

Hev b 9, an enolase and a new cross-reactive allergen from *Hevea* latex and molds

Purification, characterization, cloning and expression

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Natural rubber latex allergy is an IgE-mediated disease that is caused by proteins that elute from commercial latex products. A complementary DNA (cDNA) coding for Hev b 9, an enolase (2-phospho-D-glycerate hydrolyase) and allergen from latex of the rubber tree *Hevea brasiliensis*, was amplified by PCR. The PCR primers were designed according to conserved regions of enolases from plants. The obtained cDNA amplification product consisted of 1651 bp and encoded a protein of 445 amino-acid residues with a calculated molecular mass of 47.6 kDa. Sequence comparisons revealed high similarities of the *Hevea* latex enolase to mold enolases that have been identified as important allergens. In addition, the crucial amino-acid residues that participate in the formation of the catalytic site and the Mg²⁺ binding site of enolases were also conserved. *Hevea* latex enolase was produced as a recombinant protein in *Escherichia coli* with an N-terminal hexahistidyl tag, and purified by affinity chromatography. The yield amounted to 110 mg of purified Hev b 9 per litre of bacterial culture. The recombinant allergen bound IgE from latex, as well as mold-allergic patients, in immunoblot and ELISA experiments. The natural enolase was isolated from *Hevea* latex by (NH₄)₂SO₄ precipitation and ion exchange chromatography. The natural and the recombinant (r)Hev b 9 showed equivalent enzymatic activity. Patients' IgE-antibodies preincubated with rHev b 9 lost their ability to bind to natural (n) Hev b 9, indicating the identity of the B-cell epitopes on both molecules. Cross-reactivity with two enolases from *Cladosporium herbarum* and *Alternaria alternata* was determined by inhibition of IgE-binding to these enolases by rHev b 9. Therefore, enolases may represent another class of highly conserved enzymes with allergenic potentials.

Keywords: cross-reactivity; enolase; *Hevea* latex allergy, mold allergy; recombinant Hev b 9.

IgE-mediated type 1 allergy to natural rubber latex (NRL) is caused by proteins present in diverse latex products. Latex is the milky sap produced by the laticiferous cells of the tropical rubber tree *Hevea brasiliensis* of the family Euphorbiaceae. Sensitization occurs via the skin, mucosal or wound contact, or inhalation of airborne allergens released from powdered latex gloves [1]. NRL-allergy has been acknowledged to impose serious health problems, especially among occupationally exposed individuals, e.g. health care workers and individuals who have to undergo repeated surgeries such as patients with spina bifida [2,3].

In NRL a variety of IgE-binding proteins with molecular masses ranging from 5 to 110 kDa were detected by two-dimensional immunoblotting [4–6]. Since 1993, a total of 10 NRL allergens have received an international nomenclature

designation. With the exception of Hev b 4, full coding sequences are known for all other designated latex allergens [7–9] (S. Wagner & E. Ganglberger, unpublished data). Some of these latex allergens have been identified in latex only, while others cross-react with homologous proteins present in fruits [10]. These cross-reactivities have been termed the 'latex–fruit syndrome'. Immunological cross-reactivity has been reported between allergens from latex and banana, avocado, chestnut, peanut, papaya, passion fruit, fig, melon, pineapple, kiwi, and tomato [11]. In addition, cross-reactivities between allergens from NRL and *Ficus benjamina*, sweet pepper, grass, and weed pollen have also been described [12–14].

Prohevein from *Hevea* latex (Hev b 6.01) and its homologous proteins in vegetables and fruits have been implicated in cross-reactivity of latex with sweet pepper, chestnut, banana, peach, and kiwi [13]. Prohevein belongs to the group of chitin-binding proteins with a hevein domain. This group also includes plant class I chitinases [15]. Hevein (Hev b 6.02), the N-terminal 4.7-kDa domain of prohevein, shares epitopes with class I chitinases from avocado, chestnut, and banana, and represents one cause for their cross-reactivity [16–18]. Another protein that may play a role in the cross-reactivity of latex with pollen and fruits is Hev b 8, the latex profilin. Profilin was the first allergen to be named a 'panallergen' because of its high cross-reactivity [19]. Latex profilin was detected in a nonammoniated latex extract [20] and a certain amount of

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Abbreviations: nHev b 9, natural Hev b 9; NRL, natural rubber latex; PVDF, polyvinylidene difluoride; rAlt a 5, recombinant Alt a 5; rCla h 6, recombinant Cla h 6; rHev b 9, recombinant Hev b 9.

Enzymes: enolase (EC 4.2.1.11); superoxide dismutase (EC 1.15.1.1).

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cross-reactivity to pollen proteins was shown by inhibition studies [14]. Hev b 2, a β -1,3-glucanase, was separated from nonamminated latex and described as a latex-allergen. Hev b 2 was recognized by several atopic patients, indicating a possible cross-reactivity [21].

Based on data obtained by immunoprobings two-dimensional *Hevea* latex protein blots with sera from latex allergic patients and by protein microsequencing, several proteins from NRL have been described as putative allergens [4]. Two IgE-reactive protein spots shared over 90% sequence identity with the N-terminus of *Ricinus communis* enolase (SWISS-PROT P42896). Enolase (2-phospho-D-glycerate hydrolyase) is a highly expressed key enzyme of glycolysis and gluconeogenesis that is needed for the conversion of glycerate 2-phosphate to phosphoenolpyruvate and vice versa. Enolase occurs as a homodimer in all eukaryotic and many prokaryotic cells. Owing to its essential role as a cytoplasmic household enzyme, enolase is a highly conserved enzyme. It has been identified and characterized from diverse sources ranging from bacteria to higher vertebrates. Enolases are described as important allergens in several molds. An enolase was first identified as an allergen in *Saccharomyces cerevisiae*, but IgE cross-reactivity exists between enolases of *Cladosporium herbarum* (Cla h 6), *Alternaria alternata* (Alt a 5), *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani* [22–25].

