

Identification and Characterisation of Allergens Related to *Bet v* I, the Major Birch Pollen Allergen, in Apple, Cherry, Celery and Carrot by Two-Dimensional Immunoblotting and N-Terminal Microsequencing

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Abstract: Low temperature extracts taken from Granny Smith and Braeburn apples, cherries, celery and carrots were separated by two-dimensional electrophoresis. *Bet v* I, the major birch pollen allergen, related allergens in apple, cherry, celery and carrot extracts were detected by means of two-dimensional immunoblotting with patients' sera containing IgE antibodies specific to *Bet v* I, a rabbit polyclonal antiserum raised against *Bet v* I, and two *Bet v* I specific mouse monoclonal antibodies. The major cross-reacting allergen spots were observed with molecular weights/isoelectric points of 18.0 kDa/pI 5.5 for apple (Granny Smith and Braeburn) and 18.0 kDa/pI 5.8 for cherry, 15.5 kDa/pI 4.4–4.6 for celery and 16.0 kDa/pI 4.4 for carrot extract. Additional antibody reactivities with certain isoprotein spots were observed, which may indicate the presence of *Bet v* I-related epitopes on these proteins. Based on the first 15 N-terminal amino acid residues, the major allergen spots revealed 53% sequence identity between *Bet v* I and the Granny Smith apple allergen, 50% between Braeburn apple allergen and *Bet v* I, 67% between *Bet v* I and the cherry allergen, and 40 and 28% for celery and carrot, respectively. Furthermore, the N-terminal sequences showed identities ranging from 40% (apple/cherry) to 66% (celery) with *PcPR* 1-1, a pathogenesis-related protein in parsley.

Key words: Apple allergens, birch pollen allergens, carrot allergens, celery allergens, cherry allergens, cross-reactivity, immunoblotting, N-terminal microsequencing, two-dimensional electrophoresis.

INTRODUCTION

Most of the allergic reactions to vegetable foodstuffs are of the immediate type (type I allergy according to Coombs and Gell (1963)) which is mediated by allergen-specific IgE antibodies. The food allergy to native

apples, hazelnuts and other fresh fruits (eg cherry, plum, peach, kiwi) as well as to carrot and celery, is highly associated with IgE-mediated hypersensitivity to birch pollen (Calkhoven *et al* 1987; Dreborg 1988; Thiel 1988; Helbling *et al* 1993). Up to 70% (Eriksson *et al* 1982) of these birch pollen sensitised patients suffer from mostly local symptoms (ie oral itching, tingling, angioedema of the throat) after ingestion of the above-

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mentioned fruits and vegetables (Dreborg 1988; Ortolani *et al* 1988). The observed clustering of hypersensitivities, which usually exceed the taxonomic borders between food plant families, is due to cross-reactions of specific IgE antibodies with corresponding epitopes on allergens of the various allergen sources named (Halmepuro *et al* 1984; Halmepuro and Løwenstein 1985; Calkhoven *et al* 1987; Vieths *et al* 1992a).

Three major distinct cross-reactive structures have been identified in birch pollen and the vegetable food-stuffs in question. Besides profilins, a family of highly cross-reacting plant pan-allergens recently described (Valenta *et al* 1992; Van Ree *et al* 1992), and so-called 'cross-reactive carbohydrate determinants' (CCD) (Aalberse *et al* 1981), most of the birch pollen related food hypersensitivities are taken to be caused by a family of plant proteins sharing common epitopes with *Bet v* I, the major allergen of birch pollen (Ebner *et al* 1991; Hirschwehr *et al* 1992; Vieths *et al* 1993a, b).

Furthermore, the deduced amino acid sequence of a cDNA clone coding for *Bet v* I (MW: 17 kDa) showed a high degree of sequence identity with some plant 'pathogenesis related proteins' (PRP) (Breiteneder *et al* 1989). These proteins are produced by plants in response to stress conditions such as microbial attack, but their detailed function remains unknown (Somssich *et al* 1988; Walter *et al* 1990).

Common structures of *Bet v* I and a 17–18 kDa allergen from Golden Delicious have been identified by immunoblot and immunoblot-inhibition studies with patients' sera and monoclonal antibodies (MAb) specific to *Bet v* I (Ebner *et al* 1991; Vieths *et al* 1993a). Northern blotting, using the *Bet v* I cDNA clone as a probe, revealed cross-hybridisation of birch pollen and Golden Delicious apple allergen, suggesting significant homology at the nucleic acid level (Ebner *et al* 1991).

