

Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1

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Summary

Background Up to 70% of patients with birch pollen allergy exhibit the so-called oral allergy syndrome, an IgE-mediated food allergy. The most frequent and therefore best characterized pollen–fruit syndrome is apple allergy in patients suffering from tree pollen-induced pollinosis. The occurrence of adverse reactions to proteins present in vegetables such as celery and carrots in patients suffering from pollen allergy has also been reported. cDNAs for Bet v 1 homologous proteins have been cloned from celery, apple and cherry.

Objective The aim of the study was to identify Bet v 1 homologues from carrot (*Daucus carota*), to isolate the respective cDNA, to compare the IgE-binding capacity of the natural protein to the recombinant allergen and determine the cross-reactivity to Api g 1 and Bet v 1. **Methods** Molecular characterization of the carrot allergen was performed using IgE-immunoblotting, cross-inhibition assays, N-terminal sequencing, PCR-based cDNA cloning and expression of the recombinant protein in *Escherichia coli*.

Results A 16-kDa protein from carrot was identified as a major IgE-binding component and designated Dau c 1. Sequencing corresponding cDNAs revealed three extremely similar sequences (Dau c 1.1, 1.2 and 1.3) with an open reading frame of 462 bp coding for 154 amino acid residues.

Conclusions Purified recombinant Dau c 1.2 was tested in immunoblots displaying IgE-binding capacity comparable to its natural counterpart. Cross-inhibition assays verified the existence of common B-cell epitopes present on Dau c 1, Api g 1 as well as on Bet v 1.

Keywords: Dau c 1, Bet v 1, Api g 1, food allergen, carrot, IgE-binding, recombinant allergen

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Introduction

According to European studies food allergy as an IgE-mediated immune reaction to certain dietary antigens is supposed to affect 1–2% of the adult population [1]. These adverse reactions to food antigens seem to be more

frequent in young children (1–8% [2,3]) and may change and/or disappear during their adolescence. The panel of allergens causing these immunological reactions also changes during the lifetime. In their first year of life, food-allergic children suffer from allergic reactions to proteins from egg (49%), milk (39%) and peanuts (25% [4]), whereas children older than 10 years primarily display allergy against proteins from fruits and vegetables [5]. Food-allergic adults show symptoms when eating vegetables such as celery (43%) or proteins from milk (16%),

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carrot (13%), egg (12%) and fish (7% [6]). The prevalence of reactions to specific foods may also depend on the eating habits of a certain population, the geographical characteristics and lifestyle.

The symptoms of food allergy comprise local reactions: oral itching, inflammation of the lips and oral mucosa (oral allergy syndrome (OAS) [1], angioedema of the throat, gastroenteritis and diarrhoea and a considerable number of patients experience systemic reactions (urticaria, asthma, anaphylactic shock).

Patients suffering from allergy to certain pollens — particularly birch and mugwort pollen — often react with OAS to certain fruits, vegetables and spices. This syndrome is characterized by immediate symptoms of the lip, mouth, pharynx and larynx, yet symptoms may also be observed in organs other than the oral cavity after around 30–60 min. Usually, these hypersensitivity reactions are triggered by clusters of various plant-derived foods, such as watermelon and tomato or apple, apricot, cherry, pear, or celery, carrot, fennel and parsley. Allergy to watermelon and tomato may be associated with grass pollen allergy [7,8]. Celery, carrot, fennel and parsley seem to be associated with birch pollen, as well as with mugwort pollen allergy (Apiaceae birch mugwort syndrome [9–12]). In the recent past, homologous sequences of Bet v 1, the major birch pollen allergen, have been identified in a range of fruits and vegetables by IgE-immunoblotting [13]. The Bet v 1-related sequences from apple (Mal d 1 [14]), celery (Api g 1 [15]); and cherry (Pru a 1 [16]); have already been isolated, cloned and sequenced.

