Characterization of Cuticle Layer of *Ilex latifolia*

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Abstract

Cuticular membranes were isolated from the upper and lower surfaces of *Ilex latifolia* leaves and their morphological, chemical compositional and mechanical properties were characterized. The upper cuticular membrane contained 18.6% wax, 53.5% cutin and 17.5% cutan with low contaminating carbohydrates (10.4%), while the lower cuticular membrane had the values of 17.9%, 49.2%, 15.8% and 17.1%. Both membranes were rich in aliphatic CH_2 groups, and the main monomer of the cutin in the upper cuticular membrane was 9(10),18-dihydroxyhexadecanoic acid while 9,10,18-trihydroxyoctadecanoic acid in the lower membrane. Mechanical analyses indicate clear orientation differences in breaking stress and strain between the two membranes with higher breaking stress in the upper cuticular membrane and in the longitudinal direction parallel to the midvein. Additive such as a cellulosic hydrogel from *Salvia viridis* to make a composite influenced the mechnical properties of the cuticular membranes. Mechanical properties of the isolated cuticular membranes may be more influenced by their morphological properties than chemical compositions; thick and smooth structure of the upper cuticular membrane, while wrinkled and scattered stoma-rich structure of the lower cuticular membrane.

Key words: cuticular membrane, cutin, chemical properties, mechanical properties, *Ilex latifolia*.

Introduction

Aerial surfaces of leaves of higher plants are covered by a thin layer, the cuticle or cuticular membrane consisting of wax fraction, biopolyester cutin and insoluble non-hydrolyzable cutan (Jeffree 1996; Nawrath 2002). The cuticular membrane is the interface between the plant tissue and the environment and plays many roles in the survivability of the plant; formation of a barrier to protect leaves from the environmental hazard and pathogenic attack, controlling the movement of substances between the plant and its environment and wettability of the plant by chemicals. Previously**,** we showed that protection of the leaves by cutin was important for a mangrove, *Bruguiera gymnorrhiza* (L.) Lamk., to have a weathering tolerance (Azuma *et al.* 2006). For extending research work on the importance of cuticular membranes on woody plants and also for finding new source of cuticular membranes from plants, we surveyed the source of plants which have harder cuticular membranes enough to use as a kind of biomaterials. In this line of research work, we selected *Ilex latifolia* Thunb. (Tarajo Holly) as a new source of cuticle membranes. It is a medium-sized evergreen tree growing up to about 10 m tall with <24 cm (L) × <8 cm (W) leaves. Since drawing letters and illustrations is possible on the lower surface of its leaf by blackening of the compressed tissue due to polymerization of chlorogenic acid when pressed at its surface with pen (Shiroya *et al*. 1955), the filled up leaf is usable as a "Post Card" and the tree is also called as "Post Card Tree" in Japan. This hardness of their leaves suggests stiffness of their cuticular membranes. The aim of this study is to investigate isolation and chemical characterization of the cuticular membranes of the leaves of *Ilex latifolia* Thunb. Mechanical properties of the isolated cuticular membranes and their composites with cellulosic hydrogel from *Salvia viridis* are also investigated.

Materials and Methods

Isolation of Cuticular Membranes

One year old matured leaves of *Ilex latifolia* Thunb. grown in Kamigamo Experimental Station, Field Science Education and Research Center of Kyoto University were harvested in April to June, 2008. Immediately after harvest, their raw weight, size and thickness were measured and marginal edge portions having width of <1mm were cut off by a pair of scissors. The remained portions were autoclaved for 20 min at 120°C and treated with a mixture of cellulase from *Aspergillus niger* (100 mg, MP Biomedicals, Inc.) and pectinase from *Aspergillus niger* (2.0 mL, Sigma Chemical Co.) for 2 to 5 days at 36°C under stirring at 50 rpm in 100 mL of 50 mM sodium acetate buffer (pH 5.0). A few drops of toluene were used as antiseptic reagent. Two (upper and lower) sheets of cuticular membranes were separated from a sheet of internal reticulate vascular bundle (vein) and their inner surfaces were cleaned up with a paint brush in warm water. Enzymatic and brushing treatments of the two cuticular membranes were repeated once more. Translucent cuticular membranes were then thoroughly washed with warm water, distilled water and dried in a sandwiched manner between two meshed flat sheets.

