

Characterization of a New IgE-Binding 35-kDa Protein from Birch Pollen with Cross-Reacting Homologues in Various Plant Foods

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The present investigation was undertaken to obtain molecular data of a new immunoglobulin (Ig)E-binding birch pollen protein with a mass of 35 kDa. In a previous study, this protein showed IgE cross-reactivity with 34- and 35-kDa proteins in apples, pears, carrots, bananas and other exotic fruits. Since the protein was N-terminally blocked, it was purified by preparative SDS-PAGE, and multiple proteolytic fragments were subsequently generated by in-gel digestion with the endoproteinases Glu C, Lys C and Clostripain. After electrophoretic separation and blotting onto polyvinylidene difluoride (PVDF), the resulting polypeptides were subjected to N-terminal amino acid microsequencing. The internal sequences obtained showed a high degree of sequence identity to isoflavone reductases (IFR) and isoflavone reductase-like proteins (IRL) from several plants which 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 IRL from maize, to 80% for a tobacco IRL. A 453 bp fragment was amplified from total birch pollen RNA by polymerase chain reaction (PCR) using primers derived from the nucleotide sequence of the tobacco IRL. The deduced 151 amino acid sequence represented $\approx$ 50% of the protein and confirmed the sequence identities obtained by Edman degradation. Moreover, the 25 amino acid sequence was included in the cloned fragment. Deduced and determined amino acids showed only one mismatch, which was due to a single nucleotide exchange. At the antibody level, the immunological relationship of the birch pollen protein to IRL and IFR was demonstrated by immunoblotting with a rabbit antiserum against a pea IFR which recognized the same birch protein as patients' IgE. The rabbit antiserum also reproduced the cross-reactivity pattern previously observed with patients' IgE by recognizing related proteins in specific plant foods, including some exotic fruits. We therefore suggest that the 35-kDa birch pollen protein belongs to the IFR/IRL family and represents a minor allergen, possibly being responsible for less common pollen-related food allergies in patients allergic to birch pollen.

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INTRODUCTION

Recently we isolated and characterized a new 35-kDa protein from birch pollen which bound immunoglobulin (Ig)E from nine out of 76 (12%) sera of patients allergic to birch pollen [1]. Interestingly, IgE antibodies from these patients also recognized cross-reacting proteins of similar size in a range of fruits and vegetables such as apples, pears, oranges, mangos, lychee fruit, bananas and carrots. 'Cross-allergies' against specific plant foods occur in a high percentage of patients allergic to birch pollen [2]. These are due to homologues of Bet v 1, the major birch pollen

allergen, which are present in many plants [3, 4]. Examples of cross-reactive major food allergens belonging to this family are Mal d 1, Pru a 1 and Api g 1, from apples, cherries and celery tubers, respectively [5–8]. All these proteins have molecular masses (MM) between 16 kDa and 18 kDa. However, no allergens from the Bet v 1 family have been identified in the exotic fruit mentioned above. On the other hand, the apparent MM of 35 kDa led us to suspect the new protein to be a dimer of Bet v 1. Indeed we identified an additional band at $\approx$ 34 kDa which caused diffuse staining with sera of most patients who presented IgE against Bet v 1. This band also occurred in purified Bet v 1

and showed the N-terminal amino acid sequence of the major birch pollen allergen when subjected to Edman degradation [1]. This dimeric structure could be separated from the 35-kDa protein by two-dimensional electrophoresis: the first had an isoelectric point (pI) of 5.7, similar to Bet v 1, whereas the latter showed a main spot at pI 7.4. Our studies further revealed that the 35-kDa birch protein showed no immunological relationship to the 17-kDa Bet v 1 [1]. Thus, we hypothesized that this protein represents a new cross-reactive structure responsible for uncommon food sensitizations in a minority of patients allergic to birch pollen.

However, several attempts of N-terminal amino acid sequencing were unsuccessful, most probably due to N-terminal blockage of the proteins' N-terminus. Application of four different deblocking strategies [9] did not solve the problem. Thus the aim of the present investigation was to characterize this potential allergen further and, in particular, to obtain sequence data which may highlight its function in the plants. The results indicate that the cross-reactive birch protein belongs to a plant protein family which is related to enzymes involved in specific phytoalexin responses.

MATERIALS AND METHODS

Patients' sera and antibodies. All individual sera were collected at the Hospital Borkum Riff, Department of Dermatology and Allergology (Dr H. Aulepp, Borkum, Germany). The sera were taken from patients allergic to birch pollen who reported oral allergy-like symptoms after the ingestion of fresh apples. Diagnosis was based on a positive skin prick test to both birch pollen and apple extract, and serological data [enzyme allergosorbent test (EAST) class ≥ 2]. Patients with positive test results in these assays, a clear anamnesis of spring pollinosis, and oropharyngeal symptoms after ingestion of apples, were classified as being allergic to both birch pollen and fruit. Due to the limited amount of serum, these samples could not be used for the preparation of large pools. Thus, two additional pooled sera from seven and 12 patients allergic to birch pollen were purchased from Allergopharma (Reinbek, Germany). Except birch pollinosis, no further clinical data of the donors were available. Serum from a non-allergic subject was used as a negative control. Monoclonal antibodies (MoAbs) against Bet v 1 were raised at the Paul-Ehrlich-Institut and supplied by Dr K. Fötisch. A rabbit antiserum against an isoflavone reductase (anti-IFR) from peas [10] was applied for immunodetection of cross-reacting 35-kDa proteins.

Allergen extracts. Freeze-dried birch pollen extract was purchased from Allergopharma. All food extracts were prepared from fresh material as described elsewhere [11].

