

Cloning, expression and characterization of the major latex allergen prohevein

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Summary

Background About 70–80% of latex allergic health care workers are sensitized to prohevein (Hev b 6.01), a 20 kDa cysteine-rich chitin-binding protein of *Hevea* latex.

Objective This study reports on the bacterial cloning, expression and immunochemical characterization of rHev b 6.01.

Methods Prohevein was expressed in the periplasmic space of *Escherichia coli* as maltose binding protein (MBP) fusion protein and purified to homogeneity after factor Xa cleavage. The IgE binding capacity of both rHev b 6.01 and prohevein isolated from fresh *Hevea* latex was compared by immunoblotting experiments using sera of latex-allergic patients. The diagnostic value of rHev b 6.01 was analysed by enzyme allergosorbent test (EAST).

Results Two different cDNA clones of rHev b 6.01 were established. The deduced amino acid sequence of both clones revealed two and three amino acid differences in the C-terminal domain of prohevein compared with the original database entry. Purified rHev b 6.01 bound with high affinity to chitin as its natural counterpart isolated from natural latex. In IgE-immunoblotting using sera of affected subjects binding intensity to both proteins was comparable indicating a very high antigenic similarity. The diagnostic value of MBP-prohevein was tested in EAST using sera of 33 latex-allergic subjects. The *in vitro* test showed high sensitivity and specificity and proved the diagnostic value of uncleaved MBP-prohevein.

Conclusions The production of recombinant latex key allergens with defined quality like prohevein is a straightforward strategy for the development of standardized *in vitro* test systems.

Keywords: Hev b 6.01, IgE, latex allergy, prohevein

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Introduction

Latex type I allergy is a serious hazard, especially for health care workers (HCW) and patients undergoing repeated surgeries like spina bifida (SB) children since allergenic proteins of natural rubber latex (NRL) are present in a variety of latex products [1,2]. Enormous progress has recently been made in the identification and molecular characterization of the responsible IgE binding latex

proteins [3–6]. There exist different major allergens for both risk groups, namely Hev b 1 (rubber elongation factor) for SB patients [7] and Hev b 6.01 (prohevein) for HCWs [8]. The 20 kDa prohevein is a two-domain protein that is processed into the N-terminal domain called hevein (4.7 kDa) and a C-terminal domain (14 kDa). According to Alenius *et al.* [9] and Chen *et al.* [10] the allergenicity of prohevein which is stabilized by seven disulphide bridges is mainly due to the hevein domain. About 80% of latex-allergic HCW are sensitized to hevein, while the prevalence of sensitization to the C-terminal domain of prohevein is 15–21%. The hevein domain (43 aa) is found in several

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plant and fungal proteins like wheat germ agglutinin, endochitinase and potato stress proteins win 1 + 2 that have a binding specificity for N-acetylglucosamine. Recently, the IgE binding epitopes of prohevein were identified by selective cloning and overlapping synthetic peptides. Banerjee *et al.* [11] found a total of 10 IgE-binding epitopes in both domains and confirmed the high prevalence of hevein-specific IgE antibodies in HCWs as stated in prior studies.

It is generally accepted that recombinant production of allergens is a straightforward strategy to obtain defined material with high yields for diagnosis and treatment of IgE-mediated allergic disorders. In latex allergy the proteins Hev b 1 [12], Hev b 5 [13,14] and Hev b 6.01 [15] have been cloned and expressed as fusion constructs but data are missing about their immunological behaviour in comparison with their respective natural counterparts and potential in diagnosis of latex allergy. In this paper the bacterial cloning, expression and purification of r-prohevein are discussed. Further, the physicochemical and immunological properties of rHev b 6.01 with prohevein isolated from natural rubber latex are compared and its value for *in vitro* diagnosis of latex allergy are analysed.

Material and methods

cDNA synthesis and cloning of prohevein

RNA was extracted from shock frozen leaves of *Hevea brasiliensis* clone RRIM 600 which were kindly provided by the Institut für Nutzpflanzenkunde gemäßigter subtropischer und tropischer Regionen, Witzenhausen, Germany. After grinding one gram of the leaves in a mortar filled with liquid nitrogen and immediately drying the material, RNA isolation was performed according to the Total RNA protocol of Qiagen (Hilden, Germany). The *Hevea* RNA was dissolved in 100 µL distilled water. The final concentration of RNA was 2.5 µg/µL. Five micrograms of *Hevea* RNA were used for prohevein cDNA synthesis with prohevein specific primers:

PROHEV_5-1: 5'-GAGCAATGTGGTCGGCAAG-3',
PROHEV_3-1: 5'-CAGAATTCTTAATTAATTACT-
GATGATTCATAACGG-3'.

