

Molecular cloning and immunoglobulin E reactivity of a natural rubber latex lecithinase homologue, the major allergenic component of Hev b 4

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Summary

Background Hev b 4 is an allergenic natural rubber latex (NRL) protein complex that is reactive in skin prick tests and *in vitro* immunoassays. On SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Hev b 4 is discerned predominantly at 53–55 kDa together with a 57 kDa minor component previously identified as a cyanogenic glucosidase. Of the 13 NRL allergens recognized by the International Union of Immunological Societies, the 53–55 kDa Hev b 4 major protein is the only candidate that lacks complete cDNA and protein sequence information.

Objective We sought to clone the transcript encoding the Hev b 4 major protein, and characterize the native protein and its recombinant form in relation to IgE binding.

Methods The 5'/3' rapid amplification of cDNA ends method was employed to obtain the complete cDNA of the Hev b 4 major protein. A recombinant form of the protein was over-expressed in *Escherichia coli*. The native Hev b 4 major protein was deglycosylated by trifluoromethane sulphonic acid. Western immunoblots of the native, deglycosylated and recombinant proteins were performed using both polyclonal antibodies and sera from latex-allergic patients.

Results The cDNA encoding the Hev b 4 major protein was cloned. Its open reading frame matched lecithinases in the conserved domain database and contained 10 predicted glycosylation sites. Detection of glycans on the Hev b 4 lecithinase homologue confirmed it to be a glycoprotein. The deglycosylated lecithinase homologue was discerned at 40 kDa on SDS-PAGE, this being comparable to the 38.53 kDa mass predicted by its cDNA. Deglycosylation of the lecithinase homologue resulted in the loss of IgE recognition, although reactivity to polyclonal rabbit anti-Hev b 4 was retained. IgE from latex-allergic patients also failed to recognize the non-glycosylated *E. coli* recombinant lecithinase homologue.

Conclusion The IgE epitopes of the Hev b 4 lecithinase homologue reside mainly in its carbohydrate moiety, which also account for the discrepancy between the observed molecular weight of the protein and the value calculated from its cDNA.

Keywords allergen, glycosylation, Hev b 4, IgE antibody, lecithinase homologue, MBP-lecithinase homologue fusion protein, natural rubber latex

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Introduction

Natural rubber latex (NRL) is exuded from the ramified laticiferous cells of the rubber tree *Hevea brasiliensis*. Besides the commercially important elastomer — rubber, NRL being the laticifer cytoplasm contains a multitude of metabolites and proteins. Some residual NRL proteins withstand the harsh processing and manufacturing conditions, and remain in diverse finished products. Thirteen of these proteins, Hev b 1 to Hev b 13, have been recognized by the International Union of Immunological Societies (IUIS) as the causal agents of latex allergy. Allergy to NRL is an IgE-mediated type I

hypersensitivity that manifests in sensitized persons. The symptoms vary from mild local skin irritation to severe systemic reactions, particularly among healthcare workers and patients with spina bifida or urogenital abnormalities who frequently come in contact with latex devices [1–4].

Latex allergens are predominantly found in the B-serum [5], which is the fluid content of the relatively heavy organelles (mainly vacuole-like organelles called lutoids) that precipitate when NRL is centrifuged at high speed [6]. The Hev b 4 protein complex, which has been shown to be reactive in skin prick tests (SPT) [7], is an important allergenic constituent of the B-serum. The allergen was first described as a part of the microhelical structures within the lutoids, and resolved as a 57 kDa and a 53–55 kDa protein on SDS-PAGE [8]. The 57 kDa protein has been identified as a cyanogenic glucosidase [9], and its corresponding cDNA (GenBank accession number AY297039) has been cloned. On the other hand, both

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N-terminal and internal peptide sequence analyses of the 53–55 kDa protein component of the complex revealed a similarity to myrosinase-associated proteins and GDSL lipases [4] and some unpublished data). Occurrence of such glucosidase protein complexes is not uncommon in plants, see [10–12].

In the present study we report cloning of the transcript encoding the 53–55 kDa protein, the main IgE binding component of the Hev b 4 allergenic protein complex. Characterization of the native protein and its recombinant form that lacked glycans to elucidate the significance of carbohydrate in IgE binding is also described.

