

Molecular cloning and characterization of a birch pollen minor allergen, Bet v 5, belonging to a family of isoflavone reductase-related proteins

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Background: Birch pollen is a major cause of pollinosis and is responsible for cross-reactive oral allergies to fruits, nuts, and vegetables. The major allergen, Bet v 1, has been extensively characterized, and 3 minor allergens, Bet v 2, Bet v 3, and Bet v 4, have been cloned and sequenced. Recently, another birch pollen protein with an apparent mass of 35 kd was described as a new IgE-binding protein in birch pollen with cross-reacting homologues in plant foods.

Objective: The aim of this study was to determine the primary structure of the 35-kd birch pollen allergen and to investigate its immunologic properties.

Methods: On the basis of a known complementary DNA fragment, a PCR strategy was applied to obtain the full-length nucleotide sequence of the coding region. The protein was expressed as His-Tag fusion protein in *Escherichia coli* and purified by Ni-chelate affinity chromatography. Nonfusion protein was obtained by cyanogen bromide treatment of the fusion protein. IgE-binding characteristics and potential allergenicity were investigated by immunoblot, immunoblot inhibition analysis, rat basophil leukemia-cell mediator release assay, and basophil histamine release and compared with those of natural (n) Bet v 5, recombinant (r)Bet v 1, and rBet v 2. **Results:** Recombinant Bet v 5 has a mass of 33 kd, an isoelectric point of 9.0, and sequence identity of 60% to 80% to isoflavone reductase homologue proteins from various plants. On immunoblots the recombinant Bet v 5 bound IgE from 9 (32%) of 28 sera from patients allergic to birch pollen with a CAP class of at least 3; Bet v 1 was detected by 89% of these patients. IgE immunoblot and inhibition experiments showed that nBet v 5 and rBet v 5 shared identical epitopes. A rabbit antiserum raised against pea isoflavone reductase and patients' IgE reacted with Bet v 5 and proteins of similar size in several vegetable foods, including exotic fruits. A similar reaction pattern was obtained with 2 Bet v 5-specific mAbs. Furthermore, Bet v 5 triggered a dose-dependent mediator release from rat basophil leukemia 2H3 cells passively sensitized with murine anti-birch pollen IgE and from basophils of a Bet v 5-reactive subject with birch pollen allergy. In contrast, no mediator release could be induced from basophils of a subject who was monosensitized to Bet v 1.

Conclusions: This 33-kd protein, designated as Bet v 5, is a new minor allergen in birch pollen and may be responsible for pollen-related oral allergy to specific foods in a minority of patients with birch pollen allergy. Amino acid sequence comparison and immunoreactivity to anti-isoflavone reductase serum indicate that Bet v 5 is related to isoflavone reductase, a protein family that is involved in plant defense reactions. (*J Allergy Clin Immunol* 1999;104:991-9.)

Key words: Birch pollen allergens, Bet v 5, complementary DNA, isoflavone reductase, cross-reaction

Birch pollen is a main cause of airborne allergies, particularly in northern and central Europe. Recently, the Swiss Study on Air Pollution and Lung Diseases in Adults study revealed that in Switzerland 7.9% of the population are sensitized to birch pollen, as determined by skin prick testing.¹ Four birch pollen allergens have been identified and expressed as recombinant proteins. The major allergen Bet v 1 binds IgE from at least 95% of patients with birch pollen allergy and shares high homology with a group of pathogenesis-related proteins.^{2,3} The tertiary structure of Bet v 1 was determined by radiographic crystallography and by multidimensional nuclear magnetic resonance spectroscopy.^{4,5} Bet v 1 exists in up to 20 isoforms, which differ in their IgE-binding capacity.⁶⁻¹⁰ Birch pollen profilin, a 14-kd regulatory protein with homologues present in almost all eukaryotic cells, was identified as the birch pollen minor allergen Bet v 2.¹¹ The tertiary fold of Bet v 2 is known, and its major IgE epitopes appear to be located in the C- and N-terminal α -helices.¹² The 24-kd Bet v 3 belongs to a class of Ca^{2+} -binding proteins with significant sequence identity to calmodulin, troponin, and parvalbumin from different eukaryotes.¹³ Another Ca^{2+} -binding protein with a mass of approximately 9 kd has been identified as a birch pollen minor allergen, Bet v 4.^{14,15} The protein contains 2 EF-hand Ca^{2+} -binding sites, and similar to Bet v 3, the IgE-binding capacity was to some extent dependent on the presence of protein-bound Ca^{2+} . Bet v 2 and Bet v 3 are recognized by IgE from about 10% of patients with birch pollen allergy, whereas Bet v 4 reacts with serum IgE from approximately 20% of individuals allergic to pollen.^{11,13,14}

Bet v 1 and Bet v 2 are elicitors of well-known cross-reactivity patterns between birch pollen and a wide range of vegetable foods, leading to birch pollen-related food allergies. The occurrence of Bet v 1-related proteins is

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Abbreviations used

CNBr:	Cyanogen bromide
IFR:	Isoflavone reductase
IFRH:	Isoflavone reductase homologue proteins
RACE:	Rapid amplification of cDNA ends
RBL:	Rat basophil leukemia
RT:	Reverse transcription

restricted to foods such as apple, pear, stone fruit, hazelnut, celery tuber, carrot, potato, and some spices. In contrast, profilins with structural similarity to Bet v 2 are present in almost all eukaryotic cells, and IgE antibodies to profilins were highly cross-reactive among a wide variety of plant species. Occurrence and properties of cross-reactive allergens in fruits and vegetables have recently been reviewed.¹⁶⁻¹⁸ As a consequence of their highly cross-reactive nature, profilins from celery,¹⁹ apple,²⁰ peach,²⁰ and exotic foods, such as lychee fruit,²¹ can cause oral allergy in subjects with birch pollen allergy.

