CHAPTER FOUR

# Natural Rubber Biosynthesis in Plants: Rubber Transferase

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## Contents



## Abstract

Rubber biosynthesis in plants is a fascinating biochemical system, which evolved at the dawn of the dicotyledoneae and is present in at least four of the dictolydonous superorders. Rubber biosynthesis is catalyzed by a membrane complex in a monolayer membrane envelope, requires two distinct substrates and a divalent cation cofactor, and produces a high-molecular-weight isoprenoid polymer. A solid understanding of this system underpins valuable papers in the literature. However, the published literature is rife with unreliable reports in which the investigators have fallen into traps created by the current incomplete understanding of the biochemistry of rubber synthesis. In this chapter, we attempt to guide both new and more established researchers around these pitfalls.

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## 1. INTRODUCTION

Natural rubber is a *cis*-1,4-polyisoprene polymer composed of isopentenyl monomers derived from isopentenyl pyrophosphate (IPP), usually with a C15 *trans* tail derived from the original initiating allylic pyrophosphate (APP), farnesyl pyrophosphate (FPP). Rubber is an end-product of one branch of the ubiquitous plant isoprenoid/terpenoid synthesis pathway. Natural rubber is made and compartmentalized in microscopic particles produced in the cytosol of some plants and fungi, but the ontogeny and development of these particles is only poorly understood. However, all species produce rubber in essentially the same way, via a membrane-bound *cis* prenyl transferase called "rubber transferase" (EC 2.5.1.20), found at the surface of cytoplasmic rubber particles. Natural rubber product quality is related, on the molecular level, to the polymer molecular weight, branching, gel content (insoluble, cross-linked rubber), and the composition of the nonrubber components of the latex. On the cellular level, quality is related to the rubber particles in which the rubber polymers are coproduced, the cellular compartment into which the rubber particles are targeted, and the developmental stage and health of the plant from which the rubber is obtained.

## 2. PREPARING SAMPLES FOR ASSAYING RUBBER TRANSFERASE ACTIVITY

Enzymatically active washed rubber particles (WRPs) have been purified in our laboratory from a number of species, using slight species-specific modifications of a basic centrifugation/flotation method. The methods for active rubber particle isolation for specific species are described: *Parthenium argentatum* (Cornish & Backhaus, 1990); *Hevea brasiliensis* (Cornish & Siler, 1995); *Ficus elastica (*Cornish & Siler, 1996; Espy, Keasling, Castillon, & Cornish, 2006*)*; *Taraxacum kok-saghyz*, *Euphorbia lactiflua* (Cornish, Wood, & Windle, 1999); and *Helianthus annuus* (Pearson et al., 2010).

In laticiferous species, such as *H. brasiliensis* and *F. elastica*, rubber particle harvest is a relatively easy protocol, as plants can be tapped to release latex for subsequent rubber particle purification. This method minimizes plant wounding and results in very stable enzyme preparations. In contrast, *P. argentatum* makes rubber in parenchyma cells, which must be homogenized to release the rubber particles. Unfortunately, this homogenization

also releases copious amounts of proteases and other degradative enzymes, with a resultant decrease in enzyme stability and half-life (Cornish & Backhaus, 1990).

## 2.1. Rubber particle-bound activity

### *2.1.1 Purification of enzymatically active rubber particles*

The method described below includes all the precautions needed to yield enzymatically active purified rubber particles from *P. argentatum* (guayule).

1. Harvest guayule plants and keep on ice prior to processing.
2. Remove surface soil and record plant fresh weight. Remove dead leaves and branches and record the fresh weight of the remaining tissues.
3. Peel the bark from samples and place them into a large container with ice-cold extraction buffer (100 m*M* Tris–HCI (pH 7.5), 5 m*M* magnesium sulfate, 50 m*M* potassium fluoride, 1% ascorbic acid).
4. Grind bark for 2 min in a 1 l Waring blender with a mixture of 1:1.5 (weight:volume) ice-cold extraction buffer, plus 70 g polyvinylpolypyrrolidone, 50 µl antifoam A (Sigma)/L extraction buffer, 5 m*M* mercaptoethanol. Pass homogenates through four layers of fine cheesecloth and record the volume.
5. Centrifuge samples at 4 °C using a swinging bucket rotor. Centrifugations are done consecutively, for 10–15 min each, at 432, 1202, 2350, 3895, 5818, and 9425 × *g*.
6. After the first spin, scoop floating rubber particles into a centrifuge tube on ice, containing wash buffer (1 ×) (100 m*M* Tris–HCI (pH 7.5), 5 m*M* magnesium sulfate, 5 m*M* DTT, 0.1 m*M* AEBSF or 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) at about 1/5 volume of original extraction buffer. This volume depends on rubber particle concentration, and a high concentration would entail a larger volume than a low concentration. If WRP are diluted too much, they become difficult to scoop when floated.
7. Wash rubber particles twice more by centrifuging from 432 × *g* to 9425 × *g*, at 4 °C for 15 min, consecutively, as described in step 5. As above (step 6), transfer particles to a new centrifuge tube containing fresh wash buffer.
8. Transfer 3 × WRPs into a fresh tube containing wash buffer at about 1/20 volume of original extraction buffer used and record the total volume.

9. Separate out three 50-µl aliquots of the rubber particle slurry generated in step 8. Pipet these into preweighed weighing boats for dry weight determination and incubate the samples overnight in a 60 °C oven.
10. To the remainder of the rubber particle slurry, add glycerol to a final concentration of 10% and gently stir until thoroughly mixed. Flash-freeze, drop by drop, in liquid nitrogen; transfer into cryogenic storage tubes; and store either in liquid nitrogen or at −80 °C.
11. Calculate final 3× WRP concentrations from the weights in step 9 and the dilution in step 10. Record as mg/ml of dry rubber particle weights.

