STRUCTURE-FUNCTION RELATIONSHIP BETWEEN NATURAL RUBBER POLYMER AND PROTEINS IN THE LATEX OF MALAYSIAN HEVEA CLONES

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

°C	Degree celsius
°N	Degree north
°E	Degree east
%	Per cent
+	Plus
-	Minus
±	Plus-minus
µm	Micrometer
µg	Microgram
µg/ml	Microgram per milliliter
µL	Microliter
µL/min	Microliter per minute
µA	Microampere
Ð	Polydiversity index
10 ×	Ten times

LIST OF ABBREVIATIONS

10yoPB350	10-year-old, clone PB350
10yoRRIM2025	10-year-old, clone RRIM2025
10yoRRIM3001	10-year-old, clone RRIM3001
2-DE	Two-dimensional gel electrophoresis
5yoPB350	5-year-old, clone PB350
5yoRRIM2025	5-year-old, clone RRIM2025
5yoRRIM3001	5-year-old, clone RRIM3001
7yoPB350	7-year-old, clone PB350
7yoRRIM2025	7-year-old, clone RRIM2025
7yoRRIM3001	7-year-old, clone RRIM3001
ACAT	Acetyl-CoA acetyltransferase
APP	Allylic pyrophosphate
AUC	The area under the curve
BP	Biological process
BPP	Borax/PVPP/Phenol
BSA	Bovine serum albumin
CC	Cellular component
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulfonate
C-serum	Clear serum
Da	Dalton
DAP	Differentially accumulated protein
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DXP	1-deoxy-D-xylulose-5-phosphate
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
FPP	Farnesyl pyrophosphate
FPS	Farnesyl pyrophosphate synthase

g	Gram
g	Gravity
g/tree/tap	Gram per tree per tapping
GGPP	Geranylgeranyl pyrophosphate
GGPS	Geranylgeranyl pyrophosphate synthase
GO	Gene ontology
GPC	Gel permeation chromatography
GPP	Geranyl pyrophosphate
GPS	Geranyl pyrophosphate synthase
h	Hour
HCD	Higher energy collisional dissociation
HCl	Hydrochloride
IAA	Iodoacetamide
ICAT	Isotope-coded affinity tags
IPP	isopentenyl diphosphate
ITRAQ	Isobaric tags for relative and absolute quantitation
KCl	Potassium chloride
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg/ha/year	Kilogram per hectare per year
kV	Kilovolts
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LFQ	Label-free quantification
LGM	Lembaga Getah Malaysia
LTC	Latex timber clone
M	Molar
m/z	Mass to charge ratio
M4PC	Mercator4 protein categories
MEP	2-C-methyl-D-erythritol 4-phosphate
MF	Molecular function
mg/ml	Milligram per millilitre
min	Minute
mm	Millimetre

mM	Millimolar
Mn	Number-average molecular weight
MRB	Malaysian Rubber Board
MRFA	Met-Arg-Phe-Ala acetate salt
ms	Millisecond
MS	Mass spectrometry
MVA	Cytosolic mevalonate
MVA-5P	Mevalonate-5-phosphate
MVA-5PP	Mevalonate pyrophosphate
Mw	Weight-average molecular weight
MWD	Molecular weight distribution
NCBI	National Centre for Biotechnology Information
NR	Natural rubber
PDI	Polydiversity index
PMF	Peptide mass fingerprinting
PMSF	Phenylmethylsulfonyl fluoride
PPC	Panther protein class
ppm	Parts per million
PTFE	Polytetrafluoroethylene
PVPP	Polyvinylpolypyrrolidone
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
QQQ	Triple-quadrupole
QTOF	Quadrupole-time-of-flight
REF	Rubber elongation factor
RI	Refractive index
RNA	Ribonucleic acid
RRIM	Rubber Research Institute Malaysia
RT	Retention time
sd	Standard deviation
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SILAC	Stable isotope labelling with amino acids in cell culture

SRPP	Small rubber particle protein
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
ThFFF	Thermal field-flow fractionation
TMT	Tandem mass tag
Tris	Trisaminomethane
USM	Universiti Sains Malaysia
V	Volts
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
XIC	Extracted ion chromatography

LIST OF APPENDICES

HUBUNGAN STRUKTUR-FUNGSI ANTARA POLIMER GETAH ASLI DAN PROTEIN DALAM LATEKS KLON HEVEA MALAYSIA

ABSTRAK

Getah asli yang diperoleh daripada lateks *Hevea brasiliensis* adalah sangat penting bagi peradaban manusia. Analisis proteomik lateks *H. brasiliensis* telah mendedahkan protein utama yang bertanggungjawab untuk pelbagai laluan biologi seperti biosintesis getah, pertahanan terhadap patogen, toleransi terhadap tekanan, dan penyembuhan luka. Ciri-ciri beberapa klon Hevea di Malaysia masih belum diterokai, terutamanya dari segi berat molekul getah dan kelimpahan protein lateks yang berbeza. Analisis proteomik dan pengedaran berat molekul yang komprehensif telah dijalankan ke atas lateks tiga klon Hevea yang ditanam di Malaysia; PB350, RRIM3001 dan RRIM2025 mengikut kumpulan-kumpulan umur iaitu 5, 7 dan 10 tahun. Berat molekul getah daripada semua sampel menunjukkan kecenderungan penurunan seiring dengan pertumbuhan umur tumbuhan, dengan julat antara 9.76×105 Da hingga 29.26×105 Da. Pengedaran berat molekul menunjukkan pengedaran unimodal di kalangan semua sampel. Sebanyak 619 protein unik telah dikenalpasti dari semua sampel, dengan julat antara 127 hingga 286 protein. Kuantifikasi tanpa label mengenal pasti 50 protein yang terkumpul secara berbeza di antara klon dan kumpulan umur yang berbeza. Analisis perbandingan menunjukkan bahawa berat molekul getah berkorelasi negatif dengan tahap kelimpahan protein yang berkaitan dengan biosintesis getah. Analisis ontologi gen bagi protein yang terkumpul secara berbeza menunjukkan bahawa protein tersebut diklasifikasikan kepada beberapa kumpulan; protein metabolisme RNA, chaperone, pengawal selia transkripsi spesifik gen, enzim interkonversi metabolit, enzim pengubah protein, dan protein translasi. Prosedur pemantauan reaksi berganda telah ditubuhkan

