

Temporal analysis of natural rubber transferases reveals intrinsic distinctions for *in vitro* synthesis in two rubber-producing species

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ABSTRACT

The temporal relationship of rubber molecule initiation, polymerization and termination, as affected by limited, optimal, and non-limiting initiator concentrations, and in optimal and or non-limiting isopentenyl pyrophosphate (IPP) monomer concentrations, was investigated *in vitro* using enzymatically active rubber particles purified from *Hevea brasiliensis* and *Parthenium argentatum*. Polymer initiation occurred at the beginning of the experiments and reinitiation in excess farnesyl pyrophosphate (FPP) occurred more quickly in *P. argentatum* than *H. brasiliensis*. The number of FPP binding sites (six per rubber transferase complex, RT-ase), and thus the number of RT-ase complexes, was similar in both *P. argentatum* and *H. brasiliensis* per gram of rubber particles. Since the RT-ase complexes are bound to the rubber particle surface, the difference in mean rubber particle size, and the abundance of small particles in *H. brasiliensis* washed rubber particles (WRP) implies at least double the surface density of active RT-ases in *H. brasiliensis* WRP than in *P. argentatum* WRP in these samples. The rate of rubber biosynthesis was approximately linear with time in *H. brasiliensis*, but in *P. argentatum* IPP incorporation slowed between 1.5 and 4 h, under some conditions, and

then generally accelerated between 4 and 8 h to even higher rates. Under most conditions, it took between 1.5 h and 4 h to produce mature rubber polymers. The chain transfer reaction of both RT-ase's was accelerated by excess initiator resulting in much lower molecular weight polymers than typically extracted from living plants. However, in both species, rubber molecules kept growing when synthesized in limited initiator concentrations even at non-saturating monomer concentrations. The *P. argentatum* RT-ase was able to make higher molecular weight rubber than the *H. brasiliensis* RT-ase, and under extremely limited FPP (0.001 μ M), rubber molecular weights above 50 Mg/mol were achieved. However, in addition to primary biochemical substrate concentration effects on rubber molecular weight and, thus termination, spatial constraints appear to be a factor in the as yet undefined rubber polymer termination mechanism.

KEYWORDS: *Hevea brasiliensis*, *Parthenium argentatum*, guayule, natural rubber, rubber particles, rubber transferase activity *in vitro*.

INTRODUCTION

Rubber is a critical raw material in today's economy and 13.8 million tons of natural rubber (NR) were produced and consumed in 2019 (Association of Natural Rubber Producing Countries).

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NR (*cis*-1,4-polyisoprene) biosynthesis is catalyzed by a rubber particle-bound rubber transferase complex (RT-ase) (EC.2.5.1.20) and requires an allylic pyrophosphate (APP) initiator molecule, a monomer (isopentenyl pyrophosphate, IPP) from which the polymer is synthesized, and a divalent cation activator [1]. *In vivo*, the initiator is usually farnesyl pyrophosphate (FPP) [2] and Mg^{2+} is the activator [3, 4]. The concentration and ratio of APP, IPP and Mg^{2+} affect the reaction rate and the mature molecular weight of the polymer produced [1, 5, 6, 7]. It has previously been shown that molecular weights can be radically altered *in vivo* [8, 9] and while the molecular weight of rubber *in vivo* is generally species-specific, there can be some inter-genotype variation. Rubber polymer size in laticiferous species, such as the para rubber tree (*Hevea brasiliensis*, Müll. Arg.), and the Indian rubber tree (*Ficus elastica*, Roxb.), appears to be primarily determined by the relative concentrations of APP, IPP and Mg^{2+} in the laticifer cytosol [5, 6, 10]. In contrast, guayule (*Parthenium argentatum*, Gray) produces rubber in bark parenchyma cells, and, in addition to substrate and co-factor controls, its RT-ase exhibits negative cooperativity for initiator (FPP) binding over a wide concentration range [1, 11]; i.e. initiation of one active site in the multimeric rubber transferase complex inhibits the chain transfer reaction making it more difficult for subsequent initiations at the same site. This suggests an additional level of control over polymerization rate and rubber polymer size *in vitro*. Outside of this negative cooperativity range (above 2 μ M) guayule rubber polymer size is likewise determined by the relative concentration of substrates and Mg^{2+} cofactor/activator.

However, previous *in vitro* studies were usually carried out at a single incubation time and there is very little information on temporal formation of rubber molecules. In this report, we investigate the temporal relationship of rubber biosynthesis initiation, polymerization, and termination in purified, enzymatically active rubber particles from *P. argentatum* and *H. brasiliensis*, as affected by limited, optimal and non-limiting initiator concentrations in either optimal or non-limiting IPP monomer concentrations. This report also explores the upper molecular weight limits of rubber synthesized by rubber particles of *H. brasiliensis* and *P. argentatum*, *in vitro*.

MATERIALS AND METHODS

Plant materials

Parthenium argentatum (line 11591) plants were grown at the USDA-ARS Arid Lands Agricultural Research Center, Maricopa, AZ, and harvested at maturity. Living latex from mature *H. brasiliensis* line PB260 was donated by the Rubber Research Institute of India and was stored in buffer as previously described [12].

Chemicals

Unlabeled IPP and FPP were obtained from Echelon Biosciences Incorporated (Salt Lake City, UT, USA), [^{14}C]IPP (55 mCi/mmol) and [3H]FPP (60 Ci/mmol) were obtained from American Radiolabeled Chemical Inc., St. Louis, MO. Ready Safe scintillation fluid was purchased from Beckman Instruments, Fullerton, CA, USA. All other chemicals, unless noted otherwise, were purchased from Sigma (St. Louis, MO, USA).

Purified rubber particles

Enzymatically active washed rubber particles (WRP) were purified, as described, from *H. brasiliensis* [13, 14] and *P. argentatum* [15]. It has previously been shown that this method eliminates free APP, IPP-isomerase, and prenyl transferase enzymes and prevents their potential confounding effects on the assay results [13, 15]. The purified WRP were stored at -80 °C, after being adjusted to 10% glycerol and flash frozen as droplets in liquid nitrogen [16].

