

Anita Herndl^{1*}
Gorji Marzban^{1*}
Daniel Kolarich²
Rainer Hahn³
Donato Boscia⁴
Wolfgang Hemmer⁵
Fatemeh Maghuly¹
Elitza Stoyanova¹
Hermann Katinger¹
Margit Laimer¹

¹Plant Biotechnology Unit,
Department of Biotechnology,
BOKU, Vienna, Austria

²Glycobiology-Division,
Department of Chemistry,
BOKU, Vienna, Austria

³Downstream Processing Unit,
Department of Biotechnology,
BOKU, Vienna, Austria

⁴Universita degli Studi di Bari,
Bari, Italy

⁵Floridsdorfer Allergy Center,
Vienna, Austria

Received June 1, 2006

Revised July 24, 2006

Accepted July 27, 2006

Research Article

Mapping of *Malus domestica* allergens by 2-D electrophoresis and IgE-reactivity

The importance of apple allergens has been repeatedly emphasized, and their presence has been confirmed both in pollen and in fruits. In the present study, a combination of proteomic tools have been used to build a complete allergen map of apple. The water-soluble fraction of an apple extract was precipitated using a phenol-based procedure and separated by 2-DE. Initially four previously classified allergens, Mal d 1, Mal d 2, Mal d 3 and Mal d 4, could be identified in Western blots with polyclonal rabbit antibodies directed to the four respective allergens, and subsequently matched to the bands recognized by several patient sera. Further, all four known apple allergens were localized on a 2-DE map and they were matched with spots recognized by sera of patients with different allergic patterns. Moreover, a new, putative allergen could be identified using MS. We evaluated the influence of post-translational modifications and the immunoreactivity under different analytical conditions. The comparison of different visualization methods for 2-DE gels and blots revealed that even very low concentrations of the intact epitopes are detectable by IgEs of patients, and therefore might be sufficient to trigger allergic symptoms in sensitized individuals.

Keywords: Apple proteins, Mal d 1–4 / Apple allergen map / Fruit allergens / Pathogenesis-related proteins
DOI 10.1002/elps.200600342

1 Introduction

Apple is the most widely grown and consumed fruit in the Northern hemisphere. Together with other plant-derived food, apple has been shown to have a positive effect on human health by reducing the risk of chronic diseases such as heart disease, strokes and some forms of cancer [1, 2]. However, the consumption of apples may also impose a serious allergenic risk for sensitized individuals.

So far, four main apple allergens have been reported named according to the rules of the WHO/IUIS Allergen Nomenclature Subcommittee (www.allergen.org). These are (i) Mal d 1 (pathogenesis-related protein; PRP-10), homologous to Bet v 1 with a molecular mass of

17.5 kDa, (ii) Mal d 2 (PRP-5), a thaumatin-like protein (TLP) with a molecular mass of 23 kDa, (iii) Mal d 3 (PRP-14) a lipid transfer protein (LTP) with a molecular mass of 9 kDa and (iv) Mal d 4 (profilin), homologous to Bet v 2 with a molecular mass of 14 kDa.

The importance of apple allergens, in particular Mal d 1, in Northern Europe, and Mal d 3 in Southern Europe, has been repeatedly emphasized [3–5]. Mal d 1, a homologous protein to the major birch pollen allergen Bet v 1, is known as the main cause for pollen-associated apple allergy. Symptoms related to Mal d 1 are generally mild and local, representative for a chemical labile protein [3, 5]. Mal d 3, on the other hand, is a highly stable protein due to eight cysteine residues forming four disulfide bridges [6]. Mal d 3 and its homologues in other fruits and vegetables have been repeatedly mentioned as main elicitors for true food allergy [7]. LTPs resist denaturation and degradation by thermal food processing and digestive proteases, long enough to induce sensitization in the gastrointestinal tract and to provoke systemic symptoms [8].

Correspondence: Univ. Professor Dr. Margit Laimer, Plant Biotechnology Unit, Department of Biotechnology, Muthgasse 18, House B, BOKU, A-1190 Vienna, Austria
E-mail: Margit.laimer@boku.ac.at
Fax: +361-3697615

Abbreviations: CCD, cross-reactive carbohydrate determinant; LTP, lipid transfer protein; PRP, pathogenesis related proteins; TLP, thaumatin-like protein

* Both authors have contributed equally to this work.

Little is known about the way of sensitization to Mal d 2. However, due to the presence of 16 conserved cysteine residues, forming eight disulfide bridges, TLPs are expected to be resistant to pH- or heat-induced denaturation [9]. Mal d 4 is a minor allergen and seems to be pollinosis-related. Bet v 2, the birch pollen profilin, sensitizes approximately 20% of the pollen-allergic patients [10]. Profilins have recently been described as highly cross-reactive with other fruits and vegetables of the *Rosaceae*, *Vitaceae* and *Solanaceae*, as well as with pollen. Although profilins were shown to be strong sensitizers and highly crossreactive, frequently they do not provoke symptoms and are believed to be of limited clinical relevance [11, 12].

Apart from their importance as food allergens, there is growing evidence for the biological functions of these proteins in plant cells. Based on sequence and structural similarities, the major apple allergens can be classified as PRP, suggesting an important role in the plant defence system [13]. Unlike other PRP-10, Mal d 1 appears to be constitutively expressed in apple tissues, but differentially during different stages of development, suggesting an involvement in defined physiological changes [14, 15]. The interaction of low allergenic birch Bet v 1 isoforms with phytosteroids suggests a similar function for the homologous proteins in apples [16]. An antifungal activity of recombinant Mal d 2 has recently been demonstrated [17]. The expression of Mal d 2 has also been shown to be developmentally regulated [18]. Experimental data point to a role of LTPs in plant defence due to antifungal and antibacterial properties [19] and participation in the formation of extracellular lipophilic surface polymers. Profilins, ubiquitous proteins expressed in all eukaryotes regulate the polymerisation of cytoskeletal actin mediating signal transduction in the cells [20].

