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Cutin from Plants

Prof. Dr. Pappachan E. Kolattukudy
The Ohio State University, Columbus, Ohio 43210, USA; Tel.: +01-614-292-5682;
Fax: +01-614-292-5379; E-mail: kolattukudy.2@osu.edu

CD	circular dichroism
CMC	critical micellar concentration
CPMAS-NMR	cross-polarization magic angle spin nuclear magnetic resonance
CRE	cutin- responsive element
CREBP	cutin-responsive element binding protein
CTF	cutinase transcription factor
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FTIR	Fourier transform infrared
GC/MS	gas-liquid chromatography/mass spectrometry
HPTLC	high-performance thin-layer chromatography
K_i	inhibition constant
LSIMS	liquid secondary ion mass spectrometry
NMR	nuclear magnetic resonance
PBP	palindrome binding protein
Radio-GLC	radioactivity detector gas-liquid chromatography
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TLC	thin-layer chromatography

1 Introduction

The outer envelope of organisms consists of a polymeric structural component which, in terrestrial organisms, is made waterproof with a complex mixture of nonpolar lipids collectively called waxes. Proteins or carbohydrate polymer (chitin) serves as the structural component in animals, whereas a biopolyester (cutin) – which is derived from cellular lipids – serves as the structural component of the outer envelope (the cuticle) of higher plants. Development of the cuticle, in which cutin is embedded in waxes to make an efficient barrier against desiccation, allowed plants to move to the land about 400 million years ago. The widespread occurrence of polyesters in plants is not widely known because textbooks in biochemistry and other general fields of biology rarely mention cutin. Indeed, if the natural

occurrence of polyesters were known, then synthetic polyesters would have been developed earlier than polyamide polymers.

2 Historical Outline

Studies on the nature of plant cuticle were started in the 19th century. In these early studies, the thin films which remained after treatment of the aerial parts of plants with strong acids were considered to be the true cuticles. Since the cuticles of leaves were known to be readily attacked by alkaline solutions, the acid-resistant material from the leaves was treated with alkali, and this resulted in the generation of soluble soaps. The term cutin came from the name cutose, this being used originally to describe the material that formed part of the epidermis of leaves and which resisted the action of strong acids (Martin and Juniper, 1970).

3 Occurrence and Ultrastructure of Cutin

Cutin constitutes the structural component of the cuticles of higher plants. Even the higher plants that live under water such as the sea grass, *Zoestra marina*, which grows submerged on coastal shorelines, have cutin composed of the same type of monomers as those found on land plants. There is evidence that cuticle exists in lower plants such as mosses, the lycopods, the ferns, and liverworts (Holloway, 1982a). Extensive examination of the cuticular structures made during the modern era demonstrates that the cutin-containing layer is attached to the epidermal cell wall with a pectinaceous glue layer (Figure 1). However, this is an oversimplified general picture, and in reality the boundaries between the cell wall, pectin and cutin layers are not always clearly defined because there is usually some intermingling, especially near the boundaries. A microscopically distinct cuticular layer can be distinguished in most plant organs. Cutin-containing layers are found not only on the surfaces of all aerial parts of plants including stems, petioles, leaves, flower parts, fruits, and some seed coats, but also on internal parts such as juice sacks of citrus (Kolattukudy, 2001). The thickness of the polymer layer varies among species and among organs in the same plant. In higher plant leaves, the thickness ranges from 0.5 to 14 μm, with < 20 to 600 μg cutin per cm^2 of the surface area. In some fruits with a well-developed cuticle, the cutin content may reach 1.5 mg cm^{-2}. In lower plants the cuticle in usually very thin (~ 0.1 μm).

Electron microscopic examination of the cuticular area shows that the cutin-containing layer has a mostly amorphous appearance (Figure 2A). Scanning electron microscopic examination of the cuticular surface that is attached to the cell wall shows cellular outlines and protrusions of the polymer matrix into the intercellular junctions, and demonstrates how the polymer is molded to fit into the intercellular spaces (Figure 2B). Within this matrix, lamellae and fibrillae

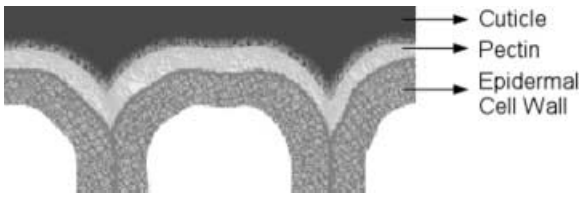

Fig. 1 Schematic representation of the cuticle.

may be found and, when present, such structures form an anastomosing system. Based on the presence of such features and their sites of occurrence within the cuticle, the cuticular ultrastructures have been classified into six groups (Holloway, 1982b). The biological significance of such different appearances is not clear, but the reticular and the anastomosing structures may play a role in the transport of materials through the cuticle.

