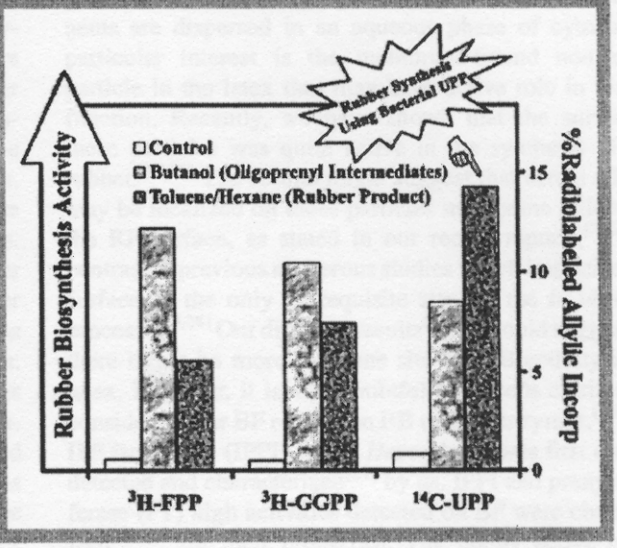
Summary: Washed bottom fraction (BF) membrane-bound particles of centrifuged fresh *Hevea* latex were found to be very active in rubber biosynthesis (RB). The washed BF membrane (WBM) showed higher RB activity and is strongly stimulated by anionic surfactants – more by DOC than SDS. WBM enzymes system can synthesize rubber either with allylic isoprenes (higher RB) or without (lower RB). Washed rubber particles (WRP), used generally in RB assays, had very low RB activity compared to the much higher activity observed for WBM. Bacterial undecaprenyl diphoshate (C_{55}-UPP) was very active allylic initiator for rubber synthesis by WBM. Comparisons of allylic UPP with the shorter ones (C_{15}-FPP, C_{20}-GGPP) showed that UPP was the most effective. WBM activity orders were UPP ≫ GGPP > FPP. The DOC activated WBM synthesized less polyprenyl intermediates (butanol extractable) but more final rubber product (toluene/hexane extract), different than FPP and GGPP. WBM enzymes were highly versatile in using diverse different allylics, but UPP was most preferable. WRP was found a little active for UPP with DOC, but still much lower than WBM. Rubber product analysis by RP-TLC with acetone/hexane solvent system showed that WBM was mostly rubber, but WRP was mainly the intermediates. Quantitative analysis showed that WBM labeled rubber was confined to the origin spot, different than WRP as mainly labeled intermediates. It was thus confirmed that the WBM plays the key role in RB functions, and not WRP as mostly reported. WBM served as the actual rubber synthesis site, and bacterial UPP was very good RB initiator.



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

Atiya Rattanapittayaporn,[1] *Dhirayos Wititsuwannakul,**[2] *Rapepun Wititsuwannakul*[1]

[1] Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkla 90112, Thailand
[2] Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
Fax: 662-248-0375; E-mail: scdwt@mahidol.ac.th

Received: July 4, 2004; Accepted: August 24, 2004; DOI: 10.1002/mabi.200400096

Keywords: bacterial UPP; *Hevea* latex enzymes; membranes; rubber biosynthesis; surfactants

Introduction

It has been demonstrated and reported for quite some time that the *Hevea* latex was active[1,2] in the synthesis of rubber molecules. Study on rubber biosynthesis (RB) process is of much interest and has appeared in several reviews.[3–7] The most extensive and up to date review covering most aspects of the *Hevea* latex structure and biochemistry[8] appeared recently with full details, discussions and the perspective outlooks. Decent understanding of the RB process is still ambiguous and the clear evidence has yet to be convincingly presented. Most studies focused mainly on rubber particles (RP) surface and always reviewed[3–7,9] as the only prerequisite site required for rubber synthesis. This might seem puzzling, an obvious paradox, on how the RP was originated. If the new rubber has to be synthesized on its surface, the question remains of the origin of RP. In layman term, one might say this is the question of chicken and egg that has long been overlooked or ignored. The actual 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 rubbers

 DOI: 10.1002/mabi.200400096

molecules that will eventually aggregate to form the RP. If one considers the complex nature of *Hevea* latex and its myriad compositions, it might possibly be that RB can take place at specific sites other than the RP surface. This is an active area of our research.

The rubbers in latex from various plants[10] are polyisoprenes of high molecular weight hydrocarbon polymers consisting of the five-carbon isoprene (C_5H_8) units. Rubber is major component of latex formed by special differentiated cells of plants. Synthesis is by series of enzyme polymerization[1] of isoprene units to various degrees, resulting in a wide range of MW. The high MW rubbers are produced in the latex of about 300 genera of Angiosperms. *Hevea brasiliensis* (Brazilian rubber trees) is the best rubber producer, and commercially cultivated for natural rubber production used industrially for various products. *Hevea* latex is accumulated in specialized cells called laticifers. Double bonds in *Hevea* rubber are in *cis* configuration as *cis*-1,4-polyisoprene, with a wide range of MW distribution. In addition to rubber particles, two other specialized particles (lutoids and Frey-Wyssling) are also present as major part of *Hevea* latex. Presence of the two particulate components provides unique characteristic to *Hevea* latex properties. *Hevea* latex biochemistry was thoroughly reviewed recently.[8]

