

Complementary DNA cloning and expression of a newly recognized high molecular mass allergen Phl p 13 from timothy grass pollen (*Phleum pratense*)

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Summary

Background Grass pollen extracts contain a range of different allergenic components that can be classified as having low, middle or high molecular mass. Almost 75% of patients allergic to grass pollen display immunoglobulin (Ig) E-reactivity to allergens in the high molecular mass range of 55–60 kDa. These proteins have not yet been fully characterized on the protein and DNA level.

Objective The aim of this study was to identify and characterize an allergen of the high molecular mass fraction of *Phleum pratense* pollen by N-terminal protein sequencing and molecular cloning.

Methods A previously uncharacterized allergen which migrates as a double band with a molecular mass of 55–60 kDa was biochemically purified and investigated by N-terminal sequencing. Subsequently, a DNA primer was designed to amplify the corresponding cDNA using PCR. The cloned cDNA and deduced amino acid sequence were compared with sequence data bases. Immunoblots carrying the recombinant expression product were developed with monoclonal antibodies and sera derived from allergic subjects. The IgE-binding capacity of natural and recombinant allergen was determined using EAST.

Results The nucleic acid sequence as well as the deduced amino acid sequence consisting of 394 amino acids indicated homology with pollen specific polygalacturonases. Four potential sites for glycosylation and 16 cysteine residues were found. The recombinant expression product exhibited the same molecular size as the natural allergen and was clearly IgE-reactive.

Conclusion The newly characterized allergen Phl p 13, which shows homology with polygalacturonases, is clearly different from the allergen designated as Phl p 4 and therefore the high molecular mass fraction is composed of at least two different allergens. A possible reason why this important allergen has not been detected until now is that Phl p 13 and Phl p 4 are hardly separable by one dimensional SDS-PAGE.

Keywords: cDNA cloning, immunoglobulin binding, Phl p 13, *Phleum pratense*, recombinant allergens, timothy grass pollen

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Introduction

Grass pollen allergens are among the most frequent inducers of allergic disease accounting for symptoms of rhinoconjunctivitis and bronchial asthma in up to 40% of patients with type I allergy [1]. Until now, total or partially purified

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extracts from pollens have been used for diagnosis and immunotherapy. An improvement might be achieved by the use of recombinant allergens [2]. Furthermore mutagenesis techniques allow these cloned allergens to be engineered to create hypoallergenic variants that may offer a safer and more efficacious immunotherapy [3]. Rapid advances have been made in the characterization of grass pollen allergens and sequence determination by chemical and molecular biological approaches have made such options a reality [2].

Several allergens of *Phleum pratense* have been described in detail, such as the major allergens Phl p 1 [4] and Phl p 5 [5], which exhibit molecular masses between 25 and 35 kDa, and smaller allergens with molecular masses around 12 kDa including Phl p 2 [6] and Phl p 6 [7]. In contrast, little is known about the allergens with molecular masses between 50 and 60 kDa, although 75% of grass pollen-allergic patients produce immunoglobulin (Ig) E specifically directed against these components [8]. The allergen Phl p 4 with a molecular mass of 55 kDa is modestly described as a basic and trypsin-resistant glycoprotein [9]. Recently, another timothy pollen allergen with a similar molecular size as Phl p 4 has been purified [10]. Since this new allergen exhibits different immunological properties than Phl p 4, it was assumed that at least two different groups of allergens are present in the high molecular mass fraction of timothy pollen extract. The cDNA of this newly recognized allergen has now been cloned, characterized and expressed in bacteria. It should now be possible to discriminate between this new allergen and the other high molecular mass allergen Phl p 4 and hence draw conclusions about their relative importance.

Methods

Purification of the new allergen (Phl p 13) and Phl p 1

The purification of the natural allergens was performed as described previously [10]. Briefly, a timothy grass pollen extract was separated by hydrophobic interaction chromatography (HIC) using phenyl Sepharose HP (Pharmacia Biotech, Freiburg, Germany). The flow-through fraction was subsequently concentrated with ultrafiltration devices Centriprep-10 (Millipore, Eschborn, Germany). Proteins of the flow-through fraction were finally purified using gel filtration with Superdex 75 (Pharmacia Biotech).

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE with the buffer system of Laemmli [11] under reducing conditions. After electrophoresis proteins were subsequently transferred onto a supported nitrocellulose membrane (Sartorius, Göttingen, Germany) by semidry blotting for 30 min at 2 mA/cm².

