with a paring machine, shock frozen, and powdered in liquid nitrogen. Frozen powder was mixed in a blender with extraction buffer (10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 0.27 mM EDTA, 13.3 mM DIECA, 2% PVPP, pH 7) in a ratio of 1:2 (w/v) and extracted by stirring at 4 °C. The mixture was filtered through two layers of Miracloth (Calbiochem, United States) and centrifuged at 10000g for 30 min at 4 °C and the supernatant immediately frozen at -80 °C. Additional parameters such as the firmness and sugar and titratable acid contents, which indicate the maturity of the apples, were measured. The firmness of the apples is measured by a penetrometer, sugar content by refractometry, and titratable acid content by titration with 0.1 M HCl (Table 1).

Apple-derived food products were obtained from the local market: (1) dried apple chips, (2) sulfured apple rings, (3) nonsulfured apple rings, (4) baby apple puree, (5) clear apple juice, (6) cloudy apple juice, (7) clear organic apple juice. Dried apple samples (2 g) were precipitated by phenol, and the obtained protein pellets were resolved in 8 N urea.¹⁸ Due to the low protein contents of apple juices, the enrichment of proteins required a precipitation step with 10% TCA/

Table 1. Parameters Such as Firmness and Sugar and Titratable Acid Contents Were Measured in Tested Apple Cultivars as Indicators of Maturity and Physiological State

cultivar	firmness (kgf)	sugar content (%)	titratable acid content (g/L)
G198	9.1	14.5	7.5
Braeburn	7.5	12.1	6.7
Fuji	7.2	13.8	3.7
Golden Delicious	6.6	14.9	5.2
Granny Smith	7.7	11.8	6.8
Pink Lady	8.7	15.1	6.7
Topaz	7.8	12.8	2.3

acetone. A 20 mL volume of apple juice was incubated with the same volume of 10% TCA/acetone overnight. The pellet was washed three times with pure acetone and dried at -20 °C. The total protein content was determined using the BCA kit (Pierce) according to the manufacturer's instructions.

Purification of Apple Allergens. Apple cultivar Topaz was used for extraction of Mal d 2 and Mal d 1.⁸ A 1 kg mass of apple peels was homogenized in carbonate extraction buffer (20 mM Na_2CO_3 , 13.3 mM diethyldithiocarbamic acid, 1 mM ethylenediaminetetraacetic acid, 2 mg/mL PVP, pH 9.0) and stirred for 30 min at 4 °C.⁸ The extract was filtered through two layers of Calbiochem Miracloth and centrifuged at 28000g (Beckman Coulter, Avanti J-25, JLA-10500) for 30 min at 4 °C. The supernatant was lyophilized and stored at -20 °C. The lyophilizate was dissolved in carbonate extraction buffer at a ratio of 1:10 (w/v), centrifuged for 30 min at 4000g and 4 °C, and buffer-exchanged using PD-10 columns (GE Healthcare) into equilibration buffer (10 mM Tris, 2 mg/mL PVP, pH 8). The obtained samples were loaded onto an anion exchange column, 30Q HR 10/10 (GE Healthcare). The purification was performed by an ÄKTA 100 Explorer chromatography system (GE Healthcare). Elution was carried out with a linear gradient from 0 to 1 M NaCl and the product fractionated for further analyses. To identify which fraction contained Mal d 1 and Mal d 2, IgE Western blotting was performed using Mal d 1- or Mal d 2-reactive patient sera. Fractions containing Mal d 1 and Mal d 2 were subjected to size-exclusion column chromatography on a Superdex 75 column (GE Healthcare) equilibrated with 25 mM sodium phosphate buffer, pH 7.0.

The identity and purity of purified Mal d 2 were confirmed by mass spectrometry. For the tryptic and GluC digestions of Mal d 2, 10 μL of the protein solution (1 mg/mL H_2O) was adjusted to 50 mM NH_4HCO_3 and 7.5 mM DTT (final concentration) by addition of 10 μL of 100 mM NH_4HCO_3 containing 15 mM DTT and incubated for 45 min at 56 °C to reduce disulfide bridges. Trypsin cleaves proteins and peptides at the amino acids arginine and lysine, and GluC cleaves proteins and peptides at glutamate. Thiol groups were carbamidomethylated by the addition of 40 μL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 . Proteins were precipitated by the addition of acetone (4:1), and the pellet was resuspended in 20 μL of NH_4HCO_3 buffer containing 0.1 μg of trypsin and GluC. All proteolytic reactions were performed at 37 °C overnight. The digested samples were directly injected into the LC-MS system (LC, Dionex Ultimate 3000 LC instrument; MS, Bruker, AmaZon ETD, equipped with the online nanosource). The peptides were separated on a C-18 column (Dr. Maisch GmbH, C-18 HPLC column, ReproSil-Pur, 200 $\times$ 0.1 mm, 3 μm packing, 200 Å pore diameter, flow 0.4 $\mu\text{L}/\text{min}$). A gradient from 95% solvent A and 5% solvent B (solvent A, 0.1% FA in water; solvent B, 0.1% FA in ACN) to 32% B in 40 min was applied, followed by a 15 min gradient from 32% B to 75% B that facilitates elution of large peptides. The peptides were analyzed by MS with data-dependent acquisition. Data were processed using the freeware program X!-tandem combined with GPM (Global Proteome Machine). The purity of Mal d 2 was determined by mass spectrometric analysis of the whole protein. A 1 μg mass of Mal d 2 was directly injected into the LC-MS system (LC, Dionex Ultimate 3000 LC instrument; MS, Bruker, Maxis

4G, equipped with the standard ESI source). The protein solution was eluted by developing a linear gradient from 10% to 60% ACN (Supelco Discovery Bio wide-pore C5 column, 50 × 0.32 mm, 3 μm packing). Data were processed using Data Analysis 4.0 (Bruker), and the spectrum was deconvoluted by the maximum entropy equation.

