

Amino acid sequence similarity of Hev b 3 to two previously reported 27- and 23-kDa latex proteins allergenic to spina bifida patients

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Separate studies have reported spina bifida patients to be especially allergic to proteins of 27 and 23 kDa found in the serum of centrifuged natural rubber latex. An insoluble latex protein located on the surface of small rubber particles, Hev b 3, has similarly been found to be allergenic to spina bifida patients. In this study, internal amino acid sequences of Hev b 3 showed similarity to the published sequences for the 27- and 23-kDa latex proteins. The latter allergens are hence identified as Hev b 3. Determination of the molecular weight of Hev b 3 revealed various species of 22–23 kDa. The consistent gaps of about 266 Da observed between various forms of the intact protein suggest that the protein undergoes post-translational modification. To determine whether Hev b 3 also occurs in a soluble form in the latex serum, its presence in molecular-filtered serum was checked by ELISA and Western blot. The results showed Hev b 3 to be largely absent in the C-serum from fresh latex. The protein is therefore insoluble in its native state. However, a small amount of the solubilized protein was detected in ammonia-stabilized latex (commonly used in the manufacture of latex products).

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Latex allergy has most frequently been reported among health-care workers in frequent contact with latex devices such as surgical or examination gloves. Besides health-care workers, another group of patients susceptible to latex allergy is children with spina bifida (1–3). Allergy to the 14.6-kDa rubber-particle protein, Hev b 1, has been found to be associated with spina bifida (3–6), whereas latex-sensitive adults are less commonly sensitive to this protein (4–6). A second latex allergen to which spina bifida patients are especially sensitive has been variously reported as a 27-kDa protein (2, 7), a 23-kDa protein (8), and a 24-kDa protein (5). The 27- and 23-kDa proteins were described as latex-serum proteins (2, 7, 8).

On the other hand, the 24-kDa protein, designated Hev b 3, was characterized as an insoluble peptide located on the surface of very small rubber particles in natural rubber latex (5). A protein of similar size associated with rubber particles has also been reported by Dennis & Light (9) and by Hasma (10).

In the present study, internal amino acid sequences of Hev b 3, isolated independently in two laboratories, were compared with those of the 27- and 23-kDa latex allergens to determine whether the three proteins were in fact identical. Should they turn out to be identical, the discrepancies in molecular weights would need to be resolved. A further objective of the study was to determine whether the proteins occurred as both water-soluble and -insoluble forms in natural rubber latex.

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Material and methods

Latex collection

Malaysian latex was collected from regularly tapped rubber trees (*Hevea brasiliensis*, clone RRIM 600) growing in the Rubber Research Institute of Malaysia's Experimental Station, Sungei Buloh, Selangor. Latex exuding from the tapped trees was collected into chilled containers and brought back to the laboratory for centrifugation and subsequent processing. Latex from Costa Rica was obtained by tapping uncultivated rubber trees that had not been previously tapped, or not tapped for several months. The exuded latex was immediately diluted at seven parts latex to three parts 300 mM Tris-HCl buffer, pH 7.5, containing 0.03% sodium azide; quickly frozen; and shipped to the laboratory in South San Francisco, CA, USA, on dry ice.

Preparation of latex C-serum and ammoniated latex serum

Fresh *Hevea* latex was centrifuged at 44 000 g in a Sorvall RC 2B high-speed centrifuge at 4°C for 1 h, and the aqueous phase, the C-serum, was extracted. The centrifugation process was repeated on the C-serum to obtain a clear serum generally free of flocculated rubber particles. Ammoniated latex containing approximately 0.7% ammonia as preservative was similarly centrifuged to extract the serum phase. Ammoniation damages the luteoids in the latex, and the luteoid content (the B-serum) is mixed with the C-serum. Hence, this serum obtained after centrifugation is termed "ammoniated latex serum" rather than "C-serum". Membrane ultrafiltration of C-serum and ammoniated latex serum was carried out in an Amicon stirred cell under nitrogen pressure using an Amicon ZM500 membrane with a 500-kDa molecular weight cutoff. Before ultrafiltration, the sera were diluted to 50% with distilled water while the membrane was pre-treated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

