Phospholipase D (PLD) is a biocatalyst in the synthesis of bioactive compounds and a key enzyme in a variety of biological signal transductions. A combination of unnatural phosphatidyl acceptor, N,N,N-triethyl-N-2-hydroxyethylammonium bromide 6, as a substrate for PLD, and tandem electrospray ionization mass spectrometry (ESI MS) was found to provide information as to whether a given phospholipid serves as a substrate for the PLD-catalyzed reaction. Thus 2-(13'-hydroperoxy-octadecadienoyl)-1-palmitoylglycerophosphocholine 1, and its degradation products 2-(13'-oxo-octadecadienoyl)-1-palmitoylglycerophosphocholine 9 and 2-(13'-hydroxy-octadecadienoyl)-1-palmitoylglycerophosphocholine 11, in a mixture were found to be a substrate of the PLD-catalyzed transphosphatidylation. The sensitivity of this method was exemplified by the observation that PLD activity in cabbage leaves was detected using a small amount of crude crushed leaves with little pretreatment. This simple method can be used in screening for PLD activity and searching for inhibitors of the enzyme from various natural sources.

Key words: phospholipase D; ESI MS; phosphatidylcholine; hydroperoxide

Phospholipase D (PLD) is an enzyme that is ubiquitously distributed in biological systems. It catalyzes the hydrolysis of phosphate esters in glycerophospholipids, producing phosphatidic acid (PA) and alcohol, and in transphosphatidylation reaction, affording various phospholipids, except for PA. It has been documented in recent years to be involved in a variety of important biological signal transduction processes.1–3) PLD has also been employed as a biocatalyst in the preparation of various bioactive compounds via transphosphatidylation because of its broad substrate specificity.4–7) Since the catalytic activity of PLD is crucial in application, a number of reports have been published about the enzymatic reaction conditions,8–10) peroxide-mediated activation of PLD11–13) and method of determining PLD activity.14,15) Morris et al. reported a review of a method of measuring PLD activity.16) The method includes head structure (choline-like phosphate part) from natural of measuring PLD activity.17) Therefore, if the product formed from phospholipids by PLD-catalyzed transphosphatidylation is also a PC analog, such as 3–6 for PLD (Scheme 1). In this report, we will describe a method of realizing this approach.

Among several phosphatidyl acceptors, 3–6, N,N,N-triethyl-N-2-hydroxyethylammonium bromide (TEAEBr) 6 was found to be the best candidate due to easy synthesis and handling as a stable crystalline form. The salt was prepared simply by refluxing a solution of N,N-diethyl-N-2-hydroxyethylamine and ethyl bromide in dry acetonitrile for 2 h. The crystalline product, after filtration and washing with a small amount of acetonitrile, this was found to be pure enough for the enzymatic reaction.
Scheme 1. PLD-Catalyzed General Reaction from 1 to 2 Using Unnatural Phosphatidyl Acceptors (3–6); PLD-Catalyzed Reaction Using Unnatural Phosphatidyl Donor (TEAEBr 6) from 1 to 8 through 7; PLD-Catalyzed Reaction Using TEAEBr 6 from 9 to 10, and from 11 to 12; Cabbage PLD-Catalyzed Phosphatidyl Transfer Reaction Using TEAEBr 6 from 13 to 14, and from 15 to 16.

As a model PC to test the validity of this approach, a phosphatidylcholine hydroperoxide (PC-OOH, 1) was employed as a phosphatidyl donor for PLD-catalyzed transphosphatidylation. PC-OOH 1 was synthesized according to our reported method. This sample contained small amounts of degradation products such as 9 and 11, formed from 1.

