

Mal d 2, the Thaumatin-Like Allergen from Apple, Is Highly Resistant to Gastrointestinal Digestion and Thermal Processing

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Key Words

Allergenicity · Circular dichroism · Food allergen · Protein stability

Abstract

Background: The stability of food allergens to proteolysis and thermal denaturation contributes considerably to their allergenicity. **Methods:** Mal d 2, an allergenic thaumatin-like protein (TLP) from apple, was isolated and purified by anion exchange chromatography. Its IgE reactivity was tested by ELISA and immunoblotting using sera from apple allergic patients. The proteolytic stability of Mal d 2 was investigated using two in vitro digestion models. Effects of thermal treatment at different pH values on the secondary structure of the protein were recorded by circular dichroism. The level and IgE reactivity of Mal d 2 present in industrially prepared foods were assessed. **Results:** Purified Mal d 2 consisted of two isoforms. Both harbored carbohydrate moieties and bound serum IgE from apple allergic individuals. Mal d 2 showed remarkable stability to proteolysis and thermal treatments. The allergen remained intact after 2 h each of gastric and subsequent duodenal digestion retaining its full IgE-binding capacity. Mal d 2 was unfolded at neutral and acidic pH at 70°C. Refolding after cooling was only observed at acidic pH. Mal d 2 detected by an anti-TLP antibody in cloudy apple juice did not bind IgE of a serum pool of apple allergic patients. **Conclusion:** Our findings suggest that Mal

d 2 maintains its structure in the gastrointestinal tract, a feature essential for sensitizing the mucosal immune system and provoking allergic reactions.

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Introduction

Apple trees (*Malus domestica*) are members of the Rosaceae family and their fruits are consumed all over the world either fresh or in processed foods. To date, four apple allergens have been identified: the Bet v 1 homologue Mal d 1 [1], the thaumatin-like protein (TLP) Mal d 2 [2], the nonspecific lipid transfer protein (nsLTP) Mal d 3 [3], and the apple profilin Mal d 4 [2]. While in Northern and Central Europe the occurrence of allergy to apple is frequently related to birch pollinosis and sensitization is due to cross-reactivity between Bet v 1 and Mal d 1, in Southern Europe this fruit allergy is observed together with allergy to peach caused by the allergens Pru p 3 and Mal d 3 [4]. Recent data based on RAST results revealed that patients from Spain and Italy demonstrated higher IgE reactivity to rMal d 2 than those from the Netherlands and Austria [4].

Mal d 2 was the first TLP described as a plant food allergen [5]. Hsieh et al. [5] identified Mal d 2 as an in vitro reactive allergen among 75% (25/34) of apple allergic subjects recruited for the study, and therefore designated it

as a major apple allergen. In 2000, its cDNA was isolated from apple fruit and characterized [6] followed by the tobacco mosaic virus (TMV)-directed expression of recombinant Mal d 2 in *Nicotiana benthamiana* plants [7]. The deduced amino acid sequence, with a calculated molecular weight of 23.2 kDa and a pI of 4.55, included two putative glycosylation sites [7]. Mal d 2 is one of the major protein constituents of mature apple [6] and distributed equally throughout the pulp of the fruit [8].

The family of TLPs, also referred to as pathogenesis-related (PR)-5 proteins, not only plays an important role in the plant's defense against pathogens but is also of high interest for food allergy research [9]. Several members of the TLP family have been identified as major allergens in Cupressaceae pollen such as Jun a 3 [10], Cup a 3 [11], and Cry j 3 [12] as well as in plant foods such as cherry [13], apple [5], kiwi [14], banana [15], orange [16], grape [17] and bell pepper [18, reviewed in 19]. PR-5-like sequences were also discovered in nematodes and insects revealing an ancient multigene family [20]. The majority of TLPs features a molecular mass of about 22 kDa, showing a characteristic pattern of 16 cysteine residues, forming 8 disulfide bonds that are responsible for protein stability and compactness [15, 21–23]. This stabilized structure contributes to the protein's resistance to low pH conditions, heat-induced denaturation, and proteolysis [19]. Numerous studies [4–7] have been performed on the frequency of sensitization to Mal d 2, but only limited information on the biochemical and immunological properties of the purified natural protein is available.

The aim of the present work was to investigate the physicochemical characteristics of Mal d 2 with a focus on its resistance to heating and proteolysis. Circular dichroism (CD) methods were used to gain a better understanding of the structural properties of Mal d 2 during heating that may contribute to its allergenicity and stability. The digestion assays were performed following two different in vitro digestion models [24, 25] to gain insight into the role of protein stability contributing to allergenic potential.

Materials and Methods

Extract Preparation and Protein Purification

Ripe apple fruits (*M. domestica* cv. Golden Delicious) were purchased from a local store (Vienna, Austria). Protein extracts from peeled apple fruits as source material for the isolation of Mal d 2 were prepared according to the method of Björkstén et al. [26]. In brief, proteins were extracted in 20 mM Tris/HCl buffer, pH 8.0, containing 5% (w/v) suspended solid polyvinylpyrrolidone, 2 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM sodium diethyldithiocarbamate trihydrate for 30 min, at 4°C. The

