Initiator-independent and initiator-dependent rubber biosynthesis in *Ficus elastica*

Stephanie C. Espy [a], Jay D. Keasling [a], Javier Castillón [b], Katrina Cornish [b,*]

[a] *Department of Chemical Engineering, University of California, Berkeley, CA 94720, USA*
[b] *USDA-ARS, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710, USA*

Abstract

The rubber-producing tree, *Ficus elastica* (the Indian rubber tree), requires the same substrates for rubber production as other rubber-producing plants, such as *Hevea brasiliensis* (the Brazilian or *Para* rubber tree), the major source of commercial natural rubber in the world, and *Parthenium argentatum* (guayule), a widely studied alternative for natural rubber production currently under commercial development. Rubber biosynthesis can be studied, in vitro, using purified, enzymatically active rubber particles, an initiator such as FPP, IPP as the source of monomer, and a metal cofactor such as Mg^{2+}. However, unlike *H. brasiliensis* and *P. argentatum*, we show that enzymatically active rubber particles purified from *F. elastica* are able to synthesize rubber, in vitro, in the absence of added initiator. In this paper, we characterize, for the first time, the kinetic differences between initiator-dependent rubber biosynthesis, and initiator-independent rubber biosynthesis, and the effect of cofactor concentration on both of these processes.
© 2006 Elsevier Inc. All rights reserved.

Keywords: Cofactor; *Ficus*; Guayule; *Hevea*; Latex; Magnesium; Natural rubber; *Parthenium*; Polymerization

Although some 2500 plant species are known to produce natural rubber, most do not produce the high quality rubber that is required for high performance commercial products. Currently, by far the most prevalent commercial rubber-producing plant species is *Hevea brasiliensis* Muell. Arg., otherwise known as the Brazilian or *Para* rubber tree [1]. Originating in South American countries, particularly Brazil, plantation-grown, genetically narrow *H. brasiliensis* suffered pathogenic attack and crop failure in South America and today is predominantly cultivated in Asian countries [1–4]. *H. brasiliensis* has strict growing conditions, limiting its cultivation to specific tropical regions. Due to this factor and others, including diminishing acreage, increasing global demand, changing political positions, and the widespread occurrence of latex allergy to *H. brasiliensis* rubber products affecting millions of people, additional rubber-producing species are currently being studied in the hope of providing alternative sources of commercially viable natural rubber. *Parthenium argentatum* Gray (guayule) is currently the most promising alternative source of economically viable rubber, making large amounts of high molecular weight rubber [3–5], without proteins that cross-react with type I latex allergy [6].

In contrast to *H. brasiliensis* and *P. argentatum*, *Ficus elastica* Roxb. is a readily available rubber-producing plant that yields low quality rubber of no commercial value today [7,8]. Its laboratory use has been primarily for comparative purposes, as the basic biochemical process for producing natural rubber is common amongst evolutionarily divergent rubber-producing plants [3,4]. Thus, *F. elastica* may be used to determine information that may be applied to commercially viable rubber-producing plant systems such as *H. brasiliensis* and *P. argentatum*.

Natural rubber is synthesized from cytoplasmic substrates by a rubber transferase (RuT)[1] enzyme (EC 2.5.1.20) bound to the surface of the free-floating rubber particles; this integral protein catalyzes rubber polymerization [5,7,9–12]. An allylic diphosphate, such as farnesyl diphosphate (FPP), acts as the initiator in the reaction; isopentenyl diphosphate (IPP) provides the monomer for the *cis*-1,4-polymerization of the rubber polymer [5,7,9–12]. The monolayer membrane of the rubber particle [13,14] is made up of species-specific lipids and proteins [15]. The hydrophilic head groups of the lipids and membrane-bound proteins enable the rubber particle to interact with the aqueous cytosolic environment, the location of the rubber substrates. The rubber is subsequently compartmentalized within the interior of the rubber particle [3,4]. In addition to the rubber particle, RuT, FPP, and IPP, metal cofactors, such as magnesium cations, also are necessary for the production of natural rubber in plants [3–5,7,9–12]. Such cations are activators for the catalytic activity of RuT, aiding in the alkylation by prenyl transfer of the diphosphate in the IPP monomer [16].

In all studies of rubber biosynthesis reported to date the synthesis of entirely new rubber molecules has been studied. This is because the process of purifying enzymatically active rubber particles seems to release, from the active site, rubber molecules that were only partially polymerized at the moment of harvest. Thus, RuTs on purified particles are devoid of polymer and all in vitro natural rubber biosynthesis has to be initiated with an allylic diphosphate before polymerization can proceed. Such systems cannot be used separately to study the polymerization reaction in the absence of initiation, and the release of a polymer from the active site followed by re-initiation (the chain transfer reaction). As seen in a previous report using enzymatically active, purified, *F. elastica* rubber particles [7], ^{14}C-IPP incorporation into rubber is high in reactions when both FPP and IPP are present.

However, unlike other species investigated, we have found that magnesium-requiring IPP incorporation also is significant in initiator-independent reactions [7], suggesting that partially formed rubber molecules remain associated with the *F. elastica* RuT during particle purification. In this paper, enzymatically active rubber particles purified from *F. elastica* are used to establish first the existence of initiator-independent rubber biosynthesis and then separately to analyze the substrate kinetics and the effect of cofactor concentration on initiator-independent and initiator-dependent rubber biosynthesis.

---

[1] *Abbreviations used:* RuT, rubber transferase; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetic acid; THF, tetrahydrofuran; OMBPP, 3,4-oxido-3-methyl-1-butyl diphosphate; APP, allylic diphosphate; DMAPP, dimethyl allyl diphosphate; GPP, geranyl diphosphate; GGPP geranylgeranyl diphosphate.

Experimental methods

Materials

Mature *Ficus elastica* plants were grown in a greenhouse at the United States Department of Agriculture in Albany, California. ^{14}C-IPP (55 mCi/mmol), IPP, and FPP were obtained from American Radiolabeled Chemical Inc., St. Louis, MO. Siliconized 1.5 ml microfuge tubes were supplied by USA Scientific, Ocala, FL. Chemicals were purchased from Sigma, St. Louis, MO. A site-directed inhibitor of IPP isomerase, 3,4-oxido-3-methyl-1-butyl diphosphate [17] was generously supplied by C. Dale Poulter, University of Utah, USA.

Collection of F. elastica rubber particles

Latex was collected in the morning hours usually before the sun appeared; at these hours, plant root pressure is high and latex is easier to collect. Using a razor blade to make an angled cut severing the stems or the petioles, latex was tapped from the plants and collected in a buffer containing 100 mM Tris–HCl (pH 7.5), 5 mM $MgSO_4$, 0.1 mM Pefabloc, and 5 mM dithiothreitol (DTT). Latex was mixed with buffer approximately 1:1 v:v, and collection tubes were placed on ice until used.

