

Stabilisation of Particle Integrity and Particle Bound *cis*-Prenyl Transferase Activity in Stored, Purified Rubber Particles

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Biochemical studies of rubber particle bound *cis*-prenyl transferase (rubber transferase, EC 2.5.1.20) activity have been complicated due to instability of the enzyme in purified rubber particles. *Parthenium argentatum* rubber transferase activity represents an extreme case of instability, which necessitated fresh preparation of enzymatically active particles from living plant tissue for every experiment. This problem was compounded because rubber transferase activity is environmentally induced in *P. argentatum*, and is only strongly active during the winter months thus restricting biochemical experimentation to this season.

In this paper, we describe a method which permits long term storage of enzymatically active rubber particles of *P. argentatum*, and validate its use in two contrasting laticiferous rubber producing species, *Hevea brasiliensis* and *Ficus elastica*. © 1997 by John Wiley & Sons, Ltd.

Phytochem. Anal. 8, 130–134, (1997)

(No. of Figures: 4. No. of Tables: 0. No. of Refs: 20)

Keywords: *cis*-1,4-polyisoprene; *cis*-prenyl transferase; natural rubber; rubber particles; rubber transferase.

INTRODUCTION

About 2,500 plant species produce the polymeric, secondary product, natural rubber (*cis*-1,4-polyisoprene) from isoprene monomers (C₅H₈) derived from isopentenyl diphosphate (IPP) (Archer *et al.*, 1963; Bonner, 1991). The rubber polymers are packaged in subcellular rubber particles that contain a variety of species specific proteins (Cornish *et al.*, 1993) and lipid components (unpublished data). The enzyme that catalyses rubber formation (rubber transferase; EC 2.5.1.20) has been characterized and shown to be firmly associated with the rubber particles in the three evolutionarily divergent (Cornish *et al.*, 1993) plant species, *Hevea brasiliensis* Muell. Arg (the Brazilian rubber tree; rubber produced as a latex by laticifers) (Berndt, 1963; Archer and Audley, 1987; Cornish, 1993), *Ficus elastica* Roxb. (the Indian rubber tree; a laticiferous latex producer) (Siler and Cornish, 1993, 1994; Cornish *et al.*, 1994; Cornish and Siler, 1996a), and *Parthenium argentatum* Gray (guayule; rubber produced in bark parenchyma cells) (Madhavan *et al.*, 1989; Cornish and Backhaus, 1990). Rubber transferase activity *in vitro* requires intact particles, is IPP-dependent, requires a divalent cation cofactor (Mg²⁺), and an allylic diphosphate, such as farnesyl diphosphate (FPP), to initiate the biosynthesis of new rubber molecules (Berndt, 1963; Lynen, 1969; Archer and Audley, 1987; Madhavan *et al.*, 1989; Cornish and Backhaus, 1990; Cornish, 1993; Cornish and Siler, 1995).

Biochemical investigations of rubber formation have been hampered, especially in *P. argentatum*, by the necessity of purifying enzymatically active rubber particles from living plant material. *P. argentatum* rubber transferase activity is very unstable *in vitro* (Cornish and Backhaus, 1990). Also, highly active particles can only be obtained

from winter harvested bark, thus restricting biochemical experimentation with field grown material to this season (Appleton and van Staden, 1989; Ji *et al.*, 1993; Cornish and Siler, 1996b). Activity in *H. brasiliensis* and *F. elastica* is more stable, but activity declines with freezer storage. Although *F. elastica* (a popular ornamental plant) can be easily grown in the United States, living latex from *H. brasiliensis* (a tropical tree, and the global, sole commercial source of rubber at the present time) is considerably more difficult to obtain on a regular or frequent basis.

In this paper, we demonstrate that an adaptation of a method developed for freezing purified nitrogenases in liquid nitrogen (Kelley *et al.*, 1967, Klucas *et al.*, 1968) effectively maintains rubber particle integrity and rubber particle bound rubber transferase activity in particles purified from all three species.