Rubber transferase activity assays

Time courses

IPP and FPP incorporation rates were assayed in WRP using a modification of a previously described method [17]. The reactions took place in individual wells of a 96-well plate (MultiScreen – R1; 1 mm Hydrophilic PTFE Membrane; Glass-Filled PP Plate; Non-Sterile with Lid, Millipore, Bedford, MA, USA; catalogue number MAR1N1010). The wells were siliconized with Sigmacote (Sigma-Aldrich, Corp., St. Louis, MO, USA, #SL-2) for 2 min, rinsed with deionized water and with 95% ethanol, and dried at room temperature overnight. The 50 μ L reaction volume (100 mM Tris-HCl pH 7.5; 5 mM DTT; 8 mM $MgSO_4$; IPP, FPP, [^{14}C]IPP, and [3H]FPP as indicated) was first loaded

into each well. The rubber biosynthesis reaction was then initiated by the addition of 0.25 mg WRP into each well. The 96-well plate was then placed on a ceramic cooling plate (Amersham Biosciences, Piscataway, NJ, USA) equipped with a circulating water bath to control the temperature. RT-ase activity assays were performed at 16 °C for *P. argentatum*, and 25 °C for *H. brasiliensis*. It has previously been shown that RT-ase activity was stable and approximately linear with incubation time up to for 8 h at 25 °C in *H. brasiliensis* PB260 [13] and 16 °C in *P. argentatum* line 11591 [15] which is why these assay temperatures were selected. Reactions were stopped after 0, 0.5, 1.5, 4 and 8 h by the addition of 25 µL of 500 mM EDTA, which chelates the Mg²⁺ cofactor making it unavailable. The filters were washed using a Millipore 96-well plate vacuum manifold by successively passing a series of washes through each well to remove residual reagents: 100 µL 95% ethanol, 3 x 150 µL 95% ethanol, 2 x 150 µL 0.1% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate-hydrate (CHAPS), 2 x 150 µL 95% ethanol, 2 x 150 µL 0.1% CHAPS, 2 x 150 µL 95% ethanol, 2 x 150 µL 0.1% CHAPS and 2 x 150 µL 95% ethanol. Filter plates were oven-dried at 37 °C for 30 min, then the filters containing the rubber product were removed from the plate and placed individually into vials with 2.5 mL ScintiVerse BD Cocktail (Fisher Scientific, Santa Clara, CA, USA). The amount of [¹⁴C]-IPP and [³H]-FPP incorporated into new rubber molecules was determined by liquid scintillation spectroscopy using Beckman LS6500 (Beckman Coulter, Fullerton, CA, USA). The mean molecular weights (MW_{rubber}) were calculated based on the IPP incorporation rate (IPP Inc) and the FPP incorporation rate (FPP Inc), as shown by equation 1. All values are the mean of three replicates:

$$MW_{rubber} = \frac{IPPInc + (3 \times FPPInc)}{FPPInc} \times MW_{Isopentenyl} + MW_{PP} \quad (1)$$

where MW_{Isopentenyl} is the molecular weight of the isoprene monomer (68) derived from IPP and MW_{PP} is the molecular weight of the pyrophosphate group (176).

The binding constants for the FPP initiator are not the same for the RT-ases from *H. brasiliensis* and *P. argentatum* and so the FPP substrate concentrations used to assay RT-ase activity were chosen to reflect the K_m (where the enzyme complex should be most responsive to changes in substrate concentrations), 1/10th K_m (a severely limiting substrate concentration) and 10x or 100x K_m (a nonlimiting substrate concentration) in *H. brasiliensis* and *P. argentatum*, respectively (Table 1). The nonlimiting FPP concentrations were chosen to be the same concentration for both species and it has previously been shown that 15 µM FPP does not inhibit RT-ase activity in these species [1]. In addition, the 1/10th K_m^{FPP} in *H. brasiliensis* is the same concentration as the K_m^{FPP} in *P. argentatum* (1.5 µM FPP) which allows two direct concentration effects to be distinguished from K_m^{FPP}-related concentration effects. The K_m for IPP is very similar for both species and so IPP concentrations equivalent to K_m and 10x K_m were selected. 1/10th K_m was not included because IPP is the rubber monomer and 1/10th K_m^{IPP} would, by itself, severely limit rubber biosynthetic rate [10, 15]. Assays were performed in all combinations of these concentrations (Table 1) and were stopped by the addition of the Mg²⁺ chelator EDTA after 0.25, 0.5, 1.5, 4 and 8 h.

Table 1. Concentrations of farnesyl pyrophosphate and isopentenyl pyrophosphate tested in time courses of rubber biosynthesis by enzymatically active rubber particles purified from *Hevea brasiliensis* and *Parthenium argentatum*.

Species	FPP (µM)			IPP (µM)	
	1/10 th K _m	K _m	10 [∞] 100*x K _m	K _m	10x K _m
<i>H. brasiliensis</i>	0.15	1.5	15 [∞]	375	3,750
<i>P. argentatum</i>	0.015	0.15	15*	375	3,750

Rubber biosynthesis in multiple substrate and cofactor concentrations

An additional assay was performed to expand a previously studied interaction of FPP, IPP and Mg^{2+} cofactor on rubber initiation, polymerization and molecular weight in *P. argentatum* [6] and to determine what molecular weights could be achieved *in vitro* within the assay time of the current temporal study. The assay conditions were as described above except that EDTA was used to chelate pre-existing Mg^{2+} and then was added back as previously described [6]. These assays were performed at 16 °C for 4 h.

RESULTS AND DISCUSSION

IPP incorporation

The incorporation of IPP monomer was used to quantify the RT-ase enzyme activity. Due to the low IPP incorporation rates under limiting FPP, which makes graphical detail difficult to see, the IPP incorporation rates (Fig. 1) also were plotted as a fraction of their maximum rate (Fig. 2) so that their rate changes over time could be more clearly seen. The rate of IPP incorporation by *H. brasiliensis* and *P. argentatum* rubber particles did not decline over time (up to 8 h, Figs. 1 and 2), indicating that RT-ase activity remained stable and that changes