Fresh *Hevea* latex can be fractionated into three fractions by centrifugation, the top rubber phase, aqueous C serum and bottom fraction (BF) of membrane-bound organelles. BF content is quite considerable, constituting about 20% by volume[8] compared to ca. 30% of the rubber phase. The BF is composed of membrane-bound organelles, lutoids and Frey-Wyssling particles. Fresh latex is a colloidal mixture of theree different particles with cell soluble substances in an aqueous suspension. Lutoids first described by Homans et al.[11] as single layer membrane-bound vacuoles, found rich in phosphatidic acids,[12,13] rendering them negatively charged vesicles. Lutoids contents (B-serum) are proteins, enzymes and a wide range of metabolites, considered as a type of phytolysosomes.[13] Frey-Wyssling are double layer membrane organelles with lipid globules, membrane vesicles and β-carotene.[14,15] The high carotenoid content suggested it probably contain enzymes for isoprenoids synthesis pathway.[14] So far, few studies were made to suggest the related metabolic roles of BF particles in the isoprenoids and/or RB pathways.[14,16] HMG-CoA reductase (HMGR), one of the rate-limiting enzymes in RB pathway,[17,18] was purified from the washed BF membrane.[16] It was shown to be under control by calmodulin,[19] heat stable Ca^{2+} binding protein in C-serum, as HMGR activator.

It was commonly believed that RP surface was RB active, as extensively characterized and reported in a series of four consecutive papers with lot of extensive details.[20–23] However, RB activity at certain membrane site is more likely with good rationale reconciling on the origin of RP. This was earlier postulated,[14,24] but received little attention and no investigation was carried out to substantiate it. Study conditions, free of preexisting rubber particles, 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 cytosol. Of particular interest is the membrane-bound non-rubber particle in the latex that may have active role in the RB function. Recently, we have shown that the surface of these particles was quite active in the synthesis of new rubber.[25,26] The results might suggest that actual RB site may be localized on these particles membrane rather than the RP surface, as stated in our recent reports,[26,27] in contrast to previous numerous studies which implicated RP surface as the only prerequisite site for the *in vitro* RB process.[2,9,28] Our different results[25–27] could suggest that there might be more than one site for RB activity in the latex. However, it is still doubtful and needs clarification considering our BF reports on RB related enzymes.[25,29,30] IPP isomerase (IPPI) in the *Hevea* latex was first directly detected and characterized[29] by us. IPPI and prenyltransferase (PT) high activities detected on BF were characterized[29,30] for their properties. The highly active rubber formation by fresh BF particles[25] was clearly shown. Kinetic study on RB activity and products analyses showed new appearance of the low MW rubber.[25] The results suggested the synthesis of new rubber being initiated and formed by these particles enzymes. Further careful studies on RB activity of the membrane[26] from the BF particles were carried out with detailed properties characterized. Extensive washed BF membrane (WBM) showed high RB activity, clearly indicating that the RB activity was located on the isolated membrane. The RB stimulation of WBM by surfactant is probably resulting from the increased active surface area of RB active mixed micelles. In a recent report,[27] the delayed use of fresh latex led to rupture of BF particles and membrane debris bound to the WRP was shown. This may be the case for those using the preserved WRP with RB activity[3–7,9] as resulted from the bound debris. The most noticeable result was the serial acetone extracted WBM proteins being still RB active.[27] Recently, two *Hevea* latex genes[31,32] 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.

It was quite intriguing that an exquisite idea proposed recently on microbes might be capable of producing rubber.[33] This coincides with our ongoing research on rubber synthesis from bacterial undecaprenyl diphosphate (C_{55}-UPP). Among family microbial prenyltransferases, UPP synthase (UPS)[34,35] has been most extensively studied. It was purified and characterized from several bacteria (*S. newington*,[36] *B. subtilis*,[34] *E. coli*,[37,38] *L. plantarum*,[39–43] and *M. luteus*[44,45]). 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 in the literature[46] and provided to us for using as allylic initiator of rubber synthesis by WBM.

In this report we 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 are also reported. Surfactant DOC effect was tested and compared to SDS on RB activation.[26,27] Comparisons of WBM and WRP activities with UPP are also made. In addition product analyses, qualitative and quantitative on the rubbers formed with UPP are extensively presented here.

Experimental Part

Materials

Isopentenyl diphosphase (IPP), farnesyl diphosphate (FPP), sodium dodecyl sulfate (SDS), deoxycholic acid (DOC) and organic solvents are all of analytical grade. Other analytical chemicals and reagents used in this study were mainly purchased from Sigma-Aldrich (St. Louis, MO). [1-^{14}C] Isopentenyl diphosphase (^{14}C-IPP, 54 mCi mmol^{-1}) was from Amersham Biosciences. [1-^{3}H] Farnesyl diphosphate (^{3}H-FPP, 20 Ci mmol^{-1}), [1-^{3}H] Geranylgeranyl diphosphate (^{3}H-GGPP, 20 Ci mmol^{-1}) were from American Radiolabelled Chemicals Inc. Uniformly labeled undecaprenyl diphosphate (^{14}C-UPP) and the UPP synthase (UPS) were generously provided by Prof. Dr. Koyama (Tohoku University, Japan). They were also prepared by us according to the published procedure[46] using the UPS enzyme with the same quality and purity as provided.

Collection of Fresh Latex for Centrifugation

Fresh latex used in this study was obtained from regularly tapped rubber trees (clone RRIM 600) at the adjoining Songkla Rubber Research Center, Thailand. These trees were tapped in a half-spiral 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 containers and was immediately subjected to centrifugation within less than 10 min from the tapping collection time.

