Study on glycosylinositol phosphoceramide and glycosylinositol phosphoceramide-specific phospholipase D in plants

(植物におけるグリコシルイノシトールホスホセラミドおよびグリコシルイノシトールホスホセラミド特異的ホスホリパーゼ D に関する研究)

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General introduction

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Phospholipids (PLs) are the most abundant constituents of membrane of all living organisms. The membrane PLs of the cells are classified into glycerophospholipids (GPLs) and sphingophospholipids (SPLs). A typical GPL is composed of glycerol, two fatty acids and phosphate having an alcohol or an amino acid. Whereas, a SPL contain a long chain amino alcohol called sphingosine or sphinganine. The long chain amino alcohol in SPL is *N*-acylated with long chain or very-long chain fatty acid to form ceramide. The polar head groups attached to the ceramide are different between animals and plants [1]. In animals, sphingomyelin (SM) is major SPL, where a phosphorylcholine is attached to 1-position of hydroxy group of ceramide (Fig. 1). In contrast, a predominant SPL in plants and fungi is glycosylinositolphosphoceramide (GIPC), whose polar head group is composed of glycan(s)-inositol-phosphate (Fig. 1) [2].

The number of sugars in GIPC in plants have been shown to 2 to 14. GIPCs are classified into various series according to the number of sugars in the head group. For example, series A contains 2 sugars (typically glucuronic acid and a hexose) after the inositol-phosphate head group, and series B contains 3 sugars after the inositol-phosphate. It has been known that different plants have GIPCs with different glycans [3]. GIPC in *Arabidopsis thaliana* (*A. thaliana*) and cabbage comprise two sugars. On the other hand, GIPC in welsh onion has three sugars. In tobacco, GIPCs are consisted of many species containing up to 14 sugars [4]. These structural variety of the sugar chain has been considered to relate with GIPC function in plants, such as cell wall anchoring, interaction with host-pathogen, protein anchoring and salt sensing for activation of Ca^{+2} influx channels etc. [5]. However, exact structural requirement of GIPC in these functions in plants is largely unknown at present.

Sphingomyelinase is an enzyme that hydrolyzes the C-position of SM to form ceramide. The Sphingomyelinase is distributed widely in animals [6]. On the other hand, sphingomyelinase D (SMaseD), which cleaves the D-position of SM, are found only in spider toxin and some bacteria species, but not in higher animals [7]. The enzyme that hydrolyzes the C-position of GIPC to produce ceramide and a phosphoinositol is called GIPC-phospholipase C (IPC-PLC) [8]. The GIPC-PLC was found only in fungi but not in plants. Recently, we found an uncharacterized sphingolipid in cabbage leaves and identified it as phytoceramide 1-phosphate (PC1P). PC1P was found to be generated by the cleavage of the D position of GIPC [9]. Interestingly, the enzyme

activity in cabbage hydrolyze exclusively GIPC but not SM or other glycerophospholipids, namely, phosphatidylcholine, phosphatidylethanolamine at a substantial level. Based on these findings, we called the enzyme activity to "GIPC-PLD activity". We also revealed that GIPC-PLD activity is higher in root of brassica plants and sprouts of soybean [10]. At present, the amino acid sequence and its encoding gene of the GIPC-PLD have not been elucidated yet. Although the enzyme activity is localized a specific tissue in plants, physiological role of GIPC-PLD is also unknown. Several researchers including us hypothesized signaling roles in the hydrolysate of GIPC, namely PC1P and inositol glycan [11]. However, there is no supportive evidence on this hypothesis.

GIPC has been shown to be the most abundant sphingolipids in plants. Indeed, it is reported that major sphingolipids in *A. thaliana* is GIPC, glycosylceramide and ceramide [12]. They accounted for ca 64%, 34% and 2% in total sphingolipids, respectively, in *A. thaliana* [13]. Similarly, the percentage of GIPC, glycosylceramide and ceramide in total sphingolipids in cabbage leaves were found to be 71%, 19% and 2%, respectively (our unpublished data). Despite its abundance in plants [14], digestibility and nutritional significance of vegetable-derived GIPC in animals are largely unknown. This is quite contrast to the plant-derived glycosylceramide whose digestibility, absorption and metabolism in animal body has been already clarified [15]. One of reasons of limited information on GIPC is underdevelopment of basic methods for biochemical experiments on GIPC, such as extraction, isolation, quantification and structural analysis of GIPC. The water soluble and organic solvent insoluble properties of GIPC would have alienate lipid researcher from GIPC. In this study, I established an isolation method of GIPC from plants and clarified its chemical properties as well as PC1P.

As mentioned above, PLD is an enzyme that cleavages between phosphate and alcohol at other side of phospholipid backbone. A common characteristic of PLD is transphosphatidylation, where phosphatidyl groups of PLs are transferred into various phosphatidyl alcohols to produce modified phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) [16]. The PLD-mediated PL modification has been extensively studied and, predominantly used in industrial purpose. But it is applicable only glycerophospholipids. SMaseD from spider toxin, which hydrolyzes SM to produce ceramide 1-phosphate, shows transphosphatidylation activity [17]. In contrast, transphosphatidylation activity of GIPC-PLD has not characterized yet. A concentration of alcohol needed for transphosphatidylation reaction performed by PLD is useful information for PLD classification.

Most of identified PLDs have a conserved motif HxKxxxxDx6G(G/S)xN (HKD). The PLD that is devoid of HKD motif are called non-HKD type [18]. It has been shown that transphosphatidylation performed by non-HKD type PLD requires higher concentration of alcohols (8-10 M) [17,19] whereas, HKD type PLD performs the reaction under low concentration of alcohols (1-2 M) [17]. To gain more insights into GIPC-PLD activity, I examined whether GIPC-PLD activity catalyzes transphosphatidylation. I also examined substrate specificity of GIPC-PLD using GIPC with different head sugar groups.

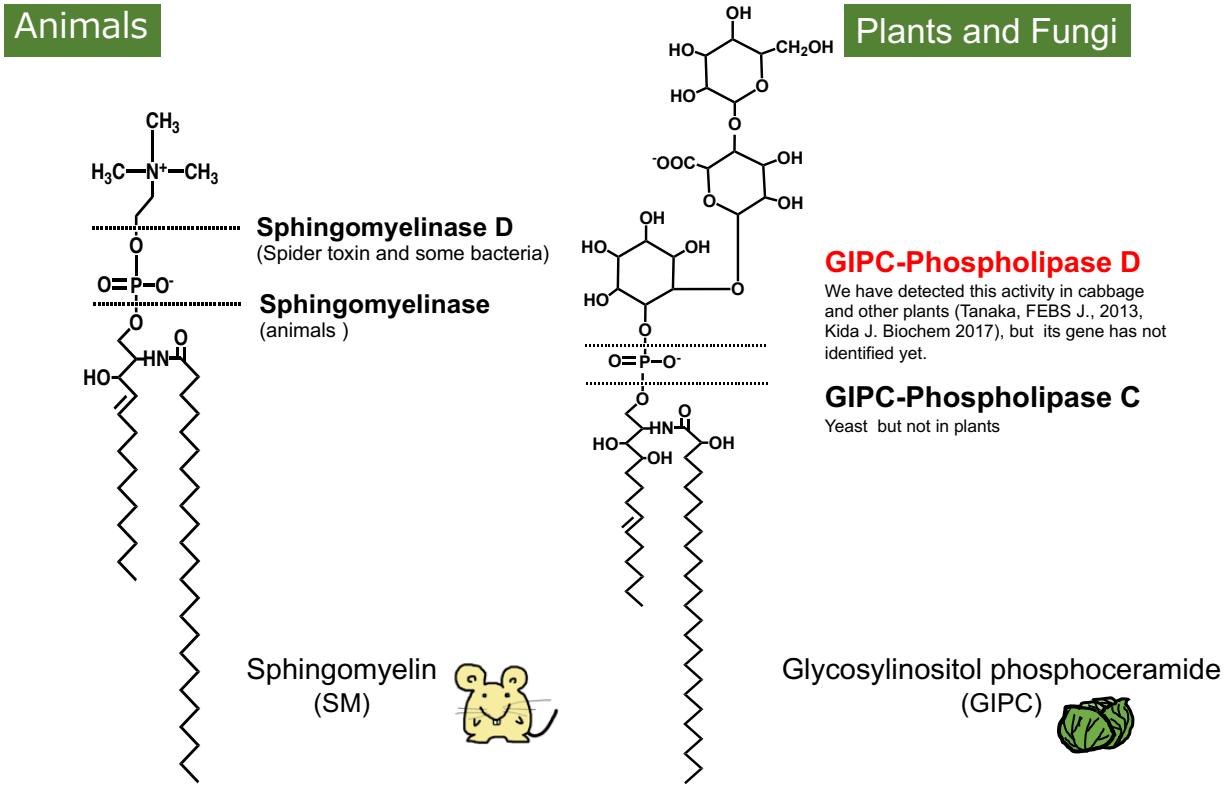

Fig. 1 Catabolism of sphingophospholipids in animals and plants

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CHAPTER-1

Isolation of glycosylinositol phosphoceramide and phytoceramide 1-phosphate in plants and their chemical stabilities

1.1 Introduction

Sphingolipids are ubiquitous constituents of eukaryotic biomembranes. In animals, the major sphingophospholipid is sphingomyelin (SM), whereas glycosylinositol phosphoceramide (GIPC) is the predominant sphingophospholipid in plants and fungi [1-4]. GIPC contains a glycan head structure linked to inositol which is bonded to the ceramide backbone through a phosphodiester bond (Fig. 2). Although GIPC is widely distributed in the plant kingdom [5-10] as the most abundant sphingolipids [11-15], little is known about its metabolism in plants.