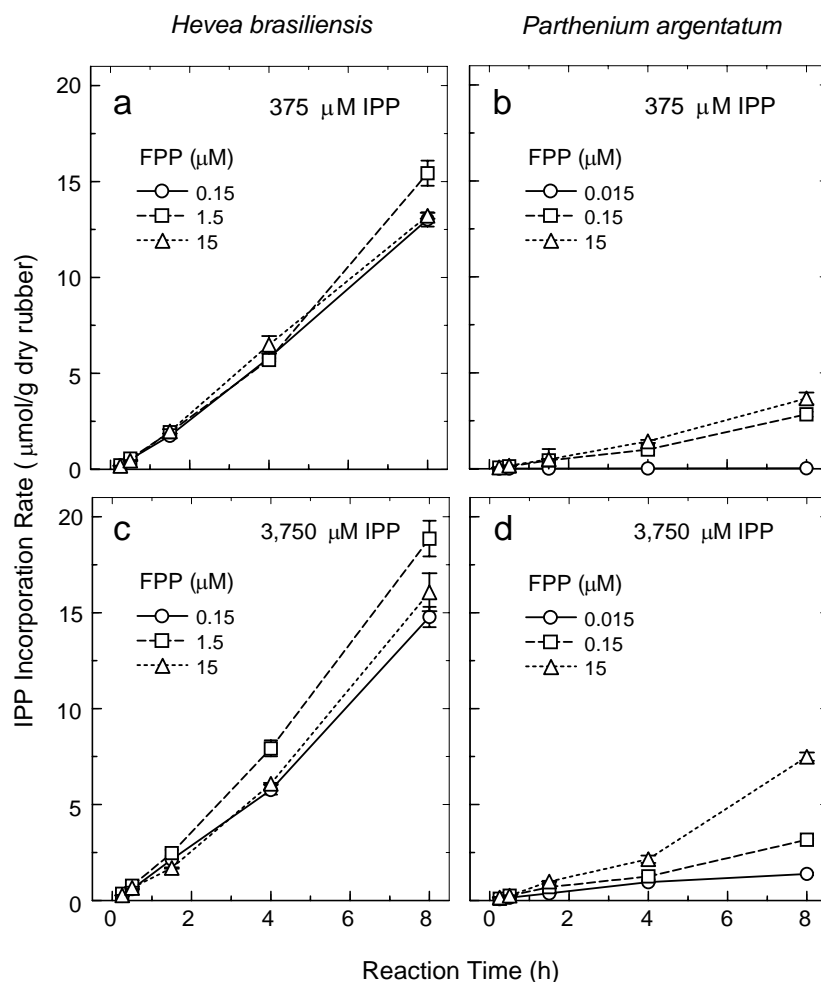


Fig. 1. Time courses of [^{14}C] isopentenyl pyrophosphate (IPP) incorporation into rubber by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10 \times K_m^{IPP}$, lower panels), and three [3H] farnesyl pyrophosphate (FPP) concentrations $10\% K_m^{FPP}$, K_m^{FPP} and nonlimiting FPP. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm$ s.e.

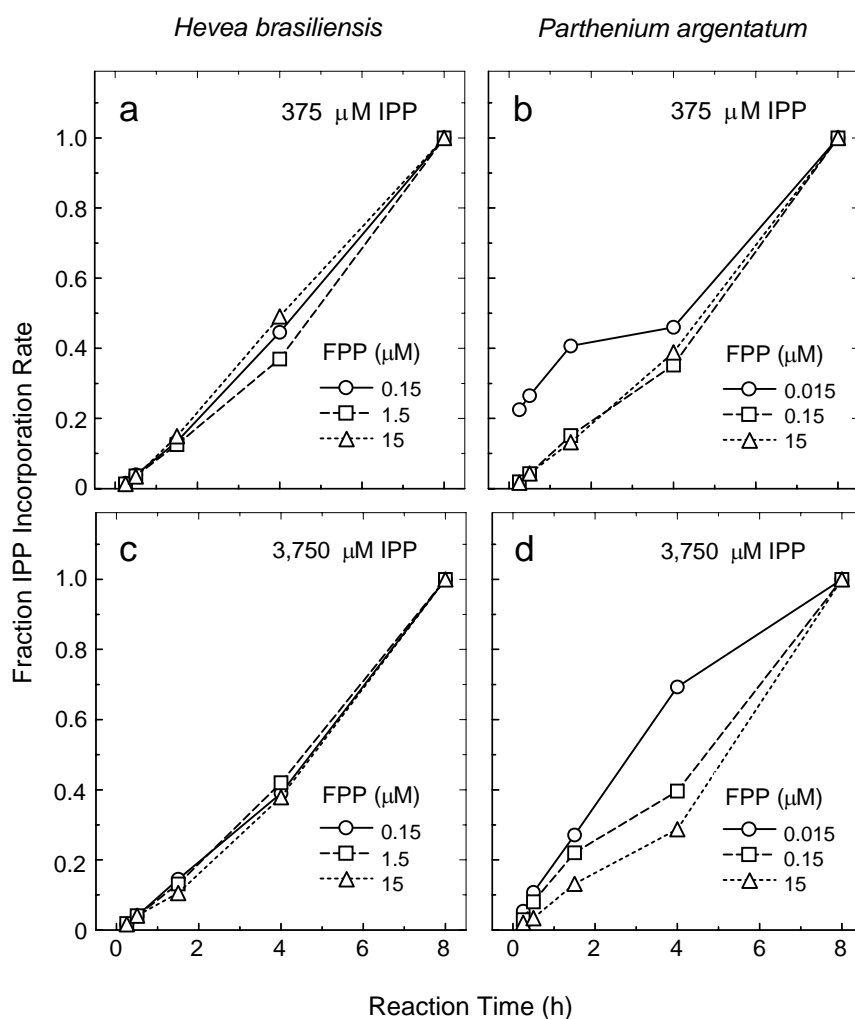


Fig. 2. Time courses of [^{14}C] isopentenyl pyrophosphate (IPP) incorporation into rubber by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10\times K_m^{\text{IPP}}$, lower panels), and three [^3H] farnesyl pyrophosphate (FPP) concentrations 10% K_m^{FPP} , K_m^{FPP} and nonlimiting FPP, expressed as a fraction of the maximum rate for each FPP and IPP concentration and species. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm \text{s.e.}$

in substrate incorporation rates and rubber molecular weight were not caused by losses in intrinsic RT-ase activity during the reaction period. Furthermore, rubber particle purification releases all rubber molecules from the RT-ases bound to the flexible particle membranes of *H. brasiliensis* and *P. argentatum* that were being elongated at the point of harvest or extraction [13, 15]. This is proved by IPP only being incorporated in the presence of initiator. This is in contrast to the RT-ases bound to the stiff rubber particle membranes of *F. elastica* which retain incomplete rubber polymers during

particle purification, and which can be further elongated *in vitro* [18]. In this system IPP is incorporated when added without additional initiator. Thus, the *in vitro* RT-ase assays in this report only quantify rubber biosynthesis in newly initiated polymers.

The RT-ase activity of the *H. brasiliensis* WRPs used here was higher than that of the *P. argentatum* WRP preparation (Fig. 1) even accounting for the assay temperature difference - the Arrhenius equation shows that enzyme activity doubles with every 10 °C increase in reaction temperature [19]. However,

activity varies by the particular preparation [20, 21] and does not directly reflect an inter-specific difference. RT-ase activity also varies according to the genotype or line [14, 15].