Immobilized proteins were either stained using india ink (Pelikan, Hannover, Germany) and afterwards AuroDye (Janssen Biochimica, Beerse, Belgium) or incubated with monoclonal antibodies (mAbs) or serum.

N-terminal amino acid sequencing

Separated proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Munich, Germany). The membrane was washed with double-distilled water, stained by 0.1% (w/v) Coomassie R-250 (Merck, Darmstadt, Germany) in 50% methanol for 1 min, destained in 50% methanol and air dried. Microsequencing of excised protein bands was performed using a 473-A protein sequencer with on-line PTH amino acid analyser (Applied Biosystems, Weiterstadt, Germany).

In order to determine internal amino acid sequences the allergen was digested proteolytically using *Staphylococcus aureus* V8 protease (Promega, Mannheim, Germany) according to the method of Cleveland [12].

First strand complementary-DNA (cDNA) synthesis

After isolating RNA from 20 mg of timothy grass pollen by using a GlassMax RNA microisolation system (Life Technologies, Karlsruhe, Germany), reverse transcription of poly (A⁺) RNA was performed using a 3'RACE System (rapid amplification of cDNA ends) and an oligo (dT)-containing adaptor primer (Life Technologies) according to the manufacturer's instructions.

PCR-based cloning and sequencing of timothy allergen cDNA

A degenerate oligodeoxynucleotide primer (Biometra, Göttingen, Germany) was designed according to the reverse translated N-terminal amino acid sequence: 5'-GGIAAIAAIGAIGAIAAIAAIGAIGA-3'. PCR was performed by adding 1 µL of each degenerate primer and abridged universal amplification primer (Life Technologies) with a concentration of 0.1 nmol/µL to 90 µL 'ready to use' supermix (Life Technologies) containing recombinant Taq DNA polymerase. After addition of 2 µL cDNA solution the volume was made up to 100 µL with sterile double-distilled water. Conditions for the PCR in a Personal Cyler (Biometra) were as follows: initial denaturation of 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C; 30 s at 50 °C, 2.15 min at 72 °C and a final extension at 72 °C for 8 min. Analysis of the amplification product was performed in a 0.8% agarose gel in the presence of ethidiumbromide (Roth, Karlsruhe, Germany) according to the method of Sambrook *et al.* [13]. The reaction product was cloned into a pCR2.1 vector using the original TA cloning kit (Invitrogen, Groningen, Netherlands).

