BIOSYNTHESIS OF A LIPID POLYMER, CUTIN: THE STRUCTURAL COMPONENT OF PLANT CUTICLE

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Received September 8, 1970

Summary: Cutin, the lipid polymer of plant cuticle, when treated with $LiAlH_4$ in tetrahydrofuran gave better than 95% conversion into reduced monomers which were separated by thin-layer chromatography into fatty alcohols, alkanediols, alkanetriols and alkanetetraols. The major component from Vicia faba leaf cutin was identified by TLC, GLC, NMR, IR and mass spectrometry to be hexadecan-1,7,16-triol, most probably derived from 10,16-dihydroxy palmitic acid of the cutin. Palmitic acid-1-^{14}C was readily incorporated into this component and lesser amounts into monohydroxy acids and fatty acids of the cutin. Experimental evidence suggests the reaction sequence palmitic acid → 16-hydroxy palmitic acid → 10,16-dihydroxy-palmitic acid → cutin. Palmitoleic acid-10-^{14}C was not incorporated into dihydroxy palmitic acid suggesting the noninvolvement of epoxide intermediate. Acetate-1-^{14}C, oleic acid-1-^{14}C and stearate-1-^{14}C also labeled the cutin.

Plant cuticle consists of a meshwork of polymerized cross-esterified hydroxy fatty acids called cutin which is embedded in wax. The chemistry and biosynthesis of wax components have been studied in recent years (1,2,3). The structure of cutin is not well understood, although its alkaline hydrolysis products have been examined recently (4). However, biosynthesis of this polymer remains merely speculative. This report, to the best of my knowledge, describes the first direct study on the biosynthesis of cutin. A novel method of cleaving the polymer quantitatively into monomers was used in conjunction with specifically labeled precursors, to show that fatty acids are hydroxylated and incorporated into Vicia faba leaf cutin.

Scientific Paper 3494, Project 2001, College of Agriculture, Washington State University, Pullman, Washington 99163.

EXPERIMENTAL

Cuticles of apple fruit and V. faba leaves were isolated by a combination of chemical and enzymatic methods. The cuticle released by treatment with a solution of ammonium oxalate (1·6%) and oxalic acid (0·4%) was repeatedly treated with a pectinase-cellulase combination each time followed by thorough extraction with chloroform. Dry powdered cutin was refluxed with $LiAlH_4$ in tetrahydrofuran (dry) for 24 hours. The products of this hydrogenolysis were separated by thin-layer chromatography on silica gel G with ethyl ether:hexane:methanol (8:2:1 v/v) yielding primarily alkanols, alkanediols, alkanetriols, and alkanetetraols which could be further analyzed by GLC-mass spectrometry. This novel method of structure analysis of cutin will be described in detail elsewhere.

Twenty discs (12 mm in diameter) cut from young leaves of V. faba were washed well with water and incubated at 30° with 0.5 ml, 5μc, of palmitic acid solution (55 mC/mmole) prepared as described before (5). At the end of the incubation period the discs were washed with water and homogenized in water (Broeck). The homogenate was centrifuged at 15,000 x g for 10 minutes, and the residue was treated with 25 ml. of a 2:1 mixture of chloroform and methanol and recentrifuged at 20,000 x g for 10 minutes. The residue was treated 4 more times in a similar manner. The treatment was repeated twice with tetrahydrofuran.

The insoluble residue was refluxed with $LiAlH_4$ in tetrahydrofuran (dry) for 24 hours. The product hydroxyalkanols were recovered after decomposition of the excess $LiAlH_4$ with water and dilute HCl, by extraction with ethyl ether 5 times. They were subjected to thin-layer chromatography. After detecting the components by a spray of 2'7'-dichlorofluoresceine 1 cm bands were scraped into counting vials and radioactivity determined as described before (5). The soluble lipids isolated from the chloroform:methanol extracts were also hydrogenolysed and analyzed in a similar manner.