Preparation of Washed Bottom Fraction Membrane (WBM)

The freshly tapped latex was immediately fractionated by centrifugation to obtain the three distinct fractions as described[16,26,27] with maximum sediment bottom fraction (BF) and minimum rubber particle (RP) associated or contaminated protein. The collected BF was washed five times by careful suspension in 50×10^{-3} M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl (w/v) so that the intact washed bottom fraction particle (WBP) was obtained with no accompanied small RP. The washed BF membrane (WBM) was then prepared from the intact WBP as described.[26,27] The cleaned WBP pellet was suspended in 3 volumes of distilled water and stirred for hypotonic lysis of WBP. All contaminants were then eliminated from the membrane by three times repeated washing. All operations were carried out at 0–5 °C for membrane integrity and stability. The WBM was kept in the ice-bath until use.

Preparation of Washed Rubber Particles (WRP)

Rubber particles was prepared from the centrifuged zone 2 rubber as described[24] by three repeated washing with 5 volumes of 50×10^{-3} M Tris-HCl buffer (pH 7.4) to obtain the WRP for assays. All operations were carried out at 0–5 °C. The prepared WRP was kept cool in icebox until use. The rubber quantity was determined by measuring the absorbance at 280 nm. The rubber content was calculated as described by Light and Dennis.[20]

Rubber Biosynthesis (RB) Assays and Incubation Conditions

The incubation mixture contained designated amount of samples (WBM or WRP) in Tris-HCl buffer, pH 7.7. The 50×10^{-3} M Tris-HCl buffer (pH 7.7) for the RB assays included reagents (30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 10×10^{-3} M DTT). The substrate unlabeled IPP (or ^{14}C-IPP) and allylic initiators (^{3}H-FPP, ^{3}H-GGPP or ^{14}C-UPP) were added as indicated in the figure and table captions. 20×10^{-3} M EDTA was added in the incubation uses as control as mentioned in figure captions. All the RB incubation mixtures were carried out at 30 °C for 2 h. After 30 °C optimum incubations, the reaction was chill stopped by placing the incubation tubes in an icebox and was immediately processed for the radiolabeled products extraction and product analysis.

Two-Steps Differential Solvents Extractions of Radiolabeled Products

Right after the reaction was stopped, 300 µl saturated NaCl solution was added and mixed thoroughly. The mixture was then treated (three times) with 500 µl H_2O saturated 1-butanol. The radioactivity in 1-butanol phase was subjected to a liquid scintillation counter to estimate the amount of radiolabeled polyprenyl intermediate. The aqueous layer (with membrane at the interphase) left after 1-butanol extraction was again treated (three times) with 500 µl of toluene/hexane (1:1, v/v). The amount of radioactivity in toluene/hexane phase was determined for the RB activity by measure the ^{14}C-labeled rubber with liquid scintillation counter. The product analysis of 1-butanol and toluene/hexane extracts were then performed on RP-TLC as described below.

Product Analysis by Using 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.[47] The products were subjected to RP-TLC plate (LKC-18, Merck) using acetone/hexane (19:1, v/v) as the solvent system.[48] The RP-TLC plates were then exposed overnight on a Fuji film BAS-III imaging plate at room temperature. The

distributions of radiolabeled products were visualized with bioimage analyzer (Fuji BAS 1000 Mac). The sizes of the radiolabeled products were determined comparing with the authentic standard alcohols that were run along with the samples and visualized with iodine vapor.

Results and Discussion

The results presented in this report are further investigations with more refined and well designed experiments to delineate the results we previously reported.[27] This is for a better understanding of the RB process and activity of the latex WBM. The results and observations are logically interpreted for rationale explanation of the possible underlying RB mechanism. New methodology and experimental procedures are 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 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[25,26] and are extensively discussed as presented in this report.

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[8] as compared to an average of ca. 30% of the rubber phase. We have recently shown that the cleanly washed surface of these particles was quite active in the synthesis of new rubber molecules.[25,26] This is in contrast to the numerous previous studies in which the washed rubber particles (WRP) surface was implicated as the one and only prerequisite site for the in vitro RB process.[2,9,28] That there might be more than one site for RB activities in the latex is still doubtful and needs clarification considering our BF particles reports on RB related enzymes.[25,29,30] 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.[27]

The result presented in Figure 1 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, as we have previously shown the SDS on RB activation of WBM.[26,27] The RB incubations assays were either with ^{14}C-IPP alone (Figure 1, A) or ^{14}C-UPP and IPP (Figure 1, 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, the 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 1, B2). All the assays with their specific controls are by the presence of 20×10^{-3} M EDTA that can completely inhibit RB activity. The overall results (Figure 1-A, 1-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 1-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[26,27] that WBM was RB active with IPP. Addition of allylic UPP to RB incubations (Figure 1-B) was even more

