Proteomics Analysis of Latex from Hevea brasiliensis (Clone RRIM 600)

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2016-0144.R1
Manuscript Type:	Article
Date Submitted by the Author:	24-Aug-2016
Complete List of Authors:	Habib, Mohd Afiq Hazlami; Universiti Sains Malaysia, Analytical Biochemistry Research Centre Gan, Chee Yuen; Universiti Sains Malaysia, Analytical Biochemistry Research Centre Othman, Fazilah; Anti-Doping Lab Qatar Zainudin, Nurul Nabilah; Universiti Sains Malaysia, Analytical Biochemistry Research Centre Abdul Latiff, Aishah; Anti-Doping Lab Qatar ISMAIL, MOHD NAZRI; Universiti Sains Malaysia, Analytical Biochemistry Research Centre
Keyword:	Hevea brasiliensis, Rubber latex, Plant proteomics, Mass spectrometry, Biosynthetic pathways

SCHOLARONE™
Manuscripts

Proteomics Analysis of Latex from *Hevea brasiliensis* (Clone RRIM 600)

Mohd Afiq Hazlami Habib^a, Gan Chee Yuen^a, Fazilah Othman^b, Nurul Nabilah Zainudin^a,
Aishah Abdul Latiff^b & Mohd Nazri Ismail^{a,*}

^aAnalytical Biochemistry Research Centre, Universiti Sains Malaysia, 11800 USM, Pulau
Pinang, Malaysia.

^bAnti-Doping Lab Qatar, Sports City St, Doha, Qatar

*Corresponding author

Dr. Mohd Nazri Ismail

Analytical Biochemistry Research Centre,

Universiti Sains Malaysia,

11800 USM, Pulau Pinang, Malaysia.

+604-6532694/4694

mdnazri@usm.my

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 particle. Previous studies showed that a large number of proteins were present inside these three 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 *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 spectric data obtained from the 3 layers of the latex. Peptides from these proteins were fragmented using collision-induced dissociation (CID), higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD) activation methods. A large percentage of proteins from the biosynthetic pathways were successfully identified, ranging from 63% to 100%. In addition, a total of 1839 unique proteins were identified from the whole translated draft genome database (AnnHBM).

Keywords: *Hevea brasiliensis*; Rubber latex; Plant proteomics; Mass spectrometry; Biosynthetic pathways

1. Introduction

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

For example, rubber particles are the colloidal components that are naturally present in latex in which comprising 30-40% of the whole latex volume (Southorn and Yip, 1968; Gomez and Hamzah, 1989). The particles have homogenous 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 μ m and has been distinguished into three different Moir's zone according to sizes (Southorn, 1969; Yeang et al., 1995; Hamzah and Gomez, 1982; Dickenson, 1969). Two different major proteins have been identified in rubber particles at molecular masses of 14.6 and 24 kDa. These proteins were classified as proteolipids (Hasma, 1987; Wititsuwannakul et al., 2004) and were identified as latex allergens (i.e. Hev b1 and Hev b3) (Czuppon et al., 1993; Alenius et al., 1993; Yeang et al., 1996). The latter has been proposed to be tightly associated with small rubber particle and also involved in the biosynthesis of rubber (Oh et al., 1999). Lectin-like protein was also found in the recent years that are responsible for latex coagulation and mediating rubber particle aggregation.

The aqueous phase of laticiferous cytoplasmic, 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. α -globulin is the highest abundant protein that contributes to the colloidal stability of the latex and rubber particles (Archer et al., 1963; Archer and Cockbain, 1955). Apart from that, several glycolytic enzymes and other common cytosolic enzymes were found in C-serum in which they are actively involved in different metabolic processes (d'Auzac and Jacob, 1969; Wititsuwannakul et al., 1990; Suvachitanont and

Wititsuwannakul, 1995; Koyama et al., 1996). For instances, 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 lutoid or membrane fraction (Wititsuwannakul et al., 1990). In addition, several allergens (i.e. Hev b5, Hev b7, Hev b8 and Hev b9) were also identified.

Lutoids, the bottom layers after ultracentrifugation, play important roles in latex coagulation and protect the tree from exterior invasion (Wei et al., 2008; Kongsawadworakul et al., 2009). They are vacuolar organelles that contain a number of pathogenesis-related proteins that obtain antifungal properties (Stintzi et al., 1993). Research showed that hevein is the major protein (approximately 50%) of lutoid body. It is formed via a proteolytic process from a precursor protein (19 kDa) into a domain (5 kDa) and a C-terminally located protein (14 kDa) in which resulted in a ragged structure after the removal of residues at the C-terminus of hevein (Soedjanaatmadja et al., 1994). Second and third major proteins, hevamine and β -1,3-glucanase, with molecular mass of 29 kDa and 32-35 kDa, respectively, were also identified (Breton et al., 1995; Churngchow et al., 1995; Subroto et al., 1996; Sunderasan et al., 1995). The cDNA sequences of these proteins were determined and they were found to be post-translationally modified via removal of an N-terminal signal peptide and a vacuolar targeting sequence from the C-terminus (Bokma et al., 2001; Chye et al., 1995). Other proteins such as osmotin-like protein (25 kDa, antifungal protein) and citrate-binding protein (responsible for citrate accumulation in lutoids) were also found.