Although the protein sequence of Mal d 2 shows two potential *N*-glycosylation sites [17], this protein was not found to carry any post-translational modifications. Thus, any involvement of Mal d 2 in carbohydrate-mediated cross-reactivity is rather unlikely (D. Kolarich, personal communication). Glyco-structures containing core α 1,3-linked fucose and β 1,2-linked xylose are typical features of the *N*-glycans from many plant-derived allergens and are generally termed as cross-reactive carbohydrate determinants (CCDs) [21].

Despite the clinical importance of fruit allergens, proteomic studies are rare due to the complexity of the plant-specific tissue matrix and the low protein content [22–24]. For this reason limited data on allergen expression profiles in fruits exist. In the frame of the present study, apple proteins have been chosen as a model for analysis to accomplish two main aims: (i) to separate as many apple

proteins as possible and (ii) to identify the allergenic proteins among them. The combination of 2-DE with IgE immunoblotting appeared to be the appropriate tool for the creation of a complete apple allergen map. Proteins most frequently binding to patient IgEs on 2-D-blots described the sensitisation pattern. Further alterations in IgE-binding affinity to separated proteins under different analytical conditions and changed post-translative modifications could be elaborated.

The results allowed to determine a finalized profile of IgE-reactive proteins in *Malus domestica*, and furthermore showed that the currently discussed groups of apple allergens seem to be far from being complete.

2 Materials and methods

2.1 Protein preparation from apple

Water soluble proteins were extracted from apples of the cultivar 'Vienna' according to a protocol described previously [15]. In order to isolate the proteins, 30% w/v sucrose was added to the apple extract to ensure a phase inversion before mixing it with an equal volume of cold phenol (Tris-buffered, pH 8, Sigma, MO, USA), and vortexing for at least 15 min at 4°C. After centrifugation for 15 min at 5500 rpm (Beckman Coulter, Allegra R21, S4180 rotor) and 4°C, the phenol phase was collected. Proteins solved phenol solution were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at –20°C and then pelleted by centrifugation at 5500 rpm and 4°C for 30 min. The precipitate was washed twice in 0.1 M ammonium acetate in methanol (–20°C) and twice in acetone (–20°C) and subsequently air-dried at –20°C. Finally, the protein precipitate was resolved in 8 M urea and the total protein content was determined by using the BCA Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.2 Protein separation by SDS-PAGE

Samples were diluted in a sample buffer containing 0.125 M Tris-HCl, pH 6.8, 20% v/v glycerol, 2% w/v SDS, 0.005% v/v bromophenol blue in the ratio 1:2 and denatured for 1 h at room temperature. When reducing conditions were required, 0.05% DTT was added to the mixture. Samples were loaded on 4–20% gradient gels (4–20% Tris-glycine gel; Invitrogen, Carlsbad, USA). Electrophoresis was carried out on the Novex System (Invitrogen).

2.3 2-DE and electroblotting

Samples were diluted in rehydration buffer (6 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 2% IPG buffer and traces of bromophenol blue), and 70 µg of protein of the total protein extract or 25 µg of protein of purified Mal d 3 was loaded on 7 cm IPG strips, pH 3–10 (Amersham Biosciences, Piscataway, USA), using in-gel rehydration. Focusing was carried out in a Multiphor II System (Amersham Biosciences) at 20°C for a total of 7.2 kV·h with a voltage maximum at 3500 V. Prior to the second-dimensional separation, strips were equilibrated for 12 min in equilibration solution (50 mM Tris-HCl buffer, pH 6.5 containing 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 2% DTT and subsequently for 8 min in equilibration buffer containing 2.5% iodoacetamide. When nonreducing conditions were required, all steps were performed as described above without DTT and iodoacetamide. Alternatively, proteins were reduced but not alkylated. The strips were then transferred onto 4–20% gradient gels (4–20% Tris-glycine ZOOM gel, Invitrogen) and SDS-PAGE was performed on the Novex System (Invitrogen) at a constant voltage of 125 V. Gels were either stained with CBB or transferred onto suitable membranes. Electroblotting was performed on the XCell II Blot Module (Invitrogen) using a buffer containing 50 mM Na₂B₄O₇·10H₂O, 0.1% w/v SDS, 20% v/v methanol.

2.4 Mal d 3 purification and characterisation

Apples of the cultivar ‘Topaz’ were washed with hot water, surface-sterilized with Danchlor and dried with a paper towel. Apples were peeled with a peeling machine (ca. 0.1 mm), weighed and immediately frozen in liquid nitrogen. The frozen material was mixed with the precooled extraction buffer (ratio 1:4) in a blender for 3 min, and stirred for 2 h in a cool room. To remove apple debris, the extract was filtered through two layers of Calbiochem Miracloth (Merck, Darmstadt, Germany) and centrifuged at 10 000 rpm (Beckman Coulter, Avanti™ J-25, JLA-10500) for 30 min at 4°C. The supernatant was lyophilized prior to separation to reduce the volume and stored at –20°C. The lyophilisate was resolved in 20 mM NaH₂PO₄, pH 7.5, in a ratio of 1:8 and centrifuged at 5500 rpm (Beckman Coulter, Allegra R21, S4180 rotor) for 30 min, at 4°C. The supernatant was filtered using a 0.2 µm filter (Minisart Flow High, Sartorius, Goettingen, Germany), and buffer-exchanged into 20 mM phosphate buffer (pH 7.5) using PD-10 column (Amersham Bioscience). Chromatographic purification was performed on an AEKTA 100 explorer chromatography system (GE Healthcare, Uppsala, Sweden). A Cation-exchange Mono S column (GE Healthcare) was used for purification. Column dimensions were 0.5 cm inner diame-

ter and 5 cm height, resulting in a bed volume of ~1 mL. The column was equilibrated with 20 mM phosphate buffer, pH 7.5 for five column volumes at a flow rate of 0.5 mL/min. Four milliliters of buffer-exchanged apple extract was loaded in a single run at a flow rate of 0.5 mL/min. After removal of unbound proteins and other compounds with five column volumes of equilibration buffer, elution was carried out with a linear gradient from 0 to 1 M NaCl in equilibration buffer over ten column volumes. Fractions were collected and subjected to further analysis.

For protein identification and characterization, the protein was digested after SDS-PAGE separation with trypsin and subjected to LC-ESI-Q-TOF-MS/MS/MS as described by Kolarich *et al.* [25]. Prediction of potential signal sequences was performed using the SignalP 3.0 software available online on <http://www.cbs.dtu.dk/service/SignalP> [26].