The PROHEV_5-1 primer represents the sequence coding for the first six amino acids of the N-terminus of prohevein. The 3'-primer PROHEV_3-1 contains the last 29 nucleotides of the coding sequence for prohevein (stop codon in bold) with an attached EcoRI restriction site (underlined) and two additional nucleotides allowing better access of the restriction enzyme. These primers were designed for directed ligation into the XmnI/EcoRI digested fusion vector pMAL-p2 [16] which enables the export of the target protein to the periplasm of *Escherichia*

coli as part of a maltose binding protein (MBP) fusion protein. RT-PCR conditions used to isolate prohevein cDNA were: 95 °C, 5 min; (94 °C, 1 min; 51 °C, 2 min; 72 °C, 3 min) × 35 cycles; 72 °C, 10 min. The correct length of the prohevein cDNA was analysed by agarose gel electrophoresis. After PCR the prohevein cDNA was incubated with Klenow enzyme (2 units, 20 min at 22 °C) to create blunt end fragments and subsequently digested with EcoRI for directed ligation into the XmnI/EcoRI-restricted pMAL-p2 vector. The ligated pMAL/prohevein was transformed into *E. coli* DH5α which was grown on isopropyl-1-thio-β-D-galactoside/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar plates containing 100 µg/mL ampicillin. The plasmid DNA of three out of six white colonies contained the full length gene encoding prohevein as analysed by gel electrophoresis after restriction with the respective enzymes and sequencing on an Perkin Elmer ABI Prism 310 DNA sequencer, using the universal and pMAL-specific malE primers with the 'Taq FS Dye Deoxy Terminator Cycle Sequencing' protocol. The cDNA sequence of each of the established prohevein clones were sequenced twice in both directions. Similarity calculation was performed using the CLUSTAL-W sequence alignment program of Thompson *et al.* [17].

Expression and purification of r-prohevein

Cultures in logarithmic stage of the *E. coli* DH5α containing pMAL-p2/prohevein were induced by addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 0.3 mmol/L and grown for 60 h at 30–32 °C. The bacteria were harvested by centrifugation for 20 min/4000 g at 4 °C. The supernatant was discarded and the cells were resuspended in 10 mL column buffer (20 mmol/L Tris/HCl, 200 mmol/L NaCl, 1 mmol/L EDTA) per gram of wet cell weight and subsequently frozen overnight at –20 °C. After thawing in a water bath, the cells were sonicated in a ice-water bath using a Branson sonifier model 250 with pulses of 15 s/intensity set to eight for a total time of 3.5 min and subsequently centrifuged at 9000 g for 30 min. The supernatant was diluted 1 + 4 with degassed column buffer and loaded onto a Pharmacia XK 16/20 column packed with amylose beads (bed volume 10 mL). Bound fusion protein was eluted with 10 mmol/L maltose in column buffer. Protein concentration was determined by ESL-assay of Boehringer Mannheim (Germany). Cleavage of MBP-prohevein at a concentration of 1 mg/mL was performed by incubation with Factor Xa (2–5%, w/w) in the presence of 0.02% SDS for 72 h at 22 °C under gentle agitation.

Preparative SDS-PAGE (491 Prep Cell, Bio Rad) was used for initial purification of r-prohevein. The round gel (37 mm inner diameter) consisted of a 2 cm stacking (T = 6%) and 10 cm running gel (T = 14%) prepared with

the buffer system according to Laemmli [18]. Four millilitres crude extract of cleaved MBP-prohevein (1 mg/mL) were mixed with 1.5 mL $3 \times$ concentrated sample buffer (150 mmol/L TRIS/HCl, pH 6.8, 10% glycerol, 6% SDS, 0.03% bromophenol blue) without reducing agent. The unheated sample was loaded onto the stacking gel and electrophoresis was started at 27 mA for 16 h. Subsequently the current was increased to 70 mA and fractions of 4 mL were collected. Fractions containing r-prohevein as monitored by SDS-PAGE and silver staining [19] were pooled, dialysed against distilled water and lyophilized. r-prohevein was further purified by reversed phase-chromatography (3 mL RPC-column, Pharmacia, Uppsala, Sweden).