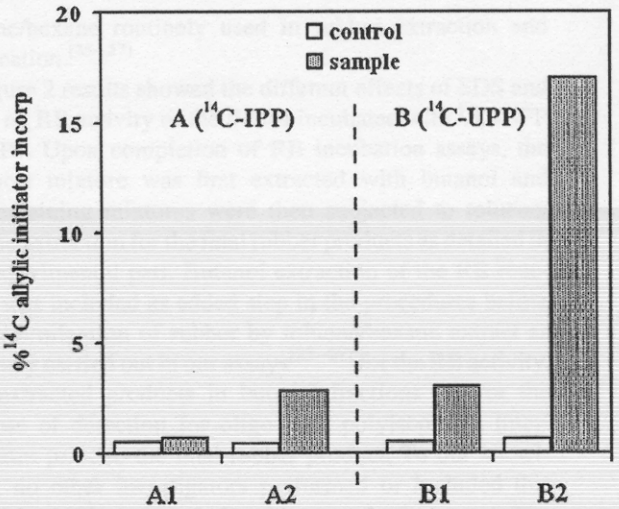


Figure 1. RB activity of WRP (1) and WBM (2) in the incubations with ^{14}C-IPP (A) or ^{14}C-UPP (B) as allylic initiators. The activity was shown as percent of the ^{14}C-allylic initiators incorporation into rubber molecules. Each tube of incubation mixture (300 µl) contained WRP or WBM (approx. 30 mg dry weight) in 50×10^{-3} M Tris-HCl buffer (pH 7.7), 2% SDS (w/v), 30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 10×10^{-3} M DTT and 40×10^{-6} M ^{14}C-IPP (5 ci mol^{-1}). In the case of using ^{14}C-UPP, unlabeled IPP (60×10^{-6} M) was added together with the ^{14}C labeled bacterial UPP (245,000 cpm). 20×10^{-3} M EDTA was added in the incubations used as controls.

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[21] and that the RB activity inhibited by SDS.[22] 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.[2,9,28] 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 we recently demonstrated [26,27] 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 quite 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. Products of the WBM activities could also be further characterized by employing more refined two-steps differential solvent extractions. This will help in 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.

Effect of Anionic Surfactants on RB Activities 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.[26,27] Since the 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 surfactants effect on WBM activities, deoxycholic acid (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[49] assays and

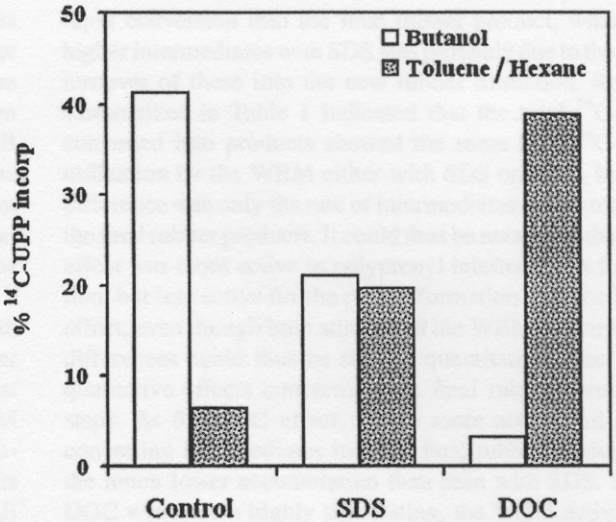


Figure 2. Effect of anionic surfactants (SDS and DOC above critical micelle concentrations) on RB activity of WBM enzymes. The results were shown as percent ^{14}C-UPP incorporation into polyprenyl intermediates that were extracted by 1-butanol. The RB activity for rubber products were shown as percent ^{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 50×10^{-3} M Tris-HCl (pH 7.7), 30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 10×10^{-3} M DTT, 60×10^{-6} M unlabeled IPP, ^{14}C-UPP (100 900 cpm) and the surfactants (SDS or DOC). No detergent was added in the control condition.

toluene/hexane routinely used in rubber extraction and purification.[25–27]

Figure 2 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 the experimental part. 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[25–27] 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. The accuracy of those RB studies with WRP[2,9,20–22,28,50] is therefore debatable and will be shown and discussed later on the TLC analyses of the products results.

As shown in Figure 2, the RB activation by DOC on WBM activity was 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 almost 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 1) that showed UPP as highly suitable for the RB activity of WBM.

The overall calculated results are summarized in Table 1 and show the distributions of the ^{14}C-UPP labeled products. The butanol extraction with SDS yielded 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, was 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 in the ratios, which was a bit puzzling. However, this can be explained by 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 2 and Table 1 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 1 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 the rate of intermediates converted into the final rubber products. It could thus be noted that the SDS effect was more active 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.[2,9,20–22,28,50]

Table 1. 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).

samples	^{14}C-UPP incorporation[a)]		
	%		
	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

[a)] The data represent the average of three determinations.

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 (Figure 1, 2) indicated the preference of WBM enzymes for UPP in the synthesis of new rubber molecules. The results so 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 2. 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 with several discussion aspects for a clearer understanding with minimum ambiguity.

Results in Figure 3 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

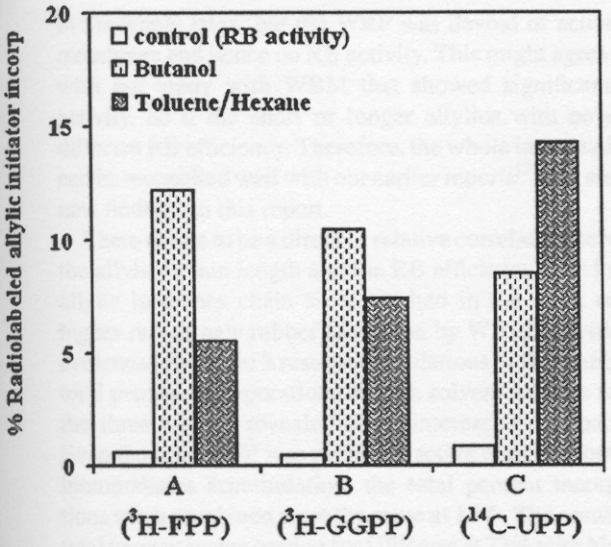


Figure 3. Enzyme activity of WBM shown in percent radiolabeled allylic initiators incorporation into polyisoprene intermediates in 1-butanol extract and rubber products in toluene/hexane (1:1, v/v) extract. The incubation mixture (200 µl) contained WBM (approx. 20 mg dry weight) in 50×10^{-3} M Tris-HCl (pH 7.7), 30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 10×10^{-3} M DTT, 40×10^{-3} M DOC, 60×10^{-6} M IPP and radiolabeled allylic initiators [12.5×10^{-6} M ^{3}H-FPP (7 ci mol^{-1}), 12.5×10^{-6} M ^{3}H-GGPP (7 ci mol^{-1}) and 100 900 cpm ^{14}C-UPP as prepared]. 20×10^{-3} M EDTA was added in the incubations used as controls.

