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Notes & Tips

A chromogenic substrate for solid-phase detection of phospholipase A_2

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ABSTRACT

A method for solid-phase detection of phospholipase A_2 (PLA_2) was developed. The method uses 1-octanoyloxynaphthalene-3-sulfonic acid, which was found to be a good substrate of PLA_2. The substrate is hydrolyzed by PLA_2 into 1-naphthol-3-sulfonic acid, which is spontaneously coupled with coexisting diazonium salt to form a red-purple azo dye. *Streptomyces* and bovine pancreatic PLA_2 spotted on a nitrocellulose membrane could be detected by this method with considerable sensitivity. In addition, colonies of recombinant *Escherichia coli* producing bacterial PLA_2 were distinguishable from those producing an inactive mutant PLA_2, facilitating high-throughput screening in directed evolution of the enzyme.

Phospholipase A_2 (PLA_2; EC 3.1.1.4) hydrolyzes phospholipids to lysophospholipids and fatty acids. PLA_2 is a useful enzyme with many applications in lipid industries such as production of lysolecithin from lecithin [1], enzymatic degumming for edible oil refinement [2], and synthesis of phospholipids having specific acyl residues [3,4].

Our previous work [5] demonstrated successful extracellular production of a PLA_2 from *Streptomyces violaceoruber,* the first bacterial PLA_2 identified [6], using a recombinant *Escherichia coli.* The success of the expression of the *Streptomyces* enzyme enabled us to perform protein engineering, especially by directed evolution, of this enzyme to improve its properties. Generally, directed evolution of an enzyme involves construction of a mutant gene library, expression of the mutated genes in the host cells, and high-throughput screening to isolate the desired mutant enzymes. Thus, to perform directed evolution of the PLA_2, a high-throughput detection method of the enzyme was necessary.

Various methods for colorimetric detection of PLA_2 have been devised so far (reviewed in [7]). An example includes quantification of the released fatty acids, by converting it to acyl-CoA with acyl-CoA synthetase and then oxidizing it to 2,3-enoyl-CoA by acyl-CoA oxidase with the formation of hydrogen peroxide, which, in the presence of peroxidase, leads to oxidative coupling of 4-aminoantipyrine and 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)aniline to generate a spectrometrically measurable purple dye [8]. Another example uses a thioester analogue of phosphatidylcholine as a substrate, from which, after hydrolysis, the generated free thiol groups reduce the disulfide bond of coexisting 5,5′-dithiobis(2-nitrobenzoic acid) to form yellow-colored 2-nitro-5-mercaptobenzoic acid [9,10]. The third example uses acyloxynitrobenzoic acid, an artificial substrate, which is hydrolyzed by the enzyme to generate yellow-colored hydroxynitrobenzoic acid [11,12].

As we intended to perform a colony-based high-throughput screening from a library for mutant enzymes with improved properties (e.g., enhanced stability to extreme conditions), it was favorable to detect the enzymatic activity on a solid support such as nitrocellulose membrane. For this reason, the above-mentioned colorimetric methods do not fulfill our requirements, because the colored dyes to be detected are water soluble and therefore diffusible into the aqueous phase. In addition, it might be difficult to visually recognize the yellow color of the nitrobenzoic acids over a white background (i.e., nitrocellulose membrane).

This paper describes a simple protocol for the solid-phase detection of PLA_2 using a new chromogenic substrate, 1-octanoyloxynaphthalene-3-sulfonic acid (Fig. 1A, compound **1**). In this method, compound **1** is hydrolyzed by the action of PLA_2 to release 1-naphthol-3-sulfonic acid, which is spontaneously coupled with a coexisting diazonium salt (Fast Blue B salt) to generate a red-purple azo dye (Fig. 1C). Since the dye adsorbs on the surface of the solid support, the positions of the enzyme can be visualized as colored spots.