Until present, research works that have been performed resulted in very limited number of proteins identified. A proteome analysis on the rubber particles of *H. brasiliensis* has revealed 186 proteins, ranging from 3.9-194.2 kDa in molecular mass (Dai L. J. et al., 2013). A study on the different subcellular fractions of the latex proteome has also been conducted on the rubber producing plant *Taraxacum brevicorniculatum* which reveals that the proteins involved in rubber biosynthesis were distributed among the fractions (Wahler D. et al., 2012). Information on the post-translational modifications (PTM) is even more limited. Therefore, it was suggested to have a new strategy in order to identify as much protein as possible to better understand the various biological pathways in rubber latex. In our collaborative research works, we have used a

genomic approach to identify the proteins in the rubber latex. Databases of these proteins in different pathways were also established, namely the photosynthesis, latex allergens, rubberwood formation, latex biosynthesis, and disease resistance. The details in each pathway of other databases were discussed in details in a previous work (Rahman et al., 2013). Therefore, using proteomic approach to search the expressed proteins in the fresh rubber latex would validate the results of the genome analysis. An efficient strategy with good quality of data is essential for the study in order to obtain the promising results.

The main objective of this study was to use proteomics to validate the result of the genomic analysis from the previous work (Rahman et al., 2013), so the results and discussions of this article will heavily depends on their work. In order to detect both the high and low abundant proteins, the activation protocols collision-induced dissociation (CID), high-energy collision dissociation (HCD) and electron-transfer dissociation (ETD) were used instead of just one activation protocol to generate characteristic fragments in the MS/MS experiment which ultimately establish amino acid sequences. In addition, we also employed a mass spectrometry method developed by Julian Saba and colleagues that uses HCD and ETD techniques to detect the low abundant glycoproteins (Saba et al., 2012). An Orbitrap mass spectrometer that has the capabilities to perform CID, HCD and ETD analyses coupled with a nano liquid chromatography was utilized and massive identification of protein in each subcellular fraction was then performed using PEAKS Studio V6 (PEAKS). Hence, this approach would assist in understanding the pathways in different phases of the biological cycle.

2. Methodology

2.1 Latex collection

Rubber tree (*H. Brasiliensis*, Clone: RRIM600) grown in Baling, Kedah, Malaysia was tapped in the early morning. The fresh latex was collected in a plasticware and immediately froze in liquid nitrogen. The latex sample was stored in liquid nitrogen while it was sent to the laboratory.

2.2 Protein extraction and purification

The extraction of protein from the rubber latex was performed according to Wang et al. (2010) with slight modifications. The fresh latex was thawed and ultracentrifuged for 30 mins at 40,000

g 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 stated below:

2.2.1 Purification of different layers

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

2.2.2 Extraction of protein from different subcellular fractions

These three different fractions (rubber particles, C-serum and lutoids) were respectively added in Borax/PVPP/Phenol (BPP) buffer containing 100 mM EDTA, 100 mM Tris (pH 8.0), 50 mM Borax, 50 mM Vitamin C, 1% PVPP (w/v), 1% Triton X-100 (v/v), 2% β-mercaptoethanol (v/v) and 30% sucrose (w/v) in a ratio of 1:5 (w/v). The mixtures were vortexed for 30 mins.

For rubber particles, the mixture was sonicated for 5 min at 70 W/cm² in an ice bath and subsequently ultracentrifuged for 15 mins at 15,000 and 4 °C. The clear phase was collected and two volumes of Tris-saturated phenol (pH 8.0) were added. The mixture was then vortexed for 15 mins. For C-serum and lutoids, two volumes of Tris-saturated phenol (pH 8.0) were added and vortexed for 15 mins.

All the mixtures were subsequently centrifuged for 15 mins at 15,000 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.

2.3 Gelfree Protein fractionation

Extracted protein samples from each subcellular fraction were then fractionated based on the molecular masses using GELFREE™ 8100 fractionation system (Protein Discovery, U.S.A.). Mid-mass cartridge kit (fractionation of proteins with molecular masses between 3.5 – 150 kDa) was used in this experiment. Protein sample of 250 µg (in solution) was mixed with acetate sample buffer (30 µl) and DTT (8 µl) to the final total volume of 150 µl prior to fractionation.

The fractionation conditions are as below:

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

Total of 12 fractions were collected and were stored at -20 °C prior to further use.

2.4 In-solution digestion

All Gelfree fractions of protein were digested using trypsin separately according to the method of Ru et al. (2006). Samples were first undergone reduction and alkylation prior to digestion. Briefly, protein samples were solubilized in 500µL of denaturing buffer (6M guanidine-HCl/25mM ammonium bicarbonate, pH 8.5). 250 µL of 1 mg/ml DTT/25 mM Ammonium bicarbonate was then added to the protein solution and incubated at 55°C for 30 minutes. Following, 500 µL of 1mg/mL iodoacetamide/25 mM ammonium bicarbonate was added, covered with aluminium foil and incubated at 55°C for 15 minutes.

The reduced and alkylated protein sample was then buffer exchanged with 25 mM ammonium bicarbonate using spin-column with molecular cut-off of 3 kDa for 3 times. 250 µL of digestion buffer (25 mM ammonium bicarbonate) and trypsin (ratio 1:50) were then 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.