Chemical Compositional Analysis of Cuticular Membranes

Two sheets of cuticular membranes were dewaxed by thorough extractions with mixed solutions of chloroform and methyl alcohol (2 : 1 and 1 : 1, v/v) and decutinized similarly by refluxing with 1% KOH in methyl alcohol at 70°C. Neutral sugar composition was analyzed by high performance anion exchange chromatography (HPEAC) equipped with an ED40 electrochemical detector in a pulsed amperometric detector mode on a column of Carbopac PA1 (4.0mm x 25.0cm) and 1mM NaOH solution as an elution solvent after hydrolysis with 72% sulfuric acid followed by 3% sulfuric acid at 121°C for 1 hour. Neutral sugar and uronic acid contents in the cuticular membranes were estimated by the phenol-sulfuric acid and *m*-hydroxydiphenyl methods using a mixed neutral sugar solution whose composition was determined by HPEAC and glucuronic acid as standards, respectively (Fournier 2005; Melton *et al*. 2005).

Monomer composition of the cutins in the cuticular membranes were determined by the method of Walton and Kolattukudy (Walton *et al.* 1972). Briefly, the cutins were purified by treatment of the dewaxed cuticular membranes with $ZnCl_2$/HCl and cutin monomers were solubilized by reduction with $LiAlH_4$ and $LiAlD_4$ in tetrahydrofuran for 2 days at 70°C. Composition of cutin monomers released was analyzed by GC/MS (Shimadzu PARVUM2, 70eV) as TMS derivatives transformed by *N,O*-bis(trimethylsilyl)-acetamide. A column of DB-1 (0.25mm x 30m; J & W Scientific) was used at a condition of linear temperature gradient from 195°C to 240°C at 2°C/min and maintaining at this temperature for 10 min.

Preparation of Composite with Cuticular Membranes and Cellulosic Hydrogel and Mechanical Test

Since the cuticular membranes are associated with polysaccharides including cellulose, hemicellulose and pectin in the native state, we tried to make artificial composites with the isolated cuticular membranes and cellulosic hydrogel isolated from seeds of Salvia (*Salvia viridis* L.) (Yudianti *et al*. 2005) and effects of composite formation on breaking stress and maximum strain were analyzed. The inner surfaces of two cuticular membranes isolated from the outer and lower surfaces of the matured leaves were attached with hydrogels of 0.5, 1.0 and 2.0 %(w/w) in water and pressed at 25 g/cm^2 at 70°C. The composites were cut into rectangular segments (5mm x 20mm) at two directions, longitudinal direction parallel to midvein and lateral direction perpendicular to midvein, and stress-strain curves were obtained by using a E4000, UBM Co., Ltd.) with a stress-strain mode at 30°C. The breaking stress and maximal strain at the breaking stress were then measured.

Instrumental Analysis of Cuticular Membranes

Solid state CP/MAS ^{13}C-NMR spectra were obtained by Chemagnetics CMX-300 (7.05T, 75.3 MHz for ^{13}C) (Shiroya *et al*. 1955). Morphological images of the isolated cuticular membranes were analyzed by a low voltage scanning electron microscope (VE-8800, Keyence Co.) on an amorphous carbon stage at 1.3-1.7 kV and x 500-1000. Differential scanning calorimetry (DSC) was conducted by Rigaku Thermolex DSC 8240 equipped with Thermal Analysis Station TAS 100. Warming thermograms were measured by heating at 10°C from -40°C to 80°C.

