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RESEARCH PAPER

The micromorphology and protein characterization of rubber particles in *Ficus carica*, *Ficus benghalensis* and *Hevea brasiliensis*

Adya P. Singh[1], Seung Gon Wi[1], Gap Chae Chung[2], Yoon Soo Kim[1] and Hunseung Kang[2,3,4]

[1] *Department of Forest Products and Technology, College of Agriculture and Life Sciences, Chonnam National University, 300 Yongbong-dong, Buk-gu, Gwangju, 500-757 Korea*

[2] *Division of Applied Plant Science and Agricultural Plant Stress Research Center, College of Agriculture and Life Sciences, Chonnam National University, 300 Yongbong-dong, Buk-gu, Gwangju, 500-757 Korea*

[3] *Kumho Life and Environmental Science Laboratory, 1 Oryong-dong, Buk-gu, Gwangju, 500-712 Korea*

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Abstract

Rubber biosynthesis takes place on the surface of rubber particles. These particles are surrounded by a monolayer membrane in which the rubber transferase is anchored. In order to gain better insight into whether rubber particles from different plant species share common structural characteristics, the micromorphology of rubber particles from *Ficus carica*, *Ficus benghalensis,* and *Hevea brasiliensis* was examined by electron microscopy. Rubber particles of all three species were spherical in shape, and the size of rubber particles of *H. brasiliensis* was much smaller than those of *F. carica* and *F. benghalensis*. In addition, investigations were undertaken to compare the cross-reactivity of the antibody raised against either the *H. brasiliensis* small rubber particle protein (SRPP) which is suggested to be involved in rubber biosynthesis, or the *cis*-prenyltransferase (CPT) which has an activity similar to rubber transferase. Both western analysis and TEM-immunogold labelling studies showed that rubber particles of *F. carica* and *F. benghalensis* do not contain the SRPP. None of the rubber particles in *F. carica*, *F. benghalensis* and *H. brasiliensis* contained the CPT, suggesting that the CPT itself could not catalyse the formation of high molecular weight rubber. These results indicate that rubber particles in the three different plant species investigated share some degree of similarity in architecture, and that the SRPP and CPT themselves are not the core proteins necessary for rubber biosynthesis.

Key words: Electron microscopy, *Ficus benghalensis, Ficus carica, Hevea brasiliensis*, immunolocalization, micromorphology, rubber particle, rubber particle protein.

Introduction

The surface of rubber particles contains the enzymes and/or factors necessary for rubber biosynthesis, and is the place where rubber biosynthesis occurs (Benedict *et al*., 1990; Cornish and Backhaus, 1990; Cornish, 2001). Rubber particles are globular particles in which the hydrophobic rubber polymers are surrounded by a monolayer membrane containing various kinds of lipids, proteins, and other components (Hasma and Subramaniam, 1986; Hasma, 1991; Siler *et al*., 1997; Cornish *et al*., 1999; Wood and Cornish, 2000). Since rubber transferase, the key enzyme for rubber biosynthesis, is one of the proteins associated with the monolayer membrane, it is considered a membrane protein. It loses its activity once it is isolated from rubber particles. Therefore, in an effort to identify the rubber transferase and/or additional factors important for rubber biosynthesis, many research groups have investigated rubber particles and the proteins associated with them in *Hevea brasiliensis* (Attanyaka *et al*., 1991;

[4] To whom correspondence should be addressed at Chonnam National University: Fax: +82 62 530 2047. E-mail: hskang@chonnam.chonnam.ac.kr
Abbreviations: CPT, *cis*-prenyltransferase; CPD, critical point drying; IDP, isopentenyl diphosphate; SEM, scanning electron microscopy; SRPP, small rubber particle protein; TEM, transmission electron microscopy.

Cornish, 1993; Dennis and Light, 1989; Goyvaerts *et al*., 1991; Light and Dennis, 1989; Oh *et al*., 1999), *Parthenium argentatum* Gray (guayule) (Backhaus *et al*., 1991; Benedict *et al*., 1990; Cornish and Backhaus, 1990; Cornish *et al*., 1994; Pan *et al*., 1995; Siler and Cornish, 1993), *Ficus elastica* (Cornish and Siler, 1996; Siler and Cornish, 1994), *Ficus carica* (Kang *et al*., 2000*a*), and *Ficus benghalensis* (Kang *et al*., 2000*b*).

