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RUBBER TRANSFERASE ACTIVITY IN RUBBER PARTICLES OF GUAYULE

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Key Word Index—*Parthenium argentatum*; Asteraceae; stembark; rubber transferase; isoprenes; prenyl transferase; natural rubber biosynthesis; rubber particle proteins.

Abstract—Rubber transferase (RuT) activity, measured as incorporation of radiolabelled isoprene from [^{14}C]isopentenyl pyrophosphate (IPP) into rubber, was assayed in suspensions of washed rubber particles (WRPs) purified from stembark tissue of *Parthenium argentatum*. Isolated WRPs had high RuT activity which was not diminished even after repeated washing, demonstrating the firm association of the enzyme with the particles. The activity of RuT was characterized with respect to substrate and WRP concentration. The rate of IPP-incorporation was dependent upon the concentration of two substrates, IPP and the allylic pyrophosphate starter molecule *E,E*-farnesyl pyrophosphate (FPP). The enzyme present in 6×10^{10} WRPs per ml was saturated by 1 mM IPP and 20 μM FPP. Under saturating cosubstrate concentrations the apparent K_m of RuT was *ca* 300 μM IPP and 3 μM FPP. Analysis of WRPs by SDS-PAGE revealed a simple protein profile characteristic of guayule rubber particles. A successful and facile assay for IPP-polymerization by isolated rubber particles is described.

INTRODUCTION

Rubber formation in plants results from an enzyme-mediated, *cis*-1,4-polymerization of isoprene into long chain molecules of polyisoprene rubber. The isoprene monomers, which are derived from isopentenyl pyrophosphate (IPP), are polymerized in a head-to-tail fashion by the enzyme rubber transferase (RuT) (EC 2.5.1.20) [1]. This enzyme is the most crucial biosynthetic step in the formation of rubber which occurs in over 2000 plant species [1]. Assays for RuT activity in *Hevea brasiliensis* latex have been used since the 1960's [2–7] and are based on the incorporation of radiolabelled IPP into rubber. Standard assay mixtures contain [^{14}C]IPP, Mg^{2+}, a thiol reducing agent, the plant protein extract of interest, and have always required a fresh preparation of washed rubber particles (WRPs). The WRPs are essential for rubber biosynthesis, but their role in the biosynthetic process was debated. The existence of an insoluble form of RuT associated with rubber particles was first established in 1963 by Berndt [6] and Archer *et al.* [2]. The WRPs would form new rubber if an initiator, dimethylallyl pyrophosphate (DMAPP), was added to the assay mixture [2, 4, 6]. In addition, a soluble protein extract from *Hevea* latex was thought to catalyse the extension of pre-existing rubber pyrophosphate chains in the WRPs [2]. Evidence that a soluble form of RuT existed was presented [5] and a method for its purification was published [3]. However, these soluble extracts cannot synthesize rubber when used alone; they have an absolute requirement for WRPs. When it was discovered that the soluble factor could be replaced by a series of initiator molecules [6–9], it became possible that the soluble enzyme was actually part of the initiating system, synthesizing allylic pyrophosphate instead of polyisoprene. The effect of the soluble protein on rubber biosynthesis, in the presence of WRPs and an initiator, was not examined.

In this paper, we show that WRPs isolated from guayule stembark contain high RuT activity, even after repeated washing, when the WRPs are assayed in the presence of the allylic farnesyl pyrophosphate (FPP). An improved and simplified assay for RuT is described which enabled the characterization of this activity with regard to temperature, substrate (IPP, FPP) and WRP concentration. Comparisons were also made of RuT activity from WRPs isolated from high and low rubber-yielding guayule lines suggesting a correlation between RuT activity and rubber yield.

RESULTS AND DISCUSSION

In vitro assays for RuT activity measure the incorporation of ^{14}C into rubber following incubation of the enzyme mixture in the presence of [^{14}C]IPP. Early procedures required that, following incubation, the labelled rubber in the reaction mixture be coagulated with ethanol or acetone and that this rubber coagulum be chemically purified, solubilized and measured via scintillation counting [2, 3, 5]. This procedure was cumbersome, allowing few simultaneous assays to be run, and often resulted in substantial losses of rubber during the coagulation and purification steps. A major improvement, introduced by Light and Dennis [10], employed the use of filters to trap WRPs following [^{14}C]IPP-incorporation; this allowed rapid processing of multiple samples for chemical purification and counting. Ozonolysis and gel permeation chromatography revealed that radiolabelled rubber was the reaction product measured [8–10]. Our method also uses filters to trap WRPs, but in

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addition, quantifies RuT activity on the basis of the number of rubber particles present in WRP preparations, thus making it possible to standardize RuT activity between separate experiments. In guayule, 10^{10} WRPs are equivalent to 6.7 mg of rubber, dried overnight at 37°.

