

## Activity of Rubber Transferase and Rubber Particle Size in Hevea Latex

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*Rubber particles in fresh latex of Hevea brasiliensis showed a bimodal distribution with a mean diameter of 1.07  $\mu\text{m}$ . The fractions containing small particles had much higher rubber transferase activity than those composed of bigger ones. The maximum incorporation of isopentenyl diphosphate went up to as high as ca. 20%. On the other hand, the fractions consisting of bigger rubber particles exhibited a small enzyme activity. The quantity of ester group linked to rubber molecules was about 2.4 mol/rubber chain for the biggest rubber particles, while the small rubber particles had almost no ester group. The small rubber particles are presumed to be composed of linear-polymers having an active diphosphate group.*

Prenyltransferases catalyse the head-to-tail condensation between isopentenyl diphosphate (IDP) and an allylic-diphosphate to produce prenyldiphosphates with various chain-lengths and geometric isomerisms. The mechanism controlling chain-length has been discussed for enzymes isolated from several bacteria<sup>1–3</sup>. Biochemical studies on the biosynthesis mechanism of *Hevea* rubber provided some evidence on the process of rubber formation. The enzyme that catalyses rubber formation has been identified as rubber transferase. The conversion of IDP to rubber was presumed to take place only on the surface of pre-existing rubber particles<sup>4,5</sup>. It has been assumed that rubber transferase is bonded to the rubber particle in *H. brasiliensis*<sup>6</sup>. The presence of

rubber transferase bound to rubber particles has been demonstrated in rubber-bearing plants such as *Parthenium argentatum*<sup>7,8</sup> and *Ficus elastica*<sup>9</sup>. It is noteworthy that the activity of the enzyme increases with an increase in the concentration of washed rubber particles<sup>10</sup>.

The molecular weight of *Hevea* rubber from fresh latex of different clonal origins has been shown to have either a distinctly bimodal distribution or a unimodal distribution with a shoulder in low molecular weight region<sup>11</sup>. The high and low molecular weight peaks are usually centered around  $10^6$  and  $10^5$ , respectively. On the other hand, the molecular weight distribution of *P. argentatum* rubber shows a single peak, irrespective of the origin and the number average

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molecular weight<sup>12</sup> of  $2-8 \times 10^5$ . The bimodal distribution of particle size has been reported for *H. brasiliensis*<sup>13</sup>. In the case of washed rubber particles, the mean particle diameter of *E. elastica* rubber is substantially larger than that of *H. brasiliensis* and *P. argentatum* by three times<sup>14</sup>. Recent studies on two distinct zones for rubber cream separated by high speed centrifugation indicated that smaller rubber particles (lower zone) showed a unimodal molecular weight distribution, while larger ones (upper zone) exhibited a typical bimodal curve<sup>15</sup>. This finding suggests that the size of rubber particle is associated with the molecular weight of rubber.

This study describes the relationship between the activity of rubber transferase and rubber particle size to clarify the mechanism controlling the chain length of rubber molecules.

## EXPERIMENTAL

### Materials

Fresh *Hevea* latex was collected in an ice-chilled flask from tappings of *Hevea brasiliensis* trees (clone RRIM 600) at Rubber Research Institute of Thailand, Hat-Yai, Thailand. [ $1-^{14}\text{C}$ ] IDP was obtained from Amersham Corp. All other reagents were of analytical grade.

### Preparation of Fractionated Rubber Particles

The latex sample was ultracentrifuged at  $4^\circ\text{C}$ , 49 000 g for 45 min to separate into three main latex fractions: rubber cream at the top of the centrifuge tube; followed by C-serum and bottom fraction. The resulting rubber cream phase was scooped off and was re-suspended in an equal volume of buffer comprising 50 mM

Tris-HCl of pH 7.4 and 0.2 % Triton  $\times$ -100<sup>®</sup>. The rubber particles were fractionated into six. The biggest rubber particle fraction (Fraction 1), was isolated by centrifuging at 500 g for 45 min. The rubber cream was removed from the tube and the serum fraction was re-centrifuged at 1000 g to get the second biggest fraction (Fraction 2). The residual four fractions, (Fraction 3) to (Fraction 6), were obtained by successive fractionation with 2000 g, 8000 g, 20 000 g and 49 000 g.