Despite continued efforts to identify rubber transferase, the nature of this extremely interesting enzyme has not yet been verified. In *H. brasiliensis* a 14 kDa protein tightly associated with the large rubber particles was suggested to be a rubber elongation factor (Dennis and Light, 1989), and its gene has been cloned (Attanyaka *et al*., 1991; Goyvaerts *et al*., 1991). However, the direct role of this protein in rubber biosynthesis has not been proven. More recently, Oh *et al*. (1999) have isolated a cDNA from *H. brasiliensis* which encodes a major rubber particle protein of 24 kDa, tightly associated with small rubber particles (designated SRPP). They demonstrated that the SRPP plays a positive role in isopentenyl diphosphate (IDP) incorporation into the rubber polymer. In guayule, the most abundant rubber particle protein of 52 kDa was sequenced to be a P450 allen oxide synthase (Pan *et al*., 1995). Based on a series of cross-specific immunoinhibition analyses, a 375 kDa protein was suggested to be a rubber transferase in *F. elastica* (Cornish *et al*., 1994; Siler and Cornish, 1993, 1994). Proteins present in the latex and rubber particles of *F. benghalensis* have been analysed, and the protein of 31 kDa was found to be the most abundant in catalytically-active rubber particles (Kang *et al*., 2000*b*).

Due to the presence of the enzymes for rubber biosynthesis in rubber particles, it is important to investigate the structure of rubber particles and to characterize the rubber particle-associated proteins in various plant species in order to identify the rubber transferase and/or additional important factors for rubber biosynthesis and also to understand the relationship between the size or composition of rubber particles and the commercial value of rubbers. The microstructure and particle size distribution of rubber particles from *H. brasiliensis*, *P. argentatum*, *F. elastica*, and *Euphorbia lactiflua* have been characterized by various electron microscopic techniques (Gomez and Hamzah, 1989; Yeang *et al*., 1995; Cornish *et al*., 1999; Wood and Cornish, 2000). However, studies involving immunolocalization of the membrane proteins are limited. Bahri and Hamzah (1996) used an immunogold labelling technique in conjunction with electron microscopy to confirm the presence of the 14 kDa protein and SRPP on the surface of *H. brasiliensis* rubber particles.

In this study, the micromorphology of rubber particles was examined from *F. carica* and *F. benghalensis*, the *Ficus* species recently identified as rubber-producing plants by this group (Kang *et al*., 2000*a*, *b*), and compared it with that from *H. brasiliensis*. In addition, in order to understand whether rubber particles of these three plant species contain similar proteins, the presence of those rubber particle proteins deemed important was investigated by western analysis and transmission electron microscopy (TEM)–immunogold cytochemistry. One of the proteins investigated in this study was the SRPP. This major rubber particle protein in *H. brasiliensis* has been suggested to be involved in rubber biosynthesis (Oh *et al*., 1999). The other protein was the *cis*-prenyltransferase (CPT) that has a catalytic activity similar to rubber transferase. It catalyses a sequential condensation of IDP with allylic diphosphate, but produces the shorter chain length polyprenyl diphosphates ranging in carbon number from C50120 (Ogura and Koyama, 1998). It was, therefore, important to investigate the presence of these proteins on the surface of rubber particles in *H. brasiliensis*, *F. carica*, and *F. benghalensis*, and biochemical and TEM-immunogold techniques were used to obtain the information.

Materials and methods

Plant material

The latex samples of *F. carica* and *F. benghalensis* were obtained from the plants grown in a greenhouse maintained at 30 °C under constant light. *H. brasiliensis* latex was collected from regularly tapped rubber trees (*H. brasiliensis* RRIM600) at the Rubber Research Institute of Malaysia, and the latex samples on ice were shipped to this laboratory. The latex was collected directly into the ice-cold buffer containing 100 mM TRIS-HCl, pH 7.5, 5 mM $MgSO_4$, and 5 mM dithiothreitol.

Preparation of washed rubber particles

The latex of *H. brasiliensis* was centrifuged at 20 000 *g* for 30 min at 4 °C, and the latex of *F. carica* and *F. benghalensis* was centrifuged at 10 000 *g* for 5–10 min at 4 °C. The top creamy fraction of rubber particles was collected, resuspended in the same buffer, and recentrifuged. The rubber particles of *F. carica* and *F. benghalensis* sedimented during the washing cycle, whereas the *Hevea* rubber particles remained afloat. The non-rubber fractions were discarded, and this washing procedure was repeated three times as described previously (Cornish and Backhaus, 1990; Siler and Cornish, 1993; Kang *et al*., 2000*a*). The washed rubber particles were used directly for western blot and immunogold label analyses.