Electrophoresis, protein transfer and immunodetection. Allergen extracts and proteolytic polypeptides were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE, T = 13%, C = 2.7%), gel size 180 × 170 × 1 mm³, according to the method of Laemmli [12] under reducing conditions. This system was also used for monitoring the purity of isolated allergens and the efficiency of fragmentation. After separation, proteins and peptides were either subjected to Coomassie Brilliant Blue or silver staining or used for immunoblotting. Proteins and proteolytic fragments were transferred to nitrocellulose (NC) membranes (Schleicher und Schüll, Dassel, Germany) by semi-dry blotting as described [13], corresponding to the method originally developed by Khyse-Andersen [14]. Transfer was controlled

by staining single NC strips of 4 mm with 0.1% (wt/vol) Indian ink (Pelikan, Hannover, Germany) in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 and 1% (v/v) acetic acid, or with an Auro Dye staining kit (Auro DyeTM forte; Amersham, Buckinghamshire, UK). Immunodetection was performed with 0.1 ml of patient serum diluted to 1 ml as described [13]. Briefly, specific IgE was detected by a tertiary antibody-amplified system with streptavidine horseradish peroxidase (HRP) conjugate as the final detection reagent. When used as primary probes, MoAbs were applied as hybridoma supernatants (1:100). Rabbit anti-IFR was diluted 1:6000. Bound IgG MoAbs and specific rabbit antibodies were detected as described [15]. Bound HRP activity was either stained with 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate or by enhanced chemiluminescence (ECL) (Amersham). All dilutions were made in 0.01 mol/l PBS containing 0.1% bovine serum albumin (BSA) and 0.05% (v/v) Tween 20. Blocking buffer was 0.3% Tween 20 in PBS. Prior to microsequencing, proteins and peptides were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Munich, Germany) as described [11].

Purification of allergens. The isolation of allergens has been described elsewhere [1]. Briefly, birch pollen extract was separated by preparative SDS-PAGE (conditions as above) [15]. Bands were excised after staining with CuCl₂ and the protein was eluted using a Centrilotur device (Amicon, Witten, Germany). Fractions were tested for purity by analytical SDS-PAGE and with sera from patients allergic to birch pollen as well as with a MoAb raised against Bet v 1 as described [1]. As the proteins' N-terminus was blocked, further evidence for purity was obtained by the absence of any sequenceable peptides when 15 µg of the purified material was subjected to Edman degradation. Those fractions which contained the desired protein exclusively were pooled. The protein content was estimated by a commercial dye binding assay (Pierce, Rockford, IL, USA).

Glycan detection. Glycosylation of the target protein was tested by means of the Carbohydrate Detection Kit (Oxford Glycosystems, Abingdon, UK). The assay is based on periodate oxidation of sugar residues which are subsequently reacted to biotin-hydrazide conjugate. In the final step, protein-bound biotin was detected with streptavidine HRP (1:20000, 2 h) and ECL.

Partial proteolysis by in-gel digestion. The following procedure is a modification of the method originally described by Rosenfeld *et al.* [16]. After preparative SDS-PAGE and Coomassie staining of birch pollen extract the target protein band was excised from the gel. Gel slices with a length of ≈ 0.5 cm were treated with 50% acetonitrile in 0.2 mol/L ammonium carbonate to remove the bulk of SDS, dye, and most of the water. Rehydration was performed with 15–50 µl of endoproteinase solution in PBS at a pH of 7.4. All enzymes were obtained from Promega, Madison, WI, USA and were applied in reaction buffer according to the instructions of the manufacturer at 37°C. The gel was either treated with Glu C, Lys C, or Clostripain. The C-terminal cleavage sites of these proteases are Glu-X, Lys-X and Arg-X, respectively. Limited proteolysis was optimized to obtain multiple fragments in the mass range above 14 kDa. The following conditions were applied: Glu C, 1:50 (µg per µg of allergen), 20 min; Lys C, 1:50 (µg per µg of allergen), 10 min; and Clostripain, 1:20 (µg per µg of allergen), 5 h. Approximately 5.5 µg of allergen was subjected to one digestion experiment. Digestion was terminated by heating the gel slices in SDS-PAGE sample buffer at 100°C for 3 min under non-reducing conditions. After transfer to 5.5 cm preparative slots, the products were again separated by SDS-PAGE, blotted onto NC and used for immunological detections or transferred to PVDF and subsequently subjected to amino acid microsequencing.

Amino acid analysis and microsequencing. For amino acid analysis, PVDF membranes with the Coomassie-stained proteins were treated with 6 N HCl for 70 min at 150°C. After derivatization with phenyl isothiocyanate, phenylthiocarbamoyl amino acid derivatives were analyzed by RP-HPLC using a 420 A amino acid analyzer (Applied Biosystems, Weiterstadt, Germany). Microsequencing was performed according to the method of Matsudaira [17] as described [8] using a 476 A pulsed liquid protein sequencer with an online phenylthiohydantoin amino acid analyzer (Applied Biosystems).

Cloning and sequencing of DNA fragments. Total RNA was isolated from birch pollen (Allergon, Engelholm, Sweden) by means of the RNeasy Plant Total RNA kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of RNA was estimated from the OD₂₆₀ and was reverse transcribed by the First Strand cDNA Synthesis kit (Pharmacia, Freiburg, Germany) using an oligo dT-deg primer (5'-TTT TTT TTT TTT TTT TNN-3') according to the protocol of the supplier. A total of 3 µg of total RNA was used for each transcription. Products of the reverse transcription were directly subjected to polymerase chain reaction (PCR). Since the internal amino acid sequence obtained by Edman degradation seemed to suggest that the 35-kDa protein belongs to a family of specific isoflavone reductases (IFR) or isoflavone reductase-like proteins (IRL) the following primers were selected on the basis of known cDNAs of proteins from these groups: Alf3ECOR1, 5'-ACG CGA ATT CTT AGA CAA ATT GAT TCA AAT A-3'; and Alf5ECOR1, 5'-CGG GAA TTC ATG GCA ACT GAA AAC AAA ATC CTC-3'. These primers had been successfully applied by Paiva *et al.* [18] for amplification of an IFR from peas. In addition to these terminal primers, sets of internal primers were chosen on the basis of a cDNA of a tobacco IRL (EMBL Acc. No. D28505; see Ref. 19) which showed a highly similar partial amino acid sequence (Table 1): TAB5a, 5'-GCA ATT AAA GAA GCT GGA-3'; TAB5b, 5'-GAG GCW GAA GGA ATT CCW TAC AC-3'; TAB 5c, 5'-AAA GTT GTC ATT YTT GGM GAT GGA AAT-3'; TAB3c, 5'-ATT TCC ATC KCC AAR AAT GAC AAC TTT-3', with W = A/T, Y = C/T, K = G/T, M = A/C, R = A/G. Finally, oligo dT-deg was also applied. Various combinations of these primers were applied as described in the Results section. As a positive control, the primers Betv15, 5'-ATC ATG GGT GTT TTC AAT TAC G-3' and Betv13, 5'-AAT AGA ACA TTT ATT ATC ATT CTC-3' were chosen for amplification of the coding region and the 3' untranslated region of the major birch pollen allergen [20]. The first strand cDNA amplification was carried out in a final volume of 100 µl containing 100 pmol dNTPs (Pharmacia), 50 pmol of each primer and 0.5 µl Taq

