

REGULAR ARTICLE

Novel isoforms of Pru av 1 with diverging immunoglobulin E binding properties identified by a synergistic combination of molecular biology and proteomics

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Birch pollen-related food allergies are mainly associated to Bet v 1. Little is known about isoforms of Bet v 1 homologous in fruit of the *Rosaceae* family. We attempted to identify novel isoforms of Pru av 1, the major cherry allergen, at the cDNA and the protein level by a combination of molecular biology and proteomic tools. A cDNA library was screened with patients immunoglobulin E (IgE) and a specific hybridization probe. Edman sequencing, mass spectrometry (MS), and MS/MS were performed after detecting Pru av 1 on 2-D maps by immunoblotting using patients IgE and a monoclonal antibody. Partial amino acid sequences were completed with a polymerase chain reaction (PCR) strategy. The IgE-binding properties of the Pru av 1 spots were analyzed by 2-D blot inhibition. cDNA library analysis revealed a novel Pru av 1 isoform. MS and N-terminal sequencing confirmed the cDNA sequences at the protein level. A series of spots were confirmed as the already known Pru av 1. One spot, exclusively detected with patients sera, was identified as the novel isoform. A partial amino acid sequence detected with MS/MS was completed by PCR-cloning. The 2-D blot inhibition revealed epitope differences between the novel isoform and the previously published Pru av 1. Our data demonstrate that a synergistic combination of molecular biology and proteomics represents a powerful tool for reliable and comprehensive identification of allergen isoforms and variants. The newly identified isoform showed diverging IgE-binding properties and may be relevant for the diagnosis or therapy of cherry allergy.

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1 Introduction

In Central and Northern Europe sensitization to birch pollen is a wide-spread phenomenon. The majority of birch pollen-allergic subjects shows immunoglobulin E (IgE) reactivity to the major birch pollen allergen, Bet v 1 [1]. The cDNA

sequence of Bet v 1 was determined in 1989 [2]. Sensitization to birch pollen and other tree pollen is frequently accompanied by adverse reactions after ingestion of several kinds of plant derived food and is mainly caused by Bet v 1 homologous in food [3]. Several food allergens belonging to the Bet v 1 family have been identified so far [4–10]. Little is known about isoforms of Bet v 1 homologous in fruit of the *Rosaceae* family. Within this family only three major isoforms of Mal d 1, the Bet v 1 homologous allergen in apple, with an amino acid sequence identity ranging from 89.3% to 97.5% to a published Mal d 1 sequence [5] were detected using a PCR strategy [6]. Another Mal d 1 isoform was found by applying MS on natural (n) Mal d 1 [11]. We selected sweet cherry for studying the isoform variation of Pru av 1, the

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Abbreviations: AP, alkaline phosphatase; n, natural; pfu, plaque-forming units; r, recombinant

major cherry allergen, which is one of the best-characterized food allergens of the Bet v 1 family: its three-dimensional structure has been resolved by nuclear magnetic resonance spectroscopy [12], epitope data are available [13, 14], and recombinant (r) Pru av 1 proved to be suitable for both *in vitro* and *in vivo* diagnosis of cherry allergy. In areas with birch trees more than 90% of cherry allergic patients are sensitized to Pru av 1 [15, 16] which identifies Pru av 1 as an interesting target molecule to reduce the allergenic potential of a plant food by genetic engineering. For such an approach, information on isoforms of Pru av 1 is required.

PCR-cloning and screening of cDNA libraries, techniques which are frequently used for identification of allergen isoforms, often lack information about their presence at the protein level. To address this, quadrupole-time of flight mass spectrometry (Q-TOF-MS) was applied on rMal d 1, rBet v 1a, rBet v 1d, and nMal d 1 [11]. nMal d 1 was affinity purified from the cultivar Granny Smith by using a monoclonal antibody (mAb). A discrepancy between studies undertaken at the cDNA and protein level was reported. Only Mal d 1b was detected in nMal d 1, whereas Son *et al.* [6] found both Mal d 1b and Mal d 1a as cDNA sequence. The authors discussed whether Mal d 1a was not present at the protein level or at least to an extent below detection limit [11]. However, purification of proteins with a mAb may not acquire all isoforms which are present in a protein extract. Analyses with proteomic tools often do not cover the complete amino acid sequence when they are applied individually [11, 17, 18].

