PROTEOMIC PROFILING OF *Hevea* LATEX SERUM INDUCED BY ETHEPHON STIMULATION

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ABSTRACT

Production of rubber tree latex is closely related to the duration of latex flow after tapping. Ethephon, an ethylene releasing compound is a stimulant used to increase the latex yield and prolong the latex flow. The effect of ethephon on latex serum proteome and its significance to latex flow was investigated using quantitative proteomic analysis namely Isobaric Tags for Relative and Absolute Quantification (iTRAQ). For this experiment, latex from RRIM 2023 was harvested during high-yielding period. Yield of freshly tapped latex was measured and fractionated using high speed centrifugation to obtain the desired serum. These non-stimulated and ethephon-stimulated trees yielded 42.74 g/t/t and 94.66 g/t/t, respectively. Latex serum proteins from trees under ethephon stimulation were identified and their expression levels were quantified. Results showed that more than 300 proteins were identified in latex serum and 116 proteins were differentially expressed. These differentially expressed proteins were further categorised into cellular component, biological process and molecular function categories. The analysis indicated that ethephon considerably induced numerous latex serum proteins that are involved in multiple biological processes and molecular functions. Most of the identified proteins are located in the organelles, membrane and extracellular region. Under molecular function category, most of the proteins were involved in binding activity, followed by catalytic activity as well as antioxidant activity. From the analysis, it revealed that carbohydrate metabolisms were predominant under the metabolic pathways affected by ethephon treatment. These findings could provide further insights into ethephon effect on latex serum proteome and its role in latex yield production.

INTRODUCTION

Hevea brasiliensis latex is the major source of commercial natural rubber. It is produced within the cytoplasm of laticiferous cells. The laticifer or simply known as latex vessels develop from the vascular cambium cells, anastomose, and form a concentric network within the phloem tissue (Dickenson 1965, Hebant 1981). *Hevea* latex is a colloidal suspension and biochemically a living cytoplasm that has a variety of molecules such as rubber particles, proteins, carbohydrates and other cell contents (Archer et al. 1969). It consists of three major fractions which are rubber cream, cytosol and bottom fraction, when separated using ultracentrifugation (Moir 1959).

Latex is harvested from rubber tree by tapping the bark in half spiral to ensure the latex vessels are fully wounded to produce maximum yield output. However, production of *Hevea* latex is highly affected by other factors as well such as duration of latex flow after tapping and the capability of latex regeneration between two consecutive tappings (d'Auzac et al. 1997). Hence, ethephon which is an ethylene releasing compound is used as stimulant to increase the latex yield and prolong the latex flow (Chrestin et al. 1997). The discovery was made in 1960s and has been continuously improved to optimise its potential to increase latex production and ironically, its detrimental consequences on rubber tree.

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Ethephon is commonly used as plant growth regulator and also known to have a diverse effect in plant physiology and development. Extensive studies have been conducted to understand the mechanism of ethephon and its consequences to the plant system. For example, it is implied that ethephon influenced growth, photosynthesis and nitrogen accumulation of *Brassica juncea* depending on the amount of nitrogen in the soil (Khan et al. 2008). Meanwhile, ethephon also has other profound effects on plant which is to enhance production of secondary metabolite and regulation of plant disease such as gum formation and regulation of pathogenesis related proteins, respectively (Li et al. 2014).

Application of ethephon on rubber tree implicates various metabolic responses and biochemical processes such as carbohydrate transport and metabolism, sucrose and glucose loading, protein synthesis**,** compartmental pH and nitrogen assimilation (Coupé and Chrestin 1989, Wang et al. 2015). Studies of ethephon effects on *Hevea* also create a centre of attention. A number of research had been performed for examples, its effect on the expression of protein allergen such as hevein (Hev b6) (Arokiaraj and Yeang 2006), rubber biosynthesis pathway (Zhu and Zhang 2009) and its connection to latex flow (Low and Yeang 1985).

