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รายงานวิจัยฉบับสมบูรณ์

สัญญาเลขที่ BGJ/4580007

โครงการ "การศึกษาขั้นตอนการสังเคราะห์ในเชิงชีวเคมีของพอลิไอโซพรีนที่ได้จากยางธรรมชาติ"

Biosynthesis of Natural Polyisoprenes

-Initiation and termination steps of rubber formation-

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Biosynthesis of Natural Polyisoprenes

-Initiation and termination steps of rubber formation-

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Executive Summary

Little is known on the mystery structure of natural rubber and also the rubber formation in *Hevea* trees including with the initiating and terminating step biosynthesis mechanism. The present work attempts to elucidate the how and the why of rubber formation by *in vitro* synthesis comparing with *in vivo* system and analyze the structure using NMR techniques as well as the study on the participated enzyme in the rubber biosynthesis to give the conclusive evidence on the mechanism step of biosynthesis process in *Hevea* tree. In addition, the structure of low molecular weight rubber will provide the information of terminal end groups, α- and ω-, as model compounds for natural rubber. First, *in vitro* polyisoprene rubber was studied in order to produce high rubber yield from fresh bottom fraction in the presence of isopentenyl diphosphate (IDP) and farnesyl diphosphate (FDP). High rubber yield could be achieved by the addition of IDP 4.175 nmol and FDP 5 nmol on fresh bottom fraction. The high amount of FDP causes the low rubber yield, which give the high production of polyprenol instead. However, the low reproducibility of *in vitro* rubber on fresh bottom fraction was governed by many factors, *i.e.*, fresh bottom fraction, centrifugation condition as well as the rubber extraction. The preparation of bottom fraction was performed on the gradient centrifugation by 2 steps in order to remove all rubber components. The storage of fresh latex and fresh bottom fraction at low temperature before synthesis led to the low rubber yield as well as the difficulty for succeed in completely rubber extraction. Secondly, study on enzyme involved in the

rubber formation in mushroom rubber, which can be used for model in rubber formation of *Hevea* rubber due to the similarity in fundamental structure of both rubbers. The farnesyl diphosphate synthase (FPS) was selected, which involved in initiation step of rubber formation. It was isolated from *Lactarius chrysorrheus* mushroom and clarified the nucleotide and deduced amino acid sequence. The FPS homolog was expressed in E. coli with pGEX6P-1 vector and purified in order to assay its activity. The pure protein (FPS) gave the FPS activity to produce farnesyl diphosphate (FPP) with DMAPP or GPP as substrate, which was examined by reverse-phase TLC. Last, the structure of low molecular weight rubbers from Jackfruit (*Artocarpus*), Mushroom (*Lactarius sp.*) as well as from seedlings of *Hevea* tree was studied. It was revealed that the structure of Jackfruit rubber consisted of dimethylallyl group and two-*trans* units at ω-terminal end while the α-terminal group composed of phosphate group. Moreover, the low molecular weight rubber from seedlings can be clearly detected the α-terminal end but the ω-terminal still be unclear.

ผลงานวิจัยที่ทำในรอบปี

1. วัตถุประสงค์ (Objective)

1.1 To study the biosynthesis conditions for high new rubber formation by fresh bottom fraction.

1.2 To study the related enzyme of rubber formation.

1.3 To analyze the structure of Jackfruit rubber and mushroom rubber as a model of natural rubber.

2. การดำเนินงานในรอบปีที่ผ่านมา (Experimental procedures)

2.1 *In vitro* study on biosynthesis of newly formed rubber and polyprenol by fresh bottom fraction

บทคัดย่อ

ยางโพลิไอโซปรีนสามารถถูกสังเคราะห์ได้ในหลอดทดลองโดยใช้ส่วนล่างสุดที่ปั่นแยกได้จากน้ำยางสด (fresh bottom fraction) ร่วมกับไอโซเพนทีนิลไดฟอสเฟต (isopentenyl diphosphate; IDP) และฟาร์เนซิลไดฟอสเฟต (farnesyl diphosphate; FDP) ซึ่งเป็นสารตั้งต้นในการเกิดปฏิกิริยาโดยทำการสังเคราะห์ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง ซึ่งผ่านการบ่มปฏิกิริยาค้างคืนที่อุณหภูมิ 4 องศาเซลเซียสแล้ว ปฏิกิริยาที่มีการเติม IDP 4.175 นาโนโมล และ FDP 5 นาโนโมลใน bottom fraction สามารถสร้างยางใหม่ได้ในปริมาณสูง ยางใหม่ที่สังเคราะห์โดยใช้ bottom fraction มีปริมาณการผลิตไม่คงที่เนื่องจากมีปัจจัยต่าง ๆ เข้ามาเกี่ยวข้อง อาทิเช่น จากตัว bottom fraction จากการปั่นแยก bottom fraction โดยใช้เซนทริฟิวส์ อีกทั้งวิธีการสกัดยางที่เกิดใหม่ การเตรียม bottom fraction ทำได้โดยการปั่นแยกแบบค่อย ๆ เพิ่มความเร็วอย่างต่อเนื่องเพื่อที่จะแยกโมเลกุลยางออกได้อย่างมีประสิทธิภาพ การเก็บน้ำยางสดและ bottom fraction ไว้ที่อุณหภูมิต่ำก่อนที่จะนำมาสร้างยางใหม่จะทำให้ได้ผลผลิตยางใหม่ในปริมาณต่ำ นอกจากนี้วิธีการสกัดยางใหม่ให้ได้อย่างสมบูรณ์ก็เป็นอีกปัจจัยหนึ่งด้วย

