

Isolation and Characterization of the Early Nodule-specific Protein Homologue (Hev b 13), an Allergenic Lipolytic Esterase from *Hevea brasiliensis* Latex*

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Recurring reports of a highly allergenic 42–46-kDa protein in *Hevea brasiliensis* latex appeared to have been resolved with the discovery of a 43-kDa allergenic latex protein that was a homologue to patatin. However, the low to moderate prevalence of sensitization to the protein, designated Hev b 7, among latex-allergic patients could not adequately explain the frequent observations of the 42–46-kDa allergen. This led to the hypothesis that another, more allergenic protein of a similar molecular mass existed in *Hevea* latex. We report the isolation and purification of a 42.98-kDa latex glycoprotein showing homology to the early nodule-specific protein (ENSP) of the legumes *Medicago sativa*, *Medicago truncatula*, and *Glycine max*. The protein is allergenic, being recognized by immunoglobulin E (IgE) in sera from latex-allergic patients. The IgE epitope resides on the carbohydrate moiety of the protein, and the presence of a similar carbohydrate component on potato tuber patatin enables the latter to inhibit IgE binding to the ENSP homologue. The cDNA encoding the ENSP homologue was isolated by reverse transcription-PCR and cloned. The protein predicted from the cDNA sequence has 391 amino acids, the first 26 of which constitute a putative signal peptide. The deduced molecular mass of the mature protein is 40.40 kDa, while its isoelectric point is estimated at 5.0. The discrepancy between the predicted and observed molecular mass might be due to glycosylation, for which three N-sites on the protein are predicted. The purified protein showed lipase and esterase activities and may be involved in plant defense.

Latex exudes from the bark of the commercial rubber tree, *Hevea brasiliensis*, when the tree is tapped. A large proportion of the harvested latex is coagulated for manufacture into “dry rubber” industrial products such as motor vehicle tires. Latex can be stabilized in an uncoagulated state by ammoniation for subsequent processing into various “dipped” latex products

such as examination and surgical gloves. About one-third by weight of *Hevea* latex is natural rubber, the polymeric hydrocarbon *cis*-polyisoprene that is synthesized in specialized organelles, the rubber particles. Another specialized organelle found in latex is the luteoid, which is vacuole-like in structure and function. In fresh latex, rubber particles, luteoids, and other minor cellular organelles are suspended in an aqueous serum called the C-serum. *Hevea* latex contains hundreds of proteins, making up 1–2% of its weight (1, 2). Among these, a small number of latex proteins can cause an allergic reaction in sensitized persons, particularly health care workers who routinely wear latex gloves in the course of their work. Many latex allergens originate from the B-serum (3), which is the fluid derived from the heavy “bottom fraction” of centrifuged latex. The bottom fraction comprises mainly luteoids, but other minor organelles are also deposited in the bottom fraction when latex is centrifuged at high speed (4, 5).

Much effort has focused on the isolation and identification of the allergenic proteins in natural rubber latex. From the early years of latex allergy research, there have been reports of a 42- to 46-kDa allergenic (IgE-reactive) latex protein(s) that was encountered frequently (6–9). In 1995, a 46-kDa latex allergen that had partial protein homology to patatin, the major storage protein of potatoes, was identified in the United States (10). This protein was assigned the World Health Organization-International Union of Immunological Societies (WHO-IUIS) name Hev b 7. Around the same time, work in The Netherlands and Indonesia uncovered a 43-kDa protein from the latex B-serum (11). This also had amino acid homology to patatin. Although originating from the latex bottom fraction, the protein did not show structural features expected of a vacuolar protein, and there is no explanation yet for its presence in the latex B-serum (12). About this time, researchers in England were investigating a 44-kDa proteinaceous rubber biosynthesis inhibitor isolated from the latex C-serum (13, 14). Again, homology to patatin was observed. When the proteins that were independently isolated were all shown to be allergenic, they were classified as Hev b 7, with the protein originating from the latex C-serum designated Hev b 7.01 and that from the latex B-serum designated Hev b 7.02. This deduction received further support when the recombinant protein produced from the DNA was shown also to be allergenic (15, 16). These developments notwithstanding, one discrepancy that surfaced was the observation that the numbers of patients who were sensitized to native or recombinant Hev b 7 were far fewer than expected, and this did not reflect the frequent observation of 42–46-kDa IgE-reactive protein. Studies showed that 8–49% (median 32%) of latex-allergic health care workers were sensitized to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[™]/EBI Data Bank with accession number(s) AY283800.

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Hev b 7 (10, 16, 17–20), depending on the analytical sensitivity of the serologic tests employed. On the other hand, the occurrence of IgE reactivity to the 42- to 46-kDa protein detected in unpurified whole latex was commonly above 60% (median 71%) (6, 9, 19). This led to the hypothesis that another, more allergenic protein of a similar molecular mass existed in *Hevea* latex.

We report the isolation and purification of a 42.98-kDa latex glycoprotein that is immunoreactive with IgE antibody in a high proportion of latex-allergic patients. This protein has been assigned the allergen name Hev b 13 by the International Union of Immunological Societies (IUIS). The protein is a homologue of early nodule-specific proteins (ENSPs)¹ from legumes and may have functions in plant defense. We describe the cloning of the cDNA encoding the *Hevea* ENSP homologue, its molecular characteristics, and its allergenic and enzyme properties.

MATERIALS AND METHODS

Latex Collection and Preparation of the C-serum and B-serum—Fresh latex tapped from *H. brasiliensis* trees (clone RRIM 600) was collected into chilled containers. The latex was centrifuged at 19,000 rpm ($43,000 \times g$) on a Sorvall RC 5C high speed centrifuge for 1 h at 4–7 °C to separate it into three main fractions: the top fraction, which was the rubber cream; the heavy bottom fraction; and the C-serum located in between (4). Latex B-serum was prepared based on the method of Hsia (21). Briefly, the latex bottom fraction from centrifuged latex was washed by resuspension in 0.4 M mannitol and recovered by centrifugation. The washed bottom fraction was then subjected to repeated freeze-thawing to rupture the luteoids that were its main constituents. The B-serum, comprising mainly the luteoid fluid, was recovered as the supernatant after recentrifugation.