Recently, these results were confirmed by further characterisation of the Golden Delicious apple allergen by two-dimensional immunoblotting and *N*-terminal microsequencing (Vieths *et al* 1994a). Based on 26 amino acid residues, *N*-terminal sequencing of the Golden Delicious apple allergen revealed 62% sequence identity with *Bet v* I, supporting the view that both proteins express common IgE-reactive epitopes.

The results of additional one-dimensional immunoblot and immunoblot inhibition experiments pointed to *Bet v* I-related allergens in pear (Vieths *et al* 1993a), celery (Vieths *et al* 1994b), carrots and cherries (Schöning and Vieths, unpublished).

In order to confirm the presumed relationship between *Bet v* I and the corresponding allergens in fruits and vegetables, two-dimensional immunoblotting and *N*-terminal microsequencing analysis with some vegetable foods were carried out. This paper reports the identification and characterisation of *Bet v* I-related allergens in two additional frequently cultivated apple

strains (Granny Smith and Braeburn), cherries, celery and carrot.

MATERIALS AND METHODS

Patients' sera

The birch pollen pool serum Wn 13/32 ($n = 7$) had RAST classes of 4.0 to birch pollen, 3.6 to Granny Smith apples, 2.8 to Braeburn apples, 3.8 to cherry, 2.7 to celery and 3.7 to carrot (RAST: radioallergosorbent test, semi-quantitative non-competitive immunoassay for the determination of allergen-specific IgE in human sera. RAST class 0 = no specific IgE, RAST 4 = very high content of specific IgE).

Patient B was allergic to birch pollen, fresh apples and several other fruits, to hazelnuts, celery, carrots and some spices. Serum B had RAST classes of >4.0 to birch pollen, 3.7 to Granny Smith apples, 3.9 to Braeburn apples, 3.8 to cherry, 3.4 to celery and 2.7 to carrot. Patient 230 was allergic to birch pollen, fresh apples and several other fruits, celery and carrots. Serum 230 had RAST classes of >4.0 to birch pollen, >4.0 to apples and >4.0 to celery.

Serum B and Serum 230 exclusively reacted to *Bet v* I on one-dimensional birch pollen immunoblots, whereas pool serum Wn 13/32 had additional IgE-antibodies against a 35 kDa component of birch pollen.

RAST classes were determined at the Institut für Lebensmittelchemie [Institute of Food Chemistry] by a commercially available ELISA (EAST) containing a monoclonal anti-IgE alkaline phosphatase conjugate and *p*-nitro-phenylphosphate as the substrate, according to the manufacturer's instructions (Allergopharma, Reinbeck, Germany).

Polyclonal antiserum and monoclonal antibodies

The rabbit polyclonal antiserum raised against *Bet v* I (rb-anti-*Bet v* I) and the mouse monoclonal anti-*Bet v* I antibody MAb 'D' were kindly supplied by Allergopharma (Dr B Weber, Dr R Wahl, Reinbeck, Germany). The *Bet v* I-specific MAb 'BIP 1' was a gift from Prof Dr D Kraft (Vienna, Austria). Culture supernatants of both MAb were used.

Allergenic extracts

Commercial birch pollen extract was obtained from Allergopharma (Reinbek, Germany) and contained 1100 µg of protein per ml. Apples, cherries, celery roots

and carrots used for this study were bought at the local market. Because some allergens of fruits and vegetables are highly labile, food extracts were prepared from uncooked material by a low-temperature acetone powder method developed in the authors' laboratory (Vieths *et al* 1992b). The acetone powders were extracted with 0.01M potassium phosphate buffer containing 0.15M sodium chloride, pH 7.4 (PBS) and centrifuged. The supernatants were stored freeze-dried at -20°C until use.

Prior to two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), the freeze-dried extracts were reconstituted, washed twice with double-distilled water and concentrated three times using disposable micro-concentrators (Centricon3, cut-off: 3000 Da, Amicon, Witten, Germany). For 2-D PAGE, 35 μg of total protein were applied in 2-D PAGE sample buffer (Vieths *et al* 1992c).

