Spectrophotometric determination of lipases, lysophospholipases, and phospholipases


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Abstract Spectrophotometric techniques for determining the activities of lipases, lysophospholipases, and phospholipases are reviewed. These methods involve the use of thioester substrate analogs as well as \( \omega \)-nitrophenyl derivatives of the corresponding lipids. The most promising results are obtained with the thioester substrate analogs. Mono- and diacylglycerol lipases are assayed by using rac-1-S-decanoyl-1-mercapto-2,3-propanediol and rac-1,2-S-O-didecanoyl-1-mercapto-2,3-propanediol, respectively. Phospholipases \( A_1 \) and \( A_2 \) are determined by using rac-1,2-S-O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol and 2-hexadecanoylthio-1-ethyl-phosphocholine, respectively. Lysophospholipases are assayed by using 2-hexadecanoylthio-1-ethyl-phosphocholine. Phospholipase C is assayed with rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercapto-2,3-propanediol. Thioester substrate analog assay procedures are more rapid, sensitive, convenient, continuous, and less expensive than the classical radiochemical techniques.—Farooqui, A. A., W. A. Taylor, C. E. Pendley II, J. W. Cox, and L. A. Horrocks. Spectrophotometric determination of lipases, lysophospholipases, and phospholipases. J. Lipid Res. 1984. 25: 1555–1562.

Supplementary key words \( \omega \)-nitrophenyl derivatives of lipids • thioester analogs • lipolytic enzymes • fluorescent substrate

Different phospholipids in neuronal tissues turn over at different rates with respect to their cellular structure and location and the membranes involved (1–5). The catabolism of membrane phospholipids consists of a stepwise process catalyzed by various phospholipases and lysophospholipases (6). Triacylglycerols and diacylglycerols, although quantitatively minor components of mammalian brain tissue (7, 8), are very active metabolically and are hydrolyzed by lipases (9–11). Studies on the isolation and characterization of lipases, lysophospholipases, and phospholipases are complicated by two major problems. They tend to have quite low activities when compared with other hydrolytic enzymes, and rapid and sensitive assay procedures for the estimation of their enzymic activities have not been available.

Lipolytic enzyme activities are generally estimated by titrimetric (12, 13) or radiochemical techniques (14). Titrimetric methods are not sensitive enough to be used for the determination of lipases, lysophospholipases, and phospholipases in homogenates or subcellular fractions. Radiochemical techniques are sensitive, but suffer from the disadvantages of being discontinuous, time-consuming, and expensive. Furthermore, handling radioactivity is very undesirable. In 1976, Aarsman, van Deenen, and van den Bosch (15, 16) developed a spectrophotometric assay for the estimation of lysophospholipase and phospholipase \( A_2 \) activities using a thioester substrate. They synthesized a glycol lecithin analog with a thioester linkage in place of an oxyester linkage. Hydrolysis of this substrate by the phospholipase resulted in exposure of the thiol group, which then reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form a yellow color. The color formation was continuously monitored spectrophotometrically at 412 nm. In other studies, Aarsman and van den Bosch (17, 18) compared the hydrolytic activities of thioester substrates with the corresponding oxyester substrates, using lipolytic enzymes. They found that the \( K_m \) values for thioester substrates are 5–10 times lower than for the oxyester substrate. However, the \( V_{max} \) values are 2–5 times higher for the thioester substrate than for the corresponding oxyester substrate. The higher \( V_{max} \) values increase the sensitivity of the assay which, at present, is only limited by the availability of the substrate.

The purpose of this paper is: 1) to review the methods available for the spectrophotometric determination of lipolytic enzymes, and 2) to stimulate further studies on the isolation and characterization of lipolytic enzymes using thioester substrate analogs.

SPECTROPHOTOMETRIC DETERMINATION OF MONO- AND DIACYLGLYCEROL LIPASES USING THIOESTER SUBSTRATE ANALOGS

Thioester substrate analogs of mono- and diacylglycerol are prepared according to the method of Cox and Horrocks (19).