Recently, we discovered an uncharacterized sphingolipid in cabbage and identified it as phytoceramide 1-phosphate (PC1P). PC1P is produced from GIPC by a novel phospholipase D (PLD) activity (Fig. 1) [16], which we previously identified to exhibit a higher activity in the roots of Brassica plants and the sprouts of soybean. Further, more than half of the GIPC is converted to PC1P following tissue homogenization [17]. We have previously reported the enzymatic properties of the GIPC-PLD from cabbage and Arabidopsis [17-18]. However, its encoding gene and biological functions have not been clarified yet. To enable a biological study on GIPC-PLD or these sphingophospholipids, purified GIPC and PC1P are essential. However, these lipids are not commercially available at present, and useful methods for their isolation have not been established yet. In this regard, a predominant obstacle for the isolation of GIPC from plant tissues is its hydrophilic property due to the presence of a bulky hydrophilic polar group.

Herein, we describe a practical method for the isolation of GIPC using Sephadex column chromatography followed by TLC. We also describe a method for the isolation of PC1P. Using the isolated GIPC and PC1P, we then examined their chemical stabilities in plants.

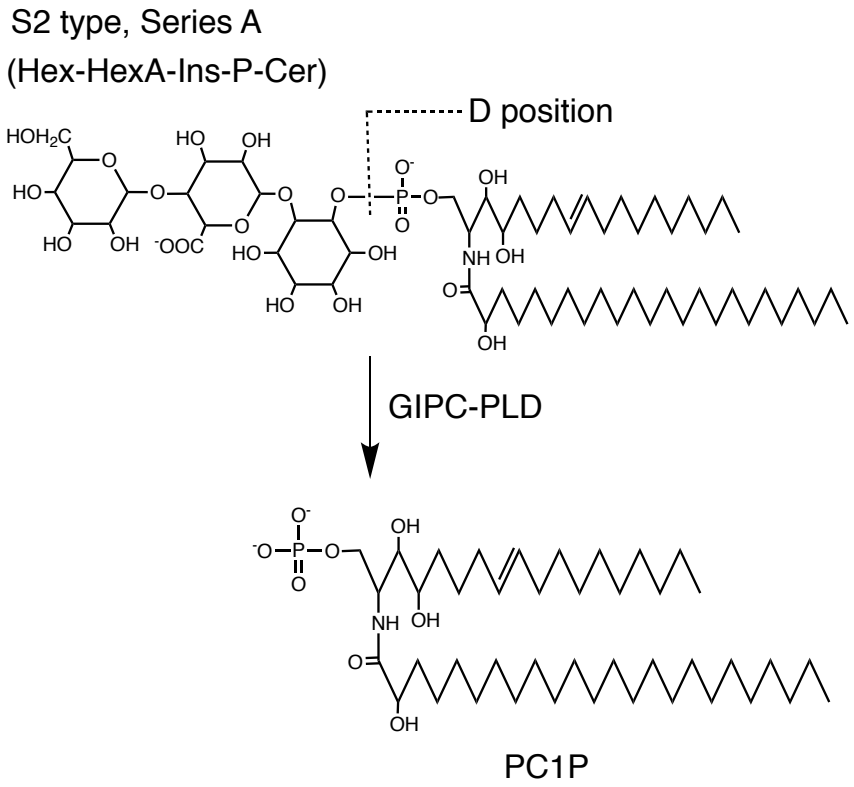


Fig. 2 Structures of glycosylinositol phosphoceramide (GIPC) and phytoceramide 1-phosphate (PC1P).

GIPC isolated in this study is Series A (sugar 2 type), hexose (Hex)-hexuronic acid (HexA)-inositol (Ins)-phosphoceramide (P-Cer), as shown above. We also purified Series B (sugar 3 type) Hex-HexN-HexA-Ins-P-Cer. The ceramide backbone of GIPC is composed of a 1,3,4-trihydroxy analogue of long chain base (LCB) (dehydrophytosphingosine, t18:1) and an α-hydroxy fatty acid with very long chain, such as h24:0 as shown here. The hydrolysis of the D-position of GIPC by GIPC-PLD produces PC1P.

1.2 Materials and methods

1.2.1 Materials

2,4,6-Trihydroxy-acetophenone (THAP) was obtained from Sigma-Aldrich (St. Louis, MO). Phos-tag with Zn [^{68}Zn] was obtained from Wako Pure Chemical Industries (Osaka, Japan). Sephadex LH-20 was purchased from Sigma-Aldrich (St. Louis, MO). All organic solvents used in this study were obtained from Sigma (Tokyo, Japan) and Wako (Osaka, Japan). Cabbage (*Brassica oleracea* L. var. *capitata*), radish (*Raphanus sativus* L.), Welsh onion (*Allium fistulosum* L.), and rice (*Oryza sativa*) bran were purchased from a local market.

1.2.2 Extraction and isolation of PC1P

Plant tissues were cut into small pieces and homogenized in water using an ultradisperser (LK-21; Yamato Scientific, Tokyo Japan). Because PC1P is produced during homogenization [16], extensive homogenization was conducted to increase the yield. The homogenates were boiled in hot water for 10 min at 80 °C to inactivate the lipolytic enzymes. The total lipids were extracted from the homogenates according to Bligh and Dyer method [19] under acidic conditions. The extracted lipids were treated with 0.1 M KOH in 95% methanol for 10 min at 80 °C for hydrolysis of glycerophospholipids. After cooling, the alkali lysates were extracted according to Bligh and Dyer method under acidic conditions. Aliquots of lipids were next subjected to preparative TLC (Merck 5721, Germany) with chloroform/ methanol/ 28% aqueous ammonia (60:35:8, v/v/v) as the solvent system. The PC1P was extracted from the silica gel according to Bligh and Dyer method with acidification. The isolated PC1P was then quantified by colorimetric method based on phosphomolybdenum-malachite green formation [20]. The purified PC1P was subjected to MALDI-TOF MS for structural elucidation.

1.2.3 Extraction of GIPC

Total lipids were extracted from plant tissues followed by alkaline hydrolysis with 40% methylamine/ethanol (5:7, v/v; solvent B) by the modified procedure reported by Markham *et al.* [21]. Briefly, 10 g of plant tissues were heated in boiling water at 80 °C for 10 min to inactivate the lipolytic enzymes. The boiled tissues were homogenized with the lower layer of isopropanol/hexane/water (55:20:25, v/v/v; solvent A) using an ultradisperser (LK-21; Yamato

Scientific, Tokyo Japan). The supernatant was collected by centrifugation at 1100 x *g* for 10 min. The pelleted material was additionally subjected to an extraction with solvent A. The supernatants were combined and the solvents were removed using a rotatory evaporator at 50 °C. After evaporation, the residual lipid was dissolved in 40% methylamine/ethanol (5:7, v/v; solvent B) for 60 min at 50 °C to hydrolyze the glycerophospholipids. After removing the solvents, the resulting extracts were suspended in a small volume of solvent A for subsequent experiments as described below.

1.2.4 Isolation of GIPC

Lipids extracted from plant tissues according to procedure 2.3. were fractionated by Sephadex column chromatography. First, Sephadex LH-20 was suspended with solvent A. A glass column (1.5 cm diameter) was plugged with cotton, filled with 10 mL of Sephadex LH-20 suspension, and then washed with 10 mL of solvent A. The height of Sephadex column was approximately 10 cm. Lipids extracted from plant tissues according to procedure 2.3. were dissolved in 0.5 mL of solvent A. This was then loaded onto the column and eluted with approximately 15 mL of solvent A for fractionation. After ten eluate fractions (1 mL each) were collected, an aliquot of each fraction was checked by TLC (Merck 5721, Germany) developed with chloroform/methanol/7% aqueous ammonia (45:35:10, v/v/v). Lipids were visualized under UV light by spraying the TLC plates with primulin to identify the GIPC-rich fractions, which were combined and dried under nitrogen. GIPC was isolated from the combined GIPC-rich fraction by preparative TLC developed with the solvent described above. The silica gel corresponding to GIPC was scraped off the TLC plate, mixed with solvent A and centrifuged (1300 x *g*, 5 min). GIPC was collected from the supernatant and quantified by the colorimetric method based on phosphomolybdenum-malachite green formation [20]. The structure of the purified GIPC was determined by MALDI-TOF MS in negative-ion detection mode as described below. It should be noted that cotton used for plug in column chromatography does not contain detectable GIPC.

