Review

Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer

Antonio Heredia *

Facultad de Ciencias, Grupo de Caracterización y Síntesis de Biopolímeros Vegetales, Departamento de Biología Molecular y Bioquímica, Universidad de Málaga, Campus de Teatinos, E-29071 Málaga, Spain

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This work is dedicated to Prof. Ignacio Nuñez de Castro on occasion of his retirement.

Abstract

Cutin is a support biopolyester involved in waterproofing the leaves and fruits of higher plants, regulating the flow of nutrients among various plant cells and organs, and minimizing the deleterious impact of pathogens. Despite the complexity and intractable nature of this biopolymer, significant progress in chemical composition, molecular architecture and, more recently, biosynthesis have been made in the past 10 years. This review is focused in the description of these advances and their physiological impacts to improve our knowledge on plant cutin, an unusual topic in most plant physiology and biochemistry books and reviews.

Keywords: Plant cuticle; Cutin; Chemical composition; Molecular architecture; Biosynthesis

1. Introduction

Most epidermal cells of the aerial parts of higher plants such as leaves, fruits and nonwoody stems, as well as some bryophytes, are covered by a continuous extracellular membrane of soluble and polymerized lipids called the cuticle or cuticular membrane. The structure and composition of the cuticle varies largely among plants, organs and growth stages [1] but it is basically composed by a cutin matrix with waxes embedded in (intracuticular) and deposited on the surface of the matrix (epicuticular). The cutin consists largely of esterified fatty acids hydroxylated and epoxy hydroxylated with chain lengths mostly of 16 and 18 atoms of carbon [2,3]. Cuticular waxes is a general term to describe complex mixtures of homologue series of long-chain aliphatics like alkanes, alcohols, aldehydes, fatty acids and esters, with the addition of varying proportions of cycloidal compounds like triterpenoids and hydroxycinnamic acid derivatives [2,4]. In addition, trace amounts of polysaccharides and amino acids may also be present in the plant cuticle [5]. Based on their constituents, the cuticle can be defined as a hydrophobic and nonreactive polyester with associated waxes.

Plant cuticular material occurs in large amounts in both natural and agricultural plant communities: between 180 and 1500 kg per hectare [6]. Considering that the weight of an isolated cuticle ranges from 2000 \( \mu \text{g cm}^{-2} \) (fruit cuticles) to 450–800 \( \mu \text{g cm}^{-2} \) (leaf cuticles), of which the 40–80% corresponds to cutin, it can be placed as the major lipid plant polymer.

While the morphology and related nomenclature of the cuticle is still disputed, most researchers agree that it is essentially a layered structure [1,5]. In a cross section, the cuticle appears to blanket the outer epidermal cell wall. In some species, pectic material of the subcuticular lamella is layered between the epidermal cell wall and the cuticular membrane. This layer can be chemically or enzymatically degraded allowing the isolation of the cuticle samples. In terms of nomenclature, and from the innermost to the outermost layer, the cuticle consists of the secondary cuticle (cuticular or cutinized layer), the primary cuticle (cuticle proper or cuticularized layer) with embedded waxes, and the

* Tel.: +34-952-131-940; fax: +34-952-132-000.
E-mail address: heredia@uma.es (A. Heredia).
epicuticular wax layer. These cuticular parts are shown in Fig. 1.

A suite of physical, chemical, mechanical and morphological properties gives the plant cuticle the characteristics of a unique and complex biopolymer. Because vascular plants managed to establish themselves on dry land around 400 millions years ago, they have been protected by this complex biopolymer. From a physiological point of view, the main function ascribed to the cuticle is to minimize water loss [7,8]. However, from a more general point of view, this role in the regulation of plant water is accompanied by other important functions: the cuticle limits the loss of substances from plant internal tissues and also protects the plant against physical, chemical and biological aggressions. In this sense, the cuticle has been well characterized for its function in gas exchange, as a lipophilic sorption compartment, and in protecting against mechanical and irradiation damage and herbivore and pathogen attack [7,9].

In recent years, some efforts have been directed toward the elucidation of the structural characteristics, biosynthesis, and molecular biology of components of the plant cuticle. It should be pointed out that the work carried out in different laboratories with mutants with altered epicuticular wax composition has led to meaningful progress in the understanding of the wax biosynthesis [10]. However, except for the studies done by Kolattukudy [11] in the 1970s, the progress in the biochemistry and molecular biology of the cutin has been much more limited. This update is focused on the current knowledge of the physical and structural characteristics, together with the biochemistry and molecular biology, of the plant cutin.

