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# Enzymatic measurement of ether phospholipids in human plasma after hydrolysis of plasma with phospholipase A<sub>1</sub>



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<sub>1</sub> (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  $\gamma$ -counter by use of a radioactive iodine is reported [17].

\* Corresponding author.

E-mail address: [mawatari@rheology.po-jp.com](mailto:mawatari@rheology.po-jp.com) (S. Mawatari).<https://doi.org/10.1016/j.plabm.2018.01.003>

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

Phospholipase A<sub>1</sub> (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<sub>1</sub> (PLA1) from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae* was purchased from Sigma-Aldrich Co. (Tokyo, Japan). Phospholipase A<sub>1</sub> 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 *Streptomyces 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 *Arthrobacter 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 ( $76.3 \pm 5.7$ ,  $n = 12$ ) were drawn into a tube containing heparin, and plasma was separated by using a clinical centrifuge at 1000g 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  $-80^{\circ}\text{C}$  until use. PLA1 (Sigma) was diluted with an equal volume of 0.1 M citrate buffer (pH 4.5), and 20  $\mu\text{L}$  of the diluted PLA1 was added to 80  $\mu\text{L}$  of plasma and incubated at  $45^{\circ}\text{C}$  for 60 min.

### 2.3. Extraction of lipids

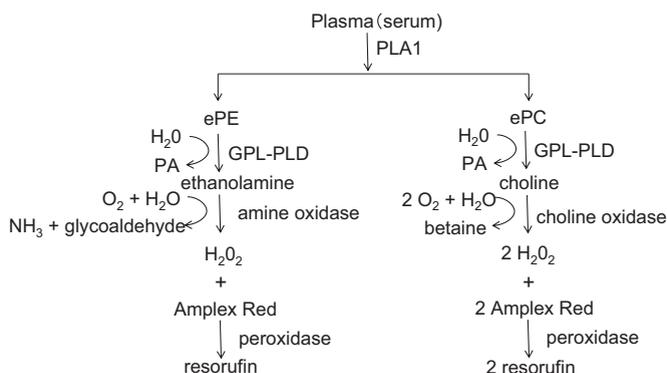
Lipid extraction after the treatment of plasma with PLA1 was accorded to the reported method [19]. Eight hundred (800)  $\mu\text{L}$  of n-hexane/isopropanol (3:2, v/v) was added to the PLA1 treated plasma (100  $\mu\text{L}$ ), and after vigorous mixing, it was placed in an ultrasound bath for 5 min. Then 400  $\mu\text{L}$  of Na<sub>2</sub>SO<sub>4</sub> solution (1 g of anhydrous Na<sub>2</sub>SO<sub>4</sub> dissolved in 15 mL of water) was added. 400  $\mu\text{L}$  of the upper hexane layer was transferred to a new conical glass tube. Then 400  $\mu\text{L}$  of hexane/isopropanol (7:2, v/v) was added to the lower phase and vigorously mixed. After brief centrifugation, 300  $\mu\text{L}$  of hexane layer was recovered. The combined hexane layer was dried under N<sub>2</sub> gas, and stored at  $-30^{\circ}\text{C}$  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  $\mu\text{L}$  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  $\mu\text{L}$  of chloroform, 100  $\mu\text{L}$  of water was added. After brief centrifugation, 160  $\mu\text{L}$  of lower chloroform phase was transferred to a new tube. Lipids were re-extracted from the remaining lower phase with 100  $\mu\text{L}$  of chloroform and 100  $\mu\text{L}$  of the lower phase was recovered. The combined chloroform phase was dried under N<sub>2</sub> gas, and stored at  $-30^{\circ}\text{C}$  until use.

### 2.4. Enzymatic measurement

Strategy for enzymatic measurements of ether phospholipids in serum or plasma is showed in Fig. 1. Phospholipase A<sub>1</sub> (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<sub>2</sub>O<sub>2</sub> 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  $\mu\text{M}$  Amplex Red, 0.75 mM CaCl<sub>2</sub>, 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  $\mu\text{L}$  of 0.5% triton X-100 just before use [21–23]. An aliquot of the sample (40  $\mu\text{L}$ ) was added to the reagent (100  $\mu\text{L}$ ) and incubated at  $37^{\circ}\text{C}$  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.



