

Lipid Transfer Proteins from Fruit: Cloning, Expression and Quantification

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Food allergy · Apple allergy · Lipid transfer protein · Recombinant allergens · Measurement

Abstract

Background: Lipid transfer proteins (LTP) are stable, potentially life-threatening allergens in fruits and many other vegetable foods. The aim of this study was to clone and express recombinant apple LTP (Mal d 3), as has previously been done for peach LTP (Pru p 3) and set up quantitative tests for measuring fruit LTPs. **Methods:** cDNA for Mal d 3 and Pru p 3 was cloned, expressed in the yeast *Pichia pastoris* and the resulting proteins were purified via cation exchange chromatography. The immune reactivity of rMal d 3 was compared to nMal d 3 by RAST (inhibition), immunoblotting and basophil histamine release testing. To obtain monoclonal and monospecific polyclonal antibodies, mice and rabbits were immunized with purified nMal d 3. **Results:** The deduced amino acid sequence of Mal d 3 was identical to the published sequence, Pru p 3 differed at two positions (S9A and S76H). The rMal d 3 had an IgE-binding potency and biological activity close to its natural counterpart. One sandwich ELISA selectively detecting apple LTP and another cross-reactive with cherry, nectarine and hazelnut LTP were developed. In addition, a competitive RIA was developed with polyclonal rabbit antiserum and labeled

nMal d 3. **Conclusion:** rMal d 3 (as shown before for rPru p 3) may be a useful tool for application in component-resolved diagnosis of food allergy. Assays for the measurement of LTP will increase the traceability of this potentially dangerous allergen.

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Introduction

Allergy to fruits of the family of the Rosaceae, like apple and peach, has been described most frequently as a cross-reactive phenomenon linked to birch pollinosis [1, 2]. More recently, it has been reported that allergy to these fruits can also occur independently of pollen sensitization [3]. The nonspecific lipid transfer proteins (LTP) were identified as major allergens for non-pollen-related allergy to Rosaceae fruits [4, 5]. It is now clear that the highly conserved structure of plant LTP justifies their designation as true pan-allergens [6] as was earlier described for profilin [7]. Although IgE binding to LTPs from a broad variety of foods was reported, it is clear that recognition of LTP from fruits like peach and apple is most frequent. So far, no patients were described that recognize non-Rosaceae LTP without IgE antibodies against peach LTP. This observation suggests that LTP from peach is most likely the starting point for sensitization in nonpollinosis patients. On the other hand, cross-reactivity to

pollen LTP (plane, mugwort, olive) has been reported and primary sensitization to pollen LTP leading to fruit allergy can still not be ruled out completely [8–11].

Bet v 1-homologous structures in vegetable foods are very labile and do not survive the harsh proteolytic environment of the stomach and intestines [12]. Therefore, it is generally accepted that they almost exclusively induce local symptoms of the oral cavity, the so-called oral allergy syndrome (OAS) [13]. More recently, however, Bet v 1-related allergens in carrot and celery and also in soy have been implicated in more severe allergic symptoms as well [14, 15]. Members of the LTP family are low-molecular-weight proteins, typically around 90–95 amino acids, containing 8 cysteine residues forming 4 disulfide bridges. They are extremely stable, resisting thermal denaturation [12] and pepsin digestion [6, Sancho et al., pers. commun.]. The latter property allows the allergen to reach the intestinal immune system in an immunogenic way, i.e. with the potential to sensitize. Moreover, a conserved IgE-binding conformation is probably the most important factor contributing to the observation that LTP-reactive patients have a higher incidence of severe systemic reactions than the patients with the classical birch-related food allergy [16, 17]. Although the cDNA cloning of peach and apple LTP was described before [18], to our knowledge the expression of Mal d 3 as a recombinant protein has not been reported yet. The availability of high-quality purified recombinant allergens as described for peach LTP [19] will help improve diagnostic tests for food allergy, in this case fruit allergy. Valenta et al. [20] introduced the term 'component-resolved diagnosis' for the diagnosis of inhalant allergies. For food allergy, this approach was proposed as well [21]. Clearly, the distinction between IgE responses to Mal d 1 and to Mal d 3 has clinical implications. Extract-based diagnostic tests will not provide such information. The first aim of this study was to clone and express LTP from apple and peach to support the development of component-resolved diagnostic tests for fruit allergies.

