

Pyr c 1, the major allergen from pear (*Pyrus communis*), is a new member of the Bet v 1 allergen family

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Abstract

Pear is known as an allergenic food involved in the ‘oral allergy syndrome’ which affects a high percentage of patients allergic to birch pollen. The aim of this study was to clone the major allergen of this fruit, to express it as bacterial recombinant protein and to study its allergenic properties in relation to homologous proteins and natural allergen extracts. The coding region of the cDNA was obtained by a PCR strategy, cloned, and the allergen was expressed as His-Tag fusion protein. The fusion peptide was removed by treatment with cyanogen bromide. Purified non-fusion protein was subjected to allergenicity testing by the enzyme allergosorbent test (EAST), Western blotting, competitive inhibition assays, and basophil histamine release. The deduced protein sequence shared a high degree of identity with other major allergens from fruits, nuts, vegetables, and pollen, and with a family of PR-10 pathogenesis related proteins. The recombinant (r) protein was recognised by specific IgE from sera of all pear-allergic patients ($n=16$) investigated in this study. Hence, the allergen was classified as a major allergen and named Pyr c 1. The IgE binding characteristics of rPyr c 1 appeared to be similar to the natural pear protein, as was demonstrated by EAST-inhibition and Western blot-inhibition experiments. Moreover, the biological activity of rPyr c 1 was equal to that of pear extract, as indicated by basophil histamine release in two patients allergic to pears. The related major allergens Bet v 1 from birch pollen and Mal d 1 from apple inhibited to a high degree the binding of IgE to Pyr c 1, whereas Api g 1 from celery, also belonging to this family, had little inhibitory effects, indicating epitope differences between Bet v 1-related food allergens. Unlimited amounts of pure rPyr c 1 are now available for studies on the structure and epitopes of pollen-related food allergens. Moreover, the allergen may serve as stable and standardised diagnostic material. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In 1982, Eriksson et al. described a specific cluster of food hypersensitivities occurring in patients with birch pollen allergy [1]. Typically, the patients experienced local symptoms at the oral mucosa

immediately after ingestion of fresh fruits, nuts, and vegetables. In 380 patients with birch pollen allergy, the following incidences of food allergy were reported: hazelnut 53%, apple 47%, peach 34%, cherry 29%, almond 27%, walnut 26%, pear 26%, and carrots 23%. In addition, it became evident that oral allergy to celeriac (celery tuber) is associated with birch pollen as well as with mugwort pollen allergy [2]. Since apples were among the most important elicitors of this kind of adverse reaction, apple

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allergy was investigated as a model for the so-called 'pollen-related food allergy'. As a first indication of the molecular basis of this clinical phenomenon, immunological inhibition assays showed that IgE antibodies specific for birch pollen allergens cross-reacted with allergens in apple extract [3–5]. Ebner et al. were the first who identified the proteins responsible for this cross-reaction [6]: They identified common epitopes on Bet v 1, the major birch pollen allergen, and a 17–18-kDa allergen in apple extract. We determined the N-terminal protein sequence of this apple allergen by Edman degradation [7]. A stretch of 26-amino acid residues showed 62% identity with the N-terminus of Bet v 1, indicating the high homology of the two proteins [8]. The cDNA of the major apple allergen, Mal d 1 has been sequenced and cloned [9–12]. Several isoforms of Mal d 1 have been expressed in *E.coli*, and these recombinant allergens presented allergenic activities similar to their natural counterparts [9,12]. Both Bet v 1 and Mal d 1 were found to be highly homologous to a group of PR-10 pathogenesis related proteins which occur in many food plants, for example in parsley [13] or soybean [14].

Cross-reactive allergens belonging to this protein family have been identified not only in fruits and nuts such as cherry, pear, and hazelnut [15–17], but also in vegetables such as carrots, celeriac, and potato [15,16,18]. The major allergens Pru av 1 from cherry [19], Cor a 1.0401 from hazelnut (GenBank accession no. AF136945), Api g 1 from celeriac [20], and Dau c 1 from carrot [21] have been cloned and expressed as bacterial recombinant proteins. All these allergens presented a high biological activity in patients suffering from the respective food allergies, and they bound IgE from sera of at least 80% of these patients. Surprisingly, studies on the cherry allergen Pru av 1 and the celeriac allergen Api g 1 showed epitope differences between these molecules, although both proteins strongly cross-reacted with IgE antibodies against Bet v 1 [22]. To get a better insight in the fine specificity of the interaction of IgE with pollen-related food allergens and into the structural basis of this kind of food allergy, we wanted to extend the number of recombinant Bet v 1-related food allergens. Therefore, we cloned the entire coding sequence of the cDNA of the major pear allergen by a PCR strategy. A gene-specific

primer was selected on the basis of related cDNA sequences and a known N-terminal protein sequence of the pear protein. After expression in *E.coli*, the recombinant allergen showed strong reactivity with IgE from pear-allergic patients in a battery of allergen assays, and unlimited amounts of pure Pyr c 1 are now available for further studies.

Little is known about minor allergens in pear. A member of the profilin family which cross-reacts with IgE against the birch pollen minor allergen Bet v 2 has been identified in pear [16,23]. Moreover, a pear allergen related to the minor birch pollen allergen Bet v 6 has recently been identified by immunoblotting [24]. Hence, rPyr c 1 offers the opportunity to investigate the extent by which the major allergen contributes to the IgE binding capacity of a fruit.

2. Experimental

2.1. Human sera and blood samples

Sera from 16 patients allergic to birch pollen and with allergic symptoms after ingestion of fresh pears were selected for this study. Most of them reported also symptoms after ingestion of fresh apples, hazelnuts and other birch pollen associated allergenic foods. Apart from anamnesis, allergy diagnosis was based on a positive skin prick test to birch pollen extract and pear extract or the determination of specific serum IgE antibodies against pear by the enzyme allergosorbent test (EAST) (Allergopharma, Reinbek, Germany). The sera from pear-allergic patients were kindly supplied by Dr. H. Aulepp (Hospital Borkum Riff, Borkum, Germany) or taken from the serum collection of the Paul-Ehrlich-Institute, Langen, Germany. Seven additional sera including two pooled sera from seven and 12 patients allergic to birch pollen were selected on the basis of a positive IgE assay to pear without further information about history of food allergy in these patients. These sera were purchased from Allergopharma, and from MAST Diagnostika (Reinfeld, Germany). Two sera from non-allergic volunteers were included as controls.

For histamine release assay whole blood samples were taken from two patients who were allergic

according to the above criteria and from two non-allergic control subjects. Written informed consent was obtained from all participants.