Two-dimensional electrophoresis

2-D PAGE was carried out as previously described (Vieths *et al* 1992c) with some modifications: All polyacrylamide (PAA) gels were prepared from a commercial stock solution containing 300 g litre⁻¹ acrylamide (AA) and 8 g litre⁻¹ methylene-bis-acrylamide (BIS) (Rotiphorese Gel 30, Roth, Karlsruhe, Germany). The isoelectric focusing (IEF) step (first dimension) was performed under native conditions in PAA rod gels (diameter 3.0 mm, $T = 4.8\%$, $C = 2.6\%$) with different pH gradients resulting from 94 ml litre⁻¹ ampholine-carrier (Servalyt T from Serva, Heidelberg, Germany). Servalyt T 3-10 was used for the separation of apple, cherry and carrot extracts, Servalyt T 4-6 for the separation of the birch pollen proteins and Servalyt T 4-5 gave the best results for celery extract. For the second dimension, vertical discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (disc-SDS-PAGE according to Lämmli (1970)) was carried out under non-reducing conditions as described (Vieths *et al* 1992c), with the modification of using 130 g litre⁻¹ resolving gels (1.0 mm; $T = 12.8\%$; $C = 2.7\%$).

Semi-dry blotting

For immunodetection, the separated proteins were transferred onto nitrocellulose (NC) membranes by semi-dry blotting (Khyse-Andersen 1984) for 45 min at 0.8 mA cm^{-2} as described (Vieths *et al* 1992c). For microsequencing of the allergens, semi-dry blotting onto polyvinylidene difluoride (PVDF) membranes (TransBlott, Bio-Rad, Munich, Germany) using a Pegasus blotter (Phase, Lübeck, Germany) was used for 45 min at 0.8 mA cm^{-2} . The transfer buffer was 10 mM 3-(cyclohexylamino)-1-propane-sulphonic acid (CAPS)

with methanol/water (1 : 9 v/v), pH 11.0 (Matsudaira 1987).

Protein staining and immunodetection

The dried NC membranes were blocked twice for 15 min in 0.01M PBS, pH 7.4 containing Tween 20 (3 ml litre⁻¹). To minimise non-specific binding, no protein was added to the blocking solution (Vieths *et al* 1992c). For total protein staining, the blocked NC membranes were incubated in India Ink (1 ml litre⁻¹) (Pelikan, Hannover, Germany), acetic acid 10 ml litre⁻¹, Tween 20 (0.5 ml litre⁻¹) in PBS (Hancock and Tsang 1983).

All sera and immunoreagents were diluted in PBS including Tween 20 (0.5 ml litre⁻¹) bovine serum albumin (BSA) (1 g litre⁻¹) (incubation buffer). In order to identify the *Bet v* I-related allergens in our fruit and vegetable extracts after separation by 2-D PAGE and semi-dry blotting, immunodetections were performed with (A) patients' antisera (diluted 1 : 25 in 10 ml total volume), (B) rabbit anti-*Bet v* I antiserum (diluted 1 : 7000 in 35 ml total volume), and (C) the *Bet v* I-specific MAb (diluted 1 : 175 in 35 ml total volume). Primary antibody incubations were carried out overnight and followed by biotin/streptavidin-amplified immunodetection using horseradish peroxidase (HRP) as the marker enzyme and 3,3',5,5'-tetramethylbenzidine as the substrate (Vieths *et al* 1992c). Bound IgE antibodies (A) were detected with (i) rabbit polyclonal anti-human IgE (1 : 4000, 1 h), (ii) goat polyclonal biotinylated anti-rabbit IgG (1 : 6000, 1 h) (both from DAKO, Copenhagen, Denmark), and (iii) streptavidin-HRP conjugate (1 : 20 000, 20 min) (Medac, Hamburg, Germany). For detection of (B) rabbit anti-*Bet v* I antibodies, the anti-IgE incubation step was omitted. The bound MAb (C) were detected with rabbit biotinylated anti-mouse IgG (1 : 3000, 1 h) (Jackson, obtained from Dianova, Hamburg, Germany), followed by streptavidin-HRP and the substrate as described. After each incubation step, the blots were washed three times with Tween 20 (0.5 g litre⁻¹) in PBS.

Edman degradation

The PVDF membranes containing the allergens were washed with double distilled water for 30 min, followed by a total protein stain with Coomassie Brilliant Blue (1 g litre⁻¹) (Merck, Darmstadt, Germany) in water/methanol (1 : 1 v/v) for 10 min, destained in water/methanol (1 : 1 v/v), again washed with double-distilled water and air-dried (Petersen *et al* 1992).

Protein spots containing the allergens were cut out. Four protein spots per allergen were used for the microsequencing analysis (Matsudaira 1987). Microsequencing was performed by Edman degradation using

a 473 A pulsed liquid protein sequencer with an on-line phenylthiohydantoin (PTH) amino acid analyser (Applied Biosystems, Weiterstadt, Germany).

