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THIN-LAYER CHROMATOGRAPHIC ASSAY METHOD FOR CYCLOPROPANE SYNTHETASE

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SUMMARY

A method using thin-layer chromatography has been developed for the assay of cyclopropane synthetase. The procedure combines specificity of product identification with the convenience necessary for routine assay work. The method has been used to assay cyclopropane synthetase from *Lactobacillus plantarum* (ATCC 8014). The enzymatic reaction was found to be linear with respect to both time and protein concentration.

INTRODUCTION

The presence of cyclopropane synthetase has been implicated in a number of different bacterial species[1–5]. This enzyme catalyzes the transfer of a methylene group from S-adenosylmethionine to the double bond of an unsaturated phosphoglyceride fatty acid to form a cyclopropane ring-containing fatty acid. Depending upon the particular bacterial species and the conditions of growth, cyclopropane fatty acids may constitute the majority of membrane phospholipid fatty acids[1]. The functions that these fatty acids fulfil in bacteria are not known.

Two methods for the assay of cyclopropane synthetase have been reported in the literature. Both methods use radioactive S-adenosylmethionine labeled in the active methyl group. The more specific method[2] involves separation of the reaction products by gas chromatography; the radioactivity of each fatty acid species is then measured with a scintillation counter. Error may be introduced by incomplete recovery of the esters from the chromatographic column and the residual radioactivity of the column itself[2]. In addition, the procedure is quite lengthy for use in routine assays.

The second reported assay involves counting the radioactivity of products precipitated onto filter paper disks with trichloroacetic acid[6]. This procedure is faster than the gas chromatographic method, but may present difficulties in certain cases. The scintillation counting efficiency of the disks is reduced by more than 50% for lipids labeled with ^{3}H; counting efficiencies are also reduced for ^{14}C and ^{32}P. Perhaps

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a more serious disadvantage is that this method measures radioactivity of any species that is insoluble in trichloroacetic acid and in water. Complications can arise, therefore, when radioactivity is incorporated into lipid moieties other than the cyclopropane ring. Such incorporation does occur in certain bacterial species[7] and may be expected in plant and animal systems which synthesize phosphatidyl choline from phosphatidyl ethanolamine.

Thin-layer chromatography (TLC) provides an assay method which is specific for radioactivity incorporated into fatty acids, and which is faster than the gas chromatographic method. The method reported herein requires only one extraction step, no liquid transfers from the reaction vessel, and no esterifications. Scintillation counting is maintained at near normal efficiency.

EXPERIMENTAL

Materials

The radioactive substrate used was [^{3}H]methyl-S-adenosylmethionine (New England Nuclear, Boston, Mass., U.S.A., specific activity = 8.02 Ci/mmole). Unlabeled S-adenosylmethionine was obtained from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Lipid substrate was obtained from cultures of *Lactobacillus plantarum* (ATCC 8014) collected during the early log phase of growth. The lipid was extracted and washed by the method of Folch *et al.*[8]. No attempt was made to separate the individual lipid components. Lipid dispersions were prepared in the presence of sodium dodecyl sulfate[9] by sonic oscillation under a nitrogen atmosphere. The dispersions were stored under nitrogen at 5° in the dark.

Crude enzyme was obtained from cell-free extracts of *L. plantarum*. Late logarithmic cells were suspended in twice their volume of 0.1 *M* potassium phosphate buffer at pH 7.0. The cells were disrupted in a Braun Model MSK mechanical cell homogenizer using glass beads of 0.10–0.11 mm diameter (Bronwill, Rochester, N.Y., U.S.A.). After centrifugation at 20,000 *g* to remove cell debris, the supernatant solution was adjusted to pH 7.0 and stored in small aliquots at −40° .

TLC was performed on Eastman Chromagram silica gel plates. Pre-activation of the plates by heating was not necessary. Hamilton microsyringes (50 μl capacity) were used to apply measured volumes of liquid to the thin-layer plates. The chromatographic solvent was petroleum ether (60–90°)–diethyl ether–glacial acetic acid (90:10:1).

Methods

Enzyme incubations and assays were conveniently carried out in 1-dram glass shell vials supplied with tightly fitting polyethylene caps. A typical assay mixture had a total volume of 0.1 ml, and contained 0.1 to 1.0 mg of protein extract, 70 μg of lipid extract, 20 nmoles of sodium dodecyl sulfate, 50 nmoles (0.5 μCi) of [^{3}H]methyl-S-adenosylmethionine, and 6.8 μmoles of potassium phosphate buffer at pH 7.0. After incubation for 30 min at 30°, the reaction was terminated by addition of an equal volume of a saturated NaOH–methanol solution. The mixture was saponified by heating for 15 min at 60°. The contents were then acidified to pH 1 by the addition of 50 μl of 3 *N* HCl, and 400 μl of methylene chloride was then added. The two resulting phases were intimately mixed for 2 min on a Vortex mixer and centrifuged

briefly to obtain complete separation of the two layers. The vials were then cooled in ice and kept cold throughout the spotting procedure to prevent evaporative losses. A total of 200 μl from each assay vial was applied in a narrow streak to a thin-layer plate and then overlaid with stearic acid. This spotting required about 4 min per vial: much less liquid can be applied provided there has been sufficient radioactive incorporation to be counted.

The plates were chromatographed for about 15 min at room temperature and then were developed in iodine vapors. Neither choline, a possible radioactive product, nor methionine, a possible radioactive degradation product of S-adenosylmethionine, migrate from the baseline in the solvent used. After the vapors had evaporated from the plates, the spots corresponding to fatty acids were cut out, placed in scintillation vials, and counted for radioactive incorporation using a Beckman liquid scintillation system (Fullerton, Calif., U.S.A.). The thin-layer plates were free of radioactivity in the regions surrounding the fatty acid spots. The counting efficiency for tritium-labeled fatty acids on the thin-layer plate was about 90%, that for the fatty acids dissolved in the scintillation fluid.