Abstract

In vitro polyisoprene rubber was synthesized by fresh bottom fraction in the presence of isopentenyl diphosphate (IDP) and farnesyl diphosphate (FDP), after pre-incubation at 4°C overnight, followed by incubation at 37°C for 6 hr. High rubber yield could be achieved by the addition of IDP 4.175

nmol and FDP 5 nmol on fresh bottom fraction. The reproducibility of *in vitro* rubber on fresh bottom fraction was governed by many factors, *i.e.*, fresh bottom fraction, centrifugation condition as well as the rubber extraction. The preparation of bottom fraction was performed on the gradient centrifugation by 2 steps in order to remove all rubber components. The storage of fresh latex and fresh bottom fraction at low temperature before synthesis led to the low rubber yield as well as the difficulty for succeed in completely rubber extraction.

Introduction

Natural rubber (NR) from *Hevea brasiliensis* is mainly composed of isoprene units linked in the *cis*-1,4 configuration. *Hevea* tree exudes latex containing about 35% rubber as small rubber particles in a diameter of 0.1 to 1.0 μm. By ultracentifugation, fresh latex can be separated into three main fractions, i.e. rubber phase, C-serum (CS) and bottom fraction (BF).

The study on rubber formation was done for *in vitro* synthesis by using wash rubber fraction (WRP). It has been revealed isopentenyl diphosphate (IDP) can be converted into rubber molecule on the surface of rubber particles. The incorporation of ^{14}C-IDP into *Hevea* rubber molecule decreased in the order $C_{20} \geq C_{15} > C_{10} > C_5$ and almost independent of the geometric isomerism of the isoprene units for *in vitro* rubber synthesis. Farnesyl (C_{15}) and Geranyl geranyl (C_{20}) diphosphate are expected to be initiating species in rubber biosynthesis as in the case of biosynthesis of terpenoids. Recently, the rubber formation from BF and CS has been studied using radioactive tracer techniques. The highest incorporation of ^{14}C-IDP and rubber yield was

observed for the incubation of fresh BF, while small amount of rubber was formed on incubation of CS with ^{14}C-IDP. In the case of BF, the rubber yield was observed without the addition of isopentenyl diphosphate (IDP) or farnesyl diphosphate (FDP), but it increased about 3.2- and 4.7-fold by the addition of IDP and FDP, respectively.

Two ways of the conversion of IDP to isoprene units are expected *in vitro* synthesized rubber, i.e., formation of new rubber molecules and chain extension of pre-existing rubber. The former requires the enzymes for the conversion of IDP to initiating species such as DMADP or FDP that are presumed to be initiating species of rubber formation. On the other hand, it was confirmed in natural rubber latex that the latter could proceed only in the presence of rubber transferase. The formation of new rubber molecules has been postulated for *in vitro* synthesis using washed rubber particles from *Hevea* and Guayule. At the present, however, these biochemical observations have not been confirmed yet by structural evidence.

Objective

1) To find out the best condition for high rubber production by fresh bottom fraction and determine the structure of in vitro rubber.

2) To study the effect of *in vitro* rubber synthesis on rubber fomation from fresh bottom fraction.

Experimental

Fresh *H. brasiliensis* latex (RRIM600) was collected in ice-chilled flasks by tapping. The latex was first filtered through a muslin cloth to remove some coagulants and then immediately centrifuged 2 steps at 12,096 g for 30 min followed by 48,384 g for 60 min at 4°C. The bottom yellowish fraction (BF) was separated from the upper rubber phase and the middle clear CS then subjected to synthesize new rubber molecule. The incubation mixture contained 4.175 nmol of [1-^{14}C] IDP (55 mCi mol^{-1}) with various amounts of farnesyl diphosphate (*t,t*-FDP) and 2.5 g (wet weight) of the fresh BF. The incubation mixture was pre-incubated overnight at 4°C, followed by incubation at 37°C for 6 hr. The control incubations were treated in the same way, except that BF was boiled at 100°C for 30 min before use. All the incubation mixtures were terminated by the addition of ethanol 3 ml. The ethanolic solution was immediately centrifuged out, which was further analyzed polyprenol fraction. The precipitant was dissolved in toluene, and then re-centrifuged for three times to extract rubber. The toluene solution was taken down in a rotary evaporator to small volume and purified by reprecipitation from toluene solution with ethanol three times. The purified *in vitro* rubber was dried up *in vacuo* and weighed.

