

Cloning and molecular characterization of the *Hevea brasiliensis* allergen Hev b 11, a class I chitinase

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Summary

Background In the last 10 years type-I allergy against proteins from *Hevea brasiliensis* latex has become an acknowledged medical issue. Fruit-allergic patients represent one risk group for developing latex allergy. Class I chitinases have been identified from chestnut, avocado and banana as relevant allergens. The chitin binding (hevein) domain from these class I chitinases has been postulated to bear the important IgE binding epitopes.

Objective To clone the cDNA of an allergenic latex class I chitinase, to express the recombinant protein and to determine its IgE cross-reactivity with hevein (Hev b 6.02).

Methods A full-length cDNA coding for a class I chitinase has been isolated from *Hevea* latex RNA by reverse transcription followed by PCR. The chitinase encoding sequence has been subcloned into the pMAL expression vector and expressed in *E. coli* as a fusion protein to maltose binding protein. The highly enriched recombinant protein fraction has been tested for its IgE binding capacity in immunoblots and ELISA. Furthermore, the pathogenesis-related function of the recombinant protein was tested in a fungal growth inhibition assay.

Results The *Hevea brasiliensis* latex chitinase, designated Hev b 11, displays 70% identity to the endochitinase from avocado and its hevein-domain 58% to hevein (Hev b 6.02). The recombinant Hev b 11-maltose binding protein is recognized by latex- and fruit-allergic patients with IgE binding in both, ELISA and immunoblots. Pre-incubation of sera with rHev b 11-maltose binding protein showed an overall 16% inhibition of subsequent binding to rHev b 6.02-maltose binding protein on solid phase. The growth of *F. oxysporum* was inhibited in a dose dependent manner by addition of rHev b 11-maltose binding protein to the culture.

Conclusions Hev b 11, a class I chitinase, is another allergen from *Hevea* latex with a chitin binding domain and displays a different IgE binding capacity compared with hevein.

Keywords allergy, class I chitinase, Hev b 11, latex–fruit syndrome

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Introduction

Latex allergy is an increasingly significant medical issue. Among health care workers the prevalence is between 5% and 15%, with less than 1% among the general population [1]. Other latex allergy risk groups include workers in the rubber industry and patients with spina bifida and urogenital abnormalities [2]. A further potential risk group for developing allergic reactions to latex are fruit-allergic patients [3]. Latex-allergic patients show a high rate of hypersensitivity to fruit, around 50% [4], while fruit-allergic patients show 86% sensitization to latex in one study [3]. Cross-reactivity between latex and fruit is designated the latex–fruit syndrome [5]. Implicated fruits include

avocado, chestnut and banana. Kiwi, papaya and other taxonomically unrelated plants may also be involved. The extent of sensitization of latex-allergic patients was shown to be 45% for banana [6] and 50% for avocado [7]. Allergens of different molecular masses were detected in banana extract, which react with latex-allergic patients’ antibodies [6]. Some *Hevea* allergens show high structural homology to fruit proteins, such as hevein, heveamine and profilin- and patatin-like proteins [3].

The class I chitinases are major panallergens associated with this syndrome. Several reports have identified class I chitinases as important IgE binding components in sera from patients with latex–fruit syndrome in banana [8], avocado [9] and chestnut [10]. The avocado chitinase has been produced as a recombinant protein in yeast. Both the banana and chestnut chitinases have been isolated from natural extracts but only the N-terminal amino acid sequences have been determined [8]. The N-terminal sequences of these chitinases are very similar and also show sequence identity with the hevein domain of prohevein. It

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has been proposed that the major IgE epitopes are located only on the hevein domain and that it plays the essential role in the allergenicity of these plant chitinases [11]. Thus, the existence of a class I chitinase in latex on the one hand, and its cross-reactivity with certain fruit chitinases on the other hand, have long been postulated. In the literature there are reports of studies involving the immunoblotting of latex extract and the subsequent demonstration of cross-reactivity between latex and various fruit [6,12]. Posch et al. identified the N-terminus of a possible endochitinase in latex [13].

Hence, the identification and characterization of this allergen in latex is necessary to qualify the nature of the latex–fruit syndrome and for assisting in its diagnosis and therapy. We report the identification of a 33-kDa protein in *Hevea* latex. The cDNA sequence coding for the mature protein was cloned and produced as a highly enriched recombinant protein in *E. coli*. The protein demonstrated both biological and immunological activity. Recombinant Hev b 11 was shown to be a class I chitinase and a relevant allergen for latex- and fruit-allergic patients.