The primary challenge encountered in this method is caused by biological variations among preparations and the need to adjust spin speeds and times to minimize coagulation, which results in irreversible loss of particles. The cleaner the WRPs become, the more prone to coagulation they are, requiring a reduction in centrifugation speed. Also, WRPs are more prone to coagulation during the first centrifugation of each washing cycle than during the remaining centrifugations. Upon successful completion of the protocol, frozen rubber particles can be thawed and used in enzymatic assays as required for several years. If stored correctly, the glycerol prevents coagulation, but the amount of glycerol present does not inhibit rubber transferase activity (Cornish & Bartlett, 1997).

### 2.1.2 Enzyme stability

Rubber transferase stability varies with species, accession, degree of purification, assay conditions, and assay temperature. In any new system, the researcher must determine the linear range of incorporation rate with time so that kinetic comparisons can be made. For example, enzyme activity in rubber particles purified from *P. argentatum* line 593 decreases below limits of detection in 1 h at 25 °C, whereas activity is stable for much longer in particles from line 11591 (Cornish & Backhaus, 1990). This observation led to most *P. argentatum* assays being run at only 16 °C, a temperature at which even the most unstable enzymes exhibit linear incorporation rates for over 4 h.

### 2.1.3 Assay system purity

Extensive testing has demonstrated that thrice-WRPs are sufficiently free of soluble enzymes (which can compete for the same substrates as the rubber transferase) for the purposes of most enzymatic assays—a linear plot of $v$ versus $v$/[IPP] in nonlimiting APP initiator confirms the presence of a single enzyme (rubber transferase) capable of using IPP (Cornish, 2001a, 2001b).

If this plot were curved, it would indicate the presence of contaminating, competing enzymes, which would invalidate kinetic studies of the rubber transferase.

### 2.1.4 *Sources of competing enzymes and the separate roles of* cis *and* trans *prenyl transferases in rubber biosynthesis*

Enzyme reactions can only be characterized in systems in which there are no other enzymes competing for the same substrates (Fig. 4.1). Investigations of rubber biosynthesis have to be closely attuned to competing enzymes. Rubber biosynthesis is catalyzed by rubber transferase (which seems to be an enzyme complex) firmly associated with the monolayer biomembrane surrounding rubber particles (Cornish et al., 1999; Siler, Goodrich-Tanrikulu, Cornish, Stafford, & McKeon, 1997; Wood & Cornish, 2000). The reaction is initiated by an APP and then isopentenyl monomers from IPP are polymerized by a condensation reaction into long-chain *cis*-1,4-polyisoprene polymers (Cornish, 2001b). The IPP is made primarily by the mevalonate pathway in the cytosol, but this pool may be supplemented by IPP produced by the methyl-erythritol plastidic pathway and translocated into the cytosol (Bick & Lange, 2003). The APP initiators are synthesized by soluble cytosolic *trans* prenyl enzymes, beginning with dimethylallyl pyrophosphate (DMAPP) and IPP, with FPP

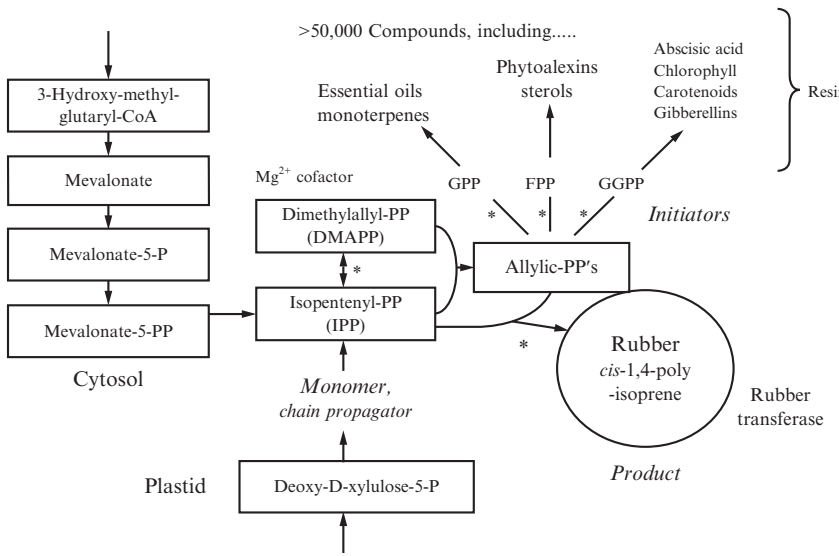


**Figure 4.1** The isoprenoid pathway, including the enzymes that can also compete with rubber transferase for isopentenyl pyrophosphate (monomer), allylic pyrophosphate (initiator), and magnesium ions (activator).

being the usual *in vivo* initiator. The DMAPP isomerase, a very active cytoplasmic enzyme, isomerizes IPP into DMAPP, with an equilibrium of 1 IPP:2 DMAPP. All these enzymes are magnesium requiring. In all experiments, EDTA is used as a control to bring enzyme activity to baseline and determine nonspecific background and to stop the reaction at the end of the reaction time.

Rubber biosynthetic activity is usually quantified by the incorporation of radiolabeled IPP into rubber by enzymatically active latex or purified rubber particles. This works well because the newly synthesized rubber can be detected in the background of previously synthesized unlabeled rubber. However, kinetics can only usefully be examined in the purified rubber particle system. In latex, the IPP isomerase will convert $^{14}$C-IPP into $^{14}$C-DMAPP, and then the *trans* prenyl transferases can make $^{14}$C-labeled FPP with label coming from the initiator and the IPP monomer in unknown proportions. Even if the isomerase is inhibited, the prenyl transferases can still incorporate the $^{14}$C-IPP into FPP, which has a considerably higher binding affinity for the rubber transferase active site than the smaller APPs. Incorrect control of competing enzymes has led to erroneous interpretation of rubber biosynthetic assay results. For example, a stereochemical shift of FPP synthase (FPS) from a C15 *trans* prenyl transferase enzyme to a high-molecular-weight *cis* prenyl transferase, via recombination of FPS with a sole membrane-bound 14.6 kDa protein, was deduced (Dennis & Light, 1989; Light & Dennis, 1989; Light, Lazarus, & Dennis, 1989). It was subsequently shown, however, that the *trans* prenyl transferase preparation used also contained IPP isomerase (Cornish, 1993), and the Coomassie stain used to indicate the presence of a single rubber particle-bound protein failed to detect many other bound proteins. See Section 5 for a comparison of Coomassie- and silver-stained gels.