Optical Microscopic and FT-IR Analyses of Cuticular Membranes

Cross sections of the matured leaves having 15 μm in thickness were made by using Plant microtome MT-2A (NK System Co., Ltd.) and the cuticular membranes were stained for 10 min with 0.5% Sudan III. Thickness of the cuticular membranes was measured by Optical Microscope (Olympus BX51). Analysis of FT-IR spectra of the specimen was carried out by FT/IR-4100 (Jasco, Co., Ltd.) equipped with IRT-5000 Infrared microscope (32 fold Cassegrain), MCT-N narrow band detector (8 cm^{-1}) and an aperture (5 x 50 μm) under transmission mode with a diamond window. Each spectrum was taken by integration for 16 times. FT-IR analysis of the sheets of cuticular membranes were also done with Shimadzu FT-IR 8600 (4 cm^{-1}).

Results and Discussion

Optical Microscopic and FT-IR Analyses of Matured *Ilex latifolia* Leaves

First of all we analyzed morphology of the cuticular membranes in the matured (1-yeared) leaves of *I. latifolia*. Figure 1 indicates a cross sectional view after staining with Sudan III. Both upper and lower surfaces of the leaf were covered with a pair of cuticular membranes stained in orange color (thickness: 19.7 ± 3.5 μm, upper layer and 13.0 ± 3.3 μm, lower layer, n=10), inside of which a single epidermal cell layer, palisade parenchyma and spongy parenchyma rich in aerial spaces including scattered veins were seen. *I. latifolia* is characteristic in thick cuticular membranes especially in the upper (adaxial) region with an abnormal wideness of aerial space in spongy parenchyma region.

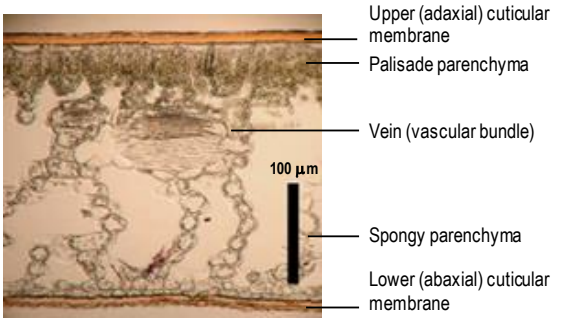


Figure 1. A cross sectional view of a matured leaf of *Ilex latifolia.*

Figure 2a showed FT-IR spectra of three cross sectioned portions at X=40 µm, 210 µm and 530 µm of a whole optical microscopic view (Figure 2b, summed up a series of measured regions, size of each area being 5 x 50 µm). Only at the upper and lower cuticular membrane reagions had strong absorptions at 2925 and 2854 cm^{-1} due to asymmetric and symmetric vibrations of aliphatic CH_2 groups, 1732 cm^{-1} due to C-O stretching vibration due to ester carbonyl groups, 1470 cm^{-1} due to deformation vibrations of aliphatic CH_2 groups and 1167 cm^{-1} due to C-O ester in good accordance with the absorptions of tomato (Holloway 1982), lime (Pacchiano *et al*. 1993) and *Clivia miniata* (Villena *et al*. 2000) cuticles. In Figure 2c, an absorption at 2925 cm^{-1} was plotted against thickness from upper to lower surfaces of a leaf supporting the accumulation of aliphatic carboxylic acids at these narrow regions. Ridged absorptions appeared at X=180 to 300 cm^{-1} may be originated from suberin or lignin-like materials deposited in the cell walls of vein. The present results indicate the presence of thick cuticular membranes in the upper and lower surfaces of *I. latifolia* leaves with much thicker in the former region.

Isolation and Characterization of Cuticular Membranes from *Ilex latifolia* Leaves

Preliminary experiments on digestibility of green matured leaves of *I. latifolia* by cellulolytic and pectinolytic enzymes and extractability with ammonium oxalate indicated that they had a high tolerance against these treatments. Stability of the leaves against the treatments did not improved by cutting off their marginal edges by a pair of scissors. Usually in all cases, darkening of the leaves occurred by oxidative polymerization of chlorogenic acid (Shiroya *et al*. 1955). We therefore tried many methods including treatments with ethyl alcohol, acetone, ethyl acetate, and so on to prevent darkening of the leaves, and found that boiling in water was the most convenient method for inactivation of peroxydases. In addition softening of the tissues inside the leaves also occurred with changing the color of the leaves from green to dark brown, leading to make degradation of the contents of the leaves much easier.