Materials and methods

Isolation of total RNA

High-quality RNA was isolated from fresh latex obtained from regularly tapped trees (*Hevea brasiliensis*, clone RRIM 600) at the Rubber Research Institute of Malaysia's Experimental Station, Sungai Buloh, Selangor. Latex milk exuding from the tapped trees was simultaneously mixed with an equal volume of extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 1 mM EDTA, 10% SDS, pH 9.5) on collection. The resulting solution was centrifuged at 100 000 g for 30 min at 15 °C. The rubber plug was removed and the aqueous phase was further extracted with chloroform/phenol. Finally, total RNA was precipitated with LiCl.

cDNA synthesis

Reverse transcription was performed using 0.5 µg of total RNA and a modified oligo dT primer, T₂₅NN (5'-GGAGAAGGA (T)₂₅(AGC)N-3'), at 42 °C for 60 min followed by a heat denaturation step at 95 °C for 5 min in a Perkin Elmer thermocycler. Further PCR experiments were carried out using 2 µL of the first strand cDNA.

Isolation of a cDNA encoding a class I chitinase

Degenerate oligonucleotides corresponding to two motifs of class I chitinases were designed. From motif I, which is CX {4,5}FY(S,T)X3(F,Y)(L,I,V,M,F)XAX3(Y,F)X2F(G,S,A), the following primer, GOR2, 5'-TTYTAYACITAYGAYGCYTTYAT-3', was constructed (degenerate positions are designated according to the IUPAC code, I designates inosin). From motif II, which is (L,I,V,M)(G,S,A)FX(S,T,A,G)2(L,I,V,M,F,Y)W(F,Y)W(L,I,V,M), primer, GOR3, 5'-GTCATCCARAACCARADVGC-3', was constructed. PCR was carried out for 10 cycles, starting at 60 °C, descending 1 ° each cycle, followed by 25 cycles at the lower temperature, 50 °C, with 3 min extension time each cycle. DNA fragments of the appropriate length were isolated (QIAEX II Agarose Gel Extraction Kit, Qiagen, Hilden, Germany) and subsequently ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). The

PCR products were sequenced using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Life Science, Little Chalfont, UK) and analysed on an automatic sequencer (Licor, Lincoln, NB, USA). The 3' segment was completed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). To do this, further internal primers were synthesized according to the now known sequence. Primer CH303e was 5'-GGCAAGCCTTAGGATTAGAC-3'. Primer CH304e was 5'-GAACAATCCAGACCTTGTCAGC-3'. The protocol was followed according to the manufacturer's instructions. The second round PCR products were cloned and sequenced.

The 5' segment was also completed using Marathon, as described above. Internal primers for this were as follows: primer CH301b was 5'-GTATCAACATCTCCGGTGG-3'; primer CH302b was 5'-CATATGGTCCATCTGGTGC-3'.

The full-length cDNA sequence and its deduced amino acid sequence were compared to known sequences in the European Molecular Biology Laboratory (EMBL) and SWISS-PROT databases.

Expression of recombinant proteins

The coding sequence of the mature Hev b 11 protein was amplified using the primers: CH30exp1, with Bam HI site (shown in italics), 5'-GGATCCGAGCAATGTGGAAGGCAAGCTG-3' and CH30exp7r, containing a Hind III site (shown in italics) after the stop codon, 5'-AAGCTTTCACCCTAATCCGAATGGCTTTTG-3' and subcloned into the *E. coli* expression vector pMAL-p2 (New England Biolabs, Beverly, MA, USA). Recombinant Hev b 11 was produced as a fusion protein with maltose binding protein ('MBP') according to the manufacturer's instructions. Briefly, an overnight culture was diluted 1:100 into fresh Rich Broth medium; 10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, 2 g/L glucose, 100 mg/L ampicillin. When the culture reached an optical density of 0.5 at 600 nm, protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.3 mM. After 6 hours cells were harvested by centrifugation and extracted using a French Pressure Cell (SLM Instruments, Rochester, NY, USA). Recombinant Hev b 11-MBP was purified from the resulting supernatant by an amylose resin column according to the manufacturer's protocols. The elution fractions were examined on SDS-PAGE gels to check their purity. Recombinant Hev b 11-MBP containing fractions were pooled.

Hev b 6.02 encoding cDNA was isolated from latex RNA by RT-PCR (EMBL acces. no: M36986). Subsequently, Hev b 6.02 cDNA was ligated into *E. coli* expression vector pMAL-p2 (New England Biolabs) and expressed as a recombinant protein fused to maltose binding protein upon IPTG induction. Recombinant Hev b 6.02-MBP was purified from the cell lysate by an amylose resin column according to the manufacturer's protocols.

Recombinant Hev b 8 (latex profilin) was expressed in *E. coli* as a non-fusion protein and purified from cell lysate by chromatographic procedures as described previously [14].

