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Enzymatic measurement of ether phospholipids in human plasma after hydrolysis of plasma with phospholipase A_1

Shiro Mawatari*, Seira Hazeyama, Tomomi Morisaki, Takehiko Fujino

Institute of Rheological Functions of Food, 2241 Kubara, Hisayama Chou, Kasuya-gun, Fukuoka 811-2501, Japan

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ABSTRACT

Objectives: Ethanolamine ether phospholipids (ePE) and choline ether phospholipid (ePC) are present in human serum or plasma. Decreases in ether phospholipids (plasmalogens) in serum (plasma) have been reported in several diseases such as Alzheimer's disease, Parkinson's disease, metabolic syndrome, schizophrenia. Therefore, need for assay of ether phospholipids in plasma may increase in the future. Nowadays, measurement of the ether phospholipids in human plasma seem to depend on tandem mass spectrometry (LC/MS/MS), but a system for LC/MS/MS is too expensive for most of ordinary clinical laboratories, moreover, use and maintenance of the system are time consuming.

Design and methods: Phospholipase A_1 (PLA1) hydrolyzes ester (acyl) bond at the sn-1 position of glycerophospholipids, but it does not act on ether bond at the sn-1 position. We confirmed by a HPLC method that treatment of plasma with PLA1 causes complete disappearance of all diacyl phospholipids, but ether phospholipids remain intact. On the basis of these observations, we developed an enzymatic assay method for ePE and ePC in human plasma by use of a fluorescence plate reader.

Results: The amount of ePE in human plasma measured by the enzymatic method was well correlated to that by LC/ESI-MS method ($R^2 > 0.94$), but the correlation of ePC between the two methods was bit poorer ($R^2 > 0.77$) than that of ePE.

Conclusion: The enzymatic method may be applied to assay of ether phospholipids (ePE and ePC) not only in human plasma but also to assay of ePE and ePC in the other tissues.

1. Introduction

Ether phospholipids constitute a special class of phospholipids characterized by the presence of an ether bond at the sn-1 position of glycerol backbone. There are two types of ether bonds in ether phospholipids: the ether (alkyl) bond and the vinyl ether (alkenyl) bond. Phospholipids with the vinyl ether bond are called plasmalogens [1–4].

Ethanolamine ether phospholipid (ePE) and choline ether phospholipid (ePC) are found in human plasma (serum), but the relative concentration of the ether phospholipids in the phospholipids of human serum are very low as compared to those in tissues such as leukocytes and erythrocytes. Functions or physiological roles of the ether phospholipids in serum (plasma) are not well elucidated, however, decreases in plasmalogens in serum (plasma) have been reported in several diseases such as Alzheimer's disease [5,6], Parkinson's disease [7], metabolic syndrome [8–10], schizophrenia [11,12] and uremic patients [13].

Nowadays measurement of serum (plasma) plasmalogens seems to depend on liquid chromatography-tandem mass spectrometry (LC/MS/MS) [5,6,8–16]. An HPLC method for detection with a flow γ-counter by use of a radioactive iodine is reported [17].

* Corresponding author.
E-mail address: mawatari@rheology.po-jp.com (S. Mawatari).

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However, these methods are time consuming and require expensive apparatus.

Phospholipase A_1 (PLA1) hydrolyzes ester (acyl) bond at the sn-1 position of glycerophospholipids, but it does not act on ether bonds at the sn-1 position. We confirmed by high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) that treatment of plasma with PLA1 causes complete disappearance of all diacyl phospholipids, but ether phospholipids and sphingomyelin (SM) remain intact [18]. Therefore, we previously reported that the HPLC-ELSD and LC/ESI-MS method can be used for measurement of ether phospholipids (ePE and ePC) and SM in human plasma after treatment of plasma with PLA1 [18]. On the basis of these observations, we developed an enzymatic assay method for ePE and ePC in human plasma by use of a fluorescence plate reader. The method may be applied to measure ePE and ePC in the other tissues other than plasma.