Rosaceae fruits are consumed both as fresh fruits and as processed food products. Many patients sensitized to fruit LTP also react to fruit-based processed foods, which suggests that these allergens are highly resistant to food-processing steps such as homogenization, heating and high-pressure treatment [12, 22]. Their extreme stability and consequently their capacity to induce severe systemic reactions designate them as dangerous allergens that need to be traceable in food products. The second target of this study was therefore the development of assays for the determination of Mal d 3 content.

Materials and Methods

Sera

Sera in this study were selected on the basis of specific IgE antibodies against peach and/or apple LTP (n = 33) and originated from a cohort of patients from Northern Italy (Milano) and Central Spain (Madrid) that were studied in the frame of the European SAFE project (EC: SAFE QLK1-CT-2000-01394). Inclusion in the SAFE cohort was based on a convincing history and positive SPT (DBPCFC was also performed in 13/33 patients) for apple. Most patients also reported clinical allergy to peach. In addition, two in-house reference sera with IgE antibodies directed at Rosaceae LTPs were used in this study.

A serum pool composed of 4/33 and 8 additional Italian sera with IgE antibodies against Mal d 3 (total n = 12) was used for RAST inhibition analysis. The specific IgE response of the pool serum to apple and nMal d 3 were 9.8 and 12.1 IU/ml, respectively.

Peach and Apple Extracts

Peach (unknown cultivar, imported from Australia) and apple (Golden Delicious) were obtained from a local food store. Extracts were prepared essentially as described by Björkstén et al. [23].

Cloning of LTP from Peach and Apple

For RNA isolation from peach and apple, the method described by Clendennen et al. [24] was followed. For technical reasons, peach RNA was isolated from a combination of peel and pulp (5 g), apple from peel alone (6.4 g). This protocol yielded 7.6 µg of RNA from peach and 46 µg from apple. cDNA was synthesized using the First Strand cDNA Synthesis Reverse Transcriptase Kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Peach-derived cDNA was used in a first PCR with primers (table 1) designed on the basis of the published sequence of LTP from the closely related *Prunus dulcis* (almond, accession. No. X96714 [25]). Amplified cDNA was purified and sequenced. The correct 5' and 3' ends were amplified using the respective RACE kits (Invitrogen). Primers were designed on the basis of the obtained sequences (table 1) to clone the correct peach LTP sequence introducing appropriate restriction sites for ligation in yeast expression vector pPICZα (Invitrogen).

Apple LTP was cloned in a similar way using primers (table 1) based on the published sequence of LTP from *Malus domestica* cv Fuji (accession No. AF221502 [26] and AJ277164 [18]). Apple LTP was cloned with and without its signal sequence.

Expression of Apple LTP in *P. pastoris*

cDNA coding for apple LTP was ligated into pPICZαA using *XhoI*-*NotI* restriction sites. After electroporation of *P. pastoris* strain GS115 the yeast was grown and expression of recombinant protein production was induced exactly as described in the protocol supplied by the manufacturer. The culture supernatant was harvested after 120 h by centrifugation and filtration. Production of rMal d 3 was monitored by immunoblotting (not shown) with a monospecific polyclonal rabbit antiserum against natural carrot LTP kindly provided by Sacco de Vries (Wageningen, The Netherlands).

Purification of Recombinant Mal d 3

For purification of rMal d 3, the culture supernatant was concentrated 10 times by ultrafiltration over a YM1 or YM5 membrane (Amicon Corp., Danvers, Mass., USA) with a 1- or 5-kDa

Table 1. Primer sequences used to clone the Pru p 3 and Mal d 3 sequences from cDNA

Name	Sequence (5' → 3')	Use	Position in published sequence
IB67	<i>ATAACATGTGGCCAAGTG</i>	clone Pru p 3 from cDNA	nt. 79–96
IB68	<i>CTTCACG/AT/GTGGCGCAGTT</i>		nt. 334–351
IB74	<i>CCATACATACCCTACGTG</i>	3' RACE	nt. 112–129
IB77	<i>GGAGGCGCTAATCTTGTA</i>	5' RACE	nt. 313–330
IB78	<i>GGGCTCGAGAAAAGA↓ATGGCTTACTCTGCCATG</i>	cloning of pre- and mature rPru p 3 and rMal d 3 to pPICZαA	nt. 1–18
IB79	<i>GGGCTCGAGAAAAGA↓ATAACATGTGGCCAAGTG</i>		nt. 79–96
IB81	<i>GGGGGCGGCCGCTCACTTCACGGTGGCGCA</i>		nt. 337–354
LZ1	<i>GGGCTCGAGAAAAGA↓ATGGCTAGCTCTGCAGTG</i>		nt. 1–18