4 Isolation of Cutin

Disruption of the pectinaceous glue that attaches the cuticle to the epidermal layer by

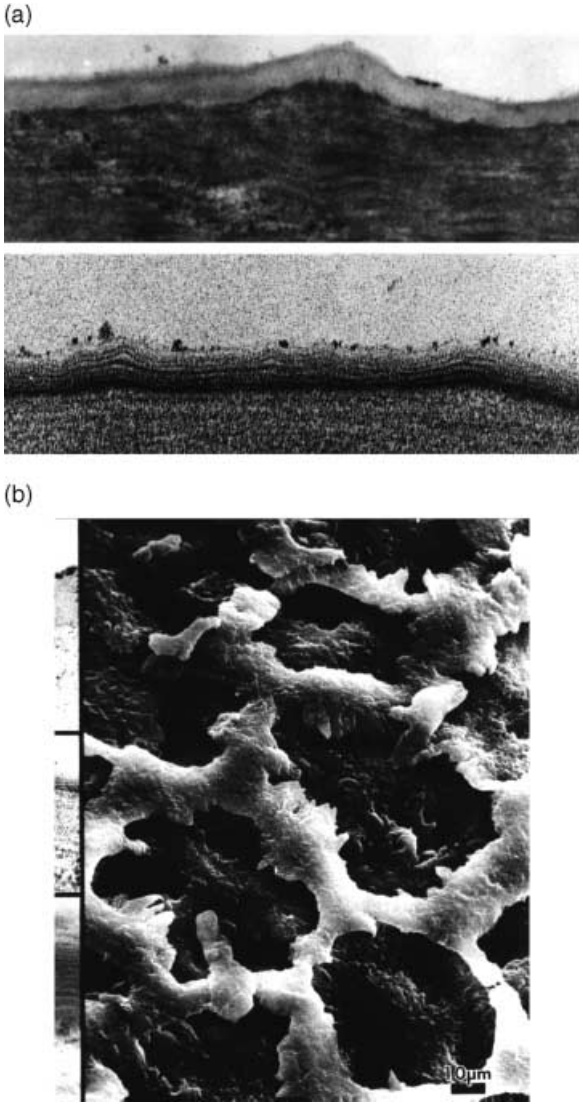

Fig. 2 (a) Electron micrographs illustrating amorphous (top, *Tropaeolum majus*) and lamellar (bottom, *Atriplex semibaccata*) cuticle. (b) Scanning electron micrograph of the underside of tomato fruit cutin showing the protrusions that help to anchor the polymer to the fruit by fitting into the intercellular grooves. *Cu*, cuticle; *CW*, cell wall.

enzymes or chemicals is required to release the cuticular layer. The most commonly used, gentle methods are treatment with ammonium oxalate/oxalic acid or pectin-degrading enzymes (Kolattukudy and Walton, 1973; Holloway, 1982a). The released cuticular layer can be physically separated and subjected to further treatment with carbohydrate-hydrolyzing enzymes to remove additional carbohydrates. A thorough extraction with chloroform is required to remove the soluble waxes embedded in the polymer matrix. Even after several days of extraction, apple cutin showed X-ray diffraction caused by the residual waxes that remained trapped within the polymer matrix (P. E. Kolattukudy, unpublished results). Additional Soxhlet extractions for many more days gradually decreased the diffraction caused by the crystalline waxes buried in the polymer matrix. This problem is more significant in the case of the thicker cuticle found in fruits. The final product can be powdered and used for chemical and/or physico-chemical studies.

5
Depolymerization of Cutin

Cutin can be depolymerized by cleaving the ester bonds chemically to release free monomers or their derivatives, depending on the chemical cleavage method used. This polyester can also be depolymerized with enzymes that catalyze the hydrolysis of ester bonds.