### 2. Cutin composition

Cutin is the major constituent (between 40% and 80% of weight) of the cuticle and, from a chemical point of view, is defined as a polymeric network of oxygenated C\textsubscript{16} and C\textsubscript{18} fatty acids cross-linked by ester bonds. Cutin can be depolymerized by cleavage of the ester bonds by alkaline hydrolysis, transesterification and other methods [2,12]. These chemical methods yield monomers and/or their derivatives depending on the reagent used. The 9- or 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid are the major components of the C\textsubscript{16} cutins. Only in some cases, 16-hydroxy-10-oxo-C\textsubscript{16} acid and 16-oxo-9 or 10-hydroxy C\textsubscript{16} acid are monomers. Major components of the C\textsubscript{18} family of monomers are 18-hydroxy-9,10-epoxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid together with their monounsaturated homologues (Fig. 2).

It is noticeable that in the cuticle of some species, once all the wax and cutin components have been removed, there is some remaining residual material. This depolymerization-resistant residue is thought to represent cutin monomers held together by nonester bonds. The residue, significantly large in weight in some species, is named cutan [1]. It has been suggested that cutan may be ubiquitous in plants since it has been reported to be a constituent of fossilized plant cuticles, terrestrial sediments and coals covering a large part of the geological past [13]. Although the cuticles of some species appear to completely lack cutans, in a number of species, the two biopolymers, cutin and cutan, may occur in any ratio differing in their relative abundance at different stages of cuticle development [13]. The structure and monomeric composition of cutan isolated from leaf cuticles of *Agave americana* and *Clivia miniata* has been elucidated in the last years [14,15]. Using Fourier transform infrared and \textsuperscript{13}C-nuclear magnetic resonance (NMR) spectroscopic analyses, X-ray diffraction and exhaustive ozonolysis, it was

### FAMILY C\textsubscript{16}:

\[
\begin{align*}
\text{CH}_3\text{(CH}_2\text{)}_{14}\text{-COOH} \\
\text{CH}_2\text{OH}\text{(CH}_2\text{)}_{14}\text{-COOH} \\
\text{CH}_2\text{OH}\text{(CH}_2\text{)}_{14}\text{-CHOH}\text{(CH}_3\text{)}_x\text{-COOH} \\
x, y = 6, 7; x+y = 13
\end{align*}
\]

### FAMILY C\textsubscript{18}:

\[
\begin{align*}
\text{CH}_3\text{(CH}_2\text{)}_{16}\text{-COOH} \\
\text{CH}_2\text{OH}\text{(CH}_2\text{)}_{16}\text{-COOH} \\
\text{CH}_2\text{OH}\text{(CH}_2\text{)}_x\text{-CHOH}\text{(CH}_2\text{)}_{16}\text{-COOH} \\
x, y = 7, 8; x+y = 15
\end{align*}
\]

Fig. 2. Structure of the most common fatty acids that act as monomers of the plant biopolyester cutin. For more details, see text.
concluded that the unsaponifiable polymeric core material consists of an amorphous three-dimensional network of polymethyleneic chains linked by ether bonds with the presence of double bonds and free carboxyl groups.

It is remarkable that cutin and cutan behave differently when subjected to the biochemical degradation processes that occur during diagenesis, with cutan being a durable constituent in most of the fossilized cuticles studied. In contrast, cutin is less stable probably due to the presence of hydrolyzable intermolecular linkages.

### 3. Physical characteristics of plant cutin

There are two physical properties of particular interest, which have been recently revised: the rheological and thermal characteristics. They concern the water relationship with the cuticle and, consequently, with the cutin. The role of plant cuticles, and more specifically the cuticular waxes, as barriers against the transport and diffusion of water, has been extensively studied [9]. However, questions such as the exact relationship between the molecular transport properties and the mechanical characteristics, in other words, the rheological properties, of the plant cuticles are still unraveled. Connected with these research lines are the studies on the thermal properties of plant cuticles.

The debate on the existence of polar pores in the cuticular membranes that may contribute to the permeability of water and polar solutes still remains open. Riederer and Schreiber [8] have recently reviewed this controversial topic and they have concluded that the bulk of water diffuses as single molecules across the lipophilic barrier that constitutes the cutin and waxes, while a only minor fraction moves through the more polar pores present in the cutin matrix.