These assays must take into account the passive trapping of radiolabel by WRPs on the filters. As particle densities increase they trap increasingly large quantities of labelled IPP (Fig. 1). However, this amount is usually less than 0.5% of the amount incorporated by active WRP preparations. Moreover, no label is retained by the filters in the absence of WRPs (Fig. 1). In all subsequent experiments, the amount of IPP incorporated passively was determined from EDTA controls using comparable WRP densities; this background amount was subtracted to determine active [^{14}C]IPP incorporation rates.

The activity of RuT at both 16 and 26°, expressed per unit of rubber, was unaffected by the repeated washing of rubber particles (Fig. 2). This demonstrates that RuT is firmly associated with guayule WRPs and exists as an insoluble form similar to the *Hevea* enzyme [2, 4, 6–8]. Additions of soluble enzyme extracts are not required for IPP-polymerization to occur. Earlier researchers, who concluded that a soluble RuT was polymerizing IPP in the absence of initiator molecules in the WRP reaction mixtures [3, 5], overlooked the possibility that the soluble enzyme being assayed was not RuT, but could have been the enzyme making an initiator molecule.

Soluble fractions from *Hevea* latex are known to contain enzymes capable of synthesizing the initiators FPP [11] and geranyl geranyl pyrophosphate (GGPP) [6, 7]. Light and Dennis claimed to have purified a soluble form of RuT from the latex serum of *Hevea* [10]. Although the existence of a soluble form of RuT is possible, their enzyme actually proved to be a soluble prenyl transferase, FPP synthetase [10]. When this enzyme was added to *Hevea* WRPs, it greatly stimulated rubber biosynthesis *in vitro*, as did a number of other

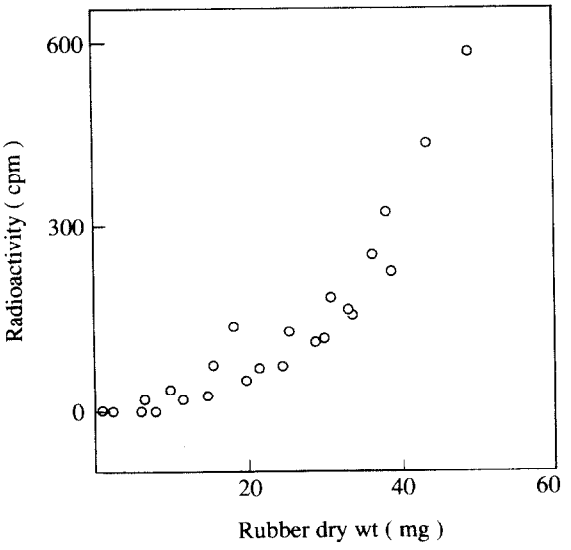

Fig. 1. The influence of WRP concentration on passive trapping of ^{14}C-IPP. WRPs were incubated in the standard assay mixture (see Experimental) except that 50 mM EDTA was added to all reactions to prevent rubber biosynthesis.

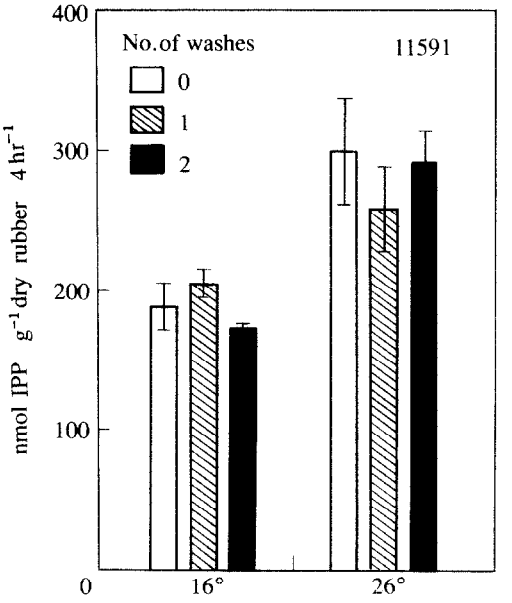

Fig. 2. Rubber transferase activity in rubber particles isolated from stembark of guayule line 11591, left unwashed, washed once or twice, and incubated at 16° or 26° for 4 hr in the standard assay mixture (see Experimental). Each value is the mean of 3 ± s.e.