1.2.5 Recovery of GIPC and PC1P

The homogenates of cabbage leaves (5 g) were spiked with 32 nmol of purified GIPC. GIPC was isolated from the GIPC-spiked sample and from the non-spiked sample by solvent A

extraction followed by preparative TLC, and quantified. The recovery was calculated with the following equation:

Recovery (%) = {(A − B)/C} × 100

Where, A is the amount of GIPC found in the spiked sample, B is the amount of GIPC found in the non-spiked sample, and C is the amount of GIPC spiked to the sample. The same methodology was applied to determine the recovery of PC1P. The recovery of GIPC and PC1P in each step of purification was conducted in the similar methodology as mentioned above.

1.2.6 Heat stability of GIPC and PC1P

Purified GIPC (65 nmol) dissolved in solvent A in long glass test tubes was heated at various temperatures (room temperature; R.T., 50 °C, 100 °C, 125 °C and 150 °C) for 60 min under reflex. The solvents were dried under nitrogen, dissolved in a small amount of solvent A, and then subjected to TLC. The band corresponding to intact GIPC was isolated and quantified via phosphomolybdenum-malachite green formation [20]. The same experiments were conducted with PC1P, except for use of water as solvent. After heating, PC1P was recovered by the method of Bligh and Dyer under acidic conditions and then applied to TLC plates. The isolated intact PC1P was quantified as described above [20].

1.2.7 Alkaline stability of GIPC and PC1P

Purified GIPC (65 nmol) was incubated in 1 mL of ethanol containing 40% methylamine at 50 °C for 60 min, 180 min, and 300 min. After drying, the lipid was dissolved in solvent A and subjected to TLC. The resulting intact GIPC was isolated and quantified as described above. The purified PC1P (65 nmol) was dissolved in 1 mL of 0.1 M KOH with 95% methanol and heated at 80 °C for 15 min, 30 min and 60 min. After cooling, PC1P was recovered by the Bligh and Dyer method under acidic conditions and then applied to TLC plates. The isolated intact PC1P was quantified as described above.

1.2.8 Acid stability of GIPC and PC1P

Purified GIPC or PC1P (65 nmol each) was treated with 1 mL of various concentrations (0.01 M, 0.1 M, 1 M and 10 M) of HCl at room temperature for 30 min. After evaporation with N_2 flow,

lipids were dissolved in a small amount of solvent A or chloroform/methanol (C:M) (2:1, v/v), and subjected to TLC purification. Isolated GIPC or PC1P was quantified as mentioned above.

1.2.9 MALDI-TOF MS of PC1P and GIPC

Molecular species of PC1P was determined by MALDI-TOF MS using Phos-tag, as previously described [16]. An aliquot of PC1P was dissolved in 100 µL of methanol containing 0.3% ammonia. The resulting solution (10 µL) was mixed with 5 µL of 0.1 mM ^{68}Zn phos-tag solution and a small portion (0.5 µL) of this mixture was spotted on MALDI sample plate. Immediately, 0.5 µL of 2,4,6-trihydroxyacetophenone (THAP) solution (10 mg/mL in acetonitrile) was layered on the mixture as a matrix solution. After drying the sample plate for a few minutes, the matrix/analyte was subjected to MALDI-TOF MS using a Voyager DE STR mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive-ion detection mode. GIPC, prepared from plant tissues, was analyzed by MALDI-TOF MS in negative-ion detection mode. In this case, THAP solution (10 mg/mL in 75% acetonitrile containing 0.1% trifluoroacetic acid) was used as the matrix. In both cases, the wavelength of the nitrogen-emitting laser, the pressure in the ion chamber, and the accelerating voltage were 337 nm, 3.7×10^{-7} Torr, and 20 kV, respectively. To enhance the reproducibility, 256 laser shots were averaged for each mass spectrum.

1.2.10 Statistical analyses

All results were expressed as mean ± SD. The significant differences between two means were carried out using Student's t-test. $^{*}P<0.05$, $^{**}P<0.01$, *and* $^{***}P<0.001$ were defined as statistically significant.

1.3 Results and discussion

1.3.1 Solubility of GIPC and PC1P

Various solvents were added to GIPC and PC1P which had been dried on the bottom of glass tubes. After vortex mixing followed by centrifugation, the quantity of GIPC present in the supernatant was determined. In this condition, theoretical concentration of GIPC dissolved in the supernatant is 30 μM. GIPC was confirmed to be soluble in water but not in absolute alcohols with the exception of methanol. The solubility of GIPC was enhanced by the addition of water in the alcohol. In fact, GIPC was found to be dissolved in isopropanol/water (55:25, v/v) and upper phase of butanol/water (1:1, v/v). GIPC was also dissolved in tetrahydrofuran (THF)/methanol/water (2:1:2, v/v/v), that has been previously reported by Ishikawa *et al* [21]. Among the solvents tested, the lower layer of isopropanol/hexane/water (55:20:25, v/v/v; solvent A, utilized by Markham *et al*. [22]) dissolved GIPC at the highest efficacy (Fig. 3A). Although GIPC was insoluble in chloroform, PC1P was soluble both in chloroform and water to the same extent. A solvent mixture consisting of chloroform and methanol was determined to be the best solvent to dissolve PC1P (Fig. 3B).

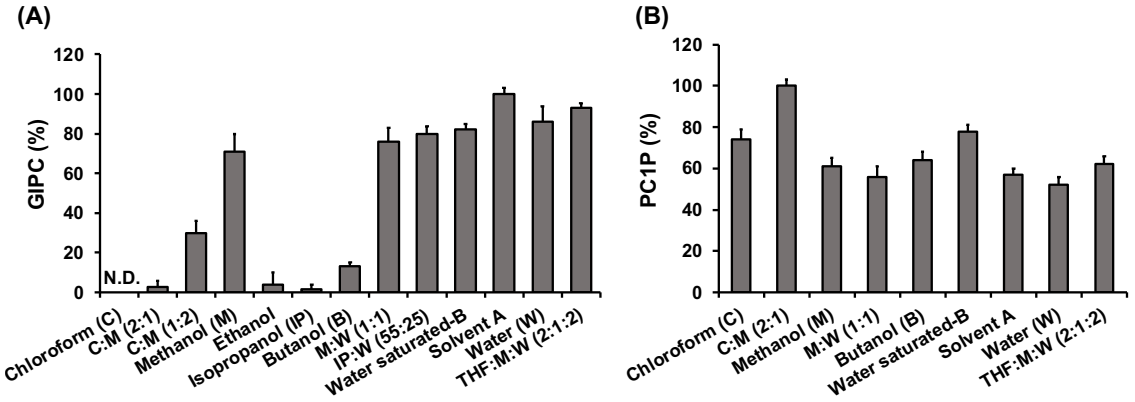


Fig. 3 Solubility of GIPC and PC1P in various solvents.

Solvents were added to dried GIPC (A) and PC1P (B) and then vortexed. After centrifugation, the quantity of GIPC and PC1P present in the supernatants was determined. The solubility of lipids was expressed as a percentage (100%, 32 nmol/mL), mean ± S.D (n= 5). N.D., not detectable; THF, tetrahydrofuran; Water saturated-B, the upper phase of butanol/water (1:1, v/v) mixture.

1.3.2 Isolation of GIPC from cabbage leaves

Results on solubility of GIPC clearly indicated that GIPC could not be extracted by conventional methods for phospholipid extraction, such as the Bligh and Dyer method. In fact, the amount of GIPC in lipids extracted by the Bligh and Dyer method from cabbage leaves was $1/10^{th}$ to that extracted by the method developed herein. The extraction method reported by Markham *et al.* [22] was utilized with minor modifications. This procedure consists of two steps: (1) tissue homogenization and lipid extraction with solvent A and (2) alkali treatment with 40% methylamine (solvent B) [22-23]. Because phase separation was not included in these steps and hydrophilic solvent was used for extraction, the extract obtained from the cabbage leaves by this method contained hydrophilic sticky materials to a large degree. These sticky materials were separated from lipids via Sephadex LH-20 column chromatography. First, the recovery of GIPC from Sephadex was determined, and solvent A was identified to be the best eluent among all those tested (Fig. 4A). The elution profile of the Sephadex column chromatography (Fig. 4B) with solvent A showed that lipids containing GIPC eluted faster than the hydrophilic sticky materials; this indicates that Sephadex column chromatography could separate GIPC from sticky materials by solvent A. The TLC of the GIPC-rich fraction obtained from Sephadex column chromatography successfully separated GIPC from other lipids or hydrophilic compounds (Fig. 4D). This was contrasted with the TLC development of the lipid extract in the absence of Sephadex fractionation in which GIPC failed to separate due to the presence of sticky materials (Fig. 4C).