The emergence of multiple options for protein analysis using proteomics-based technology is promising. As more advanced techniques are developed, protein could be identified and quantified simultaneously. Among them, an innovative MS-based approach known as Isobaric Tag for Relative and Absolute Quantification (iTRAQ) which uses isobaric mass reagents for labeling and covalently bonded to the N-terminus and side chain amines of peptides or proteins (Zieske 2006). Quantification is based on the relative intensities of reporter ions that appear in the low mass range of MS/MS spectra (Pichler et al. 2010). This quantitative proteomics method allows a comparison of protein level in complex samples and can be applied to study protein expression between two or more samples. In recent years, iTRAQ has been successfully employed to study many plant responses towards various biotic and abiotic stresses. For instance, such analyses were conducted to understand the regulation of ripening on the non-climacteric grape induced by ABA treatment (Giribaldi et al. 2010) and changes in protein of maize seedling in response to cold stress (Wang et al. 2016).

The evolution of proteomics analysis should be benefited by examining latex serum proteins implicated by ethephon as a whole. For that reason, latex serum from control and ethephon-treated trees were analysed using quantitative proteomics approach. The main objective of this study was to identify differentially expressed proteins in the latex serum upon ethephon treatment on the rubber tree. The differential expression obtained from the latex serum proteome could contribute to further research for better understanding of the molecular mechanism of ethephon effect on the rubber tree and help to shed light on its connection to latex yield production.

MATERIALS AND METHODS

Plant material and ethephon treatment

A total of ten trees of 15 year-old rubber trees (*H. brasiliensis*) clone RRIM 2023, were selected. These trees were planted at Field 101, Pelepah Experimental Station, Kota Tinggi, Johor (1°43'45.75"N 103°53'57.22"E). Rubber trees were treated with 2.5% (v/v) Ethephon two days before tapping.

Latex collection and preparation of latex serum protein

Latex was tapped from control (untreated) and ethephon-treated trees and collected in chilled flasks. Fresh latex was centrifuged at 44 000g for 1 h at 4^{o}C and three distinct fractions were obtained, the rubber cream, C serum and bottom fractions (Moir 1959). The soluble C serum was obtained directly from the

fraction while the bottom fraction which contained lutoids was further washed with 0.4 M Mannitol to remove excessive C serum from the lutoids. Centrifugation was carried out prior to the freeze thaw method to rupture lutoids membrane and release the fluid content, B serum. The ruptured lutoids were subjected to centrifugation at 5 000 rpm for 30 mins at 4^{o}C to separate the supernatant (the B serum) and the pellet (the lutoids' membrane). The concentration of all serum protein obtained was estimated using Bradford assay. Briefly, 10 µL of each protein sample and BSA standard were mixed with 20% (v/v) Bradford reagent (Bio-Rad, Hercules, CA, USA). The quantity of solubilised protein was measured at 595 nm absorbance using spectrophotometer. Protein separation was then performed with a total of 20 µL of protein loaded onto 15% SDS-PAGE and the gels were visualised using Coomassie Brilliant Blue staining method.

Determination of latex fresh weight, dry weight and total water content

The latex was aliquoted to determine the latex fresh weight (FW) and dried for 72 h in an oven at 60 ^{o}C to obtain the dry weight (DW). The total water content (TWC) was calculated as follows: TWC= [(FW-DW)/FW] x 100.

Tree productivity

Tree productivity (g/t/t) is the average dry yield of tree obtained from a single tapping over a period of time which can only be calculated after the value of dry rubber content (DRC) of the latex has been obtained.

Sample preparation of iTRAQ and mass spectrometry analysis

Latex serum was dialysed overnight against water at 4^{o}C using 3500 MWCO dialysis tubing (Thermo Fisher Scientific, USA) to remove inherent salt. Subsequently, serum proteins were quantified using Bradford Assay (Bio-Rad, Hercules, CA, USA) to determine protein concentration. In addition to that, 50 µL of dialysed protein for each serum was lyophilised.