Compound **1** was synthesized as follows: 1-naphthol-3-sulfonic acid sodium salt (984 mg, 4 mmol) and 4-dimethylaminopyridine (48 mg, 0.4 mmol) were dissolved in dry pyridine (20 ml). To this solution, octanoic chloride (576 mg, 4 mmol) was added dropwise, and the mixture was left at room temperature for 2 days with stirring. Thirty milliliters of 12 M aqueous HCl was added slowly with cooling in an ice bath. The acidified mixture was extracted with

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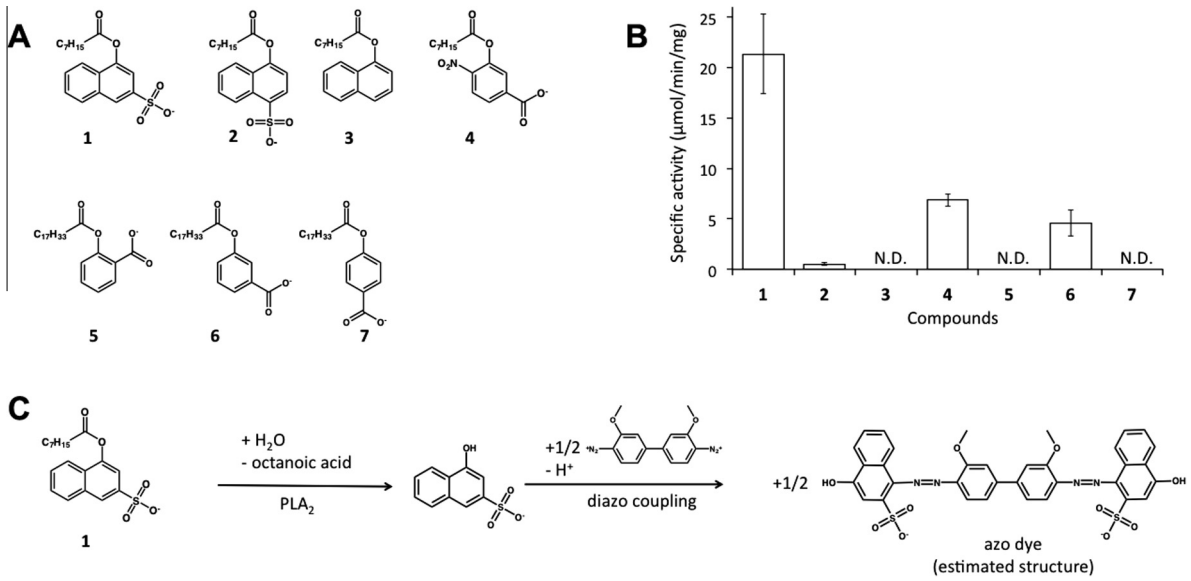

Fig. 1. Synthetic substrates for PLA_2 hydrolysis. (A) Structures of the esters tested for PLA_2 hydrolysis. (B) Hydrolytic activity of *Streptomyces* PLA_2 toward the synthetic esters. The activities were measured in triplicate, and the average values are shown. The error bars show the standard deviation. (C) Reaction scheme of the PLA_2 detection using compound **1**.

40 ml of diethyl ether four times. The ether solution was washed with saturated NaCl and dried with anhydrous Na_2SO_4. During this drying step, a white fluffy crystal formed. This crystal was recovered by filtration, washed with ice-cold diethyl ether, and then dried at room temperature to afford compound **1** as a white powder (ESI–MS *m*/*z* 349 $(M-H)^-$). Similarly, 1-octanoyloxynaphthalene-4-sulfonic acid (Fig. 1A, compound **2**) was synthesized using 1-naphthol-4-sulfonic acid sodium salt (ESI–MS *m*/*z* 349 $(M-H)^-$). 1-Octanoylnaphthol (Fig. 1A, compound **3**) was synthesized as follows: 1-naphthol (144 mg, 1 mmol) was reacted with octanoyl chloride (163 mg, 1 mmol) in dry tetrahydrofurane (6 ml) in the presence of *N*,*N*-diisopropylethylamine (259 mg, 2 mmol) for 2 days at room temperature. The mixture was filtered, and the residue was washed with diethyl ether (20 ml). The filtrate was washed with saturated $NaHCO_3$ and dried with anhydrous Na_2SO_4, and the solvent was removed by evaporation. The residual material was chromatographed on a silica gel column with *n*-hexane/ethyl acetate (95/5) to afford compound **3** (ESI–MS *m*/*z* 271 $(M+H)^+$, 288 $(M+NH_4)^+$). 4-Nitro-3-octanoyloxybenzoic acid (Fig. 1A, compound **4**) was synthesized as described elsewhere [11]. 2-Oleoyloxybenzoic acid (Fig. 1A, compound **5**) was synthesized as follows: 2-hydroxybenzoic acid (138 mg, 1 mmol) was reacted with oleoyl chloride (360 mg, 1.2 mmol) in dry diethyl ether (10 ml) in the presence triethylamine (240 mg, 2.4 mmol) and 4-dimethylaminopyridine (12 mg, 0.1 mmol) for 20 h at room temperature. The mixture was acidified with 10 ml of 2 M aqueous HCl, and the ether phase was recovered. The ether solution was washed with saturated NaCl and dried with anhydrous Na_2SO_4, and the solvent was evaporated. The residue was chromatographed on a silica gel column with *n*-hexane/ethyl acetate (9/1) to afford compound **5** (ESI–MS *m*/*z* 401 $(M-H)^-$). Similarly, 3-oleoyloxybenzoic acid (Fig. 1A, compound **6**) and 4-oleoyloxybenzoic acid (Fig. 1A, compound **7**) were synthesized from 3-hydroxybenzoic acid and 4-hydroxybenzoic acid, respectively.