extract was cleared by centrifugation and ammonium sulfate was added to 80% saturation. The precipitate was dissolved in 20 mM Tris/HCl, pH 8.0, and desalted by dialysis. The protein extracts were then cleared from pectin by precipitation with 40 mM CaCl₂ overnight at 4°C. Precipitated pectin was removed by centrifugation at 45,000 g for 60 min at 4°C. After dialysis overnight, the supernatant was precipitated with acetone (1:3, v/v) for 20 min at –20°C and the precipitate was dissolved in 20 mM Tris/HCl, pH 8.0. Mal d 2 was purified by anion exchange chromatography using a Resource Q column (GE Healthcare, Little Chalfont, UK). Bound proteins were eluted by a linear gradient from 0 to 100% of 20 mM Tris/HCl, pH 8.0, 1 M NaCl, over 20 min at a flow rate of 1 ml/min. The purification process was followed by immunoblotting aliquots of 1 ml fractions with rabbit antiserum raised against grape TLP (provided by Elizabeth Waters, Australian Wine Research Institute, Adelaide, Australia). Protein concentrations of the flow through and the eluted fractions were determined by the bicinchoninic acid assay (Pierce, Rockford, Ill., USA) as described by the manufacturer.

Detection of Mal d 2 in Apple Derived Products

Apple derived products (apple sauce, clear apple juice, cloudy apple juice) were purchased from local stores (Vienna, Austria) and the pH of each product was measured. Apple sauce contained 95% apple. Clear apple juice was reconstituted from concentrates whereas cloudy apple juice was directly squeezed from fresh fruits. Apple sauce was diluted with double distilled water at a 1:1 (v/v) ratio and stirred for 20 min at room temperature. After centrifugation, the supernatant was freeze-dried and stored at –20°C. Samples of apple derived liquid products were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent analysis by diluting them in double distilled water at a 1:1 (v/v) ratio after freeze-drying.

Immunoblotting

Samples were subjected to denaturing 15% SDS-PAGE under reducing conditions according to the method described by Laemmli [27] and stained with Coomassie brilliant blue (CBB) or blotted onto nitrocellulose membranes (Pall Life Sciences, Ann Arbor, Mich., USA). For detection of Mal d 2, blots were treated with Tris-buffered saline, 0.5% (v/v) Tween 20 (TBST) and 3% (w/v) nonfat dry milk. Immunoblotting was performed by using rabbit antiserum raised against the grape TLP or a rabbit antiserum raised against Bet v 1 in a 1:1,000 dilution. Bound antibodies were detected by incubating the strips with alkaline phosphatase-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark), diluted 1:1,000, and finally with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

N-linked glycan moieties of separated proteins were examined using a rabbit anti-horseradish peroxidase (HRP) antibody (Sigma-Aldrich, St. Louis, Mo., USA) which binds to N-linked glycan moieties with α -1,3-fucosyl and β -1,2-xylosyl substitutions [28, 29]. The antibody was diluted 1:1,000 in TBST containing 0.5% (w/v) BSA. Bound antibodies were detected as described.

For visualization of IgE-binding proteins, membranes were cut into strips and blocked with 50 mM sodium phosphate buffer, pH 7.5, containing 3% (w/v) nonfat dry milk and 0.5% (v/v) Tween 20. Strips were incubated overnight with patients' sera (diluted 1:7) at 4°C. Bound IgE was detected by incubating the strips with 1:20 diluted ¹²⁵I-labeled anti-human IgE (MALT Allergy System

Isotope Reagent, IBL Hamburg, Germany) overnight at room temperature. Strips were exposed to BioMax MS-1 films (Eastman Kodak, Rochester, N.Y., USA) overnight at -80°C .

Two-Dimensional Electrophoresis

Mal d 2 (4 μg) dissolved in isoelectric focusing solubilization buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG stock solution 3–10, 0.002% bromophenol blue, and 65 mM dithiothreitol (DTT) was loaded by overnight rehydration on immobilized pH gradient strips (Immobiline DryStrip pH 3–10 and Immobiline DryStrip pH 3–5.7, GE Healthcare, Little Chalfont, UK). Isoelectric focusing was conducted for 10 kVh, with a maximum of 7,000 V at 20°C using the EttanTM IPGphor (GE Healthcare). Strips were then equilibrated in 50 mM Tris/HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 65 mM DTT (10 min upon gentle stirring) followed by alkylation of thiol groups upon addition of 25 mg/ml iodoacetamide for 10 min. Two-dimensional SDS-PAGE was performed as described above. The proteins were then either stained with CBB or blotted onto nitrocellulose membranes (Pall Life Sciences). Immunoblot analysis was performed as described above.

In vitro Incubation in Simulated Gastric Fluid

The stability to proteolytic digestion of Mal d 2 was tested as described earlier [24, 30] with some modifications. α -Lactalbumin from cow's milk (Sigma, Poole, UK) was added to the reaction mixture as internal control of the enzymatic activity and to monitor the reaction. In brief, simulated gastric fluid (SGF) was prepared as recommended in the United States Pharmacopeia [31] and consisted of 0.03 M NaCl at pH 1.2. Four hundred micrograms of each α -lactalbumin and Mal d 2 were digested simultaneously in 1,700 μl of SGF containing pepsin (Sigma, activity: 3,300 U/mg) at a ratio of enzyme to substrate of about 13:1 (w/w). Digestion was performed at 37°C with continuous shaking and aliquots were taken at 0, 5 and 30 s and 2, 5, 15, 30, 60 and 120 min and the reaction stopped by adding 9 μl of 1 M NaOH.

