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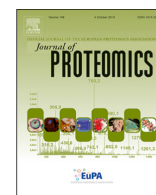

Subcellular proteome profiles of different latex fractions revealed washed solutions from rubber particles contain crucial enzymes for natural rubber biosynthesis

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A B S T R A C T

Rubber particle (RP) is a specific organelle for natural rubber biosynthesis (NRB) and storage in rubber tree *Hevea brasiliensis*. NRB is processed by RP membrane-localized proteins, which were traditionally purified by repeated washing. However, we noticed many proteins in the discarded washing solutions (WS) from RP. Here, we compared the proteome profiles of WS, C-serum (CS) and RP by 2-DE, and identified 233 abundant proteins from WS by mass spectrometry. Many spots on 2-DE gels were identified as different protein species. We further performed shotgun analysis of CS, WS and RP and identified 1837, 1799 and 1020 unique proteins, respectively. Together with 2-DE, we finally identified 1825 proteins from WS, 246 were WS-specific. These WS-specific proteins were annotated in Gene Ontology, indicating most abundant pathways are organic substance metabolic process, protein degradation, primary metabolic process, and energy metabolism. Protein-protein interaction analysis revealed these WS-specific proteins are mainly involved in ribosomal metabolism, proteasome system, vacuolar protein sorting and endocytosis. Label free and Western blotting revealed many WS-specific proteins and protein complexes are crucial for NRB initiation. These findings not only deepen our understanding of WS proteome, but also provide new evidences on the roles of RP membrane proteins in NRB.

Significance: Natural rubber is stored in rubber particle from the rubber tree. Rubber particles were traditionally purified by repeated washing, but many proteins were identified from the washing solutions (WS). We obtained the first visualization proteome profiles with 1825 proteins from WS, including 246 WS-specific ones. These WS proteins contain almost all enzymes for polyisoprene initiation and may play important roles in rubber biosynthesis.

1. Introduction

Natural rubber is a plant-derived long chain *cis*-1,4-polyisoprene polymer produced by more than 2,500 plant species, but the para rubber tree (*Hevea brasiliensis*) is the only commercially cultivated one [1–3]. Natural rubber cannot be replaced by chemical synthetic alternatives owing to its unique properties [4,5]. Natural rubber latex, as the cytoplasm of a specialized cell called laticifer, is usually collected by regular tapping the trunk bark of the rubber tree [1,6]. Tapping, as a non-destructive method for latex harvesting, can facilitate continual rubber production [3,7]. During the regular tapping, latex regeneration is an important limiting factor for rubber yield, which relies on both the complex rubber biosynthesis process on rubber particle (RP) and various organic sources supplied to laticifer cells [8,9].

After ultracentrifugation, latex can be divided into three different fractions: the top part, RP; the middle part, C-serum (CS); and the bottom fraction, lutoid [10,11]. RP is a specific latex-producing organelle, and it is recently called as a natural rubber biosynthetic machinery [12,13]. It is encapsulated by a phospholipid monolayer and contains a high molecular weight rubber core [14]. The fresh latex system contains 30–45% weight of RP [15,16]. RP diameter is ranged from 0.02 to 3.0 μm [8,17,18]. It is supposed to originate from rough

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endoplasmic reticulum [19]. Rubber molecule biosynthesis is performed by various enzymes and protein factors, which are bound to or embedded in RP membrane. Among them, cis-prenyltransferase (CPT, also named rubber transferase), rubber elongation factor (REF) and small rubber particle protein (SRPP) are crucial [5,13,14,20,21]. REF is anchored inside RP membrane by its auto-assembly ability, whereas SRPP largely covers RP surface in an oriented anisotropic manner [5,22–25].

Recently, proteomics has been performed to identify proteins from rubber latex and RP. Comparative proteomics of large and small RPs resulted in 53 differential spots corresponding to 22 gene products, and the protein abundance of SRPP and 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) in small RPs are higher than in large RPs, but REF abundance is lower in small RPs [18]. Moreover, 186 proteins were identified from RPs by shotgun [25]. Recently, we developed a phenol-based method for protein extraction from different latex fractions [26], and a method for protein extraction (REP) from the washing solution (WS) of RPs [16]. By comparison of the proteomes of primary and secondary lutoids, we also found that chitinase and glucanase play crucial roles in RP aggregation [11]. Proteomics of latex revealed ethylene inhibits the expression of enzymes for RP aggregation to prolong latex flowing, and finally improves latex production. We also found that specific isoforms of REF and SRPP are mainly phosphorylated at serine residues, which might be important for ethylene-stimulated latex production [8]. A latex proteomic study indicated that proteins associated with latex regeneration and latex flow can be affected by both ethylene and methyl jasmonate [27]. Recently, 1,839 unique proteins were identified from rubber latex by *de novo* sequencing from mass spectral data [28].

