Periploca sepium Bunge as a Model Plant for Rubber Biosynthesis Study

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Z. Naturforsch. **62c**, 579–582 (2007); received December 27, 2006/February 12, 2007

Periploca sepium Bunge (Chinese silk vine) is a woody climbing vine belonging to the family Asclepiadaceae. It originally comes from Northwest China. *Periploca* resembles the Para-rubber tree, *Hevea brasiliensis*, regarding a similar body plan to produce a milky exudate containing rubber latex. The *Periploca* plant was assessed as a rubber-producing plant by rubber structure elucidation and its molecular weight distribution. A rubber fraction purified from the milky exudate was subjected to ^{1}H NMR analysis, and a characteristic signal derived from *cis*-polyisoprene was observed. In addition, when the molecular weight distribution of rubber components in the exudate was measured (using size-exclusion chromatography), the number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity (Mw/Mn) were estimated to be $Mn = 1.3 \times 10^5$, $Mw = 4.1 \times 10^5$, and $Mw/Mn = 3.1$, respectively. Furthermore, the presence of polyisoprene, with $Mn = 4.0 \times 10^4$, $Mw = 7.6 \times 10^4$, and $Mw/Mn = 2.5$, was also confirmed in plantlets obtained from shoots as a result of tissue culture.

Key words: Polyisoprene, Rubber, *Periploca sepium*

Introduction

Natural rubber is the most abundant and the most important hydrocarbon source produced by living plants. A practical rubber production has been performed mostly in the Southeast Asia area. Only the Para-rubber tree, *Hevea brasiliensis*, has been used as a rubber-producing plant in commercial rubber plantation. For both enhancement of rubber content and improvement of rubber quality, an elucidation of the biosynthetic mechanism for rubber to create transgenic plants will be required. Generally, *H. brasiliensis* has been used as an experimental plant for rubber biosynthetic studies. However, *H. brasiliensis* is not a good material because procurement of samples is difficult due to its large size and its propagation area, the tropics, and because tissue culture and introduction of genes are difficult. Accordingly, a proper rubber-producing plant that can be grown in a conventional growth chamber in the laboratory is eagerly required as an experimental model plant. Guayule (*Parthenium argentarum* Gray) is known to be a small size model plant (Cornish, 2001). However, its body plan for plant production is completely different from the Para-rubber tree. Therefore, we investigate the rubber-producing ability of various plants to obtain a rubber-producing plant in smaller size that grows in temperate zones. The requirements for model plants include the following criteria: 1.) small body size; 2.) growth chamber propagation; 3.) short generation

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Abbreviations: SEC, size-exclusion chromatography; *Mn*, number-average molecular weight; *Mw*, weight-average molecular weight; *Mw/Mn*, polydispersity; THF, tetrahydrofuran; BHT, 2,6-di-*t*-butyl-4-methylphenol.

0939–5075/2007/0700–0579 $ 06.00

time; 4.) similar body plan to the Para-rubber tree; 5.) high-rubber producer; 6.) transformation is available. Through surveillance in the north-western part of China, we selected *Periploca sepium* Bunge as a possible model plant for the experimental model plant.

The semi-woody climbing vine, *P. sepium* Bunge, is a dwarf plant of the family Asclepiadaceae that grows in the semi-arid zone of Northwest China. Milky liquid exudes from the stem cross-section of year-old stems. The dried root bark of this plant is called "Xiangjiapi (cortex periplocae)", and is used as a Chinese medicine because of its strong insecticidal and cardiac action. In addition, it was used as firewood to light signal fires on the Great Wall of China. Because it burns well, it is assumed to contain a number of hydrocarbons. Although pregnanes and cardenoides have been reported to be components of *P. sepium* (Itokawa *et al.*, 1988; Xu *et al.*, 1990), there are no reports on rubber components. Therefore, the structure and molecular weight distribution of polyisoprene in the milky exudate were analyzed in detail in order to evaluate *P. sepium*'s potential to produce rubber. Additionally, the rubber contained in the plantlet obtained from shoots as a result of tissue culture was also analyzed.

Materials and Methods

Chemicals and plant materials

Ethanol and toluene for extraction was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For size-exclusion chromatography (SEC) analyses, tetrahydrofuran (THF) containing 0.03% 2,6-di-*t*-butyl-4-methylphenol (BHT) as stabilizer was used (Wako Pure Chemical Industries, Ltd.). To obtain the calibration curve in SEC analysis, seven synthetic *cis*-1,4-polyisoprene standards (*Mn* 1199400, *Mw*/*Mn* 1.10; *Mn* 138000, *Mw*/*Mn* 1.05; *Mn* 30000, *Mw*/*Mn* 1.04; *Mn* 12000, *Mw*/*Mn* 1.04; *Mn* 6000, *Mw*/*Mn* 1.04; *Mn* 2560, Mw/M_n 1.08; *Mn* 1150, *Mw*/*Mn* 1.11, polymer source) were used.