2.5 Mass spectrometry

All LC MS and MS/MS analyses were carried out using Thermo LTQ/Orbitrap Velos (Thermo Scientific, San Jose, CA, USA) coupled with Easy-nLC II system (Thermo Scientific, San Jose, CA, USA). Chromatographic separation of tryptic-digested peptides was performed using Easy-Column C18-A2 (100 × 0.75 mm i.d., 3 μ m; Thermo Scientific, San Jose, CA, USA) coupled with pre-column (Easy-Column, 20 × 0.1 mm i.d., 5 μ m; Thermo Scientific, San Jose, CA, USA) at flow rate of 0.3 μ l/min and injection volume of sample was 10 μ l. Pre-column was equilibrated for 15 μ l at flow rate of 3 μ l/min whereas analytical column was equilibrated for 4 μ l at a flow rate of 0.3 μ l/min. Running buffers used were: (A) deionised 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 minutes and hold for another 15 minutes. 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 PPG 2700 with 7 mM sodium acetate in 70% methanol for high mass calibration. Data acquisition was performed using Xcalibur ver. 2.1 (Thermo Scientific, San Jose, CA, USA) with mass tolerance threshold of 5 ppm. The eluant was electrosprayed into mass spectrometer at 2.3 kV (source voltage) and capillary temperature of 200 °C was used.

Two MS/MS analysis methods were used for the peptide detection. The first method, dubbed as the proteomic approach, was able to analyze peptides in both high and low abundance while the second method, dubbed as the glycoproteomic approach, was able to analyze low abundant peptides and glycopeptides. For the proteomic approach, peptides were detected by full scan mass analysis from m/z 300-2,000 at resolving power of 60,000 (at m/z 400, FWHM; 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 state. 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. Whereas, HCD was conducted with isolation width of 2 Da, normalized collision energy of 35, activation time of 0.1 ms and FT 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 and 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 only acquired when glycan oxonium ions from HCD fragmentation are detected. The oxonium ions are hexose, hexosamine, hoxosamine fragments and hexose-hexosamine.

2.6 Analysis of mass spectrometric data

PEAKS were used to construct predicted peptide sequences using MS/MS data without the needs to refer to the known protein databases. PEAKS reads the RAW files generated by LC-MS/MS for all fractions of CID, HCD, and ETD of each C-serum, lutoids, and rubber particles and extracted the peptide information during *Data Refinement*. The charge options were set to 2-10 with filter quality of >0.65.

Automated peptide de novo sequencing was executed on each sample, combining all fractions in one de novo result. Parent mass error tolerance 0.1 Da and fragment mass error tolerance 0.8 Da with Trypsin digestion enzyme 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 databases identification (PEAKS DB + PEAKS PTM + SPIDER) against the rubber genome (RG) annotated databases (<http://bioinfo-ccb.usm.my/cgi-bin/gb2/gbrowse/Rubber/>), PlantGDB (<http://www.plantgdb.org/>) and SwissProt (<http://www.uniprot.org/>). The workflow started with PEAKS DB search, where the list of candidate peptides was aligned against protein of annotated databases. The parameter includes carbamidomethylation of cysteine and oxidation of methionine as variable modifications. The search continued with the second process in the workflow, PEAKS PTM, where post translation modifications were predicted and got included in the alignment. The parameters were set to all 670 types of modification as variable. Lastly, the SPIDER was used to find more mutations on the candidate peptides to identify more proteins.

In all three workflow process, the peptide-spectrum matches (PSM) results were filtered using high confidence peptide identification at 1% FDR for databases with huge number of sequences (>1000); AnnHBM, PlantGDB and SwissProt, while for the databases with small number of sequences (<1000); latex allergens, disease resistance, rubberwood formation, latex biosynthesis, and photosynthesis, the filter $-10\lg P = 20$ was used instead. The protein filter were set to $-10\lg P = 0$ 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 from each C-serum, lutoids, and rubber particles were compared using Venny (Oliveros, 2007) to develop Venn diagrams in order to determine the number of unique proteins found on each databases.

For the glycoproteomic method, the PEAKS PTM step was skipped; with selected glycosylation masses were included into the PEAKS DB step. The list of selected glycosylation and their masses is displayed in **Table 1**. All the result filter parameters from proteomic method were also use. The standard workflow results of glycoproteomic from C-serum, lutoids, and rubber particles were compared using Venny before comparing both results of proteomic and glycoproteomic.

3. Results and Discussion

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

Figure 1 displays the representative MS spectra from this experiment. Result showed that ions peaks with high resolution ranging from 24,000 to 63,000 were produced. The high resolution is essential for the identification and confirmation of the molecular formula of the peptide sequences and thus determines the mass accuracy of the molecular ions. Ion peaks with doubly or higher charge were then selected to be fragmented. **Figure 2 (Panels A-D)** shows the

representative MSMS fragments and the ion tables. These spectra have high signal-to-noise ratio and show complete or near-complete backbone fragmentation. Low error (<0.8 Da) was also found in the data.

Figure 3 shows the coverage of 87% of a whole protein sequence as an example of high quality protein de novo sequencing that has been acquired. The blue line underneath the protein sequence indicates the identified peptide sequence which shows the high repeatability of the peptide sequences in the analysis. The list of the supporting peptides is displayed in **Table 2**. It could be observed that high accuracy (low in mass differences, ppm) were found in the data. Note that identification of these proteins was not just high in percent coverage, also all these MSMS spectra were collected at high resolution and mass accuracy. Therefore, the quality the data collected was convincing.

3.1 Protein Identification

Table 3 shows the summary of the total proteins found (proteomic method) in each subcellular fraction according to different databases. C-serum, lutoids and rubber particles subcellular fractions contributed to high amount of protein in each database. C-serum contributed to the most amount of proteins identified in every genome transcribed database except for the latex biosynthesis database. For the 5 genome transcribed databases, it was shown that 97.3% (36 out of 37 proteins), 90.5% (38 out of 42 proteins), 100% (43 out of 43 proteins), 90.3% (290 out of 321 proteins), and 61 % (372 out of 610 proteins) of the total non-redundant proteins were identified from the photosynthesis database, latex allergens database, rubberwood formation database, latex biosynthesis database, and disease resistance database, respectively. It could also be observed in **Figure 4** that generally, C-serum, lutoids and rubber particle fractions shares the majority of common proteins. The number of proteins common to C-serum, lutoids and rubber particle for each of the genome translated database are 18, 28, 36, 115, and 103, respectively.