Polyclonal antibodies were raised in rabbits by immunization with 500 μg of native Mal d 1 and Mal d 2 and Freund's adjuvant.^{8,17} The specificity of the Mal d 2 antiserum was confirmed by Western blotting.

SDS-PAGE and Western Blotting. All 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) bromophenol blue) in a ratio of 1:2 (v/v) and denatured for 10 min at 90 °C or chemically reduced by 50 mM DTT. Total protein was determined via BCA assay after buffer exchange using PD-10 columns (GE Healthcare). For cultivar screening, 10 μg of total protein per lane was loaded to precast 4–20% Tris–glycine gel (Novex System, Invitrogen). As positive controls, two different concentrations of purified Mal d 2 (0.5 and 1 μg) were applied to each gel. Gels were electroblotted onto nitrocellulose membranes using a blotting buffer containing 50 mM Na₂B₄O₇·10H₂O, 0.1% (w/v) SDS, and 20% (v/v) methanol. After overnight blocking with 3% skim milk powder, polyclonal antibodies against purified Mal d 2 were diluted to 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 Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) and added to the membranes. The immunoreaction was detected using goat antirabbit IgG conjugated with alkaline phosphatase in dilution buffer in a ratio of 1:2000 (v/v), followed by visualization with BCIP and NBT in alkaline phosphate buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris).

Influence of Different Processing Conditions on Purified Mal d 2. To study the influence of food preservatives on the immunoreactivity of Mal d 2, purified Mal d 2 was treated by reducing agents such as vitamin C (1% and 5%, w/v) as well as sulfur (1% and 5% (w/v) Na₂S₂O₅) in 10% (v/v) acetic acid at pH < 3 and different temperatures. Room temperature (rt) and 90 °C were selected, since they simulate the processing conditions in dried fruit production. Lowered pH is able to increase the effectivity of the treatments. Additionally, the purified Mal d 2 was treated for three incubation times: (i) 2 min, (ii) 20 min, and (iii) overnight (>16 h). Purified Mal d 2 reduced with 50 mM dithiothreitol for 1 h at rt was used as a control for positive IgE-binding.

Patient Selection. Six of eighteen apple-allergic patients showing positive reactivity to Mal d 2 in IgE Western blotting were selected for these studies. These patients, who were enrolled for in vivo testing, gave written informed consent for participating in the study, which was approved by the local Medical Ethical Committee of Vienna and conducted according to guidelines for Good Clinical Practice.

IgE Western Blots Using Patient Sera. Electroblotted membranes were blocked with 3% BSA for 2 h at 37 °C and incubated overnight at 4 °C with sera in a ratio of 1:10 (v/v). Goat antihuman IgE antibodies conjugated with horseradish peroxidase (1:20000, v/v) were used for visualization. Purified Mal d 2 was applied to the gel in reduced and not reduced form. Reacting protein bands were detected with the ECL-Advanced Western blotting kit according to the manufacturer's guidelines. In summary, horseradish peroxidase present on a Western blot is detected by its reaction with peroxide, an enhancer molecule, and the chemoluminescent substrate, all of which are present in the substrate reagents provided and visualized with a chemoluminescence imager.

Skin Prick and Oral Smear Tests. Only the influence of vitamin C addition on altered allergenicity of Mal d 2 could be examined by skin prick and oral smear tests. Due to severe skin irritations caused by DTT and Na₂S₂O₅ on healthy skin, sulfur-treated Mal d 2 could not be used for patient skin testing. Skin prick tests (SPTs) were performed on the forearm with different concentrations of purified Mal d 2 (100, 20, 10, and 5 μg/mL). The samples were provided as native, heat-denatured (20 min at 90 °C), and treated (2 min at 90 °C, 1% vitamin C). Positive skin reactions were recorded after 20 min by copying the wheal reaction onto a transparent adhesive tape, and wheal diameters were measured as soon as a positive reaction was visible. Fresh apple

and histamine (10 mg/mL) were used as positive controls and 1% vitamin C in saline solution and saline solution as negative controls. Oral smear tests (OSTs) were applied as a minivariant of oral provocation to test the mucosal reaction inside the mouth. OSTs were performed using increasing concentrations of purified Mal d 2 (10, 20, and 100 μg/mL) in 1% vitamin C saline solution. To start, a 1% vitamin C saline solution (negative control) soaked cotton swab was smeared for 1 min on the mucosal side of the lower lip, and after 20 min native Mal d 2 was smeared on the inner side of the upper lip. Thereafter, increasing concentrations of native Mal d 2 were smeared, alternating on the lower and upper lips until the highest concentration was reached.

β-Hexosaminidase Secretion Assay from Human FcεRI Receptor Protein Transfected Rat Basophilic Leukemia Cells.