The pH optima of rubber transferases can be determined using universal buffering systems, and the acidic and basic thresholds for denaturation, in contrast to inhibition, determined.

## 3. THE RUBBER TRANSFERASE ASSAY

Rubber transferase activity can be measured by IPP incorporation rates using a multiwell filtration system for high-throughput transferase microassays (Mau, Scott, & Cornish, 2000; Xie et al., 2008). Alternatively, microfuge tubes can be used (Cornish & Backhaus, 1990).

## 3.1. Assay method

A typical reaction volume is 50 μl of 1 m*M* unlabeled IPP, 0.9 nmol labeled [1-$^{14}$C]IPP (55 mCi/mmol, American Radiolabeled Chemicals, Inc.), 15 μ*M* FPP, 0.25–0.5 mg WRPs in a reaction buffer of 100 m*M* Tris–HCI, 1.25 m*M* magnesium sulfate, 5-m*M* DTT, using a multiscreen 96-well plate (Millipore, Cat# MSDVN6B10); WRPs must always be added last. Several controls should be included, such as an EDTA control, which will be treated as background and subtracted from all values accordingly, and a non-APP initiator (FPP, GPP, or DMAPP) control, which is used to evaluate the residual APP activity of different batches of purified WRPs.

1. Label and place a 96-well microplate on ice. Add 5 μl 10 m*M* unlabeled IPP, 0.75 μl 0.1 m*M* FPP, 0.25 μl labeled [1-$^{140}$C] IPP onto the wall of each well of the microplate. Alternatively and preferably, add all premixed components into each well using a multichannel pipet.
2. Thaw WRP on ice, dilute in assay reaction buffer, and add 34 μl diluted WRP toward the wall of each well using a multiple channel pipet.
3. Cover the microplate lid and gently tap the microplate to allow all components to be mixed at the bottom of the microplate. Wrap the microplate within aluminum foil and place it on a ceramic cooling plate (GE Healthcare, Cat# 18-1103046) on a shaker with temperature controlled by a circulating water bath. Incubate for 4 h at 16 °C for *P. argentatum*, 16 °C for *H. brasilensis* and *F. elastica*.
4. Add 50 μl 80 m*M* EDTA into each well, mix well, and then filtrate using vacuum.
5. Wash each well with 150 μl water and filtrate. Repeat this water wash once.
6. Dry the microplate membrane by adding 150 μl 95% ethanol into each well and filter.
7. Remove the microplate and dry completely in a 50 °C oven for 30 min.
8. Remove the bottom supporting part of the microplate, punch out the membrane from each well, and place it into scintillation vials containing 2 ml ScintiVerse™ BD cocktail (Fisher Science) per vial. Count each vial twice using a scintillation counter.
9. Calculate the IPP incorporation rate of each sample and convert this into the rate of IPP incorporation (μmol/g dry rubber/h).

## 3.2. Kinetic analyses

Rubber biosynthesis depends on the concentrations of APP (initiator), IPP (monomer), and magnesium ions (activator). Kinetic constants are best

determined for each by varying the concentrations one at a time, while the other two are present in nonlimiting but noninhibitory concentrations. These constants can vary over several orders of magnitude in different rubber-producing species for the initiator and over at least one order of magnitude for the magnesium ion activator, and other activators can be different again. Thus, several *species-specific* experiments may be required to find the appropriate concentration ranges for good kinetic data. Depending upon which aspect of rubber biosynthesis is under investigation, the initiation reaction (Espy et al., 2006), the polymerization reaction (Espy et al., 2006), or both simultaneously (as is most common) (Cornish, 2001a, 2001b), different kinetic analyses are appropriate (Segel, 1993). We have found that the Michaelis– Menton plot of $1/v$ versus $1/[\mathrm{APP}]$ generally results in a curved plot, leading to a subjective linear regression and doubtful $K_m$s. For the IPP polymerization reaction, in the presence of nonlimiting initiator concentrations, the Eadie–Hofstee plot of $v/[S]$ versus $[S]$ generates a linear plot over most concentrations but very low IPP concentrations and nonlimiting IPP concentrations should be deleted. The gradient of the plot is $-K_m^{\mathrm{IPP}}$, and the $y$-axis intercept is $V_{max}^{\mathrm{IPP}}$ for IPP in the particular initiator used. However, owing to the nonspecific hydrophobic binding region in the rubber transferase active site, short initiators also generate curved $v/[S]$ versus $[S]$ plots. In these circumstances, we suggest using the Hill plot of $\log(v - V_{max})/v$ plotted against $\log[S]$. In this plot, the $K_m^{\mathrm{APP}}$ is the $x$-axis value where $y = 1$.

Similarly, cofactor investigations can be problematical. It is difficult to wash out sufficient $Mg^{2+}$ to bring activity low enough to determine reaction kinetics, because of its tight affinity with the active site, without taking so much time that appreciable enzyme activity is lost during the purification. This problem can be solved by adding EDTA to chelate the essential magnesium cation activator and bring the enzyme active to baseline (da Costa, Keasling, McMahan, & Cornish, 2006; Kang, Kang, & Han, 2000; Scott, da Costa, Espy, Keasling, & Cornish, 2003). Titrating back, magnesium will indicate the true origin as activity rises above the baseline value. That particular magnesium concentration becomes the true origin and is subtracted to adjust the $x$-axis values to the origin. Informative kinetic plots can then be constructed and rate constants determined (Espy et al., 2006; Scott et al., 2003).

