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WAX ESTER FORMATION CATALYSED BY ISOENZYMES OF LIPOLYTIC ACYL HYDROLASE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; wax ester formation; lipolytic acyl hydrolase; isoenzymes.

Abstract—Wax ester formation by esterification of a long chain fatty acid (palmitic acid) with a long chain fatty alcohol (octadecanol) was enzymically catalysed by acetone dried powder preparations of potato tubers. The enzyme responsible for wax ester formation had multiple isoenzymic forms and was identical with lipolytic acyl hydrolase, a lipid deacylating enzyme. Tubers from different varietiees of potato (*Solanum tuberosum*) demonstrated markedly different levels of activity and electrophoretic patterns for both wax ester formation and lipid deacylation.

INTRODUCTION

THE ENZYME lipolytic acyl hydrolase‡ (LAH) occurs widely in plants but the richest source so far found is in tubers of potato (*Solanum tuberosum*). This enzyme catalyses the deacylation of a range of naturally-occurring lipids including mono- and diacyl phospholipids, mono- and diglycerides, mono- and digalactosyldiglycerides and some artificial lipid substrates (e.g. *p*-nitrophenyl- and methyl-esters of long chain fatty acids).[1,2] In addition, LAH catalyses acyl transfer reactions between lipids and some alcohol acceptors, e.g. methanol.[1,3] Preliminary studies[1] had indicated that the same LAH enzyme possibly catalysed a reverse hydrolysis reaction in the formation of wax esters from long-chain fatty acids and long-chain alcohols.

A similar wax synthesis reaction is catalysed by acetone powder preparations of broccoli leaves in which the enzyme activity was ascribed to an "esterase type" enzyme.[4,5] The present work demonstrates that the different isoenzymes of LAH present in tubers from three varieties of potato are responsible for the formation of wax esters from free fatty acids and fatty alcohols.

RESULTS

Wax synthesis activities of different potato varieties

Previous work[6] with 23 varieties of potato established that the LAH activity was high in all but one (Désirée) of the varieties tested. Table 1 compares the LAH and wax ester

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‡ Abbreviation: LAH—lipolytic acyl hydrolase.

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[6] GALLIARD, T. and MATTHEW, J. A. (1973) *J. Sci. Food Agr.* **24**, 623.

forming activities of two varieties (Golden Wonder and Majestic) having high LAH activities with these enzyme activities in the Désirée variety. The wax ester forming activities of the two high-LAH varieties were similar and approximately 50 × higher than that of the low-LAH variety. Thus a possible correlation between LAH and wax ester forming activities in tuber tissue was indicated.

TABLE 1. WAX ESTER SYNTHESIS AND LAH ACTIVITIES OF ACETONE-DRIED POWDERS FROM TUBERS OF DIFFERENT POTATO VARIETIES

Potato variety	Wax ester formation (% of added octadecanol)	LAH activity (μmol *p*-nitrophenylpalmitate hydrolysed/min/mg protein)
Golden Wonder	14·8	2·7
Majestic	11·2	2·1
Désirée	0·3	0·01
Golden Wonder (boiled enzyme)	0	0

Assay systems for wax ester formation contained [1-^{14}C]octadecanol (0·5 μCi; 23 nmol), palmitic acid (0·1 μmol), Triton X-100 (0·5 mg) and acetone-dried enzyme preparation (160 μg protein) in 0·1 M potassium phosphate buffer, pH 6·5. The mixture (total vol. 2·4 ml) was incubated for 2 hr at 25° before analysis of reaction products (see Experimental). The above results represent the means of duplicate assays. The LAH activity was determined as in the preceding paper.[7]

Electrophoretic patterns of wax ester forming enzyme activities

In the preceding paper[7] it was established that different varieties of potato exhibited characteristic LAH isoenzyme electrophoretic patterns. In the present work acetone-dried powder preparations from the above high- and low-LAH varieties were subjected to electrophoresis on polyacrylamide gels. After electrophoretic separation, portions of the gels were sectioned and analysed for both LAH and wax ester forming activities. Duplicate gels were stained to show protein electrophoretic patterns. Fig. 1 illustrates results obtained with the three potato varieties. A close correlation was observed between the electrophoretic patterns for both enzyme activities for a given variety, whereas different patterns between varieties were observed. These results support a conclusion that the isoenzymic forms of the enzyme demonstrate proportionality between LAH and wax ester forming activities.

This is quite different from the relationship between LAH and esterase activities of potato tuber (described in the preceding paper[7]) where different electrophoretic fractions demonstrated markedly different capacities for LAH or esterase activities.

The electrophoretic patterns for both protein and for LAH activity of acetone powders from the three varieties used in this work (Fig. 1) are very similar to those observed for these same varieties grown in the previous year at a different location as described in the paper.[7]

Properties of the wax-synthesizing enzyme

Previous studies[2] showed that the relative activities of LAH and wax synthesis in an enzyme isolated from potato tubers remained constant throughout purification of the enzyme. These earlier studies also showed that the pH optimum of the LAH enzyme varied

[7] GALLIARD, T. and DENNIS, S. (1974) *Phytochemistry* **13**, 2463.

with the substrate used and, more markedly, with the addition of surface active agents.[2,8] In the present work, a broad optimum between pH 4 and 7 was observed for the formation of wax esters from octadecanol and palmitic acid in the presence of Triton X-100; the activity fell sharply above pH 7 such that only 10% of optimal activity was obtained at pH 8.