The binding of treated and untreated Mal d 2 to IgE receptor FcεRI was studied using cell-based the rat basophilic leukemia assay (RBL assay) in vitro. RBL-703/21 cells, derived from RBL-2H3, contained genes of the human high-affinity IgE receptor FcεRI (kindly provided by Dr. Lothar Vogel, Paul-Ehrlich Institute, Langen, Germany). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 1% penicillin/streptomycin, and 100 μM 2-mercaptoethanol at 37 °C and 5% CO₂. RBL cells (4 × 10⁴ cells per well in 100 μL of medium) were seeded in a 96-well round-bottom microtiter plate (Nunc) and incubated overnight at 37 °C and 5% CO₂. Cells were passively sensitized with sera from six patients (diluted 1:25) for 2 h at 37 °C and 5% CO₂. Cells were washed twice with Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 1.4 mM CaCl₂, 1.7 mM MgCl₂, 5 mM D-glucose, 10 mM HEPES, 0.42 mM NaH₂PO₄·H₂O, 12 mM NaHCO₃) at pH 7.2 supplemented with 0.1% BSA. Native Mal d 2 (0.05 μg in 100 μL of Tyrode's buffer per well), heat-denatured Mal d 2 (90 °C for 5 min, 0.05 μg in 100 μL of Tyrode's buffer per well), Mal d 2 with 1% vitamin C (90 °C for 5 min, 0.05 μg in 100 μL of Tyrode's buffer per well), and 1% vitamin C solution alone as the control were added to control wells and incubated for 30 min at 37 °C in a 5% CO₂ atmosphere. As a negative control, RBL cells were incubated with Tyrode's buffer to calculate spontaneous release of β-hexosaminidase. Cell lysis was performed by addition of 10 μL of 10% Triton-X in Tyrode's buffer for 5 min for total release of β-hexosaminidase (100% release) as a positive control. A 50 μL volume of supernatant was mixed with 50 μL of assay solution (5 mL of 0.1 M citric acid, pH 4.5, and 80 μL of 4-methylumbelliferyl N-acetyl-β-D-glucosaminide) and incubated at 37 °C for 1 h. The reaction was terminated by addition of 100 μL of glycine buffer (200 mM glycine, 200 mM NaCl, pH 10.7). Fluorescence signals were read at 360 nm/465 nm (Cytofluor 2350, Millipore), and the results were calculated as a percentage of total release. The values of the negative controls were subtracted.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy. ATR-FTIR measurements were performed using a VERTEX 70 spectrometer (Bruker) and a BioATRcell IITM (Harrick). The BioATRcell IITM was cooled with a Haake K20 water bath at 20 ± 0.05 °C. The sample chamber of the spectrometer was continuously purged with dry air. Spectra of 20 μL volume samples from 4000 to 850 cm⁻¹ were acquired by using a nitrogen-cooled MCT (photovoltaic mercury cadmium telluride) detector (spectral resolution 4 cm⁻¹, 64 scans, forward–backward). A Blackman-Harris three-term apodization function was used along with a zero-filling factor of 1. Purified Mal d 2 at a concentration of 1.8 mg/mL was dissolved in 15 mM phosphate buffer at pH 7.0 containing 0%, 1%, and 5% (w/w) vitamin C. Samples were incubated for 1 h at rt before the measurements. For background measurements 15 mM phosphate buffer at pH 7.0 without vitamin C and the same buffer with 1% (w/w) or 5% (w/w) vitamin C in Milli-Q water (SGWater) were used. The samples were prepared so that the concentrations of the buffer and the vitamin C were identical to those of the blank used for the background. Sample preparation with identical buffer concentrations for the sample and blank is a critical error-prone step. However, a correction of small differences between sample and background concentrations is possible. For each sample three to five spectra were collected, and the averages were used for

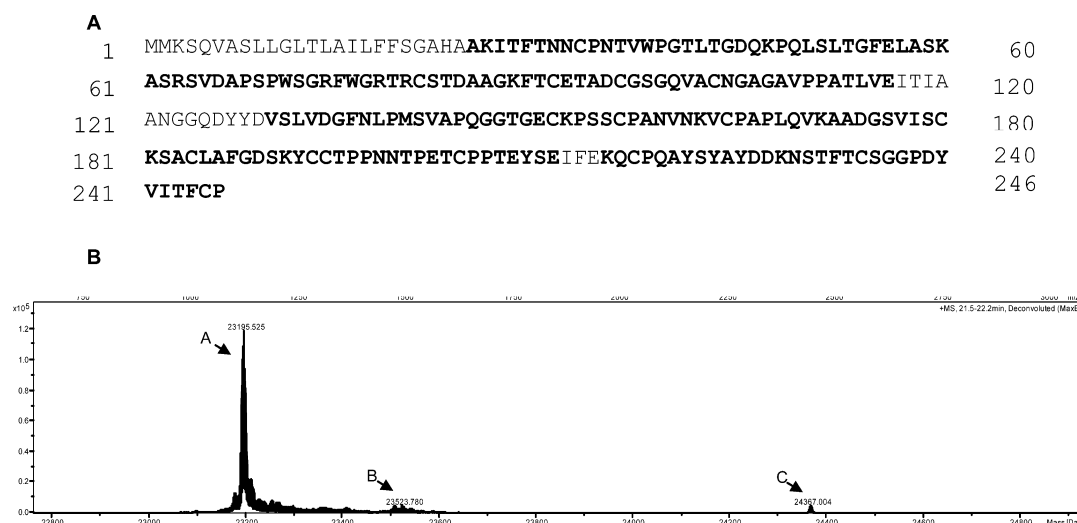


Figure 1. (A) Sequence coverage of identified peptide fragments obtained by trypsin and GluC digestion of Mal d 2. The fragments in bold were found by MS/MS analyses and showed high sequence coverage of Mal d 2. (B) Mass spectrometry of the Mal d 2 molecule. The measured mass of the major peak corresponds to the theoretical mass of 23 195.7 Da beginning from the N-terminal sequence AKITFTNNCPN (UniProt accession no. Q9FSG7). Key: (A) main peak, (B) Mal d 2 isoform, (C) glycosylated Mal d 2.

analysis. OPUS software (version 7.2, www.brukeroptics.de) was used for signal processing. An atmospheric correction of the full spectrum and a rubber-band baseline correction of the resulting spectrum were performed in the wavelength ranges between 1100 and 1900 cm^{-1} .