The two RT-ases responded differently to different substrate concentrations. For example, the IPP incorporation rate in *P. argentatum* was more sensitive to FPP and IPP concentration changes than in *H. brasiliensis* (Figs. 1 and 2). The plots of IPP incorporation rate (Fig. 1) and the accompanying fraction plots (Fig. 2) also demonstrate that the IPP incorporation rate was approximately linear over the 8 h of the reaction (Figs. 1a, b and 2a, b) in *H. brasiliensis*, which was not the case for *P. argentatum*. Moreover, unlike *P. argentatum* (Fig. 1b, d) *H. brasiliensis* WRP made similar amounts of rubber in all three FPP concentrations (Fig. 1a, c), although 15 μM FPP appeared to be slightly inhibitory. In contrast, *P. argentatum* WRP-bound RT-ase made dramatically more rubber at the highest FPP concentration (15 μM , Fig. 1b, d) than at the lower concentrations. At the lowest FPP (0.015 μM FPP) and lowest IPP (375 μM IPP) conditions, the IPP incorporation rate was not linear, decelerating between 1.5 and 4 h, and then accelerating between 4 and 8 h beyond the initial rates (Fig. 2b). Under the K_m initiator concentration (0.15 μM) and higher (15 μM), and K_m IPP, the incorporation rate was more linear but accelerated to higher incorporation rates after 4 h (Fig. 2d). This general pattern, although less pronounced, also was seen in excess IPP at 0.015 and 15 μM FPP (Fig. 2d) where rates slowed between 1.5 and 4 h and then accelerated after 4 h. These rate changes may be due to competition for the IPP and FPP binding sites as the RT-ases went through their first reinitiation event.

FPP incorporation

In both *H. brasiliensis* and *P. argentatum*, all RT-ases were immediately initiated (within the first 15 mins) in excess FPP (15 μM) (Fig. 3). This is apparent because there was a lag phase before additional FPP was incorporated, seen most clearly in the fraction plot (Fig. 4). This lag reflects the time needed for rubber molecule maturation prior to the first chain transfer reaction, i.e. the displacement of the first rubber chain made *in vitro* from the

RT-ase active site, by a new FPP molecule initiating the synthesis of a new rubber molecule by the same enzyme. A lag phase was also apparent in *H. brasiliensis* under lower initiator (1.5 μM FPP) and monomer (375 μM IPP) concentrations (Fig. 4a) even though much less FPP was actually incorporated (Fig. 3a).

However, at other nonsaturating FPP concentrations, new rubber molecules were not all initiated immediately by the RT-ase active sites in the presence of excess IPP, because no rate plateau occurred (Figs. 4c, d) probably due to competition for the binding sites between the IPP and FPP. It has previously been shown that FPP can compete with IPP for the IPP binding site [6, 10, 11], and so reciprocal competition seems likely.

In *H. brasiliensis*, at 0.15 μM FPP ($1/10^{\text{th}}$ K_m^{FPP}) in both IPP concentrations and at 1.5 μM FPP (K_m^{FPP}) in 3,750 μM IPP, initiation of all the available RT-ases did not happen immediately and the sites were filled over time (Fig. 4). A similar pattern of initiation to the *H. brasiliensis* RT-ase was observed for the *P. argentatum* RT-ase in the saturating IPP concentration of 3,750 μM , with gradual filling of initiation sites in the lower FPP concentrations and immediate saturation of RT-ase initiation sites in 15 μM FPP (Fig. 4c, d). However, the lag before reinitiation in 15 μM FPP was considerably shorter in *P. argentatum*, occurring between 0.5 and 1.5 h instead of between 1.5 and 4 h as seen in *H. brasiliensis*.

The fraction plots were generated by dividing maximum rate achieved in the experiment by actual rate at each time and substrate condition, so the lack of re-initiation in 0.015 μM FPP in *P. argentatum* (Fig. 4b) appears to reflect there being too little FPP available to trigger a chain transfer reaction in addition to FPP negative cooperativity inhibition of this reaction. However, FPP incorporation rates were very low from this concentration and this observation may reflect a detection limitation of our method.

In both species, with 15 μM FPP (Fig. 4), it appeared to take 1.5-4 h before the first chain transfer occurred, i.e. it took 1.5-4 h to make a complete rubber molecule under these substrate and assay conditions. Moreover, the initial re-initiation time in excess FPP appears to be at least twice as fast