**Fig. 1.** Strategy for enzymatic measurement of ethanolamine ether phospholipid (ePE) and choline ether phospholipid (ePC). Phospholipase A<sub>1</sub> (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<sub>2</sub>O<sub>2</sub> to produce fluorescent resorufin. PA; phosphatidic acid.

### 2.5. HPLC-ELSD method and LC/ESI-MS method

Details of HPLC-ELSD-method and LC/ESI-MS method were described in our previous report [18,24].

## 3. Results and discussion

We previously confirmed by acid hydrolysis of lipids after PLA1 hydrolysis of human plasma that the chromatographic peaks of ether phospholipids contain some alkyl bond together with alkenyl bond [18]. Therefore, it may be reasonable that the ether phospholipids in human plasma detected with HPLC-ELSD method and LC/MS-ESI method are called ether phospholipids (ePE and ePC) instead of plasmalogens.

### 3.1. Hydrolysis of plasma with PLA1

Preparation of ether phospholipids in human plasma was accorded to our previous report [18]. The brochure from the provider shows that the optimum temperature and the optimum pH for PLA1 activity are 45 °C and pH 4.5, respectively. Therefore, we diluted PLA1 with 0.1 M citrate buffer (pH 4.5) and the incubation of plasma with PLA1 was done at 45 °C. PLA1 purchased from Mitsubishi Kagaku Food Co. was suspended in 0.1 M citrate buffer (50 mg/mL). Forty (40)  $\mu$ L of the enzyme solution was added to 80  $\mu$ L of plasma, and it showed identical results to those with PLA1 purchased from Sigma by monitoring with HPLC and LC/ESI-MS method.

Time courses of main phospholipids in plasma during PLA1 hydrolysis were described in our previous study [18]. Briefly, PC which was the most abundant phospholipid in plasma was hydrolyzed completely within 40 min, and lysophosphatidylcholine (LPC), which was the product from hydrolysis of PC by PLA1, increased rapidly within 10 min and then decreased gradually until 50 min. On the other hand, ether phospholipids (ePE and ePC) and SM remained unchanged for 60 min incubation at 45 °C. Furthermore, levels of ePE and ePC were constant by incubation with different amounts of PLA1 for 60 min at 45 °C. The typical chromatograms of total phospholipids of human plasma and chromatogram after PLA1 hydrolysis of the plasma were presented (Fig. 2).

### 3.2. Lipid extraction

Extraction of lipids after the treatment of plasma with PLA1 is essential for the enzymatic method. We found that recovery of lysophospholipid (LPC) were different according to lipid extraction method [18]. Chloroform /methanol method retained large amount of LPC, but the hexane/isopropanol method removed almost all of LPC (Fig. 2) [18]. However, both lipid extraction methods can be used for the enzymatic method.

GPL-PLD activity toward LPC and SM are reported to be very low [23].

### 3.3. Enzymatic method

Recently, a report of enzymatic method for measurement of ethanolamine plasmalogen in plasma have been reported [25]. In that method, plasma was hydrolyzed with a PLA1 from *Streptomyces albidoflavus* which hydrolyze ethanolamine plasmalogen to produce lysoplasmalogen, and resulting lysoplasmalogen was further hydrolyzed with lysoplasmalogen specific phospholipase D (LyPLs-PLD) to produce free ethanolamine, and the free ethanolamine was measured by colorimetric enzymatic method. The lysoplasmalogen specific phospholipase D (LyPLs-PLD) as well as PLA1 from *Streptomyces albidoflavus* which hydrolyze ePE to lyso PE are not commercially available.