In our study, we attempted to identify unknown isoforms of Pru av 1 with a powerful combination of molecular biology and proteomic tools. A phage cDNA expression library was established to identify Pru av 1 isoforms and screened with pooled sera of cherry-allergic subjects and a Pru av 1-specific hybridization probe. At the protein level two-dimensional electrophoresis (2-DE) followed by Edman sequencing, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) and nanoelectrospray ion-trap tandem MS (nano ESI-MS/MS) were performed after detecting putative Pru av 1 spots on the 2-DE maps by immunoblotting. The results obtained at the protein and the cDNA level showed excellent correlation, were highly synergistic, and indicated that the sequence variation of Bet v 1 homologous allergens in plant derived food may be bigger than previously described. Interestingly, the newly identified isoform showed epitope differences to the previously published Pru av 1.

2 Materials and methods

2.1 Establishment of a phage cDNA expression library

Cored cherry fruits, cultivar Schneiders, were ground under liquid nitrogen, and freeze-dried. Poly(A) mRNA extraction from this source material and cDNA synthesis were per-

formed as published elsewhere [19]. The cDNA was size-fractionated (Mini Column Fractionation Kit, Novagen, Madison, WI, USA). The average size of the cDNA was monitored by agarose gel electrophoresis. cDNA fractions containing fragments smaller than approximately 300 bp were discarded. A cDNA expression library was established by using the λ SCREEN-1 system (Novagen) according to the manufacturer's instructions (user manual: TB11902/99). Differing from this, top agarose containing 10 mM magnesium sulfate and 0.2% maltose was used to enhance the infection rate of bacterial cells. The primary library showed a titer of 3.5×10^6 plaque-forming units per milliliter (pfu/ml) and was amplified to 1.1×10^{10} pfu/mL.

2.2 Screening of the cDNA library

Unless otherwise noted, the immunoscreening was done according to the manufacturer's instructions (Novagen, TB11902/99). Briefly, 2×10^4 pfu were plated on 90 mm plates, 10 mM magnesium sulfate and 0.2% maltose were added to the top agarose. The incubation was continued immediately after the membranes were layered on the plates for an additional 3.5 h; no isopropyl- β -D-thiogalactopyranoside induction was used. For immunodetection, a pooled serum consisting of equal volumes of sera from five cherry-allergic subjects was employed. All subjects were monosensitized to Pru av 1 and a positive clinical history of cherry allergy was reported. To reduce nonspecific binding, the serum was preincubated with equal volumes of both suspension and lysate of BL21(DE3)pLysE-plating cells. The membranes were incubated overnight at room temperature with a 1:10 dilution of serum in 50 mM Tris-buffered saline with 0.05% Tween 20 (TBST) and 0.1% bovine serum albumin (BSA). The IgE-binding recombinant phages were detected with an alkaline phosphatase (AP)-conjugated monoclonal mouse anti-human IgE antibody (Pharmingen, San Diego, CA, USA), diluted 1:750 in TBST with 0.1% BSA. An AP-substrate solution (Bio-Rad Laboratories, Hercules, CA, USA) was used for visualization. Positive phage recombinants obtained from the immunoscreening were enriched by further rounds of screening until pure clones were isolated. Conversion of recombinant phages to plasmid clones was accomplished by cre-mediated autosubcloning according to the manufacturer's instructions (Novagen, TB11902/99) and plasmid preparation (QIAprep8 Plasmid Mini Kit; Qiagen, Hilden, Germany). Plasmid DNA suitable for sequencing was obtained after transformation of *Escherichia coli* electro-competent cells (XL1-Blue; Novagen) and plasmid preparation as described above. A specific hybridization probe was derived from the cDNA sequence of Pru av 1 [7] (GenBank accession number: U66076) starting from position 56 to 185. The probe synthesis was carried out with the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Plaque lifts for screening with nucleic acid probes were prepared on nylon membranes on

