Chemical and Mechanical Properties of Cuticular Membranes Isolated from Young Matured Leaves of *Sonneratia alba*

Yuki Takahashi, Shuntaro Tsubaki, Masahiro Sakamoto, Shin Watanabe, Wahyu Dwianto, and Jun-ichi Azuma

Abstract

Leaf cuticular membranes (CMs) of *Sonneratia alba* are considered to play an important role in environmental tolerance, and chemical and mechanical properties of their CMs are crucial factors which provide protective barrier and mechanical supports. Leaf CMs were enzymatically isolated from both adaxial and abaxial sides of young matured leaves (L_3), and their chemical and mechanical properties were compared. Chemical compositions of both CMs were similar reflecting their morphological similarity. The adaxial CM was consisted of 23.4% wax, 64.2% cutin, 7.4% cutan and 6.1% polysaccharides with thickness of 9 μm, and the abaxial CM had the values of 23.9%, 63.2%, 9.4%, 4.2% and 8 μm, respectively. Major monomers in both cutins were 9,10-epoxy-18-hydroxyoctadecanoic acid and 9(10),16-dihydroxyhexadecanoic acid. Mechanical properties of both CMs were also similar reflecting their morphological and chemical compositional similarities.

Key words: mangrove, *Sonneratia alba*, young matured leaves, cuticular membrane, cutin, biomechanics.

Introduction

Mangrove trees grow intertidal zones of tropical and subtropical areas showing zonal distributions parallel to the coastline. *Sonneratia alba* is a kind of mangrove species which widely distributed from East Africa to Southeast and East Asia and tropical Australia. They are generally distributed outermost area of intertidal zones with high environmental tolerance and often form a seaward fringe (Tomlinson 1986).

Aerial parts of higher plants are covered with a protective barrier, cuticle or cuticular membrane (CM), which consists of soluble wax fraction, aliphatic biopolyester cutin, persistent polymer called cutan and polysaccharides (Jeffree 1996; Heredia 2003). Plant CMs minimize solute diffusion and uncontrolled water loss, prevent pathogen penetration and also contribute to mechanical support of plant body (Bargel *et al*. 2006; Heredia 2003). Therefore, chemical and mechanical properties of CMs are determinants of their functions. In dorsiventral dicotyledonous plants, adaxial and abaxial sides of leaves have clearly different structure and chemical and mechanical properties (Azuma *et al*. 2010). In contrast, *S. alba* is known to have isolateral leaves (Tomlinson 1986). This may give a merit to live in the frontier of intertidal zone under strong sunlight, wind and tide. In this study, CMs were isolated from both adaxial and abaxial sides of leaves of *S. alba* and their chemical and mechanical properties were compared to give a proof of high environmental tolerance of *S. alba*.

Materials and Methods

Plant Material and Isolation of the CMs

Leaves of *S. alba* were obtained at Iriomote Island, Okinawa, Japan in May 2009 and February 2010. Leaves after budburst were consecutively numbered from the top in the same shoot. Matured and not aged leaves of L_3 were used in this study. Leaf CMs were enzymatically isolated with fungal pectinase (2%, w/v, *Aspergillus niger*, Sigma-Aldrich, St. Louis, MO, USA) and Meicelase (0.5%, w/v, *Trichoderma viride*, Meiji Seika Kaisha Ltd., Tokyo, Japan). After incubation for 72 h at 36ºC in sodium acetate buffer (5 mM, pH 5.0) with addition of 6% ethanol to prevent microbial growth, CMs were peeled off, washed with distilled water, and air-dried.

Morphological and Spectroscopic Analysis

Isolated CMs were morphologically characterized with low-voltage scanning electron microscopy (LV-SEM, VE-8800, Keyence Co., Osaka, Japan), at 500-fold magnification with 1.7 kV of accelerating voltage. Chemical properties of CMs were characterized with FT/IR (FT/IR-4100, JASCO Co., Tokyo, Japan) in 2.0 cm^{-1} of resolution, and analytical range of 400~4000 cm^{-1} by the thin-film method. The CMs were also analyzed with solid-state CP/MAS ^{13}C NMR (Chemagnetics CMX-300 spectrometer, JEOL Ltd., Tokyo, Japan) operating at 74.7 MHz. The spinning rate was 4.5 kHz, contact time 2 ms, acquisition time 34 ms, sweep width 30 kHz and pulse delay time 5 s.

