

Short communication

Molecular cloning, purification, and IgE-binding of a recombinant class I chitinase from *Hevea brasiliensis* leaves (rHev b 11.0102)

Background: Class I chitinase in natural rubber latex (NRL) has been assumed to be an important allergen, especially concerning its cross-reactivity with fruits like avocado and banana.

Objectives: The present study aimed to produce a recombinant latex class I chitinase from *Hevea brasiliensis* leaves and to study its immunoglobulin (Ig)E-binding reactivity.

Methods: A class I chitinase-specific complementary DNA from *H. brasiliensis* leaves was synthesized, subcloned, sequenced and overexpressed in fusion with the maltose-binding protein (MBP) in *Escherichia coli*. The IgE-binding reactivity of this protein was studied by the Pharmacia CAP SystemTM and by immunoblot experiments using sera from latex-allergic patients.

Results: The rHev b 11.0102 was found to have a length of 295 amino acid residues and contains an N-terminal hevein-like domain with a 56% homology to hevein. Analysis by the CAP method revealed the presence of rHev b 11.0102-specific IgE antibodies in 17 of 58 sera (29%) of IgE-mediated latex-allergic subjects tested. Immunoblot analysis of the MBP–rHev b 11.0102 fusion protein and the MBP carrier protein as a negative control confirmed the IgE-reactivity of rHev b 11.0102.

Conclusion: Due to its IgE-reactivity rHev b 11.0102 represents an allergen of intermediate prevalence in NRL. Its property to cross-react with certain fruits makes it an important supplement in the diagnostic panel of recombinant NRL allergens.

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Key words: class I chitinase; *Hevea brasiliensis*; latex allergy; natural rubber latex (NRL); recombinant allergen.

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Natural rubber latex (NRL) is widely used in medical devices and in certain everyday articles. Prolonged exposure to latex articles can result in sensitization involving specific immunoglobulin (Ig)E antibodies and subsequently the development of clinically manifested latex allergy [reviewed in Ref. (1)]. So far, 16 latex allergens have been included in the latest IUIS nomenclature list (2).

Class I chitinases are basic proteins with a molecular mass between 25 and 35 kDa, found in certain seed-producing plants. Although their physiological role is unknown, evidence exists that class I chitinases serve as plant defence-related proteins (3, 4). A common characteristic of all class I chitinases is a small N-terminal domain with high sequence homology to hevein (5). Due to this conserved hevein-related structure, patients previously sensitized by latex prohevein or hevein are candidates for a potential cross-reaction with fruit and vegetables containing class I chitinase and/or a lectin with a hevein-like domain (6).

As it is difficult for cross-reactivity studies or diagnostic purposes to isolate appropriate amounts of pure latex class I chitinase by conventional separation methods, the alternative way to solve these problems is the cloning,

expression and isolation of a class I chitinase from *Hevea brasiliensis* leaves using recombinant techniques.

For this purpose, the nucleotide sequence AJ329578 (7) was used for the design of derived primers and the successful amplification of a latex class I chitinase from *H. brasiliensis* leaves to decode the primary sequence of this new NRL class I chitinase isoform (EMBL accession number AJ431363) and to elucidate its IgE-binding properties using sera from latex-allergic individuals after successful overexpression of the protein in *Escherichia coli* and its purification by affinity chromatography.

Materials and methods

Human sera

Serum samples were obtained from 53 NRL-allergic health care workers (HCW) comprising 46 females and seven males (age: 19–53 years, mean age: 32 years) and five NRL-allergic spina bifida (SB) patients comprising (three females and two males) (age: 7–32 years, mean age: 15 years). All HCW had latex-associated symptoms and/or showed positive skin reactions to latex extracts in skin prick testing (SPT). Immunoglobulin E antibodies to NRL proteins as tested by the Pharmacia CAP SystemTM (Pharmacia

Diagnostics AB, Uppsala, Sweden) were present in all HCW. All SB patients had latex-associated symptoms and also showed IgE antibodies to NRL proteins when tested in the same manner as HCW. The concentrations of specific IgE antibodies to NRL were found to be between 0.41 and >100 kU/l. The determination of specific IgE antibodies to avocado, banana and kiwi replaced SPT in all SB patients and in one HCW for medical reasons. Six sera from subjects without NRL allergy were used as controls.