The rheology of the plant cuticle and cutin is of particular interest. It is known that the diffusion and sorption across polymers is influenced by the mechanical properties of the polymer itself. Some factors that affect these properties are the polymer density, the presence of fillers and plasticizers in the polymer matrix and the temperature. Two important physiological problems are related to these properties. One of them is the use of foliar applied chemicals, which could modify the permeability of the biopolymer. The other physiological issue is the fruit cracking as a consequence of an insufficient flexibility of the cutin. Cuticle cracking is a persistent and widespread problem in some greenhouse-grown fruits, that causes degradation of fruit appearance and subsequently serious economic losses [16]. Despite the importance of cuticle in the potential elucidation of these physiological problems, there are only very few studies on cuticular rheology. From stress-strain studies, Petracek and Bukovac [17] described the cuticle as a viscoelastic polymer network. These authors also reported that isolated tomato fruit cuticles expanded and became more elastic and susceptible to fracture after hydration, suggesting that water plasticizes the cuticle.

Some authors have used atomic force microscopy and solid-state NMR to investigate the effect of water sorption on the elastic properties of isolated tomato fruit cutin [18]. One interesting conclusion can be formulated from this singular study: water absorbed by the cutin acts as a plasticizer, promoting molecular flexibility and softening the polymer network. One can visualize that water disrupts hydrogen-bonded cross-links between chains and also diminishes chain–chain methylene hydrophobic interactions.

Temperature-dependent changes in isolated plant cuticles, waxes and cutin have also been performed [19–21]. Isolated plant cuticles and cutins from several species showed a significant high specific heat. This high value means that the cuticular material requires greater amount of heat to raise their temperature by 1° of temperature. Specific heat value of cutin was around 2–2.5 J K⁻¹ g⁻¹, whereas cellulose, the main component of plant cell wall, has a specific heat of 1.5 J K⁻¹ g⁻¹. Although the cuticular material contributes only as a minor mass fraction to the whole leaves and fruits, it could play an important role as a thermoregulator in the course of the biophysical interaction between the plant and the environment.

### 4. Cutin monomers biosynthesis

The elucidation of the biosynthetic pathway of cutin monomers required great effort in the past [11,12]. Of both monomer families, the biosynthesis of the C₁₈ family has been more thoroughly studied.

Pioneering work showed that the oxygenated octadecanoates (Fig. 1) are derived from oleic acid (and also from linoleic acid) by ω-hydroxylation and epoxidation of the double bond followed by the hydroxylation of the epoxide [11,12]. It was suggested that the epoxidation step is catalysed by a cytochrome P450-dependent enzyme, while the hydrolysis occurred within a particulate fraction from apple skin [22]. More recently, the group of Pinot studied in more detail the enzymes involved in the abovementioned pathway. They showed that in *Vicia sativa* microsomes, oleic acid was converted into 9,10-epoxyoctadecanoic acid, which could be then hydrolyzed to the diol 9,10-dihydroxyoctadecanoic acid by an epoxide hydrolase [23,24]. Enantioselectivity of the epoxidation reaction was also studied: the epoxides (9R,10S) and (9S,10R) were produced in a 9:1 ratio [25]. An enzyme system located in the microsomes generated the 9,10,18-trihydroxyoctadecanoic and 18-hydroxy-9,10-epoxyhexadecanoic acids by ω-hydroxylation of both the epoxide and the diol [23]. Further studies demonstrated the involvement of a cytochrome P450-dependent ω-hydroxylase in these reactions. The expression in yeast of this cytochrome P450 CYP94A1 gene from *V. sativa* catalyzed the ω-hydroxylation of both saturated and unsaturated fatty acids [26]. The involvement of fatty acid ω-hydroxylation in plant cutin synthesis will be discussed below.
Cutin monomers seem to serve as potential messengers in plant–pathogen interactions. It has been demonstrated that the release of α-hydroxy fatty acids is a major event during the infection of plants by fungal pathogens. Cutin monomers have been shown to enhance the transcription of cutinase (which hydrolyses cutin) genes [27,28]. Interestingly, some authors recently have demonstrated that pathogen-challenged plants perceive cutin monomers as endogenous molecules for the induction of resistance [29,30]. Thus, treatment of *V. sativa* seedlings with the plant hormone methyl jasmonate led to a rapid accumulation of the CYP94A1 transcripts, suggesting a possible role of the cutin monomer in plant defense response [31].