under the same optimum conditions with DOC. The products formation, from these radiotracer allylic isoprenes, was 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 3, 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 3, 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, 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 3 were somewhat similar or analogous to the results observed for SDS and DOC comparisons on WBM assays with UPP (Figure 2). 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 2). 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.

The effect of allylics chain length on rubber synthesis efficiency of WBM was thus clearly shown by results (Figure 3) 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. Higher accumulation of the intermediates was thus shown, but was nevertheless still lower than the rubber products from UPP. The chain length observation was also previously studied (C_{10}–C_{20}) with WRP[9] with only a small difference on RB effect and no clear explanation was given. It should be noted that in that study, chain lengths were almost similar, FPP and GGPP were implicated as the required RB allylic initiators with WRP[2,9,20–22,28,50] 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.[9] It might probably also be due to the intermediates bound onto the WRP and hence the small degrees of RB efficiency[9] might be considered insignificant and debatable. Ours was the first attempt including both the short allylics commonly believed as the required initiator[2,9,20–22,28,50] and the longer chain UPP[27] studies with WBM, which was different from those WRP studies[3,9] that still need clarification and be verified.

It should be noted that in this study there are two major different aspects than the earlier studies-[2,3,9,18,28]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.[21] 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[21] 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 with 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[21] can be reconciled well with our earlier reports[25–27] and the new findings in this report.

There seems to be a direct or relative correlation 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 3 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 the calculated percentage of the total labeled allylics of different radiospecificity added to each 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)[9] on WRP 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 possibilities 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, we have cloned one of the most active prenytransferase enzymes in the *Hevea* latex, GGPP synthase gene,[31] and the key rubber transferase genes termed HTR-1 and HTR-2[32] that was RB active with the WBM assays. As for the allylic chain length effect in this study, the WBM activity orders were UPP $\gg$ 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 contrast to the common belief and reports[2,9,20–22,28,50] 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[33] 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 reliable, accurate, qualitative and quantitative analyses with suitable TLC assays will be presented.

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.[27] In this study, SDS was compared with another anionic surfactant DOC for the effect on WBM and found with higher RB activation than SDS as shown in Figure 2. 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 in Figure 4 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 4, B2), but WRP was also slightly activated (Figure 4, A2) even though quite 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

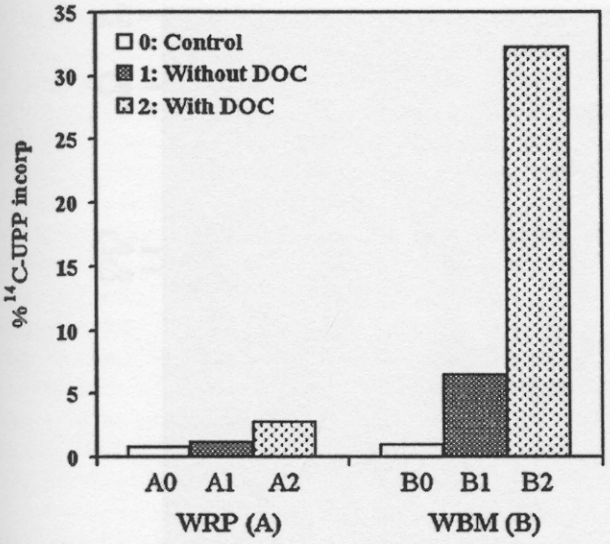


Figure 4. RB activity of WRP (**A**) and WBM (**B**) compare between incubation in the absence (**1**) and presence of DOC (**2**). The activity was shown in percent ^{14}C-UPP incorporation into rubber which was extracted by toluene/hexane (1:1, v/v). The incubation mixture (500 µl) contained WRP or WBM (approx. 30 mg dry weight) in 50×10^{-3} M Tris-HCl buffer (pH 7.7), 30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 10×10^{-3} M DTT, 60×10^{-6} M IPP and ^{14}C-UPP (354 000 cpm) with and without 40×10^{-3} M DOC. The control incubations (**A0** and **B0**) were done with 20×10^{-3} M EDTA added.

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.[7]

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 a 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 a previous study,[26] 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.[26] 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.[21] Our results in this study agree well as compared to the SDS effect on WRP with allylic ^{14}C-UPP as previously reported.[27] On the contrary, WRP without DOC showed very little or without any significant RB activity, about equal to the control inhibited with 20×10^{-3} M EDTA for the control RB activity assays. The slight activation seen with WRP could possibly be attributed to the bound rupture BF membrane debris[8] 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.[50] 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 the 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.[27] 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 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.