Western blot analysis

Rubber particle proteins were solubilized by incubating the rubber particles in a detergent solution containing 0.1% (w/v) Triton X-100 and 1% (w/v) SDS. The detergent-treated suspension was centrifuged at 13 000 rpm for 10 min, and the supernatant fraction was separated by SDS–12% PAGE. The gels were subsequently used for western blotting where the proteins in the gel were transferred to a polyvinylidene difluoride membrane. For the detection of the SRPP, the membrane was incubated with the buffer containing the polyclonal rabbit antibody raised against the SRPP from *H. brasiliensis* as described earlier (Kang *et al*., 2000*a*). For the detection of the *cis*-prenyltransferase, the polyclonal rat antibody was raised against the synthetic peptide (DLMIRTSGEQRISNF) in which the sequence was designed based on the conserved amino acid sequence of CPTs from plants and micro-organisms, which corresponds to the positions from 246 to 260 of *Arabidopsis* CPT (Oh *et al*.,

2000). After three cycles of washing with TBS-T buffer or PBS-milk, respectively, the membrane was incubated for 1 h with anti-IgG antiserum conjugated to horseradish peroxidase (Amersham Pharmarcia Biotech). After a further three cycles of washing with the same buffer, the proteins on the membrane were detected by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

Electron microscopy

The shape and diameter of rubber particles were determined by scanning electron microscopy (SEM). Initial attempt to characterize particle shape and diameter using critical point drying (CPD), a widely used method for studying biological materials, proved unsuccessful because this method caused the severe collapse of particles. A novel air-drying method that was developed specifically for this work, and which proved most suitable, is described here. The purified rubber fraction from freshly collected latex was fixed in 3% glutaraldehyde in 50 mM sodium cacodylate buffer (containing 1% tannic acid) for 1 h at room temperature. After washing in several changes of buffer, the samples were post-fixed in 1% aqueous osmium tetroxide also for 1 h at room temperature, and subsequently washed in several changes of distilled water. A small drop of the suspension (appropriately diluted) was air-dried on a piece of cover glass. The cover glass was then placed on a stub applied with a double-sided tape, sputter-coated with gold and examined with a Hitachi S-2400 SEM.

Immunocytochemistry

The initial attempts to process samples using the fixation procedures employed routinely for immunocytochemical work were not successful. Neither the paraformaldehyde–glutaraldehyde (2.5%: 0.5%) combination nor glutaraldehyde (3%) alone could stabilize the spherical form of rubber particles. The glutaraldehyde–tannic acid combination, as used for SEM work, was effective in keeping the natural form of rubber particles: however, gold-labelling was poor and non-specific. The method that worked best was fixation with osmium tetroxide.

The purified rubber fraction from *H. brasiliensis*, *F. carica*, and *F. benghalensis* was fixed with 1% osmium tetroxide (in 50 mM sodium cacodylate buffer) for 1 h at room temperature. After washing in buffer, a small drop of the rubber was placed on formvar-coated nickel grids and air-dried. Procedure for immunogold labelling was similar to that described by Kim *et al*. (2002). Prior to treatment with the primary antibody, grids were sequentially floated on drops of glycine (in PBS buffer), distilled water, PBS buffer, and normal goat serum (NGS)–PBS buffer. Grids from all samples were treated with the SRPP antibody (1:200 dilution) and CPT antibody (1:100 dilution) for 90 min at 37 °C. After sequential washes in PBS–non-fat milk–Tween 20, PBS, and TRIS-HCl, samples were treated with the following gold conjugated (10 nm) secondary antibodies (1:20 dilution) for 2 h at room temperature. Samples exposed to the SRPP antibody were treated with anti-rat IgG and those exposed to the CPT antibody were treated with anti-rabbit IgG. Samples were then washed sequentially with TBS-tween 20, TBS, TRIS-HCl, and distilled water. For the control, samples were processed as above, except that they were not incubated with the primary antibodies. Grids were air-dried and examined with a JEOL JEM1010 TEM.

