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Research Article

Purification and characterisation of relevant natural and recombinant apple allergens

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Apple (*Malus domestica*) is the most widely cultivated fruit crop in Europe and frequently causes allergic reactions with a variable degree of severity. So far, four apple allergens Mal d 1, Mal d 2, Mal d 3 and Mal d 4 have been identified. Mal d 1, a Bet v 1 related allergen, and Mal d 4, apple profilin, are sensitive to proteolytic degradation, whereas Mal d 2, a thaumatin-like protein and Mal d 3, a non-specific lipid transfer protein, are rather stable to proteolytic processes. Mal d 1 and Mal d 4 were purified after expression in *Escherichia coli* expression system, while Mal d 2 and Mal d 3 were purified from apple fruit tissue. All purified proteins were subjected to detailed physicochemical characterisation to confirm their structural integrity and maintained IgE binding capacity. Detailed investigations of carbohydrate moieties of Mal d 2 demonstrated their involvement in the overall IgE binding capacity of this allergen. It was concluded that the folded structure and IgE binding capacity of all four allergens were preserved during purification.

Keywords: Apple / Apple allergens / Food allergens / *Malus domestica* / PR proteins

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1 Introduction

The most frequently observed type I hypersensitivities to fruits include those induced by the consumption of apples (*Malus domestica*), a member of the Rosaceae family. In Northern and Central Europe the occurrence of allergy to apple is related to birch pollinosis, whereas in the South, apple allergy is associated with food allergy to peach [1]. So far, four apple allergens have been identified: Mal d 1 (Bet v 1 homologous protein), Mal d 2 (thaumatin-like protein; TLP), Mal d 3 (nonspecific lipid transfer protein; nsLTP) and Mal d 4 (profilin).

Mal d 1 the major apple allergen belongs to the group 10 of the pathogenesis related (PR) proteins. The primary sensitisation to Mal d 1 predominantly occurs *via* Bet v 1, the major birch pollen allergen, and homologues in pollen of

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Abbreviations: CD, circular dichroism; **FT-IR,** Fourier transform-infrared; **HRP,** horseradish peroxidase; **nsLTP,** nonspecific lipid transfer protein; **OAS,** oral allergy syndrome; **PR,** pathogenesis related; **TLP,** thaumatin-like protein

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related species of the Fagaceae family, resulting in cross-reactivity of IgE to Mal d 1 affecting 70–88% of apple allergic patients from Central Europe [1–3]. Symptoms caused by the major apple allergen Mal d 1 are usually mild and local and together referred as oral allergy syndrome (OAS). Mal d 1 was isolated, characterised and cloned in 1995 [3, 4]. Subsequently, a mixture of Mal d 1 isoforms was identified from apple fruit [5] and the respective numerous gene loci within the apple genome were described [6]. Like other Bet v 1 homologues, Mal d 1 appears to be both thermolabile and unstable to proteases such as pepsin. This property explains the fact that many individuals with allergy to Mal d 1 react to raw but not cooked apples [7].

In contrast, Mal d 2, a TLP belongs to PR-5 group of PR proteins, a group of highly stable proteins with eight intramolecular disulphide bonds [8]. Apple TLP displays endo-β-1,3-glucanase activity, implying a function in plant defence against pathogens [9]. IgE prevalence data range from 75% of apple allergic patients' sera tested in the US [10] to 15% of apple allergic patients in Spain and Italy and 5% in Austria and The Netherlands [1].

Mal d 3, a nsLTP, belonging to family 14 of PR proteins [11] is thought to be involved in the transport of suberin monomers during cutin synthesis [12]. It is extremely stable resisting thermal denaturation [13] and pepsin digestion which suggests that it is able to sensitise *via* the gastrointestinal tract [14]. In Mediterranean countries, apple allergic patients without birch-pollen allergy react to a nsLTPs showing mild to severe symptoms [15]. Apple LTP was first identified as an allergen by Sanchez-Monge *et al*. [16] in fruit peel. A recent study showed that the expression levels of Mal d 3 depended on the cultivar and orchard, and on the physiological maturity of the apple fruit [17]. Two genes encoding mature nsLTP proteins have been identified, designated Mal d 3.01 and Mal d 3.02 [6].

Mal d 4, the fourth allergen from apple is a member of the profilin family of ubiquituous proteins associated with the cytoskeleton. Profilins are recognised by IgE from about 10 to 20% of birch pollen and related food-allergic patients. Consequently, it has been implicated in the birch-Rosaceae fruit and the birch-mugwort-celery-spice syndromes [18–20]. Apple profilin is unstable when subjected to heat treatment or enzymatic digestion and evokes rather mild symptoms related to OAS [21, 22]. Profilin is classified as a minor allergen but based on amino acid sequence similarity, ranging from 70 to 85% [23] among profilins from different species, clinically relevant crossreactivity should not be neglected in allergic patients.

The aim of the present study was to characterise and authenticate recombinant (Mal d 1 and Mal d 4) and natural (Mal d 2 and Mal d 3) apple allergens in detail to improve food allergy diagnosis as part of the development of the EuroPrevall Allergen library project.

2 Materials and methods

2.1 Cloning, expression and purification of recombinant apple allergens Mal d 1 and Mal d 4

Complementary DNA from an individual Mal d 1 isoform, Mal d 1.0108 (EMBL Genbank Database access no. AJ417551) and from apple profilin, Mal d 4.0101, respectively (EMBL Genbank Database access no. AF129428) were obtained as previously described [24, 25]. For heterologous expression these cDNAs were subcloned into the T7-based expression plasmid pMW172 and expressed in *E. coli* BL21 [DE3] [26].