For iTRAQ analysis, proteins were dissolved in iTRAQ dissolution buffer and precipitated using the ITSI Bio's ToPREP kit to remove impurities in the sample. One hundred microgramme of protein was used for iTRAQ analysis. Proteins were reduced, alkylated and digested using trypsin and then labeled with iTRAQ 4- plex. The labeled peptides were cleaned and eluted by strong cation exchange (SCX) column using ammonium acetate gradient. The SCX was used to separate the peptides in the first dimension. These labeled peptides were dried and redissolved in 20 µL 5% acetonitrile in 0.1 % formic acid. Samples were loaded onto a PicoFrit C18 nanospray column (New Objective) using a Thermo Scientific Surveyor Autosampler operated in the no waste injection mode. Peptides were eluted from the column using a linear acetonitrile gradient from 5 to 45% acetonitrile over 230 minutes followed by high and low organic washes for another 20 minutes into an LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). A data-dependent Top 3 method was used where a full MS scan from m/z 400 to 1500 was followed by MS/MS scans on the three most abundant ions. Each ion was subjected to CID for peptide identification followed by PQD for iTRAQ quantitation.

Database search parameters and bioinformatics analysis

RAW data files were searched using Proteome Discoverer 1.3 (Thermo Scientific) and the SEQUEST algorithm against the most recent species-specific database for *Euphorbiaceae* downloaded from UniProt. Trypsin was the selected enzyme allowing for up to two missed cleavages per peptide. Methylthio Cysteine, N-terminal iTRAQ 8-plex, and Lysine iTRAQ 8-plex were used as a static modifications and

oxidation of methionine as a variable modification. Proteins were identified when two or more unique peptides had X correlation scores greater than 1.5, 2.0, and 2.5 for respective charge states of +1, +2 and +3. False Discovery Rate (FDR) analysis was performed and FDR ≤ 1.0% was estimated for protein identification by using target-decoy search strategy. iTRAQ Ratio (Treated (114 or 116) / Control (113 or 115)) is indicated as up-regulated when the ratio is more than 1.5, below than 0.67 as down-regulated and moderate to no change when ratio is between 1.5 to 0.67.

Immuno-blotting

SDS-PAGE was carried out on 15 % gel according to Laemmli (1970) and the separated proteins were transferred electrophoretically on to the nitrocellulose membrane. The membrane was blocked with 5 % non-fat milk in phosphate buffered saline (PBS) and then incubated with USM/RC2, the monoclonal antibody against the small rubber particle protein (SRPP/ Hev b 3) and polyclonal antibodies against the rubber elongation protein (REF/ Hev b 1) and Agrisera sucrose phosphate synthase protein as the primary antibody. After washing in PBS-milk, the membrane was incubated with rabbit anti-mouse IgG and goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Aldrich, MO, USA), respectively. After a further washing with PBS-milk, the nitrocellulose membrane was incubated for 10 minutes in Tris buffered saline (TBS) before being immersed in 5-bromo-4-chloro-3-indolyl phosphatase (BCIP/NPT) (Sigma Aldrich, MO, USA) reaction product. A matching gel was stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

The rubber tree clone RRIM 2023 which is a latex timber clone was selected for this experiment and it is one of five other RRIM 2000 series clones in Malaysian Rubber Board (MRB) 2013 Clone Recommendation. Basically, the Clone Recommendation is divided into two specific groups, Group 1 and Group 2. RRIM 2023 falls under Group 2 because secondary information such as disease resistance, response to stimulation and bark thickness of these clones are incomplete and research is still on-going.