RESULTS AND DISCUSSION

Characterization of Purified Mal d 2. To perform *in vivo* and *in vitro* experiments under simulated food processing conditions, Mal d 2 was purified from the apple cultivar Topaz due to its high content of Mal d 2.⁸ Mass spectrometry and peptide sequencing were used for the verification of the identity, molecular mass, and purity of purified Mal d 2 (Figure 1). The results showed high sequence coverage (UniProt accession no. Q9FSG7) and confirmed the purity of the protein at 23 195 Da. The peak at about 23 500 Da is a Mal d 2 isoform which is present in trace amounts (Figure 1B). One minor peak is detected at 24 367 Da which may contain a single glyco-MMXF residue and compared to the major Mal d 2 peak is of very low quantity. In fact, Mal d 2 was found to possess one putative N-glycosylation site.⁴ Purified Mal d 2 was used for raising specific anti-Mal d 2 polyclonal antibodies and for *in vitro* and *in vivo* experiments.

Influence of Different Processing Conditions on Purified Mal 2. SDS-PAGE and the Western blotting technique were applied to investigate the influence of different temperatures and addition of sulfur or vitamin C at different concentrations (Figure 2). A temperature of 90 °C was used for the chemical reduction of Mal d 2. Mal d 2 was not detected by Coomassie after heat treatment at 90 °C and with sulfur (1% or 5% (w/v) $\text{Na}_2\text{S}_2\text{O}_5$, Figure 2A). Considering that the detection limit of Coomassie staining for Mal d 2 is 1.0 ng or 50 fmol,¹⁹ under these conditions, the protein was completely degraded and only traces of a protein band could be visualized. In IgG Western blot and SDS-PAGE only faint bands of sulfur-treated Mal d 2 indicate the presence of only traces of intact protein (Figure 2B). Purified Mal d 2 was stable after treatments with sulfur at rt, and in Western blotting the 23 kDa band of native Mal d 2 remained constantly reactive with polyclonal antibodies, indicating the integrity of IgG epitopes (Figure 2B). No significant band migration shift was detected after

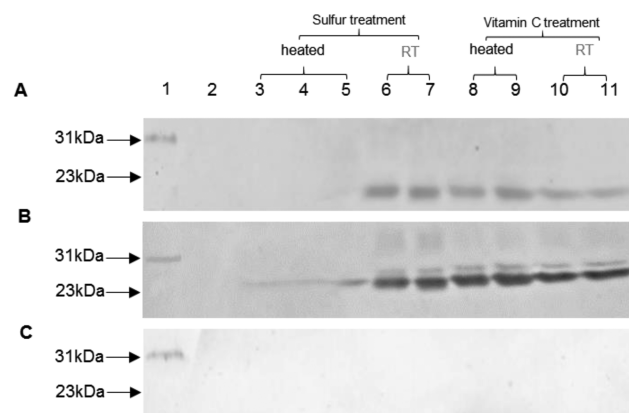


Figure 2. Influence of different treatments on immunoreactivity of Mal d 2: (A) Coomassie-stained gel, (B) Western blot using polyclonal rabbit antibody against Mal d 2, (C) IgE Western blot using the serum pool of the six selected patients. Lane 1: chemically reduced Mal d 2 as positive control. Lane 2: negative control. Lanes 3–11: Mal d 2 with different treatments: (3) 5% $\text{Na}_2\text{S}_2\text{O}_5$, 20 min at 90 °C and 1 h at rt, (4) 5% $\text{Na}_2\text{S}_2\text{O}_5$, 2 min at 90 °C, (5) 1% $\text{Na}_2\text{S}_2\text{O}_5$, 20 min at 90 °C and 1 h at rt, (6) 5% $\text{Na}_2\text{S}_2\text{O}_5$, 2 min at rt, (7) 1% $\text{Na}_2\text{S}_2\text{O}_5$, 2 min at rt, (8) 5% vitamin C at 90 °C for 2 min, (9) 1% vitamin C incubated at 90 °C for 2 min, (10) 5% vitamin C incubated overnight at rt, (11) 1% vitamin C incubated overnight at rt.

treatment at rt with sulfur or with vitamin C at different temperatures. In IgG Western blotting a diffuse faint band at approximately 28–31 kDa can be visualized after treatments with 1% or 5% sulfur at rt and 1% or 5% vitamin C at 90 °C or rt and might be attributed to a minimal denaturation of Mal d 2. The diffuse faint band was not detectable in Coomassie staining or reactive with serum pool IgE's (Figure 2).

The results showed that neither sulfur nor vitamin C is able to reduce or linearize Mal d 2, so the mobility of the Mal d 2 band in the SDS-PAGE gel is shifted to the level of a 31 kDa protein. IgE Western blotting confirmed that Mal d 2 remained in its native conformation in all treatments and therefore was not reactive with the serum pool of the selected patients (Figure 2C). The absence of *in vitro* IgE reactivity to treated

Mal d 2 can be explained by the presence of the protein in its natural conformation (Figure 2C). Taking into consideration that Mal d 2 can refold very quickly almost to its natural conformation after heat denaturation, it is unlikely to bind to serum IgE's.⁸

Conformational Stability of Mal d 2 in the Presence of Vitamin C. Biophysical measurements were performed to analyze the stability of Mal d 2 in the presence of vitamin C. Circular dichroism (CD) and differential scanning calorimetry (DSC) failed to determine the conformational stability of Mal d 2 in the presence of vitamin C because of the high ellipticity of vitamin C that overlapped with the maxima and minima of the protein. DSC could not be performed since vitamin C is destroyed by heat treatment and the thermograms were not reproducible.