2.1.1 Mal d 1 expression and purification

Expression of Mal d 1.0108 was performed at 25°C in a 10 L fermenter (MoBiTec, Goettingen, Germany) after addition of isopropyl-β-D-thiogalactopyranosidase (IPTG) to a final concentration of 1 mM at an OD_{600} of 2.7. After 19 h the cells were harvested by centrifugation. Recombinant Mal d 1.0108 was purified from the soluble fraction by hydrophobic interaction chromatography, anion exchange chromatography and gel filtration as the final step as described elsewhere [24]. The Mal d 1.0108 fractions were pooled, dialysed against 5 mM Na-phosphate buffer pH 7.4, 1 mM 2-mercaptoethanol, freeze dried and stored at –20°C.

2.1.2 Mal d 4 expression and purification

Expression of rMal d 4.0101 was performed at 37°C as described elsewhere [25]. After addition of IPTG (0.4 mM final concentration) cells were incubated for 4 h and subsequently harvested. Recombinant Mal d 4.0101 was obtained from the soluble cell lysate, and subjected to poly-L-proline affinity chromatography (Sigma, Steinheim, Germany). Mal d 4.0101 positive fractions were checked by SDS-PAGE with Coomassie staining and immunoblotting. Finally, the protein was dialysed against 5 mM Na-phosphate, pH 7.2 lyophilised and stored at –20°C.

2.2 Purification of Mal d 2 and Mal d 3 from apple protein extract

2.2.1 Purification of Mal d 2

Proteins from peeled apple (*M. domestica* cv. Golden Delicious) were extracted in 10 mM Na-phosphate buffer, pH 7.0 containing 3% w/v suspended solid polyvinylpolypyrrolidone (PVPP) (Sigma–Aldrich), for 30 min, at 4°C. The extract was cleared by centrifugation and ammonium sulphate was added up to 80% saturation. The precipitate was dissolved in 20 mM Bis Tris-HCl, pH 6.5 and desalted by dialysis. Afterwards, the sample was precipitated with 40 mM $CaCl_2$ overnight at 4°C and frozen for 24 h at –20°C. Precipitated pectin was separated by centrifugation at 26 000 × *g*, 1 h at 4°C. Proteins were loaded onto a concana-

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valin A Sepharose column (GE Healthcare, Little Chalfont, UK) equilibrated with binding buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl). Flow through fraction enriched with Mal d 2 was dialysed against buffer A (20 mM Tris-HCl, pH 8.5) and loaded onto a Resource Q anion exchange column (1 mL, GE Healthcare). After washing with buffer A, proteins were eluted by a linear gradient from 0 to 50% of buffer B (buffer A, 500 mM NaCl) over 20 min at a flow rate of 1 mL/min.

2.2.2 Purification of Mal d 3

Mal d 3 was purified as previously described, omitting dialysis steps [13]. Briefly, Mal d 3 was extracted from apple peel (cv. Golden Delicious from South Africa) in 40 mM Na-phosphate buffer pH 7.0 containing EDTA (600 mg/L), DIECA (4.56 g/L), sodium azide (0.4 g/L) and PVPP (4%) (Sigma–Aldrich). After adjusting the pH to 5.6 the clarified extract was applied to a cation exchange column (SP Sepharose Fast Flow, 1.6×10 cm^2; GE Healthcare) equilibrated with 20 mM MES, pH 5.6, and the bound protein eluted using a salt gradient (0–0.6 M NaCl). LTP containing fractions were pooled, concentrated by pressure dialysis using a 1000 Da cut-off membrane and subjected to one round of size-exclusion chromatography (Superdex 75, 1.6×60 cm^2; GE Healthcare) in 25 mM Na-phosphate pH 7.0, 150 mM NaCl. Fractions containing Mal d 3 were pooled and concentrated using a 1000 Da cut-off membrane prior to being stored frozen in aliquots.

Protein concentration of rMal d 1.0108, Mal d 2, Mal d 3 and rMal d 4.0101 were determined using BCA Protein Assay Reagent Kit (Pierce, Rockford, Ireland).

2.3 SDS-PAGE, immunoblotting and immunoblotting inhibition assays

Purified Mal d 1, Mal d 2, Mal d 3 and Mal d 4 or total apple protein extract (prepared as described in Section 2.2) were separated by reducing (and nonreducing in the case of Mal d 2) SDS-PAGE and blotted to nitrocellulose membrane. For inhibition assay a serum pool ($n = 5$) with reactivity to Mal d 1 and Mal d 4 was pretreated with 100 µg/mL rMal d 1 and rMal d 4, respectively. Detection of IgE binding proteins by immunoblotting was performed as previously described [25]. For detection of Mal d 2, immunoblotting was performed using rabbit antisera raised against grape TLP (provided by Elizabeth Waters, Australian Wine Research Institute, Adelaide, Australia). Glycosylation of Mal d 2 was determined using rabbit anti-horseradish peroxidase (HRP) antibodies (Sigma–Aldrich).

2.4 Periodate oxidation

Periodate oxidation was performed after proteins were separated by 12% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. The nitrocellulose strips were incubated in 30 mM sodium acetate pH 4.5 containing 10 mM sodium metaperiodate for 20 h at 4°C in the dark. Strips were washed with water and the IgE-, anti-HRP binding as well as TLP binding capacity were determined.

2.5 IgE ELISA

Microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with 1 µg of purified Mal d 2 *per* well. ELISA experiments were performed as described elsewhere [25]. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs. For ELISA inhibition experiments, individual patients' sera which had been preincubated with HRP at final concentrations of 50 µg/mL were added to wells coated with Mal d 2.

2.6 N-terminal sequencing

N-terminal sequencing of purified proteins was performed with an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, USA). Fifty picomole of rMal d 1, Mal d 3 and rMal d 4 respectively, were adsorbed on a Prosorb cartridge and subjected for sequence analysis. Mal d 2 was separated by 12% SDS-PAGE under reducing conditions and blotted to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, USA). The membrane was stained with 0.1% w/v CBB R-250 in 50% v/v methanol, 1% v/v acetic acid. Bands (31 and 33 kDa) were excised and subjected to sequence analysis using the pulsed liquid cleavage program with the manufacturer's chemistry version 1.1.1. Sequence data were compared with the protein databases using the BLAST program.