In order to determine the significant difference of the yield of latex between the stimulated and non-stimulated tree, the tree productivity was measured based on gram per tree per tapping (g/t/t). The results indicated that ethephon-stimulated and non-stimulated trees yielded about 94.66 g/t/t and 42.74 g/t/t, respectively, which were significantly different at $p \leq 0.05$. In general, ethephon stimulation helped to increase the fresh latex yield by two-fold. Previous study on the other RRIM 2000 series clones showed positive response to ethephon stimulation as latex yield was increased after the stimulation**.** It was also suggested that bark treatment with 2.5 % ethephon is sufficient and optimum to promote higher production of latex yield (Abang et al. 2007). On another note, the water content of latex from ethephon-treated trees (55.6 %) was increased in comparison with the control (49.1 %). This result is consistent with other reports where ethephon was found to be involved in latex dilution reaction (An et al. 2015) and hence prolonged the latex flow (Zhu and Zhang 2009).

Subsequently, to understand the effect of ethephon on rubber tree and its implication on latex production, latex serum protein was isolated from fresh latex collected during high-yielding period (HYP) that occurs between October until December (Figure 1). Serum proteome from ethephon-treated and control trees were analysed for protein identification and their differential expression using iTRAQ proteomic analysis system to describe global changes on the latex serum protein in response to ethephon treatment.

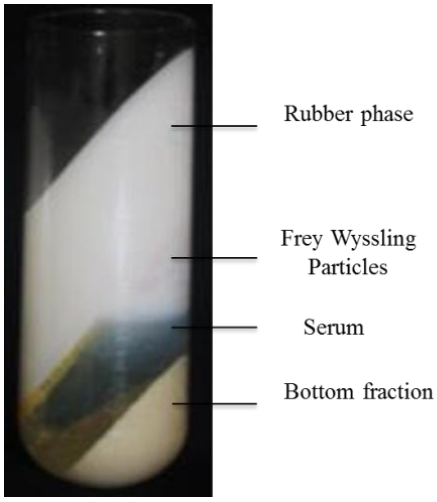

Figure 1. Fractionation of fresh latex by high-speed centrifugation yielded four main fractions which are rubber phase, Frey-Wyssling, serum and bottom fraction.

Protein identification was based on the nearest sequenced homologue alignment results with statistically significant at $p<0.05$. The proteomic analysis identified more than 300 proteins. However, only 116 proteins had significant quantitative iTRAQ ratios and were further analysed using Blast2GO (Conesa et al. 2008, Conesa et al. 2005) program against the non-redundant protein database (NR) at NCBI for their functional annotations. Based on protein score from the protein identification, the result showed that the most abundant protein cluster in the latex were small rubber particles protein (SRPP), beta-1,3-glucanase, hevamine, elicitor-responsive protein and type II metacaspase (Table 1). A proteomic analysis on other rubber tree clones also reported that these proteins were among the most abundant protein clusters found in latex (Dai et al. 2015).

Table 1. Example of the most abundant *Hevea* latex serum proteins from the protein identification analysis.

Protein Identity	Accession Number	Protein Score	Calc. p*I*	Predicted Molecular Weight (kDa)
Small rubber particles protein (*Hevea brasiliensis)*	14423933	323.93	4.91	22.3
1,3-beta glucanase (*Hevea brasiliensis*)	387778880	791.96	8.92	41.2
Hevamine (*Hevea brasiliensis)*	157831407	699.20	8.19	29.5
Elicitor-responsive protein (*Hevea brasiliensis)*	334854634	1436.46	4.18	15.2
Type II metacaspase (*Hevea brasiliensis)*	305387431	221.18	5.17	45.9

Meanwhile, Blast2Go analysis has classified the identified proteins as those located mostly at the organelles (plastid, vacuole, mitochondrion, Golgi apparatus), membrane and cytosol. Majority of the identified proteins were involved in biological regulation, cellular process, metabolic process as well as response to stimulus. Under molecular function category, most of the proteins were involved in binding

activity, followed by catalytic activity and antioxidant activity. The analysis also mapped metabolic pathways for the identified proteins, and revealed that carbohydrate metabolisms were predominant (Figure 2).