The milky exudate of *P. sepium* was collected in Yangling, Shaanxi, China. The *P. sepium* plantlets used were clones derived from plants grown in Huashan, Huayin County, Shaanxi Province, China (Miyabashira *et al.*, 2003a). The clone plantlet were cultivated aseptically at 25 °C for about 2 months in MS medium (containing 2% sucrose and 0.24% gelite, pH 5.8); light periods, 16 h light; optical intensity, 50 μmol m^{-2} S^{-1}.

Preparation of polyisoprene from milky exudate and plantlet of P. sepium

The preparation of rubber components from the milky exudate was carried out by solvent precipitation. First, methanol was added to the milky exudate and the lipophilic components were precipitated. Afterwards, purification by solvent precipitation using toluene/methanol was carried out twice, and a crude rubber fraction was finally obtained as a benzene-soluble fraction.

The rubber components were extracted from the whole plantlet of *P. sepium* by Soxhlet extraction with toluene. After being frozen and homogenized in liquid nitrogen, low-molecular weight isoprenoids were first extracted by Soxhlet extraction with ethanol. Next, high-molecular weight polyisoprene was obtained by Soxhlet extraction with toluene.

Analysis of polyisoprene

^{1}H NMR spectra were measured using an ECP-400 NMR spectrometer (JEOL Ltd., Akishima, Japan) at 50 °C in benzene-d_6; trimethylsilane was used as an internal standard.

SEC of polyisoprene was carried out using two 300 × 7.5 mm I. D. columns packed with non-polar poly-(styrene-*co*-divinylbenzene) gel (PLgel, MIXED-B, particle size 10 μm, maximum porosity 1×10^7; Polymer Laboratories Ltd., Shropshire, UK). THF was used as an eluent at a flow rate of 0.8 mL/min. The column temperature was set at 40 °C. The column effluent was monitored by a refractive index detector (L 3350, Hitachi High-Technologies Co., Tokyo, Japan). The detector signal was collected on-line by a SIC-480II data station (System Instruments Co., Hachioji, Japan). A calibration curve was made using *cis*-1,4-polyisoprene standards and SIC-480II GPC software (System Instruments Co., Tokyo, Japan). The polyisoprene content was calculated based on the area ratio of the SEC chromatograms from both the amounts of raw material and extracted polyisoprene.

Results and Discussion

The milky exudate of *P. sepium* was collected at Yangling, Shaanxi, China. The preparation of rubber components from the milky exudate was carried out by solvent precipitation (see Materials and Methods). The sample prepared by solvent precipitation was subjected to ^{1}H NMR analysis, and components were analyzed. In addition to numerous signals of other compounds (data not shown), four types of signals stemming from polyisoprene were confirmed (Tanaka and Kawahara, 1989).

Next, the rubber fraction obtained by solvent precipitation was subjected to SEC, and the molecular weight distribution of rubber components was measured. Results revealed the presence of a high-molecular weight fraction with $Mn = 1.3 \times 10^5$ and a low-molecular weight fraction with $Mn = 9.0 \times 10^2$ (Fig. 1). In order to identify the components in each fraction, the rubber fraction was purified by preparative SEC and then subjected to ^{1}H NMR analysis. ^{1}H NMR spectra of the high-molecular-weight fraction sample indicated four types of signals stemming from polyisoprene (Fig. 2). In addition, a methyl group signal was observed at 1.77 ppm; so this macromolecule was clearly *cis*-polyisoprene (Tanaka and Kawahara, 1989). Since the macromolecule has a molecular weight that exceeds 1.0×10^5, the terminal group signal was not observed (data not shown). ^{1}H NMR analysis confirmed the presence of *cis*-polyisoprene in the high-molecular weight fraction, and the content of polyisoprene in the milky exudate was estimated to be about 0.2% (vol/vol) based on the area ratio of the SEC chromatogram. In contrast, there were no polyprenols such as dolichol in the low-molecular weight fraction; results clarified the fact that this fraction is a mixture of various compounds (data not shown).

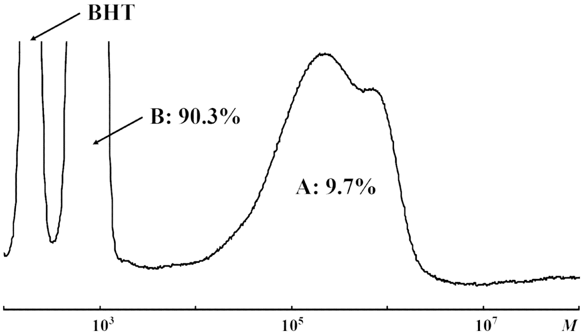

Fig. 1. SEC analysis of rubber derived from *P. sepium* milky exudate. Conditions: column, 2× PLgel, MIXED-B (300 × 7.5 mm I. D.; Polymer Laboratories, Shropshire, UK); eluent, THF; flow rate, 0.8 mL/min; column temperature, 40 °C; detection, refractive index (calibrated against *cis*-1,4-polyisoprene standards). A, High-molecular weight fraction; B, low-molecular weight fraction; BHT.