polymerase (5 U/µl: Beckmann, Munich, Germany). After a hot start the PCR (35 cycles) was performed for 1 min at 94°C, 1.5 min at 50°C and 3 min at 72°C. After a final extension for 5 min at 72°C, PCR products were subjected to agarose gel electrophoresis as described [8]. Bands of the expected size were excised from the gel and desalted by the QUIAEX II kit (Quiagen). Fragments of cDNA were ligated into the pGEM-T vector (Promega) and used for transformation of *Escherichia coli* strain JS5 (Stratagene, Heidelberg, Germany) by electroporation (2.5 kV, 25 µF, 200 Ω) using the *E. coli* gene pulser (Bio-rad). Positive clones were selected by blue/white screening and controlled by PCR screening using the vector-specific primers T7, 5'-TAA TAC GAC TCA CTA TAG G-3' and SP6, 5'-GAT TTA GGT GAC ACT ATA GA-3'. Plasmids were isolated from overnight cultures by means of the Plasmid Midi kit (Quiagen) and subjected to sequence analysis using an ABI 373 'stretch' automated fluorescent sequencer (Applied Biosystems) using SP6, T7, TAB5b and oligo dT as sequencing primers.

Similarity searches and alignments of sequences. Similarity searches and alignments of amino acid sequences, deduced amino acid sequences and nucleotide sequences were performed by non-redundant searches using the basic local alignment search tool (BLAST) server and the following databases: non-redundant gene bank CDS translations, PDB, SwissProt, SPupdate, and PIR.

RESULTS

Allergen purification

The 35-kDa protein and Bet v 1 were isolated from birch pollen extract by preparative SDS-PAGE/electroelution as described [1]. The high degree of purity obtained by this one-step procedure is demonstrated by Fig. 1. We found that N-terminal microsequencing yielded no results for the band at ≈ 35 kDa. The N-terminus of Bet v 1 (GVFNYE) [20] was obtained when the 17-kDa isolate shown in lane 4 was subjected to sequence analysis.

Immunoblotting and selection of sera

For extended characterization of the 35-kDa protein, sera from patients allergic to birch pollen were selected on the basis of the

Table 1. Degree of sequence identity of the 25-residue internal amino acid sequence with partial sequences of IRL and IFR proteins from plants

Source	Classification of protein	Molecular mass (kDa)	Number of amino acid residues	Position of corresponding internal sequence	Identity with birch sequence %	EMBL Acc. No.
Potato (<i>Solanum tuberosum</i>)	IRL	33.8	308	175–199	80	X92075
Tobacco (<i>Nicotiana tabacum</i>)	IRL	34.7	310	177–201	72	D28505
Mouse-ear cress (<i>Arabidopsis thaliana</i>)	IRL	33.7	310	178–202	72	Z49777
White lupine (<i>Lupinus albus</i>)	IRL	35.1	312	180–204	56	U48590
Garden pea (<i>Pisum sativum</i>)	IFR	35.4	318	186–210	64	S72472
Chick pea (<i>Cicer arietinum</i>)	IFR	35.4	318	186–210	56	X60755
Maize (<i>Zea mays</i>)	IRL	32.8	309	176–200	56	U33318
Alfalfa (<i>Medicago sativa</i>)	IFR	35.5	318	186–210	56	X58078

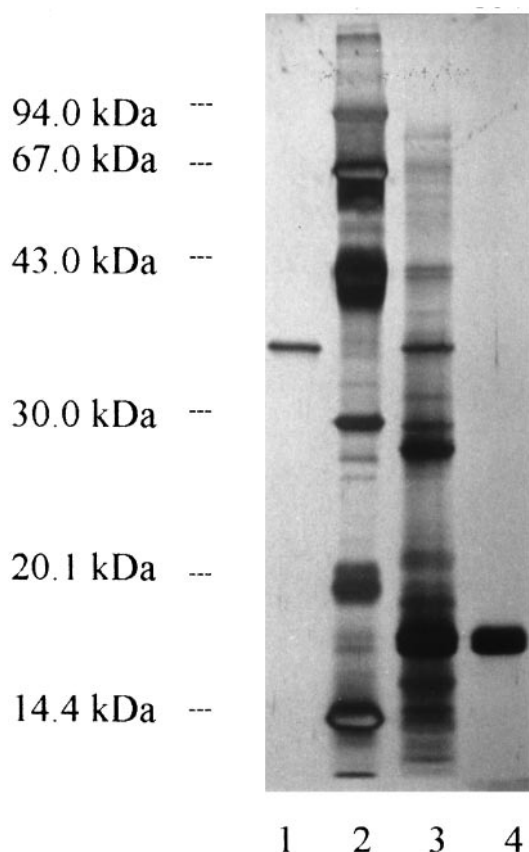


Fig. 1. Silver-stained SDS-PAGE gel of birch pollen extract and the purified proteins. 1, 35-kDa protein; 2, molecular mass marker; 3, birch pollen extract; 4, major birch pollen allergen Bet v 1.