In the present research, we finally succeeded to isolate two sheets of cuticular membranes having original shape from the upper and lower surfaces of the matured leaves of *I. latifolia* by treatment of a mixture of cellulase and pectinolytic enzymes after cutting off their marginal edges by a pair of scissors and autoclaving in water for 20 min at 120°C (Fig3). Yields of cuticular membranes from the upper and lower surfaces were 6.0 ± 0.5 %(w/w) and 4.7 ± 0.2 % (w/w)(n=10), on the basis of the raw leaves. Figure 4 indicates typical morphological images of the isolated cuticular membranes. Outer surface of the upper membrane was quite smooth (Figure 4a), while that of the lower membrane was wrinkly and had scattered stomata (Figure 4c). In the lower surface of the raw leaves, similar wrinkled structure was also observed (data not shown). Over views of inner surfaces of both cuticular membranes showed remaining skeletons of the upper and lower side epidermal cells (Figure 4b and d). No wrinkled structure was seen in the inner surface of lower cuticular membrane (Figure 4d). Thickness of the isolated cuticular membranes were 35.4 ± 6.7 µm (outer cuticular membranes) and 27.4 ± 3.9 µm (inner cuticular membranes) (n=10), respectively. These values were larger than the estimated ones from optical microscopic analysis as described above. Thicker properties of the isolated cuticular membranes may be due to containing the skeletons of epidermal cell walls (Figure 4b and d).

After isolating gram order amounts of the cuticular membranes, chemical analysis was carried out. Contents of carbohydrates remained in the cuticular membranes were estimated to be 10.4% (w/w) (neutral sugar 7.7% and uronic acid 2.7%) in the upper cuticular membrane and 17.1% (w/w) (neutral sugar 12.1% and uronic acid 5.0%) in the lower cuticular membrane, both being rich in glucose (about 64% (w/w)) from cellulose accompanied by a small amount of hemicellulosic and pectinic heterogenous carbohydrates. Relative neutral carbohydrate compositions of arabinose, rhamnose, galactose, glucose, xylose and mannose were 15.1, 5.5, 6.2, 63.6, 5.0 and 4.7% (w/w) for the upper cuticular membrane and 12.6, 5.4, 4.7, 64.3, 3.9 and 9.1% (w/w) for the lower cuticular membrane, respectively.

Figures 5 and 6 indicate FT-IR and solid state ^{13}C-NMR spectra of the isolated cuticular membranes. FT-IR spectra indicate abundance of alkyl and esterified carbonyl groups as described above for the cross sectioned view. Solid state ^{13}C-NMR spectra also support the expected carbon signals derived from hydroxylated fatty acids such as bulk methylenes (29~33 ppm), hydroxylated alkyls (60~110 ppm) including carbons due to carbohydrates, esterified carbonyl groups (175 ppm) (Zlotnik-Mazori *et al*. 1998)