RESULTS

2-D PAGE

Separation of the fruit and vegetable extracts (Granny Smith and Braeburn apple, cherry, celery and carrot) was accomplished by 2-D PAGE followed by semi-dry electroblotting. To keep denaturation of the proteins as low as possible, IEF (first dimension) was performed without using urea, disc-SDS-PAGE (second dimension) was carried out under non-reducing conditions and no alkylating agents were used.

After protein staining with India Ink, more than 50 protein spots were detectable in each of the different extracts having molecular weights of 14–80 kDa, and pI values ranging between 4.3 and 9.0. Protein spots with identical or similar molecular weights but different pI occurred in the molecular weight regions 14–20 kDa and 30–80 kDa. As an example, the result with carrot extract is shown in Fig 1.

The presumed cross-reactive allergens of the fruit and vegetable extracts consisted of one intensive major spot accompanied with two or three smaller protein spots (see Fig 1 and Table 1). These smaller accompanying spots revealed similar molecular weights but different isoelectric points and are therefore termed isoproteins of the major protein spot in the following text (in accordance with the term isoallergen used in the aller-

gological literature (Aas 1988)). Separation and staining of commercial birch pollen extract revealed three major spots with several isoproteins (see Table 1), which reacted with *Bet v* I-specific antibodies in subsequent immunoblotting experiments.

Detection of *Bet v* I-related allergens by immunoblotting

In order to localise and identify the *Bet v* I-related allergens, the 2-D NC blots were incubated with two different *Bet v* I-specific MAb ('BIP1' and 'MAB D'), one rabbit anti-*Bet v* I antiserum and two human sera. The rabbit antiserum applied contained *Bet v* I-specific IgG antibodies, the two MAb (IgG-type) recognized different single epitopes on the *Bet v* I protein. Cross-reactions with proteins from our fruit and vegetable extracts therefore indicated the existence of *Bet v* I-related epitopes on these proteins. To decide whether these cross-reactive proteins were allergens related to *Bet v* I or not, immunoblotting was practised with different human sera which contained specific antibodies to *Bet v* I.

As examples, the reactions of the human serum B and the rabbit anti-*Bet v* I antiserum with carrot extract after 2-D immunoblotting are presented in Figs 2 and 3, respectively. IgE antibodies from patient B as well as the rabbit antibodies recognized a major protein spot of 16 kDa and pI 4.4. The rabbit polyclonal antiserum also showed additional binding to some isoproteins.

The binding patterns of the different immunoreagents to the presumed allergens of the various plant food extracts are summarised in a somewhat simplified form in Table 1 and compared with birch pollen *Bet v* I. The

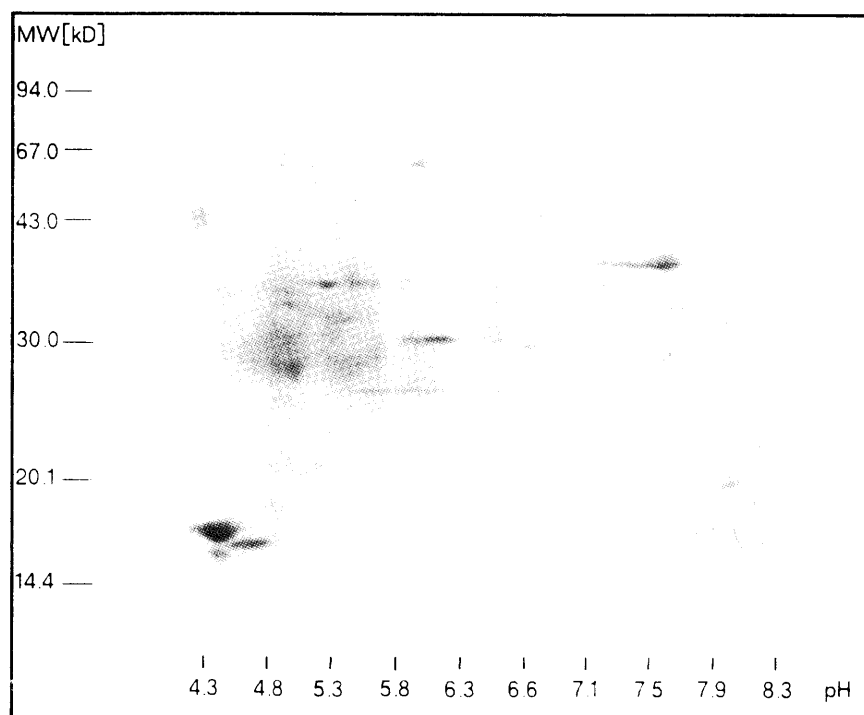


Fig 1. Protein staining of carrot extract with India Ink after 2-D PAGE and semi-dry electroblotting onto NC membranes.