2.7 Size exclusion chromatography

The homogeneity of the individual protein preparations was determined by HPLC-SEC. The purity of Mal d 1 was determined by using a Superdex 75 prep grade 26/60 column (GE, Healthcare) in 20 mM MOPS, pH 7.4, 500 mM NaCl, whereas Mal d 3 was subjected to Superdex 75 preparative grade (1.6×60 cm^2; GE, Healthcare), equilibrated and eluted in 25 mM Na-phosphate, pH 7.0 150 mM NaCl.

2.8 MS

For determining molecular mass, purified rMal d 1.0108 and rMal d 4.0101, respectively, were dialysed against water and reduced with a 50-fold molar excess of DTT, incubated for 2 h at 37°C and directly subjected to mass analysis. Data were acquired using ESI coupled to a Quadrupole TOF MS (ESI-QTOF MS; Micromass QT of Global Ultima mass spectrometer, Waters, Milford, USA). Protein samples were diluted in aqueous 50% v/v HPLC-grade ACN in 0.1% v/v formic acid at a concentration of approxi-

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mately 100 fmol/μL and directly infused at a rate of 5 μL/min. The multiply charged spectra were deconvoluted with the Micromass MaxEnt1 software. For mass determination of intact Mal d 2, approximately 2 μg protein were reduced, alkylated and digested with the Proteoextract trypsin digestion kit (Calbiochem, San Diego, USA). Proteins were eluted from the RP material (Nanoease Atlantis dC18; Waters) with 50% v/v ACN in 0.1% v/v aqueous formic and directly infused into a Global Ultima Q-TOF instrument (Waters) with ESI at a rate of 1 μL/min. The instrument was calibrated with horse heart myoglobin (Sigma–Aldrich). Spectra of multiple charged ions were recorded for 3 min in a mass/charge range from 400 to 2900, combined and deconvoluted using the MaxEnt1 software.

For sequence analysis, 5 μg of Mal d 2 were reduced, alkylated and digested with the Proteoextract trypsin digestion kit (Calbiochem). Resulting peptides were separated by capillary RP-HPLC (Nanoease Symmetry 300 trap precolumn and Nanoease Atlantis dC18 separating column; Waters) directly coupled to the mass spectrometer. The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an ACN gradient (solvent A 0.1% v/v formic acid/5% v/v ACN, solvent B 0.1%v/v formic acid/95% v/v ACN; 5–45% B in 90 min). For sequence analysis, the instrument was calibrated with the fragment ions of [Glu]-Fibrinopeptide B (Sigma–Aldrich). Data were acquired in the Data Directed Analysis (DDA) mode. Survey and fragment spectra were analysed using the software PLGS version 2.2.5 (Waters) with automatic and manual data verification. A mini database consisting of the trypsin and Mal d 2 sequences, and a combined Swiss-Prot/Trembl database were used for sequence identification.

Mal d 3 was subjected to RP-HPLC with on-line ESI-MS analysis according to a modified method by Moreno *et al.* [27]. Mal d 3 was dissolved in water and applied to a Jupiter 5 mm, C4, 300 Å column (4.6×250 mm^2; Phenomenex, Macclesfield, UK) equipped with a guard cartridge (3×4 mm^2, wide-pore C4, "Security Guard", Phenomenex). Solvents were 0.1% w/v TFA as solvent A and 0.085% w/v TFA/ACN (10:90 v/v) as solvent B. The elution was performed as follows: 0–2 min, 0.9% solvent B, 2–15 min in a linear gradient by increasing the concentration of solvent B from 0.9 to 90%.

Native Mal d 3 was analysed by MALDI-TOF-MS after in-gel trypsin digestion at the joint Institute of Food Research-John Innes Centre (IFR-JIC) proteomics facility as described by Moreno *et al.* [27].

2.9 NMR analysis

Allergen solutions were prepared with final concentrations of 0.24 mM for Mal d 1, 0.26 mM for Mal d 2, 0.08 mM for Mal d 3 and 0.42 mM for Mal d 4 in H_2O/D_2O (9:1). The solutions were transferred to high-quality NMR tubes with Ar as head-space gas and two high Resolution NMR experiments were carried out using a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla), at 25°C.

The two experiments are different in the method to manage the water signal: the zgpr experiment minimises it, while the zgesgp experiment suppresses the water peak. For each experiment 256 scans were programmed for Mal d 1 and Mal d 4, 400 scans for Mal d 2 and 6504 scans for Mal d 3.

2.10 Circular dichroism (CD) spectroscopy

Secondary structures of the purified apple allergens (Mal d 1–4) were determined using CD spectroscopy. The individual allergens were dialysed against 10 mM sodium phosphate, pH 7.0 and analysed at concentrations of 0.1 mg/mL using a J-810 or a J-710 spectropolarimeter (for Mal d 3) (Jasco, Tokyo, Japan) in a 0.2 cm quartz cuvette. The instruments were calibrated with ammonium D-10-camphorsulphonate. Far UV spectra were recorded in the range between 190 and 260 nm at room temperature and collected at 100 nm/min, with a 2 s time constant, 0.5 nm resolution and sensitivity of ±100 mdeg.

2.11 Fourier transform-infrared (FT-IR) spectroscopy

Mal d 3 solution was prepared in distilled water and FT-IR spectra recorded as previously described [27]. FT-IR spectra were recorded using a BioRad FTS 175C spectrometer equipped with a liquid N_2-cooled mercury cadmium telluride detector. Spectra were averages of 256 scans recorded at a resolution of 2 cm^{-1}. Self-deconvolution was carried out using the spectrometer software (WINIR Pro).

2.12 Patients' sera

Apple allergic patients ($n = 50$) from Austria and The Netherlands with convincing case histories (positive to skin prick test and giving positive RAST assays to apple) were identified and sera obtained with written consent. In addition, most of the patients were RAST positive (>3) to birch pollen. All patients reported food allergic symptoms when eating apples, mostly restricted to OAS.

