EPOXY ACIDS IN THE LIPID POLYMER, CUTIN AND THEIR ROLE IN THE BIOSYNTHESIS OF CUTIN

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Summary: In apple skin slices oleic acid-1-^{14}C was incorporated specifically into 18-hydroxyoleic acid, 10,18-dihydroxystearic acid and 9,10, 18-trihydroxystearic acid of cutin, whereas stearic acid-1-^{14}C was not converted into these acids. Palmitic acid-1-^{14}C, but neither palmitoleic acid-10-^{14}C nor palmitelaidic acid-10-^{14}C, was converted into 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid. Based on this tracer evidence a pathway for the biosynthesis of hydroxy C_{18} acids is proposed which involves hydration of the double bond of ω-hydroxyoleic acid and epoxidation followed by hydration. The proposed epoxy intermediate was identified in cutin by $LiAlD_4$ treatment followed by mass spectrometry.

Cutin, a polymer of hydroxy fatty acids, is the structural component of plant cuticle. Recently alkaline hydrolysis products of cutin have been analyzed by a combination of chromatographic techniques and mass spectrometry (1,2). Thus 16-hydroxy, and 10,16-dihydroxy C_{16} acids and 18-hydroxy, 10,18-dihydroxy and 9,10,18-trihydroxy C_{18} acids were identified. Cutins from very few species have been examined by these workers and very little is known about its biosynthesis. We have recently reported on the pathway for the biosynthesis of the hydroxy C_{16} acids in Vicia faba (3).

In this report we propose a pathway for the biosynthesis of mono, di and trihydroxy C_{18} acids of plant cutin based on tracer work, and mass spectrometric identification of an epoxy acid which is an hypothetical intermediate involved in the proposed pathway.

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Methods

Cylindrical tissue sections were cut from young apple fruits (2" in diameter) with a cork borer (12 mm diameter) and the skin layer (0.5 mm) was excised with a razor blade. Sixteen well worked slices were incubated with 0.5 ml of appropriate substrate solutions usually for 4 hrs, in a gyratory waterbath shaker at 30°. At the end of the incubation the slices were washed, homogenized, soluble lipids removed, and the residue was hydrogenolyzed with $LiAlH_4$ in tetrahydrofuran as described earlier (3). The hydrogenolyzed products were chromatographed and radioactivity in each fraction was determined as previously described (3).

Finely powdered cutin (3) was refluxed with excess $LiAlH_4$ or $LiAlD_4$ in tetrahydrofuran (dry) for 24-72 hrs, and the ether soluble products were isolated as described elsewhere (3).

The C_{18} triol fraction, isolated by thin-layer chromatography, was subjected to $AgNO_3$-Silica gel thin-layer chromatography to remove the unsaturated components. The saturated fraction was silylated with BSA (N,O-bis-(trimethylsilyl)acetamide) and analyzed by a combination of gas-liquid chromatography and mass spectrometry. (Perkin-Elmer-Hitachi RMU 6).

Results and Discussion

A thin-layer chromatogram of the hydrogenolysis products of apple cutin shows (fig. 1) four major fractions; the alkane tetraol fraction (T) derived from saturated and unsaturated 9,10,18-trihydroxy C_{18} acids, the C_{16} triol fraction (D_1) derived from 10,16-dihydroxy C_{16} acid, the C_{18} triol fraction (D_2) derived from saturated and unsaturated 10,18-dihydroxy C_{18} acids, and a diol fraction (M) derived from saturated and unsaturated ω-hydroxy C_{16} and C_{18} acids. Apple skin slices which metabolized palmitic acid-1-^{14}C for 4 hrs incorporated about 9% of the fed ^{14}C into cutin. When this cutin was subjected to hydrogenolysis followed by thin-layer chromatography the C_{16}-triol, C_{16}-diol and alkanol fractions were found to be labeled (fig. 1). Radiogas liquid chromatography of the TMS derivatives