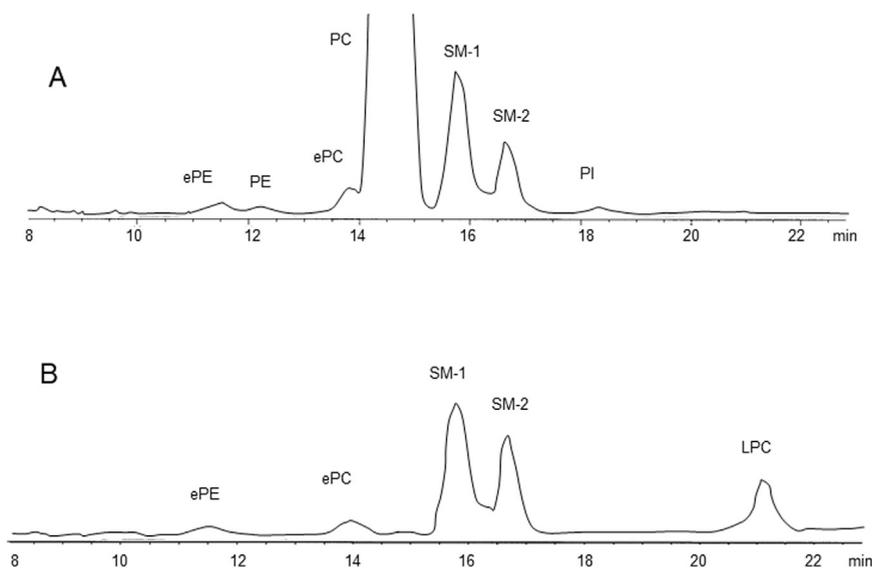


Fig. 2. HPLC chromatograms of human plasma lipids. A, Total lipids, B, After hydrolysis of plasma with PLA1, which was used to enzymatic assay of ether phospholipids (ePE and ePC). SM; sphingomyelin, LPC; lysophosphatidylcholine which was product of hydrolysis of PC with PLA1.

Our scheme for the enzymatic measurement of ether phospholipid is different from that of the reported method (Fig. 1). We used PLA1 from *Aspergillus oryzae* to remove diacyl phospholipids in plasma, and remaining ether phospholipids were hydrolyzed with glycerophospholipid specific phospholipase D (GPL-PLD) from *Streptomyces sp.* to produce free ethanolamine and free choline. Free ethanolamine and free choline were measured as fluorescence intensity of resorufin by using Amplex Red, respectively. All enzymes used in our method are commercially available.

When the plasma sample after PLA1 hydrolysis was subjected to our enzymatic method for ePE, the fluorescence intensity from the PLA1 treated sample were constantly smaller than those without PLA1 treatment (PE + ePE) (Fig. 3, A). This result indicates that only ePE was measured after PLA1 treatment. Because human plasma contains much larger amount of PC than ePC (Fig. 2), total choline phospholipids (PC + ePC) were out of measurable range by the reagent for ePC. However, the fluorescence intensity from ePC was linearly increased by increasing amounts of the PLA1 treated plasma (Fig. 3, B). To avoid possible contamination of fluorescence from free choline and free ethanolamine on the calculation of amount of the ether phospholipids, a reaction mixture in which phospholipase D was absent was used as a blank reaction, and fluorescence intensity of the blank was subtracted from that of samples.