In vitro Gastric Digestion (Phase I) and Duodenal Digestion (Phase II)

Mal d 2 and α -lactalbumin from cow's milk (Sigma) were digested as described by Moreno et al. [25]. Briefly, in vitro gastric digestion of Mal d 2 and α -lactalbumin (each 0.57 mg/ml) was performed at 37°C for 120 min at pH 2.5 in the presence of pepsin (Sigma, activity: 3,300 U/mg) at an enzyme:substrate ratio of 1:20 (w/w; 182 U of pepsin/mg of protein). Aliquots were taken at 0, 2, 5, 15, 30, 60 and 120 min. The digestion was stopped by raising the pH to 7.5 with 1 M NaOH.

In vitro duodenal digestion was performed using the 120 min gastric digesta as starting material. The pH of the digesta was adjusted to 6.5 and duodenal digestion was performed following the addition of 7.4 mM of a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and glycodeoxycholic acid (Sigma), 9.2 mM CaCl_2 , and 25 mM Bis-Tris, pH 6.5. Finally, solutions of trypsin (Sigma, activity: 13,800 U/mg) and α -chymotrypsin (Sigma, activity: 40 U/mg) in 0.15 M NaCl, pH 6.5, were prepared and added at approximately physiological ratios of Mal d 2:trypsin:chymotrypsin = 1:400:100 (w/w/w). The digestion was performed at 37°C and aliquots were taken at 0, 2, 5, 15, 30, 60 and 120 min. The digestion was stopped by adding a solution of Bowman-Birk trypsin-chymotrypsin inhibitor (0.25 mg/ml)

from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

CD Spectroscopy

CD spectra of Mal d 2 were measured at a concentration of 0.1 mg/ml in 10 mM sodium phosphate, pH 7.0, or 20 mM potassium phosphate, pH 3.2, in a J-810 spectropolarimeter (Jasco, Easten, Md., USA). Far UV spectra in the range between 190 and 260 nm were recorded using a 0.2 cm path length quartz cell. Thermal denaturation of Mal d 2 was recorded from 20 to 95°C at $1^{\circ}\text{C}/\text{min}$ intervals. The reversibility of the temperature effect was monitored by measuring the restoration of the CD signal after the sample had been cooled to 20°C . Typically, three spectra were measured and subsequently averaged.

Patients' Sera

Serum samples were taken from a group of 46 Austrian patients (Allergy Clinic Reumannplatz, Vienna, Austria) with a positive case history of immediate type reactions to apple fruits. The patients reported oral allergy-like symptoms after the ingestion of fresh apples and were tested positive to apple in skin prick tests. A serum pool from nonallergic healthy donors was used as a control.

IgE ELISA and ELISA Inhibition Experiments

Microtiter plates (MaxiSorb Immuno Plate, Nalge Nunc International, Roskilde, Denmark) were coated with 5 $\mu\text{g}/\text{ml}$ protein (native or treated Mal d 2) diluted in 25 mM sodium carbonate, pH 9.5, overnight at 4°C . After blocking with TBST containing 3% (w/v) nonfat dry milk, 1:7 diluted sera were applied to the coated plates and incubated overnight at 4°C . The plates were then incubated with a 1:1,000 diluted alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD Pharmingen, San Diego, Calif., USA) for 2 h at room temperature. Each incubation step was followed by washing 3 times with TBST. Color development was performed using disodium *p*-nitrophenyl phosphate substrate tablets (Sigma-Aldrich, Steinheim, Germany), and the optical density was measured at 405 nm (550 nm as reference wavelength).

For ELISA inhibition experiments, individual patients' sera were preincubated with proteins at a concentration of 100 $\mu\text{g}/\text{ml}$ for 2 h at room temperature and then added to the protein-coated wells. Bound IgE was detected as described. Buffer and three sera of nonallergic subjects were used as negative controls. Values were counted positive if they exceeded the mean OD value by more than three standard deviation.

Results

Purification and Biochemical Characterization of Mal d 2

Natural Mal d 2 was purified from apple fruit extracts (fig. 1a, lane 1) by a combination of precipitation and chromatographic methods. After anion exchange chromatography, Mal d 2 detected in the flow through was at