EXPERIMENTAL

Materials. *H. brasiliensis* buffered latex (made up by mixing two volumes of 0.1 M sodium bicarbonate, 50% w/v glycerol, 0.3% w/v sodium azide and 5 mM cysteine, with one volume of fresh latex), from clone PB260, was generously provided by J. R. Bugansky (Senior Botanist, Goodyear Tire & Rubber Co., Akron, OH, USA) from plantation trees grown in Sumatra. *F. elastica* plants were obtained from a local nursery and grown in a greenhouse in Albany, CA, USA. Mature *P. argentatum* plants (lines 593 and 11591) were generously provided by F. Nakayama (US Water Conservation Laboratory, Phoenix, AZ, USA); stems (including young and mature tissue) were harvested during the winter months from field grown plants, dipped in cold 1% ascorbate and shipped overnight on wet ice to Albany, CA, USA.

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Unlabelled IPP and FPP as well as $[1-^{14}\text{C}]$ IPP (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc., St. Louis, MO, USA.

Purification of rubber particles. Purified rubber particles, thrice washed with ice cold buffer containing 100 mM Tris-HCl (pH 8), 1 mM magnesium sulphate, 5 mM dithiothreitol, and 0.1 mM Pefabloc (4-(2-aminoethyl)-benzenesulphonyl fluoride), hydrochloride powder; Boehringer Mannheim (Indianapolis, IN, USA) were prepared from *P. argentatum* stem bark by a centrifugation/flotation procedure as previously described (Cornish and Backhaus, 1990), and from *H. brasiliensis* latex by the same method with some modification of centrifugation speed and duration (Siler and Cornish, 1993). Enzymatically active rubber particles from *F. elastica* latex were purified (four times washed) as previously described (Cornish and Siler, 1996a).

Preparation of liquid nitrogen frozen rubber particle beads.

Aliquots of buffered rubber particles were filtered, dried overnight at 37°C and weighed. The remaining rubber particles were suspended in different concentrations of glycerol, ranging from 0 to 30% depending upon the particular experiment. The samples were subdivided and one set was stored at 4°C in a refrigerator. The second set of rubber particles were pipetted as droplets from Pasteur pipettes (9 in) directly into open vats of liquid nitrogen. The frozen beads were collected and stored in cryotubes in a liquid nitrogen Dewar.

Assay of rubber transferase activity. Rubber transferase activity of rubber particles from *H. brasiliensis*, *F. elastica* and *P. argentatum* was determined by a method previously described for *P. argentatum* (Cornish and Backhaus, 1990). Rubber particles were incubated in buffer with 80 or 100 μM $[^{14}\text{C}]$ IPP (specific activities of 12 and 9.6 mCi/mmol, respectively), 20 μM FPP, and 1 mM magnesium sulphate. The FPP and Mg^{2+} concentrations were non-limiting. Rubber particle beads were removed from liquid nitrogen and thawed, or removed from the 4°C storage, and diluted with buffer (as described in section entitled Purification of rubber particles) to similar rubber particle concentrations. The reactions were incubated at either 25°C, pH 7.5 or 16°C, pH 7.8, depending upon the particular experiment, and were halted by the addition of 25 mM ethylene-diamine tetraacetic acid (EDTA). The rubber particles were harvested by filtration, the filters washed to remove unincorporated radiolabelled IPP, and the radioactivity on the filters (the newly synthesized polyisoprene) was determined by liquid scintillation spectroscopy.

RESULTS AND DISCUSSION

The stability of rubber transferase activity in *P. argentatum* has been shown to be sensitive to increasing temperatures above 4°C, and to vary among lines (Cornish and Backhaus, 1990), which led to our standard 16°C assay temperature in this species (Cornish and Backhaus, 1990; Cornish *et al.*, 1994; Cornish and Siler, 1995). Rubber particles from a particularly unstable line, 593, became inactive after only 2 h at 25°C (Fig. 1). Another line, 11591, lost 35% of its activity over 4 h at 25°C and 63% after 7 h. Enzyme activity was labile even when the particles were stored at 4°C, with 593 losing about 50% of activity in 4 h