Isolation and Purification of the ENSP Homologue—The ENSP homologue was prepared based on the method of Subroto *et al.* (11). Latex B-serum was dialyzed overnight against 0.3 M sodium borate and 0.016 M boric acid (pH 7) in the cold room. Dialyzed B-serum was filtered through Whatman No. 1 paper and loaded onto a carboxymethyl cellulose CM32 (Whatman) column equilibrated with 0.09 M sodium borate and 0.016 M boric acid, pH 8.6. Proteins were eluted on a gradient of 150 ml of 0.09 M sodium borate and 0.016 M boric acid, pH 8.6, to 0.9 M sodium borate and 0.16 M boric acid, pH 8.6. The unretarded material was loaded into a diethylaminoethyl (DEAE) DE52 (Whatman) column equilibrated with 0.1 M Tris-HCl, pH 8. Protein elution was carried out on a gradient from 0 to 0.5 M NaCl in the same buffer. Fractions containing proteins of about 43 kDa, as determined by SDS-polyacrylamide gel electrophoresis (22), were tested for immunoglobulin E (IgE) binding using serum from latex-allergic patients. In later experiments, monoclonal or polyclonal antibodies against the ENSP homologue (developed for this study by one of us, R. G. H.) were used for protein detection as they became available.

Immunoaffinity Purification of the ENSP Homologue—Further purification of the ENSP homologue was performed by immunoprecipitation. The ENSP homologue was bound to its monoclonal antibody by incubating for 1 h at 4 °C. The protein-antibody was then captured onto protein G coated on Sepharyl beads (Protein G-Sepharose 4 Fast Flow, Amersham Biosciences) by incubating for another h at 4 °C. After washing successively with phosphate-buffered saline (PBS) and wash buffer (50 mM Tris, pH 8) to remove unbound (irrelevant) protein, the Sepharyl beads were heated to 95 °C for 3 min in reducing electrophoretic sample buffer to elute the ENSP homologue. The protein was then loaded onto an SDS-polyacrylamide gel for electrophoretic separation.

Preparation of Hev b 7—Native Hev b 7.01 and Hev b 7.02 were prepared from the latex C-serum and B-serum, respectively, according to the method of Yusof *et al.* (14). Briefly, C-serum or B-serum was fractionated on a Sephadex G-150 (Amersham Biosciences) column equilibrated in 250 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol and 1% Tween 20. The reactive fractions that were identified

using a polyclonal antibody against the protein were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, before anion-exchange chromatography onto a DEAE cellulose (Whatman) column equilibrated in the same buffer, with 5 mM 2-mercaptoethanol added. After washing unbound protein from the column with the Tris-HCl buffer, immunoreactive fractions were recovered using a linear gradient of NaCl (0–0.3 M) in the buffer. The reactive fractions were pooled and dialyzed against a buffer comprising 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, and 0.5 M NaCl for further purification by hydrophobic interaction chromatography on a column of phenyl-Sepharose (Amersham Biosciences). After removing unbound protein from the column with the buffer, the immunoreactive protein was eluted using the same buffer but with the NaCl omitted.

Determination of Molecular Mass and Internal Amino Acid Sequences—The molecular mass of the protein was determined by matrix-assisted laser desorption ionization mass spectrometry. Briefly, 5 μ l (5 pmol) of purified protein was concentrated and desalted in a C4Zip Tip and eluted with a sinapinic acid matrix in 50% acetonitrile, 0.05% trifluoroacetic acid. Bovine serum albumin was used for external calibration.

Two methods were used in the determination of internal amino acid sequences of the protein. In the first approach, the protein was separated on a 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The target protein band was excised and subjected to trypsin digestion followed by HPLC. N-terminal sequencing by Edman degradation was carried out on three digestion products at the Keck Facility, Yale University (New Haven, CT). In the second method, trypsin-digested peptide mixtures were desalted on a Poros R2 sorbent (PerSeptive Biosystems) in GELoader tips (Eppendorf) and eluted into nanoelectrospray needles (Protana Engineering, Odense, Denmark) as described previously by Neubauer and Mann (23). Mass spectrometry was performed on a QSTAR Pulsar hybrid quadrupole time-of-flight mass spectrometer (AB-MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (Protana Engineering) to determine the internal amino acid sequences.

Isolation and Cloning of cDNA—Standard methods as detailed below were used in the preparation and purification of DNA, DNA mini- and maxipreps, restriction endonuclease digestions, agarose gel electrophoresis, ligations, and transformations.

Preparation of Latex mRNA—Latex was collected by tapping the rubber tree that was fitted with a sterilized drainage spout. Immediately upon tapping, the incision and spout were washed with about 20 ml of 2 $\times$ RNA extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 0.01 M EDTA, 10% SDS, pH 9.5). The latex was then washed down with 100 ml of RNA extraction buffer to a total collected volume of 200 ml in a sterile conical flask. In the laboratory, the latex was mixed well and centrifuged in polyallomer tubes at $112,700 \times g$ for 30 min at 15 °C. The aqueous phase was gently decanted into sterile centrifuge tubes, and subsequent processing of the aqueous phase to isolate total RNA was performed according to the method of Prescott and Martin (24).

Reverse Transcription-PCR and Molecular Cloning—First strand cDNA synthesis was prepared by reverse transcribing 1 μ g of total latex RNA in a volume of 20 μ l using the GeneRacer kit (Invitrogen) per the vendor's instructions. Synthesis of the cDNA was accomplished by PCR amplification using degenerate primers based on internal amino acid sequences. The PCR product (~1.4 kb in size) was ligated into the TOPO® vector (Invitrogen), transformed into One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen), and selected for ampicillin resistance following the vendor's instructions. After incubating overnight at 37 °C in agar medium containing ampicillin, transformants were picked by Xgal/isopropyl-1-thio- β -D-galactopyranoside color selection. Transformants were screened by miniprep assay using the Wizard® Plus SV Minipreps DNA purification system (Promega, Madison, WI) per the vendor's instructions. The ends of the cDNA clone were extended by 5'- and 3'-RACE techniques using the SMART RACE cDNA amplification kit (Clontech, BD Biosciences) per the vendor's instructions.

DNA Sequence Analysis—*E. coli* strains containing plasmids of interest were grown overnight, and the plasmids were isolated using the Wizard® Plus SV Minipreps DNA purification system (Promega) according to the manufacturer's instructions. The DNA in the plasmids was sequenced according to the protocol stated in the ABI PRISM BigDye Terminator v3.0 reaction cycle sequencing kit (Applied Biosystems, Foster City, CA) using internal primers plus the M13 reverse and forward primers.

Electrophoretic Separation of Proteins, Western Blot, Antibody Reactivity, and Carbohydrate Detection—Twenty-two individual sera were identified as being IgE-immunoreactive with the ENSP homologue

¹ The abbreviations used are: ENSP, early nodule-specific protein; PBS, phosphate-buffered saline; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high pressure liquid chromatography; RACE, rapid amplification of cDNA ends; TFMS, trifluoromethane sulfonic acid; ELISA, enzyme-linked immunosorbent assay.