Results

Comparison of the shape and size of rubber particles

Rubber particles of all three species, *H. brasiliensis, F. carica*, and *F. benghalensis*, were predominantly spherical in shape (Fig. 1). Only in *F. benghalensis* were some particles also elongated, but this appears to have been induced by centrifugation and thus is not a normal shape. Although several size populations of particles were observed for all species examined, the range was greatest for *Hevea*, which also had the smallest size particles (Figs 1A, 2). Only marginal differences were observed between *F. carica* and *F. benghalensis* size populations as well as in the size of individual particles (Figs 1B, C, 2). For *Hevea*, it was possible to categorize particles into three size groups: largest particles with a diameter range of 0.4–0.75 μm, intermediate size particles with a diameter range of 0.25–0.35 μm, and the smallest particles with a diameter range of 0.08–0.2 μm. The particles of *F. carica* and *F. benghalensis* could be categorized into two size groups, although the size range for the largest particles of *F. benghalensis* was somewhat greater compared with *F. carica*. Also, the largest particles of *F. carica* were slightly larger than those of *F. benghalensis*. Smaller sized particles were less abundant than larger sized particles for both species. The largest particles of *F. carica* measured 3.7–6.5 μm in diameter, and those of *F. benghalensis* measured 3.0–6.0 μm. The smaller particles of *F. carica* measured 1.6–3.0 μm and those of *F. benghalensis* measured 1.6–2.3 μm.

Western analysis of the proteins on the surface of rubber particles

The presence of the two proteins, the SRPP and CPT that are suggested to be involved in rubber biosynthesis, was investigated by western analyses. Specifically, it was necessary to probe whether the rubber particles of *F. carica*, *Hevea* rubber tree, and *F. benghalensis* contain the two proteins on their surfaces. As shown in Fig. 3A, the SRPP and the 14 kDa protein on *Hevea* rubber particles cross-reacted with the SRPP antibody. The cross-reactivity of both the SRPP and the 14 kDa protein with the polyclonal antibody raised against the SRPP was well established, since the SRPP and 14 kDa protein share a high degree of sequence homology in their amino acid sequences (Oh *et al*., 1999; Kang *et al*., 2000*a*). By contrast, neither the *F. carica* proteins nor the *F. benghalesis* proteins reacted with the SRPP antibody. These results indicate that the SRPP, abundantly present on the rubber particles of *Hevea* rubber trees, is not a common protein necessary for rubber biosynthesis in rubber-producing plants.

For the detection of the CPT, the polyclonal rat antibody was raised against the synthetic peptide (DLMIRTSGE-QRISNF) in which the sequence was designed based on the conserved amino acid sequence of CPTs from plant and micro-organisms, and corresponds to the positions from 246 to 260 of *Arabidopsis* CPT (Oh *et al*., 2000). Figure 3B shows that the CPT antibody did not react with any of the proteins isolated from the rubber particles of *F. carica*, *H.*

