Rubber Biosynthesis by *Hevea* Latex Organelle Membrane Proteins

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ชื่อวิทยานิพนธ์ ชีวสังเคราะห์ยางโดยโปรตีนจากเมมเบรนของออร์แกเนลล์ในน้ำยางพารา
ผู้เขียน นางสาวอธิยา รัตนพิทยาภรณ์
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บทคัดย่อ

น้ำยางสดสามารถแยกได้เป็น 3 ส่วนโดยการปั่นเหวี่ยงความเร็วสูง ทั้งนี้ 20% ของปริมาณทั้งหมดจะเป็นส่วนก้นหลอด (WBF) ซึ่งประกอบไปด้วยออร์แกเนลล์ในน้ำยาง อย่างไรก็ดีถ้าน้ำยางที่ใช้ไม่สดพอหรือเก็บไว้โดยใช้สารรักษาสภาพ การแยกโดยการปั่นเหวี่ยงความเร็วสูงจะแยกออกมาได้เพียงแค่ 2 ส่วนเท่านั้น เนื่องจากออร์แกเนลล์ในส่วนก้นหลอดเดิมมีการแตกทำลายไป และส่วนเมมเบรนของออร์แกเนลล์ดังกล่าวจะลอยไปเกาะติดอยู่กับส่วนของชั้นยางด้านบนแทน ยังผลให้ส่วนก้นหลอดหายไป การศึกษาและรายงานมาก่อนหน้านี้ เป็นการศึกษาและรายงานเกี่ยวกับการชีวสังเคราะห์ยางบนผิวของอนุภาคยาง (RP) เท่านั้น แต่เราพบว่าเมมเบรนของออร์แกเนลล์จากส่วนก้นหลอด (WBM) มีคุณสมบัติในการชีวสังเคราะห์ยางด้วยเช่นกัน และเรายังพบว่าดีเทอร์เจนชนิดที่มีประจุลบ (SDS และ DOC) สามารถกระตุ้นให้ส่วนเมมเบรนของออร์แกเนลล์ดังกล่าว มีชีวสังเคราะห์ยางในหลอดทดลองมากขึ้นกว่าเดิม ทั้งนี้การกระตุ้นจะเกิดขึ้นเฉพาะกรณีที่ใช้ดีเทอร์เจนในปริมาณมากกว่า critical micelle concentration (cmc) เท่านั้น สาเหตุที่เป็นเช่นนี้น่าจะเป็นเพราะมีการรวมของเมมเบรนและ micelle ของดีเทอร์เจนดังกล่าว เป็นการช่วยเพิ่มให้มีพื้นที่ในการเกิดชีวสังเคราะห์ยางมากขึ้น จากการนำเมมเบรนของออร์แกเนลล์ไปแยกด้วยการตกตะกอนโดยอะซีโตน (acetone fractionation) พบว่าส่วนที่แยกออกมายังมีความสามารถในการชีวสังเคราะห์ยางอยู่ และส่วนที่แยกออกมาบางส่วนมีความสามารถในการกระตุ้นให้เกิดชีวสังเคราะห์ยางของส่วนที่มีอยู่แล้วให้มีมากขึ้น ในขณะที่บางส่วนจะทำหน้าที่ยับยั้ง ผลการทดลองดังกล่าวน่าจะเป็นสิ่งที่แสดงให้เห็นว่าเมมเบรนของออร์แกเนลล์มีความเกี่ยวข้องกับกระบวนการสร้างยางในธรรมชาติ โดยที่เมมเบรนของออร์แกเนลล์นั้นมีความสามารถควบคุมตัวเองในการทำชีวสังเคราะห์ยางได้อีกด้วย

นอกจากนี้เรายังสามารถนำ C_{55}-PP (UPP) ที่สังเคราะห์ได้จากแบคทีเรียมาเป็นตัวตั้งต้นสำหรับชีวสังเคราะห์ยางโดยเมมเบรนของออร์แกเนลล์ และพบว่า UPP มีคุณสมบัติในการเป็นสารตั้งต้นได้ดีกว่า C_{15}-PP (FPP) อีกด้วย ยางที่ได้นี้มีขนาดโมเลกุลประมาณ 3-4 x 10^5 และเมื่อนำยางที่ได้นี้ มาใช้เป็นตัวตั้งต้นในการทำชีวสังเคราะห์ยางโดยอนุภาคยาง (RP) ก็จะได้ยางที่มีขนาดโมเลกุลใหญ่ขึ้น 3 เท่า (9 x 10^5) ผลการศึกษานี้นำมาสู่แนวคิดเกี่ยวกับการสังเคราะห์ยางในธรรมชาติ โดยเริ่มต้นมีการสร้างยางโมเลกุลเล็กโดยอาศัยเอ็นไซม์และตัวควบคุมในส่วนเมมเบรนของออร์แกเนลล์ หลังจากนั้นจึงเป็นการต่อสายของโมเลกุลยางให้ยาวขึ้นโดยอาศัยเอ็นไซม์บนอนุภาคยาง นอกจากนั้นการที่เมมเบรนของออร์แกเนลล์ซึ่งแยกได้จากพืช สามารถนำ UPP ที่สร้างจากแบคทีเรียไปใช้สร้างยางได้นั้น ยังนำไปสู่แนวคิดที่จะนำทั้งสองอย่างนี้มาใช้ร่วมกันเพื่อทำชีวสังเคราะห์ยางชนิดพิเศษในหลอดทดลองต่อไป

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Abstract

Ultracentrifugation of fresh *Hevea* rubber latex yields three distinct fractions. Sediment bottom fraction (BF) content of membrane-bound organelles is ~ 20% (v/v) of latex. Only two fractions obtained if delay or using preserved latex as the osmotic sensitive BF rupture and its membrane debris tightly bound to top rubber particles (RP) phase. Washed BF membrane (WBM) was clearly found as active RB initiation site, which was in contrast to previous reports that RB only occurs on the RP surface. WBM was highly activated by anionic detergents (SDS and DOC) for RB in a biphasic manner, likely WBM micelle formation of high increased active surface area for RB. Serial acetone extraction of WBM proteins showed that isolated fractions still have RB activity under appropriate conditions. And RB regulatory protein was also detected. It indicated that WBM has a well controlled metabolic function for the latex RB process.

Bacterial C_{55}-PP (UPP) was very active allylic initiator for WBM in RB function. UPP was found much more active than C_{15}-FPP for RB by WBM enzymes. RP-TLC autoradiogram of toluene/hexane extract rubber at origin showed high ^{14}C-UPP incorporation. MW determination by GPC of ^{14}C-UPP labeled rubber was 3 - 4 x 10^5 with skewed unimodal MWD. RB activity of washed RP (WRP) with allylic ^{14}C-UPP was much lower than WBM. The ^{14}C-UPP labeled rubber from WBM assay incubated with WRP showed discrete finite increased MW to 9 x 10^5, 3 folds higher. The appearance of low MW rubber with allylic ^{14}C-UPP suggested that WBM serving as initiation site for the new rubber molecules formation prior to subsequently further elongate to become mature rubber of higher MW as present in the rubber particles. Study with UPP suggested that bacterial isoprene was highly suitable initiator for synthesis new rubber molecules by WBM enzymes, and WRP elongated it to be higher MW rubber.

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PR 436 members made the laboratory enjoyable place to work deserve a debt of gratitude for all their help, companionship and friendship during this work. Many thanks are also due to all Biochemistry Department (PSU) members, C 609 (Chemistry, MU) and B303 members (Biochemistry, MU) for their helps and friendship.

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Atiya Rattanapittayapron

Contents

Contents (Continued)

List of Tables

List of Figures

List of Figures (Continued)

List of Figures (Continued)

List of Abbreviations

IPP	=	isopentenyl diphosphate
DMAPP	=	dimethyallyl diphosphate
GPP	=	geranyl diphosphate
FPP	=	farnesyl diphosphate
GGPP	=	gerenyl geranyl diphosphate
UPP	=	undecaprenyl diphosphate
^{14}C-IPP	=	^{14}C-labeled isopentenyl diphosphate
^{14}C-UPP	=	^{14}C-labeled undecaprenyl diphosphate
^{3}H-FPP	=	^{3}H-labeled farnesyl diphosphate
^{3}H-GGPP	=	^{3}H-labled geranyl geranyl diphosphate
UPS	=	undecaprenyl diphosphate synthase enzyme
IPPI	=	isopentenyl diphosphate isomerase enzyme
RT	=	rubber transferase enzymes
MVA	=	mevalonic acid
MEP	=	methylerythritol 4-phosphate
SDS	=	sodium dodecyl sulfate
DOC	=	deoxycolic acid
EDTA	=	ethylenediamine tetraacetic acid
EGTA	=	ethylene glycol-bis (β-aminoethyl ether) N, N, N', N', - tetraacetic acid
DTT	=	ditriotheritor
Tris	=	tris (hydroxymethyl) aminomethane

List of Abbreviations (Continued)

TEMED	=	tetramethyl ethylenediomine
PPO	=	2,5- diphenyloxazole
POPOP	=	1,4-bis [2-(5-phenyloxazyl)]-benzene
BuOH	=	1- buthanol
THF	=	tetrahydrofuran
UC	=	ultracentrifugation
WBP	=	washed bottom-fraction particles
WBM	=	washed bottom membrane
BF	=	bottom fraction
WRP	=	washed rubber particles
RP	=	rubber particles
SRP	=	small rubber particles (from Zone 2 rubber)
RP-TLC	=	reverse phase – thin layer chromatography
Ori.	=	origin
s.f.	=	solvent front
v/v	=	volume by volume
w/v	=	weight by volume
approx.	=	approximate
cmc	=	critical micelle concentration
cpm	=	counts per minutes
ci	=	curie
MW	=	molecular weight

List of Abbreviations (Continued)

kD	=	kilo Dalton
MWD	=	molecular weight distribution
GPC	=	gel permeation chromatography
HPLC	=	high performance liquid chromatography
SDS-PAGE	=	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
^{o}C	=	degree Celsius
UV	=	ultraviolet
Abs	=	absorbency
λ	=	wavelength
nm	=	nanometer(s)
min	=	minute(s)
h	=	hour(s)
g	=	gravitational acceleration
gm	=	gram(s)
mg	=	milligram(s)
nM	=	nanomolar(s)
μM	=	micromolar(s)
mM	=	millimolar(s)
M	=	molar(s)
μl	=	microliter(s)
ml	=	milliliters(s)
l	=	liter(s)

List of Abbreviations (Continued)

%	=	percentage
incorp.	=	incorporation
Conc.	=	concentration

Chapter 1

Introduction

Rubbers in the latex from various plants are polyisoprenoids of the high molecular weight hydrocarbon polymers consisting almost entirely of the five-carbon isoprene (C_5H_8) units. The polyisoprene rubber is major components of latex synthesized by special differentiated cells of the plants. They are synthesized by series of enzymes-catalyzed polymerization (Lynen, 1967) of isoprene units to various different degrees, resulting in a wide range of molecular weight (MW). The rubbers of high MW are produced in the latex of about 300 genera of Angiosperms. Of these, *Hevea brasiliensis* (Brazilian rubber trees) is the best rubber producer, and commercially cultivated for the natural rubber production used industrially for various products. *Hevea* latex contains rubber particles is accumulated in specialized cells known as laticifers. The double bonds in *Hevea* rubber are in the *cis* configuration as *cis*-1,4-polyisoprene, with a wide range of molecular sizes distribution. In addition to rubber particles, two other specialized particles (lutoids and Frey-Wyssling particles) are also present as major part of the *Hevea* latex. The presence of these two major particulate components provides the unique characteristic to the *Hevea* latex properties. Structure of *Hevea* latex and its detailed biochemistry is of recent thoroughly and extensively reviewed (Wititsuwannakul & Wititsuwannakul, 2001).

Fresh *Hevea* latex can simply be fractionated by centrifugation into three fractions as the top rubber layer, middle aqueous C serum, and the sediment bottom fraction (BF) of membrane-bound organelles. BF content is quite considerable, constituting about 20% by volume of the fresh latex as compared to an average of ~ 30% of the rubber phase. The BF is composed almost entirely of membrane-bound organelles, the lutoids and Frey-Wyssling particles. The fresh latex is thus a colloidal mixture of the different particles together with the cell soluble substances in an aqueous suspension. Lutoids was first described (Homans *et al*, 1948) as membrane-bound vacuoles, with the single layer membrane rich in phosphatidic acids (Dupont *et al.*, 1976), thus rendering them as negatively charged vesicles. The lutoids contents (called B-serum) are proteins, enzymes and a wide range of metabolites, considered as a type of phytolysosomes. Frey-Wyssling particles are double layer membrane organelles containing lipid globules, membrane vesicles and β-carotene (Dickenson, 1969). The high carotenoid content suggested it might contain enzymes for isoprenoids synthesis pathway. So far, only few studies were made that might suggest the related metabolic roles of these particles in isoprenoids and rubber biosynthesis (RB) pathways (Dickenson, 1969; Wititsuwannakul *et al.*, 1990a). HMG-CoA reductase (HMGR), presumably one of the rate-limiting enzymes in the RB pathway (Benedict, 1983), was purified from the washed BF membrane (Wititsuwannakul et al, 1990a). It was shown to be under control by calmodulin (Wititsuwannakul et al, 1990b), the Ca^{2+} binding heat stable protein in the C-serum, as the activator of this HMGR enzyme.

Hevea latex was shown to be active (Lynen, 1967; Archer *et al.*, 1963) in the synthesis of rubber for quite some time. Study on rubber biosynthesis (RB) process is

of much interest as appeared in several reviews (Archer & Audley, 1987; Ohya & Koyama., 2001). Details or understanding of the RB process is still ambiguous and the clear evidence has yet been convincingly presented. Most of studies focused mainly on the surface of rubber particles (RP) and was always reviewed (Archer & Audley, 1987; Audley & Archer, 1988; Kekwick, 1989; Kush, 1994; Tanaka et al., 1996; Ohya & Koyama, 2001) as the only prerequisite site required for synthesis of rubber molecules. This might seem a paradox to address the question on how and where the original RP was formed if the new rubber has to be synthesized on its preexisting surface. And this ambiguous aspect has long been overlook. The true RB initiation sites other than the RP surface with active rubber formation need to be sought out. It is therefore still an open question as to the actual specific site for synthesis of new rubbers that will eventually aggregate to form the RP. If one considers the complex nature of *Hevea* latex and its myriad compositions, it might be possible that the RB can take place at certain specific site other than the RP surface. This has been earlier postulated (Dickenson, 1969; Moir, 1959) but has received little attention and no careful investigation was carried out to substantiate this suggestion. Study with the condition that is free of rubber particles should be attempted which will serve as an ideal system to solve this query. *Hevea* latex is regarded as the living cytoplasm in which the rubber particles, the non-rubber particles and other cell components are dispersed in an aqueous phase of the cytosol. Of particular interest is the membrane-bound non-rubber particles in the latex that may have active role in the RB process. The results in Part I of this report will assure the idea that surface of membrane-bound particles was quite active in the synthesis of new rubber molecules (Tangpakdee, 1997b, Wititsuwannakul *et al.*, 2003). The results might thus suggest

that the actual RB site could be localized on these particles membrane other than the RP surface.

This aspect seems quite contrast to the previous numerous studies in which the RP surface was implicated as the one and only prerequisite site for the *in vitro* RB process (Archer *et al*., 1963 & 1982; Audley & Archer, 1988). The different results in this report could suggest that there might be more than one site for RB activities in the latex. However, it is still doubtful and need clarification considering our reports on other RB related enzymes (Koyama *et al.*, 1996; Tangpakdee et al, 1997a & 1997b). Isopentenyl diphosphate isomerase (IPPI) in the *Hevea* latex was clearly detected for the first time by our group and characterized (Koyama *et al.*, 1996). We also detected the high IPPI and prenyl transferase (PT) activities in the BF particles and their properties thoroughly characterized (Koyama *et al.* 1996; Tangpakdee *et al.*, 1997a). Consequently, we clearly showed highly active rubber formation by fresh BF particles (Tangpakdee *et al.*, 1997b). The presences of high IPPI, PT and rubber transferase (RT) activities in the same BF particles were also determined (Tangpakdee *et al.*, 1997a). Kinetic study on ^{14}C-IPP incorporation into the rubber and the product analyses showed new appearance of the low molecular weight rubber molecules (Tangpakdee *et al.*, 1997b). These results suggested quite likely the synthesis of new rubber molecules being initiated and formed by these particles enzymes. The results also pointed out the function of the BF particles surface or membrane in carrying out the rubber synthesis in this study.

Subsequently, further careful studies on RB activities of the membrane from the BF particles were carried out and detailed properties characterized. Extensive

washed BF particles (WBP) and the derived washed BF membrane (WBM) on RB activities were compared. Results in Part 1 clearly show that the RB activity located on the isolated membrane. Parameters affecting the membrane functions were investigated on the RB process and shown in Part 2. Detergents and heat treatments of the WBM before subjected to the optimum RB assays conditions were characterized in details (Wititsuwannakul *et al.*, 2003). The RB stimulation results thus suggested the possible increase of active surface area by formation of micelle caused by these parameters. The active membrane RB assays clearly indicated that synthesis of new rubber could effectively occur with no requirement of the RP prerequisite site. Moreover, recently, two *Hevea* latex genes (Takaya *et al.*, 2003; Asawatreratanakul *et al.*, 2003) were successfully cloned with high activity. One was dominant PT enzymes in latex, GGPP synthase, rubber transferase was also cloned (termed HRT) that was RB active with WBM, strongly support WBM role.

Part 2 is the further characterization of the washed BF membrane (WBM) RB activity. We could show that the RP rapidly isolated from the freshly tapped latex contains only two proteins with very low or no RB activity, but the delayed use of fresh latex can lead to rupture and disappearance of the BF particles. The RP thus obtained has higher and different protein contents, along with increased RB activity. Most importantly, we can demonstrate that the isolated proteins and enzymes from the WBM can carry out the rubber synthesis when suspended with 2% SDS. The micelle postulation for the enhanced RB activity as earlier reported is thus being strongly supported by the results in this study. Of more interesting are experiments on mixing or reconstitution of WBM fractionated proteins that provide a good indication for the RB control mechanism by the proteins present or localized in the membrane.

It was quite intriguing that an exquisite idea was proposed recently on microbes might be capable of producing rubber (Steinbüchel, 2003). This is coincided with our ongoing research on rubber synthesis from bacterial undecaprenyl diphosphate (C_{55}-UPP). Among family microbial prenyltransferases, UPP synthase (UPS) (Muth & Allen, 1982; Takahashi & Ogura, 1982) has been most extensively studied. It was purified and characterized from several bacteria [*S. Newington* (Christenson *et al.*, 1969), *B. subtilis* (Takahashi & Ogura, 1982), *E. coli* (Baba *et al.*, 1985; Fujisaki *et al.*, 1986), *L. plantarum* (Allen *et al.*, 1976 & 1985; Allen & Muth, 1977; Baba & Allen, 1987; Keenan & Allen, 1974), and *M. luteus* (Baba & Allen, 1980; Kurokawa *et al.*, 1971)]. UPP is required as a lipid carrier of glycosyl residues in synthesis of bacterial cell wall. For this RB study, we employ ^{14}C-UPP prepared as described (Shimizu *et al.*, 1998) and provided to us for using as allylic initiator of rubber synthesis by WBM. Part 3 will describe the significant role of UPP in RB process. Comparisons with other shorter allylics (C_{15}-FPP, C_{20}-GGPP) on the RB levels and efficiency were also reported. Detergent DOC effect was tested and compared to SDS on RB activation (Wititsuwannakul *et al.*, 2003 & 2004). Comparing WBM and WRP activities with UPP was also made. The Products analyses, qualitative and quantitative, on the rubbers formed with UPP will be extensively presented on the molecular analysis as shown in Part 4.

It was found that rubber biosynthesis activity was higher in the smaller rubber particles though no convincing report about the initiation of those small rubber particles as yet. Nobody has ever reported *in vitro* rubber biosynthesis directly from IDP and dimethyallyl diphosphate (DMAPP) without using rubber particles, although biosynthesis of natural rubber should start from these 2 initiators (Allen *et al.*, 1997).

Initiation of rubber biosynthesis starts by condensation of DMADP and IDP and elongation step continues by adding IDP in *cis*-configuration (Archer & Audley, 1967; Lynen, 1969). The termination step is to replace the diphosphate by hydroxyl terminal group. From theory, one end of rubber polymer should be dimethyallyl and another end is hydroxyl group. However the dimethyallyl group has never been reported from *Hevea* latex until 1997, Tangpakdee *et al.* reported this group from *in vivo* rubber biosynthesis of bottom fraction and this rubber had smaller size than from *in vivo* (Tangpakdee *et al.*, 1997). From this report, it is possible that bottom fraction plays the role in initiation and early elongation step. The short-chain rubbers produced from bottom fraction might serve as substrates for the late elongation step and become long-chain rubbers on rubber particles.

From our point of view, the former studies of rubber biosynthesis from rubber particle suspended in C-serum or rubber layer (Archer *et al.*, 1961, 1963 & 1967; Archer & Audley, 1990; Yusof *et al.*, 1998) might represent the examining of late elongation step. There are reports show that almost of membrane bound prenyl transferases need detergent to activate the reactions (Allen et *al.*, 1976; Allen & Muth, 1977; Keenan & Allen, 1974). Hence, it may be possible that specific types of amphipathic micelles are essential for different step polyisoprene biosynthesis in initiation step of rubber biosynthesis. As different phospholipids compositions on the rubber particles and lutoids were reported (d'Auzac & Jacop, 1989). They may act as cofactor of the reaction and also play supportive roles for initiation and elongate the rubber chain length. The presence of isopentenyl diphosphate isomerase (IPP isomerase) and prenyl transferase as well as stimulatory effects of detergent stimulatory on rubber biosynthesis were previously demonstrated in the bottom

fraction (Koyama et al, 1996; Tangpakdee et al., 1997; Wititsuwannakul et al., 2003; Asawatreratanakul, 2003). This report also shows the further evidence of short-chain rubber biosynthesis by bottom fraction membrane.

Literature Review

1 Rubber latex

Rubber (cis-1,4-polyisoprene) is produced in varying quantities and qualities by about 2,000 plant species. This isoprenoid polymer has no identified physiological function in plants, but it has many important industrial uses due to its elasticity, flexibility, and resilience. The diminishing acreage of rubber plantation and life-threatening latex allergy to *Hevea* rubber, coupled with increasing demand, have prompted research interests in the study of rubber biosynthesis and development of an alternative rubber source. Although natural rubber is present in many species but only that from the tree *Hevea brasiliensis* is used commercially. This is due to its combination of high quality, high yield, and ease of harvest (Allen & Jones, 1988).

2 *Hevea brasiliensis*: the rubber tree

Although natural rubber is present in many species but only that from the tree *Hevea brasiliensis* is used commercially. This is due to its combination of high quality, high yield, and ease of harvest. The *Hevea* rubber tree is a tropical tree native to the Amazon Basin in Brazil and adjoining countries. However that area has no longer played any significant part in the world natural rubber tread. *Hevea* was taken from the Amazon to South Asia (Sri-Lanka) and South East Asia (Singapore and Malaysia) by British Colonial Office where it was grown experimentally and later on plantations. Subsequently, cultivation spread to Vietnam and Cambodia, Indonesia and Thailand, and subsequently to Africa (Liberia, Nigeria, and Cote d'Ivoire). Nowadays, the most world wild natural rubber is provided by some South East Asia countries (Thailand, Indonesia, Malaysia and Sri-Lanka). Among these countries, Thailand is the first producer and exporter. A majority of rubber plantations are located in southern part of the country due to the suitable climatic factors.

Hevea rubber tree is a tropical tree. It grows best at temperatures of 20-28°C with a well-distributed annual rainfall of 1,800-2,000 mm. The tree is the tallest species of the genus, in the wild these trees may grow to over 40 metres and live of over 100 years. But in plantations, they rarely exceed 20 metres because the growth is reduced by tapping. Commercially, they are usually replanted after 23-25 years when yields fall to an uneconomic level.

3 *Hevea* rubber tree classification

The rubber tree, *Hevea brasiliensis*, synonym Siphonia brasiliensis is a member of the Euphorbiaceae family. There are many common names associated with this plant. Some of the names are: Rubber tree, Jebe, Para rubber, Arbre de Para, Parakautschukbaum, Cauchotero de Pará, Árbol del Caucho, Seringueira, seringueira-branca, siringa, etc. The classification chart of the *Hevea* rubber tree is shown below.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euphorbiaceae
Genus	Hevea
Species	Brasiliensis

4 Latex of *Hevea brasiliensis*

The *Hevea* latex is accumulated in speciallized cells or vessels known as laticifers. In *Hevea*, the rubber is formed and stored in the rings of laticifers in the bark. Anatomoses between adjacent vessels in the rings allow the latex from a large area of the cortex to drain upon tapping. The opening of the latex vessels from tapping cuts cause the latex to flow out due to the high turgor pressure inside. The latex flow will continue for a certain length of time and subsequently stop due to rubber particles coagulation and flocs formation leading to plugging of the vessel ends. The latex is specialized cytoplasm containing several different organelles in addition to rubber particles. Organelles include nucleus, mitochondria, fragments of endoplasmic reticulum and ribosome. In addition to these minor components, there are two major speciallized particles which are unique characteristics of *Hevea* latex, namely the lutoids and Frey-Wyssling particles.

The *Hevea* latex collected by regular tapping consists of the cytoplasm expelled from the latex vessels and is similar to the latex *in situ*. The cytoplasmic nature of tapped latex was firmly established by electron microscope studies (Dickenson, 1969). Latex is the cytoplasm of an anastomosed cell system which is specialized in the synthesis of *cis*-polyisoprenes. The latex usually contains 25 to 50% dry matter, 90% of which is made up of rubber. Tapping severes a number of latex vessel rings and the latex which flows out comprises the contents of vessels at different stages of development. All the organelles in the latex vessels can be found in the tapped latex. The major particles most common in latex are the rubber particles, the lutoids, and

Frey-Wyssling paricles which are less numerous than the other two. The composition of latex is about 30-40% rubber, 10-20% lutoids, and 2-3% other substances. The structure and composition of fresh latex has been elucidated by high-speed centrifugation (Moir, 1959). Generally, the latex can be fractionated into 3 distinct zones. The top fraction consists almost entirely of rubber, the middle fraction is the metabolic active aqueous phase of latex called C serum, and the relatively heavy bottom fraction consists mainly of lutoids. The yellow, lipid containing Frey-Wyssling complexes are normally found at the upper border of the bottom fraction (Figure 1).

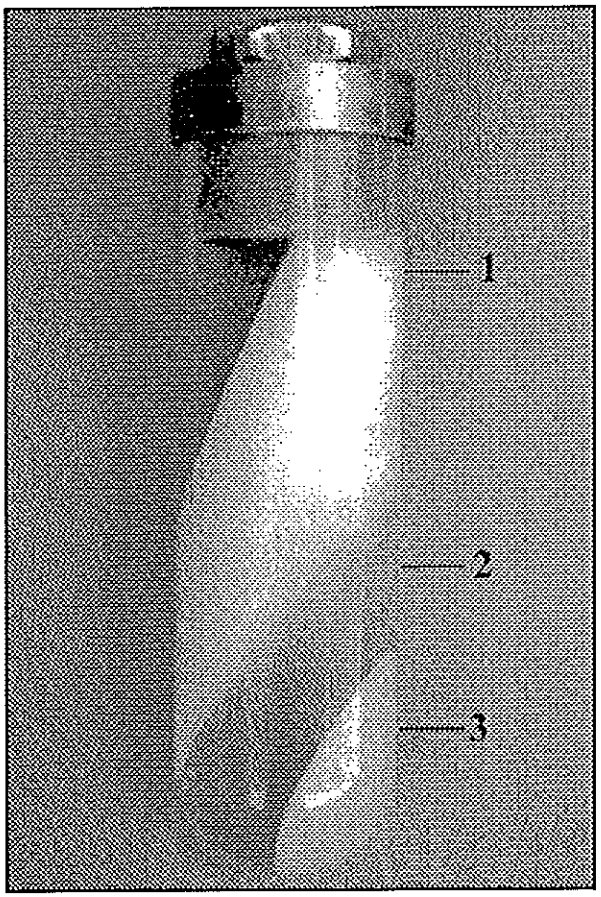


Figure 1 Fractionation of fresh *Hevea* rubber latex by untracentrifugation into three major zones. (1: rubber layer, 2: C-serum, 3: bottom fraction)

5 Composition of *Hevea* latex

The fresh latex can be separated into three major zones by ultracentrifugation as shown in Figure 1. The top rubber fraction contains, in addition to the rubber hydrocarbon, the proteins and lipids associated with the rubber particles. The serum phase contains most of the soluble substances normally found in the cytosol of plant cells. The bottom fraction can be studied by repeated freezing and thawing of the lutoids. In this manner the membranes of the lutoids are ruptured and their liquid content, B serum, can be analyzed. B serum has been found to contain proteins and other nitrogen compounds as well as metal ions. It can be visualized that the latex is a cytoplasmic system consisting of particles of rubber hydrocarbon dispersed in an aqueous serum phase. There are also numerous nonrubber particles called lutoids. The rubber particles are made up of rubber hydrocarbon surrounded by a protective membrane layer consisting of proteins and lipids. Besides from the rubber hydrocarbon which is the major component of the latex, various other components (protein, lipids, carbohydrates and inorganic substances) are also present which play important roles in the latex metabolism, include rubber biosynthesis.

6 Nonrubber constituents of *Hevea* latex

The latex contains numerous nonrubber constituents besides the rubber as were reviewed recently (Subramaniam, 1995). The nonrubber constituents are present and being distributed in all the three latex fractions. Proteins and lipids are found

associated with the rubber particles. C serum contains substances normally found in the cytosol (carbohydrates, proteins, amino acids, inositols, enzymes and intermediates of various biochemical processes, including rubber biosynthesis). Lutoids contain specific substances unique to its functions. The brief discussion will be on proteins, carbohydrates, lipids, and the inorganic substances.

6.1 Proteins

Apart from rubber hydrocarbon and water, proteins and carbohydrates are present in highest proportion in latex. The proteins content in latex shows clonal variations and can range from 1% to more than 1.8% in different samples of latex. About 25-30% of the proteins are found in the rubber phase and 45-50% in the serum phase, and about 25% in the bottom fraction in a typical latex sample. The amount of proteins in the rubber phase is less variable than the total amount of proteins in different samples of latex. The serum proteins consist of around 19 anionic and 5 cationic proteins (Tata & Moir, 1964). The major protein is α-globulin with an isoelectric point (pI) of 4.8. There are 7 anionic and 6 cationic proteins in the bottom fraction. The major proteins are hevein (>50%) of pI 4 and hevamine (≈30%) of pI 9. The amino acid sequences of hevein (Walujono *et al.*, 1975) and rubber elongation factor (REF), a 14 kD protein in the rubber phase (Dennis & Light, 1989), have been established. Recent report showed more than 200 different proteins are present in the latex (Alenius *et al.*, 1994), suggesting that the proteins composition in the latex is very complex.

6.2 Carbohydrates

Sucrose supply and utilization for latex production plays a very important role in the metabolism of latex and has been reviewed (Tupy, 1989). Of important note and quite unique to the latex is the presence of quebrachitol, an inositol derivative. Quebrachitol (1-methyl inositol) is the most abundant and makes up about 75% to 95% of the total carbohydrates present in latex. It is found mainly in the serum phase. The large amount and ubiquitous presence of this compound is unique characteristic of the *Hevea* latex. The reason for its accumulation and its physiological function in latex is not known but has been postulated to serve active role in rubber biosynthesis (Bealing, 1976). About 6 to 7 other carbohydrates components are found in small amounts. These are mostly common sugars for various metabolic processes in the latex.

6.3 Lipids

Lipids in the latex play important role in the stability of rubber particles. They not only are found associated with the rubber particles, but are also found throughout the latex fractions. The lipids content of latex also shows clonal variations (Ho *et al.*, 1976). The neutral lipids constitute more than 59% of the total lipids. The other components are mainly phospholipids and glycolipids. The presence of proteolipids has also been reported. The lipids are mainly in the rubber particles and the bottom fraction. Several neutral lipids found are triglycerides (also mono- and diglycerides), free fatty acids and esters, sterols, and lipid soluble vitamins (carotenoids and tocotrienols). Phosphatidyl choline is the major

phospholipid while phosphatidyl ethanolamine and phosphatidyl inositol are found in a smaller amounts. Phosphatidic acids are also reported as the important components of the lutoid membrane (DuPont *et al.*, 1976). Fatty acids in the latex occur mostly in the esterified form. The major fatty acids are C-16, C-18, and C-20 (palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidic acids). Also present is a rare furanoic acids (10,13-epoxy-11-methyloctadeca-10,12-dienoic acid) which is found mainly in the triglycerides fraction. The rubber latex is only the second known plant source of this furanoic acid (Hasma & Subramaniam, 1978). It is a bit unusual to find this fatty acid in the latex and its physiological function is not yet known. This is somewhat like the presence of quebrachitol in large quantity of which functions have yet to be found for their presence.

6.4 Inorganic substances

In addition to the above major nonrubber constituents, several inorganic components are also present. Of these, potassium is the most abundant element in the latex and its concentration is of the order of a few thousand part per million (ppm). The next most common element with a few hundred ppm is magnesium which is mainly contained in the lutoids. The effect of magnesium was found to decrease mechanical stability of the latex (Philpott & Westgarth, 1953). These two elements also show clonal variations. Others occurring in much smaller concentrations are the common cellular elements (sodium, calcium, iron, copper, manganese and zinc). Also present is rubidium, but the function of this element in latex is not yet known.

7 Rubber particles in *Hevea* latex

Rubber hydrocarbon is the major component of *Hevea* latex. The rubber content may vary from 25 to 45% as dry content of latex. The number average molecular weight ranges from 200 to 600 kD. The rubber molecules are found as particles in the latex. The particles consist mainly of rubber (90%) associated with lipophilic molecules, mainly lipids and proteins, forming the film enclosing rubber particles (Ho *et al.*, 1976). This film carries negative charges and is responsible for the stability of rubber particles suspended in aqueous serum. The particles size ranges from 5 nm to 3 μm with spherical shape. They also show plasticity as having polyglonal shape in mature laticifers where the particles are numerous. The size distribution as determined by the electron microscope showed maximum distribution of 0.1 μm particles (Gomez & Moir, 1979). The 0.1 μm particles may contain several hundred of rubber molecules. Molecular weight analyses using gel permeation chromatography showed the bimodal distribution of rubber for low and high molecular weights, with the average values of 100-200 kD and 1000-2500 kD, respectively (Subramaniam, 1976). The other main component of rubber particles is the enclosing membrane consisting of lipids, proteins, and enzymes. These components contribute colloidal charge to the rubber particle and their stability in the latex. Each component will be described for more details.

8 Rubber particle membrane

The rubber particles are commonly found associated with lipids which are thought to be of membrane nature. The particles appeared to have a uniform structure of the rubber molecules enclosing by a thin film seen under the microscope (Southorn, 1961). When examined under electron microscope, the rubber particles have a homogeneous and the uniform internal structure but are surrounded by a film that is more opaque than the polyisoprenes inside (Lau *et al.*, 1986). Analyses of the nature of the film enclosing the rubber particles show the presence of phospholipids and proteins together with neutral lipids similar to the membrane structure. Detailed description on rubber particles and other components has been reviewed (d'Auzac & Jacob, 1989). The composition of the membrane of rubber particles separated and purified by ultracentrifugation has been analyzed. The membrane components consist of lipids, proteins, enzymes, and charges which will be described in details.

8.1 Rubber particle lipids

Analyses of the rubber particle purified by ultracentrifugation revealed that it contained up to 3.2% in total lipids of which about 2.1% are neutral lipids expressed as rubber weight (Ho *et al*, 1976). Separation of neutral lipids showed that it was composed of at least 14 different substances. Triglycerides are the most abundant and accounted for almost 45% of the neutral lipids. Next to triglycerides are sterols, sterol esters and fatty acid esters which consitute about 40%. Other neutral lipids present in trace amount are diglycerides, monoglycerides, and free fatty

acids. In addition, tocotrienols and some phenolic substances are also found associated with rubber particle (Ho *et al*, 1976).

Phospholipids are important components of the rubber particles. Marked differences between clones in neutral lipids content of the rubber phase were noted. On the other hand, phospholipids content was not much varied among the different clones (Ho et al., 1976). Analyses of the phospholipids always consistently show the three spots on TLC separation. It was found to be a considerable amount of phosphatidyl choline and smaller amount of phosphatidyl ethanolamine and phosphatidyl glycerol. Phosphatidic acid was found to be predominant in membrane of lutoids but could not be detected on the rubber particles even though precaution of inhibiting phospholipase D activity was taken in the study (Dupont *et al.*, 1976). In addition, the presences of sphingolipids and glycolipids have also been reported. The stability of rubber particles suspension in latex is dependent on the negative charges film of proteins and phospholipids (Philpott & Weagarth, 1953).

8.2 Rubber particle proteins

Proteins are found as indigenous component of the film enclosing rubber particle. Together with lipids, these proteins form the membrane of particles which contribute to their stability. The pI of these proteins ranges from 3.0 to 5.0 which is characteristic value of the surface proteins. Particles in electric field will move toward the anode, indicating they have net negative charge on the surface (Verhaar, 1953). Anionic soaps do not affect the particles colloidal stability but cationic soaps cause the flocculation, probably due to neutralization of the surface charge. These

proteins can be considered as intrinsic or peripheral depending on their binding and affinity with rubber particles. One of the most plentiful proteins in latex is α-globulin with pI of 4.5. It was found both in cytosol and adsorbed on particles surface and might contribute to their colloidal stability in latex (Archer *et al.*, 1963). Proteins group of hydrophobic nature was also found in rubber particles. Proteolipids have been isolated and characterized (Hasma, 1987). This protein was suggested to be the component of polar lipid backbone which is part of the membrane of rubber particles. The proteins content of rubber particles has been recently refined for a more accurate quantitative analysis (Yeang *et al.*, 1996) in light of the concern over rubber protein allergens.

Other proteins with enzyme activity have been described. One of the interesting and well characterized enzymes is rubber transferase on the washed rubber particle surface (Audley & Archer, 1988). This enzyme involves in synthesis and formation of rubber molecules on the particles surface. It was found to be distributed between cytosol and the rubber particles similar to α-globulin. It has been isolated and purified from latex C serum but was active only when adsorbed onto particle for chain elongation process of the rubber molecules (Light & Dennis, 1989). Another important protein actively involved in rubber synthesis was a 14 kD protein referred to as rubber elongation factor (REF). The amino acid sequence has been determined and the molecular cloning of REF gene also carried out (Attanyaka *et al.*, 1991). More recently, molecular cloning of a protein tightly bound on the small rubber particles has been reported (Oh et al, 1999). The cloned cDNA encodes a 24 kD protein which is tightly bound on rubber particles. This protein was

suggested to be active in synthesis of rubber together with the REF. The 24 kD has previously been reported as a potent latex allergen and always found together with the 14 kD protein as the tightly bound proteins and remain with the particles even after extensive washings. Besides these tightly bound and well characterized proteins, some peripheral proteins have also been described but the function is not known.

8.3 Rubber particle enzymes

Washed rubber particles and the bound rubber synthesis enzymes have been studied and reviewed (Audley & Archer, 1988). The formation of rubber molecules, at least in elongation steps, occurs at the particles surface (Archer *et al.*, 1982). Rubber transferase is the enzyme responsible for this process. The enzyme was also found in the C serum and probably being distributed between the two fractions (McMullen & McSweeney, 1966). It has been isolated from the C serum and purified for enzyme characterization (Archer & Cockbain, 1969). The enzyme was without activity in the absence of the washed rubber particles. It remains inactive so long as the enzyme has not been adsorbed onto the particles, even when the particles have been purified by gel filtration and repeated washings. The reaction catalyzed by this enzyme appears to be essentially the chain extension or elongation of the preexisting rubber molecules even though the formation of new rubber molecules has been suggested (Lynen, 1969). However, the formation of new rubber particles is still not known at present.

So far nobody has been able to demonstrate the polymerization of IDP *in vitro* except on the preexisting rubber particles. The reaction as occurring in vivo must be at other sites as a prelude to formation of the new rubber molecules. Some findings strongly suggest that membrane phospholipids might have a key role in enabling the combined functions of different transferase enzymes to operate in vivo for new rubber formation (Keenan and Allen, 1974; Baba and Allen, 1980). It appears that some phospholipids, membranes, or other amphipathic micelles are essential for the initiation and formation of new rubber molecules. The rubber particles surface was also suggested as the site of IDP isomerase (Lynen, 1969), the enzyme catalyzing conversion of IDP into DMADP. The IDP isomerase is essential for the formation of DMADP and the chain initiation of new rubber molecules. It has long been suggested to be present in latex but only the indirect evidence was given for its detection. More recently, the direct detection and characterization of this enzyme was reported (Koyama et al., 1996). The isomerase was located in the cytosol or C serum of latex, not with the rubber particles as previously suspected. The enzyme was activated in the presence of reducing agents and detergents (Koyama *et al.*, 1996). The findings might provide a partial support for the initiation of rubber chain probably at the site other than the rubber particles.