2×10^4 pfu per plate according to the manufacturer's instructions (Novagen; TB11902/99). Differing from this the DNA was fixed by UV irradiation. Prehybridization was done for 15 min at 60°C in 10 mL of a 5-fold concentrated sodium chloride/sodium citrate buffer ($5 \times \text{SSC}$) with 0.1% *N*-lauroylsarcosine. Hybridization was performed overnight at 60°C after adding 2 μL of the specific hybridization probe. Positive phage recombinants were detected by means of the DIG Wash and Block Buffer Set and DIG Luminescent Detection Kit (Roche Diagnostics). Signals were visualized on X-ray films. Prior to enrichment, positive recombinant phages were analyzed with a gene-specific PCR. PCR products were purified from agarose gels (QIAquick Gel Extraction Kit; Qiagen) and sequenced.

2.3 PCR cloning of cDNA

Copy DNA was synthesized by using the RNeasy Plant Total RNA Kit (Qiagen), Oligotex mRNA midi Kit (Qiagen), and First Strand Synthesis Kit (Pharmacia, Freiburg, Germany) with oligo(dT) primer. To remove sequences corresponding to the already published Pru av 1 [7], the cDNA was digested with the restriction nuclease *SapI* for 16 h at 37°C. A gene-specific forward primer (5'-ATGGGTGTCTTCACATACG-3') deduced according to the *N*-terminal amino acid sequence of spot 5 (see Figs. 1 and 2) and a oligo(dT)_{NN} as reverse primer were used in a touch-down PCR. The PCR parameters were

as follows: hotstart 94°C, 15 min; 30 cycles of 94°C, 50°C, 72°C, 30 s each; 20 cycles of 94°C, 46°C, 72°C, 30 s each; final extension at 72°C, 5 min. PCR products were purified from agarose gels (QIAquick Gel Extraction Kit; Qiagen) and ligated into pCRII-TOPO-Vector (TOPO TA Cloning Kit; Invitrogen, Karlsruhe, Germany). Plasmid DNA was purified from transformed *Escherichia coli* cells as described above.

2.4 Allergen extracts

Protein extracts from ripe cherry fruits, cultivar Schneiders, were prepared by a low-temperature method [20] using 15 mL carbonate buffer per 1 g of acetone powder as extracting solution (100 mM $(\text{NH}_4)_2\text{CO}_3/\text{NH}_4\text{HCO}_3$, pH 8.1). The extract was freeze-dried before storage at -20°C .

2.5 Two-dimensional electrophoresis, immunoblot, and immunoblot inhibition analysis

Isoelectric focusing (IEF) on immobilized pH gradients (IPGs) was carried out according to a method of Görg [21], with several modifications, using ReadyStrip IPG strips, 11 cm, pH 3–10 on a Protean IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA). Freeze-dried extracts were reconstituted in 20 μL lysis buffer (9 M urea, 1% DTT, 2% CHAPS, 0.8% Bio-Lyte (pH 3–10)) and 180 μL rehydration buffer (8 M urea, 10 mM DTT, 1% CHAPS, 0.25% Bio-Lyte pH 3–10) and