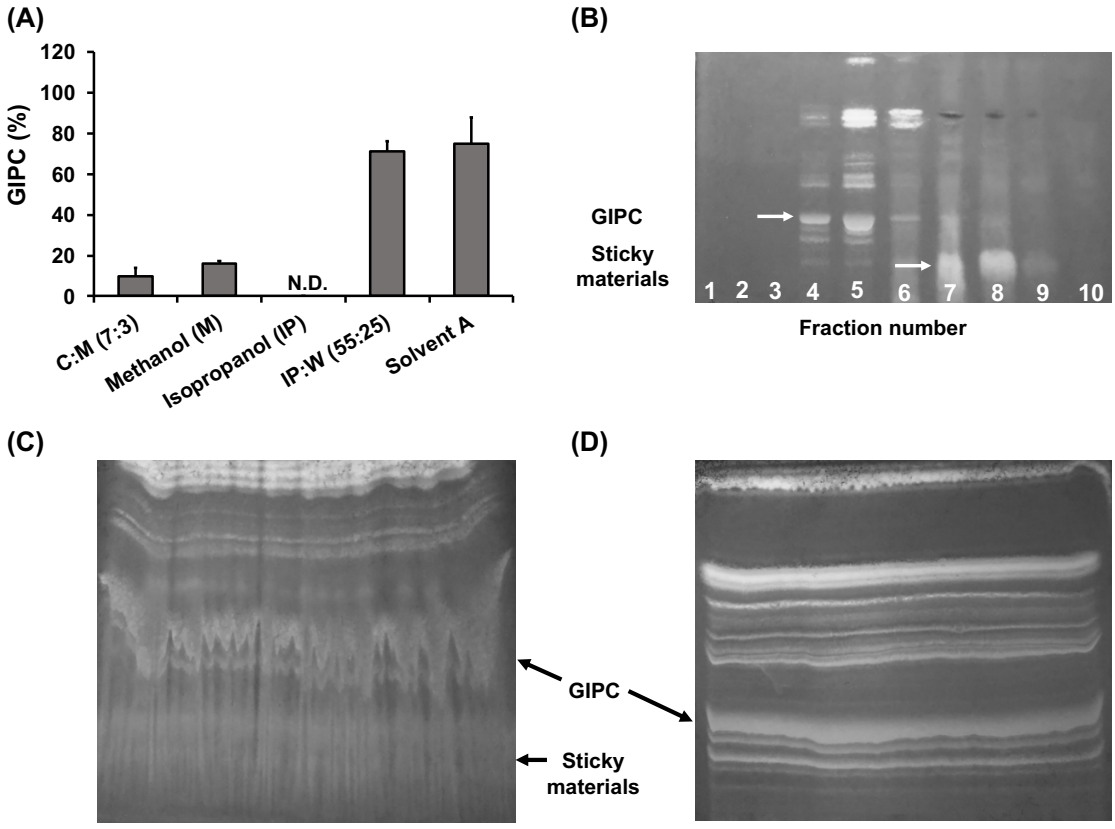


Fig. 4 Purification of GIPC by Sephadex column chromatography and TLC.

GIPC (32 nmol) absorbed in Sephadex LH-20 was mixed with the indicated solvents. After vortexing for a few seconds and centrifugation, GIPC in the supernatant was collected and quantified. The recovery of GIPC was expressed as a percentage (100%, 32 nmol), mean ± S.D (n= 3). N.D., not detectable (A). Lipid extract prepared from cabbage leaves with solvent A was subjected to Sephadex column chromatography for fractionation using solvent A as eluate. An aliquot of the resulting ten fractions was subjected to TLC to detect the GIPC (B). Typical TLC of lipid extract prepared from cabbage leaves with solvent A (before Sephadex column chromatography) (C). Typical TLC of GIPC-rich fraction after Sephadex column chromatography. GIPC and sticky materials that hamper migration of developing solvent are indicated by arrows (D).

The recovery of GIPC was assessed in each step. In the first step involving extraction of GIPC with solvent A from cabbage leaves, the yield of GIPC was 90%. In the next step involving alkaline hydrolysis with 40% methylamine in ethanolic solution, GIPC was recovered to approximately

95%. The yield of GIPC was around 72% and 77% by Sephadex column chromatography and TLC, respectively. The overall yield of GIPC throughout of these steps was between 50-70% (Fig. 5A).

1.3.3 Isolation of PC1P from cabbage leaves

Since chloroform/methanol (2:1, v/v) efficiently dissolves PC1P as mentioned above, conventional methods of phospholipid extraction, such as the method of Bligh and Dyer, was available for the extraction of PC1P from cabbage leaves. As shown in Fig. 4B, PC1P was successfully isolated by TLC of alkali-treated total lipids which were prepared by the method of Bligh and Dyer from cabbage leaves. The yield of PC1P in the extraction step by the Bligh and Dyer method was found to be approximately 82%. Around 90% and 70% of PC1P were recovered after alkali treatment and TLC purification, respectively. The final yield of PC1P throughout of these steps was between 55-70% (Fig. 5B).

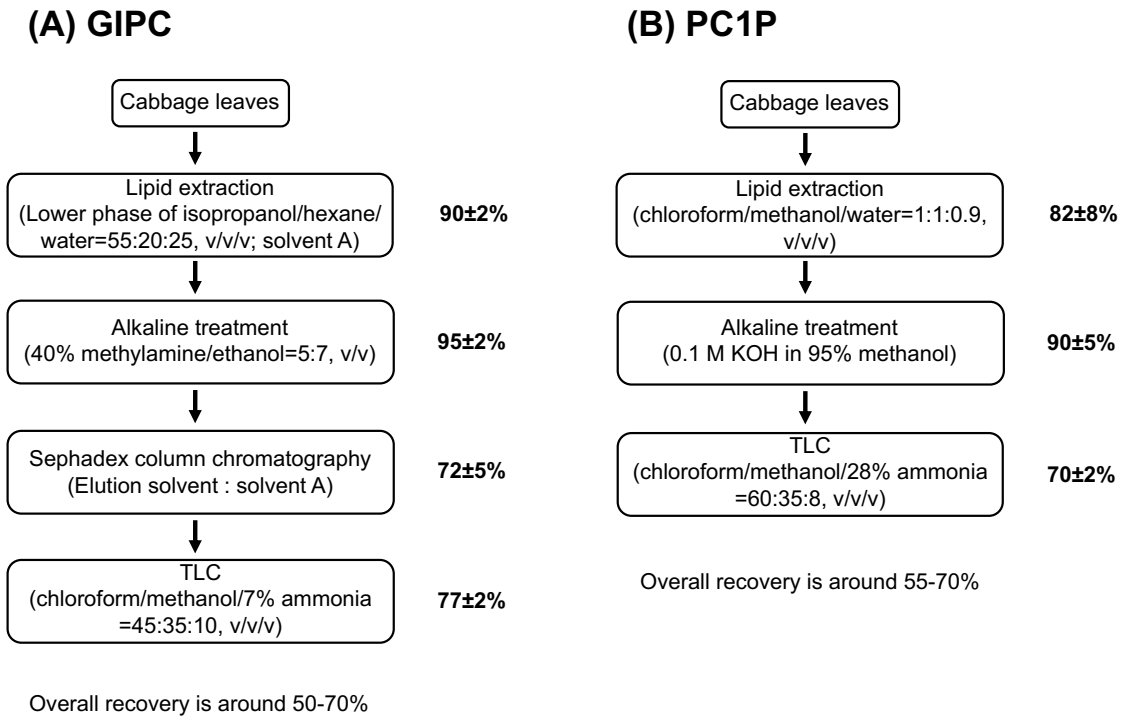


Fig. 5 Isolation methods of GIPC and PC1P.

The flow charts of the isolation steps of GIPC (A) and PC1P (B) are shown. Values indicated are recovery (%) of GIPC or PC1P in each step.

1.3.4 Isolation of GIPCs and their MALDI-TOF MS analyses

GIPCs containing 2-3 sugar chains were isolated by the methods described herein. It is well established that GIPC with two sugars (Series A) is the predominant species present in Brassica plants, whereas, GIPC with three sugar residues (Series B) is the major species in rice [21]. The Series A GIPC from radish roots (Fig. 6A) and Series B GIPC from rice bran (Fig. 6B) were successfully isolated by TLC of GIPC-rich fractions obtained by Sephadex column chromatography. The structures of the isolated GIPCs were confirmed by MALDI-TOF MS. The ions at *m/z* 1148.6, 1232.8, 1246.8, 1258.7, and 1260.7 in the mass spectrum of radish root GIPC were assigned to hexose (Hex)-hexuronic acid (HexA)-inositol (Ins)-phosphoceramide (P-Cer) with ceramide moieties of t18:1/h16:0, t18:1/h22:0, t18:1/h23:0, t18:1/h24:1, and t18:1/h24:0, respectively. The ion at *m/z* 1282.8 could be assigned to the sodium adduct of GIPC with t18:1/h24:0 ceramide structure (Table 1). Four major ions were detected in the MALDI-TOF mass spectrum of rice bran GIPC, namely, *m/z* 1407.9, 1423.9, 1437.9, and 1451.9. They were considered to corresponds to Hex-HexN-HexA-Ins-P having t18:1/h23:0, t18:0/h24:0, t18:0/h25:0, and t18:0/h26:0 ceramide moieties, respectively (Table 1). These structural assignments are based on the literature reported so far [24-25] and our previous study [16-18]. These two GIPC types were also obtained by Sephadex column chromatography followed by TLC from Welsh onion, the same genus of Allium Porum which is reported to contain both series A and B GIPCs [24-25]. Since the saccharide units of GIPC in Allium Porum were reported as HexN-HexA (Series A) and Hex-HexN-HexA (Series B) [24-25], this enabled ion assignment in the MALDI-TOF mass spectra of Series A and Series B GIPC, as shown in Table 1.