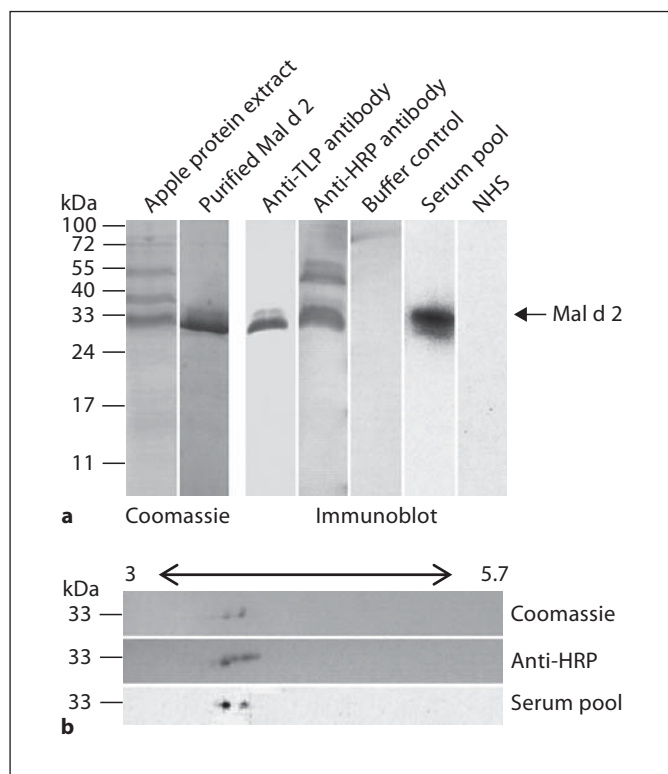


Fig. 1. Mal d 2 separated by SDS-PAGE (**a**) and 2-dimensional electrophoresis (**b**). **a** (1) Coomassie stain of apple fruit protein extract and (2) purified Mal d 2, (3) immunoblot with rabbit anti grape TLP and (4) rabbit anti-HRP antibodies, (5) buffer as a negative control for rabbit antibodies, (6) IgE immunoblot with a serum pool of apple allergic patients, and (7) IgE immunoblot with a serum pool from nonallergic donors (NHS). **b** (1) Coomassie stain of purified Mal d 2, (2) immunoblot with rabbit anti-HRP antibody, and (3) IgE immunoblot with a serum pool of apple allergic patients.

least 95% pure as determined by CBB staining after SDS-PAGE (fig. 1a, lane 2). We obtained a total of 10 mg of purified Mal d 2 from 1.5 kg apples. Purified natural Mal d 2 was separated into a predominant band at about 31 kDa and a minor band at 33 kDa after SDS-PAGE analysis (fig. 1a, lane 2). Both bands showed binding to a rabbit antiserum raised against grape TLP (fig. 1a, lane 3). The presence of N-linked glycan moieties containing α -1,3-fucosyl and β -1,2-xylosyl residues on the upper Mal d 2 band was confirmed by immunoblotting with anti-HRP antibodies (fig. 1a, lane 4). The IgE immunoblotting with a pool of sera from apple allergic patients revealed IgE binding mainly to the upper, glycosylated band of Mal d 2 but not with a pool of nonallergic donors (fig. 1a, lanes

6 and 7, respectively). When analyzed by 2-dimensional PAGE followed by subsequent CBB stain, Mal d 2 was separated into two spots of slightly different molecular weights and isoelectric points of approximately 4.5 and 5.0, respectively (fig. 1b, lane 1). Both isoforms of Mal d 2 showed binding to anti-HRP antibodies and were capable to bind IgE from a pool of four sera from apple allergic patients (fig. 1b, lanes 2 and 3, respectively). In both cases, binding to the more acidic isoform occurred with a higher intensity, although, according to the CBB analysis, its amount was lower compared to the other isoform.

Immunological Characterization of Mal d 2

The capability of isolated Mal d 2 to bind specific IgE was evaluated by immunoblot and direct ELISA using 46 sera from apple allergic patients (data not shown). Mal d 2 was IgE reactive with 39% (18/46) of the sera. Nine of the sera of these 18 patients (50%) contained IgE exclusively reactive to N-glycan epitopes shown by complete inhibition of IgE binding to Mal d 2 after preincubation of sera with HRP, a protein containing N-linked glycans carrying α -1,3-fucosyl and β -1,2-xylosyl residues. Four sera showed inhibition values in the range of 12–45% and therefore contained IgE specific for both carbohydrate and protein epitopes. The remaining five sera (27.7%) showed no inhibition and therefore only possessed IgE reactive against protein epitopes.

In vitro Digestion of Mal d 2

The effect of in vitro digestion on Mal d 2 was initially analyzed by SDS-PAGE under reducing conditions (fig. 2, 3). As an internal control, bovine α -lactalbumin was added to the same reaction vial as Mal d 2 to monitor enzyme activity. Following gastric digestion as proposed by Astwood et al. [24] with a 13:1 w/w ratio of pepsin to test protein, Mal d 2 was stable for the full 120 min with no apparent fragmentation (fig. 2). α -Lactalbumin was rapidly degraded by pepsin accompanied by the appearance of low-molecular-weight fragments of $M_r < 6,100$.

Similarly, Mal d 2 was found to be unaffected by pepsinolysis with a prominent band corresponding to the intact protein evident on SDS-PAGE after 2 h of gastric digestion (fig. 3a), using a protocol that reproduces physiological conditions at a 1:20 ratio w/w of pepsin:protein [25]. Subsequent duodenal digestion did not change the digestion patterns observed for phase I digested Mal d 2 (fig. 3b). α -Lactalbumin, used as positive control, was degraded within 5 min to fragments of the same mobility as those observed following gastric digestion as proposed

Fig. 2. SDS-PAGE analysis showing digestion of Mal d 2 and α -lactalbumin in SGF. Incubation times in seconds and minutes are given above each lane. α -L = α -Lactalbumin; P = pepsin.