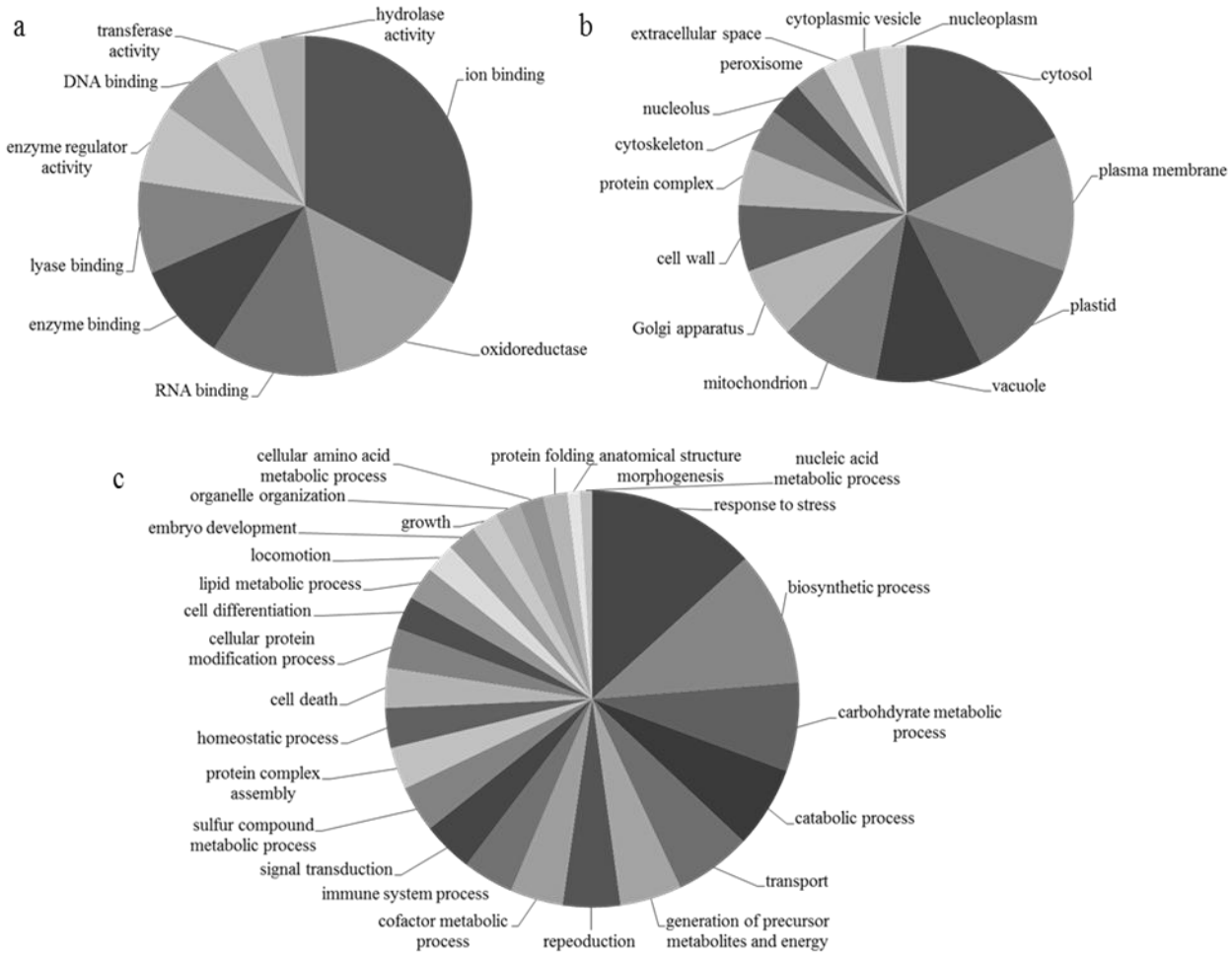

Figure 2. Blast2Go analysis of proteins identified from quantitative proteomic analysis. Classification of identified proteins in fresh latex serum by a. molecular function, b. cellular compartment, and c. biological process.