For MS on the purified, intact protein, purified Mal d 3 was diluted to a concentration of approximately 10 pmol/µL in 50% ACN containing 0.1% formic acid. This sample solution was subjected to offline ESI-Q-TOF-MS. Spectra were deconvoluted using MaxEnt 1 function of MassLynx 4.0 SP4. All experiments were done on a Q-TOF Ultima Global (Waters Micromass, UK).

2.5 Production of antibodies and IgG Western blotting

Polyclonal antibodies against Mal d 1 were raised in rabbits as described by Marzban *et al.* [15]. Purified Mal d 3 was used for raising polyclonal antibodies in rabbits by immunization with 500 µg native protein and Freund's Adjuvans. Polyclonal antibodies for the detection of Mal d 2 and Mal d 4 were kindly provided by Drs. H. Breiteneder and I. Swoboda. Proteins were blotted on PVDF membranes (Millipore) and blocked overnight with 3% skim milk powder. Membranes were incubated with the primary Ab (see above) at a ratio 1:2000 v/v and the immunoreaction was detected using mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) in a ratio 1:2000 v/v, followed by bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma) in alkaline phosphate buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris).

2.6 Patient sera and IgE Western blotting

Sera of mono- and polysensitized patients were used to identify distinct allergens and to confirm the apple allergen map. For this purpose allergic individuals were clinically characterized by anamnesis and IgE assays, and in

some cases additionally by skin prick tests (SPTs) (Table 1). The allergic reactivity of patient 2 to profilin was characterised by immunoblotting and inhibition assay.

Proteins were blotted onto NC membranes (BioRad, Hercules, USA) and blocked with 3% BSA for 2 h at 37°C. Membranes were first incubated overnight at 4°C with patient sera at a ratio of 1:50 v/v, and subsequently with goat anti-human IgE conjugated with horseradish peroxidase (ICL, Newberg, USA) at a ratio of 1:1000 v/v for 4 h at room temperature. Reacting spots were visualized by ECL (Amersham Biosciences) reaction and chemiluminescence using the Lumi-Imager TM apparatus (Boehringer Mannheim/Roche, Penzberg, Germany). To study the influence of carbohydrate residues on the immunoreaction, glyco-residues were treated prior to detection according to the method of Woodward *et al.* [27].

3 Results

3.1 Localization of the apple allergens on 2-DE maps

In order to identify the allergens previously described in apple, fruit protein extracts were enriched using a phenol-based procedure. This method has allowed the successful performance of 2-DE and Western blotting. After separation under reducing and nonreducing conditions, the 2-D gels were Coomassie-stained to visualize the apple proteins (Fig. 1). Despite being similar, the protein profiles under these two different electrophoretic condi-

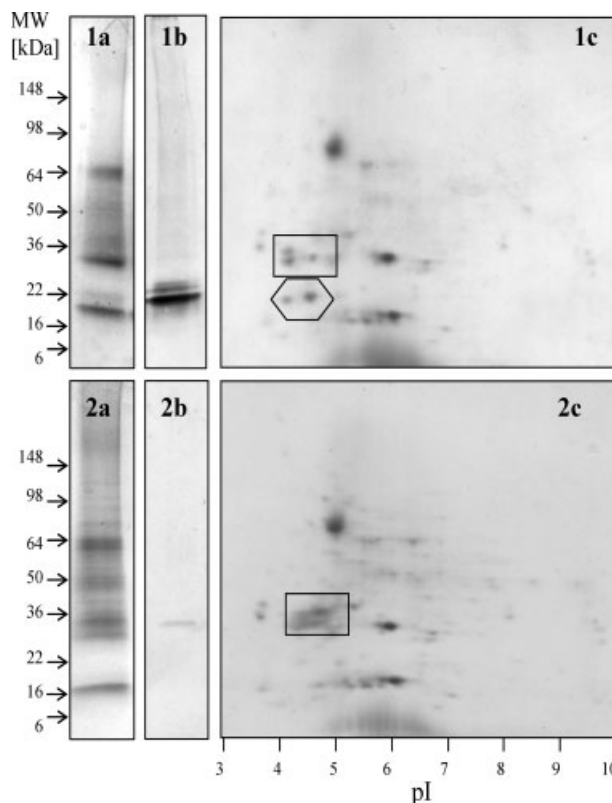


Figure 1. SDS-PAGE and 2-DE pattern of apple extracts under nonreducing (1) and reducing (2) conditions. The SDS-PAGE gel was stained with CBB (a) and blotted onto PVDF membranes and detected by pAbs to Mal d 2 (b). 2-DE gels were stained with CBB (c). The rectangle highlights the reduced form of Mal d 2 and the hexagon the nonreduced form.

Table 1. Clinical data of the allergic patients, whose sera have been used for 2-DE maps of apple allergens

	Anamnesis	IgE reactivity	SPT
P1	Apple, peach, cherry, strawberry, kiwi, peanut, almond, hazelnut, grape, carrot	Birch, apple, peach, cherry, raspberry, blueberry	n.a.
P2	Various pollen	Birch, mugwort, grasses	Birch, ragweed, grasses, mugwort, almond, peanut, hazelnut, banana, potato
P3	Peach, hazelnut, latex	Apple, peach, cherry, strawberry, raspberry	n.a.
P4	Birch pollen	Birch, apple, peach, cherry, strawberry, raspberry, blueberry	n.a.
P5	Apple	Birch, apple, peach, cherry, strawberry, raspberry	n.a.
P6	Apple and citrus fruits	n.a.	Birch, apple, citrus fruits, banana, grape, paprika
P7	Apple, peach, cherry, kiwi, almonds, peanuts, hazelnut	Birch, apple, peach, cherry, strawberry, raspberry, blueberry	n.a.

tions showed some explicit differences. The intensity of an abundant spot at 31 kDa and acidic *pI* decreased drastically under nonreducing conditions, in favor of two spots at 23 kDa missing under reducing conditions (Fig. 1c). This phenomenon was already observed in the SDS-PAGE (Fig. 1a). Both, the 23 and the 31 kDa band, under nonreducing and reducing conditions, respectively, were identified as Mal d 2 by Western blotting, using a

polyclonal antiserum (Fig. 1b). Moreover, the running conditions affected not only the spot position in the gel, but also its antibody binding intensity (Fig. 1b). In contrast to the nonreduced protein, reduced Mal d 2 shows weak IgG-reactivity and cannot be visualized on reduced and alkylated 2-D blots (data not shown). Under nonreducing conditions, however, Mal d 2 can be easily detected by specific antibodies (Fig. 2b).