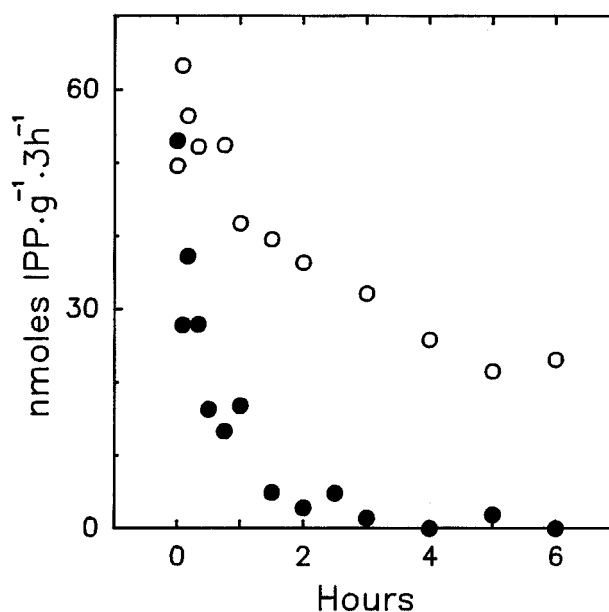


Figure 1. Rubber transferase activity in particles purified from *P. argentatum*, line 593, after storage at 25°C (●) or 4°C (○). Residual activity in rubber particles (89 ± 7 mg/mL) was assayed for 3 h at 25°C in the presence of 80 μM $[^{14}\text{C}]$ IPP and 20 μM FPP.

of storage (Fig. 1), and 11591 lost 41% in 8 h. Stability variations were also observed between different isolates from the same line (data not shown), which may reflect differences in degree of purification or the time it took to reach the desired degree of purification.

The stability of rubber transferase activity also appears to vary between isolates from *H. brasiliensis*. One rubber particle isolate stored at 0°C lost 50% of its rubber transferase activity in 1 day, and 75% after 2 days (Audley and Archer, 1988). Rubber transferase activity in our *H. brasiliensis* rubber particle preparations lasted considerably longer than the reported value when stored at 4°C (Fig. 2), again possibly reflecting the degree of purification prior to storage. Nevertheless, all of the *H. brasiliensis* preparations

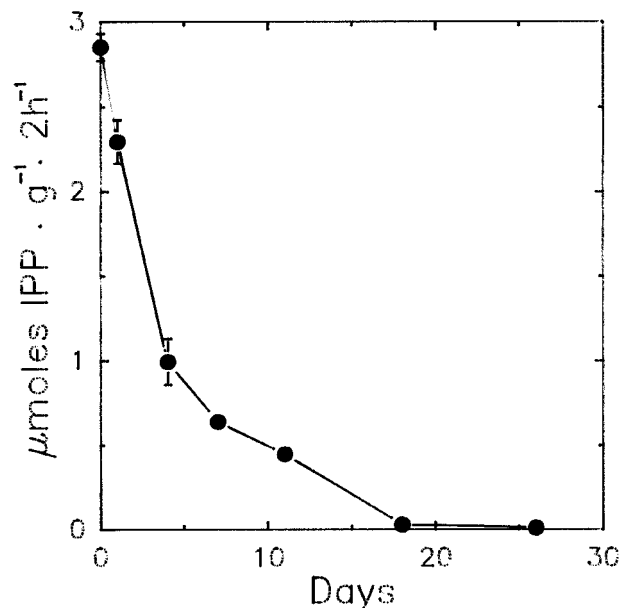


Figure 2. Rubber transferase activity in particles purified from *H. brasiliensis* and stored at 4°C. Residual activity in rubber particles (11.2 ± 1.5 mg/mL) was assayed in the presence of 100 μM $[^{14}\text{C}]$ IPP and 20 μM FPP for 2 h at 25°C. Each value is the mean of three assays \pm SE, (SE values smaller than the symbol size are not shown).

were considerably more stable than those for *P. argentatum*. Rubber transferase activity was also relatively stable in *F. elastica* particles which showed no loss of activity after 12 h at either 25°C or 4°C (Cornish and Siler, 1996a). Activity in *H. brasiliensis* and *F. elastica* also declines with storage at -20°C, and the enzyme is readily inactivated with repeated freeze-thaw cycles (data not shown).