Purification of F. elastica rubber particles

Ficus elastica rubber particles were purified as described previously for buoyant particles [7]. The collected rubber particle suspensions were adjusted to 12% glycerol and centrifuged at $2500g_n$ for 10 min at 4 °C using a Sorvall RC-5B centrifuge with an HS-4 bucket rotor. The particles that floated in the presence of 12% glycerol (light particles) were collected and the supernatant was decanted. Note that the particles that sank to the bottom of the tube (heavy particles) are enzymatically inactive and therefore not useful in this study.

Once collected, the particles were resuspended in 12% glycerol buffer and recentrifuged. The washing procedure was repeated twice more in a 0% glycerol buffer in which all rubber particles sank to the bottom of the tube. Once purified, 50 μl of particles were weighed and dried to determine the concentration of the washed rubber particles. Glycerol was added to the remaining particles to a final concentration of 10%; the particles then were frozen as beads in $N_2(l)$ to preserve enzymatic activity and stored in cryotubes in $N_2(l)$ dewars until further use [18].

For the washing experiment assayed in the presence of DMAPP, subsamples of the rubber particles were collected at each purification step for activity assays.

Microfuge tube assay of RuT activity

The amount of rubber particles used per assay varied according to the experiment, but was usually 2.5 mg in a

100 μl reaction volume with 200,000 dpm of [14C]-IPP. Except for reactions where substrate concentration was varied, typical substrate concentrations were 20 μM FPP and 200 μM [14C]-IPP. The reaction buffer contained 12.5 mM MgSO$_4$ (except for experiments with varying Mg^{2+} concentrations), 50 mM DTT, 100 mM Tris–HCl (pH 8), and DDW. All experiments with varying Mg^{2+} concentrations, and all those performed after the experiments to determine the cause of the initiator-independent reaction, also included pretreatment with 20 mM ethylene-diaminetetra-acetic acid (EDTA), to chelate any pre-existing Mg^{2+} before the remaining ingredients were added. The reaction mixtures, including buffer, substrates, and rubber particles, were incubated in siliconized microfuge tubes in a 25 °C water bath for 4 h. At this temperature, RuT activity is linear over the 4 h incubation period [4,9,19]. The reaction was stopped by addition of 25 μl of 1 M (EDTA) to chelate the Mg^{2+} added to activate the reaction, vortexed for 2 s, and centrifuged for 5 s at high speed in a microfuge.

The reaction mixtures then were diluted with 200 μl distilled water, and the rubber particles were collected on acetate/cellulose nitrate filters (Millipore; 25 mm diameter; 0.22 μm) using a vacuum manifold (Hoefer Scientific Instruments model no. FH 225V). Each reaction tube was rinsed with another 200 μl of distilled water, and the rinse fluid was added to the original filter. The filters were air dried for 40 min or until they turned opaque. Each filter was then transferred to a scintillation vial and incubated in 5 ml of 1 M HCl for 15 min to protonate unincorporated [14C]-IPP. The HCl was removed, and the filters were washed three times in 4 ml 95% ethanol to remove all unincorporated [14C]-IPP. After all the ethanol was removed, 5 ml of Scintiverse scintillation fluid (Fisher Scientific Chemicals) was added to each vial, and the amount of [14C]-IPP incorporated into polymer was determined by liquid scintillation counting (Beckman LS 6500 multipurpose scintillation counter).

Note that the multiwell plate assay method described by Mau et al. [19] is not always appropriate for *F. elastica* rubber particles; *F. elastica* particles do not coagulate tightly like those of *H. brasiliensis* or *P. argentatum*, and the washing procedure described may disturb the packing of the *F. elastica* rubber particles on the filter surface and can lead to losses during washing of the plate.

Gel-permeation chromatography

Rubber transferase assays (500 μl) were run in the presence of 5 million dpm [14C]-IPP (5 mM IPP in total) and in the presence and absence of 20 μM FPP initiator. Subsamples (50 μl) were filtered and washed, as in the rubber transferase assay described above, to remove unincorporated radiolabel, and assayed to ensure activity. The remaining 450 μl of each sample was dissolved in 3 ml hexane overnight. Samples were filtered though 0.45 μm syringe filters composed of PTFE with GMF 25 mm disposal GD/X

filters (Millipore, Burlington, MA), followed by four washes with 1 ml hexane. Samples were dried, redissolved in tetrahydrofuran (THF) and analyzed on a gel permeation chromatograph consisting of a Waters pump (1 ml THF/min), two Phenominex P-gel columns in series in a Beckman column oven at 40 °C, an Alcott universal sampler, four data capture units, an INUS beta-ram detector set up for both ^{3}H and ^{14}C analysis, and an ACS/Polymer Labs mass detector. Molecular weights were determined using PL Caliber GPC Software, and the GPC was calibrated using narrow standards of synthetic *cis*-1,4-polyisoprene.

Results

Initiator-independent IPP incorporation

The binding affinity of the *F. elastica* RuT for APP was affected by initiator size (Table 1) with increasing size leading to increased affinity. In contrast, there was no trend of initiator size on affinity for IPP, although the highest affinity was clearly attained in the presence of FPP (Table 1).

When the purity of the enzymatically active light particles prepared from the *F. elastica* latex was assessed by assaying IPP incorporation in the presence of non-limiting DMAPP (Fig. 1), we found that very little difference in activity was observed after the third wash, indicating that all soluble enzymatic contaminants had been removed by this stage. In the 0 wash preparation the particles were floated to the top of the SS-34 tubes and used in the assay. The soluble latex components had been diluted at least 7-fold (0.14×) by this stage. We estimate that the minimum dilution factors for the washing series were 0, 0.14; 1, 0.020; 2, 0.0029; 3, 0.00042; and 4, 0.000059. In some rubber particle preparations, dilution factors could be twice as great due to variations among harvests. All subsequent experiments used 4× washed rubber particles. We found a very similar pattern of the effect of washing on the amount of cytoplasmic contamination during rubber particle purification from *H. brasiliensis* latex (data not shown) as from *F. elastica* latex (Fig. 1).

The lack of isomerase contamination was confirmed by the addition of OMBPP, a site-directed inhibitor of IPP

Table 1
Binding constants for the *F. elastica* rubber transferase IPP incorporation determined from Hill plots ($v/V_{max} - v$ plotted against [S])

Initiator	K_m^{APP} (μM)	K_m^{IPP} (μM)
DMAPP	33.9	222
GPP	1.6	535
FPP	0.6	115
GGPP	0.3	535