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[2,9,20–22,28,50] 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, they will be discussed separately and then assimilated interpretations made. Qualitative analysis will be done by effective reverse phase TLC that we performed on polyprenols.[49] The products as derived from TLC separation assays will then be subjected to quantitative analysis as routinely carried out in our RB studies.[25–27]

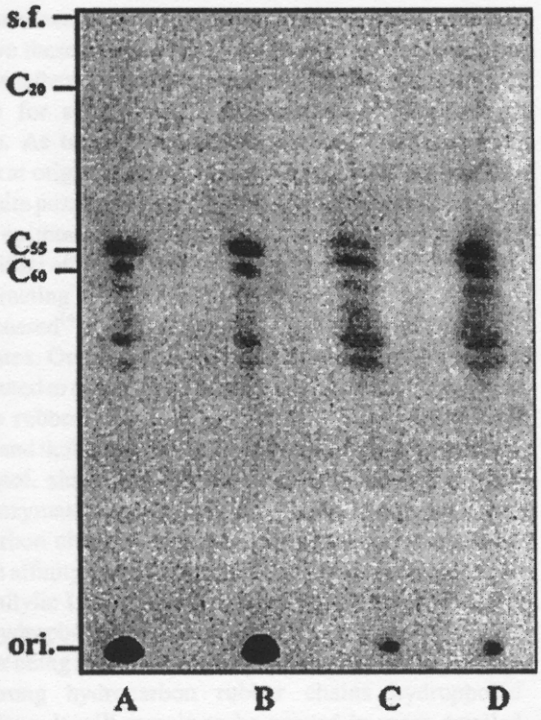


Figure 5. RP-TLC autoradiogram analysis of RB products by WBM and WRP. After product extraction and dephosphorelation as mentioned in Experimental part were performed, the products were separated on RP-TLC plate (LKC-18, Whatman) with a solvent system of acetone/hexane (19:1, v/v). The plate was exposed on image plate and analyzed by a Bio-image analyzer. Lane **A**: products from WBM incubation without DOC, lane **B**: products from WBM incubation with the presence of 40×10^{-3} DOC, lane **C**: products from WRP incubation without DOC, and land **D**: products from WBM incubation with the presence of 40×10^{-3} M DOC. On the left shows number of carbon according to authentic standards run along with the samples. **ori.** :origin, **s.f.** :solvent front.

Qualitative analysis of the WBM and WRP with allylic UPP incubation products were shown in Figure 5. The analyzed products were the toluene/hexane extract (Figure 4) 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[48] for polyisoprenes separation. However, the solvent system of acetone/water (19:1, v/v) [51] as previously used for the assay of RB products 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. The rubber did not move in this solvent system, but removed the intermediates from the origin as we previously demonstrated for the labeled rubber.[25] The acetone/water solvent showed only rubber spots with no intermediates, hence it was unsuitable for our investigations, as reported.[51] It was clear that our analyses were reliable and accurate for further quantitative analyses of 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,[2,9,20–22,28,50] which need to be reexamined. The results as shown in Figure 5 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, 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 stated earlier, 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 in contrast to 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 5) 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 4) the rubber synthesis level, it could be attributed to 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.

The results thus obtained showed the distinct patterns on TLC separation profiles of the toluene/hexane extracted products for WBM and WRP activities, which still need explanation pending further investigation and elucidation. These profiles all have symmetrical rubber spots, different than the smear diffused spot as shown in previous report.[51] 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.[47,48] 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 up more investigations. Polarity index of butanol and partition efficiency for all intermediates from the complex extracting products might be possible. This problem was discussed[49] 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 for 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.

Quantitative Analyses of Rubber Synthesis from Allylic UPP

From the TLC separation profiles and autoradiogram results (Figure 5), 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 5), be it for WBM and WRP or the assay conditions with DOC effects. Results on rubber quantitative analyses were shown in Table 2 as extended assays of Figure 5 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.[26,27] Quantitative assays (Table 2) 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 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.[26,27] Quantitative assay results revealed that WBM was very active in rubber synthesis function, but much less for WRP.

Results in Table 2 on quantitative assays of rubber products (Figure 5) was quite similar to the toluene/hexane extract results (Figure 4), 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 2 (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.[25–27] but not the WRP as commonly reported [2,9,20–22,28,50] without well defined and accurate analyses. The small insignificant activity as shown for WRP

Table 2. Quantitative analysis of the rubber synthesized by WBM and WRP with ^{14}C-UPP. The incubation condition was according to Figure 4. The total ^{14}C-UPP used in each incubation assay was 354 000 cpm.

Samples		^{14}C-UPP incorporation[a)]			
		Toluene/hexane extract		Origin spot	
		cpm × 10^3	% incorp	cpm × 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

[a)] The data represent the average of three determinations.

was actually the tiny bound BF membrane debris as we suspected all along and recently set out to prove.[27] 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 2. 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, our Table 2 data might seem contradict 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 and an extrapolating solid statement can be made 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 (Figure 1–6) as shown 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 by Steinbüchel.[33] 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. This will of course be further pursued and subsequently reported on the molecular properties of such derived rubbers.

