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Research Articles

Research Article

Optimization of Protein Extraction from Different Latex Samples of *Hevea Brasiliensis*

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Abstract

The objective of this work is to develop a method to get the protein composition of latex. Additionally to the extraction method itself, different preservation methods of whole latex (WL) were tested to prevent latex from any deterioration and/or destabilization (which might induce protein degradation) during the time elapsed between latex collection in the field and protein analysis in the laboratory. These treatments include: stabilization of whole latex with an adequate buffer to generate stabilized latex (SL) as well as freezing WL and SL in liquid nitrogen to generate frozen whole latex (FWL) and frozen stabilized latex (FSL), respectively. Proteins were extracted using three extraction buffers of different compositions and the protein composition was revealed by one-dimensional gel electrophoresis (SDS-PAGE). The SDS-PAGE gels were studied by image analysis to provide qualitative and quantitative information on protein content. The comparison of the SDS-PAGE gels allowed us to determine the most appropriatepreservation method of latex (i.e. stabilizing latex and freezing stabilized latex for short and long storage periods, respectively) as well as to determine the most efficient protein extraction buffer (i.e. 100 mM Tris, 100 mM EDTA, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8).

Keywords: Latex, Preservation Methods, Protein Extraction, SDS-PAGE Electrophoresis, Image Analysis

Introduction

Natural rubber (NR) produced from the latex of *Hevea brasiliensis* is still widely used in tyre industry due to many superior properties over its synthetic counterpart [1]. NR is made of 94% poly(cis-1,4-isoprene) and contains as much as 6% of non-isoprene components (% w/w dry rubber) including: lipids (3.4%), proteins (2.2%), carbohydrates (0.4%) and inorganic constituents (0.2%) [2]. Some studies point out these non-isoprene components, in particular lipids and proteins, as key molecules interacting with poly(cis-1,4-isoprene) chains to

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form microstructures at different scales resulting in enhanced physical properties [3-5].

To better understand the role of lipids and/or proteins on NR properties, the strategy often consists in applying diverse treatments to eliminate lipids and/or proteins from NR to compare the properties of delipidized and/or deproteinized NR to the ones of normal NR [6-8]. Although this approach is extremely useful to highlight the consequences of the absence of non-isoprenes on NR properties, it remains difficult to precisely assign these observations to specific lipids and/or proteins. Therefore it is required to link the non-isoprene compositions of latex/NR to the properties of NR.

The overall objective of our project is thus to study the protein composition of latex and NR in the form of rubber sheets (made from protein-characterized latex) to highlight possible relations between protein composition and NR properties. By comparing the compositions of latex and NR, we will identify the proteins remaining in NR and thus susceptible to affect its properties, then we will focus our future research on those specific proteins. To achieve this goal, efficient methods to extract proteins from both latex and NR materials must be developed.

In this context, the present study provides an optimized latex protein extraction protocol including: (i) a preservation method of latex to avoid any protein degradation between latex collection and protein analysis, and (ii) a protein extraction method to be applied on preserved latex samples. Several preservation methods have been tested: stabilization of whole latex with an adequate buffer and freezing of latex in liquid nitrogen just after collection in the field. Regarding the protein extraction method, we have tested three aqueous extraction buffers applied on each type of latex samples. The efficiency of each method was estimated by SDS-PAGE gels which were then studied by image analysis software to provide the protein profiles and to quantify protein contents [9].

Materials and Methods

Latex collection

Fresh latex was collected from *H. brasiliensis* (clone RRIM600, 12 years old) located in a plantation from Union Rubber Co., Ltd. (Chanthaburi, Thailand). The tapping system was S/2, 2d3. Fresh latex was collected from 9 trees. Three independent replications were prepared by pooling together the latex collected from 3 trees for each repetition. The latex was filtered through a stainless steel sieve (2 mm pore size) to obtain whole latex (WL).

Preservation methods of latex

Two preservation methods were tested: stabilization and freezing of latex. Stabilization of latex was achieved using a stabilization buffer composed of: 100 mM Tris-HCl, 10% glycerol, 0.2% sodium azide, 2 mM PMSF and 100 mM L-ascorbic acid (pH 10). Whole latex (WL) was mixed with stabilization buffer (4:1 v/v) to generate stabilized latex (SL). Both WL and SL samples were kept on ice until arrival at the laboratory.

Freezing of latex was achieved by pouring whole latex (WL) and stabilized latex (SL) in 50 mL Falcon tubes which were kept in liquid nitrogen until arrival in the laboratory and then transferred to -80°C to generate frozen whole latex (FWL) and frozen stabilized latex (FSL).