2.2. Monoclonal antibodies

Monoclonal antibodies (mAbs) directed against Bet v 1 were kindly supplied by Dr. K. Foetisch, Paul-Ehrlich-Institut. A mAb against the major peanut allergen, Ara h 1, was applied as a negative control (a gift from Dr. W.-M. Becker, Borstel Research Center, Borstel, Germany).

2.3. Allergens and allergen extracts

The recombinant major allergens from birch pollen (rBet v 1), celery tuber (rApi g 1), apple (rMal d 1) as well as the recombinant minor birch pollen allergen profilin (rBet v 2) were purchased from BIOMAY (Linz, Austria). Extract from pear (*Pyrus communis*) of the variety 'Williams Christ' was prepared as described elsewhere [7]. Birch pollen extract was obtained from MAST Diagnostica.

2.4. Cloning of cDNA

Total RNA (4 µg) was isolated from 1.5 g of fresh pear by means of the RNeasy Plant Total RNA kit (Quiagen, Hilden, Germany). RNA was reversely transcribed by the First Strand cDNA Synthesis kit (Pharmacia, Freiburg, Germany) using 0.8 µg for each transcription and oligo-dT for priming. Since the 10 N-terminal amino acid residues of the major pear allergen showed only one mismatch with the sequence of Mal d 1 [25], a 5'-gene-specific primer PYRC1-1: 5'-TAC ACA TTT GAG AAC GAG-3' was selected on the basis of the known Mal d 1 cDNA sequence. PYRC1-1 is identical with nucleotides 10–28 of the coding region of Mal d 1 [10]. Oligo-dT was used as antisense primer. The PCR conditions were: hot start (94°C, 2 min), denaturation 94°C, 1 min, annealing 50°C, 1 min, polymerisation 72°C, 2 min. After 35 cycles the final extension was 5 min at 72°C. Amplified DNA fragments were purified from agarose gels by the Quiaex II kit (Quiagen, Hilden, Germany), ligated into the pGEM-

T vector (Promega, Madison, WI, USA) and used for transformation of *E. coli* XL1-Blue (Stratagene, Heidelberg, Germany) by electroporation. Plasmids from positive clones were subjected to sequence analysis 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') as vector-specific sequencing primers. The 5'-RACE (rapid amplification of cDNA ends) method (5'-RACE System, Gibco-BRL, USA) was used for amplification of the unknown 5'-end of the pear allergen according to the manufacturer's instructions. First-strand cDNA synthesis was primed with an antisense gene-specific primer PYRC1-GSP1: 5'-CCT TCA AGG ATC TCA GCA TGC-3'. A homopolymeric dC-tail was ligated to the 3'-end of the purified cDNA by a terminal desoxynucleotidyl transferase reaction. The dC-tailed cDNA was amplified by PCR using a nested second gene-specific primer PYRC1-GSP-2 (5'-ATG AGG TTG TCA GCA TCG-3') and the 'Abridged Anchor Primer' (enclosed in the 5'-RACE System), and again cloned into pGEM-T and subjected to sequencing. For amplification of the entire coding region of the allergen, terminal primers PYRC1-2 (5'-GGA CTT TAC ACA TTT GAG AAC GAG TTC-3') and PYRC1-3 (5'-GGT GTT TAC ACA TTT GAG AAC GAG TAC-3') were selected on the basis of the sequenced DNA fragments. The purified PCR product was ligated into pGEM-T, and amplified in *E. coli* XL1-Blue cells. Plasmids were sequenced using T7 and PYRC1-3 as sequencing primers. Restriction sites (3'-*Bam*HI and 5'-*Xho*I) were added to the coding cDNA by PCR using the primers 3'-*Bam*HI-PYRC1 (5'-CGG GAT CCC GTT AGT TGT ATG CGT CGG GGT GGT CCT TAA GG-3') and 5'-*Xho*I-PYRC1 (5'-CCG CTC GAG CGG GGA CTT TAC ACA TTT GAG AAC GAG TTC 3') (restriction sites and stop codon underlined). After 2 min at 94°C, 35 PCR cycles were run at 94°C, 1 min, 55°C, 1 min, 72°C, 2 min followed by final extension for 5 min at 72°C. The product was ligated into the plasmid pET-16b (Novagen, Madison, WI, USA), amplified in XL1-Blue cells, purified and transformed into *E. coli* BL21(DE3) expression cells (Novagen). All primers were purchased from ARK (Darmstadt, Germany).

2.5. Expression and purification of recombinant allergen

Protein synthesis was induced with 1 mM isopropyl-D-thio-galactopyranoside (IPTG) for 4 h at 37°C, and cells were harvested by centrifugation. Since no soluble recombinant Pyr c 1 was obtained after cell lysis, the cell pellet was processed under denaturing conditions using 6 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0, 0.5 mg/ml lysozyme as lysis buffer. The supernatant was separated from the cellular debris, incubated with Ni-NTA-Agarose (Quiagen) at room temperature (RT) for 1 h. Purification of the fusion protein was achieved according to the protocol of the manufacturer using 6 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 5.9, and 6 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 4.5, as elution buffers. Non-fusion protein was obtained by cyanogen bromide (CNBr)-cleavage and refolded as described by Boehm and Roesch [26].

2.6. Electrophoresis, protein transfer and immunodetection procedures

Protein samples were separated by SDS-PAGE ($T=12.5\%$, $C=2.7\%$) under reducing conditions using a Bio-Rad (Munich, Germany) Mini Protean cell [27]. Gels were either stained with Coomassie brilliant blue or proteins were transferred to nitrocellulose (NC) membranes (Schleicher und Schüll, Dassel, Germany) by semi-dry blotting [28]. For the detection of specific IgE antibodies, patients' sera were diluted 1:10. Hybridoma supernatants were also applied at a dilution of 1:10. IgE antibodies and mAbs bound to recombinant Pyr c 1 were visualized as described [7] with slight modifications, using alkaline phosphatase (AP)-labeled streptavidin (Medac, Hamburg, Germany, 1:3000) instead of horseradish peroxidase-labeled streptavidin and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as the substrates. IgE binding to blotted extracts was detected with monoclonal anti-IgE-AP (1:750, Pharmingen, Hamburg, Germany).

The diluent for all reagents was 0.05 M TBS containing 0.1% bovine serum albumin (BSA) and 0.05% (v/v) Tween 20, pH 7.4. Blocking buffer was

0.3% Tween 20 in TBS. Prior to microsequencing, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) as described [7]. Inhibition assays were performed by incubation of 50 µl of patient serum with 15 µg of recombinant allergens or 100 µg of protein from allergen extract in a total volume of 0.6 ml incubation buffer for 30 min prior to immunological detection.

