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Lipase Activity of Guinea Pig Peritoneal Macrophages and Mycobacterial Lipase Inhibitor*)

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ABSTRACT

The interaction of mycobacterial lipase inhibitor (MLI), isolated from culture supernatant fluid of *Mycobacterium tuberculosis* strain H37Rv, and lipase from guinea pig peritoneal macrophages (GP-PMφs) was investigated fluorimetrically by the modified lipase assay system which had previously been proposed.

Two peaks of lipase activity were observed in the enzyme preparation from GP-PMφs. The activity of MLI against lipase from GP-PMφs was significantly high at acidic pH less than 5. 0, and the pattern of inhibition was non-competitive.

Two types of lipase were isolated from the enzyme solution prepared from GP-PMφs by an ion-exchange chromatography on a DEAE-Sepharose column. One of these acted only at pH 4. 5 and was considered to be a lysosomal acid lipase, but another showed the activity at both pH 4. 5 and 7. 0. The fomer was four times more sensitive to the activity of MLI than the latter as well as the crude enzyme preparation.

INTRODUCTION

In our previous report[3], we proposed a simple, sensitive and reproducible fluorimetric assay system for measuring lipase activity. Subsequently, we found and isolated a very strong inhibitor against lipase from guinea pig peritoneal macrophages (GP-PMφs) in the culture supernatant fluid of *Mycobacterium tuberculosis* strain H37Rv[4].

The present communication describes the purification of lipase from GP-PMφs and the interaction with the mycobacterial lipase inhibitor (MLI).

MATERIALS AND METHODS

1. *Preparation of enzyme solution from GP-PMφs.*

Collection of GP-PMφs and preparation of enzyme solution were performed according to the method described previously[3].

In brief, adherent cells obtained from guinea pig peritoneal exudates after intraperitoneal injection of casein were washed with and suspended in distilled water at a concentration of 1×10^7 cells/ml. The cell suspension was treated with freezing and thawing, and then subjected to ultrasonication at 200 W for 30 sec (Insonator model 200 M: Kubota).

The ultrasonicate was centrifuged at $1,500 \times g$ for 20 min. The clear supernatant fluid was stored at −80°C and used as the enzyme solution.

2. *Fluorimetric determination of enzyme activity.*

Lipase activity was measured fluorimetrically with 4-methylumbelliferyl (4-MU)-oleate (Koch Light Laboratories) as a substrate according to the method proposed previously[3].

The assay buffer employed was 0. 1 M acetate

*) 清谷克寛，田坂博信，築山文昭，松尾吉恭：モルモット腹腔マクロファージのリパーゼ活性とマイコバクテリアルリパーゼインヒビター

Table 1. Lipase inhibition assay method

Enzyme solution	: Supernatant of ultrasonicate after freezing and thawing of guinea pig peritoneal macrophages (GP-PMφs) (Activity: 100 pmole/min/ml)
Substrate	: 4-Methylumbelliferyl oleate (Final concentration: 0.1 mM)
Assay buffer	: 0.1 M acetate buffer, pH 4.5
Inhibitor sample	: Mycobacterial lipase inhibitor (MLI), isolated and partially purified from unheated culture filtrate of *Mycobacterium tuberculosis* H37Rv
Assay method	: Inhibitor sample 50 μl Buffered substrate 100 μl Enzyme solution 50 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 mesasured at $E_{365}F_{450nm}$
% Inhibition	: $1-\frac{\text{Enzyme activity with inhibitor}}{\text{Enzyme activity without inhibitor}}\times 100$

Table 2. Distribution of lysosomal acid hydrolases in the various lipase preparations from GP-PMφs

	Lipase activity at pH		Subcellular markers			Inhibition by MLI (50% ID : μg)
	4.5	7.0	Acid phosphatase	β-Glucuronidase	N-Ac-β-glucosaminidase	
Crude preparation	+	+	⧻	++	++	0.18
DEAE-Fr. 1	+	−	⧻	−	+	0.042
Fr. 2	+	+	−	++	−	0.21

buffer, pH 4.5. But the effect of pH on the lipase activity was examined by using GTA buffer[1], pH 3.0–7.0. Other lysosomal enzyme activities were determined also fluorimetrically[2] with 4-MU derivatives (Koch Light Laboratories) as substrates in the acetate buffer, but the activity of N-Ac-β-glucosaminidase was determined in 0.1 M citrate buffer, pH 4.5.

3. *Lipase inhibition assay.*

The outline of lipase inhibition assay is summarized in Table 1.

In brief, instead of 50 μl of the assay buffer in determining lipase activity, 50 μl of inhibitor sample to be tested was mixed with 100 μl of buffered substrate and 50 μl of enzyme solution previously adjusted to the activity of 100 pmole/min/ml. After incubation of the mixture 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. The resulting fluorescence was measured and percent inhibition was calculated as follows:

Percent inhibition =

$$\left(1-\frac{\text{Enzyme activity with inhibitor}}{\text{Enzyme activity without inhibitor}}\times 100\right)$$

The values corresponding to the doses of inhibitor sample added were plotted on a logit paper[5] and 50% inhibition dose (50% ID) of inhibitor was estimated. Dose of the inhibitor was expressed as the protein (μg) measured by the method of Lowry et al.[6], with bovine serum albumin as the standard.