Abbreviations: TLC, thin-layer chromatography; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNPAl, \( \omega \)-trinitrophenylaminolauric acid; MOPS, morpholinopropanesulfonic acid.
Monoacyl thioester substrate analogs are synthesized by the method of Aarsman et al. (15) for the monoacylation of mercaptoethanol. The sulfhydryl group of mercaptoglycerol is selectively acylated with decanoyl or hexadecanoyl chloride, under controlled conditions, to form the monodecanoylthioglycerol (V) and mono-hexadecanoylthioglycerol (VI), respectively (Fig. 1). Mercaptoglycerol (100 mmol) is dissolved in 100 ml of dry diethyl ether with 60 mmol of pyridine. Acyl chloride (25 mmol), in 30 ml of dry diethyl ether, is added dropwise with rapid stirring at 0°C. After the addition is complete, the reaction mixture is allowed to stir for 2 hr at room temperature. The mixture is then washed with four 50-ml portions of water. The organic phase is dried over Na2SO4 and rotary evaporated to yield white crystals. The crystals are redissolved in warm n-hexane and allowed to stand overnight at -20°C. The precipitate is collected on a Buchner funnel. Recrystallization is repeated until the precipitate is >95% pure by TLC with hexane-ether-acetic acid 90:10:1 (by vol). If the purity of the product after several recrystallizations is not acceptable, further purification on a neutralized silicic acid column (100 g) eluted with chloroform-methanol 98:2 or 96:4 (v/v) can be achieved. Tridecanoylthioglycerol (I) is obtained in 95% yield as a light yellow oil that migrates just ahead of trimyristin on TLC with hexane-diethyl ether-acetic acid 90:10:1 (by vol) with an RF of 0.3. Tridecanoylthioglycerol (I) is then partially hydrolyzed with R. delemar lipase using conditions described by Fukumoto, Iwai, and Tsujisaka (20). Tridecanoylthioglycerol (I) (10 mmol) is incubated with 5 mg of lipase in 60 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 10 mM CaCl2. The mixture is stirred rapidly and the reaction progress is followed by TLC with hexane-diethyl ether-acetic acid 60:40:1 (by vol). After 1 hr of incubation at room temperature, five components...
of the reaction mixture are detected. They are: unreacted tridecanoylthioglycerol (I) \((R_f = 0.98)\); 2,3-didecanoyl-1-mercapto-2,3-propanediol \((R_f = 0.95)\); decanoic acid \((R_f = 0.68)\); 1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol \((R_f = 0.47)\); and 2-O-decanoyl-1-mercapto-2,3-propanediol \((R_f = 0.26)\). The concentration of didecanoylthioglycerol \((II)\) reaches a maximum after approximately 3 hr of incubation. The reaction mixture is then extracted with four volumes of chloroform-methanol 2:1 (v/v). After rotary evaporation at 30°C, the residual oil is fractionated on a neutralized silicic acid column \((200 g)\) by elution with hexane-diethyl ether 90:10, 80:20, and 70:30 (v/v). Didecanoylthioglycerol is eluted in the last fraction in an approximate yield of 26%.

Optimal conditions for determining mono- and diacylglycerol lipase activities

I. Monoacylglycerol lipase. Strosznajder, Singh, and Horrocks (21) have assayed monoacylglycerol lipase activity using 1-S-decanoyl-1-mercapto-2,3-propanediol and its corresponding oxyester substrate. Semistable suspensions \((1.5 \text{ mM})\) are prepared by separately vortexing the thioester and oxyester substrates with 2.5 mM lysophosphatidylcholine in 0.1 M Tris-HCl buffer (pH 7.4), 0.75 \(\mu\text{mol DTNB}, 0.625 \mu\text{mol lysophosphatidylcholine}, \) and 0.375 \(\mu\text{mol} of the appropriate substrate, in a total volume of 1.0 ml. The enzyme protein \((150 \mu\text{g})\) is first reacted with DTNB, in 0.5 ml total volume, in both the reference and the sample compartments of a spectrophotometer. When the slope of the recorder tracings reaches zero, the reaction is started by adding mono-decanoylthioglycerol and monodecanoylglycerol to the sample and reference cuvettes, respectively. The formation of free thiol groups and their reaction with DTNB is monitored continuously at 412 nm. Enzymic activity is calculated from the slope of the recorder tracing using the extinction coefficient for DTNB of 12,800 \(1\text{ mol}^{-1}\cdot \text{cm}^{-1}\). Nonenzymic background activity due to substrate acyl migration is monitored at 412 nm and subtracted from the observed reaction rate to yield the actual enzymic activity.