Table 4 shows the summary of the total proteins found (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 more to selecting and sequencing glycoproteins in high throughput. This procedure also does not require any offline sample enrichment steps thus can minimize sample losses. By

using this glycoproteomic approach, although the number of proteins found is not as high as the proteomic approach but it has allowed the detection of low abundant glycoproteins that were not able to be detected via the proteomic approach. Results show clearly that the percentage of proteins identified has dropped significantly compared to proteomics method. For the 5 genome transcribed databases, it was shown that 64.9% (24 out of 37 proteins), 61.9% (26 out of 42 proteins), 69.8% (30 out of 43 proteins), 34.9% (112 out of 321 proteins), and 20.7 % (126 out of 610 proteins) of the total non-redundant proteins were identified from the photosynthesis database, latex allergens database, rubberwood formation database, latex biosynthesis database, and disease resistance database, respectively. All glycosylated peptides identified from the genome transcribed database through this approach were O-linked glycopeptides. Unfortunately, there were no glycosylated peptides identified in small rubber particle protein as declared by Wititsuwannakul (Wititsuwannakul et al., 2008). Also, Beta-1, 3-endoglucanase was identified but due to Ser-352 being replaced by Gly in cv. RRIM 600, Asn-350 was not glycosylated (Subroto et al., 2001). Generally, the majority of unique proteins identified by glycoproteomic method were found in C-serum, followed by lutoids and rubber particles (**Figure 5**).

Table 5 shows the summary of the total protein found in each method based on different database. By removing the protein redundancies found from both methods, more unique proteins were identified (**Figure 6**). Both photosynthesis and rubberwood formation database had all its protein identified. Both latex biosynthesis and disease resistance database got an increase percentage of protein identified, at 92.5% and 63.3%, respectively, while latex allergen database stays at 90.5%.

3.2 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 metabolic pathway in which rubber biosynthesis took place starting from sucrose. Six major steps that can be further divided into 12 sub-metabolic pathways which involve proteins for sucrose import and degradation, glycolysis, acetyl-CoA biosynthesis, prenyl diphosphate synthesis via mevalonate pathway in cytosol, geranylgeranyl pyrophosphate synthesis in mitochondria and rubber polymerization on the rubber particle membrane were compiled. Rahman et al. had constructed the entire metabolic

pathway of latex biosynthesis represented by 417 genes that encodes 321 proteins in the database and consists of 12 distinct sub-metabolic pathways (Rahman et al., 2013). We managed to identify a total of 297 (92.5%) non-redundant proteins thru our analysis (Table 5). The major proteins that were identified includes beta- fructofuranosidase from both the sucrose import and the fructan synthesis pathway, 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 (TCA) cycle pathway, hydroxymethylglutaryl-CoA synthase from the prenyl diphosphate synthesis via mevalonate (MVA) pathway, and also 4-hydroxy-3-methylbut-2-enyl diphosphate reductase from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. In addition, we also identified dimethylallyltranstransferase from the prenyl-PP biosynthesis pathway as well as rubber elongation factor and small rubber particle protein for the rubber polymerization pathway.

3.3 Rubberwood

The rubberwood database consists of proteins that involved in the formation of lignin in *H. brasiliensis*. As previously described by Rahman et al., 127 genes that encode 43 proteins in the database were identified for lignin formation in *H. brasiliensis* (Rahman et al., 2013). We managed to identify all 43 (100%) non-redundant proteins thru our analysis (Table 5). Among the major proteins identified includes coniferin beta-glucosidase, cinnamyl-alcohol dehydrogenase, peroxidase, caffeic acid O-methyltransferase, and coniferyl-alcohol glucosyltransferase which are proteins involved in transport, storage, and mobilization of monolignols and its final polymerization into lignins.

3.4 Disease Resistance

The disease resistance database contains all proteins that involved in the defensive mechanism against diseases in *H. brasiliensis* that emphasized on a complete salicylic acid and jasmonic acid signaling pathway. It has been dedicated to develop a novel approach for controlling plant

disease/pathogens. According to Rahman et al., the disease resistance genes includes 618 genes from the nucleotide binding site – coding R gene family, 147 genes from the pathogenesis-related proteins, and 96 genes combined from the hypersensitive response and systemic acquired resistance (Rahman et al., 2013). All of these genes encode 610 proteins in the database. We managed to identify a total of 388 (63.3%) non-redundant proteins thru our analysis (**Table 5**). Among the major proteins identified include disease resistance protein from the hypersensitive response and systemic required resistance pathway, as well as putative disease resistance protein and TMV resistance protein N.

3.5 Latex Allergens

The latex allergens database contains proteins that are regarded as allergens, substances that cause allergic reaction. According to Rahman et al., allergens from *H. brasiliensis* includes both latex based allergens and non-latex allergens, which are made up of 100 and 16 genes respectively (Rahman et al., 2013). In combination, these genes encode for 42 proteins inside the latex allergens database. We managed to identify a total of 38 (90.5%) non-redundant proteins thru our analysis (**Table 5**). Among the proteins identified are all 14 internationally recognized natural rubber latex allergens, known as Hevb1 to Hevb14 (www.allergen.org). The proteins are (in order from Hevb1 to Hevb14) rubber elongation factor, β -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.

