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# Bioluminescent Assay of Phospholipase C Using A Luminescent Marine Mutant Bacterium *Vibrio harveyi* M-17

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**A bioluminescent assay method for detecting the activity of phospholipase C (PLC; phosphatidyl choline cholinephosphohydrolase, EC 3.1.4.3) was developed using bioluminescent marine bacteria. Phospholipase C from *Bacillus cereus* and sn-1,2-dimyristoyl phosphatidyl choline (DMPC) as a substrate were used in the demonstration, and the produced sn-1,2-dimyristoyl glycerol was further hydrolyzed with lipase from *Candida cylidracea*. The hydrolyzed myristic acid was quantified using a dark mutant of *Vibrio harveyi* (designated as M-17). The *in vivo* light intensity of which was stimulated specifically up to one thousand fold in the presence of myristic acid. The rates of the hydrolysis of the DMPC substrate by the phospholipase measured by the luminescence method were linear with time and the amount of enzyme added. Activity measurement conditions (at 25°C, pH 6.5, 10 min fixed time assay) were established to detect as little as 0.1 mUnit of phospholipase C and 5 nM of myristic acid production.**

***Key words***: Phospholipase C (PLC), bioluminescence, sn-1,2-dimyristoyl phosphatidyl choline (DMPC), diacyl glycerol (DG)

Phospholipase C (PLC, phosphatidyl choline cholinephosphohydrolase, EC 3.1.4.3) is an enzyme which hydrolyzes the phosphoester bond between the glycerol backbone and phosphate head group in phospholipids (8, 26). Many different kinds of PLCs have been purified from mammalian cells including human as well as those from plants and microorganisms, such as *Bacillus, Pseudomonas, Clostridium, Listeria* and *Streptomyces* as microbial toxins (26 and references therein). Phosphatidyl inositol-specific PLC (PI-PLC) produces inositol-6-phosphate and diacyl glycerol (DG) which in turn are very important secondary messengers in the signal transduction pathway, and the PI-PLC specific inhibitors serve as possible sources of drugs in the treatment of cancer or Alzheimer's disease (1 and references therein).

The most popular assay for phospholipase C is based on the fact that the PLC reaction produces two products which are quite different in their polarities, for example, a very polar choline phosphate and nonpolar diacyl glycerol are produced from phosphatidyl choline which can be separated and detected if radioisotope labeled phospholipids are used (20). Phospholipids with $^{3}H$- or $^{14}C$- labeled fatty acids are commercially available for radioisotope assay procedures. Although this assay is a highly sensitive and reliable method which uses natural substrates, it has some drawbacks, such as the need for high-priced substrates, time and labor consuming extraction and separation procedures, as well as regulations regarding the use of hazardous radioisotope chemicals. To solve these problems, a series of synthetic substrates, such as *p*-nitrophenyl phosphatidyl choline (16), or fluorogenic substrates, such as 2-naphthyl myo-inositol-1-phosphate and fluorescein myo-inositol-1-phosphate (21, 24) or chemiluminescent synthetic substrate, namely LUMI-PI, have been designated (23). Although methods using these substrates do not cause serious problems in the radioisotopic assay, they still have a common problem because natural phospholipid substrate are not used and can give false signals by other phosphoesterase-type enzymes (9). Thus a fast, safe and reliable assay method for PLC is highly demanded.

In this report a new assay method for phospholipase C activity is presented based on bacterial bioluminescence. In this system a phosphatidyl choline containing myristic acid at the sn-1 position is used as a substrate, and after the reaction, one product (DG) was further hydrolyzed with lipase. The released myristic acid was quantified directly using the dark M-17 mutant of luminescent *Vibrio harveyi* (27). Usually, luminescent bacteria have two enzyme systems for light production *in vivo*. One of these systems is a bacterial luciferase producing light using reduced flavin mononucleotide ($FMNH_2$), long chain aliphatic aldehydes, and molecular oxygen (12). The other enzyme system, the fatty acid reductase complex, is the supplier of

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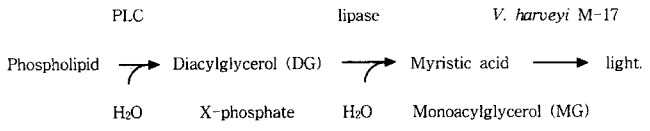
long chain aldehydes from the fatty acid moity of phospholipids, and is composed of three enzymes, acyl transferase, acyl carrier protein, and reductase. First, acyl transferase takes the fatty acid moiety from the phospholipid to the acyl carrier protein using ATP, then the reductase reduces this acyl group to the corresponding aldehyde using NADPH. Free fatty acid can bind directly to the acyl carrier protein, but the M-17 mutant has a defective acyl transferase so it cannot produce a high level of bioluminescence *in vivo*. However, in the presence of exogeneous fatty acid, especially myristic acid (C14 : 0), it can produce a normal level of light (7, 27).