The *Xho*I restriction site is indicated in bold, the *Not*I site is underlined. The peach and apple sequences are in italics. Start codons are bold/italics and stop codon is italics/underlined. The Kex 2 cleavage site is indicated with an arrow.

cut-off, respectively. During ultrafiltration, medium was replaced by a sodium acetate buffer (20 mM) pH 5.5 containing 5 mM EDTA. Concentrates were separated on an SP cation exchange column (Amersham-Pharmacia-Biotech). Elution was performed with a linear gradient from 0 to 1 M NaCl. Alternatively, rMal d 3 was affinity purified using Sepharose-coupled monoclonal antibody (mAb) 6B5 (generation of this mAb is described under 'Production of Antibodies' below), equilibrated in PBS. Concentrated yeast supernatant (50 ml) was allowed to bind on the 6B5-Sepharose column (10 ml). Subsequently, unbound material was washed out with PBS, and bound rMal d 3 was collected by elution with 15 ml 0.1-M glycine (pH 2.5). The fractions were immediately neutralized and dialyzed against PBS.

RAST and RAST Inhibition

RAST was performed as described previously [27]. For evaluation of the specificity and cross-reactivity spectrum of mouse monoclonal and rabbit polyclonal antibodies, radiolabeled goat anti-mouse IgG and sheep anti-rabbit IgG (Sanquin) were used, respectively, instead of anti-human IgE.

For RAST inhibition, serum was preincubated for 2 h with (serial) dilutions (in 50 µl PBS-AT) of inhibitor prior to addition of Sepharose-coupled extract. For the uninhibited value, serum was pre-incubated with 50 µl PBS-AT. Subsequent steps were identical to those described for the RAST. Results were expressed as percentage inhibition.

SDS-PAGE/Immunoblotting

Protein separation, detection and immunoblot (inhibition) were done as described previously [28].

Concanavalin A Binding Assay

nMal d 3 (generous gift of A.I. Sancho and N. Rigby, Norwich) and rMal d 3 were affinity blotted with concanavalin A-peroxidase as described before [29].

N-Terminal Sequencing of the Recombinant Proteins

Purified recombinant protein was separated by SDS-PAGE and transferred onto polyvinylene difluoride (PVDF) membrane as described above. Blots were stained with 0.1% Coomassie R-250 in 50% methanol. Bands with an apparent M_r around the expected 10 kDa were excised and sequenced on a Perkin Elmer/Applied Biosystems 476A gas-phase sequencer (Sequentie Centrum Utrecht, The Netherlands).

Production of Antibodies

For the production of mAbs, Balb/c mice (Charles River Laboratories, Wilmington, USA) were immunized subcutaneously with 10 µg nMal d 3 (purified from *M. domesticus* cv Golden Delicious) in complete Freund's adjuvant (Brunschwig, Basel, Switzerland). Immunization was followed by three subcutaneous and one intravenous booster (at 4-week intervals) with 10 µg nMal d 3 in incomplete Freund's adjuvant. Four days later, the spleen was removed and B cells were isolated. Hybridization was performed according to the method of Köhler and Milstein [30] with some modifications [31]. Antibody-producing hybridomas were screened by RAST with Sepharose-coupled nMal d 3. The procedure resulted in two mAbs, 5H5 and 6B5 (both IgG1κ). mAbs were purified with protein A Sepharose (Amersham-Pharmacia-Biotech). Antibody specificities were evaluated by RAST with Sepharose-coupled food extracts.

For polyclonal antibodies New Zealand White rabbits were immunized with 50 µg nMal d 3 in Montanide ISA-50 (Seppic, Paris, France). Boosters were given at 4-week intervals, and plasma was collected at day 7, 9 and 11 after boosters 4, 5 and 6. Plasma was recalcified and antibody titers were measured by RAST with Sepharose-coupled nMal d 3. On the basis of this analysis, sera were pooled and stored frozen at –80°C. Cross-reactivity was assessed by RAST with Sepharose-coupled food extracts.

Biotinylation

mAb 5H5 was biotinylated using sulfo-NHS-LC-Biotin according to the manufacturer's instructions (Pierce, Rockford, Ill., USA).