Allylic diphosphate (APP) K_m^{APP}s were determined in the presence of 1 mM IPP. K_m^{IPP}s were determined in the presence of 20 μM APP except for DMAPP (50 μM). Mg^{2+} concentrations were 1.25 mM above background.
DMAPP, dimethyl allyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP geranylgeranyl diphosphate.
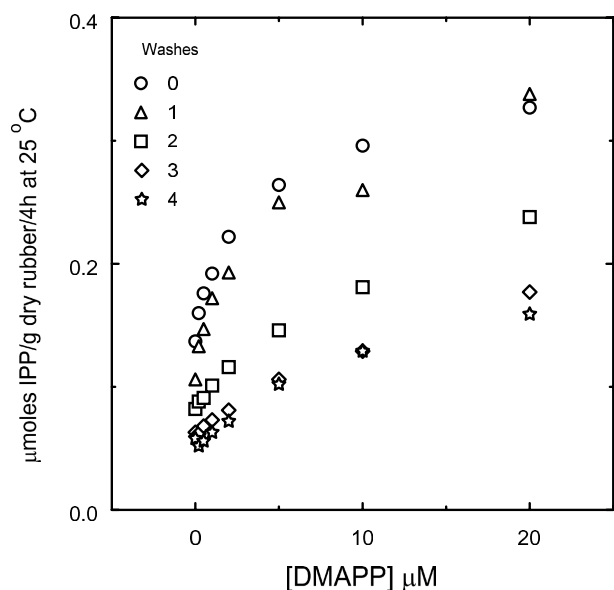
Fig. 1. Effect of degree of purification on the incorporation of IPP by *F. elastica* rubber particles in the presence of non-limiting levels of DMAPP (20 μM) initiator and 200 μM ^{14}C-IPP.

isomerase, at concentrations previously used to detect isomerase contamination in *H. brasiliensis* rubber transferase and prenyl transferase assays [11,17]. OMBPP (20 μM) did not affect IPP incorporation rates from either 1 mM IPP or from only 40 μM IPP (Fig. 2). A similar experiment was conducted on 3× washed *H. brasiliensis* rubber particles and no differences among IPP incorporation rates in 0, 5, and 20 μM OMBPP were observed (data not shown).

Initiator-independent IPP incorporation, under non-limiting IPP concentrations, was magnesium-requiring
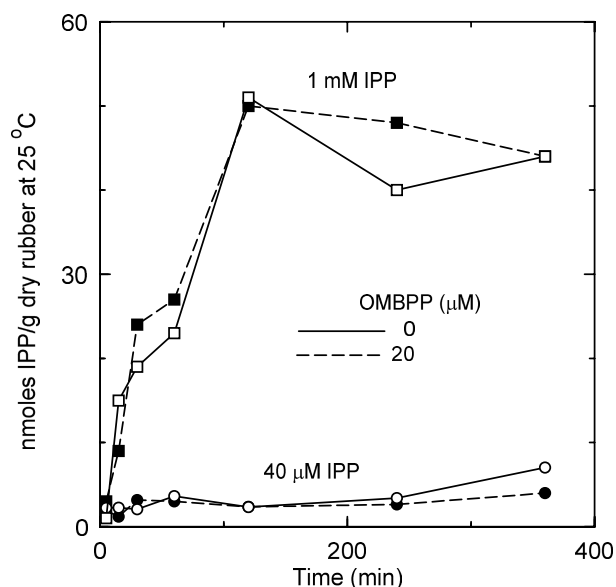
and followed the same temperature dependence as previously reported for initiator-dependent polymerization [7], with a maximum incorporation rate around 25 °C and with inhibition apparent at 37 °C after 4 h, probably due to enzyme denaturation.

Time course experiments indicated that IPP incorporation, in the absence of initiator, reached a maximum in 2 h (Fig. 3B). In contrast, IPP incorporation in the presence of FPP initiator continued for at least 6 h (and at a much higher rate than in the absence of FPP) in purified *F. elastica* rubber particles (Fig. 3A). Other experiments demonstrated that purified *F. elastica* particles, under non-limiting substrate conditions and in the presence of both IPP and FPP, continue to incorporate IPP into rubber for at least 24 h (data not shown).

Attempts were made to compare the molecular weight of the polymers produced in the absence of initiator with that produced in the presence of FPP, and with the rubber produced by the rubber particles in the living plant (Fig. 4 and Table 2). However, although radiolabeled polymer of at least as high a molecular mass as previously synthesized

Fig. 2. Comparison of the presence (- - -) or absence (—) of the isomerase inhibitor, 3,4-oxido-3-methyl-1-butyl diphosphate, on IPP incorporation by purified *F. elastica* rubber particles in either 1 mM (□) or 40 μM (○) IPP, without an allylic diphosphate initiator present in the reaction.
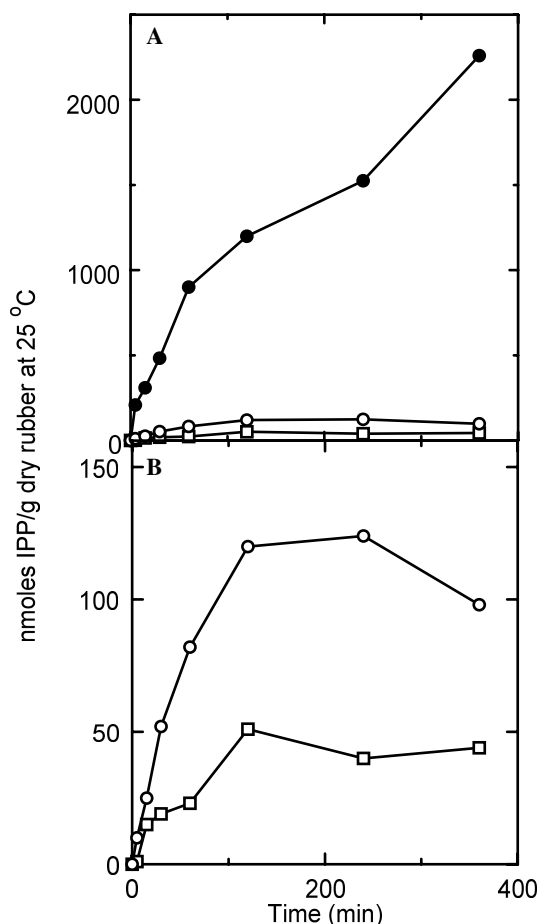