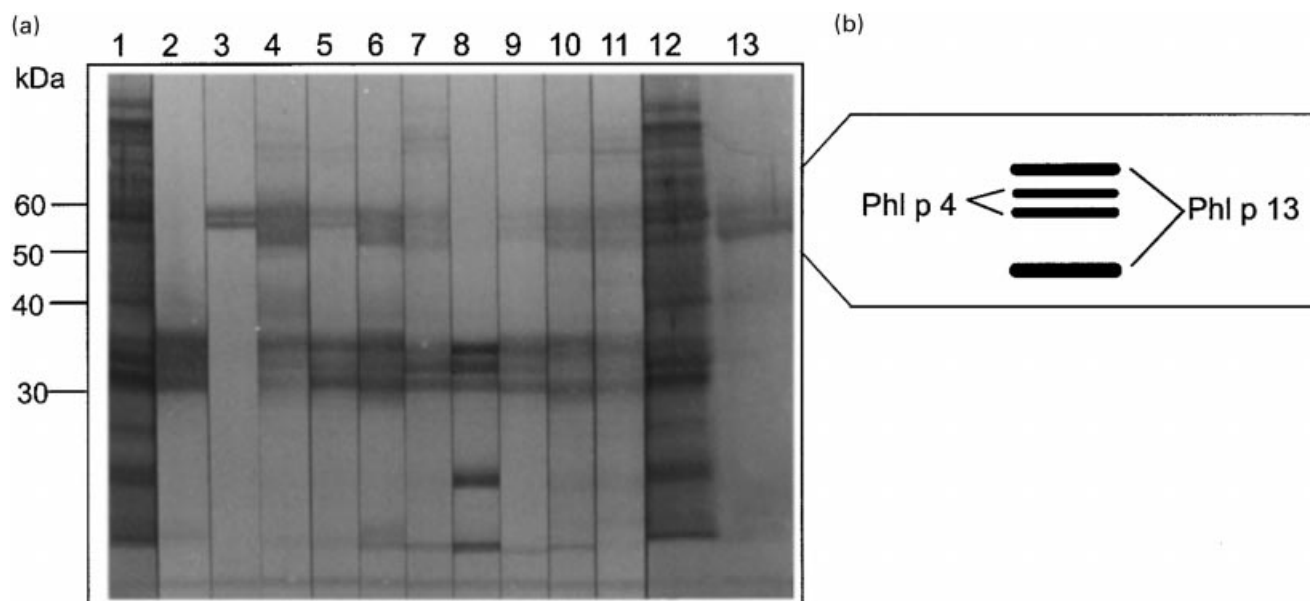


Fig. 1. Reactivity of patients' IgE with timothy grass pollen allergens. (a) Nitrocellulose strips carrying timothy grass pollen extract were either stained (lanes 1, 12) or incubated with mAbs specific for group 1 (lane 2) and group 4 (lane 3). Serum IgE from patients with grass pollen allergy was used to incubate strips 4–11. Lane 13 represents purified nPhl p 13 stained with India ink. (b) Line diagram showing the allergen pattern of the high molecular mass range between 55 and 60 kDa

Nucleotide sequences were determined by a commercial laboratory service from MWG-Biotech (Ebersberg, Germany) using the dideoxy chain termination method [14].

Sequence analysis

DNA and protein data bases (European Molecular Biology Laboratory Plant Library [EPLN], SWISS-PROT Protein sequence Database [rel37]) were searched for homologous sequences with the FASTA program version 3.2t01. Functional site searches were performed using DNASIS v.2.0 (Pharmacia Biotech).

Expression of the cloned cDNA and rPhl p 5b in *E. coli*

The cDNA was sub-cloned into the *EcoRI/XhoI* sites of a pProEx-Htb expression vector (Life Technologies). The resulting plasmid, named pRS55-1, was subsequently transformed in *E. coli* M15 pREP (Qiagen, Hilden, Germany) -competent cells. Overnight cultures were used to inoculate fresh LB (Luria-Bertani) medium supplemented with 0.1 mg/mL ampicillin and 0.025 mg/mL kanamycin. Cells were grown up to an $OD_{600} = 0.8$ and induced with 1.0 mmol/L isopropyl- β -D-thiogalactoside (IPTG). After expression for 3 h, cells were harvested by centrifugation and lysed by sonication in 6 mol/L guanidine hydrochloride, 20 mmol/L TRIS/HCl, pH 8.0, 20 mmol/L β -mercaptoethanol. Soluble

histidine-tagged recombinant fusion proteins were affinity purified using Ni^{2+} -loaded HiTrap chelating columns (Pharmacia Biotech) according to the manufacturer's instructions for denatured proteins with 6 mol/L urea containing buffers and a pH-dependent step elution. The purified recombinant proteins were directly subjected to SDS-PAGE analysis.

Recombinant production of Phl p 5b was performed as described previously [3].

Immunodetection and inhibition experiments

Identification of Phl p 4 was performed using a mixture of mAbs 5H1 and 3C4 [15], group 1 allergens with mAb 9C12 [16]. Prior to immunodetection, membranes were blocked by treatment for 30 min with TRIS-buffered saline (TBS), pH 7.4, supplemented with 0.05% (v/v) Tween20. Incubation with 1:10-diluted patients' pool serum or mAbs was carried out overnight. After washing, bound antibodies were detected with alkaline phosphatase-conjugated monoclonal mouse antihuman IgE (Allergopharma, Reinbek, Germany) or goat antimouse IgG/M with alkaline phosphatase (Dianova, Hamburg, Germany). The binding patterns were visualized by a substrate solution consisting of nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Life Technologies) in 100 mmol/L TRIS/HCl-buffered saline solution (pH 9.5). In order to examine cross-reactivities a

