Assay for triacylglycerol lipase by a rapid thin-layer chromatographic technique

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Summary A rapid and accurate assay for lipase-catalyzed hydrolysis of radioactively labeled triacylglycerols has been developed. Aliquots of reaction mixtures are applied directly, i.e., without extraction of the lipolysis products, to thin-layer chromatography plates coated with Silica Gel H containing 5% Na_2CO_3 (w/w), heated for 10 sec, and developed with diethyl ether–methanol 97:3 (v/v) to a height of 4–5 cm. About 98.5% of the fatty acids are immobilized as sodium salts at the origin of the chromatogram, whereas tri-, di-, and monoacylglycerols migrate close to the solvent front. Adsorbent at the origin and that at the remaining part of the chromatogram are then assayed for radioactivity without prior staining. — **Hills, M. J., and K. D. Mukherjee.** Assay for triacylglycerol lipase by a rapid thin-layer chromatographic technique. *J. Lipid Res.* 1988. **29:** 1397–1399.

Supplementary key words sodium salts of fatty acids • micro-TLC • sodium carbonate-impregnated silica gel

Methods for measuring lipase activity using labeled and unlabeled triacylglycerols (TG) have been recently reviewed (1, 2). In a lipase assay using radioactively labeled TG as substrate, the fatty acid produced must be separated from the unreacted TG and the mono- (MG) and diacylglycerols (DG) that are formed. In a liquid/liquid extraction system (3) about 70% of the fatty acid is partitioned from the reaction mixture into the polar phase. However, the extent of partitioning is affected by the presence of phospholipids and certain detergents. Thus, there is some uncertainty as to the proportion of fatty acid that actually partitions into the polar phase (3). If the lipids are extracted from the reaction mixture, separation of the fatty acids can also be carried out by standard TLC methods (4). With such methods, however, the different classes of acylglycerols are separated from each other and the plate must be stained before the area containing fatty acids can be removed.

We have developed a much easier and more rapid thin-layer chromatography (TLC) method whereby aliquots of reaction mixtures obtained upon lipase-catalyzed hydrolysis of radioactively labeled TG are applied directly to plates coated with silica gel containing 5% Na_2CO_3 (w/w).

MATERIALS AND METHODS

Materials

[Carboxyl-^{14}C]trioleoylglycerol (2.07 GBq · mmol^{-1}) and [1-^{14}C]oleic acid (2.2 GBq · mmol^{-1}) were purchased from Amersham International, Cardiff, U.K. Unlabeled trioleoylglycerol and oleic acid were obtained from Nu-Chek-Prep, Elysian, MN. All other substrates and chemicals were purchased from Sigma, St. Louis, MO. Distilled solvents were used throughout.

TLC plates

When a rapid lipase assay of a few samples is required, small plates can be prepared quickly by dipping microscope slides in a suspension of the adsorbent (5). We use 47.5 g Silica Gel H (E. Merck AG, Darmstadt, Federal Republic of Germany) plus 2.5 g Na_2CO_3 (powdered to pass through 50-μm mesh) in 170 ml CH_2Cl_2. A stock of this mixture is stored in the dark in a wide-mouthed 250-ml bottle with a Teflon-lined cap. When required, the suspension is mixed by a magnetic stirrer.

Standard plates (20 × 20 cm or 20 × 5 cm) can also be used for a larger number of samples. In this case silica gel layers containing 5% Na_2CO_3 (w/w) are prepared by standard procedures (4). As many as 30 assays can be performed on a 20 × 20 cm plate if the top and bottom halves of the layer are divided into lanes, each 13 mm wide. After development for 5–6 cm from one edge, the plate can be reversed and developed from the opposite edge.

Lipase assay

Lipase from germinating rape (*Brassica napus*) was assayed in a medium containing 50 mM (1,3-bis[tris(hydroxymethyl)-methylamino]-propane)-HCl (pH 7.5), 5 mM [^{14}C]trioleoylglycerol (1.25 MBq · mmol^{-1}), 2 mM dithiothreitol, and 2 mM $CaCl_2$. The emulsified [^{14}C]trioleoylglycerol was prepared as a 100 mM stock by sonication in 5% (w/v) gum arabic. The reaction was started by the addition of extracts from rape cotyledons (6).