Here, we report the nucleotide and deduced amino-acid sequence of the new latex allergen Hev b 9, which is an enolase. Further, we describe the cloning and expression of the recombinant molecule in *E. coli* and its biological activity. Characterization as an 48-kDa latex allergen was shown by IgE-immunoblot and IgE-inhibition experiments with the natural *Hevea* enolase isolated from latex C-serum. The results from IgE-immunoblots and ELISA-inhibition experiments performed with *Hevea* latex enolase and the mold allergens Alt a 5 and Cla h 6 indicate a new area of cross-reactivity of latex allergens.

PATIENTS, MATERIAL AND METHODS

Isolation of total RNA from latex

Fresh latex was collected from regularly tapped Malaysian rubber trees (*H. brasiliensis*, clone RRIM 600). Latex exuding from the tapped trees was collected while continuously mixing it with an equal volume of RNA extraction buffer (0.1 M Tris/HCl, 0.3 M LiCl, 1 mM EDTA, 10% SDS, pH 9.5) at ambient temperature. This mixture was centrifuged at 100 000 g for 30 min at 15 °C. The rubber plug was removed, the aqueous phase extracted with chloroform/phenol and total RNA was precipitated with LiCl.

RT-PCR amplification and sequencing of Hev b 9

First-strand cDNA synthesis was performed by reverse transcribing 0.5 µg of total latex RNA using a modified oligo(dT) primer, T₂₅NN (5'-GGAGAAGGA(T)₂₅(A/G/C)N-3'). For the first-round PCR 2 µL of the first-strand cDNA synthesis and for the second-round PCR 2 µL of the previous amplification reaction were used to amplify part of the coding sequence for Hev b 9. Based on sequence similarities among *Ricinus communis* enolase and enolases from other plants, degenerate primers were designed. The primers used to amplify a partial sequence of the Hev b 9 cDNA were Eno6 (5'-TT(C/T)AA(A/G)GA(A/G)GC(A/C/T)ATGAAGATGGG-3') and T₂₅NN for first-round PCR and Eno5 (5'-ATGCAGGA

(A/G)TT(C/T)ATGAT(C/T)CT(C/T)CC-3') and T₂₅NN for the following PCR. The second-round PCR products were ligated into the pGEM®-T easy vector (pGEM®-T Easy Vector System, Promega, Wisconsin, USA) and propagated in XL-1 Blue *E. coli* cells according to the vendor's instructions. The PCR products were sequenced using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Life Science, Little Chalfont, UK) with the fluorescent labelled primers fT7 forward and fM13 reverse flanking the vector's multiple cloning site.

To complete the missing 5' end of the cDNA, the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) was used according to the vendor's instructions. Complementary DNA synthesis was performed using the cDNA primer from the kit, and following first and second-round PCR with the kit's cDNA adaptor primers and the gene-specific internal primers Eno9 (5'-CAGCTTTAGCAATGGCAGTC-TT-3') and Eno7 (5'-CAACATTTGTTGCATCCTGACC-3'). The second-round PCR products were cloned and sequenced as described above.

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

Construction of the expression plasmid

The complete coding sequence of Hev b 9 was amplified by RT-PCR from total latex RNA. The primers used in the PCR were Eno10 (5'-AGGTACCGCGATTACCATTGTCTCC-3', *Kpn*I site underlined) and Eno11 (5'-ACAGCTGCTAATAGGGTTCGACAGG-3', *Pvu*II site underlined) according to the 5' and 3' end of the latex Hev b 9 cDNA determined in the previous experiments. The resulting 1339 bp long amplification product was first digested with *Sal*I, overhanging ends filled in with Klenow enzyme (Boehringer Mannheim, Germany), subsequently digested with *Kpn*I and ligated into the pQE-30 expression vector (Qiagen, Hilden, Germany). The pQE-30 expression vector provides a sequence coding for a hexahistidyl (6 × His) affinity tag at the 5' end of the Hev b 9 coding sequence. The expression construct pQE-30/Hev b 9 was transformed into competent M15[pREP4] *E. coli* cells.

Expression of rHev b 9 in *E. coli*

For the expression of rHev b 9, 1 L 2 × tryptone/yeast culture medium containing both ampicillin and kanamycin was inoculated with 10 mL of an overnight culture and grown with vigorous shaking at 37 °C to an *D*₆₀₀ of 0.8. Expression of rHev b 9 was induced by adding isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 2 mM. After growing for an additional 16 h at 37 °C, the cells were harvested and cell pellets were frozen at -20 °C.

Purification of rHev b 9 under nondenaturing conditions

Cell pellets were thawed for 15 min on ice and resuspended in 30 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). Lysozyme (Merck, Darmstadt, Germany) was added to a concentration of 1 mg·mL⁻¹ and the mixture was incubated on ice for 1 h. The highly viscous lysate was treated with DNaseI at a concentration of 200 µg·L⁻¹ on ice for 1 h. To pellet cellular debris the lysate was centrifuged for 20 min at 4 °C at 20 000 g. The supernatant was loaded onto a packed column of 3 mL of 50% w/v Ni/nitriloacetic acid agarose (Qiagen, Hilden, Germany). The column was washed with

20 mL of lysis buffer and 10 mL of wash buffer (same composition as lysis buffer plus 20 mM imidazole). Purified rHev b 9 was eluted with lysis buffer containing 100 mM imidazole. The course of the purification was monitored by SDS/PAGE on 12% gels under reducing conditions. The separated proteins were visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA).

