Separation of Phosphatidylinositol 4-Phosphate from a Mixture with Phosphatidylserine and Phosphatidylinositol by Sep-Pak C₁₈ Cartridge

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A simple and efficient method for the separation of phosphatidylinositol 4-phosphate (PI 4-P) from phosphatidylinositol (PI) and phosphatidylserine (PS) is described. A mixture of PI, PI 4-P and PS was injected onto a Sep-Pak C₁₈ cartridge. PI and PS were flushed through the cartridge with solvent 1 [methanol–chloroform (3 : 1)] while PI 4-P remained in it. Then the cartridge was inverted, and PI 4-P was eluted backward with solvent 2 [chloroform–methanol–0.5 M aqueous ammonium hydroxide (9 : 7 : 2)].

Key words Sep-Pak C₁₈ cartridge; phosphatidylinositol 4-phosphate; phosphatidylinositol; phosphatidylinositol 3-phosphate; phosphatidylserine

Phosphoinositides play important roles in cellular signal transduction.^{1,2} In this connection, the role of phosphatidylinositol 3-kinase (PI 3-kinase) is critical, because 3-phosphorylated phosphoinositides, including phosphatidylinositol 3-phosphate (PI 3-P), phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, regulate many physiological functions such as cell proliferation, differentiation, apoptosis, actin rearrangement, and vesicle transport.^{3,4}

Recently, we reported the structural requirement of phosphatidylinositol (PI) for *in vitro* phosphorylation by PI 3-kinase. In addition to natural PI purified from bovine liver, which mainly contains a stearyl group at the *sn*-1 position and an arachidonoyl group at the *sn*-2 position in the diacylglycerol substructure, PI 3-kinase phosphorylated synthetic PI contains stearic acid at the *sn*-1 position and a saturated fatty acid of carbon number 8 or less at the *sn*-2 position. The reactivity of PI decreased as the carbon number of the saturated fatty acid became larger, and PI with a fatty acid of carbon number 18 or 20 was not phosphorylated.^{5,6} These enzymatic reactions were carried out with PI 3-kinase, and a mixture of synthetic PI analogs, ATP, and phosphatidylserine (PS) added as a carrier. After the enzymatic reaction, produced PI 3-P analogs, unreacted PI analogs and PS were extracted with CHCl₃, and the extracts were analyzed by negative ion FAB-MS. PI and PI phosphates, which have polyhydroxyl groups and phosphates in the structure, give an intense [M–H][–] ion peak.^{7,8} The structures of the products were unequivocally determined by their molecular weights (M). PI 3-P analogs were detected at μ g levels.

The yields of the above enzymatic reactions were 20–30%. However, at low yields, the desired PI 3-P analog peaks are hidden behind the ion peaks of PS. Therefore, pre-purification of the produced PI 3-P analogs in the reaction sample is necessary to get clear FAB-MS spectra of the PI 3-P analogs. We report here an easy and convenient pre-treatment of the enzymatic reaction sample to remove PS, as well as starting PI, by solid-phase extraction using a Sep-Pak C₁₈ cartridge, which is suitable for small-scale separation and purification.

MATERIALS AND METHODS

Sep-Pak C₁₈ original type cartridges, which contain 360 mg of C₁₈ bonded silica, were purchased from Waters Co. Before use, the cartridges were treated with 10 ml of methanol, 10 ml of chloroform, and 2 ml of solvent 1 [in the case shown in Fig. 2, methanol–chloroform (3 : 1)]. Bovine liver PI ammonium salt, bovine brain PI 4-P sodium salt, and bovine brain phosphatidylserine were purchased from Sigma Chemical Co. Reverse-phase TLC plates RP-18F₂₅₄ and TLC plates of Silica gel 60 F₂₅₄, both 0.25 mm thick, were purchased from Merck.

Each Silica gel 60 TLC plate was pre-treated by developing with a solution of 1.5% potassium oxalate and methanol (2 : 3), followed by drying and activation at 110 °C for 15 min. After samples were spotted, the plate was developed with a solvent system of water–acetic acid–methanol–acetone–chloroform (8 : 12 : 13 : 15 : 40).⁹ Spots on TLC were visualized by exposure to iodine vapor.