Here we present three complete coding sequences of Bet v 1 homologues (Dau c 1.1, 1.2 and 1.3) from carrot (*Daucus carota*). The respective recombinant proteins were expressed in *E. coli*, and the allergenic features of one of these proteins were investigated in detail in comparison to its natural counterpart and in relation to Api g 1 and Bet v 1.

Materials and methods

Extraction and isolation of poly(A)-enriched RNA

Total RNA was extracted from 100 g carrot tuber (*D. carota* var. Nantaiser) according to the acidic guanidinium thiocyanate method [17]. The tuber and green leaves from carrot were chopped, frozen in liquid nitrogen and ground to a fine powder in a grinding machine. Subsequently, the powder was transferred to cooled tubes, the extraction buffer was added and the RNA was extracted as described in detail [17]. Poly (A)-RNA was isolated from total RNA by biotinylated oligo(dT) and streptavidin coupled to paramagnetic particles (PolyAtract mRNA Isolation System, Promega, Madison, WI, USA).

Synthesis of first-strand cDNA

Two micrograms poly(A)-enriched RNA were reverse transcribed using 50 U Moloney murine leukaemia virus reverse transcriptase (Gene Amp RNA PCR Kit; Perkin Elmer, Cetus, Norwalk, CT, USA). A synthetic oligonucleotide was used as a primer for first-strand cDNA synthesis (5'-T₁₄NN-3') which was performed at 42 °C for 60 min followed by a denaturation step at 99 °C for 5 min.

N-terminal sequence of Dau c 1, and selection of primers for DNA amplification

Proteins were extracted from carrots and subjected to SDS-PAGE analysis and immunoblotting. A predominant IgE-binding component, migrating at $\approx$ 16 kDa was identified as a major allergen and designated Dau c 1. This protein was purified by RP-HPLC (C8) as described previously [18] and the N-terminal sequence was determined by Edman degradation. Degenerated oligo-deoxynucleotides were synthesized according to the first eight amino acid residues: GAQSHSLE, 5'-GGATCC GGN GCN CAG/A AGC CAT/C TCN C/TTN G-3' and a *Bam* H I restriction site (underlined) was added. The downstream primer was either 5'-T₁₄NN-3' or according to the C-terminal sequence of the pathogenesis-related (PR) protein from carrot (Accession No. D88388 GenBank database). A *Pvu* II restriction site (underlined) was added (5'-CAGCTG TTA ATT AGC AAT GAG GTA GGC C-3').

Amplification of Dau c 1 cDNA by PCR

The products of the first strand cDNA synthesis were subjected to DNA amplification by PCR (Ampli Taq DNA Polymerase Perkin Elmer, Cetus). After a hot start (94 °C for 3 min), 30 cycles were performed using 94 °C for denaturation for 45 s, annealing temperature of 55 °C (30 s) and elongation at 72 °C for 30 s and additional 7 min at 72 °C. PCR products were analysed on an 1.2% agarose gel and the predominant bands of $\approx$ 480 bp and 650 bp, respectively, were excised, purified by silicamatrix (GENE Clean II Kit, Bio101, La Jolla, CA, USA) and cloned into the pCRTMII vector and transfected in the *E. coli* strain INV α F' (TA cloningTM kit, Invitrogen Corporation, San Diego, CA, USA). Inserts were sequenced by the dideoxy method by the use of fluorescence-labelled primers according to the vector sequence and analysed on an automatic sequencer (Licor, Lincoln, NE, USA).

Expression and purification of recombinant Dau c 1

The complete Dau c 1-encoding sequence (Dau c 1.2 and Dau c 1.3) was subcloned into the high level expression

plasmid, pDS 56 [19]. By ligating the coding sequences into the *Bam* HI and *Pvu* II restriction sites of the expression plasmid, Dau c 1 was expressed as a hexahistidine-tagged fusion protein in transformed *E. coli* M15 cells. Briefly, cells were grown in 2xYT medium to an optical density of $A_{600}=0.6$. Expression of the recombinant protein was

induced overnight by inactivation of the *lac* repressor via addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. Subsequently, cells were harvested, lysed and the protein fraction was processed as described [19]. Cell lysates were resuspended in 6 M guanidinium hydrochloride, 0.1 M NaH_2PO_4 , pH 8.0, and purified by Ni-chelate affinity chromatography [19]. Determination of protein concentration of purified rDau c 1 was performed by the micro Kjeldahl assay [20].