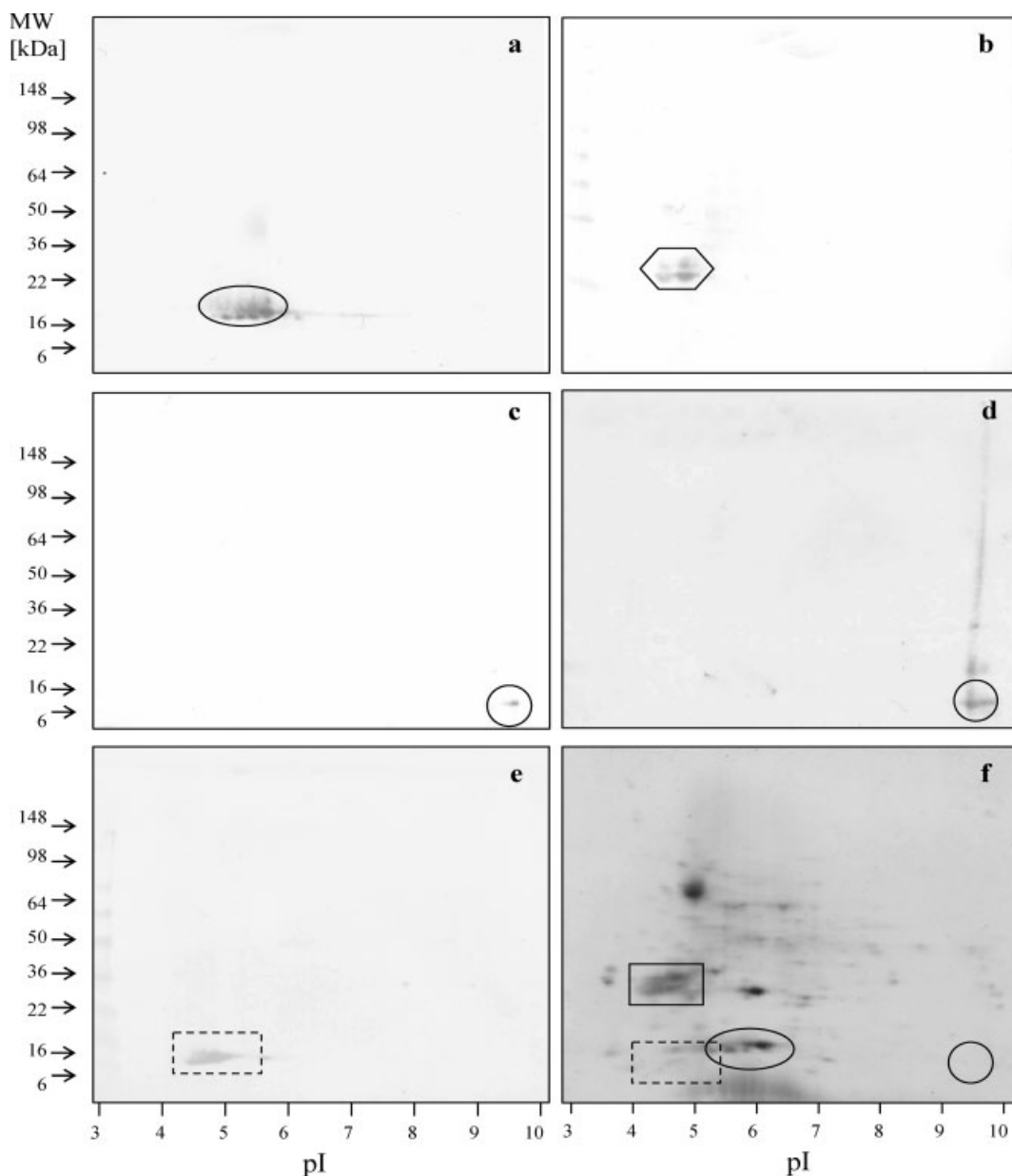


Figure 2. Localization of the major apple allergens using specific pAbs. Apple extract separated by 2-DE was blotted onto PVDF membranes. Samples were reduced and alkylated (a, d, e and f). The allergens are highlighted using an ellipse showing Mal d 1, a hexagon for nonreduced Mal d 2, a circle for Mal d 3 and a dotted rectangle for Mal d 4. The not alkylated, reduced Mal d 3 (c) and purified Mal d 3 as a positive control (d) are demonstrated in the picture above. The Coomassie-stained 2-DE gel of the apple extract is shown in (f).

The position of the known apple allergens on 2-DE maps is shown in Fig. 2. All four allergens were localized with specific antisera (Fig. 2a–d). Based on these findings, the respective spots could be attributed to the spots in the Coomassie-stained gel (Fig. 2f). In the case of Mal d 1, one reactive spot and at least three minor spots are clearly recognizable at a molecular mass of 17.5 kDa and acidic to neutral pH of 5–7. The two 23 kDa spots in the nonreduced sample (Fig. 2b) were assigned to the 31 kDa spot of the reduced sample in the Coomassie-stained gel (Fig. 2f), due to the discrepancies in the electrophoretic mobility of Mal d 2 mentioned before. Mal d 3 cannot be visualized in the Coomassie-stained 2-DE, neither under reducing (Fig. 2f) nor under non-reducing conditions (data not shown). Its position on the 2-DE map could only be determined by Western blotting (Fig. 2c). To confirm these results, purified Mal d 3 was separated by SDS-PAGE as a positive control and characterized by MS analysis. The excised band could be clearly identified as the apple allergen Mal d 3 (accession no. AY374225) (Table 2). The Mal d 3 sequence was subjected to the peptide signal cleavage prediction program SignalP 3.0. A high probability for a peptide signal cleavage was indicated between positions A24 and I25 (data not shown). These *in silico* data were confirmed by the clear identification of the peptide 25–42, which would not result from a tryptic cleavage, thus signal peptide cleavage must occur between A24 and I25. This was additionally confirmed by offline ESI-Q-TOF-MS of the purified and intact protein, whereby the protein mass of the mature protein could be determined with very high accuracy. The spectrum in Fig. 3 shows a major peak of 9076 Da and minor peaks of 9356 and 9638 Da, which

