Natural rubber (NR) stored for several months becomes progressively harder, a phenomenon called storage hardening affecting the processing properties of NR. The impact of lutoid stability on NR storage hardening was studied. Lutoid stability was determined by a qualitative parameter (lutoid integrity after centrifugation) and by a quantitative parameter (the bursting index or BI). The storage hardening of samples made from washed rubber particles (WRP) was compared to samples made from whole field latex. Lutoid bursting had a significant effect on the storage hardening of NR as shown by a significant correlation between the increase in Wallace plasticity ($\Delta P$) after an accelerated storage hardening test and the BI of lutoids. The BI test appeared a better indicator of lutoid stability than the qualitative observation of lutoids after centrifugation. It was also shown that the ADS prepared with purified particles have a much lower PRI (70) than the controls ADS prepared with the entire latex (PRI=110).

Keywords: Natural rubber; washed rubber particles; storage hardening; lutoids

Unlike synthetic elastomers, NR stored for several months becomes progressively harder, a phenomenon called storage hardening. This phenomenon is of considerable importance since it affects the processing properties of NR, especially causing higher energy consumption during the mixing step. Many studies have been undertaken to identify the mechanisms and biochemical compounds of latex involved in crosslinking between macromolecules of the polyisoprene provoking NR hardening during storage$^1$-$^8$. The latex produced by *Hevea*

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*brasilienensis* is a complex colloidal suspension containing mainly rubber particles (~30-45% w/w latex) and lutoids (complex lysosomal vacuoles, ~10-20% w/w latex) dispersed in the cytoplasmic serum (C-serum). Rubber particles are made of a hydrophobic core of poly(cis-1,4-isoprene) surrounded by a complex monolayer membrane of lipids and proteins. The main three fractions or compartments (rubber particles, C-serum and lutoids) can be separated by high speed centrifugation of latex. Lutoids contain many components in a water phase called the B-serum of which hevein, a lectin-like protein, is the major component. Several biochemical compounds of lutoids are assumed to be involved in latex destabilisation and several mechanisms have been proposed to explain this coagulation. The stability of lutoids can be estimated by a parameter called the “bursting index” (BI). Bursting index is defined by the ratio of free acid phosphatase, a specific enzyme present in lutoids, to total acid phosphatase activity. In addition to this quantitative parameter (BI), several authors have shown that it is possible to visually observe the bursting of lutoids qualitatively, after ultra-high speed centrifugation. Though many studies have investigated the influence of lutoid stability in latex coagulation, to our knowledge the effect of lutoid stability on the storage hardening of natural rubber (NR) has never been studied before.

The main objective of this paper was to evaluate the effect of lutoid stability on the storage hardening of NR. Thus, this article firstly presents changes in lutoid stability over the latex storage time at room temperature, by monitoring lutoid bursting. It then assesses how bursting of the lutoid fraction affects the storage hardening of NR samples. Air dried sheet (ADS) made from whole field latex and washed rubber particles were used in this study.

**MATERIALS AND METHODS**

**Sample Preparation**

The latex was tapped from *Hevea brasilienensis* trees of clone RRIM 600, located in Surat Thani, Thailand. Rubber latices were collected for one hour in a plastic bag placed inside a cup containing ice to prevent lutoid bursting. These latices were kept in a tank containing ice before filtering through a stainless steel sieve (1 mm pore size) into a beaker placed in another tank containing ice. The homogenised latex was then kept at room temperature.

**Preparation of washed rubber particles.** The rubber particles, C-serum and lutoids were separated by centrifuging 180 mL of fresh latex (30 mL/tube, 6 tubes) at 35,000 xg by a refrigerated high speed centrifuge machine (Z36HK, Hermle Labortechnik, Germany) for 1 h at 4°C. The rubber particles in each tube were collected and dispersed in distilled water and adjusted to about 20% dry rubber content (DRC). This latex sample made of washed rubber particles was called WRP in the figures.