2.7. N-Terminal amino acid sequencing

Microsequencing was performed by Dr. A. Petersen (Borstel Research Center) according to the method of Matsudaira [29] using a 473-A pulsed liquid protein sequencer with an online phenylthiohydantoin amino acid analyser (Applied Biosystems, Weiterstadt, Germany).

2.8. Semi-quantitative determination of allergen-specific IgE in human sera

Pear extract and recombinant non-fusion Pyr c 1 (rPyr c 1) were covalently coupled to cyanogen bromide activated filter paper disks as described for apples and extracts from other plant foods [30]. The protein concentrations in the coupling solution were 12 µg/ml for pear extract and 0.6 µg/ml for rPyr c 1. The allergen disks were overnight incubated with 50 µl of human serum at room temperature. After washing, the amount of IgE specifically bound to the immobilised allergens was semi-quantitatively determined by a commercial EAST (Allergopharma, Reinbek, Germany), using the calibration system of the manufacturer. The results were expressed as EAST classes or as kU/l (class 0, no specific IgE; class 4, very high content of specific IgE corresponding to >17.5 kU/l). All samples were analyzed as duplicates. Specific IgE to birch pollen extract and the birch pollen allergens rBet v 1 and rBet v 2 were determined by the CAP-System (Pharmacia, Uppsala, Sweden) and expressed in CAP classes (0–6) or international units/ml (0–100 U/ml, 1 U/ml corresponds to 2.4 ng/ml of IgE).

2.9. EAST-inhibition

A quantitative estimation of the cross-reactivity of IgE with recombinant and natural pear allergens was

derived from a competitive ELISA inhibition assay. Patient sera were diluted to an optical density of approximately 50% of the EAST signal (usually 1:2–1:10) in 0.05 M Tris buffer, pH 7.4, 0.15 M NaCl, 0.3 g/l bovine serum albumin (BSA) and 1 ml/l Tween 20 (TBS–BSA–Tween). A 10-fold dilution series of the inhibitor extracts was prepared in six steps in TBS–BSA–Tween. Volumes of 50 μ l diluted serum and 50 μ l inhibitor solution were incubated with an allergen disk overnight at room temperature. Subsequently, the EAST was proceeded according to the standard procedure. The results were expressed as percent inhibition. Control inhibitions were performed with extract from low-fat milk at the two highest protein concentrations of 100 and 1000 μ g/ml. Generally, all determinations were run in duplicate.

2.10. Basophil histamine release

The assay was carried out on whole blood samples as described by Siraganian [31,32] and Siraganian and Brodsky [33], respectively.

3. Results

3.1. Cloning of cDNA and sequence analysis

Four μ g total RNA (*Pyrus communis*) were isolated from 1.5 g fresh pear. Using the primer oligo-dT and the gene-specific primer PYRC1-1 an incomplete Pyr c 1 cDNA was amplified. DNA fragments of a length of approximately 700 base pairs (bp) were purified from agarose gel, cloned into plasmid pGEM-T and sequenced. The 5'-RACE method was applied to obtain the unknown 5'-end of the cDNA and to confirm the sequence of primer PYRC1-1. The 5'-RACE PCR resulted in one single fragment of approximately 250 bp length. Both sequences matched with an overlap of 47 nucleotides. Thereafter, the entire encoding sequence was amplified from RT-RNA using the 5'-primer PYRC1-2 (5') and PYRC1-3 as 3'-primer. A single band of about 500 bp was again cloned into pGEM-T. Eleven clones yielded a 550-bp product in a PCR screening with the primers T7 and PYRC1-3 (Fig. 1). Full-length sequences were obtained from two clones.

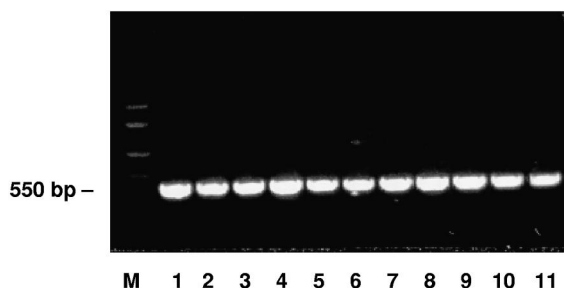


Fig. 1. PCR-screening of 11 *E. coli* XL-Blue clones containing the full-length Pyr c 1 coding region in the plasmid pGEM-T. T7 and PYRC1-3 were used as primers. Amplified fragments were separated by agarose gel electrophoresis. The expected size of the amplicon is 550 bp.

The entire coding sequence of Pyr c 1 clone 5 that was selected for expression and the deduced amino acid sequence are depicted in Fig. 2. Clone 6 contained two single nucleotide exchanges leading to amino acid exchanges I38V and Y64H (counting without Met). The coding region of the Pyr c 1 cDNA consists of 480 bp (including stop codon), describing a protein of 158 amino acid residues (excluding N-terminal Met). The deduced protein has a calculated molecular mass of 17.4, a theoretical isoelectric point of 5.5, and contains no *N*-glycosylation site. No cysteine residues are present, excluding the formation of disulfide bonds. The sequence was submitted to the GenBank database (accession no. AF057030). The deduced protein sequence was subjected to a BLAST similarity search [34]. A high degree of amino acid identity was indicated with

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ATGGGACTTTACACATTGAGAACGAGTTCACCTCTGAGATTCACCAAGATTGTTTC 60
M G L Y T F E N E F T S E I P P P R L F 20

AAGGCCCTTGTCTCGATGCTGACAACTCATCCCCAAGATTGACCCCAAGCAATCAAG 120
K A F V L D A D N L I P K I A P Q A I K 40

CATGCTGAGATCCTTGAAGGAAACGGTGGCCCGGAACCATCAAGAAGATCACTTTTGGT 180
H A E I L E G N G P G T I K K I T F G 60

GAAGGCAGCCAGTACGGCTACGTGAAGCACAGGGTCGACTCGATTGACGAAGCAAGTTAC 240
E G S Q Y G Y V K H R V D S I D E A S Y 80

TCATACGCCTATACCTTTGATTGAAGGAGATGCTTTGACAGACACCATCGAGAAAATATCT 300
S Y A Y T L I E G D A L T D T I E K I S 100

TACGAGGCCAAGTTGGTGGCATCTGGAAGTGGTTCAACCATCAAGAGCATCAGCCACTAC 360
Y E A K L V A S G S G S T I K S I S H Y 120

CATACCAAGGAGACATTGAAATTAAGGAAGAACACGTCAAGGCTGGAAGAGAGAGGCC 420
H T K E G D I E I E G E H V K A G K E K A 140

CATGGTTTGTTCAGCTTATTGAGAGCTACCTTAAGGACCAACCCGACGCATACAATAA 480
H G L F K L I E S Y L K D H P D A Y N * 160

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Fig. 2. Nucleotide sequence of the coding region and deduced amino acid sequence of Pyr c 1.