## 3.3. Regulation of molecular weight

The molecular weight of the rubber made during *in vitro* assays is primarily governed by the rate of the chain transfer reaction, that is, the termination and release of an existing rubber polymer molecule, coupled with the

initiation of a new molecule in the same active site. Its regulation is strongly dependent upon substrate and activator identity, concentration, and ratios, and especially, the relationship of substrate concentration to $K_m$. Fortunately, it is possible to determine the mean molecular weight of the rubber made under any set of conditions by dual label experiments, in which the initiator is tritiated and $^{14}$C-IPP is used as the source of monomer (Castillon & Cornish, 1999; da Costa et al., 2006). The mean molecular weight is calculated based on the ratio of $^{3}$H:$^{14}$C because each rubber polymer contains a single initiator. The molecular weight distribution of new rubber requires a gel permeation chromatograph coupled to both a mass detector of some type and a radioisotope detector (Benedict, Madhavan, Greenblatt, Venkatachalam, & Foster, 1990; Espy et al., 2006). However, very few laboratories have such equipment, whereas the mean molecular weight method is readily accessible by any basic biochemistry laboratory with a radiation license.

## 4. IDENTIFICATION AND PURIFICATION OF RUBBER TRANSFERASE

Active rubber transferase has not been purified, reproducibly, to date (Benedict et al., 1990), and so biochemical investigations have relied on using purified, enzymatically active rubber particles from which all cytoplasmic components have been removed (Cornish, 1993; Espy et al., 2006). This appears to be a valid approach because kinetic analysis indicates that only one IPP-using enzyme is present (linear IPP plots of $v$ against $v$/[S]) when rubber is synthesized, which must, therefore, be the rubber transferase (Cornish, 2001b; Cornish & Scott, 2005). Thus, purified rubber particles present a single enzyme system, positioned on a suitable aqueous–organic interface, valid for biochemical investigation. Further, the study of biochemical parameters on the native particles is likely to provide kinetic information directly applicable to the *in vivo* situation, something that can present a significant challenge in the study of solubilized membrane-bound enzymes.

### 4.1. Rubber transferase is a distinct *cis* prenyl transferase

Rubber transferase is not the only *cis* prenyl transferase in plants capable of producing *cis* polyisoprene (Espy et al., 2006; Kharel & Koyama, 2003; Oh, Han, Ryu, & Kang, 2000; Post et al., 2012). However, the substrate-binding constants are quite different among different types of *cis* prenyl transferases (Asawatreratanakul et al., 2003; Cornish, 2001b; Cunillera, Arró, Forés,

Manzano, & Ferrer, 2000; Lu, Liu, Teng, & Liang, 2010; Oh et al., 2000; Post et al., 2012) and rubber transferase is the only one capable of indeterminate polymer lengths of at least 1 million g/mol under appropriate substrate conditions (da Costa, Keasling, & Cornish, 2005, 2006, da Costa et al., 2006). *Cis* prenyl transferase contamination is common in rubber particle preparations, especially from latex-producing species which have copious amounts of soluble *cis* prenyl transferases. Given the same substrates as rubber transferase, the *cis* prenyl transferases also can make product—but this is always of low molecular weight of determinant size. *In vitro* assays should employ limiting APP concentrations and excess IPP to permit the enzyme in question to produce its maximum product length. This is one approach to distinguish the two classes of *cis* prenyl transferases. Checking the linearity of the IPP incorporation reaction in an IPP plots of $v$ against $v/[S]$ is another approach—a linear plot means that a single binding constant and, thus, a single enzyme is present. A curved plot means that multiple binding constants are operating which may, or may not, mean multiple enzymes. A third method entails determining the IPP $V_{max}$ under constant substrate concentrations in rubber particle preparations purified to different extents, while the *cis* prenyl transferase amount in question is correlated using SDS-PAGE. A combination of these methods is likely required in the absence of an expression system proving high-molecular-weight rubber synthesis with binding constants matching those of the rubber particle-bound system.

## 4.2. Photoaffinity labeling

Photoaffinity labeling is a method used to covalently bind chemical tags to the active site of a protein. Ultraviolet light is used to activate a motif that then covalently binds to the closest amino acid. The label is then used to identify the protein containing the covalently linked label. For example, benzophenone analogs of APPs have been used to identify farnesylated proteins. Radiolabels, such as tritium or $^{32}P$, can be used, and biotin also is a useful tag. Photoaffinity labels have been used in attempts to label the rubber transferase in enzymatically active WRPs of *H. brasiliensis* (Degraw et al., 2007), but the caveats in the previous section remain a concern. It is also necessary to choose a U/V exposure time that does not also instantly inactivate the enzyme in an unlabeled particle preparation (Fig. 4.2). The concentration of rubber particles in the preparation also should be optimized for the researcher's species of interest. It is also required that kinetic experiments

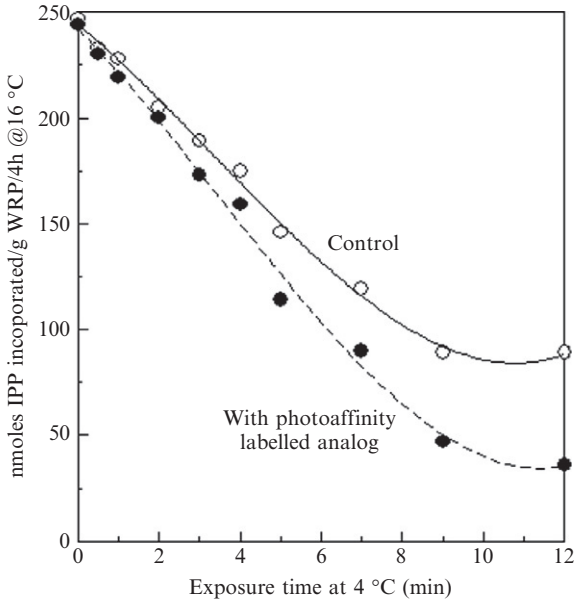


**Figure 4.2** Rubber transferase activity in purified rubber particles of *P. argentatum* assayed after U/V irradiation for different times in the presence and absence of a photoaffinity-labeled substrate analog.

be performed to determine whether the substrate analog is a true substrate or if it is a substrate competitor.

