STUDIES ON THE ACTIVATION FACTORS OF RUBBER BIOSYNTHESIS IN SMALL RUBBER PARTICLES OF *HEVEA BRASILIENSIS* LATEX

PAVEENA NAWAKITKOSOL

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.....Paveena Nawakitkosol.....
Miss Paveena Nawakitkosol
Candidate

.....Kanjanee Nawamawat.....
Miss Kanjanee Nawamawat,
Ph.D. (Polymer Science and Technology)
Chair

.....Jitladda Sakdapipanich.....
Assoc. Prof. Jitladda Sakdapipanich,
Ph.D. (Material Systems Engineering)
Member

.....C. Krittanai.....
Assoc. Prof. Chartchai Krittanai,
Ph.D. (Biochemistry and Biophysics)
Member

.....B. Mahaisavariya.....
Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....Skorn Mongkolsuk.....
Prof. Skorn Mongkolsuk,
Ph.D.
Dean
Faculty of Science
Mahidol University

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Paveena Nawakitkosol
Miss Paveena Nawakitkosol
Candidate

Jitladda Sakdapipanich
Assoc. Prof. Jitladda Sakdapipanich,
Ph.D. (Material Systems Engineering)
Major-Advisor

C. Krittanai
Assoc. Prof. Chartchai Krittanai,
Ph.D. (Biochemistry and Biophysics)
Co-Advisor

B. Mahaisavariya
Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

P. Sunintaboon
Lect. Panya Sunintaboon,
Ph.D. (Polymer Science)
Chair
Doctor of Philosophy Programme in
Polymer Science and Technology
Faculty of Science
Mahidol University

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Paveena Nawakitkosol

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PAVEENA NAWAKITKOSOL 4937464 SCPO/M

M.Sc. (POLYMER SCIENCE AND TECHNOLOGY)

THESIS ADVISORY COMMITTEE: JITLADDA SAKDAPIPANICH, Ph.D. (MATERIAL SYSTEMS ENGINEERING), CHARTCHAI KRITTANAI, Ph.D. (BIOCHEMISTRY AND BIOPHYSICS)

ABSTRACT

Small rubber particle (SRP) was prepared from rubber in zone 2 of *Hevea brasiliensis* latex by continuous steps of high-speed centrifugation at 12,000 rpm and then 19,000 rpm for 30 min and 60 min, respectively, at 4^{o}C. SRP was proved to be composed of linear and active rubber molecules, so it is suitable for the *in vitro* rubber biosynthesis. Washed SRP (WSRP) prepared by ultracentrifugation or gel-filtration chromatography was used as material for synthesis of the *in vitro* rubber. In the preparation of WSRP, washing serum fraction was separated from rubber fraction. This washing serum was added back to the incubation of WSRP for testing the activation effect. It is found that the washing serum could activate the *in vitro* rubber biosynthesis.

The study was an attempt to elucidate the factors in washing serum separated from SRP of *Hevea brasiliensis* latex that can give the highest IPP incorporation into the *in vitro* rubber. The main activation factors were found to be allylic diphosphates or proteins. In the testing of allylic diphosphate, thin layer chromatography (TLC) showed that the medium-chain allylic diphosphate was not detected in the lower fractions separated from washing serum by Microcon. In addition, there were no differences in the activity of the butanol extracts of lower fraction separated by TLC in each fraction. This indicates that the allylic diphosphate could not be detected from washing serum of SRP. In the case of protein testing in washing serum, it was investigated by heat and trypsin procedures, which are known as good methods to degrade protein. After heat and trypsin treatments, there was no more activity of rubber biosynthesis. This implies that the activity in SRP predominantly resulted from the proteins. Thus, the activator protein was further purified from washing serum by the chromatography techniques. These were anion-exchange chromatography, gel-filtration chromatography and hydroxyapatite chromatography. It is found that the activator protein can activate the IPP incorporation into the *in vitro* rubber. However, the activator protein band cannot be detected by SDS-PAGE due to its low amount.

KEY WORDS: NATURAL RUBBER/ *IN VITRO* BIOSYNTHESIS/ SMALL RUBBER PARTICLES

90 pages

การศึกษาตัวแปรที่มีผลกระตุ้นการสังเคราะห์ยางในอนุภาคยางขนาดเล็กของน้ำยางฮีเวีย บราซิเลียนสิส

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ปวีณา นวกิจโกศล 4937464 SCPO/M

วท.ม. (วิทยาศาสตร์และเทคโนโลยีพอลิเมอร์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: จิตต์ลัดดา ศักดาภิพาณิชย์, Ph.D. (MATERIAL SYSTEMS ENGINEERING), ชาติชาย กฤตนัย, Ph.D. (BIOCHEMISTRY AND BIOPHYSICS)

บทคัดย่อ

อนุภาคยางขนาดเล็กสามารถเตรียมได้จากยางในโซน 2 ของน้ำยางฮีเวีย บราซิเลียนสิสโดยการปั่นอย่างต่อเนื่องด้วยเครื่องปั่นความเร็วสูงที่ 12,000 รอบต่อนาที เป็นเวลา 30 นาที และ 19,000 รอบต่อนาที เป็นเวลา 60 นาที ที่อุณหภูมิ 4 องศาเซลเซียส อนุภาคยางขนาดเล็กประกอบด้วยโมเลกุลของยางที่เป็นเส้นตรงที่สามารถเกิดปฏิกิริยาต่อได้ ดังนั้น จึงเหมาะสำหรับศึกษาการสังเคราะห์ยางในหลอดทดลอง อนุภาคยางขนาดเล็กที่ได้จากการล้าง (WSRP) โดยการปั่นด้วยความเร็วสูง และจาก gel-filtration chromatography เป็น 2 ระบบที่นำมาใช้สำหรับการสังเคราะห์ยางในหลอดทดลอง ซึ่งการเตรียม WSRP สามารถแยกส่วนของเซรั่ม (washing serum) ออกจากส่วนของยางได้ เมื่อเติมเซรั่มกลับลงในการบ่มกับ WSRP เพื่อทดสอบการกระตุ้น พบว่าเซรั่มนี้สามารถกระตุ้นการสังเคราะห์ยางในหลอดทดลองได้

ดังนั้น งานวิจัยนี้ คือความพยายามที่จะทำให้ชัดเจนว่าตัวแปรใดในเซรั่มที่แยกจาก SRP ของน้ำยางฮีเวีย บราซิเลียนสิสสามารถกระตุ้นการสังเคราะห์ยางในหลอดทดลองได้สูงที่สุด ซึ่งตัวแปรหลัก 2 ชนิดที่สนใจคือ allylic diphosphate และโปรตีน ในส่วนของการทดสอบ allylic diphosphate ด้วยเทคนิคโครมาโตกราฟี (TLC) แสดงให้เห็นว่าไม่พบ allylic diphosphate สายโซ่ขนาดกลางในสารละลายส่วนล่างที่แยกจากเซรั่มโดยการกรองผ่านไมโครคอน นอกจากนี้ ยังไม่พบผลการกระตุ้นที่ชัดเจนจากการนำสารสกัดบิวทานอลของส่วนล่างที่แยกจากเซรั่มไปแยกโดยเทคนิค TLC จากผลการทดลองทั้งสองชี้ให้เห็นว่าไม่มีการตรวจพบ allylic diphosphate จากเซรั่มของ SRP ในกรณีของการทดสอบโปรตีนโดยวิธีการให้ความร้อน และการใช้เอนไซม์ทริปซิน พบว่าเซรั่มที่ผ่านการย่อยด้วยวิธีทั้งสองแล้วจะไม่สามารถกระตุ้นการสังเคราะห์ยางได้อีก ดังนั้น ความสามารถในการสังเคราะห์ยางของ SRP จึงเป็นผลจากการกระตุ้นของโปรตีนมากกว่า ด้วยเหตุนี้ โปรตีนที่ให้ผลการกระตุ้นจะได้รับการแยกโดยเทคนิคโครมาโตกราฟี ได้แก่ anion-exchange, gel-filtration และ hydroxyapatite chromatography พบว่า โปรตีนที่แยกได้นี้สามารถกระตุ้นการสังเคราะห์ยางในหลอดทดลองได้ แต่อย่างไรก็ตาม แถบของโปรตีนไม่สามารถตรวจพบได้ด้วย SDS-PAGE เนื่องจากปริมาณของโปรตีนที่น้อยเกินไป

90 หน้า

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LIST OF ABBREVIATIONS

NR	=	Natural rubber
SRP	=	Small rubber particle
WSRP	=	Washed small rubber particle
IPP	=	Isopentenyl diphosphate
DMAPP	=	Dimethylallyl diphosphate
DRC	=	Dry rubber content
MWD	=	Molecular weight distribution
SEC	=	Size exclusion chromatography
GPC	=	Gel filtration chromatography
HMG-CoA	=	3-hydroxy-3-methylglutaryl-CoA
MVA	=	Mevalonic acid
WRP	=	Washed rubber particle
BF	=	Bottom fraction
NPP	=	Neryl diphosphate
GPP	=	Geranyl diphosphate
FPP	=	Farnesyl diphosphate
GGPP	=	Geranyl geranyl diphosphate
kDa	=	Kilodalton
NMR	=	Nuclear magnetic resonance
REF	=	Rubber elongation factor
SRPP	=	Small rubber particle protein
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
LRP	=	Large rubber particle
RBSP	=	Rubber biosynthesis stimulator protein
PET	=	Polyethylene terephthalate

LIST OF ABBREVIATIONS (cont.)

M	=	Molar
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
BSA	=	Bovine serum albumin
NaCl	=	Sodium chloride
ml	=	Milliliter (s)
^{o}C	=	Degree Celsius
min	=	Minute (s)
h	=	Hour (s)
dpm	=	Disintegration per minute
g	=	Gravitational acceleration
µl	=	Microliter (s)
rpm	=	Round per minute
mM	=	Millimolar
nm	=	Nanometer

CHAPTER I
INTRODUCTION

1.1 Introduction

There are many wild species of higher plants, more than 2,500 species, which produce natural rubber such as *Hevea Brasiliensis, Taraxacum koksaghyz, Castillo elastica* and *Ficus elastica*. However, natural rubber from *Hevea Brasiliensis* is the most practically representative *cis*-1,4-polyisoprene accounting for more than 95% of the world's natural source of rubber. This is due to the high productivity of the plant and excellent physical properties of the rubber.

The biosynthesis of rubber has been studied since 1950s. The technique used for biosynthesis study is called the *in vitro* rubber biosynthesis. Radioactive tracer techniques have been applied to investigate the biosynthesis mechanism. The rubber formation was postulated to start from the isomerization of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP), and then the IPP was reacted to DMAPP. The subsequent chain elongation step was confirmed to proceed *via* a successive addition of IPP to polyisoprenyl diphosphate to form isoprene units in *cis*-configuration. However, the termination step was not referred to the proposed biosynthesis mechanism.

The study on rubber formation was done by *in vitro* biosynthesis. In addition, the rubber particle size was found to be a possible important parameter affecting to the activation of IPP incorporation into rubber chain. It was found that the enzymatic activity of smaller rubber particles was higher than that of large rubber particles [1]. Thus, small rubber particle (SRP) was the active rubber particle used for the *in vitro* rubber biosynthesis.

The washed rubber particle (WRP) was found to be a suitable system for analysis of the enzymatic activities in *Hevea brasiliensis*. After washing of rubber particle by chromatographic technique, the IPP incorporation into rubber by WRP showed the low incorporation. However, the IPP incorporation was restored when the fraction of low molecular weight compounds eluted after rubber particles was added back to incubation of WRP. This indicates that there were the factors activating the *in vitro* rubber biosynthesis in the serum of *Hevea* latex [2].

However, there has been no report on the activation factor of IPP incorporation in SRP. Thus, the aim of this work was an attempt to elucidate the factors which were removed into washing serum of SRP of *Hevea Brasiliensis* latex by various washing methods that could activate the highest IPP incorporation into *in vitro* rubber.

1.2 Scope of Thesis

The present work was divided into two parts based on the assumption that the main activation factor can be allylic diphosphates or proteins. First part is the separation of allylic diphosphates from washing serum of small rubber particles of freshly tapped natural rubber (NR) latex. This part included the study of the activation effect of allylic diphosphates on the *in vitro* rubber biosynthesis.

The second part is concerned with the proteins testing in washing serum. After that, the proteins were purified by the chromatography techniques in order to study the effect of proteins on the activation of the *in vitro* rubber biosynthesis.

CHAPTER II
LITERATURE REVIEW

2.1 Natural Rubber (NR)

Natural rubber (NR) is a biopolymer consisting of isoprene units linked together in a 1,4-*cis* configuration. Although rubber is produced in over 2,500 plant species, commercial production of rubber is almost exclusively from *Hevea brasiliensis*, the Para rubber tree. The annual production level of NR is nearly 10^7 tonnes, over 95% of which harvested in South-East Asia, in particular Indonesia, Thailand and Malaysia [3].

NR has unique properties due to its structure, molecular weight and non-rubber components, such as proteins, lipids, carbohydrates and minerals, which are present in the latex. These properties include resilience, elasticity, abrasion and impact resistance, efficient heat dispersion and malleability at cold temperatures [4]. Although synthesis rubbers, such as styrene-butadiene or acrylonitrile-butadiene co-polymers, are commercially produced to levels similar to NR, none of them match the price-performance ratio of NR. The unique properties of NR make it essentially irreplaceable for many applications, such as heavy-duty tires for trucks, buses and airplanes, as well as latex products for medical applications [4]. While some plants can produce other types of polyisoprenes, none of them have material properties or value similar to *Hevea* latex. For example, gutta-percha and balata are flexible but inelastic materials from *Palaquium gutta* and *Manilkara bidentata*, respectively, formed of isoprene units linked together in 1,4-*trans* configuration [3].

2.2 NR Latex

NR latex is obtained from *Hevea* tree by tapping, a diagonal cut through the bark. The latex exudes from the cut and is collected in a small cup, as shown in **Figure 2.1**. After tapping latex from the rubber trees, the latex is called as fresh NR or fresh field latex.

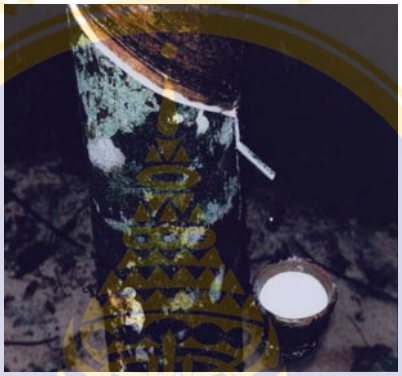


Figure 2.1 Collection of NR latex by tapping

Fresh field latex is composed of approximately 33% dry rubber content (DRC) and about 5% non-rubber components including proteins, carbohydrates, lipids and inorganic salts, depending on several factors such as weather, clone, soil, tapping frequency and season, as shown in **Table 2.1**.

Table 2.1 The composition of NR latex [5]

Constituent	% Composition (by weight)
Total solids	36
Dry rubber	33
Proteins	1-1.5
Resinous substances	1-2.5
Ash	Up to 1
Sugars	1
Water	Add to 100

2.3 The Composition of NR Latex

Fresh *Hevea* latex is a colloidal suspension of rubber particles in an aqueous serum phase [6]. As shown in **Figure 2.2**, using high speed ultracentrifugation, *Hevea* latex is separated into four main fractions [7]. There are an upper white fraction of rubber cream, a yellow layer containing Frey-Wyssling particles, a colorless serum named C-serum and a greynish yellow gelatinous sediment the bottom fraction containing mainly of lutoid particles.

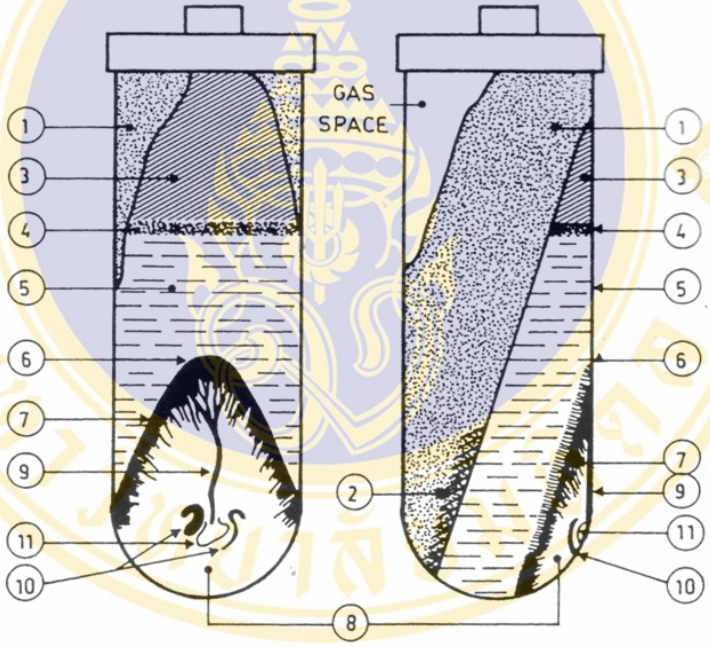


Figure 2.2 Separation of latex into ‘zones’ by refrigerated ultracentrifugation (54,000 g). Zone 1-3: rubber, Zone 4: yellow layer containing Frey-Wyssling particles, Zone 5: serum and Zone 6-11: bottom fraction [7]

2.3.1 Rubber particles

The rubber particles usually have a size ranging from 0.05 to 3 μm [8]. They are usually spherical in young trees, but the larger ones in latex from mature trees are often pear-shaped. As illustrated in **Figure 2.3**, the rubber particles comprise a sphere consisting of hydrocarbon and are surrounded by complex films which are contained phospholipids and proteins [9].

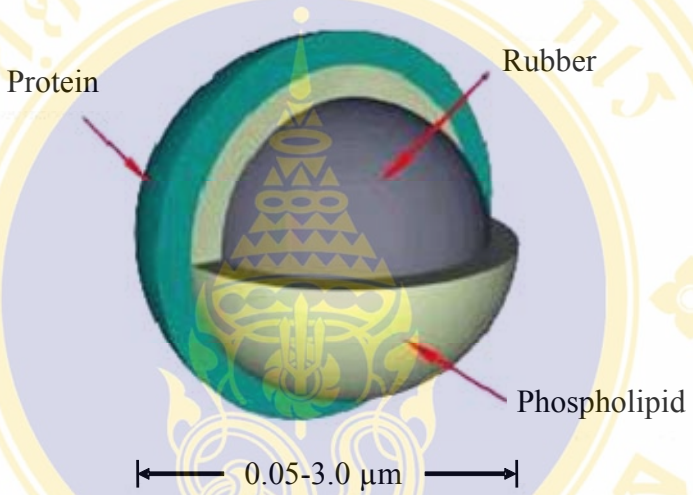


Figure 2.3 Schematic representation of rubber particle

2.3.2 Lutoid particles

Lutoids are most abundant non-rubber particles in *Hevea* latex. These are vacuoles having spherical membrane-bounded bodies with a diameter of 0.5 ± 3 μm [10]. Within the lutoids, there is an aqueous environment containing dissolved substances such as acids, minerals, proteins and sugars. Acid phosphatase and lysozyme have been detected in these particles, and in addition the lutoids contain mainly characteristic acid hydrolases. These suggest that the lutoid particles are analogous to lysosomes. Lutoids possess one of the factors that lead to cessation of latex flow, because the dilution reaction that occurs inside the latex vessel on tapping causes swelling of the osmotically sensitive lutoids remaining in the tube.

2.3.3 Frey-Wyssling particles

The Frey-Wyssling particles are spherical, 4 - 6 μm in diameter, and bound with a double membrane [10]. The particle is a composite organelle containing small particles of lipids and carotenoids; the yellow color is due to the presence of carotenoid pigments. The highly complicated structure of a Frey-Wyssling particle suggests that it has an important function in the metabolism of *Hevea* latex and may be possible sites of rubber biosynthesis. Since the particles contain plastoquinone and plastochromanol (in which β-carotene is synthesized), they are assumed to be modified plastids.