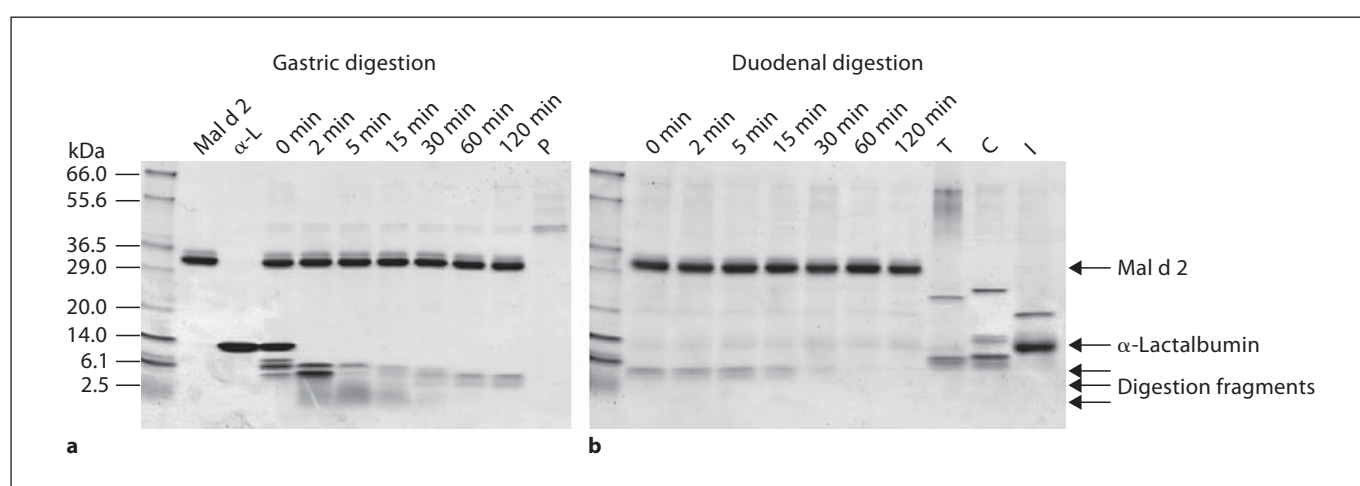
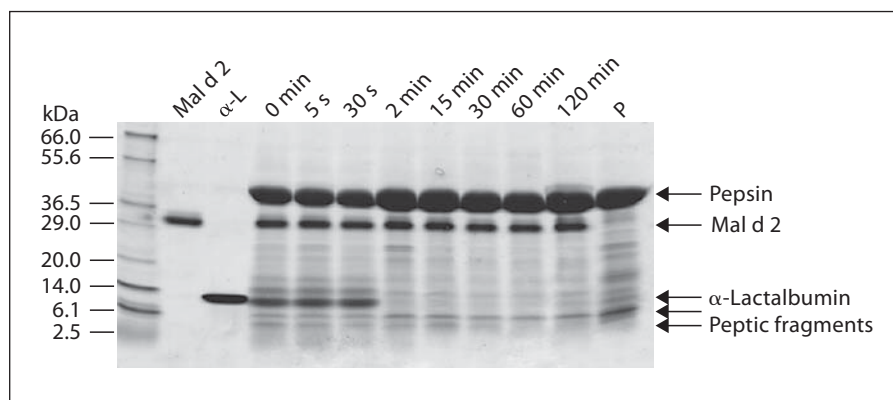
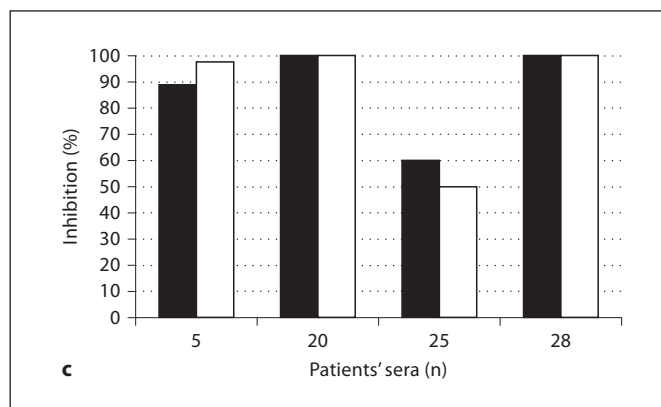


Fig. 3. SDS-PAGE analysis showing gastric digestion (phase 1; α -L = α -lactalbumin; P = pepsin) (**a**) and duodenal digestion (phase 2; T = trypsin; C = chymotrypsin; I = inhibitor) (**b**). Incubation time in minutes is indicated above each lane. **c** ELISA inhibition experiments of IgE binding to Mal d 2 using purified natural Mal d 2 (black) or Mal d 2 after 2 h of digestion (white) as inhibitors.



by Astwood et al. [24]. The fragments remaining after pepsinolysis were rapidly broken down during duodenal digestion. In addition, an ELISA inhibition assay using sera exclusively recognizing protein epitopes on Mal d 2 revealed that its IgE-binding capacity was not impaired

by treatment with gastric enzymes. Phase II digested Mal d 2 inhibited IgE binding to untreated purified Mal d 2 to an extent comparable to values obtained by self-inhibition (fig. 3c).