ATR-FTIR spectroscopy was, therefore, used as an independent methodology to detect possible conformational changes of Mal d 2 protein by the addition of vitamin C at rt. Purified Mal d 2 at a concentration of 1.8 mg/mL was dissolved in 15 mM phosphate buffer at pH 7.0 containing 1% and 5% (w/w) vitamin C. ATR-FTIR spectra of purified Mal d 2 in the absence and presence (1% or 5%, w/w) of vitamin C were recorded in the wavenumber range between 1100 and 1900 cm^{-1} (Figure 3A). Upon normalization (Figure 3B), vitamin C-induced spectral changes are obvious. The band of the amide I position around 1650 cm^{-1} is shifted to lower wavenumbers, which might reflect some loss of α -helical structure. It was shown by using electronic CD spectrometry that the secondary structure of Mal d 2 is dominated by β -sheets and has some contributions from α -helices,⁸ which could also be confirmed by ATR-FTIR-spectroscopy with a peak at about 1650 cm^{-1} (α -helices) and a shoulder at about 1630 cm^{-1} , highlighted by a black arrow (Figure 3A,B). This is due to the unfolding of the α -helices by the addition of vitamin C, comparable to thermal unfolding.⁸ As there is still a shoulder visible at about 1655 cm^{-1} in the spectrum of Mal d 2 with 5% (w/w) vitamin C (Figure 3B), the α -helical secondary structure remains intact to some extent.

The standard reduction potential of the redox couple cystine/cysteine is around -0.21 V at pH 7.0.^{20,21} DTT is a powerful reducing agent with a reduction potential at pH 7.0 of -0.33 V.²² Thus, in its presence all intramolecular disulfide bridges are in the reduced state.²⁰ DTT was used in a previous study to reduce Mal d 2.⁸ The calculated redox potential of vitamin C at pH 7.0 of $E^\circ = -0.066$ V is too low to reduce cysteine.²³

The Cys ν (stretching vibration of S–H) typically appears around 2551 cm^{-1} ²⁴ in a region without overlap by any other protein absorptions.²⁵ Strong hydrogen bonding between an SH group and a peptide carbonyl might shift the band to lower wavenumbers. In Mal d 2 no evidence for the existence of an SH group was found. The small peak at about 1760 cm^{-1} (Figure 3A) might derive from the small difference in vitamin C content between the sample and reference cell. Oxidized vitamin C (dehydroascorbic acid) has a peak at 1795 cm^{-1} which is also completely missing in the spectra. This confirms additionally that no oxidation of vitamin C and therefore most probably also no reduction of the cysteine groups of Mal d 2 took place.

Verification of Mal d 2 Allergenicity in the Cell-Based RBL Assay. To verify the allergenicity of native and heat-treated purified Mal d 2, 18 patients were screened by the means of anamnesis, positive OAS minutes after consumption

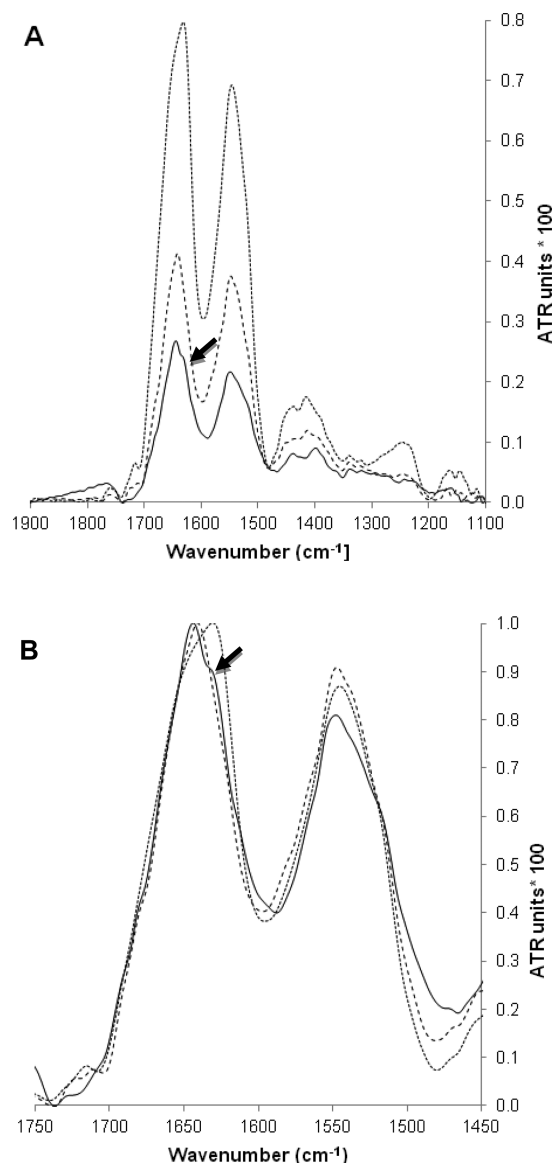


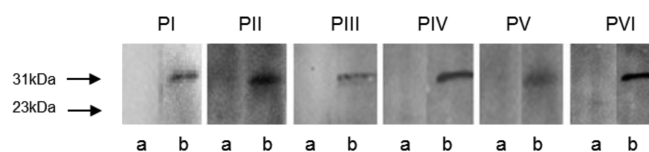
Figure 3. Spectroscopy of Mal d 2 in the absence/presence of vitamin C: (A) measured ATR-FTIR spectra in the wavenumber range from 1100 to 1900 cm^{-1} of Mal d 2 (continuous line), Mal d 2 with 1% vitamin C (dashed line), and Mal d 2 with 5% vitamin C (dotted line) using the corresponding buffer without or with vitamin C as the background, (B) ATR-FTIR spectra in the wavenumber range from 1450 to 1750 cm^{-1} which have been normalized to the amide I peak.

of apples, and serum reactivity to native and reduced Mal d 2. Six patient sera reacted positively with chemically reduced Mal d 2 in Western blotting, but none of the sera reacted with native Mal d 2. These patients were selected for in vitro RBL assay and in vivo experiments using skin prick and oral smear tests (Table 2 and Figure 4).

