Densitometric Quantification of Ether-Type Phospholipids

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Abstract: A quantification method for analysis of individual ether-type phospholipids is important in studies of the regulation of membrane lipid biosynthesis in Archaea. For ester-type lipid of Bacteria and Eucarya, a densitometric method has been established for simultaneous quantification of individual phospholipids visualized with molybdenum blue reagent on a TLC plate. In this study, we developed a TLC densitometric method for rapid quantitative determination of 6 kinds of main ether-type phospholipids in a methanogenic archaeon and an extremely halophilic archaeon. It has been reported previously that on densitometric quantification the values of molar absorptivities are approximately the same among most ester-type phospholipids. On the other hand, we found significant disparity in the molar absorptivity of archaeal ether-type lipids and serine-containing ester-type lipid. Therefore, analysis should be accomplished by use of each standard mixture. Compared with a previous method (preparative TLC method) that is measurement of inorganic phosphate of silica gel powder scraped off from spots of phospholipids on a TLC plate, the TLC densitometry is accomplished at one tenth the sample size in a short time.

Key words: archaea, ether lipid, phospholipid, densitometry.

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Introduction

More than 100 novel archaeal membrane lipid structures have been elucidated in the past 40 years [1 - 4]. All the archaeal lipids consist of ether linkages between glycerol and isoprenoid alcohols, and they are usually referred to as “ether-type lipids”. Other remarkable features of archaeal lipids are the stereo configuration of the glycerol backbone and bipolar tetraether structures. By contrast with these unique chemical structures, the polar head groups (serine, ethanolamine, glycerol, inositol) of phospholipids are shared by Archaea, Bacteria and Eucarya.

In vitro studies of the major pathway of phospholipid biosynthesis in Archaea have been demonstrated over these 15 years [5]. A quantification method for analysis of individual ether-type phospholipids will be important in studies of the regulation of membrane lipid biosynthesis in Archaea. Ether-type phospholipids composition has been determined previously by measurement of inorganic phosphate of silica gel powder scraped off from spots of phospholipid on a thin layer chromatography (TLC) plate [6, 7]. For ester-type phospholipid of Bacteria and Eucarya, a TLC densitometric method has been established for simultaneous quantification of individual phospholipids visualized with molybdenum blue reagent on a TLC plate [8, 9]. This procedure has not yet been applied to ether-type phospholipids. In this study, the quantitative determination method of the ether-type phospholipids by the TLC densitometry was established.

Materials and Methods

Lipid materials

Cells of Methanothermobacter thermautotrophicus (DSM 1053) and Halobacterium salinarum (JCM 8981) were grown and harvested at a log phase, as previously described [10]. Lipids of M. thermautotrophicus were extracted by TCA acid extraction [7]. Lipids of H. salinarum were extracted by the method of Bligh and Dyer [11].

Archaeol was prepared from total lipid extract of H. salinarum cells by HCl hydrolysis [12]. Archaeatic acid (AA) was chemically synthesized by phosphorylation of archaeol [10]. Archaealserine (AS), archaealinositol (AI) and diglucosyl caldarchaealinositol (DGCI) were purified from the total lipid of M. thermautotrophicus by TLC. Archaealglycerol (AG) and archaealglycerophosphatemonomethyl ester (AGP) were purified from the total lipid of H. salinarum. The structures of these ether-type phospholipids are shown in Fig. 1. Phosphatidylserine (PS) (porcine brain) and phosphatidylinositol (PI) (soybean) were products of Doosan Serdary Research Laboratories. Phosphatidylglycerol (PG) (1,2-dipalmitoyl) was purchased from Avanti Polar Lipids, Inc. Concentration of each standard lipid material in chloroform : methanol (2:1) solution was determined based on phosphorus measurement [13].