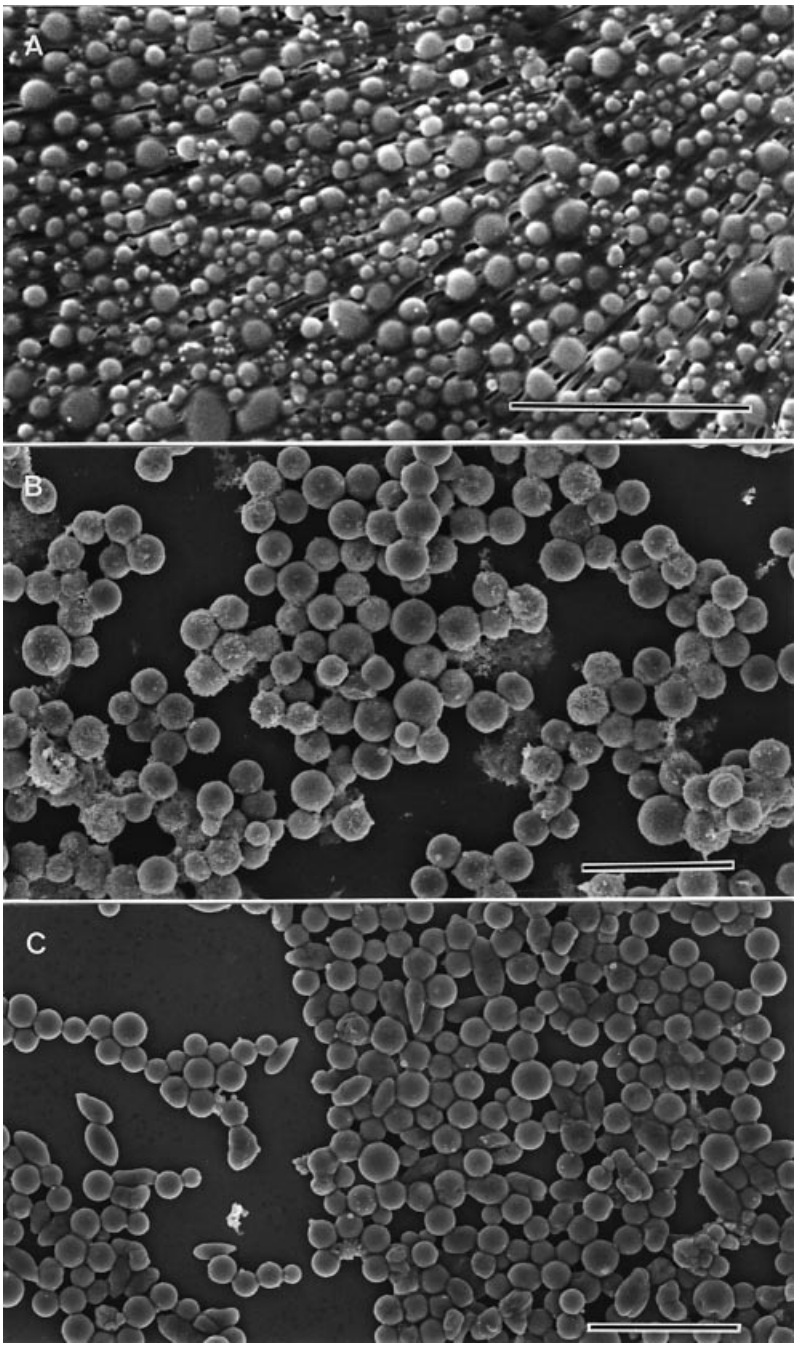

Fig. 1. (A) *H. brasiliensis* rubber particles. The particles are spherical and vary greatly in their size. SEM. Bar=5 μm, (B) *F. carica* rubber particles. The particles are much larger than *Hevea* rubber particles and less variable in their size. SEM. Bar=20 μm. (C) *F. benghalensis* rubber particles. The particles are much larger than those of *H. brasiliensis*, but are similar in size to *F. carica*. The majority of particles are spherical, but some are oblong. SEM. Bar=20 μm.

brasiliensis, and *F. benghalensis*. Since the CPT is ubiquitous in all living organisms, the protein extract isolated from tobacco leaves was used as a positive control. The tobacco leaf protein of about 32 kDa in size was clearly reacted with the CPT antibody. The absence of cross-reactivity of the proteins isolated from *F. carica*, *H. brasiliensis*, and *F. benghalensis* with the CPT antibody indicates that *cis*-prenyltransferase does not reside on the surface of rubber particles, and is not a key determinant necessary for rubber biosynthesis in plants.

Immunogold labelling of the proteins on the surface of rubber particles

The TEM examination of rubber samples, which had been incubated with the SRPP antibody and subsequently treated with gold conjugated secondary antibody, provided

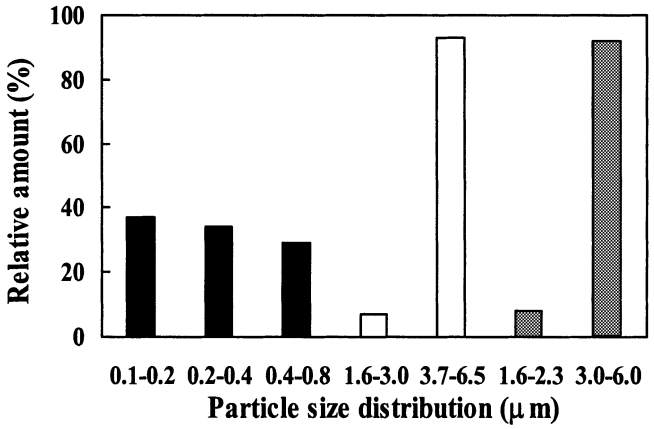

Fig. 2. Size distribution of rubber particles in *H. brasiliensis* (black bar), *F. carica* (white bar), and *F. benghalensis* (grey bar).