prenyl transferases from animal and yeast sources [12]. However, their purified prenyl transferase could not synthesize rubber by itself; rubber was only produced in the presence of WRPs. Unfortunately, their control reactions did not include WRPs plus FPP to see if their WRPs contained RuT activity. Thus, it is questionable whether they did, in fact, isolate a soluble RuT. Moreover, it has been shown by others, that *Hevea* WRPs are capable of rubber biosynthesis when supplied with any one of a number of allylic pyrophosphate initiators, such as DMAPP, GPP, FPP or GGPP [6–9].

The synthesis of FPP, by FPP synthetase, requires DMAPP and IPP. Thus, if the prenyl transferase purified by Light and Dennis [10] is really part of an initiator system, it would be necessary for their assay mixtures to contain either a low level of DMAPP or the enzyme IPP isomerase which can synthesize DMAPP from the available IPP.

Because guayule and *Hevea* WRPs possess an insoluble form of active RuT, past procedures involving heterologous systems, which utilized soluble protein extracts combined with easily isolated WRPs from other plant sources [13, 14], may have led to erroneous conclusions. It is quite possible that those soluble protein extracts merely supplied the enzyme(s) necessary to synthesize initiator molecules rather than supplying RuT itself.

The initiator used in our studies was FPP. When FPP was included in the WRP preparations, RuT activity increased 20-fold over preparations without FPP (Table 1). Although the omission of FPP greatly inhibited RuT activity, this level was slightly higher than that of the EDTA controls (Table 1). This slight residual activity may reflect the IPP incorporated by elongation of existing rubber pyrophosphate chains, as opposed to initi-

Table 1. Rubber transferase activity of rubber particles purified from stembark of guayule line 11591

Sample	Rubber transferase activity (nmol IPP g^{-1} WRP per 4 hr)*
WRPs + IPP	11.0 ± 1.7
WRPs + IPP + FPP	223.0 ± 12.0
WRPs + IPP + FPP + EDTA	2.0 ± 0.5

*Weight was determined after drying the WRP cake on the pre-weighed filter at 37° overnight. Each value is the mean of 10 assays ± s.e. carried out with WRPs isolated during the months of July and August 1988. All treatments include 300 μM $MgSO_4$. Concentrations of the listed ingredients are: WRPs, $\leq 3 \times 10^{10}$ particles per 500 μl reaction mixture; IPP, 86.5 μM; FPP, 22.7 μM; EDTA, 50 mM (see Experimental).

ation of new molecules when FPP is present in the reaction. Although not tested in this study, other initiators such as DMAPP, GPP, NPP (neryl pyrophosphate) and GGPP could be used with WRPs to assay RuT activity [7, 8]. It has been reported that FPP, the initiator we use, is one of the most effective initiators for *Hevea* WRPs [7, 8].

The observed rate of incorporation of IPP at 26° was not double the rate at 16° (Fig. 2), suggesting that the RuT activity of the WRPs was unstable. This was substantiated when the temperature and time dependence of RuT activity were examined in two different guayule lines (Fig. 3). During the first 2 hr of incubation, IPP-incorporation by WRPs progressed in a linear fashion and activity doubled with every 10° rise in temperature, which conforms to the Q_{10} expected for an isolated enzyme system. As incubation times increased, RuT activity declined progressively with increasing temperature in both guayule lines. The decline in RuT activity was much greater in WRPs from line 593 than from line 11591 (Fig. 3). This decline occurred in the presence and absence of phosphatase inhibitors (data not shown) so the effect was probably not due to phosphatase activity in the WRP preparations. The observation of RuT instability at elevated temperatures led to the adoption of a standard incubation regime, for guayule WRPs, of 16° for 4 hr.