Conclusion

Bacterial undecaprenyl diphoshate (C_{55}-UPP), lipid carrier of glycosyl residues in the cell wall synthesis, was found very suitable and highly effective for rubber synthesis by the *Hevea* latex enzymes. The washed bottom fraction membrane-bound particles of centrifuged fresh latex was rubber biosynthesis (RB) active. Washed BF membrane (WBM) showed much higher RB activity, strongly stimulated by anionic surfactants, with DOC being more effective than SDS. WBM enzymes can synthesize rubber with allylic isoprenes or without (but lower RB). Washed rubber

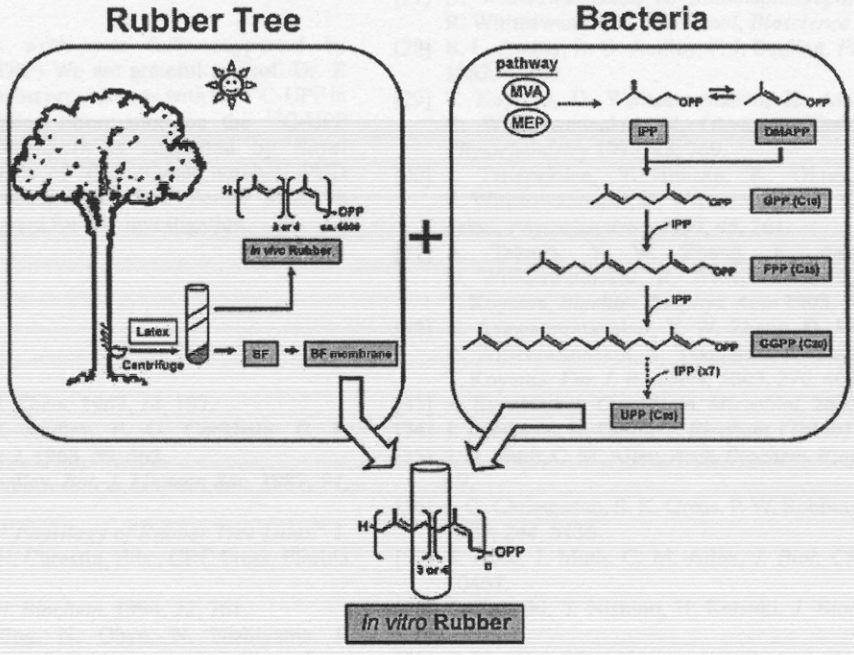


Figure 6. Schematic view proposing the interactive combinations of plant and bacteria in rubber biosynthesis.

particles (WRP) was very low on RB activity compared to the very much higher RB by WBM. Bacterial undecaprenyl diphoshate (C_{55}-UPP) was very effective as an allylic initiator for rubber synthesis by WBM. Comparisons of UPP with the shorter allylics (C_{15}-FPP, C_{20}-GGPP) showed UPP was the most effective. The RB activity orders of WBM were UPP $\gg$ GGPP > FPP. The DOC activated WBM synthesized more final rubber product (toluene/hexane extract), with less polyprenyl intermediates (butanol extractable) accumulated. This is different than FPP and GGPP, with more intermediates but less of the rubber product. Enzymes on WBM were highly versatile in using diverse different allylics, and UPP was most preferable. WRP was found little active with UPP + IPP, but inactive with IPP alone.

RP-TLC analyses of rubber product with acetone/hexane solvent system, quantitative and qualitative, were in good agreement with the WBM incubation RB assay results.

Results from this study strongly confirmed that WBM playing 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. A schematic view (Figure 6) 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 warrants study.

Acknowledgements: This work was fully supported by *Thailand Research Fund* (TRF) We are grateful to Prof. Dr. *T. Koyama* for his generous support providing us with the ^{14}C-UPP in this research and the detailed information on the ^{14}C-UPP purification. *A. Rattanapittayaporn* is supported by *Royal Golden Jubilee*-Ph.D program of TRF (Grant number: PHD 0086/2542). *R. Wititsuwannakul* is the TRF Senior Research Scholar with TRF research grant for this investigation.