results of immunoblotting. As an example, Fig. 2 presents IgE immunoblots of sera from seven patients with birch pollinosis (lanes 1–7), two pooled sera from such patients ($n = 7$ and 12, lanes 8 and 9, respectively) and a non-allergic control (lane 10). Diffuse staining of dimeric Bet v 1 can be seen in lanes 1–4, whereas a clear 35-kDa band was recognized by IgE of the sera shown in lanes 5–9. The pooled serum shown in lane 9 as well as serum from an individual patient (Bo212, not shown in Fig. 2, but shown in Figs 4 and 5) were selected for further immunological characterization of the potentially new allergen. The latter patient also presented IgE to the 35-kDa protein and an uncommon clinical pattern: the patient suffering from spring pollinosis caused by tree pollen, including birch, was not sensitized to weeds and grasses and reported local symptoms at the mucosa of lips, tongue and throat after eating apples, pears, peaches, cherries, celery, strawberries, bananas, hazelnuts and peanuts.

Glycosylation

Purified 35-kDa protein and, as controls, recombinant major cherry allergen Pru a 1 [8] (negative) and ovalbumin (positive),

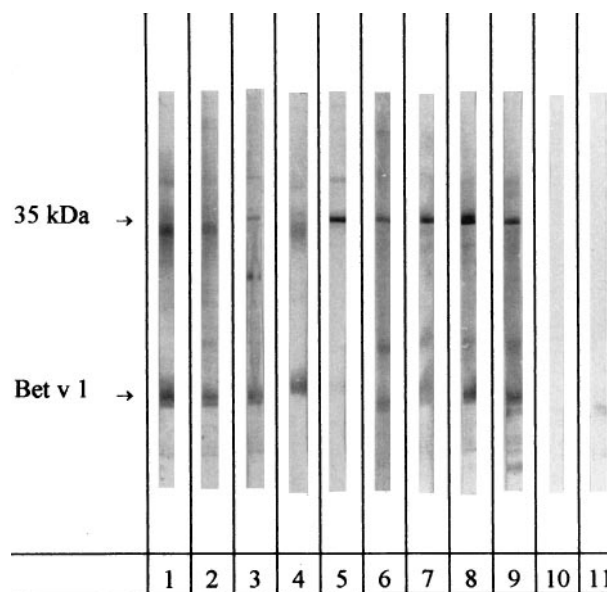


Fig. 2. SDS-PAGE-immunoblot analysis of birch pollen extract with sera from patients with birch pollinosis. Lanes 1–7, sera from single patients with birch pollen allergy and oral allergy against apples; 8 and 9, pooled sera from seven and 12 additional patients allergic to birch pollen; 10, non-allergic human control serum; 11, buffer control. Bound HRP was stained with TMB as the substrate.

were subjected to SDS-PAGE and transferred to NC membranes. After oxidation and biotinylation, blot strips with the separated proteins were incubated with streptavidine HRP. The strips were washed 3 times for 5 min and bound enzymatic activity was detected with the ECL system. Only ovalbumin caused a band at

Table 2. Comparison of amino acid compositions (mol percentage)

	35-kDa Amino birch protein acid (determined)	Bet v 1 (determined)	Bet v 1 (calculated from Ref. 20)	Tobacco IRL (calculated from Ref. 19)
D/N	10.3	12.8	11.4	9.7
Q/E	7.6	10.8	10.1	10.6
S	7.2	6.9	6.9	5.5
G	12.0	12.8	8.8	6.1
T	3.0	3.8	5.7	6.5
A	11.7	9.7	6.9	6.5
H	1.7	2.1	2.5	1.3
P	6.1	5.9	5.0	5.5
R	2.7	2.1	1.9	3.2
Y	4.3	4.6	4.4	3.5
V	6.3	6.9	7.5	8.1
M	1.5	0.4	0.6	1.0
I	6.1	5.6	8.2	9.0
L	7.6	6.4	5.7	8.7
F	5.0	3.7	5.0	5.5
K	7.5	6.9	9.4	8.1

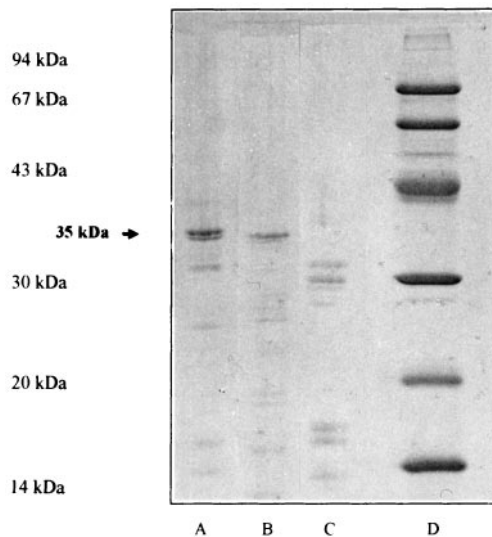


Fig. 3. Partial proteolysis of the 35-kDa birch protein as demonstrated by SDS-PAGE and Coomassie staining. Conditions were optimized for generating multiple fragments in the range between 14 kDa and 35 kDa. Treatment with, lane A, Clostripain; lane B, Lys C; and lane C, Glu C. Marker proteins are shown in lane D. Conditions of in-gel digestion as described in the experimental section.

apparent 45 kDa whereas the other samples were negative, indicating that the birch protein is not glycosylated (results not shown).