untuk protein yang terkumpul secara berbeza, di mana 4 peptida menunjukkan potensi sebagai penenda biomarker yang boleh digunakan untuk analisis MRM lateks pada masa hadapan. Data komprehensif yang dikumpulkan daripada kajian ini boleh menjadi input yang berharga untuk memahami hubungan antara protein lateks dan berat molekul mereka serta menjadi biomarker berpotensi untuk pembangunan klon Hevea yang lebih baik dan pengurusan tumbuhan.

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ABSTRACT

Natural rubber obtained from the latex of *Hevea brasiliensis* is of major importance to human civilisation. Proteomics analyses of *H. brasiliensis* latex have revealed the major proteins responsible for the various biological pathways, such as rubber biosynthesis, defence against pathogens, stress tolerance, and wound healing. The characteristics of several of the Hevea clones in Malaysia remain undiscovered, especially in the aspect of their rubber molecular weight and latex protein differential abundance. A comprehensive proteomics and molecular weight distribution analysis was conducted on the latex of three Hevea clones grown in Malaysia: PB350, RRIM3001 and RRIM2025, according to their age groups of 5, 7 and 10 years old. The molecular weight of rubber from all samples shows a decreasing trend the older the plant grows older, ranging between 9.76×10^5 Da to 29.26×10^5 Da. The molecular weight distribution shows a unimodal distribution among all samples. A total of 619 unique proteins were identified from all samples, ranging from 127 to 286 proteins. Label-free quantification identified 50 differentially accumulated proteins amongst the different clones and age groups. Comparison analysis indicated that the molecular weight of the rubber is negatively correlated with the abundance level of the rubber biosynthesis-related proteins. Gene ontology analysis of the differentially accumulated proteins shows that the proteins are classified into several groups: RNA metabolism protein, chaperone, gene-specific transcriptional regulator, metabolite interconversion enzyme, protein-modifying enzyme, and translational protein. A multiple reaction

monitoring procedure was established for the differentially accumulated proteins, where 4 peptides showed potential as viable biomarkers for future latex MRM analysis. The comprehensive data gathered from this study could serve as valuable input into understanding the relationship between latex proteins and their molecular weight, as well as becoming potential biomarkers for better Hevea clone development and plant management.

CHAPTER 1

INTRODUCTION

1.1 Background

Latex is a cloudy, coloured and often sticky fluid secreted by many plants. Latex produced from the Brazilian rubber tree, *Hevea brasiliensis* (Willd. ex A, Juss.) Mull. Arg, became one of the most important sources of rubber in modern industry (Jacob et al., 1993). The latex extracted from *H. brasiliensis* contains a high percentage (30-45%) of natural rubber (NR) as well as having high molecular weights, elasticity and resilience (Mooibroek & Cornish, 2000). The bark of the rubber tree contains laticifer cells located in the phloem, where latex is the cytoplasm. Latex is excreted when the bark of the rubber tree is tapped (Jacob et al., 1993; Zhu & Zhang, 2009).

In 2023, the global demand for NR was clocked at 15.501 million tonnes, while the global supply of NR was reported at 15.141 million tonnes (Toh, 2024). According to the Malaysian Rubber Board (lgm.gov.my), in 2022, Malaysia currently has more than 1.136 million hectares of rubber plantations, making rubber the second largest plantation area of industrial crops in Malaysia, behind palm oil (5.650 million hectares). Currently (2024), Malaysia employs a total of 7943 rubber estate workers and 97039 rubber manufacturing workers (lgm.gov.my). It has also been reported that in 2024, the Malaysian rubber industry contributed RM33.67 billion (2.2 %) to the national exports. In 2023, Malaysia was the fourth largest exporter of natural rubber (USD0.92 billion), behind Thailand (USD3.95 billion), Indonesia (USD2.91 billion), and Cote d'Ivoire (USD2.43 billion) (OEC[1], n.d.). Malaysia was also the largest exporter of rubber gloves in the world with USD1.78 billion in 2023, followed by China (USD0.99 billion) and Thailand (USD0.82 billion) (OEC[2], n.d.).

Despite being a very valuable commodity to humans, the physiological role of latex towards the rubber tree itself remains unclear. Latex was produced in excess amounts by the rubber tree while having no way to degrade it themselves (Men et al., 2019). Latex might have a role in the rubber tree's defence strategy by protecting the plant from herbivory, insects, and pathogens by sealing the plant's wounded areas (Agrawal and Konno, 2009; Konno, 2011). There are also multiple reports of defence and stress-related proteins identified in the latex, such as the proteases, protease inhibitors, lectins, chitin-binding proteins, chitinases, and oxidases (Kanokwiroon et al., 2008; Agrawal & Konno, 2009; Men et al., 2019).

The NR from the Hevea rubber tree is a polymer composed of 320 to 35,000 isoprene molecules. This cis-1,4-polyisoprene polymer is formed through the sequential condensation of isopentenyl diphosphate (IPP) units. This process is facilitated by the enzyme rubber transferase and the presence of cofactor Mg2+. The IPP units are synthesised through both the cytosolic mevalonate (MVA) pathway and the plastidic 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, with the MVA pathway being the primary source for rubber synthesis while the MEP pathway exclusively produces carotenoids (Kekwick, 2002; Ko et al., 2003; Chow et al., 2011; Kekwick, 2018).

