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Analysis of long-chain polyprenols using supercritical fluid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Abstract

The separation of long-chain polyprenols was successfully achieved using supercritical fluid chromatography (SFC). Each 100-mer greater component was separated using tetrahydrofuran as a mobile phase modifier. The molecular mass distributions derived from SFC analyses agreed with the results of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses. The number-average molecular mass calculated by MALDI-TOF-MS data were also in accord with the results of quantitative ^{1}H-NMR analysis of terminal groups. A combination of SFC and MALDI-TOF-MS analyses is a powerful tool for the elucidation of the complicated structures of natural polyprenols.

Keywords: *Eucommia ulmoides*; Supercritical fluid chromatography; Matrix-assisted laser desorption ionization mass spectrometry; Polyprenols

1. Introduction

Polyprenol is the generic name for linear 1,4-polyprenyl alcohols. The polyprenol fraction generally shows a degree of polymerization below 30, for example animal dolichols (16- to 22-mer). Naturally occurring polyprenols are classified into four categories (Fig. 1): (I) all-*trans* form. Only two such polyprenols are known, solanesol (9-mer) and spadicol (10-mer). (II) *trans*-*trans*-*trans*-poly*cis* prenols of the ficaprenol type; (III) *trans*-*trans*-poly*cis* prenols such as the bacteria prenol type and beturaprenol types; (IV) the dolichol type. Among these, only the dolichol type has the alpha terminal saturated.

Since the first report of a polyprenol isolated from tobacco, named solanesol [1], polyprenols have been isolated from animals, microbes, plants, etc. [2–4].

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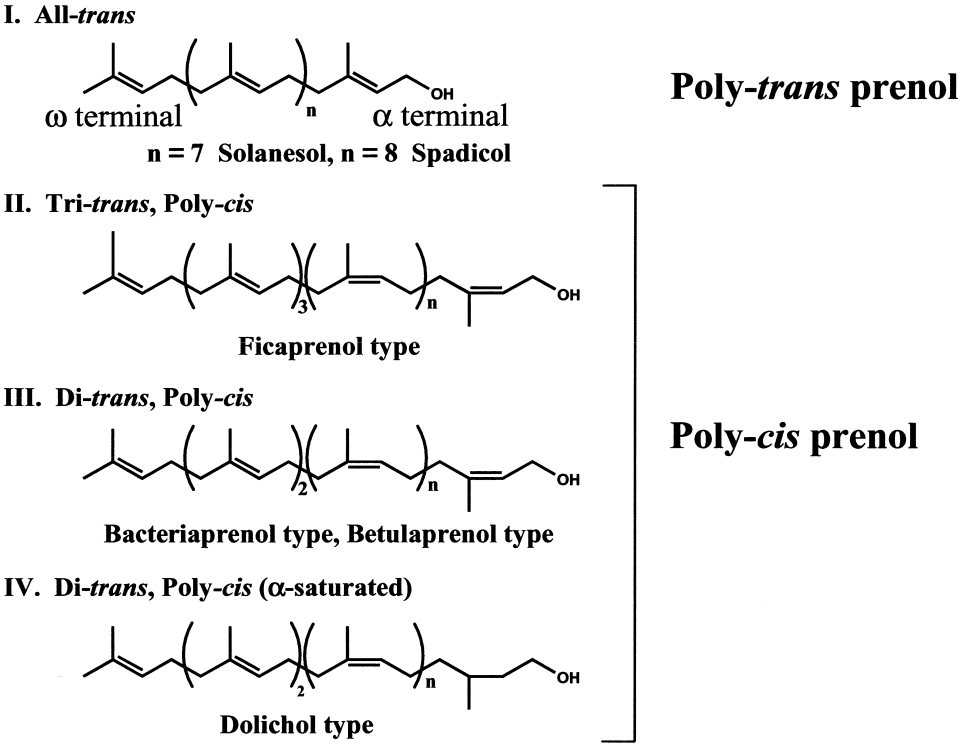

Fig. 1. Chemical structures of natural polyprenols.

There are many reports on the structure and chain-length distribution of plant polyprenols [5–10]. Reversed-phase high-performance liquid chromatography (HPLC) has been used as a conventional method for the analysis of polyprenol [6]. This analysis system is useful for separating polyprenol homologues based on polymerization degree. However, its resolution is not high enough for the baseline separation of geometric isomers and long-chain polyprenols.

