

# Proteomics analysis of latex from *Hevea brasiliensis* (clone RRIM 600)

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**Abstract:** The natural rubber latex extracted from the bark of *Hevea brasiliensis* plays various important roles in today's modern society. Following ultracentrifugation, the latex can be separated into 3 layers: C-serum, lutoids, and rubber particles. Previous studies have shown that a large number of proteins are present in these 3 layers. However, a complete proteome for this important plant is still unavailable. Protein sequences have been recently translated from the completed draft genome database of *H. brasiliensis*, leading to the creation of annotated protein databases of the following *H. brasiliensis* biosynthetic pathways: photosynthesis, latex allergens, rubberwood formation, latex biosynthesis, and disease resistance. This research was conducted to identify the proteins contained within the latex by way of de novo sequencing from mass spectral data obtained from the 3 layers of the latex. Peptides from these proteins were fragmented using collision-induced dissociation, higher-energy collisional dissociation, and electron-transfer dissociation activation methods. A large percentage of proteins from the biosynthetic pathways (63% to 100%) were successfully identified. In addition, a total of 1839 unique proteins were identified from the whole translated draft genome database (AnnHBM).

**Key words:** *Hevea brasiliensis*, rubber latex, plant proteomics, mass spectrometry, biosynthetic pathways.

**Résumé :** Le latex naturel extrait de l'écorce de *Hevea brasiliensis* remplit diverses fonctions importantes dans la société moderne d'aujourd'hui. Après une ultracentrifugation, le latex peut être séparé en 3 couches : le sérum C, les lutoïdes et les particules de caoutchouc. Des études précédentes ont montré qu'un grand nombre de protéines étaient présentes à l'intérieur de ces trois couches. Cependant, le protéome complet de cette plante importante n'est toujours pas disponible. Des séquences protéiques ont été récemment traduites de la base de données complétée de l'ébauche du génome de *H. brasiliensis*, menant à la création de bases de données protéiques annotées des voies de biosynthèse de *H. brasiliensis* ; la photosynthèse, les allergènes du latex, la formation de l'hévéa, la biosynthèse du latex et la résistance aux maladies. Cette recherche a été réalisée afin d'identifier les protéines contenues dans le latex au moyen du séquençage de novo à partir des données de spectrométrie de masse obtenues des trois couches du latex. Les peptides de ces protéines ont été fragmentés à l'aide de méthodes d'activation par dissociation induite par collision (« collision-induced dissociation »), dissociation induite par collisions à plus haute énergie (« higher-energy collisional dissociation ») et dissociation par transfert d'électrons (« electron-transfer dissociation »). Un large pourcentage de protéines des voies de biosynthèse a été identifié avec succès, allant de 63 à 100 %. De plus, un total de 1839 protéines uniques a été identifié de la base de données de l'ébauche de tout le génome traduit (AnnHBM). [Traduit par la Rédaction]

**Mots-clés :** *Hevea brasiliensis*, latex, protéomique végétale, spectrométrie de masse, voies biosynthétiques.

## Introduction

Rubber latex is a living cytoplasm from laticifer cells located in the secondary phloem of rubber trees (*Hevea brasiliensis*). This latex is formed and stored in the rings of anastomosing laticifers in the inner bark. It consists of 3 major subcellular fractions in which rubber particles, C-serum, and lutoids are dispersed in an aqueous phase of the cytosol (Moir 1959; Dickenson 1969). These 3 subcellular fractions contain a variety of proteins that are responsible for latex biosynthesis, defense, and other biological pathways.

Rubber particles, the colloidal components that are naturally present in latex, comprise 30%–40% of the whole latex volume (Southorn and Yip 1968; Gomez and Hamzah 1989). The particles consist of homogeneous and spherical rubber cores enveloped with a layer of membrane (Moir 1959; Yeang et al. 2002; Wu et al. 2008). The size of the particles varies from 0.02 to 3.00  $\mu\text{m}$ , and the particles have been divided into 3 different Moir's zones accord-

ing to size (Dickenson 1969; Southorn 1969; Hamzah and Gomez 1982; Yeang et al. 1995). Two different major proteins with molecular masses of 14.6 and 24 kDa have been identified in rubber particles. These proteins were classified as proteolipids (Hasma 1987; Wititsuwannakul et al. 2004) and identified as latex allergens (i.e., Hev b1 and Hev b3) (Alenius et al. 1993; Czuppon et al. 1993; Yeang et al. 1996). The latter protein has been proposed to be tightly associated with small rubber particles and involved in the biosynthesis of rubber (Oh et al. 1999). Recently, a lectin-like protein was identified that is responsible for latex coagulation and mediating rubber particle aggregation (Wititsuwannakul et al. 2008).

The aqueous phase of laticiferous cytoplasm, C-serum, is metabolically active. This fraction contains about 60% of whole latex proteins. Tata and Edwin (1970) showed a large number of proteins in gel electrophoresis analysis.  $\alpha$ -Globulin, the most abundant protein, contributes to the colloidal stability of the latex and rubber particles (Archer et al. 1963; Archer and Cockbain 1955). In

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addition, several glycolytic enzymes and other common cytosolic enzymes are found in C-serum and are actively involved in different metabolic processes (d'Auzac and Jacob 1969; Wititsuwannakul et al. 1990; Suvachittanont and Wititsuwannakul 1995; Koyama et al. 1996). For instance, calmodulin (a heat-stable calcium-binding protein) was found to be responsible for regulating different interrelated metabolic processes (Tangpakdee et al. 1997). It also has been shown to have the ability to activate HMG-CoA reductase in the lutoid or membrane fraction (Wititsuwannakul et al. 1990). In addition, several allergens (i.e., Hev b5, Hev b7, Hev b8, and Hev b9) have been identified.

Lutoids, the bottom layer after ultracentrifugation, play important roles in latex coagulation and protect the tree from exterior invasion (Wei et al. 2008; Kongawadworakul et al. 2009). They are vacuolar organelles that contain a number of pathogenesis-related proteins with antifungal properties (Stintzi et al. 1993). Research has shown that hevein is the major protein (approximately 50%) of the lutoid body. It is formed via a proteolytic process in which the N-terminal domain (5 kDa) and several residues at the C-terminus are removed from a precursor protein (19 kDa), leaving a 14 kDa protein with a ragged C-terminal structure (Soedjanaatmadja et al. 1994). Two other major proteins, heveamine and  $\beta$ -1,3-glucanase, with molecular masses of 29 and 32–35 kDa, respectively, have also been identified (Breton et al. 1995; Churngchow et al. 1995; Sunderasan et al. 1995; Subroto et al. 1996). The cDNA sequences of these proteins were determined and were found to be post-translationally modified via removal of an N-terminal signal peptide and a C-terminal vacuolar targeting sequence (Bokma et al. 2001; Chye and Cheung 1995). Other proteins, such as osmotin-like protein (a 25 kDa antifungal protein) and citrate-binding protein (responsible for citrate accumulation in lutoids), have also been identified.

A very limited number of proteins have been identified to date. A proteome analysis of the rubber particles of *H. brasiliensis* revealed 186 proteins ranging from 3.9 to 194.2 kDa (Dai et al. 2013). A study on the different subcellular fractions of latex of the rubber-producing plant *Taraxacum brevicorniculatum* revealed that the proteins involved in rubber biosynthesis were distributed among the fractions (Wahler et al. 2012). Information on post-translational modifications is even more limited. Therefore, a new strategy is needed to identify as many proteins as possible to better understand the various biological pathways in rubber latex. In our collaborative research, we have used a genomic approach to identify the proteins in rubber latex. Databases of the proteins in different pathways (photosynthesis, latex allergens, rubberwood formation, latex biosynthesis, and disease resistance) have been established. The details of each pathway of other databases were discussed in a previous work (Rahman et al. 2013). Therefore, using a proteomic approach to search the proteins expressed in fresh rubber latex would validate the results of the genome analysis. An efficient strategy with good-quality data is essential to obtain promising results.

The main objective of this study was to use proteomics to validate the results of the previous genome analysis (Rahman et al. 2013), so the results and discussion in this article will depend heavily on their work. To detect proteins of both high and low abundance, we used multiple activation protocols (collision-induced dissociation, CID; higher-energy collision dissociation, HCD; and electron-transfer dissociation, ETD) to generate characteristic fragments in the tandem mass spectrometry experiment, which ultimately established amino acid sequences. In addition, we used a mass spectrometry method developed by Julian Saba and colleagues that uses HCD and ETD techniques to detect glycoproteins of low abundance (Saba et al. 2012). An Orbitrap mass spectrometer that has the capability to perform CID, HCD, and ETD analyses coupled with nano liquid chromatography was utilized, and mass identification of proteins in each subcellular fraction was then performed using PEAKS Studio 6. Hence, this approach would

assist in understanding the pathways in different phases of the biological cycle.