8.4 Rubber particle charges

Colloidal stability of the latex is attributed by surface charges of the rubber particles. The film or membrane surrounding the particles gives them a negative charge as shown by surface potential or zeta potential (Southorn & Yip, 1968). The

particle membrane composition of proteins, phospholipids, and other substances have been determined as described above which are of negatively charged nature (Ho *et al.*, 1976). The lower of phospholipids content of certain clonal latex known for its instability has been noted. This observation was subsequently extended to show that lipids content of the rubber particles was positively correlated with the colloidal stability of the latex (Sherief & Sethuraj, 1978). The colloidal stability was decreased by magnesium released from the damaged or ruptured lutoids was reported (Philpott & Westgarth, 1953) as neutralization of the surface charges. The effect of inorganic cations was investigated as related to rubber particles flocculation and latex vessels plugging with negative effect on the latex flow (Yip & Gomez, 1984).

9 C-serum of *Hevea* latex

C serum fraction of centrifuged latex is the aqueous phase of laticiferous cytoplasm which can be considered as latex cytosol. This cytosol is not fundamentally different from normal cytosol of plant cells. Analyses of the latex cytosol fraction obtained by ultracentrifugation show that it contains various different organelles and particles. All glycolytic enzymes (d'Auzac & Jacob, 1969) and other common cytosolic enzymes including isoprenoid pathway (Suvachittanont & Wititsuwannakul, 1995; Koyama *et al.*, 1996) have been detected, indicating that it is active in various metabolic processes.

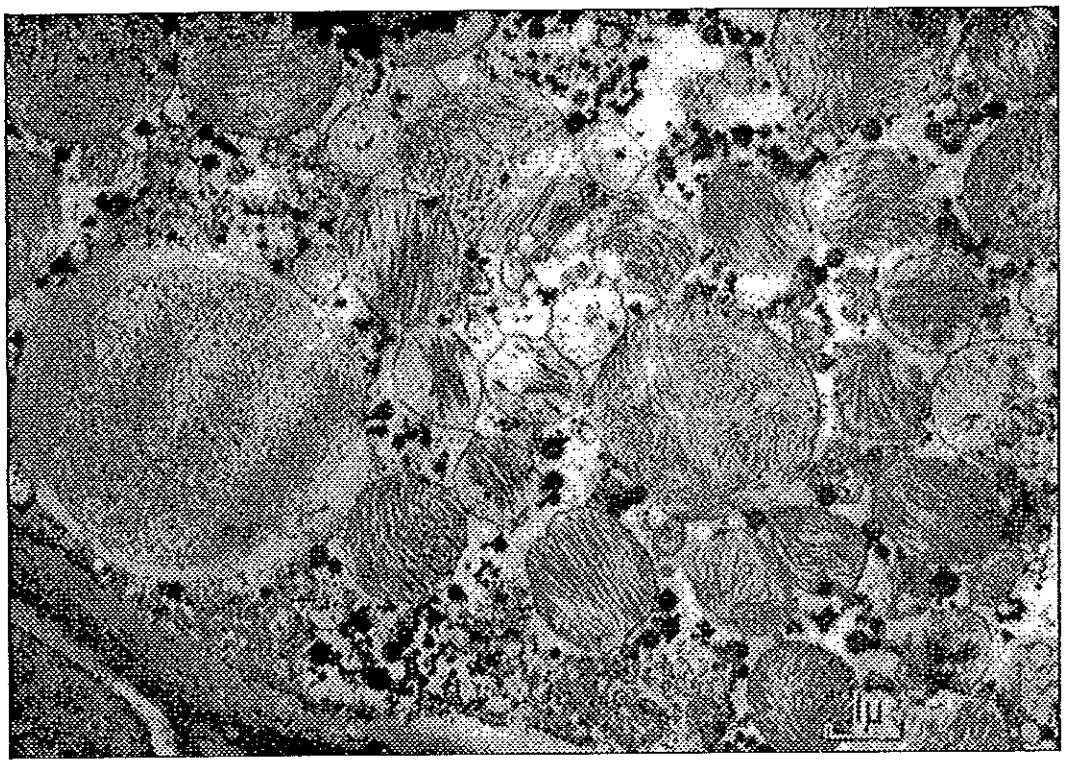
The involvement of C serum in rubber biosynthesis was noted (Tangpakdee *et al.*, 1997a). The importance of calcium binding protein (calmodulin) in contolling

many different metabolic processes was investigated. It was found to activate HMG CoA reductase in the bottom fraction. Purification and characterization of calmodulin from C serum (Wititsuwannakul *et al.*, 1990b) suggested that it had important role in the regulation of latex metabolism. Highly positive correlation between calmodulin level and latex yield was also of important note (Wititsuwannakul *et al*, 1990b). Composition of latex cytosol was reviewed (d'Auzac & Jacob, 1989).

10 Lutoids of *Hevea* latex

Lutoids are membrane bound particles sedimented in bottom fraction of centrifuged fresh latex (Moir, 1959). The electron micrograph of this organelle is showed in Figure 2. They can be considered as polydispersed lysosomal vacuoles and have been extensively discussed (d'Auzac & Jacob, 1989). The lutoids content of latex is quite considerable and found to play many important functions in the latex. They constitute about 20% by volume of fresh latex whereas the rubber phase forms approximately average of 30-40%. They are spherical in shape and larger in diameter than the rubber particles. Lutoids are enclosed by single layer membrane and the lipids composition has been determined. They play an important role in colloidal stability of latex due to negatively charged membrane which is very rich in the phosphatidic acids content (Dupont *et al.*, 1976). They are considered as similar to the lysosomes with the high content of acid hydrolases (Jacob *et al.*, 1976). Lutoids are found to play a major role in coagulation of rubber particles in colloidal

suspension of latex (Southorn & Edwin, 1968). The mechanism of coagulant effect is due to the release of cations and proteins from ruptured lutoids (Pakianathan & Milford, 1973). The storage function of lutoids was shown in the accumulation of several proteins, enzymes and solutes with active membrane transport activity (Chrestin & Gidrol, 1986). Lutoids are important latex component, both as regard to volume and the functions of their chemicals and enzymes (d'Auzac & Jacob, 1989). Lutoids compostion can be considered as two distinct components, lutoids membrane and the internal contents called B serum.



(Dickenson, 1969)

Figure 2 Lutoid particles of the fresh *Hevea* latex (EM). The picture shows the spherical shape lutoids which have larger diameter than the rubber particles, with ful development of the enclosed proteinaceous microfibrils. The unit membrane surrounding the particles is contorted because of slight plasmolysis as the lutoid are osmosensitive.

10.1 Lutoid membrane

Cytological observation, biochemical, and phyiological research on lutoids membrane have provided data about the structure, composition, and the role of these organelles in latex. Electron microscopic study showed the micellar nature of the lutoids membrane structure (Gomez & Southorn, 1969). It is very osmosensitive single layer membrane of 8-10 nm thickness. Chemical composition showed the phospholipids content forming 37.5% of the weight of proteins. It has been found to be very rich in phosphatidic acids which accounted for 82% of the total phospholipids fraction. Fatty acids composition analyzed by methanolysis of phospholipids shows predominance of saturated fatty acids as palmitic and stearic acids. The unsaturated ones are oleic and linoleic acid (Dupont *et al.*, 1976). There are practically equivalent quantities of saturated and unsaturated fatty acids in the membrane. The exceptionally high phosphatidic acids content may explain the negative surface charge of lutoid (Southorn & Yip, 1968). The relative abundance of saturated fatty acids in lutoids stand out clearly from the membranes of other plant organelles. The relative rigidity and fragility of lutoids membrane to osmotic shocks and low resistance to mechanical stress (Pakianathan *et al.*, 1966) can partly be explained by its fatty acids composition. The analysis of rubber particles membrane carried out under the same condition shows that it is totally free of phosphatidic acids but contains mainly phosphatidyl choline and phosphatidyl ethanolamine (Ho *et al.*, 1976). The other major lutoids membrane components are the proteins, several of which are enzymes important in the lutoids function. The membrane of lutoids thus plays essential and very complex role in latex as has been shown in numerous reports and reviews (Paardekooper, 1989; d'Auzac & Jacob, 1989).

10.2 Lutoid membrane proteins and enzymes

Several proteins are present in lutoid membrane and many of which are active enzymes. One of the well characterized membrane enzyme is ATPase (Moreau *et al.*, 1975). The electron transport activity of lutoids has been linked to this enzyme. It is activated by several anions. This would lead to the accumulation of anions in the lutoids compartment. It also operated as a proton pump to maintain protons gradient between the lutoids and latex cytosol. The operation of the lutoid membrane ATPase as an electrogenic proton pump has been demonstrated (Chrestin & Gidrol,1986) and extensive reviewed (Chrestin *et al.*, 1989). Other membrane enzymes included NADH-Cytochrome C reductase that function in an outward protons pumping redox system which tends to reduce the concentration of protons in lutoids and hence acidify the cytosol (Moreau *et al.*, 1975). NADH-Quinone reductase has also been described as responsible for production of superoxide ions. Generation of free radicals and the consequent effect on lutoids integrity and latex colloid stability has been the subject of recent interest and speculations.

Recently, the enzyme HMG CoA reductase has been purified from lutoids membrane by solubilization with mild detergent (Wititsuwannakul *et al.*, 1990a). Characterization of the purified enzyme was carried out on its molecular structure and properties. The native enzyme was tetrameric of 44 kD subunits similar to the findings in other plant specimens as membrane bound enzyme (Bach, 1985). It was found to be activated by reducing agents for maximum activity. Regulation of this enzyme by calmodulin in C serum was also studied in details (Wititsuwannakul *et al.*, 1990b). The findings suggest the important interactions of C serum contents for

optimal lutoids membrane activities. Rubber formation by the bottom fraction was recently noted (Tangpakdee *et al.*, 1997a & 1997b). It might thus demonstrate the participation of lutoids in rubber biosynthesis process. Other different lutoid functions were reviewed together with characterization of the various lutoid membrane enzymes (d'Auzac and Jacob, 1989). Therefore, this aspect will be described here only briefly. Structural proteins of the lutoid membrane are little known and still awaiting further characterization.

10.3 Lutoid B-serum and composition

The content inside lutoids called B serum composes of several different components. These included microfibrils, proteins and enzymes, as well as various small compounds. Microfibrils are cluster of proteins with helical structure as observed by electron microscope (Archer *et al.*, 1963; Dickenson, 1965). Characterizations of their structure showed the presence of carbohydrate up to 4% and are acidic proteins with a pI of 4 (Audley, 1966; Gomez, 1976). These fibrillar proteins may more or less completely fill the intralutoid space and was speculated that it may form nitrogen reserves (Dickenson, 1969) which can be degraded by lutoid proteases. They consist of basic proteins of 22 kD and an acidic assembling protein of around 160 kD in microhelical clusters formation (Gomez & Tata, 1977). Clonal variation of microhelices content was also observed. In addition to the clustered insoluble proteins, other soluble proteins have also been characterized. Electrophoretic analyses showed the presence of at least 7 to 8 different proteins.

Among these, hevein is most abundant constituting 70% of the total proteins (Archer, 1960; Audley, 1966).

Hevein is quantitatively the most important anionic proteins with special feature of being a very small protein of 5 kD. It has been purified, crystallized, and characterized (Tata, 1976). Amino acids composition and sequence has been determined (Walujono *et al.*, 1976) with high sulfur content contributed by cystine alone. In the light of its low molecular weight and similarity in composition with high cysteine content, it has been considered that hevein might function as the protease inhibitor similar to those detected in the vacuoles of potato and other Solanaceae. However, several tests could not show protease inhibitor activity of hevein (Walujono *et al.*, 1976). The role of these fibrillar proteins and hevein and the significance of their presence in lutoid is not clearly known. Recently, involvement of hevein in coagulation of rubber particles was reported (Gidrol *et al.*, 1994). Besides hevein, other basic proteins with high content in lutoids have been detected and characterized as hevamine A and hevamine B (Archer *et al.*, 1969). The lutoids have considerable influence on the metabolism of latex and its regulation by means of the exchanges which take place between their contents and the latex C serum. Several enzymes are present in B serum which is important in metabolic processes and homeostasis of the latex.

10.4 Lutoid B-serum enzymes

Several typical vacuolar enzymes can be detected in B serum of the lutoids. Lysozyme, commonly found in egg white which hydrolyzes mucopolysaccharides

and bacterial cell wall, was detected in the latex bottom fraction (Archer *et al.*, 1969). It was located in B serum and considered to be the same as hevamines which was abundant basic protein in lutoids (Tata *et al.*, 1976). The B serum lysozyme has been purified and characterized. The kinetics study and partial amino acids composition and sequence was determined (Tata *et al.*, 1983). The enzyme is generally implicated in the defense as antimicrobials. B serum contains several acid hydrolases (phosphodiesterase, acid phosphatase, ribonuclease, cathepsin, β glucosidase and β galactosidase (Archer *et al.*, 1969). The presences of these enzymes show similarity between animal lysosomes and lutoids. The role of lutoids content in stability and flow of latex was demonstrated (Southorn & Edwin, 1968). It was found to cause rubber particles coagulation leading to latex vessels plugging and stopping of the latex flow which will be described in details later. Acid phosphatase was found to be lutoids specific enzyme and appear in latex cytosol only after rupture or damage of the lutoids. The ratio of free acid phosphatase and the total activities reflects the stability of lutoids. The ratio is referred to as bursting index (BI) which influences the flow of latex. High correlations between BI and vessels plugging as measured in the latex flow duration has been documented and reported for the seasonal and clonal variations (Yeang & Paranajothy, 1982).

Oxidation-reduction enzymes in B serum were reviewed (d'Auzac & Jacob, 1989). These included catalase, phenol oxidase and tyrosinase. Peroxidase enzymes such as monophenol, polyphenol, and DOPA oxidases were detected in B serum as cationic proteins. These enzymes are always found together with their inhibitors and it was suggested that compartmentation in the lutoids might occur for the regulation of these enzymes. In addition, the enzymes of pathogenesis related proteins such as

chitinase and β-1,3-glucanase were also found accumulated in lutoids (Churngchow *et al.*, 1995). These enzymes appeared to be induced as wounding response due to regular tappings and serving as defense against the attack by pathogenic microbes. Normally these enzymes are absent or at very low level in the healthy plants. This observation can also be applied to the high level of lysozyme (Tata *et al.*, 1983). Listing of other B serum enzymes and more details can be found elsewhere (d'Auzac & Jacob, 1989).

10.5 Lutoids and colloidal stability of latex

Contribution of lutoids to latex colloidal stability has been briefly mentioned earlier. The colloidal stability of latex results from negative charges carried by its structural components. The more important of these are rubber particles and lutoids. Integrity of the lutoids is thus essential for maintaining the latex stability. The lutoids content of acidic serum, divalent cations, and positively charged proteins might be considered that lutoids have a destabilizing role in latex. This is certainly true only as long as the lutoids are not damaged or ruptured. However, the lutoids are very osmosensitive and therefore a certain amount of their contents are released from the ruptured lutoids into latex cytosol after tapping. A demonstration of the role of lutoids as coagulants by the formation of microflocculate consisting of lutoids fragment and rubber particles were reported (Southorn, 1969). The lutoid B serum is effectively capable of provoking the microflocculates formation of a dilute suspension of rubber (Southorn & Yip, 1968). It was shown that C serum proteins are mainly anionic while those of B serum proteins are mainly cationic (Moir & Tata,

1960). It is clear that putting into contact of the B serum proteins with negatively charged particles of rubber results in the neutralization of surface charges and the destabilization of the latex colloidal solution (Southorn & Yip, 1968; Sherief & Sethuraj, 1978). These early studies form the basis for further analysis in rubber particles coagulation caused by purified hevein (Gidrol et al., 1994). The importance of lutoids as related to latex flow duration, vessels plugging and effect on the rubber yield will be further described in details.

10.6 Lutoids and latex vessels plugging

Rubber yield can be ascribed to two parameters, latex flow duration and latex synthesis or regeneration capacity. Lutoids are implicated to influence the time of latex flow and the latex vessels plugging as reviewed (Paardekooper, 1989). Integrity of lutoids is thus essential for latex colloidal stability. Damage or rupture of lutoids causes coagulation of rubber particles leading to the stop of latex flow. Several studies provided evidence that the major cause of vessels plugging during latex flow is damage to the lutoids. This was caused by osmotic shock as a result of different turgor pressures between inside vessels and the outside during flow after tapping. The initial period of fast flow these damaged lutoids were swept out of the vessels before they suffered irreversible damage. During the subsequent slower flow the lutoids suffered greater damage within the vessels and aggregated with rubber particles to form flocs which accumulated near the cut ends, thus initiating plugging process (Pakianathan *et al.*, 1966; Pakianathan & Milford, 1973). In whole latex, breakage of lutoids by ultrasonic treatment resulted in the formation of flocs of

rubber and damaged lutoids fragment (Southorn & Edwin, 1968). Therefore, the plugging within the vessels is caused by release of B serum from ruptured lutoids. Fresh latex always contains microflocs, the formation of which is confined to the area of damaged lutoids where the contents of B serum is momentarily high, and is limited by a stabilizing effect of C serum. The latex is envisaged as a dual colloidal system in which negatively charged particles, rubber particles and lutoids, dispersed in the neutral C serum containing anionic proteins (Southorn & Yip, 1968). The two antagonistic systems can only exist so long as they are separated by the intact lutoids membrane. Lutoids damages result in the interaction between its cationic contents and anionic surfaces of the rubber particles, causing the formation of flocs.

It is observed that the enzyme acid phosphatase is released in the B serum when lutoids are damaged. The ratio of free and total acid phosphatase activities called the "bursting index" (BI) as earlier mentioned indicates the percentage of lutoids in a sample that had ruptured. This index was inversely related to osmolarity of latex and the first fraction of latex collected after tapping was higher than subsequent fractions (Pakianathan *et al.*, 1966). It was found that damage to bottom fraction was greatest in the first flow fraction after tapping. High correlations between plugging index, intensity of plugging, and lutoid BI was found in studying of seasonal variations in the flow pattern (Yeang & Paranjothy, 1982). Positive correlation between total cyclitols in C serum and plugging index with increased lutoid damage was also noted (Low, 1978). Lutoids could be disrupted by the mechanical shearing forces to which the latex is subjected when flowing through the vessels under high pressure gradients after tapping (Yip & Southorn, 1968). Difference between clones in the composition of protective film on rubber particles

may be partly responsible for the flocs formation. Rubber particles are strongly protected by complex film of protein and lipid materials as mentioned before. Marked differences between clones in neutral lipids content of rubber phase in which phospholipids content did not differ much showed the negative correlation with the plugging index. Long flowing or slow plugging clones have a high neutral lipid content in the rubber phase, as much as five times that of the fast plugging clones (Ho et al., 1976). Negative correlations between phospholipids content of lutoid membrane and BI leading to rapid plugging was documented (Sherief & Sethuraj, 1978). These findings suggested that, in addition to the effect of lutoids behavior, latex vessels plugging is also influenced by the rubber particles lipids. It can thus be seen that the plugging of vessels and hence affecting the latex yield is quite a complex process. Other factors in the process are also speculated and awaiting to be elucidated.

11 Frey-Wyssling particles of *Hevea* latex

Frey-Wyssling particles represent a minor component of latex as compared to lutoids. These particles are yellow in color because the presence of β-carotene in the organelle. The name of organelle "Lutoid" describe above (= yellow) turned out to be a misnomer, because as originally isolated they were contaminated with the yellow Frey-Wyssling particles. The Frey-Wyssling particles are bounded by a double membrane and contain many membrane or tubular structures (Figure 3) and β-carotene giving it characteristic orange to yellow color layer upon fractionation by

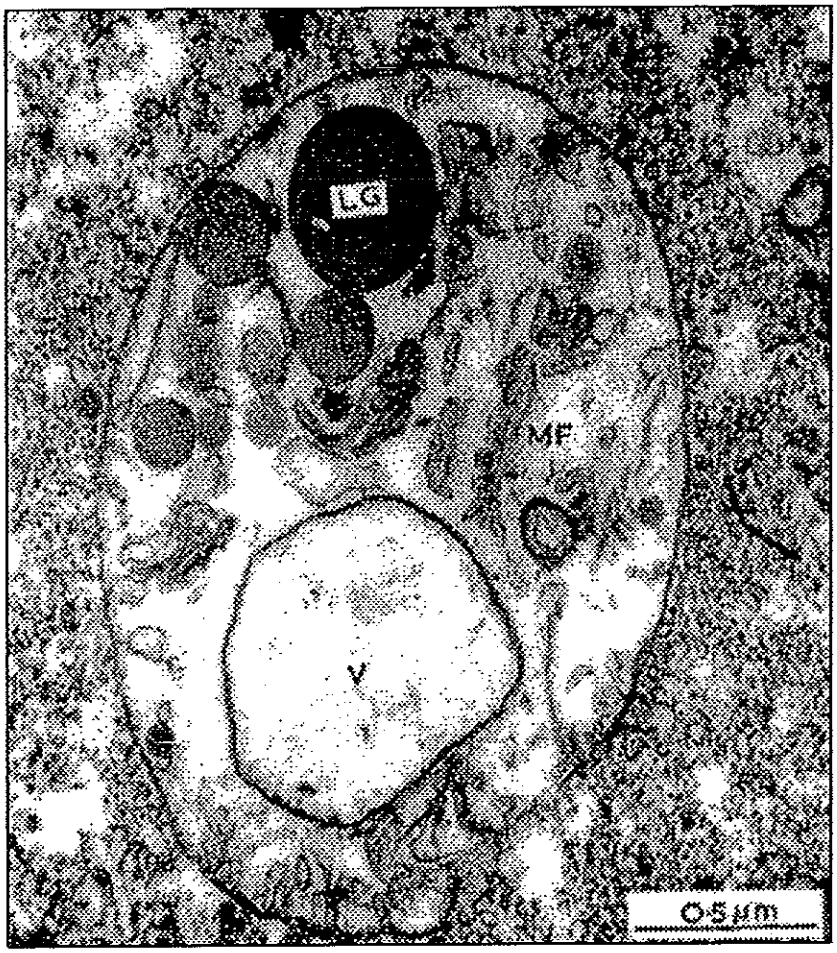
ultracentrifugation of the fresh *Hevea* latex. However, the detailed functions of these particles in latex are still very little known at the present.

12 *Hevea* latex metabolism

Hevea latex is a very unique system as consisting of specialized cytoplasm (Dickenson, 1965; Gomez & Moir, 1976). Organization and composition of this cytoplasm reflect biological functions of *Hevea* specialized laticiferous tissue. It has long been shown that fresh latex can synthesize rubber in vitro from labeled precursors acetate or mevalonate (Kekwick *et al.*, 1959; Park & Bonner, 1958). The enzymes activity and their location with substrates and effectors make it possible to study metabolism and the biochemical function of latex as "rubber factory". Latex as obtained by tapping is not so destructive compared to the general method for preparing cell cytoplasm, making it very suitable to study latex metabolic functions. Latex metabolism has been studied in various different aspects, especially the rubber biosynthesis and other related metabolic processes (Jacob *et al.*, 1989). Tapping of rubber trees for latex makes it necessary for laticiferous tissue to make up for the lost materials between successive tappings. Regeneration of cell materials is increase along with latex production. If it is not sufficiently effective, it can be limiting factor for latex production (Jacob *et al.*, 1986). It requires intense metabolic activity and numerous enzymes to be involved in at least four important processes for the latex regeneration: (1) the catabolism which provide energy and reducing capacity for the anabolic processes; (2) the activity of anabolic pathways for various syntheses

including isoprenoids; (3) the mechanisms associated with regulatory systems and homeostasis; and (4) the supply of nutrients to the zones or subcellular components in which cell materials are regenerated. The specificity of laticiferous tissue is so organized that its particular major function is directed to the production of rubber in the latex.

The formation of rubber in *Hevea* laticifers seems to be a very complex control system. Several questions regarding the regulation of rubber biosynthesis have been raised and investigated. The continuing requirement of carbon, NADPH, and the need of ATP for rubber biosynthesis must place a high degree of demands on the metabolic economy of the tissues. It has been calculated that the required rate of regeneration of rubber in alternate daily tapping is of the order of 1 μmole isoprene unit per ml latex per minute (Bealing, 1976). The capacity of latex to incorporate acetate and mevalonate has been found to fluctuate markedly with the season (Bealing, 1976). The control mechanism in the formation of rubber appears to be a complex and intricate process. The interesting feature of *Hevea* metabolism is not only that for rubber formation but also that it can be stimulated to produce rubber and other many components of latex by repeated tapping. This replenishment is not called for in the untapped tree and although a few terpenoids have been shown to suffer catabolism in plants, there is no evidence for the breakdown of rubber in vivo. The enhanced rubber yield by ethylene, a hormone associated with response to wounding in plants, has been extensively studied and reviewed (d'Auzac, 1989). Specific genes activation may shed some light on the mode of action of the tapping stimulus and ethylene activation. This will be the aspect to be described.



Gomez & Hamzah, 1989

Figure 3 Frey-Wyssling particles prepared from *Hevea* latex showing the bounded membrane with lipid globules (LG), vesicles (V) and membrane fragments (MF) inside the particles.

13 Rubber and isoprenoids biosynthesis in *Hevea brasiliensis*

Hevea rubber biosynthesis and its control has been reviewed by many authors (Archer & Audley, 1987; Kekwick, 1989; Kush, 1994) An interesting aspect of *Hevea* laticifers is the fine tuning in term of compartmentation of function or the division of labor for rubber biosynthesis pathway. It was shown that the laticifers have a differential gene expression profile (Kush *et al.*, 1990; Kush, 1994). The genes involved in rubber synthesis are highly expressed in the latex as compared to that in the leaves. The specialized differential expression serves two-fold functions. The desired enzymes for rubber synthesis are expressed in the very tissues where the formation is taking place. Localizing the rubber synthesis activity in laticifers allows the rest of other different metabolic processes in other tissues to operate at the optimum and well balanced with the whole plant functions. Specialized functions and well coordinated division of labors are thus seemed to be well organized for specific channeling of precursors and metabolites for different metabolic pathways. The tissue and cell differentiations destined to perform certain functions to best fit the metabolic distribution can thus clearly be seen in *Hevea brasiliensis*. This is somewhat different for other rubber producing plants such as guayule.

Hevea brasiliensis is unlike in some plants like guayule (*Parthenium argentatum*) where rubber synthesis is taking place in cytosol of the parenchyma tissues along with the rest of other metabolic processes needed for orderly parenchyma cell functions. Extraction of rubber from guayule plants is difficult because of the relatively low abundance of rubber particles in the cell and limited by

the cell volume (Backhaus & Walsh, 1983). Procedure of obtaining rubber from guayule is very destructive because of subcellular localization of rubber. Guayule rubber can only be obtained by crushing of the stems and extracted together with all other cellular materials and impurities of all cell types. This is one of the prime reasons that the *Hevea* rubber tree is the only commercially viable source of natural rubber in the world. Even though about two thousand species of plants producing rubber of varying types and amounts are known (Backhaus, 1985; Mahlberg, 1993), none is used or found comparable to *Hevea* rubber superior quality. This is in contrast to the *Hevea* laticifers containing high content of latex and gushed out after opening the latex vessels by small excision of the bark or tapping which is not so destructive to the plant tissues. The flow of latex is due to the high turgor pressure inside the laticifers compared to the outside. After some time of the latex flow out of the cut open vessels, the flow will stop due to coagulation of rubber particles forming the latex plugs at the vessel ends or the wounding site resulted from tapping. A lectin- like small protein, hevein, which is localized in lutoids (Gidrol *et al.*, 1994) has been shown to play an important role in latex vessels plugging. Lutoids have been shown to have very fragile membranes which burst in response to tapping because of difference in the turgor pressure as earlier discussed on the lutoid and its properties. The tapped latex contains vast number of intact organelles which makes it an excellent specimen for the study of differential and specialized metabolic functions in plants. As *Hevea* laticifers are anastomosed system, the latex in essence represents the cytoplasm of a single cell type. The rubber yields are the results of two contributing factors, the latex flow properties and regenerating synthetic capacity.

14 Isoprenoids biosynthesis

Besides the rubber, *Hevea* laticifers also synthesize several diverse isoprenoids (Kush, 1994). Different plants have capacity in synthesizing certain isoprenoid compounds for specific functions, from simple isoprenoids to the more complicated ones like natural rubber. They produce this wide range of isoprenoids in different amounts in specific organelles at different stages of growth and development. Since the diverse isoprenoid compounds are produced by a more or less conserved biosynthetic pathway (Randall *et al.*, 1993), plants must execute control mechanism to ensure the synthesis of the necessary isoprenoids in the right place at the right time. Such control is very likely mediated through some regulatory enzymes, attempts have been made to understand the regulatory mechanism of the isoprenoids and polyisoprenoids biosynthesis as well as their interrelationship (Kekwick, 1989; Mahlberg, 1993). The pathway used for the formation of isoprenoids in plants is similar to the sterol biosynthetic pathway that was worked out in animals and yeast (Clausen *et al.*, 1974; Taylor & Parks, 1978). The isoprenoid biosynthesis may be viewed as the pathway from acetyl CoA via mavalonate and IDP to long chain prenyl diphosphate. A large number of branch points can lead to a variety of diverse isoprenoids in plants (Kleinig, 1989; Randall *et al.*, 1993).

Recently, the oligoprenoid and polyprenoid in *Hevea* latex were examined (Koyama et al., 1995; Tangpakdee et al., 1997a). The chain length of B serum isoprenoids showed several components of C_{15}- C_{60} which are more or less of equal proportion. However, the C serum isoprenoids was quite different. The major chain

length in the C serum isoprenoids was the C_{20}-GGDP as analyzed by autoradiogram. Only a few isoprenoids were detected in the C serum as compared to several in the B serum. The C_{15}-FDP in the C serum was much less than the C_{20}. The differences between the two are presently still unclear. It can be assumed that the component in B serum may be the intermediates of the rubber formation as it was recently reported the rubber formation in the fresh bottom fraction of centrifuged latex (Tangpakdee *et al.*, 1997b). The major C serum of C_{20}-GGDP and to a lesser extent C_{15}-FDP might be the substrates for the prenylation of proteins as the presence of prenylated proteins has been increasingly reported to occur in the plant cells. However, the exact role of thes isoprenoids in the two latex sera is still needed to be elucidated. More recently, the polyprenoids of the dolichols group and other group in *Hevea* latex were analyzed by two dimention TLC (Tateyama *et al.*, 1999). It was found that the chain length of dolichols in *Hevea* ranges from C_{65}-C_{105}. The analysis on dolichols of the *Hevea* seeds, root, shoots, and leaves of different ages were also carried out. Comparisons on the differences were examined to understand the changes associated with growth and development. The function of dolichols is commonly known to associate with glycosylation process and it is assumed that the presence of dolichols in Hevea is no exception. The role of glycoproteins has received much attention in light of the reports on the presence of lectin in the *Hevea* latex (Wititsuwannakul *et al.*, 1997a & 1997b) which were found to have important role in latex metabolism and colloidal stability.

The enzymes HMG CoA synthase (HMGS) and HMG CoA reductase (HMGR) have been implicated as the essential regulatory enzymes in the biosynthesis of IDP (Brown & Goldstein, 1980; Bach, 1986; Goldstein & Brown, 1990). Similar roles

have been implicated in plants as well, although conclusive evidence for the regulatory role of HMGS is not yet available. Downstream of IDP in formation of specific isoprenoids varies, depending on the end products and the subcellular compartmentation. The role of HMGR in regulation of isoprenoids and rubber biosynthesis in *Hevea brasiliensis* has been well documented (Wititsuwannakul, 1986). Diurnal variations of HMGR levels and dry rubber contents of latex were conclusively shown with high corresponding and positive correlations (Wititsuwannakul, 1986), the enzyme was located as membrane bound and purified from the membrane of lutoids (Wititsuwannakul et al., 1990a). The purified enzyme was analyzed and characterized which was found to be similar to the HMGR from other plant specimens. *Hevea* HMGR was activated by calmodulin, the calcium binding protein involved in myriad regulatory processes, located in the latex C serum (Wititsuwannakul et al., 1990b). Positive correlation between the HMGR activity and calmodulin levels were demonstrated corresponding with the levels of dry rubber contents. Comparison between the high-yielding and low-yielding clones also showed the clonal variations of the calmodulin level in the same direction and correspondingly. In the rubber tree, this compartmentation is highly specialized for the syntheses of the rubber and isoprenoids as demonstrated by the regulatory mechanism of lutoid HMGR by C serum calmodulin in the control of rubber biosynthesis (Wititsuwannakul, 1986; 1990a; 1990b) and the difference in isoprenoids distribution in B serum and C serum.

15 Rubber latex regeneration capacity

Several apparent parameters were found to influence the latex regeneration metabolism. These controlling factors were commonly analyzed and compared for their effects in the tapped latex. The importance of sufficient availability of the sucrose in latex was extensively studied as precursor and having essential role for polyisoprenoids synthesis (Tupy, 1989). Sucrose metabolism and its utilization are controlled by invertase and the levels of enzyme activity were positively correlated with latex production (Yeang *et al.*, 1984; Low & Yeang, 1985). Some key enzymes affected by ions (Mg^{2+}, phosphate) and the thiols (-SH) group for reducing condition were documented as related to the rubber biosynthesis. These included invertase, puruvate kinase and PEP carboxylase. Activity levels of these enzymes influence the latex production capacity as has been detailed and summarized (Tupy, 1989; Jacob *et al.*, 1986). The intracellular pH is the essential factor in the isoprenoids metabolic regulation. Increased activity of invertase and PEP carboxylae were found under alkaline pH of the laticifer contents and was correlated with the latex and rubber yield (Yeang *et al.*, 1986; Tupy, 1989).

Significant positive correlations between these parameters and latex production were documented. The relationships between the total solids content, reduced thiols content, pH of the latex, and the latex yields among different clones and the seasons have been analyzed and extensively characterized. Highly significant positive correlations were found between these three key parameters and the latex yield (Jacob *et al.*, 1986). All the correlations analyzed are of highly

statistical significance with the very low P value ($P < 0.001$) as reported in the extensive studies (Jacob *et al.*, 1986). They thus reflect the latex regeneration capacity of the laticifers which require the coordinations and interactions of several parameters in the intricate manners. . In addition, induction and activation of the protein synthesisis was also noted for certain enzymes (Kush *et al.*, 1990; Broekaert *et al.*, 1990; Goyvaert *et al.*, 1991; Pujade-Renaud *et al.*, 1994; Kush, 1994) as the key factor on enzyme levels for latex regeneration. This is also the important key parameters in addition to the availability of sugars and the cytosol alkaline conditions of the laticifers and the latex cytosol (Tupy, 1989). It can thus be obviously seen that the latex regeneration is a very complex process requiring the participation of myriad array of parameters in a coordinated and synchronized manner for the orderly function of the laticifers and latex cytosol. And as yet some unidentified parameters which are to be added in the near future as the research on this aspect progressing along.

16 Latex of other plants

It is commonly known that rubber latex is not only formed in *Hevea brasiliensis*, but also synthesized and accumulated in over 2000 species of plants representing about 300 genera from seven families (Archer & Audley, 1973). The rubber producing plant families are *Apocynaceae, Asteraceae, Asclepiadaceae, Euphorbiaceae, Loranthaceae, Moraceae*, and *Sapotaceae*. The latex and rubber composition is varied among all these species. Rubber is plant secondary

metabolites that can be produced in large quantity in certain plant cells. However, it has no known function to the plants even though the metabolic function has been speculated but has yet to be specified. The presence of rubber in various plants does not appear to deter herbivore feeding, insect attack or diseases caused by microbes. Rubber does not serve as an energy source although tremendous resources are allocated to its formation. There is no evidence that plants have the enzymes capable of degrading rubber (Archer & Audley, 1973). It thus seems that once it is formed it is to remain in the cells. The enzyme responsible for turnover of carbon in the rubber has yet to be found and characterized. The ability of these plants to synthesize rubber is based on the presence of the key enzyme, rubber transferase, which cause the *cis*-polymerization of isoprene units (IPP) into the long hydrocarbon chain of rubber. These are assembled and accumulated as rubber particles ranging in size from 5 nm to 3 μm. These particles are enclosed or surrounded by the lipophilic film. The presence of enzymes and the processes for rubber formation are well documented. In some of these plants, *Parthenium argentatum* (guayule) and *Hevea brasiliensis* (rubber tree), detailed study on rubber synthesis process were characterized (Benedict, 1983). These two represent plant species which accumulate large quantities of high molecular weight rubber but by different intercellular routes. The rubber synthesis takes place in parenchyma cells in guayule (Backhaus & Walsh, 1983) but in specialized latex vessel cells (laticifers) in *Hevea* (Archer *et al.*, 1982). Other plants produce far less rubber than *Hevea* or guayule. They usually produce rubber of inferior quality with much lower molecular weight than *Hevea* which limit their commercial potential.

Molecular weight of rubber from various species were examined and compared. The average molecular weight of rubber molecules can vary considerably with the species. What makes *Hevea* and guayule unique among rubber producing plants is that they produce rubber with a predominantly high molecular weight average of 3-7 x 10^5, which is critical for commercial utilization. Other rubber producing plants produce inferior quality rubber with a molecular weight of ca 5 x 10^4 or less. The molecular weight distribution (MWD) of rubber extracted from *Lactuca, Carissa, Candelilla* and *Asclepias* compared to guayule as demonstrated by GPC showed that they have at least 100 times lower than guayule (10^4 or less as to 10^6 for guayule). An interesting feature of molecular weight is that *Hevea* and guayule exhibit bimodal MWD with a major peak at ca 7 x 10^5 and a minor peak at ca 0.5-1.0 x 10^5. This suggests that polymerization in these two plants either undergoes a two-step process or that two forms of rubber transferase may exist. In contrast, the sizes of rubber in other plants uniformly show a single peak of low molecular weight of ca 10^4 or less, indicating a lower degree of polymerization in these plants with the different property of rubber transferase and activity from *Hevea* or guayule.

Note: This Literature Review is adapted from a part of "Chapter 6: Biochemistry of Natural Rubber and Structure of Latex" by Wititsuwannakul, D. & Wititsuwannakul, R., in: Biopolymer, vol. 2 (Koyama, T. & Steinbüchel, A., eds.), Wiley-VCH, Weinheim (2001).

Objective

This thesis is aimed to address and answer some key questions;

1. What is the role of *Hevea* latex organelle for the *in vitro* rubber biosynthesis?
2. How is the property of the *Hevea* latex organelle membrane or their proteins that make it capable for rubber biosynthesis process?
3. What are conditions and factors that influence (activate or inhibit) the rubber biosynthesis?
4. What is the role of rubber biosynthesis in using difference allylic initiators?
5. How is the property of rubber synthesized from bacterial C_{55}(UPP)?

Chapter 2

Materials and Methods

Materials

1 Chemicals

1.1 Chemicals and reagents

Acetone, toluene and hexane were purchased from J.T.Baker (Phillipsburg, NJ). HPLC grade Tetrahydrofuran (THF) was from LabScan (Bangkok, Thailand). Undecaprenyl diphosphate synthase enzyme (UPS) was generously provided by Prof. Dr. Koyama (Tohoku University, Japan). Bio-Rad Protein Assay reagent was from Bio-Rad (Hercules, CA). Isopentenyl diphosphase (IPP), Farnesyl diphosphate (FPP), Geranylgeranyl diphosphate (GGPP), sodium dodecyl sulfate (SDS), deoxycholic acid (DOC) and potato acid phosphatase enzyme obtained from Sigma-Aldrich (St. Louis, MO). Other analytical grade chemicals and reagents used in this study were also obtained from Sigma-Aldrich (St. Louis, MO).

1.2 Radioactive chemicals

[1-^{14}C] Isopentenyl diphosphase (^{14}C-IPP, 54 mCi/mol) was from Amersham Biosciences (Amersham, UK). [1-^{3}H] Farnesyl diphosphate (^{3}H-FPP, 20 Ci mmol^{-1}), [1-^{3}H] Geranylgeranyl diphosphate (^{3}H-GGPP, 20 Ci/mmol) were from American Radiolabeled Chemicals Inc (St. Louis, MO). ^{14}C labeled undecaprenyl diphosphate (^{14}C-UPP) was prepared using the UPS enzyme with the same quality and purity according to the published procedure (Shimizu *et al.*, 1999).

2 Chromatographic materials

2.1 HPLC columns and system

TSK-GuardcolumnH$_6$ (7.5mm X 7.5cm) and TSK-Gel (7.5 mm X 30cm) which were connected tandem in a series: G7000H$_6$, G5000H$_6$, G2500H$_6$ and G1000H$_6$ (exclusion limit = 4X10^8, 4X10^6, 1X10^4 and 1X10^3, respectively) from Tosoh Corp (Tokyo, Japan). The HPLC system consisted of 126 pump connected with 166 and System Gold® Operating System from Beckman.

2.2 R-TLC and autoradiography materials

The R-TLC plate was LKC-18 from Merck (Darmstadt, Germany). The imaging plate was Fuji film BAS-IIIs and bioimage analyzer was Fuji BAS 1000 Mac from Fuji Photo Film Co (Tokyo, Japan).