possibly indicate minor amounts of differently cleaved signal peptides (Table 3). Moreover, the obtained results and the very close homology to similar proteins from other sources clearly point towards the presence of four intact disulfide bonds within the protein. Purified native Mal d 3 was used to visualize the position of this protein on the 2-D map and as a positive control as well (Fig. 2). However, Mal d 3 from apple extract reacted weakly and only in nonalkylated state with the pAb's. The purified Mal d 3 could be intensively stained even in fully reduced and alkylated state. Similar to Mal d 3, Mal d 4 was also not visualized by Coomassie staining, and its position could only be assessed by Western blotting using a specific antibody (Fig. 2e).

3.2 Serological analysis of apple allergen 2-DE maps

The characterization of the electrophoretic mobility of the four known apple allergens allowed to assign reactive proteins on the Western blots to corresponding spots on the Coomassie-stained gel. Figure 4 shows the sensitization profiles of seven fruit allergic patients. The allergens are highlighted by: ellipses for Mal d 1, rectangles and dotted rectangles for Mal d 2 and Mal d 4, while circles indicate Mal d 3. Interestingly, the sera of all seven patients recognized Mal d 1, though to a different extent. It is striking that all sera except one, also reacted with Mal d 2. In contrast to the IgG antibodies, the IgEs of patient sera seemed to have a significant reactivity with the reduced Mal d 2. The nonreduced form of Mal d 2 showed no detectable reactivity with patient sera (Fig. 5).

Table 2. Tryptic peptides from Mal d 3 identified by ESI Q-TOF LC-MS/MS/MS

Tryptic fragment	Sequence	MC	CS	Detected (m/z)	Calculated (m/z)
1–24	MACSAVIKALLVVALCMAVS VAHA			Signal peptide	
25–42	ITCGQV TSSLAPCIGYVR	0	3	661.3599	661.3326
43–56 ^{a)}	SGGAVPPACCNGIR	0	2	708.3742	708.3295
64–68	TTADR			Not detected	
57–63 ^{b)}	TINGLAR	0	1	744.4824 ^{b)}	744.4362
77–96	NLAGSISGVN PNNAAGLPKG	0	2	926.0446	925.9895
97–104	CGVNVYPYK	0	2	468.7538	468.7340
97–115	CGVNVYPYKISTSTNCATVK	1	3	700.3795	700.3468
105–115	ISTSTNCATVK	0	2	591.3082	591.2951

Sequence coverage was calculated to be 94.5% (thereof 86.8% by MS/MS). Cysteines were detected as carbamidomethyl-cysteine.

MC, missed cleavage site; CS, charge state of the most intensive detected signal.

a) Partial deamidation of N53 was detected.

b) Signal detected by MS only.

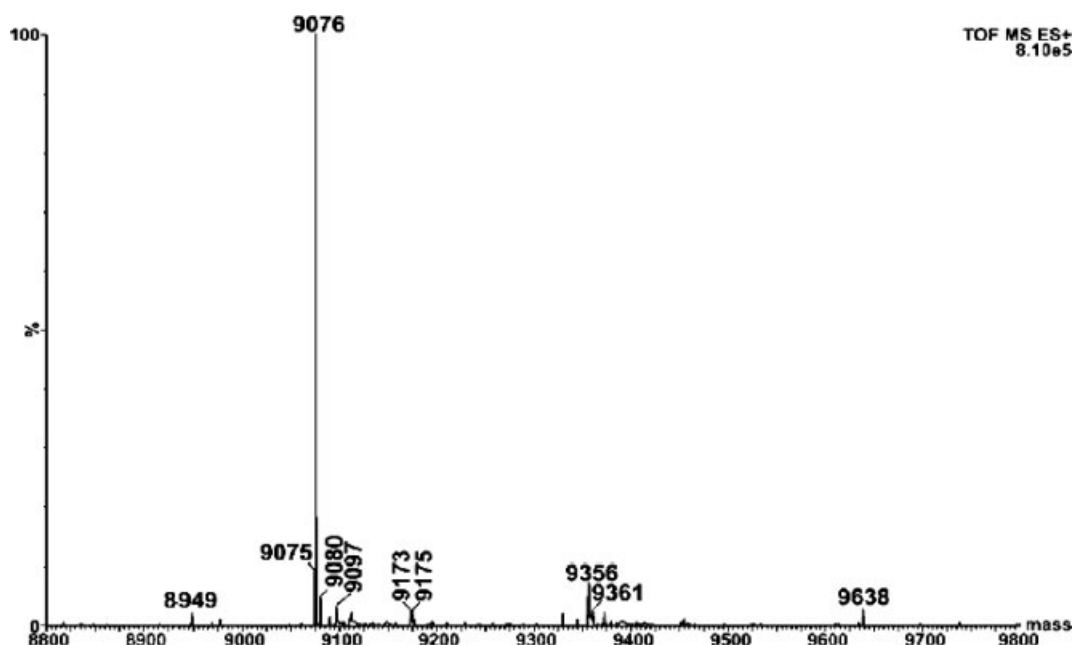


Figure 3. MaxEnt1 deconvoluted average mass spectra of Mal d 3. The determined mass is 8 Da smaller than the calculated mass of the protein, indicating the presence of four disulfide bonds within the protein.

Table 3. Theoretical and detected masses of Mal d 3 (average and monoisotopic)

Protein sequence	19–115	22–115	25–115
Calculated average mass	9641	9356	9076
Detected average mass	9638	9356	9076

As deduced from the mass only, the trace signals detected might indicate the presence of a different signal peptide cleavage.