2.3.4 C-serum

The C-serum fraction of the centrifuged latex is a major non-rubber component of the latex such as the aqueous of lactiferous cytoplasm, water, small rubber, water soluble proteins and enzymes and this can be considered as the latex cytosol. The cytosol contains various different organelles and particles. Of most importance in the C-serum containing cytosol is the presence of rubber biosynthesis pathway enzymes [11, 18] and rubber biochemical intermediates [12, 13].

2.4 Molecular Weight of Rubber Particles [14]

Rubber from *Hevea brasiliensis* is a high molecular weight polymer with broad molecular weight distribution (MWD) [15]. The variation of MWD depends on the clone from which the rubber is obtained, the age of tree, soil and climate conditions. It was reported that NR have a bimodal distribution. Exhaustive studies of the MWD of NR in fresh latex by using size exclusion chromatography (SEC or GPC) were reported by Subramaniam [15]. The distribution of all clone rubbers can be classified into one of three types as shown in **Figure 2.4**.

Type 1: Skewed unimodal distribution with a “shoulder” or a “plateau” in the low molecular weight region

Type 2: Bimodal distribution where the height of the low molecular weight peak is only half or less than the height of the high molecular weight region

Type 3: Distinctly bimodal distribution where the peak height in the low molecular weight region is nearly equal or slightly less than the peak height at the high molecular weight region

The high and low molecular weight peaks are usually centered around 10^6 and 10^5. Most rubber bearing plants produce inferior quality rubber with a low molecular weight of $\leq 5\times10^4$. The molecular weight distributions of rubbers from several species represent the degree of polymerization carried out by the rubber transferase of the particular species [16]. A molecular weight distribution of this sort may well be related in some ways to the biosynthesis mechanism or the structure of the molecular chains of *Hevea* rubber. The molecular weight distribution can vary for different clone, and appears to be genetically controlled in *Hevea* rubber [17].

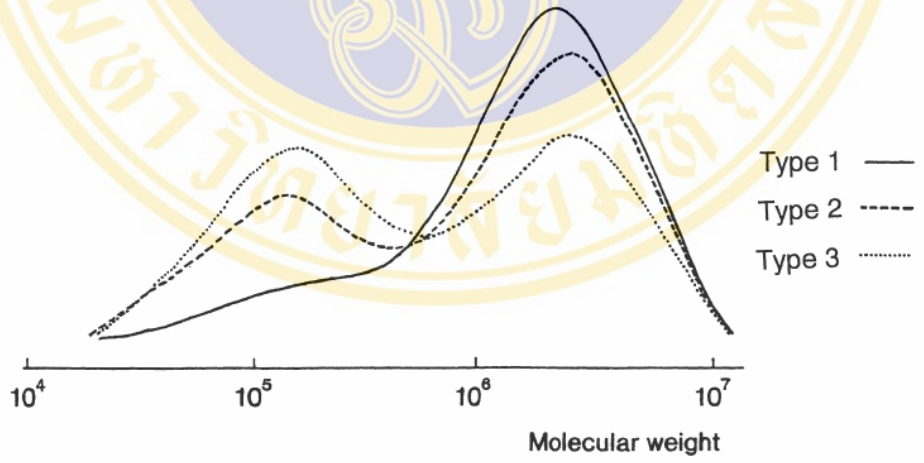


Figure 2.4 Types of molecular weight distribution curves of NR [15]

2.5 Presumed Biosynthesis Mechanism of NR

2.5.1 Biosynthesis pathway [14]

The general pathway to rubber biosynthesis was established during 1950s and 1960s. Biochemical studies presumed that rubber formation took place in the latex and started from carbohydrate *via* isopentenyl diphosphate (IPP), as shown in **Figure 2.5** [18]. This pathway is thought to involve at least 17 steps from simple sugar. Each step is naturally dependent on a particular enzyme or enzyme system. Radioactive tracer techniques have been applied to elucidate the incorporation of individual precursors [19].

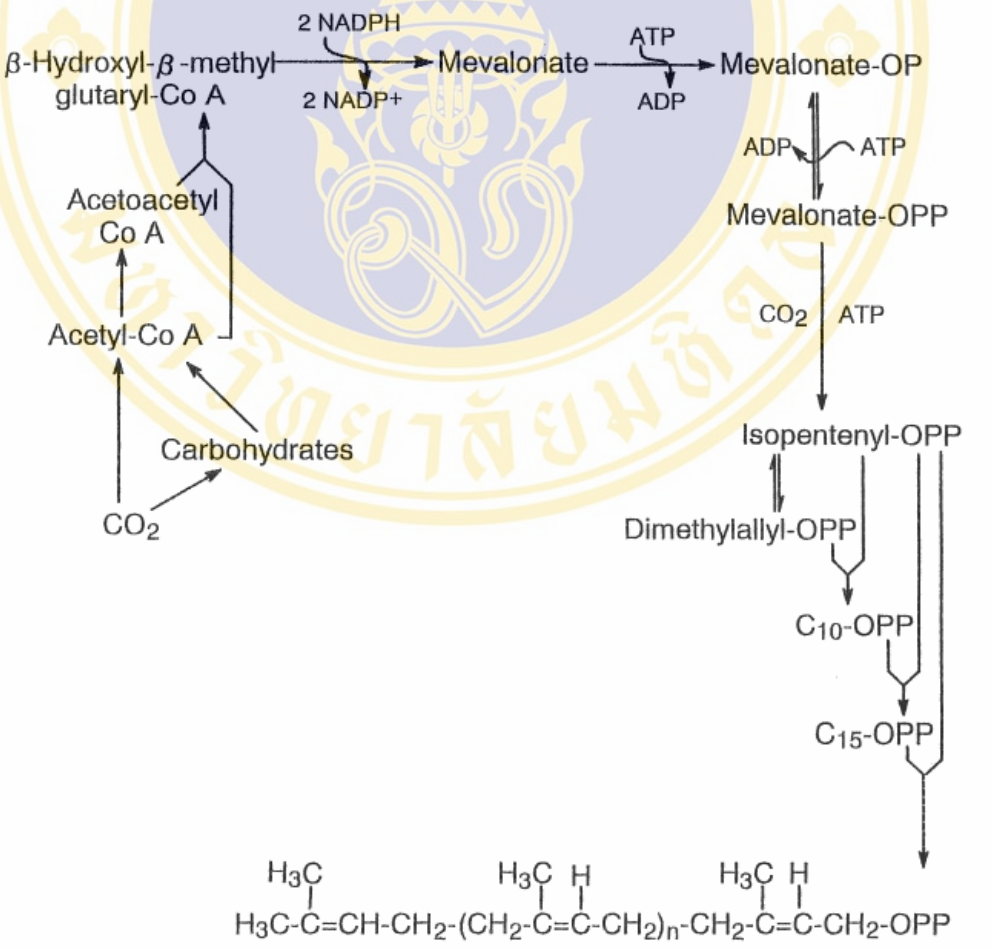


Figure 2.5 Biosynthetic pathway of NR [18]

It is generally assumed that sugars are utilized as the main source of carbon for rubber formation. A small amount of ^{14}C was incorporated into rubber when *Hevea* latex was incubated with radioactive sucrose, glucose and fructose. Although, it has been shown that sugars were converted to pyruvate in *Hevea* latex, there is little direct proof of incorporation of ^{14}C into rubber [20]. The conversion from pyruvate or acetate to rubber was demonstrated by incubation of *Hevea* latex with the radiolabeled compounds. Although *Hevea* latex contained all the enzymes and cofactors needed for rubber formation from acetate, the conversion efficiency from pyruvate or acetate to rubber is very low [21]. This poor conversion is considered to be partially due to the low activity of enzyme required for the consumption of the compounds, and also to the rapid conversion of pyruvate to ethanol in *Hevea* latex [22]. It is to be expected that acetyl-CoA and acetoacetyl-CoA are incorporated into rubber, but there is no direct proof that these compounds are converted into the rubber in *Hevea* latex.

A high level incorporation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into rubber on incubation with *Hevea* latex was reported [23]. Ozonolysis of rubber obtained from ^{14}C-labeled HMG-CoA showed that it was not decomposed to the low molecular precursors but was utilized for direct rubber molecule synthesis. Similarly it was demonstrated that ^{14}C-mevalonic acid (MVA) was incorporated into high molecular weight rubber in *Hevea* latex. By the degradation of rubber from ^{14}C-MVA, the positions of the individual carbon atoms in the repeating isoprene unit were derived from MVA [24]. The formation of MVA from HMG-CoA was estimated to be an important rate-limiting step in the regulation of rubber formation. MVA is converted into IPP *via* 5-phoshomevalonate and 5-diphosphomevalonate in *Hevea* latex, and these enzymes which catalyzed the reactions were isolated. It was demonstrated that ^{14}C-IPP was incorporated into rubber on incubation with *Hevea* latex [18]. The degree of incorporation of IPP was found to be as high as 97%, and the incorporation of IPP to rubber was faster than that of MVA. These findings indicate that IPP was the direct precursor of rubber molecule. A supply of ATP is required for these reactions, and glycolysis is generally assumed to account for this [18]. All enzymes required for IPP formation exist in the latex serum.

In the rubber biosynthesis, the first step has been postulated to be the isomerization of IPP to DMAPP *via* the enzyme IPP isomerase. The elimination of a proton leads to the first condensation product and allylic diphosphate containing ten carbon atoms. This homologous allylic diphosphate by acquisition of another IPP is converted into a C_{15}-compound. The repetition of this process, eventually, leads to high molecular weight rubber catalyzed by rubber transferase and prenyltransferase enzyme, which requires divalent cations such as Mg^{2+} or Mn^{2+} for the activity. An allylic diphosphate is required as substrate to initiate the polymerization process. Rubber formation requires three distinct biochemical processes:

(1) Initiation, i.e., synthesis of allylic diphosphate molecule catalyzed by *trans*-prenyltransferase enzymes.

(2) Chain elongation, i.e., *cis*-1,4-polymerization of isoprene units from IPP catalyzed by rubber transferase.

(3) Termination, i.e., the release of resulting polymer from rubber transferase.

2.5.2 Rubber formation on rubber particles [14]

The site of rubber biosynthesis within the tree has been extensively studied. Using electron microscopy, it was established that rubber synthesis was carried out only in the latex vessel of the rubber tree, though rubber was detected in other tissues. Studies performed during the 1960s suggested that rubber synthesis took place on the surface of pre-existing rubber particles. This hypothesis was confirmed by many experiments using washed rubber particle (WRP), which incorporated repeated dilution and centrifugation. Hence, the polymerizing enzyme was thought to bond to the surface of rubber particles.

The enzyme that catalyzes rubber formation has been identified as rubber transferase [2, 25, and 26]. It has been show that IPP is incorporated into rubber at the surface of the rubber particles in the latex by reaction with the terminal allylic diphosphate group of rubber molecules. Lynen indicated the hypothetical scheme outlining the event at the interphase, as shown in **Figure 2.6** [18]. It has been assumed that the growing hydrocarbon chain of rubber diffuses into the interior of the rubber

droplet while, on the other hand, the hydrophilic diphosphate end-group remains in the serum phase and reacts with IPP bound to the active site of the enzyme. In this way, accumulation of the water-insoluble rubber molecule on the enzyme surface, and its subsequent inhibition, is avoided.

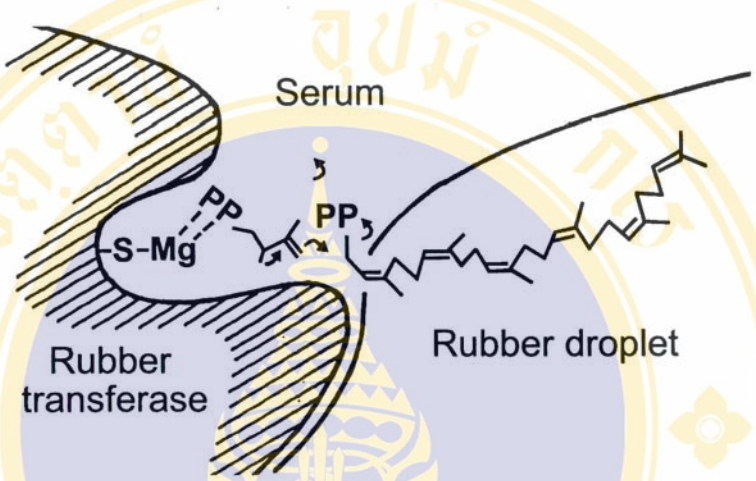


Figure 2.6 Presumed mechanism of chain elongation on the surface of rubber particles

A particle bond rubber transferase has been demonstrated in rubber bearing plants, such as guayule and *Ficus elastica* [27]. It has been reported that the rubber transferase may be bound to the rubber particle in *Hevea brasiliensis* [2, 28]. Since the rubber transferase is tightly associated with the rubber particles, the enzyme is still present on them after the particles are washed. It is noteworthy that the activity of the enzyme increases with an increase in the concentration of washed rubber particles. This finding suggests that the amount of polyprenyl diphosphate, having an active terminal group, is proportional to the concentration of washed rubber particles. The rubber transferase from *Hevea* latex [29] and guayule was partially purified and was estimated to have a molecular weight of 6×10^4.

2.6 Biosynthesis Study of NR

The biosynthesis of rubber is a process to form rubber in bio-system using enzyme as catalyst. Radioactive allylic diphosphate was used as a precursor such as mevalonates, isopentenyl diphosphate to investigate the biosynthesis mechanism. The technique used for biosynthesis study is called the *in vitro* rubber biosynthesis. The *in vitro* rubber biosynthesis has been studied in whole latex, freeze-dried latex serum, bottom fraction and washed rubber particles, which prepared by gel-filtration chromatography or high speed centrifugation/flotation.

2.6.1 Whole latex and Freeze-dried latex serum

In the case of whole latex, the incorporation of ^{14}C-IPP into rubber was found to be proportional to the amount of latex added [30], while the activity in freeze-dried latex was found to be highest, due to the presence of very small rubber particles [31].

2.6.2 Bottom fraction

A recent method for *in vitro* synthesis of new rubber molecule was established by using the freshly prepared bottom fraction (fresh BF) [32, 33]. The *in vitro* rubber was formed by incubation of fresh BF, including additional initiating species into BF. The rubber yield was as high as 120-160 mg from BF (dry weight of 790 mg) taking into account the fact that rubber was formed with the addition of very small amounts of IPP or FPP as the initiating species. This indicates that BF contains all of the enzymes and precursor to produce rubber molecule. The formation of new rubber was confirmed by the incorporation of ^{14}C-IPP into the resulting rubber [32]. The rubber from BF showed a typical bimodal MWD, which is rich in the low molecular weight fraction, compared to the endogenous rubber, which slightly incorporates ^{14}C-IPP by a chain elongation reaction, while the low molecular weight fraction is composed of newly formed rubber molecules.

2.6.3 Washed rubber particles

The initiation of new rubber molecules has been studied by using radioactive IPP and washed rubber particles (WRP) prepared by centrifugation or gel permeation techniques from latex. It has been confirmed that the addition of FPP in the *trans* configuration has a stimulating effect on the rubber formation in a system containing IPP and WRPs from *Hevea brasiliensis* [34], *Parthenium argentatum* and *Ficus elastica*. Direct incorporation of [1-^{3}H]neryl diphosphate (NPP) and [1-^{3}H]geranyl diphosphate (GPP) was reported in the case of *Hevea brasiliensis* [28]. However, allylic diphosphates of chain lengths C_5-C_{20} also show the stimulating effect, the efficiency of which increases with increasing chain length of the allylic diphosphates, i.e. $C_5 < C_{10} < C_{15} < C_{20}$, rather than the geometric isomerism of the isoprene units [2]. These finding suggest that the direct initiator is FPP and/or GGPP in the case of *Hevea brasiliensis*. Similarly, the use of C_5-, C_{10}-, C_{15}-, C_{20}-diphosphates as initiators has been reported for *in vitro* biosynthesis of rubber from IPP and WRPs from *Parthenium argentatum* [35]. It is remarkable that, in this study, these allylic diphosphates- including GPP and NPP- had equal activity in the polymerization reaction. This suggests that any short-chain allylic diphosphate is able to act as an initiator, independently of chain length or geometric isomerism.

Based on the studies of *in vitro* rubber biosynthesis, NR is synthesized by rubber transferase. By analogy with other prenyltransferases, it is believed that rubber transferase catalyzes the transfer of *cis*-polyprenyl diphosphate to IPP with elimination of inorganic diphosphate. The rubber transferase is a membrane bound onto the rubber particles [34]. Since the enzymatic activity is not diminished even after washing the particles, it has been shown that the enzyme is firmly associated with the particles. Archer and Cockbain described the method for the assay and partial purification of a soluble rubber transferase from the serum fraction of *Hevea* latex [29]. The molecular weight is estimated to be 60 kDa. The optimum pH for the enzymatic activity ranges from 6.8 to 7.5. In addition, the rubber transferase requires a divalent cation such as Mg^{2+} or Mn^{2+} as a cofactor. IPP and allylic diphosphate are needed to be the source of monomer used to elongate the polymer and the initiator of the rubber molecule, respectively [36].

A structural study by ^{1}H-NMR of low molecular weight fraction from transesterified DPNR showed two signals that corresponding to the *trans*-isoprene unit in dimethylallyl-*trans*-*trans* and *trans*-*trans*-*cis* arrangements in the spectrum of polyprenol-16, as shown in **Figure 2.7**. So *Hevea brasiliensis* contains two *trans*-isoprene units at the initiating terminus. This finding indicates that the initiating species of rubber biosynthesis in *Hevea brasiliensis* is farnesyl diphosphate (FPP) [37].

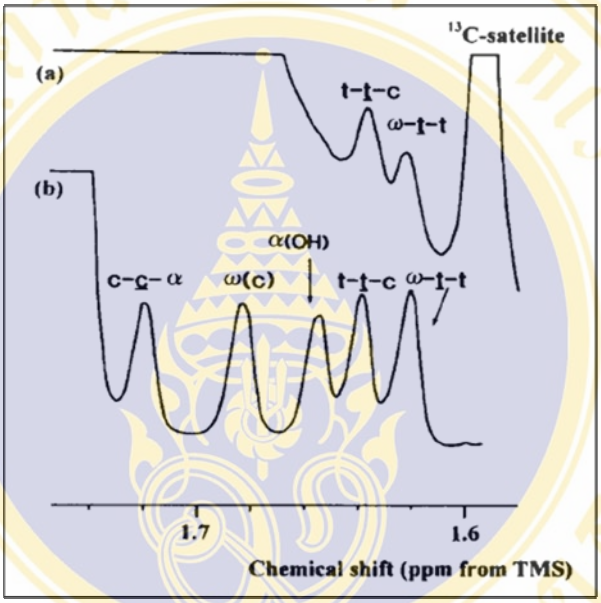


Figure 2.7 ^{1}H NMR spectra of (a) a low molecular weight fraction of TE-DPNR from field latex and (b) polyprenol-16

2.7 Rubber Biosynthesis-Related Protein

2.7.1 Rubber Elongation Factor (REF)

After many successful accumulations of efforts to investigate the *cis*-polyisoprene rubber synthesizing enzyme system from the latex of *Hevea brasiliensis* from the early 1960s-1980s, Light and Dennis reported that the purification of rubber transferase from fresh latex of the rubber tree *Hevea brasiliensis* [25]. The transferase is a dimer with a monomeric molecular mass of 38 kDa, requires Mg^{2+}, and is

stabilized by thiols, which are the similar characteristics of the rubber transferase partially purified by Archer and Cockbain [29].

Although an estimated 7000 *cis*-isoprene units are added in every second per complex of the rubber particle and the transferase, in the absence of the rubber particles and in the presence of dimethylallyl diphosphate (DMAPP), the pure enzyme catalyzes the formation of geranyl diphosphate (GPP) and farnesyl diphosphate (FPP). This means that the same prenyltransferase catalyzes the *trans* addition of IPP to DMAPP and to GPP.