The polyprenol in all ethanolic solutions were extracted with 1-butanol equilibrated with water, followed by washing the extracts with the lower phase three times. The polyprenol diphosphates were hydrolyzed by 0.1% NaOH and incubated at 37°C overnight. The resulting polyprenols were extracted polyprenols were analyzed by reversed phase TLC with a solvent system of acetone/water (19:1). The compositions of hydrolysate extracted were

identified by comparing with authentic standard and visualized with Iodine vapor.

2.2 Cloning and characterizaton of farnesyl diphosphate synthase (FPS) in *Lactarius chrysorrheus* mushroom

บทคัดย่อ

Farnesyl diphosphate synthase (FPS) เป็นเอมไซม์ที่ใช้เร่งปฏิกิริยาการสังเคราะห์ farnesyl diphosphate ซึ่งคาดว่าเป็นสารเริ่มต้นในกระบวนการสังเคราะห์ยางธรรมชาติ โดยการต่อโมเลกุลของ dimethylallyl diphosphate และ geranyl diphosphate ด้วย isopentenyl diphosphate การหาลำดับเบสของ FPS จากเห็ดซึ่งเป็นสกุลหนึ่งของราได้ทำจากวิจัยเป็นครั้งแรก โดยเริ่มจากการสกัด cDNA ที่นำรหัส FPS จากเห็ดอ่อนของสารพันธุ์ *Lactarius chrysorrheus* โดยการทำ degenerate PCR ด้วยไพร์เมอร์ที่ออกแบบจากลำดับอะมิโนที่ทราบแล้วจากราและยีสต์ชนิดอื่นๆ ซึ่งพบว่า FPS มีลำดับอะมิโนที่มีผลต่อการแสดงออกของยีน 381 ลำดับอะมิโน ซึ่งคำนวณเป็นมวลโมเลกุลได้ประมาณ 42.9 kDa โดยลำดับอะมิโนที่ได้มีความคล้ายคลึงน้อยเมื่อเทียบกับลำดับอะมิโนของรา ยีสต์ชนิดอื่นๆ รวมทั้ง FPS ที่สกัดได้จากสัตว์และพืชชนิดอื่นๆ จากนั้นทำการโคลน cDNA ที่ได้เข้าไปในแบคทีเรีย E. coli โดยการเชื่อมต่อกับเวคเตอร์ที่ทำให้เกิดการแสดงออกของยีน (pGEX6P-1) พร้อมด้วยการชักนำของสาร isopentenyl-β-D-galactopyranose (IPTG) ให้เกิดการสังเคราะห์โปรตีน จากนั้นนำโปรตีนที่ได้ไปทำให้บริสุทธิ์ แล้วจึงนำไปวัดแอคติวิตี้ของเอนไซม์ พบว่าโปรตีนที่ได้มีความสามารถในการสังเคราะห์ farnesyl diphosphate โดยการตรวจสอบผลิตภัณฑ์ที่เกิดขึ้นด้วย reverse-phase thin layer chromatography (TLC)

Abstract

Farnesyl diphosphate synthase (FPS) enzyme catalyzes the synthesis of farnesyl diphosphate, which is expected to be initiating species in rubber biosynthesis, from the sequential condensations of dimethylallyl diphosphate and geranyl diphosphate with isopentenyl diphosphate. The FPS sequence from mushroom, one genus of fungi, was first revealed. In this work, we isolated the cDNA encoded FPS from young sporophores of *Lactarius chrysorrheus* mushroom and expressed this cDNA into E. coli in order to study the participation of this enzyme in the rubber biosynthesis. A cDNA encoding FPS was obtained by degenerate PCR with designed degenerate primers from known aa sequences of fungi and yeast. The open reading frame sequence was clarified to encode a protein of 381 amino acid residues with a calculated molecular weight of 42.9 kDa. The deduced amino acid sequence of the cDNA showed low similarities with fungi and yeast as well as other eukaryotic FPS. The coding region was inserted into pGEX6P-1 vector, which expressed the target protein as GST fusion protein. The plasmids, pGEX6P-LcFPS, were cloned in E. coli, which induced the fusion protein in the addition of isopropylthio-β-D-galactopyranoside (IPTG). The purified protein gave the FPS activity, which produced the farnesol (FOH) as a main product with checking by using reverse phase thin layer chromatography.