(Hev b 13) but not with Hev b 7. Three of these sera were combined into a serum pool for the detection of IgE antibody in the Western blots. Two sera that were IgE-immunoreactive with Hev b 7 but not with Hev b 13 were combined into a second serum pool. All latex-allergic patient sera were sourced from the United States.

Proteins were separated by 12% SDS-PAGE under reducing conditions and Western blotted onto nitrocellulose membranes for immunodetection as described previously (19). Briefly, 5% nonfat milk was used for blocking, after which the nitrocellulose membranes were incubated with the relevant polyclonal rabbit antibodies, monoclonal murine antibodies, or diluted (16%) serum from latex-allergic patients. Bound rabbit IgG, mouse IgG, and human IgE were detected in separate blots with alkaline phosphatase-conjugated anti-rabbit IgG, anti-mouse IgG, and anti-human IgE, respectively. Subsequent reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate generated the colored enzyme product.

For the experiments to demonstrate the inhibition of IgE binding to latex allergen by the *Hevea* ENSP homologue or by potato (*Solanum tuberosum*) tuber patatin (a gift from Prof. Harry Gruppen, Wageningen University, The Netherlands), human serum was preincubated with 40 μ g of inhibiting protein (or buffer in the control) overnight at 4 °C. This preincubated human serum mixture was then used to probe nitrocellulose strips blotted with the antigenic protein.

Visualization of glycosylation in proteins Western blotted onto nitrocellulose membranes was 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. Glycoproteins were deglycosylated by trifluoromethane sulfonic acid (TFMS) using the Glycofree™ deglycosylation kit (Oxford GlycoSystems) following the vendor's protocol.

Quantitation of IgE Reactivity by ELISA—The frequency of positive reactions of Hev b 7.01 and the ENSP homologue to human IgE antibody was also determined using an ELISA format. In this study, sera from 78 latex-allergic Americans with latex-specific IgE antibodies were obtained. Sixty-five patients were adult health care workers, and 13 were children with spina bifida.

The test protein (Hev b 7.01 or the ENSP homologue) was coated on wells of a microtiter plate at 5 μ g ml⁻¹ in PBS for 1 h at 37 °C followed by 16 h at 4 °C. The plate was washed once with PBS, 1% Tween 20 and blocked with PBS, 1% bovine serum albumin for 1 h. Undiluted patient test sera were pipetted into duplicate wells and incubated 1 h at 37 °C followed by 16 h at 4 °C. Following four buffer washes, a 1:1 mixture of two biotin-conjugated murine monoclonal anti-human IgE Fc antibodies (clones HP6029 and Hp6061) (Calbiochem Corporation, San Diego, CA) was added to all wells (0.1 ml, 1 μ g ml⁻¹ in PBS, 1% bovine serum albumin, 1 h, 37 °C). After an additional four buffer washes, streptavidin-horseradish peroxidase (Sigma) was pipetted into the wells (0.1 ml, 1 μ g ml⁻¹ in PBS, 1% bovine serum albumin, 1 h, 37 °C). Following final buffer washes, 1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) prepared in 70 mM citrate-phosphate buffer, pH 4.3, and containing 1 μ l of 30% H₂O₂/ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added to each well. Absorbance was read at 410 nm in a microplate reader. IgE-protein binding was deemed positive when the reading was greater than 3 standard deviations above the optical density levels obtained with sera from non-latex-allergic control patients.

Lipase Activity—Lipolytic activity with Tween 80 as substrate was measured spectrophotometrically using 1% (w/v) Tween 80 in 20 mM Tris-HCl, pH 7, containing 80 mM calcium chloride (25). The rate of increase in absorbance at 450 nm was recorded upon adding the *Hevea* ENSP homologue.

Esterase Activity—Esterase activity was measured using 100 μ l of the esterase substrate, 1 mM α -naphthyl acetate in 0.1 M phosphate buffer, pH 6.5. Incubation with the *Hevea* ENSP homologue (100 μ l, 5 μ g ml⁻¹) was carried out for 20 min at 30 °C, and the reaction was stopped by adding 25 μ l of a solution of 3.4% sodium dodecyl sulfate and 0.3% Fast Blue B salt. Absorbance readings were read at 595 nm on an ELISA plate reader.

RESULTS

Protein Characterization—Two proteins of closely similar molecular weights (M_r ~43,000) were isolated from the latex B-serum. One of these was Hev b 7.02, which was purified using the method that was used to isolate Hev b 7.01 (14). Using a different experimental protocol, a second protein that was slightly smaller than Hev b 7.02 on an SDS-polyacryl-

amide gel was obtained. Their apparent molecular masses as computed based on the calibration markers were, respectively, 43 and 42 kDa. The molecular mass of the latter, the *Hevea* ENSP homologue, was 42,975 Da as determined by mass spectrometry.

Western blotted Hev b 7.01, Hev b 7.02, and the ENSP homologue were tested for the presence of covalently bound carbohydrates. The ENSP homologue was shown to be glycosylated, whereas neither Hev b 7.01 nor Hev b 7.02 were glycoproteins.

The ENSP homologue also possessed both lipase and esterase activities with the selected substrates. Using 1% Tween 80 as lipase substrate, the ENSP homologue produced 9.8 μ mol of oleic acid/mg of ENSP homologue/min. Esterase activity using α -naphthyl acetate as a substrate was demonstrated photometrically with the test reaction showing a mean absorbance of 0.861 against 0.101 for the blank control.

The N terminus of the ENSP homologue was found to be blocked. Internal amino acid sequences of the ENSP homologue were obtained from tryptic digests using two different methods. Three peptides were purified by HPLC, and their sequences were determined by Edman degradation. Amino acid sequencing of peptides resulting from trypsin digestion was also carried out by nanoelectrospray MS analysis. Peptides selected for MS/MS analysis from an initial MS analysis (Fig. 1) were subjected to collision-induced dissociation of peptide ions in the second quadrupole of the quadrupole time-of-flight mass spectrometer to generate b- and y-type fragment ions. *De novo* sequences were generated for the predicted fragment ion spectra of the doubly charged ions at m/z 550.3, 659.9, and 777.9 using BioAnalyst™ software (Applied Biosystems). These three fragment ion spectra gave sequences that were identical to the spectra obtained experimentally. This is exemplified in Fig. 2, which shows the fragment ion spectrum for m/z 659.9. The amino acid sequences of three peptides that were obtained were identical to the three obtained by Edman degradation. These were the following: Sequence 1, YLDVQYSQFR (1319 Da); Sequence 2, YSLFSEPEK (1100 Da); and Sequence 3, LPTTIIPAHGGFSSR (1556 Da). The ProBLAST script was used to search *de novo* sequences generated automatically from spectra using the MSBLAST (26) search engine at the European Molecular Biology Laboratory (EMBL) Web site. The three peptides at m/z 550.3, 659.9, and 777.9 matched the "early nodule protein" (ENSP) with a very high degree of sequence similarity in *Glycine max* (Sequences 1 and 3) and in *Medicago truncatula* (Sequence 2).

