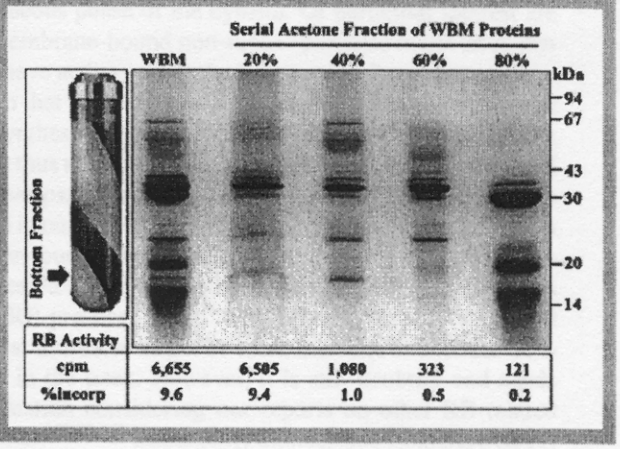
Summary: Centrifugation of fresh *Hevea* rubber latex yields three distinct fractions. The sediment bottom fraction (BF) content of membrane-bound organelles is ca. 20 vol.-% of latex. Prolonged storage or delayed use of fresh latex will result in disintegration and loss of the bottom fraction. This is due to the osmotically sensitive BF rupture and its membrane debris being tightly bound to the top rubber particles (RP) phase. The BF membrane was found to be highly active for rubber biosynthesis (RB), in contrast to previous reports that describe RB only occurring on the RP surface. It was clearly shown that washed BF membrane (WBM) was much more active than fresh RP for RB activity. WBM was highly activated by SDS for RB in a biphasic manner, but SDS strongly inhibited the RP. Probably WBM micelle formation resulted in a highly increased active surface area for RB. C_{55}-PP (UPP) was a very active allylic for WBM in RB function, but inactive for RP. Serial acetone extraction of WBM proteins showed a distinct profile of the fractions with different RB activity. WBM isolated proteins suspended in 2% sodium dodecyl sulfate (SDS) with an RB activity equal to that of intact WBM was with the 20% acetone protein fraction. The 60 and 80% fractions were inactive. Combining the 20 with 80% fractions showed a complete inhibition of RB activity. Complete RB loss was also found when WBM was mixed with the 80% fraction, indicating that WBM has both an enzyme system and a factor for regulation of the RB activity in a well controlled metabolic function for the latex RB process.



Involvement of *Hevea* Latex Organelle Membrane Proteins in the Rubber Biosynthesis Activity and Regulatory Function

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Introduction

Rubbers in the latex from various plants[1] are polyisoprenoids of high molecular weight hydrocarbon polymers consisting almost entirely of the five-carbon isoprene (C_5H_8) units. The polyisoprene rubber is the major component of latex synthesized by specially differentiated cells of the plants. They are synthesized by a series of enzyme-catalyzed polymerizations[2] of the 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. Among them, *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 that are 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 size distribution. In addition to rubber particles, two other specialized particles (lutoids and Frey-Wyssling particles) are also present as major constituents of the *Hevea* latex. The presence

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of these two major particulate components provides the unique characteristic to the *Hevea* latex properties. The structure of *Hevea* latex and its detailed biochemistry has recently been thoroughly and extensively reviewed.[3]

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. The BF content is quite considerable, constituting about 20 vol.-%[3] of the fresh latex as compared to an average of ca. 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 these different particles, together with the cell soluble substances in an aqueous suspension. Lutoids were first described by Homans et al.[4] as membrane-bound vacuoles, with the single layer membrane rich in phosphatidic acids,[5] 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.[6] Frey-Wyssling particles are double layer membrane organelles containing lipid globules, membrane vesicles, and β-carotene.[7,8] The high carotenoid content suggested it might contain enzymes for the isoprenoids synthesis pathway.[7] So far, only few studies were performed that might suggest the related metabolic roles of these particles in isoprenoids and rubber biosynthesis (RB) pathways.[7,9] HMG-CoA reductase (HMGR), presumably one of the rate-limiting enzymes in the RB pathway,[10] was purified from the washed BF membrane.[9] It was shown to be under control by calmodulin,[11] the Ca^{2+} binding heat stable protein in the C-serum, as the activator of this HMGR enzyme.