Composition of extraction buffers

For samples in the form of WL, SL and FSL, we have tested 3 aqueous extraction buffers named A1, A2, and A3.

Extraction buffer A1 was made of: 100 mM Tris, 100 mM EDTA, 1% PVPP, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8. Extraction buffer A2 was made of 25 mM Tris, 10 mM EDTA, 2% Triton X-100, 2% SDS, 20 mM DTT and 2 mM PMSF, pH 8. Extraction buffer A3 was made of: 25 mM Tris, 10 mM EDTA, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8.

Protein extraction protocols

Latex in the form of whole WL, SL and FSL was mixed with extraction buffer (1:2 v/v for WL and 1.25:2 v/v for SL). The mixture was rotated for 40 minutes at 4°C and 3 successive centrifugations were then performed (40,000 g, 30 minutes, 4°C). After each centrifugation, the liquid phase containing proteins was collected. After protein extraction, samples were directly loaded in SDS-PAGE or kept at -20°C.

SDS-PAGE electrophoresis

SDS-PAGE was performed in 13% acrylamide gels using a Thermo Fisher apparatus in a migration buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) regulated at 15°C. Running conditions were: 15 mA/gel during 30-45 minutes for stacking gel and 20 mA/gel during 90 minutes for resolving gel. Staining of the gels was done during 2 hours in staining solution (40% ethanol, 10% acetic acid, 0.1% Coomassie Blue R-250) and destaining in 10% acetic acid solution was let during overnight. Gels were scanned at 300 dpi with a Epson Expression 1680 apparatus. The gels presented in this work are representative of the 3 independent repetitions.

Before loading protein extracts, 2-beta-mercaptoethanol was added to samples to a final concentration of 0.1%. Samples were heated at 95°C for 10 minutes, mixed with Laemmli buffer (2X) (1:1 v/v) and loaded in wells. The molecular weights (given in kDa) of protein bands were compared to a standard (low range, Bio-Rad) containing 6 proteins: phosphorylase b (97.4), serum albumin (66.2), ovalbumin (45), carbonic anhydrase (31), trypsin inhibitor (21.5) and lysozyme (14.4).

Image analysis of SDS-PAGE gels

SDS-PAGE gels were analyzed using the free image analysis software 'Gel Analyzer' (http://www.gelanalyzer.com/) to provide the protein profiles and to estimate the yield of extraction. Protein profiles, i.e. intensity calculated along a line (pixel) going from the top to the bottom of each sample lane, were obtained by using automatic detection of both lane area and staining bands. Image analysis of SDS-PAGE gels was used to quantify proteins as previously described [9].

Results and Discussion

Preservation methods: impact of stabilization buffer

The SDS-PAGE gels of WL and SL samples extracted using buffer A1 are presented on Figure 1A. All samples show a large number of bands with two intense bands around 14 and 24 kDa which probably contain high amount of REF (Rubber Elongation Factor) and SRPP (Small Rubber Particle Protein), respectively [10].

The observation of these gels shows that the use of a stabilization buffer does not perturb the protein composition of whole latex which is confirmed by the protein profiles shown on Figure 1B. The profiles of WL and SL samples show the presence of 13 and 15 bands, respectively; indicating that the qualitative protein composition is not disturb by the stabilization buffer.

Image analysis of SDS-PAGE gels was used to quantify protein [9]. We obtained the following values (expressed in % w/v fresh latex): 1.31 and 1.19 for WL and SL, respectively. It was previously reported that proteins represent 1.5% w/v fresh latex [11]. Comparing our results to this 'theoretical' value, we have estimated the yield of protein extraction (buffer A1) to 88.6% and 87.2% for WL and SL, respectively.

These quantitative values must be interpreted with care as they arise from image analysis of SDS-PAGE gels. However, as all gels were treated and analyzed in the same conditions (lane area, baseline correction), the relative comparison of the samples provides information on the efficiency of each preservation and/or extraction method. The protein extraction yield obtained for WL and SL samples are similar proving that the stabilization buffer used to preserve whole latex does not disturb its protein composition. Thus, the use of a stabilization buffer as a preservative method of latex appears as an excellent option to store the latex over short period (up to few days, data not shown) at 4°C. Moreover, due to its high pH (8), this buffer prevents latex from coagulation.

Preservation method: impact of freezing

Samples in the form of stabilized latex (SL) and frozen stabilized latex (FSL) were compared to study the impact of freezing. Note that we could not study frozen whole latex (FWL) samples due to problem of latex coagulation during defrosting. Indeed, the defrosting of latex from -80°C to 4°C takes several hours and during that time, the whole latex displayed coagulation. This phenomenon was not observed on stabilized latex samples due to the highly basic pH of stabilization buffer preventing rubber particles from coagulation.