Recently, we isolated and characterized a protein from birch pollen with an apparent mass of 35 kD, which bound IgE from 9 (12%) of 76 sera of patients with birch pollen allergy.²² Interestingly, specific IgE antibodies also recognized cross-reacting proteins of similar size in a range of fruits and vegetables, such as apple, pear, orange, mango, lychee fruit, banana, and carrot. On 2-dimensional immunoblots, the isoelectric point of this allergen was determined as 7.4. Because the N-terminus of the 35-kD birch pollen allergen was blocked, internal protein sequences were determined.²³ These internal sequences showed a high degree of sequence identity to isoflavone reductase (IFR) and isoflavone reductase homologue proteins (IFRHs) from several plants that also had a similar size. For a stretch of 25 consecutive residues, this identity ranged from 56% for IFR from peas and chick peas and an IFRH from maize to 80% for a tobacco IFRH. A 453-bp fragment was amplified from birch pollen total RNA by PCR by using primers derived from the nucleotide sequence of the tobacco IFRH. The deduced 151 amino acid sequence represented approximately 50% of the protein and confirmed the sequence identities obtained by Edman degradation. A rabbit antiserum raised against an IFR from pea strongly recognized the IgE-binding birch pollen protein and proteins of similar size in apple, banana, lychee fruit, and carrot, thus reproducing the cross-reactivity seen with patients' sera. Here we report the determination of the complete complementary (c)DNA sequence of this allergen by a reverse transcription (RT)-PCR strategy. The encoded protein, designated as Bet v 5, was expressed in *Escherichia coli*, and its allergenicity and cross-reactivity were examined in immunoblot, immunoblot inhibition, and mediator release experiments.

METHODS**Patients, sera, and antibodies**

Sera from 23 patients with a documented history of spring pollinosis and CAP classes between 3 and 6 to birch pollen were taken from the serum collection of the Paul-Ehrlich-Institute. Three sera from patients with birch pollen allergy were obtained from Aller-

gopharma (Reinbek, Germany), and another serum was obtained from MAST Diagnostika (Reinfeld, Germany). Two pooled sera from 7 (Wn 13/32) and 12 (Zie 39/132) additional patients with birch pollen allergy and serum from 1 individual (PEI 11) that had been used in our previous investigations were included.^{22,23} Except birch pollinosis, no further clinical data of the donors were available. Serum from a non-allergic subject (PEI 22) was used as a negative control. Monoclonal antibodies against Bet v 1 (P6, P10, and P16) and Bet v 5 (16G10b and 14D11) were raised at the Paul-Ehrlich-Institute by conventional hybridoma technology with mice that had been immunized with whole birch pollen extract. Monoclonal antibody PN-t, directed against the major peanut allergen Ara h 1 (a gift from Dr W.-M. Becker, Borstel Research Center, Borstel, Germany), served as a negative control. A rabbit antiserum against an isoflavone reductase (anti-IFR) from pea²⁴ was used for immunodetection of the cross-reacting 35-kD birch protein. Serum from nonimmunized rabbits (Riedel de Haen, Seelze, Germany) was applied as a negative control.

Extracts and allergens

Freeze-dried birch pollen extract was purchased from MAST Diagnostika. Extracts from fruits were prepared from fresh material, as described elsewhere.²⁵ Recombinant (r)Bet v 1a and rBet v 2 were purchased from Biomay (Linz, Austria). Natural (n)Bet v 5 was purified from birch pollen extract by preparative SDS-PAGE, as described elsewhere.^{22,26}