Fig. 3. Time course of IPP incorporation by purified *F. elastica* rubber particles in the presence of 1 mM (□) or 5 mM (○) IPP, without an allylic diphosphate initiator present in the reaction, shown in (A and B) on different scales. (A) also shows the time course of IPP incorporation by purified *F. elastica* rubber particles in the presence of 1 mM IPP and 20 μM FPP as an initiator (●).
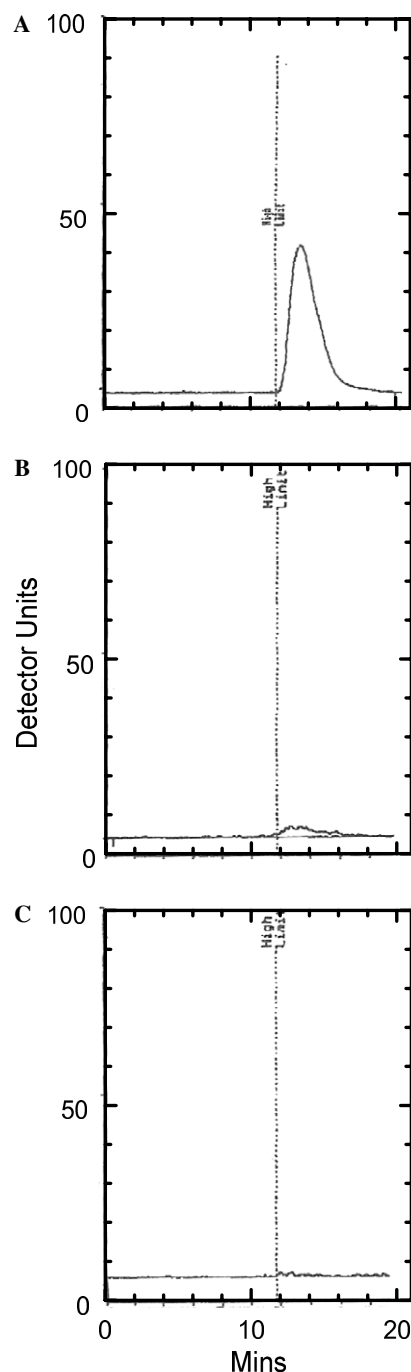
Fig. 4. Gel permeation chromatographs of (A) unlabeled rubber extracted from purified rubber particles, (B) ^{14}C-IPP labeled rubber synthesized in the presence of both IPP and FPP and (C) ^{14}C-IPP labeled rubber synthesized in IPP alone. The large amounts of unincorporated ^{14}C-IPP in samples (B) and (C) came through the detector between 22 and 26 min and are not shown on these chromatograms.

Table 2
Molecular weight parameters for rubber previously synthesized in vivo by purified *F. elastica* rubber particles and that newly synthesized in vitro in the presence of ^{14}C-IPP and FPP (samples (A) and (B) in Fig. 4, respectively)

Parameter	(A) In vivo rubber	(B) In vitro ^{14}C-rubber
Peak area	1,139,000	32,280
Polydispersity	2.21	3.33
Z-average molecular weight (M_z)	586,600	1,919,000
Molecular weight of peak maximum (M_p)	413,500	1,034,000
Weight average molecular weight (M_w)	401,000	745,700
Number average molecular weight (M_n)	131,100	223,800

analysis (Table 2) suggests that the newly synthesized rubber (in the presence of FPP) may be of higher molecular weight than the rubber produced before harvest, this is likely an artifact of the much smaller peak available for analysis and the higher polydispersity of polymer analyzed.

Effect of EDTA on RuT activity

To study the effect of metal cations on RuT activity, it was first necessary to determine the minimum amount of EDTA needed to chelate the cations associated with the rubber particle preparations in order to eliminate all RuT activity (Fig. 5). If this is not done, the pre-existing Mg^{2+} pool is sufficient to support an activity rate near the maximum, which then prevents kinetic characterization [16,20]. The ^{14}C-IPP incorporation rate was completely inhibited by 6 mM EDTA. To allow for variations in rubber particle preparations, an excess of EDTA (20 mM) was used to eliminate initial RuT activity in subsequent experiments.
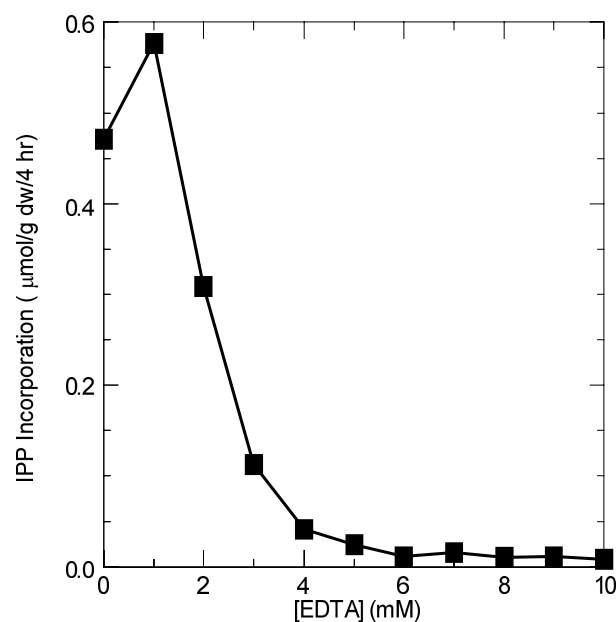
rubber was detected in the presence of FPP (cf. Figs. 4A and B, Table 2), the much smaller amount of IPP incorporated in the absence of initiator was around the detection limits and only suggestive of a similar polymer size (Fig. 4C). The radiolabeled rubber cannot be purified away from the large pool of pre-existing polymer, which prevents further concentration. Although the chromatographic

Fig. 5. Reduction of rubber transferase activity by EDTA in *F. elastica* enzymatically active (light) washed rubber particles. Incorporation of ^{14}C IPP was determined in the presence of 20 μM FPP, 200 μM IPP, and 1.25 mM MgSO$_4$ in the presence of different concentrations of EDTA.

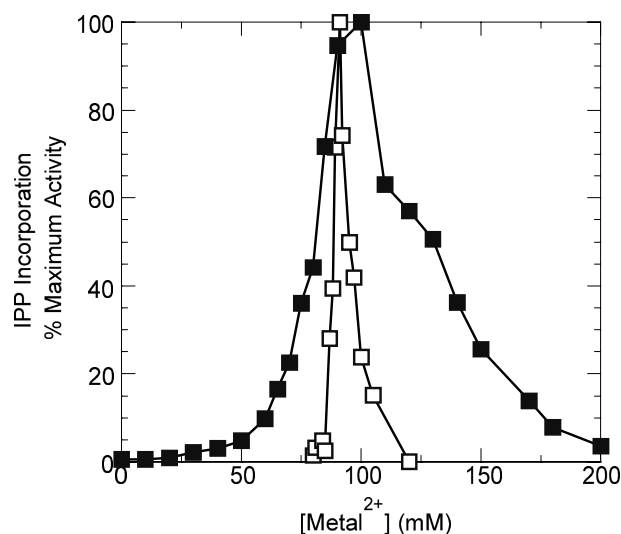Fig. 6. Concentration dependencies of magnesium and manganese cations on [14C] IPP incorporation by rubber transferase in enzymatically active purified rubber particles from *F. elastica* pretreated with 20 mM EDTA. IPP incorporation was measured in the presence of 20 μM FPP, 200 μM IPP, and for both metals [manganese, □ and magnesium, ■].

Fig. 7. [14C]-IPP Incorporation rate by enzymatically active purified rubber particles from *F. elastica*. Concentrations of the different compounds used in the reaction are 20 μM FPP, 200 μM IPP, and 20 mM EDTA.

Magnesium and manganese dependence on IPP incorporation rate

The concentration dependence of Mg^{2+} on RuT activity was determined (Fig. 6). The activity curve showed a sharp maximum at an $[A]_{max}$ (defined as the concentration of Mg^{2+} cofactor needed to achieve V_{max} [16]) of 100 mM. RuT activity was inhibited by Mg^{2+} concentrations above 100 mM.