Hevea latex has been shown to be active[2,12] in the synthesis of rubber for quite some time. The study on rubber biosynthesis (RB) process is of much interest as it appeared in several reviews.[13–17] Details or understanding of the RB process is still ambiguous and a clear evidence has not yet been convincingly presented. Most of studies focused mainly on the surface of rubber particles (RP) and was always reviewed[13–18] 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. In the layman term, one might say this is the question of chicken and egg that has long been overlooked or ignored. The true RB initiation sites other than the RP surface with active rubber formation need to be sought out. It's 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,[7,19] but has received little attention nor careful investigations were carried out to substantiate this suggestion. A study under the condition that it is free of rubber particles should be attempted because it would 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 are the membrane-bound non-rubber particles in the latex that may have active role in the RB process. Recently, we have shown that the surface of these particles was quite active in the synthesis of new rubber molecules.[20,21] The results might thus suggest that the actual RB site could be localized on these particles membrane other than the RP surface. As stated in our most recent report,[21] this was in contrast with the previous numerous studies, in which the RP surface was implicated as the one and only prerequisite site for the in vitro RB process.[12,18,22] Our different results[20,21] could suggest that there might be more than one site for RB activities in the latex. However, it is still doubtful and needs clarification considering our reports on other RB related enzymes.[20,23,24] Isopentenyl diphosphate isomerase (IPPI) in the *Hevea* latex was clearly detected and characterized for the first time by our group.[23] We also detected the high IPPI and prenyl transferase (PT) activities in the BF particles and their properties thoroughly characterized.[23,24] Consequently, we clearly showed highly active rubber formation by fresh BF particles.[20] The presence of high IPPI, PT and rubber transferase (RT) activities in the same BF particles were also determined.[24] A kinetic study on ^{14}C-IPP incorporation into the rubber and the product analyses showed new appearance of the low molecular weight rubber molecules.[20] 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[21] from the BF particles were carried out and detailed properties were characterized. Extensively washed BF particles (WBP) and the derived washed BF membrane (WBM) on RB activities were compared. It was clearly shown that the RB activity was located on the isolated membrane. Parameters affecting the membrane functions were investigated on the RB process. Effect of detergents and heat treatments of the WBM before being subjected to the optimum RB assays conditions were characterized in details.[21] The RB stimulation results thus suggested a possible increase of active surface area by formation of micelle caused by these parameters. The active membrane RB assays clearly indicated that the synthesis of new rubber could effectively occur with no requirement of the RP prerequisite site.

In this report, we have further characterized the RB activity of the washed BF membrane (WBM). We show that the RP rapidly isolated from the freshly tapped latex

contains only two proteins with very low or no RB activity, and 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 content, 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% sodium dodecyl sulfate (SDS). Thus, the postulation of micelles as the cause for the enhanced RB activity, as earlier reported,[21] is strongly supported by the results in this study. Even more interesting are experiments on mixing or reconstitution of WBM fractionated proteins, because they provide a good indication for the RB control mechanism by the proteins present or localized in the membrane.

Experimental Part

Materials

Isopentenyl diphosphase (IPP, 1 mg · ml^{-1}), sodium dodecyl sulfate (SDS) and analytical chemicals used in this RB study were mainly obtained from Sigma-Aldrich (St. Louis, MO). [1-^{14}C] Isopentenyl diphosphase (^{14}C-IPP, 54 mCi mol^{-1}) was from Amersham Biosciences. Undecaprenyl diphosphate (UPP), uniformly labeled (^{14}C-UPP) and UPP synthase (UPS) were generously provided by Dr. Koyama (Tohoku University, Japan). They were also prepared by us according to Koyama's published procedure,[25] using the UPS enzyme with the same quality and purity as provided. Other chemicals, reagents and solvents used were all of analytical grade.

Collection of Fresh Latex for Immediate 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 beakers and was immediately subjected to centrifugation within less than 10 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 BF particles.

Preparation of Washed Bottom Fraction Particles (WBP)

The freshly tapped latex was immediately fractionated by centrifugation without delay to obtain the three distinct fractions as described[9,21] with maximum sediment BF and minimum RP associated or contaminated protein. They were routinely monitored and checked to ascertain the consistency of samples before the assays. Intact BF particles were prepared as reported,[21] with a slight modification. 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 WBP was obtained, with no small RP present. All operations were carried out at 0–5 °C. The intact WBP as prepared was kept in an ice-bath. An aliquot was immediately used for the preparation of BF membrane for the RB assays. All operations were carried out at 0–5 °C.