Cloning of cDNA

Total RNA (24 µg) was isolated from 100 mg of birch pollen (Allergon, Engelholm, Sweden) by means of the RNeasy Plant Total RNA kit (Quiagen, Hilden, Germany). RNA was reverse transcribed by using the First Strand cDNA Synthesis kit (Pharmacia, Freiburg, Germany), with 3 µg for each transcription. First-strand cDNA synthesis was primed with an antisense gene-specific primer BSP-1 (5'-TCT AGG AGG AGA AGT GAG TCC TGG-3'). This primer was selected on the basis of the known partial C-terminal cDNA sequence of the target protein.²³ A PCR strategy was selected on the basis of the known cDNA fragment. Rapid amplification of cDNA ends (5'RACE) was performed with the GIBCO BRL 5'RACE system (GIBCO BRL, Eggenstein, Germany). The dC-tailed cDNA was amplified by PCR by using a nested second antisense gene-specific primer BSP-2 (5'-GAG TCC TGG TGG TGC CAA CGT AGG-3') and the abridged anchor primer provided with the 5'RACE system. The PCR conditions were as follows: hot start at 94°C for 2 minutes, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerization at 72°C for 2 minutes. After 35 cycles, the final extension was 5 minutes at 65°C. Amplified DNA fragments were purified from agarose gels by the QUIAEX II kit (Quiagen), ligated into the pGEM-T vector (Promega, Madison, Wis), and used for transformation of *E. coli* XL1-Blue (Stratagene, Heidelberg, Germany) by electroporation. Plasmids from positive clones were subjected to sequence analysis by using an ABI 373 "stretch" automated fluorescent sequencer (Applied Biosystems, Weiterstadt, Germany) using T7 (5'-TAA TAC GAC TCA CTA TAG G-3') and SP6 (5'-GAT TTA GGT GAC ACT ATA GA-3') and BSP-1 and BSP-3 (5'-CAT CCA ATT TCT TCG CTG G-3') as sequencing primers. To obtain full-length coding DNA for the expression, the RT product was amplified by using the terminal 3' primer BSP-4 (5'-CAC TGT GGT GTA TTT GAC ATC TGG-3'; based on the known fragment) and the terminal 5' primer BSP-5 (5'-GCT CAC AAA AGC AAG ATT CTG ATC ATC G-3', derived from the 5'RACE product). The purified products were ligated into the pGEM-T vector and amplified in *E. coli* XL1-Blue cells. Restriction sites (3'-Bam H 1 and 5'-Nde 1) were added to the coding cDNA by PCR by using the primers 3'-Bam (5'-CAC TGT GGT GTA TTT GAC ATC TGG TAA GGA TCC GCG-3')

and 5'-Nde (5'-GGG AAT TCC ATA TGG CTC ACA AAA GCA AGA TTC TGA TCA TCG-3', restriction sites and stop codons are underlined). After 1 minute at 94°C, 35 PCR cycles were run at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute followed by final extension for 5 minutes at 65°C. The product was ligated into the plasmid pET-16b (Novagen, Madison, Wis), amplified in XL1-Blue cells, purified, and transformed into *E coli* BL21(DE3) expression cells (Novagen).

Expression and purification of recombinant allergen

Protein synthesis was induced with 1 mmol/L isopropyl-D-thiogalactopyranoside for 6 hours at 37°C, and cells were harvested by centrifugation. Because no soluble recombinant Bet v 5 was obtained, the cell pellet was processed under denaturing conditions by using 6 mol/L urea, 0.1 mol/L NaH₂PO₄, 0.01 mol/L Tris-HCl (pH 8.0), and 0.5 mg/mL lysozyme as lysis buffer. The supernatant was separated from the cellular debris and incubated with Ni-NTA-Agarose (Quiagen) at room temperature for 1 hour. Purification of the fusion protein was achieved according to the protocol of the manufacturer by using 6 mol/L urea, 0.1 mol/L NaH₂PO₄, and 0.01 mol/L Tris-HCl (pH 5.9) and 6 mol/L urea, 0.1 mol/L NaH₂PO₄, and 0.01 mol/L Tris-HCl (pH 4.5) as elution buffers. Nonfusion protein was obtained by cyanogen bromide (CNBr) cleavage and refolded as described by Boehm and Roesch.²⁷

Electrophoresis, protein transfer, and immunodetection procedures

Protein samples were separated by SDS-PAGE²⁸ (T = 12.5%, C = 2.7%) under reducing conditions by using a Bio-Rad (Munich, Germany) Mini Protean cell. Gels were either stained with Coomassie brilliant blue stain, or proteins were transferred to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) by semi-dry blotting.²⁹ For the detection of specific IgE antibodies, patients' sera were diluted 1:10. Bound IgE antibodies were visualized as described,²⁵ with slight modifications, by using alkaline phosphatase-labeled streptavidin (1:3000; Medac, Hamburg, Germany) instead of horseradish peroxidase-labeled streptavidin and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as the substrate. Monoclonal antibodies were used as 1:10 diluted hybridoma supernatants. Rabbit anti-IFR was diluted 1:6000. Bound mAbs and rabbit antibodies were detected as described.²⁵ The diluent for all reagents was 0.05 mol/L Tris-buffered saline containing 0.1% BSA and 0.05% (vol/vol) Tween 20 (pH 7.4). Blocking buffer was 0.3% Tween 20 in Tris-buffered saline. Before microsequencing, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Munich, Germany), as previously described.²⁵ Inhibition assays were performed by incubation of 50 µL of patient serum with 15 µg of recombinant allergens or 100 µg of protein from birch pollen extract in a total volume of 0.6 mL of incubation buffer for 30 minutes before immunologic detection.

N-terminal amino acid sequencing

Microsequencing was performed by Dr A. Petersen (Borstel Research Center, Borstel, Germany) according to the method of Matsudaira³⁰ by using a 476 A pulsed liquid protein sequencer with an online phenylthiohydantoin amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

Rat basophil leukemia cell-mediator release assay

The rat basophil leukemia (RBL)-cell mediator release assay has been developed as a murine model that mimics an essential event of the type 1 allergic reaction.^{31,32} In the case of birch pollen extract,

biologic activity measured by this assay corresponded well to results of basophil histamine release in allergic patients. RBL-2H3 cells were passively sensitized with pooled murine reaginic serum raised against birch pollen extract, and the assay was performed as described previously.^{31,32}