Traditionally, total RPs in the up cream layer were collected and then resuspended and thoroughly washed three times with a Tris-buffered sucrose washing solution. The purified RPs were finally obtained by centrifugation, and membrane proteins were isolated from the washed RP for further proteomic analysis [18,25]. However, by extracting and identifying proteins with a re-extraction method with phenol (REP) from WS, we observed large amounts of proteins in these discarded solutions [16]. In the past studies, WS proteins had been neglected or discarded [18,25]. We speculated that WS may contain many proteins for regulating natural rubber biosynthesis (NRB). Combined 2-DE and shotgun mass spectrometry (MS), we performed comprehensive proteomics of WS, CS and RP, and found many WS-specific proteins. As far as our best knowledge, it is the first proteomic study of WS from RP, and our results may deepen the understanding of RP proteins in NRB.

2. Materials and methods

2.1. Plant material

Fifteen newly tapped mature (8-year-old) rubber tree (*H. Brasiliensis* Mull. Arg. Clone RY 7-33-97) were selected and randomized into three groups for latex collection. These plants were grown in an experiment farm of the Chinese Academy of Tropical Agricultural Sciences in Danzhou City, Hainan Province, China. After tapping, the first 10 latex drops were discarded, and the subsequent drops were collected in ice-chilled glass beakers and taken back for lab analysis.

2.2. Collection of rubber particles, C-serum and washed solutions from latex

The fresh rubber latex was centrifuged at 40, 000*g* for 60 min at 4 °C. The middle clear CS and upper fraction containing RP were collected as described [18,25,26]. After collecting the upper cream of RP, the remained samples were immediately put into liquid nitrogen, and the freezing middle CS icicle was cut out. The collected crude fractions of RP and CS were put into different new tubes, respectively. Then, the collected top creamy RPs were resuspended in an ice-cold washing solution (10 mM EDTA Na_2, 0.5 mM dithiothreitol, 250 mM sucrose, and 10 mM Tris-HCl, pH 7.0) in ratio of 1:10 (w/v) and stirred for 30 min and then ultracentrifuged at 30,000*g* for 15 min at 4 °C. The upper phrase and washed solution of non-rubber fractions were collected, respectively. Repeated the above steps for three times, and collected the washed solution in each time. In the following research, the upper floated RP phrase and clear non-rubber fractions in the second washing time were collected as the purified RPs and WS. Three biological replicates were performed for each sample.

2.3. Protein extraction and two-dimensional gel electrophoresis

Proteins in CS and RP were extracted as described [8,26]. For WS from RP, we collected the washed solutions from the first (WS-1), second (WS-2) and third (WS-3) washing time, then extracted their proteins by the REP method as described [16]. In brief, an equal volume of the extraction buffer was added to WS, and the mixtures were divided into six parts. Equal volume of Tris-saturated phenol (pH > 7.8) was added to one part of the divided samples. The mixtures were vortexed thoroughly for 5 min and centrifuged at 15, 000*g* for 5 min at 4 °C. Then, the phenol-based upper phase was transferred into the next portion of sample, and the extraction procedure was repeated until all WS solutions had been extracted using the same phenol. This extraction step was repeated once more to remove interfering compounds from the mixture solution. The final upper phenol phase was transferred into a new centrifuge tube, and five volumes of ammonium sulfate-saturated methanol were added to precipitate proteins.

Protein concentration was determined by Bradford assay using BSA as standard. For 2-DE, 1300 μg of proteins from WS, CS and RP were loaded onto the 24 cm, pH 4–7 linear gradient IPG strips (GE Healthcare, Uppsala, Sweden). The separation of proteins in the second dimension was performed with SDS polyacrylamide gels (12.5%). The gels were visualized by CBB-G250 staining. Gel image analysis was performed with Image Master 2D Platinum Software Version 5.0 (GE Healthcare, Uppsala, Sweden). Three biological replicates were performed for all the samples.

2.4. Protein identification via mass spectrometry

All high abundance protein spots (Vol > 0.01%) in the 2-DE gels of WS were manually excised and in-gel digested with bovine trypsin as described [8,11]. After trypsin digestion, the collected peptides were spotted on the MALDI plate, and cyano-4-hydroxycinnamic acid as matrix was added onto the dried peptides. The samples were submitted into a 5800 MALDI-TOF/TOF mass spectrometer (AB SCIEX, Foster City, CA). Twenty strongest peaks of the TOF spectra per sample were chosen for MS/MS analysis. The measured tryptic peptide masses were transferred to ProteinPilot Software (Version 5.0) and searched against a self-constructed database derived from the original *H. brasiliensis* genome scaffolds (BioProject ID: PRJNA80191, www.ncbi.nlm.nih.gov/nuccore/448814761) and the draft genome (GenBank: AJJZ01000000) as described [29] using an in-house MASCOT server (Version 2.3, Matrix Science, USA). Tryptic digestion with a maximum of 1 missed cleavage was considered. The cysteine was considered fixed modified by carbamidomethylation and a variable modification of methionine oxidation (M). Spots were considered positively identified with a Mascot score higher than 75 ($p < 0.001$, the threshold with 95% confidence is 31 for rubber tree protein identification), at least 2 matched peptides, and maximum peptide coverage higher than 5% (Table S1). In addition, an in-house BlastP searching was performed at NCBI (http://www.ncbi.nlm.) to confirm all identifications and find their homologous proteins.

2.5. Shotgun and label free quantitative proteomic analysis of WS, CS and RP

The 100 μg of proteins obtained from WS, CS and RP were

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