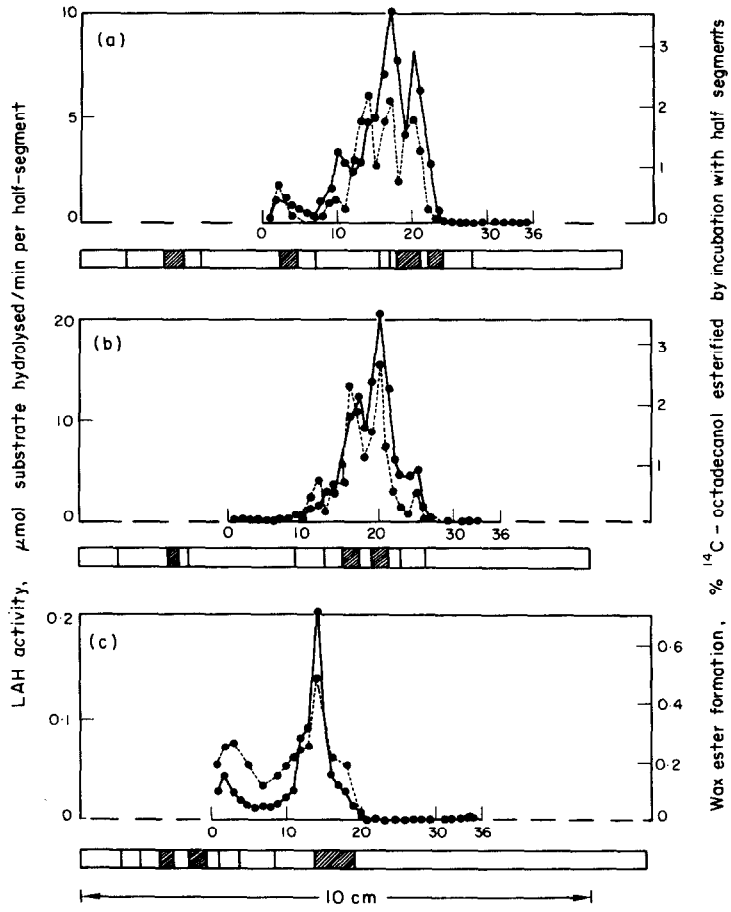

FIG. 1. DISTRIBUTION OF WAX ESTER SYNTHESIS AND LAH ACTIVITIES FOLLOWING ELECTROPHORESIS ON POLYACRYLAMIDE GELS.

Wax synthesis activities (●——●) and LAH activities (●- - -●) are shown for bisected slices taken from polyacrylamide gels after electrophoresis of acetone powder preparations of potato tubers. The tracings below each figure represent results obtained by staining duplicate gels for protein. Figures on abscissae represent segment numbers. Potato varieties used were: (a) Golden Wonder; (b) Majestic and (c) Désirée.

Wax ester formation was linear with respect to enzyme concentration up to approximately 40% esterification of added octadecanol (Fig. 2). When an acetone powder was used as enzyme source, significant wax ester formation occurred in the absence of added fatty acid (Fig. 2). Presumably, endogenous fatty acid in the enzyme preparation was available for esterification. Kolattukudy[4] observed similar wax synthesis from fatty alcohol and acetone powders of broccoli leaves in the absence of added fatty acid.

[8] GALLIARD, T. (1971) *European J. Biochem.* **21**, 90.

Preliminary experiments under incubation conditions similar to those described in the Experimental but performed at pH 7·5 had demonstrated that the wax ester formation from octadecanol and palmitic acid was linear with incubation times up to approximately 1 hr. Substrate concentration curves obeyed a linear Lineweaver–Burke reciprocal relationship giving an apparent K_m (octadecanol) of 9 mM; this is considerably higher than the apparent K_m values for the hydrolytic activity of the enzyme, e.g. 0·5 mM for *p*-nitrophenylpalmitate. No wax formation occurred if the enzyme was heated at 100° for 2–3 min. prior to incubation.

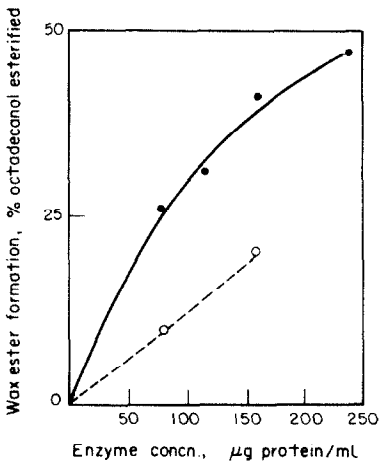

FIG. 2. DEPENDENCE OF WAX ESTER FORMATION ON ENZYME CONCENTRATION.
Incubation mixtures contained [1-^{14}C]octadecanol (0·1 µCi; 4·6 µmol), Triton X-100 (0·5 mg); acetone-dried enzyme preparation from Golden Wonder variety and, as indicated, palmitic acid (0·1 µmol) in 0·1 M potassium phosphate buffer, pH 7·5. The mixtures (total volume 2·4 ml) were incubated at 25° for 50 min. The figure presents results for incubations in the presence (●——●) and absence (○ ○) of added palmitic acid.