We have been elucidating the biosynthetic mechanism of polyisoprene in plants. Polyprenol is thought to be a possible candidate for the biosynthetic intermediate of polyisoprene [11,12] so the analyses of their structure and chain-length distribution is an important issue, demanding the development of a high-resolution analytical system for polyprenol. We have determined that supercritical fluid chromatography (SFC) is useful for polyprenol analysis [13]. The structure of polyprenols derived from *Eucommia ulmoides* Oliver has been analyzed in detail using SFC, and the chain-length of *cis* and *trans* geometric isomers and their distribution in the harvest parts of *E. ulmoides* elucidated [14]. In our previous report, the SFC separation conditions were focused on moderately low-molecular mass polyprenols, 10- to 30-mer. However, high-molecular mass polyprenols should be important biosynthetic intermediates in rubber producing plants. Therefore, separation conditions for long-chain polyprenols were developed in this study. Additionally, samples were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), size exclusion chromatography (SEC) and ^{1}H-NMR terminal group quantitative determination. The obtained analytical data are discussed in detail.

2. Experimental

2.1. Materials

HPLC-grade tetrahydrofuran (THF) for SFC modifier was obtained from Wako (Osaka, Japan). For SEC analyses, THF containing 0.03% 2,6-di-*tert*.-butyl-4-methylphenol as stabilizer was used

(Wako). *Eucommia ulmoides* Oliver were collected in July 2001, at the Hitachi Zosen Corporation experimental station (Habu 2264-1 Innoshima, Hiroshima, Japan).

To obtain the calibration curve in SEC analysis, seven *cis*-1,4-polyisoprene standards (M_n 1 199 400, M_w/M_n 1.10; M_n 138 000, M_w/M_n 1.05; M_n 30 000, M_w/M_n 1.04; M_n 12 000, M_w/M_n 1.04; M_n 6000, M_w/M_n 1.04; M_n 2560, M_w/M_n 1.08; M_n 1150, M_w/M_n 1.11; M_n, number average molecular mass; M_w, weight-average molecular mass; Polymer Source) were used.

2.2. Preparation of long-chain polyprenol

Long-chain polyprenol was prepared from *E. ulmoides* polyisoprene. Polyisoprene with a M_n of 4.6×10^3 was obtained by Soxhlet extraction (toluene) from *E. ulmoides* leaves. After hydrolysis of terminal groups by sodium methoxide, the hydrolysate was fractionated into seven fractions by preparative SEC. Among these, the six fractions (M_n 2.94×10^3, 3.99×10^3, 5.65×10^3, 8.38×10^3, 10.3×10^4, 11.8×10^4; calibrated against *cis*-1,4-polyisoprene standards), except of highest molecular mass (M_n 19.7×10^4), were subjected to analysis (Table 1). A 20-mer *trans*-polyprenol used as a marker of polymerization degree was prepared from *E. ulmoides* leaves by preparative SFC, following the reported procedure of Bamba et al. [15].

2.3. SFC analysis

SFC analyses were performed on an Inertsil Ph-3 (250×4.6 mm I.D.; particle size, 5 μm; pore size, 100 Å; surface area, 450 m^2/g; GL Sciences) column using a Super-201 Chromatograph (Jasco). The system consisted of two pumps, one for delivery of liquid CO_2 as a mobile phase (flow-rate = 3.0 ml/min) and the other for delivering THF as a modifier. The THF flow-rate (0.8 ml/min) was gradually increased to 2.0 ml/min over 30 min and held at 2.0 ml/min thereafter. The fluid pressure was kept at 19.6 MPa by back-pressure regulator. The column temperature was kept at 80 °C. Chromatograms were recorded using a UV detector (950-UV, Jasco) operating at a wavelength of 210 nm.

Table 1
Molecular mass and molecular mass distribution of *trans*-polyprenol fractions

Fraction no.	^{1}H NMR[a]	SEC[b]		MALDI-TOF-MS	
	$\overline{M}_n$ ($\times10^3$)	$\overline{M}_n$ ($\times10^3$)	$\overline{M}_w/\overline{M}_n$	$\overline{M}_n$ ($\times10^3$)	$\overline{M}_w/\overline{M}_n$
1	7.63	11.8	1.12	7.63	1.06
2	5.48	10.3	1.15	5.46	1.06
3	4.40	8.38	1.18	4.75	1.08
4	3.83	5.65	1.24	3.94	1.13
5	3.02	3.99	1.41	3.16	1.20
6	2.40	2.94	1.52	2.66	1.25

[a] Determined by terminal-group analysis.
[b] Calibrated against *cis*-1,4-polyisoprene standards.