The concentration dependence of Mn^{2+} on RuT activity was similar to that seen for Mg^{2+} (Fig. 6). In both Mg^{2+} and Mn^{2+}, RuT was activated until $[A]_{max}$ was reached, after which the activity was inhibited by additional metal. However, although both metals produced a similar A_{max} (100 mM for Mg^{2+} and 91 mM for Mn^{2+}), the Mn^{2+} plot had a narrower concentration range.

Activity assay of F. elastica purified light rubber particles under varying conditions

In the presence of both initiator and monomer, the incorporation rate of *F. elastica* washed rubber particles is high (Fig. 7), but is completely inhibited by the addition of EDTA. However, as seen earlier in this species [7], a measurable amount of IPP incorporation occurred in the absence of added initiator. This activity was also completely inhibited by EDTA, indicating the presence of existing rubber polymers associated with the RuT being elongated by the addition of IPP. Thus, it is possible to use *F. elastica* rubber particles to independently study both initiator-dependent polymerization, where FPP initiates the synthesis of new rubber molecules from IPP, and the condensation/polymerization reaction alone where rubber chains already exist and are further elongated in the presence of IPP monomer but without requiring initiation.
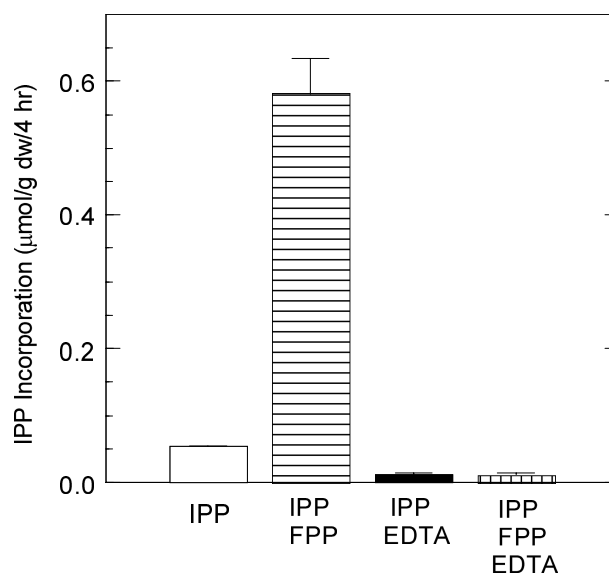
Initiator-dependent polymerization versus initiator-independent polymerization

The initiator-dependent polymerization and initiator-independent polymerization reactions were examined independently to determine the associated kinetic parameter values. The initiator-dependent reactions were examined where IPP and FPP were added in the presence of 100 mM Mg^{2+}, the $[A_{max}^{Mg^{2+}}]$ for the RuT enzyme (Fig. 8). The dependence of IPP concentration on initiator-dependent IPP incorporation into rubber (Fig. 8A) and the K_m^{IPP}, the concentration of IPP required to reach 1/2 maximum velocity ($1/2V_{max}$), were determined by plotting direct linear plots, in which velocity is plotted versus the −[IPP] for single data points (Fig. 8B). The intersection of the lines indicates the K_m^{IPP} value on the x-axis and the V_{max} value on the y-axis [21]. For this particular reaction, the K_m^{IPP} was 15.8 mM, and the V_{max} was 1.5 μmol/g dw/4 h.

Initiator-independent polymerization also was examined in the presence of 100 mM Mg^{2+}. Incorporation of 14C-IPP was dependent upon the concentration of IPP (Fig. 9), yielding a K_m^{IPP} of 0.603 mM and a V_{max} of 0.049 μmol/g dw/4 h.

To further examine the kinetics of the initiator-dependent and initiator-independent reactions, we tested the effect on IPP incorporation of the different $[Mg^{2+}]$ flanking $[A_{max}^{Mg^{2+}}]$, namely 75 mM (activating concentration) and 130 mM (inhibiting concentration) (see Fig. 6). A complete listing of all K_m and V_{max} values for the six combinations are listed in Table 3. In both reactions, the K_m^{IPP} is lowest at 100 mM Mg^{2+}. At this $[Mg^{2+}]$, the affinity of the RuT enzyme to IPP is greatest: the lower the K_m, the greater the binding affinity and the higher the IPP incorporation rate. Flanking $[Mg^{2+}]$s on either side of 100 mM result in

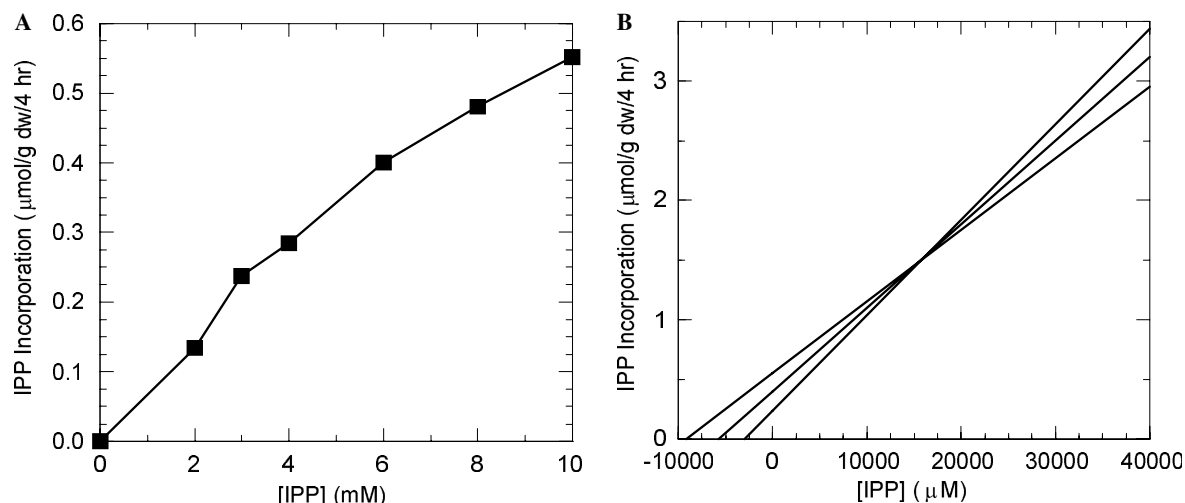Fig. 8. IPP concentration dependence of [^{14}C]-IPP-incorporation by RuT in *F. elastica* purified rubber particles pretreated with 20 mM EDTA to study the initiator-dependent reaction. Reaction carried out in the presence of 20 μM FPP and 100 mM Mg^{2+}. (A) Velocity versus [IPP]. (B) Direct linear plot of v − [IPP] where x-intercept of intersecting point denotes K_m and y-intercept of intersecting point denotes V_{max}.
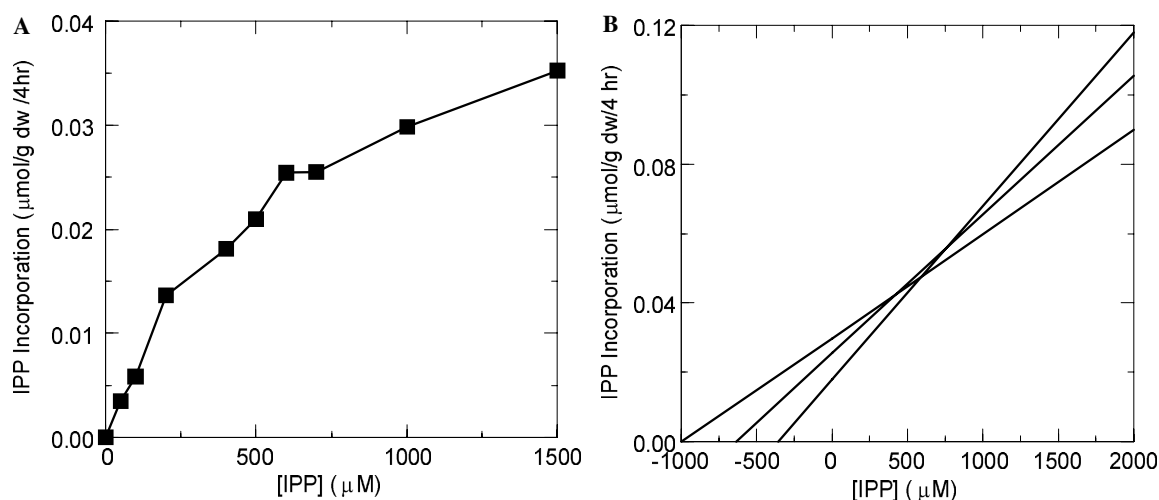