Enzymatic activity of Hev b 9

Enolase enzyme assay was performed as described previously [26]. Enolase was added to 1 mL of assay mixture (20 mM Na₂HPO₄, pH 7.4, 400 mM KCl, 0.01 mM EDTA, 2 mM 2-phospho-D-glycerate). The enzymatic activity was determined at room temperature by transferring the mixture into a quartz cuvette and measuring the absorption at A₂₄₀ for 3 min. For the determination of the specific activity an enolase from *S. cerevisiae* (Sigma-Aldrich, Steinheim, Germany) with a specific activity of 44 U·mg⁻¹ protein was used in the enolase enzyme assay. One unit is defined as the conversion of 1 μmol of 2-phosphoglycerate to phosphoenolpyruvate per minute at pH 7.4 at 25 °C.

Cloning of enolases from *Cladosporium herbarum* and *Alternaria alternata*

C. herbarum enolase (Cla h 6) was isolated by immunological screening of a cDNA expression library as described previously [27]. Using the coding sequences of *C. herbarum* enolase as a probe, the *A. alternata* cDNA expression library was screened for a positive clone [27].

Expression and purification of rCla h 6 and rAlt a 5

Allergen coding sequences were cloned into pMW172 [28] via *NdeI/EcoRI* in the case of Cla h 6 and *NdeI/HindIII* in the case of Alt a 5. Expression of the respective constructs was performed as previously described [27]. *E. coli* lysates of the recombinant proteins were analysed by SDS/PAGE, Coomassie staining, and IgE immunoblots [27]. Subsequently, the recombinant enolase of *A. alternata* was purified to near homogeneity, whereas the recombinant enolase of *C. herbarum* was enriched by an inclusion body preparation [25].

Preparation of latex C-serum

Fresh *Hevea* latex was collected in chilled containers from rubber trees (*H. brasiliensis* RRIM 600 clone) and then centrifuged at 44 000 g at 4 °C for 1 h. The aqueous phase, the C-serum, was collected and centrifugation repeated. The clear aqueous C-serum was freeze dried and stored at -20 °C.

Isolation of natural Hev b 9 from latex C-serum

Two hundred milligrams lyophilized latex C-serum was dissolved in 20 mM Tris/HCl, pH 7.5. (NH₄)₂SO₄ was added to the solution to 25% saturation and stirred for 30 min at 4 °C. The precipitated material was centrifuged and collected. (NH₄)₂SO₄ was added to the supernatant to 50% saturation, in a final step to 75% saturation, and treated as described above. The three precipitates were dissolved in 20 mM Tris/HCl, pH 7.5, and tested for enolase activity. The redissolved pellet of the 75% saturation was desalted in 20 mM Tris/HCl, pH 7.5, by passing through a PD-10 column (Pharmacia, Uppsala,

Sweden). The fraction collected in the void volume was applied to a MonoQ HR5/5 column (Pharmacia, Uppsala, Sweden), and elution performed using a linear gradient of 0–0.5 M NaCl in 20 mM Tris/HCl, pH 7.5. Each fraction was analysed for enolase activity and protein contents by SDS/PAGE.

Fraction number 9 was separated on a 8% SDS/PAGE gel and blotted onto a poly(vinylidene difluoride) membrane. The membrane was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, California). The 48-kDa band was excised and N-terminal sequencing was performed using the 491 Protein Sequencer of Procise Protein Sequencing System (PE Applied Biosystems, Foster City, CA, USA).

Study population

A total of 122 individual serum samples from adult patients with positive case histories, positive radioallergosorbent tests (RAST classes ≥ 2), and characteristic type 1 allergic reactions was used in this study. Of these sera, 103 were obtained from latex allergic patients, 7 from patients with characterized allergy to latex and *A. alternata* or *C. herbarum*, and 12 from *A. alternata* or *C. herbarum* allergic patients.

The serum pool comprised selected sera from five latex allergic and five latex and mold allergic patients. As a control group, six serum samples from nonallergic patients were used.

Immunoblot analysis

Volumes of 2 μg nHev b 9, rHev b 9, rAlt a 5, and rCla h 6 per lane were separated on 12% SDS/PAGE gels, blotted onto nitro-cellulose membranes and treated with blocking buffer (40 mM Na₂HPO₄, 7 mM NaH₂PO₄, 0.5% BSA, 0.05% w/v sodium azide, 0.5% w/v Tween-20, pH 7.5). Membrane strips were then incubated with patients' sera diluted in blocking buffer 1 : 5 overnight at 4 °C. After a washing step the blots were incubated with ¹²⁵I-labeled anti-(human IgE) Ig (IBL, Hamburg, Germany) overnight. Binding of patients' IgE to the allergens was visualized on BiomayTM MS film (Kodak, New York, USA).

For inhibition of IgE binding to nHev b 9, a pool of Hev b 9 positive sera was incubated overnight at 4 °C with 50 μg and 100 μg purified rHev b 9. The preincubated sera were then used to probe the nitrocellulose strips containing nHev b 9. Incubation of the serum-pool with 100 μg purified rHev b 10 was used as a negative control. Hev b 10 is a manganese superoxide dismutase and was produced as a recombinant protein in *E. coli* (S. Wagner, unpublished data).