The enzymatic reaction and product analyses by ESI MS were conducted as follows: PLD (5 mg, 1,500 u/g, from Actinomadura sp.) was added to a solution of PC-OOH 1 (0.2 mg, 0.3 µmol), TEAEBr 6 (2.3 mg, 10 µmol), ether (200 µl), and a trace of butylated hydroxytoluene (BHT) in a mixture of acetate buffer (60 µl, pH 5.6), and the solution was stirred at 30 °C for 3 h in an N₂ atmosphere. An antioxidant, BHT, prevented homolytic decomposition of PC-OOH. After this period, an aqueous solution of EDTA (80 µl, 0.26 mmol) was added to inactivate the enzyme, and the lipid fraction was extracted 3 times by the Bligh-Dyer method using a mixture of methanol/chloroform (2:1) (5 ml). The extract was washed with saturated aqueous sodium bicarbonate solution (0.5 ml) to remove the bromide ions, followed by drying over anhydrous sodium sulfate. Part of this solution was diluted with a mixture of CH₃CN/CH₃OH/H₂O (52:43:49) with ammonium acetate (0.1%) for tandem ESI MS measurement.

In Q1 positive ion scan mode, a peak was observed at m/z 832.6 (Fig. 1A), which coincided with the exact mass calculated for [M + H]⁺ of 8. The product ion scan mode (positive) for m/z 832.6 gave m/z 226.2 (data not shown). Since this value is equal to the mass unit (mu) of (HO)₂P(O)OCH₃CH₂N⁺(CH₃CH₃)₂, the molecular species of the compound that showed m/z 832.6 was confirmed to be product 8, formed through PLD-
catalyzed transphosphatidylolation and dehydrobromination from 7 with Na₂CO₃. In fact, as shown in Fig. 1B, when precursor ion scan mode was conducted using m/z 226, m/z 832.8 was observed. This observation demonstrated that alcohol 6 could serve well as a phosphatidyl acceptor for the PLD-catalyzed reaction. It also suggests that since PC which has a polar head group derived from 6, is unnatural, PC should be detected in a highly selective and sensitive manner even in the presence of a complex mixture of natural phospholipids and other biological samples. A small peak at m/z 850.6 was the molecular species that attached with an ammonium ion (NH₄⁺) in structure 8. We also observed a small peak at m/z 814.8 in spectrum 1B. This number is less than 832.8 by 18 mu, equal to the mu of H₂O, suggesting that the molecular species was product 10 with a carbonyl group produced by dehydration from PC-OOH 1. This dehydration is known to occur spontaneously and easily, by heating in particular.²° We conducted neutral loss (NL) scan mode in tandem ESI MS at NL = 588.5 mu which could be calculated by subtraction of the mu of the product ion from that of the precursor ion (m/z 814.7 - 226.2 = 588.5 mu). As shown in Fig. 1C, a sharp peak was observed at m/z 814.7, also indicating that the molecular species which gave a peak at m/z 814.8 in Fig. 1B was carbonyl form 10. These spectral analyses confirmed that the carbonyl form of PC 9 served as a good substrate for the PLD to afford product 10.

Another small peak was observed at m/z 816.8 (Fig. 1B). This number is less than 832.8 by 16 mu, and is equal to mu of oxygen atom suggesting that the –OOH group was replaced by –OH in 11 and 12. Neutral loss scan at NL = 590.5 mu (m/z 816.6 – 226.1 = 590.5) showed a sharp peak at m/z 816.8 (Fig. 1D). These spectral analyses confirmed that hydroxy form 11 served as a good substrate for the PLD to afford product 12. In addition, PC-OOH was confirmed to be a phosphatidyl donor for PLD (Fig. 1A and B). Since PC-OOH is known to be formed and to occur in a variety of biological systems, such as blood containing polyunsaturated fatty acids, PC-OOHs such as 1 and their degradation products such as 9 and 11 have been documented to be involved in biological systems such as inflammatory processes, pathological conditions, and aging.²¹–²³ They also influence inevitable food quality deterioration. In these biological processes, there is a possibility that phospholipid peroxidation products are subjected to enzymatic reactions by phospholipid related enzymes such as PLA₂, PLC, and PLD. These peroxidation products, however, are not always a good substrate for the enzymes, and can rather reduce or inhibit enzymatic activity. As for PLD, the present study revealed that PC-OOH 1 and its degradation products, 9 and 11, served well as a substrate for the enzyme at a small reaction scale without using any single phospholipid. Thus the combination of the unnatural phosphatidyl acceptor 6 and tandem ESI MS was found to provide information as to whether a given phospholipid could be a substrate of PLD. This new method is very simple and easy to conduct using only small amounts of phospholipid mixture.