Fig. 9. IPP concentration dependence of [^{14}C]-IPP-incorporation by RuT in *F. elastica* purified rubber particles pretreated with 20 mM EDTA to study the initiator-independent reaction. Reaction carried out in the presence of 100 mM Mg^{2+}. (A) Velocity versus [IPP]. (B) Direct linear plot of v − [IPP] where x-intercept of intersecting point denotes K_m and y-intercept of intersecting point denotes V_{max}.

an increase in the K_m^{IPP} and a subsequent decrease in the IPP incorporation rate.

The K_m^{FPP} value for initiator-dependent IPP incorporation was determined in 50, 75, 100, and 130 mM Mg^{2+} at a non-limiting concentration of IPP (1000 μM). A Woolf–Augustinsson–Hofstee plot [21] of the velocity as a function of [FPP] in 100 mM Mg^{2+} yielded a V_{max} of 0.056 μmol/g dw/4 h and a K_m^{FPP} of 0.070 μM (Fig. 10). As with K_m^{IPP}, the K_m^{FPP} was greatest at 100 mM Mg^{2+}; flanking Mg^{2+} concentrations resulted in higher K_m^{FPP}s and lower IPP incorporation rates. The $K_m^{IPP,FPP}$ and IPP incorporation rates are very sensitive to [Mg^{2+}] (Table 3). Also, it is clear that the *F. elastica* RuT has a greater affinity for FPP than for IPP under either initiator-dependent or initiator-independent conditions.

Discussion

Ficus elastica, like many rubber-producing species, produces its rubber within a living cytoplasm. Thus, other substrates and enzymes exist in the latex that may affect perceived rubber transferase activity and a system of sufficient purity must be established. For example, IPP isomerase is a soluble enzyme that rapidly interconverts IPP (the rubber monomer) and DMAPP (a rubber initiator). Isomerase contamination in a rubber particle preparation would lead to the synthesis of DMAPP, and then other allylic diphosphates, such as FPP, might be synthesized from contaminating prenyl transferase enzymes [11]. Because the allylic diphosphate binding constants are all well below the IPP ones (Table 1), such cytoplasmic contamination

Table 3
Kinetic constants at various $[Mg^{2+}]$ for the initiator-dependent reaction, initiator-independent reaction, and initiator dependence reaction for rubber transferase in *F. elastica* purified rubber particles

Polymerization reaction	Substrates present[a]	Mg^{2+} (mM)	$K_m^{IPP,FPP}$ (mM, µM)	V_{max} (µmol/g dw/4 h)
Initiator-dependent	[IPP], FPP	75	17.5	2.20
		100	15.8	1.50
		130	23.0	1.25
Initiator-independent	[IPP]	75	1.9	0.11
		100	0.603	0.049
		130	1.52	0.055
Initiator dependence	[FPP], IPP	50	0.257	0.225
		75	0.075	0.097
		100	0.070	0.056
		130	0.158	0.107

[a] [ ] denotes variation in substrate concentration.

of the rubber particles would greatly perturb the results. That these effects do occur in *F. elastica* was clearly demonstrated when the effect of washing on IPP incorporation in the presence of non-limiting DMAPP was assayed (Fig. 1). However, the washing experiment and the use of the isomerase inhibitor, 3,4-oxido-3-methyl-1-butyl diphosphate (OMBPP) [17] (Fig. 2), indicate that the IPP incorporated enzymatically by the thoroughly washed rubber particles was performed by a rubber particle-bound enzyme.

It also has been demonstrated that RuT is the only IPP-utilizing enzyme that can be detected on purified rubber particles of *F elastica*, *H. brasiliensis*, and *P. argentatum* [3,4]. Furthermore, the coincidence of the temperature dependence of the two reactions is suggestive of the same enzyme being employed by both. Thus, it seems most probable that the initiator-independent IPP-incorporation reaction (which does still require Mg^{2+}) is caused by the elongation of partially formed rubber polymers that are

not released from the active site during the purification process. Unfortunately, our attempts to attempts to assess the molecular weight of the product of the initiator-independent IPP-incorporation reaction were thwarted by the sensitivity limits of the GPC method (Fig. 4). However, the product of the IPP reaction remains an integral part of the rubber particle and cannot be washed away. Thus, it seems that a hydrophobic product has been made and transferred to the particle interior. In addition, initiator-dependent IPP polymerization continues for many hours, but the initiator-independent form stops within 2 h (Fig. 3), even though IPP was non-limiting, suggesting that the polymers have grown to a maximum size. At this stage they may either have released from the active site or simply remain in place awaiting a chain transfer agent in the form of an allylic diphosphate initiator. In either case, re-initiation is required before further polymerization from IPP is possible.

We also point out that because we were not able to characterize the product of the initiator-independent IPP-incorporation reaction, it does remain possible, although we believe unlikely, than a rubber particle-bound isomerase pool may exist which could account for our results, producing an allylic-PP initiator from the IPP which is then bound by the rubber transferase permitting subsequent polymerization. If this is the case, such an isomerase would be highly unusual in that it would be tightly bound to the rubber-particles, rather than soluble, and would also be a form insensitive to the isomerase inhibitor.