Introduction

Farnesyl diphosphate synthase (EC 2.5.1.1), is the central enzyme in the isoprenoid pathway, play an important role in both eukaryotic and prokaryotic metabolism. It catalyzes the consecutive condensations of

dimethylallyl diphosphate (DMAPP) with isopentenyl diphosphate (IPP) to resulting product, geranyl diphosphate (GPP) then subsequently addition of IPP to produce farnesyl diphosphate (FPP) as a final product. The farnesyl diphosphate is participated among several branches of the biosynthesis pathway for steroids, farnesylated proteins, sesquiterpenes, heme a and vitamin K2. Furthermore, it is the allylic diphosphate initiator to produce polyisoprene by successive addition of IPP in *trans-* or *cis*-configuration to the ultimate product, *i.e.*, chicle, gutta-percha as well as natural rubber.

Farnesyl diphosphate synthase (FPS) was first discovered enzyme of prenyl diphosphate synthase, which is responsible for squalene biosynthesis in yeast extracts. Homogeneously purified enzyme preparations have been obtained from human liver, porcine liver, rat liver, chicken liver, yeast, and *Bacillus stearothermophilus.* Recently, the FPS gene of two ascomycete fungi, *Neurospora crassa*, and *Gibberella fujikoroi* as well as two other plant pathogenic fungi, *Sphaceloma manihoticola* and *Claviceps purpurea* was isolated and sequenced. The sequence analysis demonstrated the high similarities to all four analyzed ascomycetous fungi and striking homologies to other known eukaryote FPS between 52.5%-65.6%. All analyzed fungi contain a single copy of the gene and also only one copy was found in yeast *S. cerevisiae*. On contrary, the rat liver FPS gene exists in at least five genomic copies of rat liver FPS. However, few reports was concerned with the cloning, expression and characterization of FPS isolated from fungi.

The FPS gene of *N. crassa* and *G. fujikoroi* is important for membrane sterol biosynthesis. On the other hand, both fungi are well known as producers of carotenoids and gibberellins. It is noteworthy that some species of fungi; genera *Lactarius*, *Peziza*, *Russala* and *Hygrophorus* mushroom, were

found to produce *cis*-polyisoprene, so-called natural rubber. By ^{13}C-NMR study, the farnesyl diphosphate is presumed to be an initiating species in rubber formation of *Lactarius* mushroom, which is known to catalyze by farnesyl diphosphate synthase (FPS). Furthermore, the structural study on rubber from mushroom and other natural rubber occurring as latex suggested that the biosynthesis mechanism of *cis*-polyisoprene in *Hevea* tree is fundamentally the same as that in nonlactiferous cells and in mushrooms. Therefore, the study on the FPS gene in fungi is anticipated to be a good model to clarify the occurrence of rubber. Up to now, the FPS gene in mushroom has not been verified yet.

Objective

- Study on the function of farnesyl diphosphate synthase in mushroom, which is expected to synthesize the farnesyl diphosphate as initiating species in rubber formation.

