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.