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Rubber Transferase Activity and Rubber Particle Sizes Derived from *Lactarius* Mushrooms†

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Upon cutting the fruiting body of *L. volemus*, a white latex was exuded that changed gradually to a brown colour. The rubber particles from *L. volemus* showed a bimodal or trimodal particle size distribution with a mean diameter of 1.10-1.32 μm. A comparison with *Hevea* showed that the rubber particles size in *L. volemus* latex was slightly larger than those of *Hevea*, but showed a similar distribution curve. Five *Lactarius* mushrooms were also examined and showed the characteristic particle size distributions, which varied according to species, suggesting a certain mechanism for controlling the particle size. The *in vitro* rubber biosynthesis using the rubber particles of *L. volemus* is also reported.

Key Words: Natural rubber, Biosynthesis, *Lactarius*, Substrate specificity.

INTRODUCTION

Elasticity is a property of natural rubber that has applications in tyres, gloves, balloons and sports balls. Currently, almost all commercial natural rubber is obtained from *Hevea brasiliensis* due to the high productivity of the plant and the excellent physical properties of the rubber produced. In laticiferous plants, such as *H. brasiliensis* and Indian rubber tree (*Ficus elasica*), rubber is packed in discrete particles located in latex vessels, whereas in non-laticiferous plants, such as the Guayule shrub, the rubber is found in the roots and stem parenchymal cells. Biochemical studies of rubber formation in *H. brasiliensis* showed that rubber is synthesized on the surface of rubber particles, possibly by the elongation of existing molecules[1], catalyzed by an enzyme identified as rubber transferase[2,3]. This enzyme has been demonstrated to be particle-bound in rubber-bearing plants, such as the guayule[4,5] and *F. elasica*[6].

A bimodal distribution of particle sizes has been reported for *H. brasiliensis*, with peaks at 0.30 and 0.70 μm[7]. Washed rubber particles isolated from *F. elasica* are substantially larger (approximately three times larger) than those from *H. brasiliensis*[8]. Previous studies have reported that only smaller rubber particles show a high degree of chain elongation enzymatic activity[9]. The larger rubber particles found in the cream fraction, which are separated during centrifugation, contain long-chain fatty acid esters. The small rubber particles in the serum fraction, however, are composed of linear molecules without ester groups. This suggests that a large portion of rubber particles in fresh latex is composed of terminated rubber molecules. Many biochemical and structural studies have been conducted to clarify the mechanism of rubber biosynthesis. On the other hand, the enzyme system involved in rubber formation has not been resolved. The present study was designed to provide new information on natural rubber biosynthesis using *Lactarius* mushroom models, which produce low molecular weight rubber[10,11].

EXPERIMENTAL

Synthesis of substrates: Dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), *trans,trans*-farnesyl diphosphate (FPP), *trans,cis*-farnesyl diphosphate, *tarns, trans, trans*-geranylgerany diphosphate (GGPP) and *trans,trans,cis*-geranylgeranyl diphosphate were prepared using the method reported by Davisson *et al.*[12].

Particle size distribution: The particle size distribution was determined using a Coulter LS230 light scattering particle analyzer (Beckman Coulter, USA). Distilled water was used as the dispersant for the mushroom-derived latex.

Incubation conditions: The incubation mixture for enzymatic analysis contained, in a final volume of 1 mL, 50 mM *Tris*-HCl buffer pH 7.5, 5 mM $MgCl_2$, 100 mM KF, 50 mM 2-mercaptoethanol, 0.92 μM, 1.95 GBq/mol [1-^{14}C]IPP (1.06×10^5 dpm per incubation), 2.5 μM of allylic primer and

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200 µL of the *L. chrysorrheus* rubber particle suspension. The mixture was incubated for 19 h at 27 ºC. Incubation was terminated by the addition of 1 mL of a saturated, aqueous NaCl solution. The incubation mixture was washed thoroughly with diethyl ether to remove the alcohols produced by the action of phosphatase in the *L. chrysorrheus* rubber particle suspension. The enzymatic products were then extracted with hexane-toluene (1:1) and the radioactivity of the extracts was measured using a liquid scintillation counter (Aloka, type LSC-1000, Japan).