Basophil histamine release

The assay was carried out on whole blood samples as described by Siraganian^{33,34} and Siraganian and Brodsky.³⁵

RESULTS

Cloning of cDNA

The amplification product of 5'RACE had an apparent size of 600 bp. The sequence of the amplified fragment was matched with the known 3' fragment of the cDNA in an overlap of 33 bp (excluding primer sequence). The encoding sequence of the cDNA was amplified with terminal primers BSP-4 and BSP-5. A prominent PCR product of about 900 bp was ligated in pGEM-T. Plasmids containing the insert were isolated from *E coli* XL1-Blue cells and sequenced. The encoding sequence of the suspected birch pollen allergen consists of an open reading frame of 900 bp. Four of 5 clones that were sequenced showed an identical sequence, as depicted in Fig 1. One clone (No. 3) contained 2 point mutations, leading to amino acid exchanges 120V and K26E. The cDNA sequence of clone 8 that was selected for expression was submitted to the GenBank database (Accession No. AF135127).

Sequence analysis of Bet v 5 cDNA

The deduced protein contained 299 amino acid residues (without N-terminal Met) with a theoretical molecular mass of 33.022 and a calculated isoelectric point of 9.00. The 3' region of the cDNA was identical with the known 453-bp partial sequence.²³ One N-glycosylation site was identified at amino acid position 266-269 (NHSV). The deduced protein sequence was subjected to a BLAST similarity search.³⁶ The highest degrees of 60% to 80% amino acid sequence identity were revealed with proteins described as IFRHs followed by IFRs (Table I). Moreover, 67% identity was indicated for a phenylcoumaran benzylic ether reductase from loblolly pine. All proteins with significant matches had a size similar to the birch protein, ranging from 308 to 318 amino acid residues. Interestingly, the second highest degree of sequence identity was found with an IFRH from pear (proposed denomination as allergen: Pyr c 5) that was recently identified as an IgE-binding protein (unpublished data).

Expression and purification of rBet v 5

The cDNA encoding the IFRH birch protein was ligated into the plasmid pET-16b encoding a fusion protein with 10 additional N-terminal histidine residues. Initially, the plasmid was established in an *E coli* XL1-Blue cell to obtain stable transfectants. After recloning into BL21(DE3) cells, protein synthesis was induced with 1 mmol/L isopropyl-D-thiogalactopyranoside for 6 hours.

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ATGGCTCACAAAAGCAAGATTCTGATCATCGGAGGCACCGGCTACATCGGAAAATTCATC 60
M A H K S K I L I I G G T G Y I G K F I 20

GTGGAAGCAAGTGCAAAGTCTGGCCATCCCACCTTTGCTTTGGTCAGAGAGAGTACGGTC 120
V E A S A K S G H P T F A L V R E S T V 40

TCTGATCCCGTTAAGGGAAAACCTTGTCGAGAAATTCAAGGGCTTAGGCGTCACTTTGCTC 180
S D P V K G K L V E K F K G L G V T L L 60

CATGGAGATCTGTATGACCATGAGAGTTTGGTAAAGGCGTTTAAGCAGGTGGACGTGGTG 240
H G D L Y D H E S L V K A F K Q V D V V 80

ATATCGACGGTAGGCCACCTGCAGTTAGCAGATCAGGTCAAGATTATTGCTGCCATTAAA 300
I S T V G H L Q L A D Q V K I I A A I K 100

GAGGCTGGTAATATTAAGAGATTCTTCCCTTCGGAATTCGGAACGACGTAGACCGTGTG 360
E A G N I K R F F P S E F G N D V D R V 120

CATGCTGTTGAGCCAGCAAAGACTGCATTTGCTACCAAGGCTGAAATCCGCCGCAAGACT 420
H A V E P A K T A F A T K A E I R R K T 140

GAGGCTGAAGGCATCCCCTACACTTATGTGTCATCCAATTTCTTCGCTGGATATTTTCTT 480
E A E G I P Y T Y V S S N F F A G Y F L 160

CCTACGTTGGCACAACCAGGACTCACTTCTCCTCCTAGAGAGAAAAGTCGTTATCTTCGGA 540
P T L A Q P G L T S P P R E K V V I F G 180

GATGGAAATGCCAGGGCTGTTTTTAACAAGGAAGACGACATAGGAACTTACACAATTAGA 600
D G N A R A V F N K E D D I G T Y T I R 200

GCTGTGGATGACCCAAGAACAAGTGAATAAGATAGTCTACATCAAGCCTGCCAAGAACATT 660
A V D D P R T L N K I V Y I K P A K N I 220

TACTCATTCATGAGATTGTTGCCCTTTGGGAGAAAAAGATTGGCAAAACCCTTGAGAAA 720
Y S F N E I V A L W E K K I G K T L E K 240

ATCTATGTTCCAGAGGAGAACTTTTGAAGGACATCCAAGAGTCCCCAATTCCAATCAAC 780
I Y V P E E K L L K D I Q E S P I P I N 260

GTGATATTAGCAATCAACCACTCAGTTTTTGTGAAGGGAGATCATACCAACTTTGAGATT 840
V I L A I N H S V F V K G D H T N F E I 280

GAGGCATCCTTCGGTGTGGAGGCCTCCGAGCTATACCCAGATGTCAAATACACCACAGTG*900
E A S F G V E A S E L Y P D V K Y T T V 300

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Bet v 5 (clone 8 cDNA and protein)

FIG 1. cDNA sequence of the coding region of Bet v 5 and deduced amino acid sequence.