Skin prick test

Skin prick tests were performed in all HCW with common environmental aeroallergens including tree- and grass-pollen mixtures (HAL, Duesseldorf, Germany) and with latex extract prepared in our laboratory (total protein concentrations: 1, 10 and 100 µg/ml in saline solution containing 0.4% phenol). Cutaneous reactions were read after 15 min and were considered positive when a wheal reaction was at least 2 mm surrounded by an erythema as compared with negative control values (wheal <2 mm, no erythema). Skin prick test with commercial solutions for avocado (HAL), banana (HAL), kiwi (HAL) as well as freshly prepared fruit extracts dissolved in phosphate-buffered saline in the highest possible concentration were included whenever possible and medically justified. No SPT were performed in the SB patients.

Polymerase chain reaction amplification of the cDNA

Complementary DNA (cDNA) was obtained from total RNA of *H. brasiliensis* leaves as described (8). The latex class I chitinase-specific amplification was carried out in a 50 µl reaction mixture containing: 4 ng latex cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.01% (w/v) gelatine (Sigma, Deisenhofen, Germany), 500 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each of the primers CIC-5' (5'-CGT ACG TAG AGC AAT GTG GAA GGC AAG CTG GTG GC-3') and CIC-3' (5'-CGC AAG CTT CAC CCT AAT CCG-3') and 2.5 units Taq Polymerase (Eppendorf, Hamburg, Germany). Reaction products were obtained in a thermal cycler (Applied Biosystems, Weiterstadt, Germany) with an initial denaturation step (95°C for 3 min) and a total of 40 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 2 min) followed by 10 min at 72°C.

Subcloning and sequencing of PCR products

The 891-bp polymerase chain reaction (PCR) product was first subcloned into the pDrive cloning vector (Qiagen, Hilden, Germany). Clones carrying inserts were characterized by restriction analysis and by sequencing on an ABI 310 sequencer (Applied Biosystems). Sequencing analyses revealed that the PCR products correspond to an 885-bp open reading frame named LaCIC.

For the efficient expression of the recombinant latex class I chitinase in *E. coli* strain TB-1, the complete LaCIC sequence was isolated after an *Sna* B I-*Hind* III digest from the pDrive cloning vector on a 1% agarose gel. Finally, the gel-purified *Sna* B I-*Hind* III fragment was subcloned into the expression vector pMAL-c2 (New England Biolabs, Frankfurt, Germany) downstream from the *maltE* gene coding for the maltose-binding protein (MBP). Integrity of the sequence was confirmed by sequencing as described above.

Expression and purification of MBP-rHev b 11.0102

One litre tryptone yeast (TY) medium containing 100 µg/ml (w/v) ampicillin and 2 mg/ml (w/v) glucose was inoculated with 10 ml

fresh recombinant *E. coli* cells from an overnight culture and incubated at 37°C for 2 h on a laboratory shaker (New Brunswick, Nuertingen, Germany). Expression of the fusion protein was induced by shaking in the presence of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Roth, Freiburg, Germany) for 4 h at 37°C. *Escherichia coli* cell lysates containing the expressed fusion protein were prepared by sonification in an ice-water bath with a Branson sonifier-250 (Branson Ultrasonics, Danbury, CT, USA) (eight intervals of 20 s on level 4 followed by a time stop of 30 s) followed by centrifugation at 10 000 g for 30 min. The supernatant was loaded onto a 1.5 × 20.0 cm column filled with a 7-ml bed volume of amylose (New England Biolabs). Equilibration, washing and elution were performed as recommended by the supplier's protocol (New England Biolabs).