Isolation of native prohevein by affinity chromatography using chitin beads

Lyophilized latex C-serum proteins were dissolved in 20 mmol/L TRIS/HCl, pH 8.0, to obtain a protein concentration of 3 mg/mL [5]. Two hundred and fifty millilitres of this solution were filtered through a $0.45 \mu\text{m}$ filter and applied to a Pharmacia XK 16/20 column packed with chitin beads (New England Biolabs, Beverly, USA) (bed volume 10 mL) at a flow rate of 1 mL/min. Chitin-binding proteins were eluted using 0.5 mol/L *N*-acetylglucosamine in 20 mmol/L TRIS/HCl, pH 8.0. Eluted material was dialysed against dest. water (1000 Da MWCO) and then lyophilized. A total of about 30 mg chitin-binding proteins were isolated from the initial 750 mg C-serum proteins.

Measurement of IgE antibodies

Sera were obtained from occupationally latex-exposed health care workers visiting our out-patients department. Pharmacia CAP system was used to analyse latex-specific IgE antibodies of latex allergic patients. The detection of r-prohevein specific IgE was carried out by enzyme allergosorbent test (EAST) using uncleaved MBP-prohevein (coupling concentration 0.5 mg/mL). MBP coupled paper discs (coupling concentration 0.4 mg/mL) were used as controls to detect unspecific reaction in EAST. Hevein-specific IgE antibodies were analysed as described recently [10]. According to the manufacturers instructions serum samples with IgE concentrations greater than 0.35 kU/L were regarded as positive. For inhibition experiments 50 μL serum was incubated for 12 h at 4 °C with the respective protein solution. Specific IgE measurements were then performed as usual.

Electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli [18] using 10, 12 and 15% T gels ($9 \times 7 \text{ cm}$). Runs were

performed in a Mini Protean II electrophoresis chamber of Bio Rad, at 40 mA per gel. Gels were either stained both with Coomassie and silver or electroblotted onto a PVDF-membrane using the protocol of Baker and Dunn. [20]. Two-dimensional electrophoresis of was performed according to Görg *et al.* [21].

IgE immunostaining

The dried membranes were wetted in methanol for 5 s and washed $3 \times 5 \text{ min}$ in TBS (50 mmol/L TRIS/HCl pH 7.4, 150 mmol/L NaCl). Blocking was performed with 1% BSA and 1% PVP-40 in TBS for 6–8 h. After being washed three times with TBS the blots were incubated overnight with serum diluted 1:5–1:10 in TBST-BSA (TBST containing 1% BSA) from 14 latex allergic patients. All these patients have suffered from contact urticaria, two thirds of them also reported rhinitis and one also suffered from asthma when in contact with nature rubber latex gloves. The membranes were then washed three times with TBST and incubated with alkaline phosphatase conjugated anti-human IgE (1:1000 in TBST-BSA) for 4 h. After three washes with TBST, IgE binding was demonstrated by adding a BCIP/NBT solution (each 0.35 mmol/L in 100 mmol/L TRIS-HCl, pH 9.5 containing 100 mmol/L NaCl and 5 mmol/L MgCl_2). To detect non-specific IgE immunostaining, control experiments using two sera of asymptomatic persons occupationally exposed to latex were performed in parallel.

Results

Cloning and sequence analysis of cDNA encoding prohevein

Total RNA isolated from *Hevea* leaves was used as the template for prohevein-specific cDNA synthesis, resulting in two slightly different types of plasmid clones. The sequences of the inserts consist of 564 nucleotides (clone pDHPROH 1–2 [EMBL Accession No: AJ003196], pDHPROH 1–4 and clone pDHPROH 6) (Fig. 1). Sequence comparison with EMBL database entry M36986 resulted in nucleotide sequence similarity of 99.5 and 99.3% for pDHPROH 1–2/1–4 and pDHPROH 6, respectively. The deduced amino acid sequences (187 aa each) of the prohevein clones showed similarities of 97.9% (clone pDHPROH 1–2/pDHPROH 1–4 with the following amino acids substituted: Ser₁₀₉ → Pro, Thr₁₂₆ → Ala, Ile₁₆₁ → Leu, and 98.4% (clone pDHPROH 6: Ser₁₀₉ → Pro, Ile₁₆₁ → Leu) with the published data of prohevein. The amino acid substitutions found do probably not affect IgE-binding of the C-domain, since epitopes involved in IgE-binding are located between amino acid residues 60–103 and 164–172, respectively [11].