The RBL assay allowed the characterization of individual and specific IgE response of each selected patient to native and differently treated Mal d 2. Each serum was tested, and the results were presented separately to demonstrate the individual patient response in a component-resolved manner. Sera of all patients showed no to only very weak affinity for native Mal d 2, which is in correspondence with IgE Western blotting (Figures 4 and 5). Stimulation of the RBL cells with heat-denatured as well as vitamin C heat-treated Mal d 2 induced an

Table 2. Clinical History of Six Patients with Positive OAS or SPT for Apple Whose Sera Were Selectively Reactive to Reduced Mal d 2 in IgE Western Blotting

patient no.	OAS	asthma	urticaria	SPT to inhalative allergens	SPT to nutritive allergens
I	apple, hazelnut, plum	+	—	birch	apple, hazelnut
II	apple, banana, carrot, nuts, soybean	+	—	birch, grasses	carrot, hazelnut, peanut, tomato
III	apple carrot, nuts, plum	—	—	birch, grasses, mugwort, rye	almond, apple, carrot, nuts, peach
IV	apple, kiwi, nuts	—	—	birch	apple, fig, nuts, peach
V	apple, celery, kiwi, nuts	—	—	birch, mugwort, ragweed	almond, apple, nuts, soybean, celery
VI	apple, stone fruits, nuts	+	+	ash, birch, plantain	apple, peach, hazelnut, soybean

**Figure 4.** Screening of patient sera by IgE Western blotting. The IgE Western blots of (a) native Mal d 2 and (b) reduced Mal d 2 with sera of six patients (I–VI) show a positive reaction to chemically reduced Mal d 2.

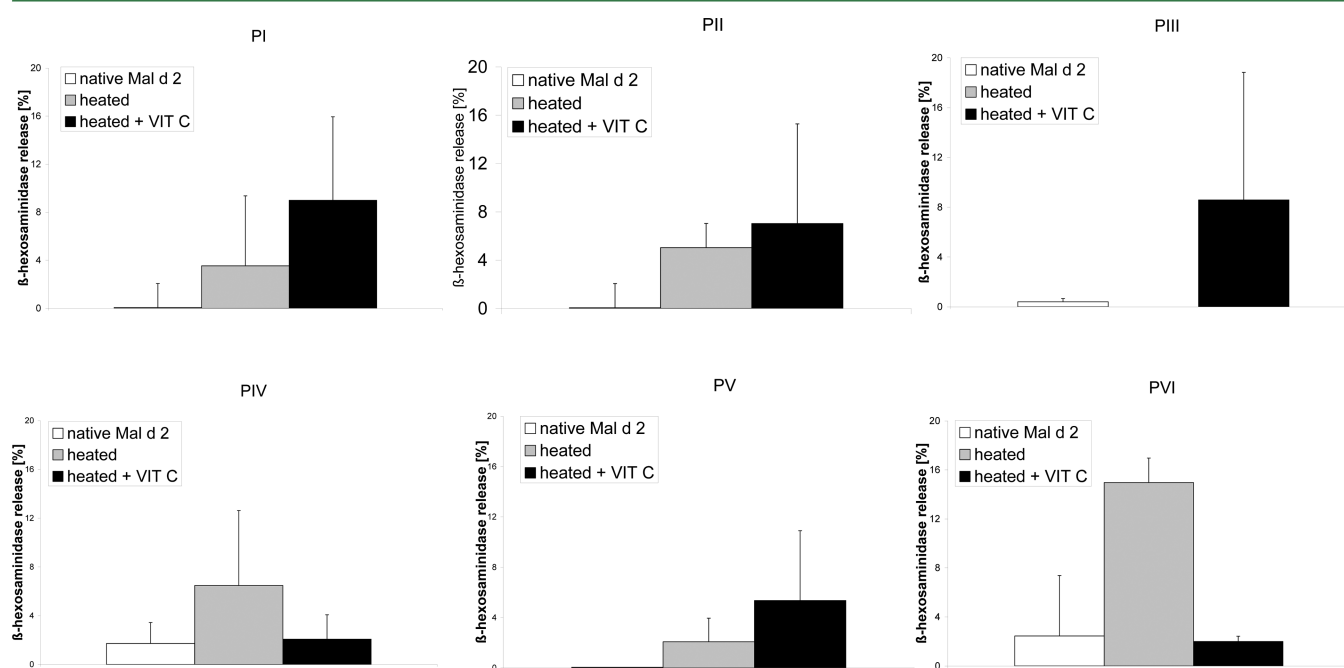
increased β -hexosaminidase release, while native Mal d 2 did not increase the mediator release (Figure 5). Heat-treated Mal d 2 caused a β -hexosaminidase release with patient sera I, II, IV, V, and VI which is higher compared to the response induced by native Mal d 2 (Figure 5). The serum of patient VI showed the highest β -hexosaminidase release by heat-treated Mal d 2 (Figure 5). The results showed that heat treatment and addition of vitamin C could not influence the IgE reactivity to Mal d 2. Due to the high toxicity of sulfur, its addition could not be examined in the RBL assay.