## Methodology

### Latex collection

A rubber tree (*H. brasiliensis*, clone RRIM 600) grown in Baling, Kedah, Malaysia, was tapped in the early morning. The fresh latex was collected in plasticware, immediately frozen in liquid nitrogen, and sent to the laboratory.

### Protein extraction and purification

Protein was extracted from the rubber latex according to Wang et al. (2010) with slight modifications. The fresh latex was thawed and ultracentrifuged for 30 min at 40 000g and 4 °C. Rubber particles (upper white rubbery layer), C-serum (middle aqueous layer), and lutoids (bottom creamy layer) were identified and separated for further purification as described below.

### Purification of different layers

The rubber particles were washed with ice-cold buffer containing 20 mmol/L Tris-HCl, 300 mmol/L mannitol, and 0.5 mmol/L dithiothreitol (DTT) at pH 7.2 at a ratio of 1:10 (w/v). The suspension was stirred for 30 min and subsequently ultracentrifuged for 15 min at 30 000g and 4 °C. The upper rubbery layer was collected. The C-serum was ultracentrifuged for 15 min at 30 000g and 4 °C. The upper clear phase was collected. The lutoids were also added to ice-cold buffer containing 20 mmol/L Tris-HCl, 300 mmol/L mannitol, and 0.5 mmol/L DTT at pH 7.2 at a ratio of 1:10 (w/v). The suspension was stirred for 10 min and subsequently ultracentrifuged for 15 min at 30 000g and 4 °C. The bottom creamy layer was collected.

### Extraction of protein from different subcellular fractions

The 3 different fractions (rubber particles, C-serum, and lutoids) were separately added to buffer containing 100 mmol/L EDTA, 100 mmol/L Tris (pH 8.0), 50 mmol/L Borax, 50 mmol/L vitamin C, 1% (w/v) polyvinylpyrrolidone, 1% (v/v) Triton X-100, 2% (v/v)  $\beta$ -mercaptoethanol, and 30% (w/v) sucrose at a ratio of 1:5 (w/v). The mixtures were vortexed for 30 min.

For rubber particles, the mixture was sonicated for 5 min at 70 W/cm<sup>2</sup> in an ice bath and subsequently ultracentrifuged for 15 min at 15 000g and 4 °C. The clear phase was collected and 2 volumes of Tris-saturated phenol (pH 8.0) were added. The mixture was then vortexed for 15 min. For C-serum and lutoids, 2 volumes of Tris-saturated phenol (pH 8.0) were added and the mixtures were vortexed for 15 min.

All mixtures were subsequently centrifuged for 15 min at 15 000g and 4 °C. The upper phase was collected and precipitated with 4 volumes of cold acetone. The precipitated protein was washed with cold acetone and air-dried prior to fractionation.

### Gelfree protein fractionation

Extracted protein samples from each subcellular fraction were fractionated based on molecular mass using the GELFREE 8100 fractionation system (Protein Discovery, USA). A mid-mass cartridge kit (fractionation of proteins with molecular masses between 3.5 and 150 kDa) was used in this experiment. A protein sample of 250  $\mu$ g (in solution) was mixed with acetate sample buffer (30  $\mu$ L) and DTT (8  $\mu$ L) to a final total volume of 150  $\mu$ L prior to fractionation. The fractionation conditions are shown in Table 1. A total of 12 fractions were collected and stored at -20 °C prior to further use.

### In-solution digestion

All Gelfree protein fractions were digested separately using trypsin according to the method of Ru et al. (2006). Samples were reduced and alkylated prior to digestion. Briefly, protein samples were solubilized in 500  $\mu$ L of denaturing buffer (6 mol/L guanidine-HCl and 25 mmol/L ammonium bicarbonate, pH 8.5). Then, 250  $\mu$ L of 1 mg/mL

**Table 1.** Fractionation conditions.

|                         |    |      |    |    |    |     |     |     |     |     |     |     |     |
|-------------------------|----|------|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|
| Voltage (V)             | 50 | 50   | 50 | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Fraction interval (min) | 16 | 41.5 | 2  | 2  | 3  | 2   | 2   | 3   | 5   | 7   | 10  | 15  | 20  |
| Fraction No.            | —  | 1    | 2  | 3  | 4  | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |

DTT/25 mmol/L ammonium bicarbonate was added to the protein solution and incubated at 55 °C for 30 min. Next, 500 µL of 1 mg/mL iodoacetamide/25 mmol/L ammonium bicarbonate was added, and the mixture was covered with aluminium foil and incubated at 55 °C for 15 min.

The reduced and alkylated protein sample was then exchanged 3 times with 25 mmol/L ammonium bicarbonate using a spin column with a molecular mass cutoff of 3 kDa. Then, 250 µL of digestion buffer (25 mmol/L ammonium bicarbonate) and trypsin (ratio 1:50) was added to the sample and incubated at 37 °C for 18 h. The protein samples were then either stored at –20 °C or directly analyzed using mass spectrometry.

### Mass spectrometry

All liquid chromatography–mass spectrometry (LC–MS) and tandem mass spectrometry (MS/MS) analyses were carried out using a Thermo LTQ/Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, Calif., USA) coupled with an Easy-nLC II system (Thermo Scientific). Chromatographic separation of trypsin-digested peptides was performed using a C18-A2 EASY-Column (100 × 0.75 mm i.d., 3 µm; Thermo Scientific) coupled with a pre-column (EASY-Column, 20 × 0.1 mm i.d., 5 µm; Thermo Scientific) at a flow rate of 0.3 µL/min. The sample injection volume was 10 µL. The pre-column was equilibrated for 15 µL at a flow rate of 3 µL/min, whereas the analytical column was equilibrated for 4 µL at a flow rate of 0.3 µL/min. The following running buffers were used: (A) deionized distilled water with 0.1% formic acid, and (B) acetonitrile with 0.1% formic acid. Gradient elution was performed from 5% to 100% buffer B over 85 min and held at 100% buffer B for another 15 min. The mass spectrometer was calibrated and mass calibration coefficients were determined using a mixture of caffeine, MRFA peptide, and Ultramark 1600 for normal mass calibration and poly(propylene glycol) 2700 with 7 mmol/L sodium acetate in 70% methanol for high mass calibration. Data acquisition was performed using Xcalibur ver. 2.1 (Thermo Scientific) with a mass tolerance threshold of 5 ppm. The eluant was electrosprayed into the mass spectrometer at 2.3 kV (source voltage) and a capillary temperature of 200 °C was used.

Two MS/MS analysis methods were used for peptide detection. The first method, dubbed the proteomic approach, was able to analyze peptides of both high and low abundance, while the second method, dubbed the glycoproteomic approach, was able to analyze peptides of low abundance and glycopeptides. For the proteomic approach, peptides were detected by full-scan mass analysis from  $m/z$  300 to 2000 at a resolving power of 60 000 (at  $m/z$  400, full width at half maximum; 1-s acquisition) with data-dependent MS/MS analyses triggered by the 8 most abundant ions from the parent mass list of predicted peptides with rejection of singly or unassigned charge states. The MS/MS analysis was performed using the same resolving power (60 000) and CID was conducted with isolation width of 2 Da, normalized collision energy of 35, activation  $q$  of 0.25, activation time of 50 ms, and charge state of 2. HCD was conducted with isolation width of 2 Da, normalized collision energy of 35, activation time of 0.1 ms, and Fourier transform first mass value ( $m/z$ ) of 100. ETD was conducted with isolation width of 2 Da, normalized energy of 35, activation  $q$  of 0.25, activation time of 100 ms, and charge state of 3 or more. For the glycoproteomic approach, the mass spectrometric acquisition parameters were based on the work of Julian Saba and colleagues (Saba et al. 2012). Briefly, the acquisition uses the complementary fragmentations of ETD and HCD intelligently. ETD spectra are acquired only when glycan oxonium ions from HCD fragmenta-

**Table 2.** List of glycosylations.

| Glycosylation                          | Mass    |
|----------------------------------------|---------|
| Biantennary                            | 1622.58 |
| Biantennary (–1 galactose)             | 1460.53 |
| Biantennary (–2 galactose)             | 1298.48 |
| Fucosylated biantennary (–1 galactose) | 1606.59 |
| Fucosylated biantennary (–2 galactose) | 1444.53 |
| Hex1HexNAc1                            | 365.13  |
| HexNAcylation (ST)                     | 203.08  |
| N-linked glycan core                   | 1216.42 |

tion are detected. The oxonium ions are hexose, hexosamine, hexosamine fragments, and hexose-hexosamine.