Amino acid composition

After blotting onto PVDF and Coomassie staining of birch pollen

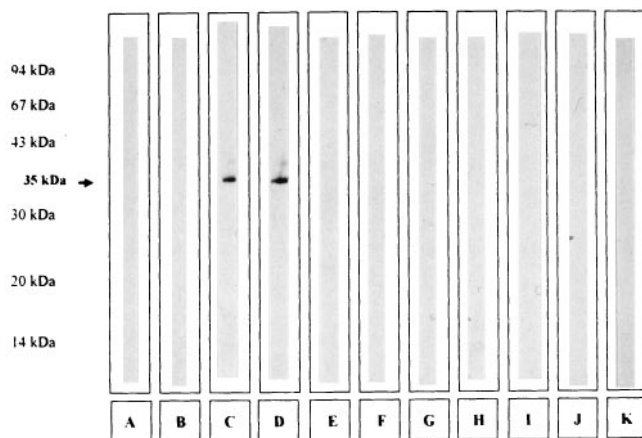


Fig. 4. SDS-PAGE immunoblot analysis of the purified 35-kDa birch protein after partial digestion with Clostripain. Despite the presence of fragments as shown in Fig. 3, the IgE antibodies of sensitized patients bound only to the undigested allergen. Lanes A and B, sera of patients Bo130 and SU, whose IgE antibodies were exclusively directed against Bet v 1; lanes C and D, human sera with IgE against the 35-kDa protein and Bet v 1 (pool, $n = 12$ and Bo212, respectively); lanes E–J, MoAbs against Bet v 1; lane K, buffer control. HRP activity was stained by ECL.

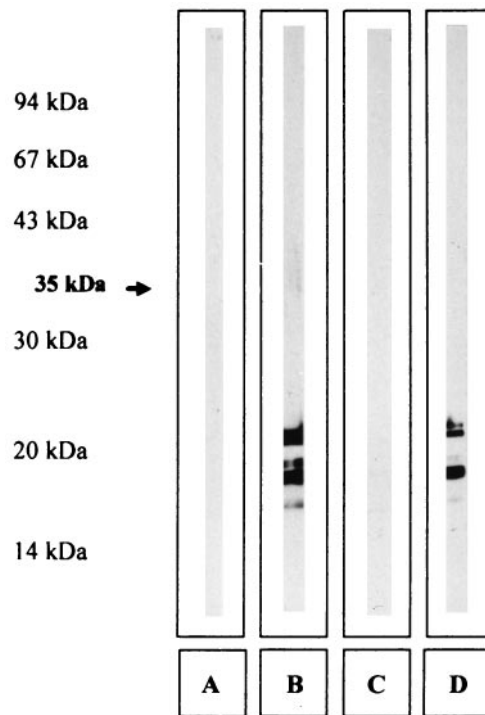


Fig. 5. Immunoblot inhibition experiment. The purified 35-kDa protein was digested with Glu C. After SDS-PAGE and transfer to nitrocellulose, immunodetection of bound IgE was performed with serum of patient Bo212. Lane A, buffer control; lane B, serum without inhibitor; lane C, serum preincubated with 10 μ g of 35-kDa protein; lane D, serum preincubated with 10 μ g of Bet v 1. HRP activity was stained by ECL.

extract, the 35-kDa protein and Bet v 1 were excised and subjected to amino acid analysis. The percentage of Trp and Cys was not determined. The results are shown in Table 2 and did not allow us to distinguish between the two allergens. In the case of Bet v 1 the results were in agreement with the amino acid composition calculated from the deduced amino acid sequence [20].

Generation and investigation of allergen fragments

Subsequent to SDS-PAGE and Coomassie staining, gel slices containing the 35-kDa band were treated with the endoproteases Glu C, Lys C and Clostripain. The digestion procedure was optimized in such a way that multiple fragments of larger size were obtained in the mass range above 14 kDa. As illustrated by Fig. 3, this resulted in a complete degradation of the 35-kDa band in the case of Glu C. In contrast, undigested allergen remained detectable after treatment with Lys C and Clostripain, respectively. As a control, pure protease solutions were analyzed by the same procedure applying identical concentration, but did not show any bands after staining with Coomassie (results not shown).

Immunological testing of the fragments was carried out after transfer to NC membranes with the 35-kDa positive serum pool

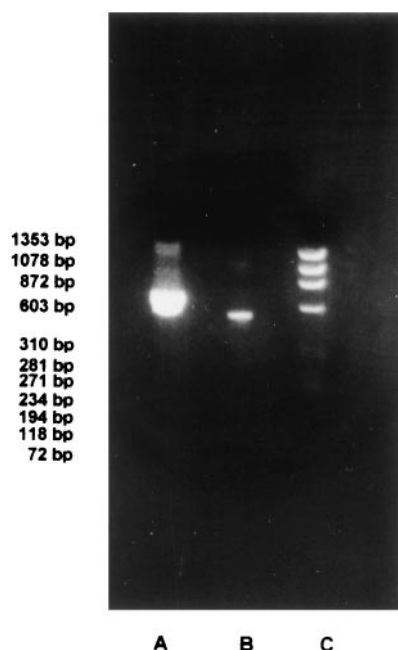


Fig. 6. Agarose gel electrophoresis of PCR products obtained from reversely transcribed birch pollen RNA. Lane A, primers BetvI3 and BetvI5; lane B, primers TAB5b/oligo dT-deg; lane C, marker.

from 12 patients (Fig. 2, lane 9) and with serum of patient Bo212. Moreover, the blots were tested with sera from two patients allergic to birch pollen who were exclusively sensitized to Bet v 1 (Bo130, SU) and with six MoAbs raised against Bet v 1 to exclude the presence of Bet v 1 epitopes. In the digests obtained with Lys C and Clostripain, only the serum pool and Bo212 caused immunostaining of residual 35-kDa protein, but no fragments were recognized by any of the probes. No immunoreactivity was seen with antibodies against Bet v 1 (patients' IgE and MoAb). The immunoblotting results obtained with Clostripain digests of the 35-kDa birch protein are presented in Fig. 4. After proteolysis with Glu C, four fragment bands in the mass range between 16 kDa and 24 kDa were exclusively detected by the serum pool and Bo212. The specificity of these results was confirmed by immunoblot inhibition with serum Bo212 as presented in Fig. 5. Binding to the fragments was completely blocked by preincubation of the serum with 10 µg of the purified 35-kDa protein (lane C). On the contrary, the immunoreactive bands were only weakly quenched by preincubation with the same amount of Bet v 1 (lane D). These results indicated that epitopes with cross-reactivity to the undigested protein were present on the proteolytic fragments. Moreover, they demonstrate that these epitopes are not related to those of Bet v 1.