The effect of WRP number on RuT activity was measured for two guayule lines (Fig. 4). Activity was much higher in line 11591 than line 593 at all WRP concentrations. Line 11591 yielded more rubber than line 593 under field conditions [15, 16] and this may be a reflection of the RuT activities observed in the WRPs. At WRP concentrations below 3×10^{10} particles per 500 μl of assay mixture, the apparent IPP-incorporation rate remained constant for both lines. However, as WRP concentrations increased above this value, (denoted by the vertical dashed line, Fig. 4) the rate of incorporation declined steadily, indicating a substrate limitation at WRP concentrations above 6×10^{10} particles per ml. If IPP were the limiting substrate, the decline in RuT rate would occur at different WRP concentrations for each guayule line because the more highly active 11591 WRPs would deplete the available IPP more rapidly than the 593 WRPs. Since the decline occurs at the same WRP concentration in both lines, FPP is implicated as the limiting substrate. This was confirmed by experiments in which FPP, IPP and WRP concentrations were varied (data not shown).

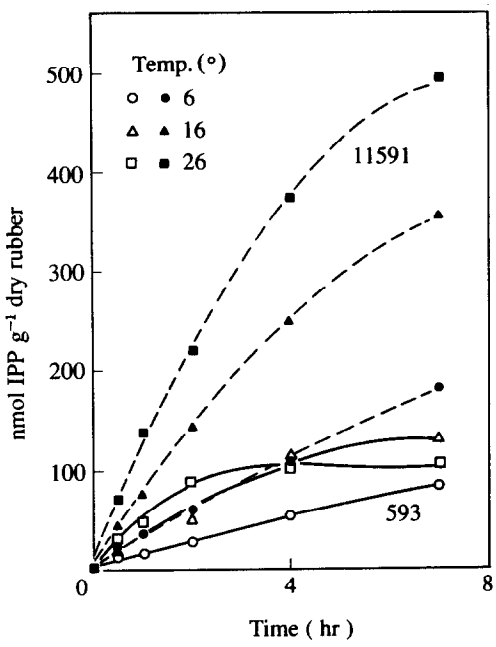

Fig. 3. Time courses of RuT activity in WRPs isolated from guayule lines 593 and 11591 and incubated at 6°, 16° or 26° in the standard assay mixture (see Experimental).

The immediate practical consequence of these results was to employ a standard concentration of $\leq 6 \times 10^{10}$ particles per ml to assay RuT activity in the presence of 20 μM FPP and 86 μM IPP. This rubber concentration is equivalent to ≤ 40 mg of dried rubber particles. This ensured that the IPP-polymerization rate was measured at saturating concentrations of the FPP starter molecule and that RuT activity was not altered by a depletion of substrate.

Since we have shown that WRPs contain RuT activity, protein analysis should reveal the RuT enzyme. Analysis, by SDS-PAGE, of a crude stembark homogenate of line 11591 and its WRPs after repeated wash cycles revealed

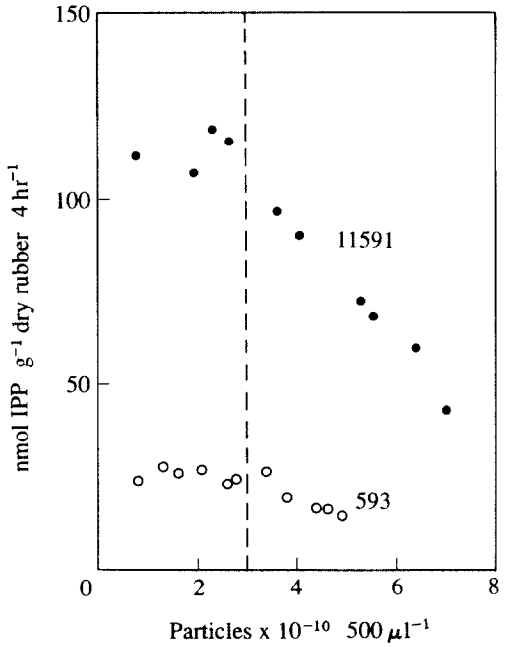

Fig. 4. The influence of WRP concentration on apparent RuT activity. WRPs were isolated from guayule lines 11591 and 593 and incubated for 4 hr at 16° in the standard assay mixture (see Experimental) in a reaction volume of 500 μl.