Immunoblot analysis of natural and rDau c 1

Total protein extract from carrot roots and purified rDau c 1.2 or rDau c 1.3 (2 μg per lane) were electrophoresed on 12% SDS polyacrylamide gels. Coomassie-stained bands of natural and recombinant Dau c 1 were scanned in an Image system, in order to use equivalent amounts of Dau c 1 for the immunoblots. The IgE-binding capacity of Dau c 1 was investigated by using allergic patients' sera followed by incubation with ^{125}I rabbit antihuman IgE (Pharmacia, Uppsala, Sweden). For control experiments, buffer and normal human serum were tested, respectively.

Cross-inhibition experiments

For inhibition assays a serum pool of carrot-allergic patients was incubated with rDau c 1.2 (30 $\mu\text{g}/\text{mL}$), carrot extract, rBet v 1 (20 $\mu\text{g}/\text{mL}$), rApi g 1 (30 $\mu\text{g}/\text{mL}$) and BSA (30 $\mu\text{g}/\text{mL}$). Subsequently, the remaining IgE-binding capacity of rDau c 1 and natural Dau c 1 was tested.

Patients, sera and antibodies

Sera were collected from five patients with established food allergy to carrots according to case history suffering from OAS and positive prick to prick tests with fresh carrot. For cross-inhibition assays a serum pool derived from six carrot-allergic patients was used. Murine monoclonal antibodies (BIP 1, BIP 4 [21]) were raised against natural Bet v 1 and polyclonal rabbit antiserum was raised against purified rBet v 1a [22].

Results

Isolation of carrot RNA and amplification of Dau c 1-cDNA by PCR

Eighteen milligrams total RNA were isolated from 100 g raw carrot (*D. carota*; wet weight) yielding 60.4 μg mRNA after poly A⁺-purification. After cDNA synthesis, PCR was performed generating cDNAs complementary to Dau c 1-encoding sequences of 465 bp and 655 bp length.