Reactive spots corresponding to Mal d 3 are present in 2-DE blots probed with sera P2, P4 and P6, whereas only P2 reacted intensively with Mal d 4. Monosensitization was observed only to Mal d 1. Moreover, five out of seven sera (71%) showed reactivity to an unknown basic protein of approximately 45 kDa. Based on the sequence homologies identified by MS/MS sequencing, this unknown protein from *M. domestica* contains several sequence stretches showing high homology to a glyceraldehyde-3-phosphate dehydrogenase from other plants (data not shown).

3.3 Effect of periodate treatment on IgE-reactivity of apple allergens

Sera from four patients were used to evaluate the influence of carbohydrate determinants on the IgE reactivity (Fig. 6). After periodate treatment, a general decrease in

the immunoreactivity of Mal d 2 was detected. The serum P1 showed some intense spots at approximately 90 kDa and one small spot at acidic pH and 18 kDa, which disappeared upon periodate treatment. The single unidentified basic spot at 45 kDa, though, was still present in all four blots.

4 Discussion

Atopic allergy is a genetically determined disorder affecting a considerable proportion of the population, whose main physiological feature is an increased ability to produce IgE antibodies in response to certain allergens by inhalation or ingestion [28]. Although IgE antibodies are produced in extremely low concentrations, they are able to cause a cascade of allergic symptoms (rhinoconjunctivitis, asthma, anaphylactic shock).

Here we demonstrated that a comparative proteomic approach using patient IgEs can be used for the mapping of apple allergens. This approach is particularly useful, since it allows a direct detection of all potential allergens present in the fruit. This has led to the identification of a novel putative allergen in apple.

Generally, proteomic studies of plant tissues face a number of obstacles, which are caused by the nature of plant material, representing a low protein content, accom-

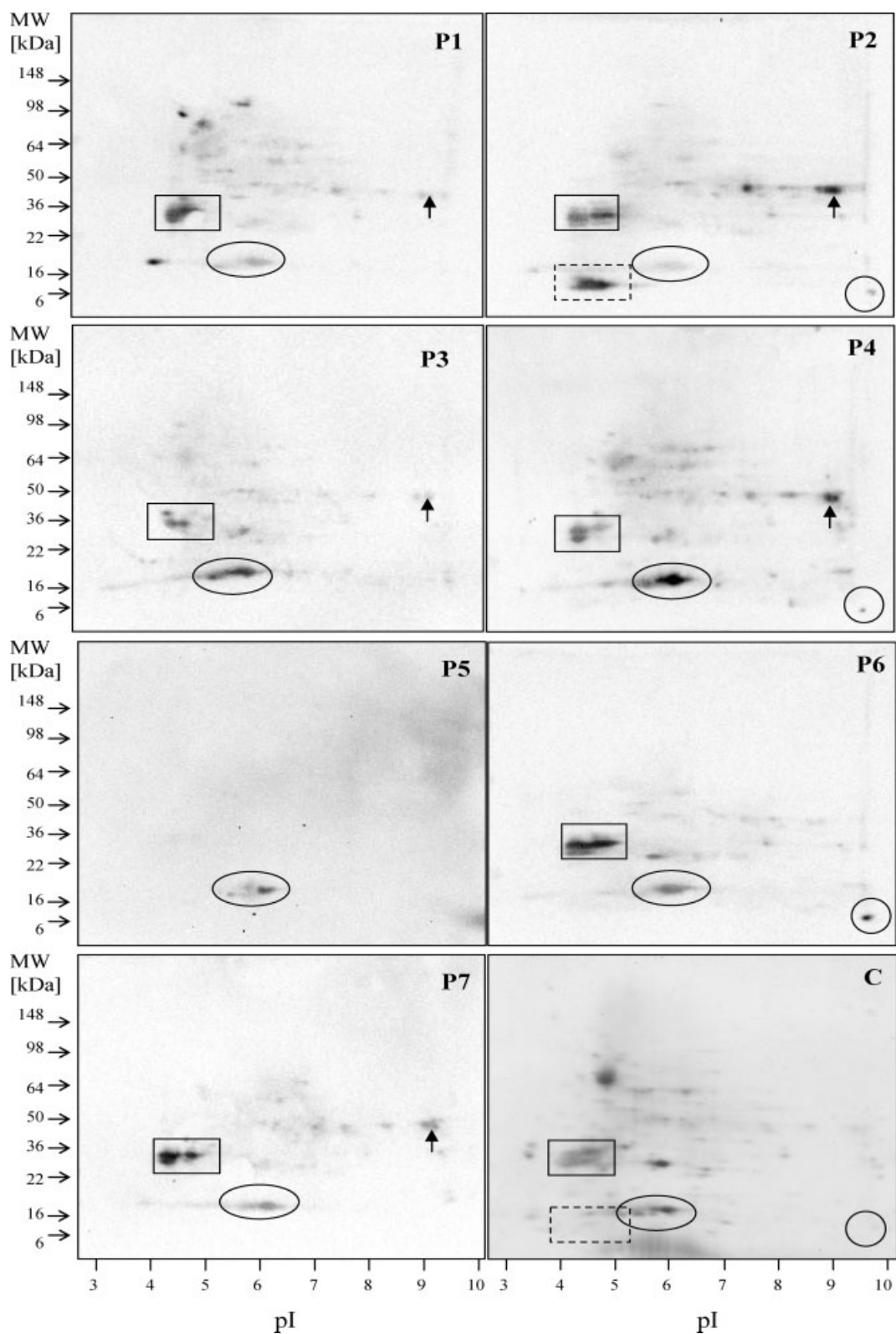


Figure 4. Identification of allergenic proteins under reducing conditions using sera of seven patients (P1–P7). Ellipses show Mal d 1, rectangles Mal d 2, circles Mal d 3 and dotted rectangles Mal d 4. An unidentified IgE reactive spot is highlighted by arrows. The Coomassie-stained 2-DE gel of the apple extract is shown in (C).