Determination of specific IgE antibodies by the CAP method

For the determination of rHev b 11.0102-specific IgE, MBP-rHev b 11.0102 fusion protein was immobilized onto a cellulose solid phase by covalent binding. The coupling concentration was adjusted to attain a linear measurement range and a background below 0.35 kU_A/l. The capture antigen (ImmunoCAPTM of rHev b 11.0102) was then used to determine IgE antibodies to rHev b 11.0102 by the Pharmacia CAP SystemTM. A specific IgE value >0.35 kU/l was regarded as positive. Natural rubber latex-specific IgE (k82) as well as IgE-specific antibodies to rHev b 6.01 and MBP were also determined by the Pharmacia CAP SystemTM as recommended by the supplier. Furthermore, determinations of total IgE and specific IgE antibodies to avocado, banana, kiwi, etc. were performed wherever possible.

Electrophoresis and immunoblotting

One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of the fusion protein was performed using 100 µg rHev b 11.0102 on a 10% NuPAGE Bis-Tris gel (NOVEX, San Diego, CA) under reducing conditions as described recently (9).

Results

Amplification of latex class I chitinase cDNA by PCR and sequence analysis

The PCR amplification of NRL cDNA using latex class I chitinase-specific primers derived from EMBL accession no. AJ238579 (7) produced a single 891-bp fragment. The cDNA (LaCIC) was obtained twice by this procedure. The nucleotide sequence and the corresponding deduced amino acid sequence of LaCIC is given in Table 1 and carries an open reading frame (885 bp) coding for 295 amino acid residues with a predicted average molecular mass of 31.55 kDa and a theoretical isoelectric point value of 5.59. Between the factor Xa recognition site on the vector and the first amino acid residue glutamatic acid (E) of rHev b 11.0102 (EMBL accession no. AJ431363) an additional primer-mediated valine (V) residue was incorporated to facilitate the optional cutting by factor Xa (data not shown). The highest amino acid identity (97%) for the whole protein was observed between the

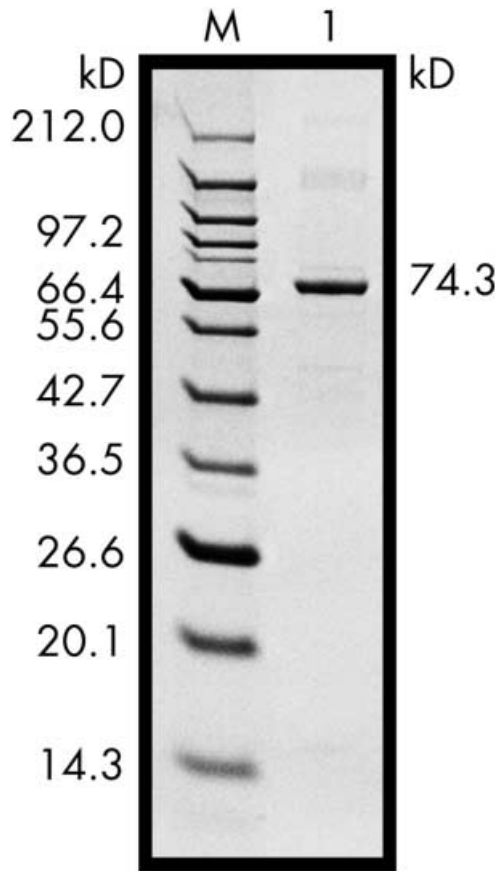


Figure 1. Purified MBP-rHev b 11.0102 on a 10% NuPAGE Bis-Tris gel. From left to right a molecular mass marker (M) as well as 5 µg affinity-purified MBP-rHev b 11.0102 (1) are shown.

(29%) had specific IgE antibodies to rHev b 11.0102 (0.38–36.5 kU/l). As a hevein domain is present in rHev b 11.0102, the 58 sera were also tested for having specific IgE antibodies to recombinant MBP-prohevein fusion protein (MBP-rHev b 6.01). In all cases, the sera showing specific IgE antibodies to the MBP-rHev b 11.0102 also had specific IgE values to MBP-rHev b 6.01, whereas MBP alone revealed negative results (<0.35 kU/l) in all measurements. However, 24 of the 41 rHev b 11.0102-negative sera (59%) showed rHev b 6.01-specific IgE antibodies.