[1] F. Lynen, *J. Pure Appl. Chem.* **1967**, *14*, 137.
[2] B. L. Archer, B. G. Audley, E. G. Cockbain, G. P. McSweeney, *Biochem. J.* **1963**, *89*, 565.
[3] B. L. Archer, B. G. Audley, *Bot. J. Linnean Soc.* **1987**, *94*, 181.
[4] R. G. O. Kekwick, in "*Physilogy of Rubber Tree Latex*" J. d'Auzac, J. L. Jacob, H. Chrestin, Eds., CRC Press, Florida 1989, p. 145.
[5] A. Kush, *Plant Physiol. Biochem.* **1994**, *32*, 761.
[6] Y. Tanaka, A. H. Eng, N. Ohya, N. Nishiyama, J. Tangpakdee, S. Kawahara, R. Wititsuwannakul, *Phytochemistry* **1996**, *41*, 1501.
[7] N. Ohya, T. Koyama, in "*Biopolymers: Vol. 2, Polyisoprenoids*", T. Koyama, A. Steinbüchel, Eds., Wiley-VCH, Weinheim 2001, Chapter 4, p. 73.
[8] D. Wititsuwannakul, R. Wititsuwannakul, in "*Biopolymers, Vol. 2, Polyisoprenoids*", T. Koyama, A. Steinbüchel, Eds., Wiley-VCH, Weinheim 2001, Chapter 6, p. 151.
[9] B. G. Audley, B. L. Archer, in "*Natural Rubber Science and Technology*" A. D. Roberts, Ed., Oxford University Press, New York 1988, Chapter 2, p. 35.
[10] R. A. Backhaus, *Israel J. Bot.* **1985**, *34*, 283.
[11] L. N. S. Homans, J. W. Van Dalfsen, G. E. Van Gils, *Nature* **1948**, *161*, 177.
[12] J. Dupont, F. Moreau, C. Lance, J. L. Jacob, *Phytochemistry* **1976**, *15*, 1215.
[13] S. Pujarniscle, *Physiol. Veg.* **1968**, *6*, 27.
[14] P. B. Dickenson, *J. Rubb. Res. Inst. Malaya* **1969**, *21*, 543.
[15] J. B. Gomez, S. J. Hamzah, *Natl. Rubb. Res.* **1989**, *4*, 75.
[16] R. Wititsuwannakul, D. Wititsuwannakul, P. Suwanmanee, *Phytochemistry* **1990**, *25*, 1401.
[17] R. Wititsuwannakul, *Experientia* **1986**, *42*, 44.
[18] C. R. Benedict, in "*Biosynthesis of Isoprenoid Compounds, Volume 2*", J. W. Porter, S. L. Spurgeon, Eds., John Wiley & Son, New York 1983, p. 355.
[19] R. Wititsuwannakul, D. Wititsuwannakul, S. Dumkong, *Phytochemistry* **1990**, *29*, 1755.
[20] D. R. Light, M. S. Dennis, *J. Biol. Chem.* **1989**, *264*, 18589.
[21] D. R. Light, R. A. Lazarus, M. S. Dennis, *J. Biol. Chem.* **1989**, *264*, 18598.
[22] M. S. Dennis, D. R. Light, *J. Biol. Chem.* **1989**, *264*, 18608.
[23] M. S. Dennis, W. J. Henzel, J. Bell, W. Kohr, D. R. Light, *J. Biol. Chem.* **1989**, *264*, 18618.
[24] G. F. J. Moir, *Nature* **1959**, *184*, 1626.
[25] J. Tangpakdee, Y. Tanaka, K. Okura, T. Koyama, R. Wititsuwannakul, D. Wititsuwannakul, *Phytochemistry* **1997**, *45*, 269.
[26] D. Wititsuwannakul, A. Rattanapittayaporn, R. Wititsuwannakul, *J. Appl. Polym. Sci.* **2003**, *87*, 90.
[27] D. Wititsuwannakul, A. Rattanapittayaporn, T. Koyama, R. Wititsuwannakul, *Macromol. Bioscience.* **2004**, *4*, 314.
[28] B. L. Archer, B. G. Audley, F. J. Bealing, *Plant. Rubb. Intl.* **1982**, *7*, 109.
[29] T. Koyama, D. Wititsuwannakul, K. Asawatreratanakul, R. Wititsuwannakul, N. Ohya, Y. Tanaka, K. Okura, *Phytochemistry* **1996**, *43*, 769.
[30] J. Tangpakdee, Y. Tanaka, K. Okura, T. Koyama, R. Wititsuwannakul, D. Wititsuwannakul, K. Asawatreratanakul, *Phytochemistry* **1997**, *45*, 261.
[31] A. Takaya, Y. W. Zhang, K. Asawatreratanakul, D. Wititsuwannakul, R. Wititsuwannakul, S. Takahashi, T. Koyama, *Biochim. Biophys. Acta* **2003**, *1625*, 214.
[32] K. Asawatreratanakul, Y. W. Zhang, D. Wititsuwannakul, R. Wititsuwannakul, S. Takahashi, A. Rattanapittayaporn, T. Koyama, *Eur. J. Biochem.* **2003**, *270*, 4671.
[33] A. Steinbüchel, *Curr. Opin. Microbiol.* **2003**, *6*, 261.
[34] I. Takahashi, K. Ogura, *J. Biochem. (Tokyo)* **1982**, *92*, 1527.
[35] J. D. Muth, C. M. Allen, *Arch. Biochem. Biophys.* **1984**, *230*, 49.
[36] J. G. Christenson, S. K. Gross, P. W. Robbins, *J. Biol. Chem.* **1969**, *244*, 5436.
[37] T. Baba, J. Muth, C. M. Allen, *J. Biol. Chem.* **1985**, *260*, 10467.
[38] S. Fujisaki, T. Nishino, H. Katsuki, *J. Biochem.* **1986**, *99*, 1327.
[39] C. M. Allen, M. V. Keenan, J. Sack, *Arch. Biochem. Biophys.* **1976**, *175*, 236.

[40] C. M. Allen, J. D. Muth, *Biochemistry* **1977**, *16*, 2908.
[41] C. M. Allen, *Methods Enzymol.* **1985**, *110*, 281.
[42] T. Baba, C. M. Allen, *Biochemistry* **1978**, *17*, 5598.
[43] M. V. Keenan, C. M. Allen, *Arch. Biochem. Biophys.* **1974**, *161*, 375.
[44] T. Kurokawa, K. Ogura, S. Seto, *Biochem. Biophys. Res. Commun.* **1971**, *45*, 251.
[45] T. Baba, C. M. Allen, *Arch. Biochem. Biophys.* **1980**, *200*, 474.
[46] N. Shimizu, T. Koyama, K. Ogura, *J. Biol. Chem.* **1998**, *273*, 19476.
[47] T. Koyama, H. Fujii, K. Ogura, *Methods in Enzymol.* **1985**, *110*, 153.
[48] S. Ohnuma, K. Hirooka, N. Tsuruoka, M. Yano, C. Ohto, H. Nakane, T Nishino, *J. Biol. Chem.* **1998**, *273*, 26705.
[49] S. Tateyama, R. Wititsuwannakul, D. Wititsuwannakul, H. Sagami, K. Ogura, *Phytochemistry* **1999**, *51*, 11.
[50] S. K. Oh, H. Kang, D. H. Shin, J. Yang, K. S. Chow, H. Y. Yeang, B. Wagner, H. Breiteneder, K. H. Han, *J. Biol. Chem.* **1999**, *274*, 17132.
[51] S. K. Oh, K. H. Han, S. B. Ryu, H. Kang, *J. Biol. Chem.* **2000**, *275*, 18482.