### Analysis of mass spectrometric data

PEAKS Studio 6 was used to construct predicted peptide sequences using MS/MS data without the need to refer to known protein databases. PEAKS reads the RAW files generated by LC-MS/MS for all fractions of CID, HCD, and ETD of C-serum, lutoids, and rubber particles and extracts the peptide information during data refinement. The charge options were set to 2–10 with a filter quality of >0.65.

Automated de novo peptide sequencing was carried out on each sample and then all fractions were combined into one de novo result. Parent mass error tolerance of 0.1 Da and fragment mass error tolerance of 0.8 Da with trypsin digestion were inserted as the sequencing parameters. De novo sequencing uses all the extracted information from MS/MS data and predicts potential peptide sequences. The process produced a list of de novo peptide sequences identified from MS/MS spectra.

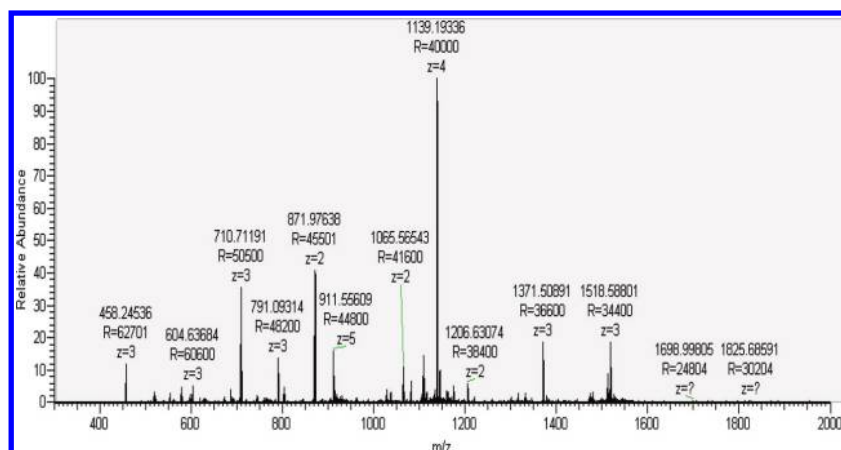
This was followed by a standard workflow of PEAKS database identification (PEAKS DB + PEAKS PTM + SPIDER) against the annotated *H. brasiliensis* genome database (<http://bioinfo-ccb.usm.my/cgi-bin/gb2/gbrowse/Rubber/>), PlantGDB (<http://www.plantgdb.org/>), and Swiss-Prot (<http://www.uniprot.org/>). The workflow started with a PEAKS DB search in which the list of candidate peptides was aligned against proteins of annotated databases. The parameters included carbamidomethylation of cysteine and oxidation of methionine as variable modifications. The search continued with the second process in the workflow, PEAKS PTM, wherein post-translational modifications were predicted and included in the alignment. The parameters were set to all 670 types of modification as variables. Lastly, SPIDER was used to find more mutations on the candidate peptides to identify more proteins.

In all 3 workflow processes, the peptide-spectrum matches were filtered using high-confidence peptide identification at a 1% false discovery rate (FDR) for databases with a huge number of sequences (>1000; AnnHBM, PlantGDB, and Swiss-Prot), while for databases with a small number of sequences (<1000; latex allergens, disease resistance, rubberwood formation, latex biosynthesis, and photosynthesis), the filter  $-\log P = 20$  was used instead. The protein filters were set to  $-\log P = 0$  and 1 or more unique peptide hits. The FDR was calculated using a decoy database search.

The standard workflow results of CID, HCD, and ETD for C-serum, lutoids, and rubber particles were compared using Venny (Oliveros 2007) to develop Venn diagrams to determine the number of unique proteins found in each database.

For the glycoproteomic method, the PEAKS PTM step was skipped and selected glycosylation masses were included in the PEAKS DB step. The list of selected glycosylations and their masses

**Fig. 1.** Representative mass spectrum of HBM\_contig260697\_s115748c12\_gene1.protein. R, resolution; z, charge state. [Colour online.]



is displayed in Table 2. All the filter parameters from the proteomic method were also used. The standard workflow results of the glycoproteomic method for C-serum, lutoids, and rubber particles were compared using Venny before comparing the results of the proteomic and glycoproteomic approaches.

## Results and discussion

Rubber latex is a complex mixture of proteins. Therefore, fractionations according to the subcellular fraction and then based on molecular mass were performed prior to mass spectrometry analysis. This protocol would allow more proteins of low abundance to be detected. The proteins found in this study were matched to several established translated genome databases including latex allergens, disease resistance, photosynthesis, rubberwood formation, and latex biosynthesis databases.

Figure 1 displays a representative mass spectrum from this experiment. Ion peaks with high resolution ranging from 24 000 to 63 000 were produced. The high resolution is essential for identification and confirmation of the molecular formula of the peptide sequences and thus determines the mass accuracy of the molecular ions. Ion peaks with double or higher charge were then selected to be fragmented. Figure 2 (A–D) shows representative MS/MS fragments and ion tables. These spectra have high signal-to-noise ratios and show complete or near-complete backbone fragmentation. Low error (<0.8 Da) was also found in the data.

Figure 3 shows 87% coverage of a whole protein sequence as an example of the high-quality de novo protein sequencing that was achieved. The blue lines underneath the protein sequence indicate the identified peptide sequences, showing the high repeatability of the peptide sequences in the analysis. The list of supporting peptides is displayed in Table 3. High accuracy (low mass differences, ppm) was found in the data. In addition to the high percent coverage, all MS/MS spectra were collected at high resolution and mass accuracy. Therefore, the quality of the data collected was convincing.

## Protein identification

Table 4 shows a summary of the total number of proteins found (by the proteomic method) in each subcellular fraction according to different databases. C-serum, lutoids, and rubber particles contributed a high number of proteins in each database. C-serum contributed the highest number of proteins identified in every translated genome database except for the latex biosynthesis database. The total non-redundant proteins represented 97.3% (36 of 37 proteins), 90.5% (38 of 42 proteins), 100% (43 of 43 proteins), 90.3% (290 of 321 proteins), and 61% (372 of 610 proteins) of the proteins from the photosynthesis, latex allergens, rubberwood formation, latex biosynthesis, and disease resistance databases, respectively. As shown in Fig. 4, the C-serum, lutoid, and rubber particle fractions

shared the majority of proteins. The number of proteins common to C-serum, lutoids, and rubber particles for each of the translated genome databases is 18, 28, 36, 115, and 103, respectively.

Table 5 shows a summary of the total number of proteins found (by the glycoproteomic method) in each subcellular fraction according to different databases. This approach, unlike the conventional glycomic methodology (Ismail et al. 2011), is not intended to rigorously characterize glycan structures but is used for high-throughput selection and sequencing of glycoproteins. This procedure also does not require any offline sample enrichment steps and thus can minimize sample losses. Although the glycoproteomic approach identified a lower number of proteins than the proteomic approach, it allowed detection of glycoproteins of low abundance that could not be detected via the proteomic approach. The percentage of proteins identified by the glycoproteomic method is significantly less than that identified by the proteomic method. The total non-redundant proteins represented 64.9% (24 of 37 proteins), 61.9% (26 of 42 proteins), 69.8% (30 of 43 proteins), 34.9% (112 of 321 proteins), and 20.7% (126 of 610 proteins) of the proteins from the photosynthesis, latex allergens, rubberwood formation, latex biosynthesis, and disease resistance databases, respectively. All glycosylated peptides identified from the translated genome databases through this approach were O-linked glycopeptides. Unfortunately, no glycosylated peptides were identified in small rubber particle protein as reported by Wititsuwannakul et al. (2008). Also,  $\beta$ -1,3-endoglucanase was identified, but owing to Ser-352 being replaced by Gly in clone RRIM 600, Asn-350 was not glycosylated (Subroto et al. 1996). Generally, the majority of unique proteins identified by the glycoproteomic method were found in C-serum, followed by lutoids and rubber particles (Fig. 5).