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5'-GGG AAG AAG GAG GAG AAG AAG GAG GAG AAG AAG GAG AGT GGA GAT GCT GCG TCC
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G K K E E K K E E K K E S G D A A S
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GGG GCC GAC GGA ACC TAC GAC ATC ACC AAG CTC GGC GCC AAA CCC GAC GGC AAG
-----
G A D G T Y D I T K L G A K P D G K
-----
ACG GAC TGC ACC AAG GAG GTG GAG GAG GCA TGG GCT TCG GCT TGC GGT GGT ACC
-----
T D C T K E V E E A W A S A C G G T
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GGG AAG AAT ACG ATC GTC ATC CCC AAG GGT GAT TTC CTG ACC GGG CCT CTG AAT
-----
G K N T I V I P K G D F L T G P L N
-----
TTC ACC GGG CCA TGC AAG GGC GAC AGC GTC ACC ATC AAG CTG GAC GGC AAC CTG
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F T G P C K G D S V T I K L D G N L
-----
CTG AGC TCC AAC GAC CTG GCA AAG TAC AAG GCT AAC TGG ATC GAG ATC ATG CGG
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L S S N D L A K Y K A N W I E I M R
-----
ATC AAG AAA CTC ACT ATC ACC GGA AAA GGC ACG CTC GAC GGC CAA AGC AAG GCC
-----
I K K L T I T G K G T L D G Q S K A
-----
GTG TGG AGC AAG AAC AGC TGC GCC AAG AAC TAC AAC TGC AAG ATC TTG CCA AAC
-----
V W S K N S C A K N Y N C K I L P N
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ACA TTG GTG CTG GAC TTC TGT GAC GAC GCT CTC ATC GAA GGC ATC ACC CTC CTA
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T L V L D F C D D A L I E G I T L L
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AAC GCC AAG TTC TTC CAT ATG AAC ATC TAC GAG TGC AAG GGC GTG ACC GTC AAG
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N A K F F H M N I Y E C K G V T V K
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GAC GTG ACC ATC ACC GCG CCC GGG GAC AGC CCC TAC ACC GAC GGC ATA CAC ATC
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D V T I T A P G D S P Y T D G I H I
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GGC GAC TCG TCC AAG GTC ACC ATC ACC GAC ACC ACC ATC GGC ACC GGC GAC GAC
-----
G D S S K V T I T D T T I G T G D D
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TGC ATC TCC ATC GGC CCC GGA AGC ACC GGC CTC AAC ATC ACC GGC GGT GCC TGC
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C I S I G P G S T G L N I T G G A C
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GGT CCA GGC CAC GGC ATC AGC GTT GGC AGC CTG GGA CGG TAC AAG GAC GAG AAG
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G P G H G I S V G S L G R Y K D E K
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GAC GTG ACC GAC ATC ACC GTC AAG AAC TGC GTG CTC AAG AAG TCC ACC AAC GGC
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D V T D I T V K N C V L K K S T N G
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CTC CGG ATC AAG TCG TAC GAG GAC GCC AGG TCG CCG CTG ACG GCG TCG AAG CTG
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L R I K S Y E D A R S P L T A S K L

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ACC	TAC	GAG	AAC	GTC	AAG	ATG	GAG	GAC	GTG	GGC	TAC	CCC	ATC	ATC	ATC	GAC	CAG
T	Y	E	N	V	K	M	E	D	V	G	Y	P	I	I	I	D	Q
AAG	TAC	TGC	CCC	AAC	AAG	ATC	TGC	ACC	TCC	AAG	GGA	GAC	TCC	GCC	AGG	GTC	ACC
K	Y	C	P	N	K	I	C	T	S	K	G	D	S	A	R	V	T
GTC	AAG	GAC	GTC	ACA	TTC	CGC	AAC	ATC	ACC	GGC	ACC	TCC	TCC	ACC	CCC	GAG	GCC
V	K	D	V	T	F	R	N	I	T	G	T	S	S	T	P	E	A
GTC	AGC	CTG	CTC	TGC	TCC	GAC	AAG	CAG	CCC	TGC	AAT	GGT	GTC	ACC	ATG	AAC	GAC
V	S	L	L	C	S	D	K	Q	P	C	N	G	V	T	M	N	D
GTC	AAG	ATC	GAG	TAC	AGC	GGC	ACC	AAC	AAC	AAG	ACC	ATG	GCT	GTC	TGC	ACC	AAC
V	K	I	E	Y	S	G	T	N	N	K	T	M	A	V	C	T	N
GCT	AAG	GTC	AGC	GCC	AAG	GGT	GTC	AGC	GAG	GCT	AAC	ACT	TGC	GCC	GCC	TGA	TGA
A	K	V	S	A	K	G	V	S	E	A	N	T	C	A	A	*	*
GCG	ACT	GTT	CTC	CAC	CTG	CTT	CCA	CGT	TCC	ATC	GAT	TCG	TCT	TAC	CTC	CCC	ACA
GAT	CCC	TCG	AGG	CAC	TCT	CTC	GCG	CGG	CAT	GAC	TTT	TTC	TCC	GTA	TTT	GTA	ATC
TAC	TAC	CAC	GTA	CAC	TAA	TTT	TCT	CTT	CTT	CTT	TTT	TTG	GTT	TCG	ATC	CTG	GTT
GTT	TCT	CTC	TCA	AGA	GAA	GAT	ACA	GAC	AGC	TCC	AGG	AGA	TCA	TCC	ATT	AGT	TTT
GTT	TCC	TCC	TTG	GTT	GAT	CGT	TCT	CCT	TTT	TTT	GTT	AAC	AAA	TCC	ATA	TTG	TAC
GTG	CCG	GCT	CAG	AGC	CAG	CCA	AAA	CTG	AGG	TGC	TTG	ATT	ATT	GAT	CTA	ATT	TTG
ACG	ATT	TAA	TTT	CTC	AAT	TTT	GAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	-3'