Light *et al.* suggested that both FPP synthases purified from avian liver and yeast could add more than 15 isoprene units to existing rubber molecules, which was consistent with the data by the *Hevea* prenyltransferase [26]. Furthermore, by employing chirally tritiated IPP ([1-^{14}C, 2S-^{3}H]IPP), they confirmed that by using the prenyltransferase purified from *Hevea brasiliensis*, avian liver or yeast, no tritium was incorporated into the rubber particles, indicating *cis* condensation. Thus, they concluded that rubber particles have the ability of the prenyltransferases to alter the stereoselective removal of the 2*R*-prochiral proton of IPP catalyzed by the FPP synthases in favor of the removal of the 2*S*-prochiral proton, indicating a quite novel example of a switch in enzyme stereospecificity.

Following further investigation into the properties of rubber particles that enable FPP synthase to make the stereochemical switch of *trans* additions of IPP to *cis* and override the usual termination after two *trans* additions to DMAPP, Light and colleagues discovered a new protein which they named “rubber elongation factor (REF)” [26]. REF is a protein that is bound tightly to serum-free rubber particles from the latex of *Hevea brasiliensis*. When washed rubber particles are extracted with 1% sodium dodecyl sulfate (SDS), only one protein is observed by SDS-PAGE. REF has a molecular mass of 14,600 Da, and is associated specifically rubber particles in whole latex. It comprises 10-16% of the total protein in whole latex, but is absent in C-serum, the supernatant fluid obtained when rubber particles are removed by centrifugation. The amount of REF in whole latex is proportional to the rubber content in latex. Prenyl chain elongation ceases upon removal of REF, and is inhibited upon binding of specific antibodies raised against REF. Thus, it was suggested that REF plays a critical role in rubber elongation by docking and positioning prenyltransferase on the rubber

molecule. Such docking may involve a reorientation of the binding sites for IPP and the allylic primer substrate.

Under the electron microscope, serum-free rubber particle preparations are seen to contain particles with diameters from 800 nm to as little as 10 nm. In the presence of 1% SDS, no particles smaller than 100 nm are observed, suggesting that the smaller particles may be mainly composed of REF molecules [38].

As REF is firmly associated with the rubber particle, it is further suggested that there is cytoplasmic assembly of the rubber particle whereby the starter molecules, short chain prenyl diphosphates, assemble with the requisite rubber particle surface components of lipids, phospholipids and proteins, mainly REF.

Against the staggering explanation of the implication of REF in the rubber transferase function, which switches the stereospecificity of the enzyme by docking and positioning the soluble *trans*-prenyltransferase from *Hevea brasiliensis* on the rubber molecule, Cornish has reported her re-examined results on the functions of prenyltransferases from latex of *Hevea brasiliensis* [34]. She demonstrated that the soluble *trans*-prenyltransferases from the latex of *Hevea brasiliensis* functions solely as FPP synthase, and plays no direct role in *cis*-1,4-polyisoprene elongation. On the other hand, the *cis*-1,4-prenyltransferase is firmly associated with the *Hevea brasiliensis* rubber particle, as is also the case in other rubber producing species. Dennis and Light reported that the plant *cis*-prenyltransferases are membrane-bound enzymes, which is comparable with the situation in animal enzyme systems [25, 26, 38 and 39].

2.7.2 Small Rubber Particle Protein (SRPP)

Rubber particles are essential components in rubber synthesis *in vitro* [18], and most experiments showing *in vitro* rubber synthesis with labeled IPP have always required the presence of such particles. It is plausible that the surface of the rubber particle is in the appropriate location for a rubber transferase, which polymerizes a hydrophobic polymer into the particle interior while obtaining hydrophilic substrates such as IPP and FPP from the cytosol. Oh *et al*. have isolated and characterized a full-length cDNA encoding a small rubber particle protein (SRPP) [40].

The amino acid sequence of SRPP is highly homologous to that of REF, suggesting its potential involvement in rubber biosynthesis. SRPP has also high sequence homology to a stress-related protein of *Phaseolus vulgaris*. SRPP is encoded by a single gene and highly expressed in latex. Although the cloned cDNA encodes the known latex allergen, an *in vitro* rubber synthesis assay showed that the protein plays a positive role in rubber biosynthesis.

2.7.3 Rubber Biosynthesis Stimulator Protein (RBSP)

In 1987, Archer and Audley detected the presence of a proteinaceous factor in C-serum of centrifuged latex [2]. Each fraction was separated by chromatographic technique of production WRP. The rubber particles were in fraction A with proteins in fraction B as expected; however fraction C, where the low molecular weight compounds were eluted, also contains some proteinaceous material.

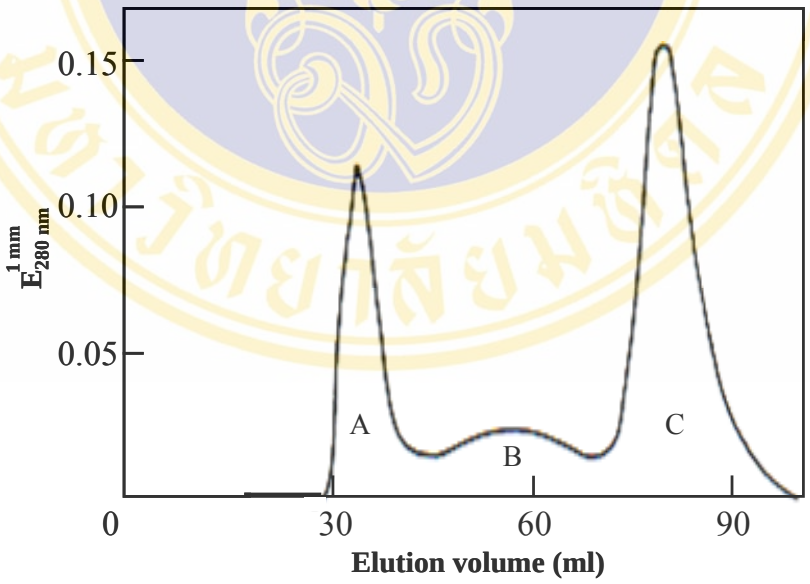


Figure 2.8 Gel-filtration chromatography of *Hevea brasiliensis* latex serum

Table 2.2 shows the data obtained when fraction C was added back to incubations of fraction A (WRP) and ^{14}C-IPP. It was found that fraction C accelerated the incorporation of ^{14}C-IPP into rubber only if neryl diphosphate (NPP) is absent and that the factor responsible is heat labile.

Table 2.2 Stimulation of incorporation of ^{14}C-IPP into rubber by fraction C

Fractions incubated	Incorporation (%)
WRP	0.6
WRP+ C	2.7
WRP+ NPP	6.2
WRP+ C+ NPP	6.0
WRP+ C_H	1.6
WRP+ C_H*+ NPP	5.6

* C_H is fraction C after heating at 100°C.

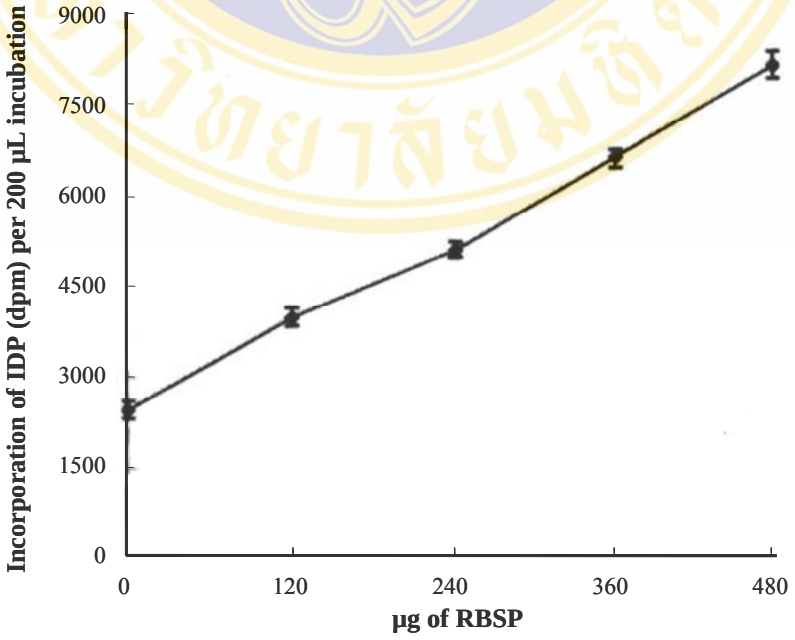


Figure 2.9 Incorporation of labeled IPP with increasing amount of RBSP (Rubber Biosynthesis Stimulator Protein)

Yusof et al. purified and characterized a stimulatory protein reported by Archer and Audley from the C-serum, which they have named as the "rubber biosynthesis stimulator protein (RBSP)" [41].

The assay in **Figure 2.9** shows the purified RBSP increased the incorporation of labeled IPP into rubber (up to 240% increase) and the relationship between the uptake and the amount of sample was linear over the range tested. The molecular weight of the RBSP was found to be about 13 kDa by mass spectroscopy.

2.8 Rubber Particle Size

It is believed that the particle size of rubber in latex is an important factor influencing the biosynthesis mechanisms controlling molecular weight of NR. In 1966, the effect of rubber particle size on the *in vitro* rubber biosynthesis has been studied by McMullen and McSweeney [36]. **Table 2.3** shows the amount of IPP incorporated per milligram of rubber by fractions II, III and IV. These fractions were obtained from the same original latex sample and were subjected to a centrifugal fractionation procedure. From the table, it can be concluded that the activity of rubber particles per unit surface area increases with diminishing particle size.

Table 2.3 Effect of rubber particle size on the incorporation of IPP

Latex fraction	Diameter of particles at size-frequency curve	Specific surface area of rubber (m^2/mg)	Isoprene residues incorporated (µg/mg of rubber)	Area of rubber surface per incorporated isoprene residue ($A^{°2}$)
II	900	50	2.0	280
II	400	140	10.0	160
IV	200	240	40.0	70

In confirmation of these results, the relationship between the particle size and radioactivity of rubber biosynthesis was reported by Ohya et al. [1], as shown in **Figure 2.10**. The fractions containing small rubber particles showed much higher

enzyme activity than bigger ones. The maximum incorporation of IPP increased to a remarkably high level. On the other hand, the fractions consisting of bigger rubber particles exhibited a very small enzyme activity, the level of which was slightly higher than that of the control samples which were prepared by boiling the fraction at 100ºC for 30 min before the incubation. For the total cream rubber particles ranging from 0.1 to 5 μm, a significant difference in the incorporation was observed between the fractions of the bigger rubber particles and others. It was presumed that the lack of an essential factor or significant inhibition for the enzyme activity can occur for particles bigger than a certain size. The relationship between enzyme activity and particle size was expressed in surface area, as listed in **Table 2.4**.

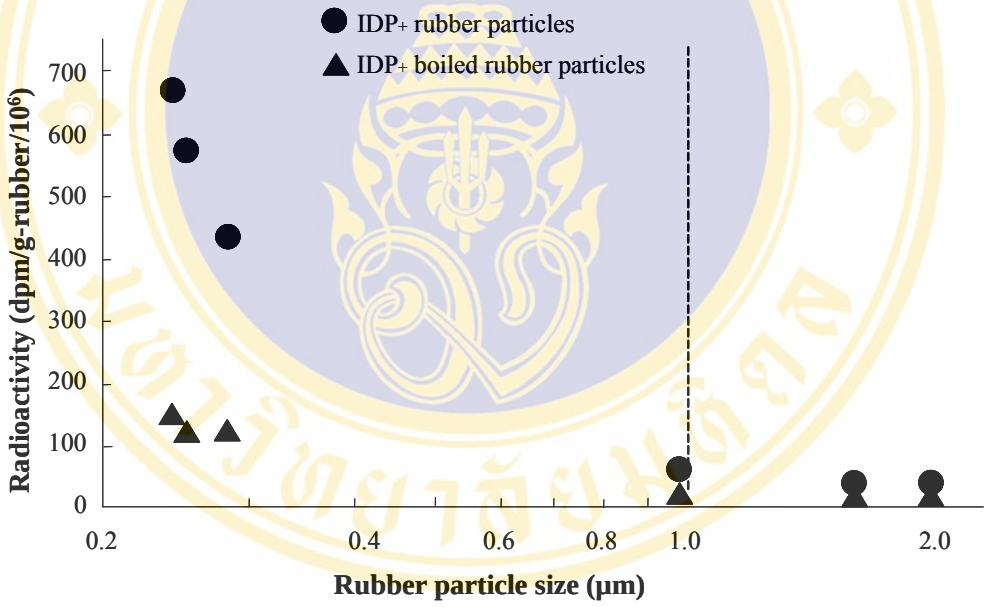


Figure 2.10 Rubber particle size and enzyme activity [1]

Table 2.4 Enzyme activity per unit surface of rubber particles

Fraction	Average diameter of particles (μm)	Specific surface area (m^2/g)	Enzyme activity per unit surface ($dpm/m^2/10^6$)
Fraction 1	1.93	2.3	15.2
Fraction 2	1.57	3.1	11.9
Fraction 3	0.98	5.1	12.6
Fraction 4	0.28	19.5	22.1
Fraction 5	0.25	21.0	27.9
Fraction 6	0.24	22.6	30.0

This was similar to that observed in **Table 2.3** showing the high enzyme activity for smaller particles. However, no difference in the enzyme activity was observed among the bigger particle fractions. These finding indicated that only small rubber particles play a significant role in the chain elongation of rubber molecules.

2.9 Small Rubber Particle (SRP)

The latex contains the rubber particles in diameter between 0.05 and 3.0 μm. Small rubber particles (SRP) and large rubber particles (LRP) can be separated by the centrifugation of rubber latex. The rubber particles in diameter less than 0.25 μm are defined as SRP and those with larger diameter are LRP.

SRP separating from the serum fraction was reported to contain high molecular weight rubber molecules showing the unimodal MWD, as shown in **Figure 2.11**. On the contrary, LRP obtaining from the cream fraction presented the bimodal MWD of low and high molecular weight rubber molecules, [42].

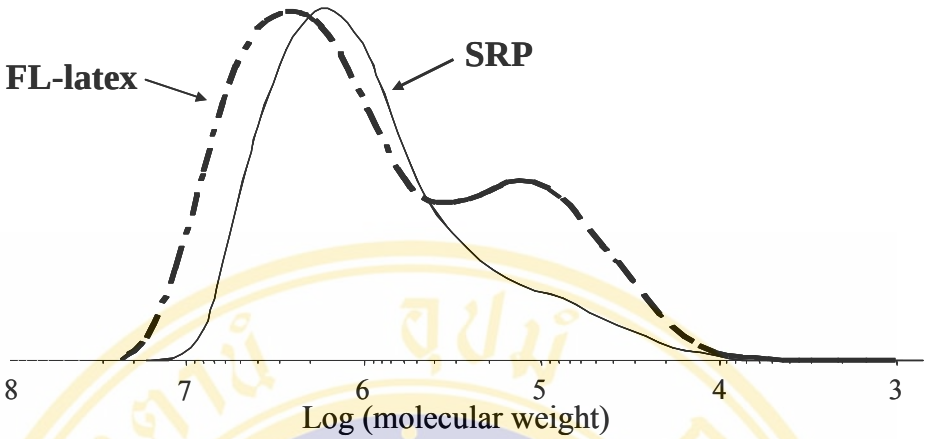


Figure 2.11 Molecular weight distribution of fresh latex (FL-latex) and small rubber particle (SRP) [42]

In addition, SRP was presumed to be composed of linear rubber molecules containing no phospholipid terminal, while LRP were polyisoprene molecules terminated by phospholipid contains the branched points [42], as shown in **Table 2.5**.

The terminating end of the rubber molecule is functionalized by phospholipid, containing 1-2 long-chain fatty acid esters [43]. Based on this finding, the ester content is regarded as an index of fatty acid molecules per rubber chain. From the table, it implies that the rubber molecules in SRP are not terminated by phospholipid groups as could be detected in the LRP molecules.

The branching characters of SRP and LRP are confirmed from the gel content of rubber. The higher gel content indicates the higher branch point. From the table, it suggests that the LRP rubber consists of branched and crosslinked molecules, while the SRP rubber is composed of linear molecules.

Table 2.5 Ester, nitrogen and gel contents of purified cream and serum rubbers

Sample	Ester content (mmol/kg)	Nitrogen content (% w/w)	Gel content (% w/w)
Original rubber	10.2	0.25	3.5
Cream rubber	8.1	0.22	7.1
	(0)	(0.21)	(0)
Serum rubber	0.1	0.02	1.3
	(0)	(0.02)	(0)

As mentioned above, it can be concluded that SRP is mainly consisted of linear molecules having both high and low molecular weight rubber chains, which are expected to be composed of active chain end for chain elongation. On the other hand, LRP is mostly comprised of the branch molecules. The branch points in LRP are expected to be derived from the aggregation of those low molecular weight rubber chains *via* the aggregation of phospholipid on the surface of rubber particle. The proposed structure of rubber chains in SRP and LRP is shown in **Figure 2.12** [44].

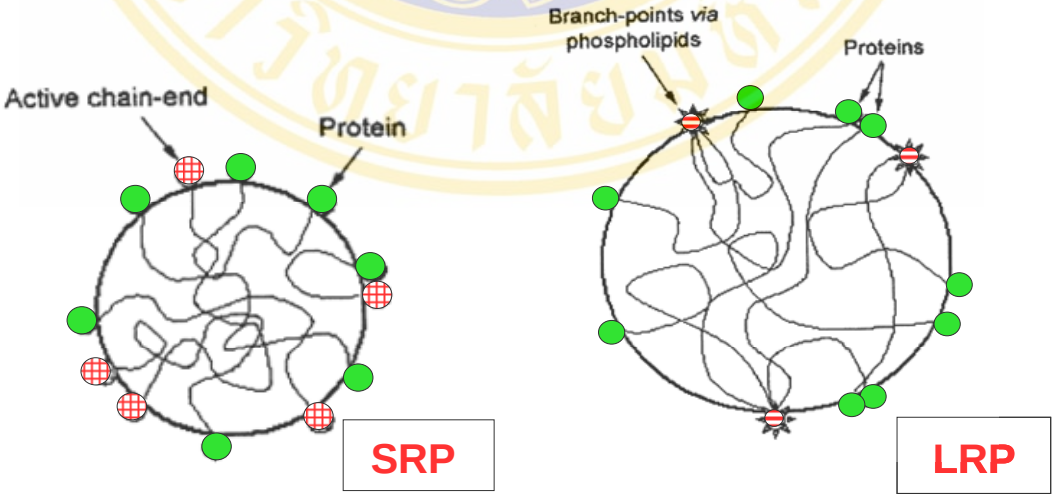


Figure 2.12 Presumed structure of SRP and LRP

CHAPTER III
MATERIAL AND METHODS

3.1 Material

Fresh latex (FL-latex) was provided from the private plantation of Thai Rubber Latex Company, Chonburi, Thailand.

The FL-latex was obtained by tapping the rubber trees of *Hevea brasiliensis* (RRIM 600). It was collected into ice-chilled plastic containers under the collecting tubes of latex drops. The latex was filtered through a muslin cloth to remove some coagulums and impurities into polyethylene terephthalate (PET) bottles and then kept cool in icebox to prevent auto-coagulation. The FL-latex was carefully transported to laboratory at Mahidol University, Salaya campus, Nakhonpathom.

3.2 Chemicals

Table 3.1 Chemicals used in the present work

Chemical	Manufacturer
Ammonium hydroxide	WAKO
Bovine serum albumin	Sigma
Butanol	Nacalai tesque
Coomassie Brilliant Blue R-250	Fluka chemical
Di-potassium hydrogenphosphate	Nacalai tesque

Table 3.1 Chemicals used in the present work (cont.)