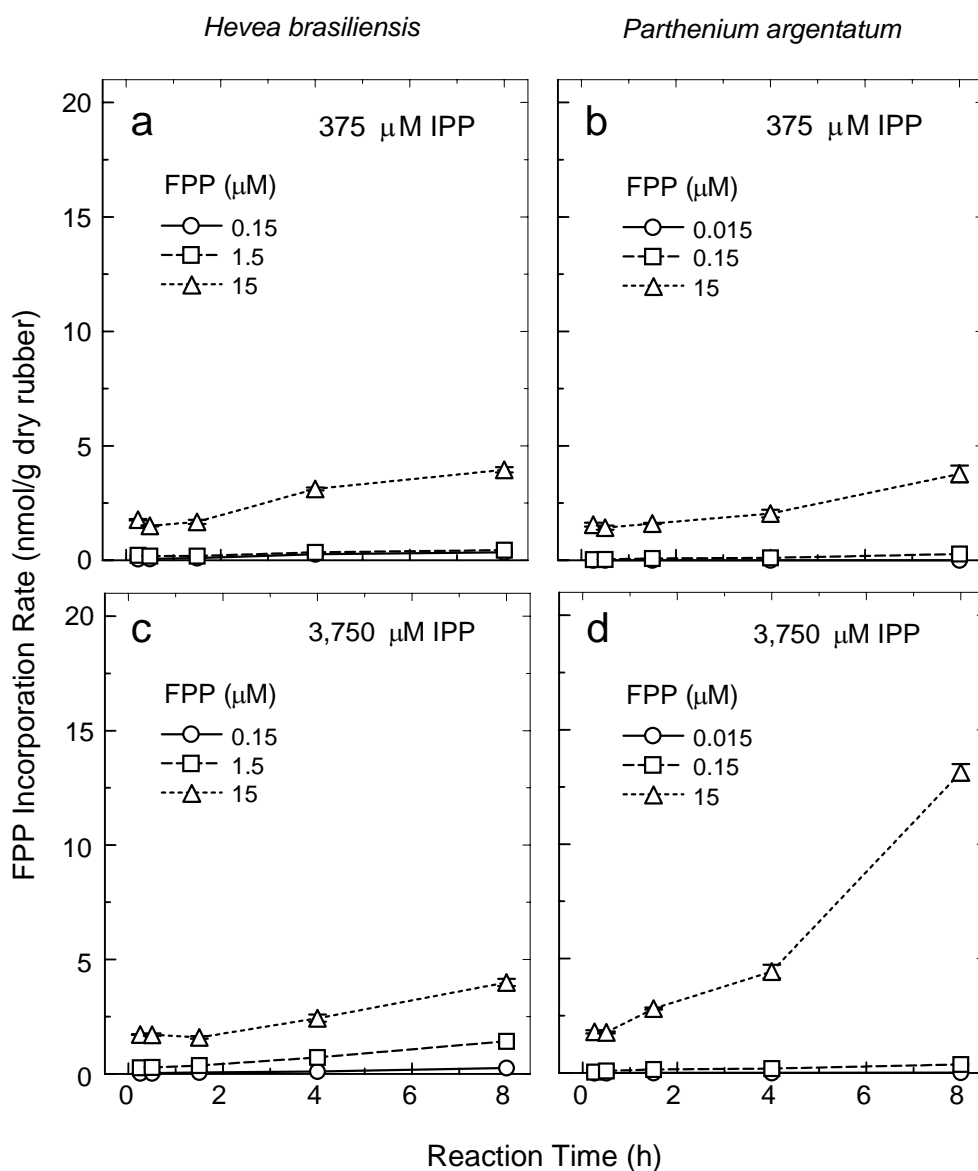


Fig. 3. Time courses of [^3H] farnesyl pyrophosphate (FPP) incorporation into rubber by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10\times K_m^{\text{IPP}}$, lower panels), and three [^3H] farnesyl pyrophosphate (FPP) concentrations 10% K_m^{FPP} , K_m^{FPP} and nonlimiting FPP. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm \text{s.e.}$

in *P. argentatum* than in *H. brasiliensis* even though the assay temperature was 9 °C lower in *P. argentatum* than *H. brasiliensis*. This suggests that, if assayed at the same temperature, the *P. argentatum* RT-ase may actually initiate rubber at four times the rate of the *H. brasiliensis* RT-ase at 375 μM IPP and eight times the rate at 3,750 μM IPP (following the Arrhenius equation).

The initial FPP incorporation rate in nonlimiting FPP (15 μM) was remarkably similar in both species at 375 and 3,750 μM IPP (~ 1.8 nmol/g dry rubber; Fig. 3) indicating that the maximum number of FPP binding sites per gram of rubber particles, and so the number of RT-ase complexes, is similar in both *P. argentatum* and *H. brasiliensis*. However, since the average size of *H. brasiliensis*

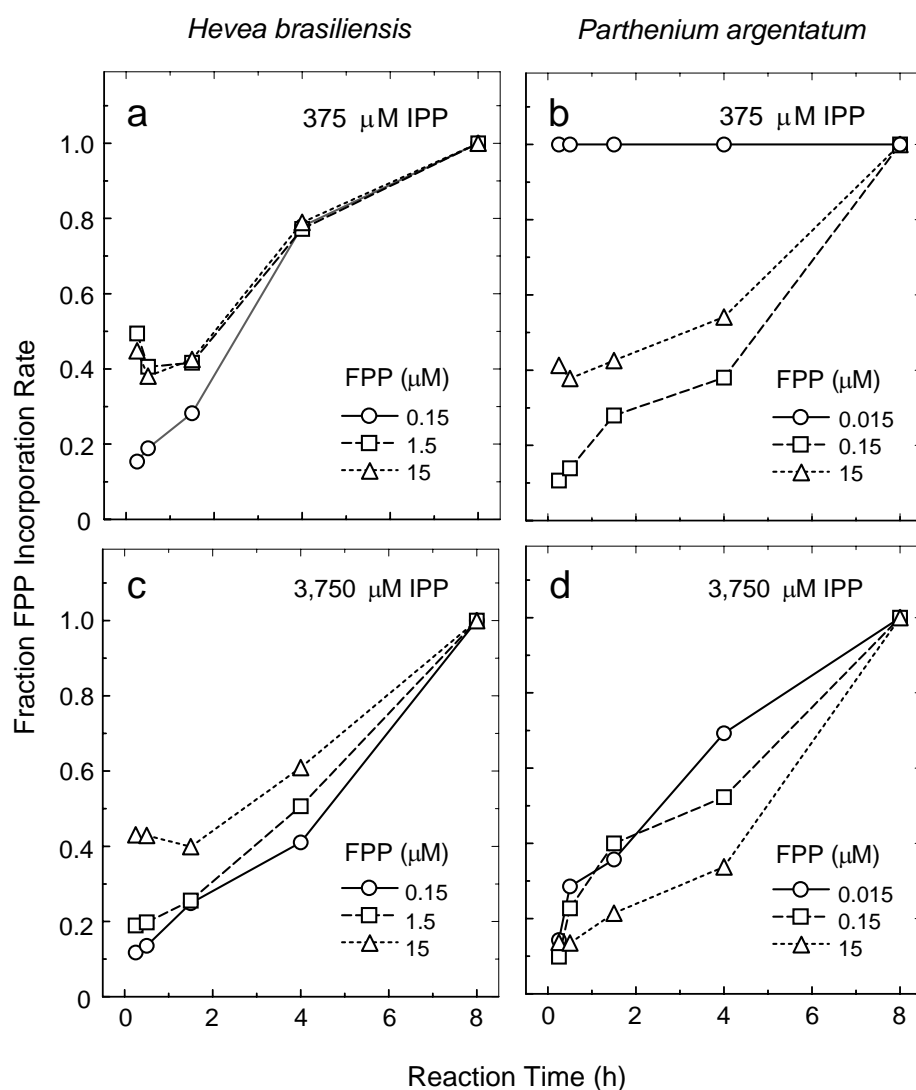


Fig. 4. Time courses of [^3H] farnesyl pyrophosphate (FPP) incorporation into rubber by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10 \times K_m^{\text{IPP}}$, lower panels), and three [^3H] farnesyl pyrophosphate (FPP) concentrations 10% K_m^{FPP} , K_m^{FPP} and nonlimiting FPP, expressed as a fraction of the maximum rate for each FPP and IPP concentration and species. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm \text{s.e.}$

WRP (1 μm in diameter) is smaller than that of *P. argentatum* WRP (1.4 μm in diameter) [22], the average WRP surface area would be 3.14 and 6.16 μm^2 , respectively, and so the surface density of active RT-ases FPP binding sites in *H. brasiliensis* WRP was at least twice that in *P. argentatum* WRP in these samples.

Photoaffinity labeling experiments indicated that each RT-ase complex appears to have six initiator

binding sites [23]. The lag phase in 1.5 μM FPP in 375 μM IPP in *H. brasiliensis* (Fig. 4a) suggests that only one of the six sites was able to bind FPP at this FPP concentration (not enough FPP to saturate all sites) whereas all six may have been initiated in 15 μM FPP. This was confirmed by the ratio of FPP incorporated in excess FPP (15 μM) to the amount incorporated in the K_m concentration (1.5 μM and 0.15 μM FPP in *H. brasiliensis* and

P. argentatum, respectively) of 6.40 ± 0.73 (mean \pm s.e. combined for both species), remarkably close to the six sites previously estimated by quite different methods [23].