Preparation of Hev b 3 from Malaysian latex

Hev b 3 was isolated from the Malaysian latex as described previously (11). Briefly, the small rubber particles (average diameter 70 nm) contained in fresh latex were obtained from zone 2 of the rubber cream (11, 12) after centrifugation for 2 h, as described above. These rubber particles, which bear relatively large amounts of Hev b 3 protein, were washed by resuspension in 30% sucrose and recovered by centrifugation. The proteins on the surface of the rubber particles were then solubilized and

extracted by adding an equal weight of detergent, comprising 0.1% Triton-X 100 and 1% sodium dodecyl sulfate (SDS), to the rubber cream. The mixture was vortexed and centrifuged to obtain the supernatant that contained solubilized Hev b 3. The protein extract was purified by SDS-PAGE and the protein band eluted out in 50 mM Tris-HCl buffer, pH 7. The sample was dialyzed against distilled water, and then against 1% glycine. Recovery of Hev b 3 was about 1.5 mg per 100 ml fresh latex. The protein yield would have been higher had an attempt been made to recover as much as possible of the zone 2 rubber from centrifuged latex. However, this was not done, as the availability of *Hevea* latex was not limiting.

Preparation of rubber particle proteins from Costa Rican latex

Rubber particle proteins from frozen, diluted Costa Rican latex were prepared as described (9, 13) from rubber particles isolated in the absence of detergent. Briefly, frozen latex was thawed and centrifuged at 2000 g for 10 min, and the latex separated from both coagulated floating rubber and a small pellet. The resulting latex was refrozen in 10-ml aliquots, which were thawed before use and a fraction run over a Sepharose S-300 column (5×50 cm) in 50 mM Tris-HCl, pH 7.5, at 0.64 ml/min without detergent. Under these conditions, the rubber particles which eluted in the void volume contained both Hev b 3 and Hev b 1 (REF [rubber elongation factor]) based on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (14). (See Fig. 2B, lane 3, or Fig. 2C, lane 3, in Dennis & Light [9].)

Preparative SDS-PAGE was used to isolate Hev b 3 by a protocol similar to that described previously for Hev b 1 (9, 15). Briefly, rubber particles containing both Hev b 3 and Hev b 1 were extracted with 1% SDS. The sample was clarified by centrifugation to remove rubber particles, the supernatant was precipitated with an equal volume of ice-cold acetone, and the resulting protein precipitation was collected by centrifugation. This protein pellet that contained Hev b 1 and Hev b 3 was redissolved in SDS-PAGE buffer, fractionated by 15% SDS-PAGE, visualized in 1 M KCl, and the gel slice containing only Hev b 3 electroeluted inside a SpectroPor dialysis bag (molecular weight cutoff 2000) in 100 mM ammonium bicarbonate, pH 8.0, 0.1% SDS. The amount of pure Hev b 3 which could be recovered (250 µg from 25-ml aliquot of latex) was less than the recovery of Hev b 1 (2 mg from 25 ml latex) due to the lower abundance of Hev b 3 on the original rubber

particles used. Care was taken to avoid contamination of Hev b 3 from the larger amount of Hev b 1 in the lower band. Samples of Hev b 3 purified in this manner were analyzed by SDS-PAGE, and no contaminating bands were detected after Coomassie brilliant blue staining of a 20- μ g load, indicating that Hev b 3 was >99% pure.

Amino acid sequencing

Hev b 3 from Malaysian latex. An aliquot of Hev b 3 (43 μ g) from Malaysian latex was loaded on to a hydrophobic sequencing column. Salts and buffers were removed by washing with 2 ml of 2% trifluoroacetic acid (TFA). Fifteen cycles of Edman sequencing chemistry were performed with a HP G1005A protein sequencer.