Preparation of Washed Bottom Fraction Membrane (WBM)

Washed BF membrane (WBM) was prepared from the intact WBP as described.[21] 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 high speed centrifugation and repeated three times after each washing to obtain WBM cleansed of any contamination. All operations were carried out at 0–5 °C for membrane integrity and stability. The washings were carried out under the same method as that used for the WBP preparation and kept in the ice-bath until used.

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 as described.[26] The acetone-precipitated protein from each step was collected by centrifugation at $10\,000 \times g$ and dried up under N_2 gas as powdered protein and stored at −20 °C until use for the assay. The protein concentration was determined by Bradford assay.[27]

Preparation of Washed Rubber Particles (WRP)

Rubber particles were prepared from the centrifuged zone 2 rubber as described[19] by repeated washing three times 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 an icebox until used. The rubber quantity was determined by measuring the absorbance at 280 nm. The rubber content was calculated as described by Light and Dennis:[28] (0.04 ± 0.002 mg of rubber)/A280.

Rubber Biosynthesis (RB) Assays and Incubation Conditions

The incubation mixture, in a final volume of 300 µl, contained designated amounts of samples (WBP, WBM, WRP, or acetone extracted WBM protein) in Tris-HCl buffer, pH 7.7, as indicated in the captions for Figure and Table. The 50×10^{-3} M Tris-HCl buffer (pH 7.7) for the RB assays was included reagents (30×10^{-3} M KF, 5×10^{-3} M $MgCl_2$, 1×10^{-2} M DTT) and 40×10^{-6} M [1-^{14}C] IPP (2.5 ci · mol), unless otherwise indicated. For the assays using C_{55}PP (UPP) as allylic acceptor for the RB activity, the ^{14}C uniformly labeled product of UPPS (^{14}C-UPP at 245 000 dpm) was used together with 40×10^{-6} M unlabeled IPP substrate. All the RB incubation mixtures were carried out at 37 °C for 6 h, unless indicated otherwise. 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.

Rubber Extraction and RB Activity Determination

The labeled rubber product was extracted by precipitation of the rubber out from the incubation mixtures. Right after the reaction was stopped, 1.2 ml cold ethanol were added into the incubation mixtures and immediately separated by centrifugation at 5 000 × *g* for 10 min. The WRP prepared as described was added as the carrier to every incubation tube before the precipitation step by the addition of cold ethanol. This step was taken to assure quantitative recovery of the labeled rubber product. This was considered necessary because only a little amount of product was formed which might not be completely self-aggregated in order to sediment out after ethanol addition and centrifugation. 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 of a 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.[7,8] The amount of radioactivity in the purified rubber was determined for the RB activity by determining the ^{14}C-labeled rubber with liquid scintillation counter.[20,21]

Results and Discussion

The results presented in this paper are extended detailed analyses of our recent report[21] on the active RB function of the washed BF membrane (WBM). Several criteria are considered for the experiments designed to ascertain and convincingly show that the RB activity can actually occur without the presence of the RP surface which is not required. This will help lending more and stronger support on our earlier reported results.[20,21] To clarify the still remaining ambiguity, some trivial, long overlooked aspect with important implications is carefully documented in experiments reported herein.

Osmotic Sensitivity of the Fractionated Latex Bottom Fraction Particles

The 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.[29,30] Addition of 0.7% ammonia or the buffered glycerol as latex preservative led to a complete loss of the BF as recently shown by Yeang et al.[31] However, the stability of intact fresh latex as fractionated immediately after tapping has never been carefully monitored and documented.

Our study on the time course of the fresh latex fractions shown in Figure 1 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 h, (tube D, E) only two fractions remained as rubber and mixed C-serum phases, while the BF diminished. The membrane debris from disrupted BF was associated with the RP as puffy rubber phase (tube E). This could probably be a hydrophobic interaction with high binding affinity to the RP.