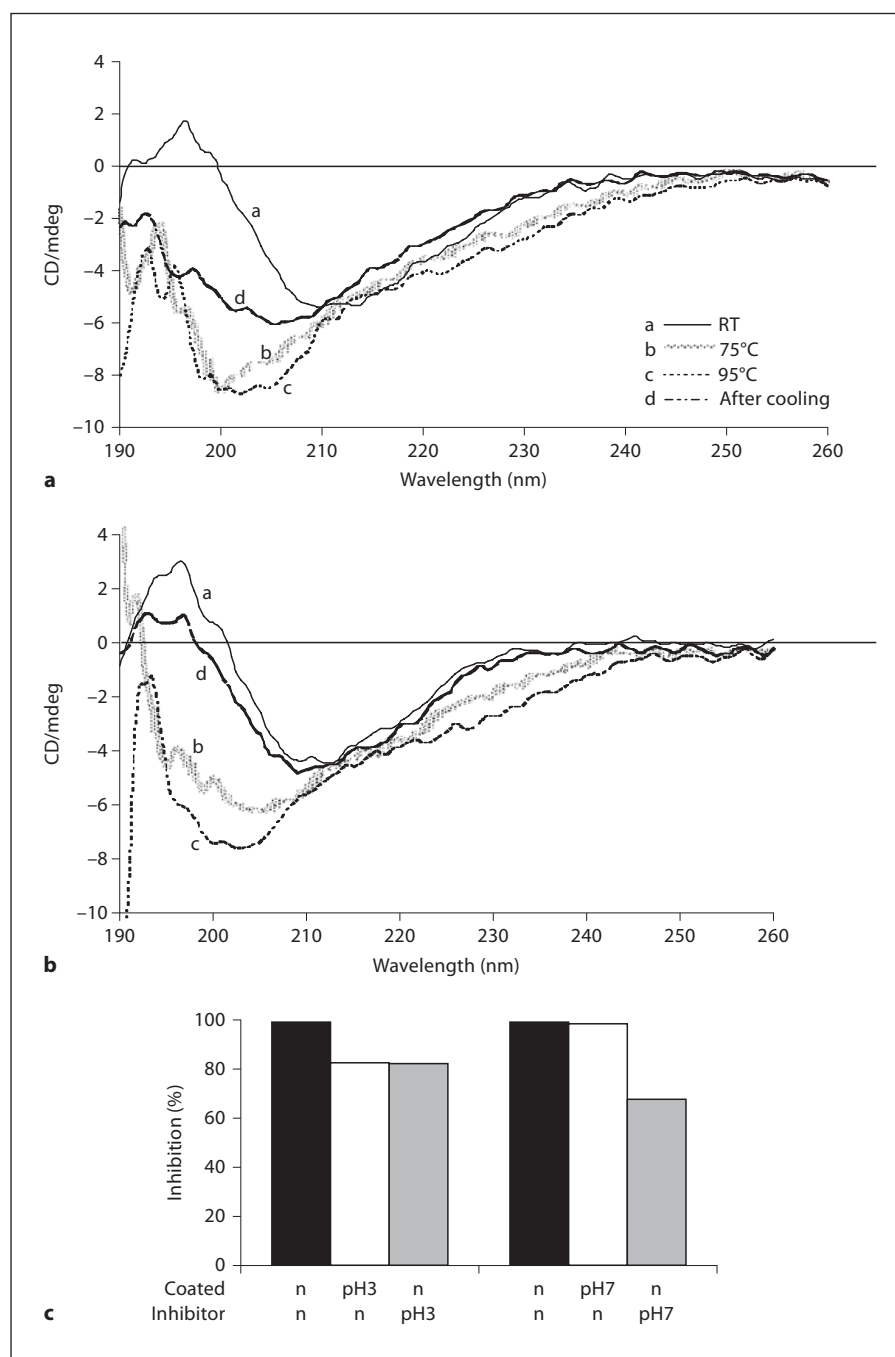


Fig. 4. CD spectra of Mal d 2 during heating from 25°C to 95°C and after cooling to 25°C at pH 7.0 (**a**) and pH 3.2 (**b**). Two rounds of binomial smoothing were applied. **c** ELISA cross-inhibition experiments: inhibition of IgE binding to native Mal d 2 with native Mal d 2 (black), heat-treated Mal d 2 (white), and inhibition of IgE binding to heat-treated Mal d 2 with native Mal d 2 (grey). n = Native Mal d 2; pH3 = Mal d 2 heated at pH 3.2; pH7 = Mal d 2 heated at pH 7.0. RT = Room temperature; mdeg = millidegree.

Stability of Mal d 2 to Heating

The effect of thermal treatment on the secondary structure of Mal d 2 at pH 7.0 and pH 3.2 was recorded using CD spectroscopy (fig. 4). Prior to heating, the CD spectrum of Mal d 2 showed a broad negative peak at 209 nm and a maximum below 200 nm. Although this appears to be quite an unusual curve for a protein consisting

mainly of β -sheet and β -turn structure, CD spectra of other TLPs (e.g. thaumatin [32, 33], zeamatin [34] and Pru av 2 [35]) suggest that this might be a common feature of this protein family [35]. After heating from 20 to 95°C at pH 7.0 the CD signal changed from one characteristic for a folded protein to one presenting a random coil. The transition between the initial conformation of

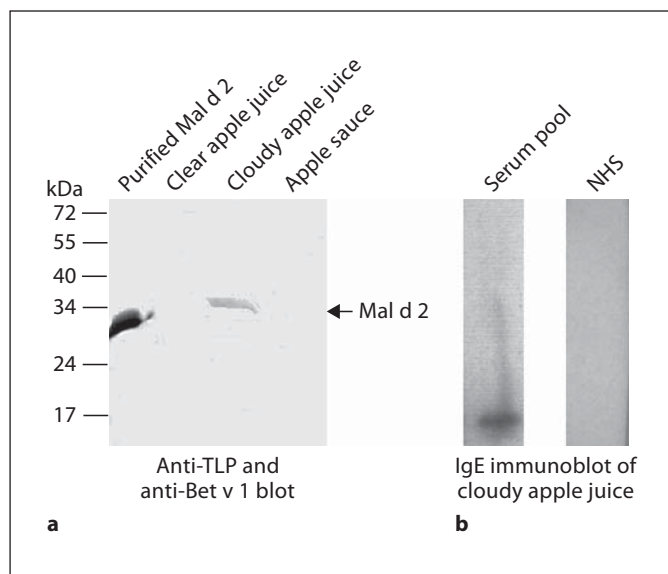


Fig. 5. Immunoblot analysis of heat-processed foods containing apple. **a** Immunoblot with both rabbit anti grape TLP and anti-Bet v 1 antisera of (1) purified natural Mal d 2, (2) clear apple juice, (3) naturally cloudy apple juice and (4) apple sauce. **b** IgE immunoblot of naturally cloudy apple juice with (1) a serum pool of apple allergic patients exclusively recognizing protein epitopes on Mal d 2 or (2) IgE immunoblot with a serum pool from nonallergic donors (NHS).