An aliquot of Hev b 3 (86 μ g) was digested with 2 μ g trypsin (Promega) in 50 mM ammonium bicarbonate (pH 8.3) at 37°C for 18 h. Then, an aliquot was injected onto a 2.1-mm Aquapore RP-300 C18 reverse-phase column. Peptide fragments were separated by gradient elution at a flow rate of 200 μ l/min beginning at 20% B and 80% A, and progressing to 60% B and 40% A, where solvent A was 0.1% TFA/H₂O and solvent B was 0.1% TFA/MeCN. Detection was at 215 nm 0.300 AUFS. The peptide fragments were collected and sequenced.

Hev b 3 from Costa Rican latex. Partial amino acid sequence and amino acid analysis data of Hev b 3 from Costa Rican latex was obtained by the methods and protocols previously described for Hev b 1 (15). Analyses were performed on a Beckman model 6300 amino acid analyzer and a Beckman model 890C sequencer, respectively. Duplicate 1.4-nmole (34- μ g) samples of Hev b 3 were hydrolyzed for 24 h in constantly boiling HCl in evacuated sealed tubes at 110°C. The samples were then analyzed for amino acid composition.

An additional 50 μ g of Hev b 3 was treated with 2.5 μ g trypsin in 50 mM Tris, 20 mM CaCl₂, pH 8.0, at 25°C, for 24 h. The resulting peptides were separated by RP-HPLC (Vydac C18), and three prominent peaks were chosen for analysis. The initial yields on the sequencer varied from 200 to 700 pmol for the first cycle.

Molecular weight determination by mass spectrometry

The molecular weight of the intact Hev b 3 protein was determined by liquid chromatography/

mass spectrometry (LC/MS) with a Platform single quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray source with an on-line LC connection to allow direct protein ionization from the eluent. An aliquot of the sample solution containing 9 μ g Hev b 3 was injected onto a Poros R/H 320- μ m internal diameter 5-cm column equilibrated in aqueous 0.1% TFA. The salts and buffers were removed by streaming aqueous 0.1% TFA through the column at a flow rate of 40 μ l/min. The protein was then eluted directly into the mass spectrometer by streaming 100% MeCN, 0.1% TFA through the column.

Detection of Hev b 3 by Western blot

SDS-PAGE was carried out on 15% gels according to Laemmli (14), and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was then blocked with PBS containing 5% nonfat milk powder for 30 min and then incubated for 2 h with the monoclonal antibody, USM/RC2, which binds specifically to Hev b 3. After three cycles of washing with PBS, detection of allergen-antibody binding was achieved by incubating for 1 h with a peroxidase-conjugated secondary antibody (Dako, Denmark). After three cycles of washing as before, the nitrocellulose membrane was incubated with peroxidase substrate (Sigma, USA) to generate an insoluble, colored product. Matching gels were stained with Coomassie brilliant blue.

Quantitation of Hev b 3 by ELISA

Latex C-serum or ammoniated latex serum (ultrafiltered or unfiltered) was serially diluted with carbonate-bicarbonate buffer, pH 9.6, and 100- μ l test samples were pipetted into the wells of an ELISA plate (Costar, USA). The proteins were allowed to bind overnight at 4°C, and, the following day, the plates were blocked for 2 h with 1% BSA in PBS. The plates were washed thrice with PBS containing 0.05% Tween 20. Polyclonal antiserum (100 μ l rabbit anti-Hev b 3, 1/500 dilution) was loaded into the wells of the antigen-coated ELISA plate and incubated for 1 h at room temperature. Unbound antiserum was removed by the same washing procedure described above. The conjugate, goat antirabbit IgG-HRP (BioRad, USA), diluted 1/2000 with 1% BSA in PBS, was dispensed in 100 μ l aliquots into each well. After incubation in the dark for 1 h at room temperature, the plate was washed

as before, and 100 µl of enzyme substrate (Bio-Rad, USA) was added per well. Color was allowed to develop for 30 min before the reaction was stopped with 2% oxalic acid. The absorbance was read at 405 nm with a spectrophotometric plate reader.

Results and Discussion

A comparison of amino acid sequences published for the 27- and 23-kDa latex allergens with that of Hev b 3 was carried out to determine whether the three proteins were identical (Fig. 1). A rubber particle protein of about 24 kDa by SDS-PAGE analysis (presumed to be Hev b 3) that was isolated from Costa Rican latex served to validate consensus in amino acid sequences between Hev b 3 from Malaysian latex and the two other latex allergens.