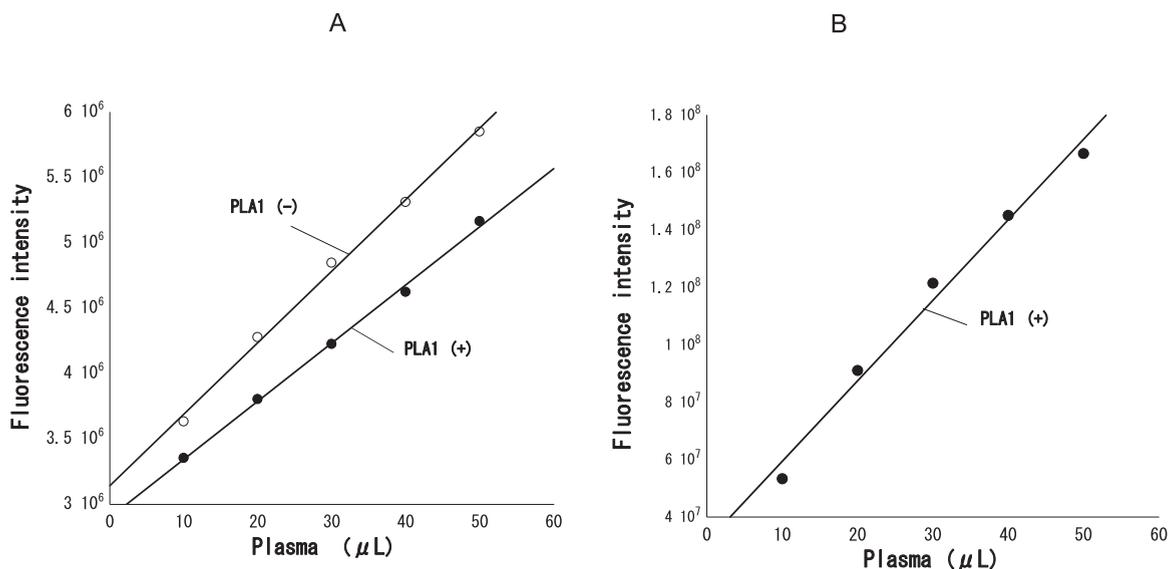


Fig. 3. Fluorescence intensity increased by increasing amounts of samples. A, plasma ePE treated with PLA1 {PLA1 (+)} showed constantly lower fluorescence intensity than those with the plasma without treatment with PLA1 {PLA1 (-)}. B, fluorescence intensity from ePC increased by increasing amount of sample after treatment with PLA1{PLA1 (+)}, total choline phospholipid (PC + ePC) was out of measurable range by the reagent for ePC.