Isolation and Characterization of the ENSP Homologue cDNA—The degenerate primers 5'-TANTCTNTNTTNNNG-ANCCAGAAAAA-3' and 5'-TACCTTGATGTCCAATATNNNC-AATTC-3' in the sense and antisense orientation were designed based on the amino acid sequences derived from the internal amino acid sequences YSLFSEPEK and YLDVQYSQF, respectively. A product of ~500 bp was sequenced and found to contain almost identical amino acid sequences when translated from the cDNA. The ends of the Hev b 13 cDNA clone were extended by 5'- and 3'-RACE techniques. For the 5'-end extension, first strand cDNA was synthesized using *Hevea* RNA for PCR with the primer sequence 5'-CTCGGGC-ACCAATTCAGCAAATATGCC-3' and a Universal Primer A mix (UPM) primer from the SMART RACE cDNA amplification kit. For the 3'-end extension, the primer 5'-TACCAACTACT-ATTATACCTGCTCATGGTGG-3' and a UPM were used instead. The DNA sequences that were obtained at the 5'-end of the 5'-RACE (5'-GCGGGGGCGTTAACACTTGGTTTTTGC-3') and the 3'-end of the 3'-RACE (5'-TAGGAAACCGAGAAAG-ACAAGTCTT-3') were then used as PCR primers to obtain the

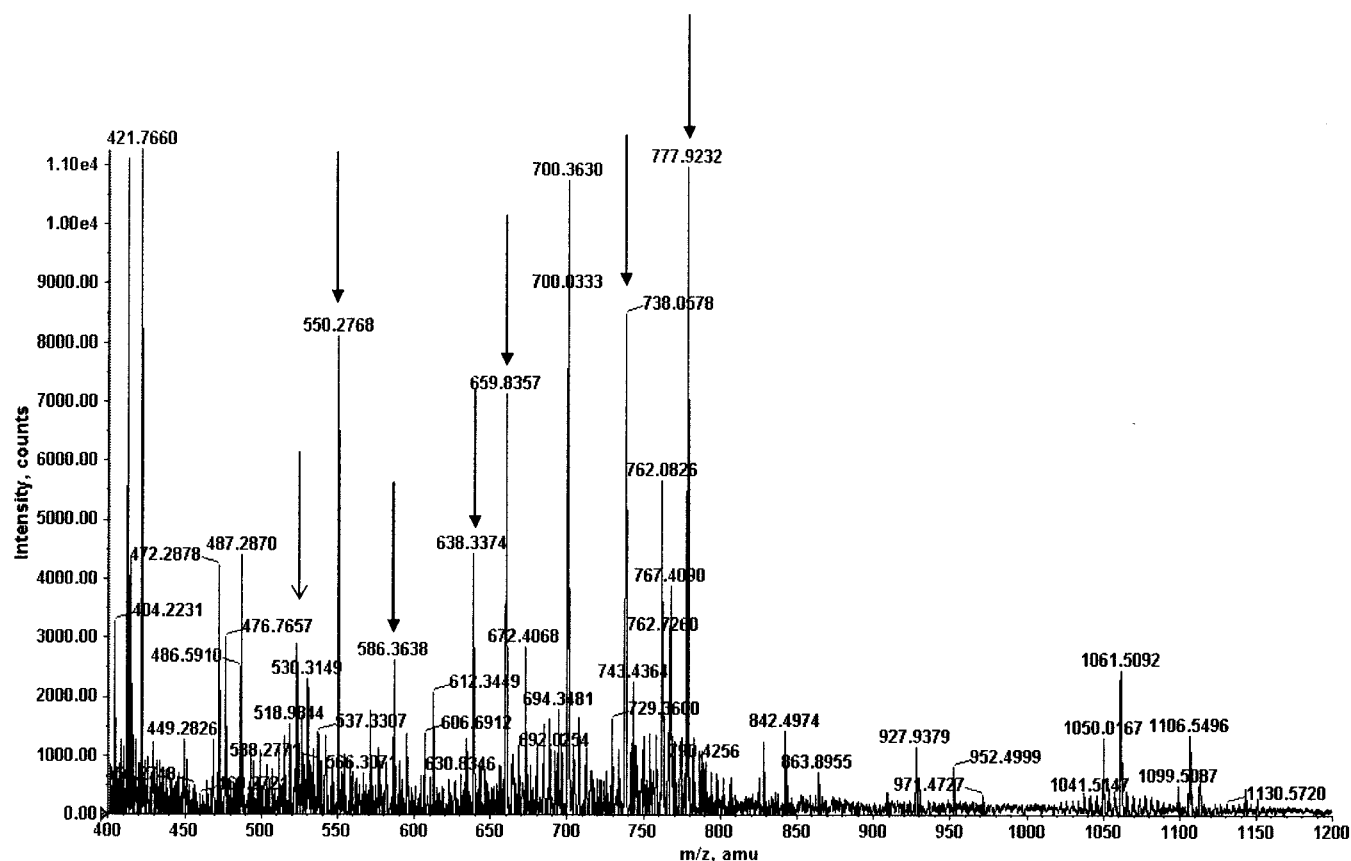


FIG. 1. Nanoelectrospray MS analysis of the tryptic digest of the ENSP homologue. Arrows indicate peptides selected for subsequent MS/MS analysis. *amu*, atomic mass unit.

full-length cDNA clone that comprised 1394 base pairs (Fig. 3). The internal amino acid sequences obtained by mass spectrometry matched the corresponding portions translated from the cDNA sequences, with the exception of two amino acids at positions 129 (Ser in place of Pro) and 130 (Arg in place of Phe) as indicated in Fig. 3.

