ENZYMATIC CONVERSION OF 16-HYDROXYPALMITIC ACID INTO 10,16-DIHYDROXYPALMITIC ACID IN VICIA FABA EPIDERMAL EXTRACTS

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Summary: A cell-free system prepared from epidermal extracts of young Vicia faba leaves catalyzed conversion of 16-hydroxypalmitic acid into 10,16-dihydroxypalmitic acid. The enzymatic product was identified by radio gas chromatographic analysis of its chromic acid oxidation products. The cofactors for the C-10 hydroxylation were ATP, CoA, NADPH and O_2 and the pH optimum was near 7.3. This mixed function oxidase type enzyme is novel in that it catalyzed hydroxylation in the middle of a saturated fatty chain of an activated fatty acid and this is a key reaction involved in the biosynthesis of cutin.

Plant cuticle is made up of cutin, a hydroxy fatty acid polymer, and waxes (1,2). Palmitic acid, 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid are found in most plant cutins and they are the major components of the cutin of many plants (3). Tracer studies with V. faba leaves (4) and apple fruit skin slices (5) indicated that palmitic acid was hydroxylated in turn at C-16 and C-10 and then incorporated into cutin. In this communication we describe a cell-free system from V. faba epidermis which catalyzed a CoA-dependent C-10 hydroxylation of ω-hydroxy palmitic acid, a key reaction postulated on the basis of tracer studies (Fig. 1).

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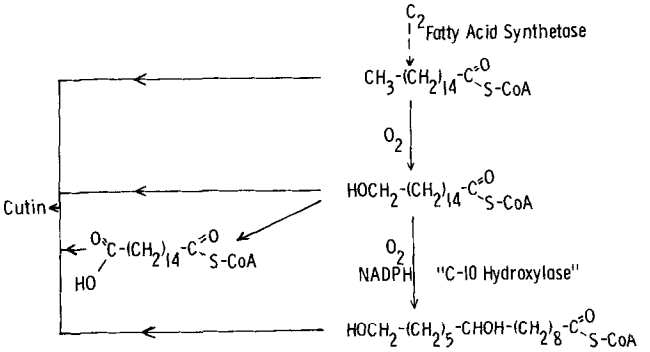

Figure 1. Proposed biosynthetic pathway for the C_{16} hydroxy acids of cutin.

METHODS

Methyl 16-hydroxypalmitate (a gift from Dr. N. Nicolaides) was tritiated by exposure to 6 Ci of $^3H_2$ at New England Nuclear Corp., Boston, Mass., according to Wilzbach's method. Rigorous purification of this methyl ester followed by hydrolysis and repurification of the acid by repetitive thin-layer chromatography gave chemically and radiochemically pure 16-hydroxypalmitic acid-R-^{3}H. An aqueous solution of the substrate was prepared by sonic dispersion (Biosonik III, needle probe at full power 3 x 30 sec) of Tween-20 solution of the substrate in water.

The epidermis excised from young, rapidly expanding V. faba leaves (6) was ground in a Potter-Elvehjem homogenizer in cold 0.01M phosphate buffer (pH 7.3) containing 0.25M sucrose and 5 x 10^{-3}M DTE. The homogenate was centrifuged at 27,000g. for 30 minutes and the supernatant was used as the enzyme source.

In the enzyme assays, flasks containing 2 ml of the supernatant with appropriate additions of cofactors and an aliquot of substrate (5.5 x 10^6 cpm) in a final volume of 4.4 ml were incubated at 30°C in a shaking water bath for 4 hrs. Regeneration system for NADPH (glucose-6-phosphate, 5 mg and glucose-

6-phosphate dehydrogenase) was included when exogenous reduced pyridine nucleotides were added. When required, the pH was adjusted with the addition of either 1N H_3PO_4 or a 5N solution of Tris.