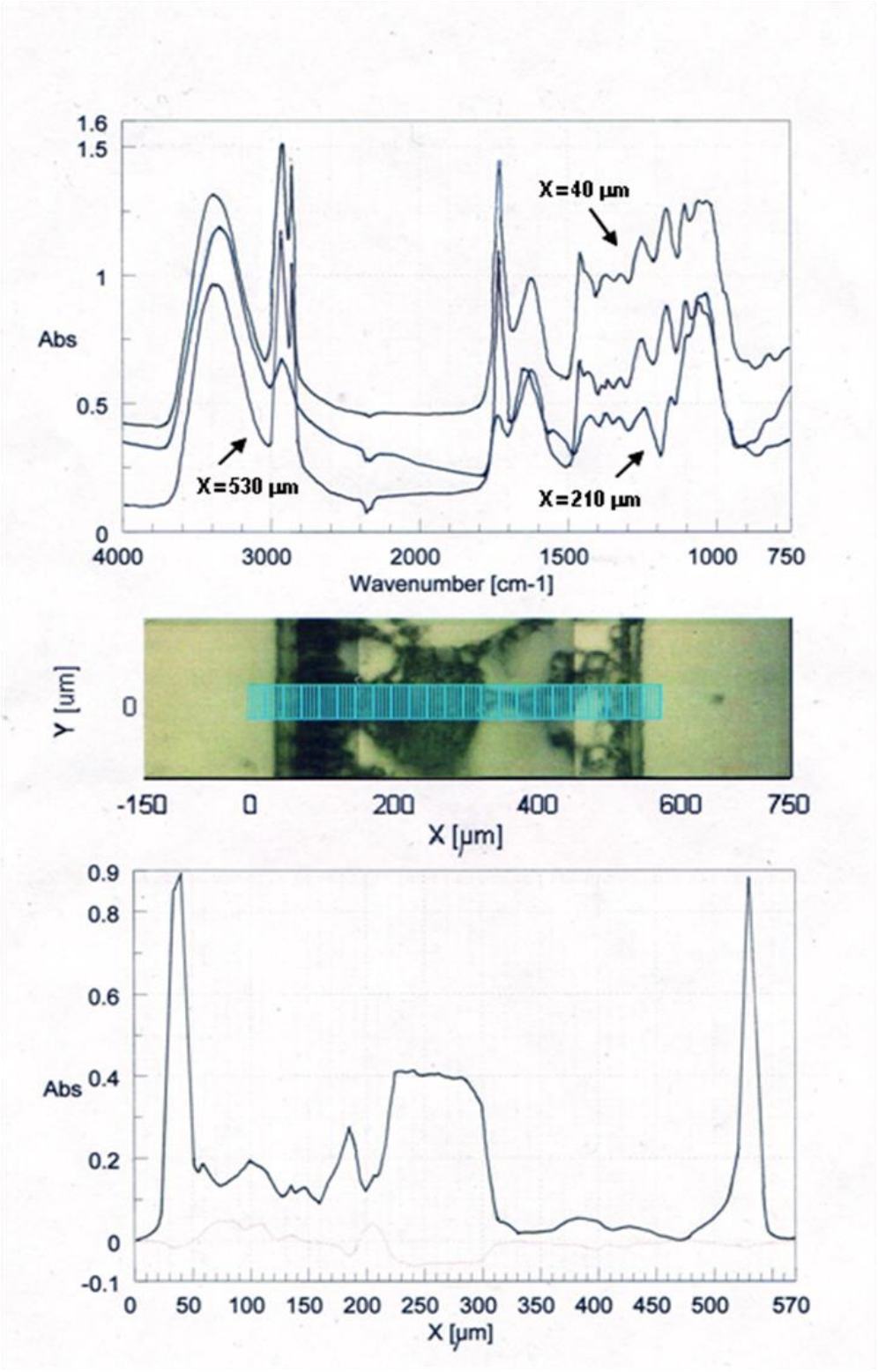


Figure 2. FT-IR spectra under optical microscope of a cross section of matured leaf of *Ilex latifolia*.
Remark: (a) Spectra at X=40, 210 and 530 µm; (b) portions for measurement; and (c) profiles of absorption at 2925 cm^{-1} due to asymmetric vibrations of aliphatic CH_2 groups from upper to lower direction within a cross-section.