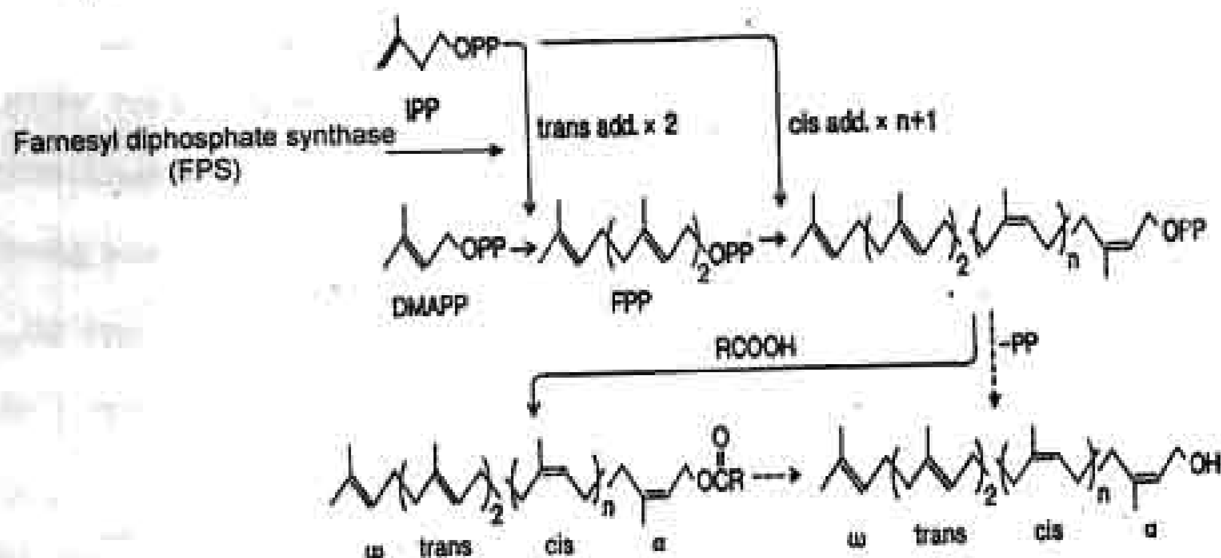

Figure 1. Biosynthesis mechanism of rubber from *Lactarius* mushroom

Experimental

Isolation and sequence analysis of FPS

Young mushrooms (*Lactarius chrysorrheus*; ki-chichitake) were collectod from Fukuoka, Kyushu prefecture, Japan. Total RNA was isolated from young sporophores of mushroom by using RNeasy® Plant Mini Kit (QIAGEN), then the single strand cDNA was synthesized by using reverse transcriptase enzyme (MMLV RT). The fragment of FPS homolog gene was amplified by degenerated PCR using degenerate primers derived from the amino acid sequences of highly conserved regions of fungi and yeasts. A clone containing a 432-bp fragment with high sequence similarity to FPS, was identified the sequence by ABI Prism® 3100 Genotic Analyzer. The 5' and 3'-end sequences of FPS homolog were analyzed by using 5'- and 3'-rapid amplification of cDNA ends (RACE) method. The open reading frame of this cDNA was designed to be LcFPS homolog gene.

Expression and purification of FPS in E. coli

The LcFPS homolog gene was introduced into expression vector, pGEX6P-1, at specific site of *Bam*H I and *Xho* I after that it was transformed into E. coli strain BL21(DE3). The pGEX6P-LcFPS transformants was growth in LB medium containing 50 $\mu g\ ml^{-1}$ ampicillin, was grown at 37°C until OD_{600} reached 0.6, followed by addition of IPTG to the final concentration of 1 mM and further cultivated at 30°C for 3 h. Cells were collected by centrifugation and disrupted by BugBuster™ HT (Novagen) reagent supplemented with PMSF 1 mM and DTT 1mM. Prior to apply the lysate to a GSTrap FF (Amersham) column, it was filtrated by 0.45 μM. The fusion proteins (GST-

LcFPS) were eluted with 10 mM reduced glutathione dissolved in 50 mM Tris-HCl pH 8.0 then changed the dissolving buffer to 50 mM Tris-HCl pH7.5, followed by the PreScission™ Protease treatment at 4°C overnight to remove the GST moiety. The pure protein (LcFPS) was achieved by washing with 1XPBS after loading on column. The purified fusion protein and pure LcFPS were checked by SDS-PAGE with coomassie brilliant blue staining and measured the protein concentration by Bradford method with BSA as standard. The pure proteins were exchanged buffer to 50 mM MOPS buffer pH 7.5 prior to use for enzymatic assay.

Enzyme activity and product analysis

FPS activity was assayed in a 200-µl reaction mixture containing 50 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT and either 50 µM DMAPP or GPP, 50 µM [4-^{14}C]IPP (37GBq mol^{-1}) and appropriate amount of enzyme. After incubation at 30°C for 30 min, the reaction was terminated by the addition of water saturated with NaCl then chilled it in an ice bath. The reaction products were subsequently extracted with 1 ml of 1-butanol layer and quantified by liquid scintillation counting.

For product analysis, the reactions were carried out in a total volume of 200 µl with similar components as describe above. After incubation for 2 h at 30°C the prenyl diphosphate products were extracted with 1-butanol then treated with potato acid phosphatase at 37°C overnight according to the method of Fuji, *et.al.* The liberated products were extracted with n-pentane and analyzed with reverse phase thin layer chromatography (LKC-18; Whatman) developed with acetone/water (9:1). The radioactive products

were identified by comparison with authentic standard visualized by iodine vapor. The radioactive spots were analyzed by using Fuji BAS1500 image analyzer.