The light intensity of the *Vibrio harveyi* M-17 mutant induced by the addition of exogeneous myristic acid is linearly correlated with the amount of myristic acid and can be used in the myristic acid quantification method (27). In this report a novel luminescence assay system for phospholipase C is demonstrated using sn-1,2-dimyristoyl phosphatidyl choline (DMPC) as a substrate, phospholipase C from *Bacillus cereus*, lipase from *Candida cylidracea* and the luminescent marine bacterium *V. harveyi* M-17 mutant.

## Materials and Methods

### *Chemicals and Enzymes*

Lipase from *C. cylidracea* (type II) and phospholipase C (type IV, from *Bacillus cereus*) were purchased from Sigma Chemical Co. and used without further purification. One unit of enzyme hydrolyzes 1.0 μmol of L-α-phosphatidyl choline to choline phosphate and DG per min at pH 8.0 and 37°C. Phosphatidyl choline (sn-1,2-dimyristoyl, DMPC) is also a product of Sigma. DMPC (10 mg) was dissolved in 1 ml of chloroform and extracted with 1 ml of 5 M NaCl aqueous solution twice to remove any contaminated free fatty acid and 0.1 ml aliquots were stored in nitrogen gas at -70°C until used. Silica gel (mesh size 200 μm) and precoated thin layer chromatography plate (Silica gel G, 0.2 mm thickness) were products of Merck Co (Germany).

### *Culture of Luminous Bacterium*

*Vibrio harveyi* mutant M-17 (7) was a kind gift from Prof. S. C. Tu (Univ. of Houston, TX, USA). This bacterium was grown in 50 ml of sea water complete medium (5 g bactotryptone, 3 g yeast extract, 3 ml glycerol, 75% aged sea water, pH 7) overnight at 25°C up to an optical density of 1.5 at 660 nm. Cells were harvested by centrifugation (10,000 rpm for 5 min), then washed twice with cold washing buffer (0.1 M phosphate buffer containing 3% NaCl, pH 6.5). The harvesting time is important because myristic acid inducing bioluminescence begins to decline after reaching a maximum value in the late logarithmic phase of growth. The washed cell pellet was resuspended in cold washing buffer to final $A_{660nm}$ of 10, stored on ice, and 0.1 ml of this suspension was diluted to 1 ml with the same buffer at room temperature just before each measurement. This cell suspension gave reproducible results for approximately 3 hrs.

### *Luminescent assay of phospholipase C*

Ten μl of phosphatidyl choline (1,2-dimyristoyl-, DMPC) stock solution (4 mM in ethanol) was added to 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0, 30 mM $CaCl_2$, 50 mM sodium deoxycholate) in a 1.5 ml Eppendorf tube and the reaction was started by addition of an appropriate amount of PLC, then incubated at 37°C. From this reaction mixture, 0.1 ml was taken and added into prepared luminous M-17 bacterium cell suspension (1 ml), vortexed briefly, and the maximum light intensity ($I_i$) was measured using a photometer equipped with a Hamamatsu R-447 photomultiplier phototube with a Mitchell-Hasting type preamplifier (18) and a high voltage DC power supply (GW-2500, Bioneer Co., Korea). Usually, light peaks were obtained within 1 min. This light intensity ($I_i$) indicates the nonspecific production of myristic acid nonenzymatically or by contaminated phospholipase A and/or lipase.

Another 0.1 ml of reaction mixture was taken and added into 0.2 ml of cold (-70°C) acetone to precipitate the remaining phosphatidyl choline, and after 10 min in the deep-freezer, centrifuged for 2 min at 13,000 × g. The supernatant containing DG was transferred to a new tube and lipase in 0.1 ml of 50 mM Tris·HCl buffer pH 8.0 was added and incubated for 10 minutes. Then, a prepared M-17 cell suspension (1 ml) was added and the maximum light intensity ($I_f$) was measured again. The peak intensity difference ($\Delta I = I_f - I_i$) was directly related to the amount of myristic acid in the mixture produced from DG by added lipase which in turn reflected the amount of DG produced from PC by PLC. Calibration was done using the continuous light source of Hastings and Weber (13). One light unit (LU) was defined as $2\times 10^7$ quanta·$sec^{-1}$. For photographic detection, a camera luminometer (Model 501, Analytical Luminescence Lab.) using a 63 multiwell plate (7×9) and Polaroid type 667(B/W) film were used. Suspension of *V. harveyi* M-17 (0.1 ml each) was added quickly to each well with an 8-channel repeating multipipet (Eppendorf, Germany).