Specific IgE antibody binding to rHev b 11.0102 could also be demonstrated by the immunoblot analysis. One hundred micrograms of MBP-rHev b 11.0102 was separated by SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The same procedure was repeated with 100-µg MBP in a parallel experiment. Analysis of three sera (HCW1–3; Fig. 2) from subjects with rHev b 11.0102-specific IgE antibodies tested by the CAP method recognized clearly the MBP-rHev b 11.0102 fusion protein band (~74.3 kDa), whereas no IgE-binding was observed to MBP (42.7 kDa) alone (Fig. 2).

Discussion

Sources of NRL are of varying quality (11) and difficult to standardize for diagnosis (12). Therefore, cloning, expression and isolation of recombinant allergens in *E. coli* is an economical, cost-effective alternative and may provide sufficient amounts of highly purified proteins for diagnostics, therapeutics as well as experimental purposes in cases where no post-translational modifications occur.

Table 2. Characteristics of the IgE-mediated NRL-allergic HCW and SB patients with rHev b 11.0102-specific IgE reactivity

Patient no.	Age/gender	NRL-associated symptoms	Positive SPT (fruit-CAP values with specific IgE >0.35 kU/l)	Total IgE (kU/l)	NRL-specific IgE (kU/l)	MBP-rHev b 6.01-specific IgE (MBP-prohevein) (kU/l)	MBP-rHev b 11.0102-specific IgE (MBP-class I chitinase) (kU/l)	MBP-specific IgE (kU/l)
HCW1	26/F	A, C, E, R, U	NRL, B	>2000	>100	>100	36.50	<0.35
HCW2	38/F	C, R	NRL, Av, B, K	267	35.8	15.60	8.08	<0.35
HCW3	28/F	A, C, U	NRL, B	635	15.3	13.30	2.26	<0.35
HCW4	33/F	C, R	NRL, B	62.2	3.7	3.18	0.65	<0.35
HCW5	45/F	A, C, R, U	NRL, Av	31.1	4.6	2.99	0.76	<0.35
HCW6	23/F	E, R, C	NRL	27.1	10.7	9.07	0.98	<0.35
HCW7	23/F	A, R, U	NRL (Av, B, K)	675	22.3	20.03	2.66	<0.35
HCW8	38/M	C, R	NRL	n.d.	13.0	10.00	0.38	<0.35
HCW9	22/F	E, R	NRL Av, B, K	343	35.0	19.60	0.76	<0.35
HCW10	25/M	C, E, R, U	NRL	221	>100	98.20	9.26	<0.35
HCW11	26/F	A, U	NRL	307	94.9	13.90	1.71	<0.35
HCW12	30/F	A, AS	Av, Ap, B, Ch	n.d.	20.5	6.06	0.50	<0.35
HCW13	36/F	A, AS	Av, B	n.d.	50.2	49.40	21.40	<0.35
SB1	12/F	A, U	n.d. (Av, B)	315	32.5	26.70	2.70	<0.35
SB2	13/M	C, R	n.d. (Av, B)	829	>100	42.10	15.20	<0.35
SB3	7/F	C, ED, R, U	n.d. (Av, B)	228	53.9	14.00	0.80	<0.35
SB4	32/F	A, AS	n.d.	n.d.	62.4	67.10	4.56	<0.35

A, asthma; Ap, apricot; AS, anaphylactic shock; Av, avocado; B, banana; C, conjunctivitis; Ch, cherry; E, eczema; ED, edema; F, female; K, kiwi; M, male; n.d., not determined; NRL, natural rubber latex; R, rhinitis; SB, Spina bifida; SPT, skin prick test; U, urticaria.