H. brasiliensis and its clones remain one of the most extensively studied trees among latex-producing plants. Some of the aspects studied include latex composition (Jacob et al., 1993), latex gene expression (Han et al., 2000) and metabolic pathways involved in rubber biosynthesis (Chow et al., 2007; Chow et al., 2011) as well as the identification of proteins that are available inside the latex (Habib et al., 2017). Among those publications, the number of research concentrating on molecular weight and

molecular weight distribution of *H. brasiliensis* latex is relatively low, even though the physical properties and processing ability of rubber products vary according to the molecular weight.

Natural rubber from Hevea trees inherently possesses high molecular weight (Mw) and has a wide range of molecular weight distribution (MWD). The Mw and MWD of the rubber are dependent on clonal type, treatment after extraction, soil and climatic factors. Age also influences the rubber produced, whereby latex tapped from young trees has a lower molecular weight as compared to mature trees (Kovuttukulrangsie & Sakdapipanich, 2005). High Mw and MWD are indications of high rubber quality (Cornish, 2001a). Rubber with high Mw is an important criterion for rubber vulcanisation during rubber processing (Coran, 2002).

Several works done on this subject incorporate the use of thermal field-flow fractionation (ThFFF) (Lee & Molnar, 1994), light scattering and osmometry in determining Mw and MWD. However, gel permeation chromatography (GPC) is a frequently used method of molecular weight determination in natural rubber, as demonstrated by Kovuttukulrangsie & Sakdapipanich (2005) and Spano et al. (2012). Hence, for this research, GPC was employed to determine the Mw and MWD of Hevea tree clones available in Malaysia.

Another important issue for the *H. brasiliensis* latex productivity is the studies into increasing the latex yield. Two ways to achieve this are by increasing the activity of rubber biosynthesis or increasing the duration of latex flow (Sreelatha et al., 2016). It was known that the rubber yield (g/tree/tap) of the Hevea tree clones from Malaysia is 46.3, 48.7, and 66.3 for Hevea clones RRIM 600, PB 217, and PB 260, respectively

(Sreelatha et al., 2016). These differences between the clones may be related to the differential protein amounts available inside the latex.

Proteins were discovered in latex in the early twentieth century. Latex proteins, largely the rubber transferase, are known to regulate various biological pathways within the *H. brasiliensis* trees, such as natural rubber biosynthesis, defence against pathogens, wound healing, and stress tolerance (Habib & Ismail, 2021). The enzymatic activity of these rubber transferases was also suggested to influence the molecular weight of rubber (He et al., 2022).

Liquid chromatography-mass spectrometry (LC-MS) has become the preferred method to study latex proteins. However, a suitable latex protein extraction method for direct analysis through LC-MS has yet to be reported.

The first part of this research is to determine the most effective protein extraction method to use on the latex samples for a direct LC-MS analysis. The second part is to profile the molecular weight and molecular weight distribution through gel permeation chromatography (GPC); to detect and quantify latex proteins; and to identify the proteomics factors influencing the molecular weight of latex, among the latex obtained from the three selected Malaysian clones, PB350, RRIM3001, and RRIM2025, across three different age groups, 5 years old, 7 years old, and 10 years old. The third part is to analyse the proteins via multiple reaction monitoring (MRM) to further validate the proteins as potential biomarkers. This study allows us to understand the fundamental properties of the different rubber clones in Malaysia, while getting insights into the rubber molecular weight correlation with biological pathways, which potential can allow us to manipulate that in the future.

1.2 Rationale of the Study

1. There's very limited fundamental research on rubber latex, let alone on the Malaysian clones.

2. The creation of different rubber clones is mostly based on the physical properties, but the cellular properties are lacking.

1.3 Problem Statements

The proteomics analysis involving the rubber clones of Malaysia is understudied. The Lembaga Getah Malaysia (LGM) produces and recommends over 20 rubber clones for high-scale plantations, including the clones PB350, RRIM2025, and RRIM3001. Only the genome of rubber clone RRIM600 were completely sequenced amongst the Malaysian rubber clones (Rahman et al., 2013). Proteomics analysis, as well as intact post-translational modification analysis, has been conducted on the latex of the rubber clone RRIM600 (Habib et al., 2016; Habib et al., 2018). The proteomics or transcriptomic studies were conducted on only three other Malaysian rubber clones: RRIM2023, RRIM3001, and RRIM712. One proteomics analysis was conducted on the latex of rubber clone RRIM2023 induced by ethephon stimulation (Abd-Rahman & Kamaruddin, 2018). Another study was the comparative transcriptome analysis performed on the bark, leaves and latex between the high-yielding rubber clone RRIM3001 and the low-yielding rubber clone RRIM712 (Bakar & Othman, 2022).

Similarly, the rubber Mw profile of Malaysian rubber clones is also understudied. Among the Malaysian clones, only the rubber clone RRIM600 was studied for its rubber Mw and MWD, ranging from 1-year-old to 7-year-old trees (Kovuttikulrangsie & Sakdapipanich, 2005). Another team studied the Mw of the

rubber clone RRIM600 in comparison to the rubber clone Reyan7-33-97 between the 1-year-old trees and 30-year-old trees (Xin et al., 2021).

The latex is rich with compounds other than proteins, such as carbohydrates, lipids, salt, and minerals that would be co-extracted with the proteins. These compounds need to be removed from the samples before the LC-MS analysis to avoid interference (Duan et al., 2006). The two-dimensional electrophoresis (2-DE) is a common method used for protein separation based on their size and charge. The protein samples were usually run on the 2-DE method before the LC-MS analysis to ensure only desired proteins were analysed with the LC-MS. Unfortunately, the non-protein compounds could interfere with the 2-DE. Additionally, among the latex proteins, the rubber elongation factor (REF) is very highly abundant and could make up to 60% of the total latex proteins. This situation often resulted in a bad separation of the protein on the 2-DE gel, showing both horizontal and vertical streaks on the highly abundant protein region (Wang et al., 2010).