Protein extracts

Protein was extracted from latex by ultracentrifugation of the freshly harvested milk at 40 000 g at 4 °C for 1 h. This resulted in an upper fraction containing the rubber particles, a translucent aqueous layer known as C-serum and a pellet containing the

Table 1. Characterization of Hev b 11-positive patients. The sera were tested by latex and banana RAST and on latex B- and C-serum by immunoblotting. In addition, IgE-binding to rHev b 11-MBP was detected by immunoblots and ELISA. Inhibition of binding to rHev b 6.02-MBP was studied by pre-incubation with rHev b 11-MBP (100 µg/mL) in ELISA and by pre-incubation with rHev b 6.02-MBP in ELISA, respectively

Patient No.	Latex RAST	Banana RAST	Latex B	Latex C	rHev b 11-MBP	rHev b 6.02-MBP	rHev b 11-MBP/ Hev b 6.02-MBP (%)	rHev b 6.02-MBP/ rHev b 11-MBP (%)
1	6	3	+	+	+	+	0	16
2	6	1	+	+	+	+	0	23
3	5	3	+	+	+	+	21	54
4	3	2	+	+	+	+	21	92
5	5	3	+	+	+	+	9	60
6	3	2	+	+	+	+	13	6
7	3	0	+	+	+	+	0	53
8	3	0	+	+	+	+	0	21
9	ND	ND	+	+	+	+	0	0
10	ND	ND	+	+	+	+	0	72

organelles designated B-serum. The B-serum was resuspended in 50 mM Tris-HCl buffer at pH 8.0 with 0.05% Triton X-100. The C-serum was re-centrifuged for 1 h at 40 000 g at 4 °C to remove residual latex particles. Both extracts were freeze dried and stored at -20 °C.

Selection of the study population

Individual serum samples from 57 patients, both female and male, were selected. The age range of the patients was from 21 to 72 years (mean 42). The clinical symptoms included local urticaria, angioedema, rhinitis and conjunctivitis. Fifty of these patients were latex allergic according to typical case history and positive RAST and had positive latex skin prick tests. They all displayed IgE-binding to latex allergens when performing immunoblots with latex B- and C-serum. Among these 50, 18 did not display allergic reactions against banana, whereas 32 patients were banana allergic according to case history and RAST. The third group comprised seven patients with exclusive fruit allergy against kiwi without latex allergic symptoms.

For immunoblot inhibition assays sera from 10 selected patients positive in binding to rHev b 11-MBP were pooled (see Table 1). A serum pool from five atopic subjects with a clinical history of birch pollen allergy but no latex or latex-fruit allergy was used as a negative control in all experiments.

Immunoblotting

For immunodetection the resolved proteins were separated by SDS-PAGE (2 µg per lane of rHev b 11-MBP, 75 µg total protein of latex C-serum per lane and 50 µg total protein of latex B-serum per lane, respectively). Subsequently, proteins were transferred to Protran nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Immunoblotting was performed as described before, using patients sera in a 1 : 4 dilution. Bound IgE was detected using ¹²⁵I-labelled rabbit anti-human IgE (diluted 1 : 20, IBL, Hamburg, Germany), and visualized by autoradiography [15]. The recombinant blotted Hev b 11-MBP was also assayed using an anti-MBP rabbit serum (New England Biolabs). Detection was carried out using alkaline phosphatase conjugated swine anti-rabbit IgG (1:2000 dilution; DAKO, Glostrup, Denmark).

For inhibition assays a serum pool was incubated with rHev b 11-MBP (50 µg/mL) and afterwards applied to membranes

coated with latex B or latex C, or rHev b 11-MBP (for self-inhibition). These membranes were then developed as described above.

ELISA assays and inhibitions

ELISA was performed in flat-bottomed 96-well maxisorp microtiter plates (Nunc-Immuno Plate, Nalge Nunc International, Roskilde, Denmark). The wells were coated with 100 µL of rHev b 6.02-MBP or rHev b 11-MBP at a concentration of 4 µg/mL in TBS. The unbound sites were blocked with 1% bovine serum albumin in TBS with 0.05% Tween 20. Patients' sera or pre-incubated patients' sera (1 : 5 dilution) were added and incubated overnight at 4 °C. Subsequently, the plate was developed using alkaline phosphatase conjugated mouse anti-human IgE antibody (Pharmingen, San Diego, California, USA) for 1 h at 37 °C followed by another hour at 4 °C. Colour development was performed using phosphatase substrate tablets according to the manufacturer's instructions (5 mg each p-nitrophenyl phosphate disodium, Sigma-Aldrich, Steinheim, Germany). The optical density was measured at 405 nm (550 nm as reference wavelength) after 10 and 30 min using an ELISA-reader (Dynatech MR 7000, Alexandria, VA, USA).