### *4.2.1 A photoaffinity labeling protocol*

A protocol for performing a 100 µl photoaffinity-labeled substrate (molecules which are substrate competitors may also be effective) experiment in *H. brasiliensis* is as follows:

1. Add 10 µl 10 × reaction buffer (1 *M* Tris–HCI (pH 7.5), 12.5 m*M* magnesium sulfate, 50 m*M* DTT), 10 µl 100 µ*M* benzophenone-containing analog of FPP tagged with biotin, $^{3}H$ or $^{32}P$, WRPs of *H. brasiliensis* (and DDW) to a final concentration of 14 or 15 mg WRP in a siliconized quartz test tube. Mix the reaction gently using a rotating shaker in the horizontal plane.
2. If substrate competition is desired as a control, add FPP to a final concentration of 100 µ*M* before adding the photoaffinity-labeled substrate.
3. Photolyze for 6 h at 4 °C in a Rayonet mini-reactor with six 350-nm bulbs and a spinning platform to spin sample to ensure an even exposure to the light.

4. After photolysis, add SDS to a final concentration of 1% and mix. Add 5-µl labeled rubber particles, 2.5-µl 4× SDS-PAGE loading buffer, 0.5 µl 1 *M* DTT, and 2 µl water, and heat for 5 min at 100 °C. Analyze by running out on a 4–12% Bis–Tris NuPAGE gel (Life Sciences, USA).

### 4.2.2 Purification of labeled proteins

Photoaffinity biotinylated proteins are solubilized from labeled WRPs and the rubber is removed and the proteins are concentrated. The labeled proteins are then purified on a streptavidin column (Rybak et al., 2004).

1. Add 1 *M* DTT into photoaffinity-labeled WRPs to a final concentration of 50 m*M*, vortex for 2 min, and then incubate for 5 min at 100 °C. Vortex for another 5 min, centrifuge at 9681 × *g* for 15 min at 20 °C to remove rubber particle layer, and repeat the centrifugation step until all rubber is removed. Filter the solubilized protein solution through a 0.2-µm filter to remove any remaining rubber particles and then concentrate at 4696 × *g* at 15 °C using a Vivaspin concentrator with a molecular weight cutoff of 5 kDa (Vivascience, USA).
2. Dilute concentrated proteins with PBS buffer to 500 µl and load onto 70 µl streptavidin Sepharose resin (GE Healthcare, USA) in a microspin column. Prior to sample loading, the streptavidin column should be washed twice with wash buffer A (1% NP-40, 0.1% SDS in PBS buffer) and then twice with 1 × PBS.
3. Incubate the sample on the column for 2 h on ice, and then remove the supernatant. Wash the resin three times with wash buffer A (200 µl/wash), twice with wash buffer B (wash buffer A plus 0.4 *M* NaCI; 200 µl/wash), and then once with 200 µl 50 m*M* Tris–HCI (pH 7.5).
4. Add 200 µl elution solution A (2%SDS, 3 m*M* biotin in 1 × PBS), mix, and then transfer the slurry into a microfuge tube and incubate for 5 min at 95 °C. Vortex samples for 2 min, then spin at 17,000 × *g* for 3 min at room temperature, and collect the supernatant into a new microfuge tube. Repeat this step twice.
5. Add 200 µl elution solution B (2%SDS, 3 m*M* biotin, 6 *M* urea in 1 × PBS), mix, and transfer the slurry into a microfuge tube and incubate for 5 min at 95 °C. Vortex samples for 2 min, spin at 17,000 × *g* for 3 min at room temperature, and collect the supernatant into a new microfuge tube. Repeat this step twice.
6. Concentrate the eluted labeled protein sample by using a Vivaspin concentrator with a molecular weight cutoff of 5 kDa (Vivascience, USA) and analyze on a 4–12% Bis–Tris NuPAGE gel.

Stain the gel with the SilverQuest Silver Staining kit (Cat#LC6070, Invitrogen, USA) (Fig. 4.3), excise stained bands, and place them into individual microfuge tubes. The gel slices are then destained and subjected to in-gel trypsin digestion followed by mass spectrometry sequencing.

## 4.3. Antibodies

### *4.3.1 Immunoinhibition and immunoprecipitation*

Antibodies have been raised against various rubber particle-bound proteins and have been subsequently used in attempts specifically to inhibit particle-bound activity. These experiments have been performed under the hypothesis that, if antibodies are able to inhibit rubber transferase activity, they are binding to members of the rubber transferase complex and may be utilized to precipitate and identify this complex (Cornish et al., 1994; Light & Dennis, 1989; Siler & Cornish, 1994). However, for these attempts to be successful, it is essential to use purified rubber particles (not latex) and purified antibodies (not crude sera). As discussed earlier, unpurified latex, or

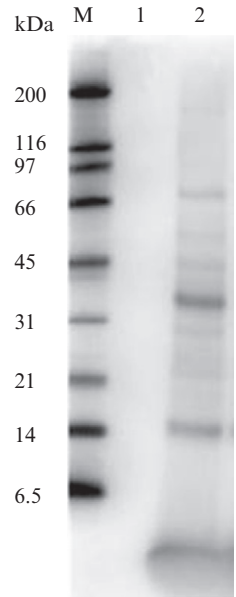


**Figure 4.3** Western blot of biotinylated proteins isolated from photolyzed washed rubber particles of *H. brasiliensis*, separated on a 4–12% Bis–Tris NuPAGE gel, transferred onto a PVDF membrane and probed with first antibiotin antibodies (2 μg/ml) and then second antirabbit-HRP (5 ng/ml) and detected by chemiluminescence. M, biotinylated makers, 12.5 ng; (A) no photolysis control; (B) photolysis for 10 min.

partially purified rubber particle preparations, still contain significant amounts of IPP isomerase and *trans* prenyl transferases which use the same substrates as rubber transferase. Also, antibodies which inhibit activity in latex could be doing this through an effect on the rubber transferase, through an effect on the initiator system, or by a nonspecific toxic effect. Crude mouse serum, for example, inhibits rubber transferase activity in purified rubber particles in a concentration-dependent manner, but this is not an antibody-dependent effect (Fig. 4.4).

### 4.3.2 Latex-free animals

It is important to raise the antibodies in animals that have been born and reared in a latex-free environment. Preimmune bleeds have shown that experimental animals (rabbits and mice) frequently contain antibodies to proteins in *H. brasiliensis*, generated presumably through response to contact with the latex gloves of their handlers and the rubber stoppers used to support their drinking tubes.