Previously it was shown that conformational changes of Mal d 2 induced by chemical reduction of disulfide bridges are responsible for enhanced binding to IgE molecules.⁸ In the cell-based RBL assay the hexosaminidase release is lightly increased

in the majority of sera by heat-denatured and vitamin C-treated Mal d 2 compared to native Mal d 2, although remaining at low levels, <10% (Figure 5). The low affinity of Mal d 2 for the patients' RBL cell receptor-bound IgE can be explained by the rapid refolding as described previously.⁸

Skin Prick Test and Oral Smear Test. To examine the clinical relevance of native and heat-treated purified Mal d 2, SPTs and OSTs were performed. Patients developed neither a skin nor an oral reaction to native, heat-treated, or vitamin C-treated Mal d 2. The lack of skin reaction in SPTs to heat- and vitamin C-treated Mal d 2 can be attributed to the stability of Mal d 2.⁸ The refolding kinetic study of Mal d 2 via CD showed that the molecule refolds after a few minutes, and the refolding spectrum overlaps almost exactly with the spectrum of the native protein.⁸

High prevalence of IgE reactivity to Mal d 2 strengthened the assumption that it might also be involved in the development of severe reactions due to high structural stability.^{6–9} However, it was reported that five out of six Mal d 2-sensitized patient sera reacted exclusively with carbohydrate epitopes on the upper glycosylated variants of the allergen.⁹ Whereas the impact of sensitization to glycosylated Mal d 2 still remains unclear, only 15% of patient sera reacted with the nonglycosylated recombinant Mal d 2, which supports the crucial role of

**Figure 5.** Results of β -hexosaminidase release in RBL assay from six tested patient sera (PI–PVI). The RBL cells are assayed using Tyrode's buffer as a negative control, native Mal d 2, heated Mal d 2, and heated Mal d 2 supplemented with 1% vitamin C. The patient sera showed variable reactivity if Mal d 2 was heated in the presence of vitamin C. The serum of patient VI displayed 2 times more reactivity to heated Mal d 2 than the other sera.

glycosylation in sensitization and IgE reactivity.⁹ The obtained MS results demonstrate that a minor fraction of Mal d 2 is glycosylated to a low extent (Figure 1B).

For patient sensitization and production of IgE's reactive to Mal d 2, natural exposure to other TLPs must also be taken into consideration since Mal d 2 shares >50% identity with other TLPs. For example, Pru p 2 from peach shows the closest homology of almost 85% with Mal d 2.²⁴ In this context, the presence of common epitopes, offering the molecular basis for cross-reactivity, is of crucial importance.²⁶ Previous observations showed that Mal d 2 reacts with human IgE's mainly after complete denaturation by destruction of disulfide bridges, which fix the intramolecular α -helical domain II.^{8,9} Lack of skin reactivity to native and heat- and vitamin C-treated Mal d 2 confirmed the previous assumption that allergenic epitopes of native Mal d 2 must be cryptic within molecular structures⁸ and the conformationally intact protein is not presenting reactive epitopes for an in vivo reaction.

Detection of Mal d 2 and Mal d 1 in Different Cultivars. To demonstrate the variations in the content of Mal d 2 and Mal d 1 in different apple cultivars, Western blotting and SDS-PAGE using polyclonal antibodies directed to these allergens under reducing conditions were performed. Bands with different intensities indicated the variable and relative allergen contents among different cultivars (Figure 6). The

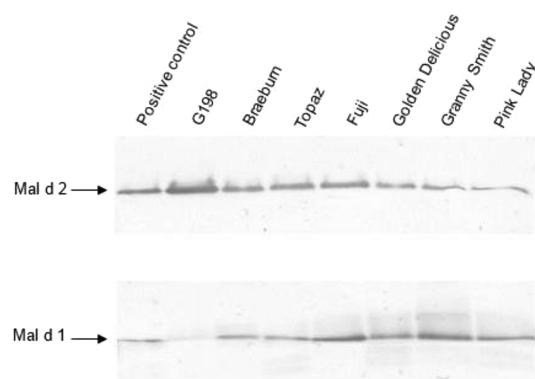


Figure 6. Semiquantitative determination of Mal d 1 and Mal d 2 in different apple cultivars (G198, Braeburn, Topaz, Fuji, Golden Delicious, Granny Smith, Pink Lady) grown under conventional conditions analyzed by reducing SDS-PAGE and Western blot. Mal d 2 and Mal d 1 were detected with polyclonal anti-Mal d 1 and anti-Mal d 2 antibodies. The positive control is 0.5 μ g of native Mal d 2 and Mal d 1.

obtained results revealed a cultivar-dependent variability of the Mal d 2 content, comparable to the previously known variation of Mal d 1, determined quantitatively by a specific enzyme-linked immunosorbent assay (ELISA).¹⁷ The protein bands for both apple allergens Mal d 2 and Mal d 1 showed almost identical intensities in the cultivars Braeburn, Topaz, Fuji, Golden Delicious, Granny Smith, and Pink Lady.

However, the band intensity of Mal d 2 was highest for the breeding line G198, which was shown previously to have a significantly lower content of Mal d 1 (3.47 μ g/g) compared to the known cultivars Braeburn, Fuji, Golden Smith, Granny Smith, and Pink Lady (Figure 6).¹⁷ Pathogen attack was reported to increase the expression of TLPs in apple apoplasts, which means that pathogen resistance is related to and responsible for the TLP content.^{28,29} Additionally, factors such as climate and environmental changes and storage and

cultivation practice influence the expression levels of TLPs in apples.³⁰

Mal d 1, the major allergen of apple, belongs to the PR-10 family and is also expressed in response to fungal or bacterial infection and stress.¹⁷ In a previous study the content of Mal d 1 was shown to be rather high in Braeburn, Fuji, Golden Delicious, Granny Smith, and Pink Lady.¹⁷ The high levels of PR-10's were considered an indication of longevity and high stress or pathogen resistance during storage.²⁷ Experimental down-regulation of PR-10 proteins in *Medicago truncatula* resulted in an antagonistic induction of TLPs and an increased tolerance to pathogens.³¹

Detection of Mal d 2 and Mal d 1 in Different Apple Products. To investigate the availability of the minor apple allergen Mal d 2 and the major apple allergen Mal d 1, Western blots were performed with extracts of different apple products obtained from local markets (Figure 7).