Materials and methods

Preparation of B-serum and protein precipitation by dialysis

Latex B-serum was prepared based on a method previously described [13, 14]. Briefly, fresh NRL was collected in chilled containers and centrifuged at 43 000 *g* for 1 h at 4 °C. The rubber cream and the C-serum were discarded while the bottom fraction was collected and re-suspended in 0.4 M mannitol to aid the removal of remnant C-serum while keeping the precipitated organelles (mainly lutoids) intact. The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing (four times) to rupture the organelles. The fluid from the organelles, the B-serum, was recovered by centrifugation. Dialysis induced protein precipitation, preparation and isolation of the Hev b 4 protein complex from latex B-serum were carried out essentially as described earlier [8]. Polyclonal rabbit anti-Hev b 4 was developed using the Hev b 4 protein complex as the immunogen; standard protocols were employed for immunization, ELISA test on the periodical bleeds and the final recovery of anti-serum.

Electrophoresis protocol and Western immunoblot

Analytical SDS-PAGE was done according to standard procedures described elsewhere [15]. The gels were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich, St. Louis, MO, USA) and molecular weight markers (14–97 kDa) were obtained from BioRad (Hercules, CA, USA). Western blotting of the separated proteins on to nitrocellulose membranes, subsequent incubation with rabbit polyclonal antibody and patient serum, and detection of antigen–antibody with a secondary antibody conjugated to alkaline phosphatase were performed essentially as previously described [8]. Adult latex-allergic patients' sera were obtained from PlasmaLab International (Everett, WA, USA). All patients had clinical symptoms of latex allergy, and the serum samples had anti-latex IgE confirmed by RAST or ELISA. Control non-latex-allergic patients' sera from adults who had no history of latex allergy were obtained from Kuala Lumpur General Hospital, Malaysia.

Carbohydrate detection and deglycosylation

Visualization of glycans in proteins Western blotted onto nitrocellulose membranes was achieved by sodium periodate

oxidation of the carbohydrate followed by successive reactions with biotin hydrazide and alkaline phosphatase-conjugated streptavidin. The GlycoTrack™ carbohydrate detection kit (Oxford GlycoSystems, Abingdon, UK) was used following the manufacturer's protocol. Deglycosylation was performed using trifluoromethane sulphonic acid (TFMS) using the Glycofree™ deglycosylation kit (Oxford GlycoSystems) following the vendor's protocol.

5'/3' Rapid amplification of cDNA ends, and reverse transcription-polymerase chain reaction and molecular cloning

Latex total RNA was isolated essentially according to the method of Prescott and Martin [16]. The complete cDNA sequence of the latex lecithinase homologue was amplified with degenerate primers designed based on its partial peptide sequences, using 5'/3' Rapid Amplification of cDNA Ends (RACE) kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The degenerate primers, 5'TATCTG TTAGTTTCGCGCAT3' (Hev 41) and 5'TCTTATCAT CACCCATATGG3' (Hev42), employed in the 3'-RACE were designed based on the previously obtained N-terminal amino acid sequence of the 53–55 kDa Hev b 4 protein (Figs 1 and 2a). 5'-RACE was performed using gene-specific primers, 5'CGAAGAAAGTGGTACCATA3' (Lip49as) and 5'TCT TCCTGTAGGATAGTCG3' (Lip48as), which were derived from the 3'-RACE product. The gene-specific primers Lip49as and Lip48as correspond to positions 220–238 and 237–255 (complimentary strand), respectively, on the lecithinase homologue complete cDNA (Fig. 1).

RT-PCR was performed on latex RNA using another set of gene-specific primers obtained from the complete cDNA of the lecithinase homologue. The primers employed in the PCR reaction were 5'TGTACCGAGCTGGTGAATATTG3' (PLgsp7) and 5'TTAACCTAATGCAGTAGCTTCGGT3' (PLgsp8). The sequences corresponded to positions 112–135 and 1123–1146 (complementary strand) on the cDNA (GenBank accession number AY437086). The amplicon was then ligated to *pGEM-T* vector and transformed into *Escherichia coli* JM109 (Promega, Madison, WI, USA).

The *pGEM-T* insert was re-amplified using 5'TTTTC TAGATGTACC-GAGCTGGATGAA3' (PLgsp9) and 5'T TTCTAGATTAACCTAATGCAGTAGCTT3' (PLgsp3) primers with *Vent*® polymerase. Primers PLgsp9 and PLgsp3 also contained the *Xba*I recognition sequences at their 5' and 3' end, respectively. The *Xba*I-digested lecithinase homologue fragment was then ligated to the pMal-c2x (New England Biolabs, Beverly, MA, USA) vector in the same translatable reading frame, and the orientation of the insert was confirmed by DNA sequence analysis.