For-cross inhibition experiments, selected Hev b 11 positive sera were tested for their IgE-binding to rHev b 11-MBP compared with that of rHev b 6.02-MBP (hevein). The inhibition of rHev b 6.02-MBP with rHev b 11-MBP and vice versa was tested by pre-incubating the serum pool with 100 µg/mL rHev b 11-MBP or rHev b 6.02-MBP (overnight, 4 °C), respectively. Negative controls were as follows: a non-latex-allergic atopic patients' sera pool, neat buffer and Hev b 8, the latex profilin as recombinant protein control. The maltose binding protein (MBP) alone was also tested in ELISA to ensure it did not interfere with the IgE binding of the rHev b 11-MBP.

Fungal growth inhibition assay

To show fungal growth inhibition, spores of *Fusarium oxysporum* were collected from 8-day-old cultures grown on potato dextrose agar plates (Difco, Detroit, MI, USA). The assay mixture contained 20 µL 3 × potato dextrose broth (Difco) and 3 × 10⁶ spores of the test fungus in 20 µL water with 20 µL sample or positive control (buffer alone). The effect of rHev b

11-MBP was tested at concentrations of 16, 40, 80 and 160 µg/mL. After an incubation period of 16 h at 25 °C, samples of each dilution were examined under the microscope and germination rates compared [16].

Results

cDNA coding for Hev b 11:

A combination of different PCR reactions as described in materials and methods was used to produce a single cDNA coding for the complete mature protein. The PCR products were cloned. Six distinct but identical clones were identified and sequenced.

The Hev b 11 encoding cDNA contained an open reading frame of 888 bp corresponding to a mature protein of 295 amino acids with a calculated molecular mass of 33 kDa. The cDNA sequence encoding Hev b 11 is available from the EMBL database under the accession number AJ238579. A chitin recognition or binding site corresponding to the consensus pattern C-X(4,5)-C-C-S-X(2)-G-X-C-G-X(4)-(FYW)-C was detected by the program MOTIFS (Wisconsin package). The sequence of Hev b 11 showed only one difference with respect to this motif present at amino acid position 25, substituting a glycine for an alanine. Two additional consensus patterns were found that are characteristic of chitinases of the family 19 of glycosyl hydrolases, consistent with the motifs used in the design of the original primers. Chitinases from the family 19 belong to endochitinases (E.C. 3.2.1.14), enzymes that catalyse the hydrolysis of β -1,4-N-acetyl-D-glucosamine linkages in chitin polymers.

Sequence homology analysis

No identical sequences were found in the databases (SWISS-PROT, Genbank and EMBL) indicating that the sequence represents both a new protein and a new allergen not described so far. The designation Hev b 11 for the latex class I chitinase was submitted to and granted by the IUIS (International Union of Immunological Sciences) allergen nomenclature committee. The complete sequence of the mature protein with significant motifs is shown in Fig. 1. The deduced amino acid sequence revealed an overall identity of at least 65% to other known plant class I chitinases. The sequence identity of Hev b 11 to Pers a 1, the endochitinase from avocado (GenBank accession number: Z78202), was 70%.

Other chitinases from edible plants showed the following sequence identity to Hev b 11: 73% for *Phaseolus vulgaris* (EMBL accession no. M13968) and *Pisum sativum* (EMBL accession no. L37876), 72% for *Oryza sativa* (EMBL accession no. D16223), 70% for *Vitis vinifera* (EMBL accession no. AF053341), 70% for *Solanum tuberosum* (EMBL accession no. U02608) and 69% for *Lycopersicon esculentum* (EMBL accession no. Q05538) over a minimum length of 293 amino acids. The similarity of Hev b 11 to hevein (Hev b 6.02) [17] is confined to the chitin binding domain with 58% identity. An alignment of the N-terminal sequences of Hev b 11 with previously published sequences from chestnut [10], avocado [9], banana [18] and the hevein domain is shown in Fig. 2. The N-terminus of Hev b 11 shows 72% identity to the hevein domain of chestnut, 77% to that of avocado and 81% to that of banana.

Protein expression and purification

The coding region of Hev b 11 was ligated into the expression vector pMAL and rHev b 11 was produced as a fusion protein with MBP. The average yield was 2 mg of rHev b 11-MBP per litre of bacterial culture. The recombinant protein was purified by affinity chromatography. This was determined by running the column fractions on SDS-PAGE gels and staining with Coomassie blue. A prominent band of approximately 70 kDa was found corresponding to the chitinase fusion protein. Figure 3 shows a Coomassie-stained SDS-PAGE and blot of rHev b 11-MBP with the bacterial extract of non-expressing cells in lane 1 and the cells expressing rHev b 11-MBP in lane 2. The enriched rHev b 11-MBP alone can be seen in lane 3.