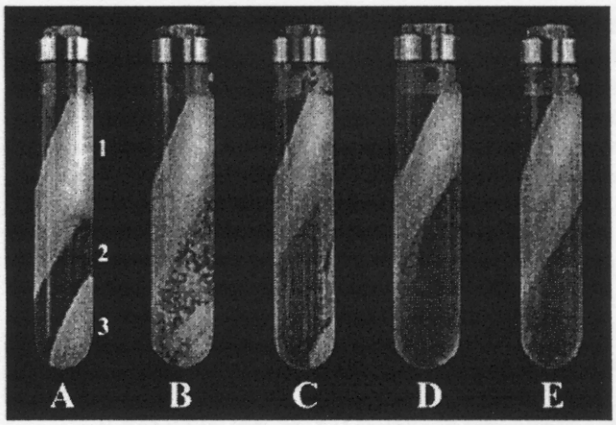

Figure 1. Fractionation by centrifugation of fresh *Hevea* latex. A: Freshly tapped latex (0 time); B: after standing at room temperature for 45 min; C: 60 min; D: 90 min; E: 120 min. (1: rubber phase; 2: C-serum; 3: bottom fraction).

The study was repeated several times on different fresh latex samples with no added preservative. The average bursting time of the isolated BF was around 40–50 min.

The results thus clearly showed that prolonged storage or delayed use of fresh latex will 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 in Figure 1 for all the RB assays, if not indicated otherwise. The next study was carried out to see how the difference on RP proteins could be observed.

Comparison of the RP Proteins Under Different Conditions

Analyses on the RP associated proteins of different RP samples 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 1, tube A and E) and the preserved latex RP commonly used in RB study by others.[13–18] The

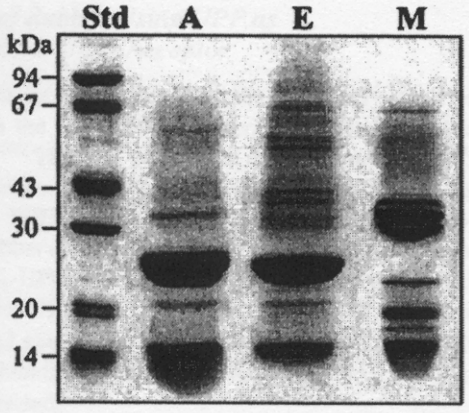

Figure 2. SDS-PAGE analyses of proteins extracted from different fractions of latex. A: Proteins from RP of freshly tapped latex (Figure 1, tube A); E: proteins from RP of the delayed use latex (Figure 1, tube E); C: proteins from WBM prepared from freshly tapped latex (Figure 1, tube A); Std: standard molecular weight proteins.

SDS-PAGE results revealed quite different protein patterns (Figure 2). The fresh RP (lane A of tube 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 showed several other prominent protein bands in addition to the two intrinsic ones (lane E). 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.[32]

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 fragments might help contribute RB activity to the RP. The preserved RP commonly used, as reviewed in the RB study[13,18] 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 BF membrane (WBM), as we have recently reported.[21] 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.

RB Activity of Fresh RP and Washed BF Membrane (WMB)

In order to clarify the actual or exact RB site in the latex, RB activity of the isolated fresh RP and the washed BF membrane (WBM) were carefully compared. 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 a factor responsible for RB activity commonly observed with the preserved washed rubber particles (WRP) in those reports.[12–14,18] Considering the high binding affinity, it is presumed that the burst BF membrane once associated with the RP, will act like a part of the RP component itself. So the isolated freshly tapped RP was used for all experiments to minimize this possibility and was compared to the BF membrane on RB activity. Results in Figure 3 clearly showed a big difference in RB function of the two specimens.

It was found that the RB activity of the isolated fresh RP was very low compared to the high RB activity of the WBM under the same assay conditions. The 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 convincingly indicated that the WBM did indeed possess the enzyme system for rubber formation. On the contrary, the fresh WRP as prepared (tube A, Figure 1) 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.[13,18] It can be explained on the different degrees of contamination on the WRP with BF membrane which was then overlooked. Only quite recently we have presented the convincing evidence that the RB site is actually located on the BF membrane.[20,21]