IgE inhibition ELISA

For cross-inhibition experiments, the Hev b 9, Alt a 5 and Cla h 6 specific serum pool was diluted 1 : 6 in 150 mM NaCl/50 mM Tris, 0.05% Tween 20, 0.5% BSA, and preincubated (overnight at 4 °C) with 1, 10, 50, and 100 μg·mL⁻¹ rHev b 9 and also rHev b 10. Microtiter plates (Nunc-Immuno Plate, Nalge Nunc International, Denmark) were coated with 4 μg·mL⁻¹ of recombinant allergens overnight at 4 °C. After blocking with NaCl/Tris, 0.05% Tween 20, 1% BSA, preincubated sera were applied onto the coated plates and incubated overnight at 4 °C. After washing five times with 150 mM NaCl/50 mM Tris and 0.05% Tween 20, the plates were incubated with alkaline phosphatase conjugated mouse anti-(human IgE) Ig (Pharmingen, San Diego, CA, USA) for 1 h at 37 °C, and for another hour at 4 °C. Color development was performed using 0.1% azinoethylbenzthiozoline sulfonic

acid substrate (Sigma-Aldrich, Steinheim, Germany) and the optical density was measured at 405 nm (550 nm as reference wavelength) after 30 min using an ELISA-reader (Dynatech MR 7000, Alexandria, VA, USA).

RESULTS

Cloning and sequence analysis of Hev b 9

Degenerate primers designed according to conserved regions of enolases from plants were used to amplify a partial sequence (coding for amino-acids 182–445) and the complete noncoding 3' end of latex enolase cDNA by RT-PCR from total latex RNA. Using a protocol to amplify cDNA ends by PCR, the complete

cDNA clone was obtained. The *Hevea* latex enolase cDNA comprises 1651 bp, containing an open reading frame from bases 30–1367. T-rich sequences dominate the last 100 bp, and the complete polyadenylation signal consists of an aggregate of multiple elements. A TTGTAA motif representing an upstream efficiency element was found 48 bp upstream and an A-rich region as a positioning element 15–18 bp upstream of the poly(A) sequence. Both motifs have been found frequently in plant sequences [29]. The complete cDNA sequence coding for latex enolase is available from the EMBL database under the accession number AJ132580.

The Hev b 9 cDNA sequence codes for a protein of 445 amino-acid residues corresponding to a predicted molecular mass of 47.6 kDa and a calculated pI of 5.6 (Fig. 1). The

1
 Hev b 9 AITIVSVRAR QIFDSRGNPT VEADVLSLSDG YLARAAPVPSG ASTGIYEAL
 Alt a 5 .M--TKIH-- SVY----- --V-IVTET- .H--I----- --SH--C-
 Cla h 6 .MP-SKIH- YVY----- --V-IVTET- .H--I----- --SH--C-
 S. cerevisiae .MAVSK-Y- SVY----- --VELTEK- .VF-SI----- --VH-----
 human ..AMQKIF- E-L----- --V-LHTAK- RF-----

 51 97
 Hev b 9 LRDGG.SDYL GKGVS KAVEN VNIIGPALV GK..DPTDQV GIDNFMVQQL
 Alt a 5 ---DKS-WG ---T---A- --DT-A-R-I KEK--VK--S AV-A-LN-K-
 Cla h 6 ---DKS-WA ---T---A- --E--A--I KEN--VK--A AV-A-LN-K-
 S. cerevisiae M---DKS-WM ---LH--K- --DV-A--F- KANI--VK--K AV-D-LI-S-
 human ---DKGR- ---L-L- I-NTL---L Q-K-SVV-E KV-K-I-E-

 98 147
 Hev b 9 DGTVNEWGWC KQKLGANAIL AVSLAVCKAG AHVKGIPLYE HIANLAGNKN
 Alt a 5 ---T----- -TN----- G-M-IA--A -AE--V--A --SD--T-
 Cla h 6 ---T----- -T-I----- G-M--A--A -AE-RV--A --SD-S-T-
 S. cerevisiae ---A----- -S----- G---ASR-A -AE-NV--K -L-D-SKS-T-
 human ---E----- -S-F----- G----- -AE--V--R --D---PD-

 148 195
 Hev b 9 ..LVLPVPAP NVINGGSHAG NKLAMQEFMI LPVGASSFKE AMKMGAEVYH
 Alt a 5 --Y-----FQ --L---D- GR-F----- V-CE-PT-S --RQ-----Q
 Cla h 6 --F-----FM --V----- GR-F----- V-S--P--T --RQ-----Q
 S. cerevisiae S-Y-----FL --L----- GA-L----- A-T--KT-A -LRI-S-----
 human ..I-----

 196 245
 Hev b 9 HLKSVIKKKY GQDATNVGD GGFAPNIQEN KEGLELLKTA IAKAGYTKGV
 Alt a 5 K--ALA--T- --S-G----- --V--D--TA E-A-D-ITK -EE-----I
 Cla h 6 K--LT--R- --S-G----- --V--D--TA E-A-D-ITD -EE-----I
 S. cerevisiae N--LT--R- -AS-G----- --V--D--TA E-A-D-IVD -KA--HD--
 human --G--A-- -K----- --L----- N-A----- -QA--PD--

 246 295
 Hev b 9 VIGMDVAASE FYGSDQ.TYD LNFKEENNNNG SQKISGEALK DLYKSFVAEY
 Alt a 5 K-A----- -KA-EKK- -D--NPDSK -KWLTY-Q-A EM---LAEK-
 Cla h 6 K-A---S- -KA-EKK- -D--NPDSK -KW-TY-Q-A -Q--QLA-K-
 S. cerevisiae K-L-C-S- -FKDG..K- -D--NP-SDK -KWLTY-Q-A --H-LMRR-
 human ---RNG.K- -D--SPDDPA RH.-T--K-G E-----IKN-