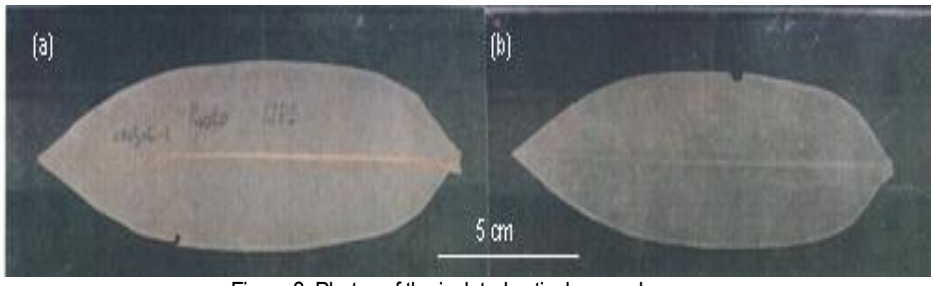
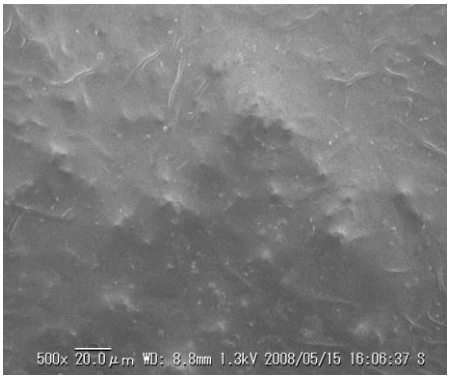
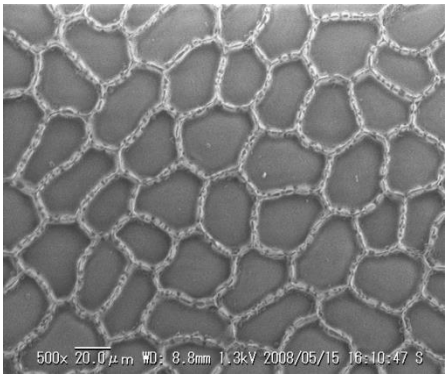


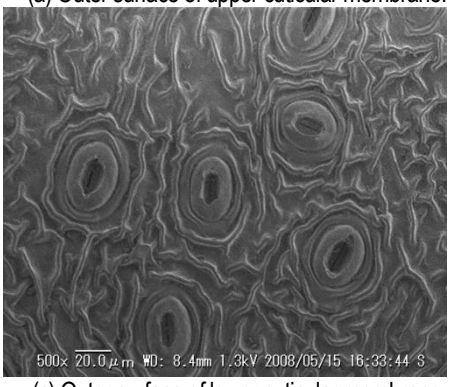
Figure 3. Photos of the isolated cuticular membranes.
Remark: (a) Lower and (b) upper cuticular membranes.



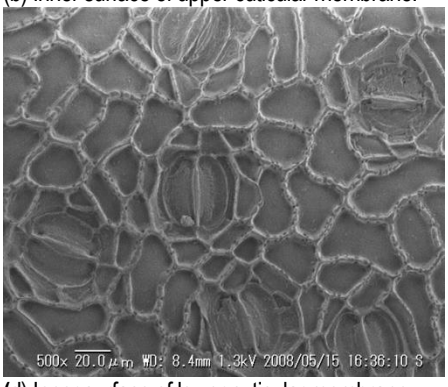
(a) Outer surface of upper cuticular membrane.



(b) Inner surface of upper cuticular membrane.



(c) Outer surface of lower cuticular membrane.



(d) Inner surface of lower cuticular membrane.

Figure 4. LV-SEM images of the isolated cuticular membranes from *Ilex latifolia.*