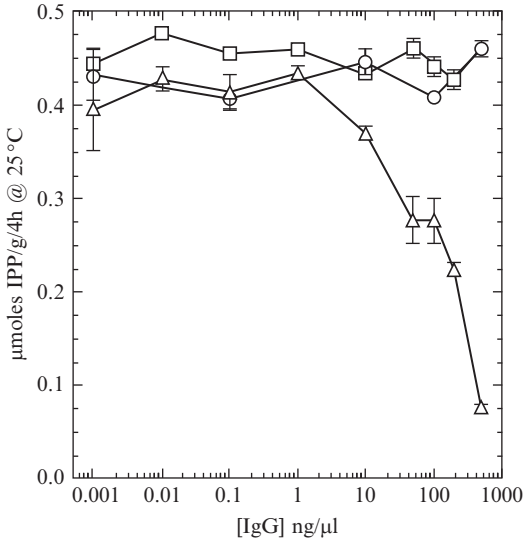


**Figure 4.4** Rubber transferase activity in purified *H. brasiliensis* rubber particles. Rubber particles were incubated for 15 h at 4 °C with different antibody preparations and then assayed (Cornish, 1993) using 1 m*M* IPP, 20 µ*M* FPP, and 1 m*M* $Mg^{2+}$. Plots are as follows: ○, control IgG; Δ, ascites fluid (crude mouse sera); □, lipoprotein IgG purified from the ascites fluid.

## 5. QUALITATIVE PROTEIN ANALYSIS

Because rubber particle-associated proteins are very strongly bound to rubber particles, harsh treatment (heat plus extensive vortexing) is used to solubilize the proteins from rubber particles. Rubber must be removed before analysis by repeated centrifugation steps and samples must be filtered to remove any remaining rubber particles. The soluble protein fractions can then be concentrated using a size exclusion membrane with a small cutoff, typically 5 kDa, to retain all rubber particle-associated proteins.

As not all proteins are detectable by Coomassie blue, silver stain is usually used to detect all proteins (Siler & Cornish, 1993, 1994).

1. Add 25 µl 4 × NuPAGE SDS sample buffer, 5 µl 1 *M* DTT, to 70 µl 3 × WRPs. Incubate for 5 min at 100 °C. Vortex for at least 3 min and then centrifuge at 17,000 × *g* for 3 min at room temperature to remove rubber. Repeat this centrifuge step until all rubber is removed (at least twice more), and then filter the supernatant using a 0.45 nm ultrafree-MC centrifugal filter (Millipore, USA).
2. Load the denatured proteins onto a NuPAGE 4–12% Bis–Tris gel and run in a buffer of 50 m*M* MES, 50 m*M* Tris base, 0.1% SDS, 1 m*M* EDTA, pH 7.3 for 35 min at 200 V.
3. Stain the gel overnight with gentle shaking at RT with 100 ml Coomassie Blue G-250 solution by mixing 80 ml of 10% ammonium sulfate, 2% phosphoric acid, 0.1% Coomassie G250, and 20 ml methanol. Destain several times with water (Neuhoff et al., 1988).
4. Alternatively, stain the gel using a silver stain kit (Bio-Rad, USA).

An example of a matched pair of gels stained with Coomassie (Fig. 4.5A) and silver (Fig. 4.5B) shows that more proteins are revealed with silver than with Coomassie.

## 6. PROTEIN QUANTIFICATION

The concentration of rubber particle proteins can be quantified by using a Micro BCA Protein Assay Kit to quantify proteins at low concentrations (Cat#23235, Thermo Scientific, USA).

1. Mix 200 µl 3 × WRPs with 250 µl 100 m*M* sodium phosphate buffer (pH 7.5), 25 µl 20% SDS, and 25 µl 1 *M* DTT; incubate for 10 min at 100 °C; and then vortex for 30 min.

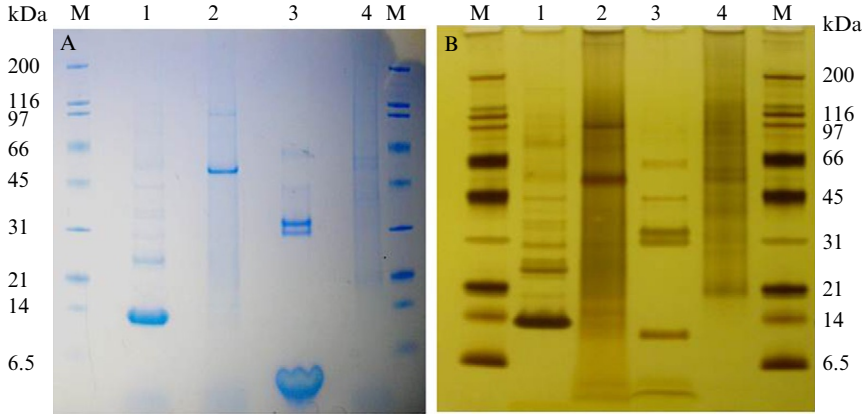


**Figure 4.5** Rubber particle proteins from 1, *H. brasiliensis*; 2, *P. argentatum*; 3, *F. elastica*; 4, *T. kok-saghyz*. Proteins were extracted using sample buffer and run on 4–12% Bis–Tris NuPAGE gels. (A) 2.5 mg (dw basis) rubber particles stained with Coomassie stain; (B) 0.3 mg rubber particles stained with silver. M contains molecular weight standards; (A) 250 ng, (B) 50 ng.

2. Centrifuge at 17,000 × *g* for 3 min at room temperature and transfer the supernatant into a new microfuge tube. Repeat this centrifugation step as necessary to remove any remaining rubber, and then filter supernatants through a 0.45-μm centrifugal filter.
3. Check the sample volume and adjust to 500 μl using 50 m*M* sodium phosphate buffer (pH 7.5).
4. Prepare bovine serum albumin standards in 500 μl of each in 50 m*M* sodium phosphate buffer (pH 7.5) at 0, 0.5, 1, 2.5, 5, 10, 20, 40, 200 μg/500 μl volume.
5. Add 50 μl 1.5 mg/ml sodium deoxycholate, mix, and stand for 10 min at room temperature.
6. Add 100 μl freshly mixed 72% trichloroacetic acid and 72% phosphotungstic acid (1:1) into each sample, mix, and incubate for 30 min at room temperature.
7. Spin for 15 min at 15,000 rpm, remove the supernatant, air dry pellet, and suspend each pellet in 100 μl 50 m*M* sodium phosphate buffer (pH 7.5).
8. Quantify protein concentrations by following the protocol described by the manufacturer (Thermo Scientific, USA).