Hev b 3 from Costa Rican latex analyzed for amino acid composition gave the results presented in Table 2. When 1.4 nmole was loaded on the sequencer, no N-terminal sequence greater than 15% of the load was obtained. This result suggests a heterogeneous N-terminus that can include a blocked N-terminus, as reported for the 27- and 23-kDa allergens (2, 8).

Tryptic digest internal amino acid sequences compared between the 27-kDa latex allergen, the 23-kDa allergen, and Hev b 3 originating from Malaysian or Costa Rican latex show good consensus in various fragments. Where discrepancies occur, the amino acid residues are often conserva-

Table 2. Amino acid composition of Hev b 3 from Costa Rican latex based on total of 220 residues

Amino acid	Number of residues
Asn/Asp	21±1.5
Thr	13±0.5
Ser	18±4
Gln/Glu	27±3
Pro	14±0.5
Gly	20±7
Ala	21±0.5
Cys	2±1
Val	23±1.5
Met	2±1
Ile	5±0.5
Leu	16±2
Tyr	10±2
Phe	6±0.5
His	2±1
Lys	13±2
Arg	8±2
Trp	Destroyed

Table 1. Comparison of amino acid sequences of 27-* and 23-kDa** latex allergens with those of Hev b 3 isolated from Costa Rican and Malaysian latex

27-kDa allergen	IVLDVASSVFNTGVQEGA
Hev b 3 (Malaysian latex)	IVLDVASSVFNT RKEKK Q Q
27-kDa allergen	AVDSFSTLY
23-kDa allergen	AAGVYAVDSFSTLYLY
Hev b 3 (Costa Rican latex)	AAGVYAVDSFSTLYLYAK
27-kDa allergen	TVVTPVYYIPLEAVK
23-kDa allergen	TVVTPVYYIPLEAVK
Hev b 3 (Costa Rican latex)	TVVTPVYYIPLE
Hev b 3 (Malaysian latex)	TVVTPVYYIPLEAVK
27-kDa allergen	TVDVSVTSLDGVVP
23-kDa allergen	TVDVSVTSLDGVVPPVVK
Hev b 3 (Costa Rican latex)	TVDVST P
Hev b 3 (Malaysian latex)	TVDVSVTSLDGVVPPVVK 1
27-kDa allergen	VSSYLPLLP
23-kDa allergen	VSSYLPLLPTEK
Hev b 3 (Malaysian latex)	VSSYLPLLPTEK

Identical amino acid residues are shaded, and residues showing conservative single DNA base changes are underlined.

*Alenius et al. (7); **Lu et al. (8).

tive single DNA base changes (Table 1). Alenius et al. reported that only one out of 14 peptide fragments bore homology to REF, but disregarded possible matches where the homology had been low (7). On the other hand, Lu et al. (8) reported a much better match of 45.6% identity between amino acid sequences of Hev b 1 and their 23-kDa allergen. Consistent with these findings, the polyclonal antibody against Hev b 3 used in the present study has previously been observed to cross-react with Hev b 1 (16). There has been previous speculation that the 23- and 27-kDa latex allergens are proteins originating from rubber particles (5, 17). From the similarities in amino acid sequences, the 27- and 23-kDa latex allergens are positively identified in this study as the rubber-particle protein, Hev b 3. The differences in molecular weights reported in the different studies could have arisen from discrepancies in the estimates by SDS-PAGE, exacerbated perhaps by the use of standard calibration markers from different manufacturers. Therefore, the Hev b 3 molecular weight was precisely determined by mass spectrometry.

Mass spectrometry of Hev b 3 (Figs. 1 and 2) gave several species of molecular weights of 22 258, 22 524, 22 791, and 23 058 Da. The consistent gaps of about 266 Da observed between various forms of the intact protein suggest that the protein is post-translationally modified. The mass of 266 Da is consistent with the addition of stearyl groups, although further work would be necessary to establish the exact nature and position of these post-translational modifications.

