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Micromorphological characterization and label-free quantitation of small rubber particle protein in natural rubber latex

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ABSTRACT

Commercial natural rubber is traditionally supplied by *Hevea brasiliensis*, but now there is a big energy problem because of the limited resource and increasing demand. Intensive study of key rubber-related substances is urgently needed for further research of in vitro biosynthesis of natural rubber. Natural rubber is biosynthesized on the surface of rubber particles. A membrane protein called small rubber particle protein (SRPP) is a key protein associated closely with rubber biosynthesis; however, SRPP in different plants has been only qualitatively studied, and there are no quantitative reports so far. In this work, *H. brasiliensis* was chosen as a model plant. The microscopic distribution of SRPP on the rubber particles during the washing process was investigated by transmission electron microscopy –immunogold labeling. A label-free surface plasmon resonance (SPR) immunosensor was developed to quantify SRPP in *H. brasiliensis* for the first time. The immunosensor was then used to rapidly detect and analyze SRPP in dandelions and prickly lettuce latex samples. The label-free SPR immunosensor can be a desirable tool for rapid quantitation of the membrane protein SRPP, with excellent assay efficiency, high sensitivity, and high specificity. The method lays the foundation for further study of the functional relationship between SRPP and natural rubber content.

Commercial quantities of natural rubber (*cis*-1,4-polyisoprene) are predominantly supplied worldwide by the Brazilian rubber tree (*Hevea brasiliensis*). High-quality natural rubber, a uniform high-molecular-weight polymer (>1000 kDa or 1000 kg/mol), is a vitally important material used in the manufacturing of industrial and medical products and cannot be replaced by synthetics in most applications [1]. With increasing consumption and supply shortages threatening natural rubber, supplementary sources such as guayule (*Parthenium argentatum*), Russian dandelion (*Taraxacum kok-saghyz*), and prickly lettuce (*Lactuca serriola*) are being sought and investigated for possible supply of natural rubber [2–5]. Meanwhile, the biosynthesis mechanism of natural rubber is being researched for the long-term goal of simulative biosynthesis of natural rubber in vitro. Thus, intensive study of rubber production-related substances is urgently needed both qualitatively and quantitatively.

Natural rubber latex from *H. brasiliensis* is a colloidal dispersion containing 30–45% rubber particles [6] and 5% non-rubber particles such as cytoplasmic organelles, proteins, lipids, cytoplasmic C-serum, and lutoids [7–9]. Natural rubber is produced on the surface of rubber particles, which are encapsulated with a monolayer lipid membrane as well as proteins [10–12]. Various components on the rubber particle surface contribute to the rubber biosynthesis, including a kind of membrane protein called small rubber particle protein (SRPP).[1] SRPP, a 24-kDa latex allergen (Hev b3) [13], has

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[1] *Abbreviations used:* SRPP, small rubber particle protein; SPR, surface plasmon resonance; RI, refractive index; TEM, transmission electron microscopy; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; SEM, scanning electron microscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RU, resonance units; LRP, large rubber particle; SRP, small rubber particle; REF, rubber elongation factor.

attracted wide interest since it was identified [14]. Previous studies suggested that SRPP played a crucial role in rubber biosynthesis in *H. brasiliensis* [14–17]. Furthermore, an in vivo study proved the role of SRPP in rubber biosynthesis in Russian dandelion *(T. kok-saghyz)* using a transgenic approach [1]. The content of SRPP in rubber-producing plants is closely associated with rubber biosynthesis given that it has a positive correlation with the plants' rubber-producing capability. However, SRPP in different plants has been only qualitatively studied, and there are no quantitative reports so far. It is imperative to develop robust analytical techniques for quantitation of SRPP. In addition, there are great challenges to obtain an accurate measure of the content of SRPP for the following reasons: (i) SRPP is a membrane protein on the surface of rubber particles, and it is difficult to quantify a membrane protein; (ii) the level of SRPP in rubber latex is generally low; and (iii) the interference of other coexisting proteins in rubber latex is generally difficult to eliminate.

Surface plasmon resonance (SPR) is one of the dominant technologies for label-free biodetection, enabling real-time monitoring of the interactions between an analyte and a ligand, with low sample consumption. SPR can achieve precise quantitative analysis of analytes through measuring the refractive index (RI) change, which is caused by molecular interaction and is related to the sample concentration or surface density [18]. On the other hand, the biorecognition element (e.g., antibody) in an SPR biosensor can offer high affinity and specificity against target molecules; thus, the matrix effect on the detector response is minimal.

In this work, *H. brasiliensis,* the current main commercial source of natural rubber, was chosen as the model rubber-producing plant. The microscopic distribution of SRPP on the surface of the rubber particles was investigated by transmission electron microscopy (TEM)–immunogold labeling (Scheme 1). A label-free immunosensor based on the SPR technique was developed as a novel tool for SRPP quantitation in rubber latex. The immunosensor was applied to preliminarily investigate several rubber-producing plant samples. The immunosensor can be applied to rapidly quantify the SRPP in different plants and predict the rubber-producing capability.