3 Instruments

3.1 Centrifuge model J2-21 (Beckman)

3.2 Centrifuge model Avanti^{TM} 30 (Backman)

3.3 Microcentrifuge model H-3 (Kokusan)

3.4 Ultracentrifuge model L8-70M (Beckman)

3.5 System Gold® - HPLC operating system (Beckman)

3.6 Spectrophotometer model DU 650 i (Beckman)

3.7 Dual Mini Slab Electrophoresis series model AE-6530 (Atto)

3.8 Prep Cell model491 (Bio- Rad)

3.9 Power Supply model Power Pac 300 (Bio-Rad)

3.10 Power Supply model 1000/500 (Bio-Rad)

3.11 Reciprocating shaking water bath model RW-1812 (Paton Industry)

3.12 Dry Bath Incubator model VH-01 (Violet BioSciences Inc)

3.13 Vortex mixture model Vortex-genie-2 (Scientific Industry)

3.14 Deep-freeze refrigerator (Scientemp Co)

3.15 –20°C refrigerator model SJ-438F (Sharp Co)

3.16 Hot air oven model 630-7 (National appliance Co)

3.17 Bioimage analyzer model BAS 100 Mac (Fuji)

Methods

1 Preparation of materials from fresh latex

1.1 Collection of fresh latex for immediate centrifugation

Fresh latex used in this study was obtained from regularly tapped rubber trees (*Hevea brasiliensis*, clone RRIM 600) under age of 20 years old, grown at the adjoining Songkla Rubber Research Center, Hat-yai, Songkhla, Thailand. These trees were tapped every other day at 7.00 a.m., in a half-spiral fashion with V-shape knife by stripping the bark (2-3 mm thick) to make cuts across the latex vessels. All preparations for fresh latex fractionation were made ready beforehand prior to the latex collection. The latex was collected in ice-chilled beakers and was immediately subjected to centrifugation within less than 20 min from the tapping collection time. As such, the fractionated fresh latex was almost similar to the *in situ* latex with minimum rupture or loss of the bottom fraction particles.

1.2 Preparation of washed bottom-fraction particles (WBP)

The chilled freshly tapped latex was immediately filtered through 4 layers of cheesecloth to remove the particulate materials and bark tissue debris. Without delay, the filtrate was fractionated by centrifugation (Beckman, model JA2-21) at 5,000 X g for 30 minutes at 10°C to obtain the three distinct fractions depend on different density of compounds consisting in the latex as shown in Figure 4. A white creamy

phase at the top was rubber layer, the middle clear aqueous latex cytosol was C-serum and the maximum sediment bottom fraction (BF) with yellowish creamy character consist of membrane bound organelles, lutoid and Frey- Wyssling particles.

The collected BF was immediately washed according to the previous reports (Rattanapittayapron, 1998; Wititsuwannakul *et al.*, 2003). The BF was washed five times by careful suspension in 50 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl (w/v) so that the intact WBP was obtained with no small rubber particle (RP) was present. All operations were carried out at 0-5°C. The washing procedure is shown in Figure 5. The amount of WBP got after the washing step was about 10% of fresh latex used. The intact WBP as prepared was kept in ice-bath and an aliquot was immediately used for the preparation of washed bottom-fraction particle membrane (WBM) for the rubber biosynthesis assays.

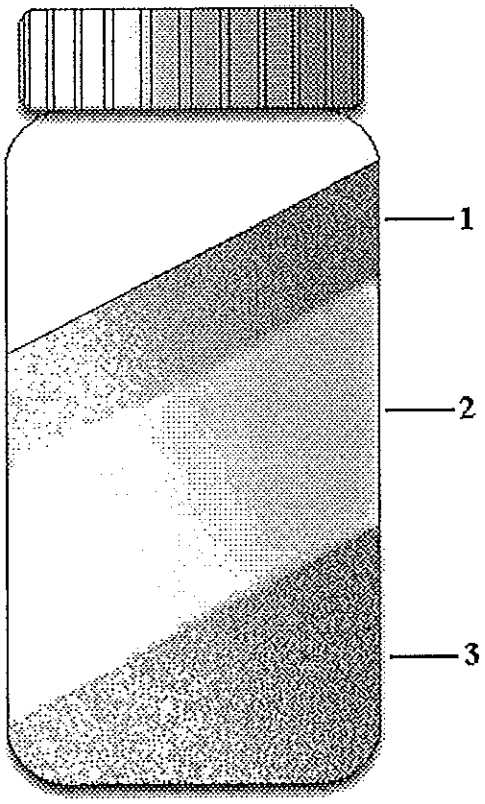


Figure 4 The centrifuged fresh latex (5,000 x g from centrifuge model JA2-21, Beckman). 1: top zone consist of white and creamy rubber layer), 2: aqueous middle zone containing C-serum and 3: yellowish at the bottom of the bottle called "Bottom fraction (BF)" containing membrane bound organelles, lutoid and Frey-Wyssling particles.

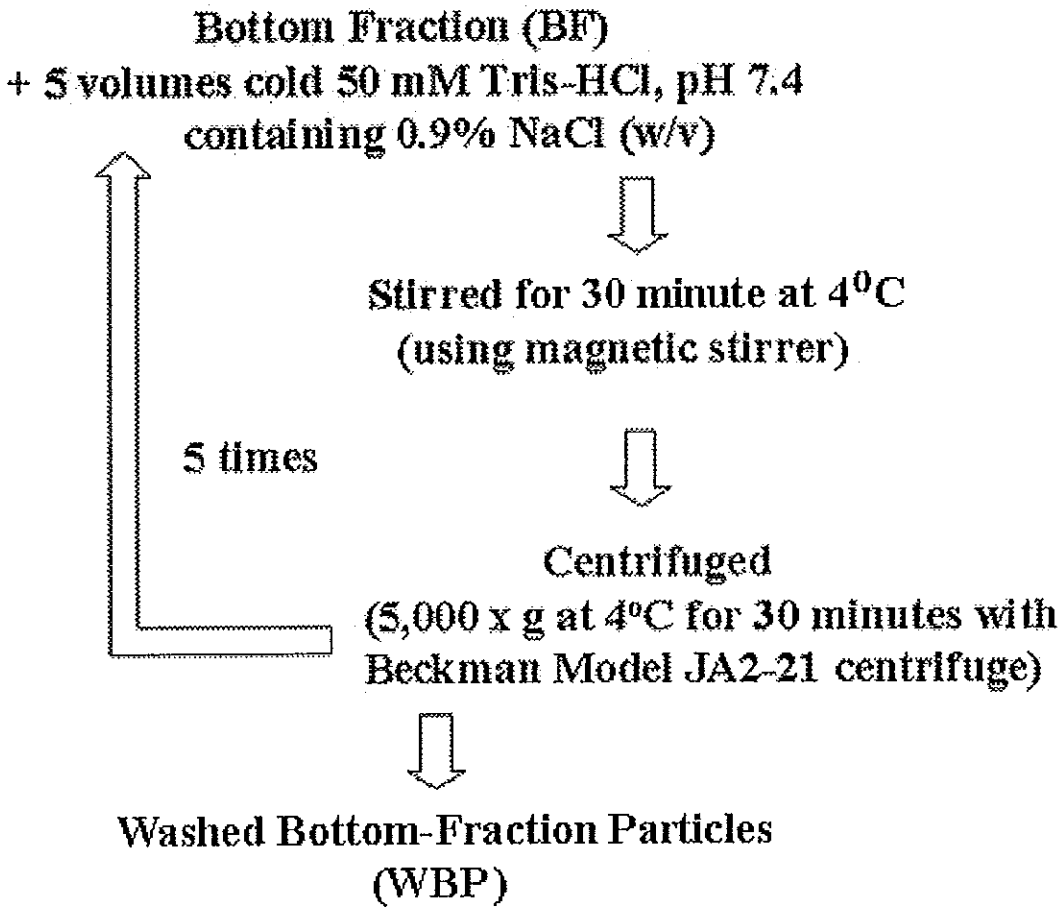


Figure 5 Summarized diagram for wash bottom-fraction particles (WBP) preparation.

1.3 Preparation of washed bottom-fraction particle membrane (WBM)

Washed bottom-fraction particle membrane (WBM) was prepared from the intact WBP as described (Rattanapittayapron, 1998; Wititsuwannakul *et al.*, 2003). The cleaned WBP pellet was suspended in 3 volumes of distilled water and stirred for hypotonic lysis of WBP. The membrane was prepared as a clean sediment fraction by centrifugation and three times repeated washing to obtain WBM cleansed of any contamination. The washing step was carried out under the same method as that used for the WBP preparation. The complete WBM preparation procedure is show in Figure 6. All operations were carried out at 0-5°C for membrane integrity and stability. The WBM was kept in an ice-bath until used.

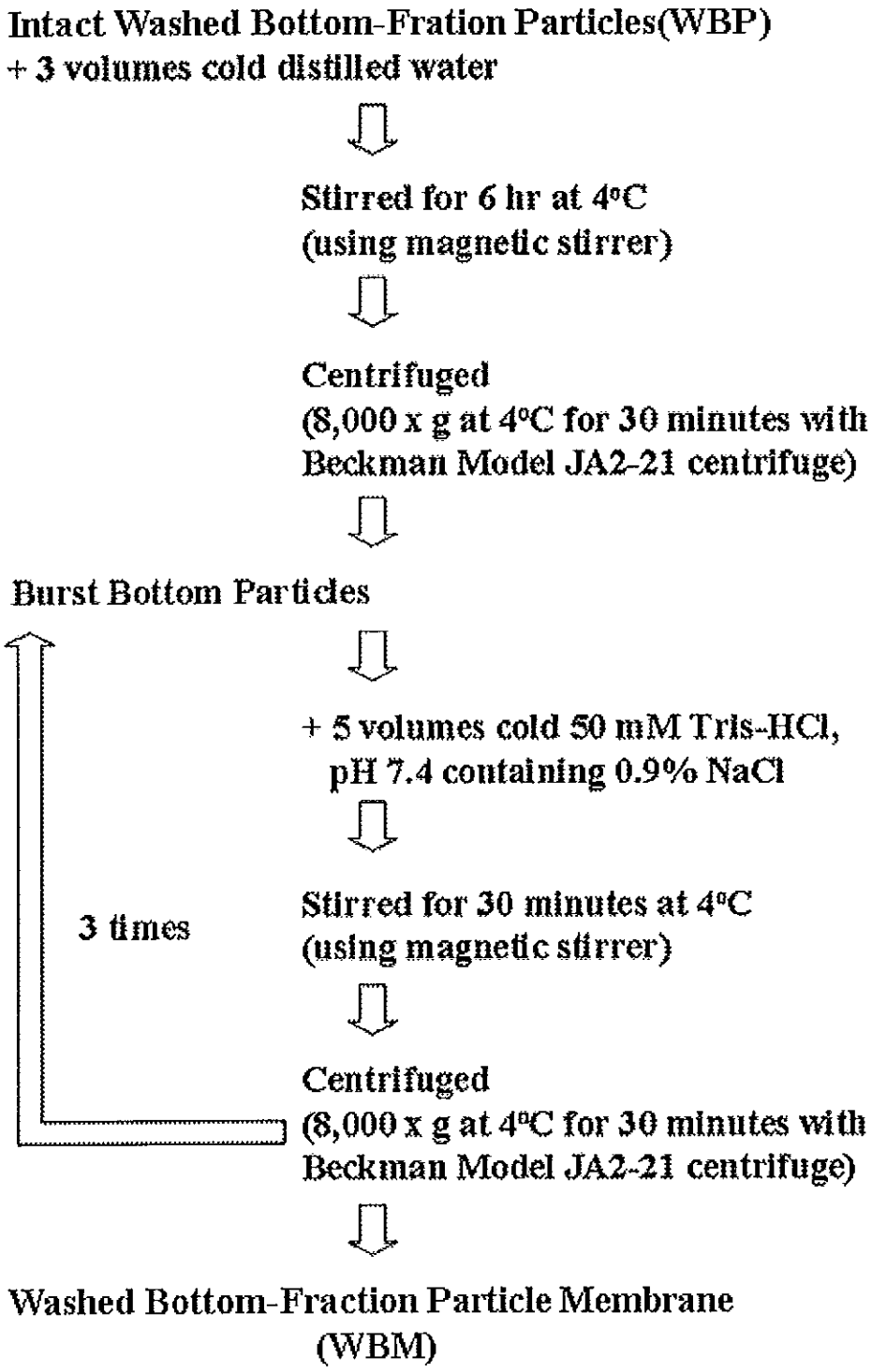


Figure 6.. Summarized diagram for washed bottom membrane (WBM) preparation.

1.4 Serial acetone fractionation of WBM proteins

The WBM was subjected to serial acetone dissolution and precipitation of proteins at the ranges of 0-20, 20-40, 40-60 and 60-80% saturation (Scopes, 1994). The entire serial acetone fractionation is shown in Figure 7 and the amount of acetone to be added to give the desired final concentration according to Table 1. The acetone precipitated protein from each step was collected by centrifugation at 10,000 x g (Beckman Model Avanti™ 30 centrifuge), dried up under N_2 gas as powdered protein and stored at -20°C until used. The WBM protein concentration was determined by Protein Assay reagent (Bio-Rad) according to Bradford assay (Bradford, 1976).

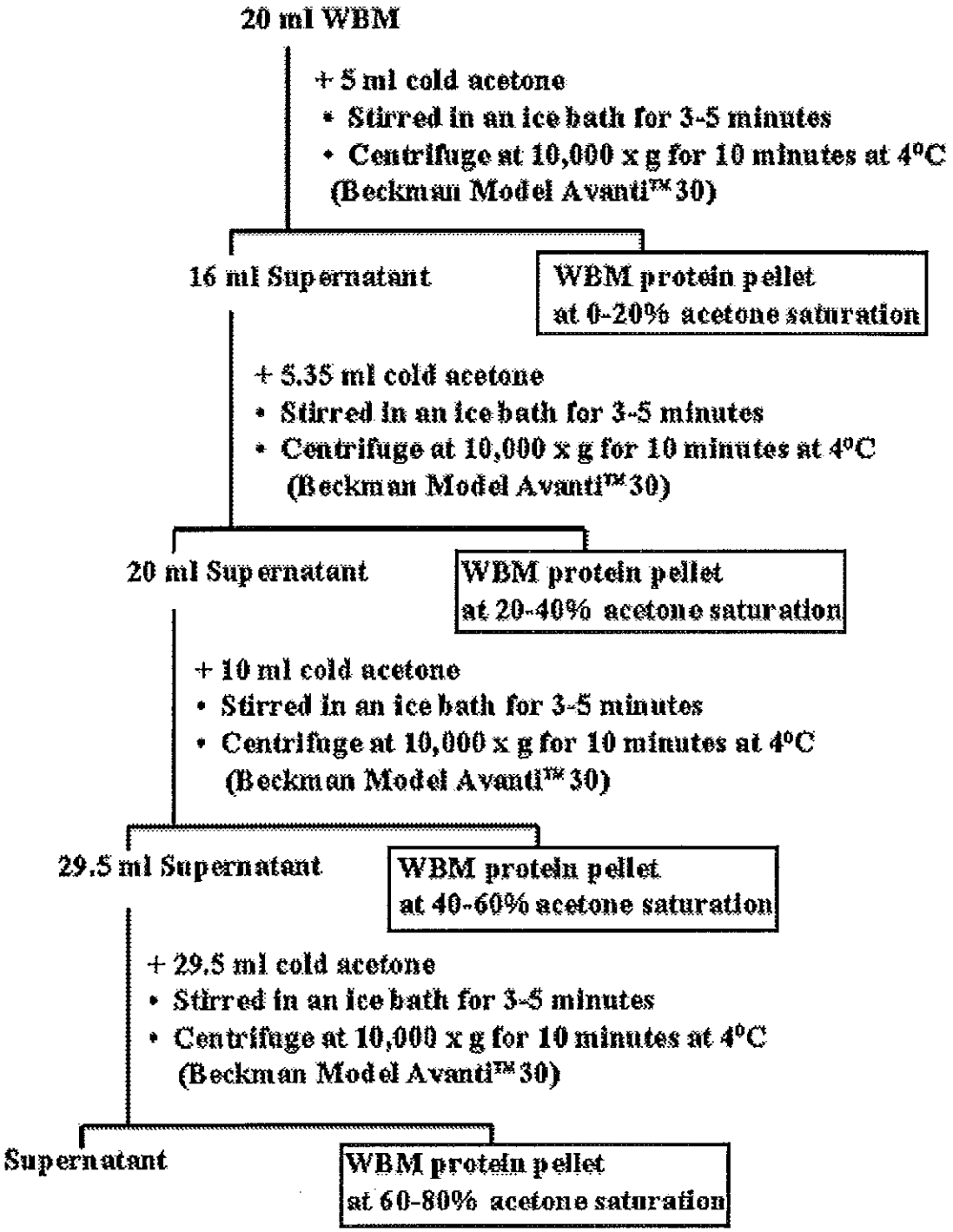


Figure 7 Summarized diagram for serial acetone fractionation of WBM proteins.

Table 1 Volume of miscible solvent, ml to be added to 1 liter (Scopes, 1994)

From C_1% \ To C_2% →	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	52	111	176	250	333	428	538	666	818	1,000	1,222	1,500	1,857	2,333	3,000	4,000	5,666	9,000	19,000
5		55	117	187	266	357	461	583	727	900	1,111	1,375	1,714	2,166	2,800	3,750	5,333	8,500	18,000
10			58	125	200	285	384	500	636	800	1,000	1,250	1,571	2,000	2,600	3,500	5,000	8,000	17,000
15				62	133	214	307	416	545	700	888	1,125	1,428	1,833	2,400	3,250	4,666	7,500	16,000
20					66	142	230	333	454	600	777	1,000	1,285	1,666	2,200	3,000	4,333	7,000	15,000
25						71	153	250	363	500	666	875	1,142	1,500	2,000	2,750	4,000	6,500	14,000
30							76	166	272	400	555	750	1,000	1,333	1,860	2,500	3,666	6,000	13,000
35								83	181	300	444	625	857	1,166	1,600	2,250	3,333	5,500	12,000
40									90	200	333	500	714	1,000	1,400	2,000	3,000	5,000	11,000
45										100	222	375	571	833	1,200	1,750	2,666	4,500	10,000
50											111	250	428	666	1,000	1,500	2,333	4,000	9,000
55												125	285	500	800	1,250	2,000	3,500	8,000
60													142	333	600	1,000	1,666	3,000	7,000
65														166	400	750	1,333	2,500	6,000
70															200	500	1,000	2,000	5,000
75																250	666	1,500	4,000
80																	333	1,000	3,000
85																		500	2,000
90																			1,000

1.5 Preparation of washed rubber particles (WRP)

Rubber particles preparation using the chilled fresh latex from 1.1 subjected to ultracentrifugation (Beckman model L8-70M) at 45,000 x g at 10°C for 45 minutes. The ultracentrifuged latex was separated into four distinct layers depend on different density of compound containing in the latex as show in Figure 8. The top layer was a white creamy layer of rubber, the yellow orange layer underneath was Frey-Wyssling membrane debris, the middle layer was a clear solution called C-serum and the pellet was bottom fraction which mainly comprised with membrane bound particles.

The rubber layer was collected by carefully scooped out from the tube without disturbing its original attribution in the centrifuge tube. By a spatula, Zone2 rubber (Moir, 1959) was carefully collected from the rubber surface which was used to contact with C-serum phase. The collected Zone2 rubber was immediately immersed in 50 mM Tris-HCl (pH 7.4) to avoid the rubber aggregation and became dry. The WBM for assays was obtained by repeated washing three times with 5 volumes of the same buffer. All operations were carried out at 0-5°C. The prepared WRP was kept cool in icebox until used. The rubber quantity was determined by measuring the absorbance at 280 nm of a dilute sample (1:1,000). The absorbance is dominated by the light scattering of the rubber particles in the sample of relatively low rubber content < 1% of the light is absorbed by protein. The conversion ratio was determined to be 0.04 + 0.002 mg of rubber/A_{280} (Light and Dennis, 1998).

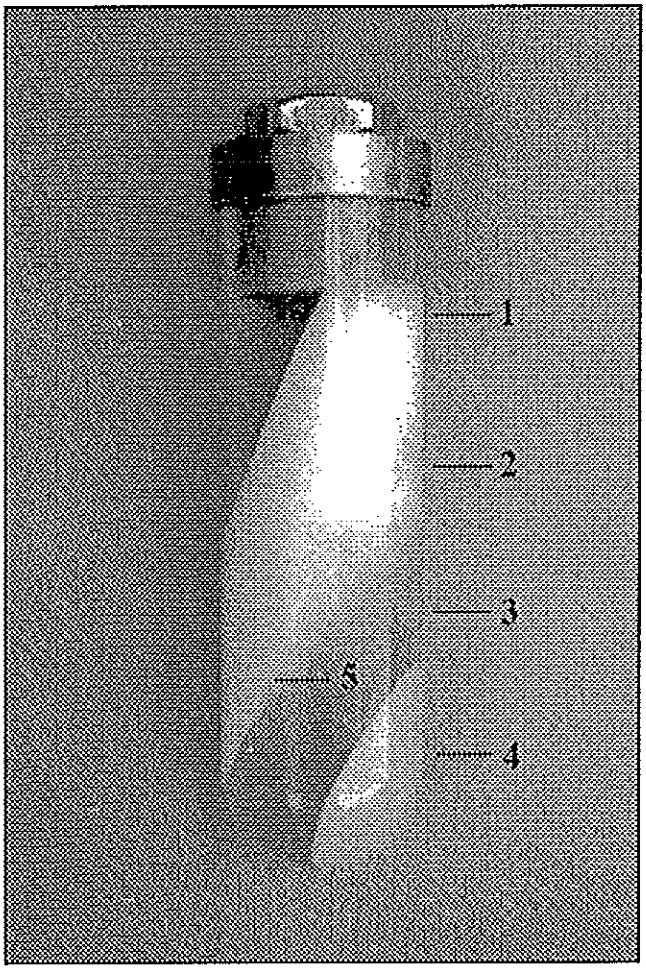


Figure 8 Ultracentrifuged fresh latex (45,000 x g from centrifuge model L8-70M, Beckman). 1: top layer consist of white and creamy rubber layer, 2: the yellow orange layer which was Frey-Wyssling membrane debris, 3: a clear solution called C-serum, 4: yellowish bottom fraction, and 5: zone 2 rubber.

2 Preparation of ^{14}C-labeled undecaprenyl diphosphate (^{14}C-UPP)

The 200 µl final volume incubation mixture contained 0.5 mM $MgCl_2$, 0.05% Triton X-100, 165 µM FPP, 165 µM [1-^{14}C] IDP (25Ci/mol) and 33 µl of undecaprenyl diphosphate synthase (UPS, 178 µg protein ml^{-1}) in 50 mM Tris-HCL buffer (pH 7.4). The reaction was carried out in a water bath at 37°C for 2 hr and chill stopped an icebox. The 200 µl saturated NaCl solution was then added, mixed thoroughly and treated twice with 400 µl 1-butanol in order to extract the radiolabeled product, composed mainly of ^{14}C-UPP. The butanol phase was separated and dried under vacuum at 35°C. Ammonia water was added to the desired concentration and the solution was kept at –20°C until used (Shimizu et al., 1998).

3 Rubber biosynthesis assays and incubation conditions

3.1 RB assay using ^{14}C-IPP as radiolabeled substrate

The incubation mixture contained designated amount of WBM or acetone extracted WBM protein or WRP in Tris-HCl buffer (pH 7.7) as indicated in the captions for Figure and Table. The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.7), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT, detergent (2% SDS or 40 mM DOC) and 40 µM [1-^{14}C] IPP (2.5-5ci/mol) as indicated in the captions for Figure and Table. In the case of SDS added, heat-preincubation treatment was carried out after mix the WBM with SDS, the other reagents were added after let the it cooled down to room temperature for 15-20 minutes. All the RB incubation mixtures were

carried out at 37°C for 6 hr, unless indicated otherwise. 20 mM EDTA was added in the incubations as controls. After 37°C optimum incubations, the reaction was chill stopped by placing the incubation tubes in an icebox and was immediately processed for the ^{14}C-labeled rubber extraction and the RB activity determinations.

3.2 RB assay using ^{14}C-UPP as radiolabeled initiator

The incubation mixture contained designated amount of WBM or WRP in Tris-HCl buffer (pH 7.7) as indicated in the captions for Figure and Table. The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.7), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT, detergent (2% SDS or 40 mM DOC), 40- 60 μM IPP and ^{14}C-UPP (amount as indicated in the captions for Figure and Table). In the case of SDS added, heat-preincubation treatment was carried out after mix the WBM with SDS, the other reagents were added after let the it cooled down to room temperature for 15-20 minutes. The RB incubation mixtures were carried out at 37°C for 2-6 hr as indicated. 20 mM EDTA was added in the incubations as controls. After the optimum incubations, the reaction was then chill stopped by placing the incubation tubes in an icebox and was immediately processed for the ^{14}C-labeled rubber extraction and the RB activity determinations.

3.3 RB assay using ^{3}H-FPP or ^{3}H-GGPP as radiolabeled initiator

The incubation mixtures contained designated amount of samples (WBM or WRP) in the reaction buffer (50 mM Tris-HCl buffer, pH 7.7 with 40 mM DOC, 30

mM KF, 5 mM $MgCl_2$, and 10 mM DTT) used in the standard assays. The substrate (unlabeled IPP) and allylic initiators (^{3}H-FPP or ^{3}H-GGPP) were added as indicated in the figure captions. 20 mM EDTA was added in the incubations as controls. The RB incubations were carried out at 30°C for 2 h, unless indicated otherwise. At the end of incubation, the reaction mixtures were chill stopped by placing the incubation tubes in icebox. They were then immediately processed for the radiolabeled products extraction and products analyses.

3.4 Incubation of WBM-derived ^{14}C-UPP rubber with WRP

The purified labeled rubber (^{14}C-rubber) obtained from WBM incubation with ^{14}C-UPP as described above was dried up in vacuo at room temperature and used for the following further RB assay. WRP (approx. 2 mg rubber) in 200 µl reaction buffer (40 mM DOC, 5 mM $MgCl_2$, and 10 mM DTT, in 50 mM Tris-HCl buffer, pH 7.7) was added 1 mM IPP and mixed well with the prepared pure ^{14}C-UPP labeled rubber (37,650 cpm as prepared) as a homogenous reaction mixture. The reaction mixtures were incubated at 37°C for 4 h. The incubations were then chill stopped by placing the tubes in icebox and immediately processed for the radiolabeled products extraction and product analyses as described above.

4 Radio labeled product extraction

4.1 Extraction of radio labeled rubber (standard method)

The labeled rubber product was extracted by precipitation of the rubber out from the incubation mixtures. Right after stopped the reaction, 1.2 ml cold ethanol was then added into the incubation mixtures and immediately separated by centrifugation at 5,000 x g for 10 min. The WRP prepared as described was added as the carrier to all every incubation tube before the precipitation step by cold ethanol addition. This step was taken for quantitative recovery of the labeled rubber product as only little amount of the product was formed and might not be completely self aggregated to sediment out by ethanol addition. The WRP addition as carrier was without any effect on the RB activity of the samples and greatly improved the quantitative product recovery. The rubber pellets were extracted and purified repeatedly 3 times using 1 ml solvent mixture of toluene and hexane (1:1, v/v). The solvent mixture was evaporated at room temperature in the fume hood to a small volume. The extracted soluble rubber was then further purified by precipitation twice with 1.2 ml cold acetone as described previously (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003). The amount of radioactivity in purified rubber was determined for the RB activity by determining the ^{14}C-labeled rubber with liquid scintillation counter.

4.2 Two-steps differential solvents extractions of radiolabeled products

After stopping the RB incubation assays, 300 µl saturated NaCl solution was added and mixed thoroughly. They were then subjected to two step differential solvents extractions of the products. The mixture was first treated (three times) with 500 µl H_2O saturated 1-butanol. The labeled products in 1-butanol phase were monitored by a liquid scintillation counter to estimate the amount of radiolabeled oligo- and polyprenyl intermediates. The aqueous layer (with white fluffy materials at the interphase) left after 1-butanol extraction was then treated (three times) with 500 µl of toluene/hexane (1:1, v/v) solvent system. The amount of labeled products in toluene/hexane phase was determined for the RB activity by measure the radioactivity of ^{14}C-labeled rubber with liquid scintillation counter. The 1-butanol and toluene/hexane extracts product analyses were then performed on RP-TLC as described (Ohnuma *et al.*, 1998) and detailed below.

4.3 Purification of Radiolabeled Rubber Products

The toluene/hexane extracts as derived from the two-steps differential solvent extractions described above were quantitatively collected and concentrated to a small volume. The purification procedure started by addition of five volumes of cold acetone into the concentrated toluene/hexane extract and thoroughly mixed well. The white rubber pellets obtained from this step were collected and dissolved in 150 µ l toluene. The completely and thoroughly dissolved rubber solution was then recrystallized with 750 µl cold acetone to obtained purified white rubber pellet. The rubber crystallized and recrystallization steps were repeated at least three times to

ascertained the purity and that all contaminants were completely removed (Tangpakdee *et al.*, 1997b)

5 Product radioactivity determination

5.1 Radioactivity determination of butanol extract

As the water saturated 1-butanal was used in the extraction process of oligo- and polyprenyl product. The polarity index of this solution is somewhat different from the conventional Toluene-base scintillation cocktail, resulting an immiscible solution which was not agree with the rule of liquid scintillation counter. So, the cocktail mixed with this sample need to be an appropriate one, the Triton-base scintillation cocktail was the choice. The suitable ratio between sample and cocktail was 1:10 which was used for all the butanol extract samples throughout the study. The recipe to the cocktail is as follow:

- Toluene 50% (v/v)
- Triton X-100 50% (v/v)
- PPO 5 gm/l
- POPOP 0.3 gm/l

5.2 Radiactivity determination of toluene/hexane extract

The radioactivity of RB product in toluene/hexane extract was analyzed by liquid scintillation counter. The extract was mixed thoroughly with Toluene-base cocktail in the satisfy ratio of 1:7. The recipe for Toluene-base scintillation cocktail is showed below:

- Toluene 1 l
- PPO 5 gm
- POPOP 0.3 gm

6 Product analysis

6.1 Product analysis by reverse phase thin layer chromatography (RP-TLC).

The radiolabeled products from the incubation mixtures extracted in 1-butanol and toluene/hexane were hydrolyzed to the corresponding alcohols with potato acid phosphatase (Koyama *et al.*, 1985). The products were applied to the RP-TLC plate (LKC-18, Merck) using acetone/hexane (19:1, v/v) as the developing solvent system (Ohnuma *et al.*, 1988). The RP-TLC plates were then exposed overnight on Fuji film BAS-III imaging plate at room temperature. The separation profiles of radiolabeled products were visualized with bioimage analyzer (Fuji BAS 100 Mac). The product molecular weight sizes were determined with the authentic standard alcohols that were run along with the samples and visualized with iodine

vapor. The radiolabeled rubber confined or remained at the origin spots were completely scraped out (quantitatively) and further extracted with toluene/hexane (1:1, v/v). The labeled rubber products were then further purified (Tangpakdee *et al.*, 1997b) as described above. The amounts of radioactivity of the extracted labeled rubber were detected and quantified by liquid scintillation counter.

6.2 Molecular weight distribution (MWD) determination of the ^{14}C-rubber

Clear solutions of purified labeled rubber in tetrahydorfuran (THF) were subjected to gel permeation chromatography (GPC). They were carried out with TSK gel (Tosho) high performance liquid chromatography columns connected in tandem series of four exclusion limit (1.01×10^6, 1.72×10^5, 1.13×10^4 and 1.30×10^3), respectively. The chromatography was carried out at 35°C using THF as an eluent at a flow rate 0.5 ml/min. Absorbance at λ210 nm was used to detect the polyisoprenes in the eluent. The eluent was collected every one minute interval, and the amount of radioactivity in each fraction was determined by liquid scintillation counter. The molecular weights of polyisoprenes and rubber were determined by comparing with the elution profile of the samples with the molecular mass distribution profiles of authentic standard polystyrenes used as MW markers.

7 Protein determination

7.1 Protein assay by Lowry's method

Protein concentration was determined according to the method of Lowry's (Lowry *et al.*, 1951) using a bovine serum albumin calibration curve (20-80 μg). A protein sample (0.1 ml) was mixed with 3 ml of freshly prepared alkaline copper reagent [0.5% copper sulfate / 2% potassium-sodium tartrate / 2% sodium carbonate in 0.1 M NaOH, 1:1:100 (v/v)]. The reaction mixture was allowed to stand at room temperature for 10 min, and than 0.3 ml of 1 M Folin-Ciocaulteu's phenol reagent was added. The mixture was vigorously mixed and left at room temperature for 30 min. The absorbance of the solution was then measured at λ 650 nm by spectrophotometer.

7.2 Protein assay by Bradford's method

In order to avoid the interference from Tris buffer, proteins were determined by using commercial Protein Assay reagent (from Bio-Rad). The principle of this reagent is according to the modified Bradford's method (Bradford, 1976). The analyzing procedures were according to the product leaflet.

7.3 Protein molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the modified method of Laemmli (Laemmli, 1970). The separation slab gel (10 x 8 x 0.1 cm) was 12 % or linear gradient fashion from 7 - 15% of acrylamide concentration with the stacking gel above. The acrylamide gel preparation recipe was according to Table 2. The protein samples were mixed with sample buffer [with final concentration of 62.7 mM Tris-HCl buffer (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 0.0005% (w/v) bromophenolblue as the dye, heated for 10min in boiling water and spun down protein solution for 5 min and loaded into the well. The electrophoresis was performed at room temperature with the anode in the lower chamber, 20 mA constant current per slab gel was applied until the tracking dye approached the bottom of the separating gel. [Electrophoresis buffer contained 25 mM Tris-HCl (pH 8.3)] The electrophoresis gel was then fixed and stain with the staining solution [0.2% (w/v) Coomassie brilliant blue R-25, 50% (v/v) methanol and 10% (v/v) acetic acid] for 2 h. Destaining of excess dye was performed by repeated changing of destaining solution [20% (v/v) methanol and 10% (v/v) acetic acid] until the background was clear.

Table 2 Recipes for polyacrylamide separating and stacking gels

Stock solutions (µl)	Stacking gel	Linear gradient of separating gel		
	3%	7%	12%	15%
30%acrylamide+0.8%bisacrylamide	300	700	1,200	1,500
1.5 M Tris-HCl, pH 8.9	-	750	750	750
0.5 M Tris-HCl, pH 6.8	750	-	-	-
10% SDS	30	30	30	30
Distilled water	1,745	1,440	940	640
0.2 M EDTA, pH 7.0	20	-	-	-
*1% ammonium persulfate	150	75	75	75
*TEMED	5	5	5	5
Total Volume	3,000	3,000	3,000	3,000

*Added to chemically the gel within 10 min

Chapter 3

Results

Part 1. The stability of intact fresh latex

1.1 Osmotic sensitivity of the fractionated latex BF particles

The study on time course of the fresh latex fractions shown in Figure 9 indicated that the sediment BF was quite unstable if not quickly separated from other fractions. Centrifugation of fresh latex right after tapping showed that BF of high contents sediment as intact particles (tube A, time 0). After standing for 45 min at room temperature, the isolated intact BF started bursting (tube B, 45 min), and releasing B-serum content together with membrane debris into the clear aqueous C-serum as a turbid phase. After 1 hr (tube C), the C-serum became clearer as the membrane debris started binding onto the rubber phase, while C-serum was mixed with the released B-serum. After 1.5 - 2.0 hr (tube D and E), only two fractions remained as rubber phase (tube E). This could probably be a hydrophobic interaction with high binding affinity to the rubber particles (RP).

The study was repeated several times on different fresh latex samples with no added preservative. The average bursting time of the isolated bottom fraction was around 40-50 min. The result thus clearly showed that the prolonged storage or

delayed use of fresh latex would yield only two fractions due to loss of the ruptured BF organelles. Therefore, a standard procedure was adopted for our experiments: to use only the RP and BF as obtained from tube A (Figure 9) for all RB assay.

1.2 Comparison of RP proteins under different conditions

Analyses on the RP associated proteins of different RP samples and WBM proteins were carried out in this study. This is to discern protein patterns comparing the RP rapidly separated from the freshly tapped latex and BF diminished RP samples (Figure 9, tube A and E) and the preserved latex RP commonly used in RB study by others. (Archer & Audley, 1987; Audley & Archer, 1988; Kekwick, 1989; Kush, 1994; Tanaka *et al.*, 1996) The SDS-PAGE results from Figure 10 revealed quite different protein patterns. The fresh RP (lane A) showed mainly two major proteins of 14 and 24 kDa with a few very faint protein bands. These two major proteins might be considered as intrinsic proteins of the RP. On the other hand, the RP from tube E (lane E) showed several other prominent protein bands in addition to the two intrinsic ones (lane A). Comparison of the two samples (the RP from tube A and tube E) clearly suggested that the extra protein bands (lane E) were likely from the tightly bound BF membrane, even though both were extensively washed before protein analyses. The protein pattern (lane E) was similar to the result of the preserved latex RP protein profile as previously shown (Hasma, 1987).

Examination of the additional bands revealed similar and common to some proteins extractable from the BF membrane (lane M). The results thus suggested that these RP extra proteins, probably derived from BF membrane fragment might help

contribute RB activity to the RP. The preserved RP commonly used, as reviewed in the RB study (Archer & Audley, 1987) might thus be attributed to the proteins or enzymes from the BF membrane. This observation might be used to distinguish the intrinsic RB activity of the RP, if ever its designated function, from RB activity of the washed bottom-fraction particle membrane (WBM). A comparison between the fresh RP and WBM on the relative RB activity would certainly be critical to support this postulation and to an understanding of the actual biological function of the RP in *Hevea* latex.

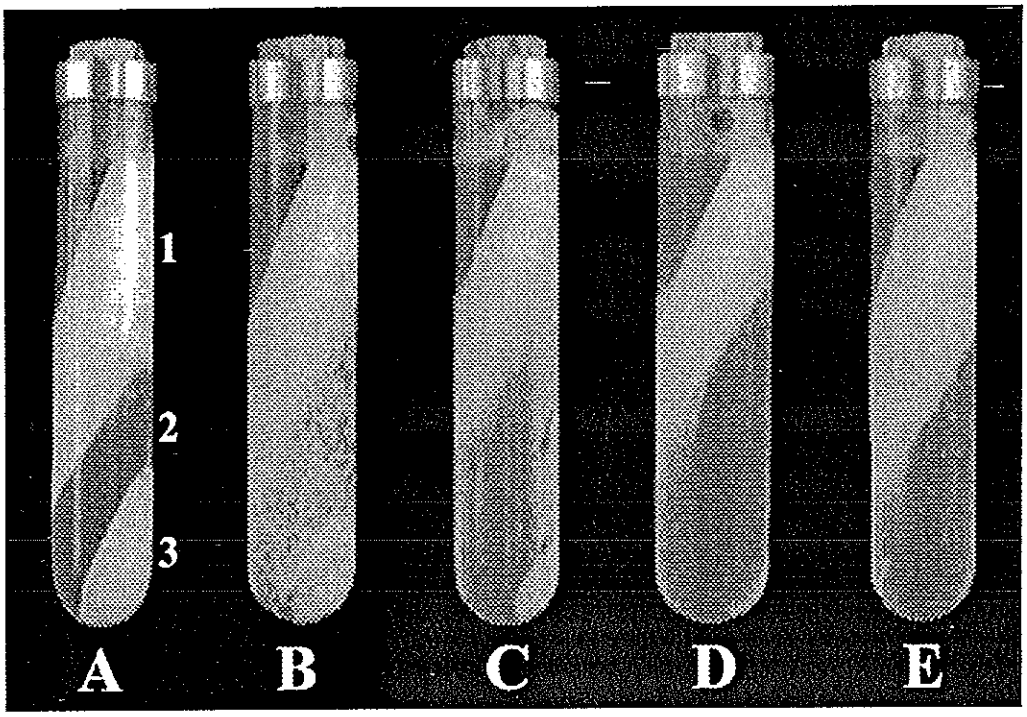


Figure 9 Fractionation by centrifugation of fresh *Hevea* latex (45,000 x g from centrifuge model L8-70M, Beckman). A: freshly tapped latex (0 time); B: after standing at room temperature for 45 min; C: 60 min; D: 90 min; E: 120 min.

(1: rubber phase; 2: C-serum; 3: bottom fraction)

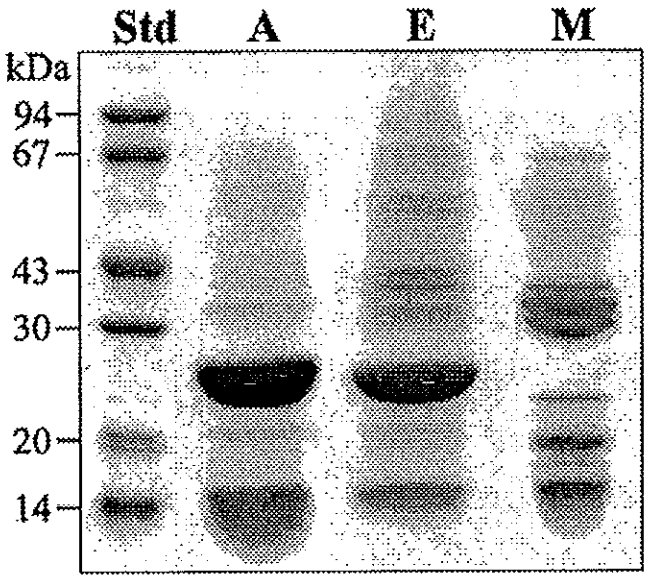


Figure 10 SDS-PAGE analyses of proteins extracted from different fractions of *Hevea* latex. A: proteins from RP of freshly tapped latex (Figure9, tube A); E: proteins from RP of the delayed use latex (Figure 9, tube E); M: proteins from WBM prepared from freshly tapped latex (Figure 9, tube A); Std: standard molecular weight proteins.