Table 6 shows a summary of the total number of proteins found using both methods based on different databases. By removing the proteins identified using both methods, more unique proteins were identified (Fig. 6). All of the proteins in the photosynthesis and rubberwood formation databases were identified. The percentage of proteins identified in the latex biosynthesis and disease resistance databases increased to 92.5% and 63.3%, respectively, while the percentage of proteins identified in the latex allergens database remained at 90.5%.

## Latex biosynthesis

The latex biosynthesis database is a collection of translated proteins involved in latex production in *H. brasiliensis*. The database consists of proteins from the entire rubber biosynthesis pathway, starting from sucrose. Six major steps, which can be divided into 12 sub-metabolic pathways involving proteins for sucrose import and degradation, glycolysis, acetyl-CoA biosynthesis, prenyl diphosphate

**Fig. 2.** Representative mass spectra of peptide sequences of HBM\_contig260697\_s115748c12\_gene1.protein: (A) AAVPSESTGLYEALR, (B) KLVLPVPAFNVINGGSHAGNK, (C) ACNALLKVNQIGSVTESTEAVK, and (D) SGETDTFIADLSVGLATGQIK. [Colour online.]

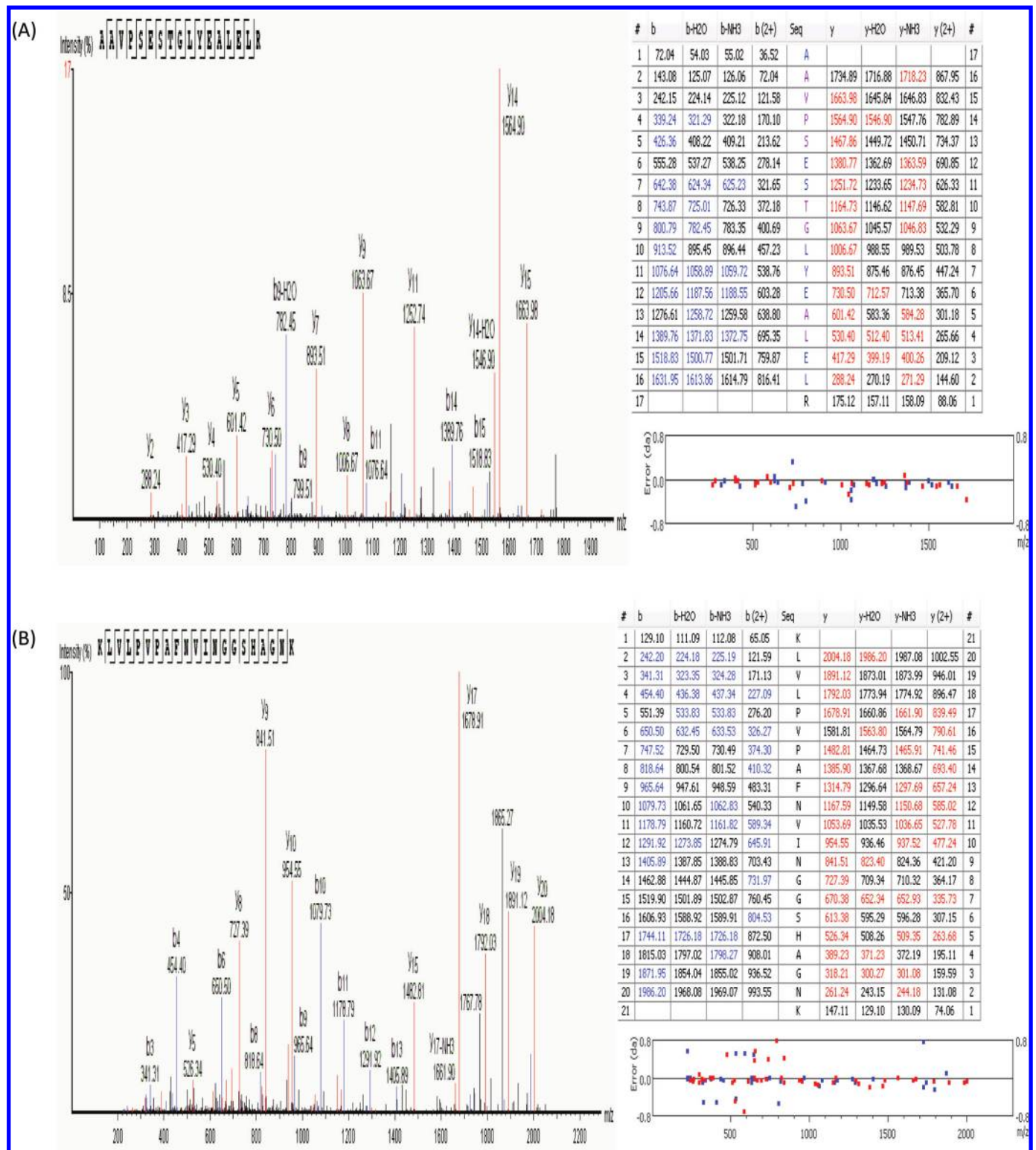
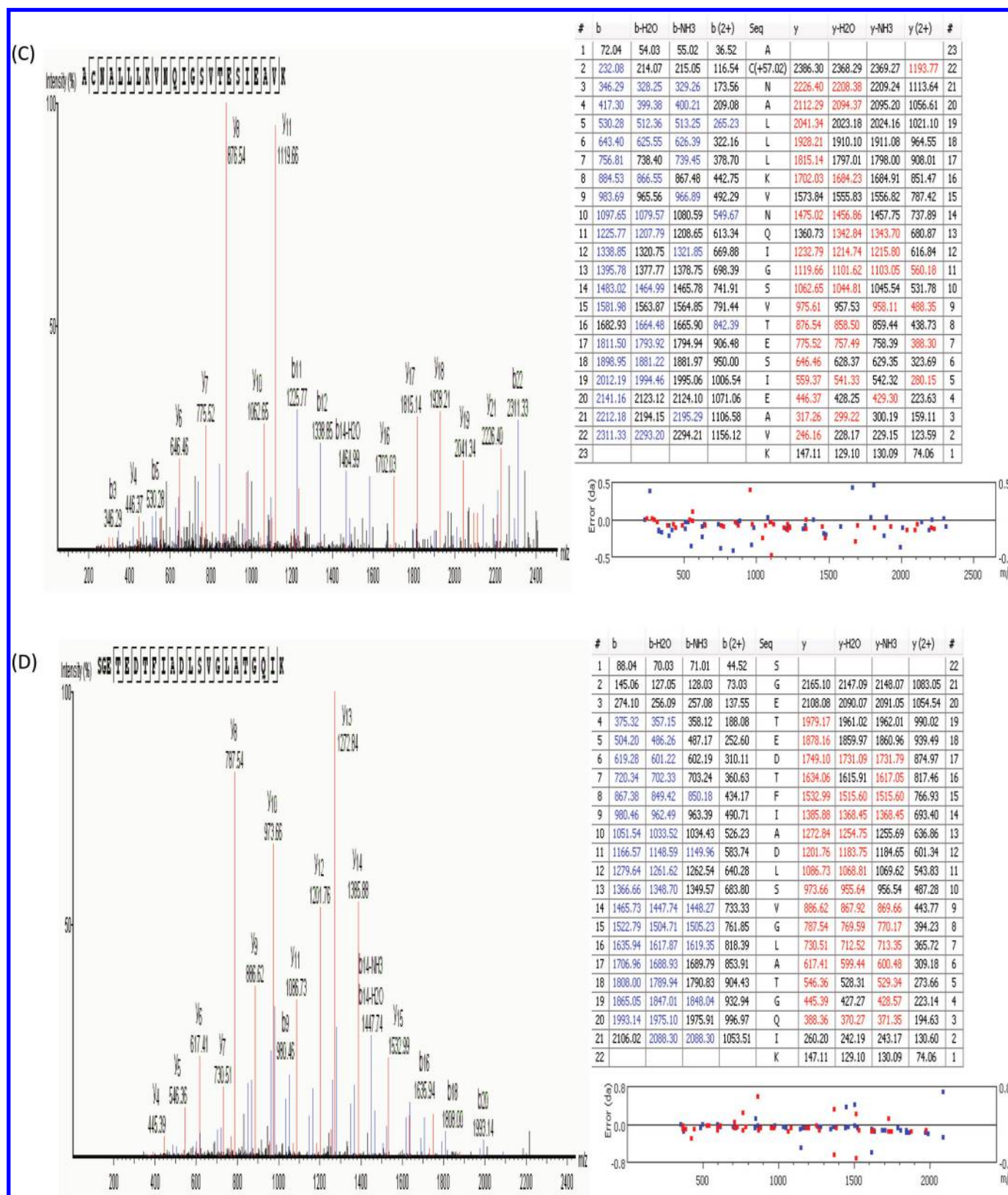


Fig. 2 (concluded).



synthesis via the mevalonate pathway in the cytosol, geranylgeranyl pyrophosphate synthesis in mitochondria, and rubber polymerization on the rubber particle membrane, were compiled. Rahman et al. (2013) constructed the entire metabolic pathway of

latex biosynthesis, represented by 417 genes encoding 321 proteins in the database and consisting of 12 distinct sub-metabolic pathways. We identified a total of 297 (92.5%) non-redundant proteins through our analysis (Table 6). The major proteins that

**Fig. 3.** Whole protein sequence of HBM\_contig338536\_s149176c3\_gene1.protein with identified peptide sequences (blue lines below the protein sequence). [Colour online.]