many proteins which were described as food allergens, tree pollen allergens or PR-10 pathogenesis related proteins or stress proteins. Representative examples are summarised in Table 1. The highest degree of 92% sequence identity was obtained with the major apple allergen, Mal d 1, followed by 84 and 79% identity with the major allergens from cherry and apricot, respectively. The major allergens from celery and carrot presented less than 40% sequence identity with Pyr c 1, but similarity with these proteins was at least 70%. A remarkably high identity of 70% was obtained with a stress protein from beech. Pyr c 1 shares 57% amino acid sequence identity with Bet v 1a, the major birch pollen allergen [8].

3.2. Expression of recombinant Pyr c 1

The cDNA coding for the suspected pear allergen was ligated into the plasmid pET-16b using *Bam*HI and *Xho*I restriction sites. This vector encodes for a fusion protein with 10 additional N-terminal histidine residues and a cleavage site for factor Xa protease. Initially, the plasmid was established in *E. coli* XL1-Blue to obtain stable transfectants; after recloning into *E. coli* BL21(DE3) cells, protein synthesis was

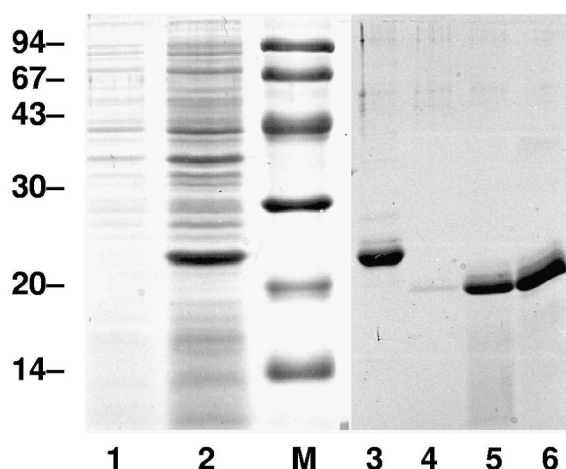


Fig. 3. Induction and purification of rPyr c 1 monitored by SDS–PAGE. Lanes: (1) non-induced *E.coli* BL21 (DE3) carrying the Pyr c 1-insert in pET-16b (5 μ l); (2) cells after induction with IPTG (4 h, 37°C); (3) rPyr c 1 fusion protein purified by Ni-chelate affinity chromatography; (4) wash; (5,6) non fusion protein obtained after CNBr treatment and refolding.

induced with IPTG for 4 h. The recombinant fusion protein showed an apparent mass of about 22 kDa and in stained SDS–PAGE gels represented about 25% of the cellular protein (Fig. 3, lane 2). For

Table 1
Amino acid sequence identity of Pyr c 1^a

Protein	Accession number	Organism	AA (no.)	I.D. (%)	SI (%)
<i>Food allergens</i>					
Mal d 1	AF074721	<i>Malus domestica</i>	158	92	97
Pru av 1	U66076	<i>Prunus avium</i>	159	84	94
Pru ar 1	AF020784	<i>Prunus armeniaca</i>	159	79	89
Cor a 1.0401	AF136945	<i>Corylus avellana</i>	159	62	85
Api g 1	Z48967	<i>Apium graveolens</i>	153	37	76
Dau c 1	Z84376	<i>Daucus carota</i>	153	37	70
<i>Tree pollen allergens</i>					
Cor a 1	Z72440	<i>Corylus avellana</i>	159	61	79
Bet v 1a	X15877	<i>Betula verrucosa</i>	159	57	83
Car b 1	Z80167	<i>Carpinus betulus</i>	159	52	81
Aln g 1	S50892	<i>Alnus glutinosa</i>	159	55	83
<i>PR-10 pathogenesis-related proteins (PRP) and stress proteins</i>					
Stress-protein	AJ130889	<i>Fagus sylvatica</i>	159	70	83
Bet v 1-Sc3	X77601	<i>Betula verrucosa</i>	159	63	78
Sam-22	X60043	<i>Glycine max</i>	158	51	82
PcPR1-3	X12573	<i>Petroselinum crispum</i>	154	38	73

^a AA, amino acid; ID, identity; SI, similarity.

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 with a purity of about 95%. Approximately 10 mg of Pyr c 1 fusion protein were purified from 500 ml of cultured bacteria. Non-fusion protein was obtained by CNBr treatment as described by Boehm and Roesch [26]. The cleaved 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 non-fusion protein presented an apparent mass of 18–20 kDa by SDS–PAGE (Fig. 3, lanes 5 and 6). To confirm the identity of the recombinant protein, the non-fusion rPyr c 1 preparation was subjected to SDS–PAGE, transferred onto PVDF membranes and analysed by Edman degradation. The N-terminal sequence of rPyr c 1 was LERGLYTFEN. This analysis revealed that the recombinant non-fusion protein contained three additional amino acid residues resulting from the vector and the nucleotides of the restriction site followed by the N-terminal sequence of the natural protein.

3.3. Allergen pattern of the patients

The allergen profile of the patients was determined by CAP and EAST. All 16 patients with a case history of pear allergy had high CAP classes of 4–6 to both birch pollen extract and the recombinant major allergen, Bet v 1. The birch pollen minor allergen profilin, rBet v 2 bound IgE from eight (50%) of the 16 patients, with four class 3 and four weak class 1 results, respectively (Table 2). Fifteen (94%) of the patients had a positive EAST for pear extract, and all showed IgE binding to rPyr c 1. Eleven of the 16 sera had a higher EAST result (U/ml) with rPyr c 1 than with pear extract, whereas four sera showed a stronger reactivity with the fruit extract. Seven additional sera were selected by the presence of serum IgE against birch pollen and pear. Three of these sera did neither react with rBet v 1 nor with rPyr c 1. These three sera had a positive CAP to rBet v 2, with two relatively high class 4 titers (Table 2). All determinations were negative with two non-allergic controls. However, it has to be noted that, owing to a higher sensitivity of the CAP

method, classes and units of CAP cannot be directly compared with EAST. IgE binding to rPyr c 1 was confirmed by immunoblotting (Fig. 4). All sera with a positive EAST to rPyr c 1 also presented IgE binding to a 17-kDa band on blot strips containing the recombinant allergen, whereas the Pyr c 1 band was not stained by the five sera with a negative EAST (Fig. 4).