Chemical	Manufacturer
Farnesyl diphosphate	Sigma
Geranyl diphosphate	Sigma
Geranyl geranyl diphosphate	Sigma
Glycine	Nacalai tesque
Hexane	Nacalai tesque
Hydrochloric acid	Nacalai tesque
[1-^{14}C]-Isopentenyl diphosphate	Amersham bioscience
Isopropanol	Nacalai tesque
Magnesium dichloride	Nacalai tesque
2-mercaptoethanol	Nacalai tesque
Methanol	Nacalai tesque
Potassium dihydrogenphosphate	Nacalai tesque
Potassium fluoride	Nacalai tesque
Silver staining solution	Nacalai tesque
Sodium chloride	Nacalai tesque
Sodium dodecyl sulfate	Nacalai tesque
Tetrahydrofuran	MERCK
Toluene	Nacalai tesque
Tris-HCl	Nacalai tesque

Table 3.1 Chemicals used in the present work (cont.)

Chemical	Manufacturer
Triton X-100	Nacalai tesque
Trypsin	Nacalai tesque
Trypsin inhibitor	Nacalai tesque
Tween-20	Nacalai tesque
Ultrogel AcA-44	Sigma

3.3 Instruments

Table 3.2 Instruments used in the present work

Instrument	Model
Anion exchange column	GE: Resource Q
Centrifugal evaporator	IWAKI: VEC-260
Freeze-dryer	EYELA: FD-5
Gel-filtration column	GE: Superdex-200
High speed refrigerated centrifuge	HITCHI: CR22G
Hydroxyapatite column	BIO-SCALE: CHT10-1
Liquid chromatography system	AKTA: AKTAexplorer
Liquid scintillation counter	BECKMAN: LS-6500

Table 3.2 Instruments used in the present work (cont.)

Instrument	Model
Rotary evaporator	TOMY: MCX-150
UV-Vis spectroscopy	HITACHI: Gene spec III

3.4 Preparation of Small Rubber Particles

The FL-latex was centrifuged by continuous steps at 12,000 g for 30 min and then 43,000 g for 60 min at 4°C. Small rubber particle (SRP) was collected from the rubber cream in zone 2. After that SRP cream was dispersed in phosphate buffer and kept at -70°C until use.

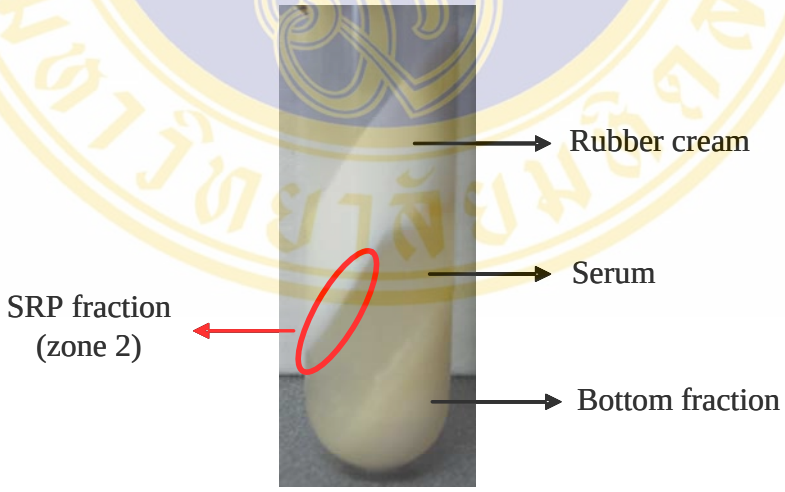


Figure 3.1 Fractions of fresh latex from ultracentrifugation

3.5 *In vitro* Rubber Biosynthesis

The activation effect of washing serum separated from SRP was investigated by the *in vitro* rubber biosynthesis. The incubation mixture, as shown in **Table 3.3**, was carried out for 12 hours or longer at 30ºC. After the incubation, the mixture was washed with saturated sodium chloride solution and extracted with butanol saturated with water and then *in vitro* rubber was obtained. The incorporation of ^{14}C-radiolabeled-isopentenyl diphosphate (^{14}C-IPP) into *in vitro* rubber dissolved in a mixture of toluene-hexane was analyzed by liquid scintillation counter.

Table 3.3 Incubation mixture used for *in vitro* rubber biosynthesis by WSRP

Incubation mixture	Volume
1 M Phosphate buffer, pH 7.5	2.5 µl
10% 2-Mercaptoethanol	1.4 µl
1 M Potassium fluoride	2.0 µl
0.1 M Magnesium dichloride	4.0 µl
0.5 mM FPP	3.0 µl
0.5 mM [1-14C]IPP	10.0 µl
SRP or washed SRP	~ 1 mg
H_2O up to	50.0 µl

3.6 Effect of Allylic Diphosphate on the Activation of *in vitro* Rubber Biosynthesis

3.6.1 Preparation of washing serum

The rubber particle was separated by centrifugation of SRP fraction to obtain washing serum, as shown in **Figure 3.2**. The SRP fraction was centrifuged at 15,000 rpm for 50 min. The buffer solution of 0.25 M Tris-HCl, pH 8.0, containing 1% Tween-20 and 5 mM 2-mercaptoethanol called as washing buffer was used to prepare washing serum. And then washing serum was separated from washed small rubber particle (WSRP) by syringe and was added back to incubations of WSRP and ^{14}C-IPP to test the activation effect.

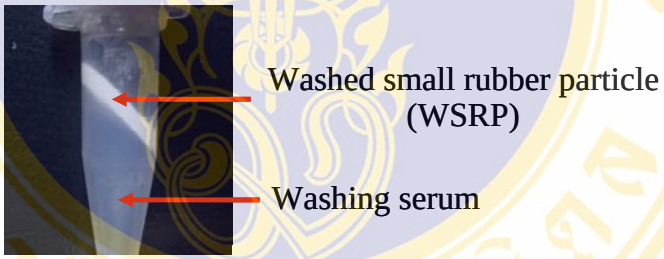


Figure 3.2 Washing serum separated from SRP by centrifugation

3.6.2 Separation of allylic diphosphate from washing serum

Washing serum was divided into two fractions. First was extracted with butanol saturated with water and another was filtered by Microcon at 14,000 g for 60 min. Filtration with Microcon, which the limitation of membrane is 10 kDa, can separate substrate into two fractions, *i.e.*, upper and lower fractions. The upper and lower fractions were presumed to be the fractions of protein and allylic substrate, respectively. The upper and lower fractions were incubated with ^{14}C-IPP. After that, the lower fraction was extracted with saturated butanol. The butanol extracts from washing serum and lower fraction were analyzed by thin layer chromatography (TLC)

with the mobile phase of (6:3:1) isopropanol: ammonium hydroxide: water and were simultaneously compared with standard allylic diphosphate, as shown in **Table 3.4**.

Table 3.4 Standard allylic diphosphate used in thin layer chromatography

Standard allylic diphosphate	Molecular weight (MW)
Geranyl diphosphate (GPP)	314
Farnesyl diphosphate (FPP)	382
Geranyl geranyl diphosphate (GGPP)	450

The butanol extracts from the lower fraction was repeatedly analyzed by TLC technique with the mobile phase of (6:3:1) isopropanol: ammonium hydroxide: water. Then the TLC plate without iodine vapor detection was scraped by spatula to 12 fractions, as shown in **Figure 3.3**. After that each substrate was extracted by saturated butanol and incubated with ^{14}C-IPP.

Figure 3.3 TLC plate scraped into 12 fractions

3.7 Effect of Protein on the Activation of *in vitro* Rubber Biosynthesis

Washing serum used to study the activation effect of protein was prepared by 2 methods. First was prepared by gel-filtration chromatography and another was prepared by centrifugation with Triton® X-100.

3.7.1 Preparation of washing serum by gel-filtration chromatography

Washing serum was prepared from SRP fraction by developed on the gel-filtration column (Ultrogel AcA-44), as shown in **Figure 3.4**. The buffer solution of 0.25 M Tris-HCl, pH 8.0, containing 1% Tween-20 and 5 mM 2-mercaptoethanol was used as the eluting buffer. The washing serum was collected after rubber fraction (WSRP) into 8 fractions and then subjected to centrifugation to remove completely rubber fraction. After that each fraction was incubated with ^{14}C-IPP and WSRP. The fraction found to have the highest activity was incubated with ^{14}C-IPP and WSRP in order to test the activation effect at the various amounts.

Each fraction of washing serum from gel-filtration chromatography was concentrated with freeze-dryer.

Figure 3.4 Gel-filtration column for preparing washing serum

3.7.1.1 The presence of protein in washing serum

The presence of protein in washing serum was confirmed by ultraviolet (UV) spectroscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques.

(1) UV spectroscopy

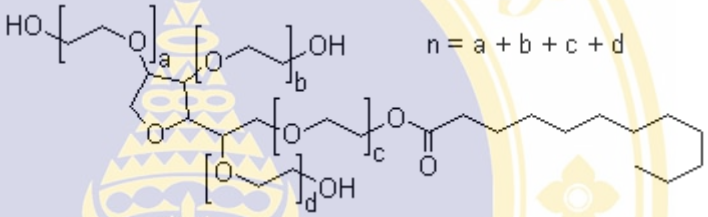
Washing serum was measured with UV spectroscopy by using the Bradford assay procedure so as to determine of its protein concentration [45]. A standard curve was made by using bovine serum albumin (BSA). To determine the protein concentration of freeze-dried washing serum, the Bradford reagent was added to the washing serum and the absorbance was measured at 595 nm.

(2) Polyacrylamide gel electrophoresis

Washing serum was analyzed by SDS-PAGE technique. Proteins in washing serum were separated by electrophoresis under denaturing conditions with 15% separating gel. Sample of 10 µl was loaded in each well of the gel. Molecular weight reference marker was run simultaneously in all experiments. SDS-PAGE was done at a constant current of 30 mA for 1 hour. The SDS-PAGE gels were subsequently stained with Coomassie Brilliant Blue R-250 staining system for 20 min and were de-stained overnight in de-staining solution.

3.7.1.2 Effect of Tween-20 on the IPP incorporation into rubber

Tween-20 in the washing buffer used to prepare washed rubber particle has been reported to be an inhibitor in the IPP incorporation into the rubber [46]. Therefore, the effect of Tween-20 on the IPP incorporation into rubber was studied by varying the amounts of buffer with and without Tween-20. The incorporation of ^{14}C-IPP into the reaction mixture was detected to evaluate this effect.

Molecular structure 

Molecular formula $C_{18}H_{34}O_6 \cdot (C_2H_4O)_n$

Scheme 3.1 Molecular structure and formula of Tween-20

3.7.1.3 Activation of *in vitro* rubber biosynthesis by protein

The activation factor of washing serum from SRP was also confirmed whether it is protein or allylic substrate by heat and trypsin treatments. Because it is known that heat and trypsin procedures are good methods to degrade protein. So, if the activator in washing serum is protein, the activity of IPP incorporation into rubber will decrease after heat and trypsin treatments.

(1) Heat treatment

Washing serum was heated in water bath for 15 min. After that heated washing serum was incubated with ^{14}C-IPP and WSRP for comparing the activity before and after the heat treatment.

(2) Trypsin treatment

Washing serum was added with typsin in the final concentration 1.5 mg/ml and was incubated at 30ºC for 90 min. After that it was added with trypsin inhibitor in the final concentration 2.0 mg/ml and incubated at 30ºC for 15 min. Finally, trypsinized washing serum was incubated with ^{14}C-IPP and WSRP for comparing the activity before and after the trypsin treatments.

3.7.2 Preparation of washing serum by centrifugation with Triton® X-100

Washing serum was prepared by incubation of SRP with 0.5% Triton® X-100 at 20ºC for 1 hour. Then the mixture was centrifuged at 15,000 rpm for 40 min and washing serum was separated from WSRP by syringe (First serum). After that WSRP was washed with 0.5% Triton® X-100 again by centrifugation without the incubation and washing serum was collected (Second serum). WSRP was washed with the washing buffer of 0.25 M Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol twice to remove Triton® X-100 by centrifugation and washing serum was collected (Third and Forth serum). Washing serum was subsequently concentrated by freeze-dryer.

3.7.2.1 Protein purification from washing serum

Proteins in washing serum were purified by the chromatography techniques [47]. These techniques were summarized in **Table 3.5**.

Table 3.5 Purification of the activator protein from washing serum

Steps	Purification
Step 1	Anion exchange chromatography (Resource-Q, 50 mM Tris-HCl pH7.5+ 5 mM 2-mercaptoethanol)
Step 2	Gel-filtration chromatography (Superdex-200, 50 mM Tris-HCl pH7.5+ 5 mM 2-mercaptoethanol+ 150 mM Sodium chloride)
Step 3	Anion exchange chromatography (Resource-Q,50 mM Tris-HCl pH7.5+ 5 mM 2-mercaptoethanol)
Step 4	Hydroxyapatite chromatography (CHT10-I, 10 mM Potassium phosphate, pH 6.8)

(1) Anion exchange chromatography

Washing serum was subjected to buffer exchange with PD-10 desalting column and then was loaded onto anion exchange chromatography. The Resource-Q column was used as an anion exchange column. The mixture of 50 mM Tris-HCl pH 7.5 and 5 mM 2-mercaptoethanol was used as the starting buffer for allows the binding of the desired solute molecules. The starting buffer and sample was filtered through 0.2 µm filter before use. Bound protein was eluted with Tris-HCl buffer containing a linear NaCl gradient of 0 to 0.5 M, as shown in **Figure 3.5**. Then the eluted sample was fractionated into 1 ml/fraction and each fraction was incubated with ^{14}C-IPP and WSRP.

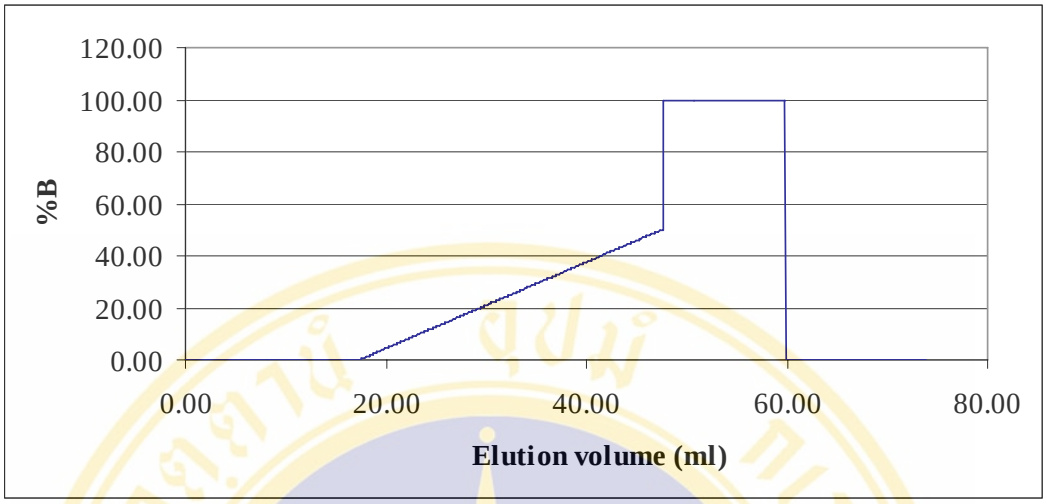


Figure 3.5 Condition of NaCl gradient of 0 to 0.5 M

(2) Gel-filtration chromatography

The fractions of high activity from anion exchange column were combined and PD-10 desalting column was used to exchange the buffer with the eluting buffer of gel-filtration column. The buffer solution of 50 mM Tris-HCl pH 7.5, 5 mM 2-mercaptoethanol was used as the eluting buffer. The eluting buffer and sample was filtered through 0.2 µm filter before use. And then the sample was loaded onto the gel-filtration column, a Superdex-200 column. The molecular weight was estimated by calibration curve of standard proteins. The standard proteins used for making calibration curve are shown in **Table 3.4**.

The eluted sample was fractionated and each fraction was incubated with ^{14}C-IPP and WSRP. The fraction of high activity from gel-filtration column was combined and analyzed by SDS-PAGE.

Table 3.6 Various molecular weight standard proteins used for making calibration curve

Standard Proteins	Molecular weight
Ribonuclease	13,700
Ovalbumin	43,000
Aldolase	158,000
Ferritin	440,000

(3) Anion exchange chromatography

The fractions of high activity from gel-filtration column were subjected to exchange the buffer to starting buffer with PD-10 desalting column. The Resource-Q column was used as an anion exchange column. The mixture of 50 mM Tris-HCl pH 7.5 and 5 mM 2-mercaptoethanol was used as the starting buffer. The starting buffer and sample was filtered through a 0.2 µm filter before use. Bound protein was eluted with Tris-HCl buffer containing a linear NaCl gradient of 0 to 0.3 M.

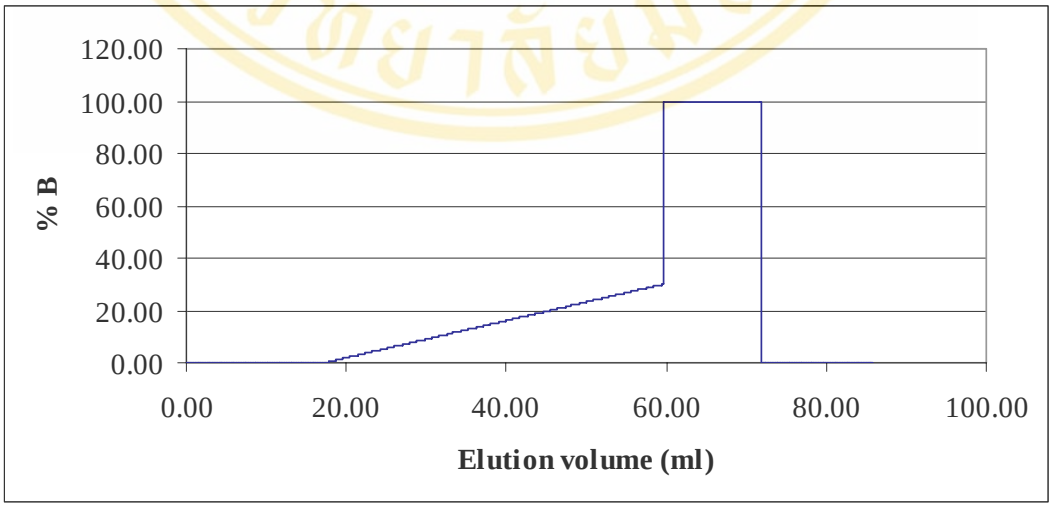


Figure 3.6 Condition of NaCl gradient of 0 to 0.3 M

(4) Hydroxyapatite chromatography

Hydroxyapatite chromatography was considered to be a mixed-mode ion exchange. It has proved to be an effective purification mechanism in a variety of processes, providing biomolecular selectivity complementary to more traditional ion exchange or hydrophobic interaction techniques [48].

Hydroxyapatite column was used to purify the fractions of high activity from anion exchange chromatography. The buffer solution of 10 mM potassium phosphate, pH 6.8 was used as the starting buffer. The sample was diluted to reduce the ionic strength to that of the starting buffer, which PD-10 desalting column was used to exchange the buffer. Buffer solution and sample was filtered through 0.2 μm filter before use. Apply the sample and allow unbound material to pass through the column. The bound material was eluted with a gradient of increasing concentration of potassium phosphate buffer from 10 to 500 mM.

(5) SDS-PAGE

The eluted fractions from each column were concentrated by Microcon, which the limitation of membrane is 3 kDa, and then the sample was analyzed by SDS-PAGE with 15% separating gel and staining with silver stain.

CHAPTER IV
RESULTS AND DISCUSSION

It was reported that small rubber particle (SRP) separated from fresh *Hevea brasiliensis* latex by high-speed centrifugation was proved to be composed of linear and active rubber molecules [44]. This indicates that SRP is the active rubber particle used for the *in vitro* rubber biosynthesis. The *in vitro* rubber biosynthesis was found to be formed by the incubation of SRP or washed SRP (WSRP) at 30ºC for 12 hours. The effect of SRP and WSRP on the IPP incorporation into rubber is shown in **Table 4.1**.