### *Product analysis*

TLC-FID (Iatroscanner MK-5, Iatron Lab., Japan) were employed for the product analysis. Reaction mixtures were extracted with chloroform : MeOH (1 : 1), evaporated

and redissolved in extraction solvent to a final concentration of 10 mg/ml. About 10 μg of each sample was loaded on a silica Chromarod III (Iatron Lab., Japan). Each Chromarods were dried, developed with phospholipid analysis solvent system (chloroform : MeOH : DW= 65 : 35 : 5) up to 50% distance, and then, with a neutral lipid analysis solvent system (n-hexane : diethylether : formic acid=80 : 20 : 0.2) to full length (19). After analysis with FID, the each peaks were integrated with an HP3396 integrator (Hewlett-Packard, USA). Calibration was performed with cholesterol acetate as the internal standard.

## Results and Discussion

The growth of the luminous bacterium *V. harveyi* dark mutant M-17 was followed by measuring both $A_{600nm}$ and the *in vivo* light intensity with 10 μM of myristic acid, because this bacterium can emit light only in the presence of long chain aldehydes or myristic acid. The level of bioluminescence was about 3 $LU \cdot ml^{-1} A_{600nm}^{-1}$ without myristic acid, but by addition of myristic acid showed a maximum value (about 3500 $LU \cdot ml^{-1} A_{600nm}^{-1}$) just before cell growth reached the stationary phase, then declined in the stationary phase (3, 4). Using a bacterial suspension in phosphate buffer (pH 6.5), the dependence of light intensity on the concentration of added myristic acid was measured, and a standard curve was generated which was linear in the range of 5 nM to 10 μM final concentration of myristic acid (4). The curve shows saturation at concentrations of myristic acid higher than 10 μM at the given cell density. Using a suspension with a higher cell density would generate a higher light intensity, but the high background signal from the cells gives no real advantage of sensitivity.

Because lipase in general has a broad spectrum of substrate specificity, phospholipids as well as diacylglycerol can be hydrolyzed to some extent by lipase. Although the degree of hydrolysis of phospholipids by lipase is very low in this assay condition, it is better to remove as much of the phospholipids in the reaction mixture before the addition of lipase to prevent a false signal from the hydrolysis of the remaining phospholipid. Usually, the whole reaction mixture was extracted with a suitable solvent, such as chloroform : MeOH (2 : 1), and concentrated. DG was separated from the phospholipid by a silica column or TLC, but this process consumes too much time and effort. In this study, a cold acetone precipitation in the presence of $CaCl_2$ which is usually included for a PLC assay system in a concentration of about 25 mM. This salt helps the precipitation of phospholipids with cold acetone because the $Mg^{2+}$ or $Ca^{2+}$ salt of phospholipid is not soluble in acetone, and had been used for the purification of phospholipid (15). The distribution of DG and PC in the precipitate and supernatant of acetone precipitation was analyzed with TLC-FID (Table 1). The best results were obtained by precipitation of phospholipid with two volumes of cold (-20°C) acetone, and most of the DG (92%) remained in the acetone/water solution while 88% of PC was precipitated which was easily separated with brief centrifugation.

**Table 1.** The proportion of dimyristoyl glycerol (DMDG) and dimyristoyl phosphatidyl choline (DMPC) in various concentrations of acetone in the reaction buffer

| Solvent used | Relative Recovery Yield (%) (n = 3) | | | |
|---|---|---|---|---|
| | DMDG | | DMPC | |
| Layer | Solvent | ppt | Solvent | ppt |
| Folch solution (2 vol) | 100[a] | -[b] | 100 | - |
| acetone (1 vol) | 72 ± 4 | 28 ± 6 | 34 ± 4 | 66 ± 5 |
| acetone (Cold, 1 vol) | 80 ± 4 | 20 ± 5 | 26 ± 3 | 74 ± 5 |
| acetone (Cold, 2 vol) | 92 ± 5 | 8 ± 3 | 12 ± 4 | 88 ± 6 |

[a] The amounts of lipids extracted with Folch solution was regarded as 100%.