Separation of fatty acids from acylglycerols in a lipase-catalyzed reaction mixture

The lipase-catalyzed reaction was stopped by adding 10% (w/v) SDS solution to the assay mixture to give a

Abbreviations: TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol.

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final concentration of 0.5% and mixed thoroughly. Approximately 10 μl of the mixture was drawn into a capillary tube and applied as a streak to the origin of the TLC plate. Larger volumes (20–30 μl) can be spotted on wider (30 mm) lanes. The plate was then placed for 10 sec on a Medax hotplate (Medax Nagel KG, Kiel, F.R.G.) thermostatically maintained at 90°C, cooled briefly, and then developed in diethyl ether–methanol 97:3 (v/v) up to a height of about 4 cm. Adsorbent at the origin containing the sodium salts of fatty acids and that from the remaining part of the chromatogram that contained the various acylglycerols was scraped into separate scintillation vials and counted in a Packard Tri-Carb C 2425 liquid scintillation spectrometer using toluene scintillator. The amount of fatty acid present was calculated by: [dpm at origin/(dpm at origin + dpm in remainder)] × nmol trioleoylglycerol in assay × 3.

RESULTS AND DISCUSSION

When radioactively labeled TG are used as substrates in lipase assays, the fatty acid product must be separated from the unreacted TG and the newly formed mono- and diacylglycerols before measurement can be made. This is achieved in the method described here by application of an aliquot of the reaction mixture directly to a silica gel layer which is impregnated with Na_2CO_3. The fatty acids are thus converted to sodium soaps which are immobilized at the origin during development of the TLC plate with diethyl ether–methanol, whereas all the acylglycerols migrate close to the solvent front.

The lipase-catalyzed reaction must be completely stopped before the mixture is applied to the adsorbent layer. When assay buffer was mixed with active enzyme and immediately applied to Na_2CO_3-impregnated silica gel layers kept on a hot plate at 110°C, as much as 8% of the substrate was hydrolyzed during the few seconds while water was driven off. The most convenient way of stopping the reaction with rape lipase was the addition of SDS. This might not deactivate all lipases, however, and this aspect should be checked for other systems. Alternatively, the lipase-catalyzed reaction can be stopped by plunging the tube into boiling water for 2 min. The addition of SDS after boiling might well be required though, since under certain circumstances, a precipitate was observed. The addition of SDS rendered the mixture homogeneous, thus ensuring accurate measurement of fatty acids and acylglycerols.

Heating of the TLC plate to 90°C for 10 sec after the application of the sample(s) serves two purposes. It drives off water from the plate, allowing it to be developed immediately after cooling, and it also ensures maximal formation of sodium salts of fatty acids. When the plates were not heated there was slight migration of [^{14}C]oleic acid away from the origin and the results were slightly more variable (**Table 1**). Breakdown of [^{14}C]trioleoylglycerol was undetectable when the TLC plate was heated for periods up to 1 min but thereafter a gradual breakdown of [^{14}C]trioleoylglycerol occurred. After 20 min heating, about 4–5% of the label from [^{14}C]trioleoylglycerol was found at the origin. Alternatively, the TLC plate can be placed in an oven at 90°C but we found this to be inconvenient when dealing with large numbers of micro-TLC plates.

In order to ensure that the acylglycerols were being well separated from the origin where the sodium salts of fatty acids were retained, the migration of trioleoylglycerol,

TABLE 1. Effect of various treatments on the retention of [^{14}C]oleic acid at the origin of Na_2CO_3-impregnated Silica Gel H TLC plates

Experiment[a]	Recovery of [^{14}C]Oleic Acid at the Origin (% of total)
Control (2 μg oleic acid)	98.03 (± 0.35 n = 4)
Control (20 μg oleic acid)	98.50 (± 0.08 n = 5)
Control (32 μg oleic acid)	98.17 (± 0.15 n = 5)
Control + BSA (2 mg · ml^{-1})	98.16 (± 0.33 n = 5)
Control + Phosphatidylcholine (2 mg · ml^{-1})	98.62 (± 0.31 n = 5)
Control + $CaCl_2$ (100 mM)	98.84 (± 0.25 n = 5)
Control + NaCl (100 mM)	98.82 (± 0.16 n = 5)
Control + Constituents of lipase assay[b]	98.84 (± 0.15 n = 5)
Control + Sodium deoxycholate (0.5%)	98.28 (± 0.18 n = 5)
Control + SDS (0.5%)	98.42 (± 0.30 n = 5)
Control + Octylthioglucoside (20 mM)	98.16 (± 0.19 n = 5)
Control + Triton X-100 (0.5%)	98.25 (± 0.06 n = 4)
TLC plate unheated, 15 min	96.60 (± 0.76 n = 5)
TLC plate unheated, 60 min	97.93 (± 0.50 n = 5)