 296 345
 Hev b 9 PIVSIEDPFD QDDWAHYAKL TSEIGEKVQI VGDDLVTNPN KRVEKAIKEK
 Alt a 5 -----A E---EAWSYF FKTYDG... ---T--- EFIK---EL-
 Cla h 6 -----A E---EAWSYF YKTS-SDF- ---T--- EFIK---ET-
 S. cerevisiae -----A E---EAWSHF FKTA...I- -A---T--- -IAT--EK-
 human -V----- -TWTSF L-GV..NI- ---T--- -IAQ-VEK-

 346 395
 Hev b 9 ACNALLVNV QIGSVTESIE AVKMSKRAGW GVMASIRSGE TELTFIADLS
 Alt a 5 S----- -TI--A-Q -A-DAFG- --V--- -VT---IV-
 Cla h 6 ----- -TI--A-N -A-D-FA- --V--- -VT---IV-
 S. cerevisiae -AD----- -TLS--K -AQD-FA- --V--- -V-----
 human --C----- -Q -C-LAQS- --V--- -V-----

 396 445
 Hev b 9 VGLATGQIKT GAPCRSERLA KYNQLLRREE ELGSEAVYAG ANFRKPVEPY
 Alt a 5 --RS----- -A----- -L-I----- --DKRL-- N--TA-NLZ-
 Cla h 6 --RA----- -A----- -L-I----- --DK--D- -TA'AINL-
 S. cerevisiae --R----- -A----- -L----- --DN--F- E--HHGDKL-
 human --C----- -M----- -A-DK-IF- RK--N-KAK-

Fig. 1. Sequence analyses of Hev b 9. Hev b 9 shows 60% identity to *A. alternata* enolase (Alt a 5), 62% to *C. herbarum* enolase (Cla h 6), 60% to *S. cerevisiae* enolase, and 72% identity to the human beta enolase. Identical amino-acid residues are indicated by dashes. Important active site residues are marked in grey, the positions of the metal binding ligands shown by arrows, and the enolase 'fingerprint' motif is boxed.

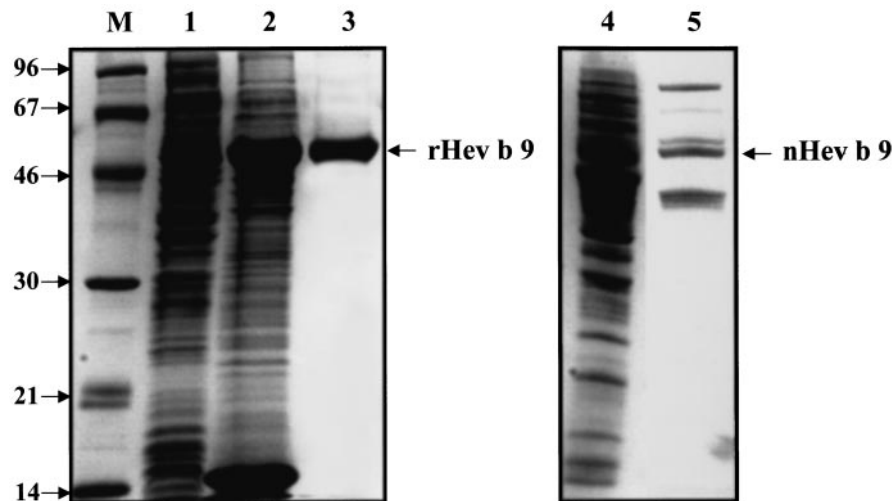


Fig. 2. Purification of rHev b 9 and isolation of nHev b 9. Protein fractions at different stages of purification were loaded onto 12% SDS/PAGE and stained with Coomassie brilliant blue R-250. Molecular mass markers in kDa as indicated are shown in lane M, total protein extract from *E. coli* harboring pQE 30 without an insert in lane 1, total protein extract from *E. coli* harboring pQE 30 with the rHev b 9 coding sequence inserted in lane 2, and elution fraction from Ni/nitriloacetic acid agarose column in lane 3. Proteins from latex C-serum precipitated by 75% $(\text{NH}_4)_2\text{SO}_4$ are shown in lane 4, and nHev b 9 purified by ion-exchange chromatography in lane 5.

deduced amino-acid sequence of Hev b 9 was compared to sequences in the SWISS-PROT database. Hev b 9 displays an overall identity of 72% to the human enolase, and approximately 60% to various fungal enolases, e.g. 60% to *S. cerevisiae* enolase, 62% to *C. herbarum* enolase, Cla h 6, and 60% to *A. alternata* enolase, Alt a 5, respectively (Fig. 1). Sequence analysis of enolases from *S. cerevisiae* and *H. brasiliensis* suggested a common active site architecture [30–32]. The crucial amino-acid residues for enolase activity have been defined in *S. cerevisiae* as Glu 211, Lys 345, and His 373. The corresponding amino-acid residues in *Hevea* enolase are Glu 215, Lys 352, and His 380 (Fig. 1). These three amino-acid residues are part of conserved regions and Lys 380 is contained within an enolase ‘fingerprint’ motif, which is highly conserved in all known enolases [31]. The three-dimensional structure determination of *S. cerevisiae* enolase reveals the presence of two Mg^{2+} in the active site. In *S. cerevisiae* enolase Ser 39, Asp 246, Glu 295, and Asp 320 are complexed to the magnesium ions, corresponding to Ser 42, Asp 250, Glu 300, and Asp 327 in *Hevea* enolase (Fig. 1).