3 Results

3.1 Recombinant apple allergens

3.1.1 Purification and physicochemical characterisation of rMal d 1

Recombinant Mal d 1.0108 (Swiss-Prot Q9SYW3) was expressed as a soluble nonfusion protein in *E. coli*. Protein expression was performed at low temperature (25°C) in order to avoid the formation of inclusion bodies. The final yield was 100 mg purified rMal d 1.0108 *per* 10 L *E. coli* culture. The purity of the recombinant protein was checked by Coomassie-stained SDS-PAGE (Fig. 1A, lane 1) and size

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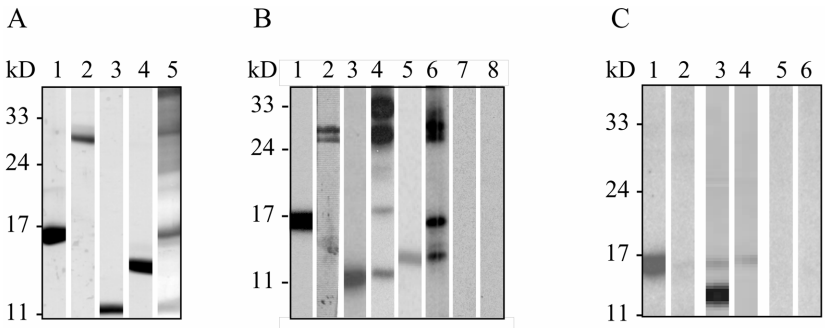

Figure 1. Coomassie stained SDS-PAGE (A) Mal d 1 (lane 1), nMal d 2 (lane 2), nMal d 3 (lane 3), rMal d 4 (lane 4) and apple protein extract (lane 5); immunoblot. (B) rMal d 1 (lane 1), nMal d 2 (lane 2), nMal d 3 (lane 3), rMal d 4 (lane 5), and apple protein extract (lanes 4 and 6) and immunoblot inhibition. (C) Apple protein extract (lanes 1 and 3), inhibition with 100 μg/mL rMal d 1 (lane 2) and 100 μg/mL rMal d 4 (lane 4). For immunoblots individual serum samples and for immunoblot inhibition assay pooled sera ($n = 5$) from apple allergic patients were used. Bound IgE was detected by ^{125}I-labelled anti-human IgE. Normal human serum (panel B, lane 7 and panel C, lane 5) and buffer alone (panel B, lane 8 and panel C, lane 6) were used for controls.

exclusion chromatography (data not shown). Recombinant Mal d 1 migrates as a single band at about 17 kDa in 15% SDS-PAGE. N-terminal sequencing gave the correct first five amino acids GVYTF, showing that the initiating methionine was cleaved off. The mass of the purified protein was determined as 17 492 Da (Fig. 2A), which is in excellent agreement with the theoretical mass of 17 491.6 Da. In addition, a second peak of mass 17 567 was detected. This was possibly due to the addition of 2-mercaptoethanol during purification and was not observed when 10 mM DTT was used for reduction. The ^{1}H 700 MHz NMR spectrum displayed a series of well resolved narrow peaks from 0.2 to 10 ppm (Fig. 3A) which indicated the presence of tertiary structure while CD spectroscopy (Fig. 3B) of recombinant Mal d 1 provided evidence of secondary structure.

3.1.2 Immunological characterisation of rMal d 1

Purified rMal d 1 was detected by specific IgE from apple allergic patients' sera in immunoblots, an example of a single serum is given in Fig. 1B, lane 1. The same serum recognised a band at 17 kDa representing nMal d 1 in apple protein extract (Fig. 1B, lane 4). Since the patient was not monosensitised to Mal d 1, specific IgE binding to Mal d 2 and Mal d 3 was also detected by the same serum (Fig. 1B, lane 4). Preincubation of rMal d 1 (100 μg/mL) with pooled sera from patients positive to Mal d 1 and Mal d 4 ($n = 5$) abolished the binding of IgE to nMal d 1 as determined in an inhibition immunoblot assay (Fig. 1C, lane 2).

3.1.3 Purification and physicochemical characterisation of rMal d 4

Mal d 4.0101 was produced as a nonfusion protein in *E. coli* and purified from the soluble cell lysate fraction. The overall yield was 70 mg purified protein/L bacterial culture. The purified apple profilin migrated as a single band at 13 kDa (Fig. 1A, lane 4) and the determined mass, 13 826 (Fig. 2D), corresponds to the theoretical mass of 13 825.7. N-terminal sequencing gave the correct first five amino acids (SWQAY) demonstrating that the initiating methionine was cleaved off. CD spectroscopy indicated the presence of secondary structure (Fig. 3H). The NMR spectra were characterised by an uninterrupted series of well resolved narrow peaks from −0.2 to 10.2 ppm (Fig. 3G), indicating the presence of a tertiary structure.

3.1.4 Immunological characterisation of rMal d 4

The IgE binding ability of rMal d 4 in immunoblots (Fig. 1B, lane 5) was similar to that of nMal d 4 as shown in Fig. 1B, lane 6. Since the patient was not monosensitised to apple profilin, specific IgE binding to Mal d 1 and Mal d 2 was also observed (Fig. 1B, lane 6). Preincubation of a serum pool of allergic patients ($n = 5$) with rMal d 4.0101 abolished further IgE binding to nMal d 4 (Fig 1C, lane 4).