Sandwich ELISAs

Two sandwich ELISA were developed for the measurement of apple LTP. One was based on mAb 6B5 as capturing and rabbit anti-Mal d 3 as detecting antibody. The second assay used mAb 5H5 as detecting antibody. Microtiter plates (Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with 100 µl mAb 6B5 at 1 µg/ml in coating buffer (50 mM NaHCO₃, pH 9.6). In the double mAb sandwich, coating antibodies were used at 4 µg/ml. After blocking with high-performance ELISA (HPE) buffer (Santquin) with 5% semi-skimmed milk, wells were incubated with (allergen) extracts. Purified nMal d 3 was used as standard [Sancho et al., in press]. Detection was performed with 1:1,000 diluted rabbit anti-Mal d 3 in conjunction with peroxidase-conjugated goat anti-rabbit IgG antibodies (Dako, Glostrup, Denmark, dilution 1:1,000) or alternatively, biotinylated mAb 5H5 (6 µg/ml) in conjunction with peroxidase-conjugated streptavidine (Amersham Biosciences, Little Chalfont, UK). All dilutions of samples, references and reagents were made in HPE buffer. All incubations were carried out for 1 h at room temperature except for the enzyme conjugate which was incubated for 30 min at 37°C. Washing was done with PBS/0.05% Tween. Finally, the plates were incubated in the dark at room temperature for 30 min with substrate (1 mM ABTS/0.03% H₂O₂ in 0.1 M citric acid/0.1 M sodium citrate, pH 4.0). Addition of 2% oxalic acid stopped the reaction. Serial dilutions of the samples were assayed in duplicate. LTP concentrations were calculated by parallel-line analysis.

Competitive RIA

As an alternative to measure Mal d 3, a competitive RIA (cRIA) [32] was developed. To this end, nMal d 3 was radiolabeled as described before [33]. Subsequently, 50 µl polyclonal rabbit anti-nMal d 3 (diluted 1:400 in 0.3% BSA) was incubated with serial dilutions of apple (or other food) extract in a volume of 50 µl PBS-AT. After 2 h, 0.5 mg protein A-Sepharose and ¹²⁵I-radiolabeled nMal d 3 (20,000 cpm) were added in a final volume of 900 µl PBS-AT. After overnight incubation and washing, bound radioactivity was measured in a γ-counter. Mal d 3 concentrations were calculated by parallel-line analysis using a standard curve of purified nMal d 3.

Basophil Histamine Release Assay

White blood cells were isolated from blood of a nonallergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere [34, 35]. Subsequently cells were resensitized with patient's serum. Histamine release was performed with apple extract (1 ng/ml–100 µg/ml) and with purified natural or recombinant Mal d 3 (100 pg/ml–10 µg/ml). Liberated histamine was measured by the fluorometric method essentially as described by Siraganian [36]. Stripped cells were used as a negative control and induced a histamine release lower than 3%. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Centre under project number MEC97/030.

Statistical Analysis

RAST results for nMal d 3 and rMal d 3 were compared by Spearman rank correlation.

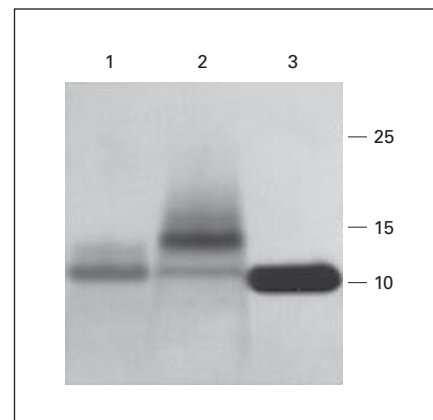


Fig. 1. Silver-stained gel of natural and recombinant LTPs. Lane 1, rMal d 3 containing the signal sequence; lane 2, mature rMal d 3; lane 3, nMal d 3. Marker sizes are indicated on the right-hand side.

Results

Cloning, Expression and Purification of Recombinant Mal d 3 and Pru p 3

cDNAs for Mal d 3 and Pru p 3 were both successfully cloned by PCR and RACE protocols. The deduced amino acid sequence for mature apple LTP and its signal sequence proved identical to the 18 published sequences (accession No. AF221502 [26] and AJ277164 [18] and AY572500 to AY572515 of isoform variant Mal d 3.0101 [37]). For Pru p 3, a new isoform was identified, differing at two positions (a.a. S9A and S76H) from the published sequence (accession No. AJ277163 [18]). The signal sequence of Pru p 3 demonstrated high or complete identity to those reported for apple, cherry and almond, i.e. 85, 96 and 100%, respectively.

