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Measurement of Lipase Activity of Guinea Pig Peritoneal Macrophages with 4-Methylumbelliferyl-oleate*)

Katsuhiro KIYOTANI, Hiromichi TASAKA and Yoshiyasu MATSUO
Department of Bacteriology, Hiroshima University School of Medicine
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ABSTRACT

Some basic conditions for fluorometric measurement of lipase activity of guinea pig peritoneal macrophages were investigated using 4-methylumbelliferyl-oleate as a substrate. The most adequate condition for the assay is as follows:

i) Enzyme preparation recommended is the supernatant of ultrasonicate after freezing and thawing of guinea pig peritoneal macrophages suspended in distilled water.

ii) Final concentration of buffered substrate should be adjusted at 0.1 mM in acetate buffer, pH 4.5, free from Triton X-100.

iii) Reaction is terminated by addition of 50 mM Tris-HCl buffer, pH 8.6, instead of 50 mM glycine buffer, pH 10.4.

INTRODUCTION

Since Mead et al.[7] described the method for determination of β-glucuronidase activity using a 4-methylumbelliferone (4-MU) derivative as a substrate in 1955, fluorometric methods for measuring enzyme activity using 4-MU substrates have been widely utilized for acid hydrolases such as exoglycosidases[4,5], acid phosphatase[1] and arylsulfatase[9]. In 1967, Jacks et el.[3] reported a method for measuring lipase activity that hydrolysis of acylated 4-MU derivatives catalyzed by lipase preparations was followed continuously for one or two min by monitoring the increase of fluorescent intensity of the enzyme reaction mixture due to the production of free 4-MU. Recently, a radiometrical method for determining lipase activity has appeared using a labelled cholesteryl ester as a substrate.

However, a simple and convenient method is still needed for assaying the enzyme activity on many samples such as each fraction tube in the purification process of some biologically active substances.

The present communication describes the fluorometric assay method for lipase activity of guinea pig peritoneal macrophages using 4-MU-oleate as a substrate, in relation to the method described by Jacks et al.[3] and other acid hydrolase assay methods[1,4,5,7].

MATERIALS AND METHODS

1. *Preparation of guinea pig peritoneal macrophages*

Peritoneal macrophages (PMφ) were obtained from a male guinea pig, Hartley strain, weighing approximately 400 g, according to the method of Oren et al[8]. Four to five days after injection with 30 ml of 1.2% casein-saline (pH 7.4), the animal was killed and peritoneal exudate cells were collceted, washed and allowed to adhere to the plastic petri dish for 2 hr at 37°C in a CO_2-incubator. Adherent cells (PMφ) were collected, suspended in distilled water (about 1×10^7 cells/ml) and treated with 2 cycles of freezing and thawing followed by ultrasonication for 30 sec at 200 W in cold (Insonator model 200 M: KUBOTA). The supernatant fluid after centrifuging at $1,500\times g$ for 20 min was used as a enzyme solution and

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stored at −80°C until use.

2. *Preparation of substrate solution*

Substrates, 4-MU-oleate and 4-MU-β-D-glucuronide (Koch light Laboratories), were dissolved in ethylene glycol monoethyl ether (methyl cellosolve) to make 10 mM stock solutions. Working solutions were prepared by diluting stock solutions with 0.1 M acetate buffer (pH 4.5).

3. *Determination of enzyme activity*

Enzyme activity was determined fluorometrically according to the method described by Mead et al.[7] for β-glucuronidase, and that described by Jacks et al.[3] for lipase. A 50-μl portion of enzyme solution was added to 50 μl of 0.1 M acetate buffer (pH 4.5) free from Triton X-100 and 100 μl of buffered substrate. The reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by addition of 3.3 ml of 50 mM Tris-HCl buffer (pH 8.6) containing 5 mM EDTA or 50 mM glycine buffer (pH 10.4) containing 5 mM EDTA. Fluorescence was measured with fluorescence spectrophotometer (Hitachi 204) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Enzyme activity was expressed as pmoles of substrate hydrolyzed per min per ml of reaction mixture.