Rubber molecular weight

The molecular weight of rubber produced *in vitro*, calculated based on initiator (one per molecule) and monomer incorporated, ranged from 10 to nearly 10,000 kg/mol. (Figs. 5 and 6). As expected [6, 10, 11], high initiator concentration (15 μ M) led to the smallest rubber molecular weights in both species (Figs. 5 and 6). Low initiator concentrations resulted in high rubber molecular weight, especially at the highest monomer concentration (3,750 μ M IPP; Figs. 5c, d and 6c, d). Remarkably, *P. argentatum* WRPs produced very high molecular weights in very short reaction times (Fig. 6d); while *H. brasiliensis* WRPs produced larger polymers with time from 15 mins to 4 h (Fig. 6c).

A molecular weight of 2 Mg/15 minutes would reflect a polymerization rate of approximately 33 monomers/s. This is certainly within normal biological operational parameters for enzymes. Bacterial undecaprenyl diphosphate synthase may polymerize this 18-unit *cis*-polyisoprene product at a rate of 2.5 to 77 isopentenyl units/s [24]. Polyhydroxybutyrate synthesis by *Alcaligenes eutrophus* synthesis proceeds at a rate equivalent to 2 molecules of monomer (D(-)-3-hydroxybutyryl-CoA)/s/polymerase [25]. This is in the same range as RubisCO – over 95% of these enzyme complexes fix CO₂ at a rate of 1-10/s, in a notoriously slow reaction. Really fast enzymes become limited by substrate diffusion rate – different forms of carbonic anhydrase range between 10,000 and 1 million reactions/s [26].

At low monomer concentration, molecular weight continued to increase up to 8 h, suggesting *in vitro* rubber biosynthesis could produce still longer chains than reported to date *in vivo* [2, 27, 2]. Maximum molecular weights achieved in this experiment were about 4 Mg/mol in *H. brasiliensis* and 7 Mg/mol in *P. argentatum*, both in 0.15 μ M FPP (Figs. 5c, d and 6c, d). In fact, a subsequent experiment demonstrated that, in severely limiting FPP (0.001 μ M), rubber molecular weights up to 50 Mg/mol could be achieved by *P. argentatum* WRP in only 4 h (Fig. 7). Importantly,

even very low levels of super high molecular weight rubber would have a large effect on the physical and rheological properties of natural rubber [28].

Molecular weight is the outcome of the amount and ratio of substrates combined with intrinsic species-specific properties of the RT-ases. It has previously been shown that the *P. argentatum* RT-ase has a broad range of FPP negative cooperativity not seen in the *H. brasiliensis* RT-ase [1, 11]. This allows the *P. argentatum* RT-ase to synthesize high molecular weight rubber throughout the year even though the rate of synthesis becomes much slower in the growing season due to competition for FPP and IPP by other pathways essential to growth and development [20, 21]. In contrast, rubber biosynthesis is compartmentalized in laticifers in *H. brasiliensis* and its rubber molecular weight appears to result from the laticiferous regulation of substrate concentrations [1, 4, 5]. In our study, interspecific comparisons of rubber biosynthesis at the same initiator concentration (0.15 μ M FPP) suggest that biology has consequences at the enzyme complex level. The rubber molecular weight produced in 0.15 μ M FPP and 375 μ M IPP was much higher after 8 h in *P. argentatum* (Fig. 5b) than in *H. brasiliensis* (Fig. 5a) because the chain transfer reaction was inhibited in *P. argentatum* and the same rubber molecules kept growing. However, *H. brasiliensis* RT-ase produced higher molecular weight rubber than *P. argentatum* in excess IPP (3,750 μ M) and 0.15 μ M FPP (Fig. 5c, d) because neither species' RT-ase has any FPP cooperativity operating in 3,750 μ M IPP and 15 μ M FPP [1]. Thus, excess IPP appeared to enhance the chain transfer reaction in the *P. argentatum* RT-ase but not in the *H. brasiliensis* RT-ase causing *P. argentatum* RT-ase to make shorter rubber than *H. brasiliensis* under the same conditions (Figs. 5 and 6) and much shorter rubber when excess FPP was combined with excess IPP (Figs. 5c, d and 6c, d). However, excess FPP also caused lower molecular weight rubber to be produced by *H. brasiliensis* RT-ase than in lower FPP concentrations, although this reduction was much less pronounced in *P. argentatum*.

The acceleration of the chain transfer reaction only in *P. argentatum*, by the combination of excess FPP and IPP, indicates that the two substrates interacted in some way in its RT-ase-binding region, enhancing