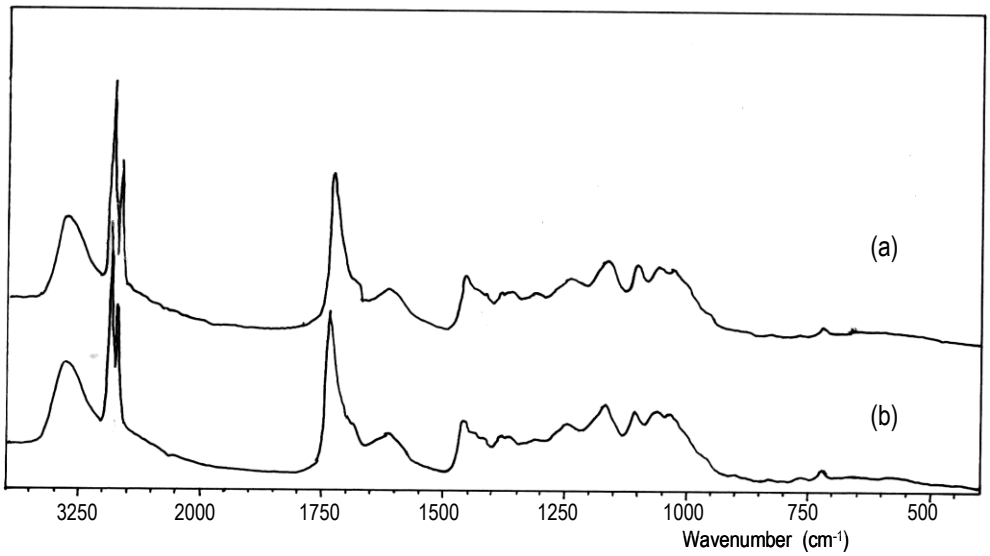


Figure 5. FT-IR spectra of the isolated cuticular membranes.
Remark: (a) Upper and (b) lower cuticular membranes.

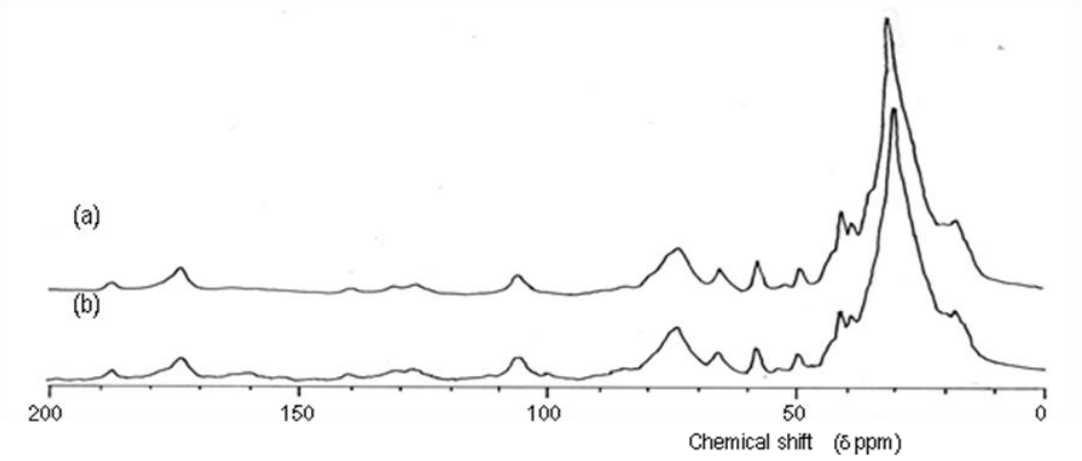


Figure 6. Solid state CP/MAS ^{13}C-NMR spectra of the isolated cuticular membranes.
Remark: (a) Upper and (b) lower cuticular membranes.

The data presented above demonstrated that the isolated two cuticular membrane sheets were derived truly from the upper and lower surface regions of *I. latifolia* leaf. We next analyzed the proportions of wax fraction, biopolyester cutin and insoluble non-hydrolyzable cutan in the isolated cuticular membranes. The data were summarized in Table 1. The contents of wax fraction, cutin and cutan in the upper cuticular membranes were slightly higher than those in the lower cuticular membranes. Contamination by carbohydrates was slightly higher in the lower cuticular membrane. We further analyzed monomeric composition of cutin by deesterification, reduction and GC/MS. Table 2 showed the presence of 16-hydroxyhexadecanoic acid, 18-hydroxyoctadecenoic acid, 9(10),18-dihydroxyhexa- decanoic acid, 9,10-epoxy-18- hydroxyoctadecanoic acid and 9,10,18-trihydroxy-octadecanoic acid as the major monomeric acids. One of the prominent differences in the monomer compositions was in that 9(10),18-dihydroxy-hexadecanoic acid was predominant in the upper cuticular membrane, while 9,10-epoxy-18-hydroxyoctadecanoic acid was the highest in the lower cuticular membrane. This difference may influence their mechanical properties, leading to their different functional roles against environment. The present monomeric acid compositions of the *I. latifolia* cutins were not in agreement with the published information, confirming the presence of wide variations of cutin composition between species and within species of plants (Baker *et al*. 1970).