Fig. 2. cDNA sequence and deduced amino acid sequence of clone pRS55-1 encoding Phl p 13. The open reading frame of Phl p 13 comprises 1182 nucleotides. Shaded areas containing bold and italic typed amino acids were determined by N-terminal sequencing. The N-terminal amino acids used for designing a degenerate oligonucleotide are underlined

Miniblot MN45 apparatus (Biometra) was used to apply natural and recombinant allergens in 20 mmol/L NaOH onto nitrocellulose membranes. After blocking, strips were cut and incubated with patients' sera that were pre-adsorbed for 30 min with natural or recombinant allergens.

Determination of allergen-specific IgE

Allergen-specific IgE levels were determined by EAST measurements with a specific 'IgE ELISA RV' kit (Allergopharma, Reinbek, Germany) containing alkaline phosphatase-conjugated monoclonal mouse antihuman IgE antibodies. Experimental allergen discs carrying purified natural and recombinant allergens were prepared according to the method of Ceska *et al.* [17]. Results are expressed as means of duplicate determinations.

Results

Amplification of allergen cDNA and sequence analysis

A new allergen that migrates as a double band with molecular masses of 55 and 60 kDa has been previously purified. In order to estimate the importance of this allergen, separated timothy grass pollen extract was incubated with individual sera derived from subjects allergic to grass pollens (Fig. 1a). Regarding the high molecular mass range from 50 to 60 kDa, Phl p 4 (Fig. 1, lane 3) represents only a part of this IgE-reactive region. Since the separation of the extract was performed under reducing conditions, possible dimerization of Phl p 1 [18] resulting in a 60-kDa dimer was efficiently prevented (Fig. 1a, lane 2). Obviously, the additional IgE-reactivity corresponds exactly to the

molecular mass of the purified new allergen (Fig. 1a, lane 13) designated as Phl p 13. Figure 1(b) displays a pattern of the probable arrangement of Phl p 4 and Phl p 13.

In order to analyse this new allergen at the primary structure level, both bands as well as a 15-kDa fragment obtained after proteolytic digestion were subjected to N-terminal sequencing (Fig. 2). Since identical N-terminal amino acids were determined for both bands, one degenerate primer was designed on the basis of the first nine amino acids for the purpose of amplifying the corresponding cDNA. After 3'RACE, using timothy pollen cDNA as template, two different sized DNA fragments with nucleotide lengths of 1490 and 1540 bp were obtained and cloned into a pCR2.1 vector. Subsequent sequence analysis of four clones carrying both variants of cDNAs revealed high identity except the 3' terminal region covering 200 nucleotides upstream of the poly(A) tail (not shown). In all cases an open reading frame from position 1 to position 1182 was found encoding a protein of 394 amino acids (Fig. 2). Heterogeneity appears at 10 positions of the primary structure compared with the sequence shown for pRS55-1: Ile²⁶ → Thr, Ser¹²⁴ → Gly, Ser¹²⁹ → Gly, Tyr¹³⁷ → His, Tyr¹⁹² → Asn, Gly²²⁶ → Ala, Arg²⁸⁰ → Lys, Thr²⁸⁹ → Ala, Lys³¹² → Arg, Ser³⁸² → Thr. Both the whole N-terminal sequence, derived from the N-terminus, as well as the internal amino acid sequence obtained from the proteolytic fragment were present in the deduced amino acid sequence from clone pRS55-1 as indicated (Fig. 2). The sequence represents a protein with a predicted molecular weight of 41 742 and an isoelectric point of 7.47. Four potential N-glycosylation sites at positions 72, 228, 332, 370 and one conserved pattern typical for polygalacturonases at position 224–234 were determined.