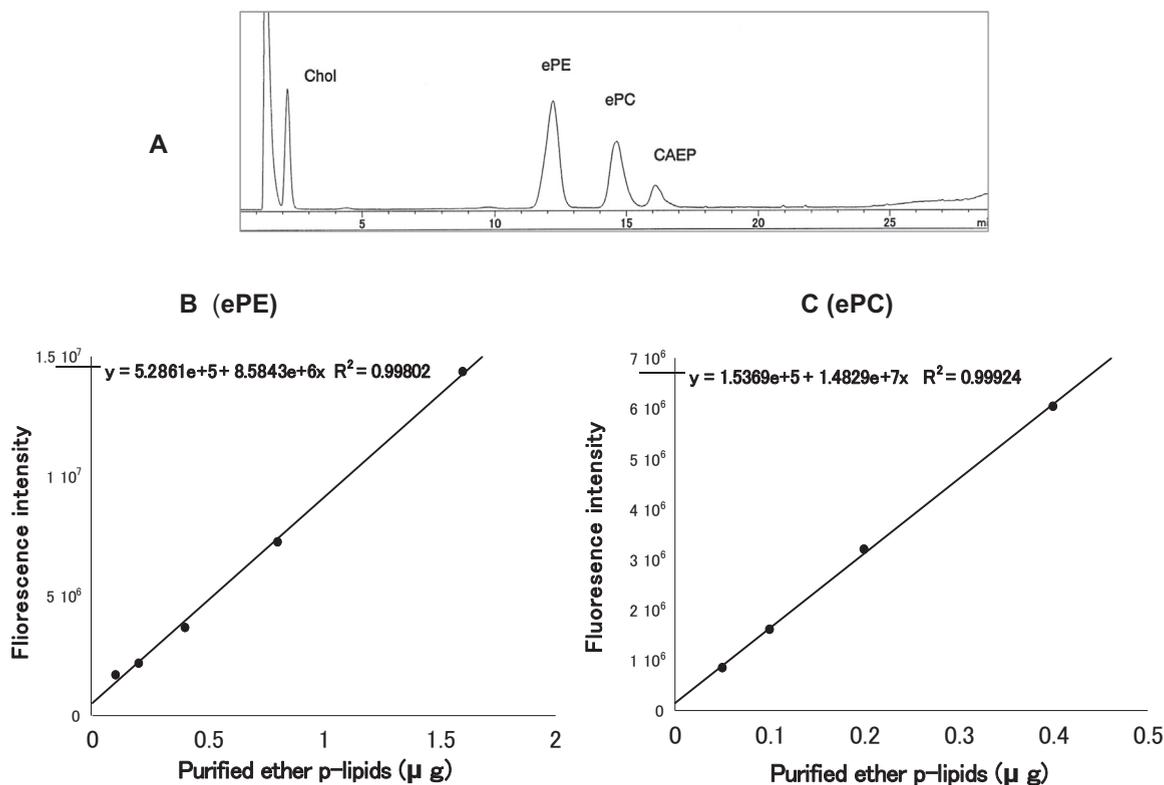


Fig. 4. A; chromatogram of purified ether phospholipids (ePE and ePC) from scallop. CAEP; ceramide aminoethylphosphonate, Chol; cholesterol. The purified ether phospholipids increased linearly by increasing amount of the sample by the enzymatic method. B; ethanolamine ether phospholipid (ePE). C; Choline ether phospholipid (ePC).

Because we did not have authentic ePE and ePC, we prepared purified ether phospholipids from scallop by hydrolysis of lipids with PLA1 according to the method described previously [26]. Fig. 4 A, showed chromatograms of the purified ether phospholipids from scallop. The lipid solution containing purified ePE and ePC from scallop were applied to the enzymatic method, and amounts of both ePE and ePC were linearly increased by increasing amounts of the samples (Fig. 4 B, C), which indicate that ether phospholipids (ePE and ePC) after PLA1 treatment of the total lipids can be measured by the present enzymatic method.

We tentatively calculated the amounts of the ether phospholipids (ePE and ePC) as diacyl phospholipids (PE and PC). Linear regression curves of PE and PC were showed in Fig. 5. A, B. For calculation as μM, following molecular weight (MW) was used; PE (stearoyl form) = 748, PC (stearoyl form) = 789. The amounts of ethanolamine ether phospholipid (ePE) and choline ether phospholipid (ePC) in human plasma measured by the enzymatic method were  $62.77 \pm 14.49 \mu\text{M}$  and  $92.2 \pm 13.9 \mu\text{M}$ , respectively. The ranges of the values of ePE in human plasma by the present method were accorded to the ranges of ethanolamine plasmalogens in

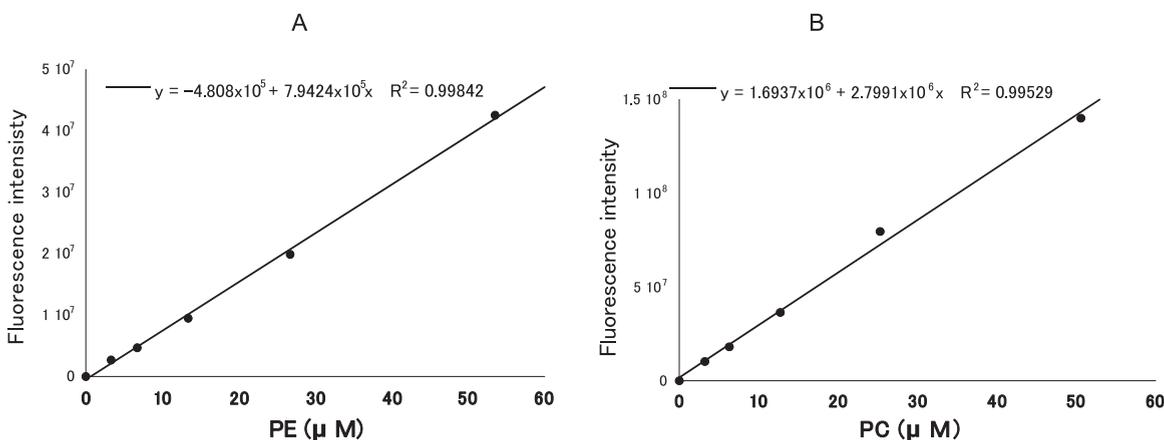


Fig. 5. Standard curves for ePE and ePC. A, linear regression for PE by the enzymatic measurement. B, linear regression for PC by the enzymatic measurement.