While Hev b 3 has been characterized as an insoluble protein located on the surface of rubber

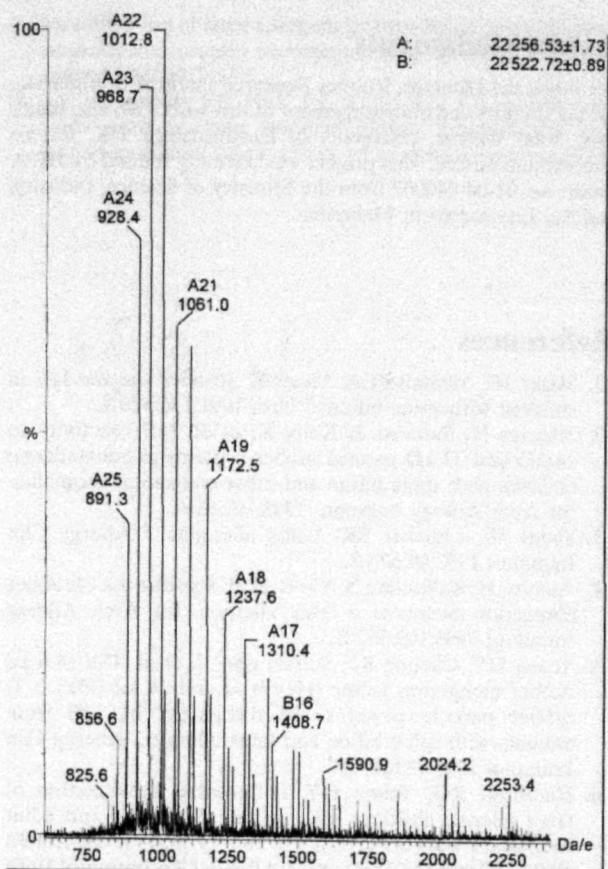


Fig. 1. Electrospray mass spectrum of Hev b 3 showing multiple charged series of molecular ions relating to species A of molecular weight 22 256.53 Da and species B of molecular weight 22 522.72 Da.

particles (5), reports on the 27- and 23-kDa latex allergens have depicted them as latex-serum proteins (7, 8), thereby implying that they are soluble in the aqueous serum. Although Lu et al. (8) had attempted to remove rubber particles from latex serum in their experiments by filtering through a 450-nm pore-size filter, this would have been largely ineffective since the very small rubber particles that bear Hev b 3 average only 70 nm in diameter (11). Experiments were therefore carried out to determine whether Hev b 3 occurs both in an insoluble form as a membrane protein and in a soluble form in the latex C-serum. The solubility of this latex allergen was also examined in the serum of ammonia-stabilized latex that is commonly used in the manufacture of latex products.

Coomassie brilliant blue staining of electrophoretically separated C-serum or ammoniated latex serum revealed a complement of proteins of varying molecular weights, including those greater than 100 kDa. Hence, there was no reason to suppose that a soluble form of Hev b 3 – had it