TABLE 1

Molecular weight (MW) and isoelectric points (pI) of Bet v I and related proteins in apple, cherry, celery and carrot. Data were obtained after 2-D PAGE, semi-dry electroblotting onto NC membranes and following immunoblotting with either one of two different mouse monoclonal anti-Bet v I antibodies (MAB BIPI and MAB D), one rabbit polyclonal anti-Bet v I antiserum (rb-anti Bet v I) or one of two patients sera (patients with sensitisation to birch pollen). Serum 230 was used instead of serum B in one case (celery). ND = not done.

Bet v I and related proteins	MW (kDa)	pI	Reaction with BIP I	Reaction with MAB D	Reaction with rb-anti Bet v I	Reaction with pool serum Wn 13/32	Reaction with patient serum B
<i>Birch pollen Bet v I</i>							
Three major spots with several isoproteins (Data not shown: pI values ranging from 4.8 to 6.5)	16.0 16.5 17.0	5.6 5.3 5.6	+	+	+	+	+
<i>Apple allergen (Granny Smith)</i>							
Major spot	18.0	5.5	+	+	+	+	+
Isoprotein spots	16.0	5.1; 5.3	-	-	-	-	+
<i>Apple allergen (Braeburn)</i>							
Major spot	18.0	5.5	-	+	+	+	ND
Isoprotein spots	17.0	5.2; 5.5	+	-	+	+	+
<i>Cherry allergen</i>							
Major spot	18.0	5.8	-	-	+	+	+
Isoprotein spots	18.0 16.0	4.9; 5.6 4.9	-	+	+	+	+
<i>Celery allergen</i>							
Major spot	15.5	4.4-4.6	+	-	+	+	(Serum 230) +
Isoprotein spots	15.5	4.9; 5.1; 6.0	-	-	+	-	+
<i>Carrot allergen</i>							
Major spot	16.0	4.4	-	-	+	+	+
Isoprotein spots	15.5 15.0	4.6 4.4	- -	- -	+	- -	- -

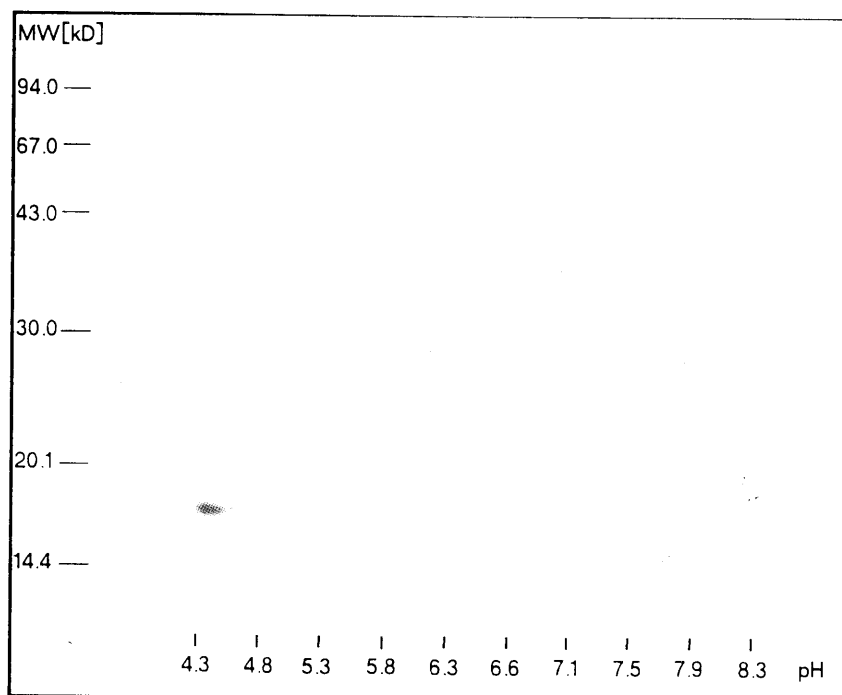


Fig 2. Reactivity of IgE antibodies of serum B to carrot extract after 2-D PAGE and semi-dry electroblotting onto NC membranes. Serum dilution was 1 : 25.

highly cross-reacting major protein spots were recognised as *Bet v* I-related allergens from these data and therefore selected for microsequencing.