3.2 Natural apple allergens

3.2.1 Purification and physicochemical analysis of Mal d 2

Mal d 2 was purified from peeled apple fruits with a yield of 18 mg of purified protein from 3 kg of apple. Mal d 2 migrated in SDS-PAGE as a prominent band at 31 kDa and a faint band at 33 kDa (Fig. 1A, lane 2 and Fig. 4B, lane 1). Both bands are recognised by a rabbit antiserum raised against grape TLP (Fig. 4B, lane 3). The nonreduced Mal d 2 sample showed a shift in its migration on SDS-PAGE with a strong band of about 20 kDa and a faint band of about 22 kDa (Fig. 4C, lane 1). Both proteins gave identical N-terminal sequences AKITF, which matched the N-terminal sequence of Mal d 2 as entered in Swiss-Prot (access no. Q9FSG7). NMR analysis (Fig. 3C) and CD spectroscopy (Fig. 3D) showed the presence of tertiary and secondary structures. Several well separated peaks can be noted in the NMR spectra of Mal d 2, where the dispersions of aromatic

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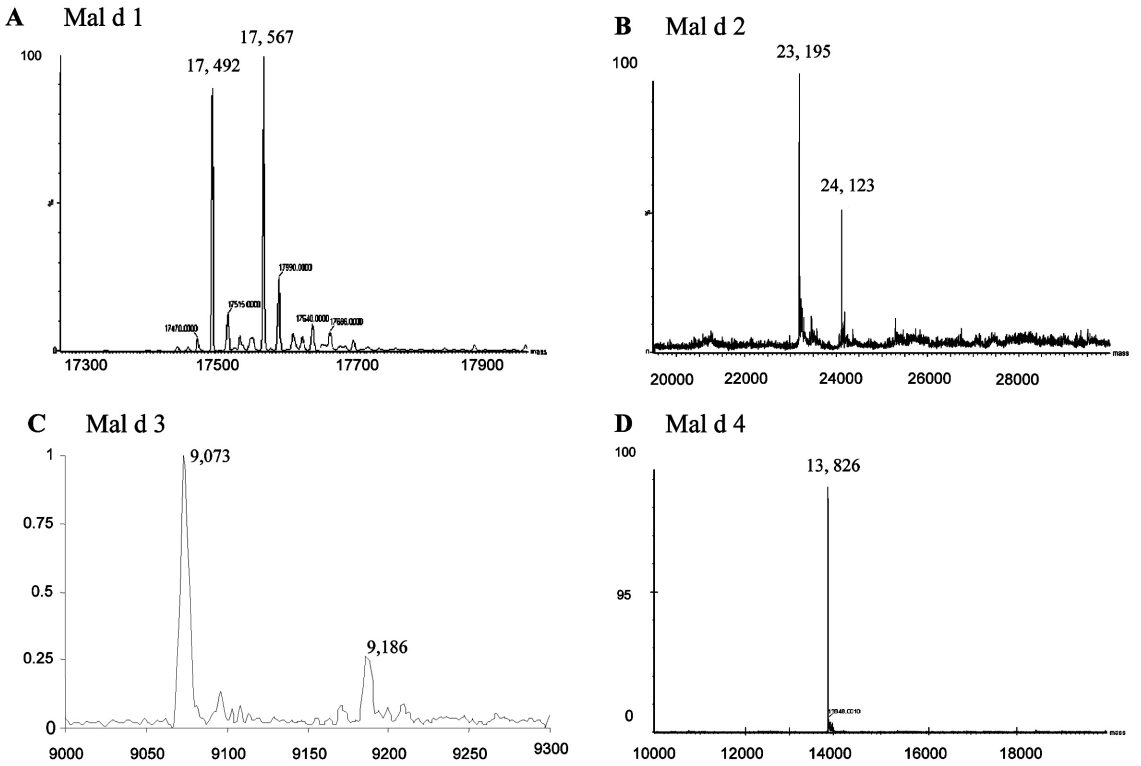

Figure 2. MS analyses of purified apple allergens Mal d 1–4.

and amide protons above 9 ppm and below 7 ppm, of alpha protons above 5 ppm and of methyl peaks below 0.5–0 ppm or more, are reliable signs of the presence of tertiary structure. Glycosylation peaks can be noted in the 3–4 ppm, region. To determine the molecular mass of purified intact Mal d 2, the sample was reduced, alkylated and desalted prior to ESI-MS analysis. The raw spectra of multiple charged ions indicated that two different protein species were present in the sample. This was confirmed by deconvolution to uncharged signals. A strong peak with a mass of 23 195 Da was observed, together with a less prominent peak (approx. 45% intensity of the major peak) with a mass of 24 123 Da (Fig. 2B). The theoretical mass of mature Mal d 2 was calculated as 24 123.7 Da after alkylation, based on the published sequence of Krebitz *et al*. [28]. This value is in excellent agreement with the experimental mass of the minor peak. The mass of the major peak at 23 195 corresponds to unreduced Mal d 2 which has a theoretical mass of 23 194.9 Da with all disulphide bridges being intact. In order to further characterise the preparation of Mal d 2, the alkylated sample was digested with trypsin and the resulting peptides were analysed by LC-MS-MS. Sixty percent of the Mal d 2 sequences are confirmed, leaving a gap in the central portion of the protein from amino acid T55 to K138. Both experiments indicated that the preparation of Mal d 2 was free of detectable amounts of contaminating proteins, and that the sequence of Mal d 2 was correct.

3.2.2 Immunological analysis of Mal d 2

IgE reactivity to Mal d 2 as a purified protein or when present in apple protein extract was determined by IgE immunoblot (Fig. 1B, lanes 2 and 4). Individual sera from 20 apple allergic patients were tested by IgE ELISA. Twelve sera (60%) showed IgE reactivity to Mal d 2, but the measured optical densities, with the exception of serum number 1, were generally low (Fig. 4A). IgE immunoblots with six representative serum samples (sera number 1, 3, 11, 17, 18 and 20) containing IgE against Mal d 2 are shown in Figs. 4B and C. IgE immunoblotting under reducing conditions showed that five out of six tested patients' sera recognised the upper band of Mal d 2, while both Mal d 2 bands were recognised by the IgE of only one serum. IgE immunoblotting under nonreducing conditions using the same set of sera revealed generally weak binding, and only three of six sera reacted with Mal d 2 (Fig. 4C, lanes 5, 7, 9). To test for glycosylation of Mal d 2, immunoblotting with anti-HRP antibodies specific for α-1,3-fucosyl and β-1,2-xylosyl residues was performed. As shown in Figs. 4B and C, lane 2, the upper Mal d 2 band was specifically recognised by anti-HRP antibodies. In order to determine the contribution of glycans to its IgE and anti-HRP binding capability, blotted Mal d 2 was deglycosylated by mild metaperiodate oxidation. Periodate oxidation destroyed *N*-glycan-dependent epitopes, as no binding of IgE and anti-HRP antibodies specific for *N*-glycans was observed in an immunoblot (data