After blotting onto PVDF and staining with Coomassie Brilliant Blue, 11 fragments were excised and processed by Edman degradation. All fragments obtained by treatment with Glu C and Clostripain (four and two, respectively) and two polypeptides generated by Lys C were again N-terminally blocked. Sequencing data were obtained for three of the Lys C fragments. Two of these, with masses of 15 kDa and 27 kDa, revealed overlaid

GTG TCA TCC AAT TTC TTC GCT GGA TAT TTT CTT CCT ACG TTG GCA	45
V S S N F F A G Y F L P T L A	
CAA CCA GGA CTC ACT TCT CCT CCT AGA GAG AAA GTC GTT ATC TTC	90
Q P G L T S P P R E K <u>V V I F</u>	
GGA GAT GGA AAC AGG GCT GTT TTT AAC AAC AAG GAA GAC GAC ACA	135
<u>G D G N A R A V F N K E D D</u> T	
GGA ACC TAC ACA ATT AGA GCT GTG GAT GAC CCA AGA ACA CTG AAT	180
<u>G T Y T I R</u> A V D D P R T L N	
AAG ATA GTC CAC ATC AAG CCT GCC AAG AAC ATT TAC TCA TTC AAT	225
K I V H I K P A K N I Y S F N	
GAG ATT GTT GCC CTT TGG GAG AAA AAG ATT GGC AAA ACC CTT GAG	270
E I V A L W E K K I G K T L E	
AAA ATC TAT GTT CCA GAG GAG AAA CTT TTG AAG GAC ATC CAA GAG	315
K I Y V P E E K L L K D I Q E	
TCC CCA ATT CCA ATC AAC GTG ATA TTA GCA ATC AAC CAC TCA GTT	360
S P I P I N V I L A I N H S V	
TTT GTG AAG GGA GAT CAT ACC AAC TTC GAG ATT GAG GCA TCC TTC	405
F V K G D H T N F E I E A S F	
GGT GTG GAG GCC TCC GAG CTT TAC CCA GAT GTC AAA TAC ACC ACA	450
G V E A S E L Y P D V K Y T T	
GTG TAA	
V	

Fig. 7. Sequence of the cDNA fragment amplified by PCR with primers TAB5b/oligo dT-deg. Deduced amino acids are additionally shown in the 1-letter code. Underlined amino acids were confirmed by Edman degradation of the 24-kDa Lys C polypeptide. In the position of the T (indicated by a break), an I was determined by amino acid sequencing.

sequences of 12 and 10 residues, each caused by two polypeptides derived from the 35-kDa protein. However, their relative amounts were considerably different and thus the overlaid sequences were distinguishable: 15 kDa, (I) TEAEGIPYTYVI + (II) VVIFGDGNARAV; 27 kDa, (III) TEAEGIPYTY + (IV) VRIRRKTEAE. A clear partial sequence of 25 amino acid residues was obtained from the third fragment which was excised at 24 kDa, (V) VVIFGDGNARAVFNKEDDIGTYTIR. Since (III) was identical with the N-terminal segment of (I), (II) represented the N-terminal stretch of (V) and (IV) showed an overlap with (I), resulting in VRIRRKTEAEGIPYTYVI, two different internal sequences were obtained. A non-redundant BLAST search using these sequences indicated high identities with corresponding sequences of IRL and IFR from several plants. The resulting identities of the 25-residue peptide (V) are summarized in Table 1. The regions of similarity were found in proteins with 308–318 amino acid residues and masses ranging from 32.8–35.5 kDa. The sequences corresponding to the longest peptide were located in regions ranging from residues 175–199 to 186–210 of the homologous proteins. Comparable identities were obtained with the other internal sequences. As an example (I) + (IV) was 88% identical with the stretch 136–153 of the tobacco IRL and to a degree of 82% identical with residues 135–152 of the IRL from mouse-ear cress.