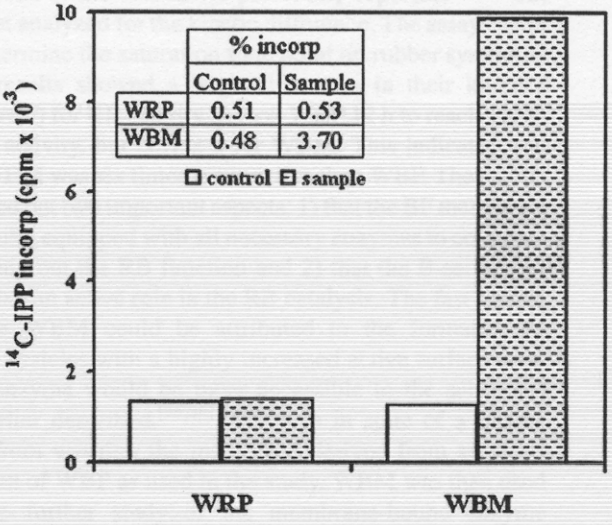

Figure 3. RB activity of WRP and WBM were shown as ^{14}C-IPP incorporation into rubber molecules. Each tube of incubation mixtures contained WRP or WBM (approx. 30 mg dry weight) and 20×10^{-6} M [1-^{14}C] IPP (20 Ci · mol^{-1}). All other conditions were followed as in Experimental Part. 20×10^{-3} M EDTA was added in the incubations used as controls. Inserted Table: Showing the percent incorporation of ^{14}C-IPP into the rubber.

Formation of Rubber Using UPP as Allylic Isoprene Units Acceptor

We have previously shown that the fresh BF particles possess both the IPPI and PT activities active in rubber synthesis.[20,24] This was also found with BF membrane[21] and as shown in the preceding results. A previous study showed that WRP could use the low MW allylic diphosphate for rubber synthesis with IPP,[18] but WRP could not use UPP (C_{55}-UPP) to form the rubber.[33] Recently, it was suggested that UPP is possibly elongated by IPP to form dolichols and eventually rubber molecules.[17] Dolichols were quite abundant in *Hevea* tissues and latex and extensive characterized.[34]

Experiments were carried out to test this postulation by incubation C^{14}-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 terms of the different magnitude of RB activities (Figure 4) between the fresh WRP and WBM. The WRP showed very low or no RB activity, but the WBM was very active in the rubber formation from UPP. This was in agreement with a previous report that WRP was not active for rubber synthesis when incubated with UPP and C^{14}-IPP substrate.[33] It was noted that WRP had a higher incorporation ratio with C^{14}-UPP than with C^{14}-IPP incubation as shown in Figure 3. This might due to some minor proteins on the fresh WRP (Figure 2) with activity for UPP condensation and because the C^{14}-UPP used in the assay was uniformly labeled.[25] The formation of UPP by UPP synthase in *M. luteus* has been extensively

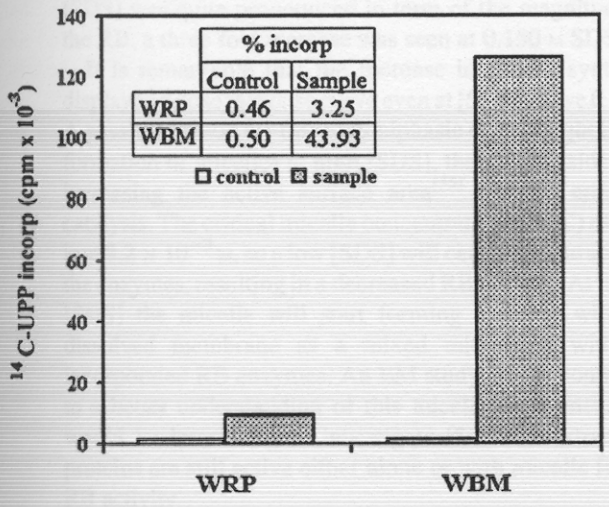

Figure 4. ^{14}C-labeled rubber formed by the activity of WRP and WBM using ^{14}C-UPP as an allylic substrate. Each tube of incubation mixture contained WRP or WBM (approx. 30 mg dry weight). The incubation condition was done according to Experimental part. 20×10^{-3} M EDTA was added in the incubations used as controls. Inserted table: Showing the percent incorporation of ^{14}C-IPP into the rubber.

characterized.[25] Recently it was suggested that UPP could also be a precursor in *Hevea* latex to form rubber.[17]