RESULTS

1. *Effect of Triton X-100 on release of 4-MU from 4-MU-oleate*

A final concentration at 0.1 mM of 4-MU-oleate in various concentrations of Triton X-100 was incubated at 37°C for 20 min. The release of 4-MU immediately after adding glycine buffer at pH 10.4, an enzyme stopper, is shown in Table 1. The fluorescence increased with dose of the detergent and the increase was 71% more in 0.2% Triton X-100, a concentration usually used solubilizing enzymes, compared with that in control without adding the detergent. The release of 4-MU further increased nonenzymatically after standing the samples at room temperature for 15min, but did not correlate with dose of Triton X-100. Since the release of 4-MU was high even in control, the effect of pH of stopper was investigated. When Tris-HCl buffer at pH 8.6 was used as the stopper, fluorescent intensity of 4-MU in incubation blanks decreased by approximately 50% comared with that in the case of glycine buffer at pH 10.4. Neither dose response of the detergent nor futher release of 4-MU after standing at room temperature was demonstrated.

Spontaneous release of 4-MU from 4-MU-β-D-glucuronide in Triton X-100 was also studied. As shown in Table 2, 4-MU-β-D-glucuronide was stable in the detergent, and Tris-HCl buffer (pH 8.6) was slightly better than glycine buffer (pH 10.4) as the stopper.

It is clear from the results obtained that stability of 4-MU derivatives varies greatly from derivative to derivative, and the use of Triton X-100 for stabilizing enzyme preparation is

Table 1. Effect of Triton X-100 on the spontaneous release of 4-MU from 4-MU-oleate

Triton X-100 (%)	Incubation blank* (pmole/min/ml) treated with pH 10.4			pH 8.6		
	(1)	(2)	(3)	(1)	(2)	(3)
0.2	192.5	258.8	34	55.0	55.6	1
0.1	185.0	218.8	18	60.0	60.4	1
0.05	153.8	185.0	20	52.5	52.9	1
0.025	141.3	165.0	17	59.1	58.5	−1
0.0125	103.1	141.3	15	51.3	49.8	−3
0	112.5	117.9	5	55.6	55.0	−1

* (1) Blanks of 4-MU-oleate (final 0.1 mM) incubated at 37°C for 20 min were treated with 50 mM glycine containing 5 mM EDTA (pH 10.4) or 50 mM Tris-HCl containing 5 mM EDTA (pH 8.6) and assayed.
(2) Blanks were read again after standing at room temperature for 15 min.
(3) % increase.

Table 2. Effect of Triton X-100 on the spontaneous release of 4-MU from 4-MU-β-D-glucuronide

Triton X-100 (%)	Incubation blank* (pmole/min/ml) treated with pH 10.4			pH 8.6		
	(1)	(2)	(3)	(1)	(2)	(3)
0.2	2.88	3.00	4	1.63	1.63	0
0.1	2.81	2.88	2	1.63	1.63	0
0.05	2.75	2.75	0	1.63	1.63	0
0.025	2.69	2.69	0	1.56	1.56	0
0.0125	2.81	2.81	0	1.56	1.56	0
0	2.75	2.81	2	1.50	1.50	0

* See legend in Table 1.

Table 3. Preparation of enzyme solution from guinea pig peritoneal macrophages for lipase assay

Treatment	Lipase activity* (1)	(2)	(3)**
Freezing and thawing (2 times)	2,238	1,046	47
〃 + sonication (30 sec)	1,668	1,255	75
〃 + 〃 (60 〃)	1,386	915	66
〃 + 〃 (120 〃)	1,215	699	58

* Lipase activity (oleate hydrolase: pmole/min/ml) (1) before and (2) after centrifuging at $1,500 \times g$ for 20 min.

** Recovery ratio (%) of lipase activity after centrifugation.

undesirable in the measurement of lipase activity.