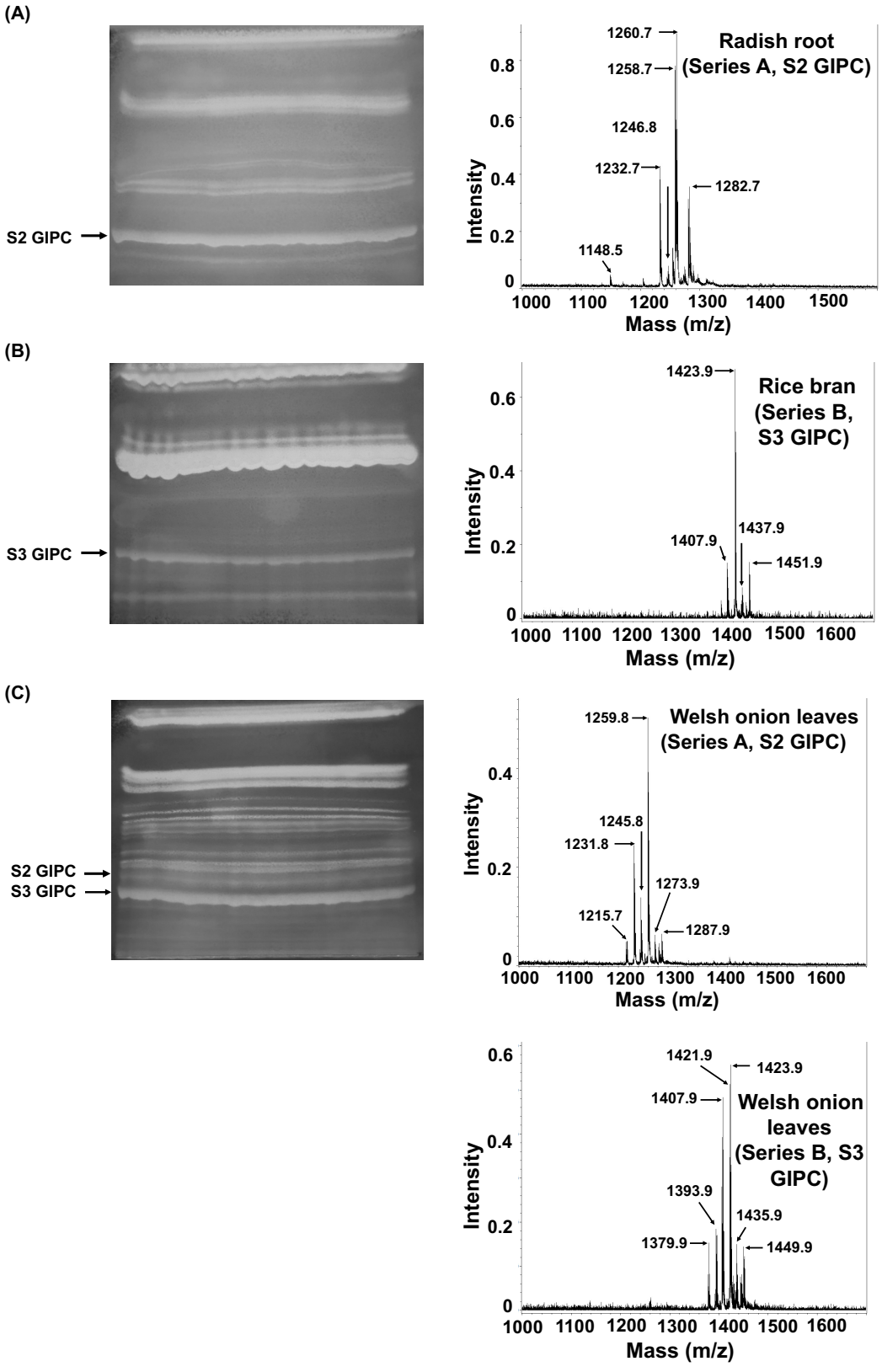

(A)
S2 GIPC
1260.7
1258.7
1246.8
1232.7
1282.7
1148.5
Radish root
(Series A, S2 GIPC)
Intensity
Mass (m/z)
(B)
S3 GIPC
1423.9
1407.9
1437.9
1451.9
Rice bran
(Series B,
S3 GIPC)
Intensity
Mass (m/z)
(C)
S2 GIPC
S3 GIPC
1259.8
1245.8
1231.8
1273.9
1215.7
1287.9
Welsh onion leaves
(Series A, S2 GIPC)
Intensity
Mass (m/z)
1421.9
1423.9
1407.9
1393.9
1435.9
1379.9
1449.9
Welsh onion
leaves
(Series B, S3
GIPC)
Intensity
Mass (m/z)

Fig. 6 Isolation of GIPCs from radish root, rice bran and Welsh onion leaves, as well as their structural confirmations by MALDI-TOF MS.

GIPC-rich fractions prepared from radish root (A), rice bran (B) and Welsh onion leaves (C) were subjected to TLC. The isolated GIPC was analyzed by MALDI-TOF MS in negative ion detection mode. Structures of GIPC of radish root and rice bran were assigned according to literatures or our previous study as shown in the Table 1. Note that GIPCs of Welsh onion leaves are possible assignments, because information on exact sugar structures and *N-acyl* residue are not available at present.

As shown in Fig. 7, isolated PC1P by TLC from cabbage leaves were analyzed by MALDI-TOF MS in positive ion mode as phos-tag complex. The ions at *m/z* 1236.5, 1320.6, 1334.6, 1346.6,1348.6,1362.6, and 1376.6 were assigned to $[M+Phos\text{-}tag]^+$ of PC1P with ceramide structures of t18:1/h16:0, t18:1/h22:0, t18:1/h23:0, t18:1/h24:1, t18:1/h24:0, t18:1/h25:0, and t18:1/h26:0, respectively (Table 1) [16].

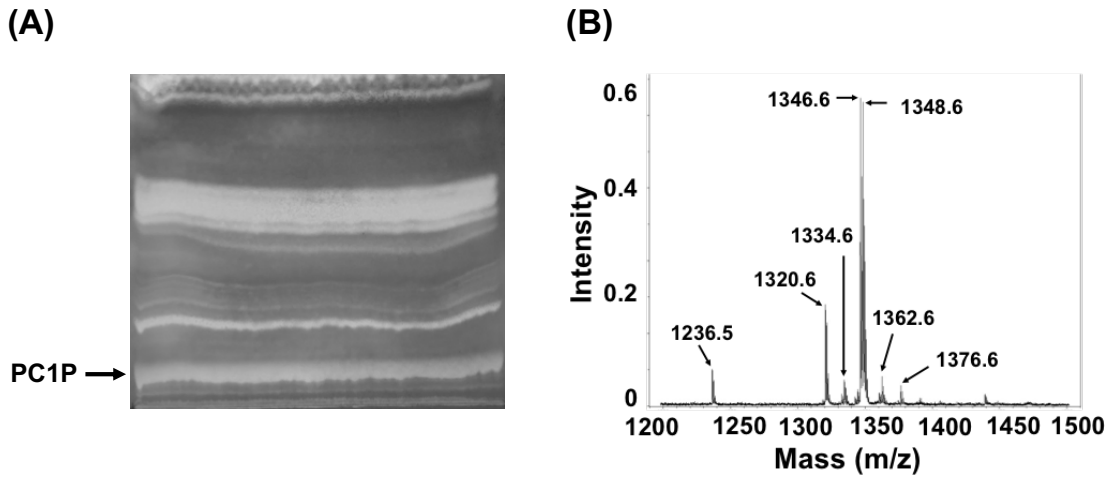
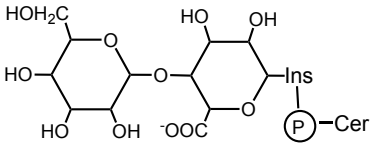
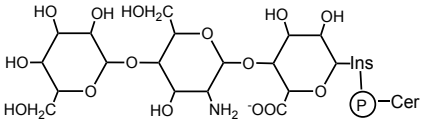
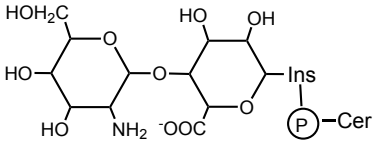
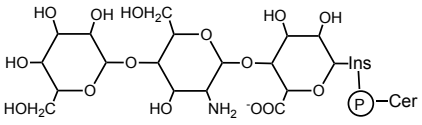
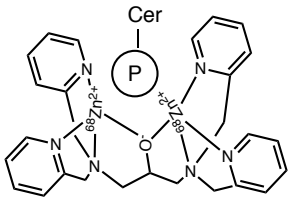


Fig. 7 MALDI-TOF MS of PC1P from cabbage leaves.