Sequence analysis of Dau c 1

The Dau c 1.1 sequence comprised 655 bp with an open

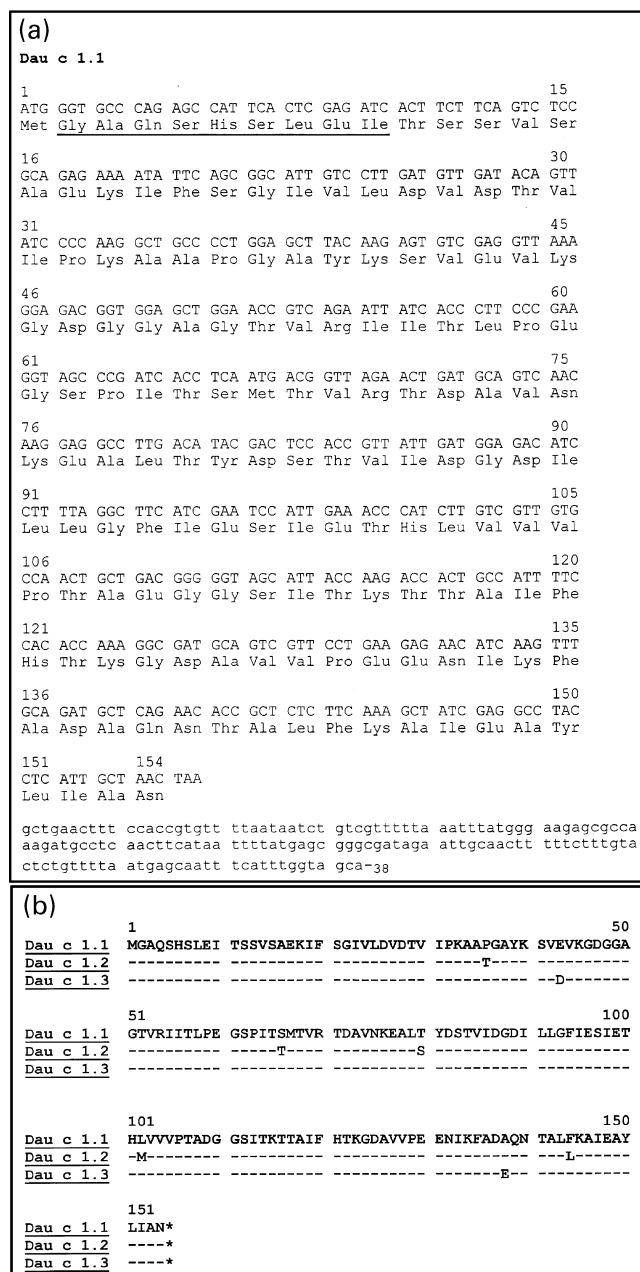


Fig. 1. (a) The nucleotide sequence and deduced amino acid sequence of Dau c 1.1 the major allergen of carrot. The N-terminal amino acid sequence, as determined by Edman degradation, is underlined. (b) The deduced amino acid sequence of Dau c 1.1, 1.2, and 1.3 with reference to Dau c 1.1.

reading frame of 462 bp coding for 154 amino acid residues with a deduced molecular mass of 16.049 kDa (Fig. 1a,b). A poly (A) tail was located 152 bp downstream from the TAA translation stop codon. A search of the nucleotide databases revealed high sequence identity to two cDNA clones isolated from carrot roots and somatic embryos [23,24]. Lin *et al.* [23] isolated a partial clone lacking two nucleotides in the coding region leading to two unidentified amino acid residues at position 21 and 22 and lacking a full 3' non-coding region. Nevertheless, this sequence seems to be identical to the full length clone with the complete 3'-region submitted by Yamamoto *et al.* [24].

The 3' non-coding region of Dau c 1.1 is 152 bp long in contrast to the downstream region of 225 bp of clone D88388 [24]. Stretches of high sequence similarity are interrupted by sequences not found in Dau c 1.1. Two additional isoforms, Dau c 1.2 and Dau c 1.3 were identified when sequencing PCR-products obtained with primers according to the N-terminus and the C-terminus of the protein. Dau c 1.2 and Dau c 1.3 sequences code for 154 amino acid residues with a deduced molecular mass of 16.071 kDa (Dau c 1.2) and 16.093 kDa (Dau c 1.3), respectively (Fig. 1b). The predicted isoelectric points are 4.47 for Dau c 1.1 and Dau c 1.2 and 4.39 for Dau c 1.3.

Sequence comparison among the individual isoforms of Dau c 1 revealed a similarity of 97–100% and identity ranging from 96 to 99% on the amino acid level (Fig. 1b). Moreover Dau c 1.1 displayed a sequence similarity of 93% (81% identity) to Api g 1, the major celery allergen (Table 1). Celery is a member of the same plant family (Apiaceae). A parsley pathogen-inducible protein (Apiaceae PcPR1 [25]); has 76% sequence similarity (60% identity) to Dau c 1.1 (Table 1).

Sequence similarity to other members of the Bet v 1

family, like Bet v 1 (Fagaceae), the major birch pollen allergen (61% similarity; 38% identity) or the major apple allergen Mal d 1 (Rosaceae) are much lower (59% similarity; 39% sequence identity), reflecting the distant taxonomical relations (Table 1).

Expression and purification of rDau c 1

Dau c 1.2 encoding cDNA was expressed as a 6 His-tagged fusion protein in *E. coli* M15. The heterologous expression of the carrot allergen resulted in the most prominent protein band from bacterial lysates as identified by Coomassie-stained 12% SDS-PAGE (Fig. 3; lane 1). Purification by Ni-chelate chromatography resulted in a homogenous protein fraction of rDau c 1.2 (Fig. 3; lane 2). An overall yield of 130 mg purified recombinant protein per litre culture was calculated. Parallel experiments were performed with Dau c 1.3 obtaining similar results of purified recombinant allergen (data not shown).