Since intact rubber particles are required for enzymatic activity, all storage methods must maintain particle integrity. The three species tested possess distinctly different rubber particle compositions. The particles differ considerably in size, protein and lipid composition, and natural rubber molecular weight (Cornish *et al.*, 1993). These differences could lead to a differential tolerance of freeze-thaw. Freezing rubber particles under conditions similar to the successful nitrogenase method (Kelley *et al.*, 1967; Klucas *et al.*, 1968: cell-free extracts in buffer, e.g. 0.1 M Tris-HCl (pH 8) or 0.05 M TES (*N*-Tris(hydroxymethyl)methyl-2-amino-ethane sulphonic acid) injected into liquid nitrogen) was ineffectual since many of the rubber particles coagulated upon subsequent thaw. Coagulated rubber is useless in biochemical investigations. Also, the degree of coagulation increases with the purity of the rubber particle preparations due to reduction of protective cytoplasmic constituents and therefore we included glycerol as an anticoagulant. Concentrations as low as 5% were found to prevent coagulation of purified rubber particles during freeze-thaw in all three species. Glycerol concentration dependencies also demonstrated that glycerol has little effect on rubber transferase activity (data not shown).

Storing the rubber particles as beads in liquid nitrogen successfully maintained rubber transferase activity for over 100 days in both *P. argentatum* (Fig. 3) and *H. brasiliensis*

(Fig. 4), even though at 4°C activity in *P. argentatum* rubber particles was much less stable than in *H. brasiliensis* particles (cf. Figs. 3 and 4). In addition, we found that glycerol has a direct protective effect, in addition to its anti-coagulant property, that was most noticeable in the *P. argentatum* rubber particles (see day 1 and 2 at 4°C in Fig. 3). The disparate *in vitro* enzymatic stability of purified *H. brasiliensis* and *P. argentatum* particles may be related to their distinct surface protein concentrations (Cornish *et al.*, 1993). *H. brasiliensis* particles contain many more proteins and at a higher overall concentration than *P. argentatum*, and these endogenous proteins may help protect particle bound enzyme activity.

The effectiveness of the method described in this paper for *F. elastica* rubber particles was examined since these particles are considerably larger (ca 3.8 µm in diameter) than those of *H. brasiliensis* (ca. 1.0 µm in diameter) or of *P. argentatum* (ca. 1.4 µm in diameter) and contain rubber of much lower molecular weight (ca. 10³ Da as opposed to over 10⁶ for the other two species; Cornish *et al.*, 1993). Thus *F. elastica* represents one extreme of rubber particle variety whilst *P. argentatum* and *H. brasiliensis* represent another. Activity was stable in rubber particle beads suspended in 5% or 30% glycerol and stored in liquid nitrogen, but when beads were stored at 4°C, 90% of the activity was lost over seven days, and this places *F. elastica* stability between that of *P. argentatum* (Fig. 3) and *H. brasiliensis* (Fig. 4). Glycerol did not appear to have an additional protective effect in these samples.

As an additional check on the validity of this storage method for maintenance of rubber transferase activity, kinetic studies were performed on all three species (IPP and FPP concentration dependencies). Enzyme activity in