The SDS-PAGE gels of SL and FSL samples extracted using buffer A1 are presented on Figure 1A. For FSL, the gel was obtained from samples stored at -80°C during 2 months after latex collection. Then, protein extracts which were kept at -20°C during 1 month before running SDS-PAGE gels.

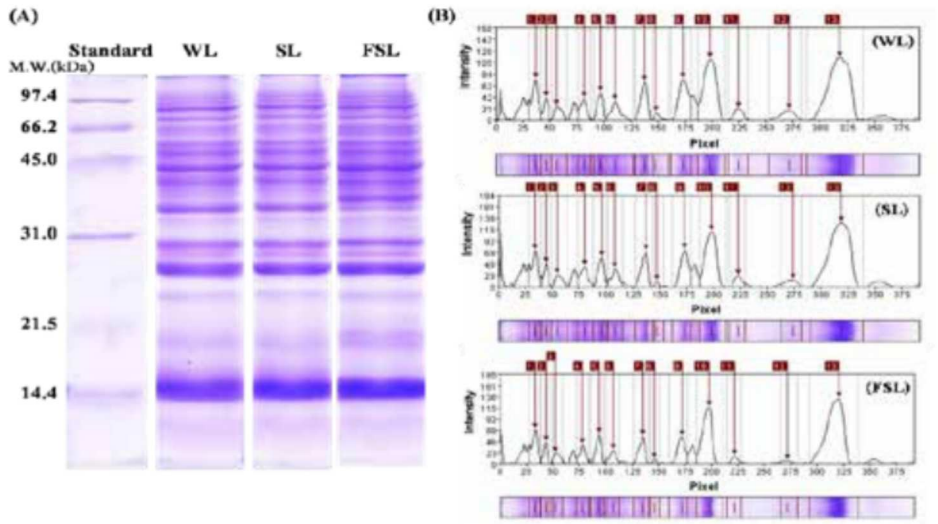

Figure 1. SDS-PAGE gels of WL, SL and FSL samples extracted with buffer A1 (A) and corresponding profiles (B).

The observation of the gels shows that freezing stabilized latex (FSL) does not perturb the protein composition as compared to non-frozen samples (SL), which is confirmed by the protein profiles shown on Figure 1B. The profiles of SL and FSL samples show the presence of 13 and 15 bands, respectively; indicating that the qualitative protein composition is not affected by freezing. Image analysis was used to estimate the yield of protein extraction (buffer A1) resulting in the following values: 87.2% and 86.0%, for SL and FSL, respectively.

The protein extraction yield obtained for SL and FSL samples are similar proving that freezing stabilized latex does not affect the protein composition. This preservative method of latex will be used to store stabilized latex over long time periods. Indeed, it is known that latex composition is susceptible to vary upon season and tapping systems [12]. Freezing latex samples thus offers a good method to study latex composition over long time periods and could be for instance a solution to get rid of the season effect.

Protein extraction method: impact of extraction buffer composition

The composition of the extraction buffer A1 was designed according to previous studies of other authors. For instance, Wang et al. [13] as well as Dai et al. [14] developed methods to extract proteins from subcellular fractions and rubber particles of laticifer latex in Hevea brasiliensis, respectively. Whereas Wang and co-authors used their previously described BPP protocol [15,16] [extraction buffer used in [13]: 100 mM EDTA, 100 mM Tris (pH 8.0), 50 mM Borax, 50 mM Vitamin C, 1% PVPP (w/v), 1% Triton X-100 (v/v), 2% β-mercaptoethanol (v/v) and 30% sucrose (w/v)], Dai and co-authors used an extraction buffer containing CHAPS detergent but without any stabilizing reagents (e.g. sucrose, glycerol) [extraction buffer used in [14]: 30

mM Tris base (pH 7.6), 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.5 % Triton-X 100, 0.5 mM PMSF and a protease inhibitor cocktail].

According to our needs (protein composition of whole latex) and constraints (simple and affordable method to be applied in routine), we defined a composition for A1 [100 mM Tris, 100 mM EDTA, 1% PVPP, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8] and we used two other buffers to study the impact of: SDS (A2) and glycerol/PVPP (A3). To validate A1 as an adequate buffer, the gel of WL sample shown on Figure 1 was compared to the one of Wang et al. obtained on whole latex [13]. Although both gels were obtained in different conditions (standards, % acryl-amide, amount of sample loaded,…), we analyzed the gel of Wang et al. by the image analysis method used in this work and we could detect 15 bands (13 in our case). Interestingly the distribution of the bands was not completely similar: Wang et al. and we detect more protein bands in the 15-30 kDa and 30-90 kDa ranges, respectively.