5.1
Chemical Depolymerization

Since cutin is largely a polyester, ester bond cleavage methods can release the monomers. Most commonly used methods include alkaline hydrolysis, transesterification with methanol containing boron trifluoride or sodium methoxide, reductive cleavage by exhaustive treatment with $LiAlH_4$ or $LiAlD_4$ in tetrahydrofuran, or with trimethylsilyl iodide in organic solvents. These methods yield monomers or their derivatives based on the depolymerization method (Figure 3). Cutin can contain functional groups that are not stable to the depolymerization methods such as epoxides and aldehydes. During depolymerization, such functional groups may be converted to derivatives that are useful for identification of the functional group(s) originally present in the polymer. For example, during reductive depolymerization with $LiAlD_4$, the epoxide and aldehyde would generate D-labeled derivatives; subsequently, mass spectrometry can readily locate the label in the reduced product and thus identify the epoxy or oxo function originally present in the polymer. In fact, such deuterium labeling is what established the 18-hydroxy-9,10-epoxy-C_{18} acid as a widely occurring cutin component (Walton and Kolattukudy, 1972; Kolattukudy and Walton, 1973). Similarly, ω-oxo acid as a major component of cutin from embryonic tissue was discovered by the deuterium labeling that occurred during the reductive depolymerization. Methanolysis of the oxirane would generate a methoxy group vicinal to a carbinol identifying the location of the epoxide in the original polymer. Functional groups such as aldehydes can also be derivatized, first by making derivatives such as an oxime, followed by depolymerization, to yield an identifiable derivative (Kolattukudy, 1974). Most cutin preparations leave behind insoluble residues after exhaustive treatments with ester-hydrolyzing reagents (see Section 7).