Part 2. Rubber biosynthesis by *Hevea* latex bottom-fraction particles and membrane

2.1 Incorporation of ^{14}C-IPP into rubber by the WBP and WBM

The incorporation of ^{14}C-IPP into rubber by the BF of centrifuged fresh latex was recently reported (Tankpakdee *et al.*, 1997b). This previous finding prompted us to investigate detail the role of BF particles in the RB process. The earlier study was carried out with unwashed and intact membrane bound BF particles and the newly formed rubber being analyzed (Tankpakdee *et al.*, 1997c). In this study, the BF particles were thoroughly cleaned and purified as intact WBP by repeated washing with an isotonic buffer. The WBM was prepared by the hypotonic lysis of WBP and again underwent repeated washings for the RB experiments. A comparison of the effects of the washed particles and the derived washed membrane on the RB activity was made.

Results shown in Figure 11 demonstrate the different rates of ^{14}C-IPP incorporation into rubber by the WBP at various ^{14}C-IPP concentrations. The experimental conditions were applied from those used for the WRP (Audley & Archer, 1998). The finding indicated the active role of latex non-rubber constituents, BF particles and the derived membrane in rubber formation. The membrane activity was about two times greater than that of the WBP. This result suggested that the WBM had higher RB activity than WRP and the membrane environment was probably a preferable condition for RB activity.

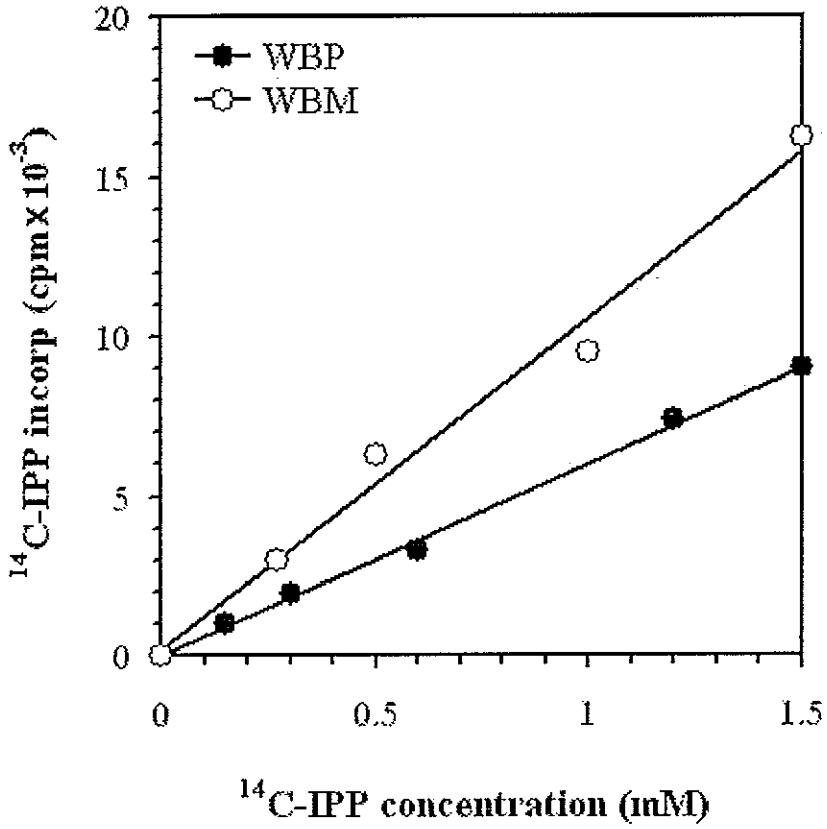


Figure 11 RB by the WBP and WBM in the presence of various concentration of [1-^{14}C] IPP. The RB incubation assay contained either a 5-gram (wet weight) of the WBP or the derived membrane WBM suspended in a 50 mM Tris-HCl buffer (pH 7.7), and the reaction was started by the addition of the substrate IPP. Reaction mixtures with 10 mM DTT and 5 mM $MgCl_2$ were added to [1-^{14}C] IPP at the concentrations indicated. The incubation assay was carried out in a shaking water bath at 37°C for 6 hr.

2.2 Comparison of the kinetics of the RB catalytic rate between WBP and WBM

The RB activity between the intact washed BF particles (WBP) and the derived membrane (WBM) was compared for the catalytic rate. The assays were to determine the saturation time point on rubber synthesis. The results in Figure 12 showed a large difference in their kinetics for RB activity. It took WBP 12 hr to reach maximum activity, but only 2 hr for WBM. This indicated that the WBM was six times more active than WBP. The results point out two important aspects: (1) that the BF membrane was fully equipped with all necessary enzymes to complete carrying out the RB function and (2) that the B-serum did not play an active role in the RB catalysis. The fast kinetic of the WBM could be attributed to the formation of small vesicles with a highly increased active surface area. The enzyme would be more accessible to the substrates as earlier described (Baba & Allen, 1980, Cullis & Hope, 1985). This was in spite of a certain loss from washing the membrane derived from an equal amount of WBP as used in the study. WBM was then used in the further study of the membrane-bound enzyme properties. The results in Figure 11 combined with this supporting kinetic study, indicated that it was an enzyme-catalyzed process being tested and verified in the following experiments on the WBM.

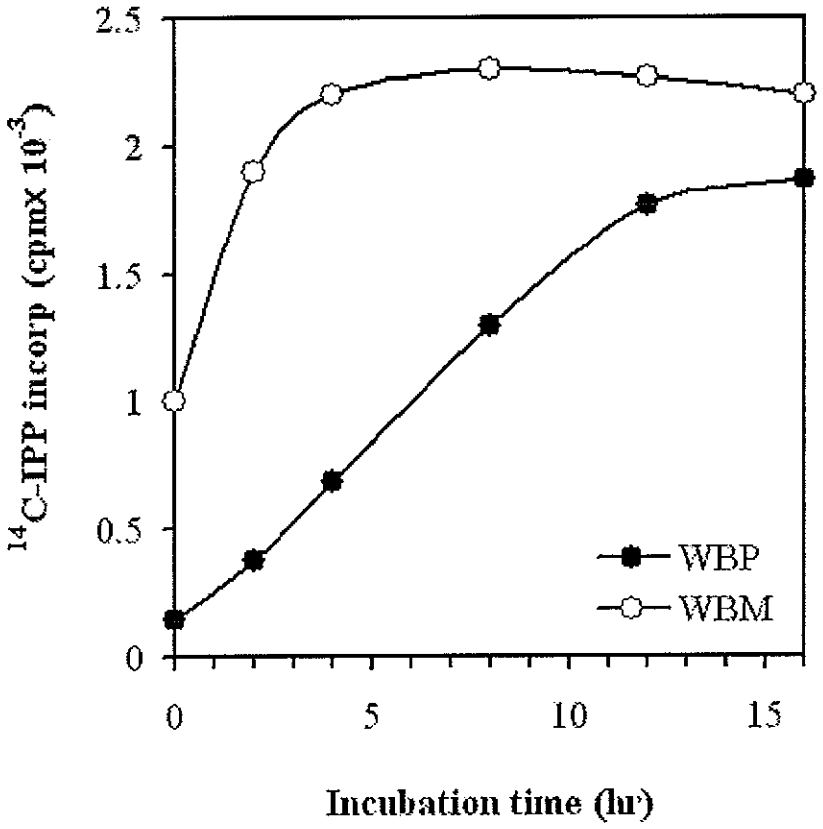


Figure 12 Kinetic of RB catalytic rate between WBP and WBM. Each incubation contained WBP (approx. 20 mg dried weight) or WBM which was prepared from the same amount of WBP. The incubation assays contained WBP or WBM suspended in a 50 mM Tris-HCl buffer (pH 7.7) with 10 mM DTT and 5 mM $MgCl_2$. The reaction was started by the addition of substrate IPP and the incubation assay was carried out in a shaking water bath at 37°C.

2.3 Effect of the chelators on the RB activity of the WBM

The buffers for both WBP and WBM assays on the RB activity contained Mg^{2+} as reported in earlier studies (Tangpakdee *et al.*, 1997b). In these WBM assays, the addition of chelating agents (EDTA and EGTA) to the incubation showed strong inhibition of rubber formation by the WBM. Figure 13 shows a sharp drop in the RB activity was observed with 2 mM EDTA, with almost complete inhibition. These results agree well with the general requirements of divalent cations for the cleavage of phosphate esters by prenyl transferase enzymes required for RB (Archer & Audley, 1987). When both chelators were present together, the degree of inhibition was not much different than that with EDTA alone. The results indicated that Mg^{2+} was an essential cofactor for the RB enzymes of the WBM, as observed for the WRP assay (Audley & Archer, 1988). The chelator inhibition of the WBM activity on rubber formation suggested that the WBM enzymes might be similar to those reported for the WRP surface. It should also be noted that the slightly higher inhibition level from the combined EDTA and EGTA might suggest a possible bound Ca^{2+} effect on WBM functions, but these remains to be further proved and elucidated.

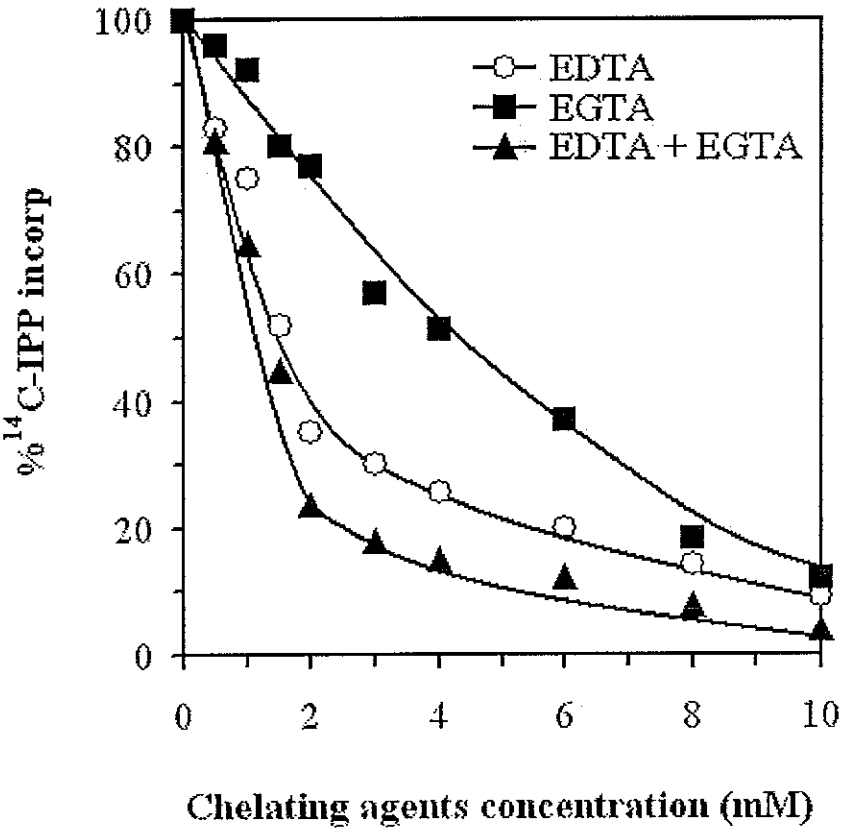


Figure 13 Effect of the chelating agents EDTA and EGTA on the RB activity of the WBM. The conditions for the RB assays were as described in the Methods section. The incorporation of [1-^{14}C] IPP into the rubber was determined for the chelator effects. The 3 ml incubation mixtures contained 2.5 gram (wet weight) of the WBM in a 50 mM Tris-HCl buffer (pH 7.7), and EDTA, EGTA, or both were added at the concentrations specified. The incubation was performed in a shaking water bath at 37°C for 1 hr.

2.4 Effect of the detergents on the RB activity of the WBM

The effects of different detergents on the WBM for RB activity were determined and shown in Figure 14. When detergents were included in the RB assay of the WBM, a significant stimulation of ^{14}C-IPP incorporation into rubber was obtained with only SDS, an anionic detergent. The nonionic detergents, Triton X-100 and Tween 20, showed no effect on RB. The level of RB stimulation increased with increasing SDS concentrations above the critical micelle concentration (cmc). A three-fold activation was detected at 5% SDS, whereas no effect was observed for non-ionic detergents. The lipids of the WBM are rich in phosphatidic acid and saturated fatty acry residues (Dupont *et al.*, 1976; d'Auzac & Jacob, 1989). SDS, being anionic in nature, might be miscible or compatible with the negatively charged membrane, having a positive effect on the RB activity. The presence of soluble amphiphiles such as SDS may alter the physical state of WBM lipids and/or induce the formation of newly mixed micelles with increased surface area. These mixed micelles were incorporated with necessary factors and enzymes required for RB. All these events might possible lead to the increased *in vitro* RB capacity of the WBM.

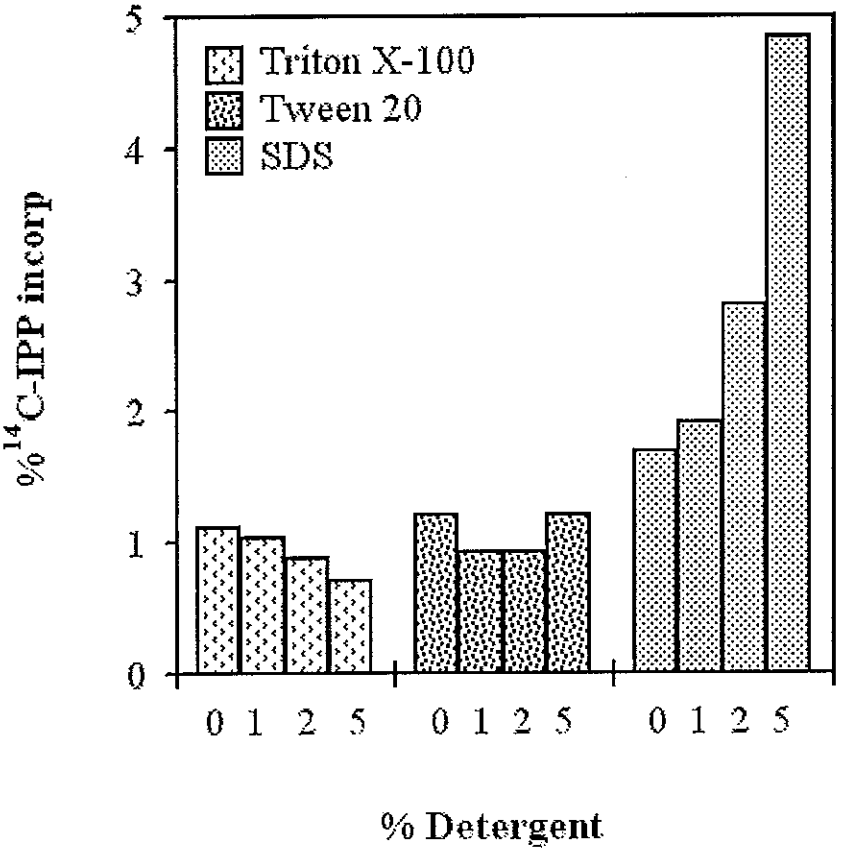


Figure 14 Effect of the detergents on the RB activity of the WBM. The standard assay mixtures were added with different amounts of SDS (0-5%, w/v), Triton X-100 and Tween 20 (v/v). The radiolabeled rubbers in the presence of different detergents were characterized at the end of 1 hr incubation. The incubation was performed in a shaking water bath at 37°C for 1 hr.

2.5 Biphasic nature of SDS activation on WBM enzymes

The results in Figure 14 show that the anionic detergent, SDS could activate the RB activity of WBM. It was commonly observed in WRP study that several detergents, but not SDS, could stimulate its RB activity (Audley & Archer, 1988). The BF membrane lipid was found with high content of phophatidic acid and thus displayed a highly negative charged character (Dupont *et al.*, 1976). It was tempting to assume that SDS might have a mimic effect on the charged environment of the membrane. In addition, protein denaturation by SDS must also be considered. Examination on this unexpected result revealed that the SDS effect was of biphasic character (Figure 15). The RB activity was inhibited at low level but then stimulating at higher concentration. This is in contrast to the WRP study that showed SDS to be a strong RB inhibitor (Dennis & Light, 1989b). Activation by high concentration of SDS was quite pronounced in term of the magnitude on the RB, a three-fold increase was seen at 5% SDS.

It is remarkable that the increase in rubber synthesis displayed a dose response curve even at above 150 mM of SDS. A possible reason for this SDS biphasic effect might be the formation of micelles at high concentration of SDS, thereby considerably increasing the active surface area (Cullis & Hope, 1985) for RB enzymes catalysis. The cmc of SDS is at 8.2 mM, so a low SDS concentration will cause denaturation of the enzymes, resulting in a decreased RB activity. At the higher concentration, the micelle will start forming together with the dissolved membrane as a mixed micelle (Baba & Allen, 1980) with the incorporated RB enzymes.

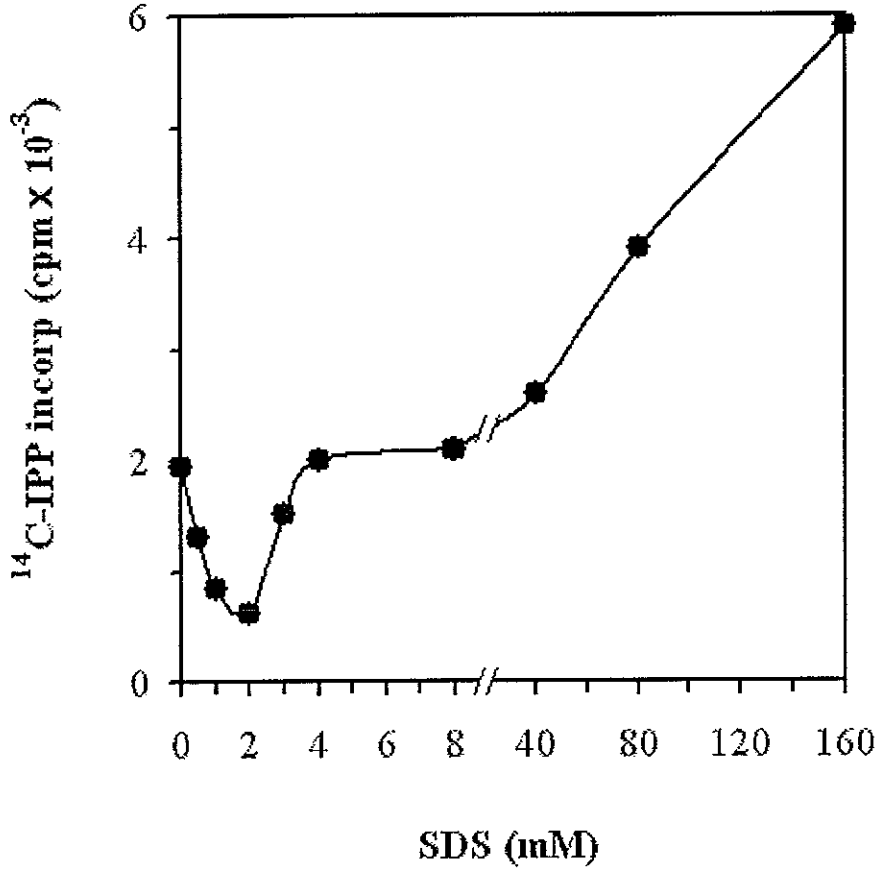


Figure 15 Biphasic nature of SDS activation on WBM enzymes. The RB activity of WBM at each SDS concentration was shown as the number of ^{14}C-IPP incorporation into the rubber. The WBM (approx. 40 mg dried weight) was mixed with the SDS before subjected to the standard incubation condition containing 10 mM DTT, 5 mM $MgCl_2$ and 30 mM KF. The incubation was performed in a shaking water bath at 37°C for 6 hr.

2.6 RB Kinetic study of WBM in the presence of SDS

A kinetic study designed to correlate the WBM levels with the rubber formation was examined. It was carried out in the presence and absence of 5% SDS (w/v) with various WBM to determine the different degrees of RB capacity. The result in Figure 16 is clearly indicated a strong positive SDS effects on RB stimulation, as shown in Figure 14 and 15. The SDS activation effect was more pronounced at higher contents of the WBM in the incubation. A steady and higher RB stimulation with increasing WBM was clearly seen in contrast to the control incubation without SDS. A continuously increasing wider gap of the different RB levels was up to ten folds at higher contents of the WBM in comparison with the smaller difference seen with the lower contents of the WBM. A direct linear relationship between the amounts of the WBM and the rubber formation was, therefore, obtained and was more pronounced in the presence of SDS. SDS could cause the kinetic difference in the WBM enzyme behavior as the control showed an early saturation curve whereas the SDS sample still showed a continuous rise in the RB activity at corresponding points.

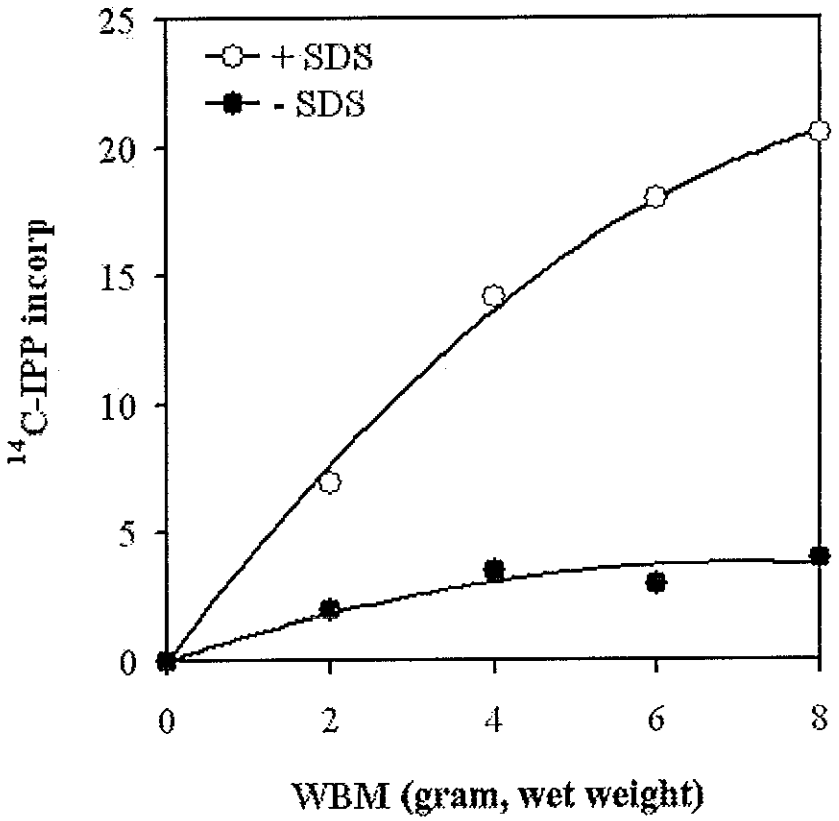


Figure 16 RB activities for different levels of the WBM in the presence or absence of SDS. The standard assay conditions were used, and the reaction mixtures contained various amounts of the WBM as indicated. A comparison was made between the incubation with 5% SDS (w/v) and the control without SDS. The RB activities were determined for radiolabeled rubber after 1-hr incubation assays. The incubation was performed in a shaking water bath at 37°C for 1 hr. The amount of WBM designated in gram wet weight.

2.7 Effect of the heat preincubation on the RB activity of the washed membrane

The temperature is the other parameter to be considered when we work with membrane function or the associated enzymes. The heat preincubation of the WBM was determined for the effect on the RB activity. The result was quite astonishing at high temperatures. In addition to SDS stimulation, the heat preincubation of the WBM could also strongly stimulate the RB activity, as clearly shown in Figure 17. Within the temperature ranges used for WBM preincubation, it was observed that the higher the temperature was, the better the yield was of rubber formation. Below 70°C, very little effect was observed. However, a sharp rise in rubber formation above 70°C was observed. A very large increase occurred at 80°C and even more at 90°C. This was quite puzzling, as one might question WBM enzyme denaturation. Several repeated assays were preformed and were still reproducible; this indicated that the effect was neither erroneous observation nor the trapping of ^{14}C-IPP by the WBM. All the assays and analyses of the results were verified as the resultant rubber was ascertained to be purified rubber by repeated and vigorous purification steps before analysis. Careful analyses of triplicate assay on the incorporation data revealed that the large increase was about 70 folds at 80°C, as shown in Figure 17. The results typically showed that 70°C was the threshold for an abrupt rise in the RB activity. This might reflect the melting temperature of stearic acid, which has been reported to be the most abundant saturated fatty acyl residual in WBM phospholipids (d'Auzac & Jacob, 1989). It has been recognized that lipid vesicles or micelles can be induced to form during incubation in the region of their gel at liquid-crystalline transition temperature (Cullis & Hope, 1985).

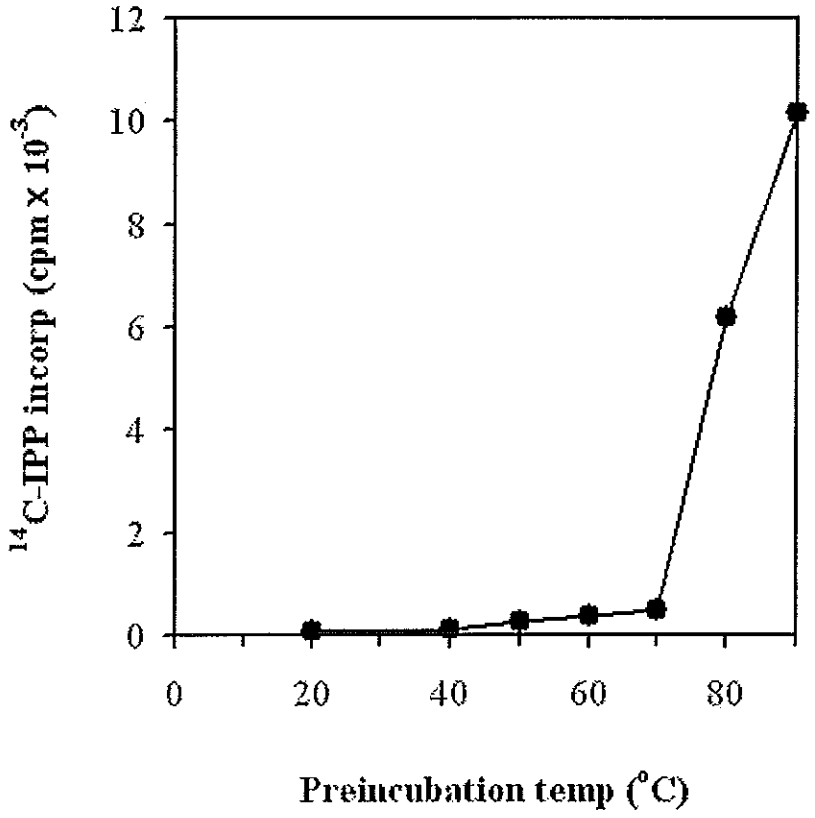


Figure 17 Effect of the heat-preincubation on the RB activity of the WBM. The heat-preincubation treatments of the WBM at adifferent temperatiures were performed for 30 min, and then it was cooled to room temperature before the standard RB assay. The incubation for the RB activity of the heat-preincubated WBM at different temperatures was carried out in a shaking water bath 30°C for 6 hr.

2.8 Effect of the heat preincubation and the presence of a detergent in the assay on the washed membrane RB activity

Because both the detergent and heat preincubation exerted a strong stimulation on the WBM activities, the combined effect was tested. The aforementioned findings indicated that heat was more effective for RB activation than a detergent, so it was interesting to see how these two parameters would affect the WBM when present together. The result was quite astonishing when the heat preincubation was performed in the presence of 5% SDS (w/v). A sharp rise in rubber formation was reduced, starting form 40°C instead of 70°C without SDS. Higher RB stimulation was observed at all corresponding points with SDS. The phenomena in Figure 18 suggested a cooperative and/or synergistic effect of SDS for the heat-preincubation WBM. Careful analyses of triplicate assays on the activation data revealed that a very large increase was seen at the lower temperatures but that the difference gap was narrowed at the higher temperature. Careful comparison and analyses of the incorporation data in Figure 18 indicated the large increase with SDS occurred from 50 to 70°C, but the increase was down to three-fold difference at 80°C when the two assays were compared. These results, therefore, indicated that SDS exerted a synergistic effect at an early stage with a lower temperature but then became additive at a later, high-temperature stage as the maximum activation was reached. The same three-fold increase was observed with 5% SDS (w/v) alone (Figure 16), which then topped the heat activation at 80°C, as shown in Figure 18.

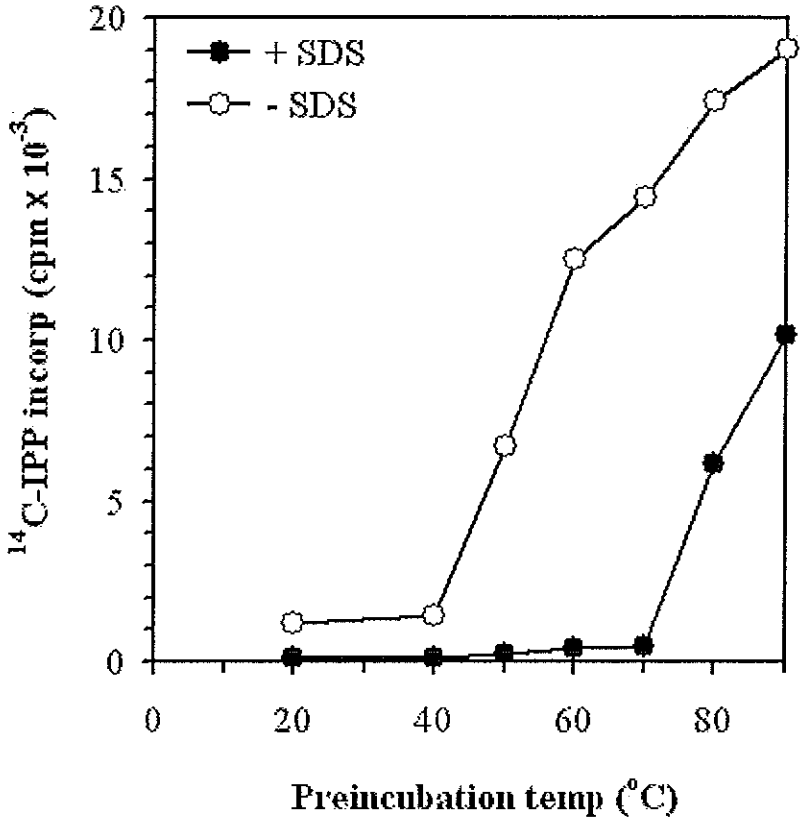


Figure 18 Effect of the heat-preincubation and SDS on the RB activity of the WBM. The heat-preincubation treatments of the WBM at different temperatures were performed for 30 min, and then it was cooled to room temperature before the RB assays. The heat-preincubated WBM for the RB assays in a standard mixture either did or did not have 5% SDS (w/v). A comparison was made for the temperature effect on the RB activity between the preincubation with SDS and the control without SDS. The radiolabeled rubber was determined for the RB activity after 6 hr reaction assays. The incubation was carried out in a shaking water bath at 37°C for 6 hr.

2.9 RB activity of fresh RP and WBM

In order to clarify the actual or exact RB site in the latex, RB activity of the isolated fresh RB and the WBM were carefully compared. Results in Figure 19 clearly showed a big difference on RB function of the two specimens. It was found that RB activity of the isolated fresh RP was very low compared to the high RB activity of the WBM under the same assay condition. RB activity of the WBM was about 6.5 up to 7.8 fold higher than RP obtained from different samples of two separated experiments. The results were done in triplicate and quite convincing to indicate that the WBM did indeed possess the enzyme system for rubber formation. On the contrary, the fresh WRP as prepared (tube A, figure 9) for this experiment had very low or no RB activity at all. This might be in contrast to the past reports and reviews that WRP was the site of rubber synthesis (Archer & Audley, 1987; Auley & Archer, 1988). It can be explained on the different degrees of contamination on the WRP with BF membrane, which was overlooked. Only the recently reports show the convincing evidence the RB site was actually located on the BF membrane (Tangpakdee *et al.*, 1977b; Wititsuwannakul *et al.*, 2003; Wititsuwannakul *et al.*, 2004).

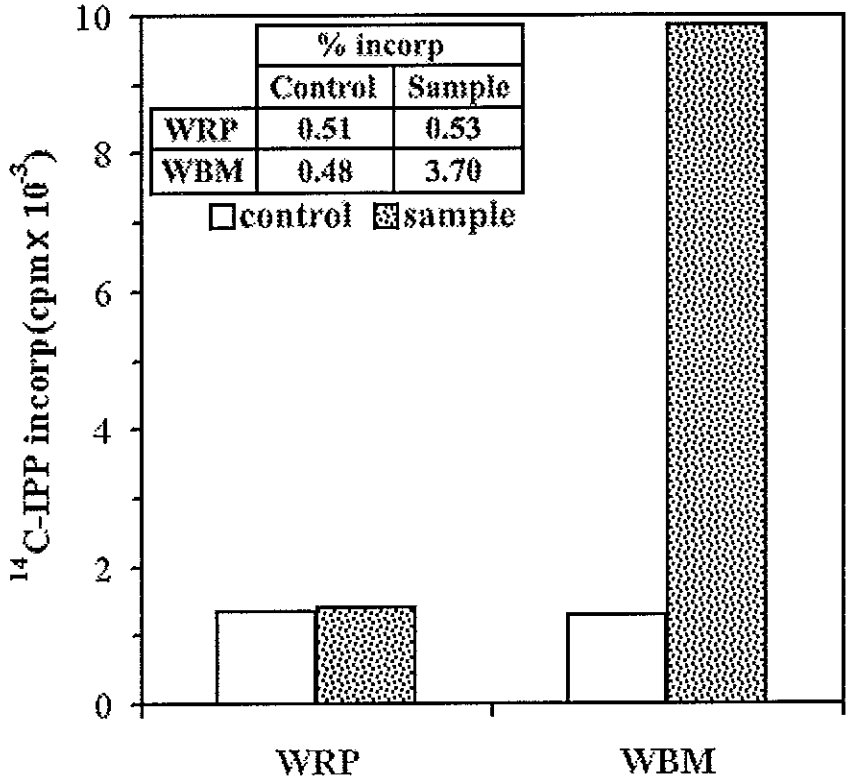


Figure 19 RB activity of WRP and WBM were shown as ^{14}C-IPP incorporation into rubber molecules. Each tube of incubation mixtures contained WRP or WBM (approx. 30 mg dry weight) in 50 mM Tris-HCl buffer (pH 7.7), 2% SDS (w/v), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT. The reactions started by addition of the substrate 20 μM [1-^{14}C] IPP (20 Ci mol^{-1}) and placed in 37°C shaking water bath for 6 hr. 20 mM EDTA was added in the incubations used as controls. Inserted table: Showing the percent incorporation of ^{14}C-IPP into the rubber.

2.10 Formation of rubber using UPP as allylic isoprene units acceptor

These experiments were carried out in order to test this postulation by incubation ^{14}C-UPP as the allylic acceptor for IPP substrate. Both the fresh RP and WBM were assayed under the same condition to test and compare RB activities. The results were quite stunning in term of the different magnitude on RB activities (Figure 20) between the fresh WRP and WBM. The WRP showed very low or no RB activity, but the WBM was very much active in the rubber formation from UPP. This was in agreement with previous report that WRP was not active for rubber synthesis when incubated with UPP and ^{14}C-IPP substrate (Light *et al.*, 1989). It was noted that WRP had higher incorporation ratio with ^{14}C-UPP than with ^{14}C-IPP incubation as shown in Figure 19 results. This might due to some minor proteins on the fresh WRP (Figure 10) with activity for UPP condensation and that the ^{14}C-UPP used in the assay was uniformly labeled (Shimizu *et al.*, 1999). Formation of UPP by UPP synthase in *M. luteus* has been extensively characterized (Shimizu *et al*, 1999) and recently was suggested that it could also be precursor in *Hevea* latex to form rubber (Ohya & Koyama, 2001).

The results in Figure 20 thus clearly showed that WBM possess the enzyme system of wide ranges for rubber synthesis, both the small allylic acceptors (GPP, FPP, GGPP) or a larger one like UPP in this study. In earlier study, it was shown that the whole latex was active for rubber synthesis when incubated with UPP and ^{14}C-IPP (Light *et al.*, 1989) but was with no activity with WRP. This gave a strong support to our results with the WBM activity. The whole latex RB activity with UPP (Light *et*

al., 1989) might likely be attributed to the BF membrane part and was in good agreement with our assays with the isolated WBM.

The difference between fresh WRP and WBM for capability in catalyzing synthesis of rubber was thus very clear in this study. The active RB function of WBM indicated it has the membrane associated enzymes system in carrying out rubber synthesis while it was absence in the fresh WRP, unless contaminated as speculated.

2.11 Protein profile of acetone-fractionated WBM proteins

Serial extraction of WBM proteins with acetone ranging from 20-80% was devised in this study. The results showed that the membrane protein could be fractionated into different fractions by acetone ranges as employed (Figure 21). Protein profiles of distinct patterns by SDS-PAGE were obtained, though overlaps of some common proteins occur. Most of the acetone precipitated proteins were in the 40% and 80% (lane B, D) acetone ranges while moderate amount were in the 20% and 60% (lane A, C) ranges. These fractions were then examined for possible RB activity.

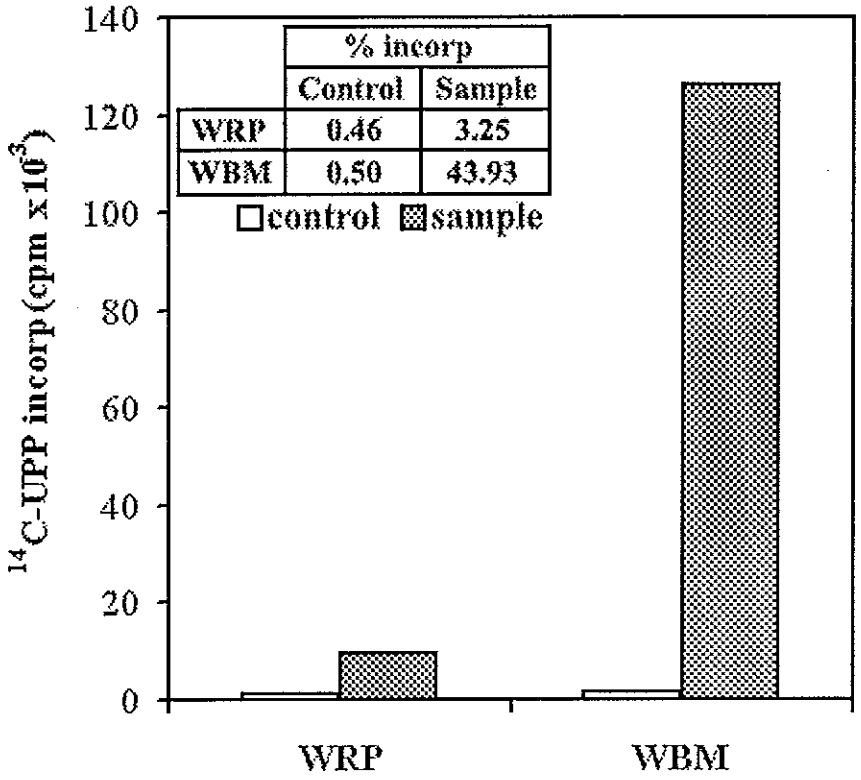


Figure 20 ^{14}C-labeled rubber formed by the RB activity of WRP and WBM using ^{14}C-UPP as an allylic substrate. Each tube of incubation mixtures contained WRP or WBM (approx. 30 mg dry weight) in 50 mM Tris-HCl buffer (pH 7.7), 2% SDS (w/v), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT. The reactions started by addition of the substrate 40 μM IPP and bacterial ^{14}C-UPP (245,000 cpm as prepared) and placed in 37°C shaking water bath for 6 hr. 20 mM EDTA was added in the incubations used as controls. Inserted table: Showing the percent incorporation of ^{14}C-UPP into the rubber.