PC1P from cabbage leaves were isolated by TLC (A) and analyzed by MALDI-TOF MS with Phos-tag in positive ion detection mode (B).

Table 1. Ions detected in MALDI-TOF mass spectra of GIPCs and their possible assignments.

Plants	Structure of GIPC	*m/z*	Possible assignments of ceramide moieties	Ref.
Radish root		1148.5	*t*18:1/h16:0	[16,17]
		1232.7	*t*18:1/h22:0	
		1246.8	*t*18:1/h23:0	
		1258.7	*t*18:1/h24:1	
		1260.7	*t*18:1/h24:0	
	Series A	1282.7	*t*18:1/h24:0 + Na	
Rice bran		1407.9	*t*18:1/h23:0	[21]
		1423.9	*t*18:0/h24:0	
		1437.9	*t*18:0/h25:0	
		1451.9	*t*18:0/h26:0	
	Series B			
Welsh onion leaves		1215.7	*t*18:1/h21:1	[16,24,25]
		1231.8	*t*18:1/h22:0	
			t18:0/h22:1	
		1245.8	*t*18:1/h23:0	
			*t*18:0/h23:1	
	Series A	1259.8	*t*18:1/h24:0	
			*t*18:0/h24:1	
		1273.9	*t*18:1/h25:0	
			*t*18:0/h25:1	
		1287.9	*t*18:1/h26:0	
			*t*18:0/h26:1	
		1379.9	*t*18:1/h21:0	
		1393.9	*t*18:1/h22:0	
		1407.9	*t*18:1/h23:0	
		1421.9	*t*18:1/h24:0	
	Series B	1423.9	*t*18:0/h24:0	
		1435.9	*t*18:1/h25:0	
		1449.9	*t*18:1/h26:0	
Cabbage leaves		1236.5	*t*18:1/h16:0	[16]
		1320.6	*t*18:1/h22:0	
		1334.6	*t*18:1/h23:0	
		1346.6	*t*18:1/h24:1	
		1348.6	*t*18:1/h24:0	
		1362.6	*t*18:1/h25:0	
	PC1P with phos-tag	1376.6	*t*18:1/h26:0	

1.3.5 Chemical stability of GIPC and PC1P

Purified GIPC and PC1P were treated at different temperatures up to 125 °C or 150 °C, respectively. As shown in Fig 7A, PC1P but not GIPC was stable up to 125 °C. Significant degradation of GIPC was observed at temperatures at 125 °C and above. Approximately 60% of GIPC was degraded by treatment at 150 °C for 60 min (Fig. 8A left). Since the structural difference between PC1P and GIPC is the inositol glycan in GIPC, this hydrophilic moiety is considered to be unstable at high temperatures. Alkali treatment was included in the methodology of sphingolipid isolation to hydrolyze glycerolipids. In this study, 40% methylamine in ethanol was utilized for preparation of GIPC, as shown above. To examine its stability to this alkali reagent, purified GIPC was incubated with 40% methylamine at 50 °C up to 300 min. Results demonstrated that GIPC was stable under this condition (Fig. 8B left). Similarly, the isolation step for PC1P includes alkali treatments with KOH. As shown in Fig. 8B right, PC1P was stable to the treatment of 0.1 M KOH in 95% methanol at 80 °C. Stability toward acid was next examined using various concentrations of HCl. As shown in Fig. 7C right, PC1P was stable up to 10 M HCl for 30 min at room temperature. In contrast, GIPC was significantly degraded following treatment with 1 M of HCl; less than 20% of GIPC remained after treatment with 10 M HCl (Fig. 8C left). When the same experiment was performed at 50 °C, GIPC was significantly degraded with 0.1 M HCl (data not shown). These results are in line with the well-established acid liability of glycosidic linkages present on the inositol glycan moiety of GIPC.

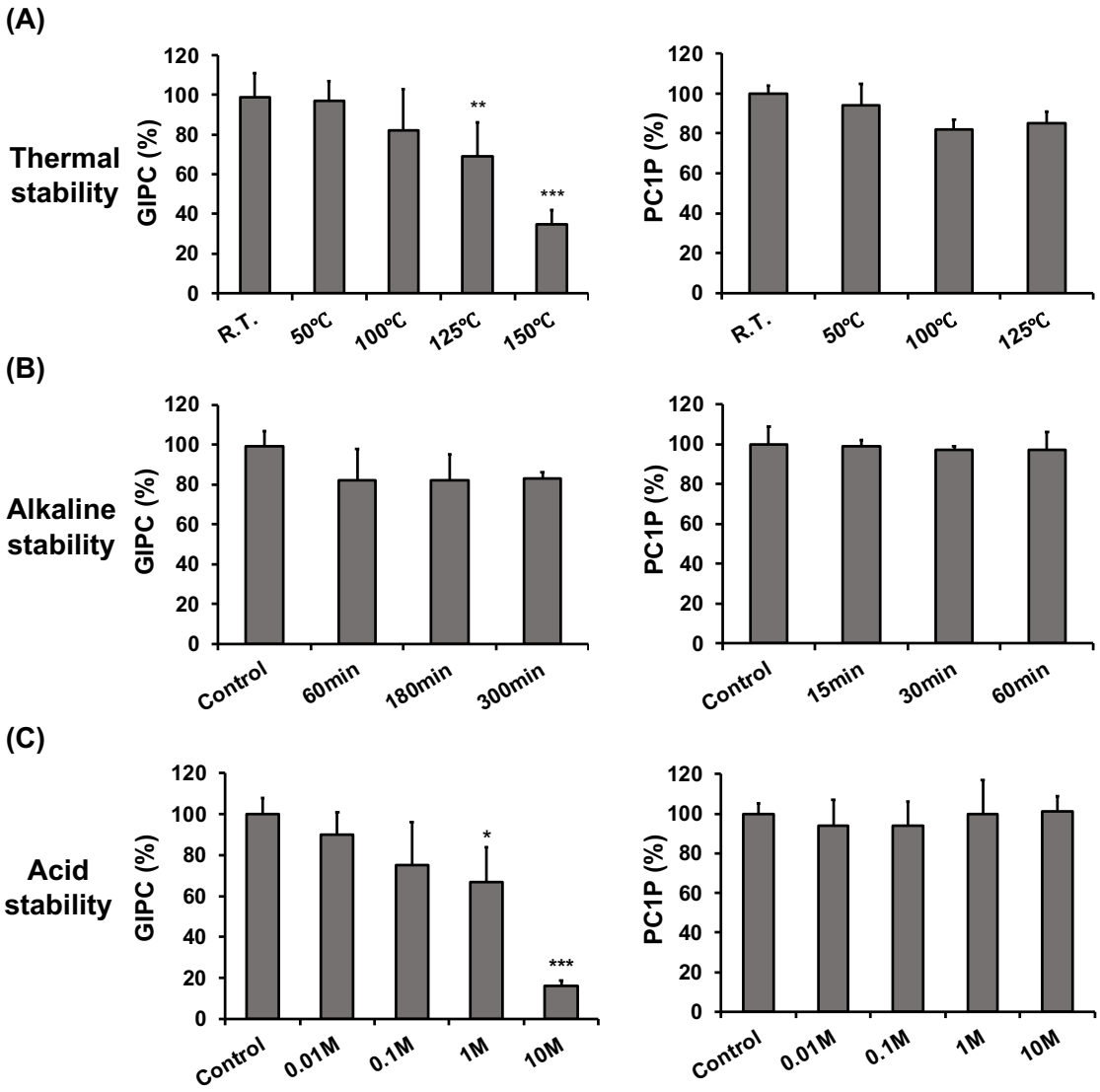


Fig. 8 Physicochemical properties of GIPC and PC1P.

GIPC and PC1P dissolved in solvent A and water, respectively, were heated at indicated temperatures for 60 min (A). GIPC was treated with 40% methylamine in ethanol (5:7, v/v) at 50 °C for indicated times (B left). PC1P was treated in 0.1 M KOH in 95% methanol at 80 °C for indicated times (B right). GIPC and PC1P were incubated with HCl at indicated concentrations for 30 min at room temperature (R.T.) (C). After these treatments, lipids were recovered from the reaction mixture and subjected to TLC to isolate the intact GIPC or PC1P. The isolated lipids were determined by the colorimetric method. Results were represented as mean ± S.D (n= 3). $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ were defined as statistically significant.