3.4. Cross-reactivity between natural and recombinant allergens

To evaluate whether the recombinant allergen was able to inhibit IgE binding to its natural counterpart and to estimate to what extent Pyr c 1 contributes to the IgE binding capacity of pear extract, dose-related EAST inhibition experiments were carried out. Sera of patients PEI 115, PEI 61, and KEN were selected on the basis of the results of the determination of specific serum IgE to birch pollen: PEI 115 was positive to Bet v 1 but negative to Bet v 2; PEI 61 was positive to both Bet v 1 and Bet v 2, and KEN was negative to Bet v 1 and positive to Bet v 2 (Table 2). Inhibition of IgE binding to pear allergen disks was assayed by preincubating the sera with birch pollen extract and pear extract (0.01–1000 µg/ml of protein) and rPyr c 1 (0.001–100 µg/ml). The results of the inhibition experiments are presented in Fig. 5A–C. In all three sera, birch pollen extract and pear extract caused an almost complete inhibition of IgE binding to pear allergen disks. With serum PEI 115, rPyr c 1 strongly inhibited IgE binding to pear extract, reaching a plateau at a level of about 80% inhibition. A similar inhibition result was obtained with serum from another pear-allergic patient who was exclusively sensitised to Pyr c 1/Bet v 1 (results not shown). In patient PEI 61 who showed additional IgE against Bet v 2 from birch pollen, the maximal applicable concentration of 100 µg/ml of rPyr c 1 resulted in a partial inhibition of 50%. In the case of serum from patient KEN who had no detectable IgE against Bet v 1 and Pyr c 1, rPyr c 1 had no inhibitory effect on the IgE binding to pear extract. The inhibition results in Bet v 2-positive sera indicated that cross-reacting profilins [23] may be present in pear extract which present an IgE binding capacity not inhibitable by the major allergen. Incomplete inhibition of the pear EAST by

Table 2
Determination of IgE specific for birch pollen and pear allergens

Patient serum	CAP class (IU/ml)			EAST class (U/ml)	
	Birch pollen	Bet v 1	Bet v 2	Pear	Pyr c 1
<i>Patients with known pear allergy</i>					
PEI 129	5 (89.5)	5 (63.9)	0 (<0.35)	3 (7.3)	3 (6.6)
PEI 11	5 (55.5)	5 (52.7)	1 (0.52)	3 (4.2)	3 (10.4)
PEI 46	6 (>100)	5 (62.2)	0 (<0.35)	2 (2.4)	1 (0.4)
PEI 47	4 (23.0)	4 (27.9)	0 (<0.35)	2 (0.9)	2 (1.7)
PEI 61	5 (74.8)	5 (61.1)	3 (13.5)	3 (3.9)	3 (7.1)
PEI 72	5 (89.4)	6 (>100)	0 (<0.35)	3 (4.7)	3 (10.5)
PEI 115	6 (>100)	6 (>100)	0 (<0.35)	4 (19.5)	4 (27.4)
PEI 117	4 (37.7)	4 (40.5)	1 (0.7)	3 (4.2)	3 (3.8)
PEI 126	5 (56.0)	4 (20.1)	0 (<0.35)	3 (9.9)	4 (25.7)
PEI 07	6 (>100)	6 (>100)	3 (9.54)	3 (14.9)	3 (24.6)
PEI 133	4 (30.1)	4 (24.8)	3 (13.1)	2 (2.3)	1 (0.5)
949-09	4 (35.0)	5 (82.7)	3 (3.8)	3 (7.2)	3 (4.9)
949-19	4 (23.0)	4 (20.9)	0 (<0.35)	2 (1.2)	2 (1.3)
Bo120	4 (18.3)	4 (19.1)	1 (0.7)	0 (<0.35)	2 (0.80)
PEI 92	6 (>100)	6 (>100)	0 (<0.35)	3 (6.5)	4 (24.7)
Bo146	6 (>100)	6 (>100)	1 (0.5)	2 (0.77)	4 (26.5)
<i>Patients with birch pollen allergy without further data about food allergy</i>					
UL	5 (61.9)	5 (77.7)	3 (4.3)	2 (2.1)	2 (2.7)
MAST114	5 (58.3)	5 (58.5)	0 (<0.35)	2 (2.8)	2 (0.9)
MAST127	4 (34.3)	0 (<0.35)	4 (31.0)	3 (7.8)	0 (<0.35)
MAST497	3 (13.8)	0 (<0.35)	1 (0.5)	4 (21.2)	0 (<0.35)
KEN	3 (7.9)	0 (<0.35)	4 (20.6)	2 (2.6)	0 (<0.35)
Pool Zie	5 (61.1)	4 (47.5)	7.8 (3)	3 (8.8)	3 (3.8)
Pool Wn	4 (45.6)	4 (48.5)	0 (<0.35)	3 (10.3)	3 (4.4)
<i>Non-allergic controls</i>					
PEI 19	0 (<0.35)	0 (<0.35)	0 (<0.35)	0 (<0.35)	0 (<0.35)
PEI 20	0 (<0.35)	0 (<0.35)	0 (<0.35)	0 (<0.35)	0 (<0.35)

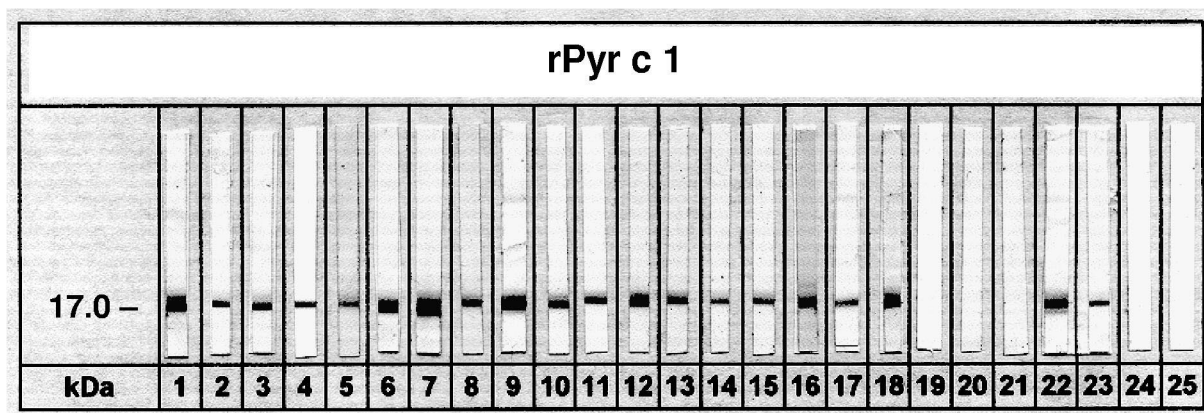


Fig. 4. Determination of IgE binding to rPyr c 1 blotted to nitrocellulose membrane. The amount of allergen subjected to SDS–PAGE was 0.6 µg/cm. Patients' sera in the same order as in Table 1.