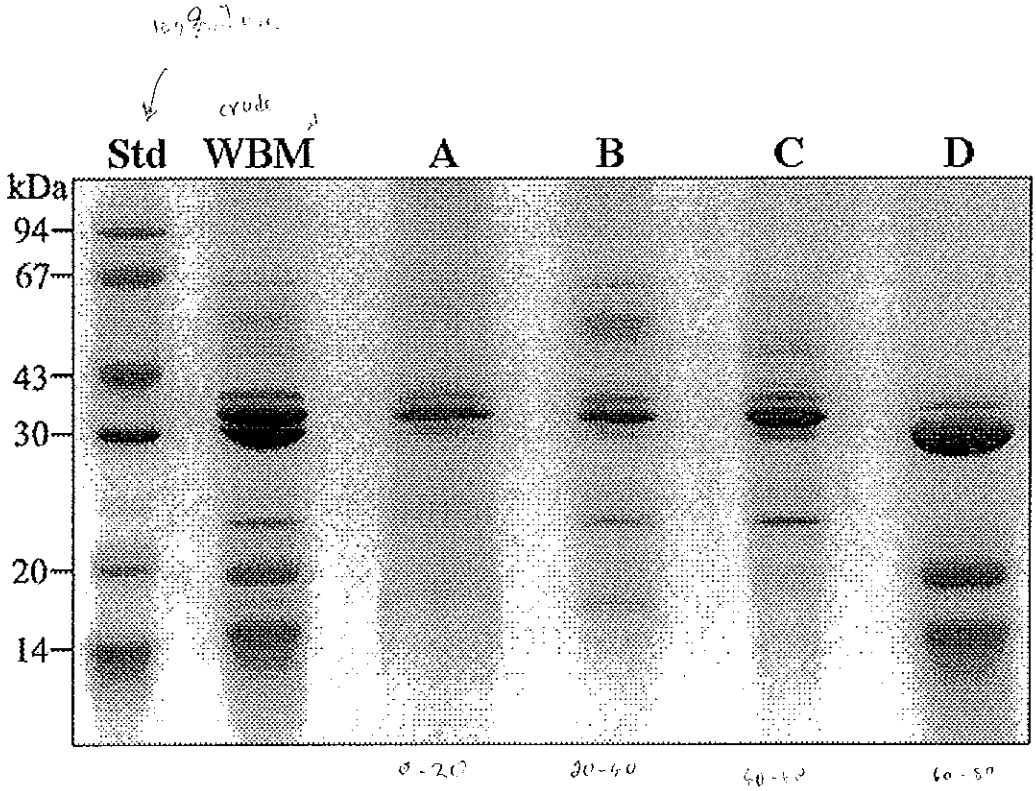


Figure 21 SDS-PAGE analyses of proteins profile from WBM proteins. WBM: proteins from WBM. The serial of acetone extract precipitated WBM proteins are shown in lane A-D. A: precipitated proteins at 0-20% acetone saturation; B: 20-40%; C: 40-60%; D: 60-80%; Std: standard molecular weight proteins.

2.12 RB activity detection of acetone-fractionated WBM proteins

The protein fractions were dissolved with assay buffer for the standard incubation of RB activity. However, no RB activity could be detected in any fractions. It seemed that they might be inactive once separated from the native membrane, or that protein composition was perturbed and different from the arrangement or topology as existing in the intact membrane. It could also be due to the absence of hydrophobic condition for them to be active. As was suggested from preceding results that high [SDS] could activate the RB activity assays, SDS was then added to the aqueous suspension of the inactive protein fractions. It was quite astonishing that activity for rubber synthesis was thus restored with the SDS addition as shown in Table 3 for the RB levels.

Different amounts of SDS were attempted, and 2% SDS was found to be optimum for RB activity assays. Results for each fraction in Table 3 showed that the maximum activity was in the 20% acetone protein fraction, whereas other fractions were much lower or without any RB activity. The RB activity was almost exclusively located in the 20% acetone fraction, which was almost equal or the same as WBM from which the protein was extracted. Repeat experiments with different preparation of samples consistently showed similar results, and thus helped confirming the results obtained.

Rationale for this could be resorted to micelle postulation. At 2% SDS (8 x cmc), a lot of micelle with highly increased active surface area as compared to WBM could be formed with the incorporated enzymes. This might be accounted for the high RB activity even with lesser enzyme in the fraction. Although the membrane

protein was distributed into fractions, it could be compensated by the highly increased active surface area for the micelle RB catalysis. Increased activity of the isolated enzyme by hydrophobic condition or micelle was previously shown for oligo-polyisoprene synthesis in the other system (Baba & Allen, 1980). Another likely reason could be the possible separation of certain inhibitory factor from this 20% protein fraction. Enzyme kinetic study was needed to substantiate the results and experiment on remix of the fractions could give support to the results and rationale for explanation as speculated.

2.13 Enzyme kinetics of the RB active protein fraction

To characterize the active rubber synthesis of this protein fraction, kinetic study on the enzyme concentrations was carried out. The results showed that RB activity was catalyzed by enzyme in this fraction, not by nonspecific trapping (Figure 22). Corresponding increases were seen with the enzymes in a dose dependent manner. Linear relationship was still observed at high enzyme level with saturated IPP substrate. It was clearly indicated that the enzyme was still highly active even when the [SDS] above cmc replaced the native or natural membrane condition. The results suggested it was possible to synthesize natural rubber *ex vivo* by protein extract from BF membrane if proper or optimum conditions being provided to support or stabilize the isolated enzyme activity.

Table 3 RB activity of the serial acetone fractionated proteins. The ranges of acetone used were 20, 40, 60, 80% (A, B, C, D fraction). The precipitated protein in each incubation tube was prepared from the same WBM (approx. 40 mg dried weight) in the serial acetone fractionation. 2% SDS (w/v) was added in all incubations before subjected to the standard incubation condition as detailed in Methods.

Sample	^{14}C-IPP incorp (cpm)	% incorp*
WBM (control)	6,655	9.6
A (20% acetone)	6,505	9.4
B (40% acetone)	1,080	1.0
C (60% acetone)	323	0.5
D (80% acetone)	121	0.2

*The data represent the average of three determinations.

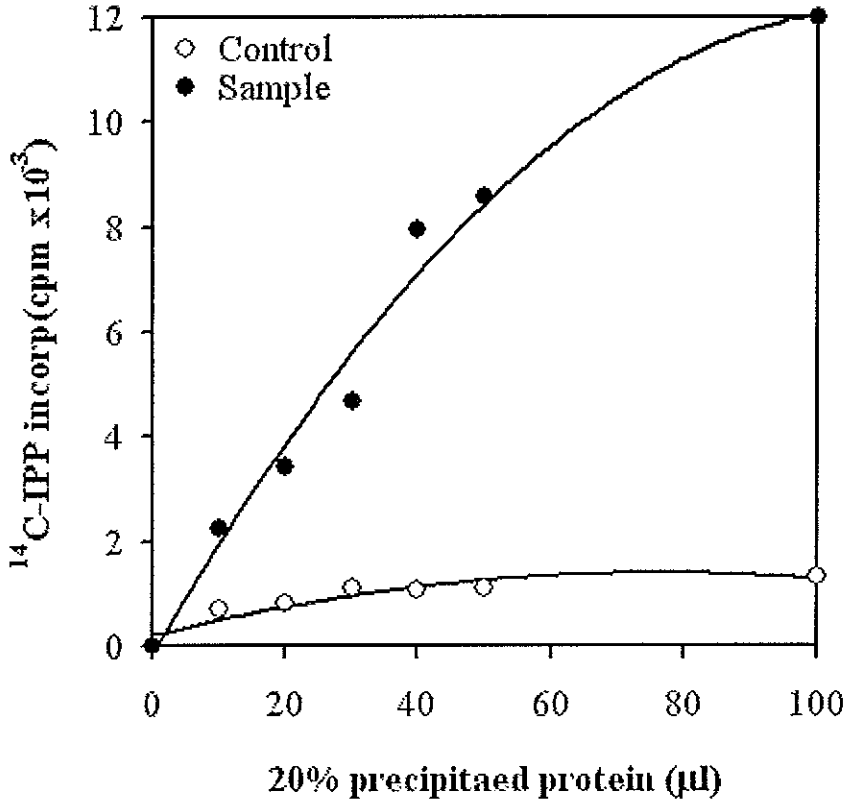


Figure 22 The activity of 0-20% acetone precipitated protein from WBM (•, 1.7 mg protein/ml). 2% SDS (w/v) was mixed with the samples before subjected to the standard incubation condition as described in Experimental part. Control (○) was carried out with the same condition as sample but with 20 mM EDTA added in the incubation assay.

2.14 RB activity of the combined or reconstituted protein fractions

The experiments were carried out by mixing the highly active fraction (fraction A) with other low or inactive RB fractions (fraction B, C, D) and follow the effect. The combining was a reconstituted assay to see if changes in RB activity were affected by the additions. When the active 20% acetone fraction (A) was mixed with any of the other fractions (B, C, D), some changes or effect on the RB activity were observed. WBM and the mixed WBM activity would be serving as controls to verify the changes as occurring in the combining isolated fractions, these results of several RB incubation assays were summarized in Table 2 as the average values of three duplicate determinations.

When the active fraction A was mixed with fraction B, an increased RB activity was observed. This indicated that the slight activity in fraction B (shown in Table 3) was overlap or spilled over from A and thus had an additive effect in the combined assay. It might also be that the fraction B contained an activator for RB activity as the total activity was a bit higher than the addition of the two isolated activity. Combining the fractions A and C or A and D showed an opposite effect, the RB activity in fraction A was inhibited by both C and D fractions. The most pronounced effect was seen with D as the fractions A activity was totally diminished. Complete inhibition exerted by the fraction D indicated that the inhibitor protein is present in this fraction upon acetone separation of the proteins from BF membrane. When the intact WBM was mixed with this fraction D, again the same complete inhibition of RB activity was also observed similar to that seen with the RB active fraction (A) (Table 4). This might lend a strong support to the result seen on the 20%

acetone protein fraction (A) that showed quite unusual high RB activity in spite of the fact that it should be expected to have lower activity due to the redistribution of enzymes upon fractionation by the acetone. It is not known if the active fraction A also contains an activator of the RB enzymes and hence the unusual high RB activity. Combining or reconstitution experiment was a common practice when studying the interaction of different fractions as previously reported for WRP activation by C-serum addition (Light & Dennis, 1989) or activation of HMGR on BF membrane by C-serum calmodulin (Wititsuwannakul *et al.*, 1990). It certainly needs further study as shown recently (Kharel & Koyama, 2003) and more reconstitution assays are in progress.

Table 4 Reconstitution experiments on RB activity of acetone fractionated proteins. Acetone ranges were as in Table 3 (fraction A was mixed with each of other fractions). The protein fraction in each incubation tube was prepared from the same WBM in serial acetone fractionation. WBM used in this study was approx. 40 mg dried weight. All incubations were mixed with 2% SDS (w/v) before subjected to the standard incubation condition as described in Method. Intact WBM and WBM+D were also tested for comparison with the isolated protein fractions.

Sample	^{14}C-IPP incorp (cpm)	% incorp*
WBM (control)	6,915	10
A (control)	6,750	9.8
A + B	8,580	12.4
A + C	990	1.4
A + D	866	1.3
WBM + D	467	0.7

* The data represent the average of three determinations.

Part 3. Significant Role of Bacterial Undecaprenyl Diphosphate (C_{55}-UPP) for Rubber Synthesis by *Hevea* Latex Enzymes

3.1 Comparison of WRP and WBM in rubber synthesis activity using IPP alone or with UPP as Allylic initiator

The result presented in Figure 23 is an attempt to clarify the roles of WRP and WBM in RB activities. Two sets of RB incubations for WRP and WBM were carried out in the presence of 2% SDS under the same conditions. The RB incubations assays were either with ^{14}C-IPP alone (Figure 23, A) or ^{14}C-UPP and IPP (Figure 23, B) for both WRP and WBM to monitor the levels of new rubber formation. This is to compare the RB activities with and without allylic isoprene and to assess the UPP function in the RB process. Besides, this experiment is also aimed at the possible use of bacterial derived oligoprenyl UPP for the *in vitro* rubber synthesis by the *Hevea* enzymes.

It was quite clear that the allylic UPP was very effective to initiate or activate the new rubber synthesis as shown with the maximum RB activity for WBM (Figure 23, B2). All the assays with their specific controls are by the presence of 20 mM EDTA that can completely inhibit RB activity (Wititsuwannakul *et al.*, 2003). The overall results (Figure 23-A, 23-B) clearly indicated that the WBM was very active using microbial UPP in the synthesis of new rubber by WBM and only slightly by WRP. In the assays with ^{14}C-IPP alone (Figure 23-A), WBM was quite active as compared to the WRP activity (A2, A1) with more than 4 folds activity over the very low or no WRP activity. The results are in good agreement with our earlier reports

(Wititsuwannakul *et al.*, 2003 & 2004) that WBM was RB active with IPP. Addition of allylic UPP to RB incubations (Figure 23-B) was even more striking as evidenced by the very much more increase of WBM activity and only moderately by WRP (B2, B1). WBM activity was about 6 folds higher than that of WRP with UPP. The WRP result was in contrast to previous report that UPP could not be used for RB by WRP (Light *et al.*, 1989) and that the RB activity inhibited by SDS (Dennis & Light, 1989). It was thus clear that UPP was very suitable for RB process.

Comparison of WRP activities showed that addition of allylic UPP resulted in about 4 folds increases over that with IPP alone (B1, A1). Numerous earlier WRP study with short allylic isoprenes (GPP, FPP, GGPP) reported the active RB function of WRP (Archer *et al.*, 1963; Archer & Audley, 1982; Audley & Archer, 1988). However, those studies were carried out with WRP prepared from the preserved latex that was quite different from our WRP immediately fractionated from the freshly tapped latex with minimum contamination by bound rupture BF membrane debris as demonstrated (Part 1) with very low RB activity. RB activity of the WBM with UPP was more than 6 folds higher than that with IPP alone (B2, A2). In fact, the WBM activity with IPP alone was already high (A2) about equal to WRP activity with UPP (B1), but was even much higher upon addition of UPP to the WBM assay (B2). These results clearly indicated that UPP is more favorable by the WBM enzymes in using allylic UPP as isoprene initiator for new rubber formation. From these results it would be very interesting to further examine and characterize the WBM activity using allylic UPP as prenyl initiator for the rubber synthesis.

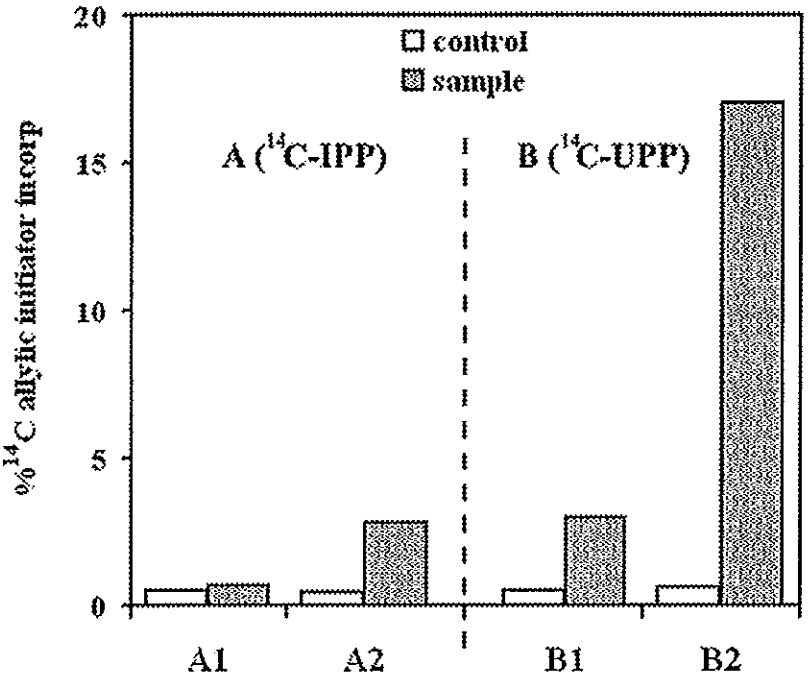


Figure 23 RB activity of WRP (**1**) and WBM (**2**) in the incubations with ^{14}C-IPP (**A**) or ^{14}C-UPP (**B**) as allylic initiators. The activity was shown as percent of the ^{14}C-allylic initiators incorporation into rubber molecules. Each tube of incubation mixture (300 µl) contained WRP or WBM (approx.30 mg dry weight) in 50 mM Tris-HCl buffer (pH 7.7), 2% SDS (w/v), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT and 40 µM ^{14}C-IPP (5 ci/mol). In the case of using ^{14}C-UPP, unlabeled IPP (60 µM) was added together with the ^{14}C labeled bacterial UPP (245,000 cpm as prepared). 20 mM EDTA was added in the incubations used as controls.

3.2 Effect of anionic detergents on RB activity of WBM

Figure 24 results showed the different effects of SDS and DOC on RB activity of the WBM incubated with ^{14}C-UPP and IPP. Upon completion of RB incubation assays, the products mixture was first extracted with butanol and the remaining mixtures were then subjected to toluene/hexane extraction for the final rubber products as detailed in Methods. The extracted products in butanol fractions are for the purpose of detection for oligo- and polyisoprenyl intermediates prior to the final rubber product. To our knowledge, no other investigators performed or included this added butanol step in their rubber synthesis assays. It is therefore debatable on the accuracy of those RB studies with WRP.

As shown in Figure 24, the RB activation by DOC on WBM activity was much more pronounced than that of SDS effects. The RB stimulation by DOC was twice that of SDS as compared on the toluene/hexane extracted rubber products between the two surfactants. However, the butanol extractable intermediate products showed opposite results to the rubber products. The polyprenyl intermediates was much higher with SDS activation than that with DOC. The lower polyprenyl or moderate chain length polyisoprene intermediates with DOC was actually converted and shown up as the final rubber products. On the other hand, the RB incubations with SDS showed about equal products in the butanol and toluene/hexane extractions. This indicated that SDS activated more for the polyprenyl intermediates formation than the rubber formation as was seen with DOC. The higher level of polyisoprene intermediates suggested that it was accumulated or lower rubber conversion rate with SDS and hence resulted in the lower level of new rubber formation. The control

without any surfactant showed that most of the ^{14}C-UPP was in the butanol phase, but the WBM was still moderately active with substantial rubber formation. This was in good agreement with preceding results (Figure 23) that showed UPP as highly suitable for the RB activity of WBM.

The overall calculated results were summarized in Table 5 that showed distributions of the ^{14}C-UPP labeled products. The butanol extraction with SDS was almost 7 folds higher products than that in the toluene/hexane extract. On the contrary, the rubber product with DOC in the toluene/hexane extract was twice higher than that with SDS. The results clearly indicated that the DOC activation was twice faster converting or turning the intermediates into the final rubber products. However, the total combined ^{14}C-UPP converted into products in both solvent extractions were similar or almost the same for both SDS and DOC activations which was amounted to total 41-42% total incorporations. Comparison of butanol extraction of RB incubations with SDS and DOC and the toluene/hexane RB extracts of both surfactants showed somewhat discrepancy ratios, which was a bit puzzling. However, this can be explained on the fact that butanol extractions were also included the unreacted ^{14}C-UPP in addition to the intermediates products, and the discrepancy ratios can thus be resolved.

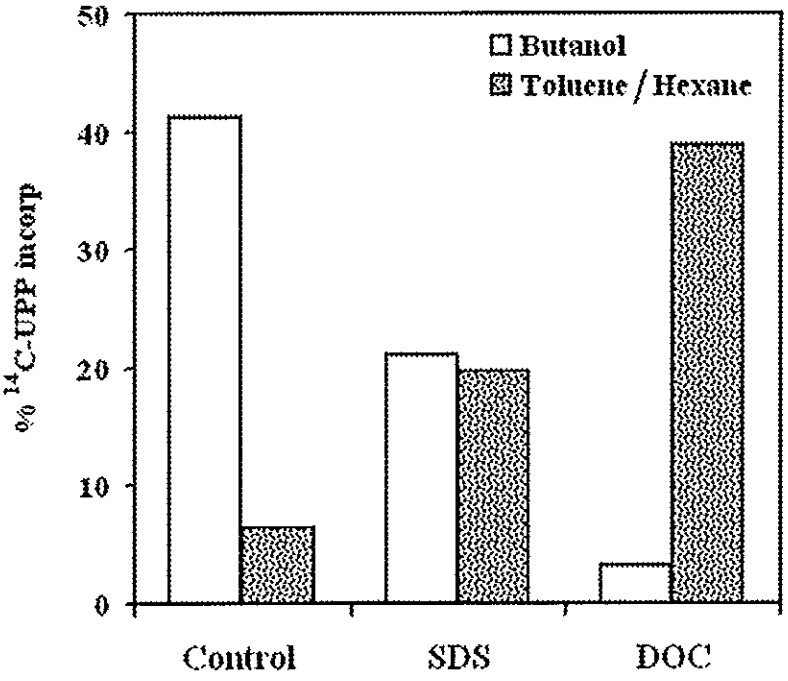


Figure 24 Effect of anionic detergents (SDS and DOC above critical micelle concentrations) on RB activity of WBM enzymes. The results were shown as percent ^{14}C-UPP incorporation into polyprenyl intermediates that were extracted by 1-butanol. The RB activity for rubber products were shown as percent ^{14}C-UPP incorporation into rubber which was detected from toluene/hexane (1:1, v/v) extract. The incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight) in 50 mM Tris-HCl (pH 7.7), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT, 60 µM unlabeled IPP, ^{14}C-UPP (100,900 cpm as prepared) and the surfactants (SDS or DOC). No detergent was added in the control condition.

Table 5 RB activity of WBM in the presence of anionic surfactants shown as percent of ^{14}C-UPP corporation into radiolabeled products extracted by 1-butanol and toluene/hexane solvent system (1:1, v/v).

	% ^{14}C-UPP incorporation*		
samples	Butanol	Toluene/Hexane	Total
Control	41.22	6.54	47.76
SDS	21.26	19.88	41.14
DOC	3.23	39.21	42.44

* The data represent the average of three determinations.

3.3 The effect of different allylic isoprenyl initiators on the rubber synthesis efficiency by WBM enzymes

The results compiled from these experiments using the different radiospecificity labeled allylic isoprenes were normalized by calculation as percent incorporation into the new rubbers formed out of the total added in the RB assays. The results being presented were compiled from several experiments with different WBM preparations and times to account for the seasonal variations being commonly observed, but were still with consistent trends. These results were then normalized by performing all assays with the same WBM preparations at the same time for accuracy and with high degree of confidence. The explanations on results need be lengthy for a clearer understanding with minimum ambiguity.

Results in Figure 25 showed the different labeled allylic isoprenes (C_{15}-FPP, C_{20}-GGPP and C_{55}-UPP) used in the study of new rubber formation by WBM in RB incubations under the same optimum conditions with DOC. The products formations from these radiotracer allylic isoprenes were analyzed both in the butanol extracts and toluene/hexane extracts for the new rubbers. They showed quite distinct and different results profiles for both solvents extractions. Comparisons of the toluene/hexane extracts showed UPP with the maximum activity, but FPP was with the maximum activity for the butanol extracts. The results (Figure 25, C) showed that UPP was the most active for rubber synthesis with highest percent incorporation as compared to other allylic isoprenes.

The other two allylics (FPP and GGPP) were about only half (GGPP) or less (FPP) as compared to UPP on the rubber synthesis activity (Figure 25, B, A, C). On

the contrary, the butanol extract results showed quite the opposite patterns on products formation from these allylic isoprenes. As pointed out earlier that the oligo- and polyisoprenyl intermediates were first separated out into the butanol phase prior to the final rubber products extracted by toluene/hexane solvent. The results showed FPP with highest intermediates formation, followed by GGPP and the lowest with UPP. These results might seem somewhat perplexing in term of the differences, but some explanations could possibly be postulated to delineate these observations and are actively sought in undergoing further investigations for the explanations to be reported soon.

It is noteworthy to point out that the results and observations shown in Figure 25 were somewhat similar or analogous to the results observed for SDS and DOC comparisons on WBM assays with UPP (Figure 24). However, the conditions in these assays were on comparisons of different chain length allylic isoprenes effects on efficiency of rubber synthesis by WBM enzymes versus the surfactants effect (Figure 24). The intermediate products were higher with FPP and GGPP than UPP similar to those seen with SDS. On the other hand, the UPP showed twice the total rubber synthesis, with concurrent decreasing of the lower MW polyprenyl products in butanol extract. This was similar to the DOC activation of WBM rubber synthesis with the allylic UPP. Conversion rate into the new rubbers formation from FPP and GGPP were much lower than that seen with UPP which showed at least two folds higher in the total final rubber formed.

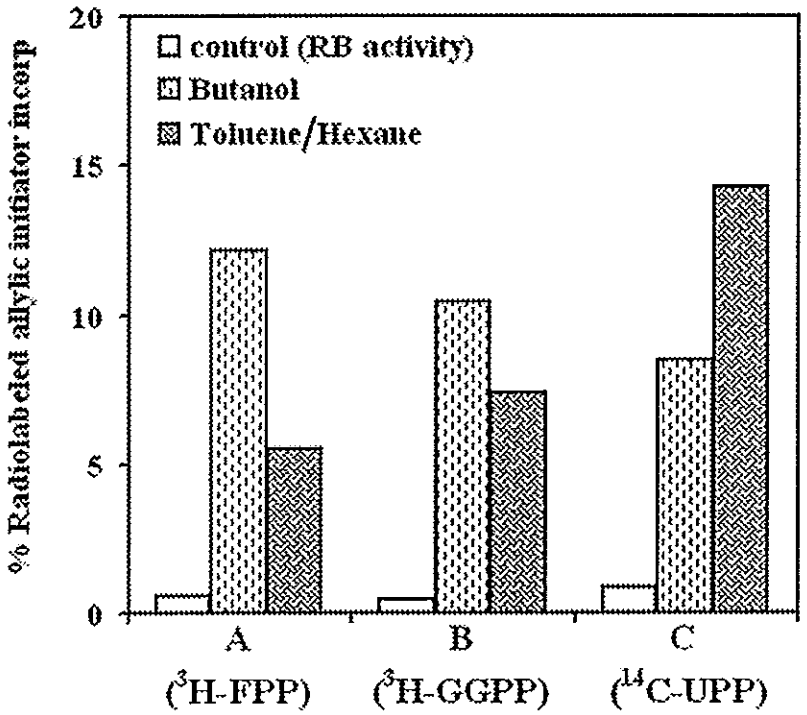


Figure 25 Enzyme activity of WBM shown in percent radiolabeled allylic initiators incorporation into polyisoprene intermediates in 1-butanol extract and rubber products in toluene/hexane (1:1, v/v) extract. The incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight) in 50 mM Tris-HCl (pH 7.7), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT, 40 mM DOC, 60 µM IPP and radiolabeled allylic initiators [12.5 µM ^{3}H-FPP (7ci/mol), 12.5 µM ^{3}H-GGPP (7ci/mol) and 100,900 cpm ^{14}C-UPP as prepared]. 20 mM EDTA was added in the incubations used as controls.

3.4 Comparison of WRP and WBM rubber synthesis functions using allylic UPP

The results in Figure 26 showed the differences of RB functions between WRP and WBM with allylic ^{14}C-UPP, both with and without DOC. It was found that the RB activity of WBM could be highly activated by DOC (Figure 26, B2), but WRP was also slightly activated (Figure 26, A2) even though very small comparing to the WBM. Even without DOC, the WBM still showed quite considerable RB activity with UPP and higher than the WRP with DOC (B1 vs. A2), a 2.5 folds difference of 6.5 % and 2.7 % ^{14}C-UPP incorporations. But when the WBM without DOC was compared to the WRP without DOC (B1 vs. A1), it was even more considerable for the difference of up to 6.5 folds higher RB activity. The results thus clearly indicated that UPP was highly favorable and preferentially suitable by WBM enzymes utilizing for the rubber synthesis. The DOC activation of WBM activity was very significant and highly substantial, an increase of 5 folds over the already high WBM activity without DOC. The levels of rubber product formation increased from 6 % to 32 % incorporations of ^{14}C-UPP (B1 vs. B2). With the presence of DOC for both specimens (B2 vs. A2), the WBM activity was almost 12 folds higher than WRP in the rubber synthesis levels. These results and observations strongly substantiate the assumption that allylic UPP derived from bacteria could be highly acceptable for the rubber synthesis by *Hevea* enzymes as was recently speculated and proposed (Wititsuwannakul *et al.*, 2004).

Although the DOC activation of WRP was small comparing to the WBM activity, but the WRP activity as seen was still quite significant. Calculation of the

WRP increased activity by DOC revealed 2.5 folds over that without DOC, from 1.1 to almost 2.8 % ^{14}C-UPP incorporations into the rubber formed by WRP activity. In past study (Wititsuwannakul *et al.*, 2003), we could not find any RB activity with WRP, and the new rubber formed by WRP could hardly be detected at all. However, in this WRP study with ^{14}C-UPP the RB activity could be significantly detected, which was different from that study with ^{14}C-IPP alone (Wititsuwannakul *et al.*, 2003). This was in contrast to the previous report that no WRP activity could be found for rubber synthesis with allylic UPP and ^{14}C-IPP substrate in RB incubation of WRP assays (Light *et al.*,1989). Our results in this study could agree well as compared to the SDS effect on WRP with allylic ^{14}C-UPP as previously reported. On the contrary, WRP without DOC showed very little or without any significant RB activity, about equal to the control inhibited with 20 mM EDTA for the control RB activity assays.

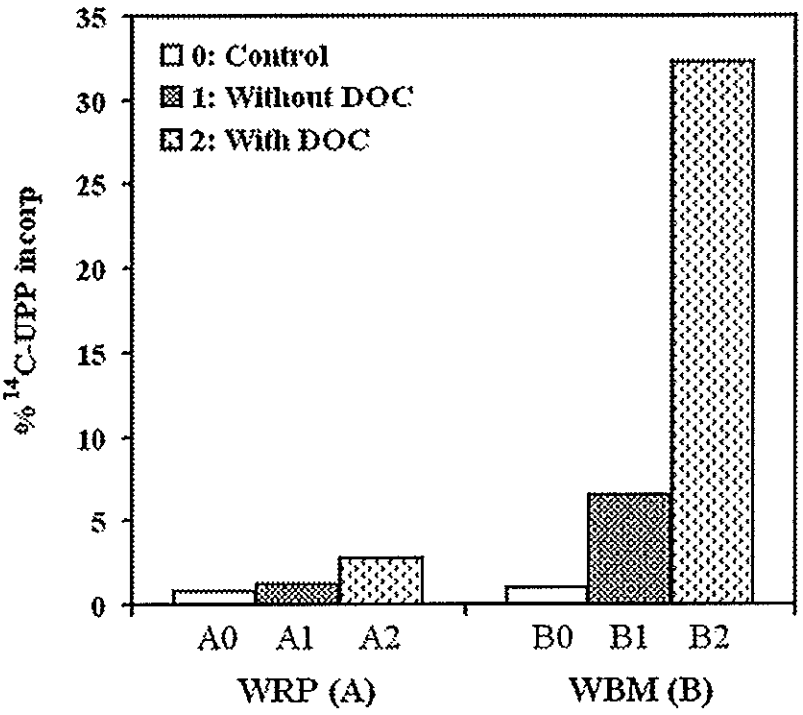


Figure 26 RB activity of WRP (A) and WBM (B) compare between incubation in the absence (1) and presence of DOC (2). The activity was shown in percent ^{14}C-UPP incorporation into rubber which was extracted by toluene/hexane (1:1, v/v). The incubation mixture (500µl) contained WRP or WBM (approx.30 mg dry weight) in 50 mM Tris-HCl buffer (pH 7.7), 30 mM KF, 5 mM $MgCl_2$, 10 mM DTT, 60 µM IPP and ^{14}C-UPP (354,000 cpm) with and without 40 mM DOC. The control incubations (A0 and B0) were done with 20 mM EDTA added.

3.5 Analyses of rubber products from UPP by WBM and WRP activities

The products from RB incubations assays of both WBM and WRP with UPP would be of great interest to determine the similarity or difference between the two enzymes system for synthesis of the rubber from allylic UPP. Since no attempt for the products analysis was ever made before by investigators (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) on rubber produced by WRP activity, this will be the first report made on WBM and WRP with allylic UPP. Besides, the DOC effects on both incubations assayed with UPP were also compared. Both qualitative and quantitative aspects are to be determined. First, Qualitative analysis will be done by effective reverse phase TLC that was performed on polyprenols (Tateyama *et al.*, 1999). The products as derived from TLC separation assays will then be subjected to quantitative analysis as routinely carried out in the RB studies.

Qualitative analysis of the WBM and WRP with allylic UPP incubation products were shown in Figure 27. The analyzed products were the toluene/hexane extract (Figure 26) on synthesis of the rubber study and the DOC effects. Rubber products from both WBM and WRP activities using UPP together with DOC effects were TLC analyzed for the products separation and identification. Four different samples were TLC assayed for WBM products (A, B) and also WRP products (C, D) without and with DOC. A few solvent systems were tested for separation suitability and the high resolution of the products identification. The solvent system of acetone/hexane (19:1, v/v) for product analyses of the new rubber synthesized from UPP was found to be the most suitable for our analyses as previously employed

(Ohnuma *et al.*, 1998) for polyisoprenes separation. However, the solvent system of acetone/water (19:1, v/v) as previously used for the assay of RB products (Oh *et al.*, 2000) was found unsatisfactory, with only one spot at the origin that might be analyses defect. With acetone/hexane solvent, both rubber product at the origin and some other polyprenyl intermediates were well separated with high resolution.

The reverse phase TLC pattern of the WBM and WRP products showed the difference with distinct profiles. The differences were seen between WBM and WRP and for the DOC effects also. For the WBM products, most of the labeled products were confined or localized as rubber at the origin (A, B), as revealed by autoradiogram profiles. But for the WRP products, most of the labeled products were as polyprenyl intermediates with very little labeled rubber. As the rubber did not move in this solvent system, but removing the intermediates from origin as we previously demonstrated for the labeled rubber (Tangpakdee *et al.*, 1997b). The acetone/water solvent showed only rubber spots with no intermediates, hence it was unsuitable for ours as reported (Oh *et al.*, 2000). It was clear that our analyses were reliable and accurate for further quantitative analyses the purified synthesized rubber with no contaminated products. The results showed clear distinction for WBM and WRP in the RB functions. Past studies always took for granted to implicate the WRP as only RB site (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999), which need to be reexamined. The results as shown in Figure 27 clearly indicated that WBM could be assumed or implicated as actual RB site. As to why other products were found with toluene/hexane extract rubber need to be further discussed later, which might provide a clearer picture of the RB process.

The anionic surfactant DOC effect also revealed the clear differences for WBM and WRP. As earlier stated, not only the WBM and WRP differences were observed, but differences on the added DOC conditions also be detected. The DOC effect profiles were quite interesting and in contrast the ones without DOC. WBM profiles (A, B) showed higher intermediates intensity without DOC (A), but lower with DOC (B) and hence the more rubber formed (B) as reported (Figure 27) which was in good agreement with TLC intensity patterns. But WRP (C, D) showed the opposite to WBM profiles. WRP without DOC (C) showed low radiotracer intensity for both rubber spot and the intermediates. The WRP with DOC (D), showed the same intensity of rubber spot, but with much higher intermediates intensity. Even though WRP with DOC showed twice (Figure 26) the rubber synthesis level, it could be attributed by the bound radiotracer as detected. Qualitative analysis of WRP and WBM from the TLC separated products would provide a clearer answer to this somewhat unexpected outcome.

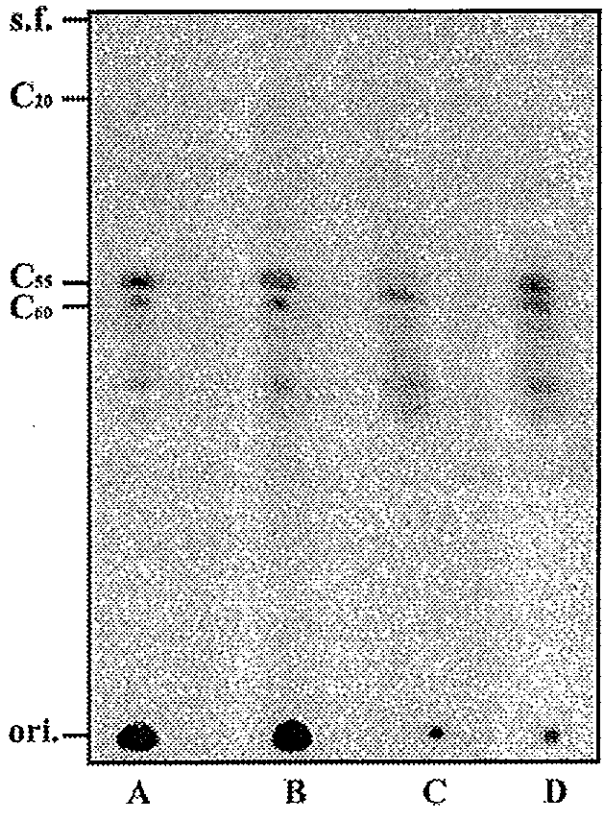


Figure 27 RP-TLC autoradiogram analysis of RB products by WBM and WRP. After product extraction and dephosphorelation as mentioned in Experimental part were performed, the products were separated on RP-TLC plate (LKC-18, Whatman) with a solvent system of acetone/hexane (19:1, v/v). The plate was exposed on image plate and analyzed by Bio-image analyzer. Lane A: products from WBM incubation without DOC, lane B: products from WBM incubation with the presence of 40 mM DOC, lane C: products from WRP incubation without DOC, and land D: products from WBM incubation with the presence of 40 m M DOC. On the left shows number of carbon according to authentic standards run along with the samples. (ori. :origin, s.f. :solvent front)

3.6 Quantitative analyses of rubber synthesis from allylic UPP

From the TLC separation profiles and autoradiogram results (Figure 27), further extended assay might provide detailed understanding of the RB process. Not only differences of WBM and WRP, but the DOC effects can also be compared to delineate active role of membrane in the rubber synthesis. Quantitative analyses may provide a logical reason and possibly better rationale to explain the results thus obtained as indicated (Figure 27), be it for WBM and WRP or the assay conditions with DOC effects. Results on rubber quantitative analyses were shown in Table 6 as extended assays of Figure 27 profiles.

The rubber products appeared as the discrete confined spots at origin. Highly purified rubber was separately tested and shown that it retained at origin in the solvent system used in this study. This is to ascertain that the analyzed products are purified rubbers. The origin spots were quantitatively scraped from TLC plate and subjected to further rubber purification as previously described. Quantitative assays (Table 6) of the labeled rubbers revealed a large difference for WBM and WRP. Besides, DOC effects differences on RB activation were also clearly indicated. Incorporation of ^{14}C-UPP in the newly formed rubbers by WBM activity were substantial and of highly significant. It is noted that even though WRP activity was quite low in this study, but the labeled rubber was still significant with allylic ^{14}C-UPP compared to the ^{14}C-IPP previously reported with no RB activity of WRP (Wititsuwannakul et al., 2003 & 2004). Quantitative assay results revealed that WBM was very active in rubber synthesis function, but much less for WRP.

Results in Table 6 on quantitative assays of rubber products (Figure 27) was quite similar to the toluene/hexane extract results (Figure 26), but with a bit lower percent incorporation for all rubber products. This was due to higher purity upon removal of the contaminants by developing solvent system. Since the intermediates in TLC profiles were not well confined, so only the rubber products were reported in the Table 6 (A, B, C, D) assay. Calculated percent incorporation of WBM synthesized rubbers were 5.8 % without DOC (A) and 30.4 % with DOC (B), about 1.5 – 2 % lower than the total extracted, but was closely comparable on the fold differences for both separated assays. WRP calculated extracted rubber results showed only 0.7 % (C) and 1.9 % (D) ^{14}C-UPP incorporations for the higher DOC effect. It was lower than the total extracts but was still similar on the difference. The results clearly indicated that the actual RB functions belong to the WBM as previously shown (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004) but not the WRP as commonly reported without well defined and accurate analyses. The small insignificant activity as shown for WRP was actually the tiny bound BF membrane debris as we suspected all along and recently set out to prove it as demonstrated in Part 2. It is very important to note that the TLC separated bands intensity has no positive correlations, whatsoever, to the quantitative results as reported in Table 6. The TLC autoradiogram as seen was very much overexposed to ascertain all non-rubber bands can be detected, no matter how low levels they are. At first glance, the Table 6 data might seem contradicts to the TLC intensity, but it is actually and absolutely not. So the data presented are highly valid. They were obtained from the averages of three separated experiments with showed a high degree of reproducibility and good consistency.

Table 6 Quantitative analysis of the rubber synthesized by WBM and WRP with ^{14}C-UPP. The incubation condition was according to Figure 26. The total ^{14}C-UPP used in each incubation assay was 354,000 cpm.

		^{14}C-UPP incorporation*			
		Toluene/Hexane extract		Origin spot	
Samples		cpm x 10^3	% incorp	cpm x 10^3	% incorp
WBM	**A**: without DOC	23.07	6.52	20.64	5.83
	B: with DOC	114.28	32.28	107.80	30.45
WRP	**C**: without DOC	4.06	1.15	2.48	0.70
	D: with DOC	9.89	2.79	6.80	1.92

*The data represent the average of three determinations

Part 4 Molecular analysis of rubber synthesized from bacterial undecaprenyl diphosphate (C_{55}-UPP)

4.1 Allylic isoprene effects on rubber synthesis activity of WBM enzymes

Results as shown in Figure 28 were designed to clarify the RB function of WBM enzymes in the synthesis of new rubbers. All the RB incubations were with anionic detergent that was earlier shown to strongly activate the RB activity of WBM (Wititsuwannakul *et al.*, 2003 & 2004). Comparison of two different RB assay conditions, one with labeled allylic initiators (^{3}H-FPP and ^{14}C-UPP) and the one without (^{14}C-IPP alone), were compared and determined for the new rubber products being formed.

The Figure 28 results showed the highly positive effect of radiolabeled allylic isoprenes (C_{15}-FPP and C_{55}-UPP) on the new rubber formation. A large difference was noted as compared to the RB incubations with no allylic (only ^{14}C- IPP alone). Incubations of WBM with ^{14}C-UPP was over 6 folds higher in new rubber formed than with ^{14}C- IPP alone under the same optimum assay conditions. Upon completion of RB incubation, the product mixture was first extracted with butanol, and then followed by toluene/hexane extraction of the products for determination of the newly formed rubber as detailed in Experimental. Butanol extraction was commonly used as differential solvent extraction (Ohnuma *et al.*, 1998) before rubber product determination in toluene/hexane extract as routinely carried out in our standard RB assays (Tankpakdee *et al.*, 1997a & 1997b; Koyama *et al.*, 1996). The products in butanol extract was for detection and separation of oligo- and

polyisoprenyl intermediates prior to the final rubber extract by toluene/hexane. To our knowledge, no other RB study groups included this butanol step in their rubber synthesis studies. It is therefore debatable on the accuracy of those RB assay results with WRP. We will subsequently show and discuss later on TLC analyses of the RB incubation products with ^{14}C-UPP.