Mal d 2 and the unfolded state occurred around a melting point of approximately 70°C. The protein was unable to refold after cooling to 20°C (fig. 4a). However, thermal unfolding of Mal d 2 appeared reversible at pH 3.2. Upon cooling to 20°C, the CD signal of native Mal d 2 was fully recovered (fig. 4b). Both native and the heat-treated Mal d 2 samples were IgE reactive but clearly differed in their signal magnitude when tested with a serum pool of 4 patients' sera recognizing protein epitopes on Mal d 2. Interestingly, the serum pool showed lower affinity to native Mal d 2 compared to heat-treated Mal d 2 at pH 3.2 and 7.0 (data not shown). Therefore, ELISA cross-inhibition experiments were performed using either native Mal d 2 as inhibitor for IgE binding to heat-treated Mal d 2 at pH 3.2 and 7.0 or heat-treated Mal d 2 at pH 3.2 and 7.0 as inhibitors for IgE binding to native Mal d 2. Inhibition values obtained for cross-inhibition of native Mal d 2 and heat-treated Mal d 2 at pH 3.2 were exactly the same with 82% but lower compared to values obtained by self-inhibition of native Mal d 2 (98%; fig. 4c). Heat-treated Mal d 2 at pH 7.0 inhibited IgE binding to native Mal d 2 to an extent comparable to values obtained by self-inhibition. In con-

trast, native Mal d 2 could inhibit IgE binding to heat-treated Mal d 2 at pH 7.0 only by 68% (fig. 4c).

Mal d 2 Does Not Retain IgE Reactivity in Processed Foods

Extracts from three heat processed foods derived from apple (clear apple juice, cloudy apple juice, and apple sauce) were analyzed for their Mal d 2 content (fig. 5a). The pH of the products was 3.2 for apple sauce and 3.5 for the juices. The identity of the protein was confirmed with a rabbit antiserum raised against grape TLP. No Mal d 2 was detected in clear apple juice or apple sauce (fig. 5a, lanes 2 and 4). In contrast, cloudy apple juice still contained Mal d 2 (fig. 5a, lane 3). IgE reactivity to Mal d 2 was tested by immunoblotting of thermally processed foods containing Mal d 2 using a pool of sera exclusively recognizing protein epitopes on Mal d 2. IgE immunodetection revealed no IgE binding to Mal d 2 but a prominent IgE-reactive protein of about 17 kDa (fig. 5b, serum pool). The identity of this protein as Mal d 1, a member of the Bet v 1 family, could be excluded as it was not recognized by a polyclonal antibody raised against Bet v 1 (fig. 5a).

Discussion

Although the mechanisms by which food allergens sensitize an individual remain unclear to date, it is obvious that not only a single characteristic is responsible for their allergenicity [36]. Relative abundance, thermal stability, and digestibility of allergenic food proteins are factors considered to have important influences on their role as allergens [9, 37]. Due to their disulfide-bonded protein structure, TLPs are expected to be resistant to pH- or heat-induced denaturation [19]. The presence of disulfide bonds was shown to increase thermostability [38], stability at ambient temperatures [39], and to reduce susceptibility to enzymatic digestion [40]. Therefore, the aim of the present investigation was to gain insight into the physicochemical properties of Mal d 2, the first allergenic TLP discovered [5]. Apart from three studies describing the allergenicity and the isolation and sequencing of the cDNA of Mal d 2 [5–7], data on biochemical and immunological features of native Mal d 2 are limited. This is in part due to difficulties in isolating Mal d 2 from fresh apple fruits.

We used ripe apple fruits to isolate Mal d 2 as it accumulates during the ripening process [6]. We devised a procedure which employed precipitation as well as chro-

matographic methods to clear the extract of undesirable pectins, the levels of which increase during ripening and which make the extract viscous [8]. Mal d 2 was found to exist as two isoforms differing in their molecular weights, isoelectric points, and relative quantities (fig. 1a, lanes 2 and 3, fig. 1b, lane 1). Although present in lower amounts, the 33 kDa isoform of Mal d 2 showed a more pronounced reactivity with IgE of sera from patients allergic to apple (fig. 1a, lane 6, fig. 1b, lane 3). Two putative N-glycosylation sites were reported for Mal d 2 according to the amino acid sequence deduced from its cDNA [7]. Using an anti-HRP antiserum, the less abundant Mal d 2 isoform was found to be recognized with a higher intensity compared to the more abundant one. Therefore, it was argued that the higher extent of IgE binding of this isoform is due to carbohydrate determinants, which is in agreement with its reactivity with anti-HRP antibodies.