Methods to quantify latex proteins extracted from latex and rubber products have also been developed by ASTM International (www.astm.org), namely,

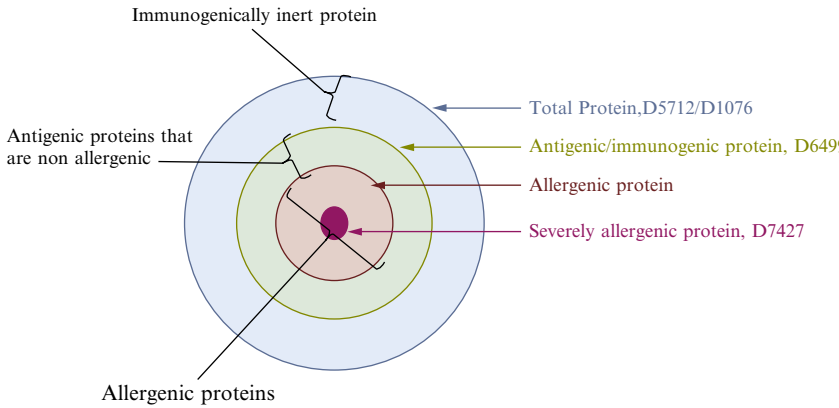


**Figure 4.6** Venn diagram indicating the subsets of proteins which are quantified with the different ASTM protein quantification methods. The diagram shows increasing immunogenic efficacy with decreasing circle diameter, which corresponds to decreasing protein number. Each ring defines the type of proteins in terms of their immunogenic efficacy.

D5712-05 "*Standard Test Method for Analysis of Aqueous Extractable Protein in Natural Rubber and Its Products using the Modified Lowry Method*," D6499-03 "*Standard Test Method for the Immunological Measurement of Antigenic Protein in Natural Rubber and Its Products,*" and D7427-08 "*Standard Test Method for Immunological Measurement of Four Principal Allergenic Proteins (Hev b* 1, 3, 5, and 6.02*) in Natural Rubber and Its Products Derived from Latex*." The differences between these methods are described pictorially in a Venn diagram (Fig. 4.6). In addition, a modification of the D5712 method for the quantification of total protein in Category 4 latex (rubber particle-bound protein, hydrophobic protein, and soluble protein) may be found in ASTM D1076-06 "*Standard Specification for Rubber—Concentrated, Ammonia Preserved, Creamed, and Centrifuged Natural Latex*." This total protein quantification method can also be used for other latices when total protein amount is needed.

## 7. SUMMARY

This chapter provides guidance in rubber biosynthesis studies. We believe it is essential for researchers in this area to properly understand the rubber biosynthetic pathway and to control for competition by contaminating cytosolic enzymes for the substrates used by the rubber transferase.

We hope that careful consideration of these methods, and the references cited within specific areas of interest, will help researchers to conduct meaningful studies and advance our knowledge of the biochemistry involved in the synthesis of an essential biomolecule.

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## REFERENCES

Asawatreratanakul, K., Zhang, Y. W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayaporn, A., et al. (2003). Molecular cloning, expression and characterization of cDNA encoding cis-prenyltransferases from Hevea brasiliensis. *European Journal of Biochemistry*, *270*, 4671–4680.

Benedict, C. R., Madhavan, S., Greenblatt, G. A., Venkatachalam, K. V., & Foster, M. A. (1990). The enzymatic synthesis of rubber polymer in Parthenium argentatum Gray. *Plant Physiology*, *92*, 816–821.

Bick, J. A., & Lange, B. M. (2003). Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: Unidirectional transport of intermediates across the chloroplast envelope membrane. *Archives of Biochemistry and Biophysics*, *415*, 146–154.

Castillon, J., & Cornish, K. (1999). Regulation of initiation and polymer molecular weight of cis-1,4-polyisoprene synthesized in vitro by particles isolated from Parthenium argentatum (Gray). *Phytochemistry*, *51*(1), 43–51.

Cornish, K. (1993). The separate roles of plant cis and trans prenyl transferases in cis-1,4-polyisoprene biosynthesis. *European Journal of Biochemistry*, *218*, 267–271.

Cornish, K. (2001a). Similarities and differences in rubber biochemistry among plant species. *Phytochemistry*, *57*, 1123–1134.

Cornish, K. (2001b). Biochemistry of natural rubber, a vital raw material, emphasizing biosynthetic rate, molecular weight and compartmentalization, in evolutionarily divergent plant species. *Natural Product Reports*, *18*, 182–227.

Cornish, K., & Backhaus, R. A. (1990). Rubber transferase-activity in rubber particles of guayule. *Phytochemistry*, *29*, 3809–3813.

Cornish, K., & Bartlett, D. L. (1997). Stabilisation of particle integrity and particle-bound cis-prenyl transferase activity in stored, purified rubber particles. *Phytochemical Analysis*, *8*, 130–134.

Cornish, K., & Scott, D. J. (2005). Biochemical regulation of rubber biosynthesis in guayule (Parthenium argentatum Gray). *Industrial Crops and Products*, *22*, 49–58.

Cornish, K., & Siler, D. J. (1995). Effect of different allylic diphosphates on the initiation of new rubber molecules and on cis-1,4-polyisoprene biosynthesis in guayule (Parthenium argentatum Gray). *Journal of Plant Physiology*, *147*, 301–305.

Cornish, K., & Siler, D. J. (1996). Characterization of cis-prenyl transferase activity localised in a buoyant fraction of rubber particles from Ficus elastica latex. *Plant Physiology and Biochemistry*, *34*, 377–384.