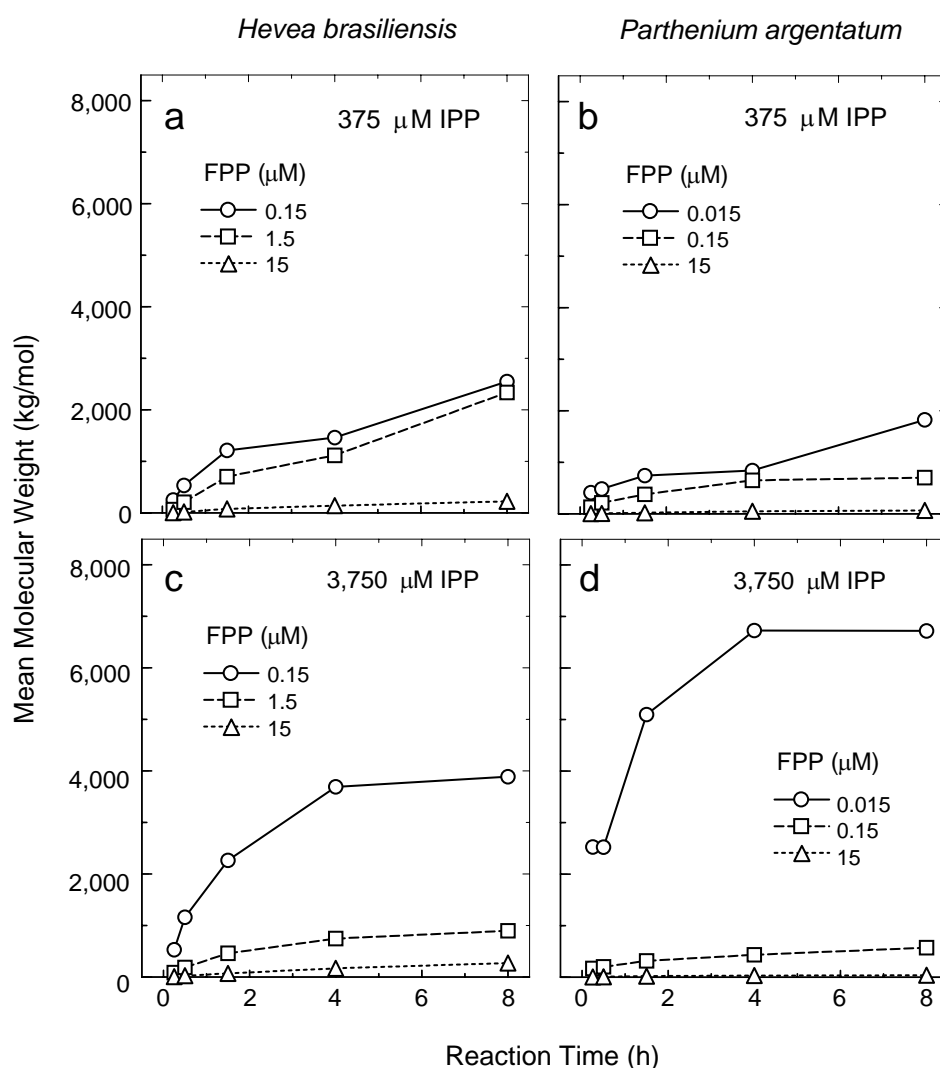


Fig. 5. Mean molecular weight time course of newly synthesized rubber produced by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10 \times K_m^{\text{IPP}}$, lower panels), and three [^3H] farnesyl pyrophosphate (FPP) concentrations 10% K_m^{FPP} , K_m^{FPP} and nonlimiting FPP, expressed as a fraction of the maximum rate for each FPP and IPP concentration and species. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm \text{s.e.}$

the displacement and reinitiation of rubber molecules. It is also clear that as re-initiation began in both species, after the initial rubber molecules, that FPP incorporation increased in a time-dependent manner, not in a step-wise increase. This indicates that rubber molecules were not all reinitiated at the same time, after the minimum time required, and may not all have been synthesized at the same rate or to the same polymer length. This may lead to the natural rubber polydispersity reported for both species [22].

Spatial considerations

The first degree of physical constraint for natural rubber biosynthesis is caused by the membrane-bound nature of the RT-ase *cis*-prenyl transferase subunit catalyzing the rubber molecule condensation reaction. Yet the RT-ase complex presents further constraints as devised in structural models [29, 30]. Remarkably, *in vitro*, simultaneous initiation at all six active sites of the RT-ase complex occurred at high initiator concentration regardless of species

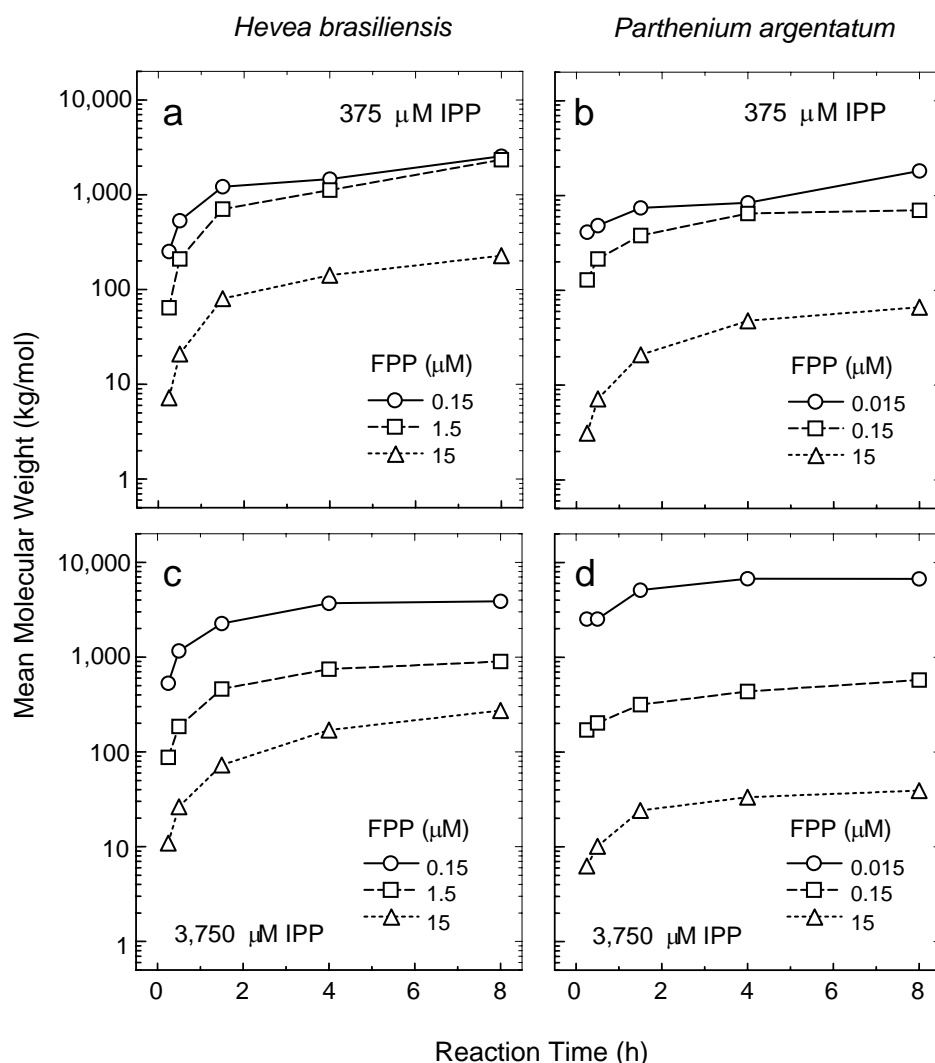


Fig. 6. Log mean molecular weight time course of newly synthesized rubber produced by washed rubber particles purified from *Hevea brasiliensis* (left panels) and *Parthenium argentatum* (right panels), in two IPP concentrations (K_m^{IPP} , upper panels and $10 \times K_m^{\text{IPP}}$, lower panels), and three [^3H] farnesyl pyrophosphate (FPP) concentrations 10% K_m^{FPP} , K_m^{FPP} and nonlimiting FPP, expressed as a fraction of the maximum rate for each FPP and IPP concentration and species. Reactions were performed at 25 °C and 16 °C for *H. brasiliensis* and *P. argentatum*, respectively. Each value is the mean of $3 \pm \text{s.e.}$

or monomer concentration (Fig. 4), suggesting unrestrained initiator binding, in agreement with analog binding studies [31, 32]. This also indicates that the central channel of the RT-ase is large enough to accommodate six simultaneously growing polymer chains. Our results indicate a lag phase (no further initiation) but continued IPP incorporation, synthesizing rubber to molecular weights of 20–25 kg/mol (*P. argentatum*) or 70–80 kg/mol (*H. brasiliensis*) before chain transfer and re-initiation occurred (Figs. 5 and 6). Entropic

considerations oblige formation of a random coil in the rubber particle interior, possibly by as little as 20 degrees of polymerization (1.5 kg/mol) as formed by dolichols [33]. Random coils of polyisoprene of 20 kg/mol and 80 kg/mol would form radii of gyration of about 5 nm and 11 nm, respectively (extrapolated from Jackson *et al.* 1996 [34]); our results imply that steric crowding at the bottom of the RT-ase channel produced sufficient physical constraint to trigger chain transfer, at multiple sites, upon release. The