DNA sequence analysis

A Perkin-Elmer 9600 thermal cycler and an ABI377 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) were employed for sequencing the cDNA clones in *pGEM-T* and *pMal-c2x* vectors using the insert internal primers as well as the T₇ and SP6 promoter primers according to the thermal cycling protocol of *Taq* Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA).

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1  ACCACAAAGA CAAAGAACCT GCACAAGCTA AATCCATATA GTGCAATGGC AAGTCTAGCT
61  TATTCCTTGT TCATTTTAAAG CCTATTTACC TTCACCCTGC TCAACCCAGT TTGTACCGAG
121 CTGGATGAAT ATTTGTTTAG CTTTGGGGAT GGACTTTATG ATGCAGGCAA TGCTAAATTT
      T ATCTGTTTAG TTTCTGGCGAT
      Hev41
181 ATATATCCTG ATAAGTATCT TCCCTCTTAT CACCATCCAT ATGGTACCAC TTTCTTCGAC
      TCTTAT CATCACCCAT ATGG
      Hev42
241 TATCCTACAG GAAGATTTTC TGATGGTCGC ACAGTTGTTG ATTTTGTTC TGAAAATGTG
301 AGCTTACCTC GTATTCCACC GTTCAAAAAC AAGGAAGCAA ATTTCACTTA TGGCGCCAAT
361 TTCGCTTCAG AAGGTGCAAC TGCTTCGGAT AGTAATCCGC TAATCGACTT TCGAAGCCAG
421 ATTAGGGAAT TCGGAGAGTT GAAACTGGAA TGGGCAGTCC AACTAGTTAA TGTAAACCGAA
481 CTAGCAAGGA GGCTGAAAAA GGCAGTTTAC TTAATTTCCCT TTGGGGCTGA TGATTATTTG
541 AATTATGAAA TACCTTCTGA AGCCAGCCGA GAACAATTGG AAAGTATTGT GGATGTAGTG
601 CTTGGCAACA TTTCGGACAG GATCAAGGAA TTGTATGATT TTGGAGCAAG GAAATTTGTA
661 GTTGAGAACG TTGCACCCCT AGGCTTAATT CCATTTATTA AACAAACGTC CGACAATTCT
721 ACACATATTT ATGAACCTGC ATCATTGCAC GCAATGAAAC TACCTCAAAT CCTCGAGAAA
781 ATACAAGATG GGTATCTTTT TCCAGAAATC AACTATACAG TCTTCAATTA CTTCGGCATC
841 ATAAAAGAAA TTATAGATGC ACCTGGCGAA CATGGCTTCA AGTACGGGGA CATTGCATGC
901 TGTGGCAATT CGACATATCG TGGCAAGCT TGTGGATTTT TGGATTATGA ATTTTGTGTG
961 TGTGGTAATA AGACTGAGTA TTTGTTTTTT GACGGTACCC ATAACACTGA TGCCGCCAAT
1021 AATCTACTAG CTGAATTAAT GTGGGACAAG GAATCAGGAT TCATATCACC TTATGGCGTT
1081 AAGGACTTCT TTCCATCTCC TACTACCATT CAGACCCTTC TTACCGAAGC TACTGCATTA
1141 GGTTAATTGA AGGCATATAT ATTATTTAAG TTACTGTGTC TGCTATATAT ATGTGTCTGT
1201 ATTTCTAATA ATTAAATAAG GCAACAAATT CGATATAGTT TTTGTTGTCC CTACTAAGGA
1261 GTTCAGAAGG ATGGATGGAT TGAACCTCTT TACGTGGTTC AGCTATTGCG TGTAGCTAGC
1321 CTATTGAAA ATCTCATCTA TGCCAAATTC AGTAAAAA AAAAAA AAAAA

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Fig. 1. Nucleotide sequence of *Hevea brasiliensis* latex lecithinase homologue. The start (ATG) and stop (TAA) signals are highlighted in bold. The degenerate sense strand primers (Hev41 and Hev42) employed in the 3' rapid amplification of cDNA ends (RACE) are aligned and the identical bases are shaded in grey. The lecithinase homologue-specific primers Lip49as and Lip48as used in the 5'-RACE correspond to positions 220–238 and 237–255, respectively, on the cDNA.