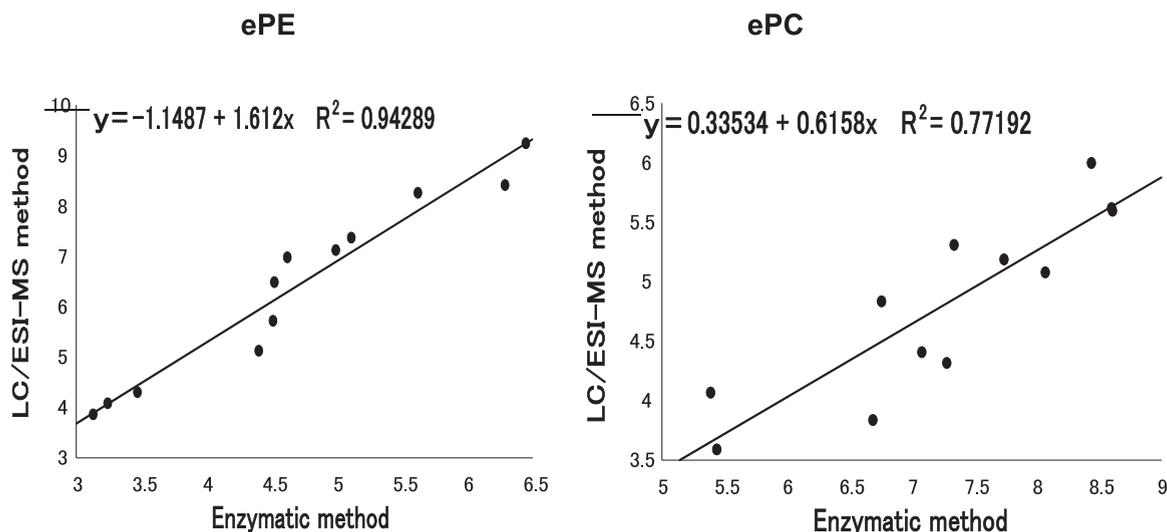


Fig. 6. Correlation of enzymatic method to LC/ESI-MS method. A, ePE ( $R > 0.94$ ), B, ePC ( $R^2 > 0.77$ ).

elderly persons of the reported methods with a flow  $\gamma$ -counter by use of a radioactive iodine ( $68.2 \pm 15.7$ ) [17], and the amounts of ePE measured by the enzymatic method was well correlated with those by LC/ESI-MS method ( $R^2 > 0.94$ ) (Fig. 6, A). However, the correlation of ePC measured by the enzymatic method to the LC/ESI-MS method was bit poor ( $R^2 > 0.77$ ) as compared to that of ePE (Fig. 6, B). We are not able to explain the poorer correlation of ePC as compared to that of ePE, and the results indicated that the enzymatic method for ePC in human plasma need further study.

This enzymatic method is applicable to assay of ether phospholipids (ePE and ePC) in the other tissues. Human erythrocyte membrane was prepared as described in our previous report [28]. PLA1 (Sigma) was diluted four times with 0.1 M citrate buffer (pH 4.5). An aliquot of human erythrocyte membrane solution (50  $\mu$ L) in 10 mM Tris-HCl (pH7.4) was directly hydrolyzed with 40  $\mu$ L of the diluted PLA1 solution at 45  $^{\circ}$ C for 60 min, and then, lipids were extracted with the HIP method. Human erythrocyte membrane