4. *Fractionation of lipase from GP-PMφs.*

About 5 mg of crude enzyme solution prepared from GP-PMφs was applied on a DEAE-Sepharose column (Pharmacia Fine Chemicals: 2.0 by 35 cm) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. After elution with 270 ml of the buffer, a linear gradient of 0 to 0.5 M sodium chloride in the buffer (400 ml in total) was started and effluent fractions were collected (3 ml). Lipase activity of each fraction was measured both at pH 4.5 and 7.0.

5. *Preparation of MLI.*

Isolation and characterization of MLI from culture filtrate of *M. tuberculosis* strain H37Rv were reported elsewhere[4].

RESULTS

Inhibition of lipase activity in the crude preparation from GP-PMϕs by MLI is shown in Fig. 1. The 50% inhibition dose of MLI was estimated to be 0.26 μg.

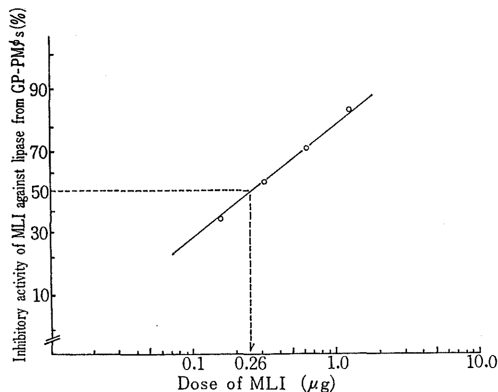

Fig. 1. Inhibitory activity of MLI against lipase from GP-PMϕs.
Lipase activity of GP-PMϕs was measured in the presence and absence of MLI and the pecent inhibition was calculated according to the method described in MATERIALS AND METHODS. Percent inhibition corresponding to the dose of MLI added was plotted on a logit paper, and 50% inhibition dose was estimated.

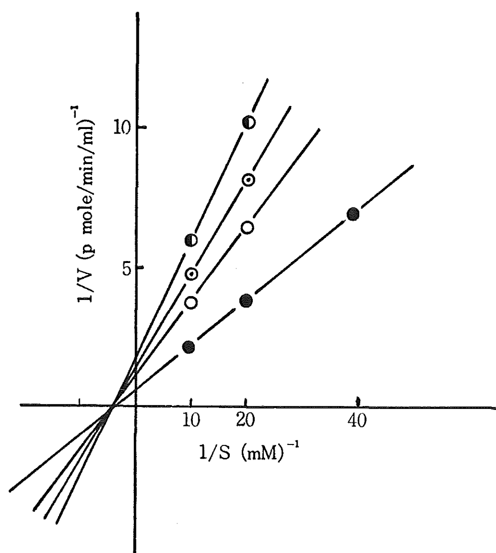

Fig. 2. Linewerver-Burk plot for lipase activity in the presence or absence (●) of 0.09 μg (○), 0.18 μg (◉) and 0.35 μg (◐) of MLI.

The inhibition pattern was non-competitive as shown in Fig. 2.

Effect of pH on the lipase activity in the crude preparation from GP-PMϕs and the inhibitory activity of MLI is shown in Fig. 3. The activity of MLI was significantly high at pH 3.0 to 4.5. There were at least two peaks of cholesteryl ester hydrolytic activity in the enzyme solution. In order to make it clear whether the enzyme solution contains different types of lipase or not, an ion-exchange chromatography was attempted.

Elution pattern of the enzyme solution on a DEAE-Sepharose column is illustrated in Fig. 4. Choresteryl ester hydrolytic activity was eluted in Fraction 1 (tube numbers 145 to 150) and Fraction 2 (tube numbers 163 to 169) separately. Fr. 2 showed lipase activity at both pH 4.5 and 7.0 in almost the same way, whereas Fr. 1 showed it only at pH 4.5 (Fig. 4).

These two fractions were pooled separately and dialyzed against 0.02 M Tris-HCl buffer, pH 8.0. Distribution of other lysosomal acid hydrolases in each fraction and the response of each fraction to MLI were measured fluorimetrically at pH 4.5.

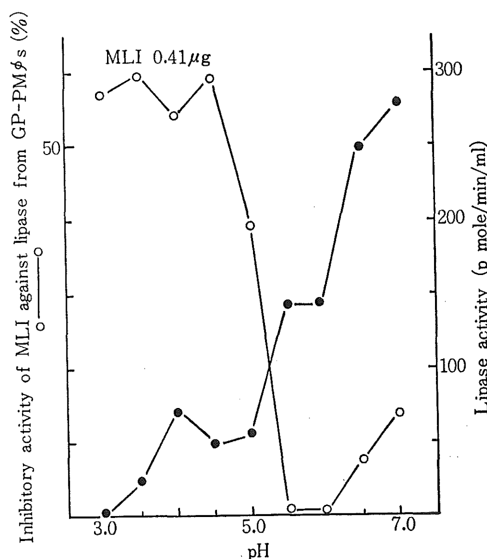

Fig. 3. Effect of pH on the lipase activity from GP-PMϕs and the inhibitory activity of MLI against the enzyme.
Lipase activity from GP-PMϕs (——●——) and the inhibitory activity of MLI (0.41 μg) against the enzyme (——○——) were measured using 0.02 M GTA buffer (pH 3.0 to 7.0) as an assay buffer according to the method described in Table 1.