Expression of recombinant lecithinase homologue fusion protein

The induction and extraction of maltose binding protein (MBP)–lecithinase homologue fusion protein were done according to the vendor's instructions (New England Biolabs). Isopropyl β -D-thiogalactopyranoside-induced *E. coli* cells were disrupted by sonication (Vibra Cell; Sonics & Materials Inc., Danbury, CT, USA) in ice-water bath with 15 s pulses for 3 min. The Bradford assay [17] was employed to verify protein content in the bacterial crude extract and the resultant insoluble material; both fractions were then analysed on SDS-PAGE.

Results

Isolation and characterization of the Hev b 4 lecithinase homologue cDNA

Distinct cDNA fragments were obtained from the 3'-RACE performed using the degenerate primers and the 5'-RACE performed using the gene-specific primers; the cDNA fragments were cloned and their nucleotide sequences were obtained. Overlap of the RACE fragments yielded the complete cDNA of 1375 bp (GenBank accession number AY437086), as shown in Fig. 1. The cDNA contained an untranslated region of 45 nucleotides at the 5' end, and another of 232 nucleotides at the 3' end. The predicted open reading frame (ORF) of the cDNA consisted of 366 amino acid residues and contained identical stretches of the previously obtained N-terminal and internal peptide sequences of the native protein (Fig. 2a).

The ORF revealed good homology to lecithinases (phospholipases) in the conserved domain database; thus, the protein was named Hev b 4 lecithinase homologue (Fig. 2b).

The ORF also matched myrosinase-associated proteins and GDSL lipases in the databases [4]. In agreement to its lutoidic (vacuolar) origin [8], an N-terminal signal peptide with a potential cleavage site between positions 24 and 25 from its first methionine was detected (ExPASy SignalP V1.1 programme). The calculated molecular weight of the mature lecithinase homologue encoded by the ORF is 38 529.29 Da, significantly lower than the 53–55 kDa observed on SDS-PAGE. The ExPASy ScanProsite programme predicted eight possible Asn glycosylation (*N*-glycosylation) sites. In addition, the NetOGlyc glycosylation prediction tool identified two potential carbohydrate attachment (*O*-glycosylation) sites on the ORF (Fig. 2a).

Immunoglobulin E binding of Hev b 4 lecithinase homologue

Western IgE immunoblot results showed that the Hev b 4 lecithinase homologue was recognized by eight out of 18 sera from latex-allergic patients. In comparison, the cyanogenic glucosidase component of Hev b 4 reacted with IgE antibody from only two patients, and they were a subset of those who reacted to the lecithinase homologue. As expected, neither components of Hev b 4 were detected by the non-latex-allergic patient's sera. The results indicated that the lecithinase homologue is the major IgE binding component of Hev b 4 (Fig. 3).

Immunoglobulin E binding to the glycans of the lecithinase homologue

The presence of glycans on Hev b 4 was confirmed using the GlycoTrack™ detection kit. The significance of glycans of