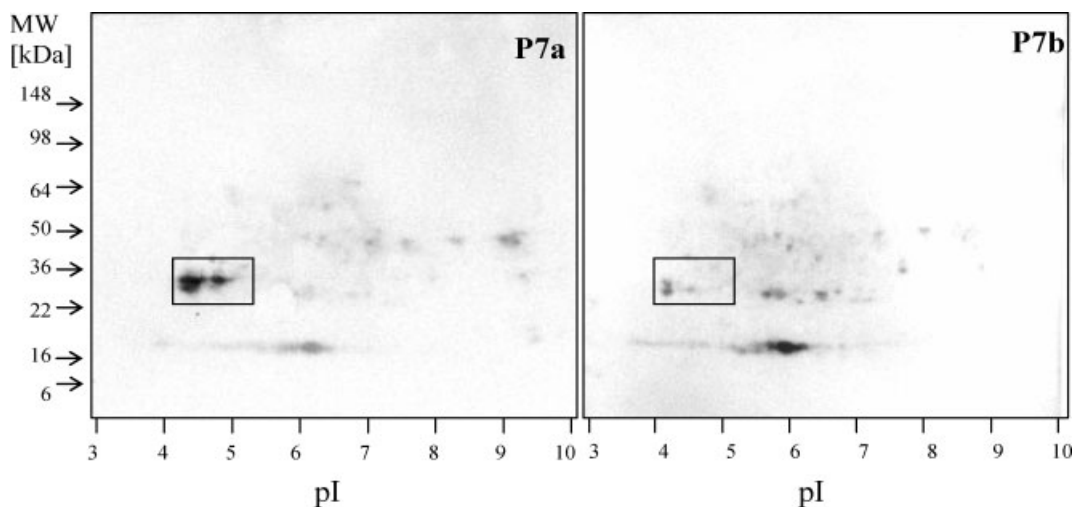


Figure 5. Influence of reducing (a) and nonreducing (b) conditions on Mal d 2 IgE-reactivity using the serum of patient 7. Rectangles highlight the serum-reactivity to reduced Mal d 2. There is no detectable binding to the unreduced form (see Fig. 1).

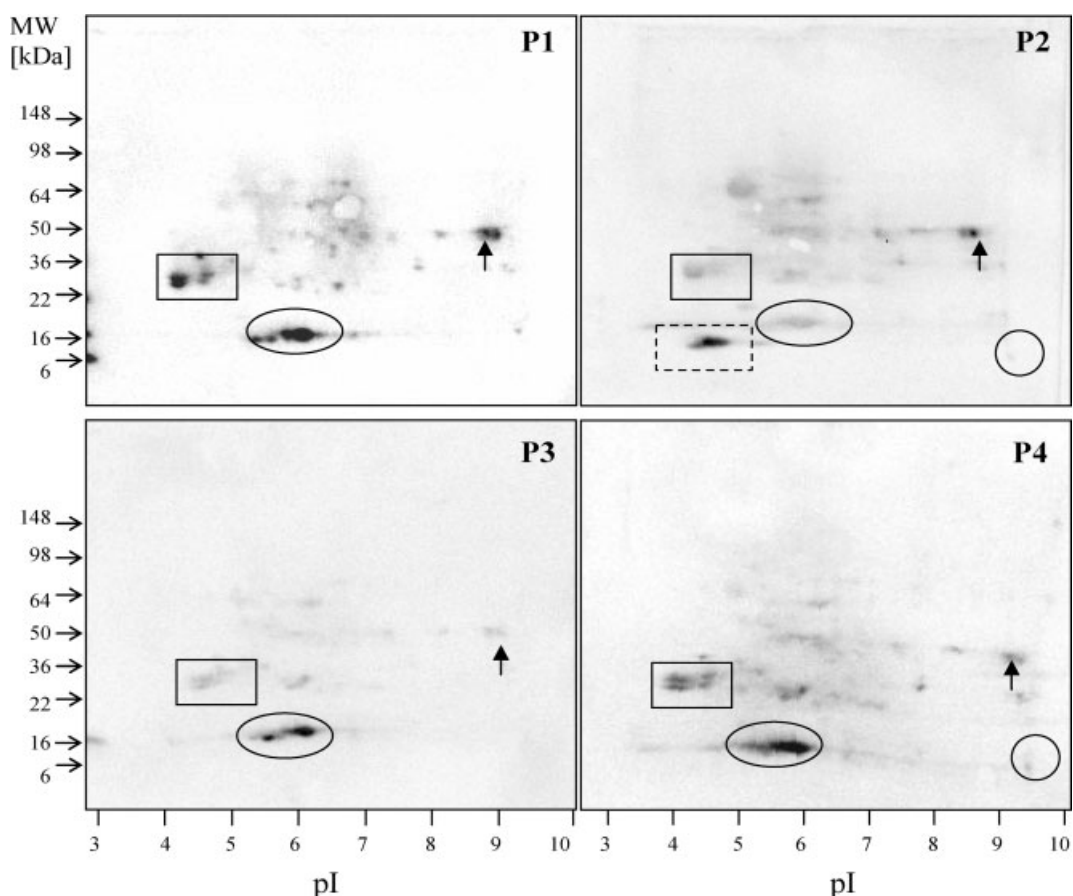


Figure 6. The influence of carbohydrate moieties on the IgE-reactivity was investigated by periodate treatment. After blotting, the membranes were subjected to a mild periodate oxidation and incubated with the sera of the patients 1–4 (P1–P4). Ellipses show Mal d 1, rectangles the Mal d 2, circles Mal d 3 and dotted rectangles show Mal d 4. The reactivity to Mal d 2 was altered after periodate treatment, whereas the other allergens remained unaffected. The unidentified spot, highlighted by arrows, demonstrated no significant alterations.

panied by a plethora of proteases and interfering compounds. These substances comprise polysaccharides, lipids, phenolic compounds and a broad range of secondary metabolites [23]. A phenol-based precipitation method as applied, overcomes these problems, and allows protein enrichment with a simultaneous removal of matrix residues. Phenol extraction has already been described as an efficient method for preparation of plant samples, combining good purification capacity with irreversible inactivation of internal proteases [29, 30].