Immunoblot analysis

Monoclonal and polyclonal IgG

Purified rDau c 1.2 (2 µg per lane) was tested in immunoblots with the murine monoclonal anti-Bet v 1 IgG, BIP 1 and BIP 4 ([21,22] Fig. 3, lane 3, 4) Both antibodies, raised against purified nBet v 1, did not bind to the Bet v 1 homologous rDau c 1. In contrast, the polyclonal rabbit antiserum, displayed binding reactivity to rDau c 1.2 (Fig. 3, lane 5). The respective buffer control was negative.

IgE

Immunoblot analyses of aqueous protein extracts from carrot roots were performed with six carrot-allergic patients (Fig. 4, panel A). They all displayed IgE-binding to a protein of ≈ 16 kDa molecular mass. In addition, patients 1, 2 and 4 revealed IgE-antibodies directed against several proteins in the range of ≈ 40–66 kDa. Patient six possessed specific IgE-antibodies against a protein of 14 kDa, possibly profilin. No IgE-reactivity was detectable when normal human serum was used (Fig. 4, panel A; lane N), nor was any unspecific binding due to the second obvious antibody (Fig. 4, panel A, lane B).

The same set of patients' sera was tested for their IgE-binding to rDau c 1.2 (Fig. 4, panel B) and displayed equal binding-capacity of rDau c 1.2 when compared to natural Dau c 1. In addition, no differences in IgE-binding capacity of rDau c 1.2 and rDau c 1.3 were detectable, when testing the same set of six patients' sera (data not shown).

Cross-inhibition assays of Dau c 1

For cross-inhibition assays, a serum pool of carrot-allergic

Table 1. Amino acid sequence comparisons of Dau c 1 to CR16 (carrot root protein; EMBL database accession no. D88388), Api g 1 (major allergen from celery; EMBL database accession no. Z48967), PcPR1 (pathogenesis-related protein from parsley; EMBL database accession no. X12574), Bet v 1 (major birch pollen allergen; EMBL database accession no. X15877) and Mal d 1 (major allergen from apple; EMBL database accession no. Z48969).

	Sequence similarity (%)	Sequence identity (%)
CR16	100	98
Api g 1	93	81
PcPR1	76	60
Bet v 1	61	38
Mal d 1	59	39

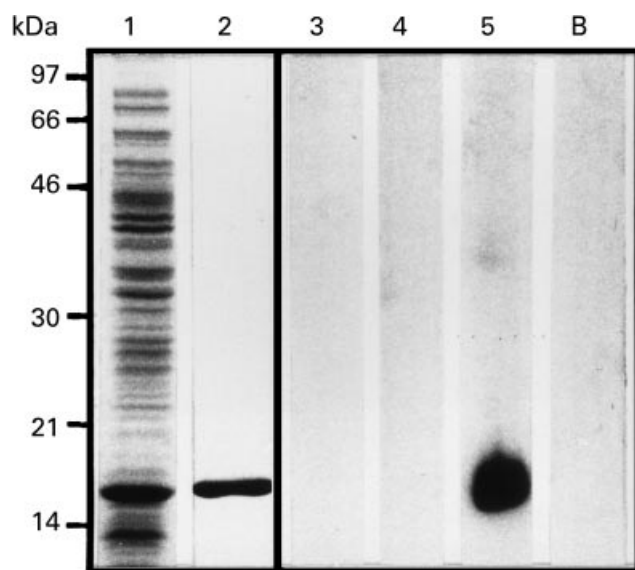


Fig. 3. SDS-PAGE and immunoblot of rDau c 1. Bacterial extract of M15 harbouring Dau c 1.2-pDS (lane 1) and purified recombinant Dau c 1.2 (lane 2) were separated by 12% SDS-PAGE and subsequently Coomassie stained. Purified rDau c 1 (2 µg per lane) was electrophoresed, electroblotted onto nitrocellulose membranes and tested with two murine monoclonal anti-Bet v 1 IgG (BIP 1, lane 3; BIP 4, lane 4), and rabbit polyclonal anti-Bet v 1 IgG (lane 5). Detection was performed with rabbit antimouse IgG and ^{125} I-labelled donkey antirabbit IgG (B: buffer control).