Table 2 highlights some of the proteins with significant differential expression between the ethephon-treated samples and control. The output indicated that ethephon considerably induced numerous latex serum proteins involved in multiple biological processes and molecular functions.

For example, elicitor-responsive protein, small rubber particle proteins (SRPP), rubber elongation factor (REF), aconitase, glutamine synthetase caffeic, acid 3-O methyltransferase, enolase and profilin were among the up-regulated proteins after ethephon treatment. Meanwhile, fructose-bisphoshate aldolase, sucrose phosphate synthase, polyphenol oxidase and malate dehydrogenase were among the down-regulated proteins after ethephon stimulation.

Table 2. Selected *Hevea* latex serum proteins with differential expression when induced by ethephon.

Protein Identity	Accession Number	Biological process	Predicted molecular weight (kDa)	Protein ratio (treated:control)
Up-regulated				
Glutathione-S-transferase (*Hevea brasiliensis*)	333690873	Metabolic process	25.5	1.535
Rubber elongation factor protein (*Hevea brasiliensis*)	530291173	Biosynthetic process	14.0	1.777
Small rubber particle protein (*Hevea brasiliensis*)	14423933	Biosynthetic process	22.3	2.092
Glutamine synthetase (*Jatropha curcas*)	802732576	Biosynthetic process / cellular process	39.2	1.549
Caffeic acid 3-O methyltransferase (*Hevea brasiliensis*)	443908531	Biosynthetic process	40.6	2.481
Proton ATPase subunit protein (*Hevea brasiliensis*)	558633439	Ion transport	59.9	1.533
Down-regulated				
Fructose-bisphosphate aldolase (*Ricinus communis*)	223531989	Biosynthetic process/ Metabolic process	38.6	0.660
Sucrose phosphate synthase (*Ricinus communis*)	223545431	Biosynthetic process	114.3	0.548
Polyphenol oxidase (*Hevea brasiliensis*)	570933638	Biosynthetic process	67.9	0.500
Malate dehydrogenase (*Jatropha curcas*)	802581654	Metabolic process	35.7	0.598
Hevamine (*Hevea brasiliensis*)	157831407	Metabolic process/ Pathogenesis-related	29.5	0.611
1,3-beta glucanase (*Hevea brasiliensis*)	387778880	Catabolic process	41.2	0.600
Phosphoglucomutase (*Ricinus communis*)	223532818	Catabolic process/ metabolic process/ biosynthetic process	63.2	0.577

Several isoforms of SRPP and REF proteins were identified on the rubber particles with different response to wounding, ethylene treatment and among rubber tree clones as well (Tong et al. 2017). Wang et al. (2015) specified that the level of REF2, SRPP1 and SRPP3 were increased upon ethylene treatment while REF and SRPP level were decreased. In this study, SRPP and REF proteins were increased upon

ethylene stimulation but no further analysis was performed to identify their isoforms. Accumulation of SRPP and REF proteins upon ethephon stimulation may display the importance of these proteins in rubber biosynthesis activity (Xiang et al. 2012). In addition, different patterns of expression for these proteins towards ethylene stimulation probably indicated discrete functions in rubber biosynthesis.

Caffeic acid 3-O methyltransferase and proton ATPase subunit protein were up-regulated upon ethephon treatment on the rubber tree. Caffeic acid 3-O methyltransferase is involved in lignin biosynthesis and accumulation of this enzyme under stress could be related to increased lignification of the cell wall as a modification to avoid water loss induced by osmotic stress (Simova-Stoilova et al. 2015) which might be linked to the increase of water content in latex (An et al. 2015). Meanwhile, proton ATPase subunit protein regulates the pH of latex cytosol and energises the transport of various solutes inside the lutoids both of which might influence rubber biosynthesis (Sreelatha et al. 2000). Similarly, glutamine synthetase (GS), the key enzyme in plant ammonium (NH^{4+}) metabolism reported to play an important role in enhancing tolerance to salt and chilling stresses in rice (Hoshida et al. 2000) was also increased in ethephon-treated serum sample. GS response to ethylene might be mediated by ammonia that increases in latex cytosol following ethylene treatment (Clément et al. 2001, Pujade-Renaud et al. 1994).