The predicted amino acid sequence contains 16 cysteine residues.

Alignments of the deduced amino acid sequence from pRS55-1 with sequences of the SWISSPROT data base indicated the highest identity of 71.5% in a 389 a.a. overlap to *Zea mays* exopolygalacturonase (SW: PGLT MAIZE P35559). Identity was displayed to a lesser extent with other polygalacturonases from various sources including the major allergen Cry j 2 from Japanese cedar (*Cryptomeria japonica*). Similar results were obtained on the nucleic acid level using EMBL plants library (EPLN).

Expression and characterization of the recombinant Phl p 13

The cDNA was sub-cloned into the expression vector pProEx-HTb using EcoRI/XhoI restriction sites present in the multiple cloning region of pCR2.1. The expression of the insert cDNA generated a fusion protein composed of an N-terminal 6xHis-tag followed by 22 additional vector-derived amino acids and the target sequence. The additional amino acids increased the predicted molecular weight by 3829. Since the fusion protein was insoluble after bacterial lysis, the complete cell pellet (Fig. 3, lane 4) was solubilized using 6 mol/L guanidine hydrochloride and subsequently subjected to Ni²⁺-chelate affinity chromatography. The elution fraction contained a large protein with an apparent molecular mass of approximately 58 kDa and several smaller proteins as judged by SDS-PAGE (Fig. 3, lane 5). Taking the fusion part into account the recombinant protein exhibits a molecular mass similar to that of the lower band of the natural allergen (Fig. 3, lane 2).

Investigation of IgE-reactivity was performed by incubating

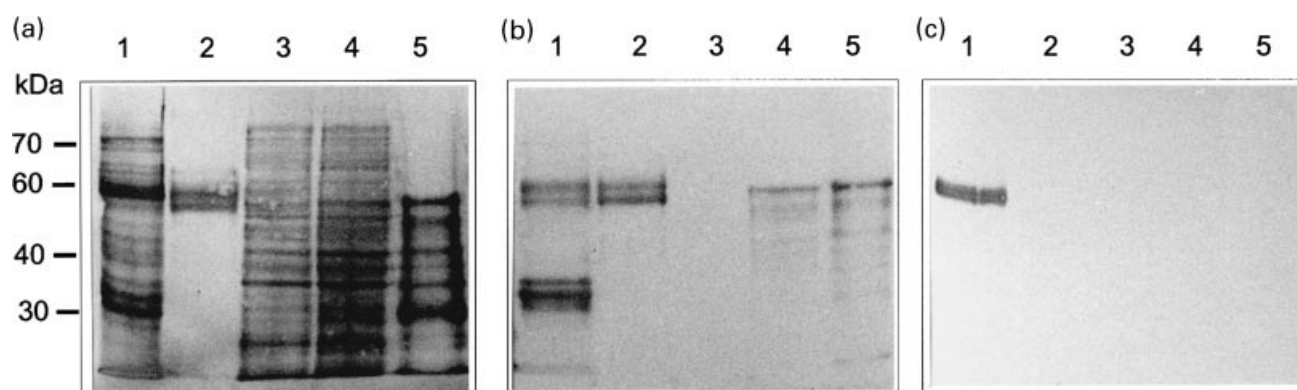


Fig. 3. Immunological analysis of nPhl p 13 and rPhl p 13. Timothy grass pollen extract (lane 1) was used to purify nPhl p 13 (lane 2). Transformed *E. coli* cells M15 containing cloned cDNA (lane 3) were lysed after induction for 3 h (lane 4). Recombinant expression product was subsequently purified using Ni²⁺ chelate affinity chromatography (lane 5). Replica gels were electroblotted onto nitrocellulose membrane and either stained with India ink and afterwards with Auro dye or immunologically investigated by incubation with patients' pool serum (b; *n* = 7) and 1 : 2 mixture of two Phl p 4 specific mAbs (c)