Mal d 3 was expressed in *P. pastoris* both with and without its endogenous signal peptide and the recombinant protein was purified from the culture supernatant by cation exchange chromatography. rMal d 3 eluted at approximately 375 mM NaCl. SDS-PAGE analysis revealed that the rMal d 3 expressed without its endogenous signal peptide migrated at a slightly higher M_r (12 kDa) than nMal d 3 (fig. 1). Surprisingly, (affinity-) purified rMal d 3 obtained upon expression with its own signal peptide showed an apparent M_r similar to its natural counterpart.

N-Terminal Sequence Analysis

N-terminal sequences of the recombinant LTP molecules were determined. Mal d 3 expressed with its endogenous signal sequence had exactly the expected N-termi-

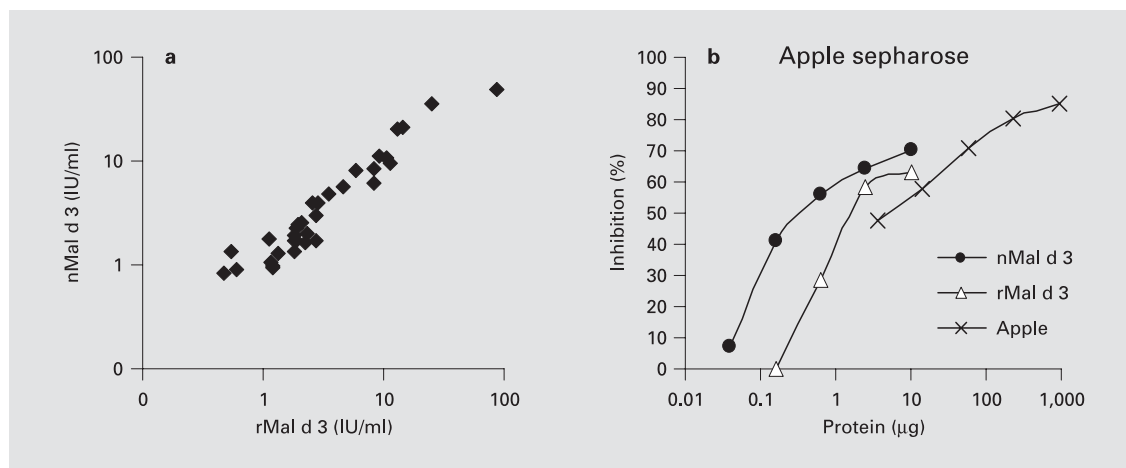


Fig. 2. **a** Specific IgE reactivity to nMal d 3 and rMal d 3 (IU/ml). **b** RAST inhibition results on apple Sepharose with nMal d 3, rMal d 3 and apple extract using a serum pool of LTP-positive patients.

nal sequence of mature LTP (ITCGQVTSSL), illustrating that the signal peptide facilitated correct cleavage of the mature allergen. In contrast, application of the mature sequence alone resulted in expression of rMal d 3 molecules with 9–14 residual vector-derived amino acids attached ((SIA)(AK)EEGVSLER). Neither of the rMal d 3 preparations stained with concanavalin A, suggesting that the lower mobility of expressed mature sequence on SDS-PAGE was not or only partly due to glycosylation (results not shown). For further analyses of immune reactivity, correctly cleaved rMal d 3 was used.

IgE Reactivity of rMal d 3

Sera with specific IgE antibodies to LTP ($n = 33$) were tested in a RAST with Sepharose-coupled natural and rMal d 3 (results not shown). The mean IgE responses were 6.8 and 7.0 IU/ml, respectively, and responses closely correlated ($R_{\text{Spearman}} 0.95$, 95% CI 0.91–0.98, $p < 0.0001$). The mean ratio of IgE reactivity to natural and rMal d 3 was 1.0 (95% CI 0.9–1.1) (fig. 2a).

To further compare the IgE-binding potency of natural and rMal d 3, RAST inhibition was performed. Both proteins inhibited the IgE binding of an LTP-positive serum pool to apple extract to a similar degree (70%). Nevertheless, nMal d 3 was around 5 times more potent as inhibitor (fig. 2b).