Cloning and sequencing of DNA fragments

Nucleotide sequencing was used to obtain extended sequence

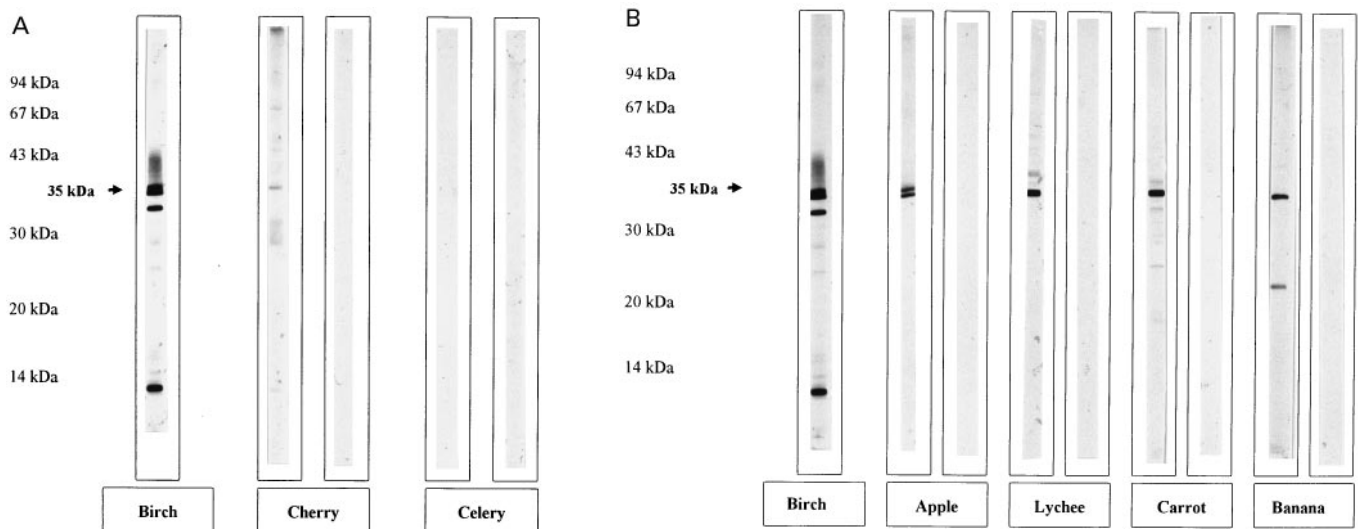


Fig. 8. (A) SDS-PAGE-immunoblot analysis of birch pollen extract and extracts from cherries and celery tubers, detected with rabbit antiserum (1:6000) raised against the purified pea IFR. Samples as indicated in the Figure. Buffer controls of the food extracts are shown in the lanes on the left side. (B) SDS-PAGE-immunoblot analysis of birch pollen extract and extracts from apples, lychee fruit, carrots and bananas, detected with rabbit antiserum (1:6000) raised against the purified pea IFR. Samples as indicated in the Figure. Buffer controls of the food extracts are shown in the lanes on the left side.

data of the proposed new birch pollen allergen. After extraction of RNA and reverse transcription various combinations of primers were tested for specifically amplifying full-length DNA or cDNA fragments. Alf3ECOR1 and Alf5ECOR1, derived from an Alfalfa sequence (Table 1), had been successfully applied to amplify the gene of a pea IFR [18, 21], but yielded no amplicon with DNA transcribed from birch pollen RNA. The same was true for the internal primers TAB5a/TAB3c, TAB5a/oligo dT-deg, and TAB5c/oligo dT-deg which were deduced from the cDNA of a tobacco IRL (Acc. No. D28505) [19]. In contrast, a product slightly below 600 bp which correlated with the fragment size expected from the known tobacco sequence (610 bp) was obtained with TAB5b/oligo dT-deg. The Bet v 1-deduced primers BetvI3 and BetvI5 always produced amplicons of the expected size (650 bp). The purity and size of the PCR products is illustrated by the agarose electrophorogram of Fig. 6. The band which appeared in Fig. 6, lane B, was excised, desalted, ligated into pGEM-T and transformed into competent *E. coli* JS5. After blue/white screening and PCR screening using primers T7 and SP6 recognizing promoter sequences of the vector, plasmids were prepared from two clones. These were subsequently processed by sequencing using T7, SP6, TAB5b and oligo dT-deg as sequencing primers. The nucleotide sequence and the deduced amino acid sequence of the open reading frame of a positive clone are presented in Fig. 7. The specific sequence contained an open reading frame of 453 nucleotides corresponding to 151 amino acids which represents $\approx 50\%$ of the 35-kDa birch protein. The fragment contained the complete 25-residue segment which was analyzed by Edman degradation (underlined in Fig. 7). Both amino acid and deduced amino acid sequence of the fragment were identical except for one residue (I/T). This point mutation is encoded by a single

nucleotide exchange. These results clearly indicated that the amplified gene product corresponded to the IgE-binding protein cleaved by proteases and analyzed by amino acid sequencing. Similarity searches confirmed the high degree of sequence identity of the birch protein encoded by the gene that was partially amplified with IRL and IFR proteins. At the amino acid level, the partial sequence from birch showed the following degrees of sequence identity: potato IRL, 69%; tobacco IRL, 62%; mouse-ear cress, 71%; lupine IRL, 45%; pea IFR, 58%; chick pea IFR, 56%; maize IRL, 57%; and alfalfa IRL, 57%.

Detection of related plant proteins by immunoblotting

In a previous publication [1] we described cross-reactivity of IgE specific for the 35-kDa birch protein with proteins from various plant foods including some kind of exotic fruit. To confirm these cross-reactivities and to prove the hypothesis that these allergens represent IFRs or IRLs a rabbit antiserum raised against the IFR from peas [10] was used for immunoblotting experiments. Birch pollen extract and extracts from selected fruits and vegetables were separated by SDS-PAGE and transferred to NC membranes. As shown in Fig. 8a, a 35-kDa band and two bands of lower masses were detected by the rabbit anti-IFR in birch pollen extract but not in extracts from cherries and celery. Apples, lychee fruit, bananas and carrots also contained 35-kDa proteins that were recognized by the serum (Fig. 8b). These results were in accordance with our data obtained by investigating the same extracts with IgE from patients allergic to birch pollen and fruit by immunoblotting and immunoblot inhibition [1]. Recognition of bands at lower masses by this serum has also been observed with extracts from peas [10, 18] and alfalfa [21] and may be explained either by binding to degradation products of IFR, by

binding to highly homologous proteins, or caused by impurities of the immunogen.

DISCUSSION

Evidence can be taken from the present investigation that the 35-kDa birch pollen protein is a candidate for a new pollen allergen with cross-reactivity to specific foods. Moreover, it is likely that the protein is related to plant IFR and IRL.

With regard to allergenicity, IgE-binding capacity of the protein and cross-reactivity to plant foods was clearly demonstrated [1]. Immunoblot inhibition with the purified 35-kDa fraction and with pure Bet v 1 showed the specific recognition of the 35-kDa band by patients' IgE as well as the presence of epitopes of the native protein and the absence of Bet v 1 epitopes on proteolytic fragments of the purified 35-kDa band (Fig. 5). Immunological independence from Bet v 1 is further underlined by the observation that the allergen and its fragments lack any reactivity with IgE from patients exclusively sensitized to Bet v 1 and with six Bet v 1-specific MoAbs. However, the clinical significance of our serological data is not proven and needs further studies in an extended group of patients. In our opinion, it is likely that the 35-kDa protein contributes to the allergenicity of birch pollen and that the related homologues in plant foods may exclusively mediate allergy to specific foods in some of these patients. For example, patient Bo212 clearly presented IgE against Bet v 1 and the 35-kDa protein. The IgE against the latter protein cross-reacted with a 35-kDa protein from bananas, against which the patient was allergic and skin test positive. No allergen with similarity to Bet v 1 has been detected in bananas to date. Several foods have been investigated by EAST inhibition in our previous study [1]. Among these were also peas, which are known to contain an IFR related to the 35-kDa birch protein. In addition to the cross-reacting foods identified by immunoblot inhibition, the 35-kDa fraction from birch significantly inhibited the IgE binding to pea allergen disks (50% at an inhibitor concentration of 25 µg/ml). This observation may now be explained by IgE binding to the pea IFR.