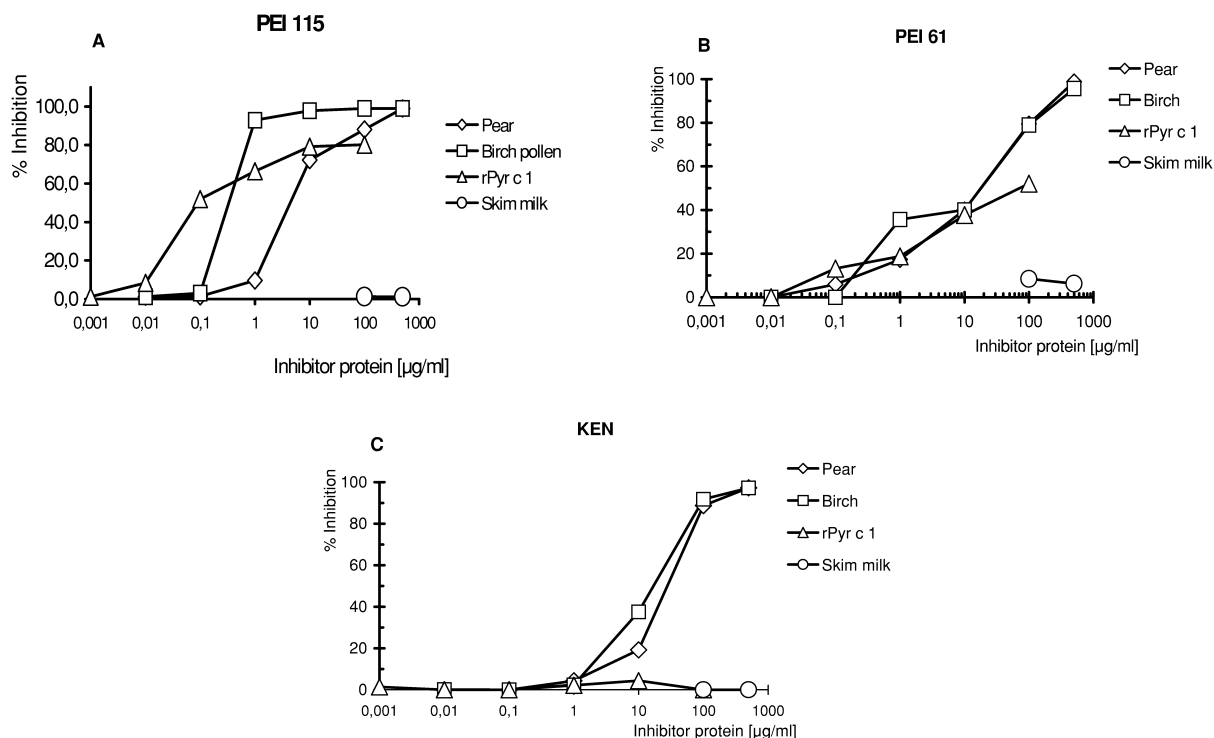


Fig. 5. Results of EAST inhibition experiments. Paper disks coupled with pear extract were incubated with diluted patient serum and increasing amounts of inhibitors as indicated. Subsequently, specific IgE bound to the solid phase was detected by a monoclonal anti-IgE-alkaline phosphatase conjugate.

rPyr c 1 may also result from epitope differences between recombinant and natural Pyr c 1. This hypothesis was tested by immunoblot inhibition with serum of patient PEI 61. In pear extract, a double band at 16–18 kDa was completely inhibited by preincubation of the serum with 15 μg of both rBet v 1 and rPyr c 1, whereas IgE binding to bands above 50 kDa remained unaffected (Fig. 6).

3.5. Detection of related allergens in extracts

Protein extracts from birch pollen, pear, and lychee fruit were separated by SDS–PAGE and transferred to nitrocellulose. Lychee fruit was chosen as control because it is not known to contain a member of the Bet v 1 allergen family. Two mAbs against Bet v 1 (Fig. 7, lanes 1 and 2) detected bands of approximately 17 kDa in birch pollen in pear, but not in lychee fruit. A similar reaction pattern was obtained with serum from the patient PEI 115 who

was allergic to pear and birch pollen, with the exception that a double band was stained in pear extract (Fig. 7, lane 4). No immunostaining was obtained with a mAb against the peanut allergen Ara h 1 (Fig. 7, lane 3) and serum from a non-allergic patient (Fig. 7, lane 5) which served as negative controls.

3.6. Cross-reactivity of IgE within the Bet v-1 allergen family

Pyr c 1 was coupled to activated paper disks which were used as solid phase for EAST inhibition experiments (Fig. 8). Binding of specific IgE from serum PEI 115 to rPyr c 1 was inhibited with 0.001 to 100 μg/ml of rPyr c 1, rBet v 1, rMal d 1, rApi g 1, and rBet v 2 as negative control. The strongest inhibition was obtained with Bet v 1, reaching 83% at maximal concentration and a 50% inhibition level caused by about 0.5 μg/ml. Self-inhibition by Pyr c 1

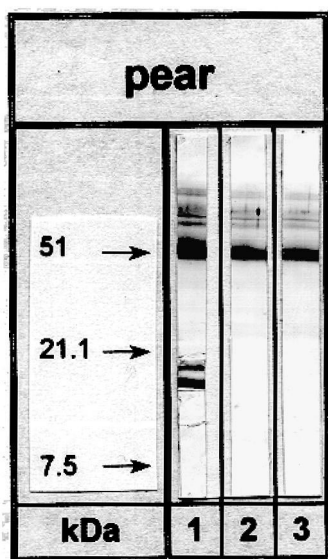


Fig. 6. Immunoblot inhibition experiment. Nitrocellulose membranes with electrophoretically separated pear extract were incubated with 50 μ l of serum from patient PEI 61 (lane 1), serum preincubated with 15 μ g rBet v 1 (lane 2), and serum preincubated with the same amount of rPyr c 1 (lane 3). A complete inhibition of all bands was obtained with 100 μ g of protein from pear extract; no inhibitory effect occurred with 100 μ g of milk protein as a negative control (results not shown).

was 78% at 100 μ g/ml, and 50% inhibition was measured at an inhibitor concentration of 10 μ g/ml. The apple allergen Mal d 1 had a lower maximal inhibition of 60%, with 50% reached at a concentration of about 50 μ g/ml. With a maximum of 16%, the celery allergen Api g 1 showed no specific inhibition, since a control experiment with the unrelated allergen Bet v 2 caused 20% non-specific inhibition in this assay.