The relationship between rubber latex proteomics and the rubber Mw is not fully explored. It is accepted that rubber is biosynthesised through the isoprenoid pathway via the MVA pathway metabolic route (Men et al., 2019). However, it is still unclear how the pathway affects the production of rubber with high or low Mw. An in vitro study on the rubber biosynthesis reported that the transcriptome variation during the isopentenyl pyrophosphate (IPP) biosynthesis and the non-rubber isoprenoid pathways may influence the ratios and levels of the IPP and the allylic pyrophosphate (APP) initiators, which also influences the Mw of rubbers among Hevea trees of similar ages (Xin et al., 2021).

There is also an absence of validated latex protein biomarkers that are specialised for rubber latex quantity and quality. The widely practised method used for the determination of rubber latex quality is the dry rubber content (DRC) test, which only calculates the percentage of rubber from the sample latex mass (Tillekeratne et al., 1988). However, the test could not be used to measure the Mw and MWD of rubber from the latex sample. Several latex protein biomarkers were already established for allergen detection, such as the Hev b 1, 2, 3, 5, 6, and 13 proteins (Jezic & Lucas, 2002). However, these protein biomarkers are not optimised for rubber quality control.

1.4 Research Questions

1. What are the latex proteome profiles of the Malaysian rubber clones PB350, RRIM3001, and RRIM2025, and how do they differ?

2. What are the rubber Mw and MWD profiles across the different ages of Malaysian rubber clones PB350, RRIM3001, and RRIM2025?

3. What is the most effective protein extraction method for the latex sample without using the 2-DE separation that is suitable for LC-MS analysis?

4. How does latex proteome variation correlate with differences in the Mw among the Malaysian rubber clones and ages?

5. Can specific latex protein biomarkers be identified and validated to serve as indicators for rubber latex quantity and quality?

1.5 Research Objectives

General objective: To understand the correlation between latex biological pathways and the quality of rubber latex.

1. To perform the rubber latex molecular weight and molecular weight distribution profiling analysis amongst different clones and ages of *H. brasiliensis*.

2. To compare the latex protein extraction method most suitable for direct LC-MS/MS analysis.

3. To identify and quantify important proteins involved in rubber biosynthesis and latex flow using LC-MS/MS amongst different clones and ages of *H. brasiliensis*.

4. To identify the proteins that influence the molecular weight of rubber latex.

5. To validate the proteins as potential biomarkers through MRM analysis.

1.6 Research Hypothesis

1. Hypothesis for Objective 1: There are significant differences in the rubber latex molecular weight and molecular weight distributions among different clones and ages of *H. brasiliensis*.

2. Hypothesis for Objective 2: One of the latex protein extraction methods evaluated yields a higher quantity and quality of proteins that are more suitable for direct LC-MS/MS analysis compared to the other methods.

3. Hypothesis for Objective 3: The abundance and types of proteins involved in rubber biosynthesis and latex flow vary significantly among different clones and ages of *H. brasiliensis* as detected by LC-MS/MS.

4. Hypothesis for Objective 4: Specific proteins have a measurable influence on the molecular weight and molecular weight distribution of latex in *H. brasiliensis*.

5. Hypothesis for Objective 5: Proteins identified as influential in rubber biosynthesis and latex molecular weight can be validated as reliable biomarkers using MRM analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Origin and History of Domestication in Malaysia

The domestication of *H. brasiliensis* in Malaysia was well documented. In 1876, Henry Wickham, a British explorer, successfully smuggled 70,000 rubber tree seeds from the Amazon to Kew Botanic Gardens in London. Only 2899 seeds germinated from those seeds. In 1877, Kew Botanic Gardens sent 100 seedlings to Sri Lanka (back then known as Ceylon), and from Sri Lanka, 22 seedlings were sent to Singapore. Under the observation of then the Director of Singapore Botanic Gardens, Henry Ridley, 9 of the 22 seedlings were planted in the Residency Gardens in Kuala Kangsar, Malaysia, where one plant is still alive to this day (https://www.kualakangsar.gov.my/en/visitors/places-interest/oldest-rubber-tree). In 1884, the High Commissioner of the Federated Malay States, Frank Swettenham, planted 400 trees in Perak that originated from the Residency Gardens trees. In 1895, Tan Chay Yan became the first rubber plantation owner in Malaysia (then Malaya) when he planted his 43-acre estate in Bukit Lintang, Melaka, with rubber trees. At the same time, Kindersley also planted 5 acres of rubber trees in Selangor. In 1897, Henry Ridley published his findings on ways of cultivating and tapping rubber trees for optimum yield and suggested to the government large-scale plantings of rubber trees. The Malayan rubber samples were also sent to England to be analysed and discovered to be much cleaner and of higher quality than their African or Amazonian counterpart (Priyadarshan, 2017a).

During the mid-1890s, the biggest commodity crop in Malaya was coffee, but there was a fungal disease epidemic, and to make matters worse, the global market price collapsed. By 1898, more planters turned to dedicated rubber plantations. In 1910, the

rubber price reached $3 per pound. By 1911, there was a total of 542,877 acres of rubber plantations across Malaya (Baxendale, 1913). During 1910-1918, Cramer and van Helten made a huge advancement in the breeding and propagation of the Hevea tree, which led to the standardisation of the Hevea vegetative propagation in 1915, and also the first commercial planting of bud-grafted Hevea clones in 1918 (Djikman, 1951; Tan et al, 1996). By the 1950s, the grafted clone's rubber trees overwhelmingly outperformed the genetically improved seedlings in latex yield, so most research started to shift to the clone's derivation, focusing on latex productivity (Priyadarshan, 2017a). To date, the total rubber plantation area in Malaysia is over 1 million hectares, where a vast majority of latex production is accounted for by smallholders (Ratnasingam et al, 2015).