The recombinant fusion protein showed an apparent mass of about 35 kd, and in stained SDS-PAGE gels represented at least 30% of the cellular protein (not shown). For purification, the cell pellet was treated under denaturing conditions, and the lysate was subjected to Ni-chelate affinity chromatography. The fraction eluted at

pH 4.5 contained the highest amount of the recombinant protein but also some impurities with lower molecular masses, which were suspected to be degradation products. The purity of this fraction was estimated at about 90%. Nonfusion protein was obtained by CNBr treatment, as described by Boehm and Roesch.²⁷ The cleaved

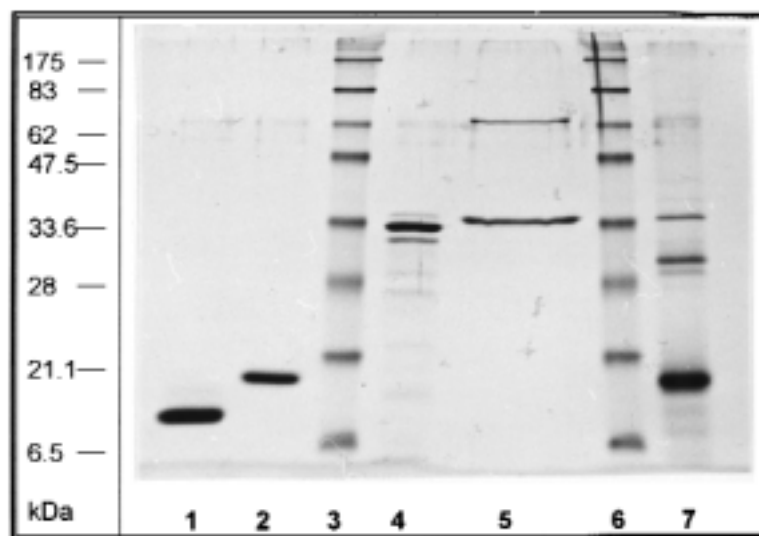


FIG 2. SDS-PAGE of allergens and extracts. *Lane 1*, rBet v 2; *lane 2*, rBet v 1; *lanes 3 and 6*, molecular weight marker proteins; *lane 4*, rBet v 5; *lane 5*, nBet v 5; *lane 7*, birch pollen extract. Bands were visualized with Coomassie brilliant blue stain.

His-tag was removed by Ni-chelate affinity chromatography, and the protein was refolded by sequential dialysis against decreasing concentrations of urea in Tris buffer. The nonfusion protein presented an apparent mass of 33.5 kd by SDS-PAGE (Fig 2, *lane 4*), and again bands were visible at lower molecular weights. To confirm the identity of the recombinant protein and to identify the low molecular weight impurities, the nonfusion rBet v 5 preparation was subjected to SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and analyzed by Edman degradation. The N-terminal sequences of the 33.5-kd rBet v 5 and a 25-kd band were identical (AHK-SKILIIGG). These analyses confirmed that the recombinant nonfusion protein contained the authentic N-terminus, and the additional bands present in the preparation represented degradation products of the protein.

Purification of nBet v 5

The nBet v 5 was isolated from birch pollen extract by preparative SDS-PAGE followed by electroelution. The purity of the material was assessed by SDS-PAGE (Fig 2, *lane 5*) and by immunoblotting with mAbs against Bet v 1 and with patients' sera. With the exception of 1 band at about 70 kd that appeared after Coomassie staining, no impurities were stained or immunodetected. The apparent mass of nBet v 5 (approximately 35 kd) was slightly higher when compared with the recombinant protein. For comparison, rBet v 1 and rBet v 2 that were applied in the subsequent immunoscreening of sera from allergic patients are also shown in Fig 2, *lanes 1 and 2*.

Allergen profile of the patient panel by immunoblotting

Twenty-seven sera from patients with birch pollen allergy with a CAP class of at least 3 and one nonallergic control subject were selected for this study. Moreover,

TABLE I. Degree of amino acid sequence identity of plant proteins with Bet v 5

Name	Origin	Accession No.	Identity (%)
IFRH	Poplar	AJ005804	80
Pyr c 5	Pear	AF071477	79
IFRH-SOLTU	Potato	X92075	76
IFRH	<i>Arabidopsis thaliana</i>	Z49777	76
Phenylcoumaran benzylic ether reductase	Loblolly pine	AF081678	67
IFRH	Tobacco	D28505	66
IFRH	Corn	U33318	60
IFR	Pea	S72472	58
IFR	Alfalfa	X58078	57
IFR	Chick pea	X60755	55

we included serum from one additional patient (PEI 11) and 2 pooled sera from patients with birch pollen allergy, which had also been used in our previous studies,^{22,23} as positive controls to investigate the IgE-binding activity of the natural and recombinant proteins. Sera from 9 (32%) of 28 individual allergic patients and the 2 serum pools bound to nonfusion rBet v 5 (Fig 3, *A*). As shown in Fig 3, *B*, all sera except one (PEI 72, *lane 11*) with IgE binding to the recombinant birch pollen IFRH also recognized the purified natural protein. Twenty-five (89%) of the 28 patients and the serum pools presented a strong IgE response to Bet v 1. Recombinant Bet v 2 was strongly recognized by 2 patients and was weakly recognized by sera from 3 additional patients. The frequency of detection for rBet v 2 was 5 (18%) of 28 (blotting results for Bet v 1 and Bet v 2 not shown). No IgE reactivity was observed with the nonallergic control (Fig 3, *lane 31*).