### Measurement of Rubber Particle Size

The rubber particle size distribution was determined using a Coulter 230 light scattering particle size analyser. About 10% latex in Triton  $\times$ -100<sup>®</sup> was initially filtered through a muslin cloth and then dispersed in an ultrasonic bath before the measurement. By assuming that all the rubber particles were spherical, the specific surface areas ( $\text{m}^2/\text{g}$ -rubber) of rubber particles were calculated as follows:

$$S = \frac{\sum 4 \cdot \pi \cdot (d_i/2)^2 \cdot x_i}{D \cdot \sum 4/3 \cdot \pi \cdot (d_i/2)^3 \cdot x_i} \quad \dots 1$$

where  $x_i$  is the frequency for  $d_i$  diameter particles and  $D$  is the density of rubber, 0.93.

### Incubation Conditions and Assay of Rubber Transferase

The incubation mixture for the enzymatic analysis contained, in a final volume of 1 mL, 50 mM Tris-HCl buffer (pH = 7.4), 50 mM NaF, 5mM  $\text{MgCl}_2$ , 50 mM 2-mercaptoethanol, 0.2 % Triton  $\times$ -100<sup>®</sup>, 0.92  $\mu\text{M}$  [ $1-^{14}\text{C}$ ] IDP ( $5.5 \times 10^4$  dpm) and 50  $\mu\text{g}$  rubber particle suspension, which had been washed by the buffer twice. The control incubations were treated in the same way except that the rubber

particle suspension was boiled at 100°C for 30 min. The incubation was carried out at 37°C for 6 h and the reaction was stopped by chilling in an ice bath. After addition of 500  $\mu$ L water, saturated with NaCl, the [ $^{14}$ C]-labeled rubber was extracted with 1 mL of mixture of hexane and toluene (1/1) three times. The extracts were thoroughly washed by water to remove low molecular weight isoprenoid contaminants. The radioactivity in the mixture was counted in a liquid scintillation counter, LS-5000TD Beckman LSC spectrometer, to estimate the activity for rubber transferase.

#### Fourier Transform Infrared (FT-IR) Analysis of Ester Groups in *Hevea* Rubber

The rubber samples for FT-IR analysis were prepared by casting off rubber solutions in chloroform on a KBr disk under a stream of nitrogen. The film was scanned with a Jasco 5300 FT-IR spectrometer. The ester content was obtained from the integrated intensity of peaks at 1738  $\text{cm}^{-1}$  (C=O) and 1664  $\text{cm}^{-1}$  (C=C of isoprene unit) according to the calibration curve which was made by using methyl stearate and synthetic *cis*-1,4-polyisoprene<sup>16</sup>.

#### Determination of Molecular Weight

The number average molecular weight of rubbers was determined by means of GPC using two columns in series packed with styrene-divinylbenzene gel having exclusion limits of  $2.0 \times 10^7$  and  $5.0 \times 10^4$ . Measurements were made using THF as eluent. Commercial standard polystyrenes were used to calibrate the elution. The molecular weight determined by using polystyrene standards was converted to the polyisoprene standard value according to the method of Subramaniam<sup>17</sup>.

## RESULTS AND DISCUSSION

### Rubber Particle Size Distribution

A light scattering analytical method clarified the bimodal distribution of rubber particles for all samples obtained from fresh latex. As shown in *Figure 1*, the rubber particles in the total cream rubber exhibited two peaks in the regions of 0.3  $\mu\text{m}$  and 1.0  $\mu\text{m}$  with a mean diameter of 1.07  $\mu\text{m}$ . Each fraction, obtained by successive fractionation, showed a unimodal particle size distribution as shown in *Figure 2*. The biggest and secondary fractions comprised 17% and 31% of the total weight of rubber cream, respectively, while the smallest and the second smallest sized fractions were less than 6%. It has been reported that large particles are predominant and a fair number of which is pear-shaped in the latex from mature trees, while the particles are spherical with the mode of 0.1  $\mu\text{m}$  in the latex from young trees<sup>18,19</sup>. These findings indicate that the rubber fractions can be classified into two groups according to the mean particle size: the particles of *ca.* 1  $\mu\text{m}$  in diameter and those smaller than 0.3  $\mu\text{m}$  in diameter.

### Rubber Transferase Activity

It is well known that the optimum pH is *ca.* 7 and  $\text{Mg}^{2+}$  is necessary as a co-factor to exhibit the activity for rubber transferase. In the assay system, the enzyme required  $\text{Mg}^{2+}$  (>5 mM) for its maximum activity. As the amount of rubber particles increased up to 200  $\mu\text{g}$ , the enzyme activity increased.