Wounding and plant hormone treatment on plants could cause overproduction of reactive oxygen species (ROS) which oxidises proteins, lipids, carbohydrates and DNA, and causes irreversible damages to plant cells (Gill and Tuteja 2010). The general radical scavenging measurements are always associated with the presence of antioxidant such as peroxiredoxin, glutaredoxin, lactoylglutathione lyase and glutathione S-transferase (Noctor et al. 2012, Gelhaye et al. 2005). Thus, changes of protein expression in latex serum when triggered by ethephon treatment implied their important role to maintain and regulate plant redox homeostasis process and defense system in rubber tree (Dalton et al. 2009).

On a different note, pathogenesis-related proteins associated with rubber particles destabilisation such as hevamine and 1,3-beta glucanase (Hao et al. 2004) were expressed lower in ethephon-treated than in control. These proteins are also known as latex allergens, Hev b 2 and Hev b 9 under PR-2 and PR-8 family (Sinha et al. 2014) which are also involved in chitin metabolism, respectively. Meanwhile, polyphenol oxidase (PPO) probably plays a role in defense against pathogen, responsible for latex browning and its activity in latex has been associated with latex coagulation and wound sealing. Hence, the decrease of PPO in ethephon-treated latex serum indicated that it was down-regulated by ethylene (Li et al. 2014). The decline in accumulation of glucanase, hevamine (chitinase III) and PPO in ethephon-treated sample might hamper rubber particle aggregation which consequently prolonged the latex flow.

A number of proteins associated with central carbohydrate metabolism were decreased upon ethephon treatment such as sucrose phosphate synthase in starch and sucrose metabolism, fructose bisphosphate aldolase and phosphoglucomutase in glycolysis pathway and malate dehydrogenase in citric acid cycle. Sucrose phosphate synthase (SPS) is the key determinant in partitioning of photosynthetically fixed carbon in the leaf and in the whole plant. From the analysis, expression level of SPS is reduced by ethephon treatment, which implied that decreased expression of SPS inhibited sucrose synthesis. Hence, with the increase of UDP-Glc/ hexose-P ratio, alternative and novel regulatory mechanisms might have been activated to compensate for the decrease in SPS expression (Strand et al. 2000).

Phosphoglucomutase (PGM) plays an important role in plant growth and is crucial in partitioning carbon between starch synthesis and carbohydrate oxidation in photosynthetic and heterotrophic tissues (Tetlow et al. 1998). Plants with reduced PGM activity displayed a decreased rate of photosynthesis, dramatic reduction in nucleotide levels and more complex changes on a range of diverse metabolic pathways (Lytovchenko et al. 2002). Fructose bisphosphate aldolase and malate dehydrogenase which are involved in carbohydrate and energy metabolism were among the most abundant protein found in latex serum

(Sunderasan et al. 2015). Malate dehydrogenase as a component of malate valves plays a key role in energy homeostasis of plant cell. Its activity has been detected in organelle such as mitochondria, chloroplast and also in cytosol. Meanwhile, fructose bisphosphate aldolase is a key enzyme in photosynthesis and a slight change in plastid aldolase activity significantly alters the level of sugars and starch (Haake et al. 1998).

To date, there is no available report yet of these carbohydrate metabolism proteins such as sucrose phosphate synthase, malate dehydrogenase or fructose bisphosphate aldolase and its significant effect on rubber biosynthesis or tree growth. Nevertheless, the differential expression of key enzymes in major carbohydrate metabolism pathways indicated that ethephon has substantial effect on these pathways for rubber tree.