Microsequencing analysis

After staining with CBB, the major spots of the allergens in question could be localised on the PVDF mem-

brane. For the analysis of each protein, spots from four different PVDF membranes were cut out and simultaneously subjected to the microsequencing analysis. The *N*-terminal amino acid sequences obtained are presented in Table 2 and compared to *Bet v* I (Breiteneder *et al* 1989). Three amino acid residues not clearly identified (Braeburn apple, cherry, carrot) are indicated with an X. Two residues, which were only weakly detected,

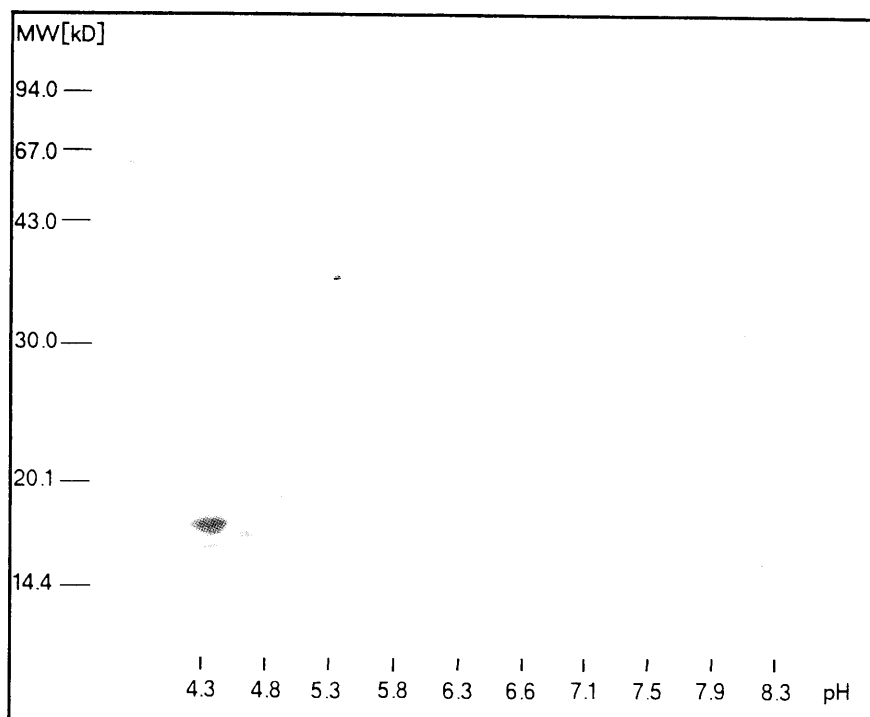


Fig 3. Reactivity of rabbit polyclonal anti-*Bet v* I antiserum to carrot extract after 2-D PAGE and semi-dry electroblotting onto NC membranes. Serum dilution was 1 : 7000.

TABLE 2^a
N-terminal amino acid sequences of the *Bet v I* related allergens of apple, cherry, celery and carrot in comparison to *Bet v I*, the major birch pollen allergen (Breiteneder *et al* 1989).

N-terminal amino acid sequence of	5					10					15					20					25				
Birch pollen <i>Bet v I</i>	G	V	F	N	Y	E	T	E	T	S	V	I	P	A	A	R	L	F	K	A	F	I	L	D	G
Apple allergen (Granny Smith)	G	V	Y	T	F	E	N	E	Y	T	S	E	I	P	P	P	R	L	F	K	A	F	I	L	D
Apple allergen (Braeburn)	X	V	Y	T	F	E	N	E	Y	T	S	E	I	P	P	P	R	L	F	K	A	F	I	L	D
Cherry allergen	G	V	F	T	Y	E	S	E	F	T	S	E	I	P	P	P?	R	L	F	X	A	F	I	L	D
Celery allergen	G	V	Q	S	T/H	V	L	E	L	T	S	S	V	S?	A	E	K	I	F	Q	G	F	I	L	D
Carrot allergen	G	A	Q	S	H	S	L	E	I	T	X	S	V	S?	A	E	K	I	F						

^a To ease the recognition of conserved amino acid residues, identities are indicated in bold.