*Figure 3* shows the relationship between the particle size and radioactivity for rubber transferase. The fractions containing small rubber particles, especially for Fraction 5 and Fraction 6, showed much higher enzyme

activity than the bigger ones. The incorporation of IDP into the rubber particles significantly increased by increasing the time of incubation up to 6 h in the case of Fraction 5 and Fraction 6. The maximum incorporation of IDP increased to a remarkably high level, as high as *ca.* 20%. On the other hand, the fractions consisting of bigger rubber particles exhibited a very small enzyme activity, the level of which was slightly higher than that of the control samples which were prepared by boiling the fraction at 100°C for 30 min before the incubation. No further increase in the incorporation of IDP was observed after 1 h of incubation. The study with unwashed rubber particles indicated that smaller rubber particles had a higher specific rate of incorporation of IDP into rubber<sup>4</sup>. It was also demonstrated, in the case of washed rubber particles smaller than 0.15  $\mu\text{m}$  in diameter, that the activity of the rubber particles per unit

surface area increased with diminishing particle size<sup>5</sup>. In our analysis for the total cream rubber particles ranging from 0.1  $\mu\text{m}$  to 5  $\mu\text{m}$ , a significant difference in the incorporation of IDP was observed between the fractions of the bigger rubber particles, Fraction 1 to Fraction 3, and others. It is presumed that the lack of an essential factor or significant inhibition for the enzyme activity can occur for particles bigger than a certain size. For comparison with a previous study<sup>5</sup>, the relationship between enzyme activity and particle size was expressed in surface area, as listed in *Table 1*. This is similar to that observed in the previous study showing the high enzyme activity for smaller particles. In our case, however, no difference in the enzyme activity was observed among the bigger particle fractions. These findings indicate that only small rubber particles play a significant role in the chain elongation of rubber molecules.

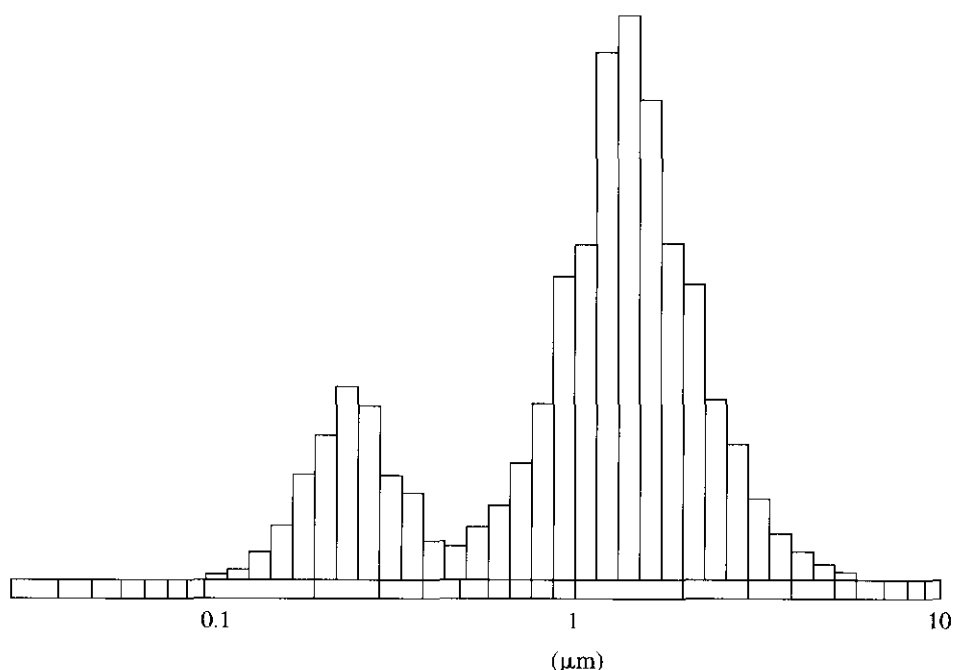


Figure 1. Particle size distribution of rubber from total cream.