**Preparation of air dried sheets.** Forty eight milliliters (mL) of the homogenised latex was diluted with 32 mL of distilled water. The diluted latex was poured into a 6.9 × 9.8 × 5.0 cm plastic box and coagulated by adding 4.8 mL of 2% formic acid. The coagulation box was covered to avoid contamination by dust. After coagulation, the coagulum was passed through a flat hand mangle 2-3 times to reduce the thickness. The rubber sheet was then passed through a rough hand mangle two times to imprint cross lines on the rubber sheet. The rubber sheets were dried in a ventilated oven at 50°C for 3 – 4 days. The ADS obtained were labeled “FL”. The ADS of washed rubber particles were prepared in the same way as field latex but dilution was not necessary. All the field latex and washed rubber particles were used in this study.
samples were prepared from latex stored for 0, 2, 6, 9, 12 and 15 h at room temperature. Triplicates of the ADS samples were prepared for each storage time.

**Bursting Index Measurement**

The methodology for determining the BI described by Ribaillier\textsuperscript{23} was adapted for our work. Free acid phosphatase (FAP) activity and total acid phosphatase (TAP) activity were measured. The measurement was divided into two sets: (i) Blank ($F_b$ and $T_b$) and (ii) Measurement ($F_m$ and $T_m$); triplicates were carried out for each measurement. A solution of 25 mM \textit{para}-nitrophenyl phosphate (\textit{p}-NPP, Sigma, UK) at pH 5.0 was prepared in 0.1 M acetate buffer (pH 5.0). The FAP medium, which prevents lutoids from bursting, was prepared by adding 18.2 g of mannitol (0.4 M, Sigma-Aldrich, China) to 100 mL of the \textit{p}-NPP solution and topped up to 250 mL with distilled water. The TAP medium used to burst the lutoid fraction was prepared by adding 0.25 g of triton X100 (0.1% w/v, Laboratoriums Reagenzien) to 100 mL of the \textit{p}-NPP solution and topped up to 250 mL with distilled water.

Test tubes containing 5 mL of FAP ($F_b$ and $F_m$ tubes) and TAP medium ($T_b$ and $T_m$ tubes) were prepared. Four milliliters of 20% trichloroacetic acid (TCA, Merck, Germany) was then added to the $F_b$ and $T_b$ tubes to immediately stop the reaction by denaturing the acid phosphatase at the initial time ($t=0$). Latex samples of 0.5 mL were added to the tubes. After 10 min of incubation 20% of trichloroacetic acid (TCA) solution in water was then added to the $F_m$ and $T_m$ tubes to coagulate the latex. Three milliliters of a solution of sodium hydroxide (NaOH, Merck, Germany) at a concentration of 100

\begin{figure}
\centering
\includegraphics[width=\textwidth]{methodology.png}
\caption{Methodology used for the fractionation of whole field latex into 3 fractions: (i) Washed rubber particles,(ii) C-serum and (iii) Lutoids.}
\end{figure}
of 2 N was then added to alkalinise the media which became yellow (colour of p-nitrophenol (p-NP)). One milliliter of each obtained solution was centrifuged at 17,000 x for 15 min to remove small rubber coagulum from the solution. The FAP solutions (F<sub>b</sub> and F<sub>m</sub>) were diluted by adding 0.4 mL of the solution to 2.6 mL of distilled water. In parallel, the TAP solutions (T<sub>b</sub> and T<sub>m</sub>) were diluted by adding 0.2 mL of solution to 2.8 mL of distilled water. The absorbance (A) at 405 nm of these diluted solutions was measured by a spectrophotometer (V-200RS, MRC). The BI was calculated using the Equation 1.

\[
BI = \frac{\text{FAP activity}}{\text{TAP activity}} 
\times 100 = 
\left( \frac{A_{Fm} - A_{Fb}}{A_{Tm} - A_{Tb}} \right) \times 100
\]

where FAP is free acid phosphatase and TAP is total acid phosphatase, \(A_{Fm}\) and \(A_{Fb}\) are the absorbance of FAP solutions (F<sub>b</sub> for the blank and F<sub>m</sub> for the measurement) and \(A_{Tm}\) and \(A_{Tb}\) the absorbance of TAP solutions (T<sub>b</sub> for the blank and T<sub>m</sub> for the measurement).