2.3 Structure characterization of low molecular weight rubber as a model of *Hevea* rubber

บทคัดย่อ

ยางโมเลกุลต่ำจากขนุน (*Artocarpus heterophullus*) และต้นอ่อนของพารา ได้ถูกทำการศึกษาโครงสร้างของยางเพื่อเป็นแบบจำลองโมเลกุลของยางโมเลกุลใหญ่จากต้นพารา พบว่า ยางที่สกัดได้จากขนุนมีปริมาณ 0.5-0.7% อีกทั้งยางน้ำหนักโมเลกุลต่ำที่สกัดได้จากขนุนมีการกระจายตัวของน้ำหนักโมเลกุลต่ำเมื่อเทียบกับยางที่สกัดได้จากต้นอ่อนของยางพารา ซึ่งมีการกระจายตัวของน้ำหนักโมเลกุลมากเป็นแบบ biomodal จากการศึกษาโครงสร้างด้วยการวิเคราะห์โปรตอนและคาร์บอนนิวเคลียร์แมกเนติกเรโซแนนซ์ พบว่ายางจากขนุนประกอบด้วย dimethylallyl และ สองหมู่ของไอโซพรีนที่มีโครงสร้างแบบทราน ซึ่งต่อกับสายโซ่ของไอโซพรีนที่มีโครงสร้างแบบซิส โดยที่ปลายสายโซ่ของยางขนุนต่อกับหมู่ฟอสเฟต (=CH-CH_2OP) โดยพิจารณาจากสัญญาณที่ 4.05 ppm ของโปรตอนนิวเคลียร์แมกนีติเรโซแนนซ์ ในขณะที่ยางจากต้นอ่อนของพารามีลักษณะโครงสร้างที่คล้ายกับโครงสร้างที่วิเคราะห์ได้จากยางน้ำหนักโมเลกุลสูงจากต้นพาราโดยที่ปลายสายโซ่ประกอบด้วยฟอสไฟไลปิดหรือสายโซ่ยาวของ fatty acid โดยที่หมู่เริ่มต้นในการสังเคราะห์เป็น หมู่ dimethylallyl ที่มีการเปลี่ยนแปลงโครงสร้างไปจากหมู่เดิมหลังการสังเคราะห์ และสองหมู่ของ ไอโซพรีนที่มีโครงสร้างแบบทราน

Abstract

The structure of low molecular weight rubber from Jackfruit (*Artocarpus heterophullus*) and *Hevea* seedlings were analyzed as a model of high molecular weight *Hevea* rubber. The rubber content of latex from Jackfruit was in the range of 0.5-0.7%. The rubber from Jackfruit latex was low molecular weight with narrow unimodal distribution, while that from *Hevea* seedlings showed a bimodal distribution with shoulder peak in high molecular weight fraction. The ^{1}H- and ^{13}C-NMR analysis showed that Jackfruit rubber is consisted of a dimethylallyl group and two *trans*-isoprene units with connected to long chain *cis*-isoprene units. The α-terminal group of Jackfruit rubber was presumed to be composed of a phosphate group based on the presence of a ^{1}H-NMR signal at 4.05 ppm corresponding to the terminal $=CH-CH_2OP$ group. The low molecular weight rubber from *Hevea* seedlings showed a similar structure observed for the rubber from mature *Hevea* tree, composed of a phospholipid and/or long chain fatty acid at the α-terminal group with a modified dimethylallyl group and two *trans*-isoprene unit as the initiating species.

Introduction

Hevea brasiliensis has been established as practically the only important natural source of rubber termed natural rubber (NR) owing to high yields with desirable physical properties. It is well-known that NR is composed of *cis*-1,4 polyisoprene. However, the detailed structure of both chain-ends has not been clarified. This may be due to the limitation derived from extreme high molecular weight of NR as well as the difficulty of purification to analyze

small amounts of functional groups in NR. Young nascent *Hevea* leaves and *Hevea* seedlings were reported to contain low molecular weight rubber enough to analyze the detailed structure. However, the ^{13}C-NMR analysis of these low molecular weight rubbers showed no dimethylallyl group at the initiating ω-end. Naturally occurring low molecular weight rubbers, originated from a mushroom (*Lactarius volemus*), sunflower (*Helianthus annuus*) and goldenrod (*Solidago altissima*), have been declared as the good models of NR. However, it was difficult to get freshly biosynthesized rubber from the higher plants as latex form, due to radical degradation of rubber in the tree during storage of latex as in the case of rubber from virgin *Hevea* tree. Jackfruit of the family Moraease (*Artocarpus heterophyllus* Lam. or *Artocarpus integrifolia*) is a kind of rubber-producing plants widely planted in Thailand. All parts of the tree contain sticky white latex. The rubber from Jackfruit is a good model for structural analysis of NR because of facility to get both low and high molecular weight rubbers from fruit and trunk.

Objective

- To study the structure of low molecular weight rubber from Jackfruit and *Hevea* seedlings as a model of NR by using high resolution NMR.

Experimental

Latex was collected from the stems of *Hevea* seedlings of strain RRIM 600 and RRIT 251, growing at the rubber plantations of Rubber Research Institute of Thailand, Chachoeng-sao, Thailand. Latex exuded by cutting with a blade was immediately dipped in acetone. The coagulated latex was

extracted with toluene and purified by reprecipitation of the rubber in toluene solution with methanol for three times. Jackfruit latex was collected directly after cutting the trunk of tree and fruit into a beaker containing hexane followed by coagulation with ethanol. The coagulum was washed with ethanol and acetone to remove impurities. The resulting rubber was purified by precipitation from hexane solution into methanol. The purified rubbers were dried *in vacuo* at room temperature. The rubbers from *Hevea* seedlings and Jackfruit rubber were subjected to fractionation by molecular weight in toluene solution with methanol in the usual way.