However, the identification of the proposed 35-kDa birch IRL as an IgE-binding and cross-reacting component is only proven in the case that the protein band isolated by preparative SDS-PAGE has a considerable degree of purity. In addition to the gel shown in Fig. 1, further evidence for purity resulted from non-reducing 2 D-PAGE of birch pollen extract supplemented by immunoblotting [1] showing a single spot at 35 kDa. Complete blockage in attempts of direct microsequencing of the purified material used in this study indicated the absence of any unblocked impurities. Fragments obtained by enzymatic cleavage were either blocked or yielded sequences of high similarity to IRL and IFR. Finally and surprisingly, the 35-kDa birch protein and Bet v 1 showed a very similar amino acid composition, also similar to that of the tobacco IRL. When the inherent variation of automated amino acid analysis from pmols of blotted proteins is taken into consideration, these proteins cannot be distinguished by their amino acid composition. For the 35-kDa

fraction, the determination of a composition corresponding to a closely related protein is not to be expected in the presence of major impurities. Taken together, these data are consistent and raise little doubt on the purity of the investigated material.

We feel that there is strong evidence that the 35-kDa protein belongs to an IFR/IRL protein family. First, sequencing of fragments resulted in two internal stretches with high degrees of identity to many IFR and IRL, all of which possess masses between 32.8 and 35.5 kDa. Second, application of a specific primer (TAB5b) that was selected on the basis of a homologue tobacco IRL sequence in a PCR with birch pollen cDNA as the template yielded an amplicon highly homologous to translation products of IFR and IRL genes. This amplicon also encoded for the partial sequence determined by Edman degradation except for one diverging residue. Third, rabbit antibodies raised against an IFR from peas strongly cross-reacted with the 35-kDa birch protein and with proteins in apples, lychee fruit, bananas and carrots which were previously identified by cross-reactivity studies with patients' IgE. As demonstrated with both IgE [1] and rabbit antiserum, these proteins were not abundant in cherry extracts or celery extracts. On the basis of our sequence data and the molecular mass of the 35-kDa birch protein, we can also exclude the theory that the protein described here is identical with the minor birch pollen allergens Bet v 3 [22] and Bet v 4 (EMBL Acc. X87153). Hence, it is likely that we have identified a new pattern of antigenic and probably allergenic cross-reactivity.

At the molecular level, three allergenic structures from birch pollen are known to cross-react with plant foods (recently reviewed in Ref. 23). The major allergen, Bet v 1, shares common epitopes with major allergens from apples, nuts, cherries and other stone fruits, celery tubers and carrots. The pattern of cross-reactivity caused by Bet v 1 is hence restricted to specific foods. In contrast, birch pollen profilin, Bet v 2, is able to mediate an almost unlimited pattern of cross-reactivity within the plant kingdom, as profilins are present in all eukaryotic cells. The same is true for the third structure, specific Asn-linked glycans containing terminal α 1,3fucose and β 1,2xylose residues which occur in many plant glycoproteins. In some patients with pollinosis IgE occurs against these structures and is highly cross-reactive. Due to our results, the 35-kDa protein was not glycosylated. This correlates with a restricted pattern of cross-reactions involving foods highly associated with birch pollen allergy, such as apples, and others such as bananas and lychee fruit that seldom affect patients allergic to birch pollen. In addition cross-reactions to other IRL- and IFR-containing foods, such as potato, are to be expected.

Antibody-reactive fragments were only obtained after Glu C treatment, whereas the digests obtained with Lys C and Clostripain lacked IgE-binding capacity. The latter observation is probably due to the destruction of conformational epitopes. On the one hand, it is remarkable that the IgE-binding fragments were not accessible to sequencing, indicating a complete N-terminus blocked by post-translational modification. On the other hand, the internal sequences were obtained from IgE-inactive

polypeptides. This could be explained by the hypothesis that the N-terminus is required to retain the proteins' IgE reactivity.

Acetylation is a very common post-translational modification leading to a blocked N-terminus of eukaryotic proteins. The amino acids Ala, Ser and Met are known to be predominantly present at the N-terminus of N- α -acetylated proteins [24, 25]. Interestingly, despite the post-translationally cleaved Met, the deduced protein sequences of the IRL and IFR from peas, chick peas, alfalfa, *Arabidopsis thaliana*, tobacco, potatoes and maize present a common Ala residue at their N-termini. Thus, N-terminal blockage by acetylation is an attractive hypothesis for explaining the blockage experimentally observed for the birch protein.

The IFR of this group are involved in generating pterocarpin phytoalexins by synthesizing chiral isoflavones from achiral precursors via an NADPH-dependent reduction step [10, 18]. These phytoalexins participate in plant defence reactions against fungal pathogens. The IRL show a high sequence identity compared to IFR [19, 26] but do not reduce 2'-hydroxyflavones, the specific substrate of IFR. Thus, the biochemical function of these IRL remains unknown. Since the partial sequence of the birch pollen protein shares a considerably higher degree of sequence identity with IRL, as with IFR, the birch gene may also encode for an IRL. After full length cDNA cloning and expression, this hypothesis will be tested by enzymatic assays using the specific substrates of IFR. Bet v 1 and its related food allergens are thought to belong to a group of intracellular pathogenesis-related proteins being involved in plant defence mechanisms [5, 20]. Our ongoing investigation will highlight whether another group of 'defence proteins' creates important allergens in pollen and food.

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