Preparation of the molybdenum blue reagent

The reagent was prepared essentially as described by Ryu and MacCoss [14], as follows. Twelve g of molybdenum oxide (MoO3) was dissolved in 300 ml of 12.5 M sulphuric acid by boiling. The solution (mixture I) was cooled to room temperature. Powdered molybdenum (0.6 g) was dissolved in 150 ml of mixture I while refluxing for 1 h (mixture II). Equal volumes
Fig. 1. Structures of ether-type phospholipids.


of mixture I and II were mixed at room temperature and filtrated with a glass microfiber filter (Whatman, GF/A). The filtrate was diluted with two volumes of water. This solution was mixed with glacial acetic acid in the ratio of 4:1 (v/v). The reagent was left at room temperature for one week before use.

Quantification of diother-type phospholipid

The standard phospholipid mixture (AA+AS+AI+DGCl, PS+PI+DGCl, AG+AGP+DGCl or PG+DGCl) and samples (5 -200 nmol of phospholipid) were applied on a Silica Gel 60 TLC plate (Merck) (5 cm x 20 cm), and developed parallel with the solvent system of chloroform, methanol, acetic acid and water (80:30:15:5). After drying for 30 min in a vacuum desiccator the plate was dipped in the molybdenum blue reagent for 5 s and then placed in a horizontal position for 5 min in air. The plate was dipped for 1 min in deionized water with gentle stirring and then for 10 s in ethanol. It was lightly dried in a stream of cold air, and was immediately covered with a glass plate of the same size. The visualized plate was usually scanned within 15 min by use of a dual wavelength flying spot scanning densitometer (Shimadzu, CS-9300PC) in the reflectance mode at 650 nm with the slit width 0.4 mm and the slit length 10 mm. The
scanning rate was 0.5 mm/s. The plates were covered with aluminum foil for long-time storage. The quantity of ether-type phospholipid component was determined by comparing with a standard curve of each standard lipid from the peak area on the densitometric chromatogram.

Results and Discussion

A typical TLC chromatogram of phospholipid standards is shown in Fig. 2. Six ether-type phospholipids (AA, AS, AI, DGCI, AG and AGP) were well separated. Figure 3 shows densitometric tracings of the TLC chromatogram. Each peak was completely separated. The peak area on the densitometric chromatogram was proportional to the quantity of 5 - 200 nmol each diether-type phospholipids with different slopes depending on the phospholipids species (Fig. 4). The straight line shows that this method can be used for quantitative measurement of diether-type phospholipids in the range of 5 - 200 nmol. In the case of tetraether-type phospholipid, DGCI, peak areas of 40 nmol or more phospholipid were lower than that expected from the linear relationship (Fig. 5).

![TLC Chromatogram](image)

**Fig. 2.** One-dimensional TLC profile of standard mixture of phospholipids.
Each compound contains 20 nmol P. The fractionated compounds are AA, AS, AI and DGCI on lane 1, phosphatidylserine (PS), phosphatidylinositol (PI) and DGCI on lane 2, AG, AGP and DGCI on lane 3 and phosphatidylglycerol (PG) and DGCI on lane 4. S.f.: Solvent front.
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Fig. 3. Densitometric scanning, A and B of lane 1 and lane 3 relative to the plate of Fig. 2, respectively.

Fig. 4. Standard curves of different diether-type phospholipids measured by densitometry within 15 min after molybdenum blue staining. The regression lines were obtained with the least squares method; the correlation coefficient is reported in brackets.
**Fig. 5.** Standard curves of DGCl measured by densitometry within 15 min after molybdenum blue staining.