	1		40
Pru av 1.01.01	<u>GVFTYESEFT</u>	<u>SEIPPPRLFK</u>	AFVLADADNLV PKIAPQAIKH
Pru av 1.02.01	*****SD*S*	*V*****	*L**E**T*I *****SV*T
Pru av 1.02.02	*****AD*S*	*V*****	*L**E**T*I *****SV*S
Pru av 1.02.03	*****AD*S*	*V*T*****	*L**E**T*I *****SV*G
	41		80
Pru av 1.01.01	<u>SEILEGDGGP</u>	<u>GTIKKITFGE</u>	GSQYGYVKHK IDSIDKENYS
Pru av 1.02.01	A**V*****V	*****S***	**H*S***R **GL**D*FV
Pru av 1.02.02	A**V*****V	*****S***	**H*S***R **GL**D*FV
Pru av 1.02.03	A**V*****V	*****S***	**H*S***R **GL**D*FV
	81		120
Pru av 1.01.01	<u>YSYTLIEGDA</u>	<u>LGDTLEKISY</u>	ETKLVASPSG GSIIKSTSHY
Pru av 1.02.01	*N***V*****	*S*KI***T*	*I*****AD* *****N*
Pru av 1.02.02	***S*V*****	*S*KV*****	*I*****AD* *****N*
Pru av 1.02.03	***S*V*****	*S*KV*****	*I*****AD* *****N*
	121		159
Pru av 1.01.01	<u>HTKGNVEIKE</u>	<u>EHVKAGKEKA</u>	SNLFKLIETY LKGHPDAYN
Pru av 1.02.01	***T*D*****	*D*****	TG*****N* *AAN***C*
Pru av 1.02.02	***T*D*****	*D*****	TG*****N* *VAN***Y*
Pru av 1.02.03	***T*D*****	*D*R*****	TG*****N* *VAN***Y*

Figure 1. Sequence alignment of all detected isoforms and variants of Pru av 1. Peptides which were detected with MALDI-TOF-MS are underlined. Bold letters indicate peptides which were sequenced by MS/MS. *N*-Terminally sequenced peptides are marked in yellow. The red color indicates theoretical trypsin cleavage sites and asterisks mark amino acid residues which are identical to Pru av 1.0101. For Pru av 1.0101, the results are given for spot 2. Pru av 1.0201 and Pru av 1.0202 were detected in spot 5.

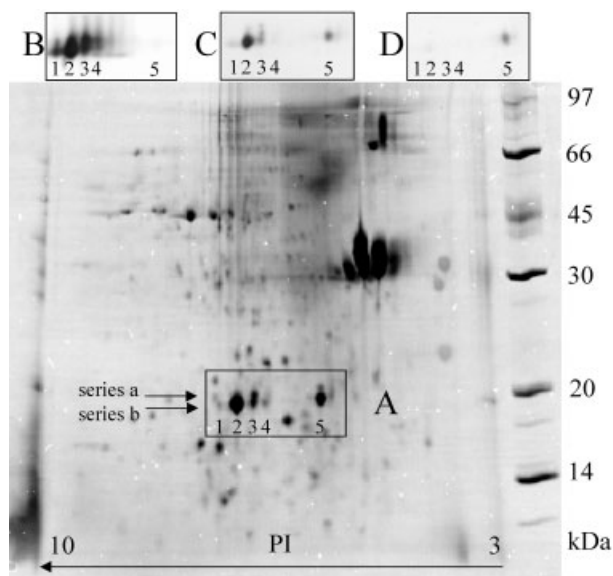


Figure 2. 2-D PAGE and 2-D immunoblot analysis. The Coomassie-stained 2-D map shows the protein pattern of ripe cherry fruit and in section A the Pru av 1-related spots. Section B displays the binding of the mAb G4A to Pru av 1.0101. Patients IgE binds to one additional spot (section C) and this IgE-binding can not be inhibited by rPru av1.0101 (section D).

subjected to active rehydration at 50 V for 12 h. During IEF the voltage was kept at 150 V, 500 V, 1200 V for 1 h, respectively, and at 8000 V for 30 000 Vh. Subsequently the IPG strips were incubated in equilibration buffer I and II for 15 min (each containing 50 mM Tris, 6 M urea, 30% glycerol, and buffer I; 65 mM DTT, buffer II; 260 mM iodoacetamide) and subjected to SDS-PAGE [22] on a Hoefer SE 600 electrophoresis unit (Amersham Pharmacia, Little Chalfont, UK). The quantity of protein separated for immunoblotting or Coomassie staining was 120 µg and 300 µg, respectively. Proteins were transferred from gels to nitrocellulose membranes by semidry blotting [23]. The detection of Pru av 1 was done as described for the plaque lifts, but without preincubation of pooled serum with *E. coli* lysate and suspension. Additionally a mouse mAb (G4A) against Pru av 1 was used. The mAb binds to several Bet v 1 homologous and mutants of Pru av 1 including Pru av 1 constructs with reduced IgE-binding capacity caused by disruption of the tertiary structure (Wiche *et al.*, in press, and Neudecker *et al.* [13]). The mAb was applied as a 1:10 dilution of hybridoma supernatant and detected as described elsewhere [20]. Immunoblot inhibition was done by using a pooled serum of cherry allergic subjects preincubated with 100 µg rPru av 1 [7]. The detection was done as described above.