The SDS-PAGE gels of WL and SL samples extracted using buffers A1, A2 and A3 are presented on Figure 2A, whereas Figure 2B shows the corresponding protein profiles. In the case of WL, the protein profile obtained using A2 buffer differs greatly from the ones obtained using A1 and A3 (this was not observed for SL). Image analysis was used to estimate the yields of extraction. For WL samples, we obtained 88.6%, 35.5% and 84.4% for A1, A2 and A3, respectively. For SL samples, we obtained 87.2%, 79.3% and 76.6% for A1, A2 and A3, respectively.

The results obtained on WL indicate that buffer A2 is not adapted to protein extraction from latex, as reveals by extraction yields. The main difference between A2 and A1/A3 is the presence of SDS which was tested to enhance the extraction of tightly membrane-bound proteins. However, SDS might also strongly disturb protein migration in acrylamide gel.

For both WL and SL samples, buffers A1 and A3 provide similar extraction yields (although slightly higher values were obtained for A1). The main differences between A1 and A3 are the presence of glycerol and PVPP in A1 as compared to A3. The PVPP is usually used to ‘trap’ impurities which can be removed through precipitation of PVPP at the bottom of the tube after centrifugation. However, we did not notice any improvement due to the presence or absence of PVPP. The glycerol is usually used to increase viscosity of the medium resulting in reduced protein aggregation. As the two major proteins of latex (REF and SRPP) are known to display strong aggregation properties [17], the use of glycerol may help to avoid any protein aggregation in protein extracts thus slightly enhancing the extraction yield.

From our results, we propose to use a buffer composition (A4) derived from both A1 and A3 buffers: 100 mM Tris, 100 mM EDTA, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8.

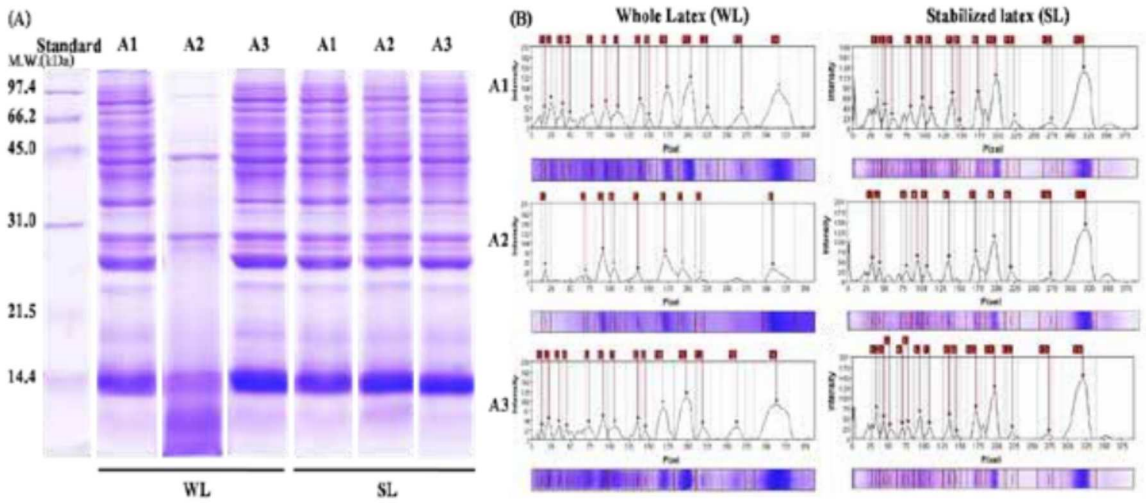

Figure 2. SDS-PAGE gels of WL and SL samples extracted with buffers A1, A2 and A3 (A) and corresponding protein profiles (B).

Conclusions

From the SDS-PAGE gels, we observed that neither stabilizing whole latex (SL) nor freezing stabilized latex (FSL) disturbs the protein composition as compared to the one of whole latex. These two methods can thus be applied to preserve the protein composition and store the latex over short (few days for SL) and long periods (few months for FSL). Moreover, the comparison of the gels obtained from samples extracted with three different buffers allowed us to propose the most appropriate extraction buffer to delineate the protein composition of whole latex: 100 mM Tris, 100 mM EDTA, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8.

As a continuation of this work, we plan to apply this optimized protein extraction method to latex samples collected from different clones of *Hevea brasiliensis*. As the clonal origin impacts NR quality, it will be interesting to observe if the different clones with different properties display different protein compositions. This study will help us to focus our efforts on specific proteins that might impact NR quality.

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