**Wallace Plasticity (P<sub>0</sub>), Plasticity Retention Index (PRI)**

Prior to any analysis, the samples were homogenised in accordance with ISO 1796 and sheeted by three passes through the mill rollers at 27ºC. The initial Wallace plasticity (P<sub>0</sub>) and the plasticity after ageing (P<sub>30</sub>) of the NR samples were determined with a Wallace Plastimeter (Wallace Rapid Plastimeter MK V P14, HW, Wallace & Co. Ltd., UK), according to ASTM D3194-9914. P<sub>30</sub> was the Wallace plasticity value after a 30 min period of oxidative ageing at 140ºC. The median of three pellets was taken as the P<sub>0</sub> or P<sub>30</sub>. The plasticity retention index (PRI) was calculated using Equation 2.

\[
PRI (%) = \left( \frac{P_{30}}{P_{0}} \right) \times 100
\]

where PH is the Wallace plasticity after ASHT and P<sub>0</sub> the initial Wallace plasticity.

**RESULTS AND DISCUSSION**

Lutoid stability over a latex storage time of 0, 2, 6, 9, 12, and 15 h at room temperature was expressed by a qualitative parameter (lutoid integrity assessed visually after centrifugation) (Figure 2a) and a quantitative parameter, BI (Figure 2b). For the qualitative parameter, lutoid status appeared stable during the short latex storage time at room temperature (0 – 6 h) (Figure 2a). For the latex stored for 9 h at room temperature, the lutoids seemed to be swollen. It is possible that some lutoid particles had already burst and released B-serum. Lutoids were completely burst after 12 h of latex storage at room temperature. The layer observed under the rubber particles may have been lutoid membrane debris. At 15 h, the two layers of rubber particles and assumed lutoid membrane debris were coagulated together to form a sole coagulum. From the results of the last two tubes (12 and 15 h, Figure 2a) it might be assumed that C-serum was mixed together with released B-serum and some non-isoprene compounds from lutoids induced rubber particle coagulation as proposed in previous...
studies\textsuperscript{16,18}. Nevertheless, Wititsuwanakul \textit{et al.}\textsuperscript{16} reported that isolated intact lutoids started bursting after 45 min of latex storage at room temperature and completely burst after 90 min of storage. Consequently, the results of lutoid stability might depend on certain environmental or agronomic parameters (genotype, tapping system, weather conditions, season, sample handling method, \textit{etc}.).

The BI is the measurement of lutoid stability expressed by the percentage of free acid phosphatase activity divided by total acid phosphatase activity in the latex (\textit{Equation 1}). The BI value reflects the phosphatase activity of the cytosolic serum at a given time and consequently the physiological state of the lutoid particles which contain this enzyme. The BI of fresh field latex stored at room temperature (time zero, control) was 26\% (\textit{Figure 2b}). Supposedly, about 26\% of the lutoids burst during latex flow from tapping because lutoids are sensitive to osmotic change and shearing\textsuperscript{24,25}. The presence of another acid phosphatase not compartmentalised in lutoids could also explain this activity threshold level in serum. After 6 h, BI slightly increased during storage time up to 9 h and considerably increased after that (45\% at 12 h and 78\% at 15 h). It can be seen that even though lutoids appeared completely burst after 12 h of latex storage (\textit{Figure 2a}), BI was only 45\%. This could be explained by the different methodology used to analyse lutoid stability. During centrifugation, lutoids can be subjected to mechanical force which might accelerate lutoid bursting, while BI was measured without any additional force. Although visual lutoid status after centrifugation and BI were not exactly correlated, BI can be a good indicator of lutoid stability.

