ENZYMATIC SYNTHESIS OF A HYDROXY FATTY ACID POLYMER, CUTIN, BY A PARTICULATE PREPARATION FROM VICIA FABA EPIDERMIS

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Summary: A 3000xg pellet preparation from epidermal extracts of young Vicia faba leaves catalyzed the incorporaton of 10,16-dihydroxypalmitic acid into insoluble material. Sequential treatment of the insoluble material with hydrolytic enzymes demonstrated that 10,16-dihydroxypalmitic acid was incorporated into cutin, the lipid polymer of plant cuticle. Cofactors required for incorporation were ATP and CoA and two pH optima, near 7.0 and near 8.5, were observed. This acyl-CoA transacylase-type enzyme is novel in that it catalyzes the formation of a hydroxy fatty acid polymer, a key reaction involved in the biosynthesis of cutin.

Plant cuticle consists of a meshwork of polymerized cross-esterified hydroxy fatty acids called cutin which is embedded in wax (1). Palmitic acid, 16-hydroxypalmitic acid and 9,16-or 10,16-dihydroxypalmitic acid are found in the cutin of most plants (1,2) and they are the major components (comprising 3.6%, 7.1% and 77.8%, respectively) of the cutin of Vicia faba (3). Previous studies with V. faba leaf slices and cell-free preparations (3,4,5) suggested that palmitic acid is hydroxylated in turn at C-16 and C-10 and then incorporated into cutin. Earlier suggestions that non-enzymatic processes are involved in the polymerization process have been seriously questioned (1,3,6), and it seems more likely that the synthesis of this important phytopolymer is under the control of specific and localized enzyme systems. However, the biological polymerization process has not been heretofore studied.

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In this communication we describe a cell-free system from V. faba epidermis that catalyzes incorporation of hydroxy fatty acids into cutin.

METHODS

Methyl 10,16-dihydroxypalmitic acid (prepared from V. faba cutin) was tritiated by exposure to 6 Ci of $^{3}H_2$ at New England Nuclear Corporation, Boston, MA., according to Wilzbach's method. Rigorous purification of the methyl ester followed by hydrolysis and repurification of the acid by preparative thin-layer chromatography gave chemically and radiochemically pure 10,16-dihydroxypalmitic acid-G-^{3}H. An aqueous solution of the substrate was prepared by sonic dispersion.

The epidermis excised from young, rapidly expanding V. faba leaves (7) was ground in a Ten-Broeck homogenizer in cold 0.05M sodium phosphate buffer (pH 7.0) containing 0.25M sucrose and 5×10^{-3}M dithioerythritol. The homogenate was centrifuged at 3000xg for 20 min and the resulting pellet rehomogenized in the same buffer and centrifuged again. This second pellet, after suspension in the appropriate buffer (sodium phosphate or Tris-Cl), was used as the enzyme source. Protein content of the pellet suspension was determined by the method of Lowry (8) with the NaOH soluble material obtained from the CCl_3COOH precipitate.

In the enzyme assays, centrifuge tubes containing the enzyme suspension, appropriate amounts of cofactors and an aliquot of substrate (1×10^{6} cpm, approximately 0.2 μmoles) in a final volume of 5 ml were incubated (sealed under N_2) at 30° in a shaking water bath for 4 hr. At the end of the incubation period, the insoluble material was removed from each sample by centrifugation at 20,000xg for 20 min. The insoluble residues were then filtered and washed with methanol in 10 x 50 mm cellulose extraction thimbles and extracted (soxhlet) with $CHCl_3$ overnight to remove any remaining soluble lipids The extracted residue was first homogenized in 5 ml H_2O and the suspension was transferred to a counting vial. After the addition of 15 ml Aquasol (New

England Nuclear Corporation, Boston, MA.) followed by shaking to form a gel, the ^{3}H was assayed directly in a liquid scintillation spectrometer. The overall counting efficiency of this gel-suspension technique was approximately 7%. The insoluble material was also hydrogenolyzed with $LiAlH_4$ in tetrahydrofuran and the products were analyzed by thin-layer chromatography (2).

In order to treat the biosynthetically labeled residue sequentially with hydrolytic enzymes, the counting gel was first liquified in hot acetone and the insoluble residue recovered by filtration. The thoroughly washed and dried residue was then treated with pectinase and cellulase as previously described (2). After this treatment, the insoluble residue remaining was recovered and the ^{3}H was measured as described above. The residue after pectinase-cellulase treatment was incubated with 8 mg of pronase (Sigma Chemical Company) in 10 ml of 0.05M phosphate buffer, pH 7.5 at 30° for 6 hr. The insoluble material was recovered and ^{3}H was measured as before. The residue after pronase treatment was incubated with a "cutinase" preparation derived from Fusarium solani pisi (9). The soluble lipids released by cutinase treatment were extracted with $CHCl_3$ and isolated by thin-layer chromatography on silica gel G using ethyl ether:hexane:methanol:formic acid (40:10:1:2 v/v) as the solvent system. The final residue was recovered and the radioactivity was measured as before.

RESULTS AND DISCUSSION

Previously it was found that in crude homogenates of V. faba epidermis the major portion of the 10,16-dihydroxypalmitic acid formed from 16-hydroxypalmitic acid was located in the insoluble polymer, indicating that an active polymerizing enzyme was present in the homogenate (4). Incubation of a 3000xg pellet suspension, prepared from excised epidermis of young V. faba leaves, with 10,16-dihydroxypalmitic acid-^{3}H and the necessary cofactors gave rise to a radioactive insoluble residue. Treatment of the residue with $LiAlH_4$ in tetrahydrofuran, followed by isolation of the $CHCl_3$-soluble products as pre-

Table I: Sequential enzyme treatment of the insoluble residue derived from 10,16-dihydroxypalmitic acid-G-^{3}H

Treatment	Weight of Residue (mg)	Radioactivity of Residue (cpm x 10^{-3})
Initial	11.5	42.5
After cellulase-pectinase	6.8	33.6
After pronase	6.1	31.8
After cutinase	5.7	6.1

Treatment conditions and assay procedures are described in Methods section.