Reactions were terminated by freezing of the reaction mixture and water was removed by lyophilization. The solid material remaining was suspended in dry tetrahydrofuran and was refluxed with excess $LiAlH_4$ for 14 hrs.

The ether soluble products were isolated and separated by thin-layer chromatography described elsewhere (4). Under some incubation conditions a β-oxidation intermediate accumulated. Its hydrogenolysis product 1,3,16-hexadecanetriol was less polar than the 1,7,16-hexadecanetriol, the reduction product of 10,16-dihydroxypalmitic acid so that the two triols were clearly separated in the thin-layer chromatographic system used.

The 1,7,16-hexadecanetriol isolated from several incubations was pooled and further purified by repetitive thin-layer chromatography. The purified triol (162,000 cpm) was dissolved in glacial CH_3COOH (2 ml), to which Cr_2O_3 (80 mg) was added and the solution heated at 75°C for 90 minutes.

The ether soluble products (110,000 cpm) were isolated and methylated overnight with BF_3/MeOH reagent. The products of this reaction were isolated and subjected to thin-layer chromatography on silica-gel G (solvent system 65:35:2 hexane:ether:formic acid), and the C_8-C_{16} dioic acid diester region was isolated (60,000 cpm). This fraction was analyzed by gas-liquid chromatography on an OV-1 column with temperature programming. The effluent was collected on glass wool in a trapping device, and was washed directly into a counting vial with scintillation fluid for radioactivity assay.

RESULTS AND DISCUSSION

Aerobic incubation of the 27000 x g supernatant with 16-hydroxypalmitic acid-R-^3H and the necessary cofactors gave rise to two products. They could be separated by $LiAlH_4$ reduction followed by thin-layer chromatography. The more polar of the two on chromic acid oxidation gave labeled nonanedioic acid

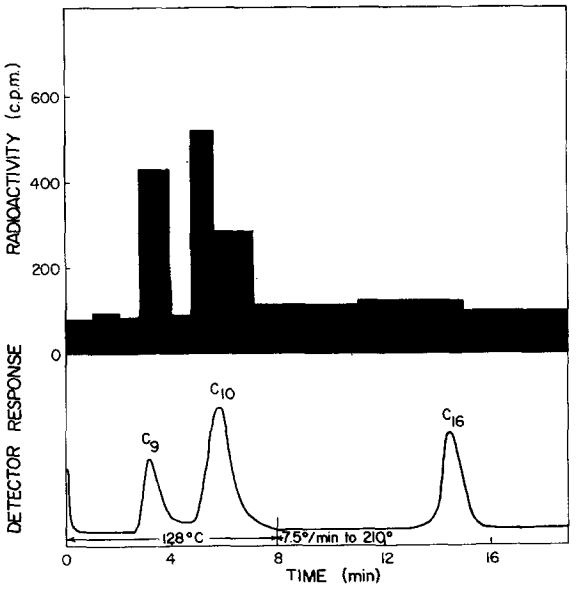

Figure 2. Gas-liquid chromatography of methyl diesters of dioic acids obtained by chromic acid oxidation of the product of C-10 hydroxylase in V. faba epidermal extracts. Top: Radioactivity, bottom: flame ionization detector tracing. Coiled copper column (4 ft. x 0.25" O.D.) packed with 3% OV-1 on 80-100 mesh gas chrom Q was used with carrier gas argon at about 70 ml/min.

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and decanedioic acid (Fig. 2). Therefore this product was derived from 10,16-dihydroxypalmitic acid proving that a C-10 hydroxylation occurred. The less polar product proved to be derived from 3,16-dihydroxypalmitic acid formed presumably by β-oxidation of the ω-hydroxy acid. For all measurements of the C-10 hydroxylase activity the thin-layer chromatographic separation of the β-oxidation product from the C-10 hydroxylase product was used.