The high IPP incorporation activity by SRP is illustrated in **Table 4.1**. It was found that the high IPP incorporation decreased from around 41,000 to 9,000 dpm after washing of SRP by centrifugation. The activity was recovered by the addition of FPP showing the IPP incorporation was in the same level as SRP. This indicates that some substrates, which required for the *in vitro* rubber biosynthesis, could be removed by washing method.

In 1993, the *in vitro* rubber biosynthesis using whole latex of *Hevea brasiliensis*, assayed in the presence of ^{14}C-IPP, was reported by Cornish [34]. Washing the rubber particles using the centrifugation/flotation procedure almost completely eliminates IPP incorporation. However, when FPP was added into washed rubber particles and IPP, the IPP incorporation restored and was more than the level observed in whole latex. FPP is known to be an effective initiator of rubber molecule formation [37].

Table 4.1 Incorporation of ^{14}C-IPP into *in vitro* rubber synthesized by SRP, SRP after washing (WSRP) and WSRP with FPP

Sample	^{14}C-Radioactivity (dpm)
SRP	41,824
WSRP	9,257
WSRP+ FPP	45,903

4.1 Effect of Allylic Diphosphate on the Activation of *in vitro* Rubber Biosynthesis

4.1.1 Preparation of washing serum

Washed rubber particles (WRP) were prepared by a centrifugal fractionation procedure, first employed in studies of IPP polymerization by McMullen and McSweeney [36]. The rubber particles were washed by diluting several times with a large proportion of buffer solution and centrifuging. The washed rubber particles prepared from centrifugation were investigated by the incorporation of ^{14}C-radiolebeled-isopentenyl diphosphate (^{14}C-IPP). McMullen and McSweeney have found that the activity largely increased, when fresh latex serum was added back to incubations of WRP and ^{14}C-IPP. This experiment demonstrated that the enzyme system responsible for the incorporation of IPP into rubber is present in latex serum as well as at the surface of unwashed rubber particles [36].

In this part, the small rubber particles were also washed by centrifugation in order to separate washing serum and WSRP. The effect of washing serum on the incorporation of ^{14}C-IPP into rubber was studied, as shown in **Table 4.2**. It was found that the IPP incorporation by WSRP without any substrate showed the low incorporation as 13,000 dpm, used as a control of the experiment. The IPP incorporation increased when washing serum and/or FPP was added. This suggests that washing serum could activate the *in vitro* rubber biosynthesis, but was lower

effective than FPP. Because FPP was found to be the initiating species of rubber biosynthesis [37], therefore, it can activate the highest IPP incorporation into rubber.

Table 4.2 Effect of washing serum on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	12,820
WSRP+ Washing serum	35,413
WSRP+ FPP	52,765

4.1.2 Separation of allylic diphosphate from washing serum

The washing serum was divided into two fractions by Microcon, which the limitation of membrane is 10 kDa. The upper and lower fractions were presumed to be the fractions containing protein and allylic substrate, respectively.

The effect of upper and lower fractions of washing serum after separation by Microcon on the incorporation of IPP into *in vitro* rubber is shown in **Table 4.3 and 4.4**. It was presented that the IPP incorporation was the lowest when no substrate was added, used as a control of the experiment. The addition of upper or lower fractions was found to increase the incorporation of IPP into rubber. The highest effect of added upper and lower fractions on the IPP incorporation was observed by the incubation with 1 µl upper and 15 µl lower fractions, respectively. However, the incorporation decreased when higher amounts of these fractions were added. In addition, the activation by these fractions was less effective than that by FPP. It was possible that there was an inhibitor in serum fractions.

Table 4.3 Effect of upper fraction of washing serum after separation by Microcon on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	13,691
WSRP+ Upper fraction 1 µl	24,567
WSRP+ Upper fraction 3 µl	18,475
WSRP+ Upper fraction 5 µl	13,712
WSRP+ FPP	52,656

Table 4.4 Effect of lower fraction of washing serum after separation by Microcon on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	13,691
WSRP+ Lower fraction 1 µl	15,971
WSRP+ Lower fraction 5 µl	21,119
WSRP+ Lower fraction 15 µl	27,659
WSRP+ Lower fraction 25 µl	22,027
WSRP+ FPP	52,656

The presence of oligo and polyprenol compounds in bottom fraction (BF) prepared by centrifugation of fresh *Hevea* latex was analyzed by Tangpakdee and co-workers [49]. BF was extracted with water-equilibrated butanol and was analyzed by reverse phase-TLC compared with oligo and polyprenol standards.

The lower fraction separated from washing serum by Microcon was also analyzed by thin layer chromatography (TLC). TLC chromatogram of butanol extracts from washing serum and from lower fraction of washing serum, compared with the standard allylic substrates is shown in **Figure 4.1**. It was seen that the butanol extracts of washing serum and the lower fractions of washing serum, after filtration by Microcon, contained more than 3 components of long-chain substrates, while the medium-chain allylic substrates such as GPP, FPP and GGPP was not observed in

these fractions (in the red rectangular). It was possible that the amount of medium-chain allylic diphosphate was too small to detect by TLC.

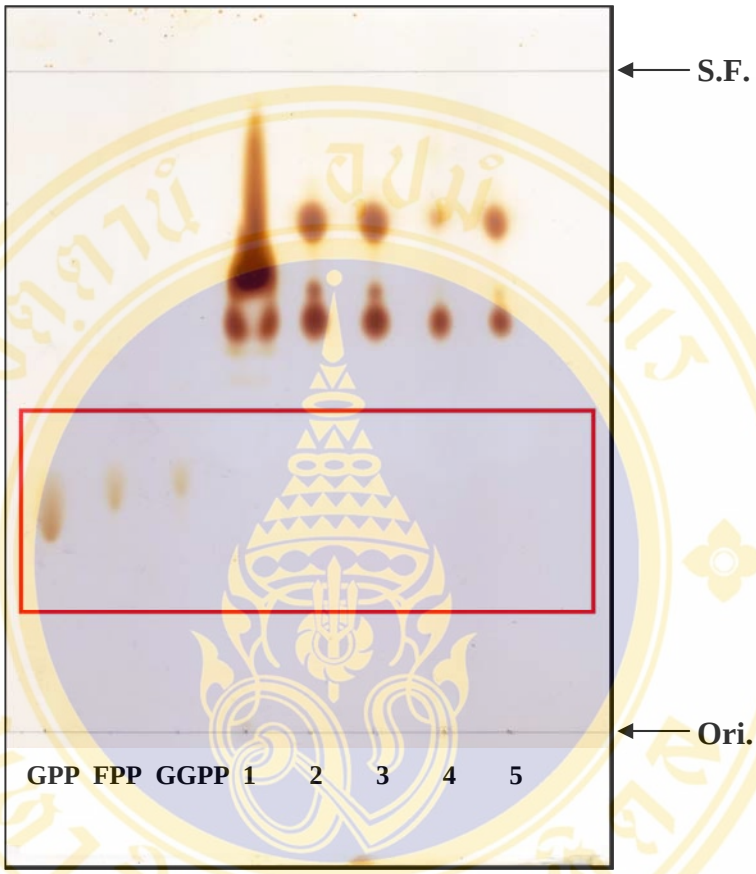


Figure 4.1 TLC chromatogram of butanol extract from washing serum (1) and from lower fraction of washing serum after filtration by Amicon (2, 3) and Microcon (4, 5) compared with GPP, FPP and GGPP

The butanol extracts from the lower fraction was subjected to TLC chromatography. The substrates on TLC chromatogram without iodine vapor detection were scraped to 12 fractions and each fraction was extracted with butanol saturated with water. The activation of the extracted substrates was analyzed by testing the *in vitro* rubber biosynthesis, showing the incorporation of ^{14}C-IPP, as listed in **Table 4.5**.

Table 4.5 Effect of substrates in fraction 1-12 of butanol extracted lower fraction of washing serum separated by TLC on incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	5,951
WSRP+ Fraction 1	17,370
WSRP+ Fraction 2	17,402
WSRP+ Fraction 3	18,180
WSRP+ Fraction 4	18,587
WSRP+ Fraction 5	13,832
WSRP+ Fraction 6	19,096
WSRP+ Fraction 7	18,270
WSRP+ Fraction 8	18,979
WSRP+ Fraction 9	15,550
WSRP+ Fraction 10	17,981
WSRP+ Fraction 11	12,908
WSRP+ Fraction 12	15,363
WSRP+ FPP	58,986

The effect of substrates in fraction 1-12 of butanol extracts from lower fraction of washing serum separated by TLC on incorporation of ^{14}C-IPP into rubber is shown in **Table 4.5**. It was found that all fractions showed the low IPP incorporation into rubber of about 18,000 dpm. Fraction 6 showed the highest IPP incorporation, but the activities in these fractions were no much different and were less effective than that by FPP. This indicates that the extracted fractions could not activate the *in vitro* rubber biosynthesis. Therefore, the allylic diphosphate such as GPP, FPP and GGPP could not be detected in washing serum of SRP.

4.2 Effect of Protein on the Activation of *in vitro* Rubber Biosynthesis

4.2.1 Preparation of washing serum by gel-filtration chromatography

Washed rubber particles (WRP) were formerly prepared by ultracentrifugation, but this was replaced in the later work by gel-filtration on columns of crosslinked hydrophilic polymers. In 1987, Archer and Audley purified rubber latex by gel-filtration chromatography (column of Ultrogel AcA-34) and collected 3 fractions [2]. Fraction A is washed rubber particle. Fraction B and C collected after rubber fraction are proteins, and low molecular weight materials, respectively. The *in vitro* rubber biosynthesis showed the activation effect of fraction C on the incorporation of IPP, when fraction C was added back to incubations of WRP and ^{14}C-IPP.

As same as the Archer and Audley method, washing serum was prepared from SRP fraction by developed on the gel-filtration column (Ultrogel AcA-44). After the elution of rubber fraction, washing serum was collected into 8 fractions and then each fraction was incubated with ^{14}C-IPP and WSRP. The effect of substrates in each fraction of washing serum prepared from gel-filtration chromatography on the incorporation of IPP into rubber is shown in **Table 4.6**.

It was found that the incorporation of IPP into rubber of almost fractions increased. The activity was found in fractions 1-6, when compared with WSRP, used as a control of the experiment. Fraction 2 showed the highest IPP incorporation which was the half of that in the case of FPP.

Table 4.6 Effect of substrates in washing serum fractions from gel-filtration chromatography on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	16,077
WSRP+ Fraction 1	35,337
WSRP+ Fraction 2	36,439
WSRP+ Fraction 3	35,371
WSRP+ Fraction 4	26,920
WSRP+ Fraction 5	24,823
WSRP+ Fraction 6	20,341
WSRP+ Fraction 7	18,636
WSRP+ Fraction 8	16,781
WSRP+ FPP	72,900

Table 4.6 indicates that there was the activation factor contained in the washing serum fractions prepared from gel-filtration chromatography. The highest effect of added washing serum on the IPP incorporation was observed by the incubation with fraction 2 of washing serum. The IPP incorporation in fraction 2 was lower than that of FPP may be because the amount of substrate was too low for activating the incorporation. Therefore, the amount of fraction 2 of washing serum was increased in the incubation mixture. After that, the activating effect was analyzed by the incubation with ^{14}C-IPP and WSRP.

Figure 4.2 shows effect of varying the amount of washing serum fraction 2 into the incubation mixture on the incorporation of ^{14}C-IPP into rubber. The activity decreased when the amount of fraction 2 increased. It may be due to the presence of an inhibitor in washing serum [2]. After that all fraction of washing serum (fraction 1-8) was separated into two fractions, i.e., upper and lower fractions with Microcon and then incubated with ^{14}C-IPP and WSRP.

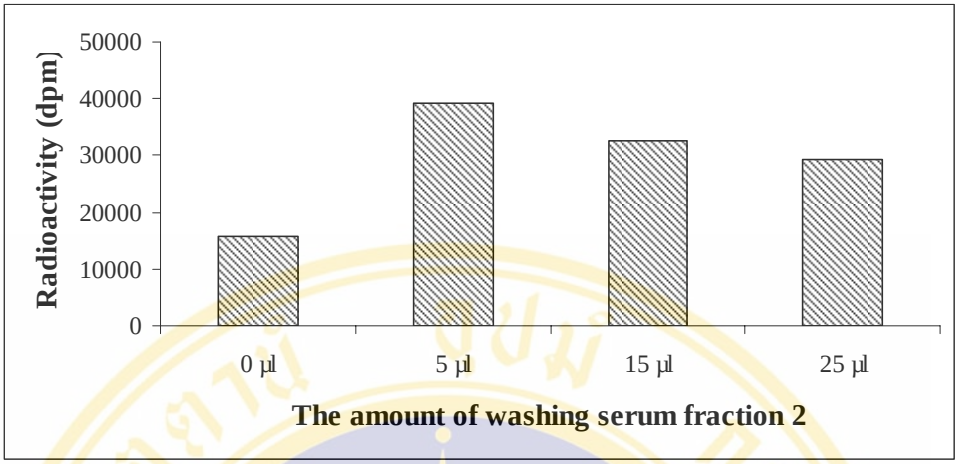


Figure 4.2 The activating effect of washing serum fraction 2, observed at various amounts of 5 µl, 15 µl and 25 µl

Table 4.7 Effect of substrates in upper and lower fraction after separated with Microcon on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity of upper fraction (dpm)	^{14}C-Radioactivity of lower fraction (dpm)
WSRP	16,077	14,445
WSRP+ fraction 1	20,071	22,548
WSRP+ fraction 2	20,214	19,753
WSRP+ fraction 3	25,426	18,124
WSRP+ fraction 4	26,217	16,326
WSRP+ fraction 5	25,053	16,760
WSRP+ fraction 6	21,470	18,245
WSRP+ fraction 7	16,945	18,330
WSRP+ fraction 8	12,865	15,929
WSRP+ FPP	72,900	70,221

The upper and lower fractions were presumed to be the fractions of enzyme and allylic substrate, respectively. From **Table 4.7**, fraction 4 of upper fraction and fraction 1 of lower fraction could activate highest IPP incorporation into rubber. The effects of varying the amount of fraction 4 of upper fractions and fraction 1 of lower fractions on the ^{14}C-IPP incorporation into rubber were observed.

The upper fraction was presumed to be the fraction of enzyme. **Figure 4.3** presents that the IPP incorporation was lowest when no substrate was added, which was a control experiment. The activity decreased with increasing the amount of fraction 4 of upper fraction. It was possible that there was an inhibitor in upper fraction. The presence of an inhibitor of rubber biosynthesis in C-serum of NR latex has been demonstrated by Archer and Audley [2]. In addition, it has been purified and characterized that it has a molecular weight of 43,700 Da by Yusof and co-workers [46].

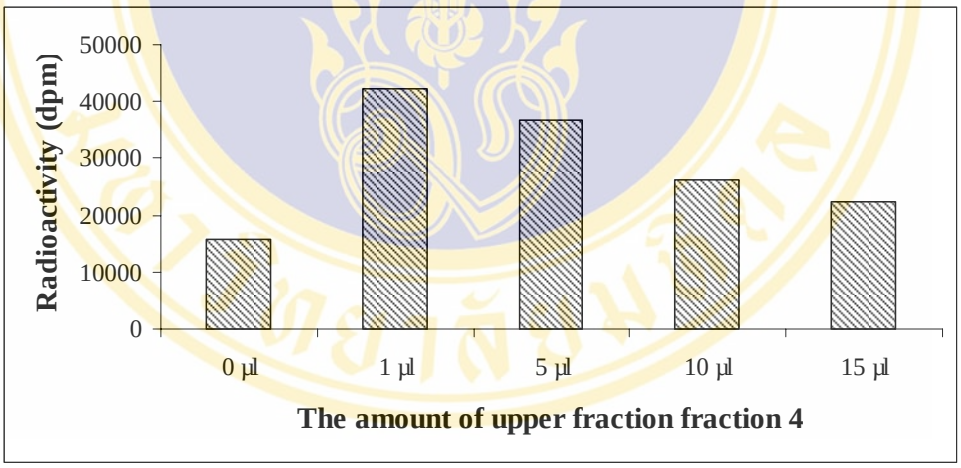


Figure 4.3 The activating effect of upper fraction fraction 4, incubated with ^{14}C-IPP and WSRP at various amounts of 1 µl, 5 µl, 10 µl and 15 µl

The lower fraction was presumed to be the fraction of allylic substrate. **Figure 4.4** shows that the activity observed from the incubation of lower fraction fraction 1 with WSRP and ^{14}C-IPP was quite low. Moreover, the activity increased with increasing the amount of fraction 1 until 25 µl. Therefore, it was possible that there was not an inhibitor in lower fraction which was the fraction of allylic substrate.

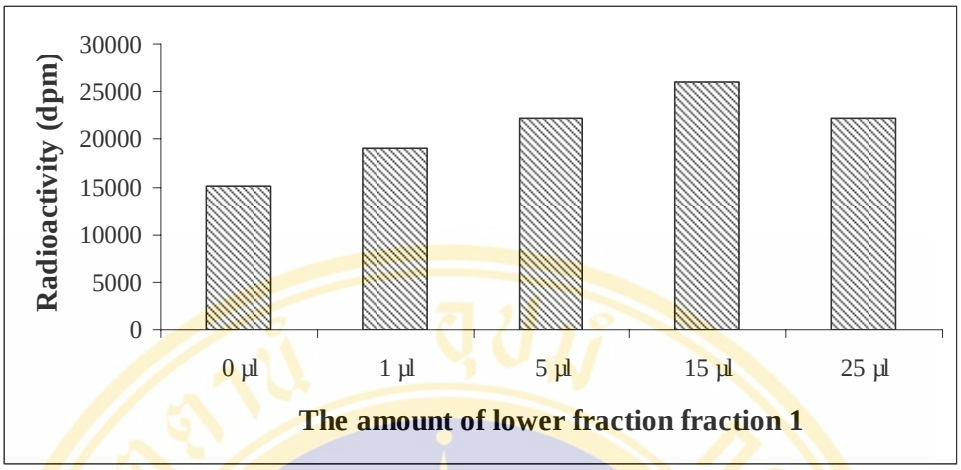


Figure 4.4 The effect of lower fraction fraction 1 on the ^{14}C-IPP incorporation into rubber at various amounts of 1 µl, 5 µl, 15 µl and 25 µl

The activation effect of washing serum, upper and lower fractions showed the low IPP incorporation into rubber. It may be due to the fact that the amount of substrate was too low for activating the incorporation. Therefore, each fraction of washing serum was concentrated by freeze-dryer and used in the further experiment.

4.2.1.1 The presence of protein in washing serum

(1) UV spectroscopy

Washing serum was measured with UV spectroscopy by Bradford assay procedure to determine the protein concentration of its. Bovine serum albumin (BSA) was used as the standard protein. **Figure 4.5** illustrates the standard curve of BSA protein.

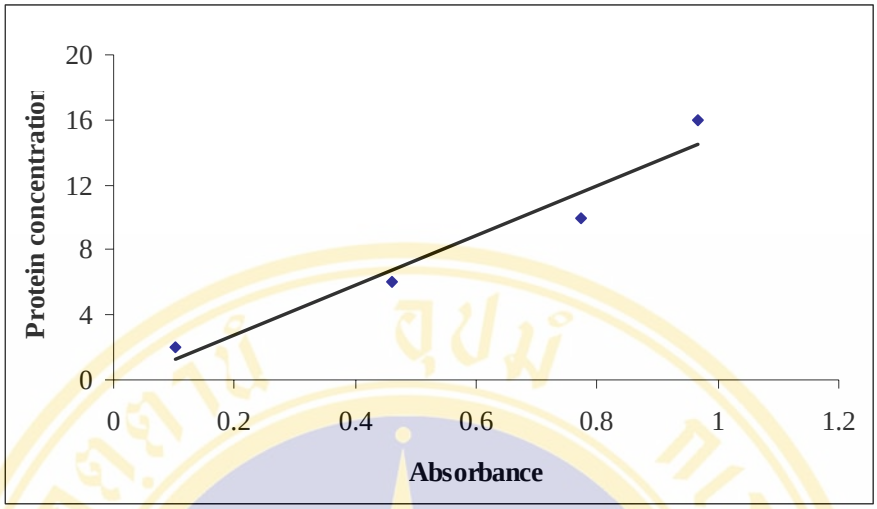


Figure 4.5 The standard curve prepared from BSA protein to determine the protein concentration of washing serum by Bradford assay procedure

The fraction 1-4 of washing serum was measured with UV spectroscopy at 595 nm and then compared with the standard curve to determine the protein concentration of each fraction, as illustrated in **Table 4.8**.