Finally, the biological potency of the rMal d 3 was compared to that of nMal d 3, using basophils sensitized with two LTP-reactive sera (fig. 3). Maximum histamine release was similar in both patients with both preparations (around 60%). Parallel-line analysis of the individ-

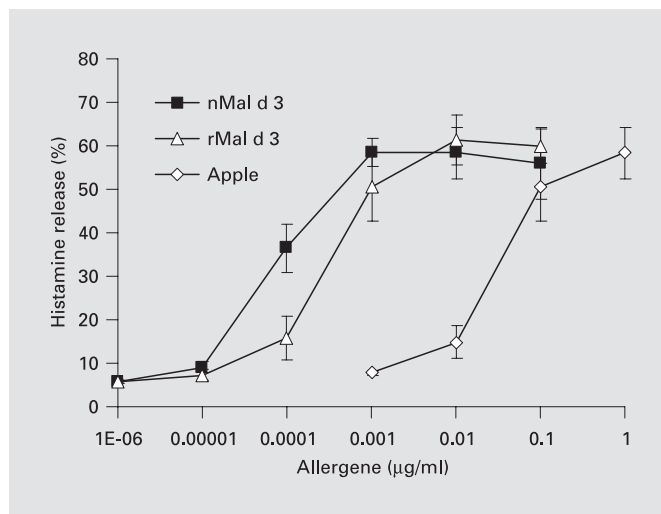


Fig. 3. Basophil histamine release assay (mean values of two patients). nMal d 3, rMal d 3 and apple. Natural and recombinant Mal d 3 induce a similar response, but the former is needed in slightly lower concentrations.

ual pairs of curves showed that nMal d 3 induced significant release at 3–10 times lower allergen concentrations than both recombinant allergens.

Detection of Mal d 3

Two mAbs against Mal d 3, 6B5 and 5H5, were produced and analyzed together with the polyclonal rabbit antiserum in a RAST for cross-reactivity to 24 foods (table 2). mAbs 6B5 demonstrated significant cross-reac-

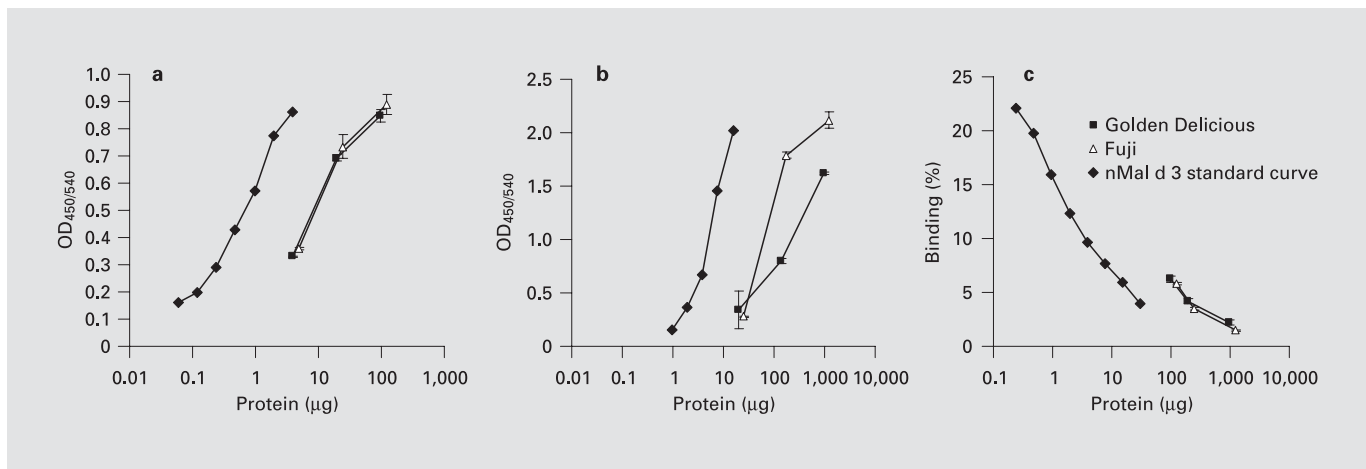


Fig. 4. Specific (a) and nonspecific (b) ELISA and the competitive RIA (c) compared for two apple cultivars, Golden Delicious and Fuji. In closed diamonds the nMal d 3 standard curve.