The results in Figure 4 thus clearly show that WBM possess the enzyme system of a wide range for rubber synthesis, be it small allylic acceptors (GPP, FPP, GGPP) or a larger one like UPP in this study. In an earlier study, it was shown that the whole latex is active for rubber synthesis when incubated with UPP and C^{14}-IPP,[33] but was with no activity with WRP. This strongly supported our results with the WBM activity. The whole latex RB activity with UPP[33] 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 in their capability to catalyze the synthesis of rubber was thus very clear in this study. The active RB function of WBM indicated that it contains the membrane-associated enzyme system carrying out rubber synthesis. The enzyme system was absent in the fresh WRP, unless contaminated, as speculated. The finding thus strongly supported our report on rubber formation by fresh BF particles[20] and a more recent study on the RB activity of WBM that did not require the presence of rubber particles.[21] The kinetic of WBM enzymes for RB catalysis was thus studied in details.

Comparison of the Kinetics of the RB Catalytic Rate between WBF and WBM

The RB activity between the intact washed BF particles (WBP) and the derived membrane (WBM) was compared for the catalytic rate. This was a further study on the RB functions of the two as we previously reported,[20,21] but not yet analyzed for the kinetic difference. The assays were to determine the saturation time point on rubber synthesis. The results showed a large difference in their kinetics (Figure 5) for RB activity. It took WBP 12 h to reach maximum activity, but only 2 h for WBM. This indicated that the WBM was six times more active than WBP. The results pointed 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.[35,36] 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.

We have earlier shown that the RB activity of WBM could be activated by the anionic detergent SDS[21] but not by any other groups. It was not clear then how SDS could bring about this RB activation. It was commonly observed in WRP study that several detergents, but not SDS,

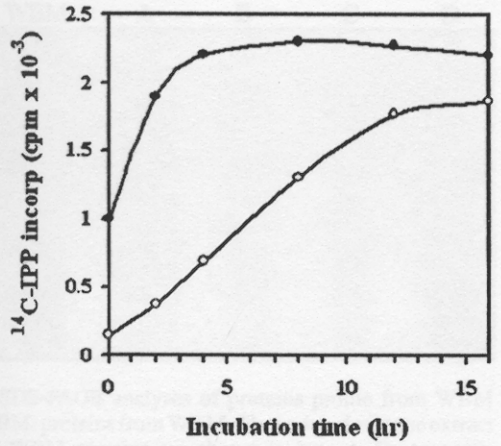

Figure 5. Kinetic of RB catalytic rate between WBP (○) and WBM (●). Each incubation contained WBP (approx. 20 mg dried weight) or WBM which was prepare from the same amount of WBP. The incubation assay condition was carried out as detailed in Experimental part.

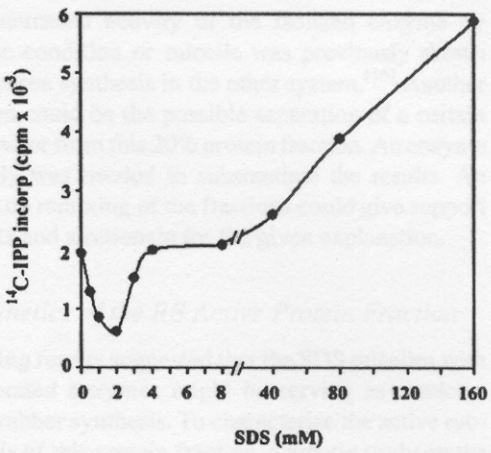

Figure 6. Biphasic nature of SDS activation on WBM enzymes. The RB activity of WBM at each SDS concentration was shown as the number of ^{14}C-IDP incorporation into the rubber. The WBM (approx. 40 mg dried weight) was mixed with the SDS before subjected to the standard incubation condition as described in Experimental part.

could stimulate its RB activity.[18] The BF membrane lipids was found with high content of phosphatitic acids and thus displayed a highly negative charged character.[5] 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 surprising result revealed that the SDS effect was of biphasic character (Figure 6), being inhibitory 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.[37] Activation by high [SDS] was quite pronounced in term of the magnitude on the RB, a three fold increase was seen at 0.150 M SDS.

It is remarkable that the increase in rubber synthesis displayed a dose response curve even at [SDS] above 0.15 M. A possible reason for this SDS biphasic effect might be the formation of micelles at high [SDS], thereby considerably increasing the active surface area[35] for RB enzymes catalysis. The critical micelle concentration (CMC) of SDS is at 8.2×10^{-3} M, so a low [SDS] will cause denaturation of the enzymes, resulting in a decreased RB activity. At higher [SDS] the micelle will start forming together with the dissolved membrane as a mixed micelle[36] with the incorporated RB enzymes. An 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.