Both the MBP alone and the MBP-rHev b 11 complex were recognized by the MBP antibody, showing the presence and reactivity of the fusion protein. The size also corresponds with the combined masses; 42.2 kDa for MBP alone and 33 kDa for Hev b 11, giving an approximate size of 75 kDa on SDS-PAGE. The blot is shown in Fig. 3, lane 4.

Immunological reactivity of natural extracts and rHev b 11-MBP

Immunoblotting of latex extracts B and C with latex-allergic patients' sera revealed binding to many bands of different sizes that varied from patient to patient. In total, 50 patients with latex allergy were tested with latex B and C serum and displayed IgE-binding to latex allergens. Patients with kiwi allergy ($n = 7$) did not bind to any of the latex allergens in immunoblotting experiments.

These sera were subsequently tested with rHev b 11-MBP. Of our study population, a total of 19% (10/57) recognized rHev b 11-MBP (Table 1 and Fig. 4). The recognition of rHev b 11 among patient subgroups was as follows: 8 out of 32 sera from patients with latex and banana allergy; 2 out of 18 sera from patients with latex allergy without banana allergy (Table 1). The control sera of birch-pollen-allergic patients or the buffer control had no evidence of binding. Figure 4 shows binding patterns of rHev b 11-MBP positive patients. A few patients showed IgE binding to additional *E. coli* proteins.

Inhibition studies

In immunoblots, inhibition of IgE binding to rHev b 11-MBP or total latex B and C extract was possible with 50 µg/mL recombinant protein using a Hev b 11-positive serum pool. In Fig. 5 the inhibition results are shown. In both latex B and C extracts the intensity of a minor band at around 30 kDa (nHev b 11) is shown to decrease after incubation with 50 µg/mL of rHev b 11-MBP. In parallel, a band of 21 kDa displays reduced IgE-binding capacity (prohevein, Hev b 6.01; Fig. 5, lane 2 and 4). Self-inhibition of rHev b 11-MBP is shown in lane 6. The discrepancy in band size between the blots can be explained by the attachment of the MBP-fusion part to rHev b 11.

In ELISA assays, IgE binding to rHev b 11-MBP was tested. The same 10 sera displayed specific IgE-binding against rHev b 11-MBP (Table 1). Furthermore, these patients' sera displayed IgE-binding to rHev b 6.02-MBP (Table 1).

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GAGCAATGTGGAAGGCAAGCTGGTGGCGCTTTGTGCCAGGAGGCCTATGTTGCAGCCAA
1  -----+-----+-----+-----+-----+-----+ 60
E Q C G R Q A G G A L C P G G L C C S Q -

TATGGCTGGTGTGCCAACACACCTGAATATTGTGGCAGTGGCTGCCAAAGCCAATGTGAC
) 66 -----+-----+-----+-----+-----+-----+ 126
Y G W C A N T P E Y C G S G C Q S Q C D -

GGTGGAGGTGGTGGTGAAGATGGCGGCATAGACCTTGGTAGTATCATCTCAAGATCAACG
) 121 -----+-----+-----+-----+-----+-----+ 180
G G G G G E D G G I D L G S I I S R S T -

TTTGAGGAGATGCTTAAACATAGGAATGATGCTGCTTGCCCTGCCAAGGGATTCTATACC
) 181 -----+-----+-----+-----+-----+-----+ 240
F E E M L K H R N D A A C P A K G F Y T -

TATGACGCTTTTATATCAGCAGCCAAAGCATTTCAGCCTTCGGCACCAGTGGAGATGTT
) 241 -----+-----+-----+-----+-----+-----+ 300
Y D A F I S A A K A F P A F G T T G D V -

GATACATGTAAAAGGGAGATTGCTGCATTCTTTGGCCAGACATCTCATGCAACTACAGGA
) 301 -----+-----+-----+-----+-----+-----+ 360
D T C K R E I A A F F G Q T S H A T T G -

GGGTGGCCAACTGCACCAGATGGACCATATGCTTGGGGATATTGCTACAAGGAGGAGCTA
) 361 -----+-----+-----+-----+-----+-----+ 420
G W P T A P D G P Y A W G Y C Y K E E L -

AATCAAGCGTCGTCTTACTGTTCTCCAAGTCCCGCTTATCCTTGTGCTCCTGGCAAGAAA
) 421 -----+-----+-----+-----+-----+-----+ 480
N Q A S S Y C S P S P A Y P C A P G K K -