1.4 Conclusion

Hydrophilic organic solvents, such as water containing isopropanol, were needed for extraction of GIPC from tissues of plants. In this case, hydrophilic sticky compounds coexisting in the extract hampered the isolation of GIPC. These compounds were successfully removed by Sephadex column chromatography, and GIPC was henceforth isolated from the resulting GIPC-rich fraction by TLC. Solvent A was the preferred solvent both for extraction and eluate in the Sephadex column chromatography. We found that the yield of GIPC was slightly higher in butanol/water method (38nmol/g of cabbage leaves) than in our method described here (32nmol/g of cabbage leaves). Our isolation method of GIPC using isopropanol has several advantages; 1) isopropanol has the capacity to inhibit lipases [25]. 2) solvent A was easy to evaporate under reduced pressure at 50 °C, a temperature that did not affect the structural integrity of GIPC. This is advantageous over the extraction method using butanol/water because butanol needs a higher temperature and a high-powered pump for its evaporation [21,25]. PC1P can be prepared using the Bligh and Dyer method, a conventional method for extraction of phospholipids. Almost 50-70% of GIPC and PC1P was recovered by our methods. GIPC was found to be degraded under acidic conditions, such as 0.1 M HCl at 50 °C. These data presented here will be useful for biochemical and nutritional studies on these sphingolipids.

1.5 References

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CHAPTER-2

Glycosylinositol phosphoceramide-specific phospholipase D activity catalyzes transphosphatidylation

2.1 Introduction

Sphingolipids are ubiquitous components in cells of animals, plants and fungi. In animals, sphingomyelin (SM) is a predominant sphingophospholipid, whereas glycosylinositol phosphoceramide (GIPC) is a major sphingophospholipid in plants and fungi [1] (Fig. 9). In general, the backbone of SM in most of mammalian cells is a C18 sphingosine (d18:1). On the other hand, a predominant backbone of GIPCs in plants and fungi is a C18 phytosphingosine (t18:0) or dehydrophytosphingosine (t18:1) [1]. It has been known that a-hydroxy fatty acid with a long (such as C16) or very long chain (such as C24) is linked as an N-acyl residue in GIPC [1]. In contrast, a-hydroxy fatty acid is rarely detected in SM except for skin [2].

Intracellularly produced ceramide is a signaling molecules that induces apoptosis or differentiation of animal cells [3]. The intracellular ceramide is supplied by de novo synthesis and hydrolysis of SM in animals [3]. The enzyme that hydrolyzes the C position of SM is called sphingomyelinase (SMase). The corresponding enzyme that hydrolyzes the C position of GIPC is GIPC-phospholipase C (GIPC-PLC), or inositol phosphoceramide (IPC)-PLC. IPC-PLC has been cloned in fungi, such as *Saccharomyces cerevisiae* and *Cryptococcus neoformans* [4-7]. Based on the alignment of the amino acid, they are considered to be orthologs of mammalian neutral SMase 2 [4]. As far as we know, GIPC-PLC has not been reported in plants.

SMase D is an enzyme that hydrolyzes the D position of SM. SMase D is known to exist only in spider toxin and some species of bacteria [8-9]. The corresponding enzyme that hydrolyzes the D position of GIPC is GIPC-phospholipase D (GPC-PLD). Recently, we identified phytoceramide 1-phosphate (PC1P) in cabbage lipid and found that the PC1P is produced by hydrolysis of D position GIPC [10]. A partially purified enzyme fraction from cabbage and *Arabidopsis thaliana* hydrolyzed GIPC specifically, but did not hydrolyze phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or SM at substantial levels [10-11]. Based on these findings, we called this activity as "GIPC-PLD". We also examined the distribution of GIPC-PLD activity in 25 tissues of 10 plants and detected it in roots in most of the plants [11].

Interestingly, the enzyme activity is higher in growing tissues, but under the detection level in differentiated tissues such as outer aged leaves of cabbage and Arabidopsis thaliana [11].

A well-known characteristic of PLD is a reaction called transphosphatidylation, which catalyzes transference of the phosphatidyl moiety to alcohol [12]. This PLD-mediated reaction is applicable to synthesis of functional phospholipids with a different head group, such as phosphatidyl ascorbic acid [13]. It is also available to synthesize PE and phosphatidylglycerol from PC, which is naturally abundant [12].

In this study, we examined whether the GIPC-PLD activity in plants catalyzes transphosphatidylation. Results showed that GIPC-PLD activities of cabbage young leaves and radish root do it (Fig. 9). We also showed that this reaction proceeds during lipid extraction when alcohol is used. GIPC-PLD will be potential catalyst for enzymatic modification of sphingophospholipids.

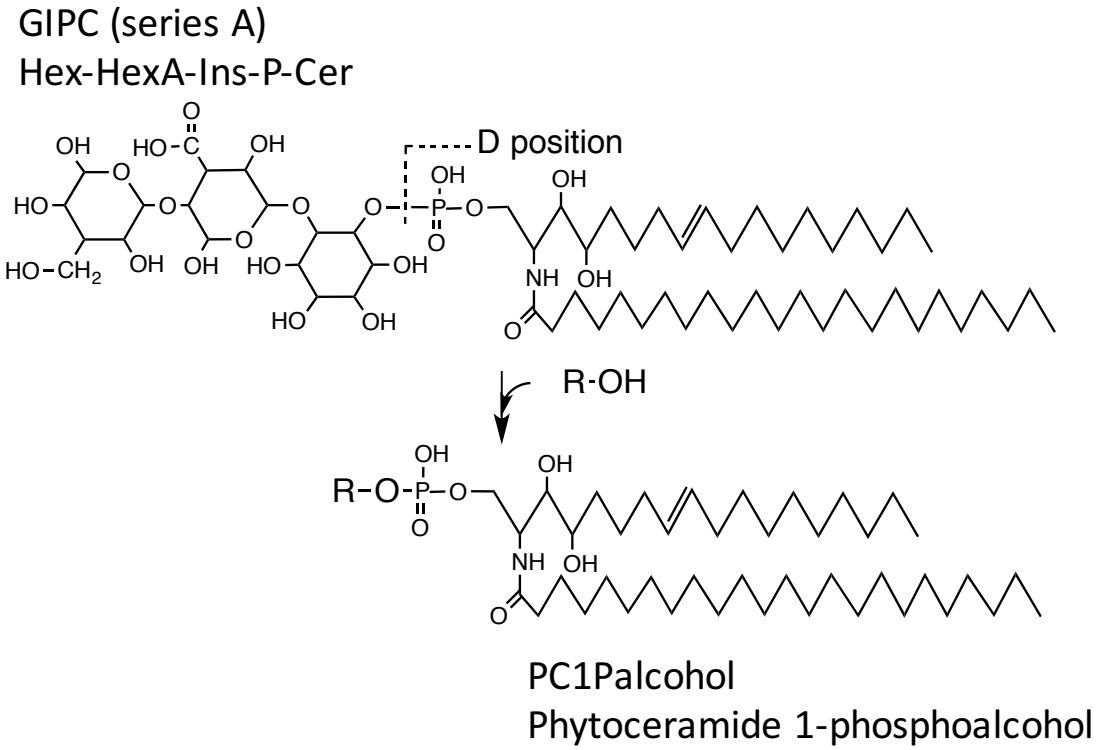


Fig. 9 Transphosphatidylation catalyzed by GIPC-PLD activity.

GIPC is classified dependent on number of sugar chain as proposed by Buré et al [23]. GIPC with hexuronic acid (HexA)-Inositol (Ino) phosphoceramide (P-Cer) is basal structure of GIPC and called as series 0 GIPC with Hexose (Hex)-HexA-Ino-P-Cer is series A as shown above. GIPC with Hex-Hex-HexA-Ino-P-Cer is series B. Hydrophobic part of typical GIPC is a ceramide composed of a 1,3,4-trihydroxy analogue of long chain base (LCB) (dehydrophytosphingosine, t18:1) and an ∝-hydroxy fatty acid with very long chain, such as h24:0 as shown here. Transphosphatidylation by GIPC-PLD produces phytoceramide 1-phosphoalcohol (PC1Palcohol) and glycosylinositol.

2.2 Materials and methods

2.2.1 Materials

2,4,6-Trihydroxy-acetophenone (THAP) and PLD from Streptomyces chromofuscus was obtained from Sigma-Aldrich (St. Louis, MO). Choline chloride, L-serine, glycerol and myo-inositol were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Zymolyase-20T was obtained from Nacalai Tesque Inc. (Kyoto, Japan). 2-(4-Hydroxyphenyl) ethanol was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). PC from egg yolk, PI from soybean and SM from bovine brain were purchased from Avanti Polar Lipids (Alabaster, AL). PC1P from cabbage was prepared as described previously [11]. All organic solvents and alcohols used in this study were of analytical reagent grade.