Expression and purification of rHev b 9

The coding region of Hev b 9 (excluding the N-terminal methionine) was ligated into the expression vector pQE-30, and rHev b 9 was produced as a recombinant His-tagged protein. After induction of transformed M15 *E. coli* with 2 mM IPTG for 16–20 h, cells were harvested, lysed by addition of $1 \text{ mg} \cdot \text{mL}^{-1}$ lysozyme (Merck, Darmstadt, Germany) and rHev b 9 was detected by SDS/PAGE. The recombinant protein was purified by Ni/nitriloacetic acid affinity chromatography and purification was analysed by SDS/PAGE (Fig. 2). The yield of purified, soluble rHev b 9 was 110 mg per litre culture.

Enolase activity

Purified recombinant Hev b 9 and *S. cerevisiae* enolase as a standard with a specific activity of $44 \text{ U} \cdot \text{mg}^{-1}$ enolase were used for the enzyme assay. Comparing the increases of absorption- $\text{mg}^{-1} \cdot \text{min}^{-1}$ of the enolases resulted in a specific activity of $22 \text{ U} \cdot \text{mg}^{-1}$ enolase for rHev b 9.

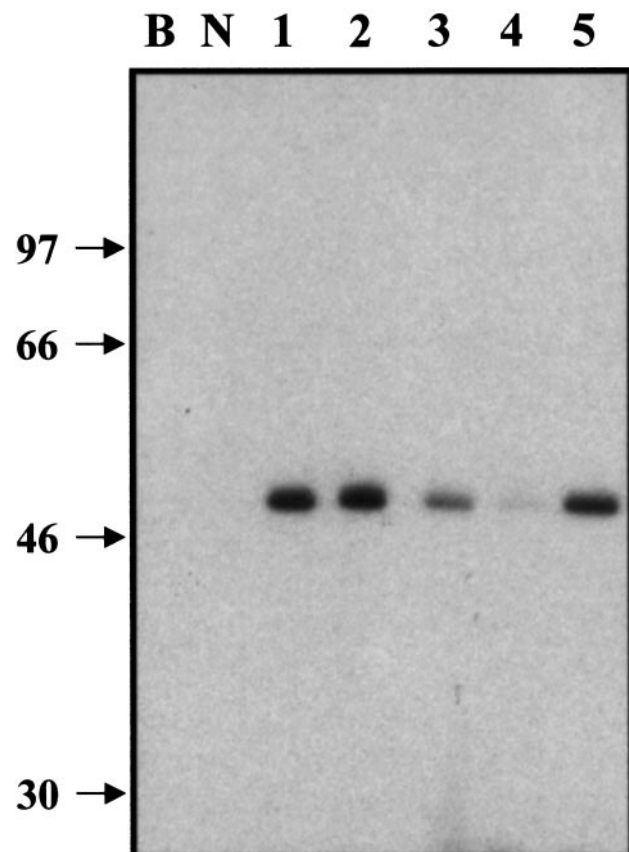
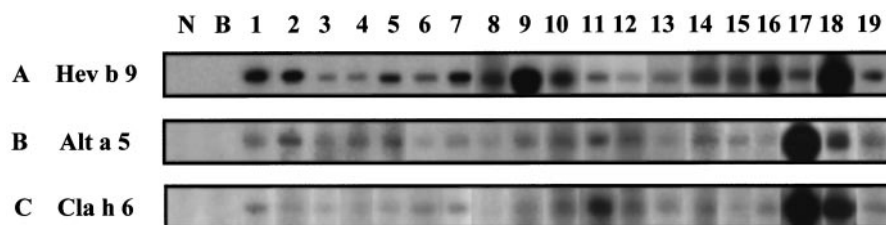


Fig. 3. IgE immunoblots and inhibition experiments with recombinant and natural Hev b 9. Blotted recombinant (lane 1) and natural Hev b 9 (lane 2) were probed with a serum pool of latex-allergic patients. For IgE inhibition, blotted natural Hev b 9 was probed with the serum pool preincubated with 50 μg (lane 3) or 100 μg (lane 4) recombinant Hev b 9, and 100 μg recombinant Hev b 10 (lane 5). Molecular mass markers in kDa are indicated. For controls nHev b 9 was probed with a normal human serum pool (lane N), and a buffer control without addition of serum (lane B).

Fig. 4. IgE immunoblots with rHev b 9 (A), rAlt a 5 (B) and rCla h 6 (C). Blotted recombinant proteins were probed with sera from single patients, with a normal human serum pool (lane N) or a buffer control without addition of serum (lane B). Lanes 1–12 show IgE reactions of latex allergic patients, lanes 13–16 display patients with latex and mold allergy, and lanes 17–19 IgE reactions of mold allergic patients.



Isolation of natural Hev b 9 and its biochemical and immunological analysis

Natural Hev b 9 was isolated from latex C-serum. Proteins of the C-serum were separated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Subsequently, each fraction was tested for enolase activity. Specific activity was present within the 75% $(\text{NH}_4)_2\text{SO}_4$ fraction. The proteins of this fraction were further separated by ion exchange chromatography, collecting 500 μL samples. Among five samples with specific enolase activity, the fraction with the highest activity (20 $\text{U}\cdot\text{mg}^{-1}$ protein) contained a distinct component migrating at 48 kDa together with additional components on SDS/PAGE (Fig. 2, lane 5). N-terminal sequencing of the 48-kDa protein revealed 15 amino-acid residues, matching the N-terminus without a methionine residue of the Hev b 9 sequence.

A serum pool of latex allergic patients was used for immunoblot and inhibition studies. No difference in IgE binding to nHev b 9 was observed when compared to purified rHev b 9 (Fig. 3, lanes 1 and 2). In order to demonstrate that rHev b 9 was able to inhibit IgE binding to nHev b 9, we examined the effect of preincubating the pooled sera with soluble rHev b 9 before adding the sera to blotted nHev b 9. At concentrations of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ rHev b 9, IgE binding to nHev b 9 was significantly reduced and almost completely abolished when 100 $\mu\text{g}\cdot\text{mL}^{-1}$ were applied (Fig. 3, lane 3 and 4). No inhibition could be observed when the serum pool was preincubated with 100 μg rHev b 10 (Fig. 3, lane 5).