Table 2. RAST results (% binding) of mAbs (6B5 and 5H5) and rabbit a-n Mal d 3 pAb on several food extracts coupled to Sepharose

Sepharose	Monoclonals		Polyclonal
	6B5	5H5	
Apple (peel)	<i>39.3</i>	<i>5.2</i>	<i>33.4</i>
Cherry	<i>15.2</i>	<i>0.2</i>	<i>18.7</i>
Nectarine	<i>12.4</i>	<i>0.2</i>	<i>24.1</i>
Peach	<i>12.4</i>	<i>0.6</i>	<i>28.9</i>
Plum	<i>4.1</i>	<i>0</i>	<i>6.5</i>
Hazelnut	<i>7.7</i>	<i>0.2</i>	<i>21.0</i>
Walnut	<i>2.0</i>	<i>0.6</i>	<i>16.0</i>
nMal d 3	<i>43.5</i>	<i>19.4</i>	<i>34.6</i>
rPru p 3	<i>37.9</i>	<i>1.7</i>	<i>24.7</i>

The values considered positive are indicated in italics. Food extracts tested were apple, peach, cherry, pear, kiwi, red currant, mango, melon, papaya, avocado, banana, orange, strawberry, grape, nectarine, plum, almond, hazelnut, walnut, cashew nut, Brazil nut, basil, chervil, parsley and celery. Extracts with values below 2% binding are not shown.

tivity to peach (confirmed with purified rPru p 3), cherry and nectarine and to a lesser extent to hazelnut and plum. mAbs 5H5 selectively recognized Mal d 3. The polyclonal antibody cross-reacted to the same foods as mAb 6B5, and in addition also to walnut. Using the available antibodies, three assays for measurement of Mal d 3 and some related LTPs were developed.

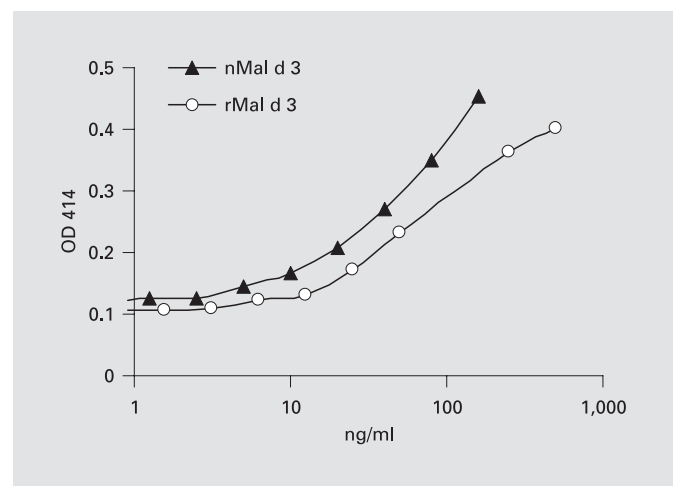


Fig. 5. Comparison of nMal d 3 and rMal d 3 in the nonspecific ELISA.

Combination of mAb 6B5 (coating) and mAb 5H5 (detection) resulted in a sandwich ELISA that selectively recognized LTP from apple. The measuring range was around 20–150 ng/ml (fig. 4a). Replacement of detecting antibody 5H5 by the polyclonal rabbit antiserum resulted in a more cross-reactive ELISA with increased sensitivity, i.e. a measuring range of around 5–20 ng/ml (fig. 4b). Two apple extracts (Fuji and Golden Delicious) were tested in both ELISAs on 3 consecutive days. In the double mAb ELISA, a mean Mal d 3 content of 71.7 μg/g apple was found for Fuji and 14.4 μg/g for Golden Delicious. The

interassay coefficient of variation (COV) was 8%. In the cross-reactive ELISA, these figures were 57.9 and 38.4 µg/g, respectively, with an interassay COV of 21%. Cross-reactivity of the latter assay was confirmed by analyzing cherry, hazelnut and nectarine extract (not shown). Both natural and rMal d 3 were recognized equally in the cross-reactive ELISA (fig. 5).

In addition, a competitive radio-immuno assay (cRIA) for the measurement of Mal d 3 was developed. The measuring range was 2–750 ng/ml (fig. 4c). Fuji and Golden Delicious extracts were analyzed and showed Mal d 3 contents close to the levels found by ELISA: 29.7 and 26.1 µg/g, respectively. As was observed for the ELISA with this rabbit antibody, the cRIA was found to be cross-reactive to related fruits like peach and cherry (not shown).