2.2 Plant Structure and Hevea Clones

H. brasiliensis belongs to the spurge family Euphorbiaceae. It is a tall flowering deciduous plant that can reach a height of 25m in cultivation. The leaves branch out in spirals and are trifoliate with darker shades of green in colour (Figure 2.1). The flowers are small yet extremely pungent, without petals and creamy-yellow, where both male and female flowers are located on the same inflorescence (Figure 2.2). The fruits are trigonous, where the three seeds are located inside a capsule that explodes when ripe (Figure 2.3). The bark is smooth on the surface and, due to bud grafting, commercial *H. brasiliensis* trunks do not taper and appear almost cylindrical with a swollen base (often bottle-shaped) (Figure 2.4) (*Hevea brasiliensis*, Retrieved December 14, 2021; Priyadarshan, 2017b).

Figure 2.1 The leaves of *H. brasiliensis*. From Hevea brasiliensis (Willd. Ex A. Juss.) Müll. Arg., by D. Sakaki et al., Plants of the World Online (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:349913-1#source-NTK). CC-BY 3.0.

Figure 2.2 The flowers of *H. brasiliensis*. From Hevea brasiliensis (Willd. Ex A. Juss.) Müll. Arg., by D. Sakaki et al., Plants of the World Online (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:349913-1#source-NTK). CC-BY 3.0.

Figure 2.3 The fruit of *H. brasiliensis*. From Hevea brasiliensis (Willd. Ex A. Juss.) Müll. Arg., by D. Sakaki et al., Plants of the World Online (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:349913-1#source-NTK). CC-BY 3.0.

Figure 2.4 The trunk of a commercial bud-grafted *H. brasiliensis*

The Malaysian clones of *H. brasiliensis* can be differentiated based on their leaves. The PB350 clone leaves are rounded and have faded colour, and the trifoliate overlaps (Figure 2.5). The RRIM3001 clone leaves are elliptic and have a shiny colour, and the trifoliate leaves are separate (Figure 2.6). The RRIM2025 clone leaves are obovate, have a clear colour, and the trifoliate is separate (Figure 2.7) (Anjomshoae & Rahim, 2018).

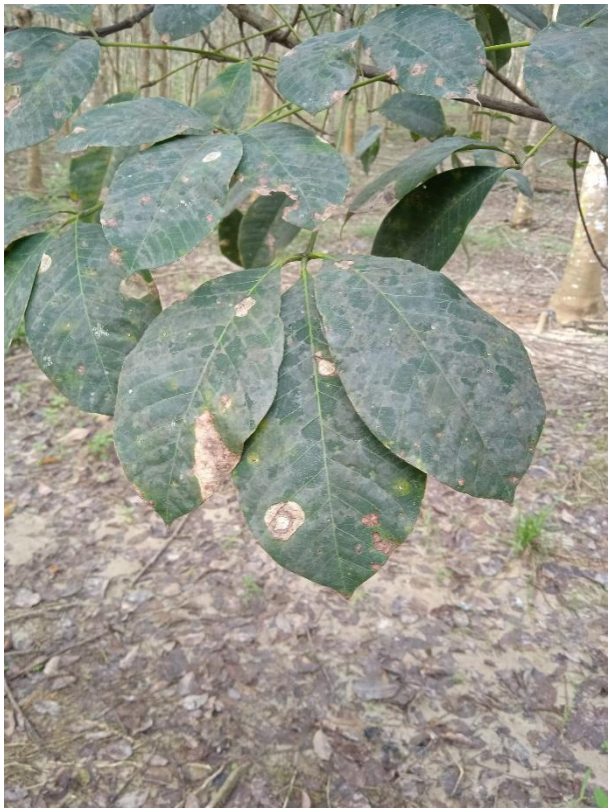

Figure 2.5 The leaves of *H. brasiliensis* clone PB350

Figure 2.6 The leaves of *H. brasiliensis* clone RRIM3001

Figure 2.7 The leaves of *H. brasiliensis* clone RRIM2025

Rubber clones of Malaysia are grouped by Lembaga Getah Malaysia (LGM), previously known as Rubber Research Institute Malaysia (RRIM). The categories for planting recommendation are based on the clone's performance (yield and clear bole volume) under different environmental conditions. Group 1 consists of 5 high-yielding clones (>1500 kg/ha/year), which include PB350. Clones from this group are the most recommended for commercial planting and receive no restrictions in the planting area. Group 2 consists of 7 high-yielding clones (>1800 kg/ha/year), which include RRIM3001. Clones from this group received a maximum planting area restriction of 40% due to limited information on their performance under environmental constraints and secondary characteristics (Malaysian Rubber Board, 2021). Table 2.1 shows the current list of *H. brasiliensis* clones recommended for commercial planting in Malaysia by the LGM (Malaysia Rubber Board, 2021).

Table 2.1 List of LGM's *H. brasiliensis* clones recommended for commercial planting in Malaysia

Group 1 **Minimum mean latex yield = 1500 kg/ha/year** **Minimum mean clear bole volume = 0.3 m^3/tree**	**Group 2** Minimum mean latex yield = 1800 kg/ha/year Minimum mean clear bole volume = 0.3 m^3/tree
RRIM928	RRIM2007
RRIM2001	RRIM2023
RRIM2002	RRIM2024
PB260	RRIM3001
PB350	PB347
	PB373
	PB374

PB350 is a latex timber clone (LTC) designed for the high production of latex as well as rubberwood. The clone originated from the Prang Besar Isolated Seed Garden, where it got the PB abbreviation in its name. This clone was recommended to Group 1 in 2006, where it shows great performance in latex yield (up to 1689

kg/ha/year) as well as high resistance to multiple diseases and wind damage (Benong et al., 2007).