3.7. Biological activity of rPyr c 1 investigated by basophil histamine release

Dose related histamine release experiments were undertaken in two patients who were allergic to birch pollen and pears and whose IgE antibodies did not react with minor allergens of birch pollen and pear (data not shown). These patients (PEI 21 and PEI 62) did not participate in the serological part of this study. Whole blood samples were stimulated with increasing amounts of extracts from birch pollen and

pear and with rBet v 1 and rPyr c 1. Released histamine was reacted with *o*-phthaldialdehyde and quantitated by means of an automated fluorometric analyser. The results are presented in Fig. 9. The response to allergen stimulation showed the same ranking in both patients, as graded by the protein concentration required to stimulate a histamine release of 50%. This concentration was highest for pear extract protein (0.05–0.1 μ g/ml), and about 10-fold lower amounts of rPyr c 1 were required to elicit the same effect. Another 10-fold lower amounts of birch pollen protein had about the same activity as rPyr c 1. Recombinant Bet v 1 had the highest allergenic potential in this system, with 100 pg/ml or even less releasing 50% of the histamine from the patients' basophils. In two non-allergic volunteers, pear extract and rPyr c 1 caused an unspecific signal corresponding to about 15% at the maximal concentration, whereas unspecific effects of Bet v 1 and birch pollen extract were generally less than 5% (not shown). These controls indicated that the release effects observed in the pear-allergic patients were specific.

4. Discussion

We have cloned, sequenced and expressed an allergen from pear which was identified as a new member of the Bet v 1 allergen family. All 16 pear-allergic patients investigated in this study had IgE that bound to the recombinant non-fusion protein. Moreover, serum IgE against this protein was detected in four of seven human sera which were selected on the basis of a positive IgE assay to pear. Hence, the pear protein represents a major allergen of this fruit and has to be named Pyr c 1 according to the revised official allergen nomenclature [35].

It is likely that the allergenic activity of rPyr c 1 is, to a high degree, similar to the natural pear protein: in patients who were not sensitised to minor allergens of the fruit such as PEI 115, PEI 62, and PEI 21, rPyr c 1 was able to inhibit most of the IgE binding to pear extract (Fig. 5A) and to trigger a histamine release similar to pear extract at concentrations corresponding to a major allergen amount of 1–10% of the total extract protein (Fig. 9A,B). Three additional amino acid residues (LER) at the

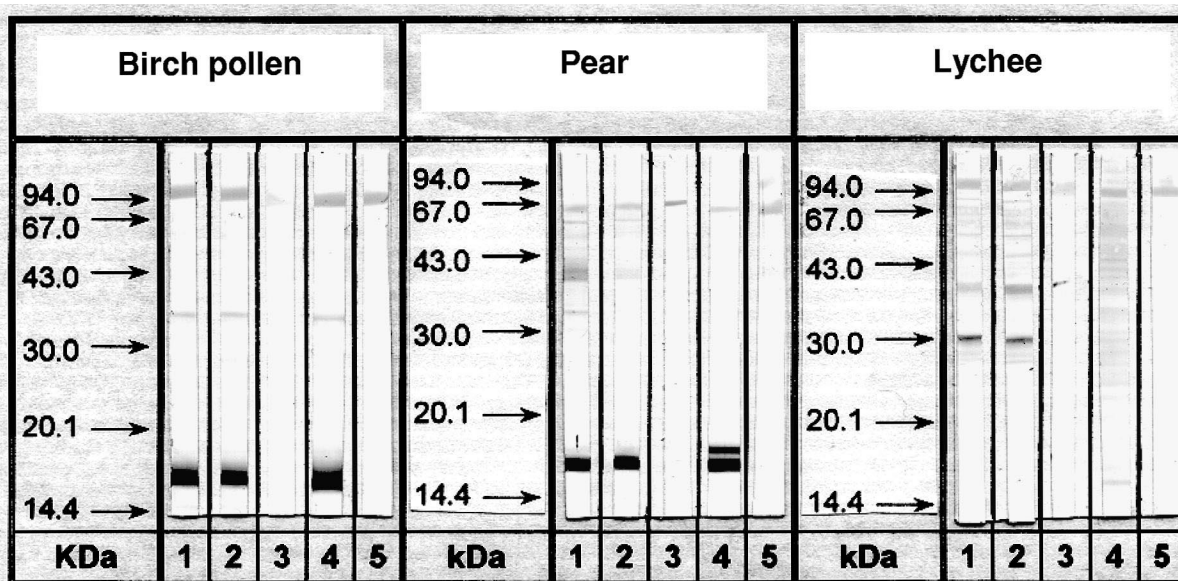


Fig. 7. Detection of cross-reacting allergens in birch pollen extract and pear extract. Blot strips of birch pollen, pear, and lychee fruit extract were probed with two mAbs against Bet v 1 (lanes 1 and 2), a mAb against the major peanut allergen Ara h 1 (lane 3), serum of patient PEI 115 (lane 4), and a non-allergic human control serum (lane 5).

proteins' N-terminus which resulted from the cloning strategy did not affect the allergenic properties. Natural Pyr c 1 was detected by IgE as a double band on immunoblots (Figs. 6 and 7). The binding of IgE to both bands was inhibited by rPyr c 1, indicating the existence of a second highly homologous isoform or truncated form of the allergen (Fig.

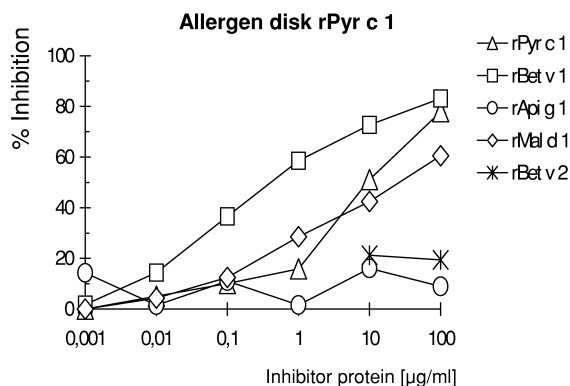


Fig. 8. EAST inhibition experiment to investigate cross-reactivity within the Bet v 1 allergen family. Allergen disks coupled with rPyr c 1 were incubated with serum of patient PEI 115 and inhibitor allergens as indicated.