II. Diacylglycerol lipase. Enzymic activity is determined by the method of Farooqui et al. (22), with 1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol as the substrate. Suspensions of didecanoylthioglycerol and dicaprin \((1 \text{ mM})\) are prepared by separately sonicating each with 1 mM lysophosphatidylcholine in 0.05 M MOPS buffer (pH 7.4). Sonicated suspensions are stable for 4–5 hr at room temperature. The assay mixture contains 100 \(\mu\text{mol} of MOPS buffer (pH 7.4), 0.75 \mu\text{mol} of DTNB, 0.25 \mu\text{mol} of thioester or oxyester didecanoylglycerol, and up to 300 \mu\text{g} of enzyme protein, in a total volume of 1.0 ml. Enzymic activity is determined as described for monoacylglycerol lipase. The reaction of the thiol group with DTNB is shown in Fig. 2. DTNB is usable only in the pH range from 6 to 10, but other thiol reagents, such as 4,4'-dithiodipyridine (23) for the pH range from 3 to 7, can also be used. With the use of these two thiol capture reagents, the complete pH range necessary for the assay of lipases can be covered.

Kinetic parameters of bovine brain mono- and diacyl-glycerol lipases using thioester substrate analogs are given in Table 1. These substrates follow simple Michaelis-Menten kinetics and show saturation at 250 \(\mu\text{M}\).

Development of continuous spectrophotometric procedures using thioester substrate analogs has aided the study of various lipases. Using 1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol, Farooqui et al. (22) have shown that bovine brain contains two diacylglycerol lipases.
lipases. One is located in the microsomal and the other in the crude plasma membrane fraction. The microsomal enzyme is markedly stimulated by Triton X-100 and by Ca2+. However, the crude plasma membrane enzyme is strongly inhibited by Triton X-100, while Ca2+ has no effect on its enzymic activity. We have also separated mono- and diacylglycerol lipases (24) using heparin-Sepharose affinity chromatography (25). Microsomal diacylglycerol lipase is completely retained on a heparin-Sepharose column and can be eluted with 0.5 M NaCl, whereas monoacylglycerol lipase is not retained and therefore washed from the column. Further studies on the isolation and characterization of mono- and diacylglycerol lipases are currently in progress in this laboratory.

SPECTROPHOTOMETRIC DETERMINATION OF LIPASES USING NITROPHENYL DERIVATIVES

Preparation of ω-nitrophenylaminolauryl-glycerol

Goldberg, Barenholz, and Gatt (26) synthesized ω-trinitrophenylaminolauric acid (TNPAL) by the method of Satake et al. (27). Glycerol esters of TNPAL were prepared by the method of Bhattacharya and Hilditch (28). Briefly, TNPAL (11 nmol) is mixed with dry naphthalenesulfonic acid (24 mg) and double vacuum-distilled glycerol (285 mg). The mixture is treated at 150°C for 6 hr at 35–55 mm Hg. The products are extracted with chloroform and the solvent is evaporated under a stream of nitrogen. Equal volumes of benzene and 0.1 M NaHCO3–ethylene glycol 1:1 (v/v) are added and the mixture is shaken to separate most of the residual unreacted fatty acid. The benzene phase is washed twice with the NaHCO3–ethylene glycol solution and concentrated in vacuo. Toluene is much less toxic and probably can be substituted for benzene. Mono-, di-, and tri-TNPAL are separated by preparative TLC on 1-mm-thick silica gel H plates developed in chloroform–methanol–acetic acid 98:2:1 (by vol). The bands that correspondingly migrate with authentic mono-, di-, and triacylglycerol standards are scraped, placed in small columns, and eluted with the above solvent system.