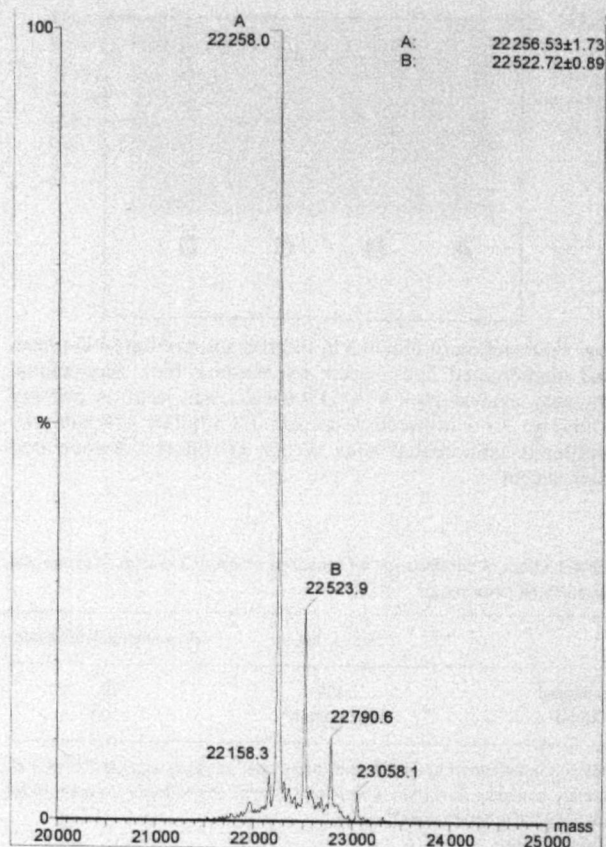


Fig. 2. Reconstructed mass spectrum of Hev b 3 to true molecular weight scale showing species A (22 256.53 Da) and species B (22 522.72 Da).

been present – would not be recovered in the filtrate that was free of rubber particles. In the Western blot, Hev b 3 in unfiltered C-serum was clearly detected by its monoclonal antibody, USM/RC2 (Fig. 3). However, the protein was no longer found in the C-serum after passing through the ultrafiltration membrane. In the case of the ammoniated latex serum, a fainter but still distinct band was observed after filtration. These results show that Hev b 3 is insoluble in its native state, but a small amount of the protein is solubilized in the presence of ammonia. Quantitation of Hev b 3 by ELISA gave essentially the same result (Table 3). Hev b 3 was present only in trace amounts in filtered C-serum, while some of the protein was detectable by ELISA in ammoniated latex serum after ultrafiltration. These observations agree with those of Hasma, who noted that ammonia solubilized a 24-kDa protein from rubber particles (10). Hev b 3 isolated by Alenius et al. (2, 7) and by Lu et al. (8, 18) from latex serum could have been derived from the small amounts of solubilized rubber particle protein. Perhaps it is more likely that the protein originated from the small rubber

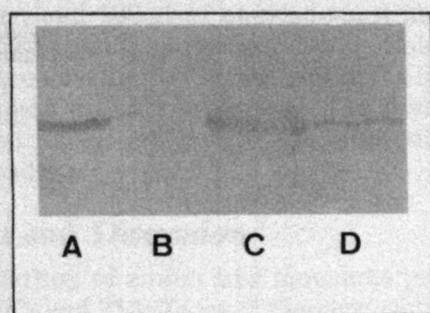


Fig. 3. Detection of Hev b 3 in filtered and unfiltered C-serum and ammoniated latex serum by Western blot. Monoclonal antibody against Hev b 3, USM/RC2, was used as primary antibody. A) Unfiltered C-serum; B) filtered C-serum; C) unfiltered ammoniated latex serum; D) filtered ammoniated latex serum.

Table 3. Effect of ultrafiltration on presence of Hev b 3 in latex C-serum and ammoniated latex serum

	Latex C-serum	Ammoniated latex serum
Unfiltered	100	25
Filtered	Undetected*	0.1

Hev b 3 quantitation by ELISA with polyclonal antibody against Hev b 3 as primary antibody. Absorbance reading (405 nm) of unfiltered C-serum (2.23) taken as 100 arbitrary units.

*Less than 10^{-3} .

particles remaining in the latex serum after centrifugation (5, 11).

The fact that Hev b 3 is only sparingly soluble even in ammoniated latex (which is used in latex product manufacture) could explain why the washing of latex gloves with water is ineffective in removing a 23-kDa allergen (shown here to be Hev b 3) even though most soluble proteins are removed (18).

The finding that Hev b 3 is largely insoluble might explain also why spina bifida patients are so susceptible to sensitization to the allergen. Unlike other water-soluble allergens that dissolve in the sweat, Hev b 3 is not so readily transmitted to adults through the use of examination or surgical gloves. However, latex devices (surgical gloves, catheters, etc.) often come into direct contact with mucosal membranes of spina bifida and other multioperated patients and might cause sensitization without the need for the allergen to be substantially present in the dissolved state (5). Nevertheless, this proposition requires further examination, in view of the fact that Hev b 3 is slightly soluble in the ammoniated latex used in glove manufacture.

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