2.6 MS analysis and N-terminal sequencing

MALDI-TOF and MS/MS analysis were performed as described elsewhere [24]. For N-terminal sequencing 300 µg of cherry extract proteins were separated by 2-D PAGE and

either blotted or passively adsorbed onto PVDF membranes [25]. For passive adsorption the desired spot (see Fig. 2) was excised from a 2-D map, dried in a Speed Vac and rehydrated with 50 µL 200 mM Tris-HCl, with 2% SDS, pH 8.5; 200 µL distilled H₂O and approximately 5 mm² PVDF membrane, pretreated in methanol, were added. After 24 h methanol was added to a final concentration of 10%. Adsorption was allowed for 5 days at room temperature. The membrane was washed 5 times with 10% methanol. The air-dried membrane was subjected to microsequencing as described elsewhere [26] using a Procise 492 protein sequencer in pulsed liquid mode (Applied Biosystems, Foster City, CA, USA).

3 Results

3.1 A novel isoform of Pru av 1 isolated from the cDNA library

The screening of the cDNA library with patients IgE and the gene-specific hybridization probe revealed 19 IgE-reactive clones and 15 clones with positive hybridization signals, respectively. All 15 clones identified by hybridization represented the previously published Pru av 1 [7] sequence (designated Pru av 1.0101 in the following), whereof 6 showed a single base exchange (A426C) that did not affect the deduced amino acid sequence. Sequencing of the recombinants isolated by immunoscreening resulted in two major full-length cDNAs with an open reading frame of 477 base pairs (bp) and a 3- and 5- nontranslated region including a poly(A) tail. Fifteen out of 19 recombinants represented Pru av 1.0101, whereof 9 recombinants showed the base exchange described above. Four clones showed the cDNA sequence of an unknown Pru av 1 isoform (termed Pru av 1.0201 in the following, GenBank AY540507). The sequence identity to Pru av 1.0101 is 78% at the DNA level. The sequence identity at the amino acid level is 73%. The amino acid sequence is shown together with the MS results in Fig. 1.

3.2 2-D immunoblotting reveals Pru av 1 isoforms with diverging IgE-binding properties

A protein extract of ripe cherry fruit was separated by means of 2-D PAGE and Coomassie-stained for total protein analysis and MS. The 2-D maps showed a reproducible protein spot pattern. A representative example is given in Fig. 2A. Proteins were transferred to nitrocellulose membranes to identify Pru av 1 isoforms and to analyze the IgE-binding properties of the different Pru av 1 spots. A series of spots were detected with the mAb G4A. All spots showed slight differences in both, pI and molecular weight. These spots are shown in Fig. 2B and numbered from 1 to 4. The spots with a higher apparent molecular weight were combined in

series b and the spots with a lower molecular weight in series a. The intensive spot No. 2 was not resolved in two different protein spots. Patients IgE bound to the same series of spots as the mAb and additionally to the protein spot No. 5, as shown in Fig. 2C. In parallel, the IgE-binding was inhibited with rPru av 1.0101. As shown in Fig. 2D, rPru av 1 inhibited the IgE-binding to spots 1–4 but not to spot No. 5. The immunoblots detected with patients IgE and with the mAb were matched with a Coomassie-stained gel and the corresponding protein spots were excised for MS analysis.