Chemical Analyses

Chemical compositions of the CMs were analyzed by solvent extraction and gravimetric analyses. First, cuticular wax was removed by refluxing the isolated CMs in a mixture of chloroform and methanol (2:1, v/v) for 2 h at 50°C. Then, cutin was saponified by refluxing dewaxed CMs with 1% KOH in methanol for 2 h at 70°C. Decutinized residues were thoroughly washed with methanol and hydrolyzed by Saeman hydrolysis (Saeman *et al*. 1945). Constitutive

monosaccharides were obtained in the filtrate, and the final non-saponifiable and non-hydrolysable residue was defined as cutan. Polysaccharide contents were quantified by the phenol-sulfuric acid method.

Analysis of cutin monomers was conducted according to the method of Walton and Kolattukudy (1972). Reduction of polyester components was carried out by refluxing the dewaxed CMs in tetrahydrofuran with an excess amount (2.5 times, w/w) of $LiAlH_4$ for 48 h at 70°C. Reduction was also carried out with $LiAlD_4$ to label carboxyl and epoxy groups with deuterium. Reduced monomers were extracted with diethyl ether, dehydrated with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. After TMS derivatization with *N,O*-bis(trimethylsilyl)-acetamide, the compositions of the derivatives were determined by GC-MS (GC/MS 2010/PURVUM 2, Shimazu Co., Kyoto, Japan) with a DB-1 (0.25 mm × 30 m, df = 0.25 µm) non-polar capillary column (J & W Scientific, Agilent Technologies, Inc., Santa Clara, CA, USA) and a helium carrier gas at a flow rate of 0.91 mL/min. The column oven temperature was programmed from 195°C to 240°C at the rate of 2°C/min, and then held for 10 min, and subsequently heated up to 300°C at the rate of 10°C/min. The interface temperature was 250°C, and electron impact (EI) ionization was conducted at 70 eV. The mass spectra were obtained in the range of 40.00~650.00 m/z by scanning mode.

Mechanical Tests

Mechanical properties of the isolated CMs were measured by the tensile test with a tensile tester (Tack Tester TA-500, UBM Co., Kyoto, Japan). Rectangular uniform segments (5 × 20 mm) of samples were clipped with stainless cramps, and deformed at a constant rate at room temperature (23°C). The data of strain and load were collected twice a second. Elastic modulus E (MPa) was obtained from linear region at the initial part of the stress-strain curve. Breaking stress σ_{max} (MPa) and maximum strain ε_{max} (%) were also determined for each sample.

Statistics

Results were presented as mean ± standard deviation (SD). Contents of wax and cutin were calculated as the average of 8~10 replicates, and 3~6 replicates were used to obtain the contents of cutan and polysaccharide. Composition of reduced cutin monomers was determined as the average of triplicate analyses. Biomechanical parameters were calculated as the average of 6~10 specimens prepared from 5~10 replication sets. Mean values of adaxial and abaxial CMs were compared by *t*-tests at the 5% level of significance.

Results and Discussion

Morphological and Spectroscopic Properties

Young matured leaves (L_3) of *S. alba* had stomatal wrinkled surface in both adaxial and abaxial sides as shown in Figure 1. Thickness of the adaxial CMs was 9 ± 2 µm and their yield was 0.76 ± 0.24% of the leaves in the dry state, and the values for the abaxial CMs were 8 ± 2 µm and 0.69 ± 0.24% (n = 20). CMs of *S. alba* leaves were well-developed throughout their surfaces. Their contents were, however, low due to succulence. As shown in Figure 2 (A), FT-IR spectra of the isolated CMs had predominant absorptions at 2920, 2850 and 1730 cm^{-1} assigned to the asymmetric and symmetric stretching vibrations of the methylene groups and the stretching vibrations of ester carbonyl groups (Ramirez *et al*. 1992; Villena *et al*. 2000), confirming aliphatic polyester-nature of the CMs. In addition, the CP/MAS ^{13}C NMR spectra showed presence of carbonyl (160~180 ppm), carbohydrate (60~110 ppm), OCH_3 (around 56 ppm) and alkyl carbons (10~45 ppm) as shown in Figure 2 (B) (Pacchiano *et al*. 1993; Tsubaki *et al.* 2008). These results clearly show that both adaxial and abaxial CMs are structurally similar.