The frequency of Mal d 2 recognition among apple allergic patients and the contribution of glycosylation to its IgE reactivity were studied using sera from 46 patients reporting adverse reactions after the ingestion of fresh apples. In contrast to already published data of a prevalence of 72% (18/24) [5], the lower prevalence of 39% (18/46) in our study indicated that Mal d 2 should be classified as a minor allergen. Furthermore, ELISA inhibition experiments using HRP as inhibitor suggested that both protein epitopes and glycan moieties contributed to the IgE binding to Mal d 2.

The resistance of Mal d 2 to proteolysis was assayed comparing two digestion systems [24, 25]. Mal d 2 was tested both in SGF with an enzyme:protein ratio of 13:1 (w/w) [24] and a more physiological system employing an enzyme:protein ratio of 1:20 (w/w) and two stages to mimic the passage of food through the stomach into the duodenum [25, 41]. α -Lactalbumin, known to be very susceptible to proteolysis, was digested with Mal d 2 in the same reaction as internal control.

Mal d 2 was demonstrated being remarkably stable in SGF, irrespective of the pepsin to protein ratio used (fig. 2, 3a). When the *in vitro* digestion was continued with duodenal enzymes, Mal d 2 remained unaffected by protease treatment maintaining the IgE reactivity of untreated Mal d 2 as confirmed by inhibition experiments (fig. 3c). We therefore concluded that the protein epitopes of the allergen were unaffected by the protease treatment. This is of particular importance since it indicates that intact allergen is likely to be present in the gut lumen as it moves down the duodenum and into the jejunum/ileum to the site of the first sampling for food antigens and allergens [36]. Similar data were published recently for the 2S albu-

mins Ses i 1 from sesame seeds [41] and Ber e 1 from Brazil nut [25] as well as for the grape LTP [42]. All three proteins were highly resistant to 2 h of gastric digestion. In contrast to Mal d 2, duodenal digestion of Ber e 1 and grape LTP resulted in a partial breakdown of the proteins. Ses i 1, however, showed only some limited proteolysis. The authors generally assumed that disulfide bonds accounted for the resistance to the gastrointestinal digestion by holding the protein structure together [25, 41, 42]. This could also apply for Mal d 2.

Another factor contributing to the allergenic potential of food proteins is resistance to denaturation by heat treatments. Processed foods like juices, sauces, and jams that are prepared from fruits are frequently subjected to thermal treatments. Thermal stability of Mal d 2 was investigated under neutral and acidic conditions. Heating at acidic pH was conducted to study possible alterations in the conformation of Mal d 2 in usually acidic apple-derived products during thermal treatment. The relatively high melting point (70°C) of Mal d 2 (fig. 4) is a result of its compact 3-dimensional structure, disulfide bridges, and β -sheets [22, 23]. In addition, our results demonstrated that thermal stability of Mal d 2 was dependent on the pH value. Mal d 2 was unable to refold on cooling after heating at pH 7.0, which was not the case at pH 3.2 (fig. 4). An explanation could be thiol-catalyzed disulfide interchange reactions at alkaline pH at ambient temperature. In the process, a free sulfhydryl residue is formed via the β -elimination of a disulfide bond and leads to aggregation of the protein [43]. This effect has also been previously reported for thaumatin [44]. Mal d 2 heat-treated at pH 3.2 and 7.0 was recognized by IgE of a patient serum pool with a higher intensity in ELISA than the untreated protein. Additionally, cross-inhibition experiments identified Mal d 2 heat-treated at pH 7.0 as a stronger inhibitor of IgE binding to native Mal d 2 than the other way round. We assume that this phenomenon is dependent on the limited access of antibodies to binding sites due to the compact folding of native TLP proteins. Unfolding of the protein, however, results in the availability of hidden epitopes which become immunologically accessible only after breakage, decomposition, or denaturation of the antigen [45]. This increases the likelihood of additional allergenic epitopes becoming accessible during food processing. Consequently, we investigated the presence and IgE reactivity of Mal d 2 in industrially prepared foods. Mal d 2 was detected by an anti-TLP antibody in cloudy apple juice but not in apple sauce and clear apple juice (fig. 5a). The processes employed in the production of cloudy apple juice are less ag-

gressive, omitting the steps involving filtration and pasteurization, and the presence of Mal d 2 is therefore more likely than is the case with clear apple juice or apple sauce. However, no IgE binding was detected to the Mal d 2 present in cloudy apple juice with a serum pool of apple-allergic patients (fig. 5b, lane 1). This result was startling as our thermal denaturation experiments at different pH values either led to full recovery of the native structure of Mal d 2 (pH 3.2) upon subsequent cooling or to the availability of cryptotopes (pH 7.0). Thus, despite the detectable presence of the allergen in a food, the food matrix could result in a modification of its allergenic potential [46]. Previously, Herndl et al. [47] described that Mal d 2 migrates as one single band at 23 kDa in an SDS gel, if the fruit was extracted at acidic pH. In this study, IgE reactivity of native Mal d 2 was significantly lower than that of the reduced form. The authors, therefore, speculated that this phenomenon possibly led to allergen inactivation during industrial juice production [47].

In summary, our data indicate that Mal d 2 is a minor allergen comprising epitopes that encompass the Mal d 2 polypeptide and its N-glycan structure. The high stability of Mal d 2 to in vitro gastrointestinal digestion may be important for its role as a food allergen. Findings from this study have shown that Mal d 2 could retain sufficient 3-dimensional structure after in vitro gastrointestinal digestion to still be IgE reactive. The overall stability of Mal d 2 is consistent with its compact structure and allows the allergen to be presented to the immune system of the gastrointestinal tract in an allergenic conformation.

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