3.3 Microsequencing and MS identified a further isoform of Pru av 1

The protein spots for *N*-terminal sequencing were identified by matching the immunoblots with a Coomassie-stained PVDF membrane. To confirm the immunoreactive proteins as Pru av 1-related, the spots 1–5 were subjected to *N*-terminal sequencing without distinguishing between series a and b. The first five amino acid residues of all five spots were analyzed and were found to be identical to the *N*-terminus of the previously published sequence of Pru av 1.0101. MALDI-TOF-MS analysis was performed on spots 1, 3, and 4 in series a and b and spot 2 and 5. The spots 1, 3, and 4, both in series a and b, and spot 2 were confirmed as Pru av 1.0101. The sequence coverage was between 31% and 98%. The spectra of spot 5 showed masses which represented 89% of the amino acid sequence of Pru av 1.0201. These data confirmed the results obtained with the cDNA expression library. The MALDI-TOF spectra of spot 5 showed several prominent signals which could neither be confirmed as Pru av 1.0201 nor as peptides representing autodigested trypsin. To determine the amino acid sequence of these peptides MS/MS *de novo* sequencing was performed. The *de novo* sequencing revealed 3 internal peptides with amino acid sequences which were very similar, but not identical to the corresponding peptides from Pru av 1.0201. These findings indicated the presence of two Pru av 1 variants within spot 5, as the peptides starting from position 77 to 94 were sequenced by MS/MS for both Pru av 1.0201 and a second variant (designated Pru av 1.0202; GenBank AY540508). Additional *N*-terminal sequencing after passive adsorption was performed on the series a of the spots 1, 3 and 4, and on spot 2 and 5. The first twenty amino acid residues of Pru av 1.0101 were confirmed in spot 1–4. For spot 5, the first twenty amino acid residues were found to be identical with the *N*-terminus of Pru av 1.0201, with the exception of one single amino acid exchange (P14T). As the sequence could not be completed by MS a PCR strategy, based on the MS/MS *de novo* sequencing and *N*-terminal sequencing results, was used to complete this sequence (see below). A re-evaluation of the MALDI-TOF spectra confirmed the PCR results and revealed that 100% of the amino acid sequence of the variant Pru av 1.0202 were covered by the MS data. A sequence alignment of all detected Pru av 1 isoforms and variants including MALDI-

TOF, MS/MS and *N*-terminal sequencing results for spot 2 and 5 is shown in Fig. 1. The results of the MALDI-TOF and MS/MS analysis for all spots are summarized in Table 1.

3.4 A PCR strategy completed the MS/MS results and revealed another Pru av 1 variant

PCR cloning was done to complete the partial sequence of the new variant Pru av 1.0202 detected by means of MS/MS and *N*-terminal sequencing in spot 5. The Pru av 1.0202 specific forward primer was designed according to the MS/MS and microsequencing results to discriminate a single base exchange to Pru av 1.0201. Pru av 1.0101 cDNA was removed by SAP I digestion of the cDNA, as this sequence could not be discriminated by the gene specific forward primer. Two different cDNA sequences were amplified and sequenced. The open reading frames again were 477 bp long, followed by the 3-noncoding region and a poly(A) tail and encoded for two Pru av 1 variants (Pru av 1.0202 and Pru av 1.0203; GenBank; AY540509). The deduced amino acid sequence of Pru av 1.0202 was in full accordance with the MS/MS and MALDI-TOF data. Pru av 1.0203 could not be confirmed by MS at the protein level. The sequence identity at the amino acid level of these variants is 98%. The sequence identities are 73%–74% to Pru av 1.0101, and 94%–95% to Pru av 1.0201. Both deduced amino acid sequences are shown together with the MS results in Fig. 1.