The highest protein concentration was observed from fraction 1 of washing serum. Fraction 2-4 of washing serum showed the low protein concentration which was about 50% of fraction 1.

Table 4.8 The protein concentration determined by Bradford assay procedure

Fraction	Absorbance	Protein concentration (µg/ml)
1	0.943	14.135
2	0.513	7.528
3	0.322	4.594
4	0.355	5.101

(2) Polyacrylamide gel electrophoresis

Washing serum was subjected to protein characterization by SDS-PAGE. The proteins were separated by 15% separating gel and subsequently stained with Coomassie Brilliant Blue R-250.

SDS-PAGE results showed the protein bands observed in washing serum fraction 1 and 2 (lane 3 and 4) compared with the molecular weight marker (lane M), as shown in **Figure 4.6**. It was found that there were two main protein bands observed at the molecular weight of 14 and 24 kDa. These may be rubber elongation factor (REF) [26] and small rubber particle protein (SRPP) [50], respectively. Moreover, there are many other protein bands observed in washing serum fraction 1 and 2.

From the results of UV spectroscopy and SDS-PAGE, it can be concluded that there were many kind of proteins contained in washing serum separated from SRP. One of the proteins may be the activation factor.

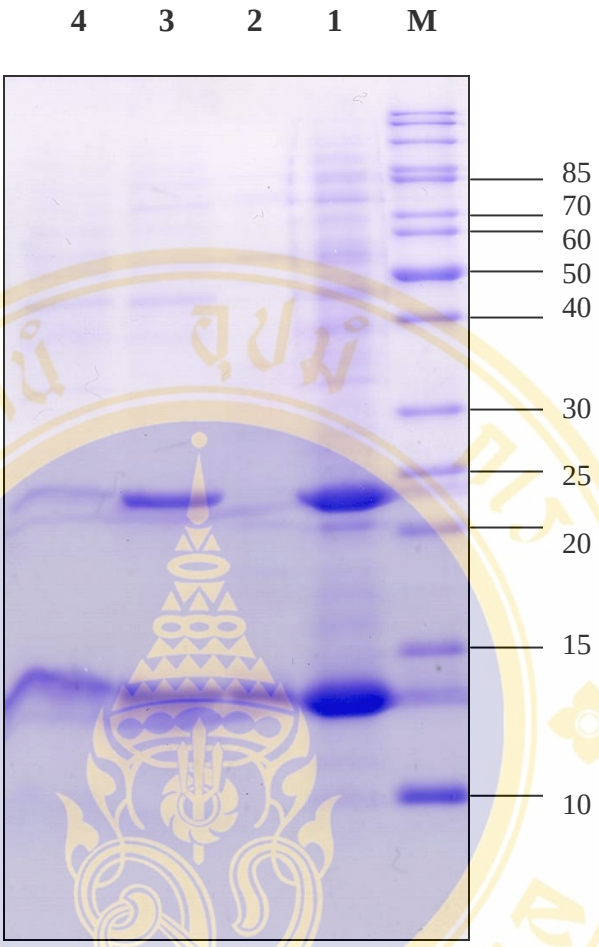


Figure 4.6 SDS-PAGE result of freeze-dried washing serum fraction 1-2; M= molecular weight marker, 1= SRP, 2= WSRP, 3 and 4= freeze-dried fraction 1 and 2

4.2.1.2 Effect of Tween-20 on the IPP incorporation into rubber

The effect of Tween-20 on the inhibitory activity has been studied. Yusof et al. has found that the inhibitory effect was more distinct if Tween-20 was added to the rubber biosynthesis assay incubation mixture [46]. Therefore, the inhibitory effect of buffer with Tween-20 was investigated. The buffer with and without Tween-20 were added to incubation mixture of WSRP and ^{14}C-IPP.

Table 4.9 Effect of buffer with and without 1% Tween-20 on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)	
	With Tween-20	Without Tween-20
WSRP	19,192	19,192
WSRP+ Buffer 5 µl	7,248	18,389
WSRP+ Buffer 15 µl	5,006	17,235
WSRP+ Buffer 25 µl	4,233	17,412

The IPP incorporation of WSRP was used as a control of the experiment. The activity markedly decreased with adding buffer with Tween-20 in the incubation mixture. Furthermore, the activity was more decrease with increasing the amount of buffer containing Tween-20. In the case of adding buffer without Tween-20, the activity was almost same in each incubation even different amount of buffer. Therefore, this result could confirm the inhibitory effect of Tween-20 when it was added in the washing buffer. But the role of Tween-20 towards the inhibitory activity is still unclear [46]. However, the buffer without Tween-20 was used as the washing buffer in the further experiment.

The buffer without Tween-20 was used to prepare the washing serum from gel-filtration chromatography. Washing serum was collected after rubber fraction into 4 fractions. Then each fraction was measured the activity by the incubation with ^{14}C-IPP and WSRP. The results are shown in **Table 4.10**.

Table 4.10 Effect of substrates in washing serum prepared from gel-filtration chromatography with buffer without Tween-20 on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	33,683
WSRP+ Fraction 1	45,190
WSRP+ Fraction 2	74,395
WSRP+ Fraction 3	74,862
WSRP+ Fraction 4	36,676
WSRP+ FPP	84,540

Table 4.10 shows the activation effect of washing serum with buffer without Tween-20 on the incorporation of ^{14}C-IPP into rubber. It was found that the IPP incorporation by WSRP without any substrate showed the lowest incorporation. The IPP incorporation increased when washing serum without Tween-20 or FPP was added. This suggests that washing serum without Tween-20 could activate the *in vitro* rubber biosynthesis, especially in fraction 2 and 3.

The effect of varying the amount of washing serum fraction 2 and 3 prepared with buffer without Tween-20 on the ^{14}C-IPP incorporation into rubber was observed. The IPP incorporation by WSRP and washing serum fraction 2 at various amounts is shown in **Figure 4.7**. The activity increased with increasing the amount of washing serum fraction 2. This indicates that there was no inhibiting effect on the IPP incorporation into rubber.

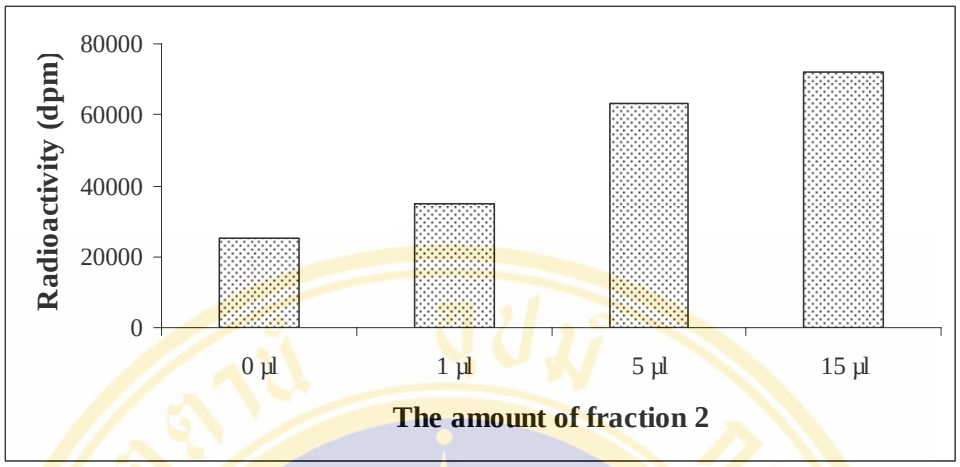


Figure 4.7 The effect of washing serum fraction 2 prepared with buffer without Tween-20 on the ^{14}C-IPP incorporation into rubber at various amounts

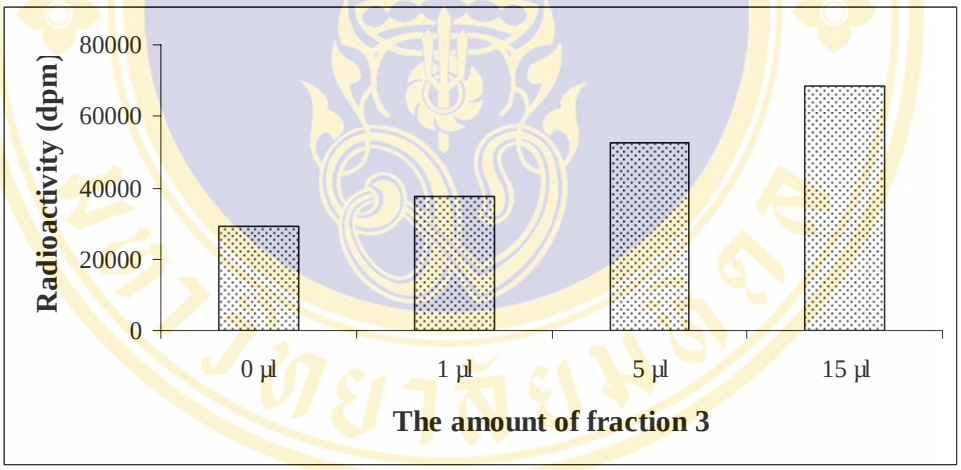


Figure 4.8 The effect of washing serum fraction 3 prepared with buffer without Tween-20 on the ^{14}C-IPP incorporation into rubber at various amounts

As same as the case of fraction 2, the effect of washing serum fraction 3 on the incorporation of ^{14}C-IPP into rubber is shown in **Figure 4.8**. The activity increased with increasing the amount of washing serum fraction 3 prepared from gel-filtration chromatography with buffer without Tween-20.

From **Figure 4.7 and 4.8**, the activation effect was observed by using fraction 2 and 3 of washing serum with buffer without Tween-20. It was

found that the activity increased with increasing the amount of washing serum fraction 2 and 3. This indicates that Tween-20 showed the inhibition effect on the *in vitro* rubber biosynthesis. Therefore the buffer without Tween-20 was suitable to use for the *in vitro* rubber biosynthesis.

The fraction 2 and 3 of washing serum with buffer without Tween-20 was subsequently lyophilized with freeze-dryer.

4.2.1.3 Activation of *in vitro* rubber biosynthesis by protein

The activation factor was analyzed whether it is protein or allylic substrate by heat and trypsin treatments. The incorporation of ^{14}C-IPP into the reaction mixture before and after these treatments was detected to evaluate this activation factor.

(1) Heat treatment

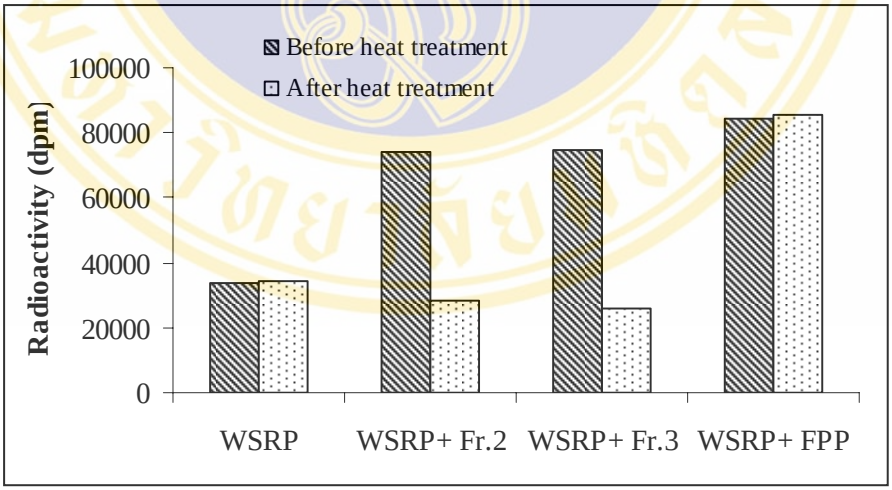


Figure 4.9 Effect of washing serum fraction 2 and 3 before and after heat treatment on the incorporation of ^{14}C-IPP into rubber

Figure 4.9 shows the effect of washing serum fraction 2 and 3 before and after heat treatment on the incorporation of ^{14}C-IPP into rubber. It was found that the activity of rubber biosynthesis after heat treatment decreased significantly when compared with the activity before heat treatment. This means that the heat treatment could inhibit the activity of fraction 2 and 3. It is known that heat treatment is a good method for degrade protein. The protein degradation was effect to the inhibition of IPP incorporation into rubber. Therefore, the activator in washing serum fraction 2 and 3 were protein. This conclusion was confirmed by the result from trypsin treatment.

(2) Trypsin treatment

As same as the case of heat treatment, the effect of washing serum fraction 2 and 3 before and after trypsin treatments on the incorporation of ^{14}C-IPP into rubber is shown in **Figure 4.10**. The activity of rubber biosynthesis drastically decreased after trypsin treatment. Trypsin treatment is also a good method for degrade protein [51]. Therefore, this result confirms that the activity of washing serum decreased after the proteins activators were degraded by this treatment.

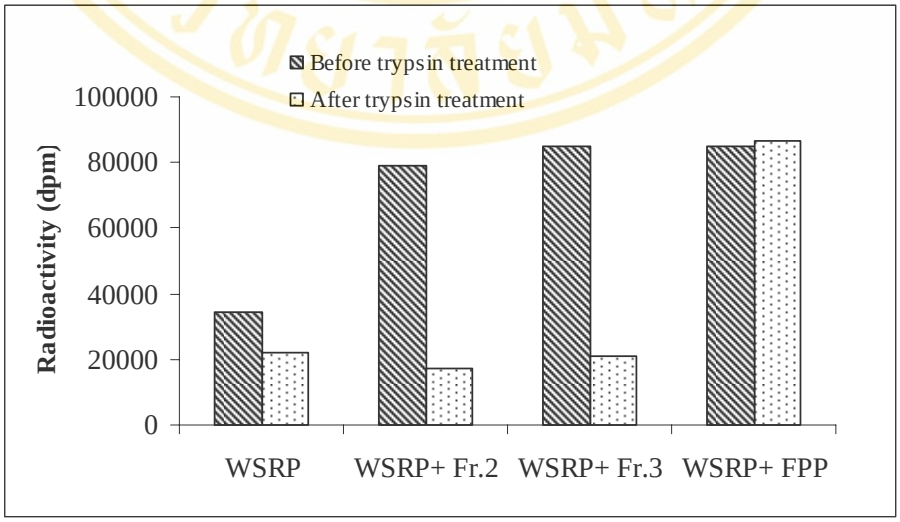


Figure 4.10 Effect of washing serum fraction 2 and 3 before and after trypsin treatment on the incorporation of ^{14}C-IPP into rubber

Table 4.11 Effect of trypsin treatment on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)	
	Fr. 2	Fr. 3
WSRP	22,119	22,119
WSRP+ Fr.+ Tryp-in	92,919	94,104
WSRP+ Fr.+ Heated Tryp+ Tryp-in	94,782	93,448
WSRP+ FPP	86,714	86,714

Tryp = Trypsin, Tryp-in = Trypsin inhibitor

Trypsin inhibitor was used to inhibit the reaction of trypsin enzyme when the incubation time reached to 90 min [52]. **Table 4.11** shows trypsin inhibitor protein itself no effect on the inhibition of the IPP incorporation into rubber. The inhibition effect observed after trypsin treatment resulted from the degradation of proteins in washing serum fractions by trypsin.

As shown in **Table 4.11**, it was found that the incubations of washing serum fractions and trypsin inhibitor show the high incorporation of IPP into rubber. The incubations of fractions, heated trypsin and trypsin inhibitor also showed the high IPP incorporation into rubber. This indicates that there was no effect of trypsin inhibitor protein on the inhibition of IPP incorporation into rubber. The activity of the incubations of trypsinized washing serum fractions decreased because the protein activator was degraded by trypsin treatment, as mentioned above.

Table 4.12 Effect of washing serum in the presence of FPP on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
(1) WSRP	34,210
(2) WSRP+ Fr.2+ FPP	85,868
(3) WSRP+ Fr.3+ FPP	82,342
(4) WSRP+ FPP	85,773

To characterize the protein activator, the washing serum fraction 2 and 3 were incubated with FPP. It was found that the incubations of washing serum fractions and FPP (2 and 3) showed the IPP incorporation into rubber similar as the incubation of FPP and no washing serum (4), as shown in **Table 4.12**. Therefore, there was no effect of fraction 2 and 3 of washing serum on the incorporation of IPP into rubber in the presence of FPP.

Table 4.13 Effect of washing serum fraction 2 and 3 after heat treatment on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
(1) WSRP	34,210
(2) WSRP+ heated fraction 2	28,411
(3) WSRP+ heated fraction 3	26,042
(4) WSRP+ FPP	85,773

The washing serum was heated in 100ºC for 15 min in order to heat treatment, as shown in **Table 4.13**. It was found that the incubation of heated fractions and WSRP (2 and 3) showed the low IPP incorporation into rubber similar as that of WSRP and no washing serum (1), which was a control experiment. This indicated that heating to 100ºC entirely inhibited the activity.

Proteins in washing serum were analyzed by SDS-PAGE technique, as shown in **Figure 4.11**. Protein bands of freeze-dried fraction 1-3 were shown in lane 3-5 of SDS-PAGE result.

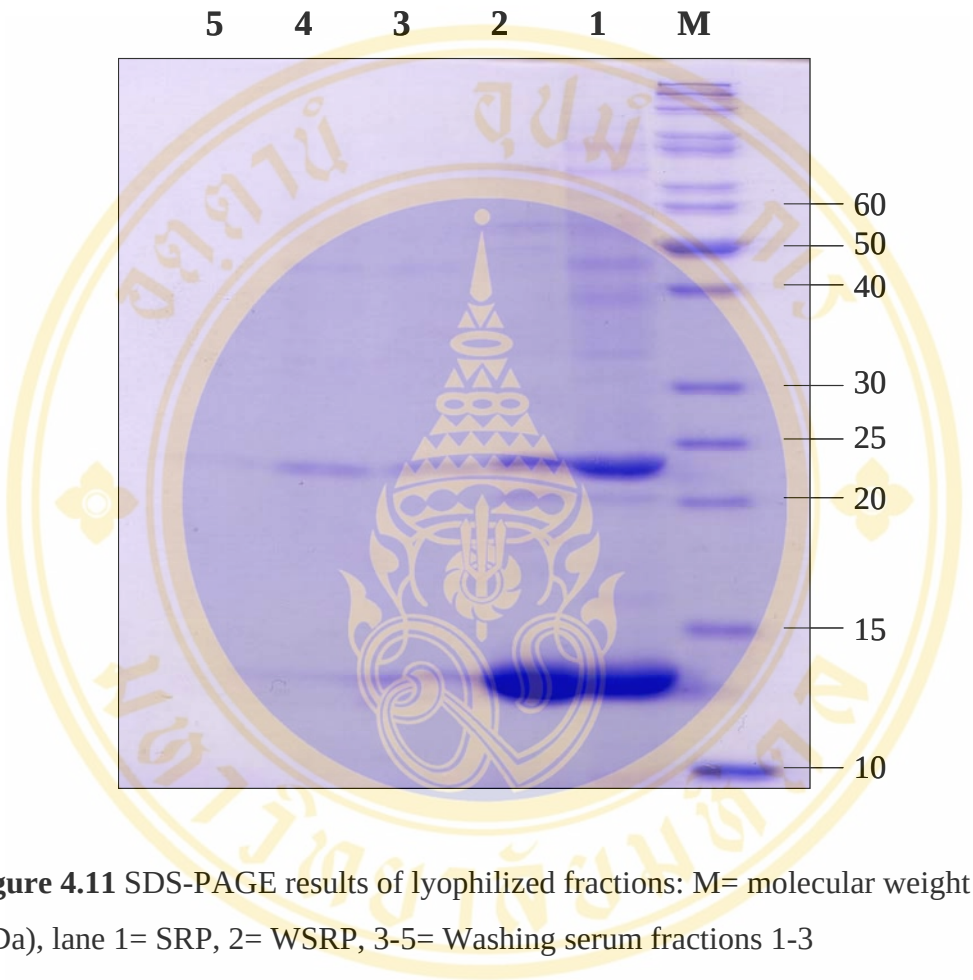


Figure 4.11 SDS-PAGE results of lyophilized fractions: M= molecular weight marker (kDa), lane 1= SRP, 2= WSRP, 3-5= Washing serum fractions 1-3

SDS-PAGE results showed only three protein bands detected from washing serum fraction 1-3. This may be due to the very low concentration of proteins. Thus, the protein concentration was determined by Bradford assay procedure.