Physicochemical and Mechanical Properties of the Isolated Cuticular Membranes and their Composites

Phase transitions of the isolated cuticular membranes were analyzed by DSC. The results indicate that both the upper and lower cuticular membranes showed a glass transition at -30°C to -20°C (Data not shown) similar to the tomato fruit cuticles (Luque *et al*. 1994). This transition was ascribed to the conformational changes of the long CH_2 chains by the same authors.

The mechanical properties of the isolated cuticular membranes were analyzed by Dynamic Viscoelasticity Measurement Apparatus with a stress-strain mode in two directions, longitudinal direction parallel to the midvein and lateral direction perpendicular to the midvein. The results shown in Table 3 showed clear orientation differences in breaking stress and strain between the two isolated cuticular membranes with higher breaking stress in the upper cuticular membrane. The values of the maximum strain (%) at the breaking point were always higher in the upper portions and in the longitudinal directions, indicating the presence of anisotropy inside the cuticular membranes. The thickness of the upper cuticular membrane may also influenced their stiffness property.

In the raw leaves the isolated cuticular membranes were attached to the networks of polysaccharides including cellulose. For analysis of the effects of attachment on mechanical property of the cuticular membranes, we made a kind of composites with the isolated cuticular membranes and cellulosic hydrogel from *S. viridis* by pasting the latter on the inner surfaces of the formers followed by compression to dryness, and their mechanical properties were compared. The results listed in Table 3 showed that addition of a small amount of the hydrogel remarkably reduced the breaking stress, but the breaking stress recovered gradually by increasing the amount of the added hydrogel. The extensibility also has a tendency to increase by addition of the hydrogel. Although this is the first report to make a composite with cuticular membranes, composite formation seems to be an attractive technique to improve the physical properties of the cuticular membranes and the cellulosic hydrogels.

In summary, present results indicate that the leaves of *I. latifolia* are an appropriate source for further characterization and functional analysis of the cuticular membranes.

Table 1. Composition of the isolated cuticular membranes from *Ilex latifolia.*

Component	Upper cuticular membrane (%)	Lower cuticular membrane (%)
Wax fraction	18.6	17.9
Cutin	53.5	49.2
Cutan	17.5	15.8
Carbohydrates	10.4	17.1

Table 2. Composition of cutin monomers present in the cuticular membranes from *Ilex latifolia.*

Component	Upper cuticular membrane (%)	Lower cuticular membrane (%)
Hexadecanoic acid	0.5	0.3
16-Hydroxy-hexadecanoic acid	13.8	8.9
18-Hydroxy-octadecenoic acid	11.3	21.4
9(10),18-Dihydroxy-hexadecanoic acid	47.6	24.4
9,10-Epoxy-18-hydroxy-octadecanoic acid	17.6	33.1
9,10,18-Trihydroxy-octadecanoic acid	9.3	11.9

Table 3. Breaking stress and strain of the isolated cuticular membranes and their composites with cellulosic hydrogel from *Salvia viridis* (Breaking stress and maximam strain at breaking position were shown in MPa and %, respectively.)

Consentration of hydrogel to make composite (%)	Longitudical direction parallel to midvein		Laterall direction perpendicular to midvein	
	Upper cuticular membrane	Lower cuticular membrane	Upper cuticular membrane	Lower cuticular membrane
0% (Original cuticular membrane)	25.4 MPa (1.9%)	7.2 MPa (1.4%)	13.5 MPa (1.3%)	5.8 MPa (1.1%)
0.5%	15.0 MPa (1.0%)	13.0 MPa (2.8%)	8.7 MPa (0.7%)	6.3 MPa (2.7%)
1.0%	13.2 MPa (2.2%)	9.0 MPa (1.8%)	15.2 MPa (1.3%)	11.9 MPa (2.2%)
2.0%	19.1 MPa (2.3%)	9.5 MPa (1.8%)	17.9 MPa (2.9%)	9.2 MPa (1.4%)

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