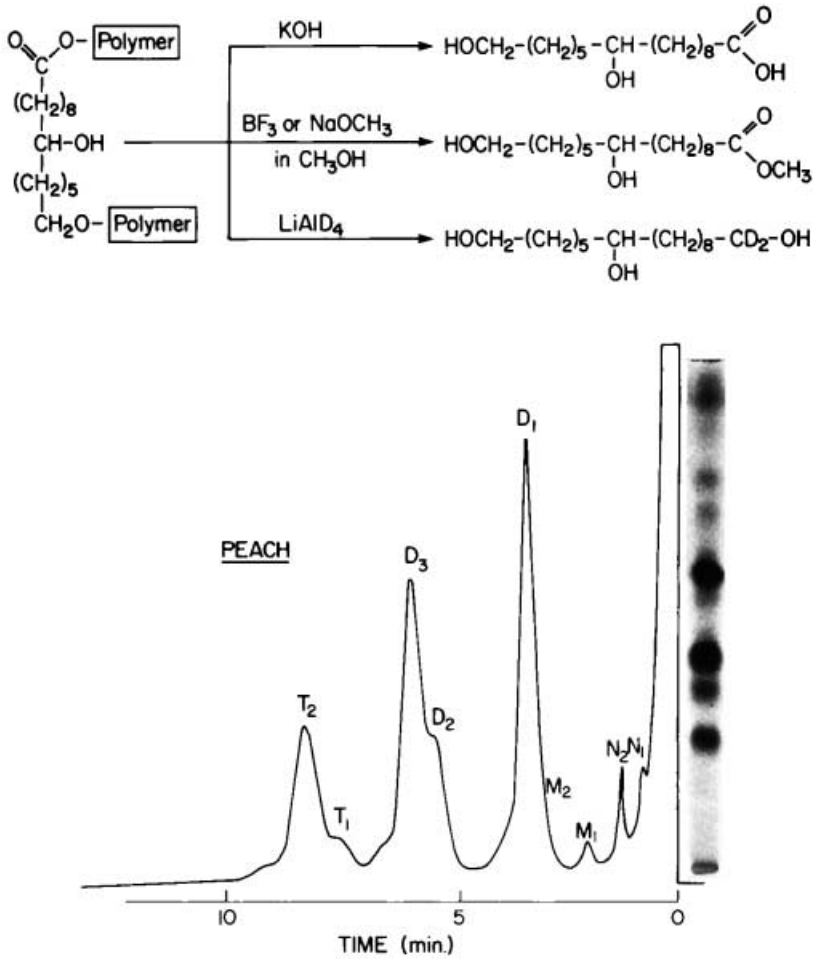

Fig. 3 Chemical methods used to depolymerize the polyesters (top); thin-layer and gas-liquid chromatograms (as trimethylsilyl derivatives) of the monomer mixture obtained from the cutin of peach fruits by $LiAlH_4$ treatment (bottom). In the thin-layer chromatogram the five major spots are, from the bottom, C_{18} tetraol, C_{16} triol, and C_{18} triol (unresolved), diols, and primary alcohol. N_1, C_{16} alcohol; N_2, C_{18} alcohol; M_1, C_{16} diol; M_2, C_{18} diol; D_1, C_{16} triol; D_2 and D_3, unsaturated and saturated C_{18} tetraol, respectively.

5.2

Enzymatic Depolymerization

Since the majority of monomers are held together by ester linkages, esterases can cleave the polymer. Pancreatic lipase can hydrolyze cutin, thereby releasing oligomers and monomers. Evidence was presented that bile salts stabilize the enzyme at the surface of the insoluble polymer and that the interaction of the polymer surface with lipase–colipase–bile salt system is similar to that observed with triglycerides (Brown and Kolattukudy, 1978b). A special polyester-hydrolyzing lipase – cutinase – has evolved in microorganisms such as bacteria and fungi (see Section 9). The first cutinase

purified and characterized was a fungal cutinase, and this enzyme was shown to release oligomers and monomers. Since cutinase preferentially hydrolyzes primary alcohol esters, enzymatically isolated oligomers are suitable for structural studies involving cross-links via secondary alcohol ester bonds (see Section 7).

6 Monomer Composition of Cutin

The complex mixture of monomers produced by the chemical depolymerization methods can be separated by thin-layer chromatography (TLC) on silica gel into different classes (depending on their polarity) such as ω-hydroxy-, dihydroxy-, trihydroxy-, and fatty acid derivatives (Kolattukudy and Walton, 1973; Holloway, 1982a). The selection of the solvent systems varies depending on the depolymerization method used. The individual fractions recovered from TLC are then subjected to combined gas-liquid chromatography/mass spectrometry (GC/MS) after making appropriate derivatives, again depending on the depolymerization method used. For example, transesterification method yields methyl esters, and therefore derivatization of the free hydroxyl groups by trimethylsilylation provides the appropriate derivatives for combined GC/MS. Hydrogenolytic cleavage with $LiAlD_4$ produces reduced monomers that can be trimethylsilylated before GC/MS. If a hydrolytic method is used, both the carboxyl groups and the hydroxyl groups need to be derivatized. Even though trimethylsilylation derivatizes carboxyl and hydroxyl groups, trimethylsilyl (TMSi) derivatives of methyl esters give more diagnostic mass spectra. Retention times are useful in identifying monomers when authentic standards are available. However, mass spectrometry is necessary to identify reliably the monomer structure; hence GC/MS is the method of choice for determining the structure and composition of monomers. Usually, the mixture of monomers obtained by one of the depolymerization methods can be derivatized and directly analyzed by combined GC/MS, without preliminary TLC. The highly preferred α-cleavage on either side of the trimethylsiloxyl groups of the mid-chain oxidized fatty acid derivatives makes it relatively easy to identify the location of the mid-chain hydroxyl groups.

The results obtained from such analysis performed on cutin from many plants show that this plant polyester is composed of mainly a C_{16} family and a C_{18} family of monomers (Figure 4) (Kolattukudy and Walton, 1973; Holloway, 1982a; Kolattukudy, 2001). The most common major components of the C_{16} family of monomers are 16-hydroxyhexadecanoic acid and 9 or 10,16-dihydroxyhexadecanoic acid. This dihydroxy acid is usually the dominant component, and usually there is a mixture of mid-chain positional isomers. In some cases, other C_{16} derivatives can be significant or major components. Examples include 16-hydroxy-10-oxo-C_{16} acid in citrus cutin and 16-oxo-9, or 10-hydroxy-C_{16} acid in the very young leaves and embryonic shoots of *Vicia faba*. In some cases (especially lower plants), a reduced C_{16} monomer, such as 1,8,16-hexadecanetriol, is a major component. The primitive cutin in ferns and lycopods is characterized by large amounts of 16-hydroxyhexadecanoic acid and other ω-hydroxy acids. The most common major members of the C_{18} family of cutin monomers include 18-hydroxy-C_{18}-9-enoic acid, 18-hydroxy-C_{18}-9,12-dienoic acid, 18-hydroxy-9,10-epoxy-C_{18} acid, 18-hydroxy-9,10-epoxy-C_{18}-12-enoic acid, 9,12,18-trihydoxy-C_{18} acid, and 9,10,18-trihydroxy-C_{18}-12-enoic acid. In some plants such as *Rosmarinus officinalis*,