2.4. SEC analysis

SEC of long-chain polyprenols was carried out using two 300 mm×7.5 mm I.D. columns packed with nonpolar poly(styrene–*co*-divinylbenzene) gel (PLgel MIXED-B, particle size 10 μm, maximum porosity 1×10^7, Polymer Labs.). THF was used as an eluent at a flow-rate of 0.8 ml/min. The column temperature was set to 40 °C. The column effluent was monitored by a refractive-index detector (L 3350, Hitachi). The detector signal was collected on-line by an SIC-480II data station (System Instruments).

A calibration curve was made by *cis*-1,4-polyisoprene standards using SIC-480II GPC software (System Instruments).

2.5. NMR and MALDI-TOF-MS measurements

^{1}H-NMR spectra were obtained with a Varian UNITY INOVA NMR spectrometer operating at 750 MHz in deuterated benzene at 50 °C, with tetramethylsilane as an internal standard. According to the ratio of methyl proton signals of the dimethylallyl terminal group at 1.68 ppm to those of the main chain at 1.64 ppm, the M_n of fractions were determined. MALDI-TOF-MS analyses were performed by a Voyger-DE PRO (Applied Biosystems).

3. Results and discussion

Long-chain polyprenol samples derived from *E. ulmoides* leaves were used in the analysis experiments. This plant produces fibrous rubber (*trans*-

polyisoprene), called *EU*-rubber, which accumulates in all parts except the seed. This unique rubber is thermoplastic and is similar to gutta-percha in terms of chemical and physical properties. Six long-chain polyprenol fractions of M_n of ca. 3.0×10^3–1.2×10^4 (see above) were used. SFC analysis of long-chain polyprenols was carried out using a column packed with a phenyl group-modified silica gel to achieve an excellent baseline separation, in which over 100-mer (MW: 6818) components were completely separated using THF as a modifier (Fig. 2). Due to the maximum absorption wavelength of polyisoprene being 210 nm, solvents suitable for a modifier are limited. As an alternative modifier, *n*-hexane was also examined. However the separation of high-molecular mass components was insufficient, because *n*-hexane was not enough to elute them. It is thought that the terminal hydroxyl group effects polyprenol separation by SFC, since the separation of polyprenol requires a somewhat polar solvent, as suggested in HPLC analysis [6].

The above-mentioned long-chain polyprenol fractions were also analyzed by MALDI-TOF-MS. The results were compared with those of SFC. After investigating various reagents, good spectra were successfully obtained using dithranol as the matrix and copper(II) nitrate as the cationization reagent. The MALDI-TOF-MS spectrum of the same sample as the Fig. 2 experiment is shown in Fig. 3. The peak

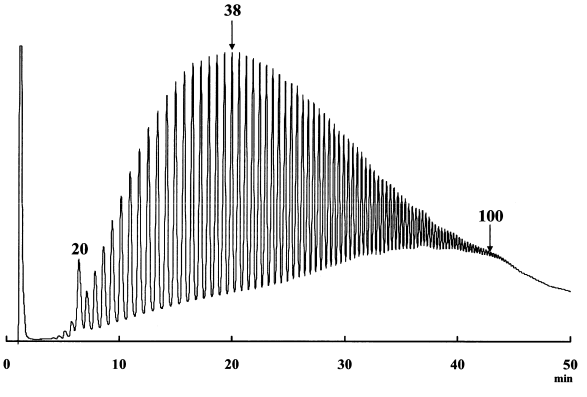

Fig. 2. SFC chromatogram of long-chain polyprenols in *E. ulmoides* leaves. The sample had M_n 3.99×10^3 (calibrated against *cis*-1,4-polyisoprene standards) and M_w/M_n 1.41. The numbers represent degrees of polymerization for polyprenol homologues.