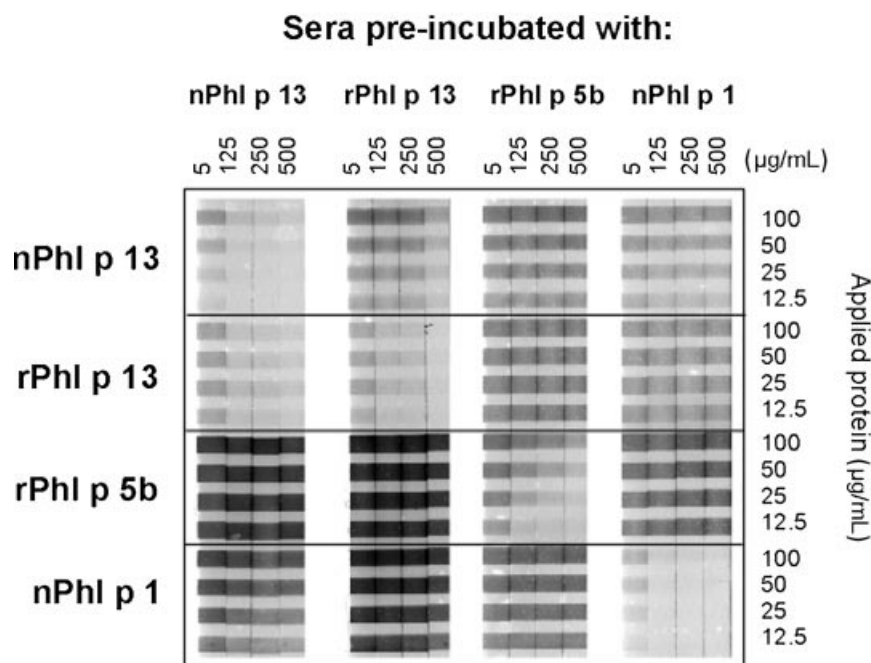


Fig. 4. Immunological cross-reactivity between natural and recombinant Phl p 13. Different amounts of nPhl p 13, rPhl p 13 and for control purposes nPhl p 1 and rPhl p 5b were immobilized on nitrocellulose membrane and strips were subsequently incubated with a pool of two preselected sera pre-adsorbed with increasing amounts of nPhl p 13, rPhl p 13, rPhl p 5b or nPhl p 1 as indicated

immunoblots carrying both the natural and recombinant allergen with a serum pool derived from six allergic subjects (Fig. 3b). The strong IgE-binding capacity demonstrated that the recombinant protein exhibits allergenic potential (Fig. 3b, lane 5). The smaller proteins were also IgE-reactive indicating that they represent truncated expression products.

A mixture of two mAbs recognizing unrelated epitopes of Phl p 4 [15] were used to investigate the immunological relationship between Phl p 4 and the new allergen (Fig. 3c). Phl p 4 was clearly recognized in the timothy grass pollen extract (Fig. 3c, lane 1), whereas neither the natural nor the recombinant Phl p 13 were bound.

Inhibition experiments were performed to verify that the recombinant allergen corresponds to the natural counterpart. Increasing amounts of nPhl p 13 and rPhl p 13 (Fig. 4) were added to two pooled sera which were subsequently used for immunological detection of immobilized recombinant and natural Phl p 13. Natural Phl p 1 and recombinant Phl p 5b were also investigated as controls. There was a dose-dependent inhibition of binding of specific IgE to both nPhl p 13 and rPhl p 13 as reflected by decreased immunostaining. No effect was observed in the case of the control allergens nPhl p 1 and rPhl p 5b. Crossover inhibition revealed that the recombinant Phl p 13 did not completely block binding to the natural counterpart. Nevertheless the presence of common IgE-epitopes was clearly demonstrated. Inhibition with nPhl p 1 and rPhl p 5b affected specifically the staining of the corresponding immobilized allergens.

An EAST assay was performed using five sera derived from grass pollen-allergic patients in order to evaluate the

specific IgE-binding capacity of recombinant and natural Phl p 13 (Fig. 5). Every patient exhibited specific IgE directed against *Phleum pratense* pollen extract (Fig. 5), purified nPhl p 1 and nPhl p 13. In contrast, only two sera contained IgE that recognized recombinant Phl p 13, and in both cases the value, expressed in EAST classes, was smaller compared with the natural allergen.