5. Molecular architecture of cutin, a complex biopolymer

The major cutin monomers shown in Fig. 2 were identified in the 1970s using chemical degradation of the biopolymer and the elucidation of their covalent linkages was based on the consideration of their chemical reactivity [11]. An important number of analytical studies have demonstrated that, while the half of the mid-chain hydroxyl groups in the polymer are involved in cross-linking, most of the primary hydroxyl groups are involved in ester bonds and, consequently, there are a very low number of unesterified carboxyl functional groups.

In spite of the high amount of information on the molecular analysis of the plant cutin, some aspects of its molecular architecture are quite elusive. Since the soluble products obtained after depolymerization do not always represent the situation in the intact polymer, the recent use of nondestructive techniques has produced more useful and complementary information. Structural studies focused on Fourier-transform infrared (FT-IR) spectroscopical analysis of isolated plant cuticles and cutin have been performed in the laboratory of the author [31,32]. This noninvasive tool can be used to check the purity of cuticle isolation and chemical characteristics of the isolates after partial depolymerization [33]. Our research group has also carried out X-ray diffraction analysis. Isolated tomato fruit cutin has been extensively studied with this technique, suggesting a model of cutin comprising an amorphous structure that acts as molecular sieve with two major hydrophobic interplanar spaces around 1.0 and 0.45 nm. These distances are large enough to accommodate molecules of low molecular weight [32].

Solid-state NMR analysis on isolated cuticles and cutin has also been performed in the last years. Most studies indicate that cutin is a moderately amorphous and flexible netting with motional constrains at concrete cross-linked sites [34]. More than half of the methylenes are in a molecular domain of rigid category, with about 36% in a molecular domain of less rigid or mobile category. More recently, 2D NMR spectroscopy has been used to investigate the changes produced by partial depolymerization, using enzymatic or chemical reagents, of the intact polyesters in a solvent-swollen form [35]. These studies confirmed the general structural features deduced from indirect and classical chemical studies: the cutin polyester is held together mostly by primary alcohol ester linkages with about half of the secondary hydroxyl groups involved in ester cross-links.

There is in the recent literature one example of theoretical study on molecular structure of plant cutin. Molecular dynamics (MD) calculations have been applied to picture an initial structure based on the molecular characteristics described above. It is known that MD calculations can provide detailed information that helps to rationalize macroscopically observed behavior from a microscopic standpoint. MD simulations suggested that cutin is a moderately flexible netting with motional constrains mainly located at the cross-link sites of functional ester groups [36]. In this sense, the molecular modeling draws a picture of cutin that agrees with their amorphous and hydrophobic characteristics. From these results, two interesting conclusions can be reached: the existence of molecular spacing of 0.4–0.5 nm between the polymer (methylene) chains and, consequently, the continuous formation of holes or cavities within the structure. MD calculations have also been applied to simulate the diffusion of water molecules through the cutin polymer. The theoretical analysis indicated that water molecules proceed by a hopping mechanism, that is, the water molecules oscillate for long periods of time around certain positions and perform quick jumps between these positions. Theoretical coefficients for the diffusion of water in the cutin model were in good agreement with the experimental data [36].

6. Toward a better understanding of cutin biosynthesis

During the 1970s, it was clearly shown that the biosynthesis of cutin is accomplished by enzymes located in epidermal cells or in outer epidermal walls [22]. Some studies reported that only leaf epidermis and fruit skin were capable of converting exogenous hydroxy fatty acid precursors into an interesterified insoluble polymer. As a consequence of these studies, they postulated the existence of a hydroxyacyl–CoA:cutin transacylase that transfers the hydroxyacyl moieties from their CoA esters to the growing cutin polymer. However, the authors never reported the purification of a cutin transacylase from any source. During the last 10 years, the majority of the work done on cuticle and cutin biosynthesis has been confined to the quantification of the incorporated labeled fatty acid precursors, mainly hexadecanoic and oleic acids, to cutins from leaf or fruit disks. From these studies, it has been possible to know that young tissues present a more active cutin biosynthesis than older ones and to establish the influence of plant hormones in the rate of cutin biosynthesis [37,38]. However, the fact
that no mutants deficient in cutin have been described so far in any species has hampered the progress in this subject.