Cornish, K., Siler, D. J., & Grosjean, O. K. K. (1994). Immunoinhibition of rubber particle-bound cis-prenyl transferases in Ficus elastica and Parthenium argentatum. *Phytochemistry*, *35*, 1425–1428.

Cornish, K., Wood, D. F., & Windle, J. J. (1999). Rubber particles from four different species, examined by transmission electron microscopy and electron-paramagnetic-resonance spin

labeling, are found to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane. *Planta*, *210*, 85–96.

Cunillera, N., Arró, M., Forés, O., Manzano, D., & Ferrer, A. (2000). Characterization of dehydrodolichyl diphosphate synthase of Arabidopsis thaliana, a key enzyme in dolichol biosynthesis. *FEBS Letters*, *477*, 170–174.

da Costa, B. M. T., Keasling, J. D., & Cornish, K. (2005). Regulation of rubber biosynthetic rate and molecular weight in Hevea brasiliensis by metal cofactor. *Biomacromolecules*, *6*, 279–289.

da Costa, B. M. T., Keasling, J. D., McMahan, C. M., & Cornish, K. (2006). Magnesium ion regulation of in vitro rubber biosynthesis by Parthenium argentatum Gray. *Phytochemistry*, *67*, 1621–1628.

DeGraw, A. J., Zhao, Z., Strickland, C. L., Nural, A. H., Hsieh, J., Jefferies, M., et al. (2007). A photoactive isoprenoid diphosphate analogue containing a stable phosphonate linkage: synthesis and biochemical studies with prenyltransferases. *The Journal of Organic Chemistry*, *72*, 4587–4595.

Dennis, M. S., & Light, D. R. (1989). Rubber elongation factor from Hevea brasiliensis. Identification, characterization and role in rubber biosynthesis. *The Journal of Biological Chemistry*, *264*, 18608–18617.

Espy, S. C., Keasling, J. D., Castillon, J., & Cornish, K. (2006). Initiator-independent and initiator-dependent rubber blosynthesis in Ficus elastica. *Archives of Biochemistry and Biophysics*, *448*, 13–22.

Kang, H., Kang, M. Y., & Han, K. H. (2000). Identification of natural rubber and characterization of rubber biosynthetic activity in fig tree. *Plant Physiology*, *123*, 1133–1142.

Kharel, Y., & Koyama, T. (2003). Molecular analysis of cis-prenyl chain elongating enzymes. *Natural Product Reports*, *20*, 111–118.

Light, D. R., & Dennis, M. S. (1989). Purification of a prenyl transferase that elongates cis-polyisoprene rubber from the latex of Hevea brasiliensis. *The Journal of Biological Chemistry*, *264*, 18589–18597.

Light, D. R., Lazarus, R. A., & Dennis, M. S. (1989). Rubber elongation by farnesyl pyrophosphate synthases involves a novel switch in enzyme stereospecificity. *The Journal of Biological Chemistry*, *264*, 18598–18607.

Lu, Y. P., Liu, H. G., Teng, K. H., & Liang, P. H. (2010). Mechanism of cis-prenyltransferase reaction probed by substrate analogues. *Biochemical and Biophysical Research Communications*, *400*, 758–762.

Mau, C. J. D., Scott, D. J., & Cornish, K. (2000). Multiwell filtration system results in rapid, high-throughput rubber transferase microassay. *Phytochemical Analysis*, *11*, 356–361.

Neuhoff, V., Arold, N., Taube, D., & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, *9*, 255–262.

Oh, S. K., Han, K. H., Ryu, S. B., & Kang, H. (2000). Molecular cloning, expression, and functional analysis of a cis-prenyltransferase from Arabidopsis thaliana. *The Journal of Biological Chemistry*, *275*, 18482–18488.

Pearson, C. H., Cornish, K., McMahan, C. M., Rath, D. J., Brichta, J. L., & Van Fleet, J. E. (2010). Agronomic and natural rubber characteristics of sunflower as a rubber-producing plant. *Industrial Crops and Products*, *31*, 481–491.

Post, J., van Deenen, N., Fricke, J., Kowalski, N., Wurbs, D., Schaller, H., et al. (2012). Laticifer specific cis-prenyltransferase silencing affects the rubber, triterpene and inulin content of Taraxacum brevicorniculatum. *Plant Physiology*, *154*.

Rybak, J., Scheurer, S. B., Neri, D., Elia, G. (2004). Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution. *Proteomics*, *4*, 2296–2299.

Scott, D. J., da Costa, B. M. T., Espy, S. C., Keasling, J. D., & Cornish, K. (2003). Activation and inhibition of rubber transferases by metal cofactors and pyrophosphate substrates. *Phytochemistry*, *64*, 123–134.

Segel, I. H. (1993). *Enzyme kinetics*. New York: John Wiley and Sons.

Siler, D. J., & Cornish, K. (1993). A protein from Ficus elastica rubber particles is related to proteins from Hevea brasiliensis and Parthenium argentatum. *Phytochemistry*, *32*, 1097–1102.

Siler, D. J., & Cornish, K. (1994). Identification of Parthenium argentatum rubber particle proteins immunoprecipitated by an antibody that specifically inhibits rubber transferase activity. *Phytochemistry*, *36*, 623–627.

Siler, D. J., Goodrich-Tanrikulu, M., Cornish, K., Stafford, A. E., & McKeon, T. A. (1997). Composition of rubber particles of Hevea brasiliensis, Parthenium argentatum, Ficus elastica, and Euphorbia lactiflua indicates unconventional surface structure. *Plant Physiology and Biochemistry*, *35*, 881–889.

Wood, D. F., & Cornish, K. (2000). Microstructure of purified rubber particles. *International Journal of Plant Sciences*, *161*, 435–445.

Xie, W., McMahan, C. M., DeGraw, A. J., Distefano, M. D., Cornish, K., Whalen, M. C., et al. (2008). Initiation of rubber biosynthesis: in vitro comparisons of benzophenone-modified diphosphate analogues in three rubber-producing species. *Phytochemistry*, *69*, 2539–2545.