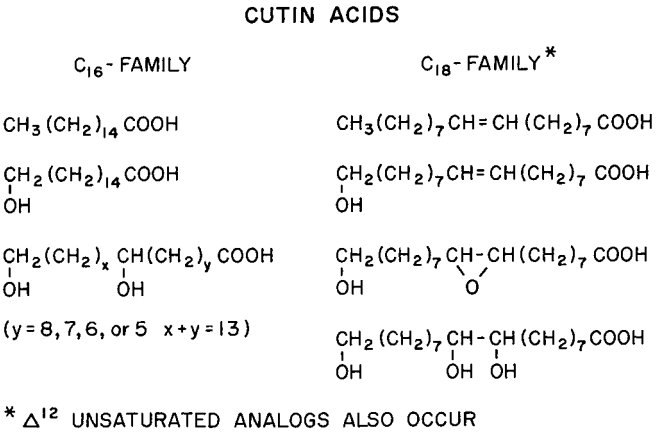

Fig. 4 Structure of the most common major monomers of cutin.

9,10,18-trihydroxy-12,13-epoxy-C_{18} acid and 9,10,12,13,18-pentahydroxy-C_{18} acids are found (Croteau and Kolattukudy, 1974a). The ω-oxo derivatives of the C_{18} family of acids may also occur in small quantities. Most plants contain different mixtures of the two families of monomers, with each species showing a characteristic mixture of components. However, the monomer composition depends on the anatomic location within a plant. For example, the content of the major C_{18} family of cutin monomers in *Malus pumila* cutin from fruit, leaf, stigma and flower petal was found to be 73%, 35%, 14%, and 12%, respectively (Espelie et al. 1979). Developmental changes in cutin composition can also occur, as demonstrated with the developing *V. faba* leaves (Kolattukudy, 1974). Comparison of cutin composition of many plants and organs within them suggest that fast-growing plants and organs tend to have a higher content of the C_{16} family of monomers. For example, *Arabidopsis* leaf cutin is composed of 9,16- and 10,16-dihydroxy-C_{16} acid as a major monomer with less amounts of ω-hydroxy-C_{16} and C_{18} acids and 18-hydroxy-9,10-epoxy-C_{18} acid and 9,10,18-trihydroxy-C_{18} acid as minor components (P. E. Kolattukudy, unpublished results). Cutin from lower plants tend to have some unusual monomers and less hydroxylated monomers. Dicarboxylic acids and very long-chain ($> C_{18}$) fatty acid derivatives that are major components of suberin, are only minor components in cutin. Even though most of the monomers are held together by ester linkages in the polymer, virtually all cutin preparations leave significant amounts of residual insoluble material after exhaustive treatments with all ester-cleaving reagents as discussed below; the composition of this material is not well understood (Crisp, 1965; Kolattukudy and Walton, 1973).

7 Structure of Cutin

Being an insoluble, amorphous polymer, only a limited number of methods can be applied to investigate the structure of this polymer. Transmission Fourier transform infra-red (FTIR) spectra of isolated cutin show absorbances indicative of hydroxyl (3300 cm^{-1}), aliphatic C–H stretch (2924 cm^{-1} and 2852 cm^{-1}), ester carbonyl (1731 cm^{-1}), and C–O ester (1167 cm^{-1}) as expected (Villena et al., 2000). Some general