2. Materials and methods

2.1. Materials

Phospholipase A_1 (PLA1) from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae* was purchased from Sigma-Aldrich Co. (Tokyo, Japan). Phospholipase A_1 from *Aspergillus oryzae* (10,000–13,000 U/g) was purchased from Mitsubishi Kagaku Foods Co. (Tokyo, Japan). Glycerophospholipid specific phospholipase D (GPL-PLD) from *Streptmyces sp*. (41.1 u/mg) was purchased from Asahi Kasei Pharma Co. (Tokyo, Japan), tyramine oxidase from *Arthrobacter sp.* (4.6 u/mg) and choline oxidase from *Arthrobactor glob.* (20 u/mg) were purchased from Asahi Kasei Pharma Co. (Tokyo, Japan). Horseradish peroxidase (460 u/mg) was obtained from Oriental Yeast Co. (Tokyo, Japan). Amplex Red reagent was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Phosphatidylethanolamine from egg yolk and phosphatidylcholine from bovine liver were obtained from Doosan Serdary Laboratory (Toronto, Canada). Scallop and chicken breast muscle were obtained at a market place.

2.2. Preparation of ether phospholipids in plasma

With a written informed consent, human venous blood of volunteers who were aged over 70 years (76.3 ± 5.7, $n = 12$) were drawn into a tube containing heparin, and plasma was separated by using a clinical centrifuge at 1000*g* for 5 min. Hemolysis was checked visually and all of the plasma with hemolysis were discarded.

The study was approved by the Institutional Review Boards of BOOCS clinic (Fukuoka, Japan). The study was implemented in compliance with Declaration on Helsinki.

Plasma was kept at – 80 °C until use. PLA1 (Sigma) was diluted with an equal volume of 0.1 M citrate buffer (pH 4.5), and 20 μL of the diluted PLA1 was added to 80 μL of plasma and incubated at 45 °C for 60 min.

2.3. Extraction of lipids

Lipid extraction after the treatment of plasma with PLA1 was accorded to the reported method [19]. Eight hundred (800) μL of n-hexane/isopropanol (3:2, v/v) was added to the PLA1 treated plasma (100 μL), and after vigorous mixing, it was placed in an ultrasound bath for 5 min. Then 400 μL of Na_2SO_4 solution (1 g of anhydrous Na_2SO_4 dissolved in 15 mL of water) was added. 400 μL of the upper hexane layer was transferred to a new conical glass tube. Then 400 μL of hexane/isopropanol (7:2, v/v) was added to the lower phase and vigorously mixed. After brief centrifugation, 300 μL of hexane layer was recovered. The combined hexane layer was dried under N_2 gas, and stored at − 30 °C until use.

In some experiments chloroform/methanol (1:2, v/v) method was used with some modification from Bligh and Dyer method [20]. After treatment of plasma with PLA1 as described, 300 μL of chloroform/methanol (1:2, v/v) was added, and the mixture was vortexed for 30 s, and was sonicated for 1 min. After mixing with 100 μL of chloroform, 100 μL of water was added. After brief centrifugation, 160 μL of lower chloroform phase was transferred to a new tube. Lipids were re-extracted from the remaining lower phase with 100 μL of chloroform and 100 μL of the lower phase was recovered. The combined chloroform phase was dried under N_2 gas, and stored at − 30 °C until use.

2.4. Enzymatic measurement

Strategy for enzymatic measurements of ether phospholipids in serum or plasma is showed in Fig. 1. Phospholipase A_1 (PLA1) hydrolyzes diacyl phospholipids in plasma (serum) and leaves ePE and ePC intact. In the second step after lipid extraction, ePE is hydrolyzed by glycerophospholipid specific phospholipase D (GPL-PLD) to ethanolamine, and ePC is hydrolyzed by GPL-PLD to choline. Oxidation of ethanolamine is catalyzed by amine oxidase, and oxidation of choline is catalyzed by choline oxidase. The last steps are catalyzed by peroxidase, and Amplex Red reacts with H_2O_2 to produce fluorescent resorufin. Reagent solution for ePE determination contained 1 U/mL of tyramine oxidase, 5 U/mL of peroxidase, 10 U/mL of PLD, 50 μM Amplex Red, 0.75 mM $CaCl_2$, 0.1% Triton X 100, and 50 mM NaCl in 50 mM Tris-HCl (pH 7.4) [21–23]. The sample (the dried lipid extract) was dissolved in 500 μL of 0.5% triton X-100 just before use [21–23]. An aliquot of the sample (40 μL) was added to the reagent (100 μL) and incubated at 37 °C for 30 min. The fluorescence intensity was measured using a fluorescence microplate reader (DTX series, Beckman Coulter, Tokyo, Japan). The excitation and emission wavelengths were set to 535 and 595 nm, respectively.

For enzymatic measurement of plasma ePC, tyramine oxidase in the reagent used for ePE was replaced by 1 U/mL of choline oxidase. The procedure was the same as that for measurement of ePE as described above.