3.6 Photosynthesis

The photosynthesis database contains proteins that are involved in the photosynthesis mechanism, particularly the Calvin cycle. There were 37 proteins in the database and we managed to identify all 37 (100%) non-redundant proteins thru our analysis (**Table 5**). Major proteins identified include phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-biphosphate aldolase, which were enzymes involved in the Calvin cycle (Diwan, 2005).

3.7 AnnHBM, SwissProt, and PlantGDB

The AnnHBM, SwissProt, and PlantGDB database serves as a control to establish estimation as to how much identifiable proteins from the rubber latex could be obtained by using both the proteomic method and the glycoproteomic method. The AnnHBM database consists of 69103 proteins is the collection of all translated proteins from the *H. brasiliensis* genome. The SwissProt and PlantGDB are both a collection of annotated proteins where SwissProt database is the collection of reviewed proteins from the UniProtKB while PlantGDB database is the collection of proteins derived from green plants. We managed to identify a total of 1839, 2324, and 4038 non-redundant proteins thru our analysis for AnnHBM, SwissProt, and PlantGDB database, respectively (**Table 5**). In both proteomic and glycoproteomic methods, more unique proteins were identified in each C-Serum, lutoids and rubber particle instead of common proteins (**Figure 4 and Figure 5**).

4. Conclusion

In general, we have successfully identified almost all rubber latex proteins in high percentage of coverage that were resulted by the high resolution and high mass accuracy mass spectrometric analysis. Therefore, the quality of the data collected was very convincing. The results shown here have successfully validated the genome databases generated in the previous study (Rahman et al., 2013).

Acknowledgment

We would like to thank the current and past members of the Centre for Chemical Biology (CCB) of University Sains Malaysia (USM) for providing the protein sequence database of genome translated *H. brasiliensis* and its subsequent biological pathways. This work was supported by grants from the APEX DE 2012 grant (1002/PDOPING/910335) and World First Discovery Innovation Fund (WFDI-1002/PDOPING/910203) from Universiti Sains Malaysia.

Conflict of Interest

The authors agree that there is no conflict of interest.

References

1. Alenius, H., Palosuo, T., Kelly, Y., Kurup, V., Reunala, T., Makinen-Kiljunen, S., Turjanmaa, K., Fink, J., 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.
2. Archer, B.L., Barnard, G., Cockbain, E.G.C., Dickenson, P.B., McMullen, A.I., 1963. Structure, composition and biochemistry of Hevea latex. In: Bateman (Ed.), *Chemistry and Physics of rubber-like substances*. McLaren and Sons, London, pp. 41–72.
3. Archer, B.L., Cockbain, E.G., 1955. The proteins of Hevea brasiliensis latex 2. Isolation of the α -globulin of fresh latex serum. *Biochem. J.* 61, 508–512.
4. Bokma E., Spiering M., Chow K.S., Mulder P.P.M.F.A., Subroto T., Beintema J.J., 2001. Determination of cDNA and genomic DNA sequences of hevamine, a chitinase from the rubber tree Hevea brasiliensis, *Plant Physiol. Biochem.* 39, 367–376.
5. Breton F., Coupé M., D'Auzac J., Sanier C., 1995. Demonstration of β -1,3-glucanase activities in laticifers of Hevea brasiliensis latex, *J. Nat. Rubber Res.* 10, 37–45.
6. Churngchow N., Suntaro A., Wititsuwannakul R., 1995. β -1,3-Glucanase isozymes from the latex of Hevea brasiliensis, *Phytochemistry* 39, 505–509.
7. Chye M.L., Cheung K.Y., 1995. β -1,3-Glucanase is highly expressed in laticifers of Hevea brasiliensis, *Plant Mol. Biol.* 29, 397–402.
8. Czuppon, A.B., Chen, Z., Rennert, S., Engelke, T., Meyer, H.E., Heber, M., 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.
9. d'Auzac, J., Jacob, J.-L., 1969. Regulation of glycolysis in latex of Hevea brasiliensis. *J. Rubb. Res. Inst. Malaya.* 21, 417–444.
10. Dai L.J., Kang G.J., Li Y., Nie Z.Y., Duan C. F., Zheng R.Z., 2013. In-depth proteome analysis of the rubber particle of Hevea brasiliensis (para rubber tree). *Plant Mol. Biol.* 82, 155–168.
11. Dickenson, P.B., 1969. Electron microscopical studies of the latex vessel system of Hevea brasiliensis. *J. Rubb. Res. Inst. Malaya* 21, 543–559.
12. Diwan, J.J., 2005. *Photosynthetic Dark Reaction. Biochemistry and Biophysics*, Rensselaer Polytechnic Institute.
13. Gomez, J.B., Hamzah, S., 1989. Particle size distribution in Hevea latex: some observations on the electron microscopic method. *J. Nat. Rubb. Res.* 4, 204–211.