IgE binding to rHev b 9, rAlt a 5, and rCla h 6

The importance of *H. brasiliensis* enolase as an allergen was assessed by direct binding to the recombinant allergen by IgE of latex and mold allergic patients. Sixteen out of 110 latex-allergic patients, that is 14.5%, revealed IgE binding to rHev b 9 in immunoblots (Fig. 4A, lanes 1–16). According to case histories, four of these patients were also allergic to molds (lanes 13–16). Out of 12 patients with characterized allergy to *A. alternata* and *C. herbarum*, three showed IgE reactivity to rHev b 9 (Fig. 4A, lanes 17–19). No IgE-binding could be detected when using a serum pool of six nonallergic individuals or a buffer control (Fig. 4A, lane N and B). The designation Hev b 9 for the latex enolase was submitted and granted from the IUIS allergen nomenclature committee.

All 19 patients' serum samples with IgE reactivity to rHev b 9 were further tested for IgE cross-reactivity to rAlt a 5 and rCla h 6. In the case of rAlt a 5, all 19 sera displayed specific IgE antibodies (Fig. 4B), in the case of rCla h 6 all sera except serum number 8 displayed IgE antibodies reacting with the recombinant allergen (Fig. 4C). All three recombinant allergens displayed different IgE binding capacities.

ELISA inhibition studies with rHev b 9, rAlt a 5, and rCla h 6

For cross-inhibition studies, 10 sera from patients displaying IgE reactivity to rHev b 9, rAlt a 5, and rCla h 6 were used as

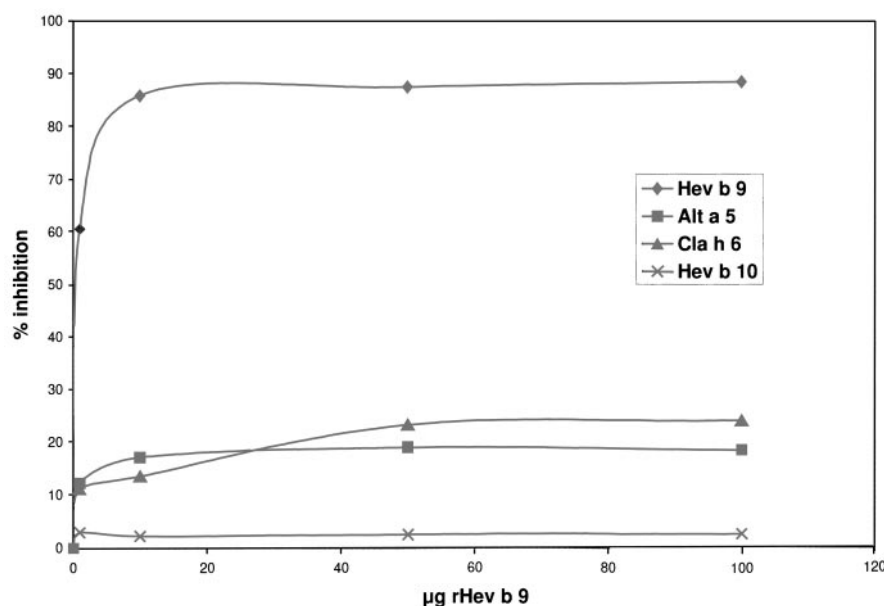


Fig. 5. IgE inhibition ELISA. Recombinant Hev b 9, rAlt a 5, and rCla h 6 were coated at 4 $\mu\text{g}\cdot\text{mL}^{-1}$. Inhibitions were determined with a Hev b 9-specific serum pool after preincubation with different concentrations of rHev b 9 or rHev b 10.

a serum-pool in ELISA experiments (sera from patients 1, 2, 5, 7, 9, 14–18 from Fig. 4). The three recombinant enolases were coated ($4 \mu\text{g}\cdot\text{mL}^{-1}$) and tested with aliquots of the serum-pool after preincubation with increasing amounts of rHev b 9 and rHev b 10 as a negative control. Recombinant Hev b 9 was able to diminish about 60% of IgE binding to rHev b 9 at a concentration of $1 \mu\text{g}\cdot\text{mL}^{-1}$ and up to 90% at concentrations over $10 \mu\text{g}\cdot\text{mL}^{-1}$. Furthermore, rHev b 9 was able to reduce IgE binding to Alt a 5 μp to 19% and to Cla h 6 μp to 24% at concentrations over $50 \mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 5). Preincubation of the serum-pool with equivalent amounts of purified recombinant Hev b 10 did not influence IgE binding to rHev b 9 (Fig. 5).

DISCUSSION

We report the cloning and sequencing of Hev b 9, a new and cross-reactive *Hevea* latex allergen. The Hev b 9 cDNA encodes a cytoplasmic latex protein of 445 amino-acid residues with a predicted molecular mass of 47.6 kDa and a calculated pI of 5.6. The protein displays 62% identity with Cla h 6, the enolase of the mold *C. herbarum*, and 60% identity with Alt a 5, the enolase of the mold *A. alternata* (Fig. 1). We have produced Hev b 9 as a recombinant His-tagged protein in *E. coli* and demonstrated that the molecule possesses IgE reactivity equivalent to nHev b 9 (Fig. 3). Furthermore, cross-reactivity with enolases from molds was tested using IgE-inhibition ELISA.