To examine the sensitivity of this method for the detection of PLD activity using molecular probe 6, an experiment was conducted using cabbage as a source of PLD. Crushed cabbage leaves (0.3 g) and diethyl ether (0.2 ml) were added to a solution of TEAEBr 6 (30 mg, 0.13 mmole) and partially purified soybean phosphatidylcholine (22 mg) that was a mixture of 2-linoleoyl-1-palmitoylphosphatidylcholine 13 and 1,2-dilinoleoylphosphatidylcholine 15 (1:0.4) in an acetate buffer (0.6 ml, pH 5.6, 10 mM with 0.1 m CaCl₂). The mixture was stirred for 4 h at room temperature. After this period, a solution of EDTA in water (0.6 ml, 10 mM) was added to quench the reaction. An excess of a mixture of chloroform/methanol (5 ml, 1:2) and anhydrous sodium sulfate (0.5 g) was added and well mixed, followed by simple filtration using a filter paper to remove the cabbage leaves and sodium sulfate. Twenty μl of the filtrate was diluted with a solvent mixture (1.0 ml, CH₃OH/CH₃CN/H₂O, 52:43:49, with 0.1% ammonium acetate), and the solution was submitted to mass spectrometry in positive ion Q1 scan mode in ESI MS.
As seen in Fig. 2A, two peaks were observed, at \( m/z \) 758.4 and 782.4, for soybean PC 13 and 15 respectively. In addition, two more peaks were observed, at \( m/z \) 800.4 and 824.4. The difference between 758.4 and 800.4 is equal to 42, and this number is equal to the difference in mass units (87 \( \text{[CH}_2\text{CH}_3\text{]} \) = 42). Hence the PC that showed a peak at \( m/z \) 800.4 was regarded as reaction product 14. Similarly, the peak at \( m/z \) 824.4 was assigned to product 16. To confirm this, neutral loss scan mode was conducted at NL = 598.4 \( m/z \) 782.4 = 574.4; \( m/z \) 800.4 = 526.2 = 574.2), and a spectrum was obtained (Fig. 2B). The peak at \( m/z \) 758.4 was that of PC 13 (calculated exact mass, 758.4), and a new peak appeared at \( m/z \) 800.4, which was assigned to reaction product 14. Similarly, neutral loss scan was conducted at NL = 598.4 \( m/z \) 782.4 = 598.4; \( m/z \) 824.4 = 226.2 = 598.2), and a spectrum was obtained (Fig. 2C). The peak at \( m/z \) 782.4 was that of PC 15 (calculated exact mass, 782.4), and a new peak appeared at \( m/z \) 824.4, which was assigned to reaction product 16. These analyses unambiguously demonstrated that the PLD activity of cabbage produced 14 and 16 from 13 and 15. The involvement of the PLD-catalyzed reaction in cabbage was also demonstrated by an experiment in which, when cabbage was heated for 5 min in boiling water, no peak appeared at \( m/z \) 800.4 or 824.4 (Fig. 2B and C), indicating that the PLD in the cabbage was inactivated. Thus the method developed in the present study can be applied to detect PLD activity with a little pretreatment of PLD-containing material.

The characteristics of the present method can be summarized as follows: By using precursor and neutral loss scan modes in collision-induced tandem electrospray mass spectrometry and an unnatural phosphatidyl acceptor, it was possible to determine whether a particular glycerophospholipid molecular species could serve as a substrate for PLD even when the sample solution contained more than one phospholipid species in the mixture. In this analysis, phospholipid hydroperoxide and its degradation products were found to be a substrate of PLD for the first time. This simple method can be used to screen PLD activity and the search for inhibitors of the enzyme\(^{23}\) from various natural sources.

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**References**

Detection of Phospholipase D Activity Using a Molecular Probe