[b] The amounts of lipids extracted from the precipitate between Folch solution and the aqueous phase was not determined.

It was not necessary to remove the included acetone for the next lipase reaction because the lipase from *C. cylidracea* was not inhibited by 70% of the acetone/buffer biphasic solution system (17). A typical light production

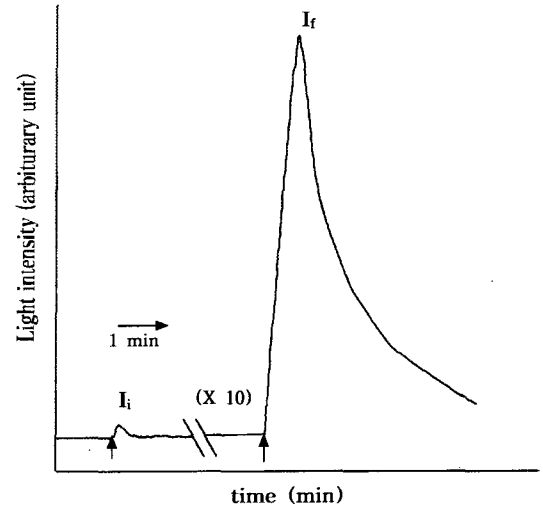



**Fig. 1.** A typical bioluminescence assay of phospholipase C. To 1 ml of 50 mM Tris-HCl buffer (pH 8.0 containing 25 mM $CaCl_2$), 25 μl of 4 mM ethanolic DMPC solution were added, and the reaction was started with the addition of 0.1 unit of PLC at 37°C. At 10 min, 100 μl aliquots were taken, then added into 1 ml of luminous bacteria suspension in 0.1 M phosphate buffer (pH 6.5) to obtain $I_i$ (indicated by first arrow under baseline). Another 100 μl aliquots were taken, and added with cold acetone, and hydrolyzed with 0.1 unit of lipase, then added into 1 ml of luminous bacteria suspension in 0.1 M phosphate buffer (pH 6.5) to obtain $I_f$ (indicated by second arrow under baseline). The $I_f$ curve was reduced to 1/10 the vertical scale to compare with the $I_i$ curve in the same pannel.

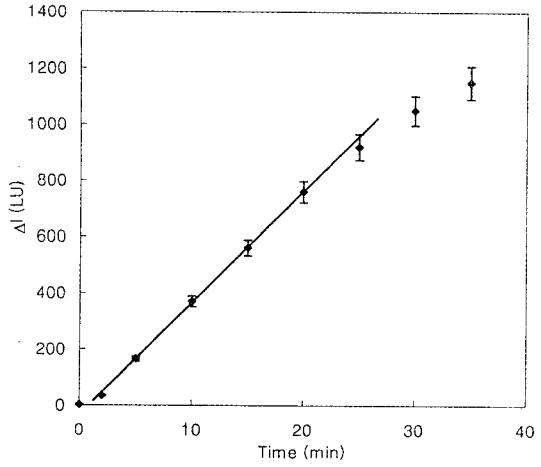



**Fig. 2.** Time dependent myristic acid production from DMPC by phospholipase C. Assayed in the same way with Fig. 1, with lipase and luminous marine bacterium *V. harveyi* M-17. To 1 ml of 50 mM Tris-HCl buffer (pH 8.0 containing 25 mM $CaCl_2$), 25 μl of 4 mM ethanolic DMPC solution was added, and the reaction was started with the addition of about 0.1 unit of phospholipase C. The reaction mixture was incubated at 37°C, and at the times indicated, 100 μl aliquots were taken and added with cold acetone, and hydrolyzed with 0.1 unit of lipase, then added into 1 ml of luminous bacteria suspension in 0.1 M phosphate buffer (pH 6.5) and then assayed as described in Materials and Methods.