2. *Preparation of enzyme solution from guinea pig peritoneal macrophages*

Solubilization of lipase from guinea pig PMφ was attemped without using Triton X-100. A considarable amount of lipase was solubilized by freezing and thawing the harvested PMφ, but recovery of the activity in the supernatant after centrifuging at $1,500 \times g$ for 20 min was less than 50% of that in the cell extract. Since the cell extract contained cell debris, ultrasonication was further performed. Although lipase activity decreased with elapsed time of ultrasonication, recovery rate of the lipase activity in the supernatant was maximum in ultrasonicate for 30 sec (Table 3).

3. *An adequate concentration of 4-MU-oleate*

A concentration of 4-MU-oleate adequate for lipase assay was investigated because of its high level of spontaneous hydrolysis and opacity at a concentration of 0.2 mM, the standard concentration of other 4-MU derivatives. Release of 4-MU and opacity of reaction mixture decreased proportionally to concentrations of the substrate, but lipase activity of guinea pig PMφ measured was almost the maximum level at 0.1 mM of the substrate (Table 4).

Table 4. Concentration of 4-MU-oleate and reaction pattern

Concentration of substrate*	Opacity	Incubaction blank**	Lipase activity**
0.2	++	62.50	100
0.1	+	31.25	98.1
0.05	+w	16.25	65.1
0.025	−	8.88	36.3

* 4-MU-oleate (mM)

** pmole/min/ml

From the results obtained, the most adequate condition for fluorometric measurement of lipase activity was settled as shown in Table 5.

DISCUSSION

Triton X-100, a non-ionic detergent, has generally been utilized in assay systems for meas-

Table 5. Lipase assay method

Enzyme solution	: Supernatant of ultrasonicate after freezing and thawing of cells
Substrate	: 4-Methylumbelliferyl-oleate (final concentration 0.1 mM)
Assay buffer	: 0.1 M acetate buffer, pH 4.5, free from Triton X-100
Assay method	: Enzyme solution 50 μl
	Assay buffer 50 μl
	Buffered substrate 100 μl
	——→incubated at 37°C for 20 min
	——→terminated by addition of 3.3 ml of 50 mM Tris-HCl buffer, pH 8.6, containing 5 mM EDTA
	——→fluorescence is masured at $E_{365}F_{450nm}$

urering enzyme activity in crude preparations such as tissue or cell homogenate in order to ensure stable and reproducible results. In the present study, however, we found that this detergent caused extremely high levels of incubation blank values in fluorometric measurement of lipase activity monitored by nonenzymatic release of 4-MU from the substrate 4-MU-oleate, while spontaneous hydrolysis of 4-MU-β-D-glucuronide was very little. We also experienced a high spotaneous release of 4-MU day by day from 10 mM of 4-MU-oleate stock solution stored in a refrigerator. In regard to stability of fatty acyl esters bound to 4-MU, Leaback[6] reported that 4-MU-acetate, a substrate for esterase, was extremely labile under basic conditions compared with other 4-MU substrates such as glycosidases. As such, the use of Triton X-100 is undesirable in fluorometric measurement of lipase activity. However, complete elimination of this detergent from the assay system might involve great difficulties in solubilizing an enzyme lipase from PMϕ. This problem was dissolved by applying freezing and thawing of the cells followed by ultrasonication for 30 sec.

Jacks et al.[3] observed nonenzymatic hydrolysis of 4-MU substrate in determination of carboxylesterase activity under a condition at pH 10.4, and recommended the use of Tris-HCl buffer at pH 7.4 as an assay buffer for lipase. Our finding was that fluorometric intensity of 4-MU spontaneously released from 4-MU-oleate decreased approximately by half when Tris-HCl buffer at pH 8.6 was used instead of glycine buffer at pH 10.4.

Thus, an improved method for fluorometric assay of lipase activity has been established, which minimizes unfavorable factors and promises reproducible results.

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