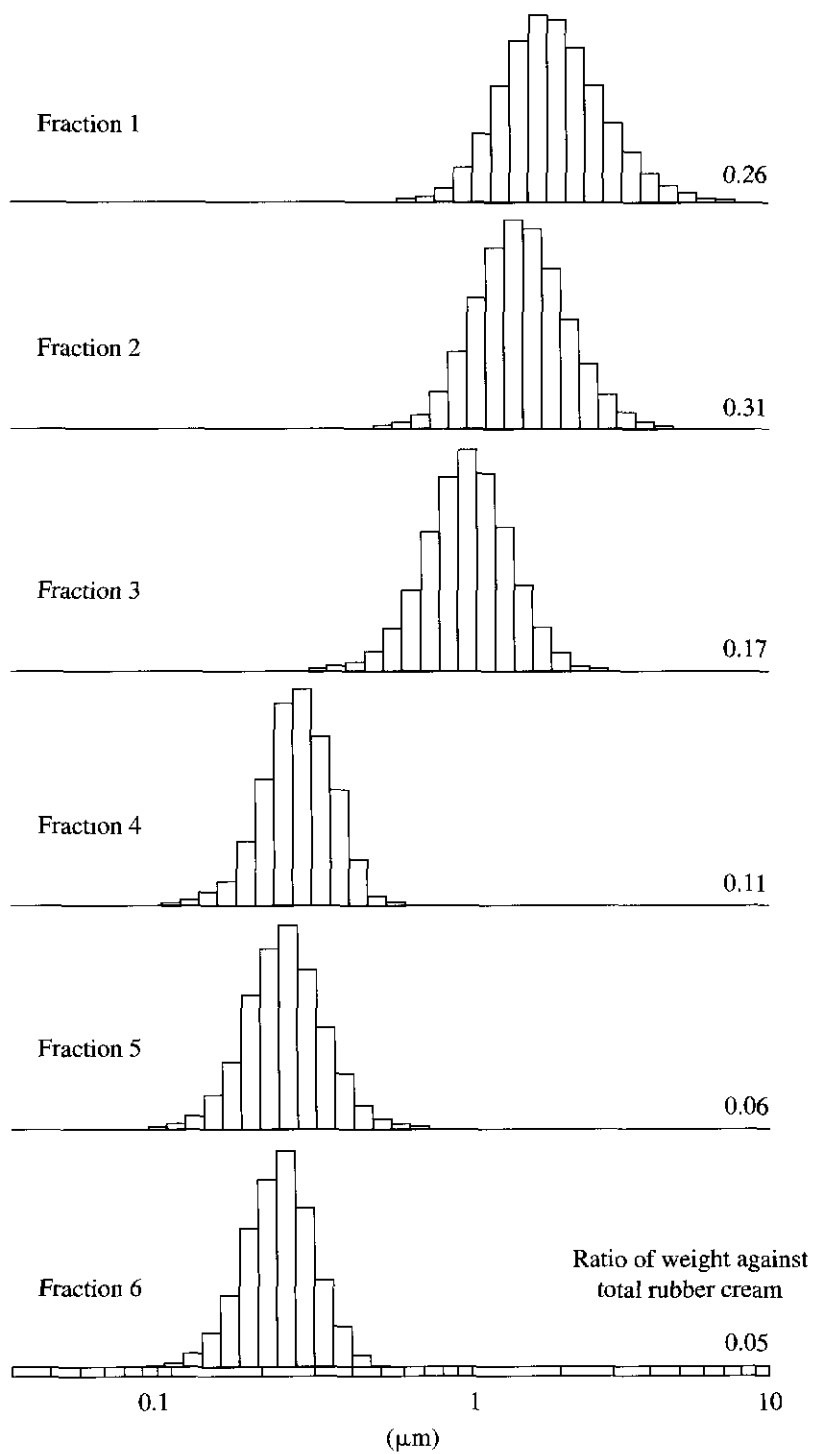


Figure 2. Particle size distribution of fractionated rubbers.