TATTACGGTAGAGGTCCCATCCAACCTTTCATGGAATTATAATTATGGGCAATGTGGGCAA
) 481 -----+-----+-----+-----+-----+-----+ 540
Y Y G R G P I Q L S W N Y N Y G Q C G Q -

GCCTTAGGATTAGACCTATTGAACAATCCAGACCTTGTAGCGACAGATCGAGTCATTTCT
) 541 -----+-----+-----+-----+-----+-----+ 600
A L G L D L L N N P D L V A T D R V I S -

TTCAAGGCAGCAATTTGGTTCTGGATGACTCCACAATTTCCAAAGCCCTCTTGTCATGAC
) 601 -----+-----+-----+-----+-----+-----+ 660
F K A A I W F W M T P Q F P K P S C H D -

GTCATCACTGGACAATGGTCACCCACCGCCACGATATTTAGCTGGAAGAGCTCCAGGT
) 661 -----+-----+-----+-----+-----+-----+ 720
V I T G Q W S P T G H D I S A G R A P G -

TATGGTGTGATAACAAACATCATCAATGGTGGTCTTGAATGTGGTAGGGGTTGGGATGCT
) 721 -----+-----+-----+-----+-----+-----+ 780
Y G V I T N I I N G G L E C G R G W D A -

AGGGTTGAAGATCGTATTGGGTTTTATAAGAGATACTGTGATATGTTTCGAGTTGGCTAT
) 781 -----+-----+-----+-----+-----+-----+ 840
R V E D R I G F Y K R Y C D M F A V G Y -

GGCAGCAATCTTGACTGCTACAATCAAACGCCATTTCGGATTAGGGTGA
841 -----+-----+-----+-----+-----+-----+ 888
G S N L D C Y N Q T P F G L G * -

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Fig. 1. Sequence of Hev b 11 showing chitin recognition or binding site in bold and chitinase motifs underlined.

	1				43
rHev b 11	EQCGRQAGGA	LCPGGLCCSQ	YGCANTPEY	CGSGCQSQCD	GGG
BANANA	-----	-----	---G---DP-	--Q--X---T	-ST
AVOCADO	-----	-----	F---GS-SD-	--PT-----G	-VT
CHESTNUT	-----	A-ANN-----	F---G--A--	--A-----S	SPT
HEVEIN	-----K	---NN-----	W---GS-D--	-SPDHNC-SN	CKD

Fig. 2. N-terminal amino acid sequence of Hev b 11 aligned with those previously reported for chitinases from avocado (Pers a 1) [9], chestnut [10] and banana [18] and hevein [17]. Differences to rHev b 11 are shown.

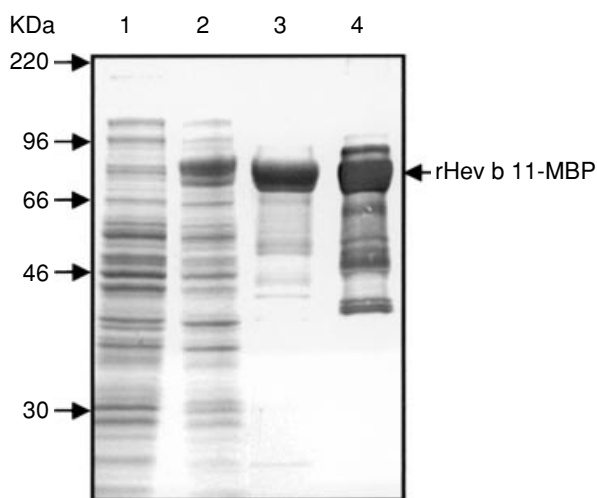


Fig. 3. SDS-PAGE and blot of rHev b 11-MBP; bacterial extract of untransformed cells (lane 1) and cells expressing rHev b 11-MBP (lane 2) and purified rHev b 11-MBP (lanes 3 and 4). Lanes 1–3: Coomassie-stained SDS-PAGE gels. Lane 4: anti-MBP immunoblot, alkaline phosphatase development. The arrow marks the rHev b 11-MBP fusion protein at approximately 75 kDa.

Self-inhibition of rHev b 11 was shown to increase with amounts of 5 to 100 µg/µL from 60 to 85% and complete self-inhibition of rHev b 6.02 was also observed (data not shown). Recombinant Hev b 6.02-MBP could inhibit IgE binding to solid-phase-bound rHev b 11-MBP varying from 0 to 92% (data now shown).

In addition, these sera were pre-incubated with rHev b 11-MBP and subsequently tested for IgE binding to rHev b 6.02-MBP. Inhibitions of 0 to 21% (patients numbers 3 to 6; Table 1) were observed, while binding of IgE from other sera was not inhibited at all under the same conditions (patients numbers 1, 2 and 7 to 10; Table 1). No binding or inhibition was detected with control sera, the recombinant protein (rHev b 8) control or the buffer control. A certain, however not significant, background of IgE-binding to MBP was observed.