patients was tested for IgE-binding to natural carrot proteins (Fig. 5, left panel) and compared to IgE-binding to rDau c 1.2 (Fig. 5, right panel). In the natural protein extract IgE-binding to the possible dimer of Dau c 1 was detectable. Preincubation of the serum pool with BSA did not influence the binding intensity neither to nDau c 1 nor to rDau c 1.2. Pretreatment with the homologous protein from celery, Api g 1, weakened the IgE-binding to nDau c 1 strongly, but abolished IgE-binding to rDau c 1.2 completely. The same result was observed when preincubating the serum pool with rBet v 1, resulting in a strong reduction of IgE-binding intensity to nDau c 1 and complete inhibition to rDau c 1.2. Equal results of total inhibition of IgE-binding were achieved when pretreating the serum pool with purified rDau c 1.2 and probing with nDau c 1 and vice versa.

Discussion

The demonstration of cross-reactivities by skin testing or specific IgE does not necessarily indicate a clinical relevance. Frequently, however, patients sensitized to pollen also suffer from adverse reactions to allergens of fruits and vegetables which cross-react with pollen allergens [13,26]. Ragweed-sensitive individuals may show symptoms when

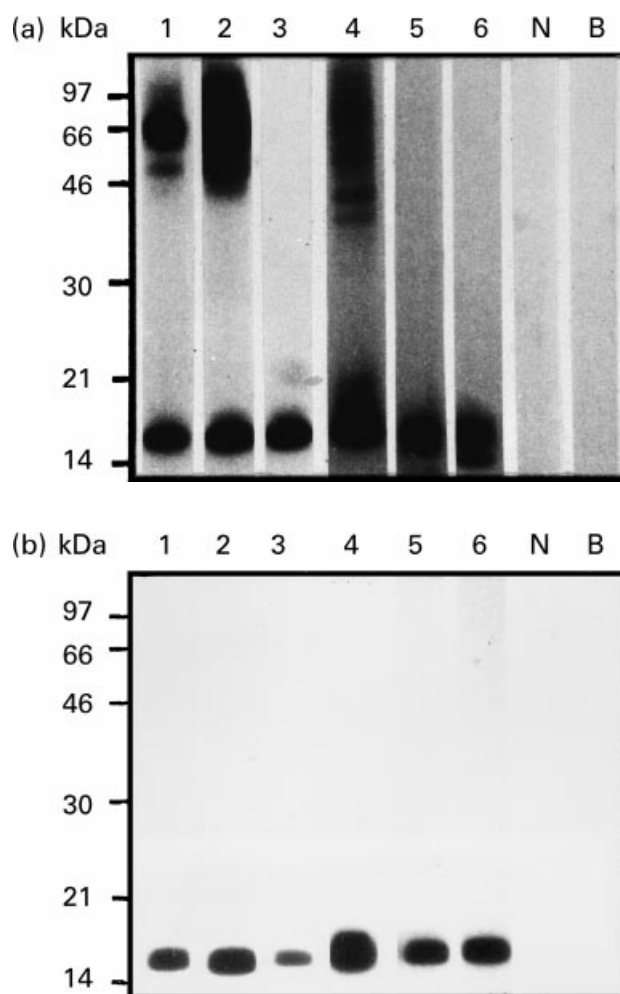


Fig. 4. Analysis of natural carrot proteins (a) and purified recombinant Dau c 1.2 (b) by SDS-PAGE and immunoblotting. Protein extract from carrot and purified rDau c 1.2 (2 µg/lane) were electrophoresed and blotted onto nitrocellulose membranes. Blotted proteins were probed with individual sera of carrot-allergic patients (lanes 1–6) or with normal human serum (lane N), or buffer (lane B). Bound IgE was detected by ^{125} I-rabbit antihuman IgE.

