Self-Assembly in Plant Barrier Biopolymers

Eva Domínguez and Antonio Heredia

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

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A new procedure is given to isolate the components that constitute the translucent lines present in some layered plant cuticles. These electron-translucent lines are mainly composed of fatty acids and *n*-alkanes. This waxy material is capable to form molecular bilayers with a constant thickness of approximately 5 nm. This special arrangement have a strong contribution in water transport across the cuticle.

Cutin, suberin and sporopollenin, the main plant barrier biopolymers, cover all aerial parts, underground parts of the plant and pollen grains, respectively. Their functions are water movement restriction, avoiding tissue dehydration, as well as protection (Walton, 1990). These common features are accompanied by a similar fine structure that reinforces the functional, chemical and physiological relatedness of these polymers (Scott, 1994). Their visualization under the electron microscope shows an arrangement of alternate electron-opaque and electron-translucent lamellae. The opaque layers are more polar and of variable thickness, 10–50 nm, while the translucent ones are hydrophobic and more uniform, ranging from 3–10 nm (Fig. 1). These polylamellate layers are always present in suberized cell walls. Although most of the plant cuticular membranes and exines display this layered region, it is dependent on the species and also on the developmental stage (Jeffree, 1996).

Suberin ultrastructure has been most extensively studied. Thus, it is widely accepted that dark bands are mainly constituted by hydroxyfatty and dicarboxylic acids forming a linear polyester together with phenolic components, whereas the light bands are formed of waxes such as very long chain *n*-alkanes and alcohols. These alcohols together with some fatty acids from the dark bands could form a bilayer joined by hydrophobic forces and with their polar functions buried in the dark lamellae (Scott, 1994). This would explain the constant thickness of the translucent laminae. Cross-links between the molecules within both layers, will form a rigid scaffold for the deposition of alkanes, that would be entrapped in the vacancies left by the packing of the hydrophobic chains.

In the case of cuticular membranes the lamellae composition has remained unclear for a long time. Wax extraction from isolated cuticles followed by cutin depolymerization still yields a solid residue with this bilayered pattern. In this paper we report the compounds obtained after the development of a procedure to purify and extract of the components of the translucent bands.

The cuticular membranes used in this work were enzymatically isolated from leaves of *Clivia miniata* Reg. obtained from greenhouse-grown plants by incubation with cellulase and pectinase (Sigma, St. Louis, Mo., USA) following standard procedures (Riederer and Schönherr, 1988). The cuticles were isolated from the adaxial surfaces of the young leaves (4–7 cm length) of the plants. Cuticular waxes were removed by refluxing in chloroform:methanol (1:1, v/v) for 4 hr. The resultant cutin was depolymerized by saponification in 1% potassium hydroxide in methanol for 3 hr under reflux conditions. Polysaccharide material, principally cellulose, was selectively removed by immersion of the cutin in anhydrous hydrogen fluoride in pyridine (Aldrich, Germany) for 5 hr at 40 °C. Phenolic material was removed by refluxing the samples by 10^{-4} M HCl in 1,4-dioxane. After these procedures, translucent bands components were extracted from the solid residue by refluxing it overnight in tetrahydrofuran.

In order to analyse the residue, after the evaporation of the organic solvent, the sample was derivatized using *N*, O-*bis*-(trimethylsilyl)acetamide (Sigma, USA) to form the corresponding trimethylsilyl esters. The esters were analysed by gas chro-

Reprint requests to Prof. Heredia.
Fax: # +34–952–132000.
E-mail: heredia@uma.es

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matography – mass spectrometry (GC-MS) in a Hewlett-Packard 5890 (USA) chromatograph with a HP-1 methyl silicon capillary column.

As it has been indicated above, after cutin depolymerization by saponification, polysaccharide and phenolic material were removed using anhydrous hydrogen fluoride and acidolysis procedure, respectively. The resultant residue was refluxed in tetrahydrofuran for 48 hours. This reflux allowed the extraction of the waxy components that are major constituents of the white lines. In isolated cuticles of *Clivia miniata* leaves, the main constituents identified were fatty acids and *n*-alkanes. The major fatty acids found were hexadecanoic (12.6%) and octadecanoic (9.1%) acids and small amounts of *cis* 9-octadecenoic acids (9.4%). In addition, *n*-alkanes contribute significantly to the total composition (41%). These *n*-alkanes range from C_{19} to C_{26} with C_{22} being the most predominant. Self-assembly of these components, taking into account the behavior above described, will provide a bimolecular lamellae of 5 nm as was observed and reported by several authors (Riederer and Schönherr, 1988). These white lines are known to alternate with dark bands formed by a branched cutin polyester mainly composed by 9,16 and 10,16-dihydroxyhexadecanoic, 18-hydroxy-9-octadecenoic and 9,10-epoxy-18-hydroxyoctadecanoic acids (Riederer and Schönherr, 1988). Fig. 2 shows a theoretical model of the bilayered arrangement of the translucent lamellae.