Determination of the molecular weight distribution (MWD) of the radiolabeled rubber: The molecular weight distribution of the polymer was determined by gel-permeation chromatography (GPC) using a GPC column packed with polystyrene gel. The measurements were made using tetrahydrofuran as the eluent at a flow rate of 1.0 mL/min at 35 ºC. Commercially-obtained standard polystyrenes were used for column calibration. The eluent from the columns was collected at 0.5 min intervals and assayed for their radioactivity. The elution profile of the radiolabeled rubber was compared with the molecular weight distribution profile of the endogenous rubber.

RESULTS AND DISCUSSION

Particle size distribution of rubber in the latex from the sporophores of *Lactarius* mushrooms: After cutting the fruiting bodies of several species of mushrooms, the latexes exuded gradually changed colour. Only the latexes from *L. vellereus* and *L. subzoarius* were stable and retained their natural colour. Table-1 lists the colours of the latexes exuded from various species. The latex from *L. volemus* showed a bimodal or trimodal particle size distribution with a mean diameter of 1.10-1.32 µm (Fig. 1). *Hevea* rubber also showed a bimodal particle size distribution[13]. The *L. volemus* latex particles were slightly larger than those derived *Hevea*, but appeared to have a similar distribution curve. Fig. 2 shows the rubber particle size distributions of the other *Lactarius* mushrooms. The *L. chrysorrheus* rubber particles showed a bimodal distribution with peaks at 0.14 and 1.92 µm. The *L. hatudake* rubber particles had a unimodal distribution with a mean diameter of 1.91 µm, which is larger than those derived from the other species. *L. subzoarius* also showed a single peak centered at 0.47 µm, which was similar to the smaller peak for the latex particles from *L. volemus*. *L. vellereus* demonstrated a unique, broad, unimodal size distribution, ranging from 0.05 to 3.01 µm.

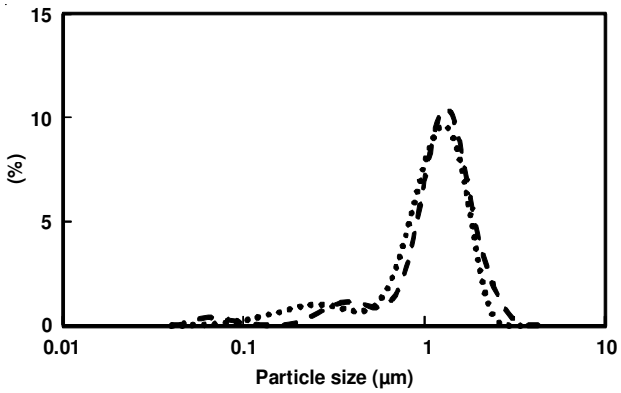

Fig. 1. Rubber particle size distributions from latex of (a) stem of *L. volemus*, (b) gills of *L. volemus*

TABLE-1
COLOUR OF LATEX EXUDED FROM *Lactarius* spp. MUSHROOMS INITIALLY UPON CUTTING OF THE FRUITING BODY AND AFTER EXPOSURE TO AIR

Mushroom species	Initial latex colour	Final latex colour
L. volemus	White	Brown
L. hatudake	Dark brown	Dark green
L. chrysorrheus	White	Yellow
L. vellereus	White	White
L. subzoarius	Brown	Brown