Some authors have recently used an elegant and indirect way to investigate the functions of the cuticle during development after disrupting the cuticle integrity by the expression of a cutin-degrading enzyme [39]. In this sense, transgenic Arabidopsis plants were engineered to express and secrete a cutinase from Fusarium solani f. pisi. These plants showed an altered ultrastructure of the cuticle and an enhanced permeability to solutes. In fact, cutinase-expressing plants showed ultrastructural changes at the location of cutin polymer, that is, at the outer extracellular matrix of shoot epidermis, resulting in a loose structure of the whole cuticle, which was sometimes totally missing. These results demonstrate that an intact cutin is critical for the physical barrier properties of the cuticular membrane.

Some additional results from this work with transgenic Arabidopsis should be emphasized. The plants showed some developmental abnormalities such as postgenital organ fusion implying an important role for the cuticle, more concretely the cutin, in the inhibition of organ fusion. These results coincide with the model described by other authors on the morphological characteristics of the fiddlehead (fdh) mutant [40,41]. This mutant is deficient in epidermal fusions and interactions exhibiting fused organs as well as pollen germination and tube growth on epidermal cells. Epidermal cell walls and cuticles in this mutant were more permeable and the plants showed a different lipidic composition. From these observations, the authors formulated the hypothesis that localized changes in the permeability of the cutin when transported out of the cell, through the cell wall, to the site of polymerization. Some hypotheses have been formulated over the years but the molecular mechanism is not yet understood. It is important to emphasize that early reports about the occurrence of pores have never been confirmed. The hypothesis of a transport in special vesicles through channels in the cell wall and the cuticle still stands [1]. Other alternative explanation is the participation of lipid transfer proteins (LTPs) in this process. This hypothesis was formulated early in the 1990s [46]. According to this hypothesis, LTPs would carry the acyl cutin monomers necessary for the biosynthesis of cutin. Different observations seem to support this alternative: (i) LTPs are secreted to the apoplasm or into the suspension medium; (ii) high levels of LTP gene expression and LTP gene products accumulation have been detected in the peripheral cell layers and epidermis [47]; and (iii) LTPs are mainly localized in the wax layer [48]. Notwithstanding this theory may seem convincing, it remains to be validated. As Kader [49] indicated in his review on LTPs in plants, the primary query concerning LTPs regards their exact biological role. The remaining question is whether lipids are transported in vivo by these proteins or not.

7. Perspectives

The ubiquitous presence of a well-developed and stable cutin in the aerial parts of soil-growing plants is a clear testimony of its essential role in the plant’s adaptation to an aerial oxidant environment. This main component of the cuticle has received scarce attention in the past. Probably, it has been one of the less intensively studied systems in plants. For example, most of the cuticles characterized correspond to leaf and fruit cuticles with only a few attempts to describe and compare the cuticles that cover other organs such as stems, stigmas and substomatal cavities. Although it has been established that there are differences among them, how different are they? Can these differences be attributed to specific functions? Another overlooked question is how,
during the course of evolution, plants managed to develop this essential biopolymer. There are only a few reports that indicate the existence of a kind of cuticle in some ferns and mosses [50,51] but this cuticle has not been properly characterized neither shown to be a common feature of these lower plants. In some green algae, the presence of a kind of extracellular lipid layer surrounding the whole plant body has been observed and needs further investigation [52,53]. Could this layer be the precursor of the cuticle? It is widely accepted that algae do not have a cuticle because it would be detrimental, which would be the exact function of this cuticle-like layer in algae if its presence is definitively confirmed.

Looking backwards, our current information on plant cuticle and cutin has been slowly obtained over the past three decades, after a great effort of a limited number of research groups. The identification of the fatty acids forming the cutin network and the elucidation of the biosynthesis pathway of these monomers, done using elegant biochemical approaches, is currently well established. However, the exact characterization of the enzymes and the encoding genes involved in the pathway remains quite elusive. On the other hand, our current knowledge of the molecular structure, physical properties, and characteristics of this biopolyester has undergone a dramatic change during the last decade, as a consequence of the application of new physical and biological tools. We now have a model of plant cutin that can partially explain the fascinating and complex properties (permeability, flexibility, and resistance to degradation) that combines with the plant cuticle. Hopefully, the application of molecular biology to the biosynthesis of cutin is just coming to and it promises, in the next future, exciting findings including potential biotechnological applications. As it has been indicated in a recent review on cutin and suberin biopolymers, tools in genomics that are now available in Arabidopsis are likely to lead to the identification of genes and proteins involved in cutin monomer biosynthesis [54]. The challenge today is to use the best of the new molecular and biophysical tools to find appropriate answers to a basic physiological question: what is the relationship between cutin composition, structure and cuticular functions?

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