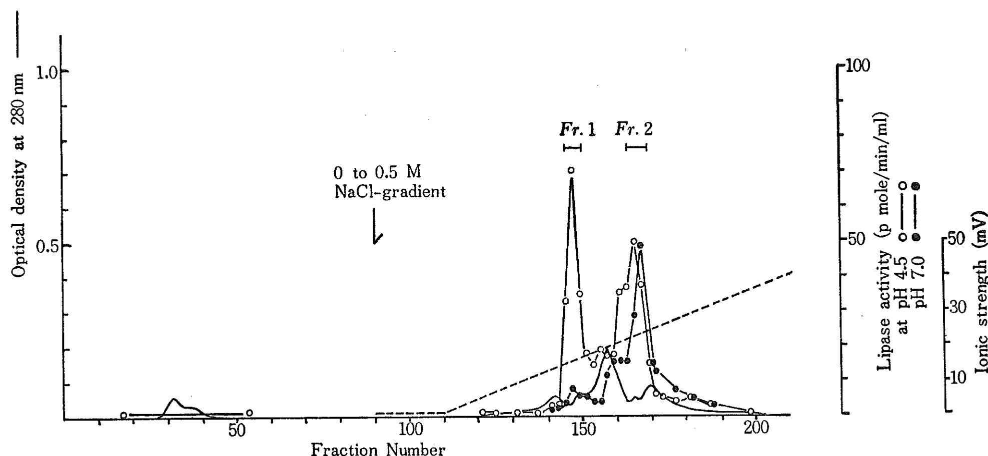

Fig. 4. Isolation of lipase active fractions from GP-PMφs by an ion-exchange chromatography on a DEAE-Sepharose CL-6B column.
About 5 mg of enzyme solution prepared from GP-PMφs were applied on a DEAE-Sepharose column (2.0 by 35 cm) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, and eluted with 270 ml of the buffer. Subsequently, elution was continued by a linear gradient of 0 to 0.5 M sodium chloride in the buffer (400 ml in total). Effluent fractions (3 ml) were collected. Each fraction was assayed for the lipase activity both at pH 4.5 (——○——) and 7.0 (——●——). Bars indicate the pooled fractions 1 and 2.

The results are shown in Table 2. Acid phosphatase and N-Ac-β-glucosaminidase activities were associated with Fr. 1, while β-glucuronidase activity was associated with Fr. 2. Activity of MLI against lipase eluted in Fr. 1 was approximately four times stronger than that eluted in Fr. 2 as well as in the crude enzyme solution.

DISCUSSION

Fluorimetric measurement of lipase activity of GP-PMφs and the activity of MLI against the enzyme was performed by the modified lipase assay method proposed previously[3]. The results showed that at least two cholesteryl ester hydrolases were present in the enzyme solution prepared from GP-PMφs.

Riddle et al.[9] thoroughly investigated the cholesteryl ester hydrolase in various tissue homogenates of a rat, and reported that both the acid and neutral cholesteryl ester hydrolases were distributed widely in tissues. Web and Cohn[13] showed lipase activity of mouse peritoneal macrophages which hydrolyzed cholesteryl linolate or palmitate at pH 4.0.

In the present study, we isolated two types of lipase from the enzyme solution of GP-PMφs by an ion-exchange chromatography on a DEAE-Sepharose CL-6B column. One of these was active only at pH 4.5 and very sensitive to MLI. Another was active at both pH 4.5 and 7.0 and was less sensitive to MLI.

Prabhakaran et al.[8] reported the elevation of the β-glucuronidase activity in mouse footpads infected with *M. leprae* and suggested that the elevation might be attributed to the enzyme of infiltrated phagocytic cells. They also stated that lysosomal enzymes of phagocytic cells and bacteria having a similar substrate specificity somewhat differed from one another in properties such as pH optima and sensitivity to specific inhibitors. It has been reported that there are some isozymes which differ from one another in molecular weight, isoelectric point and/or optimum pH, and cellular origin[7,10]. Although it is not clear where the present two lipases were derived from, at least the acidic lipase is considered to be lysosomal because of its acidic pH optimum.

Purification of lipase was reported by Teng et al.[11] from rat liver lysosomes by solubilization with Triton X-100 and by an ion-exchange chromatography on a CM-Sepharose column. Verine et al.[12] purified a lipase from human adipose tissue by affinity chromatography on a CH-Sepharose column. The former authors showed that the purified enzyme was very labile and the most stable at pH 5.0 in 30%

glycerol.

The activity of the present lipases from GP-PMϕs extremely decreased after the ion-exchange chromatography. The reason is not clear. Because lipase activity was stable at the concentration of sodium chloride used for elution from DEAE-Sepharose column (data not shown), reduction in lipase activity might be attributed to factors other than the process of chromatography.

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