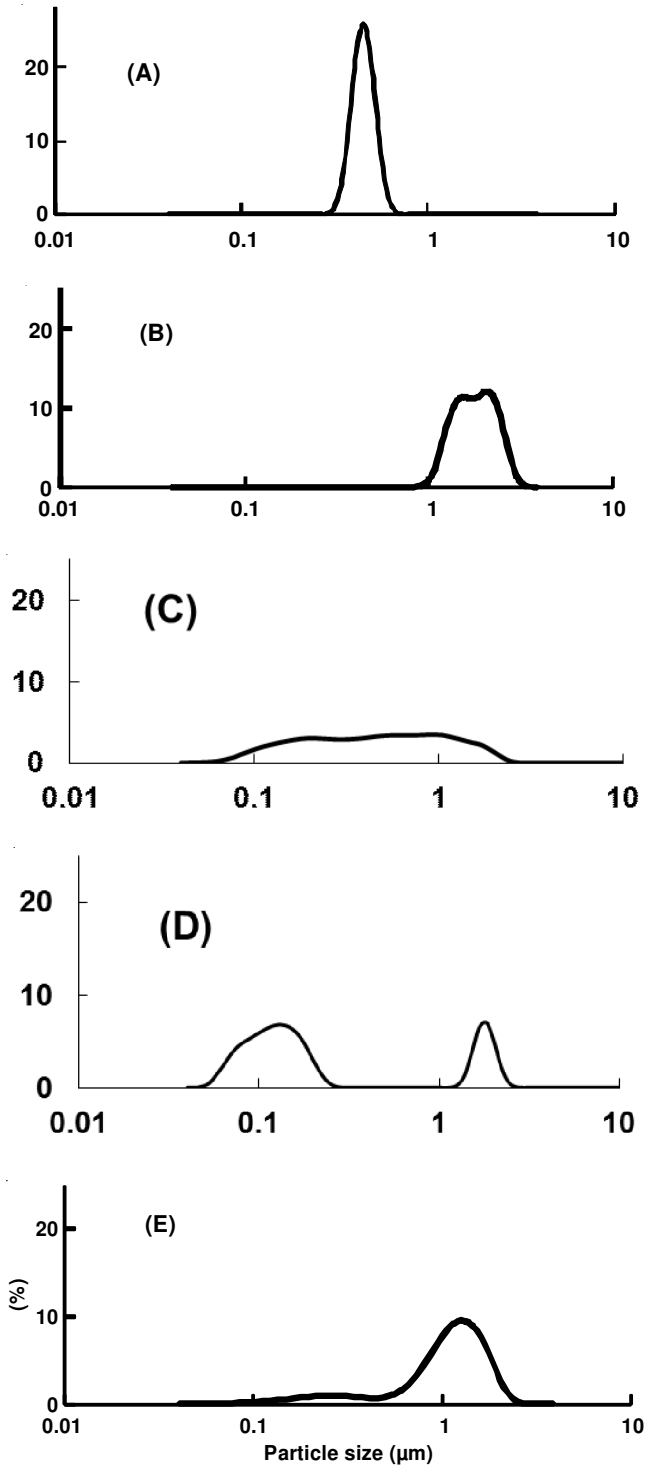

Fig. 2. Rubber particle size distributions from latex of (A) *L. subzonarius*, (B) *L. hatudake*, (C) *L. vellereus*, (D) *L. chrysorrous*, (E) *L. volemus*

Small rubber particles derived from *Hevea* sp., are composed of both low and high molecular-weight rubber molecules, whereas the larger rubber particles (> 0.25 μm) consist mainly of low molecular-weight rubber molecules[14]. The contents of the ester groups attached at the terminus of each rubber chain also changed with the diameter of the *Hevea* rubber particles[14]. The ester group appeared to participate in the branch structures of *Hevea* rubber molecules, whereas *Lactarius* rubber did not demonstrate a branching structure, producing only linear rubber molecules. Interestingly, the rubber particles derived from *L. volemus* showed a unimodal molecular weight distribution with a shoulder in the high molecular weight region (data not shown). This suggests that the characteristic particle size distribution observed in *Lactarius* rubber particles, which vary according to species, arises from a mechanism that controls the particle size, which is unrelated to the molecular weight regulation.

***In vitro* biosynthetic activity of *L. chrysorrheus* rubber particles:** Archer *et al.* reported that an allylic primer was needed to form a new rubber molecule upon incubation with the WRP of *Hevea* latex[1]. The substrate specificity of rubber biosynthesis was examined using the allylic-PPs described in the experimental section. As shown in Table-2, the use of these substrates caused a significant difference in the *in vitro* rubber biosynthesis activities. The *trans,trans*-FPP was the most active substrate, resulting in an estimated 5 % incorporation of ^{14}C-IPP into rubber. A slight enhancement of the rubber transferase activity was observed for both GPP and *trans,trans,cis*-GGPP, under identical assay conditions to those without the allylic substrates. This indicates that the enzyme exhibits some selectivity for *trans,trans*-FPP primers. NMR studies[15] revealed the presence of both two-*trans* and three-*trans* sequences in the rubber derived from the leaves of goldenrod and sunflower. In addition, ficaprenol-12 and polyprenol-16, as model compounds for natural rubber, showed a small signal due to the unexpected isomeric terminal sequences with a relative intensity of 5 % against the inherent signal corresponding to the two-*trans* type and three-*trans* type sequences, respectively. In contrast, only the two-*trans* type sequence was observed in the rubber from *Lactarus* mushrooms. Several interpretations of the meaning of these observations appear plausible. The selectivity of the primer for rubber transferase in leaves is not highly specific with respect to the chain length of the allylic initiator. The absence of three-*trans* type rubber molecules in *Lactarius* mushrooms molecules might be related to the presence of an enzyme forming *tarns,trans,trans*-GGPP in latex.