However, despite many other inter-specific similarities, the rubber-producing species *H. brasiliensis* and *P. argentatum* do not evince an initiator-independent polymerization of IPP into rubber in their enzymatically active purified rubber particles. It seems likely that *F. elastica* rubber transferase exhibits both the normal initiator-dependent incorporation of IPP into newly synthesized rubber
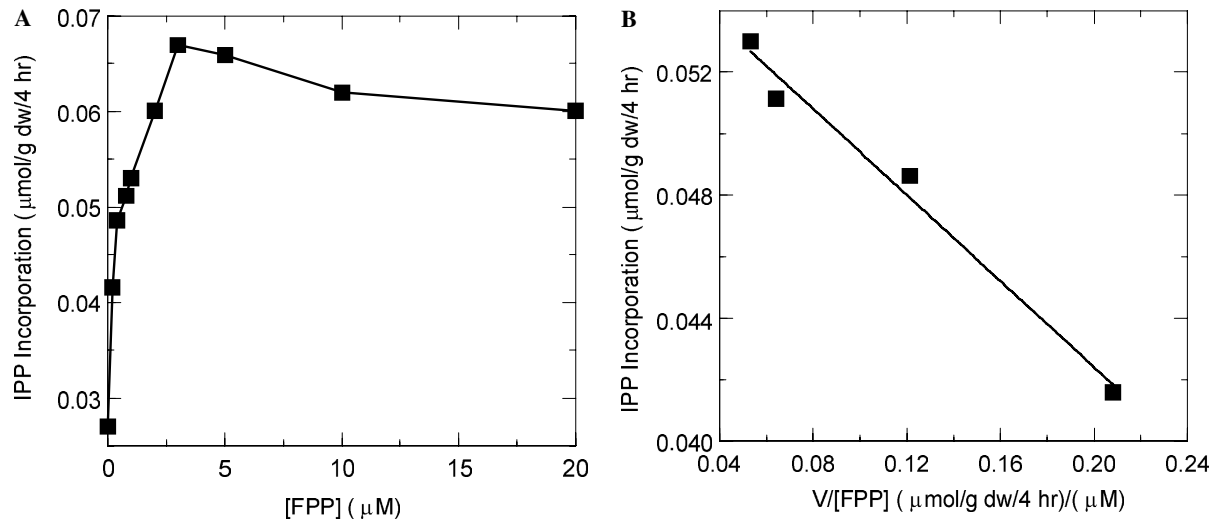

Fig. 10. FPP concentration dependence of [^{14}C]-IPP-incorporation by RuT in *F. elastica* purified rubber particles pretreated with 20 mM EDTA. Reaction carried out in the presence of 1000 µM IPP and 100 mM Mg^{2+}. (A) Velocity versus [FPP]. (B) Eadie–Hofstee plot where extrapolation of line to *y*-axis denotes V_{max} and slope of line yields K_m.

and the so far unique initiator-independent IPP incorporation because of differences in rubber particle architecture among species. The membrane of the *F. elastica* rubber particle is rigid, showing none of the flexibility seen in *H. brasiliensis* and *P. argentatum* particles [14]. This is probably due to the prevalence of long chain, waxy fatty acids in the rubber particle membrane of this species [15]. The rigid membrane may allow the partially formed rubber molecules to be held in place in the RuT active site during purification, whereas they are released from RuTs positioned in flexible rubber particle membranes as found in *H. brasiliensis* and *P. argentatum* [14], which have non-waxy fatty acid profiles [15].

As seen before, when initiator-dependent polymerization has been investigated in rubber transferases from different species, the K_m^{FPP} for the *F. elastica* RuT is two orders of magnitude, or more, smaller than the K_m^{IPP} ([4], Tables 1 and 3). However, in this study, the K_m^{IPP} for the initiator-independent polymerization indicates that the *F. elastica* RuT has a considerably higher affinity for IPP than previously measured for the initiator-dependent reaction (9–26× greater depending upon the [Mg^{2+}]).

Although different [Mg^{2+}]s were used, the large difference between the K_m^{IPP} reported here (Table 3) and those reported previously for the initiator-dependent reaction (e.g., Table 1) may result from competition between FPP and IPP for the IPP binding site, which would impede the binding of IPP and the subsequent condensation reaction, and lead to the higher observed K_m^{IPP}. This seems probable because this phenomenon also is observed in this study between the K_m^{IPP} for initiator-dependent polymerization and the K_m^{IPP} for initiator-independent polymerization where [Mg^{2+}]s were controlled (Table 3). Although such competition has not been established for the *F. elastica* RuT, competition between various natural and synthetic substrates and IPP for the IPP binding site has been reported in *H. brasiliensis* and *P. argentatum* [3,5,22]. In the initiator-independent reaction, the allylic diphosphate reactive end of the elongating rubber molecule is positioned in the active site, almost certainly in the allylic diphosphate initiator binding site, but is not fully released from the active site during each condensation (polymerization) reaction. This may protect the adjacent IPP binding site from access and inhibition by FPP. Alternatively, the higher RuT IPP affinity in the initiator-independent reaction may reflect competition between IPP and FPP at the allylic diphosphate binding site when that site does not have an elongating rubber molecule in place (i.e., the initiator-dependent reaction).

In all three reactions studied, the V_{max} and the K_m of the substrate is affected by [Mg^{2+}] (Table 3), with the affinity of the enzyme for the substrate being greatest at 100 mM Mg^{2+}, the concentration at which maximum IPP incorporation rates are seen (Fig. 8). At concentrations below A_{max}, as Mg^{2+} increases, approaching the A_{max} of 100 mM, the metal binds to IPP present in solution, forming the IPP·Mg complex that is the true monomeric substrate for rubber biosynthesis [23]. As its concentration increases, there is an increased interaction with RuT and higher IPP incorporation rates. However, at concentrations above $A_{max}^{Mg^{2+}}$, there is an excess of Mg^{2+} compared to IPP molecules in solution. The excess metal ions may inhibit the incorporation of IPP by blocking IPP molecules from entering the RuT active site and subsequently polymerizing rubber chains. This is similar to what has been reported for the rubber-producing species *H. brasiliensis* [23] and *P. argentatum* [24]. Also, it is possible that the metal alone binds to the RuT, changing its conformation so that IPP or IPP·Mg cannot bind. Thus, when Mg^{2+} is in excess, increasing [IPP] will lead to an increase in IPP incorporation rates, supporting the conclusion that excess Mg^{2+} alone interacts with RuT, increasing K_m^{IPP} and inhibiting rubber production.