Discussion

It has been suggested that recombinant allergens may be used for diagnostic and therapeutic applications of type 1

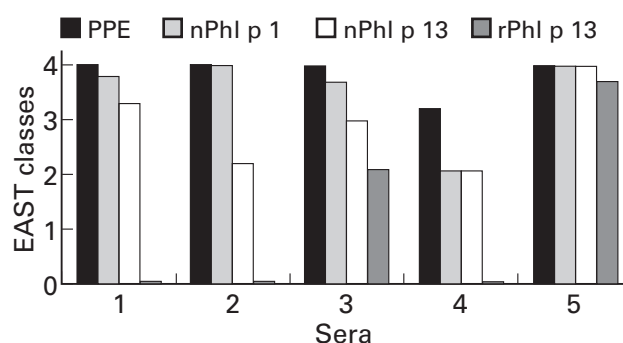


Fig. 5. Quantification of allergen-specific IgE. Sera from five grass pollen-allergic patients were analysed with *Phleum pratense* pollen extract (PPE), major allergen nPhl p 1, nPhl p 13 and rPhl p 13 by ELISA measurements. Results are displayed as EAST classes. Values below 1 indicate lack of detectable specific IgE

allergy [2]. In order to attain that objective the availability of sequence data from at least all the major allergens is essential. In the case of grass pollen allergens many cDNA and amino acid sequences have been described for proteins in the molecular mass range from 8 to 35 kDa. In contrast, little is known about the higher molecular mass range allergens of grass pollen, including group 4 allergen and others of similar molecular mass, although they are among the major components responsible for IgE binding [9,19].

A new allergen that migrates as a double band with an apparent molecular mass of 55–60 kDa has been biochemically purified previously [10]. In the present study the corresponding cDNA encoding the new allergen was amplified and cloned and its identity confirmed by supporting data. Firstly, the amino acid sequences determined from the natural allergen were found to be present in the deduced amino acid sequence of the open reading frame. Secondly, the bacterial expression product derived from the cloned cDNA sequence exhibits a similar molecular mass to that of the natural allergen. Thirdly, the recombinant protein was clearly IgE reactive. Moreover, specific IgE reactivity of the recombinant allergen was inhibited by pre-incubation of sera with the natural counterpart and *vice versa*. Therefore the recombinant and natural molecules share identical IgE epitopes. The incomplete inhibition of binding of nPhl p 13 by rPhl p 13 could be due to the glycosylation state of the former and incorrect folding of the recombinant protein [20]. This would also explain why only two of five sera contained specific IgE that was reactive with the recombinant Phl p 13, though IgE directed against nPhl p 13 was clearly detectable in every serum. Obviously, the three non-reactive sera contained IgE molecules recognizing either conformational epitopes or glycans. Another possibility is the existence of isoforms that are not covered by the recombinant allergen, which is supported by the sequence heterogeneities of analysed clones.

Regarding the calculated molecular mass based on the deduced amino acid sequence, a discrepancy is apparent with the size estimated by SDS-PAGE separation. There are various possible explanations for anomalous behaviour of polypeptides in SDS-PAGE including glycosylation, acidic or basic pIs and high proline content [21]. Alterations of even a single amino acid may change the mobility of proteins in SDS-PAGE by up to 10%. In this respect the discrepancy is not surprising. Other examples for this phenomenon are the allergens Cha o 1 [22] and Phl p 1 [4].

The natural allergen appears as a double band in SDS-PAGE. This might be due to different glycosylation states since four potential N-glycosylation sites were found in the primary sequence. Moreover, the recombinant fusion allergen, which is expressed in *E. coli* and therefore non-glycosylated, is comparable with the lower band making allowance for the fusion component.

The allergen designated as Phl p 4 exhibits a similar molecular mass but there are obvious differences. In particular, mAbs directed against Phl p 4 do not bind to Phl p 13. Moreover, Phl p 4 is a basic protein while the calculated pI for Phl p 13 is neutral. Both allergens are even unrelated on the polyclonal IgE level (Suck *et al.*, manuscript in preparation). Until now Phl p 4 was proposed to be responsible for IgE binding in the higher molecular mass range. Based on the investigations presented in this paper at least two different proteins represent the high molecular mass fraction of pollen allergens. Future studies will seek to distinguish between these allergens.

Regarding the importance of the new allergen described here, preliminary studies indicate that it is recognized by more than 50% of patients with grass pollen allergy (Suck *et al.*, manuscript in preparation). This is not surprising since more or less homology was found to polygalacturonases from various sources including the major allergen from Japanese cedar pollen Cry j 2 [23]. Polygalacturonases depolymerize polygalacturonic acid (PGA) by hydrolysis. They are known to be expressed specifically in pollen [24,25]. Since the corresponding gene is conserved, it is reasonable to suppose that they occur in pollens from many angiosperms [26]. Therefore we suggest that similar to the panallergen profilin, IgE binding to higher molecular components may be explained at least in part by the existence of polygalacturonases. Further investigations will focus on the importance of this class of allergens.

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