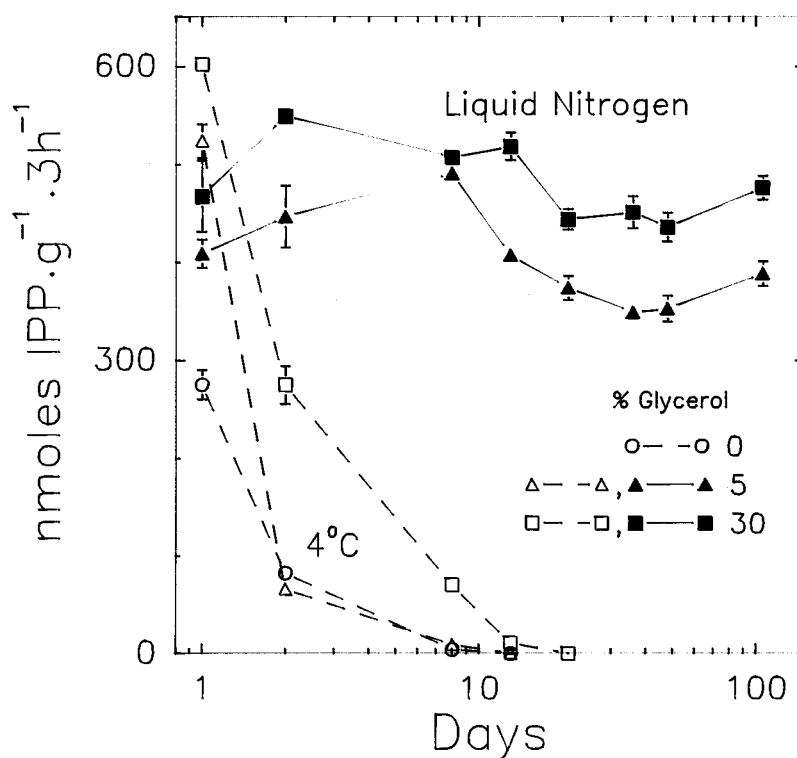


Figure 3. Rubber transferase activity in particles purified from *P. argentatum*, line 11591, and stored at different glycerol concentrations in either at 4°C (open symbols) or as beads frozen in liquid nitrogen (closed symbols). Residual activity in rubber particles (31.2 mg/mL) was assayed in the presence of 100 µM [¹⁴C]IPP and 20 µM FPP for 3 h at 16°C. Each value is the mean of four assays ± SE (SE values smaller than the symbol size are not shown).

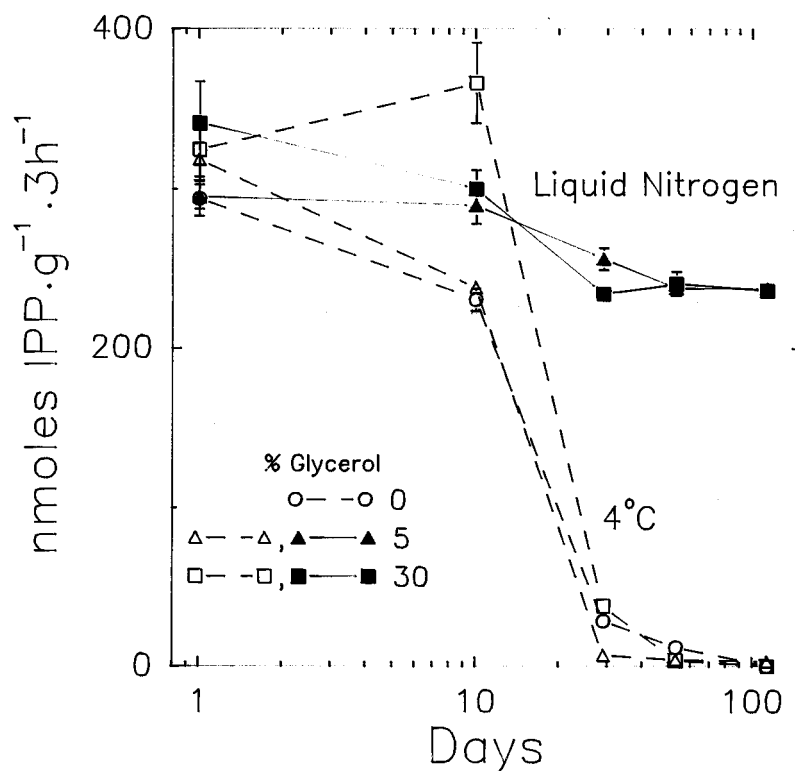


Figure 4. Rubber transferase activity in particles purified from *H. brasiliensis* and stored at different glycerol concentrations in either at 4°C (open symbols) or as beads frozen in liquid nitrogen (closed symbols). Residual activity in rubber particles (49.6 mg/mL) was assayed in the presence of 100 μM [^{14}C]IPP and 20 μM FPP for 3 h at 25°C. Each value is the mean of four assays \pm SE (SE values smaller than the symbol size are not shown).

freshly prepared particles, and liquid nitrogen-stored and thawed rubber particles, was biochemically indistinguishable (data not shown). Stability was not affected by the concentration of the purified rubber particles being stored.

The long term effectiveness of this glycerol/liquid nitrogen/rubber particle beading method is illustrated by our

successful maintenance of enzymatically active purified rubber particles (stored in 10% glycerol as beads in liquid nitrogen) for two years, in all three species (data not shown). The successful employment of this method for the dissimilar rubber particles tested, suggests a high likelihood of its practical application to all rubber producing species.

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