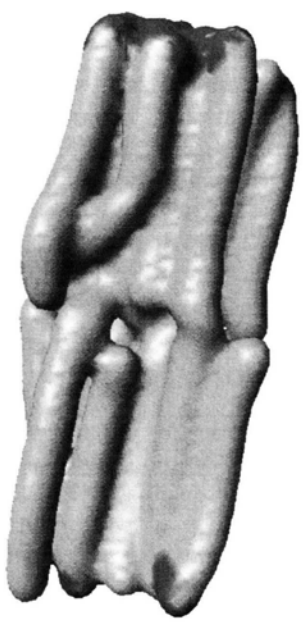

Fig. 2. Molecular model of a bilayer composed by fatty acids and *n*-alkanes showing a molecular arrangement stabilized by hydrophobic interactions between the hydrocarbon tails of both types of molecules.The picture shows the van der Waals surface of this arrangement formed by monomer constituents of the translucent lamellae. The model takes into account a high molecular packing of the fatty acids and *n*-alkanes.The polar functional groups (carboxylic acid groups) are just located at the top and bottom (darker zones) of the bilayer. Mean theoretical thickness of this bilayer is 4.9 nm. Molecular modellization was obtained by standard molecular dynamics calculations using the HyperChem program (HyperCube Inc., Ontario, Canada).

It is interesting to note that the fatty acids found in the white lines are not present in the composition of *C. miniata* cutin but are precursors or intermediates in the biosynthetic pathway of C_{16} and C_{18} hydroxyfatty acids which form the framework of the polyester cutin and cuticular waxes that cover the outer surface of plant cuticles (Kolattukudy, 1996).

The physiological significance of this layered arrangement in different barrier biopolymers seems clear. This disposition of polar and non-polar components in different laminae strongly increases the resistance of the polymer to water loss, leading to a very low cuticular transpiration in plants that show this pattern. This could be interpreted as an evolutionary advantage to maximize the water dif-

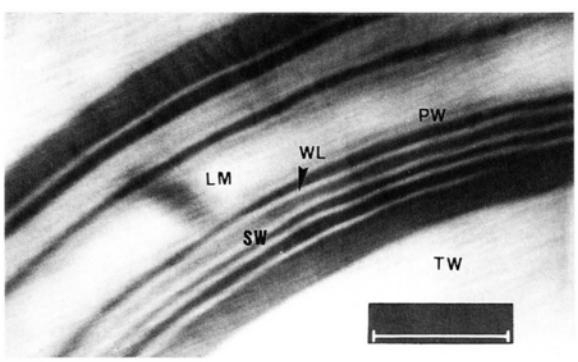

Fig. 1. Transverse transmission electron micrograph (TEM) of a thin section of suberized root exodermis cell wall of *Clivia miniata* Reg. stained with osmium tetroxide. The micrograph (x160.000) shows the characteristic suberin lamellae deposited in the secondary cell wall (SW). Opaque layers are of variable thickness, whereas the lucent wax layers appear with narrower and more uniform thickness. LM: middle lamella; PW: primary wall; TW: tertiary wall and WL: wax layers. Magnification bar, 100 nm.

fusion resistance. This fact is supported by cuticular conductance data which show that plant cuticles with this lamellae structure have a lower cuticular conductance (Kerstiens, 1996).

In the case of suberin is noticeable to point out that Soliday *et al.*(1979) reported that the specific inhibition of the synthesis of the white lines waxes resulted in severe inhibition of diffusion resistance to water vapor. It is important to stress that, as it occurs in the plant cuticle, self-assembly of nonpolar waxes in electron-lucent lamellae takes place. X ray diffraction results of *C. miniata* purified suberin obtained in our laboratory from root hypodermis, showed a diffraction peak corresponding to a basal spacing of 4.7 nm that corresponds to the arrangement of the white lines (Fig. 1). Diffraction peaks corresponding to shorter molecular distances were not observed. Although waxes are present in the translucent lines in high amounts they do not form crystals, reinforcing the idea that they are entrapped and randomly distributed in the biopolymer matrix holes.

Finally, a comment should be made on the common feature mentioned above. Self-assembly of specific lipid compounds is a valuable chemical property. It is a direct consequence of their chemical nature. From a biological point of view, this process facilitates the cellular and trans-cellular self-organization yielding, in this case, specific supramolecular structures that form the protective barriers of aerial and underground plant tissues.

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