Protein Profile of Acetone-Fractionated WBM Proteins

Several procedures for membrane protein extraction are available[35] and were tried. Among them the acetone method was found to be most satisfactory and suitable to be employed. The advantage of using acetone is to dissolve the soluble membrane while the released proteins are precipitated in the same treatment steps. Serial extraction of WBM proteins with acetone ranging from 20–80 vol.-% was devised in this study. The results showed that the membrane protein could be fractionated into different fractions by the acetone ranges as employed. A protein profile of distinct patterns by SDS-PAGE (Figure 7) was obtained, although overlap of some common proteins did occur. Most of the acetone-precipitated proteins were in the 40 and 80% (lane B, D) acetone ranges while moderate amounts were in the 20 and 60% (lane A, C) ranges. These fractions were then examined for possible RB activity.

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 the 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 thus restored by the SDS addition as shown in Table 1 for the RB levels.

Different amounts of SDS were attempted, and 2% SDS was found to be optimum for RB activity assays. Results for

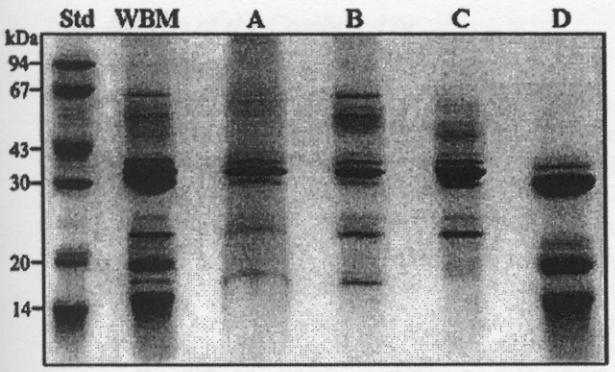

Figure 7. SDS-PAGE analyses of proteins profile from WBM proteins. WBM: proteins from WBM. The serial of acetone extract precipitated WBM proteins are shown in lane A–D. A: precipitated proteins at 0–20% acetone saturation; B: 20–40%; C: 40–60%; D: 60–80%; Std: standard molecular weight proteins.

each fraction (Table 1) 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. Repeated experiments with different preparations of samples consistently showed similar results, and thus helped to confirm the results obtained.

One explanation for this could be the formation of micelles. At 2% SDS (8 × CMC), a lot of micelles 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 polyisoprene synthesis in the other system.[36] Another likely reason could be the possible separation of a certain inhibitory factor from this 20% protein fraction. An enzyme kinetic study was needed to substantiate the results. An experiment on remixing of the fractions could give support to the results and a rationale for the given explanation.

Table 1. RB activity of the serial acetone fractionated proteins. The ranges of acetone percentage used were 20, 40, 60, and 80% (A, B, C, D fraction). The precipitated protein mixture in each incubation tube was prepared from the same WBM (approx. 40 mg dried weight) in the serial acetone fractionation. To all incubations were added 2% SDS (w/v) before being subjected to the standard incubation condition as detailed in the Experimental Part.

Sample	^{14}C-IPP incorp.	% incorp.[a)]
	cpm	
WBM (control)	6655	9.6
A (20% acetone)	6505	9.4
B (40% acetone)	1080	1.0
C (60% acetone)	323	0.5
D (80% acetone)	121	0.2

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

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 microreactor for rubber synthesis. To characterize the active rubber synthesis of this protein fraction, a kinetic study on the enzyme concentrations was carried out. The results showed that the RB activity was catalyzed by enzyme in this fraction, not by nonspecific trapping (Figure 8). Corresponding increases were seen with the enzymes in a dose dependent manner. A linear relationship was still observed at high enzyme levels 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 that it is possible to synthesize natural rubber ex vivo by a protein extract from BF membrane if proper or optimum conditions are provided to support or stabilize the isolated enzyme activity.