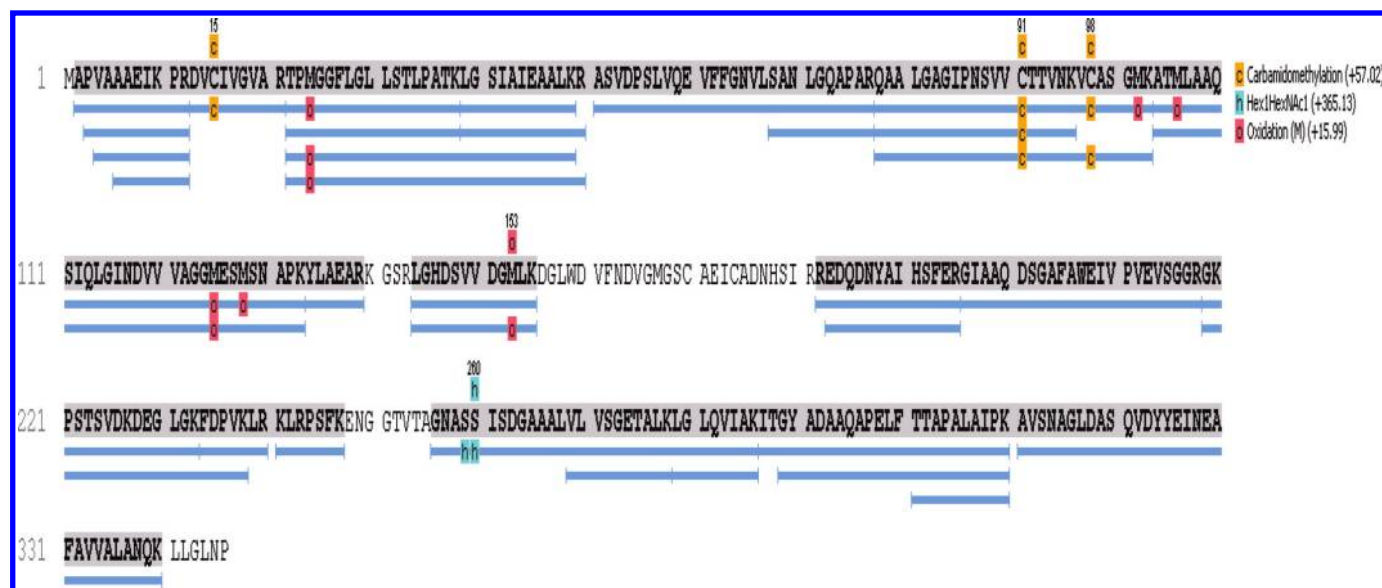


**Table 3.** Supporting peptides found in HBM\_contig338536\_s149176c3\_gene1.protein.

| Peptide                                                    | -10log P | Mass     | Start | End | PTM                                 |
|------------------------------------------------------------|----------|----------|-------|-----|-------------------------------------|
| R.TPM(+15.99)GGFLGLLSTLPATKLGSIAIEAALKR.A                  | 70.66    | 2941.668 | 22    | 50  | Oxidation (M)                       |
| R.GIAAQDSGAFWEIVPVEVSGGR.G                                 | 67.45    | 2315.155 | 196   | 218 |                                     |
| K.ITGYADAAQAPELFTTAPALAIK.A                                | 65.97    | 2429.284 | 287   | 310 |                                     |
| R.TPM(+15.99)GGFLGLLSTLPATK.L                              | 64.71    | 1718.928 | 22    | 38  | Oxidation (M)                       |
| R.REDQDNYAIHSFER.G                                         | 63.41    | 1778.797 | 182   | 195 |                                     |
| R.TPMGGFLGLLSTLPATK.L                                      | 62.02    | 1702.933 | 22    | 38  |                                     |
| R.ASVDPSLVQEVFFGNVLSANLGQAPAR.Q                            | 60.62    | 2785.44  | 51    | 77  |                                     |
| R.LGHDSVVDGM(+15.99)LK.D                                   | 59.64    | 1285.634 | 144   | 155 | Oxidation (M)                       |
| K.AVSNAGLDASQVDYYEINEAFVVALANQK.L                          | 56.65    | 3169.557 | 311   | 340 |                                     |
| R.QAALGAGIPNSVVC(+57.02)TTVNK.V                            | 55.18    | 1898.988 | 78    | 96  | Carbamidomethylation                |
| R.EDQDNYAIHSFER.G                                          | 54.03    | 1622.696 | 183   | 195 |                                     |
| R.TPM(+15.99)GGFLGLLSTLPATKLGSIAIEAALKR                    | 53.65    | 2785.566 | 22    | 49  | Oxidation (M)                       |
| A.PVAAAEIKPR.D                                             | 51.68    | 1050.619 | 3     | 12  |                                     |
| T.GYADAAQAPELFTTAPALAIK.A                                  | 49.17    | 2215.152 | 289   | 310 |                                     |
| R.GKPSTSVKDEGLGK.F                                         | 49.16    | 1516.773 | 219   | 233 |                                     |
| M.APVAAAEIKPR.D                                            | 49.16    | 1121.656 | 2     | 12  |                                     |
| R.GKPSTSVKDEGLGKFDPVK.L                                    | 48.7     | 2103.085 | 219   | 238 |                                     |
| R.QAALGAGIPNSVVC(+57.02)TTVNKVC(+57.02)ASGM(+15.99)K.A     | 47.19    | 2648.308 | 78    | 103 | Carbamidomethylation, oxidation (M) |
| R.DVC(+57.02)IVGVAR.T                                      | 44.97    | 987.5172 | 13    | 21  | Carbamidomethylation                |
| K.LGSIAIEAALKR.A                                           | 42.96    | 1240.75  | 39    | 50  |                                     |
| K.ATM(+15.99)LAAQSIQLGINDVVVAGGM(+15.99)ESM(+15.99)SNAPK.Y | 42.67    | 3050.472 | 104   | 133 | Oxidation (M)                       |
| R.QAALGAGIPNSVVC(+57.02)TTVNKVC(+57.02)ASGMK.A             | 42.02    | 2632.314 | 78    | 103 | Carbamidomethylation                |
| K.LGSIAIEAALKR                                             | 36.86    | 1084.649 | 39    | 49  |                                     |
| K.LGLQVIK.I                                                | 34.85    | 840.5433 | 279   | 286 |                                     |
| P.VAAAEIKPR.D                                              | 32.96    | 953.5658 | 4     | 12  |                                     |
| F.TTAPALAIK.A                                              | 31.98    | 981.5858 | 301   | 310 |                                     |
| R.KLRPSFK.E                                                | 31.21    | 874.5388 | 241   | 247 |                                     |
| K.FDPVKLR.K                                                | 29.19    | 873.5072 | 234   | 240 |                                     |
| L.SANLGQAPAR.Q                                             | 26.01    | 983.5148 | 68    | 77  |                                     |
| K.YLAEAR.K                                                 | 24.6     | 721.3759 | 134   | 139 |                                     |
| R.LGHDSVVDGMLK.D                                           | 24.5     | 1269.639 | 144   | 155 |                                     |
| L.VLVSGETALK.L                                             | 24.46    | 1015.591 | 269   | 278 |                                     |
| K.ATMLAAQSIQLGINDVVVAGGM(+15.99)ESMSNAPK.Y                 | 19.61    | 3018.482 | 104   | 133 | Oxidation (M)                       |
| A.AAEIKPR.D                                                | 17.03    | 783.4603 | 6     | 12  |                                     |
| A.GNAS(+365.13)S(+365.13)ISDGAAALVLVSGETALKLGLQVIK.I       | 16.57    | 3682.914 | 256   | 286 | Hex1HexNAc1                         |

Note: M, methionine; PTM, post-translational modification.