The products formations from labeled IPP alone and the labeled allylics together with IPP were determined both in butanol and toluene/hexane extracts for rubber products. They showed quite distinct result profiles for both solvent extracts. Toluene/hexane extracts showed UPP with maximum rubber synthesis (Figure 28, C), and was moderate for FPP (Figure 28, B). The rubber formation with UPP was more than two folds higher than FPP. The rubber yield from labeled FPP was 6.4 % versus 14.3 % incorporations with UPP. However, with ^{14}C-IPP alone (Figure 28, A), new rubber formation could also occur, but it was least active with less than 3 % incorporation. The rubber formation by WBM from ^{14}C-IPP could still be considered significant, as none could be seen when it was incubated with WRP (Wititsuwannakul *et al.*, 2003 & 2004) in our earlier reports.

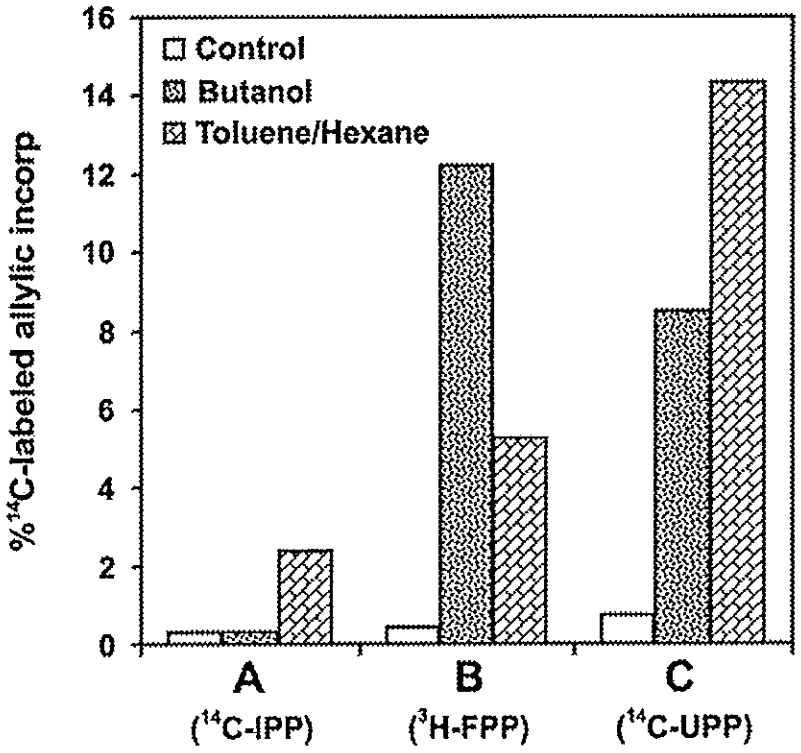


Figure 28 Allylic isoprene effects on rubber synthesis activity of WBM enzymes. Enzyme activity of WBM was shown as percent radiolabeled allylic incorporation into oligo-polyisoprene intermediates (1-butanol extract) and rubber products (toluene/hexane (1:1, v/v) extract). The incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight), 60 µM IPP and radiolabeled allylic initiators [12.5 µM ^{3}H-FPP of 7 ci/mol(B) or 100,900 cpm ^{14}C-UPP as prepared(C)] in the standard RB assay buffer. For the assay without allylic initiator (A), 15 µM ^{14}C-IPP (15 ci/mol) was added in the incubation assays. 20 mM EDTA was added in the incubations as the controls.

4.2 RP-TLC analyses of rubber synthesized from bacterial allylic UPP

The products obtained from UPP as allylic initiator for rubber synthesis by WBM enzymes system were characterized by reverse phase-TLC. Butanol and toluene/hexane extracts were analyzed, both on qualitative and quantitative assays. The developing solvent system of acetone/hexane (19:1, v/v) for product analyses (Ohnuma *et al.*, 1998) used in this study was found most suitable.

The results in Figure 29 showed patterns on the separation profiles of the products from RB assays of WBM with ^{14}C-UPP and IPP. Reverse phase TLC (RP-TLC) as reported previously (Tangpakdee *et al.*, 1997a, Tateyama *et al.*, 1999), was employed with the developing solvent system of acetone/hexane (19:1, v/v) with good separation. Butanol (lane A) and toluene/hexane extracts (lane B) were both analyzed on the same RP-TLC. Long term exposure to ascertain that all labeled products were detected was as shown in the autoradiogram. The separation profiles of both extracts were somewhat similar, but with minor qualitative difference. Unreacted substrate (^{14}C-UPP) and intermediates were first separated or removed by the butanol prior to the extraction of rubber with toluene/hexane. The rubber was further purified as described in Method. Both extracts showed the labeled bands at origin (rubber). However, more bands of higher intensity were detected with butanol (lane A). Toluene/hexane extract was expected to contain only the rubber, some minor bands of low intensity was also seen (lane B). It might probably be the tightly bound intermediates with rubber molecules and eluted out by our developing solvent system. This observation should be cautiously noted, especially the RB studies with WRP.

Quantitative analyses of the labeled rubber products, the two extracts showed large difference on the new rubber formation with ^{14}C-UPP initiator. Labeled origin bands were completely scraped from TLC plate to monitor for the ^{14}C-UPP incorporation. The labeled rubbers were again purified as previously described (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004) and subjected to radioactive measurement for analyses of the newly synthesized ^{14}C-UPP incorporated rubbers. Butanol extract (lane A) was found with little or devoid of the labeled rubber. It was calculated and found with only 4 % ^{14}C-UPP incorporation. Whether this was the rubber or just long chain isoprenes confined at origin is not known. Previous RB report on WRP with TLC labeled origin using acetone/water (19:1, v/v) solvent (Oh *et al.*, 1999 & 2000) showed it to be C_{120} polyisoprene. The slight moving diffused spot seen at the origin suggested it could be mixed polyisoprenes. On the contrary, the product analysis of toluene/hexane extract showed a very focused rubber spot at origin. The rubber was found to be the highly labeled product and was calculated as amounted to 38.5 % ^{14}C-UPP incorporation, almost 10 folds over that of butanol. This would be as expected, since it was most suitable for extraction and purification of the rubber products (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004).

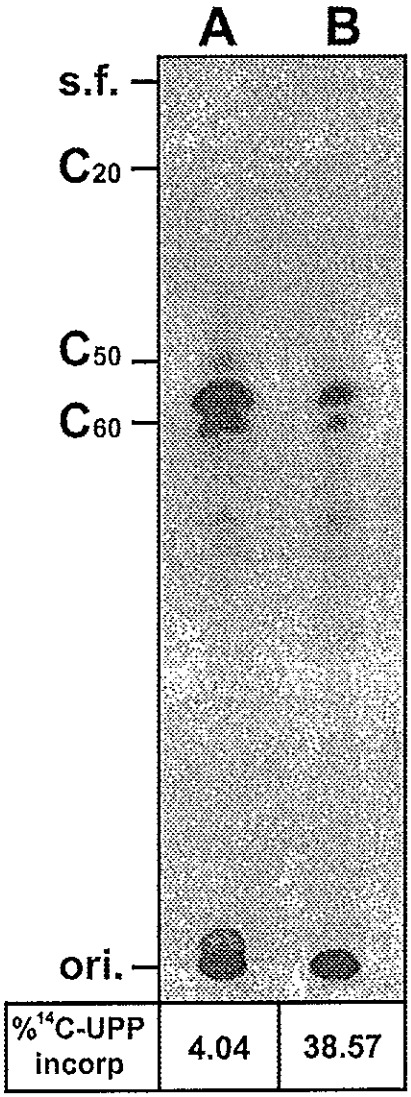


Figure 29 RP-TLC analyses of rubber synthesized from bacterial allylic UPP. Autoradiogram of the RP-TLC analysis was shown for the products of the RB incubation (WBM with ^{14}C-UPP) for butanol extract (lane A) and toluene/hexane extract (lane B). The authentic standards were run along with the samples for the isoprene chain lengths. The percent ^{14}C-UPP incorporation into the radiolabeled products at the origin spots were determined and shown at the bottom. (ori.: origin, s.f.: solvent front.)

4.3 Molecular weight determination of the rubber synthesized from UPP initiator

The labeled rubber from RB incubation of WBM with allylic ^{14}C-UPP and IPP was subjected to toluene/hexane extract and purified as described (Tangpakdee *et al.*, 1997b; Wititsuwanakul *et al.*, 2003 & 2004). Determination of molecular weight for the labeled rubber was carried out by GPC calibrated with MW standard markers as previously described (Tangpakdee *et al.*, 1997b) and detailed in Methods. Figure 30 results showed the elution profile of the labeled rubber, monitored by UV detector and radioactivity assays. The calculated MW of the labeled rubber was in the range of 3 - 4 x 10^5 (peak A), which was much lower than unlabeled UV detected rubber (peak B). The appearance of low MW labeled compound was the tightly bound contaminant as observed in the TLC autoradiogram. The GPC analysis was thus in good agreement with the TLC profiles as discussed in preceding results. The labeled rubber obtained in this study of ^{14}C-UPP with WBM was slightly of higher MW than that previously observed on the intact fresh BF particles (Tangpakdee *et al.*, 1997b) with ^{14}C-IPP. In that study, MWD was in the range of 5 x 10^4 and 4 x 10^5 with a very broad diffused distribution compared to the (almost) symmetrical MW peak of 3 - 4 x 10^5 as obtained in this study.

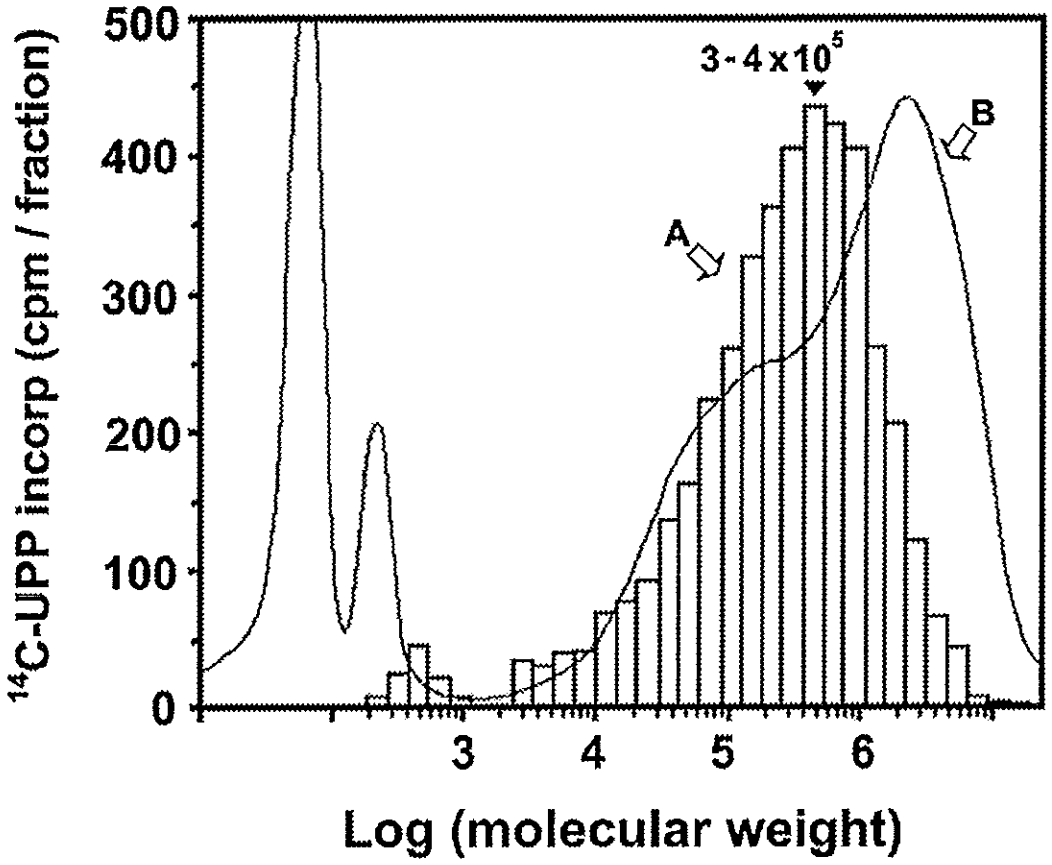


Figure 30 Molecular weight determination of the rubber synthesized from UPP initiator. MWD profile of radiolabled rubber extracted from TLC origin spot of WBM-RB reaction was analyzed by GPC. The sample from incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight), 1 mM IPP and allylic initiator [bacterial ^{14}C-UPP (117,000 cpm as prepared)] in the standard RB assay buffer. The product in toluene/hexane (1:1, v/v) extract was subjected to RP-TLC separation before GPC analysis. MWD of radiolabeled rubber remaining at the origin spot was determined by GPC as shown. Each fraction was detected by ^{14}C radioactive detector (A) and UV detector (B).

4.4 Activities of SRP and WBM in rubber synthesis with UPP allylic initiator

Comparison between WBM and WRP on rubber synthesis activities with UPP allylic initiator was carried out in this study. This was to see if UPP could also be utilized by WRP, commonly used in RB assays, in which UPP was shown to be highly RB active for WBM enzymes in preceding results. The RB activity assay of WRP with ^{14}C-UPP initiator and IPP elongating substrate was done under the same optimum conditions as WBM. The RB activities of the two were then compared for the effectiveness in using UPP initiator for the new rubber formation.

The results in Figure 31 showed the RB activities of WRP (Figure 31, A) and WBM (Figure 31, B) incubated with ^{14}C-UPP initiator and IPP substrate. They showed a large difference on the levels of the labeled new rubbers being formed. Although we could not detect RB activity of WRP in past reports with ^{14}C-IPP (Wititsuwannakul *et al.*, 2003 & 2004), but it was found with moderate activity in this study with UPP. The ^{14}C-UPP incorporation of 3.3 % was found in the labeled rubber formed by WRP activity. This was the first observation on its capability to synthesize rubber with UPP. This was in contrast to the previous report (Dennis & Light, 1989) that UPP could not be used by WRP in the RB process. On the contrary, RB activity of WBM with UPP was much more pronounced with much higher labeled new rubber formed than WRP. The calculated ^{14}C-UPP incorporation was 17 %, almost 6 folds above that of WRP. The two results thus clearly indicated that both WBM and WRP could initiate new rubber formation with allylic ^{14}C-UPP, but with much difference on the RB activity.

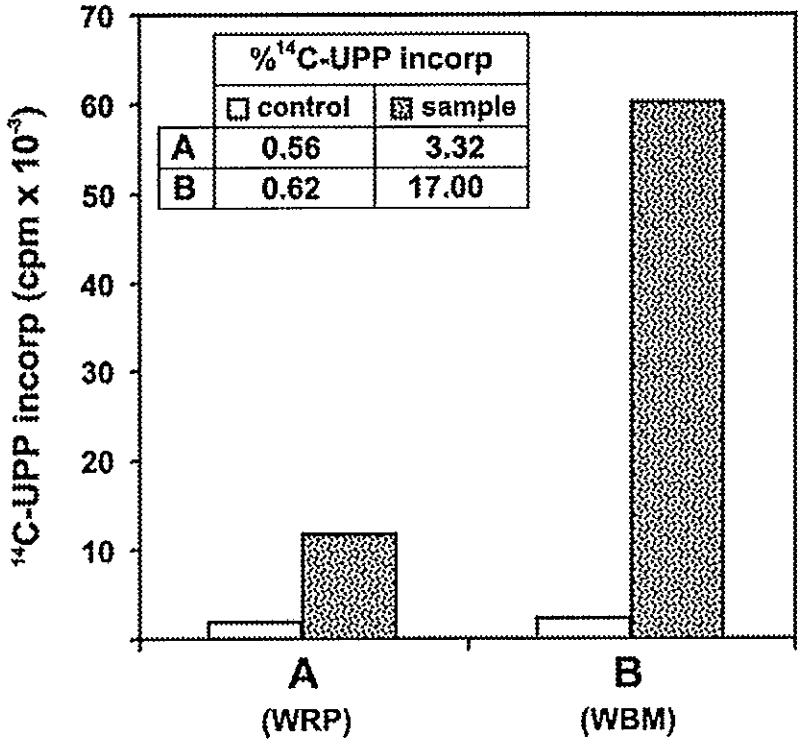


Figure 31 RB activity of WRP (A) and WBM (B) with bacterial ^{14}C-UPP as allylic initiator. The activity was shown in percent ^{14}C-UPP incorporation into rubber extracted by toluene/hexane (1:1, v/v). The incubation mixture (200µl) contained WRP or WBM (approx.20 mg dry weight) and bacterial ^{14}C-UPP (354,000 cpm, as prepared) in standard RB assay buffer. The incubations used as controls contained 20 mM EDTA.

4.5 Elongation of WBM synthesized rubber from allylic UPP by WRP

^{14}C-UPP labeled rubber obtained from the incubation of WBM and ^{14}C-UPP was extracted. The purification step as described in Method was then performed to eliminate possible oligo- and polyprenyl isoprene. This is to assure that only the pure labeled rubber being used as a substrate for the RB activity of the WRP. The resulting labeled rubber from the reaction of WRP showed a highly increased in MW from 3 - 4 x 10^5 to 9 x 10^5. The labeled rubber as catalyzed by the WRP was almost 3 folds higher than that from WRP (Figure 32). The results showed that WRP surface must be very suitable site for elongation of the preexisting rubber which still actively undergoing for chain length extension. This was the first clear and convincing evidence to show and well demonstrated the active role of WRP in the rubber elongation steps. Since the RB incubation was devoid of any labeled allylic (only pure labeled rubber as obtained in Figure 30 results), so the initiation role of WRP was ruled out from the interpreted result.

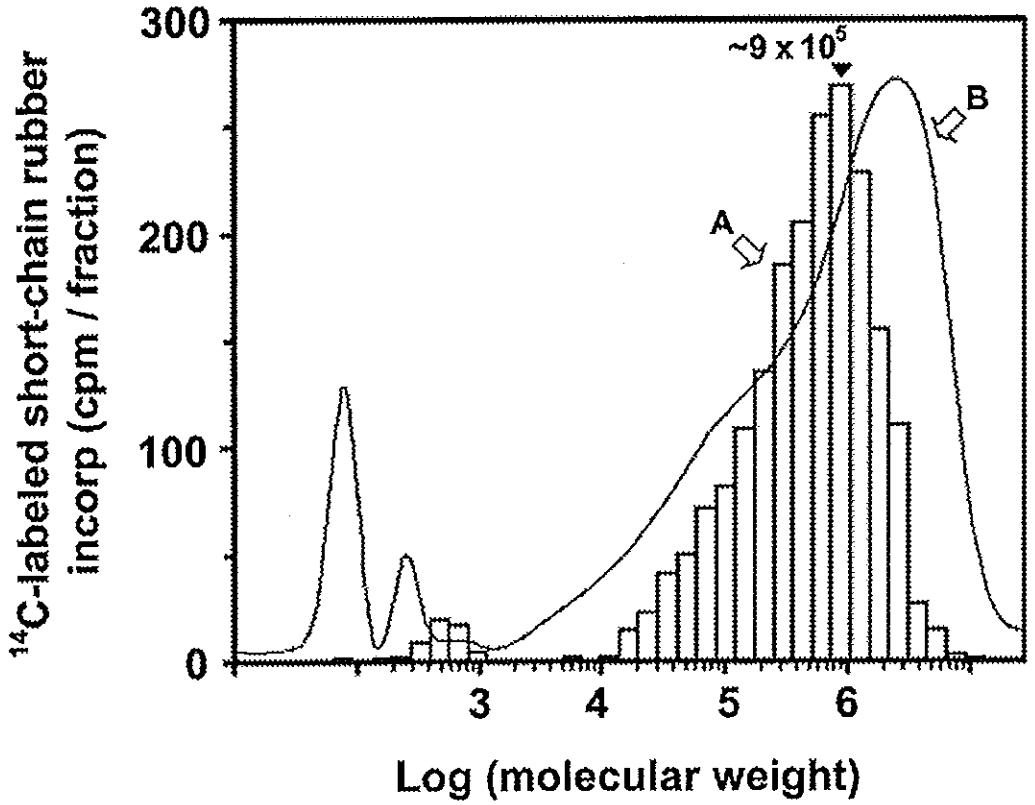


Figure 32 Elongation of WBM synthesized rubber from allylic UPP by WRP. MWD of the increased MW radiolabeled rubber was shown in the elution profile. The sample from incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight), 1 mM IPP and purified ^{14}C-UPP labeled rubber from WBM reaction (37,650 cpm as prepared) in standard RB assay buffer. The product in toluene/hexane (1:1, v/v) extract was subjected to RP-TLC separation before GPC analysis. MWD of radiolabeled rubber at the origin spot was extracted and determined by GPC as shown. Each fraction was detected for radioactivity of the ^{14}C-UPP labeled rubber product. [A; radioactivity determination, B; UV detection]

Chapter 4

Discussions

The discussions presented in this report are divided in to four separation parts according to the details in Chapter 3. Several criteria are considered for the experiment designed to ascertain and convincingly show that the RB activity can actually occur without the presence of the rubber particle (RP) surface which is not required. This will help lending more and stronger support on the earlier reported results from our group (Tangpakdee *et al.*, 1997b; Asawatreratanakul *et al.*, 2003; Wititsuwannakul *et al.*, 2003 & 2004). To clarify the still remaining ambiguity, some trivial, long overlooked aspect with important implications is intensively discussed in this chapter.

Part 1. The stability of intact fresh latex

1.1 Osmotic sensitivity of the fractionated latex BF particles

The bottom fraction (BF) membrane-bound particles are quite abundant, comprising around 20% or more of the latex volume. They are sensitive to osmotic change and some might rupture during the flow of latex from tapping (Gomez &

Southorn, 1969; Pakianathan & Milford, 1973). Addition of 0.7% ammonia or the buffered glycerol as latex preservative let to a complete loss of the BF as recently shown (Yeang *et al.*, 2002). However, the stability of intact fresh latex as fractionated immediately after tapping has never been carefully monitored and documented, except in our earlier report (Wititsuwannakul *et al.*, 2004).

The study on time course of the fresh latex fractions (Figure 9) indicated that the sediment BF was quite unstable if not quickly separated from other fractions. Centrifugation of fresh latex right after tapping showed that BF of high contents sediment as intact particles. After the latex was kept for a period of time, the isolated intact BF started bursting and releasing B-serum content together with membrane debris into the clear aqueous C-serum as a turbid phase. After that, the C-serum became clearer as the membrane debris started binding onto the rubber phase, while C-serum was mixed with the released B-serum. At last, only two fractions remained as rubber phase. This could probably be a hydrophobic interaction with high binding affinity to the RP. The result clearly showed that the prolonged storage or delayed use of fresh latex would yield only two fractions due to loss of the ruptured BF organelles.

1.2 Comparison of RP proteins under different conditions

The RP associated proteins of different RP samples and WBM proteins were analyzed by SDS-PAGE (Figure 10). This is to discern protein patterns comparing the RP rapidly separated from the freshly tapped latex and BF diminished RP samples and the preserved latex RP commonly used in RB study by others. (Archer & Audley,

1987; Audley & Archer, 1988; Kekwick, 1989; Kush, 1994; Tanaka *et al.*, 1996) The SDS-PAGE results revealed quite different protein patterns between the fresh RP, RP from the delayed use latex and proteins extracted from BF membrane. The fresh RP showed mainly two major proteins of 14 and 24 kDa with a few very faint protein bands. These two major proteins might be considered as intrinsic proteins of the RP. On the other hand, the RP from the delayed use latex showed several other prominent protein bands in addition to the two intrinsic ones. Comparison of the two samples clearly suggested that the extra protein bands were likely from the tightly bound BF membrane, even though both were extensively washed before protein analyses. The pattern of BF membrane proteins was similar to the result of the preserved latex RP protein profile as previously shown (Hasma, 1987). Examination of the additional bands revealed similar and common to some proteins extractable from the BF membrane.

The results suggested that these RP extra proteins, probably derived from BF membrane fragment might help contribute RB activity to the RP. The preserved RP commonly used, as reviewed in the RB study (Archer & Audley, 1987) might thus be attributed to the proteins or enzymes from the BF membrane. This observation might be used to distinguish the intrinsic RB activity of the RP, if ever its designated function, from RB activity of the washed bottom-fraction particle membrane (WBM). A comparison between the fresh RP and WBM on the relative RB activity would certainly be critical to support this postulation and to an understanding of the actual biological function of the RP in *Hevea* latex.

Part 2. Rubber biosynthesis by *Hevea* latex bottom-fraction particles and membrane

Previous studies on the initiation of RB indicated that washed rubber particles (WRP) surfaces could catalyze the formation of new rubber molecule with FPP, GGPP and GPP as allylic initiators. However, it still remains possible that RB can occur at certain specific sites other than the RP surfaces, as earlier suggested, but has not yet been carefully investigated. The particular interests in the non-rubber particulate (lutoid particles and Frey-Wyssling complexes) surface in the latex, which might have an important role in the RB process. Unfortunately, little attention has been paid to possible RB activity on the surfaces of these non-rubber particulate components. It seems to be a paradox if we consider the previous numerous studies in which the RP surface was implicated and suggested as the one and only prerequisite site for the *in vitro* RB process (Audley & Archer, 1998; Archer *et al.*, 1963). The question then is how and where the original or first RP are formed in the laticifers and serve as the required sites for the formation of new rubber molecules by the RP isolated from latex in those *in vitro* RB studies with WRP. No careful systematic study has yet been done or tested for the other alternative sites, except the earlier report on rubber formation by a fresh BF (Tankpakdee *et al.*, 1997b). Therefore, we carried out the experiments to test and examine fresh *Hevea* BF surfaces or membranes for their possible active rule and involvement in the RB process (Wititsuwannakul *et al.*, 2003).

This part is the further studies on *in vitro* RB activities of the membrane derived from the BF particles. A comparison of the effects of the washed bottom-fraction particles (WBP) and washed bottom-fraction particle membrane (WBM) on the RB was made first to validate further studies on the RB activities of the particle membrane. Parameters affecting the membrane functions were investigated for the determination of their interactions and effects on the RB process. Among these, the effects of detergents and the heat pre-incubation of the WBM at different temperatures on the RB activities were extensively characterized. A possible important role of WBM in rubber biogenesis was also examined.

2.1 Incorporation of ^{14}C-IPP into rubber by the WBP and WBM

The previous report on active rubber formation in the BF showed the incorporation of ^{14}C-IPP into rubber by the BF of centrifuged fresh latex (Tankpakdee *et al.*, 1997b). This finding prompted us to investigate detail the role of BF particles in the RB process. The earlier study was carried out with unwashed and intact membrane bound BF particles and the newly formed rubber being analyzed (Tankpakdee *et al.*, 1997c). In this study, the BF particles were thoroughly cleaned and purified as intact WBP by repeated washing with an isotonic buffer. The WBM was prepared by the hypotonic lysis of WBP and again underwent repeated washings for the RB experiments. A comparison of the effects of the washed particles and the derived washed membrane on the RB activity was made (Figure 11).

The result demonstrated different rates of ^{14}C-IPP incorporation into rubber by the WBP at various ^{14}C-IPP concentrations. The experimental conditions were

applied from those used for the WRP (Audley & Archer, 1998). The finding indicated the active role of latex non-rubber constituents, BF particles and the derived membrane in rubber formation. The membrane activity was about two times greater than that of the WBP. The higher WBM activity might be attributed to increased accessibility of substrates for the enzymes. The results suggested not only that the WBM was more active than the WBP but also that a membrane or membrane-like environment was required or necessary for the RB activity.

2.2 Comparison of the kinetics of the RB catalytic rate between WBP and WBM

The catalytic rate of the intact washed BF particles (WBP) and the derived membrane (WBM) was compared. The assays were to determine the saturation time point on rubber synthesis. The results (Figure 14) indicated a large difference in their kinetics for RB activity. It showed that the WBM was six times more active than WBP. This result point out two important aspects: (1) that the BF membrane was fully equipped with all necessary enzymes to complete carrying out the RB function and (2) that the B-serum did not play an active role in the RB catalysis. The fast kinetic of the WBM could be attributed to the formation of small vesicles with a highly increased active surface area. The enzyme would be more accessible to the substrates as earlier described (Baba & Allen, 1980, Cullis & Hope, 1985). This was in spite of a certain loss from washing the membrane derived from an equal amount of WBP as used in the study. The results in Figure 11 combined with this supporting

kinetic study, indicated that it was an enzyme-catalyzed process being tested and verified in the following experiments on the WBM.

2.3 Effect of the chelators on the RB activity of the WBM

Divalent cations were essential cofactors in a WRP study for RB activity (Archer & Cockbain, 1969). The buffers for both WBP and WBM assays on the RB activity contained Mg^{2+} as reported in earlier studies (Tangpakdee *et al.*, 1997b). In these WBM assays (Figure 13), the addition of chelating agents (EDTA and EGTA) to the incubation showed strong inhibition of rubber formation by the WBM. These results agree well with the general requirements of divalent cations for the cleavage of phosphate esters by prenyl transferase enzymes required for RB (Archer & Audley, 1987). When both chelators were present together, the degree of inhibition was not much different than that with EDTA alone. The results indicated that Mg^{2+} was an essential cofactor for the RB enzymes of the WBM, as observed for the WRP assay (Audley & Archer, 1988). The chelator inhibition of the WBM activity on rubber formation suggested that the WBM enzymes might be similar to those reported for the WRP surface. It should also be noted that the slightly higher inhibition level from the combined EDTA and EGTA might suggest a possible bound Ca^{2+} effect on WBM functions, but these remains to be further proved and elucidated.

2.4 Effect of the detergents on the RB activity of the WBM

The activation of prenyl transferase and RB activities by detergents has been commonly observed (Audley & Archer, 1988). The effects of different detergents on

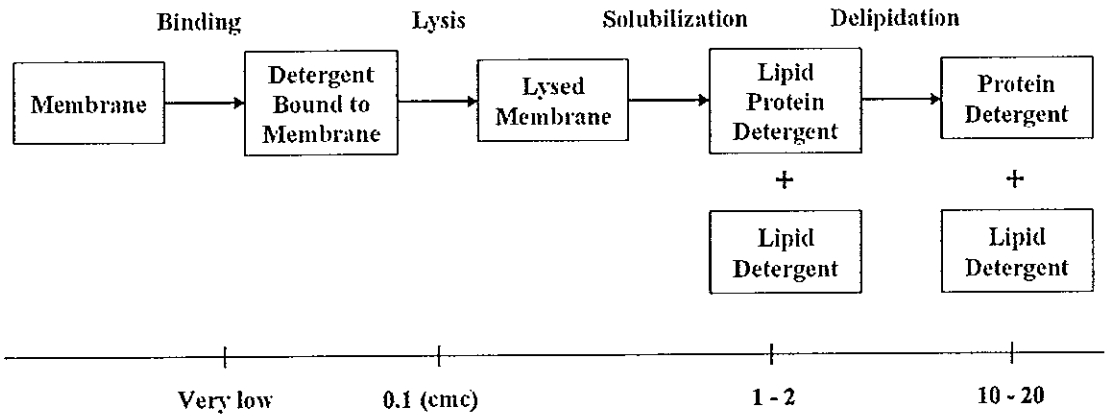
the WBM for RB activity were determined (Figure 14). When detergents were included in the RB assay of the WBM, a significant stimulation of ^{14}C-IPP incorporation into rubber was obtained with only SDS, an anionic detergent. The nonionic detergents, Triton X-100 and Tween 20, showed no effect on RB. The level of RB stimulation increased with increasing SDS concentrations above the critical micelle concentration (cmc) whereas no effect was observed for non-ionic detergents. The lipids of the WBM are rich in phosphatidic acid and saturated fatty acry residues (Dupont *et al.*, 1976; d'Auzac & Jacob, 1989). SDS, being anionic in nature, might be miscible or compatible with the negatively charged membrane, having a positive effect on the RB activity. The presence of soluble amphiphiles such as SDS may alter the physical state of WBM lipids and/or induce the formation of newly mixed micelles with increased surface area. These mixed micelles were incorporated with necessary factors and enzymes required for RB. All these events might possible lead to the increased *in vitro* RB capacity of the WBM.

2.5 Biphasic nature of SDS activation on WBM enzymes

The BF membrane lipid was found with high content of phophatidic acid and thus displayed a highly negative charged character (Dupont *et al.*, 1976). It was tempting to assume that SDS might have a mimic effect on the charged environment of the membrane. In addition, protein denaturation by SDS must also be considered. Examination on this unexpected result revealed that the SDS effect was of biphasic character (Figure 15). The RB activity was inhibited at low level but then stimulating

at higher concentration. This is in contrast to the WRP study that showed SDS to be a strong RB inhibitor (Dennis & Light, 1989b).

A possible reason for this SDS biphasic effect might be the formation of micelles at high concentration of SDS, thereby considerably increasing the active surface area (Cullis & Hope, 1985) for RB enzymes catalysis. The cmc of SDS is at 8.2 mM, so a low SDS concentration will cause denaturation of the enzymes, resulting in a decreased RB activity. At the higher concentration, the micelle will start forming together with the dissolved membrane as a mixed micelle (Baba & Allen, 1980) with the incorporated RB enzymes. These micelles might be favorable environment and condition for the WBM associated enzymes to be embedded and better express their activities. This was described by the well documented in the diagram below (Thomas & Mark, 1990). However, an electron microscopic (EM) study would contribute to a better understanding of this micelle phenomenon. It would be interesting to investigate if WBM extractable proteins are still active either alone or with micelle for the RB activity.



(Thomas & Mark, 1990)

2.6 RB Kinetic study of WBM in the presence of SDS

The result is clearly indicated a strong positive SDS effects on RB stimulation (Figure 16). The SDS activation effect was even more pronounced at higher contents of the WBM in the incubation. A steady and higher RB stimulation with increasing WBM was clearly seen in contrast to the control incubation without SDS. A continuously increasing wider gap of the different RB levels was up to ten folds at higher contents of the WBM in comparison with the smaller difference seen with the lower contents of the WBM. A direct linear relationship between the amounts of the WBM and the rubber formation was, therefore, obtained and was more pronounced in the presence of SDS. SDS could cause the kinetic difference in the WBM enzyme behavior as the control showed an early saturation curve whereas the SDS sample still showed a continuous rise in the RB activity at corresponding points. This might be assumed to be a cooperative or enhanced effect of SDS on the WBM enzymes, and so the catalytic activity increased accordingly.

2.7 Effect of the heat preincubation on the RB activity of the washed membrane

As we were working with the membrane associated enzymes, the temperature is another important factor to be considered. The experiment on heat preincubation of the WBM was done to see its effect on the RB activity. The result was quite astonishing at high temperatures. In addition to SDS stimulation, the heat preincubation of the WBM could also strongly stimulate the RB activity (Figure 17). Within the temperature ranges used for WBM preincubation, it was observed that the

higher the temperature was, the better the yield was of rubber formation. This was quite puzzling, as one might question WBM enzyme denaturation. Several repeated assays were preformed and were still reproducible; this indicated that the effect was neither erroneous observation nor the trapping of ^{14}C-IPP by the WBM. All the assays and analyses of the results were verified as the resultant rubber was ascertained to be purified rubber by repeated and vigorous purification steps before analysis. This might reflect the melting temperature of stearic acid, which has been reported to be the most abundant saturated fatty acyl residual in WBM phospholipids (d'Auzac & Jacob, 1989). It has been recognized that lipid vesicles or micelles can be induced to form during incubation in the region of their gel at liquid-crystalline transition temperature (Cullis & Hope, 1985). Therefore, the high-temperature preincubation of the WBM might lead to the formation of numerous small micelles and reversed micelles with necessary factors and enzymes to catalyze the rubber formation and, therefore, greatly increase their capacity for the RB process.

The other, more probable explanation for these unexpected but interesting findings is the renaturation of enzymes and other WBM protein components. After the return to the normal conditions after the heat pretreatment for a brief period, it is likely that certain WBM enzymes and proteins might be renatured to their original structures with the RB activity mostly retained. This has been commonly observed for numerous enzymes and proteins, including our findings (Wititsuwannakul *et al.*, 2003). The reassembling of these RB enzyme systems into the newly formed small micelles and/or reversed micelles might then occur, with something resembling the former membrane configuration. Full recovery is unlikely, and it is expected that a certain loss will occur. However, this might be compensated by the tremendous

increase in the micelle surface area far exceeding the certain denaturation loss, which leads to the highly increased RB activity, as shown. These remarkable results for the recovery and stability are indeed quite puzzling and present a challenge for a more detailed study to verify the findings and provide and better understanding of the *in vitro* RB process.

2.8 Effect of the heat preincubation and the presence of a detergent in the assay on the washed membrane RB activity

The combined effect of detergent and heat was another experiment need to be investigated as both of them displayed a strong stimulation on the RB activities of WBM. The results showed that heat was more effective for RB activation than a detergent, so it was interesting to see how these two parameters would affect the WBM when present together. The result was quite astonishing when the heat preincubation was performed in the presence of SDS (Figure 18). Higher RB stimulation was observed at all corresponding points with SDS. Very large increase was seen at the lower temperatures but that the difference gap was narrowed at the higher temperature. This phenomenon suggested a cooperative and/or synergistic effect of SDS for the heat-preincubation WBM.

In the incubation with SDS, RB activity revealed a large increase occurred from 50 to 70°C, but the increase was down to three-fold difference at 80°C when the two assays were compared. These results, indicated that SDS exerted a synergistic effect at an early stage with a lower temperature but then became additive at a later, high-temperature stage as the maximum activation was reached. The same three-fold

increase was observed with SDS alone (Figure 16), which then topped the heat activation at 80°C (Figure 18). Therefore, a synergistic effect was observed at a lower temperature as the lauryl chains in SDS became fluid at the melting temperature (ca. 44°C). Under these conditions, numerous vesicles such as mixed SDS-phospholipid micelles could be induced with the incorporated enzymes to carry out high *in vitro* RB at a relatively lower temperature. These micelles were, therefore, more effective and suitable to be more active in the RB process. They gave rise to a much higher level of RB activity than the heat-pretreated WBM alone. Another factor might be the anionic character of SDS, which is similar to that of phosphatidic acids, reported to be quite abundant in the WBM (d'Auzac & Jacob, 1989). It might promote the formation of new, small micelles and/or reversed micelles with a tremendous increase in the surface area for the highly active RB process being observed.

2.9 RB activity of fresh RP and WBM

Preceding results showed that the RP extra proteins were difficult to remove or wash out, be it intrinsic or BF membrane proteins. This might probably be factor responsible for RB activity commonly observed with the preserved washed rubber particles (WRP) in those reports (Archer *et al.*, 1963; Archer & Audley, 1987; Audley & Archer, 1988; Kekwick, 1989). Considering the high binding affinity, it is presumed that the burst BF membrane once associated with the RP, it will be acting like a part of the RP component itself. So the isolated freshly tapped RP was used for all experiments to minimize this possibility and compared to the WBM on RB activity.

The results clearly showed a big difference on RB function of the two specimens (Figure 19). It was found that RB activity of the isolated fresh RP was very low compared to the high RB activity of the WBM under the same assay condition. On the contrary, the fresh WRP prepared for this experiment had very low or no RB activity at all. This might be in contrast to the past reports and reviews that WRP was the site of rubber synthesis (Archer & Audley, 1987; Auley & Archer, 1988). It can be explained on the different degrees of contamination on the WRP with BF membrane, which was overlooked. Only quite recently reports that shown the convincing evidence the RB site was actually located on the BF membrane (Tangpakdee *et al.*, 1977b; Wititsuwannakul *et al.*, 2003; Wititsuwannakul *et al.*, 2004).

2.10 Formation of rubber using UPP as allylic isoprene units acceptor

There were previously shown that the fresh BF particles possessed both the isopentenyl diphosphate isomerase (IPPI) and prenyltransferase (PT) activities active in rubber synthesis (Tangpakdee *et al.*, 1997a; Tangpakdee *et al.*, 1997b). This was also found with BF membrane (Wititsuwannakul *et al.*, 2003; Wititsuwannakul *et al.*, 2004). Previous study showed that WRP could use the low MW allylic diphosphate for rubber synthesis with IPP (Audley & Archer, 1988), but WRP could not use UPP (C_{55}-PP) to form the rubber (Light *et al.*, 1989). Recently, it was suggested that UPP possibly elongated by IPP to form dolichols and eventually rubber molecules (Ohya & Koyama, 2001). Dolichols were quite abundant in *Hevea* tissues and latex and extensive characterized (Tateyama *et al.*, 1999).

Experiments were carried out to test this postulation by incubation ^{14}C-UPP as the allylic acceptor for IPP substrate. Both the fresh RP and WBM were assayed under the same condition to test and compare RB activities. The WRP showed very low or no RB activity, but the WBM was very much active in the rubber formation from UPP (Figure 20). This was in agreement with previous report that WRP was not active for rubber synthesis when incubated with UPP and ^{14}C-IPP substrate (Light *et al.*, 1989). It was noted that WRP had higher incorporation ratio with ^{14}C-UPP than with ^{14}C-IPP incubation. This might due to some minor proteins on the fresh WRP with activity for UPP condensation and that the ^{14}C-UPP used in the assay was uniformly labeled (Shimizu *et al.*, 1999). Formation of UPP by UPP synthase in *M. luteus* has been extensively characterized (Shimizu *et al*, 1999) and recently was suggested that it could also be precursor in *Hevea* latex to form rubber (Ohya & Koyama, 2001).