(a)	1	MASLAYSLFILSLFTFTLLNPVCT ELDEYLF SFGDGLYDAGNAKFIYDPDKYLPSYHHPYG
	61	TTFFDYPTGRFSDGRTVVDFVAEN VSLPRIPPFKNKEANFTYGANFASEGATASDSNPLI
	121	DFRSQIRDFGELKLEWAVQLV NV TELARRLKKA ~~VY~~ LISFGADDYLN ~~YE~~ I ~~P~~ SEASREQLES
	181	IVDVVLGNISDRIK ELYD FGARKFVVENVAPLGLIPFIKQTS DN STLFYELASLHAMKLP
	241	QILEKIQDGYLFPEFNYTVFN ~~Y~~ FGI I KEI IDAP GEHGFKYGDIA CCGN STYRGQACGF
	301	YEFVCV GN KTEYLFFDGTHNTDAANLLAELMWDKESGFISPYGVKDFFPST TIQ TLL T
	361	EATALG
(b)		
<i>H. brasiliensis</i>	COG3240	FGDGLYDAGN-----AKFIYDPDKYLPSYHHPYGTTFFDYPTGRFSD
<i>P. aeruginosa</i>		FGDSLSDSGNYRPAHGDPGSYGTIPGPSYQNGNGYTVTVVPETLG-
Consensus		*** * * * * * *
<i>H. brasiliensis</i>	COG3240	GRTVVDFVAENVSLPRIPPFKNKEANFTYGANFASEGATASDSNPLIDFR
<i>P. aeruginosa</i>		QLGVNHDFTYAAADPNGLYIHWAGGNLAVGGARSTEPNTGNSIGASATS
Consensus		QLGIAPGDLAASTSPVNAQQGIADGNNWAVGGYRTDQ--IYDSITAANGS
		* * *
<i>H. brasiliensis</i>	COG3240	SQIRDFGELKLEWAVQLV NV TELARRLKKA ~~VY~~ LISFGADDYLN ~~YE~~ I ~~P~~ SEA
<i>P. aeruginosa</i>		LAQQVGAF ~~LA~~ AGQGGFVWPNYPAQGLDPSALYFLWGGANDYLALPMLKAA
Consensus		LIERDNTLLRSRDGYLVDRARQGLGADPNALYYITGGGNDFLQGRILNDV
		* * * *
<i>H. brasiliensis</i>	COG3240	SREQLESIVDVVLGNISDRIK ELYD FGARKFVVENVAPLGLIPFIKQTS D
<i>P. aeruginosa</i>		AYQQLEGST---KADQSSAVQRLIAAGARNILVMTLPDLSLTPAGKAYGT
Consensus		QAQQAAGRL---V---DSVQALQQAGARYIVVWLLPDLGLTPATFGGGL
		* * * * *
<i>H. brasiliensis</i>	COG3240	NSTLFYELASLHAMKLPQILEKIQDGYLFPEFNYTVFN ~~Y~~ FGI I KEI IDAP
<i>P. aeruginosa</i>		EAIQASQATIAFNASLTSQLEQLGG-----NIIRIDTYTLLKEIMTNP
Consensus		QPF-ASQLSGTFNAELTAQLSQAGA-----NVIPLNIPLLLKEGMANP
		* * * * *
<i>H. brasiliensis</i>	COG3240	GEHGFKYGDIA CCGN STYRGQACGFLDYEFVCV GN KTEYLFFDGTHNTDA
<i>P. aeruginosa</i>		AEFGLA---NTTAPACDATVSNPACSASLPALCAAPQKYLFA DS VHPTTA
Consensus		ASFGLAADQN LIG TCFSGNGCTMNPTYGINGSTPDPSKLLF ND S VHPT IT
		* * * * *
<i>H. brasiliensis</i>	COG3240	ANNLLAELM
<i>P. aeruginosa</i>		VHHLIAEYI
Consensus		GQRLIADYTYSLLSAPWELTLLPEMAHGTLRAYQDELRSQWQADWENQ N
		* *

Fig. 2. Translated amino acid sequence of *Hevea brasiliensis* lecithinase homologue and comparative protein sequence alignment. The SignalP predicted N-terminal leader sequence in the lecithinase homologue is underlined. Asparagines predicted to be N-glycosylated in the Asn-Xaa-Ser/Thr sequons are highlighted in bold. The predicted O-glycosylated threonine residues in the sequence output are highlighted in bold italics. The N-terminal amino acid sequence that was used in designing of the degenerate primers, and two other internal peptide sequences are shaded in grey (a). The alignment of matching regions within the lecithinase homologue (depicted as *H. brasiliensis*) to the representative phospholipase/lecithinase/hemolysin gn1CDD12577 (clusters of orthologous groups, COG3240) in the conserved domain database and that of a *Pseudomonas aeruginosa* (GenBank accession number ZP_00141584) is shown. Asterisks indicate residues identical in all sequences compared.

the lecithinase homologue in IgE binding was also investigated. The purified protein complex was treated with TFMS (Glycofree™ deglycosylation kit) to strip the carbohydrate moieties. The deglycosylated lecithinase homologue migrated at circa 40 kDa on SDS-PAGE, comparable to the calculated value of 38 529.29 Da (Fig. 4). The untreated Hev b 4 and its deglycosylated form were Western blotted onto nitrocellulose membranes after separation by electrophoresis. Both the untreated and deglycosylated proteins showed binding to rabbit anti-Hev b 4 polyclonal antibody (results not shown). The blots were also tested with two IgE anti-Hev b 4-positive sera from latex-allergic patients, including one that is known to react with the cyanogenic glucosidase component. In both

Western immunoblots, IgE binding was observed on the untreated glycosylated Hev b 4, but was absent on the deglycosylated form. As expected, both patients recognized the native lecithinase homologue, while only one patient detected the cyanogenic glucosidase component of Hev b 4 (Fig. 4).