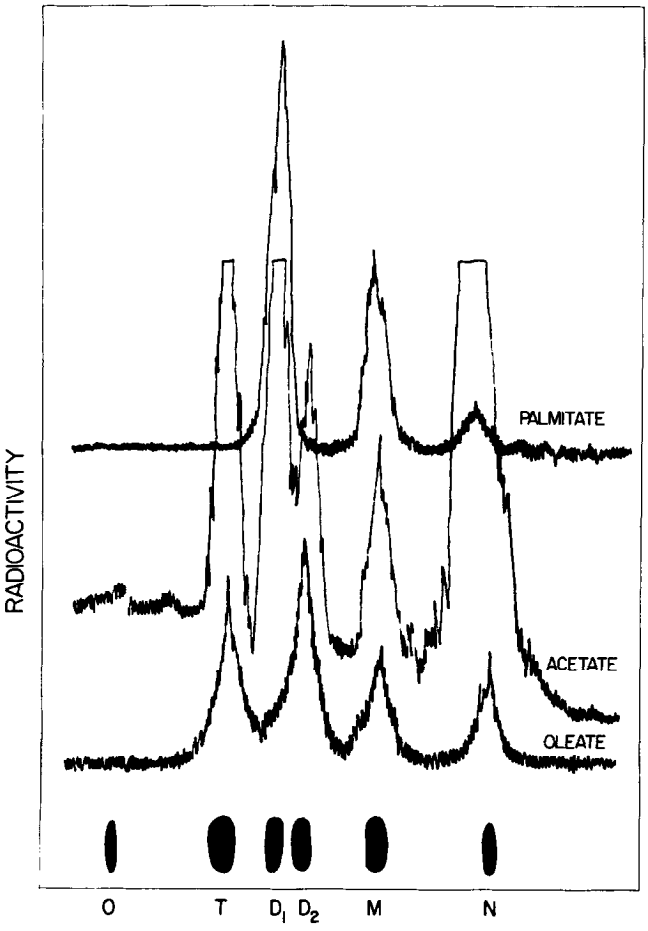

Figure 1. Thin-layer chromatogram of hydrogenolysis products of cutin from apple skin slices that metabolized the labeled substrates shown in the figure. O-origin, T-1,9,10,18-C_{18} tetraol, D_1-1,7,16-C_{16} triol, D_2-1,9,18-C_{18} triol, M-α,ω-diols, N-fatty alcohols.

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of these fractions showed that all the radioactivity of each fraction was in C_{16}-1,7,-16-triol, C_{16}-1,16-diol and C_{16}-1-ol respectively. Thus palmitic acid was converted into ω-hydroxy palmitic acid and 10,16-dihydroxy palmitic acid in apple skin. Conversion of palmitic acid into these hydroxy acids did not occur in tissue slices taken from inside the fruit, showing that the cutin acids are synthesized near the cuticle. Similar experiments with acetate-1-^{14}C showed that about 2% of the ^{14}C was incorporated into cutin. In this case all 5 TLC fractions were labeled (fig. 1). Radiogas liquid chromatography of each fraction showed radioactivity in C_{16} and C_{18} moieties.

Since acetate is the precursor of all fatty acids such a labeling pattern would be expected.

Similar experiments with stearic acid-1-^{14}C did not give rise to any radioactive chain hydroxylated C_{18} acids in contrast to the C_{16} incorporation. Therefore, oleic acid was suspected to be the precursor of chain hydroxylated C_{18} acids of the cutin. Oleic acid-1-^{14}C did indeed label the cutin (about 5% of the fed ^{14}C) and specifically the tetraol and C_{18}-triol fractions. Radiogas liquid chromatography showed that the radioactivity in these fractions was almost exclusively in 9,10,18-trihydroxy C_{18} acid and 10,18-dihydroxy C_{18} acid respectively. The radioactivity in the diol and alkanol fractions was from ω-hydroxyoleic acid and oleic acid respectively. Thus oleic acid was converted specifically into ω-hydroxy, 10,18-dihydroxy and 9,10,18-trihydroxy C_{18} acids. From these results the biosynthetic pathway shown in Figure 2 is proposed.

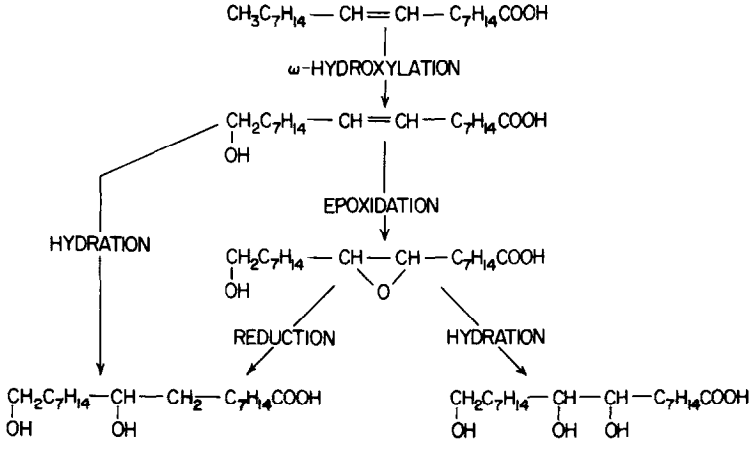

Figure 2. Proposed pathway for the biosynthesis of hydroxy C_{18} acids of cutin.