14. Hamzah, S.B., Gomez, J.B., 1982. Electron microscopy of ultracentrifuged fractions of Hevea latex. I. Top-fractions. J. Rubber Res. Inst. Malay. 30, 161–173.
15. Hasma, H., 1987. Proteolipids of natural rubber particles. J. Nat. Rubber Res. 2, 129–133.
16. Ismail, M.N., Stone, E., Panico, M., Lee S., Luu Y., Ramirez, K., Ho S., Fukuda, M., Marth J., Haslam S., Dell A. 2011. High sensitivity O-glycomic analysis of mice deficient in C2GnTs. Glycobiology. 21: 82-98.
17. Kongsawadworakul P., Viboonjun U., Romruensukharom P., Chantuma P., Ruderman S., 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.
18. Koyama, T., Wititsuwannakul, D., Asawatreratanakul, K., Wititsuwannakul, R., Ohya, N., Tanaka, Y., Ogura, K., 1996. Isopentenyl diphosphate isomerase in rubber latex. Phytochemistry 43, 769–772.
19. Moir, G.J., 1959. Ultrastructure and staining of Hevea latex. Nature, 184:1626-1628.
20. Oh, S.K., Kang, H., Shin, D.H., Yang, J., Chow, K.S., Yeang, H.Y., Wagner, B., Breiteneder, H., Han, K.H., 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.
21. Oliveros, J.C. 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.
22. Rahman A.Y., Usharraj A.O., Misra B.B., Thottathil G.P., Jayasekaran K., Feng Y., Hou S., Ong S.Y., Ng F.L., Lee L.S., Tan H.S., Sakaff M.K., Teh B.S., Khoo B.F., Badai S.S., Aziz N.A., Yuryev A., Knudsen B., Dionne-Laporte A., Mchunu N.P., Yu Q., Langston B.J., Freitas T.A., Young A.G., Chen R., Wang L., Najimudin N., Saito J.A., Alam M., 2013. Draft genome sequence of the rubber tree Hevea brasiliensis. BMC Genomics, 14, 75.
23. Soedjanaatmadja U.M.S., Beintema J.J., Bruins A.P., Hofsteenge J., Jeronimus-Stratingh C.M., 1994. Demonstration by mass spectrometry that pseudo-hevein and hevein have ragged C-terminal sequences, Biochim. Biophys. Acta 1209, 144–148.
24. Southorn, W.A., 1969. Microscopy of Hevea latex. In: Proc Nat Rubb Res Conf 1996. Rubber Research Institute of Malaya, Kuala Lumpur, pp. 766–776.
25. Southorn, W.A., Yip, E., 1968. Latex flow studies. III. Electrostatic considerations in the colloidal stability of fresh Hevea latex. J. Rubb. Res. Inst. Malaya. 20, 201–215.
26. Stintzi A., Fritig B., Geoffroy P., Heitz T., Kauffmann S., Legrand M., Prasad V., Wiedemann-Merdinoglu S., 1993. Plant ‘pathogenesis-related’ proteins and their role in defense against pathogens, Biochimie 75, 687–706.

- 505
506 27. Subroto T., Beintema J.J., Schreuder H.A., Soedjanaatmadja U.M.S., Van Koningsveld G.A.,
507 1996. Chitinase and β -1,3-glucanase in the lutoid-body fraction of *Hevea latex*,
508 *Phytochemistry* 43, 29–37.
509
- 510 28. Sunderasan E., Cardoso M.J., Hamid S., Hamzah S., Yeang H.Y., Ward M.A., 1995. Latex B-
511 serum β -1,3-glucanase (Hev b II) and a component of the microhelix (Hev b IV) are major
512 latex allergens, *J. Nat. Rubber Res.* 10, 82–99.
513
- 514 29. Suvachitanont, W., Wititsuwannakul, R., 1995. 3-Hydroxy-methylglutaryl-coenzyme A
515 synthase in *Hevea brasiliensis*. *Phytochemistry* 40, 757–761.
516
- 517 30. Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Wititsuwannakul, R., Wititsuwannakul,
518 D., 1997. Isopentenyl diphosphate isomerase and prenyl transferase activities in bottom
519 fraction and C-serum from *Hevea latex*. *Phytochemistry* 45, 261–267.
520
- 521 31. Tata, S.J., Edwin, E.E., 1970. *Hevea latex* enzymes detected by zymogram technique after
522 starch gel electrophoresis. *J. Rubb. Res. Inst. Malaya* 23, 1–12.
523
- 524 32. Wahler D., Colby T., Kowalski N.A., Harzen A., Wotzka S.Y., Hillebrand A., Fischer R.,
525 Helsper J., Schmidt J., Gronover C.S., Prufer D., 2012. Proteomic analysis of latex from the
526 rubber-producing plant *Taraxacum brevicorniculatum*. *Proteomics* 12: 901–905.
527
- 528 33. Wei F., Xiao X.Z., Liu S.Z., 2008. A methodological research on extraction and two-
529 dimensional electrophoresis of lutoid proteins in rubber tree latex. *Prog Mod Biomed*,
530 8:2258-2260.
531
- 532 34. Wititsuwannakul, D., Rattanapittayaporn, A., Koyama, T., Wititsuwannakul, R., 2004.
533 Involvement of *Hevea latex* organelle membrane proteins in rubber biosynthesis activity and
534 regulatory function. *Macromol. Biosci.* 4, 314–323.
535
- 536 35. Wititsuwannakul, D., Wititsuwannakul, R., 2001. Biochemistry of Natural Rubber and
537 Structure of Latex. In: Steinbuchel, A. (Ed.), *Biopolymers*, vol. 2. Wiley–VCH, Weiheim,
538 Germany, pp. 151–202.
539
- 540 36. Wititsuwannakul, R., Wititsuwannakul, D., Dumkong, S., 1990. *Hevea calmodulin*:
541 regulation of the activity of latex 3-hydroxy-3-methylglutarylcoenzyme A reductase.
542 *Phytochemistry* 29, 1755–1758.
543
- 544 37. Wu K.X., Wang Z., Yao M.P., Chen X.T., Chen S.C., 2008. Comparative study of
545 electrophoretic patterns of rubber particle proteins from different clones of *Hevea*
546 *brasiliensis*. *J Anhui Agri Sci*, 36:15785-15787.
547
- 548 38. Yeang H.Y., Arif S.A.M., Yusof F., Sunderasan E., 2008. Allergenic proteins of natural
549 rubber latex. *Methods*, 27:32-45.
550