**Table 4.** Summary of the total number of proteins (with at least 1 unique peptide) found in each subcellular fraction of latex (proteomics method).

|                      | C-serum | Lutoids | Rubber particles | Non-redundant total | Percentage |
|----------------------|---------|---------|------------------|---------------------|------------|
| Photosynthesis       | 31      | 28      | 27               | 36                  | 97.3       |
| Latex allergens      | 34      | 34      | 34               | 38                  | 90.5       |
| Rubberwood formation | 42      | 40      | 39               | 43                  | 100        |
| Latex biosynthesis   | 201     | 220     | 212              | 290                 | 90.3       |
| Disease resistance   | 260     | 210     | 227              | 372                 | 61         |
| AnnHBM               | 845     | 671     | 649              | 1745                |            |
| Swiss-Prot           | 1120    | 594     | 754              | 2095                |            |
| PlantGDB             | 1849    | 1201    | 1236             | 3642                |            |

**Fig. 4.** Venn diagrams of the number of proteins found in each subcellular fraction based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) AnnHBM, (g) Swiss-Prot, and (h) PlantGDB. (See Supplementary Tables 1a–1h<sup>1</sup> for the full list of proteins identified from each database.) [Colour online.]

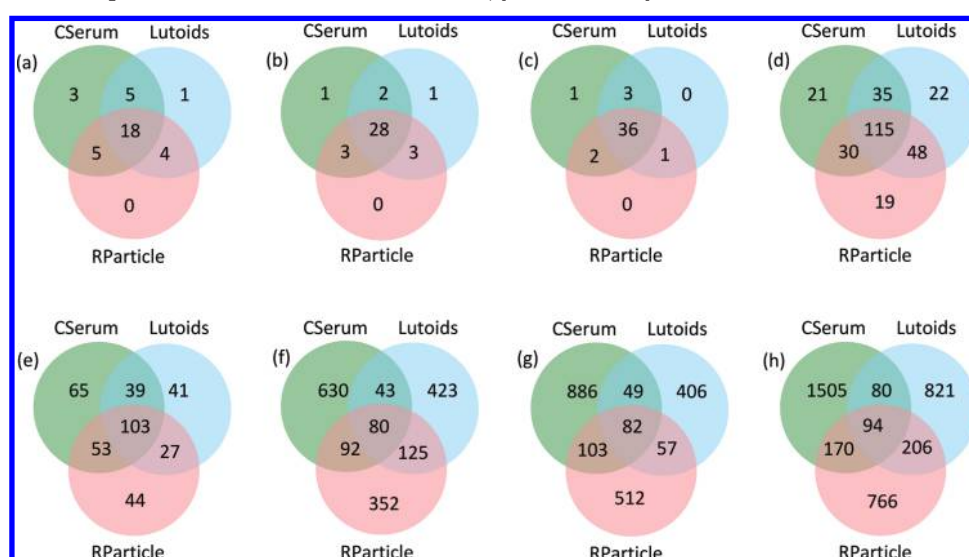


**Table 5.** Summary of the total number of proteins (with at least 1 unique peptide) found in each subcellular fraction of latex (glycoproteomics method).

|                      | C-serum | Lutoids | Rubber particles | Non-redundant total | Percentage |
|----------------------|---------|---------|------------------|---------------------|------------|
| Photosynthesis       | 15      | 6       | 9                | 24                  | 64.9       |
| Latex allergens      | 18      | 12      | 16               | 26                  | 61.9       |
| Rubberwood formation | 20      | 13      | 13               | 30                  | 69.8       |
| Latex biosynthesis   | 59      | 34      | 50               | 112                 | 34.9       |
| Disease resistance   | 43      | 50      | 49               | 126                 | 20.7       |
| AnnHBM               | 264     | 146     | 66               | 404                 |            |
| Swiss-Prot           | 400     | 117     | 74               | 557                 |            |
| PlantGDB             | 748     | 230     | 125              | 1025                |            |

were identified include beta-fructofuranosidase from both the sucrose import and fructan synthesis pathways and the glycolysis-related proteins phosphopyruvate hydratase, phosphoglycerate mutase, phosphoglycerate kinase, fructose-bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase. We also identified alpha-amylase, alpha-glucosidase, and oligo-1,6-glucosidase from the starch metabolism pathway, glucose-6-phosphate 1-dehydrogenase from the pentose phosphate pathway, acetyl-CoA C-acetyltransferase

from the acetyl-CoA biosynthesis pathway, carbonate dehydratase from the tricarboxylic acid cycle pathway, hydroxymethylglutaryl-CoA synthase from the prenyl diphosphate synthesis via mevalonate pathway, and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase from the 2-C-methyl-D-erythritol-4-phosphate pathway. In addition, we identified dimethylallyltransferase from the prenyl diphosphate biosynthesis pathway as well as rubber elongation factor and small rubber particle protein from the rubber polymerization pathway.

<sup>1</sup>Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/bcb-2016-0144>.

**Fig. 5.** Venn diagrams of the numbers of proteins and glycoproteins found in each subcellular fraction based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) AnnHBM, (g) Swiss-Prot, and (h) PlantGDB. (See Supplementary Tables 2a–2h<sup>1</sup> for the full list of proteins identified from each database.) [Colour online.]

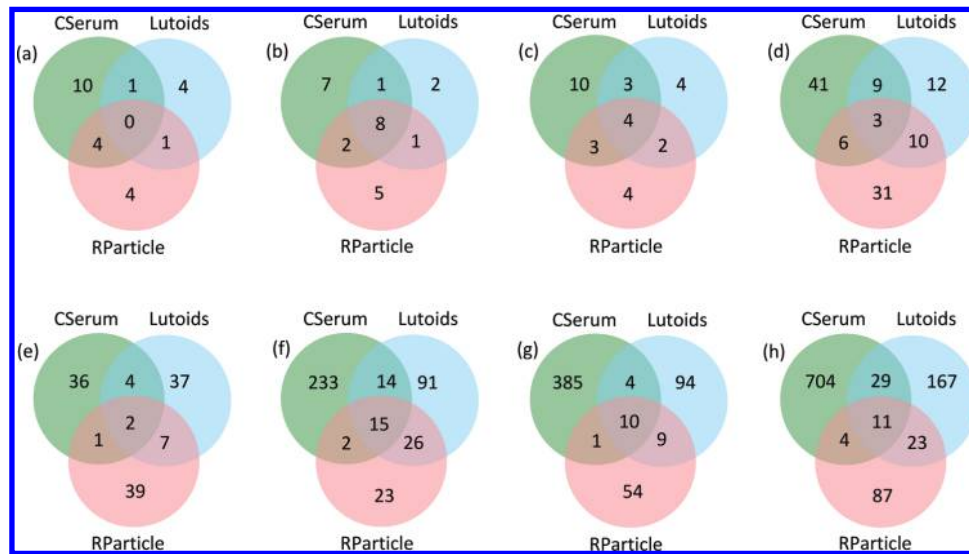
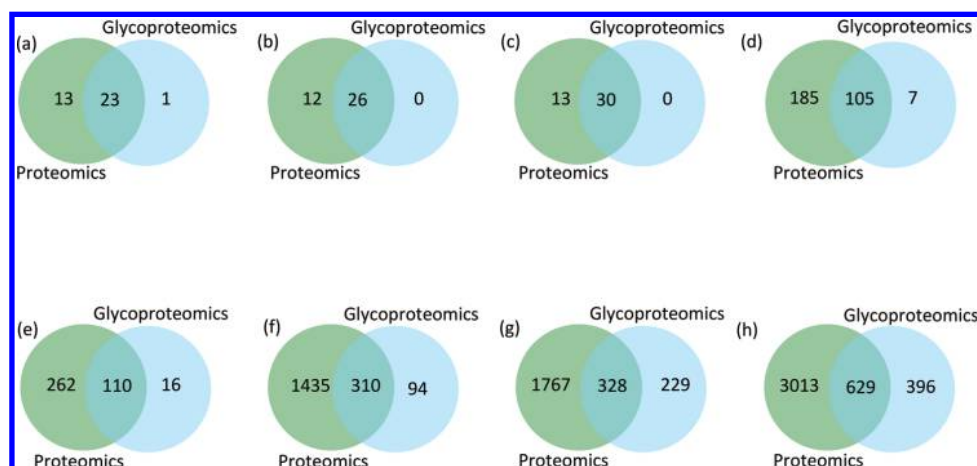