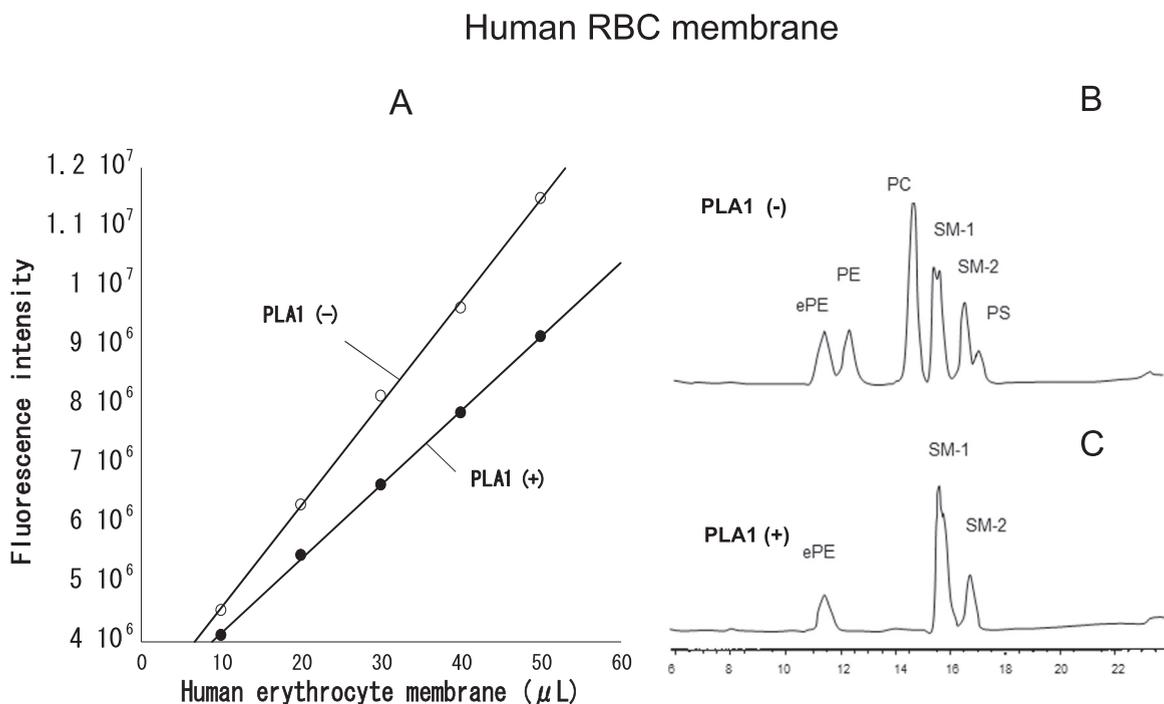
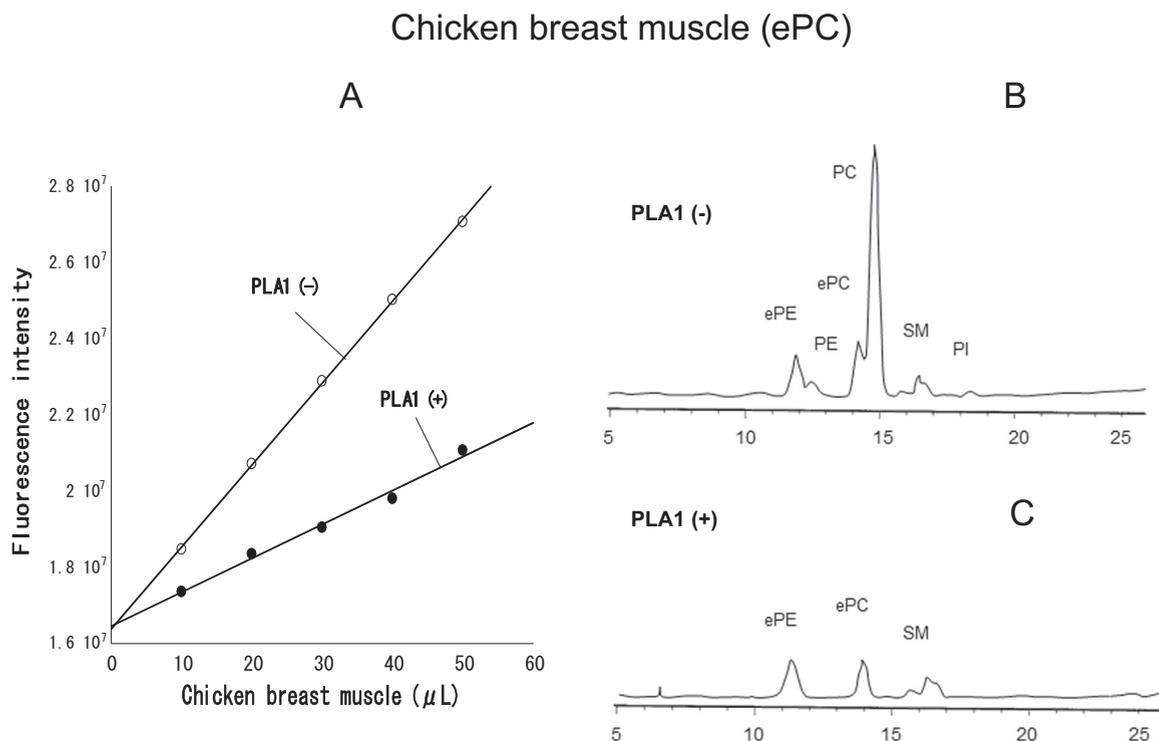