Inhibition of fungal growth by rHev b 11

The growth of *F. oxysporum* was inhibited by 16% at a concentration of 80 µg/mL rHev b 11-MBP. At a concentration of 160 µg/mL, inhibition of 34% was demonstrated. The positive controls showed 77% germination. These data correlate well, showing that a double concentration of protein causes twice as much germination inhibition. Due to the observed necrotic hyphae the effect of the addition of rHev b 11 was considered as fungicidal.

Discussion

The need to produce recombinant allergens in sufficient amounts is essential for the improvement and standardization of future diagnosis and therapy of allergies. The allergen content of natural sources of these proteins may vary depending on source and method of extraction, among other factors, and as such affect the quality of the final product. The expression of a recombinant protein in bacteria or yeast and its subsequent purification results in a product of consistent standard with IgE binding activity.

Recombinant Hev b 11 was produced as a fusion protein with MBP. This expression system allows both periplasmic targeting, necessary for correct folding, and simple single-step purification via affinity chromatography.

The calculated molecular weight, the high DNA sequence similarity and the N-terminal homology all identify this protein as a class I chitinase. The N-terminal sequence of the latex chitinase shows 72% (chestnut), 77% (avocado) and 81% (banana) identity with other known N-terminal hevein domains of fruit chitinases. Consensus patterns typical of class I chitinases were also found. This classification is reinforced by the detection of the chitin binding or recognition domain, a conserved region of 43 amino acids at the N-terminus typical for chitinases from the family 19. Further evidence of the protein's identity was collected. Inhibition of the growth of fungal hyphae has been observed as one of the proposed defence roles of chitinases in plants [19]. The demonstration of fungicidal activity of rHev b 11-MBP concurs with the protein's expected biological function as a chitinase. These results are also important from an allergologist's point of view as, by showing biological activity of rHev b 11-MBP this indicates a correct folding of the recombinant protein; thus, structures required for antibody binding *in vivo* can be assumed to be present on rHev b 11-MBP.

The immunochemical properties of the purified rHev b 11-MBP were examined by Western blotting and ELISA with sera from well-defined latex- and fruit-allergic patients. Nineteen per cent of allergic patients' sera recognized rHev b 11-MBP with variable IgE binding intensity, the majority (8/10) of these positive patients were primarily latex and banana allergic, two patients were latex allergic. In addition, one patient with kiwi allergy without latex allergy displayed no reactivity to any latex allergens, as detected in immunoblots. However, the patient's serum displayed specific IgE against Hev b 11 (data not shown).

IgE binding of rHev b 11 was compared with that of rHev b 6.02. Hevein, a 4.7-kDa protein, has been identified as a major allergen for latex-allergic patients [20]. Around 80% of latex-allergic patients display specific IgE towards it [11, 21]. The 43

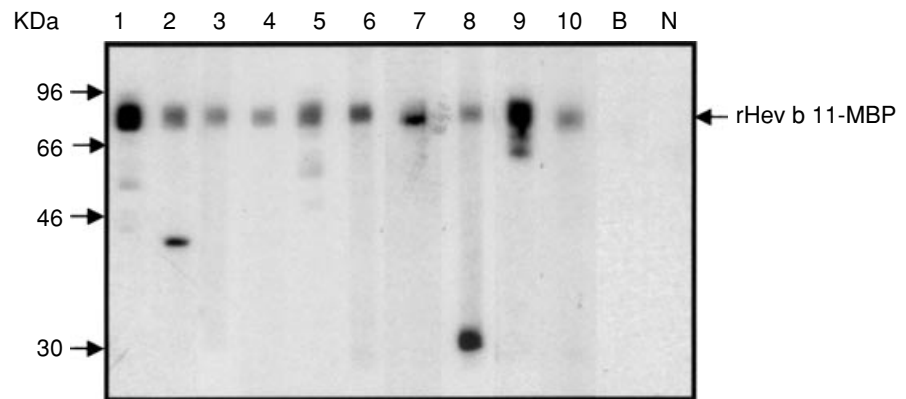


Fig. 4. Immunoblot of purified rHev b 11-MBP. Lanes 1–10: individual patient's sera. Lane B: buffer control. Lane N: birch pollen-allergic control serum pool. The arrow marks rHev b 11-MBP fusion protein at approximately 75 kDa. Standard size marker proteins in kDa.