- 551 39. Yeang, H.Y., Cheong, K.F., Sunderasan, E., Hamzah, S., Chew, N.P., Hamid, S., Hamilton,
552 R.G., Cardoso, M.J., 1996. The 14.6 kD rubber elongation factor (Hev b 1) and 24 kD (Hev b
553 3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex
554 allergy. J. Allergy Clin. Immunol. 98, 628–639.
555
- 556 40. Yeang, H.Y., Yip, E., Hamzah, S., 1995. Characterization of zone 1 and zone 2 rubber
557 particles in *Hevea brasiliensis* latex. J. Nat. Rubber Res. 10, 108–123.

558

Draft

559 Table 1: List of glycosylation

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

560

561

Draft

562 Table 2: Supporting peptide found in HBM_contig338536_s149176c3_gene1.protein.

Peptide	-10lgP	Mass	Start	End	PTM
R.TPM(+15.99)GGFLGLLSTLPATKLGSAIEAALKR.A	70.66	2941.668	22	50	Oxidation (M)
R.GIAAQDSGAFaweIVPVEVSGGR.G	67.45	2315.155	196	218	
K.ITGYADAAQAPeLFTTAPALAIPK.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.AVSNAGLDASQVDYYEINEAFavVALANQK.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)GGFLGLLSTLPATKLGSAIEAALKR	53.65	2785.566	22	49	Oxidation (M)
A.PVAAAeIKPR.D	51.68	1050.619	3	12	
T.GYADAAQAPeLFTTAPALAIPK.A	49.17	2215.152	289	310	
R.GKPSTSVdKDEGLGK.F	49.16	1516.773	219	233	
M.APVAAAeIKPR.D	49.16	1121.656	2	12	
R.GKPSTSVdKDEGLGKFDPVK.L	48.7	2103.085	219	238	
R.QAALGAGIPNSVVC(+57.02)TTVNKVC(+57.02)ASG M(+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)ES M(+15.99)SNAPK.Y	42.67	3050.472	104	133	Oxidation (M)
R.QAALGAGIPNSVVC(+57.02)TTVNKVC(+57.02)ASG MK.A	42.02	2632.314	78	103	Carbamidomethylation
K.LGSIAIEAALKR	36.86	1084.649	39	49	
K.LGLQVIAK.I	34.85	840.5433	279	286	
P.VAAAeIKPR.D	32.96	953.5658	4	12	
F.TTAPALAIPK.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)ESMSNAP K.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)ISDGAAALVLVSGETALK LGLQVIAK.I	16.57	3682.914	256	286	Hex1HexNAc1
Total 35 peptides					

563

564

565

566

Table 3: Summary of the total protein found in each subcellular fraction of latex (with at least 1 unique peptide) (proteomics method)

	C-Serum	Lutoids	RParticle	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	
SwissProt	1120	594	754	2095	
PlantGDB	1849	1201	1236	3642	

571 Table 4: Summary of the total protein found in each subcellular fraction of latex (with at least 1
572 unique peptide) (glycoproteomics method)

	C-Serum	Lutoids	RParticle	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	
SwissProt	400	117	74	557	
PlantGDB	748	230	125	1025	

573

574

Table 5: Summary of the total protein 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	
SwissProt	2095	557	2324	
PlantGDB	3642	1025	4038	

579 Figure 1 Representative MS spectra of HBM_contig260697_s115748c12_gene1.protein
580

581 Figure 2 (Panel A) Representative MSMS spectra of peptide sequence of
582 AAVPSESTGLYEALRL in HBM_contig260697_s115748c12_gene1.protein
583

584 Figure 2 (Panel B) Representative MSMS spectra of peptide sequence of
585 KLVLPVPAFNVINGGSHAGNK in HBM_contig260697_s115748c12_gene1.protein
586

587 Figure 2 (Panel C) Representative MSMS spectra of peptide sequence of
588 ACNALLLKVNQIGSVTESTEAVK in HBM_contig260697_s115748c12_gene1.protein
589

590 Figure 2 (D) Representative MSMS spectra of peptide sequence of
591 SGETDTFIADLSVGLATGQIK in HBM_contig260697_s115748c12_gene1.protein
592

593 Figure 3 The whole protein sequence of HBM_contig338536_s149176c3_gene1.protein with the
594 identified peptide sequences (blue line below the protein sequence)
595

596 Figure 4 Venn diagrams of the numbers of protein found in each subcellular fractions based on
597 database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex
598 biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See
599 Supplementary Tables 1a-1h for the full list of proteins identified from each database).
600

601 Figure 5 Venn diagrams of the numbers of protein and glycoprotein found in each subcellular
602 fractions based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation,
603 (d) latex biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See
604 Supplementary Tables 2a-2h for the full list of proteins identified from each database).
605

606 Figure 6 Venn diagrams of the numbers of protein and glycoproteins found in each extraction
607 method based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d)
608 latex biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See
609 Supplementary Tables 3a-3h for the full list of proteins identified from each database).