The cDNA clone only has a short 5'-end untranslated region (39 bp) and a long 3'-untranslated region (182 bp) with three potential polyadenylation signal sites at the 3'-end. Two methionine possible start sites were encountered at position 39 and 96. The first possible (and probable) open reading frame started at position 39 and stopped at position 1212, giving a translated protein of 391 amino acids with a calculated molecular mass of 43,334 daltons. The second possible open reading frame started at position 96, giving a protein of 372 amino acids with a calculated molecular mass of 41,155 daltons. With the longer open reading frame, a signal peptide was predicted at amino acid sequences AYA↓SE (ExPASy SignalP V1.1 program) (Fig. 3) (27). The mature protein thus had a deduced molecular mass of 40,400 Da. The discrepancy between this value and the molecular mass of 42,975 Da determined by mass spectrometry might be due to glycosylation. Two estimates of the isoelectric point of the protein were 4.9 and 5.1 using the ExPASy L'Atelier BioInformatique de Marseille program (Université Aix-Marseille I) and the ExPASy Compute pI/Mw Tool program (28), respectively. The ExPASy ScanProsite program (29) predicted three possible Asn glycosylation (*N*-glycosylation) sites at 186–188 (NLT), 193–195 (NAT), and 313–315 (NFS) as shown in Fig. 3. Comparison of the protein sequence deduced from DNA with entries in the National Center for Biotechnology Information (NCBI) data base using the BLAST algorithm revealed 60.91% identity with the *Medicago sativa* early nodule-specific protein (ENOD8) (Fig. 3).

Allergenic Characteristics of the ENSP Homologue—Because of the close similarity in molecular weight and pI, it was important to ensure that allergenicity of the ENSP homologue, as indicated by the ability of the protein to bind IgE, was not due to contamination by Hev b 7.02 that was also found in latex B-serum. Incubating Western blotted Hev b 7.02 and the latex ENSP homologue with two different pools of sera from latex-allergic patients showed that each pool was reactive to one of the two proteins (Fig. 4). This observation demonstrated that the two proteins had dissimilar IgE epitopes. To confirm further that IgE binding to the ENSP homologue was not due to contamination by some other allergenic latex allergen, the protein was immunoaffinity-purified by binding it to its monoclonal antibody that was then captured onto protein G coated on Sepharacryl beads. After washing to remove any contaminating proteins, the protein was subjected to SDS-PAGE and Western immunoblotting using latex-allergic patient serum. The protein reacted with its monoclonal antibody and with IgE, thus validating both its identity and its allergenic property (Fig. 5). In this experiment, the latex allergen, Hev b 2, and its monoclonal antibody were used as controls. In one negative control, Hev b 2 was mismatched with the monoclonal antibody specific for the ENSP homologue. The other negative control was the ENSP homologue protein that was mismatched with the monoclonal antibody specific for Hev b 2 (Fig. 5).

Assays of IgE recognition of the latex ENSP homologue by ELISA were carried out with 78 sera from latex-allergic American subjects. Of the 65 adult latex-allergic patient sera that were analyzed by ELISA, 78% were positive for IgE specific to the ENSP homologue. The proportion of positive cases among the spina bifida sera was identical, with IgE in 77% of the sera recognizing the ENSP homologue (Table I). IgE reactivity among the sera from both the adult health care workers and

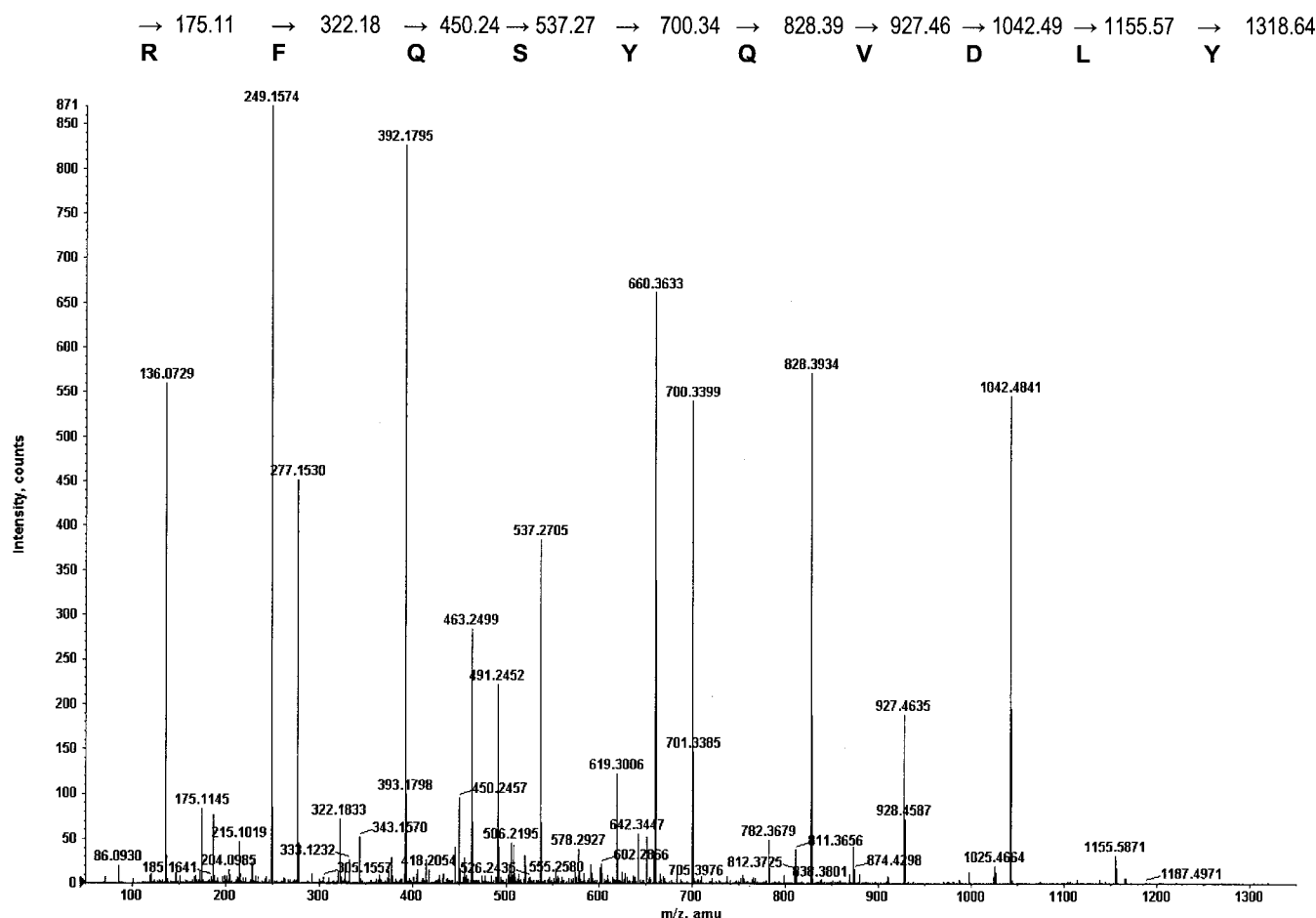


FIG. 2. MS/MS (fragment ion) spectrum of the doubly charged ion at m/z 659.9. Peptides from trypsin digest were subjected to collision-induced dissociation of peptide ions in the second quadrupole of the quadrupole time-of-flight mass spectrometer. Residues labeled with single letter codes are based on the mass difference between two adjacent C-terminal y-type fragment ions. *amu*, atomic mass unit.

pediatric spina bifida subjects to Hev b 7.01 was significantly lower ($p \leq 0.001$, Fisher's exact probability test) (30) as compared with the ENSP homologue reactivity. The frequency of IgE reactivity was 32% among the sera of health care workers and only 8% (one subject of 14) for sera from the latex-allergic spina bifida children.