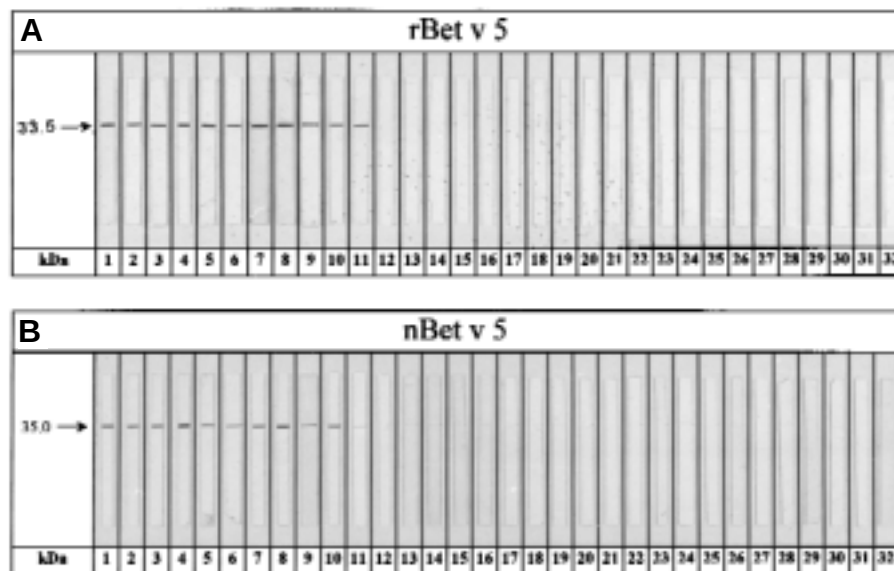


FIG 3. SDS-PAGE immunoblot analysis of the sera from patients with birch pollen allergy: **A**, rBet v 5; **B**, nBet v 5. Human sera: lane 1, pool Wn 13/32; lane 2, pool Zie 39/132; lane 3, 5253; lane 4, 5254; lane 5, 5178; lane 6, PEI 11; lane 7, PEI 46; lane 8, MAST 114; lane 9, Bo 103; lane 10, Lam; lane 11, PEI 72; lane 12, PEI 61; lane 13, Din; lane 14, Pap; lane 15, Zim; lane 16, Mai; lane 17, Med; lane 18, Mau; lane 19, Ken; lane 20, Rei; lane 21, PEI 126; lane 22, Dio; lane 23, PEI 62; lane 24, PEI 63; lane 25, PEI 117; lane 26, PEI 17; lane 27, Kob; lane 28, PEI 65; lane 29, PEI 21; lane 30, Sch; lane 31, PEI 22 (nonallergic control); lane 32, buffer control.

Immunoblot inhibition

The specificity of the IgE from all sera that recognized rBet v 5 was confirmed by inhibition experiments. Preincubation with birch pollen extract strongly inhibited IgE binding to rBet v 5 of all sera and thus demonstrated immunologic activity of rBet v 5 in comparison with its natural counterpart (not shown). The specificity of IgE binding to nBet v 5 was also confirmed by inhibition with birch pollen extract in all cases (not shown). The 2 serum pools and serum from patient Lam (Fig 3, lane 10) were used for extended inhibitions with rBet v 1, rBet v 5, and birch pollen extract as inhibitors. As presented in Fig 4, birch pollen and rBet v 5 strongly inhibited IgE binding to rBet v 5, whereas Bet v 1 had no inhibitory effect.

Immunodetection with monoclonal and polyclonal antibodies

Mice were immunized with whole birch pollen extract. Hybridoma supernatants were screened by ELISA with either rBet v 1 or rBet v 5 adsorbed to the solid phase. Several clones were established that produced mAbs of the IgG isotype against 1 of the 2 allergens. These results clearly underlined that the immune response against natural birch pollen extract can be directed against Bet v 5 in addition to Bet v 1, and that rBet v 5 contains epitopes of its wild-type counterpart. On immunoblots rBet v 1 was strongly recognized by 3 mAbs directed against this protein but not by 2 mAbs directed against Bet v 5. In contrast, rBet v 5 and nBet v 5 reacted strongly with the Bet v 5-specific antibodies

but not with the Bet v 1-specific mAbs. A rabbit anti-serum against an IFR from pea²⁴ contained antibodies that cross-reacted with rBet v 5 and nBet v 5 but not with rBet v 1. No binding or weak unspecific staining occurred with rabbit preimmune serum, which was applied as a negative control (data not shown).