The proposed epoxidation of hydroxyoleic acid is hypothetical. However, the following evidence shows that the epoxy intermediate was present in the cutins of all five species examined (fruits of apple, peach, pear, grape and Senecio odoris leaves) which contained significant amounts of C_{18}

hydroxy acids: (a) When these cutins were cleaved with $LiAlD_4$ and the TMS derivatives of the saturated C_{18} diol fractions were subjected to GC-Mass spectrometry the M^+, $(M-15)^+$, $(M-90)^+$, $(M-15-90)^+$, and $(M-199)^+$ ions were 3 atomic mass units higher than the corresponding ions from $LiAlH_4$ experiments showing incorporation of 3 deuteriums as expected from an epoxide. (b) An intense ion m/e 105 ($CD_2 = {}^+O\text{-}Si(CH_3)_3$) was found as expected from the reduction of one carboxyl function by $LiAlD_4$. Obviously the third D was a nonterminal carbon atom. (c) Prominent ions at m/e 305, 318, 303 and 320, expected from the α-cleavage at the nonterminal hydroxyl group of the $LiAlD_4$ reduction products of ω-hydroxy,9,10-epoxy C_{18} acid, were found in the mass spectrum (Figure 3). (d) The possibility that ketoacids rather than an epoxy acid are responsible for the incorporation of 3D can be ruled out since the ion at m/e 305 cannot be explained on the basis of ketoacids. A mixture of 9 and 10 ketoacids in the cutin would have given α-cleavage

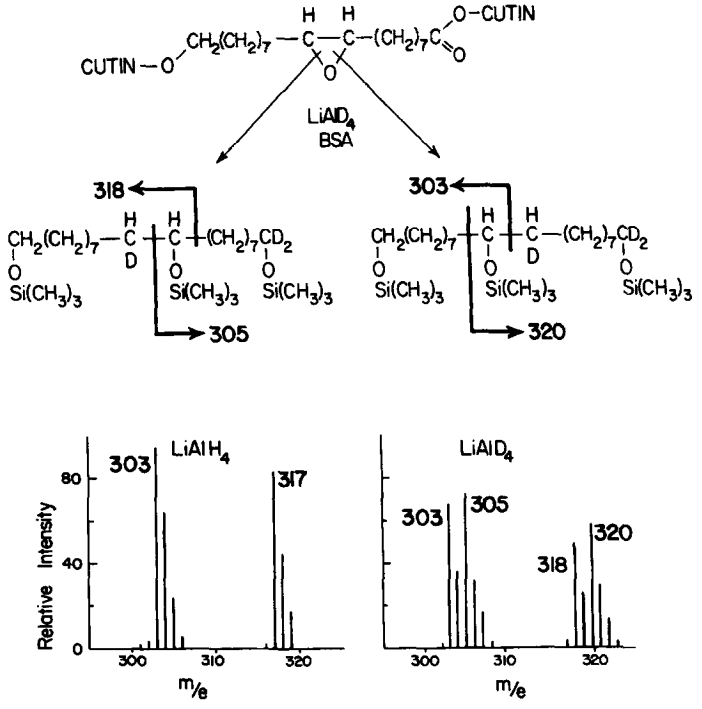

Figure 3. α-cleavage ions from the triol obtained by $LiAlD_4$ treatment of the expoxy C_{18} acid of cutin. The α-cleavage ions from $LiAlH_4$ product is also shown for comparison. Ionizing voltage 70 e.v.

ions at m/e 318, 306, 304, and 320. The naturally occurring 10-hydroxy C_{18} acid would give ions at m/e 319 and 303. A possible source of the ion at m/e 305 could be 9-hydroxy C_{18} acid in the cutin, but it can be ruled out because of the absence of an ion at m/e 317 of comparable intensity.

The pathway proposed here for the biosynthesis of C_{18} cutin acids involves ω-hydroxylation, hydration of a double bond, expoxidation at a double bond and hydration of an expoxide all of which are known types of biochemical reactions (4,5,6,7,8). In the case of hydroxy C_{16} acids however, neither palmitoleic acid (3) nor palmitelaidic was found to be a precursor of 10,16-dihydroxy palmitic acid in V. faba leaves and apple fruit skin, suggesting that chain hydroxylation in this case involves a different mechanism, presumably a direct hydroxylation. However, the possibility that 16-hydroxypalmitic acid may undergo dehydrogenation followed by hydration to give 10,16-dihydroxypalmitic acid has not been ruled out.

Acknowledgements

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