Preincubation of serum with patatin inhibited IgE binding to Western blotted ENSP homologue (Fig. 6, *strip 4*). This was despite the fact that the two proteins do not share common amino acid sequences. It was noted, nevertheless, that they were both glycoproteins. To investigate the possibility that the IgE epitope of the ENSP homologue resided in its carbohydrate component, the protein was deglycosylated using TFMS. Removal of the carbohydrate from the ENSP homologue resulted in a slight decrease in apparent molecular weight as observed on an SDS-polyacrylamide gel (Fig. 7). Significantly, IgE binding with the protein was lost upon its deglycosylation (Fig. 7). In comparison, TFMS treatment of Hev b 3 (a non-glycosylated protein that served as a positive control) did not affect IgE binding, showing that the reagent had no effect *per se* on amino acid IgE epitopes (data not presented). In IgE inhibition experiments, the binding of IgE to the ENSP homologue was no longer inhibited by either the ENSP homologue or patatin when these proteins were deglycosylated (Fig. 6, *strips 5 and 6*).

DISCUSSION

Amino acid sequence analyses and immunological reactions with specific monoclonal and polyclonal antibodies have demonstrated that the ENSP homologue is a new latex allergen

(Hev b 13). It is quite distinct physically and immunologically from Hev b 7, with which it had been confused previously. Of the two proteins, sensitization to Hev b 13 has been shown to be significantly more widespread as indicated by a higher frequency of IgE antibody reactivity from adult health care workers and pediatric spina bifida sera (Table I). The disparity between the two proteins has been confirmed in a different adult population by skin prick testing (31). The observation in the present study that a low percentage of latex-allergic children with spina bifida is sensitized to Hev b 7.01 (7.6% positive) is in agreement with the observation of Sepälä *et al.* (20) (0 positive of 7 patients). However, it varies with the findings of Wagner *et al.* (32) (39.5% positive) and Kurup *et al.* (18) (77, 23, and 15% positive in three studies). Hev b 13 was not evaluated in these above mentioned investigations.

The 78% prevalence in IgE-mediated sensitization to Hev b 13 in the present study and 63% prevalence of allergy to the protein as shown by skin prick tests (31) place the *Hevea* ENSP homologue in the category of a major latex allergen according to the definition of the International Union of Immunological Societies (IUIS) that designates major allergens as those serologically reactive with at least 50% of allergic individuals (33).

That the ENSP homologue had been misidentified as Hev b 7.02 was perhaps unsurprising. The close similarities in molecular masses of the two proteins (about 43 kDa) and isoelectric points (~ 5) required stringent purification procedures for their separation. Both proteins were closely similar in their migration on SDS and on native polyacrylamide electrophoretic

Hb	acgcggggcggttaacacttggtttttgcttccacttcatggagttccctgaaaccaataa	61
Hb	- M E F P E T N N	8
Ms	M A R I E L S R H	9
Hb	caaccctatcatcactctctctttcttattatgcatgctttccctagcttatgcttccgaa	122
Hb	N P I I T L S- F L L C M L S L <u>A Y A</u> ↓S E	28
Ms	M : L V : : IV L V : : T T P P I F : : T	30
Hb	acctgtgattttccagcaatctttaacttcggcgactccaattccgataccgggtggcaagg	183
Hb	T C D F P A I F N F G D S N S D T G G K	48
Ms	H : : : : : : : S : : A : : V : : : : L	50
Hb	cagctgccttttatcctcttaaccctccttatggagagactttctttcacaggctcgacagg	244
Hb	A A A F Y P L N P P Y G E T F F H R S T G	69
Ms	: : : : R A P P S : : : Q : Y : N : : : :	71
Hb	aaggctactctgatggaaggctcataatagattttatcgccgagagtttcaatctcccatat	305
Hb	R Y S D G R L I I D F I A E S F N L P Y	89
Ms	: F : : : : I : : : : : : Q : : R : : :	91
Hb	ctgagtcctatcttagttccctgggaagcaacttcaaactggtgcagattttgccacag	366
Hb	L S P Y L S S L G S N F K H G A D F A T	109
Ms	P : : : : N : : : : : : T : : : N : : :	111
Hb	caggatccaccattaaactaccaactactattatacctgctcatggtggatttagtcatt	427
Hb	A G S T I K <u>L P T T I I P A H G G F S P F</u>	130
Ms	: : : : : N <u>I</u> : : S : L : - K : I L : : :	131
Hb	ctaccttgatgtccaatatttcgcaattccggcaattcatacccagatcacagtttatcagg	488
Hb	<u>Y L D V Q Y S Q F R Q F I P R S Q F I R</u>	150
Ms	S : Q I : : I : : K D : : S K T K L : :	151
Hb	gaaactggaggcatatttgctgaattggtgcccgaggaatattattttgagaaagctttat	549
Hb	E T G G I F A E L V P E E Y Y F E K A L	170
Ms	D Q : : V : : T : : : K : D : : S : : :	171
Hb	acacattcgcgatatttggtcaaaatgatcttacagaaggattcttgaacttaactgtggaaga	610
Hb	Y T F D I G Q N D L T E G F L- N* L T V E E	191
Ms	: V : : : : : : : : : I : : FG : K : I Q Q	193
Hb	agtgaatgcaactgtccctgatcttgtgaatagcttctcagcaaactgtaagaaaatatac	671
Hb	V N* A T V P D L V N S F S A N V K K I Y	211
Ms	: : : : : : : : : N Y I E : I : N : :	213
Hb	gatttgggagctagaacattttggattcacacacaggaccaattggtgtctttcattca	732
Hb	D L G A R T F W I H N T G P I G C L S F	231
Ms	N : : : : S : : : : S : : : K : : A P V	233
Hb	ttttaacgtattttccctgggcagaaaaggatagtgaggtgtgcaaaagcttacaatga	793
Hb	I L T Y F P W A E K D S A G C A K A Y N E	252
Ms	: : A N : : S : I : : : Y : : : : Q : : :	254
Hb	agttgctcagcattttaatacacaagttgaaggagatcgttgcactcaggaaggatttg	854
Hb	V A Q H F N H K L K E I V A Q L R K D L	272