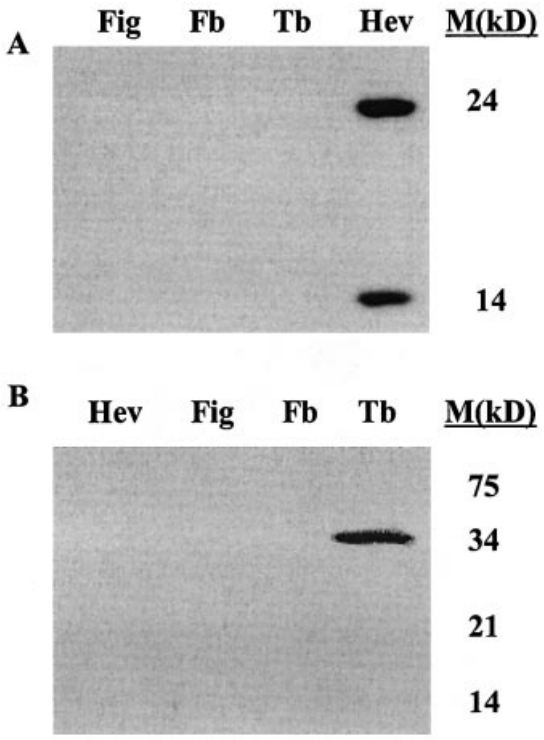

Fig. 3. Western analyses of the solubilized rubber particle proteins. The proteins were separated on SDS-12% polyacrylamide gel and transferred to the membranes, which were reacted with the antibody raised against either the SRPP (A) or the CPT (B). Hev, *H. brasiliensis*; Fig, *F. carica*; Fb, *F. benghalensis*; Tb, tobacco.

evidence for intense gold labelling of *H. brasiliensis* rubber particles, and the absence of labelling of *F. carica* and *F. benghalensis* rubber particles. Several *H. brasiliensis* rubber particles are shown at low magnification in Fig. 4A. At this magnification gold particles were just detected as small, electron-dense granules associated exclusively with rubber particles, and there were no indications of any background labelling. Gold particles were attached to the margins of all rubber particles and, in the case of the smaller particles, which are less dense, gold particles also appeared to be distributed all over the surface. Gold particle labelling is more clearly visible in the high magnification view in Fig. 4B. The size of rubber particles is variable in the population shown in this figure. All rubber particles were intensely labelled, and numerous gold particles were associated with the margins of the particles. Where resolvable, gold particles could also be seen all over the surface of rubber particles. There appeared to be no difference between large and small rubber particles of *H. brasiliensis* with regard to labelling intensity. However, confirmation of this point will require quantification, which was not undertaken in this work. The complete absence of gold labelling of *F. carica* and *F. benghalensis* rubber particles is shown in Fig. 5. TEM examination of rubber samples, which had been incubated with the CPT antibody and subsequently treated with gold conjugated secondary antibody, did not show any gold labelling of rubber particles in all the species examined (data not shown).

Discussion

Observations of the size, form and architecture of rubber particles, and characterization of the proteins on the surface of rubber particles are of great importance in understanding the mechanism of rubber chain elongation and chain length determination, and also in relation to the properties of the final rubber products. It was stated at the outset that microscopic characterization of rubber particles is difficult (Condon and Fineran, 1989) probably because the membrane which encloses rubber material within individual particles is highly sensitive to the chemical treatments used in conventional microscopy preparations. Aldehydes destabilize latex particles, rendering them susceptible to fusion, and dehydrating agents, such as acetone and ethanol, cause extraction of internal contents (Condon and Fineran, 1989). It is therefore not surprising that the initial attempts to process rubber samples by conventional preparation methods for SEM and TEM observations were unsuccessful. Tannic acid is known to stabilize some tissue components against extraction (Simionescu and Simionescu, 1976), which was used as an additional fixative mixed with aldehyde. Tannic acid has also been used to increase osmification of tissue culture cells (Katsumoto *et al.*, 1981). In the SEM work, initial fixation was with a combination of glutaraldehyde and tannic acid, and post-fixation with osmium tetroxide. It appears that a combination of these treatments stabilized rubber particles adequately for them to withstand the forces of surface tension generated during air-drying. Interestingly, the same fixation procedure used in conjunction with CPD failed to stabilize particles, large

populations of which were severely collapsed. It is likely that the bounding membrane was not completely stabilized and became leaky during the ethanol-dehydration step of CPD, resulting in the extraction of the internal contents of rubber particles, and their consequent collapse. This highlights the importance of developing a suitable method for preserving rubber particles in their natural size and form, because these, together with compositional characteristics, are likely to be important factors in determining the properties of rubber in industrial processing.