Chemical Compositions

Chemical compositions of the isolated CMs were listed in Table 1. Contents of wax, cutin, cutan and polysaccharides accounted for 23.4, 64.2, 7.4 and 6.1%, respectively, in the adaxial CM. In the abaxial CM, the values accounted for 23.9, 63.2, 9.4 and 4.2%, respectively. The CMs from leaves of *S. alba* were abundant in cutin and wax. No significant differences were detected in the values between adaxial and abaxial CMs, supporting results of spectroscopic analyses.

Since cutin was the most abundant constituent of the CM, an attention was focused on the monomer composition of the cutin. Monomeric compositions of both cutins identified according to Walton and Kolattukudy (1972) were summarized in Table 2. Octadecan-3-ol was the predominant component followed by hexadecan-3-ol. By combination with GC/MS analysis of the deuterium-labeled monomers, these components were concluded to be derived from 9,10-epoxy-18-hydroxyoctadecanoic acid and 16-dihydroxyhexadecanoic acid (obtained as a mixture of 9- and 10-isomers), respectively. They are detected as common monomers of cutins in the other dicotyledonous plants (Walton and Kolattukudy 1972; Kolattukudy 1980). Both adaxial and abaxial cutins were compositionally similar. As a summary, present results showed isolaterality of leaves of *S. alba* for the first time not only from morphological point of view but also from chemical compositional properties.

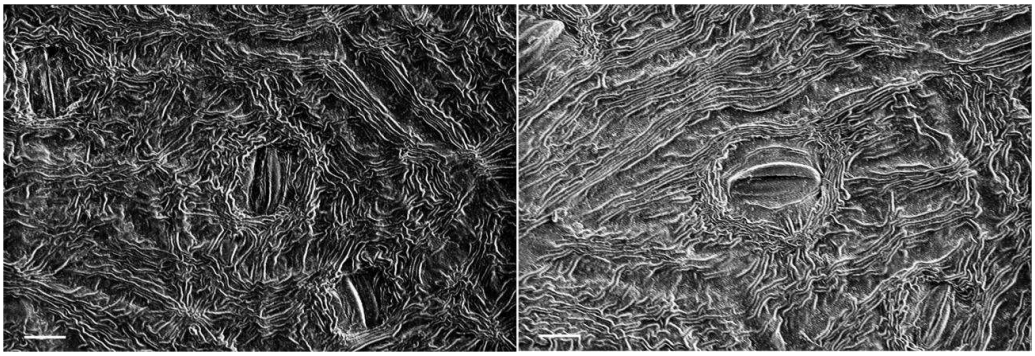

Figure 1. Typical LV-SEM images of the CMs isolated from a young matured leaf (L_3) of *S. alba* (left, adaxial side; right, abaxial side; × 500; bar equals 20 μm).

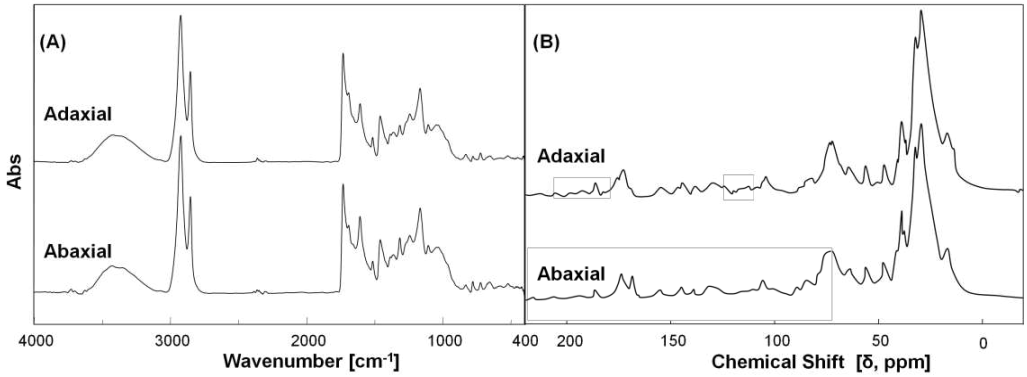

Figure 2. Typical (A) FT-IR spectra and (B) solid-state CP/MAS ^{13}C NMR spectra of the CMs isolated from young matured leaves (L_3) of *S. alba*.