Cross-reactivity to foods

Birch pollen extract and extracts from pear and lychee fruit that contain IgE-binding proteins in the mass range between 30 and 35 kd²² were tested with rabbit anti-IFR, 2 Bet v 5-specific mAbs, and serum from patient Lam who had both Bet v 1- and Bet v 5-specific IgE antibodies. All these probes recognized nBet v 5 in birch pollen extract, as well as cross-reacting proteins of similar size in both food extracts (Fig 5, lanes 1, 3, 5, and 6). With serum from the allergic patient, Bet v 1 was strongly recognized in birch pollen extract, as was the related allergen in pears, which appeared as a double band at about 18 kd. No Bet v 1-related allergen was detected by the patient serum in lychee fruit. Control experiments performed with serum from a nonallergic patient, with serum from a naive rabbit, and with an mAb specific for the major peanut allergen Ara h 1 were all negative.

Mediator release assays

RBL-2H3 cells were passively sensitized with a murine serum pool containing IgE against birch pollen extract. Cells were stimulated with rBet v 5 (1 ng/mL-10 µg/mL) and rBet v 1 (10 pg/mL-1 µg/mL). In accordance with previous experiments,^{31,32} Bet v 1 had a very high allergenic activity in this system, inducing a maximal

rBet v 5- inhibition

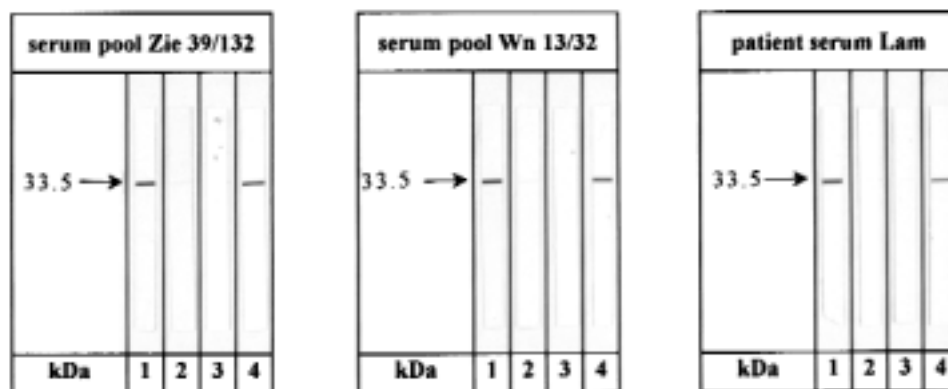


FIG 4. Immunoblot inhibition experiments. IgE binding to rBet v 5 on the membrane was inhibited by preincubation of serum with the following: *lane 1*, no inhibitor; *lane 2*, 100 µg of birch pollen protein; *lane 3*, 15 µg of rBet v 5; *lane 4*, 15 µg of rBet v 1.

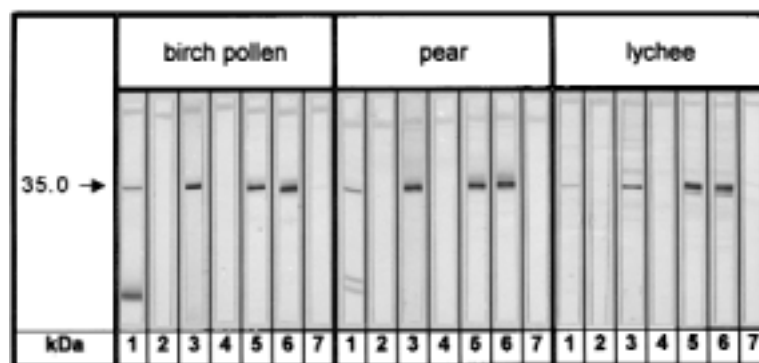


FIG 5. Immunoblotting of allergen extracts for detection of cross-reacting proteins. Extracts from birch pollen, pear, and lychee fruit were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with the following: *lane 1*, serum from patient Lam; *lane 2*, nonallergic human control serum; *lane 3*, rabbit anti-IFR; *lane 4*, rabbit preimmune serum; *lane 5*, mAb 16G10b against Bet v 5; *lane 6*, mAb 14D11 against Bet v 5; *lane 7*, mAb PN-t against the major peanut allergen Ara h 1.

release of 24% at a concentration of 10 ng/mL. Bet v 5 had a considerably lower but clearly detectable biologic activity, with a maximum of 20% release at 10 µg/mL. Less than 5% mediator release was obtained when the cells were sensitized with a murine reaginic serum pool against grass pollen extract (data not shown).

To prove the biologic activity of rBet v 5 in the human system, basophils from patient Lam with strong IgE binding to both Bet v 1 and Bet v 5 were stimulated with rBet v 1 and rBet v 5 in the concentration range between 10 pg/mL to 1 µg/mL. Another patient with birch pollen allergy without detectable IgE against Bet v 5 was included as a negative control. Patient Lam showed a maximal histamine release of about 42% at the maximal Bet v 5 concentration of 1 µg/mL without reaching the plateau. Recombinant Bet v 1 had a maximal release of 73% at 1 ng/mL, thus exceeding the biologic activity of rBet v 5 by several orders of magnitude in this assay. Identical experiments were performed with a blood sam-

ple of a donor who was monosensitized to Bet v 1. In this patient rBet v 1 showed a maximum of 72% at 1 ng/mL, whereas less than 5% release was measured after stimulating the cells with 1 µg/mL rBet v 5 (results not shown).

DISCUSSION

A previously partially characterized IgE-binding protein was identified as a novel minor allergen from birch pollen, which is now designated Bet v 5 according to the revised allergen nomenclature.³⁷ The complete primary structure was determined by cDNA cloning and sequencing.