The shape and size distribution of the rubber particles detected in this study compare well with the previously reported results (Gomez and Hamzah, 1989; Cornish *et al*., 1993; Wood and Cornish, 2000). Among the three plant species examined, *H. brasiliensis,* producing high molecular weight rubber, had the smallest rubber particles and *F. carica,* producing low molecular weight rubber, had the largest rubber particles. It has been suggested in the previous report that higher molecular weights were associated with the smaller rubber particles (Yeang *et al*., 1995). However, more extensive analysis is required for a

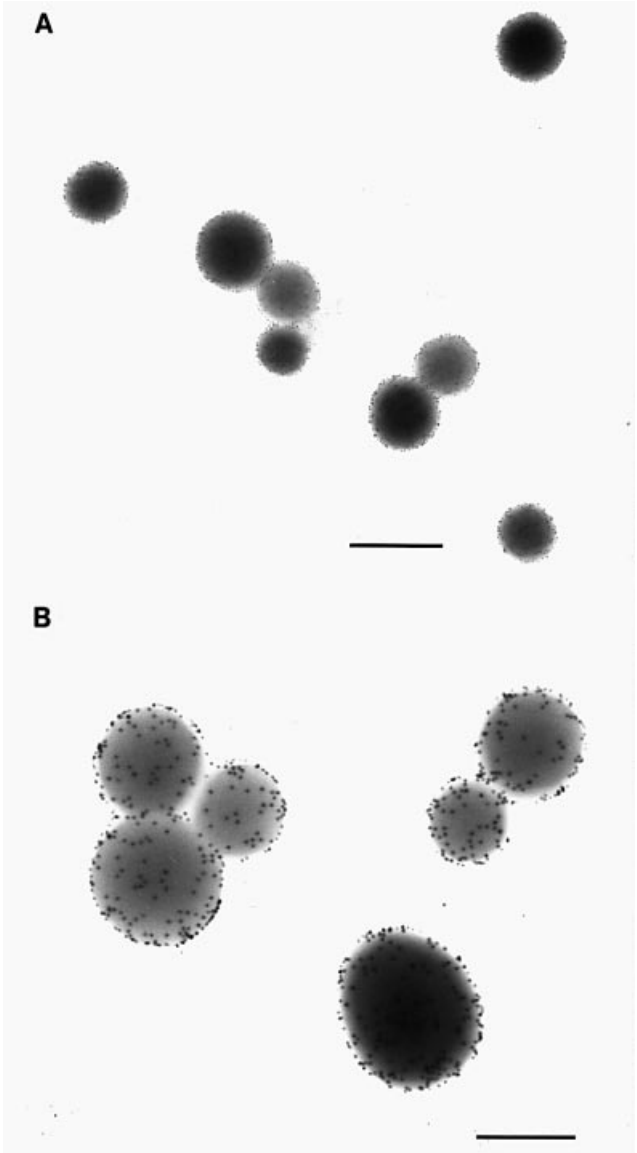

Fig. 4. *H. brasiliensis* rubber particles which had been incubated with the SRPP antibody and subsequently treated with gold conjugated secondary antibody. All rubber particles are intensely labelled, and there is virtually no background labelling. (A) Gold particles appear small at this magnification, but are distinguishable. TEM. Bar=500 nm. (B) At this high magnification gold particles are clearly visible, and are associated with the margin of rubber particles. In less dense rubber particles, gold particles can also be seen over the entire surface. TEM. Bar=200 nm.