**Fig. 6.** Comparison of molar absorptivities ($\varepsilon$) of different phospholipids. The $\varepsilon$ of AA, AS, AI, AG and AGP are the slope of standard curves of each lipid (Fig. 4). The $\varepsilon$ of DGCl is the slope of Fig. 5 at amounts ranging 0 – 35 nmol. The $\varepsilon$ of PS, PI and PG are the slope of standard curves (data not shown) of each lipid.
The slopes of standard curves of phospholipids (Fig. 4, 5), which show molar absorptivities, $\varepsilon$ (peak area / nmol P), varied depending on phospholipid species. The value of $\varepsilon$ is a densitometric detector response per nmol P in phospholipid. Figure 6 shows $\varepsilon$ values of various ether-type and ester-type lipids. It has been reported previously that on densitometric quantification the values of molar absorptivities are approximately the same among most ester-type phospholipids [8]. On the other hand, we found significant disparity in the $\varepsilon$ values of archaeal ether-type lipids and serine-containing ester-type lipid. $\varepsilon$ (AA) ($\varepsilon$ value of AA) was the maximum value. $\varepsilon$ (AI) and $\varepsilon$ (AG) were about 73% of $\varepsilon$ (AA). $\varepsilon$ (AS) was 40% of $\varepsilon$ (AA). Comparing ether-type lipid with ester-type lipid, which contains the same polar head group, $\varepsilon$ (AI) and $\varepsilon$ (PI) were almost the same; $\varepsilon$ (AS) and $\varepsilon$ (AG) were 1.7 times higher than $\varepsilon$ (PS) and $\varepsilon$ (PG), respectively.

The color intensity of spots of the AA, AI, AG, AGP, DGCI, PI and PG on the TLC plate slowly decreased during 22 h. The intensity of the AI and AG (ether-type lipids) decreased more slowly than that of ester-type phospholipids with the same polar head group (PI and PG) (Fig. 7). Serine phospholipid (ether and ester-types) (AS and PS) showed almost constant color intensity for 22 h (Fig. 7). Densitometric scanning was, therefore, conducted within 15 min after visualization of phospholipid spots with the molybdenum blue reagent.

The previous method for determination of ether-type phospholipids composition is the measurement of inorganic phosphate of silica gel powder scraped off from spots of phospholipids on a TLC plate [6, 7]. The present densitometric method has the advantage over the previous method of higher sensitivity (ten times) and shorter working time. The previous method takes 15 h to convert phospholipid to inorganic phosphate by combustion. However, for this densitometric quantification, it is essential that standard lipids are developed together with sample lipids on TLC, because $\varepsilon$ values are different depending on the phospholipid species.

Spots of phospholipids are colored blue with the molybdenum blue reagent on the TLC plate. The appearance of the blue color of the spots is probably due to changes in the oxidation state of Mo (V) to an average oxidation state of Mo (V) and Mo (VI) [15]. The mechanism by which only phospholipids take part in oxidation of the reagent is unknown at present. Therefore, the cause of the difference of the molar absorptivity ($\varepsilon$) and stability of color intensity between phospholipid types and species are not known.

In conclusion, the TLC densitometric procedure is a rapid and simple method for the quantification of individual ether-type phospholipids. This procedure should be accomplished by use of each standard mixture. It is also important that the TLC plate be scanned within 15 min after visualization using molybdenum blue reagent.
Fig. 7. Color intensity of phospholipid spots, each of which contains 30 nmol P, at different times after staining with molybdenum blue reagent. The intensity is presented as a percentage of the original value (0 hr), which is the peak area within 15 min after staining.

References

エーテル型リン脂質のTLCデシトメトリーによる定量

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要旨：古細菌のエーテル型リン脂質の代謝制御を解析する上で、リン脂質の定量が必要である。真正細菌と真核生物のエステル型リン脂質の定量法については、モリブデン酸ブルー試薬を用いた薄層クロマトグラフィー（TLC）デシトメトリー法が確立している。本研究ではメタン生成古細菌と高度好塩性古細菌の主要エーテル型リン脂質6種類もTLCデシトメトリー法で迅速に定量できる事を示した。エステル型リン脂質のモル吸光率は脂肪酸の種類や極性基の種類によらずほぼ一定であったと報告されておりがエーテル型リン脂質およびセリンを含むエステル型リン脂質のモル吸光率には大きな相違があった。従って、エーテル型リン脂質をTLCデシトメトリーで定量するには毎回それぞれの標準物質が必要である。しかし、従来のTLCのスポットを抜き取りリン定量を行う方法と比べて、このTLCデシトメトリーでは試料の必要量が1/10で済み、時間も短縮された。

キーワード：古細菌、エーテル型脂質、リン脂質、デシトメトリー。

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