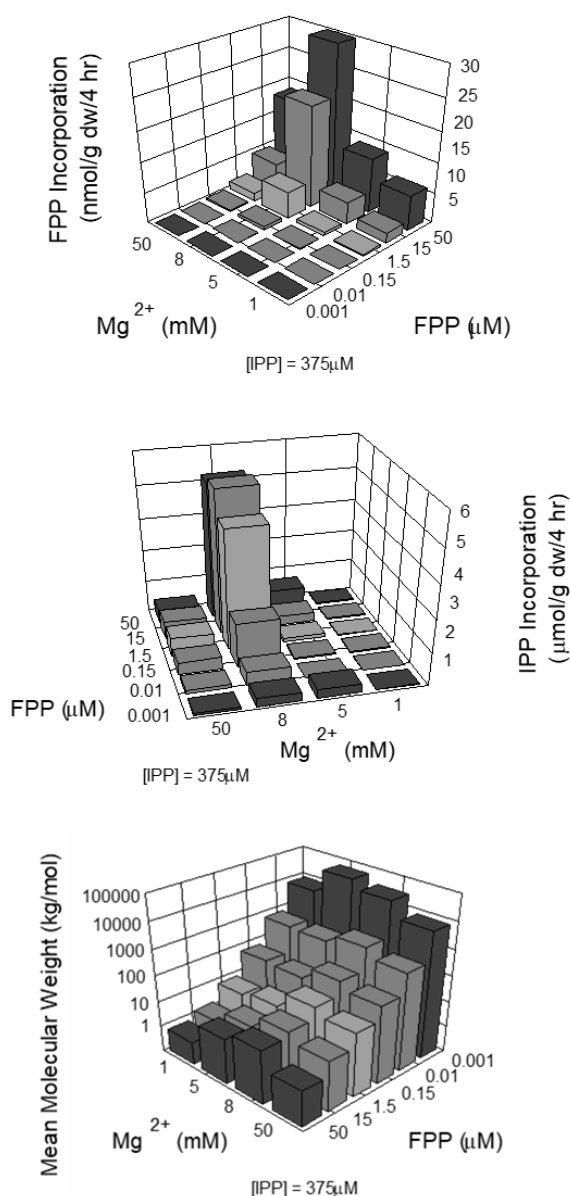


Fig. 7. Farnesyl pyrophosphate (FPP), isopentenyl pyrophosphate (IPP) and magnesium cation concentration dependence of [³H]FPP incorporation, [¹⁴C]IPP incorporation and mean rubber molecular weight made by washed rubber particles purified from *Parthenium argentatum*. Each value is the mean of 3.

subsequent series of initiation events took place quickly (in excess FPP), with repeated steric crowding limiting molecular weights (especially for *P. argentatum*) even in excess IPP. Since *H. brasiliensis* rubber molecules reached 4x higher molecular weights before the first chain transfer, and consistently reached higher molecular weights

at 15 and 0.15 μM FPP, its RT-ase may have a wider funnel upon release to the interior. It certainly has a longer channel than the *P. argentatum* RT-ase [1]. Alternatively, *H. brasiliensis* molecules may form a more compact coil, as has been suggested [35], allowing the RT-ase to accommodate higher molecular weight polymers before release.

Nevertheless, when initiation is limited, RT-ases are able to physically accommodate *in vitro* synthesis of very high molecular weight polymers: for *P. argentatum* over 67 Mg/mol (Rg 180 nm) at 0.015 μM FPP, and for *H. brasiliensis* over 38 Mg/mol (Rg 130 nm) at 0.15 μM FPP. Yet in both cases there appears to be a physical limit, since the MW reached a plateau after 4 hours (Fig. 5c, d) even with excess monomer. Our results suggest natural rubber molecular weight may be limited *in vivo* due to spatial considerations. Rubber molecules of these very large sizes may have been physically displaced from the RT-ase binding sites, triggering one or more termination events.

Biosynthesis *in vivo* clearly lies outside our experimental conditions, where initiation and polymerization occur progressively. Interestingly, molecular weights similar to those typically reported from plant extracts were found *in vitro* at/near substrate K_m (1.5 μM FPP, 375 μM IPP for *H. brasiliensis*; 0.15 μM FPP, 375 μM IPP for *P. argentatum*). Even so, physical constraints may indeed play a role in termination *in vivo*, including active site crowding by rubber particle proteins such as rubber elongation factor (REF), small rubber particle protein (SRPP) and allene oxide synthase (AOS) [36].

Rubber biosynthesis by *P. argentatum* WRP is more sensitive to extremes in substrate concentration than *H. brasiliensis* in *in vitro* assays and both very low and very high molecular weight rubber can be made (Fig. 7). This knowledge may enable genetic engineering of *P. argentatum* to produce tailored molecular weight rubbers. For example, *P. argentatum* is mostly dormant during the winter with little carbon demand for development and this is when it makes most of its rubber [37, 38]. Downregulation of the endogenous FPP synthase gene(s) with a cold-inducible promoter would reduce the FPP pool size and higher molecular weight rubber *should* result. At break of dormancy the promoter would stop working and normal FPP levels should resume. Alternatively, FPP synthase

genes could be overexpressed with a cold inducible promoter causing endogenous FPP levels to rise and lowering rubber molecular weight.

CONCLUSIONS

Temporal analysis of natural rubber polymerization in two phylogenetically distant species has proved useful in understanding rubber molecule initiation and polymerization and even sheds light on the polymer termination – the release of the mature molecule from the RT-ase membrane-bound complex caused by biochemical and physical factors. If coupled to models of the rubber particle and the RT-ase complex, such investigations may lead to a fuller interpretation of the mechanisms of this essential enzyme and the critical natural resource it produces.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

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