[a]Unless otherwise stated, a sonicated emulsion of 20 μg [^{14}C]oleic acid (5000 dpm) in 10 μl was applied to the TLC plates. Each value represents the mean ± SD.
[b]Contained unlabeled trioleoylglycerol and heat-denatured lipase preparation.

dioleoylglycerol, and monooleoylglycerol, contained in assay mixtures, was tested. When diethyl ether was used as the developing solvent, trioleoylglycerol and dioleoylglycerol migrated close to the solvent front; however, some streaking of monooleoylglycerol was observed and separation of monooleoylglycerol from the origin was difficult. With diethyl ether–methanol 97:3 (v/v) as developing solvent, monooleoylglycerol had an R_f of 0.83 and thus there was little danger of contamination of the [^{14}C]oleic acid at the origin with [^{14}C]monooleoylglycerol.

The migration of [^{14}C]oleic acid from the origin was tested in the presence of various chemicals that might affect the lipase assay. The data (Table 1) show that the retention of more than 98% of [^{14}C]oleic acid at the origin was not affected by a wide range of chemicals often used in lipase assays, such as bovine serum albumin, phospholipid, $CaCl_2$, and NaCl, or by detergents. It has been reported that phospholipids and Triton X-100 cause a significant decrease in the partitioning of labeled oleic acid into the aqueous phase of a liquid/liquid partition assay (3). The assay described here might, therefore, prove useful for purification of membrane-bound lipases. Most important, the common constituents of the lipase reaction mixture did not alter the retention of [^{14}C]oleic acid at the origin.

In order to check that there was indeed no contamination of the sodium salts of oleic acid at the origin by monooleoylglycerol, dioleoylglycerol, or trioleoylglycerol, lipase-catalyzed reactions were carried out using [^{14}C]trioleoylglycerol and aliquots of reaction products were applied to the Na_2CO_3-impregnated silica gel TLC plates. After development, the adsorbent at the origin was extracted according to the method of Folch, Lees, and Sloane Stanley (7) using acetic acid at 0.1% (v/v) in the solvent system. The extract was chromatographed on a conventional silica gel TLC plate using hexane–diethyl ether–acetic acid 80:20:1 (v/v/v). The areas corresponding to trioleoylglycerol, dioleoylglycerol, monooleoylglycerol, and oleic acid were scraped and assayed for radioactivity. ^{14}C was detected only in oleic acid and none was found in the acylglycerols although analysis of the total ^{14}C-labeled lipids in the lipase-catalyzed reaction mixture by TLC showed that 44% of the ^{14}C was present in trioleoylglycerol and 9.8% in mono- and dioleoylglycerols. It is clear that fatty acid is specifically retained at the origin and is not contaminated by acylglycerols.

The error in using this method to measure lipase activity was assessed. Lipase assays were performed using various preparations of rape seedling cotyledons. Five aliquots were taken from each reaction mixture and the method described above was followed. The amount of [^{14}C]oleic acid produced by each reaction and standard deviation are given in **Table 2.** It can be seen that the standard deviation of the mean was between 3% and 5% of the mean rate measurement.

TABLE 2. Reproducibility of lipase assay

Experiment[a]	nmol [^{14}C]Oleic Acid Released per Assay[b]
1	40.5 ± 1.98 (4.9)
2	75.0 ± 1.65 (3.0)
3	55.0 ± 3.23 (4.3)
4	8.9 ± 0.43 (4.8)

[a]Various preparations of rape lipase were used as the enzyme source as described in Materials and Methods. Five aliquots were taken from each reaction and assayed for [^{14}C]oleic acid. The results reported are means ± SD.

[b]Figures in parentheses show the standard deviation as a percentage of the mean.

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