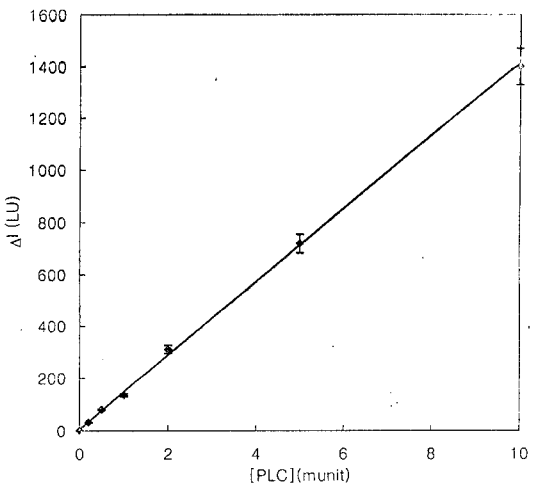



**Fig. 3.** Dependence of light production on the concentration of phospholipase C. Various amounts of phospholipase C activity were used in the presence of 0.1 mM of DMPC in 50 mM Tris-HCl buffer (pH 8.0 containing 25 mM $CaCl_2$) at 37°C, using a 10 min-fixed time assay procedure.

pattern was shown in Fig. 1. In this case in using a partially purified commercial PLC, the nonenzymatic hydrolysis of fatty acid was negligible. However, analysis of a crude enzyme source, such as screening of PLC producing microorganisms, which can be contaminated with other phospholipases such as $A_1$ or $A_2$ or lipase, can produce free myristic acid from the substrate (DMPC) directly, or the produced DG can cause a false signal. To solve this problem, a control analysis should be performed and the background signal ($I_i$) was subtracted from the final light intensity ($I_f$) to obtain ΔI as PLC activity.

The production of myristic acid by lipase from the sn-1 position of diacyl glycerol (DG) was monitored with time using the bioluminescence method (Fig. 2). A plot of light intensity (that is myristic acid released) with time was linearly increased for 25 minutes. A small delay in myristic acid production was observed during the initial incubation phase before 5 min, probably due to the binding of phospholipase C to the PC micelle. Ten minutes of fixed assay time was found to be sufficient to determine PLC activity, especially for a large number of analyses, such as screening microbial PLC production or bioactive PLC inhibior. Phospholipase C activity versus light intensity showed good linearity with this luminescent assay, down to 0.1 mUnit (Fig. 3). Phospholipase C activity versus increasing concentration of added phosphatidyl choline exhibited a sigmoidal saturation curve and did not fit in the hyperbolic curve below 20 μM, and from a double reciprocal plot of the hyperbolic part, the *Km* value of phosphatidyl choline (DMPC) for phospholipase C of *Bacillus cereus* with the luminescence assay method was estimated to be 42 μM (Fig. 4). Although there is no reported *Km* value of DMPC for the PLC of *Bacillus cereus*, the *Km* of sn-1,2-dipalmitoyl phosphatidyl choline (35 μM) is a reasonably close to the value obtained with the radioisotope assay method (14).

This bioluminescent assay of PLC is a suitable assay for analyzing large numbers of samples at a time, such as the activity assay of chromatographic fractions, the screening

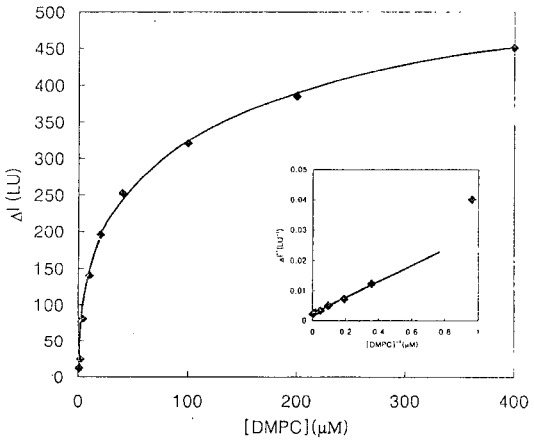



**Fig. 4.** Dependence of light production on the concentration of DMPC. Luminescent assay of phospholipase C activity was performed at various concentrations of DMPC. 0.1 unit of phospholipase C was used in 50 mM Tris-HCl buffer (pH 8.0 containing 25 mM $CaCl_2$ at 37°C), and 10 min-fixed time assays were used. Inset: the *Km* value PLC for DMPC was determined with a double-reciprocal plot of hyperbolic part of Fig. 4.