DISCUSSION

Using acetone-dried powders from broccoli leaves, Kolattukudy[4,5] has demonstrated three mechanisms by which fatty acids may be incorporated into wax esters: (a) by direct esterification with fatty alcohols or by acyl transfer from either (b) phospholipid or (c) fatty acyl CoA to an acceptor alcohol. The direct acylation of octadecanol by free fatty acid as described in the present work fits mechanism (a) above, which Kolattukudy[4,5] ascribed to an esterase-type enzyme. Our work has confirmed the direct esterification of fatty alcohol and fatty acid and has identified the responsible enzyme as lipolytic acyl hydrolase (a carboxylic ester hydrolase acting preferentially on lipid substrates[2,8]).

The specific activity of the potato LAH enzyme acting hydrolytically on natural lipid substrates (monoglycerides, phospholipids, galactolipids etc.[2]) is a factor of 10^4 higher than its specific activity in wax ester formation as described here. However, no detectable activity of LAH for wax ester hydrolysis was observed in earlier studies[2] and the incorporation of up to 50% of added octadecanol into wax ester indicates that the equilibrium is more favourable towards the reverse hydrolysis for wax esters than for the above polar lipids. The reversibility of carboxylic ester hydrolase enzymes is well established[9] and a

[9] HOFSTEE, B. H. J. (1960) in *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBÄCK, K., eds.), Vol. 4, p. 485, Academic Press, New York.

similar mechanism for wax synthesis in mammalian liver has been proposed.[10] It is possible that reactions in a hydrophobic area of the lipolytic enzyme and the insoluble nature of the wax formed may be contributory factors in the enhancement of the reversal of the hydrolytic reaction. Although the LAH enzyme readily catalyses acyl transfer reactions of lipid-bound fatty acids to short-chain acceptor alcohols,[2,3] no such transfer to fatty alcohols or sterols was observed with this enzyme.[2]

Wax esters comprise an important group of constituents of plant tissues[11,12] but the physiologically important mechanism(s) for their biosynthesis have yet to be ascertained. The physiological role of the lipid deacylating enzyme, LAH, is not yet known; the enzyme is particularly active in potato tubers and is responsible for major lipid breakdown in disrupted cells. The enzyme is present, at lower levels, in a wide range of plant tissues,[13] but a specific physiological role in living tissues either as a hydrolytic enzyme or in wax ester formation remains to be established.

EXPERIMENTAL

Materials. Potatoes (*Solanum tuberosum*) of the varieties Desiree, Golden Wonder and Majestic were grown locally under standard agricultural conditions and harvested in Sept. 1972. Tubers were stored in the dark at 10° until used. Palmitic acid, octadecanol (stearyl alcohol) and *p*-nitrophenylpalmitate were purchased from Sigma Chemical Co. and [1-^{14}C]octadecanol (21·8 μCi/μmol) was obtained from the Radiochemical Centre, Amersham. Triton X-100 (scintillation grade) was purchased from Koch-Light Laboratories.

Enzyme preparations. Acetone-dried powders were prepared as previously described[2] from 15000 *g* for 30 min. supernatant fractions of potato tuber homogenates.

Enzyme assays. Lipolytic acyl hydrolase activity was determined using *p*-nitrophenylpalmitate as substrate as described previously.[2] In initial studies (see Table 1) wax ester formation was assayed by the method used previously;[2] subsequently the following modifications were introduced to optimize conversion rates: incubation times were limited to 1 hr or less; max enzyme concn was 170 μg protein/ml and a pH of 6·5 was used. For technical and economic reasons, octadecanol substrate concns were below the saturating level of 31 mM. The percentage incorporation of radioactive octadecanol into wax ester was measured as previously by adding non-radioactive carrier lipid to reaction products before separation by TLC.[2] Radioactive compounds were detected with a modified Geiger counter or a Panax radio-chromatogram scanner and areas of silica gel containing radioactive spots and blank areas were scraped into vials and subjected to liquid scintillation counting in NE. 233 fluid (Nuclear Enterprises Ltd.) Only the wax ester fraction and unreacted octadecanol were radioactive and incorporation into wax ester was expressed as a percentage of the total radioactivity of reaction products. Polyacrylamide gel electrophoresis was performed as the preceding paper[7] and a selected portion (5·3 cm in length) of each cylindrical gel was cut into 36 sections (1·5 mm thick) using a bank of razor blades. Each section was further bisected, one half being assayed for LAH activity and the other half for wax ester forming activity. The half sections were soaked in 0·5 ml of appropriate buffer solutions overnight at 1° to permit diffusion of enzymes before assay. Duplicate gels were stained with Coomassie Blue[2] to locate protein bands. Protein content of enzyme preparations was determined by the method of Lowry *et al.*[14] using bovine serum albumin as standard.

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