The results clearly showed that WBM possess the enzyme system of wide ranges for rubber synthesis, be it small allylic acceptors (GPP, FPP, GGPP) or a larger one like UPP in this study. In earlier study, it was shown that the whole latex was active for rubber synthesis when incubated with UPP and ^{14}C-IPP (Light *et al.*, 1989) but was with no activity with WRP. This gave a strong support to our results with the WBM activity. The whole latex RB activity with UPP (Light *et al.*, 1989) might likely be attributed to the BF membrane part and was in good agreement with our assays with the isolated WBM.

The difference between fresh WRP and WBM for capability in catalyzing synthesis of rubber was thus very clear in this study. The active RB function of

WBM indicated it has the membrane associated enzymes system in carrying out rubber synthesis while it was absence in the fresh WRP, unless contaminated as speculated. The finding thus lent a strong support to our report on rubber formation by fresh BF particles (Tangpakdee *et al.*, 1997b) and more recent study on RB activity of WBM that did not require the presence of rubber particles (Wititsuwannakul *et al.*, 2003). Kinetic of WBM enzymes for RB catalysis was thus studied in details.

2.11 Protein profile of acetone-fractionated WBM proteins

Several procedures for membrane protein extraction are available (Cullis & Hope, 1985) and tried, which acetone was found to be most satisfactory and suitable to be employed. Advantage of using acetone to dissolve soluble membrane with the released proteins be precipitated in the same treatment steps. Serial acetone extractions from 20-80% were performed on WBM and the proteins derived from each fraction were analyzed by SDS-PAGE. The results showed that the membrane protein could be fractionated into different fractions by acetone ranges as employed. Protein profiles of distinct patterns (Figure 21) were obtained, though overlaps of some common proteins occur. Most of the acetone precipitated proteins were in the 40% and 80% acetone ranges while moderate amount were in the 20% and 60% ranges. The possible RB activity in each acetone fractions was then examined the results was then discussed in 2.12.

2.12 RB activity detection of acetone-fractionated WBM proteins

We found that no RB activity could be detected in any fractions. It seemed that they might be inactive once separated from the native membrane, or that protein composition was perturbed and different from the arrangement or topology as existing in the intact membrane. It could also be due to the absence of hydrophobic condition for them to be active. As was suggested from preceding results that high [SDS] could activate the RB activity assays, SDS was then added to the aqueous suspension of the inactive protein fractions. It was quite astonishing that the activity for rubber synthesis was restored with the SDS (Table 3).

The results showed that the maximum activity was in the 20% acetone protein fraction, whereas other fractions were much lower or without any RB activity. The RB activity was almost exclusively located in the 20% acetone fraction, which was almost equal or the same as WBM from which the protein was extracted. The possible reason for this evident could be restoration of micelle. At 2% SDS (8 x cmc), a lot of micelle with highly increased active surface area as compared to WBM could be formed with the incorporated enzymes. This might be accounted for the high RB activity even with lesser enzyme in the fraction. Although the membrane protein was distributed into fractions, it could be compensated by the highly increased active surface area for the micelle RB catalysis. Increased activity of the isolated enzyme by hydrophobic condition or micelle was previously shown for oligo-polyisoprene synthesis in the other system (Baba & Allen, 1980). Another likely reason could be the possible separation of certain inhibitory factor from the 20% protein fraction.

2.13 Enzyme kinetics of the RB active protein fraction

The preceding results suggested that the SDS micelles with the incorporated enzymes might be serving as a micro-reactor for rubber synthesis. To characterize the active rubber synthesis of this protein fraction, kinetic study on the enzyme concentrations was carried out. The results showed that RB activity was catalyzed by enzyme in this fraction, not by nonspecific trapping (Figure 22). It was clearly indicated that the enzyme was still highly active even when the [SDS] above cmc replaced the native or natural membrane condition. The results suggested it was possible to synthesize natural rubber *ex vivo* by protein extract from BF membrane if proper or optimum conditions being provided to support or stabilize the isolated enzyme activity.

2.14 RB activity of the combined or reconstituted protein fractions

As was discussed in the preceding results that fractionation of BF membrane proteins might lead to separation of certain inhibitory factor from the fraction that showed high RB activity (20% acetone protein), it would be interesting to validate this assumption. The designed experiments were carried out by mixing the highly active fraction with other low or inactive RB fractions and follow the effect (Table 4). The combining was a reconstituted assay to see if changes in RB activity were affected by the additions. WBM and the mixed WBM activity would be serving as controls to verify the changes as occurring in the combining isolated fractions.

When the most RB active fraction (0-20% acetone protein) was mixed with 20-40% acetone fraction, an increased RB activity was observed. This indicated

that the slight activity in 20-40% acetone fraction was overlap or spilled over from the first one, and thus had an additive effect in the combined assay. Another possibility is that the 20-40% acetone fraction contained an activator for RB activity as the total activity was a bit higher than the addition of the two isolated activity. Combining the 0-20 % acetone fractions with others showed an opposite effect, the RB activity was inhibited by both 40-60% and 60-80% acetone fractions. The complete inhibition exerted by the 60-80% acetone fraction indicated that the inhibitor protein is present in this fraction upon acetone separation of the proteins from BF membrane. This might lend a strong support to the result seen on the 20% acetone protein fraction that showed quite unusual high RB activity in spite of the fact that it should be expected to have lower activity due to the redistribution of enzymes upon fractionation by the acetone. It is not known if the active fraction also contains an activator of the RB enzymes and hence the unusual high RB activity. Combining or reconstitution experiment was a common practice when studying the interaction of different fractions as previously reported for WRP activation by C-serum addition (Light & Dennis, 1989) or activation of HMGR on BF membrane by C-serum calmodulin (Wititsuwannakul *et al.*, 1990). It certainly needs further study as shown recently (Kharel & Koyama, 2003) and more reconstitution assays are in progress.

The two inhibitory results for both WBM and the isolated active protein fraction was strong evidence that RB activity of the BF membrane was tightly controlled in a well regulated manner. The results from both were complimentary and supporting one another on the control mechanism that certainly exists on membrane enzyme system. It was something not totally unexpected on these results when one considering the fact that most or all of the biochemical processes need to have well

regulated control mechanisms, for metabolic balance and coordinated process of the overall metabolic pathways integration and the balanced cell functions.

This is indeed a totally new finding and quite an exciting result on the BF particles function in the latex. It thus strongly suggested that the RB enzyme in the membrane is not only capable of catalysis for rubber synthesis, but it also contains controlling element in regulatory mechanism for the rubber synthesis and the biogenesis control. This original preliminary finding on the control process of rubber synthesis needs a careful examination with well designed experiments to verify in detail of regulatory mechanism that pertain to the BF membrane for the control synthesis of new rubber molecules. Therefore, this certainly warrant more detailed study and further refined investigation to gain a better understanding of the BF membrane functions in their active roles for rubber synthesis and the control mechanism in the *Hevea* latex.

Part 3. Significant Role of Bacterial Undecaprenyl Diphosphate (C_{55}-UPP) for Rubber Synthesis by *Hevea* Latex Enzymes

For better understanding on the RB process and activity of the WBM, more refine experiments were investigated. The possibly underlie RB mechanisms on the results and observations is logically interpreted for rationale explanation. New methodology and experimental procedures are being employed for a more detailed study on the active role of WBM in synthesis of new rubber molecules. Different allylic isoprene pyrophosphate initiators for the rubber synthesis activity were tested and compared for their suitability and efficiency, especially the bacterial C_{55}-isoprene UPP. Surfactant study was also including deoxycolic acid (DOC) effect on RB activity as an extended study comparing to the SDS. Differential solvent extractions were employed for the separation of polyisoprene intermediates and the rubber product. In addition, separation of the products by RP-TLC was characterized on both qualitative and quantitative evaluations. The results obtained in this study strongly substantiated our earlier findings (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003) and are extensively discussed as presented in this part.

3.1 Comparison of WRP and WBM in rubber synthesis activity using IPP alone or with UPP as Allylic initiator

Fractionation of fresh *Hevea* latex by high speed centrifugation resulted in three distinct fractions as top rubber phase, middle aqueous C-serum, and the

sediment bottom fraction (BF) particles of membrane-bound organelles. The BF content of fresh latex is quite considerable, constituting about 20% by volume (Wititsuwannakul & Wititsuwannakul, 2001) as compared to an average of ~ 30% of the rubber phase. The results in Part 2 and our recent reports showed that the cleanly washed surface of these particles was quite active in the synthesis of new rubber molecules (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003). This is in contrast to the previous numerous studies in which the washed rubber particles (WRP) surface was implicated as the one and only prerequisite site for the in vitro RB process (Archer *et al.*, 1963; Archer & Audley, 1982; Audley & Archer, 1988). That there might be more than one site for RB activities in the latex is still doubtful and need clarification considering our BF particles reports on RB related enzymes (Koyama *et al.*, 1996; Tangpakdee *et al.*, 1997a & 1997b). The washed BF membrane (WBM) was unequivocally demonstrated to be highly active in the RB process when incubated with IPP alone as shown in our recent report (Wititsuwannakul *et al.*, 2004). This experiment is an attempt to clarify the roles of WRP and WBM in RB activities and also aimed at the possible use of bacterial derived oligoprenyl UPP for the *in vitro* rubber synthesis by the *Hevea* enzymes.

The results in Figure 23 indicated that the WBM was very active using microbial UPP in the synthesis of new rubber by WBM and only slightly by WRP. These results are in good agreement with our earlier reports (Wititsuwannakul *et al.*, 2003 & 2004) that WBM was RB active with IPP. Addition of allylic UPP to RB incubations was even more striking as evidenced by the very much more increase of WBM activity and only moderately by WRP. The WRP result was in contrast to previous report that UPP could not be used for RB by WRP (Light *et al.*, 1989) and

that the RB activity inhibited by SDS (Dennis & Light, 1989). It was thus clear that UPP was very suitable for RB process.

Many previous groups studies WRP with short allylic isoprenes (GPP, FPP, GGPP) reported the active RB function of WRP (Archer *et al.*, 1963; Archer & Audley, 1982; Audley & Archer, 1988). However, those studies were carried out with WRP prepared from the preserved latex that was quite different from our WRP immediately fractionated from the freshly tapped latex with minimum contamination by bound rupture BF membrane debris as demonstrated (Part 1) with very low RB activity. In fact, the WBM activity with IPP alone was already high about equal to WRP activity with UPP but was even much higher upon addition of UPP to the WBM assay. These results clearly indicated that UPP is more favorable by the WBM enzymes in using allylic UPP as isoprene initiator for new rubber formation. From these results it would be very interesting to further examine and characterize the WBM activity using allylic UPP as prenyl initiator for the rubber synthesis. Products of the WBM activities could also be further characterized by employing more refined two-steps differential solvent extractions. This will help differentiating and separation of oligoprenyl intermediates from the final rubber product to ascertain rubber purity. Besides, the anionic surfactant activation on RB activity of WBM should also further be characterized. Aside from SDS, DOC is also commonly used as anionic surfactant in most biochemical and enzyme study and should be tested on the WBM activities.

3.2 Effect of anionic detergents on RB activity of WBM

As stated in preceding results, WBM was highly capable of using microbial allylic UPP in new rubber synthesis and was activated by SDS as was shown for the RB activity with IPP (Wititsuwannakul *et al.* 2003 & 2004). Since our goal of this study is to clarify and characterize the utilization of UPP by WBM and try to understand the role of UPP influencing WBM activities, so the followed experiments will focus on this longer chain isoprene. This will also be compared to other short chain allylics in subsequent assays. Comparison results of the anionic detergents (surfactants) effect on WBM activities, DOC and SDS, will be presented. In addition, two-steps differential solvent extractions will also be used for products analyses of WBM activities with UPP. The differential solvents are water-saturated butanol that we have previously used in dolichols or polyprenols (Tateyama *et al.*, 1999) assays and toluene/hexane routinely used in rubber extraction and purification (Tankpakdee *et al.*, 1997; Wititsuwannakul *et al.*, 2003 & 2004).

The different effects of SDS and DOC on RB activity of the WBM incubated with ^{14}C-UPP and IPP were observed. Upon completion of RB incubation assays, the products mixture was first extracted with butanol and the remaining mixtures were then subjected to toluene/hexane extraction for the final rubber products as detailed in Methods. Butanol extraction of the RB reactions was included as added step in the procedures before the determination of rubber by toluene/hexane extract as routinely carried out in our assays (Tankpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004) for the RB activity. The extracted products in butanol fractions are for the purpose of detection for oligo- and polyisoprenyl intermediates prior to the final

rubber product. To our knowledge, no other investigators performed or included this added butanol step in their rubber synthesis assays. It is therefore debatable on the accuracy of those RB studies with WRP (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) that we discuss later on the TLC analyses of the products results.

The result in Figure 24 indicated that SDS activated more for the polyprenyl intermediates formation than the rubber formation as was seen with DOC. The higher level of polyisoprene intermediates suggested that it was accumulated or lower rubber conversion rate with SDS and hence resulted in the lower level of new rubber formation. The control without any surfactant showed that most of the ^{14}C-UPP was in the butanol phase, but the WBM was still moderately active with substantial rubber formation. This was in good agreement with preceding results (Figure 23) that showed UPP as highly suitable for the RB activity of WBM.

The results in Table 5 indicated that the DOC activation was twice faster converting or turning the intermediates into the final rubber products. However, the total combined ^{14}C-UPP converted into products in both solvent extractions were similar or almost the same for both SDS and DOC activations. Comparison of butanol extraction of RB incubations with SDS and DOC and the toluene/hexane RB extracts of both surfactants showed somewhat discrepancy ratios. However, this can be explained on the fact that butanol extractions were also included the unreacted ^{14}C-UPP in addition to the intermediates products, and the discrepancy ratios can thus be resolved.

Examination of the results in Figure 24 and Table 5 pointed out that lower intermediates with DOC was the result of rapid conversion into the final rubber product, while the higher intermediates with SDS was probably due to the slow turnover of these into the new rubber formation. Results summarized in Table 5 indicated that the total ^{14}C-UPP converted into products showed the same total ^{14}C-UPP utilization by the WBM either with SDS or DOC, but the difference was only on the rate of intermediates converted into the final rubber products. It could thus be noted that the SDS effect was active more in polyprenyl intermediates formation, but less active for the rubber formation than the DOC effect, even though both stimulated the WBM activity. The differences could thus be said on quantitative rather than qualitative effects concerning the final rubber formation steps. As for DOC effect, it was more active and rapid converting intermediates into the final rubber product, so the much lower accumulation than seen with SDS. Since DOC was shown highly stimulating the WBM activity in synthesis of the new rubber from UPP, it would be quite interesting to compare UPP with the shorter chain allylic isoprenes (FPP and GGPP) commonly used by others in the RB study with WRP (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999).

3.3 The effect of different allylic isoprenyl initiators on the rubber synthesis efficiency by WBM enzymes

The highly significant WBM activities with ^{14}C-UPP allylic initiator and the DOC activation in preceding results in this part indicated the preference of WBM

enzymes for UPP in the synthesis of new rubber molecules. The results thus far revealed quite a strong selective degree of WBM for using UPP initiator in the RB process. In order to assess and differentiate the preferential degrees of WBM for other allylics, some apt investigations were set up to compare the different allylic isoprenes for RB activities. Experiments were carried out using the different labeled allylic isoprenes with excess IPP substrate to monitor the levels of new rubber synthesis from these labeled allylics. Amounts of the final rubber products separated were evaluated by differential solvent extractions as used in Figure 16. The results compiled from these experiments using the different radiospecificity labeled allylic isoprenes were normalized by calculation as percent incorporation into the new rubbers formed out of the total added in the RB assays. The results being presented were compiled from several experiments with different WBM preparations and times to account for the seasonal variations being commonly observed, but were still with consistent trends. These results were then normalized by performing all assays with the same WBM preparations at the same time for accuracy and with high degree of confidence.

The effect of allylics chain length on rubber synthesis efficiency of WBM was clearly shown by results (Figure 15) in this study. These results on precursor allylics thus suggested that the longer chain was more effective in rubber formation by WBM than the short ones, shown by UPP and GGPP with more rubber formed than FPP. Shorter allylics were more suitable or preferable by WBM to form lower MW products than the rubber end product seen with the UPP. It was thus shown higher accumulation of the intermediates, but was still lower than the rubber products from UPP. The chain length observation was also previously studied ($C_{10} - C_{20}$) with

WRP (Audley & Archer, 1988) with only a small difference on RB effect and no clear explanation was given. It should be noted on that study with chain lengths were almost similar, FPP and GGPP were implicated as the required RB allylic initiators with WRP (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) but was never included or extended to the longer allylic like UPP. The very small difference on those allylics chain lengths thus could not provide clear cut results (Audley & Archer, 1988). It probably also be due to the intermediates bound onto the WRP and hence the small degrees of RB efficiency (Audley & Archer, 1988) might be considered insignificant and debatable. Ours was the first attempt including both the short allylics commonly believed as the required initiator (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) and the longer chain UPP (Wititsuwannakul *et al.*, 2004) studies with WBM, which was different from those WRP studies (Archer & Audley, 1987; Audley & Archer, 1988) that still need clarification and be verified.

It should be noted that in this study there are two major different aspects than those earlier studies (Archer *et al.*, 1963 & 1982; Benedict, 1983; Archer & Audley, 1987; Audley & Archer, 1988) one is the WBM as opposed to the WRP and the other is the use of longer allylic UPP along with the shorter ones. There was only one exception to these studies that used UPP and ^{14}C-IPP in the RB study of the whole latex (Light *et al.*, 1989). They found probably new rubber could be formed in the whole latex assay, but did not find nor could show any RB activity with the WRP. These results were still unclear on the different outcomes, and no further study (Light *et al.*, 1989) was attempted to clarify it. Our opinion is most likely the presence of BF

membrane in the whole latex, but the WRP was devoid of active BF membrane and hence no RB activity. This might agree well on our study with WBM that showed significant RB activity, be it the short or longer allylics with only the different RB efficiency. Therefore, the whole latex study (Light *et al.*, 1989) can be reconciled well with our earlier reports (Tankpakdee *et al.*, 1997; Wititsuwannakul *et al.*, 2003 & 2004) and the new findings in this report.

There seem to be direct or relative correlations between the allylics chain length and the RB efficiency. The longer allylic isoprenes chain sizes resulted in the more or the higher rate of new rubber formation by WBM activities as evidenced in Figure 25 results. Calculations of the combined total percent incorporations in both solvent extracts for all the three allylics revealed some interesting comparison. Even though GGPP was more RB active than FPP but less intermediates accumulation, the total percent incorporations when combined were the same at 17%. The combined total percent incorporation for UPP was at 23 % with highest proportion as the rubber product. WBM utilization of the UPP was thus almost ½ fold higher than the other two allylics for the overall WBM activities. The differences of 6 % in total incorporation and the 2 folds higher rubber formation pointed out the significant and important roles of UPP in the RB process. If all the 3 allylics were assumed to act as only the initiators, each rubber molecule would have or contain only one unit of each allylic. Therefore they should have similar or about equal percent incorporations in the rubber product when normalized by calculated as percent of the total labeled allylics with different radiospecificity added to each of RB incubation. The difference between FPP and GGPP was very small, within the errors limit, and thus could be reconciled with the assumption. Previous study with short allylics (GPP, FPP and

GGPP) on WRP (Audley & Archer, 1985) showed similar or almost equal percent incorporations, albeit insignificant difference, agreed well with our FPP and GGPP results. However, when they were compared to the UPP percent incorporation, a big difference was observed with 2 folds or almost 3 folds higher than GGPP or FPP. This was obviously not the case as assumed and there seems to be something special on the UPP properties, not only on its molecular nature, but might also be its specific recognition or preference by WBM enzymes. These perplexing and interesting results opened up to something that might help expanding the research on RB process that is still complex and little understood. They are now under extensive and refined investigation to elucidate the mechanism of RB process and the interactions between UPP and WBM enzymes system.

These results pointed out the selective or preferential degrees of WBM enzymes for the allylics chain length, with more activity for the longer ones. This might be attributed to the WBM enzymes active site affinity or differential recognition for the allylics. More detailed study of WBM enzymes was certainly needed. Recently, one of the most active prenytransferase enzymes in the *Hevea* latex, GGPP synthase gene have been cloned (Takaya *et al.*, 2003), and the key rubber transferase genes termed HTR-1 and HTR-2 (Asawatreratanakul *et al.*, 2003) that was RB active with the WBM assays. As for the allylic chain length effect in this study, the WBM activity orders were UPP>>GGPP>FPP for the rubber synthesis. So far, very little is known on WBM enzymes details as we are the first group starting this study to clarify the mystery of how and where molecules of rubber are initiated and eventually aggregated to form the rubber particles (RP). This is in opposite to the common belief and reports (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988;

Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) that the rubber was being synthesized by the RP enzymes. Our common sense certainly would indicate or suggest otherwise that this belief is rather a poor rationale, an obvious paradox, or might be misleading as to how we can explain the origin of the RP as being present in the latex.

It was thus clear that bacterial allylic UPP was highly active and suitable for synthesis the new rubber by the WBM enzymes. This is quite agreeable with the thoughtful and insight opinion recently proposed (Steinbüchel, 2003) on the microbes capable of producing rubber-like polymers. Therefore, more assays were conducted for better understanding to resolve the differences between WRP and WBM on the RB functions. More reliably accurate qualitative and quantitative analyses with suitable TLC assays will be presented.

3.4 Comparison of WRP and WBM rubber synthesis functions using allylic UPP

It was previously shown that the RB activity of WBM could be strongly activated by SDS with ^{14}C-UPP as allylic initiator together with IPP as elongating substrate for the synthesis of new rubber molecules (Wititsuwannakul *et al.*, 2004). In this study, SDS was compared with another anionic surfactant DOC for the effect on WBM and found with higher RB activation than SDS (Figure 24). DOC was therefore used in further studies on the rubber synthesis effects. As indicated in preceding results on the roles of WRP and WBM in RB functions, the experiments were carried out to compare the RB activities of WRP and WBM using allylic ^{14}C-

UPP for both and in the presence of DOC. The assays are of two purposes, comparing the RB functions of both with UPP and the DOC effects.

The results clearly indicated that UPP was highly favorable and preferentially suitable by WBM enzymes utilizing for the rubber synthesis (Figure 25 and 26). The DOC activation of WBM activity was very significant. These results and observations strongly substantiate the assumption that allylic UPP derived from bacteria could be highly acceptable for the rubber synthesis by *Hevea* enzymes as was recently speculated and proposed (Wititsuwannakul *et al.*, 2004).

For the WRP, although the DOC activation was small comparing to the WBM activity, but the WRP activity as seen was still quite significant. In past study (Wititsuwannakul *et al.*, 2003), we could not find any RB activity with WRP, and the new rubber formed by WRP could hardly be detected at all. However, in this WRP study with ^{14}C-UPP the RB activity could be significantly detected, which was different from that study with ^{14}C-IPP alone (Wititsuwannakul *et al.*, 2003). This was in contrast to the previous report that no WRP activity could be found for rubber synthesis with allylic UPP and ^{14}C-IPP substrate in RB incubation of WRP assays (Light *et al.*,1989). Our results in this study could agree well as compared to the SDS effect on WRP with allylic ^{14}C-UPP as previously reported. On the contrary, WRP without DOC showed very little or without any significant RB activity, about equal to the control inhibited with 20 mM EDTA for the control RB activity assays. The slight activation seen with WRP could possibly be attributed to the bound rupture BF membrane debris (Wititsuwannakul & Wititsuwannakul, 2001) due to shearing force during the flow of latex upon tapping, which is inevitable no matter how fresh the

latex from which the WRP is prepared. This tiny little contaminated BF membrane debris could or might thus be activated by DOC as seen in the results. Other plausible reason might arise from the fact that the WRP being used in this study was prepared from the small rubber particles (SRP) in zone 2 of centrifuged fresh latex that was previously reported to be RB active (Oh *et al.*, 1999). The WRP from SRP as used for assays in this study was with some RB activity but none for WRP prepared from the upper top rubber phase that was mainly mature RP with only two major associated proteins. When the WRP derived from SRP was characterized, it showed slightly different extractable proteins profile than that seen with mature WRP as we have previously shown in the SDS-PAGE analyses (Wititsuwannakul *et al.*, 2004). This could therefore be accounted for the observed activity with UPP and DOC effect.

Since both WBM and WRP could be detected for RB activities with UPP and were highly significant with DOC activation. It was therefore might be of interest to assay further for the nature of the products, even though it might seem quite obvious as the toluene/hexane extracted rubber products. Both qualitative and quantitative analyses by appropriate TLC separation assays with suitable solvent systems might yield some useful data and results that might further clarify the roles of UPP in the synthesis of new rubber products both by WBM and WRP activities.

3.5 Analyses of rubber products from UPP by WBM and WRP activities

The results obtained in Figure 27 showed the distinct patterns on TLC separation profiles of the toluene/hexane extracted products for WBM and WRP activities, which still need explanation pending on further investigation and

elucidation. These profiles all have symmetrical rubber spots, different than the smear diffused spot as shown in previous report (Oh *et al.*, 2000). It should be noted that all the samples analyzed were first extracted with water-saturated butanol to remove the unreacted substrates and short to medium chain intermediates. The butanol extraction step was found effective and suitable to remove these compounds (Koyama *et al.* 1985; Ohnuma *et al.*, 1998). This was then followed by toluene/hexane solvent dissolution of the rubber products for subsequent TLC analyses as detailed in Methods. As to why many bands other than the rubber products at origin were seen in this TLC separation profiles is still quite puzzling. A few speculations could be proposed to open for more investigations. Polarity index of butanol and partition efficiency for all intermediates from the complex extracting products might be possible. This problem was discussed (Tateyama *et al.*, 1999) in the extraction of polyprenols from the whole latex. One of the most likely cause or reasons could be attributed to the strong association of these intermediates onto the rubber molecules or rubber particles with high affinity, and thus some can neither be separated nor removed by butanol. Since the intermediates are still undergoing active enzymatic propagation or elongation of polyprenol hydrocarbon chains with strong hydrophobic interactions and high affinity on the rubber chains. This is also plausible be said of the allylic UPP exerting quite high affinity toward the WBM hydrophobic enzymes and resulting in the high yield of rubber being formed. In addition, it might also be coupled with strong hydrocarbon rubber chains hydrophobic interactions. It will remain to be proved in more detailed study with well defined and refined experiments. However, quantitative assays of the TLC separated rubber products might be helpful.

3.6 Quantitative analyses of rubber synthesis from allylic UPP

The results (Figure 26, 27 and Table 6) clearly indicated that the actual RB functions belong to the WBM as previously shown (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004) but not the WRP as commonly reported (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999) without well defined and accurate analyses. The small insignificant activity as shown for WRP was actually the tiny bound BF membrane debris as we suspected all along and recently set out to prove it as reported and demonstrated in Part 2 (Wititsuwannakul *et al.*, 2004). It is very important to note that the TLC separated bands intensity has no positive correlations, whatsoever, to the quantitative results (Table 6). The TLC autoradiogram as seen was very much overexposed to ascertain all non-rubber bands can be detected, no matter how low levels they are. At first glance, the data might seem contradicts to the TLC intensity, but it is actually and absolutely not. So the data presented are highly valid. They were obtained from the averages of three separated experiments with showed a high degree of reproducibility and good consistency.

From these results it could be deduced or making an extrapolating solid statement that the WBM was highly capable of rubber synthesis functions. In contrast, the WRP was not capable as compared to the highly active WBM with very high RB levels. WBM enzymes exhibited also a high degree of capability in utilizing diverse allylic isoprene initiators to synthesize new rubber molecules, be it the short chain (FPP, GGPP) or a medium to long chain isoprenes (UPP) as demonstrated in this report. It thus showed the high versatility of WBM enzymes in rubber synthesis

from the diverse different allylic isoprene with distinct and characteristic effective degrees and efficiency.

The overall results in this part clearly revealed that WBM enzymes system was highly capable of forming high MW polyisoprenes up to the rubber molecules. The highly active WBM enzymes system and the versatility in synthesis new rubber molecules from bacterial isoprene UPP might point the way for engineering microbes to synthesize rubber or the rubber-like polymers as exquisitely discussed and recently opinionated (Steinbüchel, 2003). It is therefore quite tempting to postulate that it may have certain potential degree or at least to construct the interactive combination of the microbe metabolites and plant enzymes to realize the possibility. The findings in this report certainly warrant further investigation. Of particular interest is determination of the rubber MW as resulted from the bacterial isoprene UPP. This would certainly hold a promise for a better understanding of the RB mechanism and potential utilization of the *Hevea* enzymes system for the *in vitro* synthesis of specialty functionalized rubber with the desired properties of superior functions. Part 4 will be the further reported on the molecular properties of such derived rubbers.

Part 4 Molecular analysis of rubber synthesized from bacterial undecaprenyl diphosphate (C_{55}-UPP)

The discussion presented in this part is the continuation of RB activity of WBM enzymes that we have initiated the characterization. Molecular analyses of the rubber product synthesized from bacterial UPP allylic isoprene as RB initiator was investigated. Both qualitative and quantitative analyses of the UPP derived rubber molecules were extensively examined. Comparative study of different allylics on their effectiveness and suitability for the WBM enzymes system in the rubber synthesis function and the degree of the rubber formation efficiency were also analyzed.

4.1 Allylic isoprene effects on rubber synthesis activity of WBM enzymes

We previously showed that the BF particles prepared rapidly from the freshly tapped latex was with high RB activity upon incubation with IPP and ^{14}C-IPP tracer, and that it was the newly formed rubber of low MW (Tangpakdee et al., 1997b). It was also clearly shown that the new rubber was synthesized via allylic initiator from IPP by BF enzymes (IPP isomerase and prenyltransferase) as we characterized and reported (Tankpakdee *et al.*, 1997a & 1997b; Koyama *et al.*, 1996). Subsequently, it was clearly demonstrated that the rubber synthesis was catalyzed by the enzymes system localized on the washed BF membrane (WBM) that was free of rubber particles. The common belief that the washed rubber particles (WRP) surface was the

only site of rubber synthesis as reported by several groups was still debatable. In our recent reports on WBM studies (Wititsuwannakul *et al*, 2003 & 2004), the RB function was examined and characterized for WBM enzymes system in rubber synthesis. This was then clear that WBM also served as the site for rubber synthesis in addition to WRP surface as reported by others. Thus, WBM might provide an explanation on how the rubber particles as present in the latex get originated as always questioned (Audley & Archer 1998).

The experiments were designed to clarify the RB function of WBM enzymes in the synthesis of new rubbers. The products formations from labeled IPP alone and the labeled allylics together with IPP were determined both in butanol and toluene/hexane extracts for rubber products. The results thus clearly indicated that WBM enzymes system was highly capable of rubber synthesis, either without or with allylics and that UPP was the most effective initiator (Figure 28). It was commonly believed and FPP implicated as allylic initiator for rubber synthesis on WRP surface. Our study with WBM indicated otherwise, the UPP was much more active as initiator with much higher new rubber formation by the WBM enzymes. RB results on UPP suggested that WBM enzymes might be different than that on WRP surface, which was earlier reported (Dennis & Light, 1989) incapable of using the UPP in RB process. It also suggested that the enzymes of WBM were quite flexible using different allylics in RB function, but with varied RB rates. It thus showed versatility of WBM enzymes to polymerize the allylics into rubber molecules, be it a shorter or longer one as well as the formation of allylic initiator from IPP monomers (Tangpakdee et al., 1997a & 1997b).

From the result (Figure 28), butanol extracts revealed the opposite results to those seen with the toluene/hexane extracts. RB incubation with ^{3}H-FPP was maximum activity for the labeled products. The UPP was most active for rubber synthesis, while the FPP was more active on formation of intermediates but less for rubber product. Ratio for intermediates formation with FPP of 12.2 % was 1.5 folds over 8 % incorporation for UPP. Postulation for the differences could be related to enzyme affinity and allylic chain length. UPP might possibly be more suitable or preferred by the WBM enzymes over FPP, as polyprenyl diphosphate size range of $C_{50\text{-}65}$ was abundantly predominant in *Hevea* latex and tissues (Tateyama et al., 1999). Therefore, they might be the common allylics elongated by latex enzymes to be dolichols and eventually to rubber as recently proposed (Ohya & Koyama, 2001; Kharel & Koyama, 2003). This might be accounted for the high rubber synthesis activity by WBM with UPP as seen in this study. One could also assume that conversion rate of intermediates from UPP to rubber was faster due to higher affinity with RB enzyme. Since our focus was on the rubber derived from labeled UPP, which has never been conducted or reported before, it was thus further analyzed.

4.2 RP-TLC analyses of rubber synthesized from bacterial allylic UPP

Properties of the products derived from UPP as allylic initiator for rubber synthesis by WBM enzymes system were characterized. Reverse phase-TLC with good resolution (Tangpakdee *et al.*, 1997a; Tateyama *et al.*, 1999) was used in the study. Butanol and toluene/hexane extracts were analyzed, both on qualitative and quantitative assays. Developing solvent system of acetone/hexane (19:1, v/v) for

product analyses (Ohnuma *et al.*, 1998) used in this study was found most suitable. Solvent system of acetone/water (19:1, v/v), commonly used in the RB assays (Oh *et al.*, 1999 & 2000), was found unsatisfactory for our UPP study. Patterns of similar separation profiles were obtained consistently for different RB incubations, so the system was quite reliable. In fact, this was the first analytical effort for WBM with UPP in the RB assay. The past RB study on WRP surface was still debatable on the rupture BF membrane debris tightly bound as contaminant, and this was substantiated by recent reports (Wititsuwannakul et al., 2004; Yeang *et al.*, 2002; Singh *et al.*, 2003).

The separation profiles of both butanol and toluene/hexane extracts were somewhat similar, but with minor qualitative difference (Figure 29). Unreacted substrate (^{14}C-UPP) and intermediates were first separated or removed by the butanol prior to the extraction of rubber with toluene/hexane. Both extracts showed the labeled bands at origin (rubber). However, more bands of higher intensity were detected with butanol. Toluene/hexane extract was expected to contain only the rubber, some minor bands of low intensity was also seen. It might probably be the tightly bound intermediates with rubber molecules and eluted out by our developing solvent system. This observation should be cautiously noted, especially the RB studies with WRP. The contrary results obtained from quantitative analyses of the labeled rubber products, the two extracts showed large difference on the new rubber formation with ^{14}C-UPP initiator. The ^{14}C-UPP labeled rubber (origin spot) from toluene/hexane extract was found almost 10 folds higher than that from butanol. It is possible that the slight moving diffused spot seen at the origin of butanol extracted product could be mixed polyisoprene. This would be as expected, since it was most

suitable for extraction and purification of the rubber products at the origin before measuring for the radioactivity (Tangpakdee *et al.*, 1997b; Wititsuwannakul *et al.*, 2003 & 2004).

It should be noted from the results obtained in this study. Minor contaminant with the toluene/hexane extract rubber seen in autoradiogram might possibly be the common tightly bound compounds eluted by the developing solvent as employed in our assay, but failed to be detected by different solvents in previous reports (Oh *et al.*, 1999, 2000). Another note was that the labeled rubber here was highly composed of ^{14}C-UPP by WBM activity, as opposed to the low incorporation with the shorter allylics (FPP and GGPP) shown on RB activities with WRP (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999). Determination of this ^{14}C-UPP labeled rubber molecular weight should give an insight on the nature of this newly found *in vitro* RB process by the BF membrane with the bacterial UPP allylic initiator.

4.3 Molecular weight determination of the rubber synthesized from UPP initiator

The labeled rubber derived from the RB activity of WBM using ^{14}C-UPP as allylic initiator was analyzed by GPC (Figure 30). The MW of the labeled rubber was in the range of 3 - 4 x 10^5, which was much lower than unlabeled UV detected rubber. The appearance of low MW labeled compound was the tightly bound contaminant as observed in the TLC autoradiogram. The GPC analysis was thus in good agreement with the TLC profiles as discussed in preceding results (Figure 29).

The labeled rubber obtained in this study of ^{14}C-UPP with WBM was slightly of higher MW than that previously observed on the intact fresh BF particles (Tangpakdee *et al.*, 1997b) with ^{14}C-IPP. In that study, MWD was in the range of 5 x 10^4 and 4 x 10^5 with a very broad diffused distribution compared to the (almost) symmetrical MW peak of 3 - 4 x 10^5 as obtained in this study. The results indicated clearly different outcomes as resulted from the RB incubation assays. Conditions here were using WBM free of rubber particles, if contaminated (as might be the case for the whole BF) it would be minimal, incubated with ^{14}C-UPP as allylic initiator with excess elongating substrate IPP. In previous report (Tangpakdee *et al.*, 1997b), it showed that rubber synthesis could occur with only ^{14}C-IPP alone, no allylic was added in the assay. However, the rubber formation was much slower as it took 6 h incubation time compared to only 2 hr in this ^{14}C-UPP study with much faster rate and higher amount of the newly formed rubber. In addition, the ^{14}C-incorporation in the rubber molecules was almost 4 folds above that of ^{14}C-IPP alone as shown in the sharp MW peak of the new rubber formed. The WBM results thus clearly confirmed what we saw previously with the BF, but with a more defined in RB process catalyzed by enzymes associated with WBM. The UPP was proved a much better than those short allylics (FPP, GGPP) commonly employed in the RB study and with a much faster kinetic in the rubber formation with WBM enzymes. The rubber of highly ^{14}C-labeled suggested some likelihood that was noteworthy for postulation and discussion.

The highly ^{14}C-labeled products suggested that a large number of rubber molecules of similar MW were formed with the allylic ^{14}C-UPP. It might also possibly be that each of the rubber molecules containing more than one UPP, as indicated by the very high total % ^{14}C-UPP incorporation. This was as found in

preceding TLC result (Figure 29), agreed well with this analysis (Figure 30). This is quite interesting for gaining a better understanding of the RB mechanism, which is now under active study as possibly being a new aspect on the RB process. It might point out diversity in using wide range of initiators by WBM enzymes, be it a shorter or longer ones, and versatility in RB mechanisms.

Since this is the first success reported on the rubber synthesis with UPP by WBM, it would be of interest to inquire further if UPP can also be used by WRP. So far, all RB studies were on WRP surface with short allylics (FPP, GGPP). The lingering question on how and where exactly the rubber molecules get initiated is still mystery (Audley & Archer, 1988). Recent report showed that small rubber particles (SRP) as prepared from aqueous C-serum or zone 2 of centrifuged fresh latex was highly RB active (Oh *et al.*, 1999), so SRP will be studied with allylic ^{14}C-UPP comparing to WBM under the same optimum conditions.

4.4 Activities of SRP and WBM in rubber synthesis with UPP allylic initiator

The RB activity of WBM and WRP with UPP was carried out and compared. The result clearly indicated that both WBM and WRP could initiate new rubber formation with allylic ^{14}C-UPP, but with much difference on the RB activity. The RB activity of WBM with UPP was much more pronounced than the WRP one as it was much higher labeled new rubber being detected (Figure 31).

In the very careful cumulative study on rubber synthesis by WRP with unlabeled short allylics (DMAPP, GPP, FPP and GGPP) and ^{14}C-IPP as the

elongating substrate (Audley & Archer, 1988), it was interpreted with meticulous analysis that the labeled rubber as determined was the elongation of preexisting rubber molecules, rather than the initiation of new rubber. In this study by using the labeled longer allylic (^{14}C-UPP) with the elongating substrate IPP, as the opposite, it could be assumed that the labeled rubbers were the newly formed ones being initiated from allylic ^{14}C-UPP. The postulation would then be reconciled with the common belief that polymerization in rubber synthesis was by repeated enzyme-catalyzed IPP condensation with the allylic primer, or else how could one explain the labeled rubber being formed by WRP with ^{14}C-UPP incubation. It would then be quite interesting to look further on the role of WRP in the RB process by studying it with the UPP.

4.5 Elongation of WBM synthesized rubber from allylic UPP by WRP

Since WRP was implicated for the role of either elongation of the preexisting rubber molecules (Audley & Archer, 1988) or initiation of the new rubber as in most reports (Archer *et al.*, 1963 & 1982; Audley & Archer, 1988; Light & Dennis, 1989; Light *et al.*, 1989, Dennis & Light, 1989; Oh *et al.*, 1999), it was still remained unsettle issue. In the RB studies with WRP as customarily reported above, it was difficult or impossible to separate and differentiate the newly formed rubber from the elongated rubber among the myriad rubber molecules in the WRP. The rationale is that the newly labeled rubber would immediately become part of the mixture among all the rubbers of WRP once being formed. It was still confusing unsettled question.

The ^{14}C-UPP results in Figure 31 suggested the possible role of initiation for the WRP (as prepared from small rubber particles of Zone 2), but it was still remained

an open question as being discussed above. To separate or distinguish the role of WRP in the RB process, an experiment was set up that might help clarifying the complex nature of the RB mechanisms. An idea is that if the pure labeled rubber, derived from RB assay system free of rubber particles, being incubated with the zone 2 WRP, what changes would occur to the pure labeled rubber? The outcomes and changes being observed would certainly provide some clue suggesting for the role of WRP as related to the RB functions and the mechanisms involved.