TABLE 3^a
N-terminal amino acid sequences of the *Bet v I* related allergens of apple (Granny Smith, Braeburn), cherry, celery and carrot in comparison to the 18 kDa apple allergen (Golden Delicious) Vieths *et al* 1994a).

<i>N</i> -terminal amino acid sequence of	5					10					15					20					25					
Apple allergen (Golden Delicious)	G	V	Y	T	F	E	N	E	F/Y	T	S	E	I	P	P	P	R	L	F	K	A	F	V	L	D	A
Apple allergen (Braeburn)	X	V	Y	T	F	E	N	E	Y	T	S	E	I	P	P	P	R	L	F	K	A	F	V	L	D	A
Cherry allergen	G	V	F	T	Y	E	S	E	F	T	S	E	I	P	P	P	R	L	F	X	A	F	V	L	D	A
Celery allergen	G	V	Q	S	T/H	V	L	E	L	T	S	S	V	S?	A	E	K	I	F	Q	G	F	V	L	D	A
Carrot allergen	G	A	Q	S	H	S	L	E	I	T	X	S	V	S?	A	E	K	I	F							

^a To ease the recognition of conserved amino acid residues, identities are indicated in bold.

TABLE 4^a
N-terminal amino acid sequences of the *Bet v I* related allergens of apple, celery and carrot in comparison to *PcPR 1-1*, a pathogenesis related protein from parsley (Somssich *et al* 1988).

<i>N</i> -terminal amino acid sequence of	5					10					15					20					25					
Parsley <i>PcPR 1-1</i>	G	V	Q	K	S	E	V	E	T	T	S	S	V	S	A	E	K	L	F	K	G	L	C	L	D	I
Celery allergen	G	V	Q	S	T/H	V	L	E	L	T	S	S	V	S?	A	E	K	I	F	Q	G	F				
Carrot allergen	G	A	Q	S	H	S	L	E	I	T	X	S	V	S?	A	E	K	I	F							
Apple allergen (Granny Smith)	G	V	Y	T	F	E	N	E	Y	T	S	E	I	P	P	P	R	L	F	K	A	F	V			

^a To ease the recognition of conserved amino acid residues, identities are indicated in bold.

TABLE 5

Degree of identity of the first 15 N-terminal amino acid residues with *Bet v* I¹, *PcPR 1-1*² and the 18 kDa apple allergen (Golden Delicious).³

	Identity of N-terminal sequence with		
	<i>Bet v</i> I ¹	<i>PcPR</i> 1-1 ²	Apple allergen (Golden Delicious) ³
Apple (Granny Smith)	53%	40%	100%
Apple (Braeburn)	50%	36%	100%
Cherry	67%	40%	80%
Celery	40%	66%	33%
Carrot	28%	57%	21%

¹ Breiteneder *et al* 1989

² Somssich *et al* 1988

³ Vieths *et al* 1994a

one most likely serine in celery/carrot and the other most likely proline residue in cherry, are marked. At position no 5 of the celery allergen, threonine and histidine were simultaneously detected. Tables 3 and 4 demonstrate identities of the N-terminal sequences of the *Bet v* I-related allergens to the 18 kDa allergen of Golden Delicious apple (Vieths *et al* 1994a) and a pathogenesis-related protein from parsley (Somssich *et al* 1988), respectively. To ease the recognition of conserved amino acid residues and sequences, identities are indicated in bold.

In addition to Tables 2–4, in Table 5 the degrees of the N-terminal sequence identities of the allergens from Granny Smith and Braeburn apples, cherry, celery and carrot with *Bet v* I, *PcPR 1-1* and the 18 kDa allergen of Golden Delicious apples are compared.

DISCUSSION

The modified 2-D PAGE applied was found to be very suitable for the analysis of our fruit and vegetable extracts. Due to the two independent parameters used for separation, high resolution was obtained (see Fig 1). In addition, sample treatment under non-reducing conditions probably preserved allergenic epitopes of the proteins on NC blots.