Fig. 7. Enzymatic method for ePE of human erythrocyte membrane. HPLC chromatogram of total phospholipids (B). The treatment of erythrocyte membrane with PLA1 caused complete removal of diacyl phospholipids (C). When the enzymatic method for ePE was applied to the lipid extract of the erythrocyte membrane after PLA1 treatment (A), the PLA1 treated sample (PLA1 (+)) showed constantly lower values than those of the untreated sample (PLA1 (-)), indicating that only ePE was measured after PLA1 treatment. PS; diacyl serine phospholipid.



**Fig. 8.** Enzymatic method for ePC of chicken breast muscle. HPLC chromatogram of total lipids (B). PLA1 treatment of the total lipid caused complete disappearance of diacyl phospholipids (C). When the lipid extract after PLA1 treatment was applied to the enzymatic method for ePC (A), the PLA1 treated sample (PLA1 (+)) showed constantly lower values than those of the untreated sample (PLA1 (-)), indicating that only ePC was measured after PLA1 treatment.

lipids consist of relatively high amount of ePE and SM (Fig. 7, B). The human erythrocyte membranes treated with PLA1 showed only ePE and SM on the HPLC chromatogram (Fig. 7, C). When the lipid extracts were applied to the enzymatic method for ePE, the PLA1 treated erythrocyte membrane showed constantly lower values of ePE than those of PLA1 untreated membrane, indicating that only ePE was measured after the PLA1 treatment (Fig. 7, A). In the case of chicken breast muscle, the total lipid extract was treated with PLA1. The diacyl phospholipids disappeared completely after the PLA1 treatment of the total lipid extract at 45 °C for 60 min (Fig. 8, C). When they were applied to the enzymatic method for ePC, the PLA1 treated sample showed lower values than those of the untreated sample, indicating that only ePC was measured after PLA1 treatment (Fig. 8, A). Concentrations of ePE and/or ePC in tissues may be calculated on the amount of protein or the tissue weight.

#### 4. Conclusions

We developed an enzymatic method for measurement of ether phospholipids in human plasma by using a fluorescent microplate reader after hydrolysis of plasma with PLA1. The method is sensitive and high-throughput for assay of plasma ether phospholipids. The method may also be performed by using a colorimetric microplate readers and/or spectrophotometers [27,28]. Therefore, the enzymatic method may be performed at many ordinary clinical laboratories without use of expensive apparatus. Furthermore, 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.

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