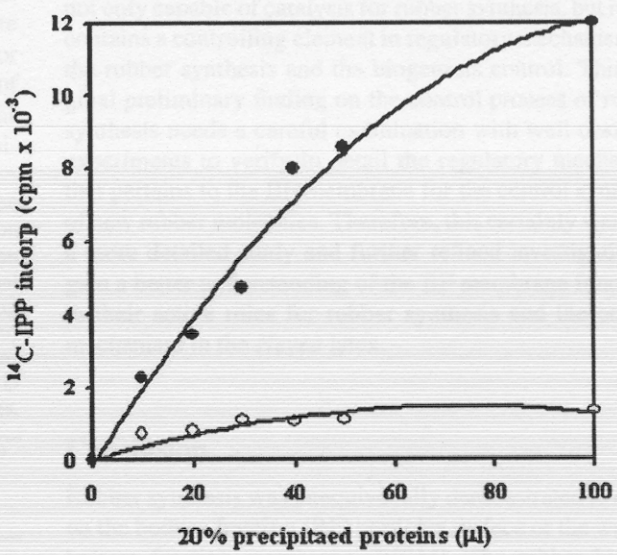

Figure 8. The activity of 0–20% acetone precipitated protein from WBM (●, 1.7 mg protein ml^{-1}). 2% SDS (w/v) was mixed with the samples before subjected to the standard incubation condition as described in Experimental part. Control (○) was carried out with the same condition as sample but with 20 × 10^{-3} M EDTA added in the incubation assay.

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, fraction A), it would be interesting to validate this assumption. The designed experiments were carried out by mixing the highly active fraction (fraction A) with other low or inactive RB fractions (fraction B, C, D), and the effect was followed. 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 1) was an overlap or spilled over from A and thus had an additive effect in the combined assay.

It might also be that fraction B contained an activator for RB activity as the total activity was a bit higher than the addition of the two isolated activities. Combining 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 fractions' A activity was totally diminished. Complete inhibition exerted by 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 2). This might lend a strong support to the result seen in the 20% acetone protein fraction A that showed quite unusual high RB activity. It was expected to have lower activity due to the redistribution of enzymes upon fractionation by the acetone. It is not known whether 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[38] or activation of HMGR on BF membrane by C-serum calmodulin.[11] It certainly needs further study as shown recently[39] and more reconstitution assays are in progress.

Table 2. Reconstitution experiments on RB activity of acetone fractionated proteins. Acetone ranges were as in Table 1 (fraction A was mixed with each of the 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 being subjected to the standard incubation condition as described in the Experimental Part. Intact WBM and WBM + D were also tested for comparison with the isolated protein fractions.

Sample	^{14}C-IPP incorp.	% incorp.[a)]
	cpm	
WBM (control)	6915	10
A (control)	6750	9.8
A + B	8580	12.4
A + C	990	1.4
A + D	866	1.3
WBM + D	467	0.7

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

The two inhibitory results for both WBM and the isolated active protein fraction was a strong evidence that the RB activity of the BF membrane was tightly controlled in a well regulated manner. The results from both were complimentary and supporting one another in the control mechanism that certainly exists in the membrane enzyme system. It was something not totally unexpected from these results considering the fact that most if not all the biochemical processes need to have well regulated control mechanisms, for metabolic balance and a coordinated process of the overall metabolic pathways integration and balanced cell functions.

This is indeed a totally new finding and quite an exciting result of 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 a 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 the regulatory mechanism that pertains to the BF membrane for the control synthesis of new rubber molecules. Therefore, this certainly warrants a 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.

Conclusion

Rubber synthesis was unequivocally demonstrated to occur on the bottom fraction (BF) particles surface or the washed bottom fraction membrane (WBM). The rubber biosynthesis (RB) activity of WBM was much higher than the rubber particles (RP) surface. Generally, RP was considered to be the only RB site. Our results suggested that the RB activity observed with the washed rubber particles (WRP) surface might be due to the associated membrane fragments from rupture of the BF particles. The WBM was highly

active with C_{55} (UPP) as an allylic acceptor for the isopentenyl diphosphate isomerase (IPP) in rubber synthesis, but was inactive for the WRP. SDS was a strong RB activator for WBM, probably due to micelle formation, thereby highly increasing the active surface area, resulting in a high RB activity with WBM. The SDS effect was of biphasic manner: inhibition at low SDS concentrations, but activation at high ones.

Serial acetone extraction of WBM proteins showed that isolated fractions still have RB activity under appropriate conditions. An RB regulatory protein was also detected in this study.

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