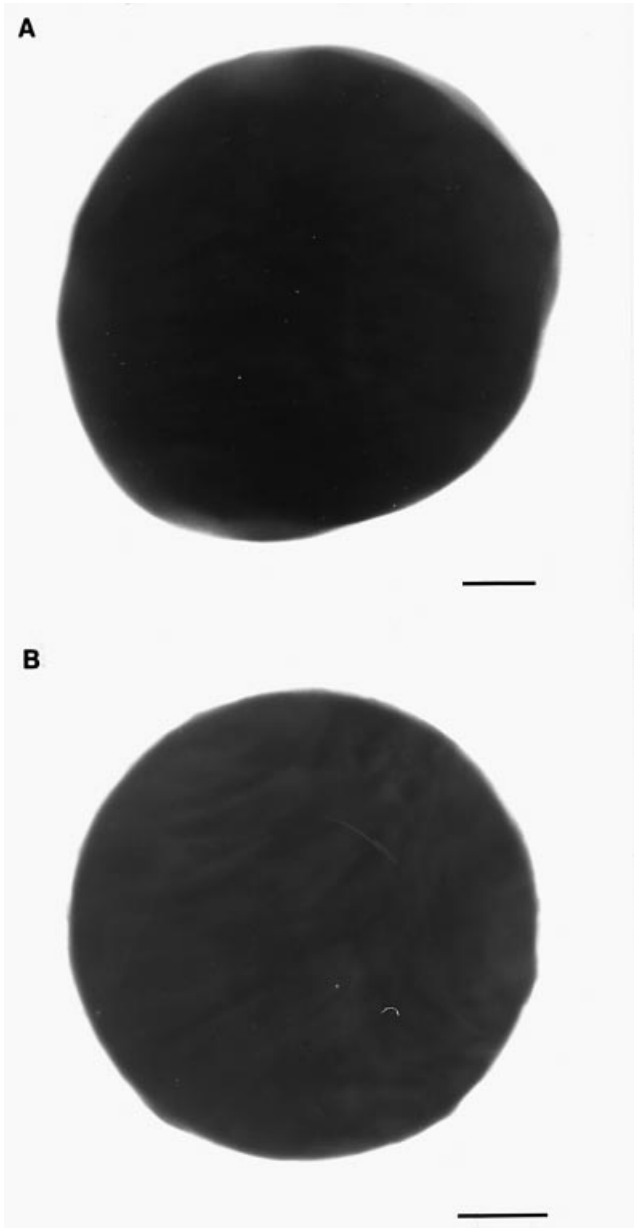

Fig. 5. The rubber particles of (A) *F. carica* and (B) *F. benghalensis*, which had been incubated with the SRPP antibody and subsequently treated with gold conjugated secondary antibody. There is complete absence of gold labelling. TEM. Bar=500 nm.

better understanding of the correlation between rubber particle size and the molecular weight of rubber in plants.

Conventionally, a mixture of paraformaldehyde and glutaraldehyde has been used to fix biological materials in immuno-TEM studies. In the fixation medium, these components are used in such a proportion that a proper balance between adequate fixation of cell components and antigenicity is achieved. However, in this immuno-TEM work, glutaraldehyde-based fixation resulted in poor stabilization of rubber particles and non-specific gold labelling. The fixation of the rubber fraction directly in osmium tetroxide proved to be the most satisfactory method. Although for immunocytochemistry this is not a fixative of choice because of concerns that it may adversely affect tissue antigenicity, the high labelling intensity of *H. brasiliensis* rubber particles, which had been incubated in the SRPP antibody, suggests that immunogenic properties of the bounding membrane of rubber particles were not affected by osmium fixation, and this is a suitable fixation method for rubber material.

The role of the SRPP in rubber biosynthesis has not been clearly verified. The study has provided additional information by investigating whether the SRPP found on the surface of *Hevea* rubber particles is also present on the rubber particles of *F. carica* and *F. benghalensis*. The observations presented here clearly indicate that the rubber particles of *F. carica* and *F. benghalensis* do not contain the SRPP found in *H. brasiliensis* (Figs 4, 5). The absence of the SRPP in *F. carica* and *F. benghalensis* implies that the SRPP, the most abundant protein in rubber tree, is not a universal protein required for rubber biosynthesis in rubber-producing plant species. It has been suggested that the SRPP plays a positive role in rubber biosynthesis (Oh *et al*., 1999). If this protein is a rubber transferase or forms a rubber transferase complex, a similar protein should be present on the rubber particles of every rubber-producing plant species. No cross-reactivity was found of the rubber particle proteins isolated from *F. carica* and *F. benghalensis* with the SRPP antibody, which implies that the SRPP is not a rubber transferase required for rubber chain elongation.

Rubber transferase belongs to a family of CPT that catalyses the synthesis of linear prenyl diphosphates involved in the biosynthesis of various isoprenoid compounds including natural rubber. The plant CPT gene has recently been cloned from *Arabidopsis,* and the analysis has shown that the *Arabidopsis* CPT catalyses the formation of polyprenyl diphosphates with the predominant carbon number C120 (Oh *et al*., 2000). Since no report confirming the function of CPT genes from rubber-producing plants has yet been published, it is of importance to investigate if the CPTs are present on the surface of rubber particles. The western and immunogold label analyses showed that the rubber particles of all three plant species tested, *F. carica, H. brasiliensis*, and *F. benghalensis*, do not contain the CPT. This implies that CPT itself is not a part of the rubber transferase complex. Although the three rubber-producing plants do not contain CPTs on their rubber particles, it would be of interest to isolate the CPT from rubber-producing plant species, and to test if the CPT is involved in catalysing the formation of higher molecular weight polymers similar to natural rubber.

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