eating banana or melon [27]. Many mugwort-sensitive persons react to vegetables of the Apiaceae, e.g. celery, fennel, carrot, parsley and spices [9,10]. A majority of patients with birch pollen allergy display allergic reactions after eating apples, hazelnut, cherry, celery and carrot. So far, extracts used for diagnosis and treatment are sometimes difficult to prepare and of varying protein and carbohydrate content. Allergens are sometimes thermolabile and unstable and may degrade in extracts. As a consequence these extracts are of changing quality and batch-to-batch inconsistent. Therefore, prick-to-prick tests with fresh material are the method of choice at the moment. Purified and

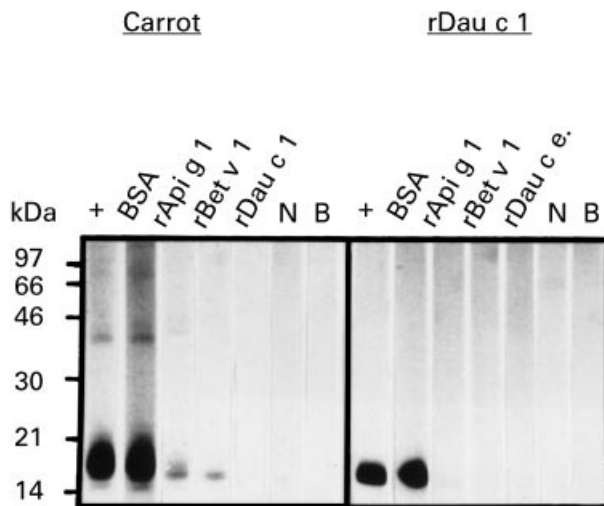


Fig. 5. Cross inhibition experiments. Protein extracts from carrot and purified rDau c 1.2 were separated by 12% SDS-PAGE and blotted onto nitrocellulose. IgE-binding of a carrot-allergic serum pool was studied after preincubation with BSA (30 μ g/mL), purified rApi g 1 (30 μ g/mL), rBet v 1 (20 μ g/mL), rDau c 1.2 (30 μ g/mL) and carrot extract, respectively. The normal human serum pool (N) and the buffer control (B) were used untreated.

well-defined recombinant allergens available in unlimited quantities and of consistent quality would offer a much better alternative as diagnostic tools.

It has been shown that a small number of recombinant proteins is sufficient, for diagnosis of grass and tree pollen allergy to achieve up to 99% accuracy [28,29]. The relevant allergens for precise diagnosis of food allergy still need to be defined. In the case of pollen-related food allergy, a number of cross-reacting allergens could be identified as being present in pollen of trees from the order *Fagales* as well as in fruits and vegetables. Profilin has been characterized as an allergen present in a range of fruits and vegetables such as apple, pear, celery, carrot and potato [13] as well as in grass and tree pollen [30]. For the 'mugwort–celery–birch syndrome', birch–celery or birch–mugwort cross-reacting allergens of a molecular mass of 46–65 kDa have been identified [10]. Wüthrich has already shown in his study that there is a small group of birch pollen-allergic patients with allergy to fruits and vegetables without IgE directed against Bet v 1 and Bet v 2. It is highly likely that these patients have been primarily sensitized against some of the cross-reacting allergens of the birch–celery–mugwort–spice syndrome [31].