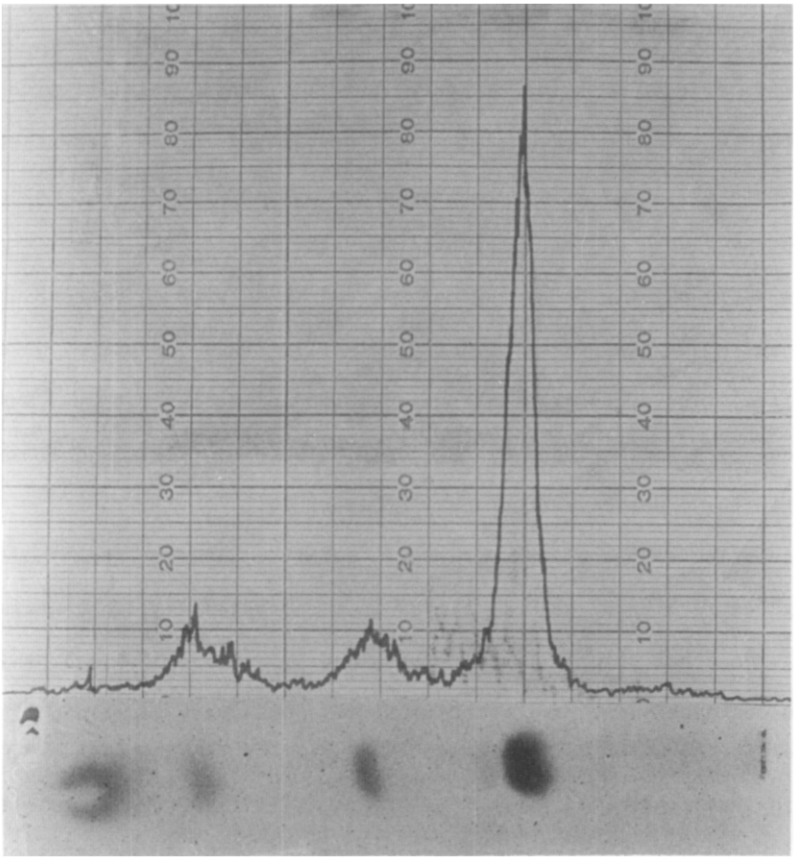

Figure 1: Thin-layer chromatogram of the ether-soluble products of hydrogenolysis of V. faba cutin. TLC on silica gel G with ethyl ether: hexane:methanol (8:2:1 v/v) as the solvent. Detection by H_2SO_4-dichromate charring. Radioactivity was monitored with a thin-layer scanner. The biosynthetic product was diluted with unlabeled material before chromatography.

Biosynthetically labeled 10,16-dihydroxy palmitic acid isolated from the hydrolysate of V. faba cutin by repeated thin-layer chromatography with ethyl ether:hexane:methanol:formic acid (40:10:1:2) as solvent, was dispersed in water with the aid of Tween 20 and ultrasonic treatment (Biosonik III, needle probe, 2 min. at maximum power). This solution was incubated with V. faba leaf discs for 4 hours, and then the discs were processed as described in the earlier experiments.

RESULTS AND DISCUSSION

Hydrogenolysis of cutin with $LiAlH_4$ gave about 95% conversion into ether soluble monomers. Alkaline hydrolysis products (under N_2) of pure

cutin when treated with $LiAlH_4$ in tetrahydrofuran gave similar products. Since the cutin contained in a few leaf discs used in the biosynthetic experiments was insufficient for isolation, the hydrogenolysis method was routinely used on the insoluble biosynthetic material.

The V. faba cutin on hydrogenolysis and thin-layer chromatography gave three major classes of products (Fig. 1). These fractions were identified by chromatographic techniques in conjunction with IR, NMR and mass spectrometry to be alkane-1-ols, alkane-α,ω-diols and alkane-triols in the order of decreasing R_f (Figure 2).

Incubation of young leaf discs with palmitic acid-1-^{14}C followed by thorough extraction of soluble lipids left a labeled residue containing cutin. The ether soluble products obtained by hydrogenolysis of this residue contained most of the radioactivity, with very small amounts of label in the water soluble products. When the ether-soluble material was thin-layer chromatographed in ethyl ether:hexane:methanol (8:2:1), three labeled components were observed (Fig. 1). The three areas of radio-activity coincided with the three major components derived from V. faba cutin. The alkan-1-ols and alkanediols contained much smaller amounts of ^{14}C than did the alkanetriol band. The following observations further show that the major part of the ^{14}C was in hexadecan-1,7,16-triol: a) When it was isolated, diluted with hexadecan-1,7,16-triol and rechromatographed, the radioactivity coincided with mass of the triol; b) Acetylation followed by thin-layer chromatography and radio gas-liquid chromatography showed that the radioactivity coincided with the mass of hexadecanetriol acetate; c) The labeled material was mixed with synthetic octadecan-1,9,10-triol and subjected to periodate cleavage in pyridine (6). The products expected from the synthetic triol were found, but all the label remained in a compound with an R_f value identical to that of the starting labeled material showing absence of vicinal diol function.