structural information was obtained by testing for the presence of free functional groups in the polymer by indirect methods. Two methods were used to modify the free hydroxyl groups present in the polymer, followed by depolymerization and measurement of the monomers that had been modified in each class of monomer. One method used oxidation of the free hydroxyl groups with CrO_3-pyridine followed by depolymerization with $NaOCH_3$ in anhydrous methanol and analysis of the carbonyl-containing monomers (Deas and Holloway, 1977). The other involved mesylation of the free hydroxyl groups in the polymer by treatment with methanesulfonyl chloride followed by depolymerization with $LiAlD_4$ (Kolattukudy, 1977). This procedure resulted in replacement of each free hydroxyl group in the polymer with a deuterium atom that could be located and measured by GC/MS analysis of the monomers. These methods have been applied to cutin containing only the C_{16} family of monomers. Both methods led to similar conclusions. Most of the primary alcohol groups are in ester linkages in the polymer, indicating that the polyester is held together predominantly by primary alcohol ester linkages. About half of the secondary alcohols were also found to be in ester linkages, indicating branching and/or cross-links. The conclusion from the mesylation analysis is that there is about 0.4 free hydroxyl groups/monomer in tomato fruit cutin, consistent with the number of free hydroxyl groups acetylated with radioactive acetylating reagents (Kolattukudy, 1977). Based on such results a model was suggested for cutin containing mainly the C_{16} family of monomers (Figure 5).

More recently, more direct structural studies have been carried out using NMR approaches (Zlotnik-Mazori and Stark, 1988; Round et al., 2000; Fang et al., 2001). CPMAS-NMR analysis indicated that cutin is a moderately flexible netting with motional constraints probably at cross-link sites. Even with citrus cutin, which contains considerable amounts of mid-chain carbonyl groups, more than half of the methylenes were found to be in the rigid category. In cutins containing primarily mid-chain hydroxyl groups, with a higher potential for cross-linking, even higher portions of the methylenes may be in the rigid category. To gain further insight into the structural details, oligomers enzymatically generated from cutin were subjected to structural studies. Pancreatic lipase and fungal cutinase preferentially hydrolyze primary alcohol ester bonds and release soluble oligomers, as demonstrated at the time of first purification of cutinase (Purdy and Kolattukudy, 1975b). Structural studies on enzymatically released oligomers using solution-state NMR and liquid secondary ion mass spectrometry (LSIMS) gave direct proof for the predicted presence of secondary alcohol esters involving the 10-hydroxy group of the major cutin monomer, 10,16-dihydroxyC_{16} acid. For example, the oligomer released by pancreatic lipase shown in Figure 6 has all of the secondary alcohols in ester linkages (Ray and Stark, 1998). A mild chemical reagent, iodotrimethylsilane, that preferentially cleaves sterically hindered esters under neutral conditions at room temperature, was used to release soluble products (Ray et al., 1998). Isolation of these compounds by HPTLC followed by structural studies using multidimensional LSIMS also showed participation of the alcohol functions at C-10 in ester linkages. These structural studies provide direct evidence for the type of cross-links or branching involving the mid-chain hydroxyl groups proposed earlier on the basis of indirect chemical evidence. However, what fraction of the secondary alcohol functions are involved in such linkages was not determined in the recent studies.