RESULTS AND DISCUSSION

Our purpose was to establish a convenient method of isolation of PI 3-P from a mixture of PI, PI 3-P and PS. However, because PI 3-P is commercially unavailable, phosphatidylinositol 4-phosphate (PI 4-P) was employed in place of PI 3-P, on the assumption that PI 4-P would chromatographically behave like PI 3-P.

First, conditions to isolate PI 4-P from PS and PI with a Sep-Pak C₁₈ cartridge were examined by comparing the *R_f* values of PI, PI 4-P and PS on a C₁₈-coated TLC plate (PLC plates RP-18F₂₅₄). The chromatograms developed with methanol–chloroform (3 : 1) [solvent 1] and (1 : 1), and chloroform–methanol–0.5 M aqueous ammonium hydroxide (9 : 7 : 2) [solvent 2] are shown in Figs. 1A–C. With a solvent system of methanol–chloroform (3 : 1) or (1 : 1), PI and PS were developed, while PI 4-P remained at the origin. With a solvent system containing aqueous ammonia, PI, PI 4-P and PS were all developed fairly well.^{10,11}

These data indicated that, using a Sep-Pak C₁₈ cartridge, PI and PS could be separated from PI 4-P with solvents 1 and 2. With solvent 1, PI and PS could be flushed through the

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Fig. 1. Thin-Layer Chromatogram of PS, PI and PI 4-P

About $2\mu\text{g}$ of each sample was applied to a reverse-phase C_{18} TLC plate (RP-18F₂₅₄), and developed with the following solvent systems. A. Methanol-chloroform (3:1) [solvent 1]. B. Methanol-chloroform (1:1). C. Chloroform-methanol-0.5M ammonium hydroxide (9:7:2) [solvent 2]. PS, PI and PI 4-P were visualized by exposure to iodine vapor.

Fig. 2. Separation of PS, PI and PI 4-P on a Sep-Pak C_{18} Cartridge

Compounds eluted from the cartridge were applied to a pretreated Silica gel 60 TLC plate, and developed with a solvent system of water-acetic acid-methanol-acetone-chloroform (8:12:13:15:40).⁹⁾ PS, PI and PI 4-P were visualized by exposure to iodine vapor. Fractions 1—5 were eluted with solvent 1. After elution with solvent 1, the cartridge was inverted, and fractions 6—10 were eluted with solvent 2. The mixture contained PS, PI and PI 4-P.

cartridge while PI 4-P remained in it. Then PI 4-P in the cartridge could be eluted with solvent 2. Therefore, a mixture of PI, PI 4-P and PS was prepared, and *ca.* $20\mu\text{l}$ of the mixture containing $50\mu\text{g}$ of each component was injected onto a pretreated Sep-Pak C_{18} cartridge. Solvent 1 was passed through the cartridge, and five fractions (0.5 ml/fraction, hold-up volume of the cartridge) were collected. At this stage, the resid-

ual PI 4-P was expected to be located at the top of the cartridge. Then the cartridge was inverted, and PI 4-P was eluted backward with solvent 2. Five fractions (0.5 ml/fraction) were collected. The flow rate through the cartridge was approximately 0.5 ml/min. The chloroform and methanol of fractions 1—5 in solvent 1 were removed with an N_2 stream and then *in vacuo*. Most of the organic solvent of the chloroform-methanol-aqueous ammonium hydroxide [solvent 2] of fractions 6—10 was removed with an N_2 stream, then about 0.1 ml of water was added, and the residual solvent was removed by lyophilization. The product of each fraction was analyzed by silica gel TLC. The chromatogram, developed with a solvent system of water-acetic acid-methanol-acetone-chloroform (8:12:13:15:40), is shown in Fig. 2. As expected, fractions 1 and 2 contained PI and PS. Fractions 7 and 8, the first and the second eluates with solvent 2 in the reversed elution, contained PI 4-P. The recovery of PI 4-P was 70 to 80%.

The use of a Sep-Pak C_{18} cartridge is convenient for the separation of PI 4-P from PS and PI. With this type of cartridge containing 360 mg of C_{18} bonded silica, up to $50\mu\text{g}$ of PI 4-P could be separated from $50\mu\text{g}$ each of PS and PI (Fig. 2). Larger cartridges are also available. The established procedure has been successfully applied to the analysis of enzymatic reaction mixture containing PI 3-P derivatives. The details will be published elsewhere.

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