Materials and methods

Materials and apparatus

Natural rubber from *H. brasiliensis* and *T. kok-saghyz* was kindly provided by Heilongjiang Academy of Sciences. *Taraxacum* sp. *1, 2,* and *3* and prickly lettuce were wild plants from a suburb of Beijing, China. Gold-conjugated anti-mouse goat antibody, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine were purchased from Sigma–Aldrich (Shanghai, China). Anti-SRPP mouse monoclonal antibody and anti-rubber elongation factor mouse monoclonal antibody were purchased from Icosagen (Tartu, Estonia). A Bradford Protein Assay Kit was purchased from Sangon Biotech (Shanghai, China). All chemicals were of analytical grade. Buffers were prepared with ultrapure water and filtered using 0.22-μm membrane filters before use.

Scanning electron microscopy (SEM) and TEM observations were carried out with a Hitachi S-4800 microscope (Hitachi, Japan) and Jeol JEM-1230 electron microscope (Jeol, Japan), respectively. SPR measurements were performed on a Biacore T200 incorporating a CM5 chip (GE Healthcare Life Sciences, Uppsala, Sweden). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was analyzed by a Smart Gel II imager from Beijing Sage Creation Science (Beijing, China).

Pretreatment of rubber latex samples

Purified rubber particle fractions

Ample rubber latex from *H. brasiliensis* was pretreated using two consecutive ultracentrifugations at 12,000 rpm and 4 °C for 20 min. Ultracentrifuged rubber latex yields three fractions. The top creamy fraction contains mostly rubber particles, the intermediate weight fraction is called C-serum, and the bottom fraction contains mostly lutoids [19]. Then the top creamy layer was spooned out to mix with an equal volume of ice-cold buffer (100 mM Tris–HCl [pH 7.5], 5 mM $MgSO_4$, and 5 mM dithiothreitol) [20], marked as purified rubber particle fraction.

Because there was limited latex in dandelions or prickly lettuce, these latex samples were pretreated using syringe filtration with 0.22-μm membrane filters.

Washing process for rubber latex from H. brasiliensis

To test the SRPP adhesion on the surface of rubber particles from *H. brasiliensis,* a washing process was carried out as follows. Natural rubber latex (1 ml) was centrifuged at 12,000 rpm for 20 min at 4 °C (marked as *round 1*). Then the top creamy layer was isolated to be resuspended in 1 ml of ice-cold buffer and was centrifuged at 10,000 rpm and 4 °C for 10 min (denoted as *round 2*). The top creamy layer and the second layer were collected and marked as *W2* and *R2,* respectively. The washing procedure was repeated another six times. The top creamy layer and the second layer were marked according to the washing round, for example, as *W8* and *R8.*

Micromorphological characterization of rubber particles and SRPP

Micromorphological characterization was performed to assist the development of an SPR immunosensor. The purified rubber particles were observed with SEM. The purified rubber particles fraction was fixed with 3% glutaraldehyde in 50 mM sodium cacodylate and 1% tannic acid for 1 h at room temperature (RT) [21]. Then the fixed rubber particles were washed twice with ultrapure water using centrifugation. The fixed creamy layer was finally diluted in ultrapure water, and rubber particles were observed using a Hitachi S-4800 microscope (SEM). The characterization of SRPP was accomplished with the TEM–immunogold labeling technique (Fig. 1A). Before TEM–immunogold labeling, the purified rubber particle fraction and the washed rubber particle samples (*W5*) were first fixed with 1% osmium tetroxide for 1 h at 4 °C [20]. After centrifugation, a small drop of each of the pretreated samples was placed on the formvar-coated nickel grids and air-dried. Then the immunogold labeling was conducted as follows. Grids carrying samples were floated on drops of PBS/T buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA], 0.05% Triton X-100, and 0.05% Tween 20) for 5 min at RT. Then grids were incubated with the anti-SRPP antibody (primary antibody, 1:100 dilution with PBS containing 1% BSA and 0.05% Tween 20) for 2 h at 37 °C. After six washes in PBS, samples were labeled with gold (5 nm)-conjugated anti-mouse goat antibody (secondary antibody, 1:40 dilution) for 1 h at RT. Then the grids were washed six times with PBS and four times with ultrapure water. For the control, samples were treated as above except for the incubation with the primary antibody. Grids were air-dried and observed with the Jeol JEM-1230 microscope (TEM).

SDS–PAGE of proteins in purified rubber particle fraction and in second layers from different washing rounds

Proteins in 10 μl of purified rubber particle fraction and second layers from different washing rounds were separated on 12%