Further evidence of carbohydrate IgE determinant was gathered from the MBP-recombinant lecithinase homologue synthesized in *E. coli*, which is therefore non-glycosylated. In our preliminary analyses, the MBP-lecithinase homologue fusion protein remained largely insoluble. It is for this reason that subsequent analyses on the MBP-lecithinase homologue were performed using the *E. coli* lysate. In Western

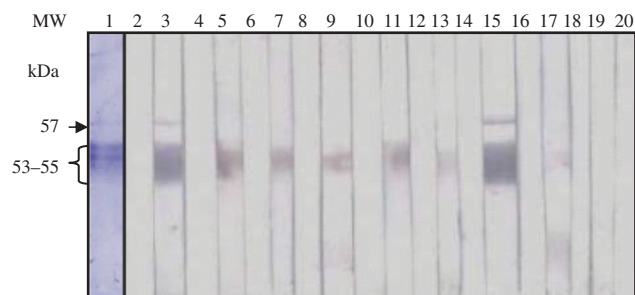


Fig. 3. Western IgE immunoblots of Hev b 4. Coomassie blue R-250-stained Hev b 4 on 12.5% SDS gel (lane 1). Blot reacted with sera from non-latex-allergic individual, negative control (lane 2). Blots reacted with sera from latex-allergic patients (lanes 3–20). Blots with IgE binding to the 52–55 kDa lecithinase homologue (lanes 3, 5, 7, 9, 11, 13, 15); blots with IgE binding to the 57 kDa cyanogenic glucosidase (lanes 3, 15).

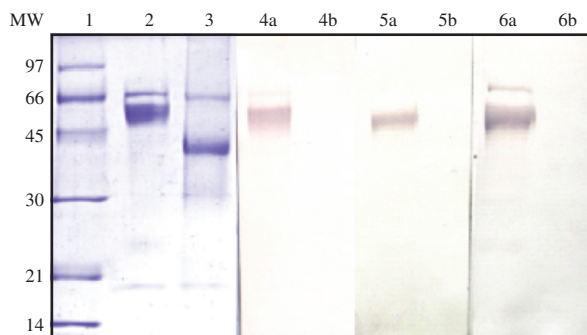


Fig. 4. Glycodetection and IgE binding to glycans of Hev b 4. Coomassie blue stained 12% SDS-PAGE showing molecular weight markers (lane 1), native Hev b 4 (lane 2), deglycosylated Hev b 4 (lane 3). Detection of glycans on native Hev b 4 (lane 4a), glycans not detected on deglycosylated Hev b 4 (lane 4b). Western immunoblots show binding of native Hev b 4 to sera from three different latex-allergic patients (lanes 5a, 6a) and loss of IgE binding after deglycosylation (lanes 5b, 6b).

immunoblots, the bacterial recombinant MBP–lecithinase fusion protein did not react to IgE in sera from Hev b 4-positive latex-allergic patients, although the protein recognized polyclonal rabbit IgG anti-Hev b 4 and MBP-specific polyclonal antibody (Fig. 5).

Discussion

Earlier use of the immunogold-labelling technique revealed that the allergenic Hev b 4 protein complex could be a part of the microhelical structures within the lutoids of NRL [8]. It is evident now that Hev b 4 consists of a cyanogenic glucosidase [9] and a lecithinase homologue. The nucleotide sequence of the lecithinase homologue also showed similarity to myrosinase-associated proteins and GDSL lipases in databases, in agreement to the information gathered previously from the N-terminal and internal peptide sequence of the protein [4, 8]. Occurrence of thio-glucosidases (myrosinases) in the complex with their associated proteins has been reported in crucifers [10–12]. Myrosinase-associated protein is also an abundant vacuolar protein in the leaves of *Arabidopsis*, although the reason for its accumulation remains unclear [18]. Elsewhere the Hev b 4 lecithinase homologue has been described as a protein involved in latex coagulation (GenBank accession number AY207386).