Discussion

In Mediterranean countries like Italy and Spain, Rosaceae fruits can cause severe allergic reactions. In these cases, lipid transfer proteins have been identified as the major allergens. In a recent study [38], purified Pru p 3 and Mal d 3 were used for skin prick testing. Strong correlations were found between the skin responses to fresh fruits, fruit extracts and purified LTP of both peach and apple. In this study, apple LTP was successfully expressed and shown to have good IgE reactivity and biological activity. Because only a limited number of patients was tested, the lower biological activity (around 5-fold) of rMal d 3 compared to its natural counterpart needs to be confirmed by additional experiments. If confirmed, the difference could point towards incorrect folding of the recombinant version. The presence of multiple isoforms in the nMal d 3 preparation with different IgE-binding capacity could play a role. In addition to rPru p 3, rMal d 3 may prove a useful tool in the development of component-resolved diagnostic tests. The advantage of this approach is that IgE responses to mild and potentially severe allergens can be distinguished. The potential diagnostic value of rPru p 3 has been previously discussed [39] and combined with the results presented here this will be a major step forward for food allergy diagnosis that are traditionally of poor clinical prognostic value. Moreover, the availability of clones that code for fruit LTPs opens the possibility to create hypo-allergenic mutants by site-directed mutagenesis as is shown by the preliminary data on hypoallergenic variants of Pru p 3 [40]. This is particularly relevant for the development of immunothera-

peutic approaches for severe food allergies. It is well known that application of immunotherapy for the treatment of severe food allergies, like peanut allergy, has been hampered by the high incidence of severe adverse reactions [41].

Expression of recombinant apple LTP without inclusion of the endogenous signal peptides was accompanied by inefficient cleavage at the Kex2 site adjacent to the yeast α -mating signal. N-terminal sequencing revealed that 9–14 residual amino acids of this signal peptide were still attached. This was also observed in our laboratory during expression of the major cat allergen, Fel d 1 [unpubl. data]. This is in contrast to the results published by Diaz-Perales et al. [39] on rPru p 3 who reported correct cleavage. This may be explained by their addition of an extra glutamic acid at the start of the protein sequence. Surprisingly, expression of Mal d 3 with its own signal peptide resulted in correct cleavage. It is currently under investigation whether the apple-derived signal peptide can facilitate correct cleavage for other non-related proteins as well. Although the extra N-terminal amino acids did not interfere with the immune reactivity of the recombinants in immunoblotting and RAST (inhibition) experiments (data not shown), the correctly cleaved rMal d 3 was used for further analyses.

Glycosylation of proteins expressed in *P. pastoris* is a common phenomenon [42, 43]. Both N- and O-linked glycans in yeast are dominated by mannose residues [44, 45]. Mal d 3 and Pru p 3 do not contain consensus sequences for N-glycosylation. A negative concanavalin A binding assay excluded O-linked glycosylation as a cause of the different mobility of nMal d 3 and the mature recombinant proteins.

Two sandwich ELISAs and a competitive RIA have been developed with adequate sensitivity to measure Mal d 3 in apple extracts. The double mAb-based ELISA was specific for apple-LTP, whereas the mixed mono-polyclonal ELISA and the cRIA were cross-reactive to cherry, nectarine and hazelnut. This ELISA is now being optimized to measure LTP in these other food extracts. Both cross-reactive assays were approximately 10 times more sensitive than the apple-specific ELISA. Their sensitivity was at the ppb level (ng/ml). Detection of trace amounts of allergen in processed foods requires sensitivity at ppm level [46]. Measurement of allergens in food products of course requires extraction. Typically, food extracts are made at weight per volume ratios ranging from 10 to 50% (i.e. 0.1–0.5 g/ml), depending on the water content of the food product [47]. This implies that 1 ppm is between 100

and 500 ng/ml. Extraction efficiency is a critical issue but the assays developed for the measurement of apple LTP can handle efficiencies below 10% to still reliably exclude the presence of Mal d 3 at ≥ 1 ppm level.

The selectivity of the assays described here is similar to that of the assay of Pru p 3 described by Duffort et al. [19] (recognition of some related Rosaceae fruits). The sensitivity for nMal d 3 is lower than described for Pru p 3 (5 vs. 0.1 ng/ml); however, all other food extracts could be tested at the same concentration as apple to produce a detectable signal (compared to a concentration about 1,000-fold higher described for the Pru p 3 assay).

In summary, this study has provided the tools for a reliable measurement of specific IgE against a major fruit LTP and for a sensitive detection of these allergens in food extracts.

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