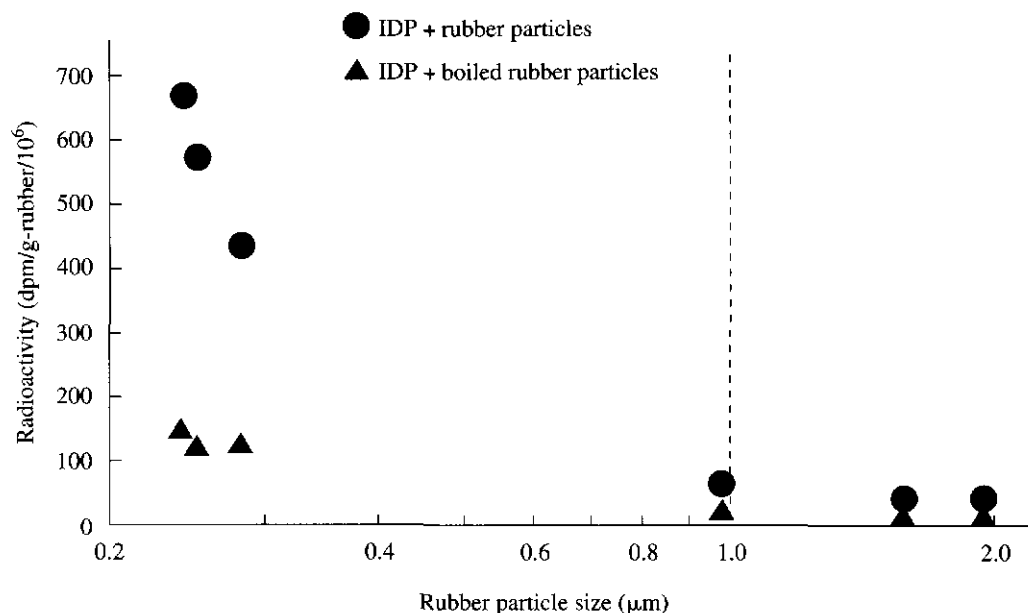


Figure 3. Rubber particle size and enzyme activity.

### Ester Group Contents in *Hevea* Rubber

NMR analysis of the highly purified natural rubber has disclosed the presence of fatty acid ester groups linked to the rubber chain<sup>20</sup>. *Hevea* rubber deproteinised by enzymatic deproteinisation followed by acetone extraction contained 1–2 ester groups per rubber molecule irrespective of the molecular weight<sup>16</sup>. This ester group has been postulated to be located at the chain-end as a phospholipid, which forms a branching point<sup>20</sup>.

The contents of the fatty acid ester group in *Hevea* rubber from Fraction 1 and Fraction 6 were 4.3 mmol/kg and 0.2 mmol/kg rubber, respectively. The quantity of the ester group linked to the rubber molecules were estimated to be about 2.4 and 0.1 per rubber chain, based on the degree of polymerisation estimated

by Mn,  $5.6 \times 10^5$  and  $3.9 \times 10^5$ , respectively. This indicates that the small rubber particles have an insignificant amount of ester groups. The small rubber particles are also obtainable from the serum fraction which is fractionated from the cream fraction during the process of commercial concentrated latex production. The rubber obtained from the serum fraction contained a very small amount of fatty acid groups<sup>16</sup> as in the case of our Fractions 4 to 6. The fact that small rubber particles have almost no ester group to form branch-points implies that they are composed of linear rubber molecules having an active terminal-end<sup>21</sup>. It has been confirmed that the enzyme activity significantly increased with the addition of allylic diphosphates in the case of the washed rubber particles<sup>7,22</sup>. It is reasonable to note that the rate of IDP-incorporation into washed rubber particles without allylic

TABLE 1. ENZYME ACTIVITY PER UNIT SURFACE OF RUBBER PARTICLES

Fraction	Average diameter of particles	Specific surface area	Enzyme activity per unit surface
	( $\mu\text{m}$ )	( $\text{m}^2/\text{g}$ )	( $\text{dpm}/\text{m}^2/10^6$ )
Fraction 1	1.93	2.3	15.2
Fraction 2	1.57	3.1	11.9
Fraction 3	0.98	5.1	12.6
Fraction 4	0.28	19.5	22.1
Fraction 5	0.25	21.0	27.9
Fraction 6	0.24	22.6	30.0

diphosphates was low because the large rubber particles, which are the major portion of cream rubber, were dead polymers having no active terminal group. The slight activity observed for the large rubber particles would be due to the incorporation of IDP into a trace amount of an existing rubber diphosphate chain in them. The present study clearly indicates that small rubber particles in the cream phase have a much higher activity for IDP incorporation than the larger ones. It seems reasonable to assume that small rubber particles are in the growing stage and have a high enzyme activity. However, the mechanism to modify the pyrophosphate terminal remains open. Certain chain termination reactions are expected when the shortage of IDP is marked by increasing the rubber particle diameter due to the low stability of the terminal polyprenyl diphosphate linkage. The formation of the phospholipid terminal group can be a result of the termination reaction.

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