4 Discussion

The relevance of allergen isoforms is frequently discussed in the context of genetic engineering of either hypoallergenic allergens for immunotherapy, or plants with reduced allergenicity. In both cases, a thorough knowledge is necessary about isoforms and their IgE-binding properties. In our study, we examined the isoform variation of Pru av 1, the major cherry allergen. The combination of proteomic and molecular biology tools in a synergistic approach revealed the existence of a novel isoform of Pru av 1 which comprises three variants, whereof at least two were shown to be present at the protein level. Pru av 1.0101 and Pru av 1.0201 were isolated from a cDNA expression library. The existence of both isoforms and the variant Pru av 1.0202 was confirmed at the protein level. Pru av 1.0203 which was only detected by the PCR strategy may not be present as polypeptide or at least to an extent below the detection limit of the mass spectrometric methods applied. The newly identified isoform shows epitope differences to Pru av 1.0101 which was confirmed as the dominant isoform of the major cherry allergen at the protein and the cDNA level. In contrast to Pru av 1.0101 and all other variants Pru av 1.0201 has a cysteine residue at position 158. As there is no further cysteine residue an effect on the conformation of this monomeric molecule is not very likely. Possibly, intermolecular disulfide

Table 1. Summary of MALDI-TOF and nano ESI-MS/MS data for the spots 1, 3, and 4 in series a and b and spot 2 and 5. A + indicates peptides which were detected with MALDI-TOF or *de novo* sequenced with MS/MS.

Spot	1a	1b	2	3a	3b	4a	4b	5					
	Pru av 1.0101							Pru av 1.0201			Pru av 1.0202		
Peptide position	MS	MS	MS	MS	MS	MS	MS	Peptide position	MS	MS/MS	MS	MS/MS	
1– 17	+	+	+	+	+	+	+	1– 17	+		+		
21– 32	+		+	+	+	+	+	18– 20	+		+		
33– 39	+	+	+	+	+	+	+	21– 32	+	+	+	+	
40– 54	+	+	+	+	+	+	+	33– 39	+		+		
40– 55			+		+			40– 55	+			+	
55– 68	+		+	+	+	+	+	40– 54			+	+	
56– 68	+	+	+	+	+	+	+	40– 55			+		
69– 97			+	+	+			55–68	+		+		
71– 97	+		+	+	+			56– 68	+	+	+	+	
77– 97	+		+	+	+	+		69– 76	+		+		
98–103	+	+	+	+	+	+	+	77– 94	+	+	+	+	
104–115	+		+	+	+	+	+	77– 97			+	+	
104–123			+					98–103			+		
116–123	+	+	+	+	+			98–115	+				
124–129			+					104–115	+	+	+	+	
124–134	+	+	+	+	+			116–129	+	+	+	+	
135–159			+	+	+			116–134	+	+	+	+	
140–145		+	+	+	+	+		130–139	+		+		
146–152			+	+	+	+	+	135–139	+		+		
153–159			+	+	+			140–145	+		+		
								146–159			+		
Sequence coverage	54%	31%	98%	98%	98%	70%	48%	Sequence coverage	89%	47%	100%	58%	

bonds may result in dimer formation and therefore change the migration characteristics in SDS-PAGE. However, cherry extract which was separated under nonreducing conditions showed no IgE-reactive proteins at 34 kDa after detection with sera from allergic patients, who were monosensitized to Pru av 1 (data not shown). Thus, a formation of dimeric molecules does not seem to occur naturally.

A cDNA library was established to detect all putative isoforms of Pru av 1 with a minimized risk of incorrect sequences caused, *e.g.*, by PCR artifacts. The screening procedure was designed to facilitate a sensitive detection of Pru av 1 without discrimination between putative isoforms. The application of a specific hybridization probe did not meet this criterion in our study, whereas a pooled serum of cherry allergic subjects was applied successfully. Nevertheless, even as the application of a pooled serum featured a broad range of epitope specificities and therefore detected the novel isoform Pru av 1.0201, the cDNA library results were very likely restricted for statistical reasons. Pru av 1.0101 and Pru av 1.0201 were detected by screening the cDNA library, whereas Pru av 1.0202 was not detected even though its presence at the protein level was demonstrated by MS/MS and Edman sequencing. In our opinion, the frequency of the corresponding cDNA was too low within the