The low molecular weight fractions were subjected to the NMR analysis. The NMR spectra of rubber from *Hevea* seedlings and Jackfruit were recorded using a Varian UNITY INOVA 600MHz and 750MHz NMR spectrometers, respectively. The ^{13}C-NMR measurements were carried out on the frequencies of 150-190 MHz in $CDCl_3$ and C_6D_6 solution of rubber from *Hevea* seedlings and Jackfruit rubber, respectively at 50°C, with pulse repetition time of 5-10 sec. The ^{1}H-NMR measurements were performed on the frequencies of 600-750 MHz in C_6D_6 solution at 50°C, with pulse repetition time of 4 sec. The molecular weight and molecular weight distribution of these rubbers were determined by GPC.

3. ผลที่ได้รับ (Results)

3.1 <u>*In vitro* study on biosynthesis of newly formed rubber and polyprenol by fresh bottom fraction</u>

The new rubber molecules were produced by the addition of [1-^{14}C] IDP and FDP as high as 64 mg at the FDP level of 5 nmol. The rubber yield

decreased by the increasing concentration of FDP, which might be resulted from interference of FDP. It can be suggested that the excess amount of initiating species will prefer to make the new rubber chain to form low molecular weight or polyprenol rather than the extensions of rubber chain at the beginning steps based on the high production of polyprenol as increasing FDP. The rubber molecules synthesized from bottom fraction were fluctuated owing to the nature of fresh bottom fraction and synthesis condition and so on.

The factors governing on the rubber yield were carried out, *i.e.*, aging of BF after obtained from freshly tapped mature *Hevea* tree, centrifugation condition, *etc.* The long-term aging of fresh latex after collection and of bottom fraction after centrifugation at low temperature gave low rubber yield. This might be caused by the coagulation of rubber without addition of preservatives and also the destroyed membrane of bottom fraction by hypotonic pressure at low temperature. The preparation of fresh bottom fraction was affected on the rubber formation by which the low centrifugal force leads to the high content of pre-existing rubber in bottom fraction. The gradient centrifugation by two steps was effective to separate the fresh bottom fraction from the small rubber molecule. Furthermore, all rubber molecules produced were obtained completely depending on the extraction procedure. Some rubber molecules were remained in residual BF even the extraction of toluene was performed for 3 times. It can be explained by the formation of soft gel during the storage of terminated BF by ethanol at low temperature.

3.2 <u>Cloning and characterization of farnesyl pyrophosphate synthase (FPS) from *Lactarius chrysorrheus* mushroom</u>

The nucleotide and deduced amino acid sequences of *L.chrysorrheus* FPS homolog was obtained by the degenerate PCR and RACE method. The sequence analysis revealed that the *Lactarius* FPS homolog is about 1.5 kbp in length, which an open reading frame encoded a protein of 381 amino acid residues. The predicted translation product of this cDNA has a molecular mass of 42,900 Da. The amino acid sequence of this cDNA contains the aspartarte-rich motif of all *trans*-prenyl diphosphate synthase, *i.e.*, FARM (DDIMD) and SARM (DDYLD) as well as other conserved regions. By comparison with other known eukaryotic FPS, the deduced LcFPS homolog gene showed the striking homologies. The peptides are low similarities between 25.4% and 56.5%. This might be the lack in mushroom sequence information. To verify this sequence is FPS, the expression in <u>E. coli</u> and assay its function as well as the product analysis were studied. The coding region of this cDNA, which contained the *Bam*H I and *Xho* I sites, was generated using PCR and inserted into pGEX6P-1 vector at same restriction sties to produce pGEX-LcFPS. The fusion protein (GST-LcFPS) was expressed in <u>E. coli</u> with the addition of IPTG to the final concentration of 1 mM as indicated at 69 kDa from SDS-PAGE. Fusion proteins were partially purified on GSTrap FF column, followed by treatment of PreScission Protease at low temperature overnight then applied on column in order to obtain pure FPS as indicated at 43 kDa from SDS-PAGE.