The similarities and differences between the kinetic properties of the RuT of *F. elastica*, reported here, and those of *H. brasiliensis* and *P. argentatum* with respect to $A_{max}^{Mg^{2+}}$, are very interesting; the *F. elastica* $A_{max}^{Mg^{2+}}$ is nearly an order of magnitude greater than the $A_{max}^{Mg^{2+}}$s of the other two species, and the endogenous latex [Mg^{2+}] appears to closely match the $A_{max}^{Mg^{2+}}$s [16]. It is believed that K_ms reflect the substrate concentration at which the RuT operates most effectively [21]. Thus, the RuT K_m^{IPP} and K_m^{FPP} for the initiator-independent reactions and initiator-dependent reactions are the conditions for which the RuT is most tightly regulated at a specified [Mg^{2+}] and the enzymes have evolved in concert with their endogenous magnesium concentration. As for the initiator-dependent reaction in *H. brasiliensis* and *P. argentatum*, the *F. elastica* K_m^{IPP} for both initiator-dependent and independent reactions are much larger than the K_m^{FPP}, supporting the interpretation that rubber is made at times when IPP is non-limiting for all other prenyl transferases. These enzymes catalyze vital biochemical reactions, all of which have much lower K_m^{IPP}s similar to that of K_m^{FPP} [3]. The only exception known to this last statement is the K_m^{FPP} for the *P. argentatum* RuT, which is extremely low (<0.01 μM) and likely reflects the parenchymatous (rather than laticiferous) location of rubber biosynthesis is this species [25]. In this species, FPP substrate limitations are prevented by negative cooperativity, which inhibits the chain transfer reaction and, by this means, prevents FPP being used up to make a pool of low molecular weight rubber [25]. Nonetheless, the overall biochemical similarity among the three species, including that initiation and polymerization both operate most efficiently at the same species-specific $A_{max}^{Mg^{2+}}$, leads these authors to speculate that rubber biosynthesis may have evolved early on in the Angiosperms, and that a considerable conservation of rubber biosynthetic mechanisms was maintained as the three species diverged phyogenetically (these species are each in a different Superorder of the Dicotyledonae).

Data also showed that Mn^{2+}, though present only in trace amounts in *F. elastica* latex [16], can be used in place of Mg^{2+} in the biosynthesis of natural rubber, as is the case in other rubber-producing species [16,23,24].

However, Mn^{2+} has a narrower concentration range and is more deactivating than Mg^{2+} (Fig. 8). Unlike the gradual increase in activity seen as the $[Mg^{2+}]$ increased, an abundance of Mn^{2+} was necessary to obtain measurable $[^{14}C]$-IPP incorporation. Rather than any difference between the ways the rubber transferase handles the two cofactors, this may be due to non-enzymatic binding of Mn^{2+} to the rubber particle surface, which has been observed with electron paramagnetic resonance spectroscopy by John J. Windle and Katrina Cornish (unpublished data). It seems possible that free Mn^{2+} only becomes available to activate the RuT after the particle surface is saturated with Mn^{2+}.

Like Mg^{2+}, the incorporation of $[^{14}C]$-IPP was inhibited by additional Mn^{2+} once the optimal cation concentration A_{max}^{metal} was reached. Thus, excess cofactor has a negative impact on rubber production regardless of the cofactor used.

Conclusions

The presence of initiator-independent incorporation of IPP by enzymatically active rubber particles purified from *F. elastica* led to the conclusion that preexisting, partially formed rubber chains are present in *F. elastica* purified rubber particles, which are further elongated in vitro upon the addition of IPP. FPP appears to serve as both the chain transfer agent and the re-initiation agent. Separate kinetic characterization of the IPP incorporation reactions showed that K_m^{IPP} is lower for the initiator-independent reaction than for initiator-dependent IPP polymerization probably because of competition between the substrates for the opposite binding site.

Metal cofactors, such as Mg^{2+}, affect the production of natural rubber in vitro; the initiation rate, the IPP incorporation rate, and both the K_m^{FPP} and K_m^{IPP} are affected by the concentration of cations associated with the RuT. At the optimal $[Mg^{2+}]$ for maximum rubber synthesis in *F. elastica* ($A_{max}^{Mg^{2+}} = 100$ mM), the K_m^{IPP} is at a minimum for both the initiator-dependent and initiation-independent IPP condensation reactions, and the K_m^{FPP} is at a minimum for the initiator-dependent IPP condensation reaction, indicating that this $[Mg^{2+}]$ results in the highest affinity of both substrates to bind to RuT. For all Mg^{2+} concentrations studied, the $K_m^{FPP} < K_{m\ initiator\text{-}independent}^{IPP} < K_{m\ initiator\text{-}independent}^{IPP}$.

References

[1] J. d'Auzac, J.-L. Jacob, H. Chrestin, Physiology of Rubber Tree Latex, CRC Press, Boca Raton, Florida, 1989.
[2] W. Davis, Fortune 4 (1997) 86–95.
[3] K. Cornish, Phytochemistry 57 (2001) 1123–1134.
[4] K. Cornish, Nat. Prod. Rep. 18 (2001) 182–227.
[5] K. Cornish, J. Castillón, D.J. Scott, Biomacromol. 1 (2000) 632–641.
[6] D.J. Siler, K. Cornish, R.G. Hamilton, J. Allergy Clin. Immunol. 98 (1996) 895–902.
[7] K. Cornish, D.J. Siler, Plant Physiol. Biochem. 34 (1996) 377–384.
[8] G. Heinrich, Protoplasma 70 (1970) 317–323.
[9] K. Cornish, R.A. Backhaus, Phytochemistry 29 (1990) 3809–3813.
[10] B.L. Archer, B.G. Audley, Bot. J. Linn. Soc. 94 (1987) 181–196.
[11] K. Cornish, Eur. J. Biochem. 218 (1993) 267–271.
[12] F. Lynen, J. Rubb. Res. Inst. Malaya 21 (1969) 389–406.
[13] K. Cornish, D.F. Wood, J.J. Windle, Planta 210 (1999) 85–96.
[14] D.F. Wood, K. Cornish, Int. J. Plant Sci. 161 (2000) 435–445.
[15] D.J. Siler, M. Goodrich-Tanrikulu, K. Cornish, A.E. Stafford, T.A. McKeon, Plant Physiol. Biochem. 35 (1997) 881–889.
[16] D.J. Scott, B.M.T. da Costa, S.C. Espy, J.D. Keasling, K. Cornish, Phytochemistry 64 (2003) 123–134.
[17] M. Muehlbacher, C.D. Poulter, Biochem. 27 (1988) 7315–7328.
[18] K. Cornish, D.L. Bartlett, Phytochem. Anal. 8 (1997) 130–134.
[19] C.J.D. Mau, D.J. Scott, K. Cornish, Phytochem. Anal. 11 (2000) 356–361.
[20] H. Kang, M.Y. Kang, K.-H. Han, Plant Physiol. 123 (2000) 1133–1142.
[21] I.H. Segel, Enzyme Kinetics, John Wiley & Sons, Inc., NY, New York, 1993.
[22] C.J.D. Mau, S. Garneau, A.A. Scholte, J.C. Vederas, K. Cornish, Eur. J. Biochem. 270 (2003) 3939–3945.
[23] B.M.T. da Costa, J.D. Keasling, K. Cornish, Biomacromol. 6 (2004) 279–289.
[24] B.M.T. da Costa, J.D. Keasling, K. Cornish, Proceedings of the International Conference of the Association for the Advancement of Industrial Crops, Murcia, Spain, September 17–22, 2004.
[25] K. Cornish, D.J. Scott, Ind. Crops Prod. 22 (2005) 49–58.