In the case of Bet v 1, the major birch pollen allergen, a number of homologous genomic as well as cDNA-derived sequences have been identified from a broad range of plant species [32]. A recent update of the so far known Bet v 1-related sequences deposited in the GenBank database

revealed nine genomic sequences and 58 cDNA sequences derived from seven plant families including dicots as well as one monocot family [32]. Genomic sequences usually consist of two exons interrupted by an intervening sequence at a well-conserved position (amino acid residue 62 for Bet v 1, Mal d 1 and PcPR1 [33]). Phylogenetic studies of Bet v 1-related sequences revealed that they represent an ubiquitous new class of evolutionarily well-conserved pathogenesis-related or defence-related proteins [34].

For the Bet v 1 homologues from hazelnut, celery, apple and cherry, the allergenic nature of the respective proteins has already been demonstrated [14–16,35]. Since the majority of tree pollen-allergic patients (up to 70%) displays allergic symptoms when eating various fruits and vegetables, it is tempting to speculate that primary sensitization by pollen proteins takes place through the upper airways. In the case of Bet v 1 and related proteins sequence comparisons revealed a 59–93% similarity on the amino acid level. These significant values are also reflected on similar structural characteristics and the presence of cross-reactive B-cell epitopes. Yet these proteins have a number of epitopes in common as well as distinct ones. Api g 1 seems to possess some but not all of the epitopes present on the Bet v 1 molecule [15]. Mal d 1 on the other hand seems to display another set of epitopes but not all of the Bet v 1 epitopes [14].

In the case of Dau c 1 the botanical relationship to Api g 1 is obvious by sequence comparison (93% similarity; 81% identity) as well as to another member of this plant family *Apiaceae* — the pathogenesis-related protein from parsley, PcPR1 (76% similarity and 60% identity). Sequence analysis of Dau c 1 revealed an open reading frame of 154 amino acid residues, the same length as for Api g 1 and one amino acid residue shorter than PcPR1 from parsley [25]. The coding sequence is six amino acid residues shorter than Bet v 1 and its related tree pollen allergens Aln g 1, Car b 1 and Cor a 1 [36–38]. These differences in amino acid composition and the shortened C-terminal part of the proteins may influence the conformation of these allergens and change their B-cell epitopes.

Cross-inhibition experiments using rApi g 1 and rBet v 1 abolished IgE-binding to rDau c 1.2 totally and weakened IgE-binding to nDau c 1 significantly. This indicates that nDau c 1 consists of a number of isoforms which possess different epitopes not present on Bet v 1 and Api g 1. Monoclonal antibodies originally raised against purified nBet v 1 (BIP 1, BIP 4) did not recognize rDau c 1.2 as it was the case with rApi g 1 (personal observation). The polyclonal rabbit antiserum raised against purified rBet v 1 bound to rDau c 1 and rApi g 1 [35].

Recently, Yamamoto and coworkers isolated and characterized a cDNA clone with high sequence identity to Dau c 1 [24]. The protein expression pattern seems to be

restricted to root tissues, especially accumulated in older roots. Southern blot experiments confirm the existence of a multigene family [24]. These data are in good agreement with the so far known Bet v 1 sequences. They also seem to represent a multigene family with a rather specific tissue and/or developmental restricted expression pattern for individual members of this gene family [33,39].

Bet v 1, Cor a 1 and Car b 1 comprise a group of closely related isoforms *in vivo*, differing by only a few amino acid exchanges [35,38,40]. In some cases these isoforms can display great differences in their IgE-binding capacity ranging from very weak to very strong IgE-binders [38,41]. In the case of Dau c 1 three different sequences were identified and two of them: rDau c 1.2 and rDau c 1.3 (differing in seven amino acid residues) were tested in IgE-immunoblots. No differences in the IgE-binding capacity of rDau c 1.2 and rDau c 1.3 were detected when testing allergic patients' sera, so far.

A panel of well-defined and purified recombinant allergens might be a useful tool for evaluating the relevant B- as well as T-cell epitopes, and may in the future lead to a small number or a single Bet v 1-related protein sufficient for precise diagnosis of Bet v 1-related food allergies. Moreover, with a spectrum of well-characterized recombinant allergens, an individual, patient-related diagnosis and possible individual immunotherapy can be envisaged.

Acknowledgements

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