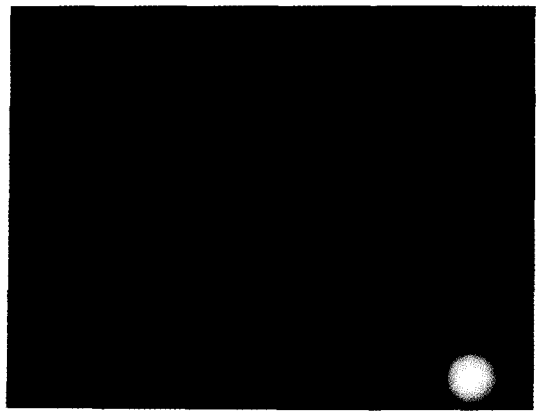


**Fig. 5.** Photographic screening of PLC producing marine bacteria with a luminescence coupled reaction. Camera luminometer which included a 63 multiwell (7×9) microplate was used. Cell free culture broth of bacteria (0.2 ml) and 10 μl of DMPC (0.1 mM) were added into each well, and after the reaction for 30 min, 10 μl of lipase was added without acetone precipitation. *V. harveyi* M-17 suspension (0.1 ml) was added after 10 min and photographs were taken (exposing time was 10 sec). The well at the bottom right corner is a positive control including myristic acid (5 μM).

of extracellular PLC producing microorganisms or PI-PLC inhibition screening for biologically active natural products (1, 26). Using a commercially avaliable camera luminometer with Polaroid type 667(B/W) film, extracellular PLC producing bacteria can be easily detected (Fig. 5). Although a direct quantification of PLC activities in each fractions was not possible, only the microwells with PLC producing bacterium (unidentified strain) showed a bright circle. For quantification of light intensity from each well, a digital camera (at least one million pixels) and proper quantification software instead of a camera luminometer could be employed.

The usual phospholipase C assay system involves quantification of released diacyl glycerol which is labeled with a radioisotope fatty acid and is, so far, the most sensitive method (20). But increasing concerns about environmental contamination and public health have resulted in a large number of regulations regarding the use of radioisotope labeled chemicals in laboratories. The time and labor cost of the separation process of the diacyl glycerol from the whole reaction mixture, such as extraction and chromatography, is another drawback of this method. Alternatively, a synthetic substrate such as *p*-nitrophenyl phosphocholine or *p*-nitrophenyl phosphoinositol can be used for analysis and the produced *p*-nitrophenol is measured with an absorbance at 405 nm (10, 16). Although these spectrophotometric assays are very easy and convenient, they are not sensitive enough and the use of synthetic substrate which are structurally very different from natural phospholipids cannot used for each PLC assay (25) and nonspecific cleavage by enzymes other than PLC can cause interference (9). Moreover, these artificial substrates do not contain a glycerol moity which is indispensible for optimal PLC activity (21). Recently, another line of synthetic substrate for PLC were designed and reported using chemiluminescent properties of the dioxethane ring system (22). This chemiluminescent synthetic substrate will give much higher sensitivity, but the synthetic substrate is also structurally different from the natural substrate and difficult to synthesize because it is not commercially available.

The bioluminescent assay of PLC using luminous bacterium reported here is in fact a triple coupled assay, PLC-lipase-bacterial bioluminescence, but practically this can be regarded as a PLC-lipase coupled assay system because bacterial bioluminescence gives the results almost instantaneously. This assay system is a straightforward, safe and easy analysis system using simple equipment and a relatively inexpensive, commercially available, natural phospholipid, such as phosphatidyl choline, phosphatidyl ethanol amine, phosphatidyl serine, and phosphatidyl inositol substrates for any substrate specific PLC with high sensitivity. Diacylglycerol is actually analyzed in this assay system and is a common product of all kinds of PLC. This assay can be applied to any type of PLC such as PC-specific PLC or PI-specific PLC by changing only the substrate. Moreover, sn-1,2-dimyristoyl glycerol (1,2-DG) produced by PLC is quickly converted into sn-1,2-dimyristoyl glycerol (1,3-DG) spontaneously, and almost all myristic acids can readily be substrates of usual lipases. Any time and labor consuming pretreatment steps such as organic solvent extraction, filtration, chromatography, and derivatization were minimized in this system.

The sensitivity of assay methods is greatly dependent on the ability to detect concentration changes of substrate(s) or product(s). The lower limit of myristic acid detection with M-17 is dependent largely on the sensitivity of the photometer used. In this work the limit was 5 *p*mol of myristic acid using a luminometer assembled in this laboratory. Luminous bacteria can be easily cultured and stored and can be used directly as an assaying reagent with relatively little cost and labor. Now *V. harveyi* M-17 is commercially available (Bioness Co., Korea) as Lumi-Tox®. Although the stability of the *V. harveyi* M-17 suspension is not high and gives reproducible results for only 3 hrs, the bacterial suspension can be prepared easily. This newly developed method can be applied to any myristic acid producing reaction, such as the phospholipase $A_1$ or $A_2$ (4), cholesterol esterase reaction (3), and alkyl aldehydes as in monoamine oxidase or α-oxidase (2, 6).

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