The calculated isoelectric point of rBet v 5 was 9.0, whereas nBet v 5 appeared at an isoelectric point of 7.4 on 2-dimensional immunoblots.²² Apart from differences between theoretical and experimental approaches, nBet v 5 was N-terminally blocked, possibly by acetylation.²³ This posttranslational modification may be responsible

for an acidic isoelectric point shift. No glycosylations were detected on the natural molecule, which could have an additional influence on the isoelectric point.²³ However, N-glycosylation site was present at amino acid position 266-269 of the recombinant protein.

After expression in *E coli* BL21(DE3), the recombinant Bet v 5 was purified, and the nonfusion protein was obtained with an authentic N-terminus after CNBr cleavage. Sera from 9 (32%) of 28 patients with birch pollen allergy contained IgE specific for the recombinant protein, as determined by SDS-PAGE immunoblotting. This frequency of recognition is not representative for the average population with birch pollen allergy because patients with high CAP classes were selected, and participants were included who were identified as being sensitized against the 35-kd birch protein in our previous investigations.^{22,23} In a random screening of 76 patients with birch pollen allergy, we found IgE binding to this protein in 12% of the sera on birch pollen extract immunoblots.²²

Recombinant Bet v 1 bound IgE from 89% of our patients. This high frequency reflects the well-known predominance of Bet v 1 as the major birch pollen allergen. In comparison with Bet v 5, the birch pollen profilin Bet v 2 was of minor importance in our group of patients.

The biologic activity of rBet v 5 was confirmed in the RBL-cell mediator release assay that served as a murine model of the type 1 allergic reaction, as well as by basophil histamine release. In both systems Bet v 5 was clearly active but had a lower potency than Bet v 1. This situation corresponds well to the view of a minor allergen not only being recognized by IgE from a lower number of patients but also showing a weaker biologic activity. Because all Bet v 5-positive patients with the exception of Bo 103 (Fig 3, lane 9) were also sensitized to Bet v 1, it is difficult to assess to what extent Bet v 5 contributes to the allergic symptoms of these participants. However, pollen-related food allergens of the Bet v 1 family present 100- to 1000-fold reduced mediator release capacity when compared with the birch pollen allergen,^{27,31,38} but there remains little doubt that these proteins are responsible for eliciting food-related symptoms in patients with birch pollen allergy. The maximal concentrations applied for release experiments in this investigation caused mediator release effects below the saturation range. Maximal concentrations were limited for practical reasons. Because the biologic activities determined for Bet v 5 with cells from the Bet v 5-sensitized patient and also with murine antibirch pollen IgE clearly exceeded those of appropriate controls, we conclude that these effects are specific.

The specificity of IgE binding to rBet v 5 and the cross-reaction with the natural counterpart was confirmed by immunoblot inhibition with birch pollen extract in all cases. Moreover, the relationship between rBet v 5 and nBet v 5 was investigated by IgE immunoblotting with the purified natural molecule. IgE antibodies of 8 of the 9 subjects reacted both with nBet v 5, as well as rBet v 5. The discrepancy seen in one patient

might be related to a higher sensitivity that is sometimes observed with recombinant allergens. We identified a strong IgE binding to the cherry allergen rPru a 1 in patients with cherry allergy whose sera did not react to nPru a 1 on cherry extract immunoblots.³⁸ Finally, mAbs were obtained after immunization of mice with natural birch pollen extract that strongly bound to both rBetv 5 and nBet v 5. Therefore we conclude that the antibody-binding characteristics of the recombinant protein are, to a high degree, similar to those of the native molecule.

Sequence alignments revealed between 60% and 80% amino acid sequence identity with IFRHs and identities between 55% and 58% with IFRs. The relationship of Bet v 5 to IFR is further underlined by the fact that a rabbit antiserum against a pea IFR strongly reacted with rBet v 5 and nBet v 5. IFRs of this group are involved in generating pterocarpan phytoalexins by synthesizing chiral isoflavones from achiral precursors by means of an NADPH-dependent reduction step.^{24,39} These phytoalexins participate in plant defense reactions against fungal pathogens. IFRHs show a high sequence identity to IFRs^{40,41} but do not reduce 2'-hydroxyflavones, the specific substrate of IFR. Thus the biochemical function of these IFRHs remains unknown. Because the sequence of the birch pollen protein shares a considerably higher degree of sequence identity with IFRHs as with IFRs, the birch gene may also encode for an IFRH. Similar to Bet v 1 and related proteins, another birch pollen allergen has thus been identified that may be involved in defense reactions of plants.

The widespread occurrence of IFRs and IFRHs in plants, including foods such as pea and chick pea, raises the question of the potential of these proteins to cause cross-reactive food allergies in patients with pollen allergies. We have shown that both the rabbit anti-IFR and anti-Bet v 5 mAb, as well as IgE from a patient sensitized to Bet v 5, recognize cross-reacting proteins in plant foods, such as pear and lychee fruit. Recently, we have cloned and sequenced an IgE-binding pear homologue of Bet v 5 (GenBank Accession No. AF071477). The detailed investigation of the allergenicity of this protein, Pyr c 5, and its counterparts from other foods will be a target of our future work.

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