FIG. 3. DNA sequence of the *Hevea* ENSP homologue (Hev b 13) cDNA clone. In the two rows marked *Hb*, lowercase letters depict the DNA sequences, whereas capital letters depict the amino acid sequences. The highlighted *aataaa* sequences are the possible signal sites for 3'-end trimming and poly(A) tail addition. Amino acid sequences obtained by Edman chemistry and by mass spectrometry are underlined. These sequences were used to design degenerate primers in the initial stages of the cDNA cloning. A signal peptide is deduced, with the cleavage site (arrow) at AYASE (double underlined). Three predicted *N*-glycosylation sites are indicated by asterisks. The row marked *Ms* shows the sequence comparison of Hev b 13 and the ENOD8 protein of *M. sativa* (BLASTX accession number S59943). Identical sequences are depicted by colons (:), and gaps (-) are introduced for maximal alignment.

gels. Both were allergenic as evidenced by their reactivity with human IgE antibody. Both Hev b 7 (16) and the ENSP homologue (this report) showed esterase activity. An 80-kDa allergenic esterase that included a 45-kDa subunit observed after SDS-polyacrylamide gel electrophoresis has been reported by Yagami *et al.* (34). However, it is not clear whether their

protein might be analogous to either Hev b 7 or the ENSP homologue. Perhaps the most persuasive circumstantial evidence for Hev b 7 and the ENSP homologue being thought to be the same protein arose from the observation that IgE binding by the latter was inhibited by patatin. Since Hev b 7 is a patatin homologue, it was a reasonable confirmation, although

Ms	: S : Y : : L : : : : A L : : : : S : :	274
Hb	ccttttagctacattcgtccacggttgacatctattctgtcaagtattcttttattcagtgagc	915
Hb	P L A T F V H V D I Y S V K Y S L F S E	292
Ms	: : : A I T Y : : : : : P : : : : : T N	294
Hb	cagaaaaacacggttttcgagtttccacttataacatggttggtgctacggaggaaagtacaa	976
Hb	P E K H G F E F P L I T C C G Y G G K Y N*	313
Ms	: K : Y : : : L : Y V A : : : : : E : :	315
Hb	tttttagtggttactgctccatgtggagatacagttacagcagacgacggtacaaaaatagtt	1037
Hb	F S V T A P C G D T V T A D D G T K I V	333
Ms	- - I G : G : : A : I N V N - : : : : :	332
Hb	gtgggttcatgtgcttgcccttcagttcgagtaaattgggatggagctcactacactgaag	1098
Hb	V G S C A C P S V R V N W D G A H Y T E	353
Ms	A : : : K N : : T : I T : : : T : : : :	352
Hb	ctgccaatgaatattttttcgaccagatttctacaggagccttctctgatccccctgttcc	1159
Hb	A A N E Y F F D Q I S T G A F S D P P V P	374
Ms	: : : K I V : : : : : : : : : N : : : I S	373
Hb	attgaatatggcatgtcataaaactgaatcattgaggacattagcctctgtataggttata	1220
Hb	L N M A C H K T E S L R T L A S V	391
Ms	: D : : : Y R K - - - - - - - - -	381
Hb	tgaagtgctttgctgaaagcccgcataataaataatgaggaataataataaataagagaacccat	1281
Hb	tgattatgtaggattcacttggtttctatcataataatctatctgttgatatatacaacag	1342
Hb	ttgtatgaaatagtttcttctaataaagacttggtcttttctccggtttcccta	1394

FIG. 3—continued

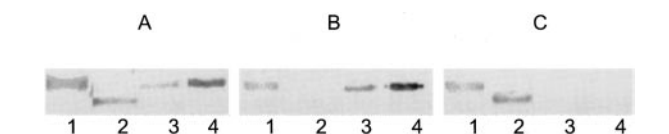


FIG. 4. Binding of IgE from different serum pools to Hev b 7 and the Hevea ENSP homologue (Hev b 13). The Hev b 7 and Hev b 13 proteins were Western blotted following SDS-polyacrylamide gel electrophoresis and then incubated with two pools of sera from latex-allergic patients. A, Coomassie Blue staining of the gel. B and C, Western blots of similar gels reacted with two pools of patient sera and detected for IgE binding. Lane 1, molecular mass marker (45 kDa); lane 2, ENSP homologue (Hev b 13); lane 3, Hev b 7.01; lane 4, Hev b 7.02.

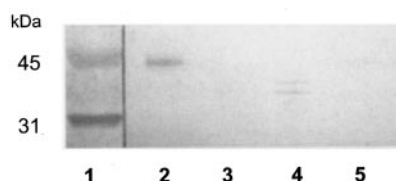


FIG. 5. IgE binding to affinity-purified Hevea ENSP homologue (Hev b 13). The Hevea ENSP homologue (Hev b 13) and Hev b 2 were captured by monoclonal antibodies bound to a solid support of Sephacryl G beads. Affinity-captured proteins were Western blotted and incubated with sera combined from latex-allergic subjects. Detection of IgE binding to the affinity-purified proteins was then performed. The latex allergen Hev b 2 (a 35-kDa doublet) and its monoclonal antibody were used as controls. Lane 1, molecular mass markers; lane 2, Hev b 13 matched with anti-Hev b 13 (test result); lane 3, Hev b 2 matched with anti-Hev b 13 (negative control); lane 4, Hev b 2 matched with anti-Hev b 2 (positive control); lane 5, Hev b 13 matched with anti-Hev b 2 (negative control).

shown subsequently to be wrong, that the two proteins were the same. Surprisingly, there is no peptide homology between the amino acid sequences for patatin and the ENSP homologue.