**Table 6.** Summary of the total number of proteins found (with at least 1 unique peptide) (proteomics + glycoproteomics).

|                      | Proteomics | Glycoproteomics | Non-redundant total | Percentage |
|----------------------|------------|-----------------|---------------------|------------|
| Photosynthesis       | 36         | 24              | 37                  | 100        |
| Latex allergens      | 38         | 26              | 38                  | 90.5       |
| Rubberwood formation | 43         | 30              | 43                  | 100        |
| Latex biosynthesis   | 290        | 112             | 297                 | 92.5       |
| Disease resistance   | 372        | 126             | 388                 | 63.3       |
| AnnHBM               | 1745       | 404             | 1839                |            |
| Swiss-Prot           | 2095       | 557             | 2324                |            |
| PlantGDB             | 3642       | 1025            | 4038                |            |

**Fig. 6.** Venn diagrams of the numbers of proteins and glycoproteins found using each method based on each database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) AnnHBM, (g) Swiss-Prot, and (h) PlantGDB. (See Supplementary Tables 3a–3h<sup>1</sup> for the full list of proteins identified from each database.) [Colour online.]



## Rubberwood

The rubberwood database consists of proteins involved in the formation of lignin in *H. brasiliensis*. As previously described by Rahman et al. (2013), 127 genes encoding 43 proteins in the database were identified for lignin formation in *H. brasiliensis*. We identified all 43 (100%) non-redundant proteins through our analysis (Table 6). Among the major proteins identified were coniferin beta-glucosidase, cinnamyl-alcohol dehydrogenase, peroxidase, caffeic acid O-methyltransferase, and coniferyl-alcohol glucosyl-transferase, which are proteins involved in transport, storage, and mobilization of monolignols and their final polymerization into lignins.

## Disease resistance

The disease resistance database contains all proteins involved in defense against diseases in *H. brasiliensis*, including the complete salicylic acid and jasmonic acid signaling pathways. It may be used to develop novel approaches for controlling plant diseases or pathogens. According to Rahman et al. (2013), the disease resistance genes include 618 genes from the nucleotide binding site-coding R gene family, 147 genes encoding pathogenesis-related proteins, and 96 genes involved in the hypersensitive response and systemic acquired resistance. All of these genes encode 610 proteins in the database. We identified a total of 388 (63.3%) non-redundant proteins through our analysis (Table 6). Among the major proteins identified were a disease resistance protein from the hypersensitive response and systemic acquired resistance pathway, a putative disease resistance protein, and TMV resistance protein N.

## Latex allergens

The latex allergens database contains proteins that are regarded as allergens, substances that cause allergic reaction. According to Rahman et al. (2013), allergens from *H. brasiliensis* include both latex-based allergens and non-latex allergens, which are encoded by 100 and 16 genes, respectively. In combination, these genes encode 42 proteins in the latex allergens database. We identified a total of 38 (90.5%) non-redundant proteins through our analysis (Table 6). Among the proteins identified are all 14 internationally recognized natural rubber latex allergens, known as Hevb1 to Hevb14 (<http://www.allergen.org>). The proteins are (in order from Hevb1 to Hevb14) rubber elongation factor,  $\beta$ -1,3-glucanase, small rubber particle, lecithinase homolog, latex major allergen, havein, patatin-like, profilin, enolase, superoxide dismutase, chitinase, lipid transfer precursor, esterase, and hevamine.

## Photosynthesis

The photosynthesis database contains proteins involved in photosynthesis, particularly the Calvin cycle. We identified all 37 proteins in the database (100%) through our analysis (Table 6). Major proteins identified include phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase, which are enzymes involved in the Calvin cycle (Diwan 2005).

## AnnHBM, Swiss-Prot, and PlantGDB

The AnnHBM, Swiss-Prot, and PlantGDB databases served as a control to estimate how many proteins from rubber latex could be identified using both the proteomic and glycoproteomic methods. The AnnHBM database, consisting of 69 103 proteins, is a collection of all translated proteins from the *H. brasiliensis* genome. The Swiss-Prot and PlantGDB databases are both collections of annotated proteins. Swiss-Prot is a collection of reviewed proteins from UniProtKB, while PlantGDB is a collection of proteins derived from green plants. We identified 1839, 2324, and 4038 non-redundant proteins through our analysis for the AnnHBM, Swiss-Prot, and PlantGDB databases, respectively (Table 6). With both the proteomic and glycoproteomic methods, more unique proteins than common proteins were identified in each of the C-serum, luteoid, and rubber particle fractions (Figs. 4 and 5).

## Conclusion

We have successfully identified almost all rubber latex proteins, with high percent coverage, using high-resolution and high mass accuracy mass spectrometric analysis. Therefore, the quality of the data collected was very convincing. Our results validate the genome databases generated in a previous study (Rahman et al. 2013).

## Conflict of interest

The authors declare that there is no conflict of interest associated with this study.

## Acknowledgements

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## References

- Alenius, H., Palosuo, T., Kelly, K., Kurup, V., Reunala, T., Mäkinen-Kiljunen, S., et al. 1993. IgE reactivity to 14-kD and 22-kD natural rubber proteins in latex-allergic children with spina bifida and other congenital abnormalities. *Int. Arch. Allergy Immunol.* **102**: 61–66. doi:10.1159/000236551.
- Archer, B.L., and Cockbain, E.G. 1955. The proteins of *Hevea brasiliensis* latex 2. Isolation of the  $\alpha$ -globulin of fresh latex serum. *Biochem. J.* **61**: 508–512. PMID:13269389.
- Archer, B.L., Barnard, G., Cockbain, E.G.C., Dickenson, P.B., and McMullen, A.I. 1963. Structure, composition and biochemistry of *Hevea* latex. In *Chemistry and physics of rubber-like substances*. Edited by L. Bateman. McLaren and Sons, London. pp. 41–72.
- Bokma, E., Spiering, M., Chow, K.-S., Mulder, P.P.M.F.A., Subroto, T., and Beintema, J.J. 2001. Determination of cDNA and genomic DNA sequences of hevine, a chitinase from the rubber tree *Hevea brasiliensis*. *Plant Physiol.* **39**: 367–376. doi:10.1016/S0981-9428(01)01247-5.
- Breton, F., Coupé, M., Sanier, C., and D'Auzac, J. 1995. Demonstration of  $\beta$ -1,3-glucanase activities in luteoids of *Hevea brasiliensis* latex. *J. Nat. Rubber Res.* **10**: 37–45.
- Churngchow, N., Suntaro, A., and Wititsuwannakul, R. 1995.  $\beta$ -1,3-Glucanase isozymes from the latex of *Hevea brasiliensis*. *Phytochemistry*, **39**: 505–509. doi:10.1016/0031-9422(95)00974-C.
- Chye, M.-L., and Cheung, K.-Y. 1995.  $\beta$ -1,3-Glucanase is highly expressed in laticifers of *Hevea brasiliensis*. *Plant Mol. Biol.* **29**: 397–402. doi:10.1007/BF00043663.
- Czuppon, A.B., Chen, Z., Rennert, S., Engelke, T., Meyer, H.E., Heber, M., and Baur, X. 1993. The rubber elongation factor of rubber trees (*Hevea brasiliensis*) is the major allergen in latex. *J. Allergy Clin. Immunol.* **92**: 690–697. doi:10.1016/0091-6749(93)90012-5.
- Dai, L., Kang, G., Li, Y., Nie, Z., Duan, C., and Zheng, R. 2013. In-depth proteome analysis of the rubber particle of *Hevea brasiliensis* (para rubber tree). *Plant Mol. Biol.* **82**: 155–168. doi:10.1007/s11103-013-0047-y.
- d'Auzac, J., and Jacob, J.-L. 1969. Regulation of glycolysis in latex of *Hevea brasiliensis*. *J. Rubber Res. Inst. Malays.* **21**: 417–444.
- Dickenson, P.B. 1969. Electron microscopical studies of the latex vessel system of *Hevea brasiliensis*. *J. Rubber Res. Inst. Malays.* **21**: 543–559.
- Diwan, J.J. 2005. Photosynthetic dark reaction. *Biochemistry and Biophysics*, Rensselaer Polytechnic Institute.
- Gomez, J.B., and Hamzah, S. 1989. Particle size distribution in *Hevea* latex: some observations on the electron microscopic method. *J. Nat. Rubber Res.* **4**: 204–211.
- Hamzah, S.B., and Gomez, J.B. 1982. Electron microscopy of ultracentrifuged fractions of *Hevea* latex. I. Top-fractions. *J. Rubber Res. Inst. Malays.* **30**: 161–173.
- Hasma, H. 1987. Proteolipids of natural rubber particles. *J. Nat. Rubber Res.* **2**: 129–133.
- Ismail, M.N., Stone, E.L., Panico, M., Lee, S.H., Luu, Y., Ramirez, K., et al. 2011. High-sensitivity O-glycomic analysis of mice deficient in core 2  $\beta$ 1,6-N-acetylglucosaminyltransferases. *Glycobiology*, **21**: 82–98. doi:10.1093/glycob/cwq134.
- Kongsawadworakul, P., Viboonjun, U., Romruensukharom, P., Chantuma, P., Ruderman, S., and Chrestin, H. 2009. The leaf, inner bark and latex cyanide potential of *Hevea brasiliensis*: evidence for involvement of cyanogenic glucosides in rubber yield. *Phytochemistry*, **70**: 730–739. doi:10.1016/j.phytochem.2009.03.020.
- Koyama, T., Wititsuwannakul, D., Asawatreratanakul, K., Wititsuwannakul, R.,