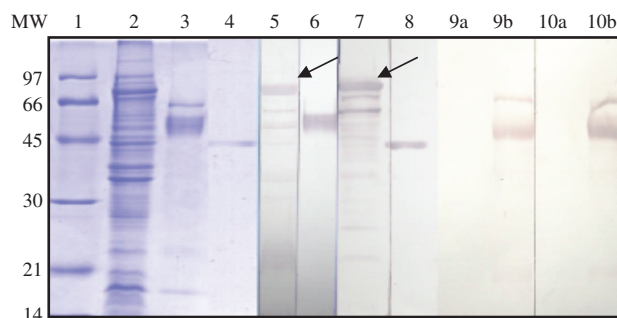


Fig. 5. Western immunoblots of recombinant Hev b 4. Coomassie blue-stained 12.5% SDS gel showing molecular weight marker (lane 1), crude *Escherichia coli* JM109 extract containing maltose binding protein (MBP)–lecithinase homologue fusion protein (lane 2), native Hev b 4 (lane 3), MBP (lane 4). Western immunoblot with polyclonal anti-Hev b 4 IgG, arrow indicates MBP–lecithinase homologue fusion protein detected at circa 80 kDa (lane 5) and native Hev b 4 (lane 6). Western immunoblot with anti-MBP IgG, arrow indicates MBP–lecithinase homologue detected at circa 80 kDa (lane 7), MBP (lane 8). Western immunoblots with sera from two latex-allergic patients, no IgE binding detected in *E. coli* crude extract containing MBP–lecithinase homologue (lanes 9a, 10a), IgE binding present in native Hev b 4 (lane 9b, 10b).

The experimental data and bioinformatics analyses presented here showed that the Hev b 4 lecithinase homologue is a heavily glycosylated IgE binding protein. Evidence of glycosylation of the lecithinase homologue was gathered when internal peptide sequencing was first attempted on the protein. Electrospray ionization coupled with mass-spectrometry (ESI-MS) analysis of the protein revealed that the majority of the tryptic-digested peptides were glycosylated. Furthermore, the heavy glycosylation appeared to direct fragmentation along the sugar chains rather than the amino acids; consequently, sequence data were not obtained for these peptides (unpublished data). The presence of glycans on the protein Western blotted onto nitrocellulose membrane was then confirmed using the GlycoTrack™ kit (Oxford GlycoSystems, Abingdon, UK).

Removal of glycans from the lecithinase homologue with TFMS helped explain the discrepancy between the apparent and calculated molecular weight of the protein. The Western IgG immunoblots on the deglycosylated lecithinase homologue revealed that the antigenic determinants of the protein remained unscathed after treatment with TFMS. In a similar way, TFMS-treated Hev b 3, a non-glycosylated latex allergen, has also been shown to retain recognition to IgE antibodies from latex-allergic patients (results not presented).

Glycans, although long known to be potentially antigenic and bind IgE, their clinical significance and ability to elicit an allergic response have been somewhat disregarded [19, 20]. The ability of Hev b 4 to induce allergic reactions, however, has been confirmed by skin prick tests and various *in vitro* immunoassays [7, 21–23]. These findings inclusive of the present study are summarized in Table 1. IgE reactivity that was sensitive to periodate treatment (implying carbohydrate IgE epitopes) had also been reported previously for latex proteins [24]. More recently, the presence of carbohydrate IgE determinants on Hev b 13, a potent latex allergen, was also conclusively demonstrated [14]. Taken together, these findings emphasize the significance of glycans in IgE recognition. The presence and significance of glycans attached to the cyanogenic glucosidase, the minor IgE binding component of

Table 1. Reactivity of adult latex-allergic patient sera to purified Hev b 4 protein complex

Reference	Assay	Sample size	Hev b 4
[7]	SPT	62	39
[21]	ELISA	65	20
[22]	ELISA	31	65
	RAST	31	23
	RAST	31	61
[23]	DIB	36	75
Present study	WIB	18	44

DIB, dot-immunoblot; SPT, skin prick test; WIB, Western IgE immunoblot. Prevalence of skin reaction (SPTs) or IgE reactivity expressed in percentage of the test population.

Hev b 4, were also confirmed from this study. Examining the histamine release property, see [25–27] of the native, non-glycosylated and deglycosylated forms of lecithinase homologue and the cyanogenic glucosidase component of Hev b 4 would further strengthen the role of glycans in latex allergy *per se*. With the cDNA cloning of the lecithinase homologue component of Hev b 4, the nucleotide sequence of all 13 IUIS recognized latex allergens are now available.

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