prohevein aa	E Q C G R Q A G G K L C P N N L C C S Q	20
prohevein	GAGCAATGTGGTTCGGCAAGCAGGTGGCAAGCTCTGCCCAATAACCTATGTTGTAGCCAG	60
pDHPROH 1-2/1-4		60
pDHPROH 6		60
prohevein aa	W G W C G S T D E Y C S P D H N C Q S N	40
prohevein	TGGGGGTGGTGTGGCTCCACTGATGAATATTGTTTCACCTGATCATAACTGCCAAAGCAAT	120
pDHPROH 1-2/1-4		120
pDHPROH 6		120
prohevein aa	C K D S G E G V G G G S A S N V L A T Y	60
prohevein	TGCAAAGACAGCGGCGAAGGTGTTGGTGGTGGAAAGTGCTTCCAACGTTCTTGCGACGTAC	180
pDHPROH 1-2/1-4		180
pDHPROH 6		180
prohevein aa	H L Y N S Q D H G W D L N A A S A Y C S	80
prohevein	CATTTGTATAATTCACAGGATCATGGATGGGACTTGAATGCCGCAAGTGCATATTGCTCT	240
pDHPROH 1-2/1-4		240
pDHPROH 6		240
prohevein aa	T W D A N K P Y S W R S K Y G W T A F C	100
prohevein	ACATGGGATGCTAACAAGCCATATTCATGGCGGAGCAAGTATGGCTGGACTGCATTCTGC	300
pDHPROH 1-2/1-4		300
pDHPROH 6	..G.....A.....	300
prohevein aa	G P V G A H G Q S S C G K C L S V T N T	120
prohevein	GGTCCCCTCGGAGCACACGGCCAATCCTCCTGTGGAAAGTGCTTGAGTGTGACAAATACA	360
pDHPROH 1-2/1-4	C.....	360
pDHPROH 6	C.....	360
prohevein aa	G T G A K T T V R I V D Q C S N G G L D	140
prohevein	GGGACTGGAGCTAAAACGACAGTGAGGATTGTGGATCAGTGTAGTAATGGAGGACTAGAT	420
pDHPROH 1-2/1-4	G.....	420
pDHPROH 6		420
prohevein aa	L D V N V F R Q L D T D G K G Y E R G H	160
prohevein	TTGGACGTGAATGTTTTCCGTCAACTGGACACAGATGGGAAAGGATATGAACGAGGTCAT	480
pDHPROH 1-2/1-4		480
pDHPROH 6		480
prohevein aa	I T V N Y Q F V D C G D S F N P L F S V	180
prohevein	ATTACAGTGAACCTACCAATTTGTTGATTGTGGAGATTCCTTCAATCCTCTATTCTCCGTT	540
pDHPROH 1-2/1-4	C.....	540
pDHPROH 6	C.....	540
prohevein aa	M K S S V I N *	187
prohevein	ATGAAATCATCAGTAATTAATTA	564
pDHPROH 1-2/1-4		564
pDHPROH 6		564

Fig. 1. Nucleotide sequence comparison of three r-prohevein clones (pDHPROH 1-2, pDHPROH 1-4; pDHPROH 6) with the sequence of prohevein (EMBL database entry M36986). The sequences representing the hevein domain are written in bold letters, the stop codon is underlined. The deduced amino acid sequence of prohevein is given as single letter code (prohevein aa).

Expression and purification of r-prohevein

The prohevein cDNA was cloned into the plasmid vector pMal-p2 and expressed as a fusion protein with the 43 kDa

protein maltose binding protein (MBP) at the N-terminus (clone pDHPROH 1-2 was used for these experiments). After induction with IPTG the cells were disrupted by sonification and the MBP-prohevein was isolated by affinity

chromatography using amylose resin. The purity and integrity of MBP-prohevein was analysed by SDS-PAGE (Fig. 2A). A single, dominant band at about 63 kDa was detected and no fragmentation of the isolated fusion protein was observed. High yields of about 40 mg of MBP-prohevein per litre medium were obtained. The endoproteinase factor Xa was subsequently used to obtain r-prohevein without fusion partner. However, cleavage under standard conditions failed, indicating a blocked or difficult accessible cleavage site of the fusion construct. Addition of 0.02–0.04% SDS improved the cleavage efficiency, but on the other hand, a quite complex protein cleavage pattern was obtained as analysed by SDS-PAGE (Fig. 2B) and 2-D electrophoresis (Fig. 3). This may be due to unspecific enzymatic activity of factor Xa leading to multiple fragmentation products of the MBP-fusion partner of prohevein.