The rubber components of *P. sepium* plantlet grown by aseptic culture for transformation were also analyzed. Since little milky exudates were produced by plantlets, rubber components were extracted from the entire plantlet by toluene Soxhlet extraction. Removal of low-molecular

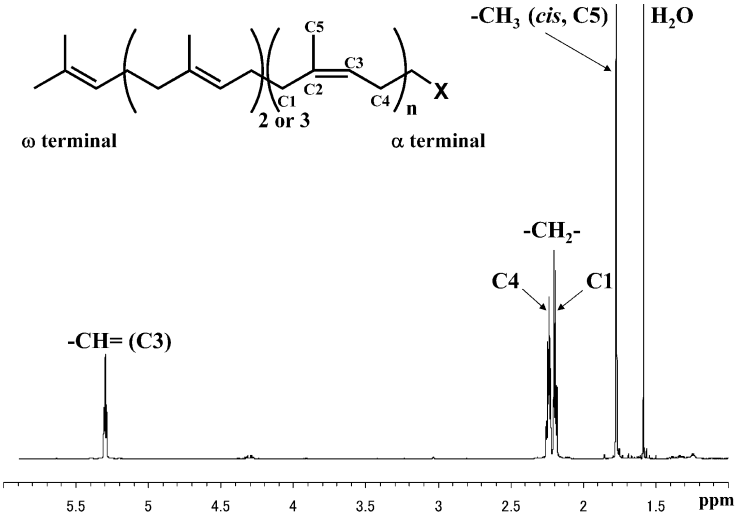

Fig. 2. ^{1}H NMR spectrum of high-molecular-weight rubber from *P. sepium* milky exudate. The spectrum was measured using an ECP-400 NMR spectrometer (JEOL, Akishima, Japan) at 50 °C in benzene-d$_6$; trimethylsilane was used as an internal standard. The signals derived from *cis*-polyisoprene are indicated by arrows.

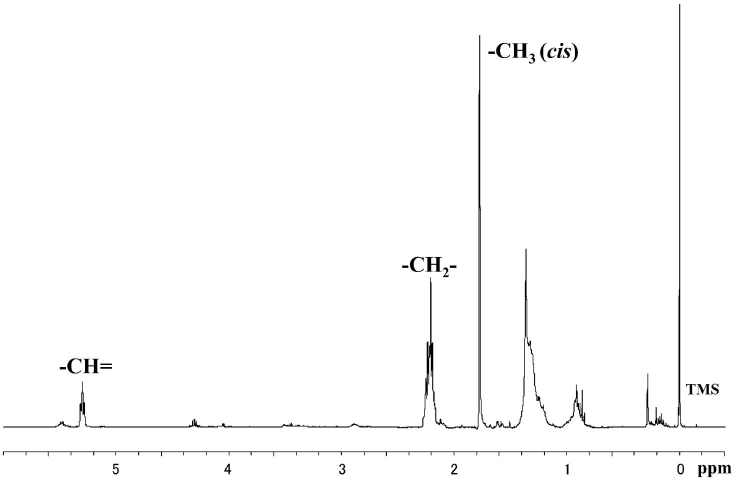

Fig. 3. ^{1}H NMR spectrum of a rubber fraction from *P. sepium* plantlet. Measurement conditions were the same as in Fig. 2.

weight compounds was done by performing Soxhlet extraction with ethanol beforehand. Results of ^{1}H NMR analysis of the toluene Soxhlet extract confirmed the presence of *cis*-polyisoprene like that in the exudates (Fig. 3) (Tanaka and Kawahara, 1989). Results of examining the molecular weight distribution by SEC confirmed the presence of a polymer with $Mn = 4.0 \times 10^4$, $Mw = 7.6 \times 10^4$, and $Mw/Mn = 2.5$. Additionally, polyisoprene content in the plantlet was estimated to be about 0.2 mg/g wet weight based on the area ratio of the chromatogram.

This research confirmed the presence of polyisoprene in the plantlets of *P. sepium*, although in small amounts, and in the milky exudate. This plant cannot be used to produce rubber because of its low rubber content, but tissue culture and transformation systems have previously been established with this plant (Miyabashira *et al.*, 2003a, b), so it is extremely useful in rubber biosynthesis research as a model plant for *cis*-form rubber production. In the future, analysis of the mechanism of polyisoprenoid biosynthesis in plants is expected using *P. sepium*.

Acknowledgement

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO), project “Development of Transgenic Plants for Production of Industrial Materials”. Thanks are due to the Instrumental Analysis Center, Faculty of Engineering, Osaka University, Japan for their assistance in performing the NMR experiments on a JEOL ECP-400.

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