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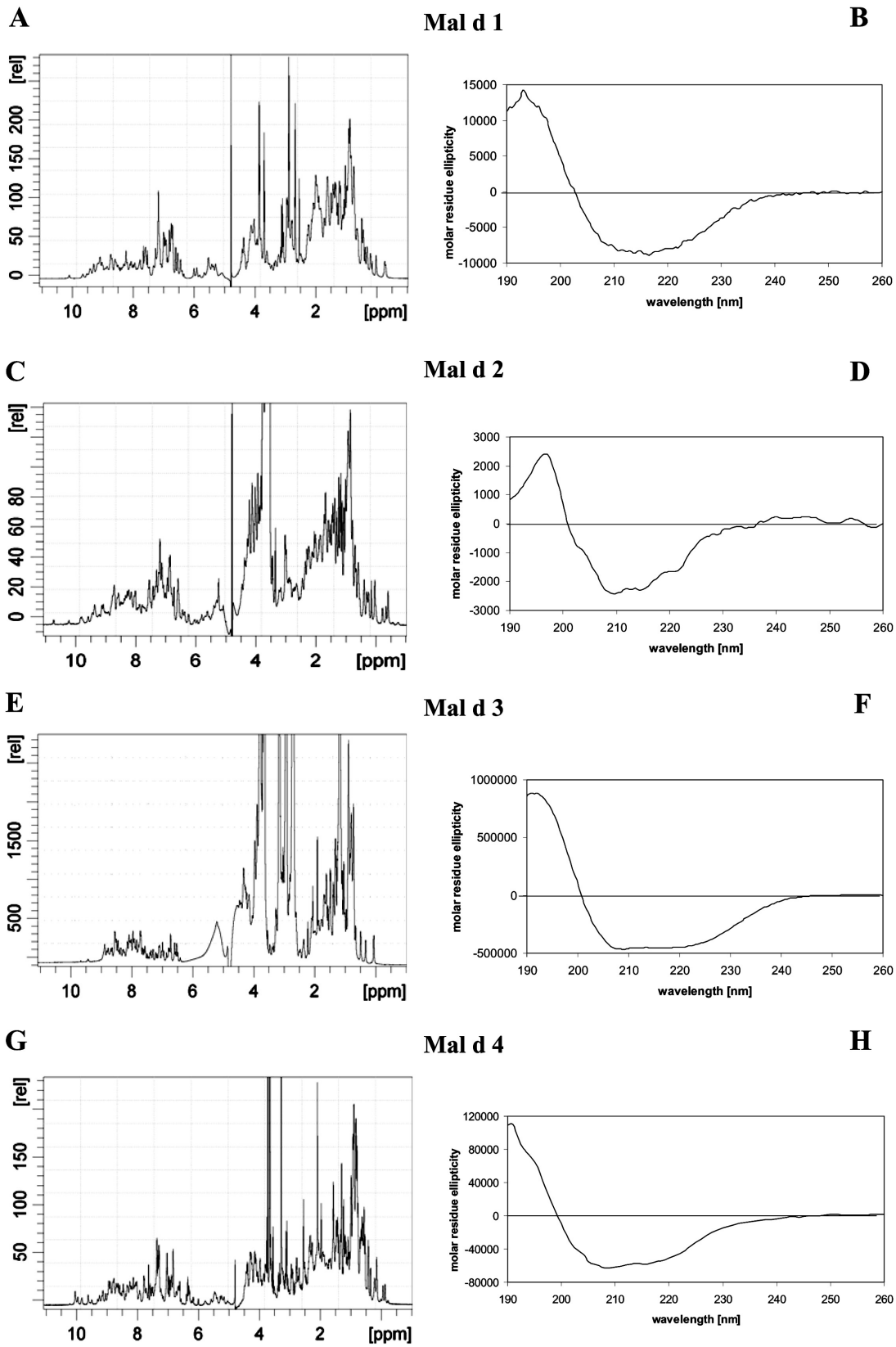

Figure 3. NMR spectra (panels A, C, E and G) and CD spectra (panels B, D, F and H) of purified apple allergens, Mal d 1 – 4.

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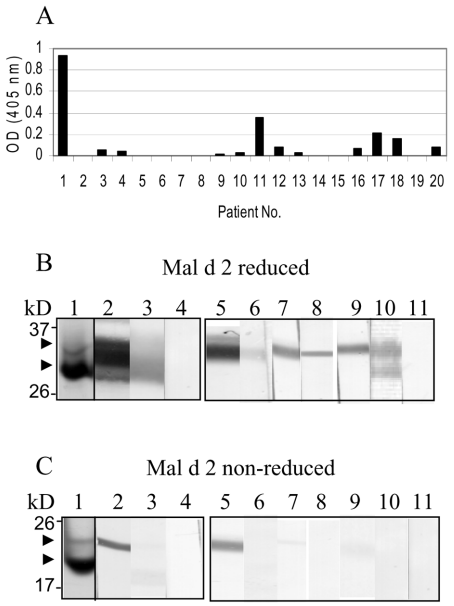

Figure 4. IgE reactivity of Mal d 2 tested by ELISA (A); SDS-PAGE and immunoblot analysis of Mal d 2 under reducing (B) and nonreducing conditions (C); Coomassie stain (lane 1), immunoblot performed with anti-HRP (lane 2) and anti-TLP antibodies (3), negative control with secondary antibody (lane 4), IgE immunoblot analysis using sera no.: 1 (lane 5), 3 (lane 6), 11 (lane 7), 17 (lane 8), 18 (lane 9) and 20 (lane 10); control with a normal human serum (lane 11).

not shown). The significance of the carbohydrate moiety for IgE binding of Mal d 2 was further determined by an ELISA inhibition assay with HRP using the same sera as for IgE immunoblotting. This showed that sera number 1, 3, 11, 17 and 18 only displayed IgE reactive against glycan epitopes on Mal d 2 (data not shown). No inhibition of IgE binding was observed when serum number 20 (Fig. 4B, lane 10) was inhibited with HRP indicating IgE binding to accessible protein epitopes present on both variants of Mal d 2 (data not shown).