An immuno-blot analysis was performed on selected proteins to evaluate their expression pattern, namely REF (Hev b 1), SRPP (Hev b 3) and sucrose-phosphate synthase (Figure 3). Monoclonal antibody was used for SRPP and it detected a ~17 kDa protein band. As mentioned by Tong et al. (2017), there are several isoforms of SRPP in latex with different p*I* and molecular weight ranging from ~4.0 to ~5.5 and ~14 kDa to ~24 kDa, respectively. Hence, the SRPP identified in this study might specifically belong to one of these isoforms.

Meanwhile, a polyclonal antibody was used to detect REF and SPS. As expected, many protein bands would be detected for REF but only one protein band was identified for SPS. Similar to SRPP, REF also has a number of isoforms but this antibody in particular should detect the ~14kDa protein as highlighted in the box. As for SPS, its original size is ~120 to 130 kDa and could be fragmented into ~30 and ~90 kDa. Apparently only ~ 30 kDa protein was detected which represented the smaller size of its fragments. Nevertheless, the expression pattern between control and ethephon-treated serum sample did not show noticeable difference. Perhaps, different method such as RT-PCR should be employed to complement the quantitative analysis.

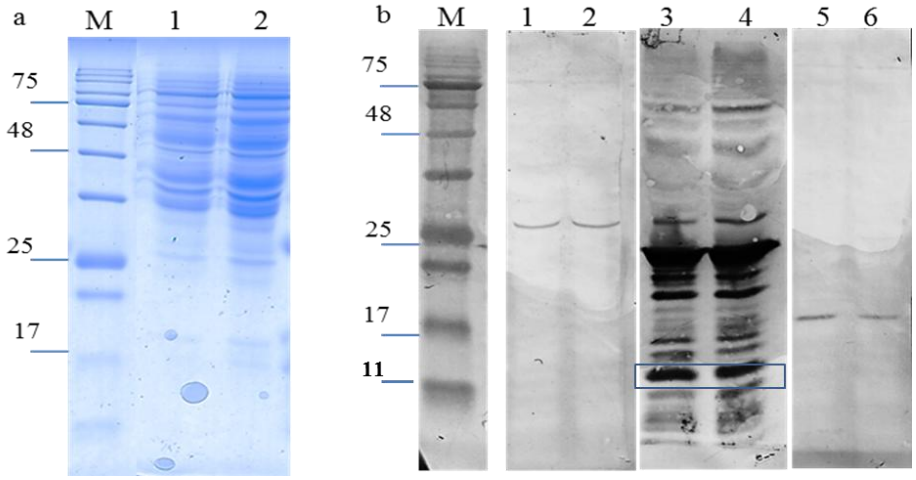

Figure 3. SDS-PAGE analysis of fresh latex serum protein of *Hevea brasiliensis.* A total of 50 µg proteins from the control and treated were run on (a) 15% SDS-PAGE and visualised using Coomassie Brilliant Blue method. M: marker, Lane 1: Control sample Lane 2: Ethephon-treated sample. (b) Immuno-detection analysis for SPS, REF and SRPP from corresponding SDS-PAGE M: marker, Lane 1 and 2: detection of SPS (polyclonal), Lane 3 and 4: detection of REF (polyclonal), Lane 5 and 6: detection of SRPP (monoclonal). Lane 1, 3 and 5: Control samples; Lane 2, 4 and 6: Ethephon-treated samples.

CONCLUSIONS

The proteomic tool utilised in this study allows identification of a series of proteins displaying differential expression patterns after ethephon treatment on rubber tree. Application of ethephon as yield stimulant can implicate various and complex biochemical and physiological changes. For instance, some of them were related to carbohydrate metabolism, plant defense and stress-related response and cell homeostasis as well as rubber biosynthesis. This study helps to gain better understanding of ethephon effects and its connection to latex yield production. Nevertheless, more information is required to elucidate the regulation mechanism of ethylene stimulation with the progress in omics technology in the future.

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