To determine whether the fusion proteins encoded functional enzyme, the FPS activity of these obtained proteins were examined. The optimum

condition fro assay of this enzyme is at 50 mM MOPS pH 7.5 with 2 mM $MgCl_2$. The pure FPS gave higer activity than the GST-FPS. In addition, it was specific to DMAPP or GPP as substrate, but it is not specific to FPP and GPP. This can be confirmed that this protein is FPS. However, the products from the reaction were identified by reverse phase TLC. It can be seen that farnesol (FOH) was a major product of the reaction for both substrate, GPP or DMAPP. Furthermore, the small quantities of geranol (GOH) was produced in the reaction mixture of pure FPS with DMAPP. This might be the result from two reaction steps are required to produce FPP with DMAPP as substrate. From TLC analysis data, it can be concluded that the obtained cDNA sequence encodes FPS.

3.3 Structure characterization of low molecular weight rubber as a model of *Hevea* rubber

The rubber content in Jackfruit latex was 0.5-0.7%, which is very low compared with that of 30-35% in the latex from *Hevea* tree. All of the Jackfruit rubbers showed a unimodal molecular weight distribution with narrow polydispersity index. The latex from *Hevea* seedlings contained low molecular weight rubber, which showed a typical bimodal distribution rich in low molecular weight fraction. The number-average molecular weight increased with the age of the tree. *Hevea* leaf gave lower molecular weight rubber than that from *Hevea* tree with a broad range of unimodal distribution in low molecular weight fraction. It is noteworthy that the molecular weight of *Hevea* rubber decreased from the trunk to leaf as similar to *trans*-polyisoprene case. All the *Hevea* rubbers showed higher polydispersity of molecular weight than

the Jackfruit rubber. The molecular weight of rubbers from Jackfruit and *Hevea* seedlings can be a good model of NR for the analysis of detail structure in connection with the mechanism of biosynthesis. The low molecular weight fractions of Jackfruit and *Hevea* rubbers in the range of 2.9 x 10^5 to 1.2 x 10^5 were obtained by fractionation from toluene solution.

In the ^{1}H-NMR spectrum of Jackfruit rubber, a small triplet signal centered at 4.05 ppm (J = 6.5 Hz) is presumed to be derived from the methylene protons linked to a phosphate group (=CH-CH_2OP). The number average molecular weight estimated from the relative intensity of this signal was comparable to that obtained from GPC, showing that the signal was derived from a terminal group. Similarity, the ^{1}H-NMR of *Hevea* seedlings shows the same signal as previous report, which contained various kinds of terminal groups. Until now, the terminal groups of *Hevea* rubber have not been clarified yet. The ^{13}C-NMR spectra of low molecular weight fraction of Jackfruit and *Hevea* seedlings rubber show the characteristic signals of C-1 methylene and C-5 methyl carbon atoms in the *trans*-isoprene units at 39.8 and 16.0 ppm. The signals due to the terminal methylene (-CH_2-CO_2-), methylene carbon atoms (-$(CH_2\text{-})_n$) and methyl carbon atoms (-CH_3) in the long chain fatty acid groups were observed at 34.4, 29.7 and 14.0 ppm, respectively. The C-5 methyl carbon signal in the dimethylallyl group of Hevea rubber, which is expected to resonate at 17.6 ppm, was not detected. However, small signals were detected at 16.9 and 43.9 ppm, which are corresponding to the terminal groups based on their intensities. It is speculated that the initiating species of rubber formation in *Hevea* rubber is a derivative of *trans-trans*-FDP, presumably a dimethylallyl group modified at the methyl-carbon. In the case of Jackfruit rubber, a small signal resonated at

17.5 ppm is assigned to one of the methyl carbon atom in dimethylallyl group by comparison with the result of model compounds. This clearly indicates that the Jackfruit rubber contains a dimethylallyl group and two *trans*-isoprene units at the ω-terminal. It is remarkable that the rubber from *Hevea* seedlings and Jackfruit showed no signal due to terminal isoprene units having hydroxyl group and fatty acid group, which are resonated at 59.1 and 60.9 ppm in the case of Sunflower, Goldenrod and *Lactarius* mushroom. The small signals at 64.4 and 69.2 ppm were detected in the rubber from *Hevea* seedlings and at 64.4 ppm in Jackfruit rubber, which is presumed to be derived from the carbon atom linked to phospholipid. According to these findings, the α-terminal group of both Jackfruit and *Hevea* rubber was presumed to link with phospholipid.

4. งานที่จะทำต่อในอนาคต (Future works)

Analysis of ω-terminal end group of natural rubber molecule by using polyprenol extracted from *Hevea* rubber.

5. ผลงานจากงานวิจัย

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2. **Dararat Mekkriengkrai**, Tomoki Sando, Jitladda Sakdapipanich, Yasuyuki Tanaka, Ei-ichiro Fukusaki, and Akio Kobayashi (2004) "CLONING AND CHARACTERIZATION OF FARNESYL DIPHOSPHATE SYNTHASE IN *LACTARIUS CHRYSORRHEUS* MUSHROOM". In preparation.