RESULTS AND DISCUSSION

Initial studies were performed to determine whether fatty acids could be applied to the thin-layer plate directly after dissolving the assay mixture in a single-phase system. An amount of 300 μl of acetone was added to an acidified, saponified assay mixture (150 μl) to dissolve the fatty acids in a single phase. Thin-layer chromatography of this liquid was not feasible, however, due to the large amount of proteinaceous material which deposited onto the plate. Therefore, studies were initiated to

TABLE I

EFFECT OF MIXING TIME ON THE EFFICIENCY OF EXTRACTION OF FATTY ACIDS INTO METHYLENE CHLORIDE

Incubation mixture contained 0.4 mg protein, 70 μg lipid, 20 nmoles sodium dodecyl sulfate, 50 nmoles [^{3}H]methyl-S-adenosylmethionine (0.5 μCi), and 6.85 μmoles potassium phosphate buffer, pH 7.0. Total volume of the reaction mixture was 0.1 ml. Incubation was carried out at 30° for 30 min.

Duration of mixing	*Radioactive counts extracted*			*Total radioactive fatty acid in 1st extraction (%)*§
	1st extraction	*2nd extraction*	*3rd extraction*	
30 sec	565*	742	332	20
	594*	963	185	34
	367*	604	350	28
1 min	638**	387	0	62
	661**	317	0	68
2 min	973**	103	0	91
	604***	0	0	100
	603***	0	0	100
	636***	0	0	100

*,**,*** Results obtained in separate experiments.

§ Extraction efficiency was measured by TLC employing the procedure described in the *Methods* section. The R_F value for the fatty acid spots was 0.45.

determine the completeness of fatty acid extraction in a two-phase solvent system. Methylene chloride was chosen for the organic solvent because it forms the lower layer in the presence of water, and thus minimizes evaporation of the organic phase.

The best mixing procedure was determined by measuring the completeness of fatty acid extraction after Vortex mixing for 30 sec, 1 min, and 2 min. After the organic phase was spotted on a plate, additional methylene chloride was added to the assay vial and the mixing procedure repeated until no additional radioactivity was extracted into the organic phase. The results are shown in Table I. When the procedure outlined in *Methods* was followed, the first extraction consistently removed 90–100% of the fatty acids into the organic phase after 2 min of Vortex mixing.

To determine that radioactivity was incorporated only into cyclopropane fatty acid, the fatty acids were extracted from the thin-layer spot, esterified[10], and collected in U-tubes from a Loenco Model 2400 gas chromatograph (Mountain View, Calif., U.S.A.) using a column of 15% diethylene glycol succinate on Chromosorb W at 180°. Virtually all of the radioactivity present in the fatty acid mixture was found incorporated into the cyclopropane fatty acid (lactobacillic acid). No significant radioactivity was found in any other fatty acid.

The assay method was subsequently used to determine the linearity of the enzymatic reaction with time (Fig. 1). It was also determined that the amount of product formed is linear with protein concentration (Fig. 2).

Thin-layer chromatography has been shown to be applicable to the assay of cyclopropane synthetase in a bacterial system. The method should be of particular value for studying this enzyme in systems capable of transferring the active methyl group from S-adenosylmethionine to the nitrogen atom of phosphatidyl ethanolamine. In these cases, the thin-layer assay offers the needed specificity of product separation along with greater speed than is obtained with gas chromatographic methods.

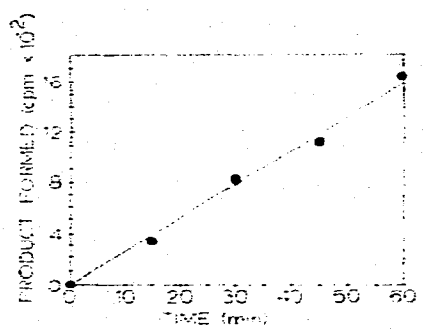

Fig. 1. TLC assay of cyclopropane fatty acid production *versus* incubation time. The incubation mixture contained 0.2 mg protein, 70 μg lipid, 20 nmoles sodium dodecyl sulfate, 50 nmoles [³H]methyl-S-adenosylmethionine (0.5 μCi), and 6.81 μmoles potassium phosphate buffer, pH 7.0. The total volume of the reaction mixture was 0.1 ml. Incubation was carried out at 30°. Extraction, chromatographic and counting procedures are described in the *Methods* section.

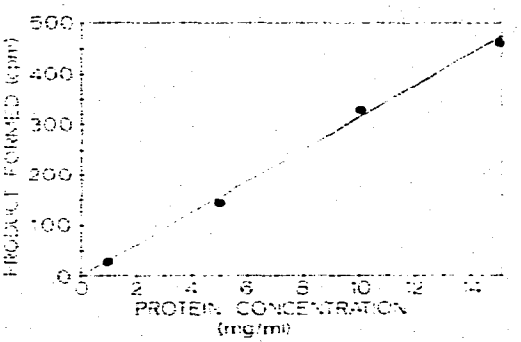

Fig. 2. TLC assay of cyclopropane fatty acid produced *versus* protein concentration. The incubation mixture contained 140 μg lipid, 40 nmoles sodium dodecyl sulfate, and 60 nmoles [³H]methyl-S-adenosylmethionine (0.5 μCi). Crude enzyme was added as appropriate. The volume was brought up to 0.1 ml by addition of 0.1 *M* potassium phosphate buffer, pH 7.0. Incubation was carried out at 30° for 15 min. Extraction, chromatographic and counting procedures are described in the *Methods* section.

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