The assay mixture contains 25 μmol of Tris-HCl buffer (pH 8.0), 0.5 μmol of CaCl2, 0.25 μmol of mono-TNPAL-glycerol, and 100–200 μg of rat brain microsomal protein in a total volume of 0.5 ml. Incubation is carried out for 5 min at 37°C. The reaction is terminated with 0.5 ml of 30 nmol of NaOH in ethylene glycol, followed by the addition of 2 ml of benzene. The mixture is vortexed for 30 sec, centrifuged for 5 min at 2000 rpm, and the upper benzene phase is removed. The lower phase is again washed with benzene and then 0.1 ml of concentrated HCl is added to the aqueous ethylene glycol phase. The yellow fatty acid is extracted into 2 ml of benzene and the absorbance of the benzene phase is measured at 330 nm.

With 1,3-di-TNPAL-glycerol, the apparent $K_m$ and $V_{max}$ values for Rhizopus delemar lipase are 16 μM and 200 μmol per min per mg protein, respectively. The hydrolysis rates of mono-, di-, and tri-TNPAL-glycerol by rat brain microsomal lipase, hog pancreatic lipase, and R. delemar lipase are of similar orders of magnitude as those determined with labeled oleoylglycerol derivatives. This method has two serious disadvantages. The trinitrophenyl derivatives have solubility properties that are different from the normal triacylglycerols. Therefore, an exact comparison of the hydrolytic rates of triolein and trinitroaminolauryl-triacylglycerol cannot be made. The other disadvantage is that these substrates provide a fixed-time discontinuous assay procedure and thus require several samples to be performed in order to assure linearity and reproducibility.

SPECTROPHOTOMETRIC DETERMINATION OF PHOSPHOLIPASES USING THIOESTER SUBSTRATE ANALOGS

I. Determination of phospholipase $A_1$ activity

Cox and Horrocks (19) have reported on the preparation of thioester substrate analogs of phosphatidylcho-
line and phosphatidylethanolamine for the determination of phospholipase A₁ activity.

**rac-1,2,5,6-O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (III) synthesis**

The didecanoylthiglycerol (II) (3.45 mmol) is dissolved in 15 ml of chloroform and added dropwise to a rapidly stirring solution of bromoethylphosphorus oxydichloride (13.8 mmol) and 28 mmol triethylamine, at 0–5°C. After the addition is complete, the mixture is constantly stirred at room temperature for 12 hr, followed by hydrolysis, trimethylamination, and purification as described by Aarsman et al. (15). Following treatment with the ion exchange resin, the crude products are taken up in chloroform and eluted from a 100 g silicic acid column with chloroform–methanol 9:1, 6:1, 5:1, and 4:1 (v/v). The thiophosphatidylethanolamine (III) elutes in the last fraction in 30% yield. On TLC, it migrates just below bovine brain ethanolamine glycero-phosphate and stains positively for both choline and phosphate.

**rac-1,2,5,6-O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (IV) synthesis**

The didecanoylthiglycerol (II) (3.45 mmol) is converted to the thiophosphatidylethanolamine by the method of Eibl (29), and purified on a 100 g silicic acid column with chloroform–methanol 4:1, 1:1, 2:3, and 1:3 (v/v). The thiodioethanolamine (III) elutes in the last two fractions in 40% yield. On TLC, it migrates identically to didecanoylglycerolphosphatidylethanolamine and stains positively for both choline and phosphate.

**Assay conditions for phospholipase A₁ determination**

Substrate suspensions are prepared in 0.05 M MOPS buffer (pH 7.5). The assay mixture contains 100 μmol MOPS buffer (pH 7.5), 0.05 μmol of thioether or oxester substrate, and 0.6 μmol of 4,4'-dithiodipyridine, in a total volume of 1.0 ml. Samples are treated as described for mono- and diacylglycerol lipases. Up to 1000 μg of *Rhizopus delemar* lipase has been used as a source of phospholipase A₁ activity for development of the assay system. Enzymic activity is calculated using the molar extinction coefficient of 4-thiopyridone (19,800 1·mol⁻¹·cm⁻¹) at 324 nm.

