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Research paper

New lipase assay using Pomegranate oil coating in microtiter plates

Serdar Ülker [a, 1], Camille Placidi [a], Vanessa Point [a], Benoît Gadenne [b], Carole Serveau-Avesque [a], Stéphane Canaan [a], Frédéric Carrière [a], Jean-François Cavalier [a, *]

[a] CNRS – Aix-Marseille Université – Enzymologie Interfaciale et Physiologie de la Lipolyse – UMR 7282, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France
[b] ITERG, Parc Industriel Bersol 2, 11 Rue Gaspard Monge, 33600 Pessac, France

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ABSTRACT

Lipases play various roles in fat digestion, lipoprotein metabolism, and in the mobilization of fat stored in lipid bodies in animals, plants and microorganisms. In association with these physiological functions, there is an important field of research for discovering lipase inhibitors and developing new treatments of diseases such as obesity, atherosclerosis, diabetes and tuberculosis. In this context, the development of convenient, specific and sensitive analytical methods for the detection and assay of lipases and/or lipase inhibitors is of major importance. It is shown here that purified triacylglycerols (TAGs) from *Punica granatum* (Pomegranate) seed oil coated on microtiter plates can be used for the continuous assay of lipase activity by recording the variations with time of the UV absorption spectra at 275 nm. UV absorption is due the release of punicic acid (9*Z*,11*E*,13*Z*-octadeca-9,11,13-trienoic acid), a conjugated triene contained in Pomegranate oil. This new microtiter plate assay allows to accurately measure the activity of a wider range of lipases compared to the similar assay previously developed with Tung oil containing α-eleostearic acid (9*Z*,11*E*,13*E*-octadeca-9,11,13-trienoic acid), including the LipY lipase from *Mycobacterium tuberculosis*. Although punicic acid is a diastereoisomer of α-eleostearic acid, the Δ^{13}*cis* double bound found in punicic acid gives a different structure to the acyl chain that probably favours the interaction of Pomegranate TAGs with the lipase active site. The microplate lipase assay using Pomegranate TAGs shows high sensitivity, reproducibility and remarkable relevance for the high-speed screening of lipases and/or lipase inhibitors directly from raw culture media without any purification step.

1. Introduction

Lipases occur widely in the microbial [1,2], plant [3] and animal kingdoms [4] and have various applications in chemistry [5–7], biotechnology [8,9], and medicine [10,11]. They catalyse the hydrolysis of triacylglycerol (TAG) ester bonds [12] and are water-soluble enzymes, whereas their substrates are insoluble in water. In this context, the catalytic reaction of lipolysis occurs at interfaces and strongly depends on the organization of the lipid substrates present in interfacial structures such as oil-in-water emulsions, membrane bilayers, monolayers, micelles and vesicles [13]. Their catalytic process mainly consists of two consecutive steps: a reversible lipase adsorption/desorption step at the water/lipid interface, followed by the formation of an interfacial enzyme–substrate complex and the release of lipolysis products [14]. As a result, the classical Michaelis–Menten model no longer applies and only "*apparent*" kinetic constants (k_{cat}, K_m, K_i) can be obtained [15].

Numerous methods are available for measuring the hydrolytic activity as well as for the detection of lipases [16], and most high-throughput screening (HTS) assays are based on chromogenic and fluorescent substrates or sensors [17–19]. Many of these substrates

Abbreviations: β-CD, β-cyclodextrin; BHT, butylhydroxytoluene; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; FFA, free fatty acid; GPLRP2, guinea pig pancreatic lipase-related protein 2; HTS, high-throughput screening; MAG, monoacylglycerol; OD, optical density; PPE, porcine pancreatic extracts; PPL, porcine pancreatic lipase; TAG, triacylglycerol; TC4, tributyrin; TLC, thin-layer chromatography; x_I, inhibitor molar excess related to 1 mol of enzyme; x_{I50}, inhibitor molar excess leading to 50% lipase inhibition.

* Corresponding author.
E-mail address: jfcavalier@imm.cnrs.fr (J.-F. Cavalier).
[1] Current address: Recep Tayyip Erdogan University, Department of Biology, 53100 Rize, Turkey.

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are however non-specific for lipases and can be hydrolysed by various esterases often present in biological samples. Unless the investigated lipases have been shown to hydrolyse both the assay substrate and TAG, the use of long-chain TAGs is highly recommended to detect a true lipase activity. As a typical example of this, *p*-nitrophenyl acyl esters (*p*NP esters) are widely used as "lipase" substrates because of their high detection sensitivity [20–22], but they are not hydrolysed by many true lipases. Moreover, pseudo-enzyme activity has been recorded with these substrates and non-enzymatic proteins like serum albumin [23,24].