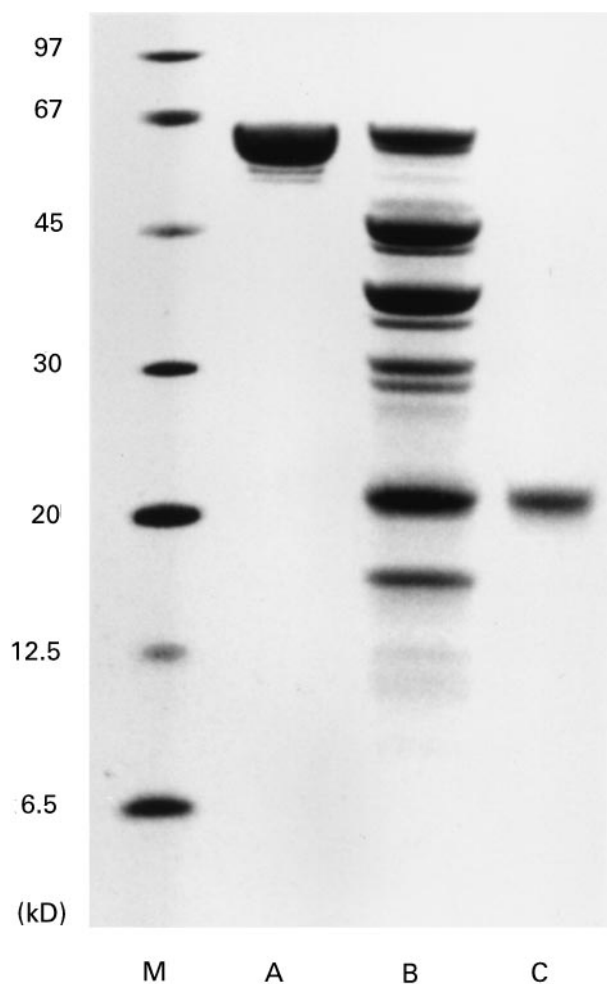


Fig. 2. SDS-electrophoresis and silver staining of (A) MBP-prohevein (63 kDa) isolated by amylose affinity chromatography, (B) factor Xa cleaved MBP-prohevein, (C) nonfusion r-prohevein (20 kDa) isolated by preparative electrophoresis and RP-HPLC.

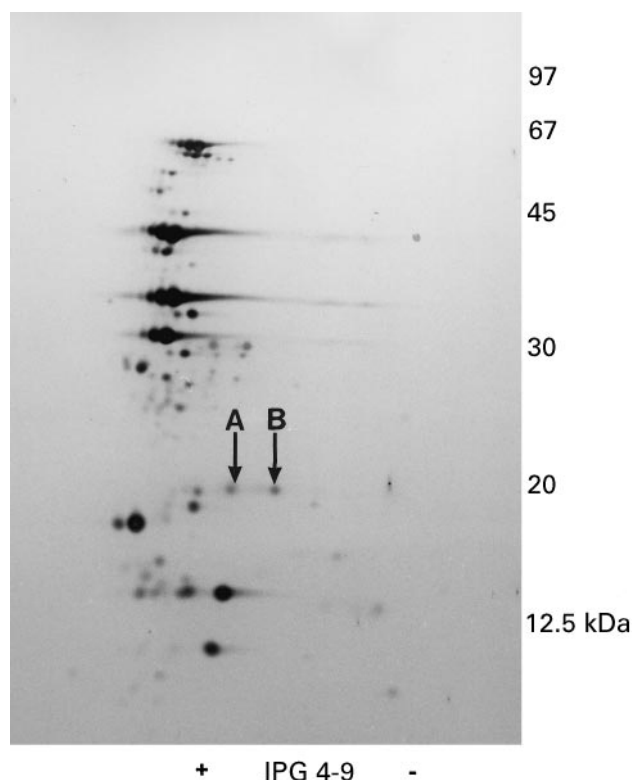


Fig. 3. 2D-electrophoresis of factor Xa-cleaved MBP-prohevein (see Fig. 2b). First dimension: IPG 4–9. Second Dimension: SDS-PAGE, T = 12%. Protein spots A and B correspond to r-prohevein as identified by N-terminal protein microsequencing.

Two protein spots were identified by 2-D electrophoresis and N-terminal protein sequencing to correspond to r-prohevein. They had the same apparent molecular weight but differed in their pI values (spot A: 5.7; spot B: 6.1). Due to the complex protein pattern no simple chromatographic purification scheme was applicable to purify r-prohevein. r-prohevein was obtained by preparative SDS-PAGE followed by RP-HPLC (Fig. 2c). The final yield of purified nonfusion recombinant Hev b 6.01 was between 2 and 3 mg per litre *E. coli* cell culture. The biological function of r-prohevein was tested by affinity chromatography. r-prohevein was shown to bind with high efficiency to chitin beads and could be specifically eluted by N-acetylglucosamine (data not shown).