3.2.3 Purification and biochemical analysis of Mal d 3

Natural Mal d 3 purified from apple peel migrated in reduced SDS-PAGE as a single band at 11 kDa (Fig. 1A, lane 3). The purified protein was shown to be monomeric with a mass of 9000 Da while a molecular mass of 9074 Da was determined by LC-ES MS. This is in close agreement with the predicted mass (9078 Da, Swiss-Prot access no. Q9M5X7). An additional small component at 9189 Da was observed which gave the same molecular ion as Mal d 3 and probably resulted from a ligand effect. MALDI-TOF analysis (Fig. 2C) identified all peptide masses as Mal d 3 with a probability of identification of 100% based on molecular weight search (MOWSE) score. The NMR spectra of nMal d 3 (Fig. 3E) show many separated peaks within the intervals 6.5–9 and 0–4.5 ppm, while no signal was detected outside these limits, except for the tryptophan signal near 9.5 ppm. The number and dispersion of the peaks and their separation are reliable indicators of the presence of tertiary structure. The CD spectrum of nMal d 3 at pH 7.0 was characteristic of an α-helical structure, with two negative minima at 208 and 219 nm and a maximum at 193 nm (Fig. 3F). The FT-IR spectrum of purified Mal d 3 (Fig. 5) shows the amide I and amide II bands at 1655 and 1540 cm^{-1}, respectively. The amide I maximum is classically assigned to α-helical structures. There are three shoulders, one at around 1640 cm^{-1} indicative of unordered structures such those found in surface loops, the other two at 1680 and 1625 cm^{-1} (Fig. 5) are normally assigned together to β-sheet. The spectrum is very similar to those of other LTPs such as that from wheat and is indicative of the protein being in its native folded state [29].

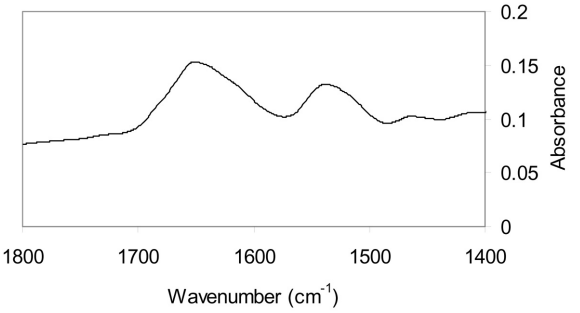

Figure 5. FT-IR spectrum of Mal d 3.

3.2.4 Immunological analysis of Mal d 3

The IgE binding capacity of purified Mal d 3 either as purified protein or present in apple protein extract was confirmed by immunoblotting as shown with selected serum samples in Fig. 1B, lanes 3 and 4. Patients with specific IgE to Mal d 3 more frequently reported respiratory symptoms, urticaria, gastrointestinal problems and even in some cases anaphylaxis when eating apple and other Rosaceae fruits than did patients with specific IgE to other allergens.

4 Discussion

4.1 rMal d 1 and rMal d 4

To eliminate degradation and loss of IgE binding ability and to ensure batch to batch reproducibility, recombinant expression was the method of choice for preparing Mal d 1 [3, 24]. In the present study the expression and purification gave a total yield of 100 mg purified rMal d 1 *per* 10 L *E. coli* culture. It is known from previous studies that purification of recombinant proteins from inclusion bodies frequently results in proteins with random coiled structure [30]. Furthermore, solubilisation and refolding methods

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using chaotropic reagents (urea) may lead to modifications of amino acid residues resulting in incorrect mass data not equivalent to the natural protein [31, 32]. Therefore, the optimal temperature and duration of expression needs to be defined aiming at the highest possible yield without the formation of inclusion bodies [26].

The production of rMal d 1 was performed at 25°C for 19 h and Mal d 1 was purified from the soluble cell lysate fraction. To determine whether the protein was folded or not, the tertiary and secondary structures were determined by NMR analysis and CD spectroscopy, respectively. Immunological characterisation revealed that rMal d 1 was recognised by IgE from apple allergic patients' sera in immunoblot. It is well documented that several Mal d 1 isoforms are present at the transcriptional level as well as at the protein level in the apple fruit [33, 34]. Individual Mal d 1 isoforms also displayed different IgE binding capacity when used to screen a number of patients' sera, with Mal d 1.0108 being recognised by all the sera tested so far [35]. Moreover, rMal d 1.0108 completely inhibited the IgE binding ability of pooled apple allergic patients' sera to the mixture of Mal d 1 isoforms present in the natural apple extract (Fig. 1C, lane 2).

Plant derived profilins are sometimes difficult to isolate from natural sources resulting in low yields and instable products (L. Zuidmeer, unpublished study). Therefore, we considered the recombinant production of profilins to be superior to the purification of the natural protein in terms of yield and quality. However, the conditions for production in *E. coli* need to be optimised for each individual protein in order to obtain a recombinant protein with a 3-D structure equivalent to the natural protein. In the case of rMal d 4 the expression was performed at 37°C for 4 h and recombinant protein was obtained from the soluble cell lysate. In contrast, when recombinant apple profilin was purified from inclusion bodies only a random coiled structure was detectable as shown for other isoforms [25]. So far, a small number of apple profilin isoforms has been identified, cloned and sequenced suggesting a small gene family. On the genomic level at least four different genes have been identified in the apple genome [36]. All sequences encoding Mal d 4 comprise an ORF of 396 nt and share sequence similarity of 77–81% with each other. As previously shown by SAXS (small angle X-ray scattering) analysis, rMal d 4 is present in solution as a monomer even at higher concentration [25]. The IgE binding capacity of the individual isoforms was found to be comparable. In addition, preincubation of specific sera with purified rMal d 4.0101 abolished further IgE binding to the mixture of natural apple profilin present in the apple extract (Fig. 1C, lanes 3 and 4).