6). This is in accordance with previous results: by two-dimensional immunoblotting and N-terminal microsequencing Schöning [25] identified two major allergen spots in pear extracts with apparent masses of 18 and 19 kDa, respectively, which had Bet v 1-related N-terminal sequences.

In patient PEI 61 with IgE against additional pear allergens, preincubation of the serum with rPyr c 1 completely inhibited immunostaining of natural Pyr c 1 on immunoblots of pear extract, but had no effects on the recognition of the minor allergens (Fig. 6). These minor allergens are also candidates to explain incomplete inhibition of the pear EAST by rPyr c 1 with serum of this patient (Fig. 5B). Since patient PEI 61 had a positive CAP for Bet v 2, we suspected that pear profilin with a mass of about 14 kDa would be the minor allergen responsible for this effect. By contrast, proteins with masses greater than 50 kDa were identified in pear extract by IgE from this patient. Thus, in this case pear profilin did either not cross-react with IgE against Bet v 2, or was not present in our pear extract.

The immunological relationship of Pyr c 1 with Bet v 1 was demonstrated by immunoblot inhibition with serum from a pear-allergic patient (Fig. 6) and

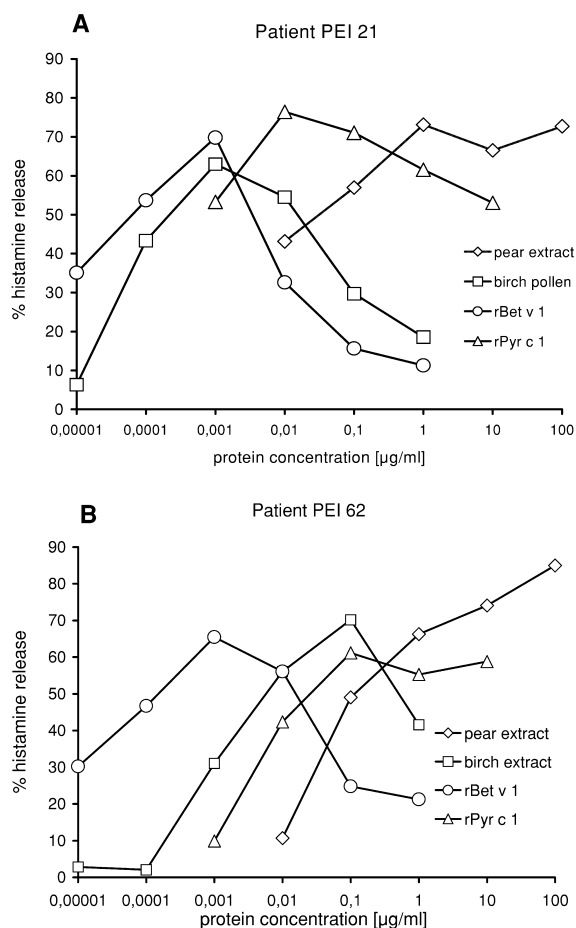


Fig. 9. Dose-related histamine release in the pear-allergic patients PEI 21 and PEI 62.

by Western blotting with mAbs directed against Bet v 1 that recognised Pyr c 1 in pear extract (Fig. 7). The histamine release data and EAST inhibition support the view that the cross-reacting IgE antibodies have a higher affinity to Bet v 1 than to Pyr c 1. Ten- to 100-fold lower amounts of Bet v 1 stimulated the same histamine release and about 20-fold lower amounts of Bet v 1 were able to inhibit 50% of the IgE binding to rPyr c 1 allergen disks (Fig. 8). These data are in accordance with published data about the cross-allergenicity of Bet v 1, Mal d 1 [36], and Pru av 1 [19,22]. Moreover, our inhibition data highlight epitope differences between pollen-related food allergens. Although IgE binding to Mal d 1, Pyr c 1, and Api g 1 can be blocked completely

with low amounts of Bet v 1 [18,20,25,36], Mal d 1 inhibited IgE binding to Pyr c 1 to a weaker extent than Pyr c 1 itself, and Api g 1 had no inhibitory activity in this system (Fig. 8). This is in accordance with results of cross-inhibition experiments with Bet v 1, Pru av 1, Mal d 1, and Api g 1 [22]. Keeping in mind the high degree of 92% sequence identity of the two allergens, small immunological differences between Pyr c 1 and Mal d 1 are particularly interesting and may serve as a basis for investigating epitope structures.

Diagnostic applications targeted to replacing extracts with recombinant allergens would require a complete panel of allergens having the immunological features of the wild-type molecules. Although our data clearly show that rPyr c 1 has a high biological activity, it is also evident that a second isoform of this molecule exists (Figs. 6 and 7) which may have a different reactivity with IgE from certain patients. Moreover, it is evident from our results that additional pear allergens have to be cloned to achieve a more complete diagnostic approach.

Sequence alignments (Table 1) identified Pyr c 1 as a typical member of a protein family related to intracellular plant pathogenesis related proteins of the PR-10 group [37,38] which contains many proteins with a high allergenic potency. Although more than 90 members of this group are known, their biological function is basically unknown [38]. The sequence identity of Pyr c 1 with other fruit allergens of this family is very high, ranging from 92% (Mal d 1) to 79% (Pru ar 1). In accordance with the results of immunological studies, the allergens from celery and carrot show a lower degree of 37% identity with Pyr c 1 (Table 1). Among the stress- and PR-proteins, Pyr c 1 shares the highest identity with proteins from trees, namely beech (70%) and birch pollen (63%). Recently, serine in position 112 of Bet v 1 and Pru av 1 and in the corresponding position 111 of Mal d 1 have been identified as being essential for the IgE binding capacity of these allergens [12,22]. Mutation of this residue to cysteine led to a reduced IgE reactivity, and replacement with proline nearly depleted IgE binding. The corresponding serine residue is conserved in position 111 of Pyr c 1 (counting without N-terminal Met), correlating well with its high allergenicity.

In summary the availability of rPyr c 1 will promote studies on the structure and cross-reactive

epitopes of pollen-related food allergens. Since fruit extracts are known to be particularly labile [5,36], rPyr c 1 may serve as stable and standardised reference material for diagnostic applications. Further studies will be undertaken to clone and express minor allergens from pear in order to cover the whole IgE binding capacity of this fruit by a panel of recombinant allergens.

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