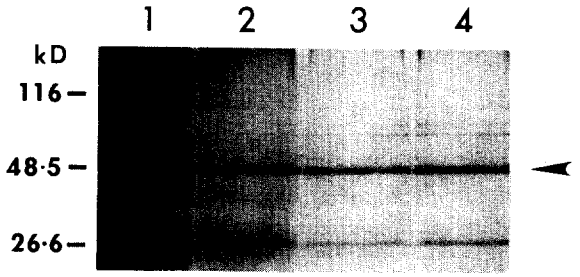

Fig. 5. SDS-PAGE profiles of stembark proteins from guayule line 11591. Lane 1, total proteins isolated from stembark tissues; lanes 2–4, guayule rubber proteins obtained from rubber particles washed 0, 1 or 2 times by flotation, respectively. The M_r of protein standards are indicated to the left of lane 1. The most abundant rubber protein has a M_r of 48 500 (arrow).

characteristic rubber particle proteins (Fig. 5). The RuT activities from each of these wash cycles was shown previously in Fig. 2 (cf. lanes 2–4, Fig. 5 with washes 0, 1 and 2 of Fig. 2). The washing procedure (cf. lane 1 with lanes 2–4 in Fig. 5) revealed a few predominant polypeptides, which are designated guayule rubber proteins. The 48500 M_r polypeptide (arrow, Fig. 5) is the most abundant rubber protein in all guayule lines examined so far, and may well be RuT [17]. It is noteworthy that no 38 000 polypeptide, characteristic of the FPP-synthetases from *Hevea* latex [10, 12], avian liver [18] and yeast [18], appears in the protein profile of guayule WRPs. The simple SDS-PAGE protein profile exhibited by guayule WRPs also suggests that rubber particles represent a distinct, compartmentalized portion of the cell and could even be considered a rudimentary organelle.

The dependence of IPP-incorporation upon IPP concentration was investigated at a saturating FPP concentration (see Experimental, data not shown). Under these conditions RuT saturated at a concentration of *ca* 1 mM IPP. Although RuT was not purified to homogeneity, the WRPs do represent a simple subcellular fraction with only a few constituent proteins (Fig. 5). It is, therefore, quite likely that the RuT activity measured does reflect a single enzyme system. If this is true, Michaelis–Menton kinetics may be applied. An Eadie–Hofstee plot demonstrated a linear dependence of V against V/S, which supports the idea that the RuT activity in isolated WRPs is the manifestation of a single system. A multi-component system would be expected to give rise to a nonlinear Eadie–Hofstee plot. The apparent K_m of RuT, determined several times in different experiments, was 300 μM IPP. This compares with the reported K_m of 120 μM IPP for *Hevea* WRPs [4]. There was little difference between the apparent K_m of RuT in WRPs from guayule lines 11591 and 593.

The dependence of IPP-incorporation upon the concentration of FPP was determined at saturating IPP (1 mM) concentrations. Under these conditions RuT saturated at a concentration of 20 μM FPP (data not shown), much lower than the 1 mM obtained for IPP. A linear Eadie–Hofstee plot was again obtained. The apparent K_m for FPP was 3 μM, two orders of magnitude less than that for IPP. Thus, the RuT enzyme system has a higher affinity for FPP than for IPP. A K_m of 13 μM DMAPP has been reported for RuT in *Hevea* WRPs [4].

We have demonstrated that RuT activity is present in guayule WRPs as is also true for *Hevea* [6–8]. The assay system developed here is easily adaptable to other natural rubber systems, regardless of whether the rubber is produced in laticifers, as in *Hevea*, or in non-laticiferous species, such as guayule. We have characterized RuT activity with respect to the substrates IPP and FPP, and as a function of WRP concentration, and have shown that activity in WRPs varies between the two guayule lines examined. Examination of RuT activities from different lines or under varying environmental conditions could be useful for crop improvement when screening new guayule lines. Finally, the simple protein profile of the guayule rubber proteins should facilitate the isolation of these polypeptides and, ultimately, the genes encoding the enzymes involved in rubber biosynthesis.

EXPERIMENTAL

Plant material. Mature, five-year-old, field-grown shrubs of guayule, *Parthenium argentatum* Gray, lines 593 and 11591, were graciously provided by Dr F. Nakayama (US Water Conservation Laboratory, Phoenix, AZ). Unless stated otherwise, plants were harvested between 1 November and 15 March (when RuT activities were greatest), the trimmed stems were immersed in ice-cold 1% ascorbic acid, and were used the same day for isolation of WRPs.

Chemicals. All chemicals, unless otherwise noted, were obtained from Sigma. Radiolabelled IPP, either [1-^{14}C]isopentenyl pyrophosphate (57 mCi mmol^{-1}) (Amersham) or [4-^{14}C]isopentenyl pyrophosphate (48 mCi mmol^{-1}) (NEN) was used. Aliquots of the NEN isotope were evapd to dryness, to remove the EtOH–NH_3 solvent in the stock soln, and redissolved in 100 mM Tris–HCl at pH 7.5. Unlabelled IPP was

the generous gift of Dr B.G. Audley (Tun Abdul Razak Laboratory, Hertford, England). Unlabelled FPP was the generous gift of Dr C. D. Poulter (University of Utah, Salt Lake City, UT, U.S.A). The unlabelled substrates appeared pure when analysed using TLC.