Specific IgE to MBP-prohevein and hevein

Sera of 33 latex-allergic patients sub-divided into two groups were used to analyse the diagnostic potential of MBP-prohevein (Table 1). Group A consisted of 15 hevein-negative and group B of 18 hevein-positive subjects. All hevein-positive sera reacted with MBP-prohevein as well.

Table 1. IgE-measurements to latex, hevein and MBP-prohevein in 33 latex allergic subjects.

Patient number	Latex (kU/L)*	Hevein (kU/L)**	MBP-prohevein (kU/L)**	MBP (kU/L)**
Hevein-negative subjects				
1	0.64	0.35	0.35	0.35
2	0.79	0.35	0.35	0.35
3	4.52	0.35	0.35	0.35
4	1.48	0.35	0.35	0.35
5	10.40	0.35	0.35	0.35
6	2.88	0.35	0.35	0.35
7	13.20	0.35	0.35	0.35
8	1.10	0.35	0.35	0.35
9	29.60	0.35	0.35	0.35
10	1.69	0.35	0.49	0.35
11	4.74	0.35	0.63	0.35
12	3.13	0.35	0.65	0.35
13	0.89	0.35	1.06	0.35
14	2.51	0.35	1.22	0.35
15	7.00	0.35	2.95	0.35
Hevein-positive subjects				
16	6.44	0.48	0.68	0.35
17	3.44	0.68	3.48	0.35
18	3.38	0.79	1.44	0.35
19	3.74	0.80	4.17	0.40
20	19.00	1.55	9.45	0.35
21	7.38	1.63	9.10	0.35
22	9.02	1.67	4.76	0.35
23	6.54	2.08	6.58	0.35
24	27.60	2.31	10.52	0.41
25	5.85	2.42	3.49	0.35
26	10.20	2.73	6.87	0.56
27	10.80	4.52	17.72	0.82
28	10.10	4.94	9.41	0.55
29	11.00	5.09	11.22	0.35
30	94.90	5.52	10.91	0.35
31	19.70	7.31	16.38	0.58
32	41.30	11.21	17.88	1.22
33	45.20	24.10	23.07	0.53

*Pharmacia CAP system; ** EAST.

Additionally, positive IgE values to MBP-prohevein in six sera out of group A were found. Interestingly, all IgE values to MBP-prohevein were significantly higher when compared with the IgE levels to hevein. The specificity of the MBP-prohevein measurements were tested by inhibition experiments. All positive IgE measurements could be totally inhibited by both MBP-prohevein (0.5 mg/mL) and hevein (0.2 mg/mL), but not MBP (0.4 mg/mL). Moreover, to analyse the influence of MBP with respect to IgE binding to MBP-prohevein, all subjects were tested in EAST with

MBP-coupled cellulose discs. All patients with RAST classes 1 and 2 to MBP-prohevein were negative to MBP. In eight out of 14 patients with RAST classes of three and four to MBP-prohevein negligible unspecific reactivity to MBP were observed (Table 1).

IgE immunoblotting of r-prohevein and n-prohevein

Both purified r-prohevein and chitin-binding latex proteins (CBLP) were subjected to SDS-PAGE and subsequently blotted onto PVDF-membranes (Fig. 4). The CBLP fraction of latex sap consisted mainly of three species and were identified in former studies [5,6] as hevein (5 kDa), prohevein (20 kDa) and endochitinase class II (29 kDa), lacking the hevein domain as analysed by N-terminal sequencing. r-prohevein was visible both as monomer (20 kDa) and dimer (40 kDa), respectively. r-prohevein and CBLPs were incubated with serum of 14 latex allergic health care workers that have been classified in EAST for IgE antibodies to hevein (nine positive subjects) and MBP-prohevein (11 positive subjects). Sera of 12 patients exhibited positive and comparable IgE binding to both r-prohevein and n-prohevein indicating that the recombinant derived prohevein has very similar IgE binding characteristics compared with its natural counterpart. Patient 4 had negative EAST values to MBP-prohevein and hevein, but reacted both with r- and n-prohevein in immunoblotting. Additionally, subject 29 exhibited a weak reaction to n-prohevein, but a distinct signal was obtained for r-prohevein. The detection of specific IgE to hevein was identical for all sera both in EAST and immunoblotting. Surprisingly, the IgE antibodies of four latex subjects reacted with an unknown 46 kDa protein that was not visible on the Coomassie-stained membrane. The 29 kDa latex chitinase lacking the hevein domain was stained only very slightly in a few cases and doesn't seem to play a major role in latex allergy.