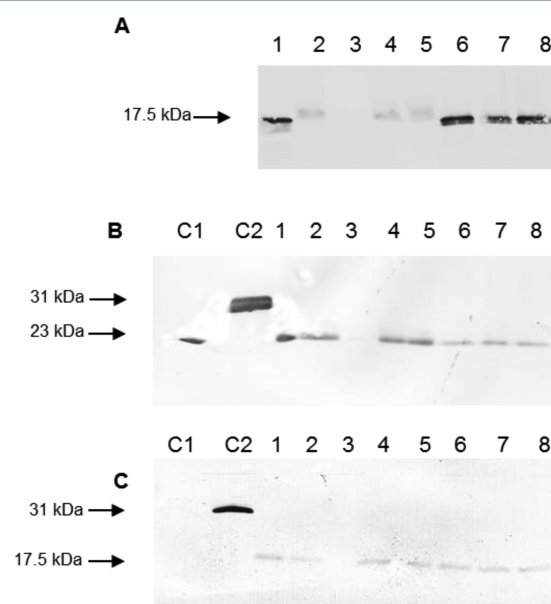


Figure 7. SDS-PAGE and Western blot analyses of different apple products: (A) Western blot using polyclonal rabbit antibody against Mal d 1, (B) Western blot using polyclonal rabbit antibody against Mal d 2, (C) IgE Western blot using the patient serum pool. Key: (C1) native Mal d 2, (C2) chemically reduced Mal d 2, (1) positive control "apple extract Topaz", (2) apple chips nonsulfured, (3) apple rings sulfured, (4) apple rings nonsulfured, (5) apple baby food, (6) clear apple juice, (7) cloudy apple juice, (8) clear organic apple juice.

Analyses of apple-derived foods revealed that Mal d 2 can be detected as the native 23 kDa band in nonsulfured dried apple products (Figure 7B). Mal d 2 was previously detected on two-dimensional electrophoresis (2-DE) gel maps of reduced and not reduced apple extracts.¹⁰ It was shown that chemically reduced Mal d 2 migrated as a protein band at 31 kDa in SDS-PAGE and was reported as IgE reactive.^{5,10} The same altered mobility was also reported for the homologous allergen Pru ap 2 from peach.³² However, the correlation between denaturation of Mal d 2 by reduction of disulfide bridges and its allergenicity is unclear so far. It could be demonstrated that the IgE reactivity of the reduced form of Mal d 2 was recovered after reoxidization of Mal d 2.⁸ In fact, the results indicate that Mal d 2 is mostly available as nonreduced native protein in nonsulfured apple products and is not reactive to patients'

IgE's (Figure 7B,C). The same samples were analyzed using patient sera to characterize the patient reactivity to Mal d 2 in the apple-derived products (Figure 7C). The sera reacted strongly only to the positive control, a reduced Mal d 2 band at 31 kDa, and weakly to Mal d 1 at 17.5 kDa in all apple products except sulfured apple rings.

Within the experiment, the major allergen from apple Mal d 1 was also investigated in parallel due to its significant susceptibility to heat treatment and high processing pressure.^{27,33} Detection of Mal d 1 is an indication that none of the analyzed products were heated or treated by pressure except sulfured apple rings (Figure 7A). The reactive bands to Mal d 1 polyclonal antibodies are diffuse and shifted in apple samples such as dried and nonsulfured products as well as baby food (Figure 7A), which show protein degradation or aggregation.

Sulfurization is used in the manufacturing of dried food as a pretreatment before hot and cold drying processes to increase the shelf life and to act as an antimicrobial agent.³⁴ Due to health risks for consumers, the addition of sulfur to food is currently limited by regulations.³⁴ The investigation of commercially available apple products confirmed the assumption that nonsulfured apple products contain detectable amounts of native Mal d 2. However, the food allergy is mediated through IgE's bound to the surface of mast cells and basophils.^{35,36} If the allergen protein binds to the specific IgE's, it can potentially cause the release of histamine and other mediators responsible for the clinical symptoms. Food pretreatments such as sulfurization and subsequent hot drying in combination with food preservatives such as vitamin C were shown to denature food allergens and lead to a reduction or an enhancement of binding to IgE-reactive epitopes.^{37,38} It has been demonstrated that irreversible heat-denatured allergens can cause increased binding to immunoglobulin by the exposure of different sets of epitopes in a mouse model.^{35,36} Remarkable structural stability and resistance to proteolysis has been linked to potentially increased risks of peptide allergens which survive food treatments and the digestive tract.³⁹ However, a prerequisite for sufficient IgE reactivity remains the presentation of IgE-binding epitopes.³⁵

In conclusion, sulfur is able to degrade or modify Mal d 2 only in combination with heat treatment. Vitamin C, however, is too weak as a reducing agent to cause breakage of the disulfide bridges or protein defolding. The result of the RBL assay confirmed that the individual patient sera can bind to heat-treated purified Mal d 2 to various extents, but the overall affinity for Mal d 2 remained significantly low to nonexistent. The ATR-FTIR results confirmed only minimal changes at α -helices comparable to those observed during thermal unfolding.⁸ However, the molecule remained tightly held by eight disulfide bridges and resisted defolding in the presence of vitamin C.

The results of our studies confirm that (a) only the treatment with hot sulfites is able to destroy Mal d 2 and (b) the lack of patient symptom development to apple Mal d 2 is attributed to the absence of defolded IgE-reactive Mal d 2 in fresh and processed apple products. Additionally, current data substantiate that Mal d 2 is present mainly in its native nonreactive conformation in the apple products and cannot be seen as a major health risk for sensitized consumers.

AUTHOR INFORMATION

Corresponding Author

*Phone: +43-1-47654-6560. Fax: +43-1-47654-6675. E-mail: margit.laimer@boku.ac.at.

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Notes

The authors declare no competing financial interest.

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DEDICATION

This work is dedicated to Manfred Schwanninger.

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