RRIM3001 is an LTC clone also known as KT or Clone 1Malaysia in honour of the then Malaysian Prime Minister Najib Razak. This clone was introduced in 2009 by the Malaysian Rubber Board (MRB) and was recommended to Group 2 in 2013. It was demoted from Group 2 in 2019 due to the widespread occurrence of trunk protuberances, but rejoined Group 2 in 2021 due to good performance and MRB's findings that the protuberances would not affect the wood volume and quality. It shows an impressive performance in latex yield (up to 2123 kg/ha/year) as well as good resistance to multiple diseases (Kavitha, 2018; Malaysian Rubber Board, 2021).

RRIM2025 was introduced in 1998 and has yet to be included in the MRB's planting recommendation group. This clone is also a part of LTC and is of particular interest due to its high growth performance. It shows a high latex yield (up to 1626 kg/ha/year) and high wood volume, making it more desirable in rubberwood production (Husin & Ahmad, 2018; Halip et al., 2022).

2.3 Importance of Natural Rubber to Malaysia's Economy

The modern world relies tremendously on rubber. As of 2023, the total area planted with rubber trees in Malaysia was estimated at 1.1 million hectares, the third largest in the world behind Thailand (3.9 million hectares) and Indonesia (3.78 million hectares). In 2022, the natural rubber production of Malaysia was estimated at 0.37 million tonnes, while in the same year, Thailand produced 4.7 million tonnes of natural rubber, the largest in the world, followed by Indonesia at 2.72 million tonnes. During that year, the rubber industry contributed a total of RM47.49 billion, accounting for 3.1% of the national exports of Malaysia. In contrast, Malaysia's rubber consumption

in 2022 amounted to 0.85 million tonnes, which covers 2.9% of the world's consumption. In 2022, Malaysia exported 0.99 million tonnes (RM7.219 billion) and imported 1.16 million tonnes (RM7.244 billion) of natural rubber. In that same year, Malaysia also exported RM27.169 billion and imported RM11.13 billion of rubber products such as tyres, inner tubes, footwear, industrial rubber goods, and general rubber goods (Malaysian Rubber Board, n.d.).

2.4 Application of Natural Rubber

The characteristics of natural rubber, its strength, compressibility, and heat resistance have made natural rubber a high-value material in engineering applications (Kovuttikulrangsie & Sakdapipanich, 2005). While natural rubber is used to produce over 40,000 types of rubber products, the majority (over 50%) is used to make tyres (Cornish, 2001b). Modern automobiles could have up to 300 components made out of natural rubber apart from tyres. Natural rubber also has adhesive properties, which have found their applications in rubber cement and road constructions as soil stabilisation materials. While most natural rubber applications are vulcanised, raw natural rubber is used in the footwear industry as shoe soles and in the medical industry as gloves and catherers (Hamilton, 2016).

2.5 Biochemical Composition of Latex

Latex from Hevea brasiliensis is the cytoplasm of laticiferous cells, forms vessels in concentric rings within the phloem and is specialised in producing cis-1,4-polyisoprene (Andrews & Dickenson, 1960; Dickenson, 1969). This cis-1,4-polyisoprene polymer is formed through the sequential condensation of IPP units (Figure 2.8). When the Hevea tree is tapped by making a small incision in the bark to

cut laticiferous cells, latex is released due to the high vessel pressure. This process is used commercially to collect latex for natural rubber production (Blackley, 1997).

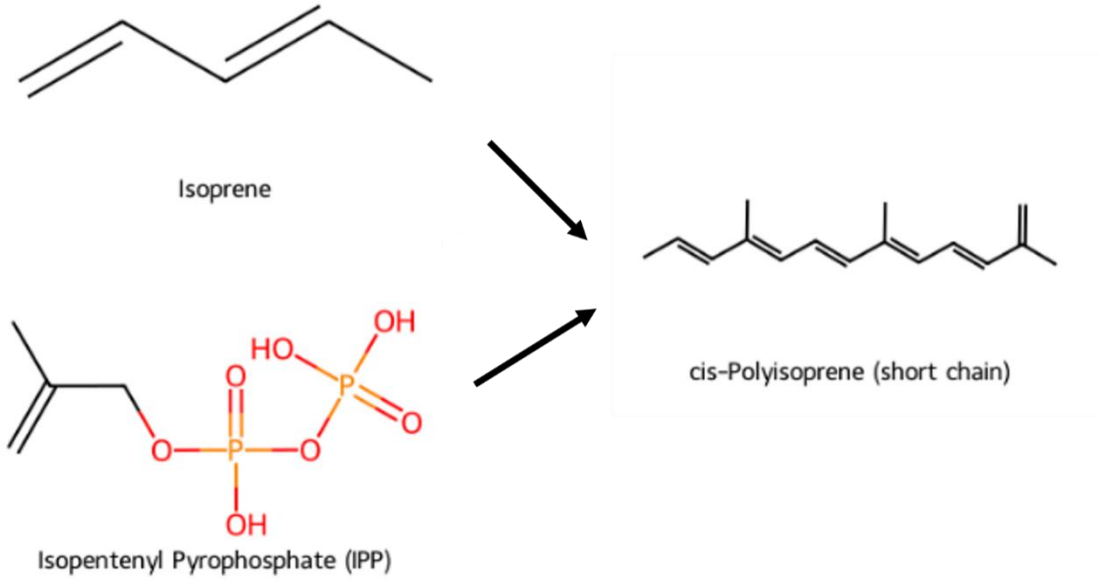

Figure 2.8 The cis-polyisoprene is derived from isoprene and IPP as precursors

Latex that was freshly tapped from a rubber tree is a white, opaque fluid, which, depending on the rubber content, would have a density between 0.97 and 0.98. It would contain 25%-50% dry matter with a slightly acidic pH between 6.5-7.0 (D'Auzac & Jacob, 1989). The main component is cis-1,4-polyisoprene, which makes up about 35% of its fresh weight or 87% of its dry weight. The remaining 5%-6% w/w fresh latex or 13% w/w dry latex consists of naturally occurring non-isoprene molecules like lipids, proteins, carbohydrates and minerals (Vaysse et al., 2012).