In this context, the development of analytical methods for the detection and assay of lipases has been an important target over many years and is still an active field of research [16,25]. A convenient, specific and continuous lipase activity assay using naturally fluorescent parinaric acid-containing purified TAGs from *Parinari glaberrimum* seed oil was developed [25]. The main drawback of this fluorescent method, however, is the high susceptibility of parinaric acid to be oxidized by atmospheric oxygen. Pencreac'h et al. further developed a sensitive UV spectrophotometric lipase assay using less oxidation-sensitive TAGs extracted from *Aleurites fordii* seeds (Tung oil) [26] and containing up to 72% α-eleostearic acid (9*Z*,11*E*,13*E*-octadeca-9,11,13-trienoic acid) located at the *sn*-1 and *sn*-3 positions of the glycerol backbone [27,28]. The three conjugated double bonds (Δ^9*cis*, Δ^{11}*trans* and Δ^{13}*trans*) present in α-eleostearic acid constitute an intrinsic chromophore, which confers strong UV absorption properties [29] on both the free fatty acid and the TAGs. This lipase assay was then based on the difference of the absorption coefficients between α-eleostearic acid esterified in TAG and the free acid released into the reaction medium. A change in the UV absorption spectrum was observed during lipolysis and the enzymatic activity could be quantified continuously by measuring the optical density (OD) increase at 272 nm. This bulk assay was further modified by coating on the wells of microtitration plates either the purified TAGs from Tung oil, or synthetic citronellol α-eleostearate esters [30,31], or a synthetic TAG containing the α-eleostearic acid at the *sn*-2 position (*i.e.*, *sn*-2 lipase assay [32]). A phospholipase A assay based on the same principle was also developed using a synthetic phosphatidylcholine with the α-eleostearic acid located at the *sn*-1 and *sn*-2 positions [33]. Under the hydrolytic action of various lipolytic enzymes, the released α-eleostearic acid is desorbed from the lipid-coated interface and solubilized in the micellar phase, therefore allowing a continuous monitoring of the enzymatic reaction *via* changes in OD measurement.

There are however two major limitations of the latter reported methods. First, the choice of hexane for TAG coating onto microtitration plate [30–32] turned out *a posteriori* to induce a significant alteration of the plastic material at the bottom of the well which could strongly alter UV adsorption of lipid substrate and lipase activity measurement, as observed with most commercial microtiter plates. Another major drawback is related to the fact that some lipases do not have significant lipolytic activity towards TAGs from Tung oil, probably due to the angled chemical structure of the α-eleostearic acyl chain (Fig. 1), therefore limiting the applications of this method, particularly for the screening of unknown lipases from various sources.

In that context, the aim of this study was to improve the lipase assay based on UV-absorbing long chain polyunsaturated TAGs in order to assay a wider range of lipases and limit interferences due to plastic alteration by organic solvents in microtiter plates. Among potential substrates, natural TAGs from Pomegranate oil (Fig. 1) contain large amounts of punicic acid, a conjugated triene-like α-eleostearic acid, and they appear as excellent substitutes to Tung oil TAGs for the high-speed detection and/or measurement of lipase activities. A new protocol has been optimized and adapted for the assay of lipases and lipase inhibitors in buffers as well as in raw culture medium without any purification step.

2. Materials and methods

2.1. Materials

All reagents, including Orlistat (tetrahydrolipstatin) and Tung oil, were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). Pomegranate oil was purchased from New Holland Extraction LTD (New Holland, United Kingdom). The compound 9(Z),11(E),13(Z)-octadecatrienoic acid (*i.e.*, punicic acid) was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). All organic solvents were purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France) and were of HPLC grade. Corning® 96-well clear flat bottom UV-transparent microplates (Product id #3635) purchased from Corning B.V. Life Sciences (Amsterdam, The Netherlands) were chosen for their lowest background absorbance (data not shown) as compared to other commercial microplates tested.

2.2. Enzymes

All lipases were produced and purified to homogeneity in the laboratory. Recombinant guinea pig pancreatic lipase-related protein 2 (GPLRP2) was expressed in *Aspergillus orizae* and purified according to [34]. *Fusarium solani pisi* Cutinase was produced and purified according to [35]. The LipY lipase from *Mycobacterium tuberculosis* was produced and purified as previously reported [36]. Porcine pancreatic extracts (PPE), also named pancreatin (P7545; 8 × USP grade) was purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France) and contained 0.95 wt% of porcine pancreatic lipase (PPL).

2.3. Synthetic culture media

Difco Middlebrook® 7H9 broth and Difco® YPD medium were purchased from BD (Le Pont de Claix, France). Luria broth (LB) and Terrific Broth (TB) were purchased from Invitrogen (Saint Aubin, France). Superior Broth (SB) was purchase from Euromedex (Souffelweyersheim, France). In addition to these commercial culture media, five synthetic culture media adapted from Ref. [37] were prepared as follow: **M1**: 15 g L^{-1} glucose; 15 g L^{-1} soy peptone; 2 g L^{-1} $CaCO_3$; 5 g L^{-1} NaCl; 2 g L^{-1} yeast extract. **M2**: 2.5 g L^{-1} 7H9 supplemented with 5 g L^{-1} glucose; 20 g L^{-1} soy peptone; 20 g L^{-1} glycerol; 3 g L^{-1} NaCl; and 5 g L^{-1} yeast extract. **M3**: 10 g L^{-1} glucose; 4 g L^{-1} soy peptone; 0.25 g L^{-1} $MgSO_4$; 5 g L^{-1} NaCl; 2 g L^{-1} yeast extract. **M4**: 25 g L^{-1} LB supplemented with 15 g L^{-1} glycerol; 2.5 g L^{-1} NaCl; 1 g L^{-1} tween 80; and 10 g L^{-1} olive oil. **YP**: 10 g L^{-1} yeast extract; 20 g L^{-1} peptone.

2.4. Thin layer chromatography

Thin-layer chromatography (TLC) was carried out on analytical aluminium sheets coated with Silicagel 60 (0.25 mm, Merck KGaA). The elution was performed with *n*-heptane/diethyl ether/formic acid (55:45:1, v/v/v) containing 0.01% (w/v) Butylhydroxytoluene (BHT) acting as antioxidant. This solvent mixture allows the separation of all TAG lipolysis products in a single run [38]. After being eluted, TLC plates were dried, sprayed with copper acetate–85.5% phosphoric acid solution (50:50, v/v), and the neutral lipids were revealed by charring at 180 °C for 10–15 min. Triolein, 1,2-diolein, 1-monoololein and oleic acid were used as reference standards for TAG, diacylglycerol (DAG), monoacylglycerol (MAG) and free fatty acid (FFA), respectively.

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