R : Resolution

Z : charge state

Figure 1 Representative MS spectra of HBM_contig260697_s115748c12_gene1.protein

Figure 2 (Panel A) Representative MSMS spectra of peptide sequence of AAVPSESTGLYEALR in HBM_contig260697_s115748c12_gene1.protein

#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	129.10	111.09	112.08	65.05	K					21
2	242.20	224.18	225.19	121.59	L	2004.18	1986.20	1987.08	1002.55	20
3	341.31	323.35	324.28	171.13	V	1891.12	1873.01	1873.99	946.01	19
4	454.40	436.38	437.34	227.09	L	1792.03	1773.94	1774.92	896.47	18
5	551.39	533.83	533.83	276.20	P	1678.91	1660.86	1661.90	839.49	17
6	650.50	632.45	633.53	326.27	V	1581.81	1563.80	1564.79	790.61	16
7	747.52	729.50	730.49	374.30	P	1482.81	1464.73	1465.91	741.46	15
8	818.64	800.54	801.52	410.32	A	1385.90	1367.68	1368.67	693.40	14
9	965.64	947.61	948.59	483.31	F	1314.79	1296.64	1297.69	657.24	13
10	1079.73	1061.65	1062.83	540.33	N	1167.59	1149.58	1150.68	585.02	12
11	1178.79	1160.72	1161.82	589.34	V	1053.69	1035.53	1036.65	527.78	11
12	1291.92	1273.85	1274.79	645.91	I	954.55	936.46	937.52	477.24	10
13	1405.89	1387.85	1388.83	703.43	N	841.51	823.40	824.36	421.20	9
14	1462.88	1444.87	1445.85	731.97	G	727.39	709.34	710.32	364.17	8
15	1519.90	1501.89	1502.87	760.45	G	670.38	652.34	652.93	335.73	7
16	1606.93	1588.92	1589.91	804.53	S	613.38	595.29	596.28	307.15	6
17	1744.11	1726.18	1726.18	872.50	H	526.34	508.26	509.35	263.68	5
18	1815.03	1797.02	1798.27	908.01	A	389.23	371.23	372.19	195.11	4
19	1871.95	1854.04	1855.02	936.52	G	318.21	300.27	301.08	159.59	3
20	1986.20	1968.08	1969.07	993.55	N	261.24	243.15	244.18	131.08	2
21					K	147.11	129.10	130.09	74.06	1

Figure 2 (Panel B) Representative MSMS spectra of peptide sequence of KLVLPVPAPFNVINGGSHAGNK in HBM_contig260697_s115748c12_gene1.protein

#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.04	54.03	55.02	36.52	A					23
2	232.08	214.07	215.05	116.54	C(+57.02)	2386.30	2368.29	2369.27	1193.77	22
3	346.29	328.25	329.26	173.56	N	2226.40	2208.38	2209.24	1113.64	21
4	417.30	399.38	400.21	209.08	A	2112.29	2094.37	2095.20	1056.61	20
5	530.28	512.36	513.25	265.23	L	2041.34	2023.18	2024.16	1021.10	19
6	643.40	625.55	626.39	322.16	L	1928.21	1910.10	1911.08	964.55	18
7	756.81	738.40	739.45	378.70	L	1815.14	1797.01	1798.00	908.01	17
8	884.53	866.55	867.48	442.75	K	1702.03	1684.23	1684.91	851.47	16
9	983.69	965.56	966.89	492.29	V	1573.84	1555.83	1556.82	787.42	15
10	1097.65	1079.57	1080.59	549.67	N	1475.02	1456.86	1457.75	737.89	14
11	1225.77	1207.79	1208.65	613.34	Q	1360.73	1342.84	1343.70	680.87	13
12	1338.85	1320.75	1321.85	669.88	I	1232.79	1214.74	1215.80	616.84	12
13	1395.78	1377.77	1378.75	698.39	G	1119.66	1101.62	1103.05	560.18	11
14	1483.02	1464.99	1465.78	741.91	S	1062.65	1044.81	1045.54	531.78	10
15	1581.98	1563.87	1564.85	791.44	V	975.61	957.53	958.11	488.35	9
16	1682.93	1664.48	1665.90	842.39	T	876.54	858.50	859.44	438.73	8
17	1811.50	1793.92	1794.94	906.48	E	775.52	757.49	758.39	388.30	7
18	1898.95	1881.22	1881.97	950.00	S	646.46	628.37	629.35	323.69	6
19	2012.19	1994.46	1995.06	1006.54	I	559.37	541.33	542.32	280.15	5
20	2141.16	2123.12	2124.10	1071.06	E	446.37	428.25	429.30	223.63	4
21	2212.18	2194.15	2195.29	1106.58	A	317.26	299.22	300.19	159.11	3
22	2311.33	2293.20	2294.21	1156.12	V	246.16	228.17	229.15	123.59	2
23					K	147.11	129.10	130.09	74.06	1

Figure 2 (Panel C) Representative MSMS spectra of peptide sequence of ACNALLKLVNQIGSVTESTEAVK in HBM_contig260697_s115748c12_gene1.protein

Figure 3 The whole protein sequence of HBM_contig338536_s149176c3_gene1.protein with the identified peptide sequences (blue line below the protein sequence)

Figure 4 Venn diagrams of the numbers of protein found in each subcellular fractions based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See Supplementary Tables 1a-1h for the full list of proteins identified from each database).

Figure 5 Venn diagrams of the numbers of protein and glycoprotein found in each subcellular fractions based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See Supplementary Tables 2a-2h for the full list of proteins identified from each database).

Figure 6 Venn diagrams of the numbers of protein and glycoproteins found in each extraction method based on database: (a) photosynthesis, (b) latex allergens, (c) rubberwood formation, (d) latex biosynthesis, (e) disease resistance, (f) annHBM, (g) SwissProt, and (h) PlantGDB. (See Supplementary Tables 3a-3h for the full list of proteins identified from each database).