By 2-D immunoblotting we were able to demonstrate allergens with *Bet v* I-related epitopes in apple, cherry, celery and carrot extracts. Microheterogeneity of the *Bet v* I-related allergens seems to be poorer when compared to birch pollen *Bet v* I. Only one major spot and two or three smaller isoprotein spots were detected for the different allergens (see Table 1). In accordance with other authors (Jarolim *et al* 1989; Ipsen *et al* 1993) and the authors' own previous results (Vieths *et al* 1992c), *Bet v* I was found to consist of many isoprotein spots with slightly different molecular weights and pI values between pH 4.8 and 6.5. This heterogeneity of *Bet v* I isoallergens is assumed to result from differences in glycosylation or multiple gene loci coding for this protein (Jarolim *et al* 1989). All of the immunodetection

reagents applied (MAb 'BIP1' and 'MAb D', rabbit anti-*Bet v* I antiserum and human sera B and Wn 13/32) showed binding to the major three spots of *Bet v* I, but different reactions with the smaller isoprotein spots. From the data recorded, the major protein spots were selected for microsequencing to obtain additional information of the molecular structure for the allergens under study.

Due to their high degree of sequence identity, the N-terminal amino acid sequences obtained confirmed the hypothesis that these allergens are *Bet v* I-related (see Tables 2–5). In addition some of the amino acid residues are similar, for example valine and isoleucine in position number 13 for carrot/*Bet v* I (see Table 2). Therefore, the degree of similarity is even higher, for example a value of 67% similarity for apple (Granny Smith)/*Bet v* I compared to only 53% sequence identity apple (Granny Smith)/*Bet v* I (degrees based on the first 15 N-terminal residues). The levels of sequence identity between the allergens in comparison and *Bet v* I may indicate different degrees of relationship, with the fruit allergens being more closely related to *Bet v* I than the vegetable ones. These findings are confirmed by the results of our immunodetections presented in Table 1. The apple allergens gave the most positive reactions with *Bet v* I-specific antibodies, followed by cherry, celery and carrot allergen in declining order. These results correspond to the anamnesis data and reports of *Bet v* I allergic patients who have been participating in these studies. Most of them show additional sensitisation to fresh apples and other stone fruits and to a lesser extent to uncooked celery and carrot. Cross-reactivity studies of birch pollen and plant food allergens already performed suggest that all allergenic determinants of the vegetable foods are present in birch pollen but that only some, but not all, epitopes of the birch pollen allergens can be found in fruits and vegetables (Björkstén *et al* 1980; Halmeupuro *et al* 1984; Ebner *et al* 1991; Vieths *et al* 1992a, 1993a). The 2-D immunoblotting and microsequencing data reported here further support this view.

A very close relationship could be stated for the three different apple allergens studied so far and the cherry allergen. Similarities are up to 100% among Granny Smith, Braeburn and Golden Delicious (see Tables 3 and 5), cherry revealed 80% sequence identity and 93% similarity to Golden Delicious respectively. All four fruit sequences studied so far showed three consecutive proline residues in positions 14–16, an uncommon sequence which is not present in *Bet v* I. On the other hand, the high degree of identity between the N-terminal sequences from celery and carrot (15 residues out of 18) indicates a close relationship for the two vegetable allergens.

The degree of sequence identity between *Bet v* I, the *Bet v* I-related allergens and some plant PRP, for example, *PcPR 1-1*, a 'pathogenesis-related protein' from parsley, is striking (see Tables 4 and 5). Synthesis

of PRP is induced by a wide variety of infectious agents and leads to acquired resistance of the plant (Somssich *et al* 1988; Walter *et al* 1990). According to Breiteneder *et al* (1989), it is not unlikely that *Bet v 1* fulfils a similar function in birch pollen. The mentioned PRP were discovered after treating plant cell cultures with different elicitors (Somssich *et al* 1988; Walter *et al* 1990), in contrast to *Bet v 1* which is constitutively expressed in birch pollen. The detailed function of this group of PRP has remained unknown but latest findings suggest that these proteins may have ribonuclease activity (Moiseyev *et al* 1994).

In previous studies we were able to demonstrate that the allergenic potency of apples depends on the occurrence of the *Bet v 1*-related 18 kDa allergen (Vieths *et al* 1993b, 1994c). The relative amount of this allergen varies greatly among fruits from different strains and increases during ripening. A comparative analysis of apple fruits of the same strain but grown under different conditions (biological/conventional cultivation) revealed no clear differences (unpublished results). Up to the present the function of *Bet v 1* in birch pollen and the related allergens in apples, other fruits and vegetables has remained unknown and needs further investigation.

CONCLUSION

The results of the present study support the hypothesis that a considerable part of the birch pollen related food hypersensitivities is caused by a family of low molecular weight allergens with a high degree of sequence identity to *Bet v 1*, all of them probably belonging to a group of PRP. Individual sensitisation patterns of different patients may be due to the sequence variations among the *Bet v 1*-related food allergens.

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