Immunoblotting using polyclonal anti-Mal d 1 antiserum revealed four distinct spots with a similar molecular weight, which could be partly visualized by Coomassie staining. It is well documented that several isoforms of Mal d 1 are expressed in apple fruits [31, 32], and therefore it was tempting to assume that these spots represent different isoforms. However, it has been shown, both at the RNA and protein level that these isoforms are not equally expressed. Indeed only one isoform (Mal d 1.02) is abundantly present in apple fruit tissue, while the others show minor expression levels [31, 33]. Previously, it has been demonstrated that four putative Pru av 1 isoforms, separated by 2-DE, represent one single isoform [24]. The authors suggested a nonenzymatic deamination of asparagine and glutamine residues as an explanation for this phenomenon. Similar to Pru av 1, it seems more likely that the putative Mal d 1 isoforms, detected by 2-DE and Western blotting, derive from one single isoform, which is predominantly expressed in the fruit tissue.

Mal d 2 showed anomalies in its electrophoretic mobility if treated by reducing agents. Interestingly, the molecular weight of Mal d 2 seems to increase under reducing conditions compared to nonreduced protein. The altered migration of TLP proteins in SDS-PAGE has previously been reported, not only for Mal d 2 [18, 34], but also for the homologous protein in cherry [35]. The predicted M_r of 23 kDa has been confirmed by MS, although the protein migrated as a 31 kDa band in SDS-PAGE [17]. However, the influence of reducing milieu on the conformational changes was generally ignored. A similar behavior was only described for a basic TLP from kiwi, which was attributed to the high number of disulfide bonds [36].

Reduced Mal d 2 was, to some extent, also present under nonreducing conditions, probably due to partial reduction during sample preparation or reducing agents naturally present in apple tissue. Previously, Mal d 2 has been described to deliver one single distinct band of 23 kDa, if the fruit was extracted at an acidic pH [37]. At this low pH-level the activity of reducing compounds available in the fruit tissue, might be reduced, so that only nonreduced protein was detectable in SDS-PAGE.

A significant increase in IgE-binding activity under reducing condition was observed, which might be related to the refolding nature and alteration of electronegatively charged cleft at the protein surface [37]. None of the sera tested, recognised the nonreduced protein at 23 kDa (data not shown). It seems of eminent importance to investigate this phenomenon during food processing. The finding that the IgE-reactivity of native Mal d 2 is significantly lower than that of the reduced form, could be an important consideration for allergen inactivation during industrial juice production.

The attribution of Mal d 1 and Mal d 2 to the respective protein spots on the 2-DE map could be easily achieved by comparing the corresponding spots on Western blots. In the case of Mal d 3 and Mal d 4, however, no corresponding spots are visible on the 2-DE gel stained by Coomassie, although a high amount of protein was loaded. According to our findings, Mal d 3 can only be stained in SDS-PAGE, if present in high amounts, *i.e.* ca. 15 μ g and without rigorous gel destaining (data not shown). This can result from the absence of basic amino acids, since there is a significant correlation between the color intensity with Coomassie staining and the number of lysine, histidine and arginine residues of a protein [38]. Additionally, the poor stainability could be due to a very low Mal d 3 content in the analyzed apple extracts. By Western blotting with pAbs Mal d 3 could be detected, though weakly and only if not alkylated. Using 25 μ g of purified Mal d 3 as a positive control led to a significant improvement of the immunoreactivity, supporting our previous assumption. A reversible refolding for Mal d 3 after mild heating has been described, whereby the disulfide bonds most likely assist in this process [6]. Therefore, we expect that Mal d 3 refolds after reduction if the cysteine residues are not blocked to allow antibody binding. The Mal d 3 positive control could probably be partially alkylated, due to the very high protein concentrations and accessibility of disulphide bridges. However, Mal d 3 was easily recognised by the sera of three patients, suggesting that low amounts of this protein are sufficient for allergen recognition and subsequent allergic symptoms. This is of particular interest, since Mal d 3 is believed to be the major elicitor of severe symptoms after fruit consumption [8]. Analogous to Mal d 3, Mal d 4, could not be detected by Coomassie staining, but easily visualized by Western blotting both with IgG and IgE antibodies. Considerations made for Mal d 3, about protein concentrations and stainability, might also be valid for Mal d 4. In contrast to LTP, however, profilins are believed to be of limited clinical relevance [10].

The inactivation of carbohydrate moieties revealed a lowered reactivity for Mal d 2. Since Mal d 2 is not glycosylated, the involvement of CCDs in this phenomenon is

unlikely. It has been shown that periodate treatment of Art v 1, the major mugwort pollen allergen, resulted in a loss of IgE binding, even from sera clearly identified to be directed against peptide moieties of the protein [39]. Thus, periodate treatment appears to induce more changes on the proteins beyond deglycosylation. There is an ongoing debate on the clinical relevance of anti-CCD IgEs. The general impression is that they are of poor to no biological activity. One reason given is that proteins carrying only one sugar moiety would not be able to elicit mediator release [40, 41]. Nevertheless, anti-CCD IgEs might be responsible for the reactivity to some spots in higher M_r ranges than 35 kDa.

The sensitization profiles of the seven patients tested were characteristic. Although all sera reacted to some extent with Mal d 1, a monosensitization to Mal d 1 could only be demonstrated in one single case. Symptoms related to Mal d 1 are described as mostly mild and local, many patients tend to ignore the impact of this allergen on the dietary behavior. However, a continued consumption of apples might increase the risk of a cosensitization to more stable allergens and the risk of anaphylactic reactions [42]. The sensitization profiles of patients in the present study support this idea. Nevertheless, it needs to be mentioned that apple allergy can also occur without concomitant pollinosis, which is mainly attributed to Mal d 3 [8].

Serological screening of 2-DE maps allowed the identification of a novel allergen candidate in apple. We found this putative allergen at a basic pI of about 9 and an M_r of 40–45 kDa, showing reactivity to 71% of tested patient sera. The reactivity to this rather high-molecular-weight protein was probably not due to CCDs, since it did not alter upon periodate treatment. The corresponding protein was identified by MS analysis as a glyceraldehyde-3-phosphate dehydrogenase, which has already been described as an important allergen in wheat flour [43]. Further analyses to characterize this protein and its clinical relevance are in progress.

The present proteomic approach allowed to unequivocally map the four major apple allergens by IgE Western blots with patient sera recognizing the spots identified by polyclonal antibodies and delivering authentic 2-DE apple allergen maps.

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