Discussion

In allergy research, pure individual proteins are needed in sufficient amounts for comprehensive functional and structural studies as well as for improvement and standardization of diagnosis tools. Natural sources like latex sap are of varying quality [22], difficult to standardize [23] and the preparative isolation of the respective key allergens may be time consuming and complicated. As this is the case for prohevein, a major latex allergen, a cloning strategy for its recombinant production in *E. coli* was developed. Hev b 6.01 which contains seven disulphide bridges was expressed as fusion protein with MBP. This expression system enables both periplasmatic targeting, which is a prerequisite for the correct folding of proteins with disulphide bridges and easy purification in a single step via affinity chromatography at

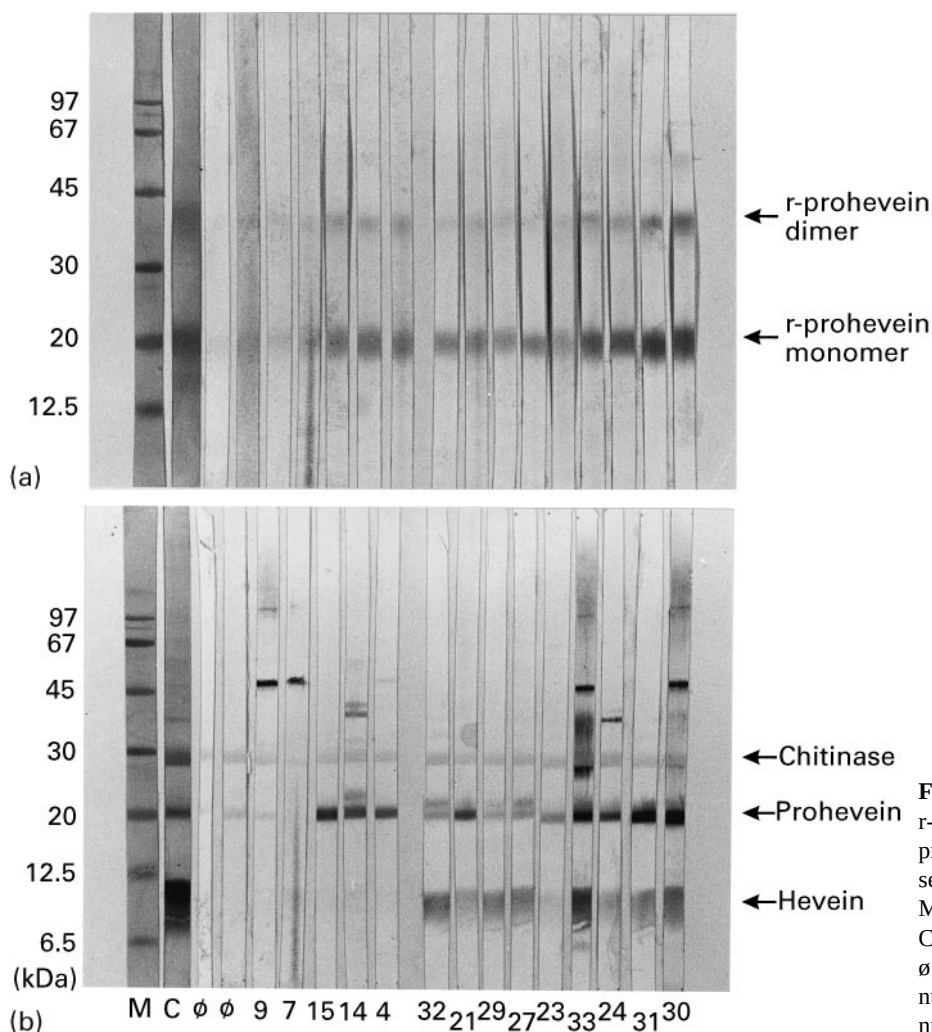


Fig. 4. IgE immunoblotting of purified r-prohevein (b) and chitin-binding proteins (b) from natural latex using sera of 14 subjects allergic to latex. M: Molecular weight standard, C: Coomassie blue-stained blotting strip, $\emptyset$: latex negative patient's sera. The numbers correspond to the patient numbers in Table 1.