The result in Figure 32 was of something as expected, at lease on the role of WRP as carefully analyzed and well commented (Audley & Archer, 1988). When the pure labeled rubber from RB incubation of WBM with allylic ^{14}C-UPP incubated with WRP as described above, a substantial change of MW occurred with definitive magnitude. The labeled rubber as used was purified (at least 3 x) before being used for assay with WRP. The RB incubation of WRP was with the pure labeled rubber and excess IPP (1 mM) substrate. The changed MW of the labeled rubber was found to increase substantially and significantly.

The labeled rubber from WBM with ^{14}C-UPP was highly increased in MW from 3 - 4 x 10^5 to 9 x 10^5, almost 3 folds higher, as catalyzed by the WRP. The results showed that WRP surface must be very suitable site for elongation of the preexisting rubber which still actively undergoing for chain length extension. This was the first clear and convincing evidence to show and well demonstrated the active role of WRP in the rubber elongation steps. Since the RB incubation was devoid of any labeled allylic (only pure labeled rubber as obtained in Figure 30 results), so the initiation role of WRP was ruled out from the interpreted result. Since the results

from past studies on RB with WRP were quite ambiguous and confusing to assign the exact role of WRP, as discussed and detailed above on the difficulties involved. So the experimental design employed as the first and quite original in this study for WRP activity could thus clearly be elucidated or interpreted for the role of WRP in the elongation steps. This could be assigned or said as the WRP main functions in RB process. Even though this might seem a bit contradict to the WRP result with the allylic ^{14}C-UPP (Figure 31), which might suggest initiation role of WRP using UPP initiator. However, the exact nature of rubber formation by WRP activity in this study was still unclear and need to be further verified. Whether it was initiation or elongation step by ^{14}C-UPP with preexisting rubber by WRP was an open question, if the enzyme catalyzing this bizarre step ever exists? It would be a breakthrough of new discovery indeed, if such enzyme could be detected and analyzed. More refined study is under active study to differentiate the possible two options.

It was noteworthy to point out that the initial labeled rubber was almost completely converted to the higher MW size with hardly the lower one remained. However, it was elongated only to a certain limited MW size of only 9×10^5, with a sharp symmetrical peak as shown for the labeled eluted rubber products. Variations of the IPP concentration or incubation time within the allowable optimum limits had no effect on the final MW size, as the MW size average of 9×10^5 range could always be consistently observed. It is quite tempting to speculate that there might be a control factor to limit the chain length elongation, or the absence and lacking of certain factor that might further extend the rubber chain length. Enzyme aspects of the isoprenoid chain elongation (Ogura & Koyama, 1998) and the factors involve (Ogura & Koyama, 1998; Zhang & Koyama, 2001; Kharel & Koyama, 2003) were

elegantly reviewed, this could fit well with our speculated factor. This observation could only be said for *in vitro* assay only, and could not be extrapolated or implied to the actual *in vivo* conditions. Since latex was a living cytoplasm with all components interact in the colloidal suspension (Wititsuwannakul & Wititsuwannakul, 2001), including the translocation of certain proteins between the rubber and non-rubber phases (Archer & Audley, 1987; Wititsuwannakul & Wititsuwannakul, 2001). The study on isolated WRP (and also WBM) must be considered with care for disparity with the whole latex. This is indeed a very important and interesting aspect in trying to design experiments as close as possible to the latex native state. This endeavor has been attempted by us before (Wititsuwannakul *et al.*, 2003 & 2004) in reconstitution assays. Better progress in understanding of the RB process and mechanisms in the regulatory or control functions certainly will ensue out of these efforts.

Chapter 5

Summary

1 It is clearly shown that washed rubber particle (WRP), washed bottom fraction (WBF) and washed bottom fraction membrane (WBM) could carry out rubber biosynthesis (RB), although with difference capacity. The highest capacity one was demonstrated in WBM prepared from WBF.

2 The RB activity of WBM was stimulated by anionic detergents (SDS and DOC), but nonionic detergents (Triton X-100) and zwitterionic (Tween 20) had no effect on its activity. The anionic detergents effect was of biphasic manner: inhabitation at low concentration, but activation at high one.

3 The RB activity of the WBM was stimulated by anionic detergents (SDS and DOC) while SDD inhibit RB activity of WRP and DOC showed little or no effect on. DOC was found more effective for RB activity of WBM than SDS.

4 The heat-preincubation treatment of the WBM led to a much higher RB stimulation than SDS. And the heat-pretreated WBM activity exhibited an even more pronounced RB stimulation in the presence of the SDS. Moreover, the heat activation of the WBM occurred at a much lower temperature with SDS and with greater RB stimulation. That is, a synergistic effect for the RB

stimulation was observed with a combination of the heat pretreatment of WBM and the SDS detergent in the incubation mixtures.

5 Serial acetone extraction of WBM proteins showed that isolated fractions still have RB activity under appropriate conditions. The RB regulatory proteins were also detected.

6 The WBM was highly active with bacterial C_{55} (UPP) as an allylic initiator in rubber biosynthesis. Comparisons of UPP with the shorter allylics (C_{15}-FPP, C_{20}-GGPP) showed UPP was the most effective, the RB activity order of WBM were UPP>>GGPP>>FPP.

7 The DOC activated WBM synthesized more final rubber product with less poly prenyl intermediates accumulated. This is different from FPP and GGPP, with more intermediates but less of the rubber product.

8 From the GPC product analysis, the ^{14}C-UPP incorporated rubber catalyzed by WBM enzyme system showed skewed unimodal fashion with MWD of 3-4 x 10^5.

9 WRP showed slight RB activity but was much less than WBM with the ^{14}C-UPP initiator. The pure ^{14}C-UPP labeled rubber from WBM assay when incubated with the WRP showed a skewed unimodal fashion but with narrower peak than that of WBM. The MWD was show at 9 x 10^5, which was 3 folds higher than that from WBM incubation.

Conclusion

Results from this study strongly confirmed that WBM plays the key role in the RB functions, not WRP as mostly reported. WBM was thus serving as the actual rubber synthesis site, and the bacterial UPP was very good RB initiator for WBM enzymes system. The appearance of low MW rubber with allylic ^{14}C-UPP suggested that WBM serving as initiation site for the new rubber molecules formation prior to subsequently further elongated to become mature rubber of higher MW as present in the rubber particles (RP). The role of WRP in the elongation steps was clearly shown in this investigation that agreed well with the well commented and meticulously analyzed (Audley & Arther, 1998) in the earlier studies. From our WBM *in vitro* RB study together with knowledge and idea from many of previous RB pilot researchers, it guides us to the idea of hypothesis on in vivo *Hevea* rubber biosynthesis as depicted in Figure 33

The results as presented were the first to show that the bacterial oligoprenyl diphosphate (C_{55}-UPP) was very active and highly suitable serving as allylic initiator for the new rubber molecules synthesized by WBM enzymes. A schematic view (Figure 34) can thus be drawn for proposing the interactive combinations of the apt microbial metabolite (UPP) and the plant enzymes capable of rubber synthesis *in vitro*, that might possibly be manipulating in formation of some specialty rubber. This idea would of course be verified and warrant for further study.

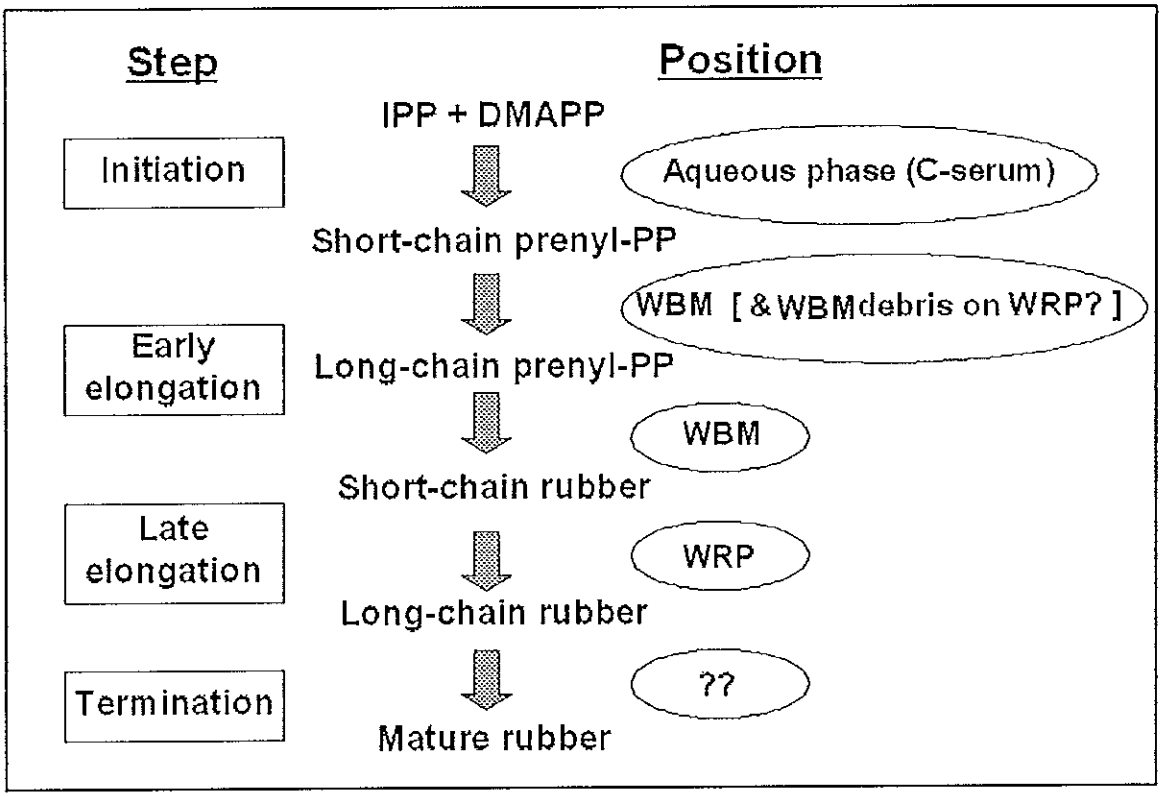


Figure 33 Schematic view of the *in vivo Hevea* rubber biosynthesis.

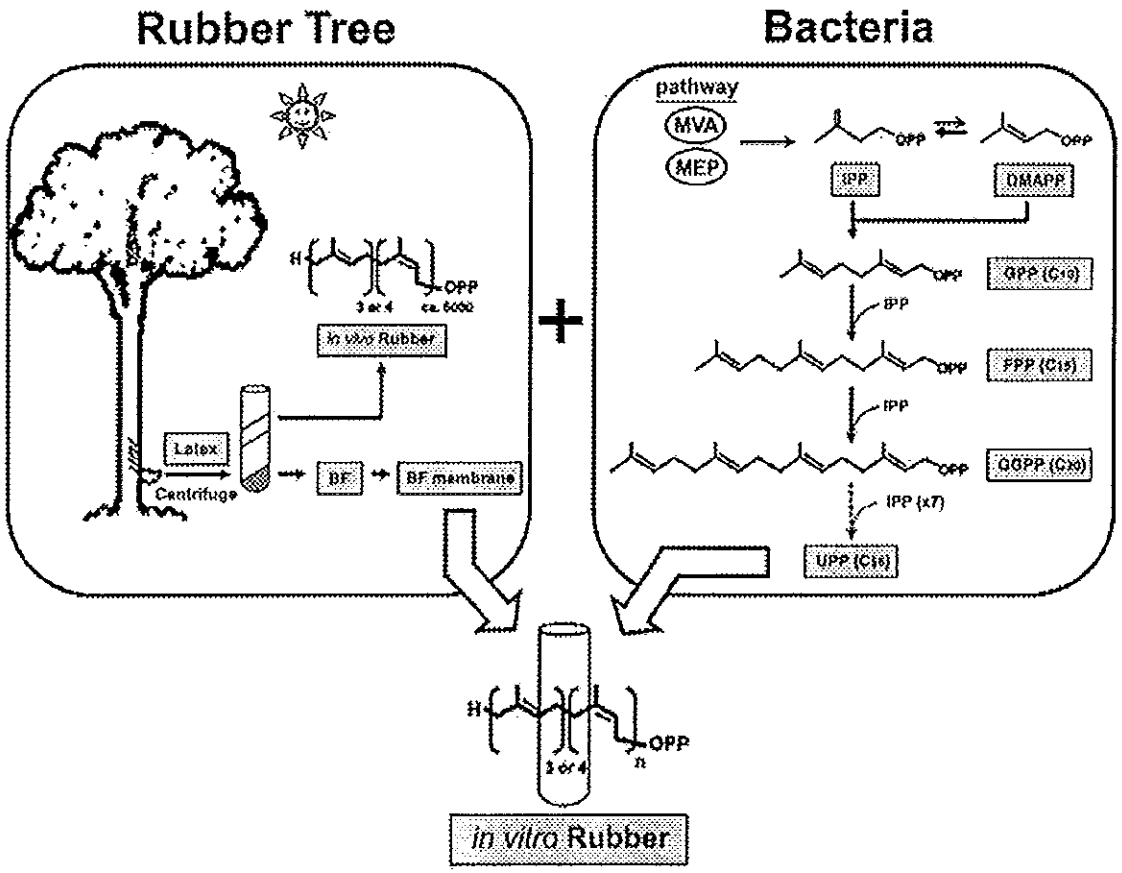


Figure 34 Schematic view proposing the interactive combinations of plant and bacteria in rubber biosynthesis

References

Allen C.M. (1985) Purification and characterization of undecaprenyl-pyrophosphate synthetase. *Methods Enzymol.* **110,** 281-299.

Allen, C.M., Alworth, W., Macrae, A. & Bloch, K. (1997) A long chain terpenyl pyrophosphate synthetase from Micrococcus lysodeikticus. *J. Biol. Chem.* **242,** 1895-1902.

Allen C.M. & Muth, J.D. (1977) Lipid activation of undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum*. *Biochemistry* **16**, 2908-2915.

Allen, C. M. & Keenan M.V. & Sack, J. (1976) *Lactobacillus plantarum* undecaprenyl pyrophosphate synthetase: purification and reaction requirements. *.Arch. Biochem. Biophys.* **175,** 236-248.

Allen, P.W. & Jones, K.P. (1988) A historical perspective of the rubber industry, in: *Natural rubber science and technology* (Robertes, A.D., ed.), pp. 1-34. New York: Oxford University Press.

Archer, B.L. & Audley, B.G. (1987) New aspects of rubber biosynthesis. *Bot. J. Linnean Soc.* **94**, 309-332.

Archer, B.L. & Audley, B.G. (1973) Rubber, gutta percha and Chicle, in: *Phytochemistry* (Miller, L.P., ed), Volume 2, pp. 310-343. New York: Von Norstrand Reinhold.

Archer, B.L. & Cockbain, E.G. (1969) Rubber transferase from hevea brasiliensis latex. *Methods Enzymol.* **15**, 476-480.

Archer, B.L., Audley, B.G. & Bealing, F.J. (1982) Biosynthesis of rubber in *Hevea brasiliensis*. *Plant. Rubb. Intl.* **7**, 109-111.

Archer, B.L., Audley, B.G., Cockbain, E.L. & McSweeny, G.P. (1963) The biosynthesis of rubber incorporation of mevalonated and isopentenyl pyrophosphate into rubber by *Hevea brasiliensis* latex. *Biochem. J.* **89**, 565-574.

Archer, B.L., Audley, B.G., Mc Sweeney, G. P. & Hong, T. C. (1969) Studies on the composition of latex serum and bottom fraction. *J. Rubb. Res. Malaya* **21**, 560-569.

Archer, B.L., Ayrey, G., Cockbain, E.G. & McSweeney, G.P. (1961) Incorporation of [1-^{14}C] isopentenylpyrophosphate into polyisoprene. *Nature*, **189**, 663-664.

Asawatreratanakul, K., Zhang, Y.W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayaporn, A. & Koyama, T. (2003) Molecular cloning, expression and characterization of cDNA encoding cis-prenyltransferases from *Hevea brasiliensis*: A key factor participating in natural rubber biosynthesis. *Eur. J. Biochem.* **270**, 4671-4680.

Attanyaka, D.P.S.T.G., Kekwick, R.G.O. & Franklin, F.H.C. (1991) Molecular cloning and nucleotide sequencing of rubber elongation factor from *Hevea brasiliensis*. *Plant Mol. Biol.* **16**, 1079-1081.

Audley, B.G. & Archer, B.L. (1988) Biosynthesis of rubber, in: *Natural rubber science and technology* (Robertes, A.D., ed.), pp. 35-62. New York: Oxford University Press.

Baba T. & Allen, C.M. (1978) Substrate specificity of undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum*. *Biochemistry* **17,** 5598-5604.

Baba T.& Allen, C.M. (1980) Prenyl transferases from *Micrococcus luteus*: characterization of undecaprenyl pyrophosphate synthetase. *Archives of Biochemistry & Biophysics. Arch. Biochem. Biophys.* **200,** 474-484.

Baba, T. & Allen, C.M. (1980) Prenyl transferase from Microcrococcus luteus. *Arch. Biochem. Biophys.* **200,** 474-484.

Baba, T., Muth, J. & Allen, C.M. (1985) Photoaffinity labeling of undecaprenyl pyrophosphate synthetase with a farnesyl pyrophosphate analogue. *J. Biol. Chem.* **260,** 10467-10473.

Bach, T.J. (1986) Hydroxylmethylglutaryl Co A reductase, a key enzyme in phytosterol synthesis. *Lipids* **21,** 82-88.

Backhaus, R.A. (1985) Rubber formation in plants - a minireview. *Israel J. Bot.* **34,** 283-293.

Backhaus, R.A., & Walsh, S. (1983) The ontogeny of rubber formation in guyule *Parthenium argentatum* Gray. *Bot. Gaz.* **144,** 391-400.

Bealing, F.J. (1976) Quantitative aspects of latex metabolism: possible involvement of precursors other than sucrose in the biosynthesis of *Hevea* rubber, in: *Proc. Intl. Rubb. Conf., Kuala Lumpur 1975*, Volume 2, pp. 543-565. Kuala Lumpur: RRIM.

Benedict, C. R. (1983) In *Biosynthesis of Isoprenoid Compounds* (Porter, J.W. & Spurgeon, S.L. eds.), Vol. 2, pp 355-369. New York: John Wiley & Son.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Broekaert, W., Lee, H., Kush, A., Chua, N.H. & Raikhel, N. (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc. Natl. Acad. Sci. USA.* **87**, 7633-7637.

Browns, M.S. & Goldstein, J.L. (1980) Multivalent feedback regulation of HMG CoA reductase, a control mechanism co-ordinating isoprenoid synthesis and cell growth. *J. Lipid. Res.* **21**, 505-517.

Chrestin, H. & Gidrol, X. (1986) Contribution of lutoidic tonoplast in regulation of cytosolic pH of latex from *Hevea brasiliensis*, in: *Proc. Int. Rubber Conf., 1986.* Volume 3, pp. 66-87. Kuala Lumpur: RRIM.

Christenson J., Gross G.S.K. & Robbins P. W. (1969) Enzymatic synthesis of the antigen carrier lipid. *J. Biol. Chem.* **244**, 5436-5439.

Churngchow, N., Suntaro, A. & Wititsuwannakul, R. (1995) β-1,3-Glucanase isozymes from latex of *Hevea brasiliensis. Phytochemistry* **39**, 505-509.

Clausen, M.K., Christansen K., Jensen, P.K. & Behnke, O. (1974) Isolation of lipid particles from baker's yeast. *FEBS Lett.* **43**, 176-179.

Cullis, P.R. & Hope, M.J. (1985) In *Biochemistry of lipids and membrane* (Vance, D.E. & Vance, J.E. eds.), pp. 25. Menlo Park: Benjamin/Cummings.

d'Auzac, J. & Jacob, J.L. (1989) The composition of latex from *Hevea brasiliensis* as a laticiferous cytoplasm, in: *Physiology of rubber tree latex* (d'Auzac, J., Jacob, J.L. & Chrestin, H., eds.), pp. 59-96. Florida: CRC Press.

Dennis, M.S. & Light, D.R. (1989) Rubber elongation factor from *Hevea brasiliensis* identification, characterization, and role in rubber biosynthesis. *J. Biol. Chem.* **264**, 18608-18617.

Dennis, M.S., Henzel, W.J., Bell, J., Kohr, W. & Light, D. R. (1989) Amino acid sequence of rubber elongation factor protein associated with rubber particles in *Hevea* Latex. *J. Biol. Chem.* **264**, 18618-18626.

Dickenson, P.B. (1969) Electron microscopical studies of latex vessel system of *Hevea brasiliensis*. *J. Rubb. Res. Inst. Malaya* **21**, 543-559.

Dupont, J., Moreau, F. Lance, C. & Jacob, J.L. (1976) Phospholipid cpmposition of the membrane of lutoids of *Hevea brasiliensis* latex. *Photochemistry* **15**, 1215-1217.

Fujisaki, S., Nishino, T. & Katsuki H. (1986) Isoprenoid synthesis in*Escherichia coli*. Separation and partial purification of four enzymes involved in the synthesis. *J. Biochem.* **99:**1327-1337.

Gidrol, X., Chrestin, H., Tan H.L., Kush, A. (1994) Hevein, a lectin like protein from *Hevea brasiliensis* (rubber tree) is involved in the coagulation of latex. *J. Biol. Chem.* **269**, 9278-9283.

Goldstein J.L. & Brown, M.S. (1990) Regulation of mevalonate pathway. *Nature*. **343**, 425-430.

Gomez, J.B. (1976) Comparative ultracytology of young and native vessel in *Hevea brasiliensis*, in: *Proc. Int. Rubber Conf., 1975.* Volume 2, pp. 143-164. Kuala Lumpur: RRIM.

Gomez, J.B. & Southorn, W.A. (1969) Studies in lutoid membrane ultrastructure. *J. Rubb. Res. Inst. Malaya* **21**, 513-523.

Gomez, J.B. & Tata, S.J. (1977) Further studies on the occurrence and distribution of microhelices in clones of *Hevea*. *J. Rubber Res. Inst. Malaya.* **25**, 120-124.

Goyvaerts, E., Dennis, M., Light, D. & Chua, N.H. (1991) Cloning and sequencing of the cDNA encoding the rubber elongation factor of *Hevea brasiliensis. Plant Physiol.* **97**, 317-321.

Hasma, H. (1987) Proteolipids of the natural rubber particles. *J. Natl. Rubb. Res.* **2**, 129-133.

Hasma, H. & Subramaniam, A. (1978) The occurrence of furanoid fatty acid in *Hevea brasiliensis* latex. *Lipids.* **13**, 905-908.

Ho, C.C., Subramanian, A. & Yong, W.M. (1976) Lipids associated with the particles in *Hevea* latex, in: *Proc. Intl. Rubber Conf.*, Volume 2, pp. 441-456. Kuala Lumpur: RRIM.

Homans, L.N.S., Dalfsen, J.W. & Gils, G.E. (1948) Complexity of fresh *Hevea* latex. *Nature* **161**, 177-178.

Jacob, J.L., Eschbach, J.M., Prevot, J.C., Roussel, D., Lacrotte, R., Chrestin, H. & d' Auzac, J. (1986) Physilogical basis for latex diagnosis of the functioning of

the laticiferous system in rubber-tree, in: *Proc. Int. Rubb. Conf.,1986.* Volume 3, pp. 43-65. Kualar Lumpur: RRIM.

Jacob, J.L., Prevot, J.C. & Kekwick, R.G.O. (1989) General metabolism of *Hevea brasiliensis* latex (with the exception of isoprenoid anabolism), in: *Physiology of rubber tree latex* (d' Auzac J., Jacob, J.L. & Chrestin, H. eds.) pp. 101-144, Florida: CRC Press.

Keenan, M.V. & Allen, C.M. (1974) Characterization of undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum. Arch. Biochem. Biophys..***161,** 375-383.

Kekwick, R.G.O. (1989) The formation of polyisoprenoids in *Hevea* latex, in: *Physiology of rubber tree latex* (d'Auzac, J., Jacob, J.L. & Chrestin, H., eds.), pp. 145-164. Florida: CRC press.

Kharel, Y. & Koyama, T. (2003) Molecular analysis of cis-prenyl chain elongating enzymes. *Nat. Prod. Rep.* **20**, 111-118.

Kleinig, H. (1989) The role of plastids in isoprenoid biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 39-59.

Koyama, T., Fujii, H. & Ogura, K. (1985) Enzymatic hydrolysis of polyprenyl pyrophosphates. *Methods in Enzymol.* **110**, 153-155.

Koyama, T., Wititsuwannakul, D., Asawatreratanakul, K., Wititsuwannakul, R., Ohya, N., Tanaka, Y. & Okura, K. (1996) Isopentenyl diphosphate isomerase in rubber latex. *Phytochemistry* **43**, 769-772.

Kurokawa, T., Ogura K. & Seto, S (1971) Formation of polyprenyl phosphates by a cell-free enzyme of *Micrococcus lysodeikticus*. *Biochem. Biophys. Res. Commun.* **45**, 251-257.

Kush, A. (1994) Isoprenoid biosynthesis: the *Hevea* factory. *Plant Physiol. Biochem.* **32**, 761-767.

Kush, A., Goyvaerts, E., Chye, M.L. & Chua, N.H. (1990) Laticifer specific gene expression in *Hevea brasiliensis* (rubber tree). *Proc. Natl. Acad. Sci. USA.* **87**, 1787-1790.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the heat of bacteriophage T4. *Nature* **227**, 680-685.

Lau, C.M., Gomez, J.B. & Subramaniam, A. (1986) An electron microscopy study of the epoxidation of natural rubber particles, in: *Proc. Intl. Rubb. Conf., 1986,* Volume 2, , pp. 525-529. Kuala Lumpur: RRIM.

Light D.R. & Dennis, M.S. (1989) Purification of a prenyltransferase that elongate *cis*-polyisoprene rubber from the latex of *Hevea brasiliensis*. *J. Biol. Chem.* **264**, 18589-18597.

Light, D.R., Lazarus, R.A. & Dennis, M.S. (1989) Rubber elongation by farnesyl pyrophosphate synthases involves a novel switch in enzyme stereospecificity. *J. Biol. Chem.* **264**, 18598-18607.

Low, F.C. (1978) Induction and control of flowing in *Hevea*. *J. Rubb. Res. Inst. Malaya* **26**, 21-32.

Low, F.C., Yeang, H.Y. (1985) Effect of ethephon stimulation on latex invertase in *Hevea*. *J. Rubber Res. Inst. Malaya.* **33**, 37- 47.

Lowry, O.H., Rosebrough, M.J., Farr, A.L. & Ramdall, R.L. (1951) Protein measurement with the folin regent. *J. Biol. Chem.* **193**, 528-544.

Lynen, F. (1969) Biochemical problems of rubber synthesis. *J. Rubber Res. Inst. Malaya.* **21**, 389-406.

Mahlberg, P.G. (1993) Laticifers: An historical prospective. *Bot. Rev.* **59**, 1-23.

McMullen, A.I. & McSweeney, G.P. (1966) Biosynthesis of rubber. *Biochem. J.* **101**, 42-47.

Moir, G.F.J. (1959) Ultracentrifugation and staining of *Hevea* latex. *Nature* **184**, 1626-1628.

Moir, G.F.J.& Tata, S.J. (1960) The proteins of *Hevea brasiliensis* latex, III. The soluble proteins of bottom fraction. *J. Rubb. Res. Inst. Malaya* **16**, 155-159.

Moreau, F., Jacob, J.L., Dupont, J. & Lance, C. (1975) Electron transport in the membrane of lutoids from the latex of *Hevea brasiliensis*. *Biochim. Biophys. Acta.* **396**, 116-124.

Muth, J.D. & Allen, C.M. (1984) Undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum*: a dimeric protein. *Arch. Biochem. Biophys.*, **230**, 49-60.

Ogura, K., Koyama, T. (1998) Enzymatic aspects of isoprenoid chain elongation. *Chem. Rev.* , **98**, 1263-1276.

Oh, S. K., Han, K.H., Ryu, S.B. & Kang, H. (2000) Molecular Cloning, Expression, and Functional Analysis of a *cis*-Prenyltransferase from *Arabidopsis thaliana*: Implications in rubber biosynthesis. *J. Biol. Chem.* **275**, 18482-18488.

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.

Ohnuma, S., Hirooka, K., Tsuruoka, N., Yano, M., Ohto, C., Nakane, H. & Nishino, T. (1998) A pathway where polyprenyl diphosphate elongates in prenyltransferase: Insight into a common mechanism of chain length determination of prenyltransferase. *J. Biol. Chem.* **273**, 26705-26713.

Ohya, N. & Koyama, T. (2001) Biosynthesis of natural rubber and other natural polyisoprenoids, in: *Biopolymer* (Koyama, T. & Steinbüchel, A., eds.), Volume 2, pp. 73-109. Weinheim: Wiley-VCH.

Paardekooper, E.C. (1989) Exploitation of rubber tree, in: *Rubber* (Webster, C.C., Baulkwill, W.J., eds) pp. 319-414. Essex: Longman.

Pakianathan, S.W. & Milford, G.F.J. (1973) Changes in the bottom fraction contents of the latex during flow in *Hevea brasiliensis*. *J. Rubb. Res. Inst. Malaya* **23**, 391-400.

Pakianathan, S.W., Boatman, S.G. & Taysum, D.H. (1966) Particle aggregation following dilution of *Hevea* latex: a possible mechanism for the closure of latex vessels after tapping. *J. Rubb. Res. Inst. Malaya.* **19**, 259-271.

Park, R.B. & Bonner, J. (1958) The enzymatic synthesis of rubber from mevalonic acid. *J. Biol. Chem.* **233**, 340-342.

Philpott, M.W. & Wesgarth, D.R. (1953) Stability and mineral composition of *Hevea* latex. *J. Rubb. Res. Inst. Malaya.* **14**, 133-148.

Pujade-Renaud, V., Clement, A., Perrot, C., Prevot, J.C., Chrestin, H., Jacob, J.L. & Guern, J. (1994) Ethylene induced increase in glutamine synthetase activity and mRNA levels in *Hevea brasiliensis* latex cells. *Plant Physiol.* **105**, 127-132.

Randall, S.K., Marshall, M.S. & Crowell, D.N. (1993) Protein isoprenylation in suspension-cultured tobacco cells. *Plant Cell* **5**, 433-442.

Rattanapittayapron, A. (1998) Rubber biosynthesis by lutoid membrane in *Hevea* latex. Master thesis in biochemistry, Mahidol University, Thailand.

Scopes, R.K. (1994) In *Protein purification principles and practice*. 3rd ed, pp. 346-348. New Delhi: Narosa Publishing House.

Sherief, P.M. & Sethuraj, M.R. (1978) The role of lipids and proteins in the mechanism of latex vessel plugging in *Hevea brasiliensis*. *Physilogia Plantarum.* **42**, 351-355.

Shimizu, N., Koyama, T. & Ogura, K. (1999) Molecular cloning, expression, and purification of undecaprenyl diphosphate synthase. No sequence similarity between *E*- and *Z*-prenyl diphosphate synthases. *J. Biol. Chem.* **273**, 19476-19481.

Singh, A.P., Wi, S.G., Chung, G.C., Kim, Y.S. & Kang, H. The micromorphology and protein characterization of rubber particles in *Ficus carica*, *Ficus benghalensis* and *Hevea brasiliensis*. *J. Exp. Bot.* **2003**, *54*, 985-992.

Southorn, W.A. (1961) Microscopy of *Hevea* latex, in: *Proc. nat. Rubb. Conf., 1960*, pp. 766-776. Kuala Lumpur: RRIM.

Southorn, W.A. (1969) Physilogy of *Hevea* latex flow. *J. Rub. Res. Inst. Malaya* **21**, 494-512.

Southorn, W.A. & Edwin, E.E. (1968) Latex flow studies, II. Influence of lutoids on the stability and flow of *Hevea* latex. *J. Rubb. Res. Inst. Malaya.* **20**, 187-200.

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.

Steinbüchel, A. (2003) Production of rubber-like polymers by microorganisms. *Curr. Opin. Microbiol.* **6**, 261-270.

Subramaniam, A. (1976) Molecular weight and other properties of natural rubber: a study of clonal variation, in: *Proc. Int. Rub. Conf., 1975*, Volume 4, pp. 3-11. Kuala Lumpur: RRIM.

Subramanium, A. (1995) The chemistry of matural rubber latex, in; *Immunology and Allergy Clinics of North America; Latex Allergy* (Fink, J.N., ed) Volume 15, pp. 1-20. Philadelphia: W.B. Saunders.

Suvachittanont, W. & Wititsuwannakul, R. (1995) 3-hydroxy-methylglutaryl-coenzyme A synthase in *Hevea brasiliensis*. *Phytochemistry*. **40**, 757-761.

Takahashi, I. & Ogura, K. (1982) Prenyltransferases of *Bacillus subtilis*: undecaprenyl pyrophosphate synthetase and geranylgeranyl pyrophosphate synthetase. *J. Biochem. (Tokyo)* **92**, 1527-1537.

Takaya, A., Zhang, Y.W., Asawatreratanakul, K., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S. & Koyama, T. (2003) Cloning, expression and characterization of a functional cDNA clone encoding geranylgeranyl diphosphate synthase of *Hevea brasiliensis*. *Biochim. Biophys. Acta* **1625**, 214-220.

Tanaka, Y., Eng, A.H., Ohya, N., Nishiyama, N., Tangpakdee, T., Kawahara, R. & Wititsuwannakul, R. (1996) Initiation of rubber biosynthesis in *Hevea brasiliensis*: Characterization of initiating species by structural analysis. *Phytochemistry* **41**, 1501-1505.

Tangpakdee, J., Tanaka, Y., Okura, K., Koyama, T., Wititsuwannakul, R. & Wititsuwannakul, D. (1997a) Isopentenyl diphosphate isomerase and prenyl transferase activities in bottom fraction and c-serum from *Hevea* latex. *Phytochemistry* **45**, 261-267.

Tangpakdee, J., Tanaka, Y., Okura, K., Koyama, T., Wititsuwannakul, R. & Wititsuwannakul, D. (1997b) Rubber formation by fresh bottom fraction of *Hevea* latex. *Phytochemistry* **45**, 269-274.

Tangpakdee, J., Tanaka, Y., Okura, K., Koyama, T, Wititsuwannakul, R. & Chareonthiphakorn, N. (1997c) Structure of *in vitro* synthesized rubber from fresh bottom fraction of *Hevea* latex. *Phytochemistry* **45**, 275-281.

Tata, S.J. (1976) Hevein: its isolation, purification and some structural aspects, in: *Proc. Int. Rubber Conf., 1975.* Volume 2, pp. 449-517. Kuala Lumpur: RRIM.

Tata, S.J. & Moir, G.F.J. (1964) The proteins of *Hevea brasiliensis* latex, V. Starch gel electrophoresis of C-serum proteins. *J. Rubb. Res. Inst. Malaya* **18**, 97-101.

Tata, S.J., Beintema, J.J. & Balabaskaran, S. (1983) The lysozyme of *Hevea brasiliensis* latex, isolation purification: enzyme kinetics and a partial amino acid sequence. *J. Rubb. Res. Inst. Malaya.* **31**, 35-48.

Tateyama, S., Wititsuwannakul, R, Wititsuwannakul, D, Sagami, H & Ogura, K. (1999) Dolichols of rubber plant, ginkgo and pine. *Phytochemistry* **51**, 11-15.

Taylor, F.R. & Parks, L.W. (1978) Metabolic conversion of free sterols and sterylesters in Saccharomyces cerevisiae. *J. Bacteriol.* **126**, 531-537.

Thomas, C.T. & Mark, G.M. (1990) Purification of membrane protein, in *Guide to protein purification.* (Murray, P.D., ed.), pp. 499-520. New York: Academic Press.

Tupy, J. (1989) Sucrose supply and utilization for latex production, in: *Physiology of rubber tree latex* (d' Auzac J., Jacob, J.-L., Chrestin, H. eds.) pp. 179-199, Florida: CRC Press.

Vehaar, G. (1959) Natural latex as a colloidal system. *Rubber Chem. Technol.* **32**, 1622-1627.

Walujono, K., Schiolma, R.A., Beintema, J.J., Mariono, A. & Hahv, A.M. (1975) Amino sequence of Hevein, in: *Proc. Int. Rubber Conf., 1975.* Volume 2, pp. 518-531. Kuala Lumpur: RRIM.

Wititsuwannakul, R. (1986) Diurnal variation of 3-hydroxyl -3-methylglutaryl coenzyme A reductase in latex of *Hevea brasiliensis* and its reletionship to rubber content. *Experientia.* **42**, 44-45.

Wititsuwannakul, R., Wititsuwannakul, D. & Suwanmanee, P. (1990a) 3-hydroxy-3-mehtyglutaryl coenzyme A reductse from the latex of *Hevea brasilensis. Phytochemistry.* **29**, 1401-1403.

Wititsuwannakul, R., Wititsuwannakul, D. & Dumkong, S. (1990b) *Hevea* calmodulin: Regulation of the activity of latex 3-hydroxy-3-methylglutaryl coenzyme a reductase. *Phytochemistry* 29, 1755-1758.

Wititsuwannakul, R., Wititsuwannakul, D. & Pasitkul, P. (1997a) Rubber latex coagulation by lutoidic lectin in *Hevea brasiliensis. Plant Physiol.* (suppl.) **114**, 71.

Wititsuwannakul, D., Wititsuwannakul, R., Pasitkul, P. (1997b) Lectin binding protein in C serum of *Hevea brasiliensis. Plant Physiol.* (suppl.) **114**, 72.

Wititsuwannakul, D. & Wititsuwannakul, R. (2001) Biochemistry of natural rubber and structure of latex, in: *Biopolymer* (Koyama, T. & Steinbüchel, A., eds.), Volume 2, pp. 151-201. Weinheim: Wiley-VCH.

Wititsuwannakul, D., Rattanapittayaporn, A. & Wititsuwannakul, R. (2003) Rubber Biosynthesis by a *Hevea* labex bottom-fraction membrane. *J. Appl. Polym. Sci.* **87**, 90-96.

Wititsuannakul, D, Rattanapittayaporn, A., Koyama, T & Wititsuwanakul, R. (2004) Involvement of *Hevea* latex organelle membrane proteins in the rubber biosynthesis activity and regulatory function. *Macromol. Biosci.* **4**, 314-323.

Yeang, H.Y. (1984) A preliminary investigation into the relationship between latex invertase and latex vessel plugging in *Hevea brasiliensis*. *J. Rubber Res. Inst. Malaya* **32**, 50-55.

Yeang, H.Y. & Paranjothy, K. (1982) Initial physiological changes in *Hevea* latex flow characteristics associated with intensive tapping. *J Rubb. Res. Inst. Malaya.* **30**, 31-36.

Yeang, H.Y., Arif, S.A.M., Yusof, F. & Sunderasan, E. (2002) Allergenic proteins of natural rubber latex. *Method* **27**, 32-45.

Yeang, H.Y., Cheong, K.F., Sunderasan, E., Hamzah, S., Chew, N.P., Hamid, S., Hamilton, R.G. & Cardosa, M.J. (1996) The 14.6 kd rubber elongation factor (Hev b1) and 24 kd (Hev b3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy. *J. Allergy Clin. Immunol.* **98**, 628-639.

Yip, E. & Southern, W.A. (1968) Latex flow studies, VI. Effects of high pressure gradients on flow of fresh lates in narrow bore capillaries. *J. Rubber Res. Inst. Malaya* **20**, 248-256.

Yusof, F., Audley, B.G., Ismail, F. & Walker, J.M. (1998) A rapid assay for the incorporation of isopentenyl diphosphate in rubber biosynthesis. *J. Rubb. Res.* **1**, 48-66.

Zhang, Y.W. & Koyama T. (2001) Biosynthetic routes Diverting from Intermediates of Isoprenoid Biosynthesis to related Products of the Metabolism, in: *Biopolymers* (Koyama, T. & Steinbüchel, A., eds.), Volume 2, pp 111-150. Weinheim: Wiley-VCH.

Publications

1. Wititsuwannakul, D., Rattanapittayapron, A. & Wititsuwannakul, R. (2003) Rubber biosynthesis by a *Hevea* latex bottom-fraction membrane. *J. Appl. Polym. Sci.* **87**, 90-96.

2. Asawatreratanakul, K., Zhang, Y.W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayapron, A. & Koyama, T. (2003) Molecular cloning, expression and characterization of cDNA encoding *cis*-prenyltransferase from *Hevea brasiliensis* : a key factor participating in natural rubber biosynthesis. *Eur. J. Biochem.* **270**, 4671-4680.

3. Wititsuwannakul, D., Rattanapittayapron, A., Koyama, T. & Wititsuwannakul, R. (2004) Involvement of *Hevea* latex organelle membrane proteins in the rubber biosynthesis activity and regulatory function. *Macromol. Biosci.* **4**, 314-323.

4. Rattanapittayapron, A., Wititsuwannakul, D & Wititsuwannakul, R. Significant Role of bacterial undecaprenyl diphosphate (C_{55}-UPP) for rubber biosynthesis by *Hevea* latex enzyme. *Macromol. Biosci.* (Accepted)

5. Wititsuwannakul, D., Rattanapittayapron, A., Koyama, T. & Wititsuwannakul, R. Molecular analysis of rubber synthesized from bacterial undecaprenyl diphosphate (C_{55}-UPP). *Biomacromolecules* (Submitted : Invited speaker, ISBP 2004)