Isolation of guayule WRPs. Guayule stembark (70 g), peeled from green stems greater than 0.5 cm diam, was homogenized in 350 ml ice-cold extraction buffer containing 100 mM Tris–HCl, pH 7.5, 50 mM KF, 1% ascorbic acid, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 17.5 μl Antifoam A, and 30 g polyvinylpolypyrrolidone. The homogenate was filtered through 8 layers of cheesecloth, distributed between eight 50 ml centrifuge tubes, and centrifuged in a swinging bucket rotor at 4000 g and 4° for 8 min. The creamy layer of unwashed rubber particles was scooped from the tubes and suspended in 15 ml ice-cold wash buffer containing 100 mM Tris–HCl, pH 7.5, 5 mM $MgSO_4$ and 10 mM DTT. The WRPs were prepared by resuspending unwashed particles, isolated as described, in 160 ml ice-cold wash buffer. These were centrifuged in a swinging bucket rotor at 2400 g and 4° for 7 min. The rubber particles were scooped from the tubes, resuspended in 80 mls of ice-cold wash buffer, and the last centrifugation step was repeated. The WRPs were retrieved from the tubes and suspended in ice-cold wash buffer at a concentration of *ca* 5×10^{10} WRPs per ml, as determined by haemocytometry. The WRP suspensions were stored on ice and used the same day they were prepared.

Another procedure for preparing WRPs, using size-exclusion CC on LKB Ultrogel AcA34, has been described previously [7–9]. We have also used this procedure to prepare guayule WRPs. We found no difference in purity of WRPs as measured by SDS-PAGE from either procedure. Moreover, the centrifugation/flotation procedure described above is more rapid and results in much higher WRP yields than the column method. This was an especially important consideration in light of our findings showing the rapid decay of RuT activity in guayule WRPs at room temperature.

Assay of RuT activity in WRPs. Reactions were performed in 500 μl containing a final concn of 86.5 μM ^{14}C-IPP (1.17 μCi-μmol^{-1}), 22.7 μM FPP, 50 mM Tris–HCl, pH 7.5, 0.3 mM $MgSO_4$, 47.7 mM DTT and $2–3 \times 10^{10}$ WRPs. Reactions were typically run for 4 hr at 16°. Reactions were stopped by the addition of 50 μl of 0.5 M EDTA. Radiolabelled rubber was quantified as follows. Each reaction mixture was filtered through a pre-weighed, 0.22 μm cellulose acetate/cellulose nitrate filter (GSWP 025 00, Millipore Corp.) to trap the rubber particles [10], as were aliquots of the original WRP suspensions. The filters and rubber cakes were dried overnight at 37° and reweighed. Each was placed into the bottom of a scintillation vial and washed for at least 15 min in 3 ml 1 M HCl to protonate any unincorporated IPP and render it soluble. The samples were rinsed × 3, for at least 5 min per rinse, with 4 ml of 95% EtOH and dried under an IR lamp. The radioactivity on each filter was determined by liquid scintillation spectroscopy using a toluene-based scintillant. Activities determined in the absence of EDTA were adjusted for ^{14}C-IPP passively trapped by similar concentrations of WRPs assayed in the presence of 50 mM EDTA. The effects on RuT activity of the washing procedure, temperature, and of WRP, IPP and FPP concentration were determined. The dependence of RuT activity upon IPP concentration was determined at saturating FPP concentrations, using $< 3 \times 10^{10}$ WRPs per ml and 20 μM FPP. The dependence of RuT activity upon FPP concentration was determined at saturating IPP concentrations using $< 3 \times 10^{10}$ WRPs per ml and 1 mM IPP.

SDS-PAGE analysis of WRPs. A 240 μl aliquot of homogenized stembark of guayule line 11591 and aliquots of its resultant WRPs after each of three washes were mixed with SDS sample buffer to a final concn of 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue and heated to 90° for 15 min. The proteins were analysed by discontinuous SDS-PAGE according to ref. [19] and visualized by silver staining (Bio-Rad Laboratories).

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