\textbf{Figure 2. Evaluation of lutoid stability over storage time for field latex at room temperature by (a) Visual appearance after centrifugation (35,000 g, 1 h, 4\(^\circ\)C) and (b) The bursting index (BI).}
The influence of lutoid stability on storage hardening of the NR samples was evaluated. NR samples were prepared as air dried sheets (ADS) made from whole field latex (control) or washed rubber particles. For 12 and 15 h of latex storage, as the rubber particles coagulated during centrifugation NR samples could not be made with washed rubber particles samples. The sensitivity of NR samples to storage hardening was assessed with ASHT, namely the increase in plasticity (ΔP). Increase in plasticity is the difference between the plasticity after and before an accelerated storage hardening test. The higher the ΔP, the greater the sensitivity of NR samples to storage hardening. Figure 3 shows that the ΔP for control samples made from whole field latex did not change significantly for up to 9 h of storage (20 < ΔP < 23). For latex storage times over 9 h the ΔP increased significantly to reach a value of 38 after 15 h of latex storage. It can be seen in Figure 4 that there is a really good correlation of the ΔP with the bursting index. This positive correlation suggests a significant influence of lutoid bursting, and release of the B-serum, on the storage hardening of the rubber samples prepared from whole field latex. The NR samples made from washed rubber particles exhibited a significant difference in storage hardening compared to the control field latex (Figure 3). Between 0 and 9 h of whole field latex storage, for field latex samples, ΔP values were around 20-23 and around 8-12 for NR samples made from washed rubber particles. Washing the rubber particles before making the NR samples dramatically reduced their sensitivity to storage hardening. Figure 4 shows a significant positive correlation between storage hardening (ΔP) and the BI for NR samples made from washed rubber particles. The higher the BI the greater was the ΔP, hence, the storage hardening of rubber samples made from field latex.

The PRI of the NR samples was also measured. PRI is used to forecast the sensitivity of NR samples to thermo-oxidation. Figure 5 gives the PRI of NR samples made from whole fresh field latex and fresh washed
Figure 4. Increase in plasticity ($\Delta P$) after an accelerated storage hardening test as a function of the bursting index (BI) for NR samples made from whole field latex (FL) stored at room temperature ($r^2 = 0.96$).

Figure 5. Plasticity retention index (PRI) as a function of the fresh field latex storage time at room temperature for NR samples made from whole field latex (FL) and washed rubber particle latex (WRP).
rubber particles (0 h storage time at room temperature). It can be seen that the PRI values for NR samples made from field latex (≈ 110) were greater than the theoretical value of 100. Indeed, during thermo-oxidation of NR samples in a PRI oven, there is always a balance between scissions and crosslinking of the polyisoprene chains. Scissions are due to oxidation and crosslinking will promote the storage hardening. For field latex samples, as the PRI was higher than 100 (maximum theoretical value), the crosslinking predominated. Figure 5 shows that the PRI of the NR samples made with washed rubber particles had very significantly lower PRI values (PRI ≈ 70) than the NR samples made from field latex (PRI ≈ 110). As seen in Figure 3, the NR samples made from washed rubber particles were less prone to storage hardening and therefore to crosslinking, which could explain their lower PRI compared to NR samples made from field latex. However, natural antioxidants are known to be present in NR latex, some being present in lutoids (tyrosine, betaines: trigonelline, ergothioneine and hercynine). These compounds have been reported to be one of the factors contributing to the high PRI of pale crepe rubber. Thus, the difference in PRI between NR samples made from field latex or washed rubber particles may therefore have been partly due to antioxidants released from lutoids by bursting. It is difficult to know about the main factor involved (scissions or crosslinking) and we need additional experiments to know the exact weight of both.

CONCLUSION

Lutoid stability during latex storage at room temperature was studied by a qualitative parameter (visual lutoid status after centrifugation) and a quantitative parameter. The two methods could not provide strictly correlated results because of the additional mechanical force provided by centrifugation which probably accelerated lutoid bursting. The BI test appeared to be a better indicator of lutoid stability than the qualitative observation of lutoids after centrifugation. For the samples prepared from whole field latex, our results showed a good correlation between the storage hardening phenomenon and lutoid stability. The main consequence of removing lutoids before preparing NR samples was a significant increase in sensitivity to thermo-oxidation (decrease in PRI).

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