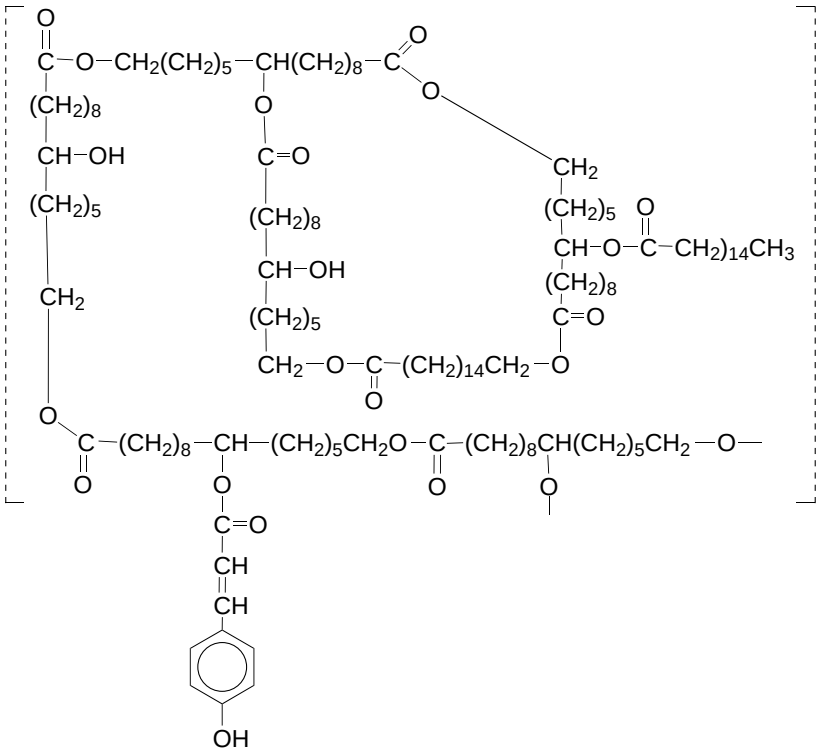

Fig. 5 Models showing the type of structures present in the polymer cutin.

The percentage of cutin remaining after the ester-cleaving depolymerization treatments show species-dependent variation. There is convincing evidence that the residue contains polymethylenic compounds, suggesting that they are derived from fatty acids. For example, NMR studies on the insoluble residues remaining after exhaustive hydrogenolysis with $LiAlH_4$ treatment of cutin from the fruits of apple, pepper, and tomato showed the presence of methylenes (Kolattukudy, 1996). ^{13}C CP-MAS NMR studies of the residue remaining after treatment of lime fruit cutin with TMSI showed the presence of polymethylene function. Pyrolysis-coupled GLC/MS of the nonester-bound residues yielded not only products expected from C_{16} and C_{18} fatty acids, but also aliphatic hydrocarbons containing 19–26 carbons (Villena et al., 1996), possibly derived from cuticular waxes contained within the cuticular matrix. The nonester-bound residual materials from *Clivia miniata* and *Agave americana* were studied by FTIR and ^{13}C-NMR spectroscopic analyses, calorimetry, X-ray diffraction, and exhaustive ozonolysis (Villena et al., 1999). On the basis of the results of such studies, it was concluded that this depolymerization-resistant core consists of an amorphous three-dimensional network of polymethylene molecules containing double bonds and free

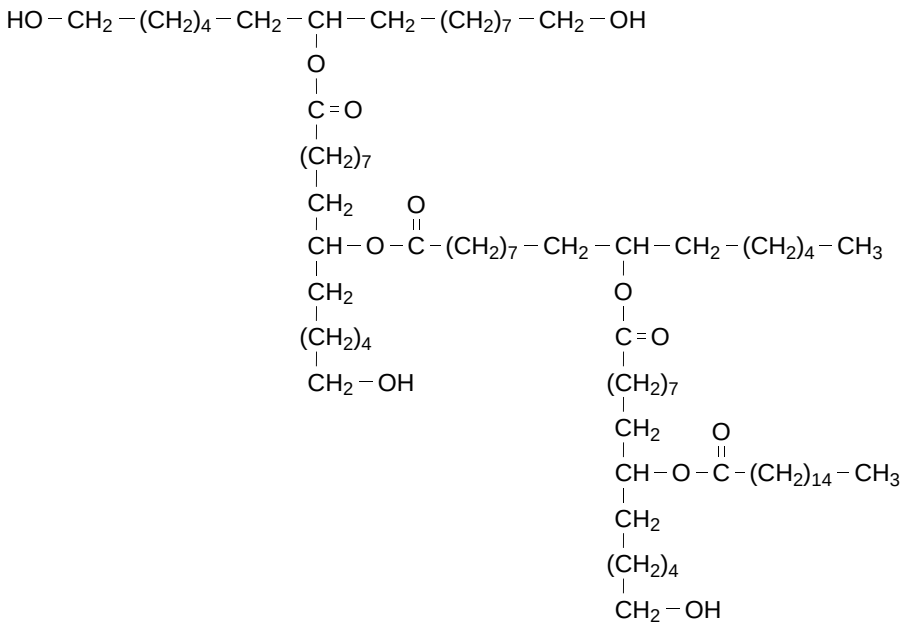

Fig. 6 Proposed chemical structure of an isolated soluble product of lime cutin depolymerization by pancreatic lipase.

carboxylic functions. That such cores contain ether linkages was suggested by the observation that HI treatment released part of the core as soluble materials (Crisp, 1965; Kolattukudy and Walton, 1973). Since structural studies have not been carried out, the exact nature of these monomers remain unknown. Some carbohydrate materials may also be part of the nonester-bound residue. Since the cuticle is known to contain some phenolics and epidermis contains peroxidases, peroxidatively coupled phenolics might be present in the cuticular polymer and may be part of the depolymerization-resistant residual material.