A time-course of incorporation of palmitic acid into cutin is shown

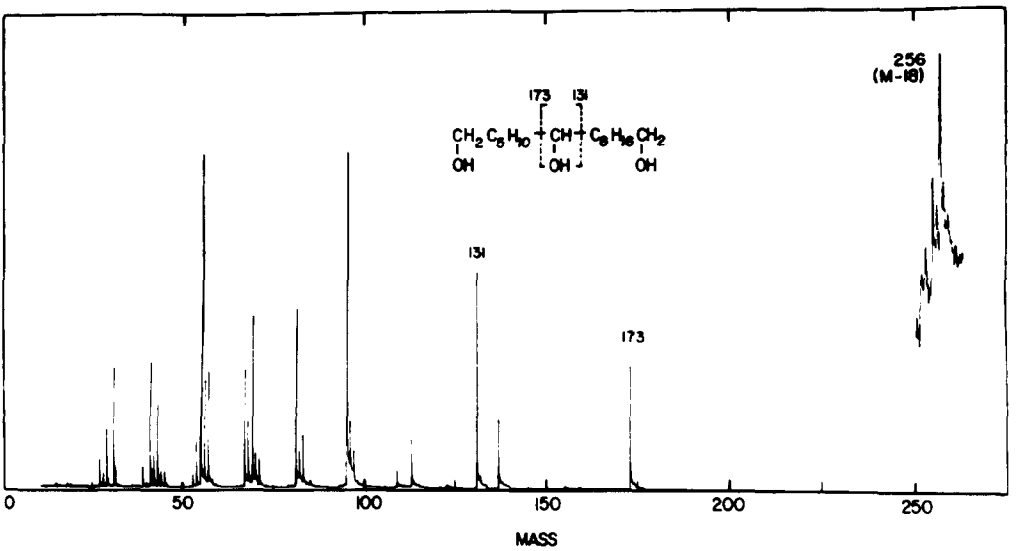

Figure 2: Mass-spectrum (70 ev) of the thin-layer chromatographically purified triol from hydrogenolysis of V. faba cutin.

in Figure 3. Under the experimental conditions incorporation leveled off after about 3 hours. Thin-layer chromatographic analysis of the soluble lipids showed that much of the administered fatty acid was converted into metabolic products such as phospholipids and cutin in about 4 hours. About 16-20% of the palmitic acid fed to the discs was converted into cutin. Boiled discs incorporated little palmitic acid into cutin. The conversion of palmitic acid into cutin required oxygen (Fig. 3) suggesting involvement of hydroxylation reactions.

During the 4 hours of incorporation of palmitic acid into the cutin acids the major labeled component was always 10,16-dihydroxy palmitic acid followed by palmitic acid and ω-hydroxy palmitic acid. Apparently, once the molecule was incorporated into the polymer it did not undergo any further oxidation. The following observations suggest that palmitic acid is probably first converted into ω-hydroxy palmitic acid and subsequently into 10,16-dihydroxy palmitic acid. The hydroxylated components are then incorporated into the polymer. 1) Hydroxylated palmitic

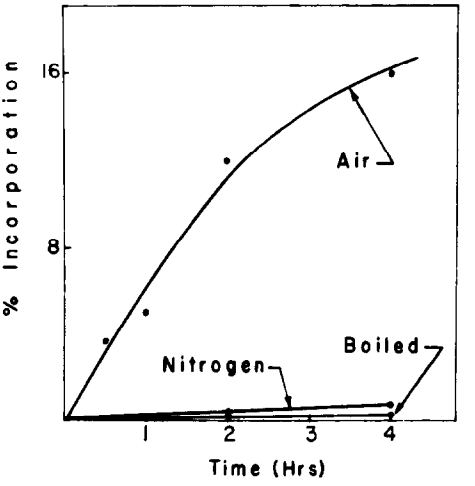

Figure 3: Time-course of incorporation of palmitic acid-1-^{14}C into the cutin of V. faba leaf. Experimental conditions as in the text.

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acid was detected in the soluble lipids, but at low concentrations at all times, indicating that once they were hydroxylated fairly rapid incorporation into the polymer occurred. 2) Biosynthetically labeled ω-hydroxy palmitic acid and dihydroxy palmitic acid when fed to leaf discs, were incorporated into cutin. The labeled cutin derived from the former contained labeled monohydroxy palmitic acid and dihydroxy palmitic acid whereas the cutin derived from the latter contained only labeled dihydroxy acid. Significant degradation of the fed acids was not detected in either case.

The introduction of a hydroxyl group at C-10 could be either by exoxidation of palmitoleic acid followed by ring opening, by hydration of a double bond in a manner similar to that occurring in a pseudomonad (7), or by direct hydroxylation on C-10 of palmitic acid itself. However, palmitoleic acid-10-^{14} did not label the dihydroxy acid significantly, and, therefore, I consider a direct hydroxylation of palmitic acid as a more likely mechanism for the synthesis of 10,16-dihydroxypalmitic acid of V. faba cutin.

ACKNOWLEDGEMENTS

I thank Mrs. Susan Grant for skillful technical assistance and Mr. Charles Oldenburg for raising the plants. Assistance from Drs. K. D. McMichael and J. A. Magnuson in obtaining the mass spectrum and NMR spectrum is gratefully acknowledged.

REFERENCES

1. Eglinton, G. and Hamilton, R. J., Science, 156, 1322 (1967).
2. Kolattukudy, P. E., Lipids, 5, 259 (1970).
3. Kolattukudy, P. E., Ann. Rev. Plant. Physiol., 21, 163 (1970).
4. Eglinton, G. and Hunneman, D. H., Phytochemistry, 7, 313 (1968).
5. Kolattukudy, P. E., Biochemistry, 6, 2705 (1967).
6. Baumann, W. J., Schmid, H. H. O. and Mangold, H. K., J. Lipid Res., 10, 132 (1969).
7. Schroepfer, G. J., J. Biol. Chem., 241, 5441 (1966).