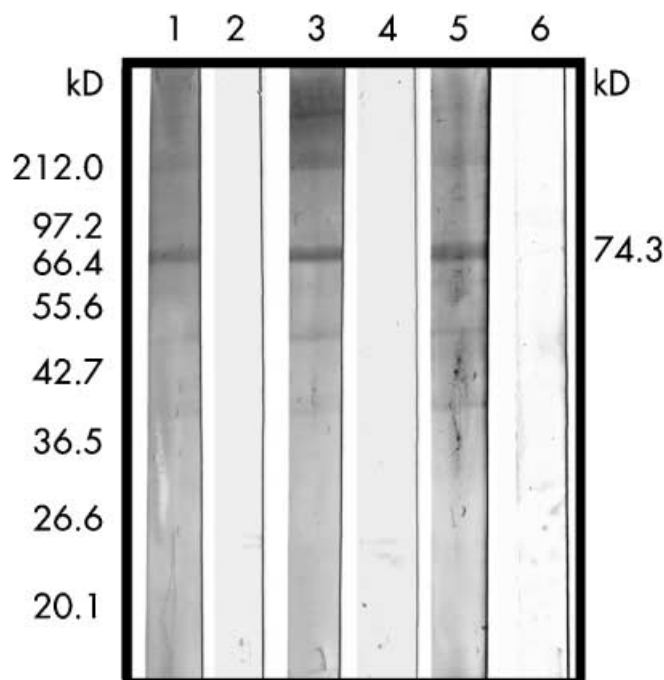


Figure 2. Results of immunoblot experiments: the target protein MBP-rHev b 11.0102 (lanes 1, 3 and 5) as well as the carrier protein MBP alone (lanes 2, 4 and 6) was used in immunoblotting and IgE-binding experiments. The MBP-rHev b 11.0102 fusion protein (74.3 kDa) is the clearly visible IgE-band in three sera having rHev b 11.0102-specific IgE antibodies when tested by the Pharmacia CAP System (lanes 1, 3 and 5). MBP (42.7 kDa) alone (lanes 2, 4 and 6) showed no reaction.

In the present study, we have chosen the latter approach to identify the NRL class I chitinase-coding sequence of *H. brasiliensis* leaves by cDNA cloning using reverse transcriptase (RT)-PCR techniques and amplification of the class I chitinase from *H. brasiliensis* leaves using NRL class I chitinase-specific primers. The resulting recombinant NRL class I chitinase has 295 amino acid residues with a calculated pI of 5.59 and a molecular mass of 31.55 kDa. In agreement with the IUIS this new allergen was named rHev b 11.0102 (2).

The allergenicity of the rHev b 11.0102 was studied by testing its IgE-binding reactivity with the well-established CAP method. Fifty-eight sera from IgE-mediated NRL-

allergic patients were tested; 17 sera (29%) revealed class I chitinase-specific IgE antibodies (Table 2).

Three sera showing IgE values of 36.5 kU/l (HCW 1), 8.08 kU/l (HCW 2) and 2.66 kU/l (HCW 3) to rHev b 11.0102 in the CAP system were used in the immunoblotting experiments and the MBP-rHev b 11.0102 fusion protein showed IgE-specific binding, whereas no IgE-binding reactivity was observed in MBP alone (Fig. 2).

Recent articles about the role of class I chitinases of various fruits revealed the presence of conserved hevein-related structures [reviewed in Ref. (6)]. For example, in the case of avocado, Posch et al. (5) and, in the case of banana, Mikkola et al. (13) reported of an allergen showing N-terminal sequence stretches with high homology to hevein-like structures. A similar domain is also present in the class I chitinase of *H. brasiliensis* leaves.

In this study, 14 of 18 latex-allergic subjects with rHev b 11.0102-specific IgE values were tested for having positive SPT reactions and/or specific IgE antibody binding to fruits like avocado, banana, kiwi, apricot and cherry (Table 2). Twelve of 14 (86%) patients tested in this manner showed a positive result to at least one of these fruits. Interestingly, each of the sera showed a higher specific IgE value to prohevein, than to the specific IgE value for the corresponding class I chitinase. These results are in agreement with the idea that conserved hevein-related structures in patients previously sensitized by NRL prohevein or hevein act as cross-reactive mediators to fruit and vegetables containing a hevein-related domain. From the present data, we conclude that the class I chitinase itself, in terms of IgE-binding reactivity, seems to play an intermediate role as an NRL allergen. Its property to cross-react with prohevein and/or hevein, both major allergens in HCW, therefore, makes it an important supplement in a diagnostic panel of recombinant NRL allergens. Nevertheless, comparisons with the natural allergen in future studies may be helpful to fully understand the role of cross-reactivity of this class I chitinase.

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