- Ohya, N., Tanaka, Y., and Ogura, K. 1996. Isopentenyl diphosphate isomerase in rubber latex. *Phytochemistry*, **43**: 769–772. doi:10.1016/0031-9422(96)00374-3.
- Moir, G.F.J. 1959. Ultracentrifugation and staining of *Hevea* latex. *Nature*, **184**: 1626–1628. doi:10.1038/1841626a0.
- Oh, S.K., Kang, H., Shin, D.H., Yang, J., Chow, K.-S., Yeang, H.Y., et al. 1999. Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*. *J. Biol. Chem.* **274**: 17132–17138. doi:10.1074/jbc.274.24.17132.
- Oliveros, J.C. 2007. VENNY. An interactive tool for comparing lists with Venn diagrams [online]. Available from <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.
- Rahman, A.Y., Usharraj, A.O., Misra, B.B., Thottathil, G.P., Jayasekaran, K., Feng, Y., et al. 2013. Draft genome sequence of the rubber tree *Hevea brasiliensis*. *BMC Genomics*, **14**: 75. doi:10.1186/1471-2164-14-75.
- Ru, Q.C., Zhu, L.A., Katenhusen, R.A., Silberman, J., Brzeski, H., Liebman, M., and Shriver, C.D. 2006. Exploring human plasma proteome strategies: high efficiency in-solution digestion protocol for multi-dimensional protein identification technology. *J. Chromatogr. A*, **1111**(2): 175–191. doi:10.1016/j.chroma.2005.06.080. PMID:16569577.
- Saba, J., Dutta, S., Hemenway, E., and Viner, R. 2012. Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int. J. Proteomics*, **2012**: 1–7.
- Soedjanaatmadja, U.M.S., Hofsteenge, J., Jeronimus-Stratingh, C.M., Bruins, A.P., and Beintema, J.J. 1994. Demonstration by mass spectrometry that pseudohevein and hevein have ragged C-terminal sequences. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1209**: 144–148. doi:10.1016/0167-4838(94)90150-3.
- Southorn, W.A. 1969. Microscopy of *Hevea* latex. In *Proceedings of the Natural Rubber Research Conference, 1996*. Rubber Research Institute of Malaysia, Kuala Lumpur. pp. 766–776.
- Southorn, W.A., and Yip, E. 1968. Latex flow studies. III. Electrostatic considerations in the colloidal stability of fresh *Hevea* latex. *J. Rubber. Res. Inst. Malays.* **20**: 201–215.
- Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., et al. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie*, **75**: 687–706. doi:10.1016/0300-9084(93)90100-7.
- Subroto, T., van Koningsveld, G.A., Schreuder, H.A., Soedjanaatmadja, U.M.S., and Beintema, J.J. 1996. Chitinase and  $\beta$ -1,3-glucanase in the luteoid-body fraction of *Hevea* latex. *Phytochemistry*, **43**: 29–37. doi:10.1016/0031-9422(96)00196-3.
- Sunderasan, E., Cardosa, M.J., Hamid, S., Hamzah, S., Yeang, H.Y., and Ward, M.A. 1995. Latex B-serum  $\beta$ -1,3-glucanase (Hev b II) and a component of the microhelix (Hev b IV) are major latex allergens. *J. Nat. Rubber Res.* **10**: 82–99.
- Suvachittanont, W., and Wititsuwannakul, R. 1995. 3-Hydroxy-3-methylglutaryl-coenzyme A synthase in *Hevea brasiliensis*. *Phytochemistry*, **40**: 757–761. doi:10.1016/0031-9422(95)00310-4.
- Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Wititsuwannakul, R., Wititsuwannakul, D., and Asawatratanakul, K. 1997. Isopentenyl diphosphate isomerase and prenyl transferase activities in bottom fraction and C-serum from *Hevea* latex. *Phytochemistry*, **45**: 261–267. doi:10.1016/S0031-9422(96)00837-0.
- Tata, S.J., and Edwin, E.E. 1970. *Hevea* latex enzymes detected by zymogram technique after starch gel electrophoresis. *J. Rubber Res. Inst. Malays.* **23**: 1–12.
- Wahler, D., Colby, T., Kowalski, N.A., Harzen, A., Wotzka, S.Y., Hillebrand, A., et al. 2012. Proteomic analysis of latex from the rubber-producing plant *Taraxacum brevicorniculatum*. *Proteomics*, **12**: 901–905. doi:10.1002/pmic.201000778.
- Wang, X., Shi, M., Lu, X., Ma, R., Wu, C., Guo, A., Peng, M., and Tian, W. 2010. A method for protein extraction from different subcellular fractions of laticifer latex in *Hevea brasiliensis* compatible with 2-DE and MS. *Proteome Sci.* **8**: 35.
- Wei, F., Xiao, X.Z., and Liu, S.Z. 2008. A methodological research on extraction and two-dimensional electrophoresis of luteoid proteins in rubber tree latex. *Prog. Mod. Biomed.* **8**: 2258–2260.
- Wititsuwannakul, R., Pasitkul, P., Kanokwiroon, K., and Wititsuwannakul, D. 2008. A role for a *Hevea* latex lectin-like protein in mediating rubber particle aggregation and latex coagulation. *Phytochemistry*, **69**(2): 339–347.
- Wititsuwannakul, D., Rattanapittayaporn, A., Koyama, T., and Wititsuwannakul, R. 2004. Involvement of *Hevea* latex organelle membrane proteins in rubber biosynthesis activity and regulatory function. *Macromol. Biosci.* **4**: 314–323. doi:10.1002/mabi.200300080.
- Wititsuwannakul, R., Wititsuwannakul, D., and Dumkong, S. 1990. *Hevea* calmodulin: regulation of the activity of latex 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Phytochemistry*, **29**: 1755–1758. doi:10.1016/0031-9422(90)85009-5.
- Wu, K.X., Wang, Z., Yao, M.P., Chen, X.T., and Chen, S.C. 2008. Comparative study of electrophoretic patterns of rubber particle proteins from different clones of *Hevea brasiliensis*. *J. Anhui Agric. Sci.* **36**: 15785–15787.
- Yeang, H.Y., Yip, E., and Hamzah, S. 1995. Characterization of zone 1 and zone 2 rubber particles in *Hevea brasiliensis* latex. *J. Nat. Rubber Res.* **10**: 108–123.
- Yeang, H.Y., Cheong, K.F., Sunderasan, E., Hamzah, S., Chew, N.P., Hamid, S., et al. 1996. The 14.6 kD rubber elongation factor (Hev b 1) and 24 kD (Hev b 3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy. *J. Allergy Clin. Immunol.* **98**: 628–639. doi:10.1016/S0091-6749(96)70097-0.
- Yeang, H.Y., Arif, S.A.M., Yusof, F., and Sunderasan, E. 2008. Allergenic proteins of natural rubber latex. *Methods*, **27**: 32–45. doi:10.1016/S1046-2023(02)00049-X.