**II. Determination of phospholipase A₂ activity**

**rac-2-hexadecanoylthio-1-ethyl-phosphocholine (thioglycol lecithin) synthesis**

This procedure was described by Aarsman et al. (15). The substrate is prepared by mixing 2-mercapto-1-ethanol (147 mmol) with 10 ml of dry ethyl ether and 5 ml of anhydrous pyridine. Palmitoyl chloride is dissolved in 10 ml of ethyl ether and added dropwise to the rapidly stirring mixture, at 0°C. After 1 hr of reaction, the mixture is diluted with 100 ml of ethyl ether and then washed with five 50-ml portions of water to remove the excess 2-mercapto-1-ethanol. The remaining ether layer is dried over anhydrous Na₂SO₄ and then rotary evaporated to dryness. The residue is dissolved in warm n-hexane and allowed to stand overnight at −20°C. The white precipitate is collected on a G2 glass filter, and the supernatant is discarded. The precipitate is redissolved in dry chloroform and stored at 0°C when not in use. To the hexadecanoyl-thiomonoacylglycerol mixture is added 20 mmol of 2-bromoethylphosphodichloride and 20 mmol of triethylamine in chloroform. This reaction is allowed to continue at room temperature for 20 hr. Hydrolysis of the 2-hexadecanoylthio-1-ethyl-phosphomonochloride-ethylbromoester to 2-hexadecanoylthio-1-ethyl-phosphoethanol-bromoester, and the introduction of the trimethylamine group is achieved by a slight modification of the method of Eibl et al. (30). Purified 2-hexadecanoylthio-1-ethyl-phosphocholine is isolated by silicic acid chromatography in yields of 33%. The isolated compound has no free thiol groups, and on weight basis the phosphorus analysis revealed a purity of 98%.

The assay mixture for the estimation of phospholipase A₂ activity contains 200 μmol of Tris-maleic acid buffer (pH 7.5), 100 nmol of 2-hexadecanoylthio-1-ethyl-phosphocholine, 1 μmol of DTNB, and 0.6 μmol of EDTA. The addition of hog pancreatic phospholipase A₂ in the presence of EDTA has no effect on the absorbance because this enzyme has an absolute requirement for Ca²⁺ (31). After the addition of Ca²⁺, a sharp linear increase in the absorbance at 412 nm is seen, which can be stopped by the addition of excess EDTA. Volwerk et al. (32) synthesized a short chain diethioester analog of phosphatidycholine (2,3-bis(hexanoylthio)-propyl-phosphocholine) in which both fatty acids were bound to glycerol by a thioester linkage. This thioester analog has an advantage over long chain diacylphospholipids in that it produces a clear suspension. However, it is a racemic mixture and therefore may not be suitable for studying enzyme kinetic properties, since the enzymically inactive enantiomer may act as a competitive inhibitor. Hendrickson, Hendrickson, and Dybvig (33) have described the synthesis of a chiral diethioester substrate analog of phosphatidycholine (1,2-bis(heptanoylthio)-1,2-dideoxy-α-glycerol-3-phosphocholine). This substrate has been used for the rapid and precise determination of cobra venom phospholipase A₂ kinetic properties (34, 35), and may be quite useful for the isolation and characterization of mammalian phospholipase A₂. The kinetic parameters of crude preparations of mammalian
phospholipase A₂ with thioester substrate analogs are shown in Table 1.

Wittenauer et al. (36) have used a fluorescent phospholipid analog, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl] phosphatidylcholine (C₆-NBD-PC) to assay porcine pancreatic phospholipase A₂ as well as bovine milk lipoprotein lipase. Hydrolysis of C₆-NBD-PC by these enzymes results in a more than 50-fold fluorescence enhancement, with no shift in the emission maximum at 540 nm.

In their recent publication, Fugman et al. (37) entrapped 6-carboxyfluorescein into liposomes prepared from egg phosphatidylcholine. They demonstrated that porcine pancreatic phospholipase A₂ and bovine milk lipoprotein lipase catalyze the hydrolysis of phosphatidylcholine, which causes the release of the entrapped dye. This procedure provides a simple, accurate, and convenient method for determining the rate of phospholipase A₂ and lipoprotein lipase catalyzed hydrolysis of phosphatidylcholine vesicle substrates.