Fig. 1B compares the hydrolytic activities of the *Streptomyces* PLA_2 purified from a recombinant *E. coli* [5] toward these synthetic esters. The hydrolytic activities were measured by conducting the reaction in 20 mM Tris–HCl (pH 8.0) containing 2 mM synthesized ester, 0.2% Triton X-100, and 10 mM $CaCl_2$ for 10 min at 37 °C, stopping the reaction with addition of EDTA to give a final concentration of 50 mM, which was followed by quantification of the released fatty acids (either octanoic or oleic acid) using a kit (NEFA-C Test; Wako, Japan). It was found that compound **1** was a good substrate with specific activity of 21 μmol/min/mg, although the activity was lower than that for dioleoylphosphatidylcholine (183 μmol/min/mg), the natural substrate of the enzyme. In contrast, compound **2** (a positional isomer of compound **1**) was only slightly hydrolyzed (0.5 μmol/min/mg), and compound **3** (a sulfonate-less derivative) was not hydrolyzed at all. The enzyme showed a moderate activity (6.9 μmol/min/mg) toward compound **4,** a known synthetic substrate for snake venom and porcine pancreatic PLA_2 [11]. Furthermore, compound **6** was hydrolyzed moderately (4.6 μmol/min/mg), but its positional isomers (compounds **5** and **7**) were not hydrolyzed at all.

These results indicated the structural requirements for the substrate of the *Streptomyces* PLA_2: (i) the presence of an anionic group is required; (ii) the anionic group is not necessarily a phosphate group as in the natural phospholipids, but can be carboxylate or sulfonate; and (iii) there must be the proper distance between the anionic group and the scissile ester bond, i.e., four chemical bonds between the anionic group and the ester oxygen. It is reported that snake venom PLA_2 can hydrolyze not only compound **4** but also its positional isomers 4-octanoyloxy-3-nitrobenzoic acid (with carboxylate at the *para* position of the octanoyloxy group) and 2-octanoyloxy-4-nitrobenzoic acid (with carboxylate at the *ortho* position of the octanoyloxy group), with a preference for 4-octanoyloxy-3-nitrobenzoic acid over the other isomers [11]. This suggests that the snake enzyme requires the carboxylic anion for capturing the substrates, but the distance between the scissile ester and the carboxylate is not very critical; the best substrate of the snake enzyme is the *para* isomer, yet the *ortho* and *meta* isomers can still be substrates. In contrast, the *Streptomyces* PLA_2 recognizes the substrates in a more stringent way, i.e., it can hydrolyze only the esters that fulfill the above-mentioned structural requirements.