Table 1. Chemical composition of the CMs isolated from young matured leaves (L_3) of *S. alba*. (w%, Mean ± SD).

	Adaxial	Abaxial
Wax*	23.4 ± 4.1	23.9 ± 4.1
Cutin*	64.2 ± 2.6	63.2 ± 4.5
Cutan**	7.4 ± 3.1	9.4 ± 3.2
Polysaccharide**	6.1 ±1.3	4.2 ± 2.3

*n = 8~10, **n = 3~6

Table 2. Composition of the reduced monomers obtained by hydrogenolysis of cutin. (R_t, retention time in min; Relative area %, Mean ± SD (n = 3)).

No.	R_t (min)	Components	Adaxial	Abaxial
1	6.5	Hexadecan-1-ol ($C_{16:0}$)	0.18 ± 0.11	0.31 ± 0.09
2	10.2	Octadecan-1-ol ($C_{18:0}$)	0.03 ± 0.03	0.11 ± 0.04
3	14.1	Hexadecan-2-ol ($C_{16:0}$)	0.77 ± 0.17	0.84 ± 0.06
4	18.7	Octadecen-2-ol ($C_{18:1}$)	1.85 ± 0.20	1.73 ± 0.15
5	19.4	Hexadecan-3-ol ($C_{16:0}$)	25.57 ± 1.14	18.14 ± 0.64
6	20.7	Tetradecen-3-ol ($C_{14:1}$)	2.91 ± 0.18	3.31 ± 0.18
7	22.3	Heptadecan-3-ol ($C_{17:0}$)	0.27 ± 0.03	0.23 ± 0.01
8	24.9	Octadecen-3-ol ($C_{18:1}$)	0.79 ± 0.05	1.60 ± 0.15
9	25.7	Octadecan-3-ol ($C_{18:0}$)	47.48 ± 1.21	47.87 ± 0.59
10	27.4	Hexadecen-3-ol ($C_{16:1}$)	3.29 ± 0.68	7.84 ± 0.24
11	31.1	Octadecan-4-ol ($C_{18:0}$)	2.75 ± 0.01	4.12 ± 0.05
		Unidentified	14.11 ± 0.31	10.12 ± 0.43

Mechanical Properties

Biomechanical parameters of the isolated CMs were analyzed and the results of breaking stress (σ_{max}), maximum strain (ε_{max}) and elastic modulus (E) were listed in Table 3. Although the values of σ_{max}, ε_{max} and E of abaxial CMs were slightly higher than those of adaxial CMs, no significant differences were statistically observed due to large variances. Both adaxial and abaxial CMs thus showed similar mechanical properties, as expected from their similarities in morphological and chemical properties.

Table 3. Mechanical properties of the CMs isolated from young matured leaves (L_3) of *S. alba* (Mean ± SD (n = 6 ~ 10)).

	Adaxial	Abaxial
Breaking Stress σ_{max} (MPa)	6.47 ± 1.99	7.34 ± 1.37
Maximum Strain ε_{max} (%)	4.34 ± 1.96	4.50 ± 1.55
Elastic Modulus E (MPa)	303 ± 43	364 ± 48

Conclusions

Both adaxial and abaxial CMs enzymatically isolated from the leaves of *S. alba* (L_3) were found to have similar spectroscopic and chemical compositional and mechanical properties. These properties may participate to the high environmental tolerance of *S. alba* enough to survive at the frontier of intertidal zones.

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Yuki Takahashi, Shuntaro Tsubaki, Masahiro Sakamoto, and Jun-ichi Azuma*
Division of Environmental Science and Technology,
Graduate School of Agriculture, Kyoto University
Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502,
Japan.

*Corresponding Author
Tel/Fax : 81-75-753-6465/6471
E-mail : azumaji@kais.kyoto-u.ac.jp

Shin Watanabe
Iriomote Station, Tropical Biosphere Research Center,
University of Ryukyus
870, Uehara, Taketomi-cho, Yaeyama-gun,
Okinawa 907-1541, Japan.
Tel/Fax : 81-980-85-6560/6830
E-mail : nabeshin@lab.u-ryukyu.ac.jp

Wahyu Dwianto
Research and Development Unit for Biomaterials,
Indonesian Institute of Sciences, Bogor 16911, Indonesia
Tel/Fax : 62-21-87914509 / 87914510
E-mail : wahyudwianto@yahoo.com