Table II: Cofactor requirements for the incorporation of 10,16-dihydroxypalmitic acid into cutin by V. faba epidermal extracts

Additions	Incorporation into Cutin (cpm x 10^{-3})
ATP, CoA	10.8
ATP	0.73
CoA	0.40
None	0.28
Boiled Control	0.56

Reaction mixture: 16.5 μmoles ATP, 0.6 μmole CoA, 4 μmoles $MgSO_4$, 1 x 10^6 cpm (approximately 0.2 μmoles) 10,16-dihydroxypalmitic acid, 3 ml of enzyme suspension containing about 0.6 mg protein, in a total volume of 5 ml 0.05M sodium phosphate buffer (pH 7.0) containing 0.25M sucrose and 5 x 10^{-3}M dithioerythritol. Incubation was at 30° for 4 hr.

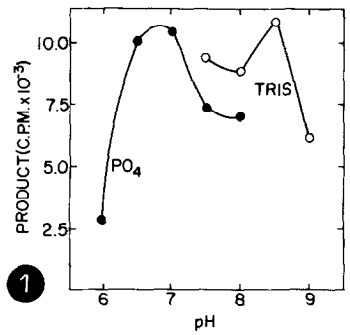
viously described (2) gave, exclusively, the expected product, hexadecanetriol-^{3}H, showing that unmodified acyl chains were incorporated. In order to test whether this incorporation represented synthesis of cutin or a nonspecific acylation of some other cellular polymer, the biosynthetically labeled residue

was sequentially treated with several hydrolytic enzymes (Table I). Cellulase, pectinase and pronase treatments resulted in the loss of 50% of the residue and only 25% of the radioactivity, while treatment with a cutinase preparation (9) solubilized only 4% of the residue but released over 60% of the radioactivity. The radioactivity released was exclusively 10,16-dihydroxypalmitic acid, as shown by thin-layer chromatography of the soluble lipid fraction. Thus, the substrate was incorporated predominantly into the hydroxy fatty acid polymer, cutin.

The 3000xg supernatant fraction, either alone, with boiled pellet, or with added purified V. faba cutin, was devoid of "cutin polymerase" activity. The 3000xg pellet did not incorporate 10,16-dihydroxypalmitic acid into soluble polymers. Therefore, it appears that both the polymerizing enzyme and the polymer which functions as the primer are contained in the particulate preparation.

Cofactor requirements for the incorporation of 10,16-dihydroxypalmitic acid are shown in Table II. There was an absolute requirement for ATP and CoA, suggesting that an activated derivative was the true substrate for the esterifying enzyme. Therefore, the hydroxy acyl chain was probably esterified to the cutin polymer via an acyl-CoA transacylase-type mechanism. This demonstration of the requirement for activation of the carboxyl group prior to esterification helps to explain the efficient esterification process observed at the very low monomer concentrations that are found in tissues which synthesize cutin (3). The present results, together with the observation that activation of the carboxyl group is required for the C-10 hydroxylation of 16-hydroxypalmitic acid (5) strongly support the hypothesis that a palmitoyl thioester undergoes hydroxylations after which the products are transferred to the growing polymer without involving free intermediates.

The effect of pH on 10,16-dihydroxypalmitate incorporation is shown in Figure 1. The bimodal nature of the curve may be due to the different pH requirements for activation and polymerization. The incorporation of 10,16-



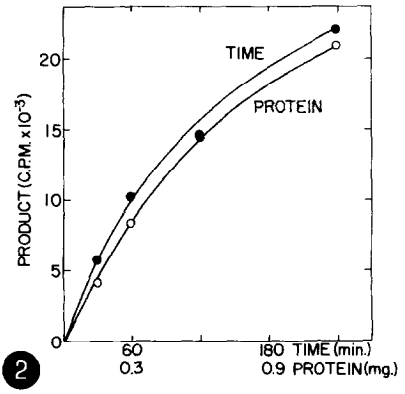


Figure 1. Effect of pH on the incorporation of 10,16-dihydroxypalmitic acid-G-^{3}H into cutin. Buffers are 0.05M sodium phosphate and Tris-HCl. Other conditions and assay procedures are described in Methods section.

Figure 2. Effect of time and protein concentration on the incorporation of 10,16-dihydroxypalmitic acid-G-^{3}H into cutin. Conditions and assay procedures are described in Methods section.

dihydroxypalmitate was linear for over 2 hr and was proportional to the amount of enzyme used, at least up to 0.6 mg protein (Figure 2). An enzyme system similar to the leaf preparation described here was also prepared from young V. faba flowers. In addition to 10,16-dihydroxypalmitic acid, both preparations also catalyzed the CoA-dependent incorporation of palmitic acid and 16-hydroxypalmitic acid into cutin. The enzyme(s) described in this communication is novel in that it catalyzes the formation of a large hydroxy fatty acid polymer.

Incorporation of palmitic acid into the hydroxy acids of cutin in V. faba leaves ceased when the leaf reached full expansion (7) suggesting regulation of synthesis of the polymer. Therefore, the cutin polymerase activity described here was also measured in particulate preparations from mature leaves. In such leaves the polymerase activity was found to be less than 12% of that in the rapidly expanding leaves. Thus, regulation of cutin synthesis also involves regulation of the cutin polymerase.

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