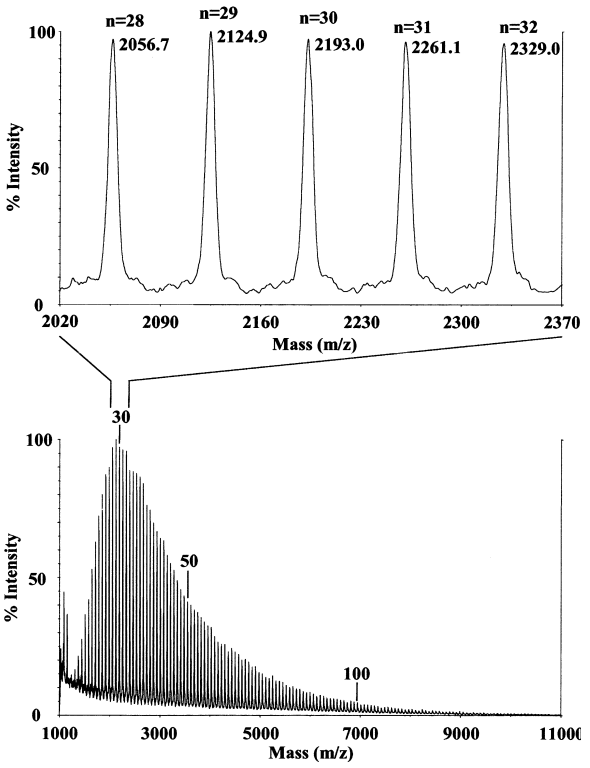

Fig. 3. MALDI-TOF-MS spectrum of long-chain polyprenols (the same sample shown in Fig. 2).

at mass number 2193.0 corresponds to the copper(II) ion (atomic mass 63.5) adduct of polyprenol 30-mer (molecular mass 2119.7). The mass numbers of the other peaks were in good agreement with the copper(II) ion adduct of other polyprenol homologues. The M_n calculated from the MALDI-TOF-MS spectrum was consistent with the results of a terminal group quantitative determination by ^{1}H-NMR. This suggests that MALDI-TOF-MS is useful for the accurate measurement of molecular mass in the case of small sample amounts. However, it is apparent that the distribution maxima of the MALDI-TOF-MS spectra differed from those of SFC chromatograms of the same samples (Fig. 4). The cause of the discrepancy is that a MALDI-TOF-MS spectrum reflects a distribution of the molecular number, while a SFC chromatogram reflects that of mass. Additionally, a mass discrimination effect occurs as the ionization efficiency of a high molecular number component decreases in MALDI-TOF-MS when the

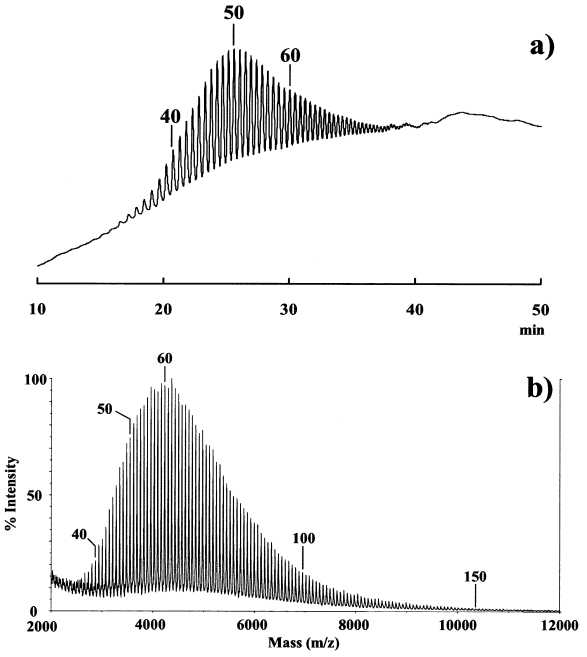

Fig. 4. Comparison of SFC chromatogram and MALDI-TOF-MS spectrum of long-chain polyprenols in *E. ulmoides* leaves. The sample had M_n 8.38×10^3 (calibrated against *cis*-1,4-polyisoprene standards) and M_w/M_n 1.18.

polydispersity of sample exceeds 1.2. Hence the molecular mass distribution maximum shows a lower molecular mass than the real value. This is one of the reasons for the difference in the distribution maximum between the SFC chromatogram and the MALDI-TOF-MS spectrum.

The combination of efficient SFC for separation and isolation of polymerization homologues or geometric isomers and MALDI-TOF-MS for determining efficiently an accurate molecular mass with a small amount of sample enables the detailed analysis of natural polyprenols.

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