TABLE-2
REACTIVITY OF ALLYLIC SUBSTRATE FOR RUBBER TRANSFERASE OF *L. chrysorrheus*

Substrate	Enzymatic activity (dpm)
None	480
DMAPP	578
GPP	1,570
trans,trans-Farnesyl diphosphate	6,540
trans,cis-Farnesyl diphosphate	650
trans,trans,trans-GGPP	730
trans,trans,cis-GGPP	1,320

Molecular weight distribution (MWD) of radiolabeled rubber after the incubation of rubber particles derived from *L. chrysorrheus* with ^{14}C-IPP: Fig. 3 shows the molecular weight distribution of the radiolabeled rubber after incubation of ^{14}C-IPP with rubber particles from *L. chrysorrheus*. The molecular weight distribution of these particles was compared with the molecular weight distribution of unlabeled rubber extracted from the rubber particles. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of the unlabeled rubber were estimated to be 2.9×10^4 and 2.2×10^5, respectively, with broad polydispersity. On the other hand, the radiolabeled rubber showed much lower MWDs, with $M_n = 1.4 \times 10^3$ and $M_w = 1.2 \times 10^4$. The average molecular weight of the radiolabeled rubber was *ca.* 5% of that of the unlabeled material. The maximum molecular weight of the radiolabeled rubber was only 0.5×10^4. In the case of *L. chrysorrheus*, rubber could not be synthesized from rubber particles with IPP, whereas new rubber formed during the incubation of washed *Hevea* rubber particles with ^{14}C-IPP[16]. This suggests that only fresh *Hevea* latex has enzymatic activity or a certain cofactor protein is needed to biosynthesize high molecular weight rubber in *L. chrysorrheus*. The most obvious explanation is the labeled rubber newly formed in vitro might be formed from shorter chain length rubber molecules with an active terminal group and/or initiating substrates, such as FPP, with the incorporation of monomers, ^{14}C-IPP. This was attributed to the unstable latex from *Lactarius* mushrooms.

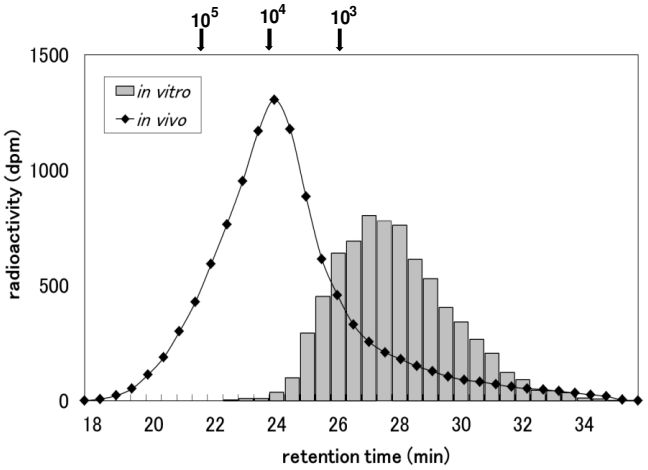

Fig. 3. Molecular weight distribution of *in vitro* and *in vivo* rubber from *L. chrysorrheus*

Conclusion

Rubber particles in the latex from *L. volemus* showed similar distribution curves to *Hevea* latex, but the M_w of *L. volemus* rubber was much smaller than *Hevea* rubber. Incubation with ^{14}C-IPP and rubber particles in *L. chrysorrheus* latex gave a much smaller MW rubber than the *in vivo* synthesized rubber in *L. chrysorrheus* latex. The addition of an allylic primer had a stimulatory effect on rubber formation.

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