Enolase is a highly expressed key enzyme of glycolysis and gluconeogenesis and a highly conserved enzyme in all eukaryotic and most prokaryotic cells. Total latex RNA was used to amplify the cDNA coding for the *Hevea* enolase. The deduced amino-acid sequence of the enolase from *H. brasiliensis* shows an identity in the range of 60% to enolases from several molds. The important amino-acid residues responsible for the active site of enolases have been described for the enolase of *S. cerevisiae*. Interestingly, sequence alignments of enolases showed that these amino-acid residues are located in conserved regions [31], which are also present in the Hev b 9 sequence.

Enolases are described as allergens in several molds. Alt a 5, the enolase from *A. alternata*, is the second most important allergen of this mold, and recognized by 22% of all *A. alternata* allergic patients [25]. The same has been reported from the enolase of *C. herbarum*, which is also the second most important allergen of this mold, with 22% of the patients reacting positively [25,33]. In addition, a 46- to 48-kDa enolase is also a main allergen and cross-reacting component of *C. albicans*, *S. cerevisiae*, and *C. utilis* [22,24,34]. Comparing several mold protein extracts, a 46-kDa band appeared as the only dominant band present in all the protein extracts [34]. Therefore, enolases are not only important allergens but also predominant proteins in mold extracts.

Two-dimensional electrophoresis and N-terminal sequences lacking the N-terminal methionine provided the first evidence that enolase is an allergen of *Hevea* latex [4]. We have produced Hev b 9 as a recombinant His-tagged protein in *E. coli* and were able to purify rHev b 9 from *E. coli* cells at a yield of 110 mg per litre culture medium.

The biological activity of rHev b 9 was tested with biochemical and immunological assays to confirm that the recombinant protein was equivalent to its natural counterpart and correctly folded. Therefore, in a first step the enzymatic activity of rHev b 9 was tested in an enolase assay. In this assay, the enzyme catalyzed the conversion of glycerate 2-phosphate to phosphoenolpyruvate and the extinction coefficient of the

produced phosphoenolpyruvate was measured. Recombinant Hev b 9 showed an activity of $22 \text{ U}\cdot\text{mg}^{-1}$ enolase in this enzyme assay. Thus, the recombinant protein possessed an active site because of its correct folding. Furthermore, enolases are described to be enzymatically active only as dimers with identical subunits [32]. Our rHev b 9 was enzymatically active, indicating its ability to form homodimers.

In latex C-serum natural Hev b 9 is a minor component. Detection of IgE binding to Hev b 9 in latex C-serum is difficult because a high percentage of IgE reactivity is directed to proteins in the 43–50 kDa range [5,6]. Therefore, we isolated natural Hev b 9 from C-serum. The protein showed enzymatic activity of $20 \text{ U}\cdot\text{mg}^{-1}$ protein, which is comparable with the activity found for the recombinant protein. Both natural and recombinant Hev b 9 bound IgE from a serum-pool of latex allergic individuals with comparable intensities in immunoblots. Furthermore, rHev b 9 was able to abolish IgE binding to nHev b 9 when the serum-pool was preincubated with the recombinant protein (Fig. 3). Consequently, the highly purified and standardized recombinant protein is a superior tool compared to the natural protein.

We tested the IgE binding capacity of the recombinant Hev b 9 by IgE immunoblots using 110 individual serum samples from latex-allergic patients. Sixteen patients (14.5%) revealed IgE-binding to the recombinant Hev b 9. Four out of these 16 patients were also allergic to molds. Three out of 12 mold allergic patients showed IgE-binding to rHev b 9. All rHev b 9 positive patients were also tested for their IgE-binding to rAlt a 5 and rCla h 6. Except one patient (Fig. 4, lane 8), all others showed IgE reactivity to the mold enolases (Fig. 4), but IgE binding intensities to the three recombinant enolases were different when tested in IgE immunoblots. The cross-inhibition ELISA studies confirmed these results, that rHev b 9 was only able to diminish IgE binding to rAlt a 5 and rCla h 6 in the range of 19 and 24%, respectively. From these data we conclude that the three enolases share a number of epitopes. It has been described that enolases from molds contain several common IgE binding epitopes, but inhibition occurs only partially [24]. This implies that the mold enolases also have unique epitopes. Therefore, it becomes evident that sensitization to Hev b 9 may occur from *Hevea* latex and does not represent an epiphenomenon of sensitization to the homologous mold allergens.

Some *Hevea* latex allergens seem to have a high potential for cross-reactivity, including well-conserved proteins such as manganese superoxide dismutases, chitinases, profilins, and enolases. Sequence comparisons, three-dimensional structure determinations and cross-inhibition assays could help to predict immunogenic regions of the individual proteins. These cross-reactivities often occur with proteins from several fruits and were described as the 'latex–fruit syndrome' [11]. The latex allergens involved in this syndrome are often defence-related enzymes such as Hev b 2 and class I chitinases [21]. In contrast, enolases are house-keeping enzymes that have been described as important allergens of several molds. Another important cross-reactive allergen from molds is a manganese superoxide dismutase [35,36]. MnSOD from *Hevea* latex is also a described allergen and has been designated Hev b 10 (IUIS Allergen Nomenclature Subcommittee: Official list of allergens available at <ftp://biobase.dk/pub/who-iuis/allergen.list>). Although Hev b 9 is a minor latex allergen, its cross-reactivities and the possible cross-reactivities of Hev b 10 to mold allergens indicate the possible existence of a 'latex–mold-syndrome' that will have to be confirmed in the future by the use of the relevant recombinant allergens.

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The complete cDNA sequence coding for the latex enolase is available from the EMBL database under the accession number AJ132580

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