Latex is a complex colloidal dispersion containing particles of colloidal size (several nm to several microns). It is made up of several fractions of rubber particles, lutoids and Frey-Wyssling particles dispersed in the cytoplasmic serum (C-serum). Rubber particles are the major constituent, representing ~50%-70% w/w of fresh latex. They are spherical with a core of cis-1,4-polyisoprene surrounded by a monolayer of

lipids and proteins. Rubber particles exhibit a bimodal size distribution with large (0.4-1.0 mm) and small (0.1-0.4 mm) particles, called cream and skim fractions, respectively. Lutoids are complex lysosomal vacuoles of 2-5 mm in diameter, representing ~12%-22% w/w of fresh latex. Frey-Wyssling particles are less abundant (~2%-3% w/w fresh latex), yellowish-coloured non-rubber particles of 5-6 mm in diameter (Bottier, 2020).

2.5.1 Proteins in Latex

Latex from the Hevea trees is rich in proteins (Archer et al., 1963). In the author's previous study from the latex of Hevea clone RRIM600, 1839 unique proteins were identified using mass spectral data through *de novo* sequencing (Habib et al., 2016). Proteins are available in all fractions of latex, although not homogeneously distributed (Bottier, 2020).

Rubber particle proteins are adsorbed on the surface of the rubber particle fraction (Bottier, 2020). A study conducted on the rubber particle fraction proteins identified 186 proteins using shotgun tandem mass spectrometry (Dai et al., 2013). Another study identified 123 proteins, this time using two-dimensional gel electrophoresis (2-DE) before shotgun tandem mass spectrometry (Wang et al., 2018). Proteins that are involved in rubber biosynthesis were among those identified in the rubber particle fraction, such as rubber elongation factor (REF), small rubber particle protein (SRPP), isopentenyl pyrophosphate polymerase, rubber transferase, as well as two latex allergens Hev b1 and Hev b3 (Archer & Cockbain, 1969; Alenius et al., 1993; Yeang et al., 1996; Chow et al., 2007).

Lutoids proteins are dispersed in the serum (B-serum) located in the core of the lutoids fraction (Bottier, 2020). The lutoids fraction proteins are dominated by a single protein, hevein. The protein covers 50%-70% of the total proteins from the lutoid

protein fraction (Archer et al., 1969). Multiple allergens were identified among the proteins of the lutoids fraction. This includes hevein (Hev b6.02), prohevein (Hev b6.01), prohevein C-terminal (Hev b6.03), and β-1,3-glucanase (Hev b2) (Archer, 1960; Soedjanaatmadja et al., 1995a; Sunderasan et al., 1995; Rozynek et al., 1998). Both forms (A and B) of hevamine are also abundant in the lutoids fraction (Archer, 1976). A study conducted on the lutoids fraction proteins identified 57 proteins using SDS-PAGE and 2-DE before tandem mass spectrometry (Wang et al., 2013).

C-serum proteins are dispersed in the C-serum fraction (Bottier, 2020). C-serum fraction is rich in protein, which makes up 60% of the whole latex protein content (Li et al., 2009). The majority of the proteins in the C-serum are involved in the glycolytic pathway and rubber biosynthesis (Archer & Audley, 1967; Bealing, 1969; d'Auzac & Jacob, 1969). Multiple allergenic proteins were identified in the C-serum fraction. This includes heat-stable 15-kDa protein (Hev b5), 42-kDa patatin-like protein (Hev b7), 15-kDa latex profiling (Hev b8), and 51-kDa enolase (Hev b9) (Yeang et al., 2002). A study conducted on the C-serum fraction proteins identified 1837 unique proteins based on 1824 protein spots in 2-DE, before tandem mass spectrometry (Wang et al., 2018). Other proteins identified in the C-serum fractions include calmodulin, α-globulin, and lectin (Archer & Cockbain, 1955; Wititsuwannakul, 1990a; Wititsuwannakul et al., 2008).

2.6 Latex Protein Extraction Methods

The activities of proteins peroxidase and catalase were first described in dialysed latex (Spence, 1908). The rubber latex was reported to contain about 14 mg/ml of protein concentration by a direct analysis of the Bradford protein assay (Yagami et al., 2004; Yeang et al., 2002). However, latex also contains other compounds that would

interfere with the MS analysis, such as the rubber polymer and carotenoids. Latex protein extraction methods were developed to isolate the latex proteins from the other compounds. While the latex proteins can be extracted from the latex as it is (whole latex), the latex itself can be separated into different fractions by ultracentrifugation. The proteins can then be extracted from these fractions independently. One team from China described three different fractions of latex: rubber particle, C-serum, and lutoids, after ultracentrifugation (Wang et al., 2010). Another team from Thailand described four different fractions of latex: cream, skim, serum, and bottom, after ultracentrifugation.

Three different approaches can be taken for the latex protein extraction methods. They are the phenol extraction method, the trichloroacetic acid (TCA)/acetone precipitation method, and the agitation method. The phenol extraction method has been used to extract proteins since the 1950s (Wu et al., 2007). It was observed that this method was effective against recalcitrant plant tissues (Faurobert et al., 2007). This method involves the addition of phenol solution to the buffer and latex mixture solution to separate the proteins from other components of the latex. A team from China was the first to demonstrate the phenol extraction method on the latex C-serum fraction (Dejun et al., 2009). Then another team, also from China, improved the extraction buffer used previously to also be suitable for the whole latex and the other latex fractions (Wang et al., 2010). The same team later added a re-extraction with phenol step into their experiment to extract low-concentration proteins from the washing solution of the rubber particle fraction of latex (Wang et al., 2016).