The fact that compound **1** was a good substrate for the *Streptomyces* PLA_2 encouraged us to design the solid-phase detection method of the enzyme as illustrated in Fig. 1C. The wild-type *Streptomyces* PLA_2, its activity-less H64A mutant (having a mutation at its catalytic His 64 to Ala), and bovine pancreatic PLA_2 (Sigma)

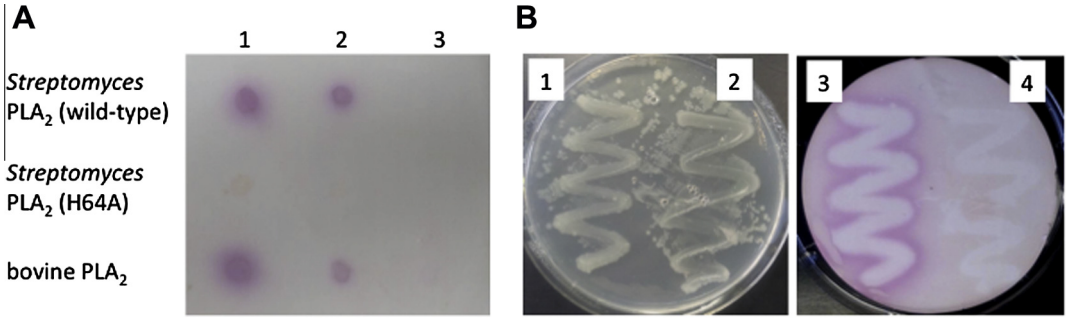

Fig. 2. PLA_2 detection on nitrocellulose membrane. (A) Dot-blot analysis: (lane 1) 700 ng, (lane 2) 70 ng, (lane 3) 7 ng of the wild-type *Streptomyces* PLA_2 (top row), its H64A mutant (middle row), and bovine pancreatic PLA_2 (bottom row) were spotted on a membrane and subjected to detection using compound **1**. (B) Colony-based detection of PLA_2-producing recombinant strains of *E. coli:* the recombinants producing the wild-type PLA_2 (1) and the inactive PLA_2 (2) were grown on an agar medium and subjected to the solid-phase detection (3 for the wild-type and 4 for the inactive enzymes).

were spotted on a piece of nitrocellulose membrane (Hybond-C; Amersham) and air-dried. The membrane was soaked in the substrate solution containing 0.02% compound **1,** 0.02% Fast Blue B salt (Aldrich), 10 mM $CaCl_2$, 100 mM NaCl, and 10 mM Tris–HCl (pH 8.0) and incubated at room temperature. Soon after soaking, the positions of the wild-type PLA_2 became visible by the formation of red-purple spots (Fig. 2A). In contrast, the spots with the inactive enzyme did not turn red-purple; instead, the inactive enzyme appeared as light yellow-brown spots, possibly due to a side reaction with the protein and the diazonium salt. The method was applicable also for the detection of bovine pancreatic PLA_2. As little as 70 ng of PLA_2 could be detected in 10 min of incubation. The reaction was terminated by rinsing the membrane with water, which did not wash away the colored spots.

Two recombinant *E. coli* strains [5], BL21 (DE3) having pET22b-PLA_2 (encoding the wild-type *Streptomyces* PLA_2 gene) and one having pET22b-PLA_2 (H64A) (encoding the inactive H64A mutant gene), were grown at 37 °C for 16 h on an LB-agar medium containing 100 μg/ml ampicillin. A sterilized piece of nitrocellulose membrane was put on the LB-agar plate to transfer the colonies, and the membrane was placed on another LB-agar plate with the colony side up and incubated at 37 °C for 8 h to grow the colonies on the membrane. Then, the membrane was placed onto another LB-agar plate containing 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubated for another 16 h to induce the expression of the target genes. Since the recombinant strains produce PLA_2 extracellularly [5], the synthesized enzymes were immobilized on the surface of the membrane around the colonies.

After the induction, the membrane was washed briefly by shaking in 10 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ and 100 mM NaCl to remove the cell debris. Then, the membrane was soaked into the substrate solution and incubated at room temperature. As shown in Fig. 2B, the recombinant colonies having pET22b-PLA_2 could be recognized by the formation of the red-purple dye. In contrast, those with pET22b-PLA_2 (H64A) were not stained. The result indicated that colonies expressing the active PLA_2 could be selectively identified by this method.

In conclusion, this paper demonstrates a simple method for detecting PLA_2 activity on nitrocellulose membrane. The color of the azo dye was stable for several weeks at room temperature without fading. We believe that the method is useful for high-throughput screening of mutant PLA_2 with improved properties from mutant libraries. Also, it may be applicable for biochemical studies related to PLA_2.

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