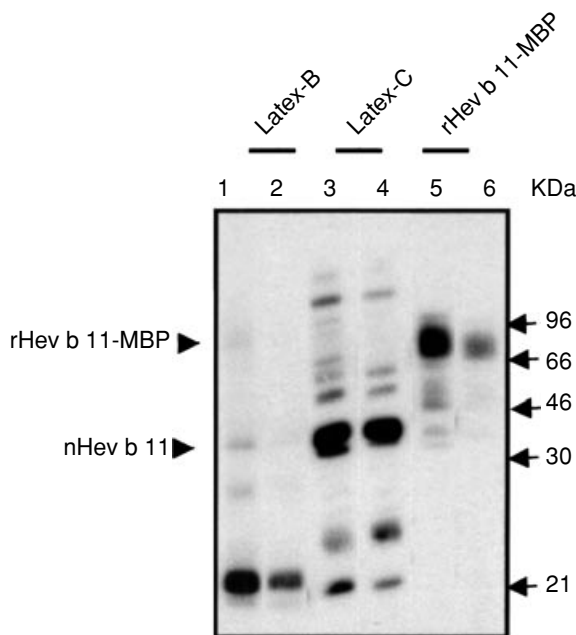


Fig. 5. Inhibition tests. A Hev b 11-positive serum pool was tested for IgE binding without (lanes 1, 3 and 5) and with pre-incubation of 50 µg rHev b 11-MBP to latex B extract (lanes 1 and 2), latex C extract (lanes 3 and 4), and rHev b 11-MBP (lanes 5 and 6). Detection was performed with ^{125}I -labelled anti-IgE. Buffer and birch pollen-allergic sera control were negative and not shown. Standard size marker proteins in kDa.

amino acid long chitin binding or recognition domain is a ubiquitous motif found in different families of plant proteins and it has been proposed that it is responsible for the development of food allergies by latex-allergic subjects [11]. In 1998 the essential role of the hevein domain in the allergenic potential of plant chitinases was demonstrated [10]. The question is, is the hevein domain solely responsible for this cross-reactivity? Purified hevein inhibits IgE binding to avocado chitin binding proteins in six out of seven avocado-sensitized latex patients. Posch et al. [7] and Sanchez-Monge et al. [8] proposed that the cross-reactivity of homologous class I chitinases from different fruit is caused by IgE binding to the hevein domains of these proteins.

No evident inhibition of the IgE binding to hevein by avocado in 50% (8/16) of patients tested was observed by Chen et al.

[11]. They concluded that the hevein molecule contains individual IgE binding epitopes that are not present on the avocado proteins and that these allow hevein to bind strongly with IgE antibodies, even if some binding sites have been blocked by the shared epitopes on avocado proteins. The recombinant chitinase from latex seems to confirm this conclusion.

The latex class I chitinase shows 58% sequence identity with hevein in the chitin binding domain. Our results showed that some rHev b 11-MBP-positive patients' sera, when pre-incubated with rHev b 11-MBP, displayed inhibition of binding to natural Hev b 11 from latex B- and C-serum as well as weakened binding to a band of a molecular mass corresponding to Hev b 6.01 in immunoblots. Furthermore, some Hev b 11-positive sera displayed 16% decreased binding to solid phase rHev b 6.02-MBP after pre-incubation with rHev b 11-MBP, while others showed no inhibition. These data seem to contradict the hypothesis that the hevein domain of the latex class I chitinase is the only location of the relevant IgE binding epitopes responsible for cross-reactivity with fruit. We conclude that the chitinase molecule contains other IgE epitopes that are not present on the chitin binding domain of Hev b 6.02. In addition, the epitopes of the hevein domain of prohevein are different compared with the epitopes of Hev b 11 on the corresponding region. Experiments expressing Hev b 11 lacking the chitin binding domain could give a better insight into this complex issue.

Soil-borne microorganisms that produce chitinases have been considered as potential biocontrol agents against fungi and nematodes that cause diseases of agricultural crops. Chitinases can also be expressed in transgenic plants and confer better resistance against chitin-containing pathogens [19]. Since the demonstration that enhanced chitinase levels in transgenic plants are able to reduce pathogen damage [22], research into the commercial potential in this field has increased [23]. The establishment of chitinases as allergens and their use in transgenic plant breeding poses serious issues, including a possible conflict of interests between public health and agricultural production [24, 25]. A further issue is the use of ethylene to accelerate ripening of fruit and vegetables. For example bananas, apples, avocados and tomatoes are treated with ethylene after harvesting. As a consequence the levels of chitinases in these foods are elevated and hence also the levels of allergens [26].

The availability of rHev b 11 should facilitate the further investigation of the latex–fruit syndrome and could assist in

the research of possible cross-reactivities and paths of sensitization. Thus its future use for diagnosis of patients seems highly recommendable.

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