The washing serum fraction 1-3 was measured with UV spectroscopy by Bradford assay procedure and compared with the standard curve, **Figure 4.5**, to determine the protein concentration of each fraction, as listed in **Table 4.14**. Washing serum fraction 1-3 showed the low protein concentrations, when compared with that of washing serum prepared by gel-filtration column with buffer contained Tween-20 (**Table 4.8**), as shown in **Figure 4.12**.

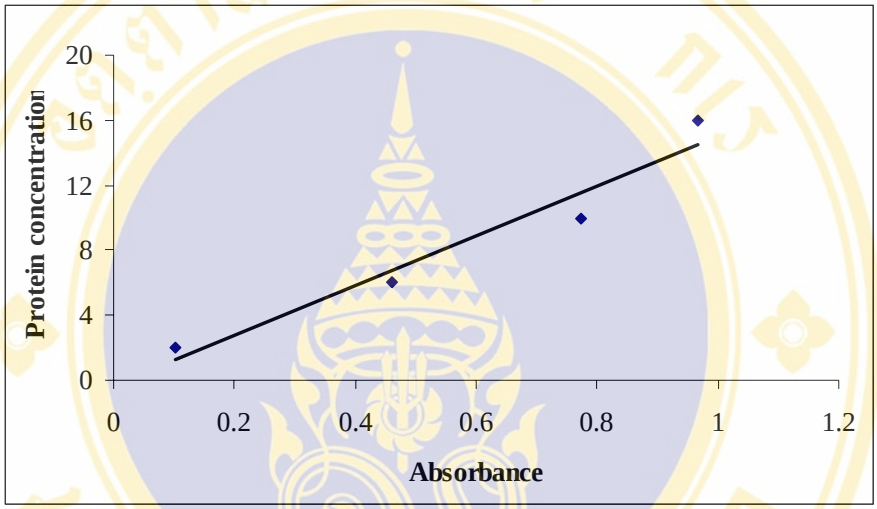


Figure 4.5 The standard curve prepared from BSA protein to determine the protein concentration of washing serum by Bradford assay procedure

Table 4.14 The protein concentration determined by Bradford assay procedure

Fraction	Absorbance	Protein concentration (µg/ml)
1	0.335	4.794
2	0.231	3.196
3	0.174	2.320

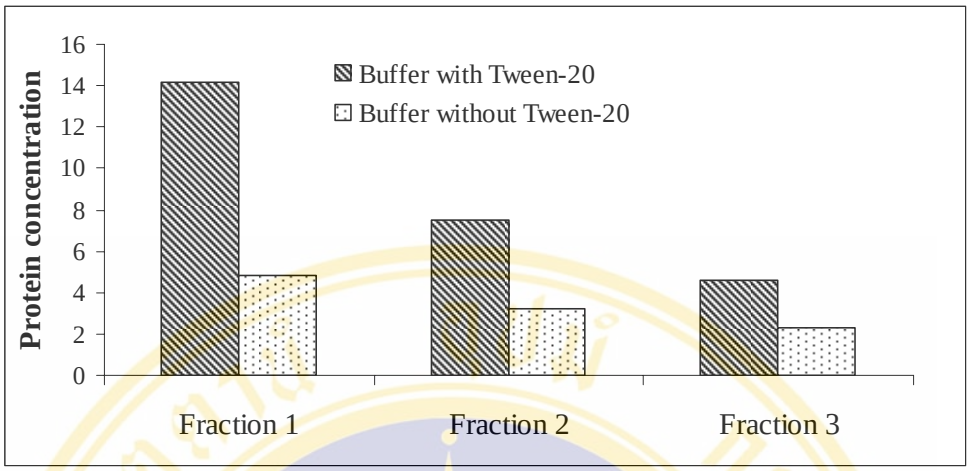


Figure 4.12 The comparison of protein concentration between washing serum fraction 1-3 prepared by buffer with and without Tween-20

Tween-20 was found to be an inhibitor, when was added into buffer that used to prepare washing serum. Therefore, the buffer without Tween-20 was used for preparing washing serum. Washing serum prepared by gel-filtration column with buffer without Tween-20 showed the high incorporation of IPP into rubber. However, the very low protein concentration was observed from washing serum prepared by buffer without Tween-20. This may be due to no surfactant in buffer resulting in proteins could not be removed from rubber fraction.

Triton® X-100 was used as surfactant in order to removed proteins from rubber fraction. Washing serum was prepared by centrifugation with Triton® X-100 and investigated the incorporation of ^{14}C-IPP into rubber.

4.2.2 Preparation of washing serum by centrifugation with Triton® X-100

Washing serum was separated by centrifugation of SRP latex with 0.5% Triton® X-100. It was collected into 4 fractions after each centrifugation. The activation effect by some factors in washing serum was detected by the incubation with ^{14}C-IPP and WSRP. The result is shown in **Table 4.15**.

Table 4.15 Effect of washing serum on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
SRP	86,273
WSRP	9,134
WSRP+ First serum	80,815
WSRP+ Second serum	39,028
WSRP+ Third serum	37,077
WSRP+ FPP	89,223

The high IPP incorporation activity by SRP was detected, as shown in **Table 4.15**. The activity of rubber biosynthesis drastically decreased after washing method. However the addition of washing serum fraction, the activity was recovered. The highest IPP incorporation was observed by the incubation with first washing serum. It indicates that this washing method could remove some factors which required for the *in vitro* rubber biosynthesis into washing serum.

Washing serum prepared by centrifugation with Triton® X-100 showed the high IPP incorporation into rubber. The activation factor of washing serum from SRP was confirmed whether it is protein by trypsin treatment. Fraction 1-3 of washing serum were combined and used for testing trypsin treatment.

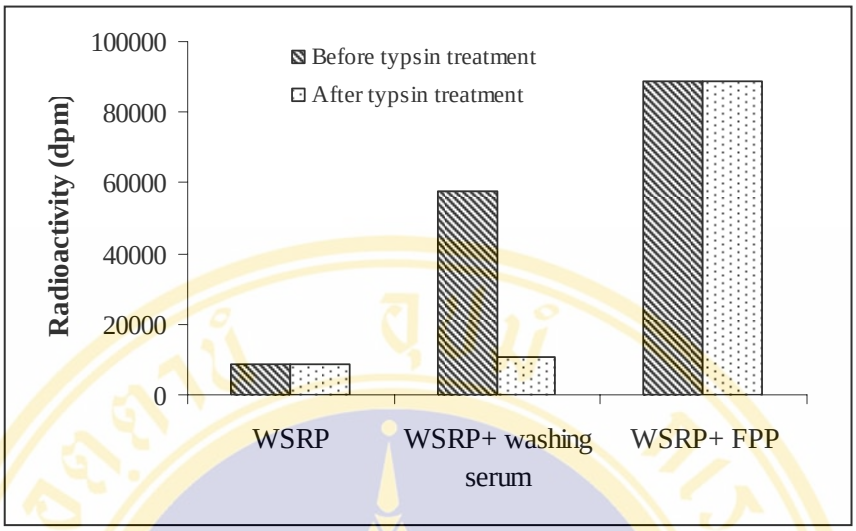


Figure 4.13 Effect of washing serum before and after trypsin treatment on the incorporation of ^{14}C-IPP into rubber

The effect of washing serum before and after trypsin treatment on the incorporation of ^{14}C-IPP into rubber is shown in **Figure 4.13**. The activity of rubber biosynthesis drastically decreased after trypsin treatment. Therefore, this result confirms that the activity of washing serum decreased after the protein activator was degraded. Thus, the activator in washing serum prepared by centrifugation with Triton® X-100 was protein.

4.2.2.1 Protein purification from washing serum

Yusof and co-workers purified and characterized the RBSP from the C-serum [41]. RBSP were purified by the various chromatography techniques. In the case of the activator protein in washing serum from SRP, it was also purified by the chromatography techniques [47].

(1) Anion exchange chromatography

Washing serum was preliminary purified by anion exchange chromatography. It was loaded onto a Resource-Q column and the buffer of 50 mM Tris-HCl pH 7.5 plus 5 mM 2-mercaptoethanol was used to elute the unbound protein. When the protein detection of the fractions at 280 nm had returned to zero, bound protein was eluted with the same Tris-HCl buffer containing a linear NaCl gradient of 0 to 0.5 M.

The effect of the activator fraction eluted from Resource-Q column on the *in vitro* rubber biosynthesis was studied. As shown in **Table 4.16**, it was found that the IPP incorporation by WSRP without any substrate showed the low incorporation, used as a control of the experiment. The IPP incorporation increased when the activator fraction was added. This suggests that the activator protein separated by a Resource-Q column could activate the *in vitro* rubber biosynthesis.

Table 4.16 Effect of the activator fraction eluted from Resource-Q column on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	8,011
WSRP+ Activator fraction	39,574
WSRP+ FPP	94,800

Figure 4.14 shows the elution profile of the protein from the Resource-Q column. The fraction fractionated from the column was incubated with ^{14}C-IPP and WSRP to investigate the activation effect. The activator fractions were eluted between 25 and 35 ml. It was combined and loaded onto the further chromatography technique.

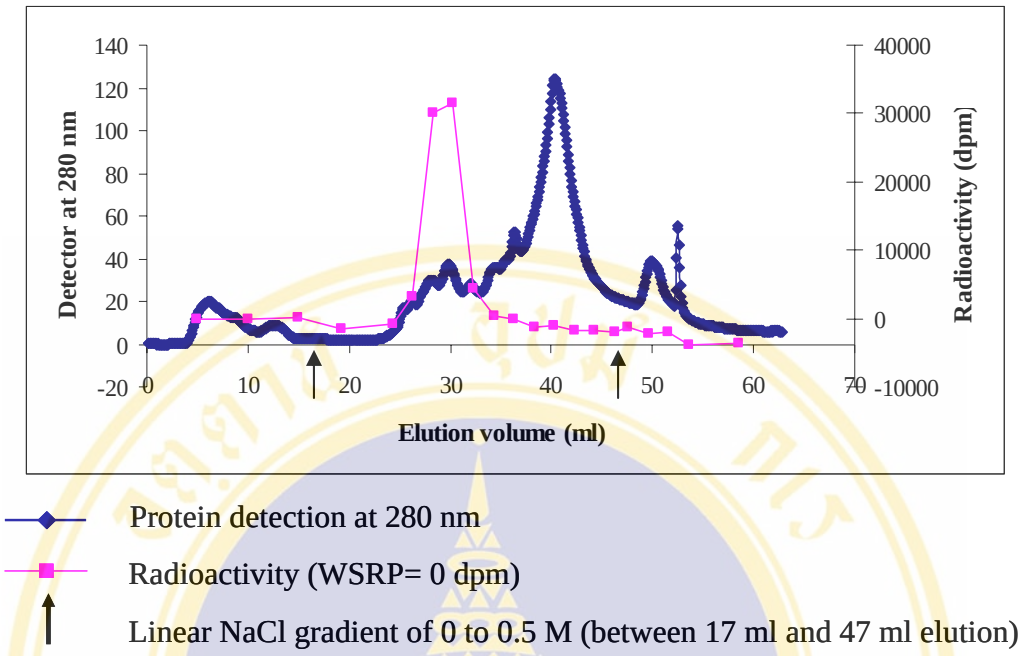


Figure 4.14 Elution result of activator protein from Resource-Q anion exchange chromatography

(2) Gel-filtration chromatography

The activator fraction was loaded onto a Superdex-200 gel-filtration column. The buffer of 50 mM Tris-HCl pH 7.5, 5 mM 2-mercaptoethanol was used to elute the protein.

From **Table 4.17**, it was found that the activator fraction showed the high IPP incorporation into rubber of about 66,000 dpm. This indicates that the activator fraction eluted from gel-filtration column could activate the *in vitro* rubber biosynthesis.

Table 4.17 Effect of the activator fraction eluted from Superdex-200 column on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	14,088
WSRP+ Activator fraction	66,176
WSRP+ FPP	93,941

The elution profile of the activator protein from Superdex-200 column is shown in **Figure 4.15**. The activator fractions were eluted between 80 to 90 ml by detection of radioactivity.

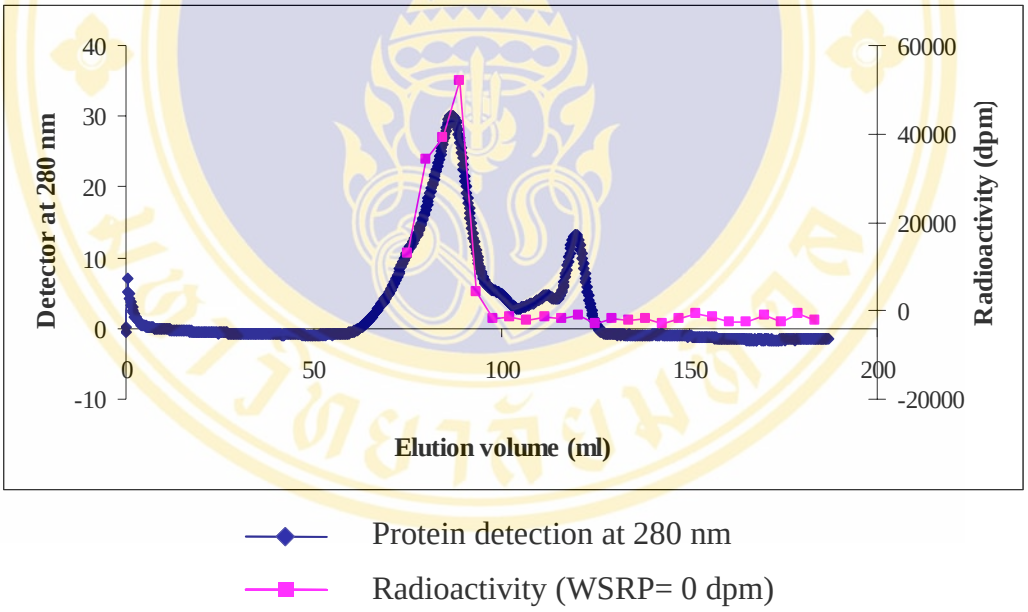


Figure 4.15 Elution result of activator protein from gel filtration chromatography

The molecular weight of the activator protein was estimated by using the calibration curve of standard proteins, as shown in **Figure 4.16**. It was about 48,000 g/mol and different from the activator protein observed in C-serum by Yusof *et al* [41]. The RBSP observed in C-serum was shown to have a molecular weight of 13,068 Da by mass spectroscopy.

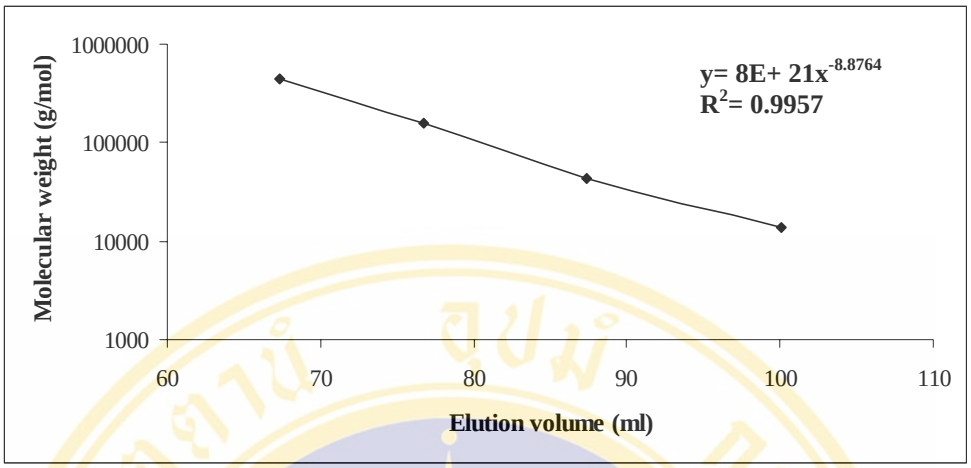


Figure 4.16 Calibration curve for gel-filtration chromatography

(3) Anion exchange chromatography

Each of the activator fractions eluted from Superdex-200 gel-filtration column was mixed and loaded onto a Resource-Q column. The buffer of 50 mM Tris-HCl pH 7.5 plus 5 mM 2-mercaptoethanol was used to elute the unbound protein. When the protein detection of the fractions at 280 nm had returned to zero, the bound protein was eluted with the same Tris-HCl buffer but this time containing a linear NaCl gradient of 0 to 0.3 M.

The effect of the activator fraction eluted from Resource-Q column on the incorporation of ^{14}C-IPP into rubber was investigated. The incubation of activator fraction and WSRP showed the high IPP incorporation into rubber. This suggests that the activator fraction eluded from Resource-Q column could activate the *in vitro* rubber biosynthesis.

Table 4.18 Effect of the activator fraction eluted from Resource-Q column on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	10,028
WSRP+ Activator fraction	51,387
WSRP+ FPP	91,280

Figure 4.17 shows the elution profile of the activator fractions from Resource-Q column. The activator fractions were detected in the fractions of the bound protein, eluting between 30 to 42 ml.

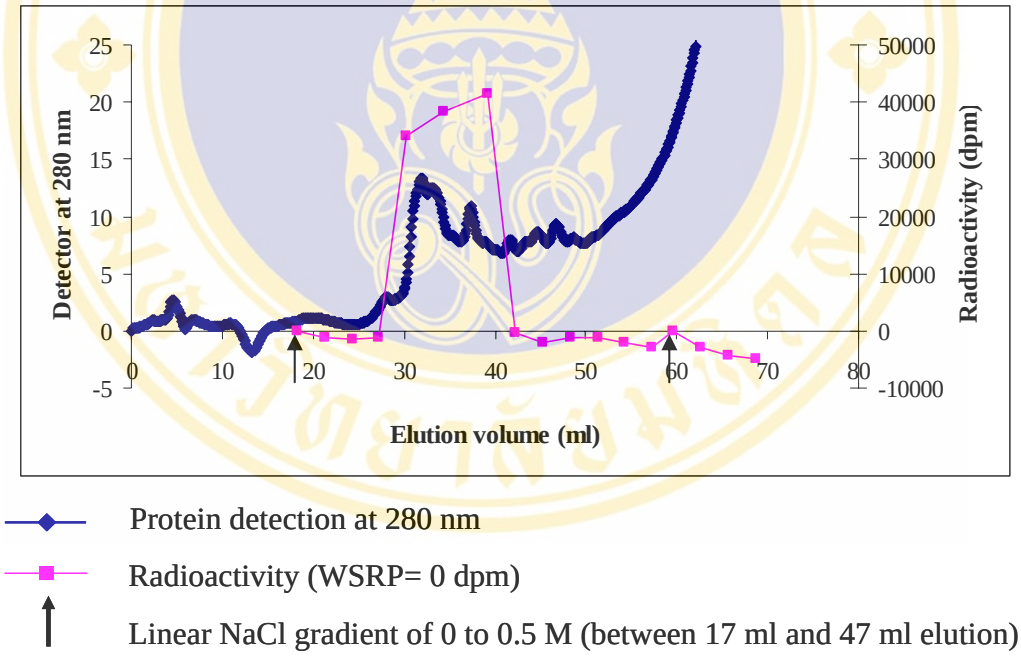


Figure 4.17 Elution result of activator protein from Resource-Q anion exchange chromatography

(4) Hydroxyapatite chromatography

The activator fractions eluting between 30 to 42 ml elution volumes was combined and was loaded onto the CHT10-I hydroxyapatite column in a low ionic strength buffer of 10 mM Potassium phosphate, pH 6.8. The bound protein was eluted with a phosphate gradient of 10 to 500 mM.