The TCA/acetone precipitation extraction method is the most common protein extraction method due to its simple procedure and has been proven suitable for extracting proteins from various plant species and tissues (Wu et al., 2014). This method

involves the addition of the TCA/acetone solution to the buffer and latex mixture solution to precipitate the proteins from other components of the latex. A team from China was the first to perform the TCA/acetone precipitation method to extract proteins from the C-serum and lutoid fractions of latex (Yan and Chen, 2008a, b; Yan et al., 2008). Then another team from China used the TCA/acetone precipitation method to extract proteins from the C-serum fraction of latex and compared the results with the phenol extraction method (Dejun et al., 2009). Another team from Thailand conducted the TCA/acetone precipitation method on the rubber particle and C-serum fractions of latex to discover that the major proteins in each fraction are different (Sansatsadeekul et al., 2011). One more team from China performed the TCA/acetone precipitation method on the whole latex to observe protein differences between rubber trees treated with ethrel and methyl jasmonate (Dai et al., 2015).

The agitation extraction method is where the agitation from sonication of the rotating machine was used on the mixture of the extraction buffer and latex. This step would break the cell wall, and proteins can be obtained. The agitation method does not have an additional step to separate the proteins from the extraction buffers since all agitation extraction methods would undergo 2-DE afterwards for protein separation. A team from China developed the protocol for protein extraction from the rubber particle fraction of latex, followed by 2-DE and mass spectrometry analysis (Duan et al., 2006). Another team, also from China, then extracted and analysed proteins from the small and large rubber particle fractions of latex using a modified buffer from the previous work (Xiang et al., 2012). A team from Thailand demonstrated a protein extraction buffer suitable for the latex in whole, stabilised, and frozen whole, and frozen stabilised conditions, followed by 2-DE and mass spectrometry (Srisomboon et al., 2014). Another team, also from Thailand, conducted a protein profiling of latex separated into

4 fractions of cream, skim, lutoids, and serum using a similar extraction buffer from the previous work (Liengprayoon et al., 2017).

2.7 Biosynthesis of Natural Rubber

Natural rubber (NR) synthesis occurs through the isoprenoid pathway, which produces the building blocks isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors can be synthesised through two metabolic routes - the mevalonate (MVA) pathway in the cytosol, and the 1-deoxy-D-xylulose-5-phosphate/ 2-C-methyl-D erythritol-4-phosphate (DXP/MEP) pathway in plastids (Figure 2.1) (Kirby & Keasling, 2009). In *H. brasiliensis*, IPP units for rubber production are generated solely through the MVA pathway, while the MEP pathway generates carotenoids (Chow et al., 2012).

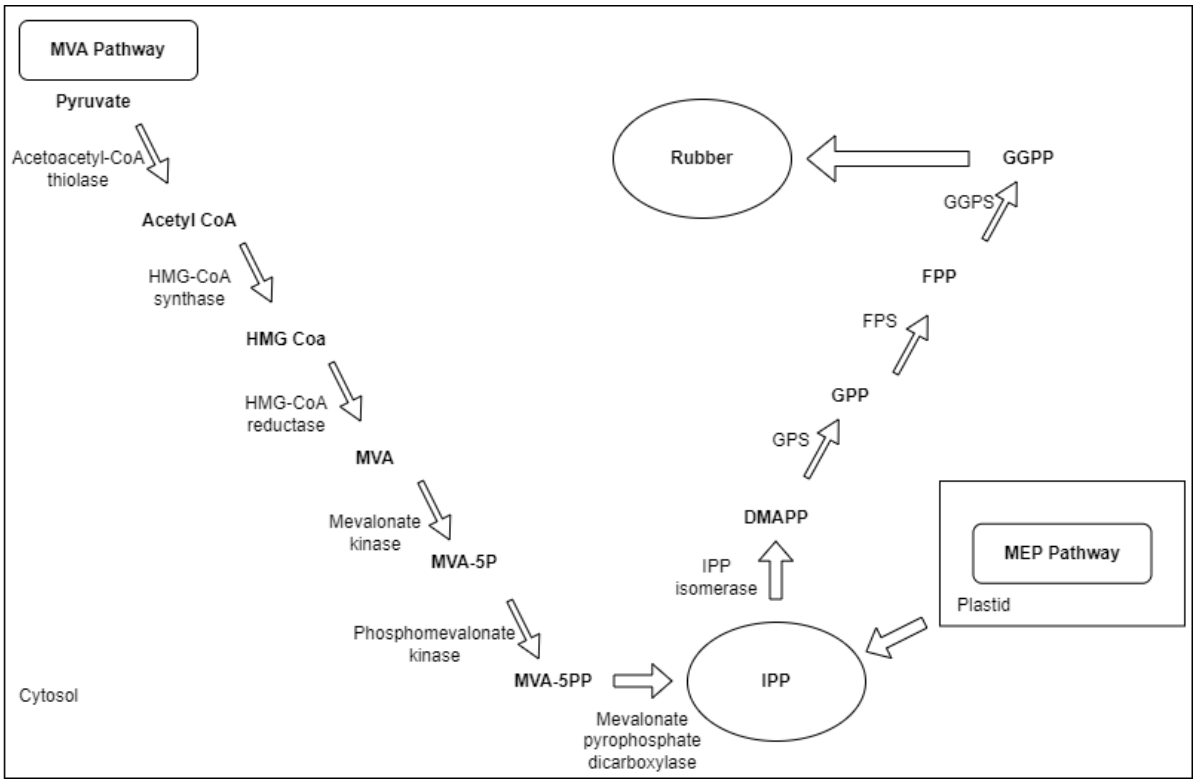

Figure 2.9 Biosynthesis of natural rubber from the MVA pathway