It was noted, nevertheless, that both were glycoproteins. Experiments were therefore performed to determine whether the IgE epitope for the ENSP homologue might be located on the carbohydrate moiety of the glycoprotein and whether patatin might possess a similar carbohydrate component that could explain its IgE inhibition effect. IgE reactivity that was sensitive to periodate treatment (implying a carbohydrate IgE epitope) had been reported previously for latex proteins (35). When deglycosylated, the ENSP homologue no longer bound IgE, and neither was it capable of inhibiting the binding of IgE with its untreated glycosylated form. Similarly, deglycosylated patatin lost its ability to inhibit IgE binding with the ENSP homologue. These observations are consistent with the proposition that the specific IgE epitope of the ENSP homologue is located on its carbohydrate moiety and that patatin has a similar carbohydrate component. Further confirmation of the role of carbohydrate could be seen from the fact that the recombinant form of this protein synthesized in *E. coli*, and therefore non-glycosylated, was not reactive with IgE (results not presented). The carbohydrate components of many allergenic plant glycoproteins are not known to have a significant role in allergenicity. Where these carbohydrates do interact with IgE, it is widely believed that such cross-reactive carbohydrate determinants commonly have no clinical significance in allergies (36, 37). The ENSP homologue (Hev b 13), on the other hand, has been shown to be active by skin prick tests (31). Recent research has also shown the carbohydrates of some plant glycoproteins, *e.g.* Ole e 1 of olive pollen (38) and Lyc e 2.02 of tomato (39), to be instrumental in precipitating the allergic reaction. Such observations have led to a call for the critical reconsideration of the general claim that cross-reactive carbohydrate determinant-specific IgE is clinically irrelevant (39).

TABLE I
Frequency of IgE in sera binding to Hev b 7.01 and to the Hevea ENSP homologue

Source of sera	Sample size	Hev b 7.01	Hevea ENSP homologue (Hev b 13)
Latex-sensitized adult health care workers	65	21 (32%)	51 (78%)
Latex-sensitized spina bifida children	13	1 (7.7%)	10 (77%)

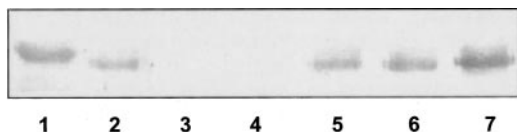


FIG. 6. Inhibition of IgE-Hevea ENSP homologue (Hev b 13) binding by patatin. Hevea ENSP homologue (Hev b 13) was Western blotted following SDS-polyacrylamide gel electrophoresis. Strips 3–7 of nitrocellulose membrane carrying the protein were reacted with IgE from serum of latex-allergic subjects. The serum had been preincubated with various proteins. Strip 1, 45-kDa molecular mass marker; strip 2, Coomassie Blue staining; strip 3, serum preincubated with ENSP homologue; strip 4, serum preincubated with patatin; strip 5, serum preincubated with deglycosylated ENSP homologue; strip 6, serum preincubated with deglycosylated potato patatin; strip 7, serum preincubated with buffer (uninhibited serum).

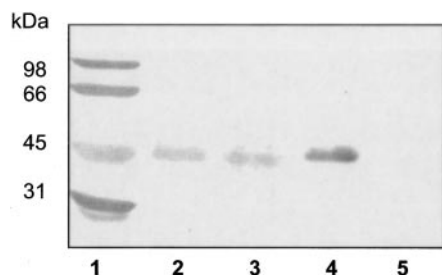


FIG. 7. Loss of IgE binding with ENSP homologue (Hev b 13) after deglycosylation with TFMS. Hevea ENSP homologue and its deglycosylated form were Western blotted following SDS-polyacrylamide gel electrophoresis, and IgE detection was performed. Lanes 1–3 are stained with Coomassie Blue; lanes 4 and 5 show IgE reactivity. Lane 1, molecular mass markers; lanes 2 and 4, untreated protein; lanes 3 and 5, deglycosylated protein.

A protein data base search of the partial protein sequences and the translated cDNA sequences showed significant similarity of the ENSP homologue to several nodule-specific proteins (notably ENOD8 of the legume *Medicago*). The Hevea ENSP homologue was isolated from the latex B-serum that was derived from the bottom fraction of centrifuged latex. Hence, the presence of a putative signal peptide in the cDNA sequence was consistent with its location in the lutoids, a latex organelle that makes up most of the bottom fraction in centrifuged latex. The *Medicago* sequence was nine residues shorter at its C terminus than in the case of the Hevea ENSP homologue. Further work is required to determine whether these nine residues might represent the vacuolar targeting signal that directs the protein to the lutoids. Such sequences were demonstrated in four other B-serum proteins by comparing C-terminal sequences of mature proteins with those derived from cDNA studies (40). Nine cysteine residues that were found in the protein may be involved in disulfide bridges, or they may be present as free cysteines. They did not appear to influence trypsin proteolytic susceptibility. The deduced amino acid sequence from the cDNA was closely similar to that obtained from direct amino acid sequencing of the trypsin digests, with the exception of two amino acids at positions 129 (Ser) and 130 (Arg). The SR sequence that was obtained in our testing is in better agreement with trypsin specificity. Various isoforms of the Hevea ENSP homologue may therefore exist.

Esterase activity of the ENSP homologue has been observed earlier. A latex B-serum protein (identified as the ENSP homo-

logue in the present study) was shown by Jekel *et al.* (12), who used *p*-nitrophenyl palmitate as the esterase substrate. The Hevea ENSP homologue carried in its protein sequence FGD-SNSDTGGKAAAFYPLNPPY, which was consistent with the motif (F/T)G(N/D)SXXDXG(N/G)X(10–20 of any amino acid)PY, which identified a family of lipolytic plant enzymes proposed by Brick *et al.* (41). As a lipase, the protein was substrate-specific, being reactive with Tween 80 but having little detectable activity with olive oil (data not presented). In leguminous plants, the early nodule-specific protein is thought to have a role in nodule organogenesis or in infection of the emergent nodule by symbiotic bacteria (42). In non-leguminous plants, similar proteins were detected in *Arabidopsis* shoots and anthers (41, 43). The function of its homologue in Hevea latex is unknown, but its lipase and esterase properties might be involved in plant defense. Lipolytic esterase activity has been linked to the protection of bean leaves from disease (44). Expression of an ENSP-like gene from *Brassica napus* (45) was increased in response to wounding. In fact, the plant defense function frequently occurs in allergenic proteins of plant origin (46). This trait is common among the latex allergens (47). Besides the ENSP homologue (Hev b 13) that is the subject of this study, eight of the remaining 12 recognized allergenic latex proteins (Hev b 2 (glucanase), Hev b 3 (stress-induced), Hev b 4 (cyanogenic glucosidase), Hev b 6 (wound-induced, chitin-binding), Hev b 7 (lipid acyl hydrolase), Hev b 10 (superoxide dismutase), Hev b 11 (endochitinase), and Hev b 12 (lipid transfer protein)) are stress- or pathogenesis-related proteins.

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