8 Biosynthesis of Cutin

The early notion that cutin was generated by spontaneous oxidation and polymerization of cellular lipids was replaced with the idea that lipoxygenase action on unsaturated fatty acids could generate cutin. However, such hypotheses were not consistent with even the monomer composition of cutin. For example, the C_{16} family of monomers and much of the C_{18} family of monomers in many plants are saturated and not obviously derived from the action of lipoxygenases that require *cis*-1,4-pentadiene structures in their substrates. Modern biosynthetic studies started with the observation that rapidly expanding leaves of *Vicia faba* incorporated ^{14}C-labeled acetate and palmitate into the insoluble residue remaining after extraction

of all soluble lipids (Kolattukudy, 1970a,b). When the insoluble material was subjected to exhaustive $LiAlH_4$ treatment, ^{14}C-labeled ether-soluble compounds were obtained which could be subjected to TLC and radio-GLC. The ^{14}C-distribution in the monomer clearly showed that the observed incorporation represented cutin biosynthesis. Furthermore, the most rapidly expanding young leaves incorporated the exogenous precursors most rapidly into cutin, and incorporation was very low in fully expanded leaves. In expanding fruits such as apple and grape berries, the incorporation of exogenous precursors was also dependent on the rate of growth, with little incorporation occurring in fully grown fruits. The epidermis was shown to be the site of cutin biosynthesis. Excised epidermis from the leaves of *V. faba*, *Senecio odoris* (*Kleinia odora*), and pea incorporated ^{14}C-labeled acetate and palmitate into cutin, showing that the epidermal layer of cells contains all of the enzymes needed for the synthesis of fatty acids, subsequent hydroxylations, and incorporation of monomers into the polymer. In fruits, only the skin and not the internal tissue incorporated labeled precursors into cutin. Clearly, the biosynthesis of cutin – just like the biosynthesis of the cuticular waxes – is a specialized function of the terminally differentiated epidermal cells (Kolattukudy and Walton, 1973; Kolattukudy, 1996). Thus, early conclusions on cutin biosynthesis based on observations on the effects of fatty acids on wounded tissue (viewed as cutin resynthesis) were not valid, as wounding does not cause resynthesis of cutin. Since enzymatic activities truly involved in cutin synthesis would be present only in the epidermal cells, enzymes and their mRNA should meet this localization criterion before their role in cutin biosynthesis can be established.

8.1 Biosynthesis of the C_{16} Family of Cutin Monomers

Chromatographic analysis of the depolymerization products derived from ^{14}C-labeled acetate or palmitate in rapidly expanding *V. faba* leaves showed that the major part of the label incorporated into the insoluble polymer was in 9 or 10,16-dihydroxy C_{16} acid, reflecting the composition of cutin monomers in this tissue (Kolattukudy, 1970a,b; Kolattukudy and Walton, 1972). A similar labeling pattern was observed when young, rapidly expanding pea leaves, *S. odoris* leaf disks of skin disks from rapidly expanding apple fruits or grape berries were incubated with labeled C_{16} acid (Kolattukudy et al., 1973). In the tissues that have cutin composed mainly of C_{16} monomers, exogenous labeled stearic acid and oleic acid were hardly incorporated into cutin, and the very small amount of label that was incorporated was in unhydroxylated acids or ω-hydroxy acids. The time-course of labeling showed that at all times the dihydroxy C_{16} acid was the most heavily labeled monomer, with much less label in the ω-hydroxy acid. Soluble monomers did not accumulate, but they could be detected at very low levels by autoradiography. Thus, the monomers are incorporated into the polymer as soon as they are synthesized. Exogenous labeled 16-hydroxy-C_{16} acid was incorporated directly and after mid-chain hydroxylation into cutin in *V. faba* leaves (Kolattukudy and Walton, 1972). Exogenous labeled dihydroxy-C_{16} acid was incorporated into cutin without any modification, and no other components of cutin contained any label. These results, together with the fact that mid-chain-hydroxylated C_{16} acid without any ω-hydroxyl group was not found in *V. faba* cutin or any other plant cutin, strongly suggested that biosynthesis of the C_{16} family of monomers involves