III. Determination of phospholipase C activity

Cox, Snyder, and Horrocks (38) have extended the use of thioester substrate analogs by describing their use in the determination of phospholipase C activity. They prepared rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercaptopropanediol and 1-S-phosphocholine-2-O-hexadecanoyl-1-mercaptopropanediol. Incubation of Clostridium perfingens phospholipase C with 0.05 mM thioester substrate analogs in the presence of 10 mM CaCl₂ and 0.1 M MOPS buffer (pH 7.4) demonstrated that these substrates are rapidly hydrolyzed by phospholipase C. The rate of enzymic hydrolysis is continuously monitored with 4,4’-dithiodipyridine at 324 nm.

SPECTROPHOTOMETRIC DETERMINATION OF LYSOPHOSPHOLIPASES USING THIOESTER SUBSTRATE ANALOGS

van den Bosch’s group (15, 16) prepared thiodeoxylysolecithin(3-hexadecanoylthio-1-propyl-phosphocholine) by a procedure similar to that described for the phospholipase A₂ substrate, except that 3-mercaptopropanol is substituted for 2-mercaptopropanol. This substrate is rapidly hydrolyzed by beef liver lysophospholipases, and has been successfully used in the isolation and characterization of these enzymes (39).

Our laboratory has also obtained homogeneous preparations of two forms of bovine brain lysophospholipases (22, C. E. Pendley and L. A. Horrocks, unpublished results) using 2-hexadecanoylthio-1-ethyl-phosphocholine. The purified enzyme has a molecular weight of 36,000, and is strongly inhibited by EDTA, Triton X-100, and Tween-20. The kinetic parameters of bovine brain lysophospholipase with thioester substrate analogs are shown in Table 1. Our results (22), as well as those of Aarsman et al. (15, 39) strongly support the view that the rates of hydrolysis of 2-hexadecanoylthio-1-ethyl-phosphocholine by lysophospholipases are proportional to their true activities.

Dithioester substrate analogs of phosphatidylcholine with hexanoyl groups (32) have been used for determining the activity of bovine milk lipoprotein lipase (40). The substrate was prepared essentially as described by Cox and Horrocks (19). Shinomiya et al. (40) report an apparent Vₘₐₓ for the dihexanoylithio analog of phosphatidylcholine of 0.12 μmol per min per mg protein, which is considerably lower than the Vₘₐₓ for the dihexanoylthioform of phosphatidylcholine of 5.0 μmol per min per mg protein. Their assay blanks did not include a blank for light scattering. When light scattering is reduced by hydrolysis of the substrate, the apparent absorbance is decreased, thus cancelling out part of the increased absorbance due to the reaction of DTNB with free sulfhydryl groups. The apparent Kₘ values are also considerably lower at 1900 μM versus 4000 μM, respectively (Table 1). Shinomiya, Epps, and Jackson (40) suggest that the lower Vₘₐₓ may be due to differences in the nature of the chemical bond (C=S–C vs. C=O–C) and to steric factors.

DISCUSSION

Thioester substrate analogs have proved to be useful for the assay of both crude and purified preparations of lipases, lysophospholipases, and phospholipases. The rates of oxyester to thioester hydrolytic activity remain constant during purification of beef liver lysophospholipase (39). Constant rates of oxyester to thioester activities have also been obtained for phospholipase C (38) as well as phospholipase A₂ (32). The thioester substrate assays have several advantages over the traditional radiochemical methods. Assays with thioester substrates are fast, specific, continuous, and convenient. The relatively short acyl chains make substrate suspension easier and obviate the problem of unsaturated side chain oxidation. However, it is also recognized that the relatively shorter length side chains are not necessarily physiological and may not represent exact physiologic Kₘ and Vₘₐₓ values. The color formed with the thionitrobenzoate product permits the discrimination of spectrophotometric enzyme activity from light scattering by visual inspection of the incubation mixture.
The most serious drawback of the thioester substrate assay is the necessary use of thiol capture reagents. These compounds may inhibit enzymic activity, and also exclude measurement with samples containing mercaptoethanol or dithiothreitol.

It is expected that thioester substrate analogs will be highly exploited for the isolation and characterization of lipolytic enzymes in the near future.

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REFERENCES