As shown in **Table 4.19**, the IPP incorporation into rubber increased when the activator fraction eluted from Hydroxyapatite column was added. This indicates that the activator fraction could activate the *in vitro* rubber biosynthesis.

Table 4.19 Effect of the activator protein eluted from Hydroxyapatite column on the incorporation of ^{14}C-IPP into rubber

Sample	^{14}C-Radioactivity (dpm)
WSRP	9,015
WSRP+ Activator fraction	28,353
WSRP+ FPP	88,371

The elution profile of the activator protein from hydroxyapatite column is shown in **Figure 4.18**. The activator fractions were eluted between 40 to 50 ml by detection of radioactivity.

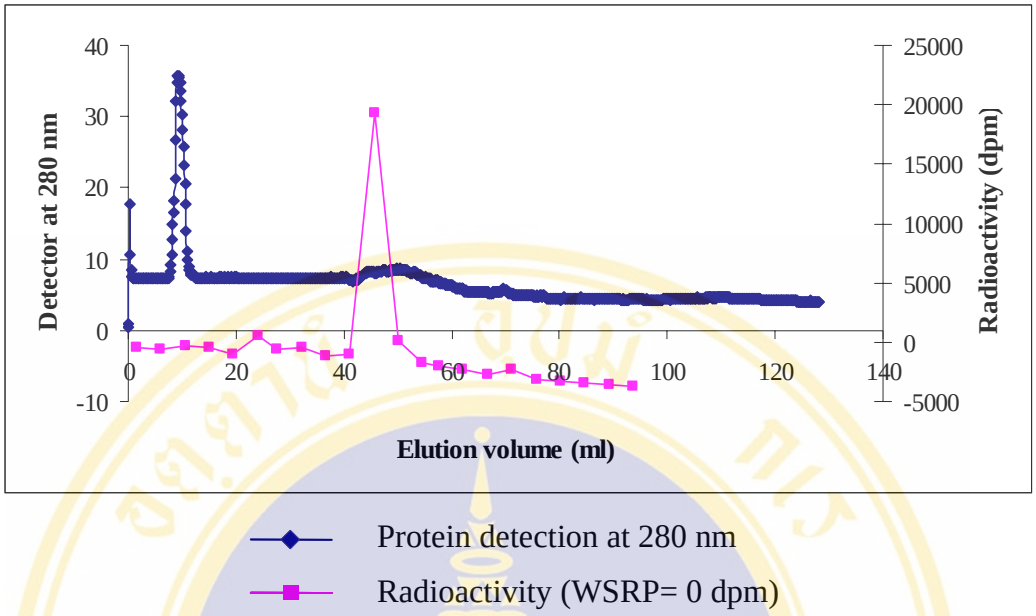


Figure 4.18 Elution result of activator protein from Hydroxyapatite chromatography

(5) SDS-PAGE

The activator fractions eluted from each column were analyzed by 15% separating gel of SDS-PAGE. **Figure 4.19** shows SDS-PAGE result of the activator fractions eluted from Resource-Q column and Superdex-200 column. Many bands of protein were detected from SDS-PAGE result, especially in a range of molecular weight of 30 to 60. The protein bands decreased after the activation factor was purified by Superdex-200 column (lane 3-6), when compared with the protein bands eluted from the first Resource Q column.

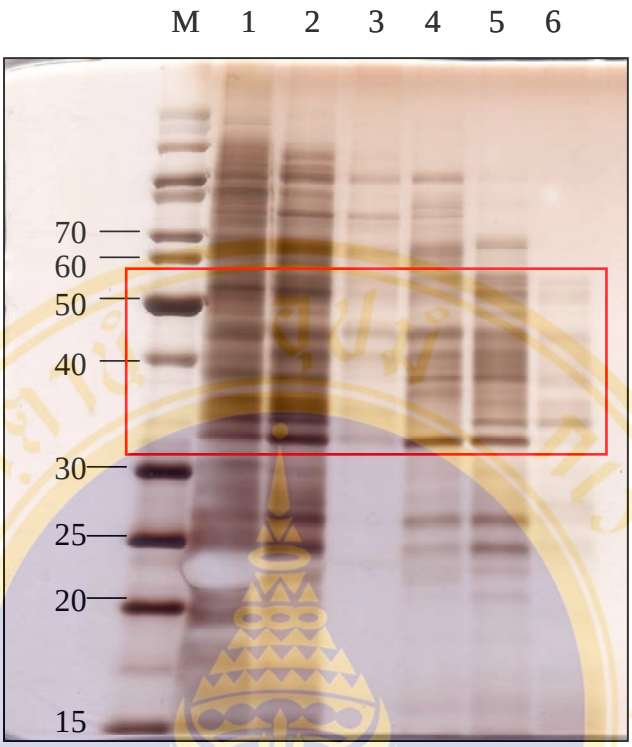


Figure 4.19 SDS-PAGE results, M = molecular weight marker (kDa), 1= washing serum (1st centrifugation), 2= the activator fraction from Resource Q column, 3-6= the activator fraction from gel filtration column

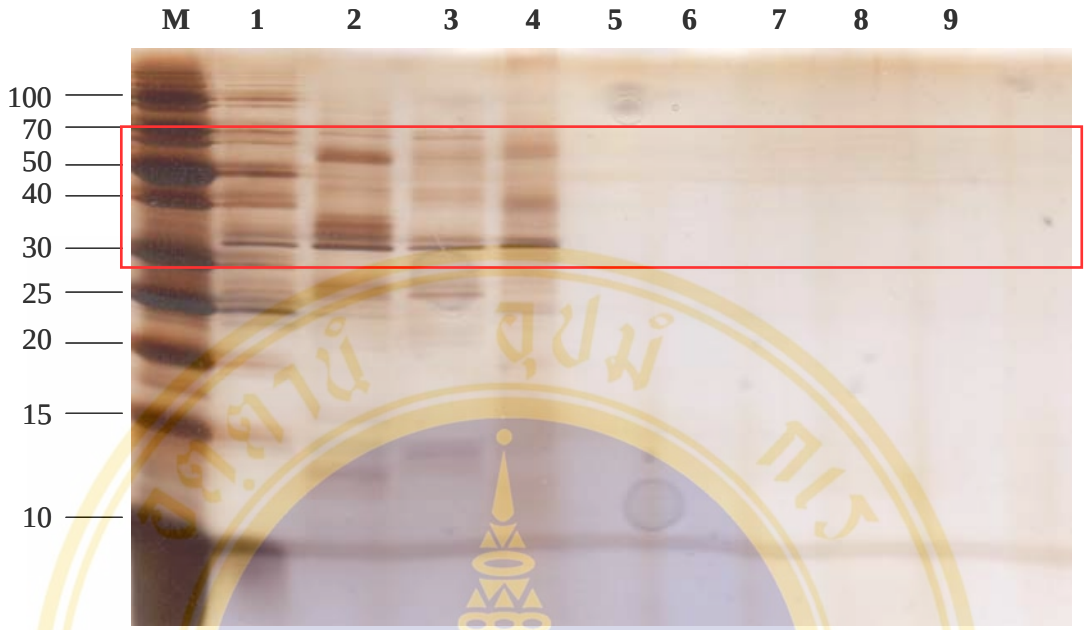


Figure 4.20 SDS-PAGE results, M = molecular weight marker (kDa), 1-4= the activator fraction from Resource Q column, 5-9= the activator protein from hydroxyapatite column

Figure 4.20 shows SDS-PAGE result of the activator fractions eluted from the second Resource-Q column and hydroxyapatite column. The protein bands were detected in the activator fraction eluted from the second Resource Q column. However, the activator fraction eluted from hydroxyapatite column could not be detected by SDS-PAGE due to the low amount of the activator protein. As shown in **Figure 4.18**, the activator protein eluted from hydroxyapatite column showed the low amount of protein detected at 280 nm (blue line). This means that the amount of the activator protein was too low in washing serum of SRP for the present study.

CHAPTER V
CONCLUSION

Based on the *in vitro* rubber biosynthesis, washing serum separated from SRP was proved to activate the IPP incorporation into rubber. The activation factors in washing serum were elucidated whether it is protein or allylic substrate. In the case of allylic substrate, it was separated from washing serum by Microcon. However, TLC showed that the medium-chain allylic diphosphate was not detected in the lower fractions separated from washing serum by Microcon. The activation effect of allyllic substrate separated from butanol extracts of lower fraction of washing serum by TLC showed that there were no differences in the activity of the extracted fractions from TLC in each fraction. This indicates that the extracted fractions could not activate the rubber biosynthesis. Thus, it can be concluded that the allylic diphosphate could not be detected from washing serum of SRP.

In the case of protein testing in washing serum, separated from SRP, it was investigated by heat and trypsin procedures in order to confirm the presence of the activator protein. After heat and trypsin treatments, which are known as good methods to degrade protein, it was found that there was no more activity of rubber biosynthesis. This implies that the activity in SRP was predominantly resulted from the proteins. The activator protein was purified by the various chromatography techniques. The molecular weight of the activator protein was about 48,000 g/mol, which was estimated by gel-filtration chromatography. The activation effect of activator protein separated from washing serum showed the high IPP incorporation into rubber. This indicates that the activator protein could activate the *in vitro* rubber biosynthesis. However, this activator protein band could not be detected by SDS-PAGE. This means that the amount of the activator protein was too low in washing serum of SRP for the present study. So, as for the further work, it is necessary to start from large amount of washing serum. And after getting the purified protein, it should be subjected to studying for DNA sequence to get the clear structure of the activator protein.

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APPENDIX

APPENDIX A
BRADFORD PROTEIN ASSAY PROCEDURE

The Bradford assay procedure can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Coomassie Brilliant Blue G-250, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The dye is prepared as a stock solution in either phosphoric or perchloric acid, the latter reagent being more stable. The method is a rapid, simple one-step procedure in which the reagent is added to the sample and the absorbance measured at 595 nm. The amount of absorption is proportional to the protein present.

The procedure is very sensitive, working in the range 0.2-1.4 mg protein ml^{-1} for the standard assay and 5-100 µg protein ml^{-1} for the microassay procedure. It exhibits a significant dependence on protein amino acid composition and this has recently been shown to be a consequence of the dye binding primarily to basic and aromatic amino acid residues. A difficultly observed in performing the assay is the tendency of the protein-dye complex to bind to glass surfaces. Disposable cuvettes can, therefore, be used to advantage, but it is relatively easy to remove the dye from glass cuvettes by soaking in 0.1 M HCl or by washing with concentrated detergent followed by water and acetone.

APPENDIX B
EXCHANGE OF BUFFER

Gel-filtration method is only applicable to small volumes. The maximum sample volume should not exceed 25-30% of the volume of the column to ensure adequate resolution between the protein and salt. A gel-filtration matrix with a small pore size is poured into a column to give a bed volume of approximately five times the volume of sample to be desalted. A syringe plugged with glass wool or a glass fibre disc can be used or small disposable columns. Pre-packed columns for desalting and buffer exchange are available from Pharmacia (PD-10 columns).

APPENDIX C
ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange is the most commonly practiced chromatographic method of protein purification. This stems, in part, from its ease of use and scale-up, wide applicability and low cost in comparison with other separation methods. Ion-exchange of proteins involves their adsorption to the charged groups of a solid support followed by their elution with fractionation and/or concentration in an aqueous buffer of higher ionic strength.

Theory of ion-exchange

Proteins carry both positive and negative charged groups on their surface, due largely to the side chains of acidic and basic amino acids. Positive charges are contributed by histidine, lysine, arginine and to a lesser extent, N-terminal amine. Negative groups are due to aspartic and glutamic acids, C-terminal carboxyl groups and to a lesser extent, cysteine residues. The net charge on a protein depends on the relative numbers of positive and negative charged groups; this varies with pH. The pH where a protein has an equal number of positive and negative charged groups is termed its isoelectric point (pI). Most proteins have a pI between pH 5 and 9. Above their pI proteins have a net negative charge while below it their overall charge is positive.

Ion-exchange is the separation of proteins on the basis of their charge and can be used to resolve proteins which differ only marginally in their charged groups. Separation of proteins is achieved by their difference in equilibrium distribution between a buffered mobile phase and a stationary phase consisting of a matrix to which charged inorganic groups are attached. For the effective use of ion-exchange in protein purification stationary phase must therefore be capable of binding either

positively-charged or negatively-charged proteins. To this end ion-exchange matrices are derivatized with positively-charged groups for the adsorption of anionic proteins (termed anion exchangers) or negatively charged groups for the adsorption of cationic proteins (cation exchangers).

Associated with both stationary phase and protein charged groups are counter-ions which are simple, low molecular weight ions. In order for the protein to bind to the stationary phase, therefore, the counter-ions of both groups must become electrolytically dissociated.

Counter-ions 'screen' the exchanger groups, preventing their binding with a protein. Na^+ and H^+ are the common counter-ions for cation exchangers while Cl^- and OH^- are usually used with anion exchangers. Counter-ions can be arranged in an 'activity' series according to their strength of interaction with their respective ionogenic groups at equal concentration. Consequently chloride would replace hydroxide ions at equal strength as the counter-ion for an anion exchanger. Counter-ions do not permanently bind to an ionogenic group but stay in a state of equilibrium, continually shifting between the bulk solution and the exchanger groups. It follows that the ionogenic groups can become uncovered to allow the binding of a protein. The higher the counter-ion concentration, the less frequently do the ionogenic groups become uncovered. Prior to the use of an ion exchanger the counter-ion may require replacement with a different ion more suitable to the particular application.

APPENDIX D
GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography (GFC) is a form of partition chromatography used for separating molecules of difference sizes. GFC has been described by several other terms including gel permeation, gel exclusion chromatography, molecular sieve chromatography or even simply as gel chromatography. The basis principle of GFC is that molecules are partitioned between solvent and a stationary phase of defined porosity. The separation process is carried out using a porous gel matrix (in bead form) packed in a column and surrounded by solvent. Consider a sample containing a mixture of molecules smaller and larger than the pores of the stationary phase matrix, as well as molecules intermediate in size. The smaller molecules can enter the matrix pores and hence move more slowly through the column, appearing as the last components in the chromatogram. The larger molecules are excluded from the stationary phase and hence eluted first from the column. Molecules intermediate in size can enter the stationary phase, but spend less time within it than smaller molecules do. Thus, all the molecules are eluted in order of their decreasing size. Some proteins show anomalous behavior on GFC; these include proteins which are not globular in shape. Generally long thin proteins elute earlier than globular proteins of the same size. Denaturing agents such as urea or guanidine hydrochloride minimize the effects of tertiary and quaternary structures of proteins by transforming them to a random coil configuration, and hence allow a more accurate assessment of their molecular weight. However, the effective exclusion limit of a given matrix will be reduced due to the increased hydrodynamic radius (viscosity) of a random coil conformation. The separation mechanism of GFC involves both molecular shape and mass of the molecules, thus GFC can be used to determine molecular shape in solution and consequently as a monitor for denaturation.

Determination of molecular weights

GFC is a valuable alternative to SDS-PAGE for the determination of molecular weights of proteins. The elution volumes of globular proteins are mainly determined by their hydrodynamic radius which is related to their molecular weight; thus the elution volume is an approximately linear function of the logarithm of the molecular weight.

(1) A calibration graph is usually constructed for each column to be used for the determination. Apply a mix of suitable standard proteins (in the correct fractionation range) to the column. Determine the elution volume of each standard from the chromatogram.

(2) Plot these values against the logarithm of the molecular weight (**Figure A**).

(3) Run the protein of unknown molecular weight on the same column, and note its elution volume. Use this value to obtain the molecular weight from the calibration graph.

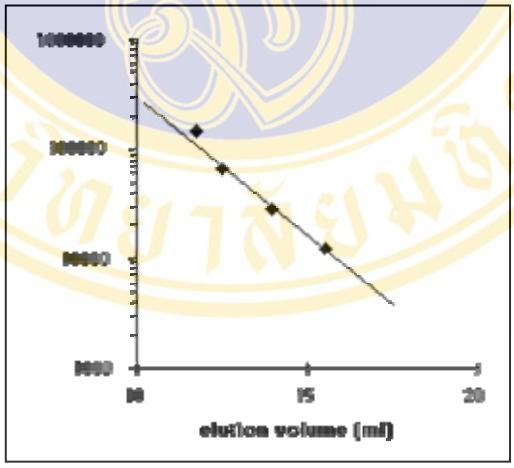

Figure A Plot of molecular weight of proteins against their elution volume from a gel filtration column. Proteins are yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

APPENDIX E
HYDROXYAPATITE CHROMATOGRAPHY

Hydroxyapatite chromatography was introduced in 1956, but despite numerous publications describing its merits as a unique chromatography tool, it has failed to attain the popularity of ion exchange. A number of reasons account for its neglect, most of which are artificial and the remainder obsolete. In practice, it offers a unique assemblage of process characteristics that merit serious evaluation.

Structure and Composition

The formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$. The functional groups comprise positively charged pairs of crystal calcium ions (C-sites) and clusters of six negatively charged oxygen atoms associated with triplets of crystal phosphates (P-sites). C-sites, P-sites, and hydroxyls are distributed in a fixed pattern on the crystal surface.

Hydroxyapatite made by the Tiselius method or adjustments thereto often have excess phosphate in the structure. This leads to the formation of unstable rectangular plate-shaped crystals with poor flow, pressure, and stability characteristics. Recently developed synthesis methods yield hexagonal cross-section columnar crystals with the ideal Ca:P ratio of 1.67. They can be agglomerated to form particles, and sintered at high temperatures to fuse the particles into a stable porous “ceramic” mass. This yields media that possess flow properties, capacity, and scale-up attributes that are competitive with other popular methods. The available surface area and pore size are affected by the sintering process used during manufacturing, and this will affect the selectivity, capacity, and separation characteristics.

Separation Mechanism

The hydroxyapatite:biomolecule interactions are complex. The following simplified discussion is an attempt to clarify the most significant features. Amino groups are attracted to P-sites but repelled by C-sites. The situation is reversed for carboxyls.

Although amine-binding to P-sites and the initial attraction of carboxyls to C-sites are electrostatic, the actual binding of carboxyls to C-sites involves formation of much stronger coordination complexes between C-sites and clusters of protein carboxyls. This has been proven experimentally by evaluating the retention of proteins on which the carboxyls have been replaced by sulfo groups. Binding is reduced dramatically even though net charge is unaltered. Further proof that carboxyl/C-site binding does not reflect a classical anion exchange interaction is found in the fact that binding capacity diminishes for acidic proteins with increasing pH.

Phosphoryl groups on proteins and other solutes interact even more strongly with C-sites than do carboxyls. This is reflected in extremely strong binding by phosphoproteins. DNA does not bind as strongly as expected for a phosphoryl-rich solute. The spacing of the phosphoryl groups along the backbone apparently prevents an ideal match with the steric distribution of C-DNA binds well nonetheless and the strength of the interaction increases with its size. Endotoxins bind by the numerous phosphoryl groups on their core polysaccharide and lipid-A moieties.

BIOGRAPHY

NAME Miss Paveena Nawakitkosol
DATE OF BIRTH 21 August 1984
PLACE OF BIRTH Samutprakarn, Thailand
INSTITUTIONS ATTENDED Thammasat University, 2005:
Bachelor of Science (Chemistry)
Mahidol University, 2008:
Master of Science
(Polymer Science and Technology)
HOME ADDRESS 48/4 M. 3, Theparak Rd., Bangplee-yai,
Bangplee, Samutprakarn, Thailand
10540
Tel. 0-2755-5349
E-mail: kung_paveena@hotmail.com