library to detect it with a reasonable effort. Application of PCR with degenerated primers is generally considered to be sensitive enough to amplify even low-abundant cDNA sequences, but this may not be true in every case. The cDNA sequences of the newly identified isoform of Pru av 1 and its variants were identical within the first 25 base pairs and showed only an exchange of one single base at position 19. Even compared to Pru av 1.0101 the difference is only 5 or 6 bases, respectively, within the first 25 base pairs. Neither a PCR with a 3'-primer specific for Pru av 1.0202 under stringent conditions nor with a 3'-primer specific for Pru av 1.0101 under low stringency conditions on non-SAP I digested cDNA was applied successfully. The sequencing of about 28 clones only revealed the dominant cDNA sequence of Pru av 1.0101 (data not shown). In this case, only the detailed knowledge of the primer binding site based on the MS/MS and N-terminal sequencing and the enzymatic removal of the Pru av 1.0101 cDNA sequence facilitated the amplification of Pru av 1.0202 and Pru av 1.0203. Thus, it seems to be unlikely that a PCR strategy without a specific knowledge of the primer binding sites is optimal for a comprehensive identification of allergen isoforms, leading to the conclusion that isoform identification can be biased if it is exclusively based on PCR.

Application of MS on proteins identified by 2-D immunoblotting proved to be very suitable to confirm the results obtained with the cDNA library and in addition, to detect proteins which correspond to low-abundant cDNA sequences. Even though MS and MS/MS sequencing are powerful tools to identify or confirm novel isoforms at the protein level, these methods often do not display the complete amino acid sequence. The above particular obstacles of MS and cDNA analysis were overcome by synergistic effects when both methods were combined. Nevertheless, the most crucial factor is the choice of the detection antibodies for identifying isoforms with 2 D-PAGE/MS or a cDNA library. This is particularly important for identification of allergen isoforms which may show epitope differences. Helsper *et al.* [11] applied MS on mMal d 1, which was purified by means of mAb affinity chromatography to analyze which isoforms of the major apple allergen derived from a PCR-based analysis of cDNA were present at the protein level. The authors only picked up Mal d 1b at the protein level and discussed whether Mal d 1a and Mal d 1c were present at an amount below their detection limit, or if Mal d 1c did not exist and was a PCR or sequencing artifact. In that context, one should consider that a mAb may not bind to all isoforms which are present in a protein extract. In fact, we detected the novel Pru av 1 isoform only by applying a pooled serum of cherry-allergic patients and not with the mAb. Moreover, the relevance of artifacts generated by PCR or sequencing may be commonly overestimated, as the sequencing of 28 PCR-derived Pru av 1 clones showed only faultless cDNA sequences of Pru av 1.0101. Interestingly, the mAb showed a string of spots in 2-D immunoblotting which turned out to represent only Pru av 1.0101. Thirty-one to seventy percent of the sequence were detected in spots 1 and 4. But as 98% of the sequence were covered for the spots 2, 3a, and 3b by MS and as for all three spots the same missing peptide was covered by microsequencing, differences in the amino acid sequence were not the cause for this series of spots. Post-translational modifications were also not detected. It is more likely that a nonenzymatic deamidation of asparagine or glutamine residues brought up this spot pattern. Sarioglu *et al.* [27] reported this phenomenon for human blood plasma proteins. A mixture of proteins in each spot was detected by the authors and described to differ only in the position of the deamidated asparagine residue. Even if we did not test this hypothesis for Pru av 1, this same phenomenon very likely is the cause for our observations, since no post-translational modifications or changes in the amino acid sequence could be detected.

A large number of allergens and allergen isoforms were described in recent years and most of them have not been confirmed at the protein level, despite the fact that protein analysis is essential for the identification of the clinically relevant forms of allergens. The data presented in our study show that a comprehensive and reliable analysis of allergen isoforms can be achieved by a combination of proteomic and molecular biology tools, both combined with a carefully selected detection system. In our study, the synergistic

effects facilitated the identification and cloning of novel Pru av 1 isoforms and variants, which may, due to their diverging IgE-binding properties, be of interest for diagnostic purposes or immunotherapy.

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