Cofactor requirements for the 10-hydroxylase are shown in Table I. There was an absolute requirement for ATP and CoA suggesting that an activated derivative presumably 16-hydroxypalmitoyl-CoA was the true substrate for this enzyme. NADPH was the preferred reductant, NADH being ineffective. Anaerobic conditions inhibited the reaction almost completely showing the requirement for molecular oxygen. The effect of pH on C-10 hydroxylation of ω-hydroxypalmitic acid is shown in figure 3. A rather broad pH optimum with maximal activity

Cofactor Requirements for the 10-Hydroxylation of 16-Hydroxypalmitic Acid in Cell-free Extracts in V. faba Epidermis

Additions	10,16-Dihydroxypalmitic Acid Formed (cpm x 10^{-5})
ATP,CoA,NADPH	2.45
NADPH	0.49
ATP,CoA	0.43
ATP,CoA,NADH	0.26
None	0.56
Boiled Control	0.21
ATP,CoA,NADPH under N_2	0.20

Reaction mixture: 10 mg. ATP, 1 mg. CoA, 1 mg. $MgCl_2$, 3 mg. NADPH, 3 mg. NADH in a total volume of 4.4 ml. 0.01M potassium phosphate buffer (pH 7.3) containing 0.25M sucrose and 5 x 10^{-3}M dithioerythritol.

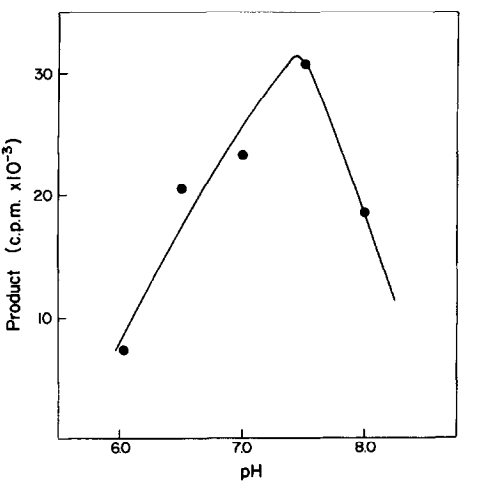

Figure 3. Effect of pH on the formation of 10,16-dihydroxypalmitic acid from 16-hydroxypalmitic acid in the extracts of V. faba epidermis.

near 7.3 was obtained. Since activation of the ω-hydroxy fatty acid is required before hydroxylation the observed pH optimum may not represent optimum condition

for the hydroxylase reaction per se. The requirements for O_2 and NADPH for C-10 hydroxylation are expected of a mixed function oxidase.

The observations that in tissue slices neither palmitoleic acid nor palmitelaidic acid was converted into 10,16-dihydroxypalmitic acid, that C-10 hydroxylation was inhibited by chelating agents such as phenanthroline, and that this inhibition was reversed by Fe^{+2} (4,7) are all consistent with the mixed function oxidase mechanism. Incorporation experiments with palmitic acid-1-^{14}C,9,10-^{3}H indicated that only one of the 4 hydrogens at 9,10 position was lost during C-10 hydroxylation suggesting that a Δ^9 double bond is not involved. These results also support a direct hydroxylation mechanism (7).

Activation of the carboxyl group far removed from the hydroxylation site is intriguing. Ricinoleic acid formation from oleic acid in castor beans also required an activated carboxyl group (8). However in that case the hydroxylation occurred at a carbon atom one methylene removed from a double bond and the corresponding saturated acid was not hydroxylated. Hydroxylation in the middle of the carbon chain of an activated saturated fatty acid observed in our C-10 hydroxylase system is a novel reaction. After hydroxylation at C-16 and C-10 the acyl moiety may be transferred to the growing polymer thus giving a plausible mechanism for efficient synthesis of cutin. In support of this contention is our unpublished observations that a major portion of the 10,16-dihydroxy acid-1-^{14}C formed from 16-hydroxypalmitate-1-^{14}C in epidermal homogenates of V. faba was in the insoluble material.

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