mild elution conditions. This approach resulted in the production of full-length MBP-prohevein at high yields (40–50 mg/L medium). However, the site-specific cleavage of the fusion protein by factor Xa to remove MBP failed under standard conditions. Prolonged incubation time and addition of 0.02% SDS improved cleavage efficiency of factor Xa but unspecific protease activity was observed which complicated the purification of rHev b 6.01. Subsequently a two-step purification scheme including preparative electrophoresis and RP-HPLC to remove trace impurities was established to obtain r-prohevein at yields of 2–3 mg per litre *E. coli* cell culture. r-prohevein was identified and characterized by 2D-electrophoresis and protein sequencing. Two protein spots with apparently identical molecular weight but different pI values were detected on the 2D map. The pI value of spot A (≈ 5.7) is very similar to its theoretical point of 5.64 and corresponds well with n-prohevein as recently analysed by Posch *et al.* [5]. The pI value of spot B was estimated to be 6.1. The

observed charge heterogeneity may represent an artifact generated during isoelectric focusing of the 2D procedure. This phenomenon of isobaric isoallergens was also reported by Spangfort *et al.* [24] characterizing rBet v 1 by 2D electrophoresis and mass spectrometry.

Despite the rough conditions applied during isolation the biological activity of r-prohevein — namely its chitin-binding capacity — remained unchanged. r-prohevein bound quantitatively to chitin beads and was eluted with *N*-acetylglucosamine in the same manner as n-prohevein.

The immunochemical properties of purified rHev b 6.01 were tested in immunoblotting. Binding intensity of 12 latex allergic patient's serum IgE was comparable between naturally occurring and recombinant protein indicating that the antigenic properties are preserved in the recombinant derived protein. However, for application in routine diagnosis, the strategy of producing nonfusion prohevein via enzymatic factor Xa cleavage is not economical. Therefore, the IgE binding activity of MBP-prohevein in EAST was

analysed in parallel. As reference in terms of specificity and sensitivity EAST measurements with hevein that can be easily isolated in sufficient amounts from *Hevea* latex were used. Results indicated that EAST with MBP-prohevein as the target antigen is much more sensitive compared with the hevein test, since IgE to MBP-prohevein but not hevein in six sera were detected. This phenomenon can probably be explained by the fact that during coupling of the 4.7 kDa hevein using BrCN-activated cellulose discs, IgE-specific epitopes will be destroyed or due to the small size of hevein coupling will lead to steric hindrances. Both possible cases will cause decreased IgE reactivities in EAST. In contrast with hevein, the 62 kDa MBP-prohevein is more likely to be coupled by amino acid residues located in the MBP or C-terminal domain of prohevein. Although the C-terminal domain of prohevein seems to play a negligible role in latex allergy, it cannot be excluded that some of the patients have C-domain-specific IgE antibodies leading to slightly increased EAST values when comparing hevein and prohevein. No IgE antibodies to MBP-prohevein could be measured after preincubation of these six sera with MBP-prohevein and hevein, respectively. The fact that hevein was also able to completely inhibit the IgE reaction to MBP-prohevein supports the results of Alenius *et al.* [9] and Chen *et al.* [10] that the sensitizing power of prohevein is mainly caused by hevein and the C-terminal domain has only marginal effects in developing latex allergy. MBP had no inhibitory effect in EAST measurements to MBP-prohevein which proves again the specificity of the test. All hevein-positive sera reacted with MBP-prohevein as well but the MBP-prohevein EAST showed significantly higher IgE values. A low-grade reactivity to MBP in EAST in our study group was observed. However, this unspecific binding was exclusively observed in some subjects with RAST classes >3 to MBP-prohevein and did not influence the validity of the test system. Similarly results were reported by Lynch *et al.* [25] for glutathione-S-transferase as fusion partner for Der p 2, Der p 5 and Der p 7.

In summary, the cloning approach presented here for recombinant production and purification of the major latex allergen, prohevein, was shown to be successful. Biologically active nonfusion r-prohevein that exhibits comparable immunochemical characteristics as its natural counterpart isolated from latex sap was obtained. Purified r-prohevein can now be used to perform comprehensive structure-function studies that may help to explain its enormous sensitizing power. Additionally, the applicability of uncleaved MBP-prohevein in *in vitro* latex allergy diagnosis was investigated. Preliminary results indicated that MBP obviously has no effect leading to false positive results when measuring prohevein-specific IgE antibodies. However, this has to be confirmed by investigating a larger number of patients. The applicability of uncleaved

MBP-prohevein in *in vitro* latex allergy diagnosis was also confirmed. As recently reported by Lundberg *et al.* [26], commercially available *in vitro* tests differ in their quality for specific IgE antibodies to latex. Recombinant key latex allergens with defined quality may therefore either serve as supplement helping to improve existing test systems or for the development of new, standardized *in vitro* test systems without using extracts from natural sources.

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