4.2 Mal d 2

Mal d 2 contains 16 conserved cysteine residues involved in the formation of eight disulphide bridges and is therefore difficult to produce it as a properly folded recombinant protein with high expression levels [28, 37, 38].

The yield of Mal d 2 is closely related to the ripeness of apple fruits [39]. Therefore, ripe apple fruits were used to isolate Mal d 2. It has been shown that Mal d 2 is present throughout the apple pulp and peel. Further, the peel of an apple contains different proteins to those in pulp as well as other components such as wax [40]. Therefore, peeled apple fruits were used to isolate natural Mal d 2. The overall yield was about 5 mg of pure protein *per* 1 kg of apple, showing that Mal d 2 is an abundant apple protein. Purified Mal d 2 migrated in SDS-PAGE as a double band, with the lower band being abundant and the upper band a minor form (Figs. 4B and C, lane 1). Although the theoretical molecular weight of 23 194.9 Da for purified Mal d 2 was confirmed by ESI-MS analysis (Fig. 2B), the protein clearly migrates differently, at about 31 and 33 kDa in SDS-PAGE under reducing conditions. Anomalous migration of TLPs in SDS-PAGE has been previously reported for other PR-5 proteins such as thaumatin [41], cherry TLP and kiwi TLP [42] and may be ascribed to the unusually high number of disulphide bridges and/or the presence of carbohydrate chains [43]. A shift to lower molecular mass (from approximately 31 and 33 kDa to 20 and 22 kDa) was observed when Mal d 2 was analysed by SDS-PAGE under nonreducing conditions, thus maintaining the disulphide bridges. Further, the presence of two Mal d 2 isoforms was confirmed by N-terminal sequencing and immunoblotting with rabbit anti-grape TLP. Immunoblotting with anti-HRP antibodies and IgE immunoblotting of native and metaperiodate oxidised Mal d 2 indicate the presence of *N*-glycans carrying α-1,3-fucosyl and β-1,2-xylosyl residues on the upper Mal d 2 band.

The mature Mal d 2 was found to posses one putative *N*-glycosylation site [28]. Also, the presence of peaks in 3–4 ppm region in the NMR spectra of Mal d 2 indicates the presence of glycosylation (Fig. 3C) [44]. However the glycosylated variant of Mal d 2 could not be detected by ESI-MS analysis, possible due to the low amount in the sample.

Sixty percent (12/20) of apple allergic patients' sera tested in ELISA contained IgE specific for Mal d 2. Immunoblot analysis revealed that the IgE binding of five out of six Mal d 2 sensitised patients was exclusively to the carbohydrate epitopes present on the upper glycosylated variant of Mal d 2, whereas only one of six sera contained IgE specific for protein epitopes present on both variants of Mal d 2 (Fig. 4B, lane 10). Altogether, these results agree with previously published data showing 72% (18/24) IgE reactivity when the sera were tested by immunoblotting using apple protein extract containing both glycosylated and nonglycosylated variants of Mal d 2 [10]. However, only 15% of sera were reactive to nonglycosylated rMal d 2 in RAST [1]. Interestingly, all of the antibodies tested (IgE, rabbit antigrape TLP or rabbit anti-HRP) showed lower affinity to nonreduced Mal d 2 (ELISA and SDS-PAGE under nonre-

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ducing conditions) compared to the reduced Mal d 2 (SDS-PAGE under reducing conditions) (Fig. 4). We suggest that this phenomenon is due to limited access of antibodies to binding sites due to the extremely compact conformations of the native TLPs in general [8].

4.3 Mal d 3

Mal d 3 (nsLTP) is abundant in apple peel although the yields are generally higher from freshly picked apples rather than those that have been stored or transported under modified atmosphere, as might be expected from the effect of post-harvest treatments on expression of this protein [17]. Four disulphide bridges, typical for members of the nsLTP family contribute to the overall stability of Mal d 3 which facilitates purification of the protein from natural sources. MS showed the expected theoretical mass, while CD, NMR and FT-IR analyses indicated the presence of secondary and tertiary structures. The IgE binding capacity was also retained.

4.4 Concluding remarks

The present paper describes the purification and characterisation of four apple allergens, Mal d 1, Mal d 2, Mal d 3 and Mal d 4. Mal d 1, a Bet v 1 homologue, and Mal d 4, apple profilin, were produced as recombinant proteins, since these molecules lack posttranslational modifications and have previously been expressed in *E. coli*. In contrast, glycosylation and the formation of disulphide bridges are important for Mal d 2 and Mal d 3. Furthermore, these proteins are abundant in apple fruit tissue and stable during purification. Therefore, Mal d 2 and Mal d 3 were purified from natural fruit tissue.

N-terminal sequencing and MS of Mal d 1, Mal d 2, Mal d 3 and Mal d 4 were used to confirm the authenticity of the purified apple allergens. CD spectroscopy showed the presence of secondary structure while well-resolved NMR spectra showed the presence of tertiary structure.

Two distinct sera recognition patterns can be observed which relate to different symptom patterns. Birch pollen-associated apple allergy is predominant in Central Europe based on IgE reactivity mainly to Mal d 1 and results in rather mild food allergic symptoms. In contrast, the majority of Southern European apple allergic patients display specific IgE reactivity to LTP accompanied by more severe food allergic symptoms. Mal d 4 and Mal d 2 sensitisation differ when comparing apple allergic patients' sera from Southern Europe *versus* Central Europe [1]. However, whether these different sensitisation patterns also contribute to the different patterns of symptoms remains to be investigated in more detail.

In summary, the well characterised individual apple allergens described here will contribute to improved food allergy diagnosis and will facilitate the development of detailed dietary recommendations.

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The authors have declared no conflict of interest.

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