2.2.2 Preparation of IPC, MIPC and GIPC

Inositol phosphoceramide (IPC) and mannosylinositol phosphoceramide (MIPC) was prepared from yeast (*Saccharomyces cerevisiae*). The yeast protoplasts were prepared by treatment of zymolyase-20T and homogenized in a Potter-Elvehjem type homogenizer as described in [14]. Lipids were extracted from the homogenate by the Bligh and Dyer method [15] with acidification of the upper layer with HCl. The extracted lipids were treated with mild alkali (0.1 M KOH in 95% methanol) at 60 ℃ for 0.5 h to degrade glycerolipids, but not sphingolipids. The lipids were recovered from the reaction mixture by the Bligh and Dyer method with acidification of the upper layer with HCl and subjected to TLC, which was developed with chloroform/methanol/28% ammonia (60:35:8, v/v) for isolation of IPC and MIPC.

GIPC having two sugars (series A type) and that having three sugars (series B type) were prepared from cabbage leaves and rice bran, respectively, as described previously [11]. First, the plant tissues were heated in boiling water for 5 min to inactivate GIPC-PLD which reduces the GIPC content in the plant tissue. Lipids were extracted from the boiled tissues with mixed solvent [solvent A: lower layer of a mixed solvent consisting isopropanol/hexane/water (55:20:25, v/v)] as described by Markham et al. [16]. The extract was heated at 50 ºC for 1 h in 40% methylamine/ethanol (5:7, v/v). After evaporation, the alkali lysate of the plant lipids was subjected to TLC using chloroform/methanol/7% ammonia (45:35:10, v/v) as the developing

solvent. The silica gel corresponding to series A type and series B type GIPCs were scraped off the plate and extracted with solvent A. Isolated IPC, MIPC and GIPCs were quantified by the colorimetric method based on phosphomolybdenum-malachite green formation [17]. The structures of the purified IPC, MIPC and GIPCs were analyzed by MALDI-TOF/MS as described below.

2.2.3 Preparation of crude PC-PLD and GIPC-PLD fractions

The crude PC-PLD fraction was prepared from cabbage leaves using a method described by Davidson and Long [18]. In brief, cabbage leaves were mixed with an equal volume of saline and homogenized. The filtrate of the homogenates was heated at 55 ºC for 5 min and centrifuged at 11,000 X g for 30 min. A cold acetone precipitate was obtained by mixing the supernatant with double the volume of acetone and cooled at -20 ºC. The precipitate was used as the crude PC-PLD fraction. The PLD activity prepared by this procedure prefers PC and does not hydrolyze GIPC, as shown previously [10]. The GIPC-PLD fraction was prepared from a 100,000 X g (100K) pellet fraction of cabbage leaves homogenates. The 100K pellet was solubilized with 0.2 M Tris-HCl buffer (pH 7.4) containing 0.6% Triton X-100 and centrifuged at 100,000 X g for 30 min. The protein in the supernatant fraction was precipitated with ammonia sulfate (60% saturation). The precipitate was dialyzed and subjected to DEAE-cellulose column chromatography. The active faction was obtained by elution with 200 mM NaCl and used for the GIPC-PLD assay.

2.2.4 GIPC-PLD assay and PC-PLD assay

The PLD assays were conducted essentially as described previously [10-11]. The mixture of GIPC-PLD assay contained 48 nmol purified GIPC, 0.1 ml of the enzyme fraction and 3 mg (10 mM) sodium deoxycholate (NaDOC), in the presence or absence of various alcohols up to 20% (v/v) in 0.2 M Tris/HCl buffer (pH 7.4) in a total volume 0.7 ml. The reaction mixture was incubated at 30ºC with continuous stirring. After incubation for 30 min, lipids in the reaction mixture were extracted by the method of Bligh and Dyer [15] with acidification. The resulting PC1P or PC1Palcohol was isolated by TLC developed with chloroform/methanol/28% ammonia (60:35:8, v/v), and quantified by a colorimetric method based on phosphomolybdenum-malachite green formation [17]. The structure of PC1Palcohol was confirmed by MALDI-TOF/MS as described below. The mixture of the PC-PLD assay contained 48 nmol of soybean lecithin, 0.15

ml enzyme solution, 0.1 ml 0.1 M calcium chloride and 1 ml diethylether in 0.1 M acetate buffer (pH 5.6) in a total volume of 1.7 ml. Methanol up to 10% (v/v in total water volume) was added to the reaction mixture. The reaction mixture was incubated at 30ºC for 30 min. The resulting products, PA and phosphatidylmethanol (PAmethanol), were isolated and determined by the same method as used for GIPC-PLD products. The structure of PAmethanol was confirmed by MALDI-TOF/MS as described below.

2.2.5 MALDI-TOF/MS

MALDI-TOF/MS was applied for structural elucidation of the transphosphatidylation product of PLD activity. The product isolated by TLC was dissolved in a small amount of methanol. A small portion of the solution (0.5 µL) was spotted on a sample plate. Then, it was layered with 0.5 µL THAP solution (10 mg/mL acetonitrile, 0.1% trifluoro acetic acid) on the plate. The matrix/analyte cocrystal was analyzed by a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in negative mode. IPC and MIPC isolated from yeast and GIPCs prepared from plant tissues were also analyzed by MALDI-TOF/MS in negative detection mode [11]. MALDI-TOF/MS using Phos-tag was applied for analysis of PC1P [2, 10]. Briefly, PC1P was dissolved in a small volume of methanol containing 0.1% aqueous ammonia. Ten µL of this solution was mixed with 5 µL of 0.1 mM ^{68}Zn Phos-tag solution and 2 µL of silica suspension (100 mg/mL in methanol). A small portion of the mixed solution (0.5µl) was spotted on a sample plate. Then, it was layered with 0.5 µl THAP solution (10 mg/mL acetonitrile) on the plate. In all cases, the wavelength of the nitrogen-emitting laser, pressure in the ion chamber, and accelerating voltage were 337 nm, 3.7 X 10^{-7} Torr, and 20 kV, respectively. To enhance the reproducibility, 256-512 single laser shots were averaged for each mass spectrum.

2.3 Results

2.3.1 GIPC-PLD catalyzes transphosphatidylation

A GIPC-PLD assay was conducted in the absence or presence of increasing concentrations of methanol. Because we used the crude enzyme fraction from cabbage leaves, endogenous lipids, such as PA and PI, were contained in the enzyme fraction. Those endogenous lipids and the NaDOC used as the activator appeared in all lanes of TLC (Fig. 10A). The remaining substrate GIPC was scarcely detected at the origin on the TLC plate. When an increasing concentration of alcohol was added to the reaction mixture, intensities of bands of PC1P decreased dependent on the alcohol concentration used. Instead, uncharacterized lipid bands emerged just above the digalactosyldiacylglycerol (DGDG) bands. The intensities of the newly emerged bands increased dependent on the concentration of methanol. We extracted the lipid from the silica gel and analyzed it by MALDI-TOF/MS. Three major ions (m/z 746.6, 772.6 and 774.6) were detected in the negative ion detection mode (Fig. 10C). Because t18:1/h22:0, t18:1/h24:1 and t18:1/h24:0 were the major molecular species of PC1P produced from the GIPC by GIPC-PLD activity (Fig. 10D), the three major ions were assigned to phytoceramide 1-phosphomethanol (PC1Pmethanol) with ceramide structures of t18:1/h22:0, t18:1/h24:1 and t18:1/h24:0, respectively. They were the major molecular species of the series A GIPC of cabbage used as a substrate (Fig. 10B). Quantification of PC1P and PC1Pmethanol in each experiment revealed that the concentration of methanol producing PC1Pmethanol equimolar to PC1P was 10% (v/v) in our assay condition (Fig. 10E). It should be noted that 20% of PC1P remained even in the presence of 20% methanol.

Cabbage leaves are known to have high PLD activity, which hydrolyzes glycerophospholipids [12]. Tentatively, the term "PC-PLD" is used for this conventional PLD activity in cabbage, because PC is the best substrate for this activity. We prepared the PC-PLD fraction from cabbage leaves by an established method using acetone precipitation [12], and conducted similar experiments using soybean lecithin as the substrate. We confirmed that the PC-PLD fraction catalyzed transphosphatidylation. It was evident that the transphosphatidylation of PC-PLD proceed at lower concentrations of methanol than that of GIPC-PLD. The concentration of methanol producing PAmethanol equimolar to PA was found to be 1.4% in our assay condition (Fig. 10F). The requirement for a relatively lower concentration of alcohol is consistent with those reported earlier [19]. It is known that some lipases catalyze condensation reaction [20]. We

examined a possibility that GIPC-PLD catalyzes condensation of PC1P and methanol. Results showed that PC1Pmethanol was not formed at appreciable level when PC1P was treated with GIPC-PLD in the presence of 10% methanol.

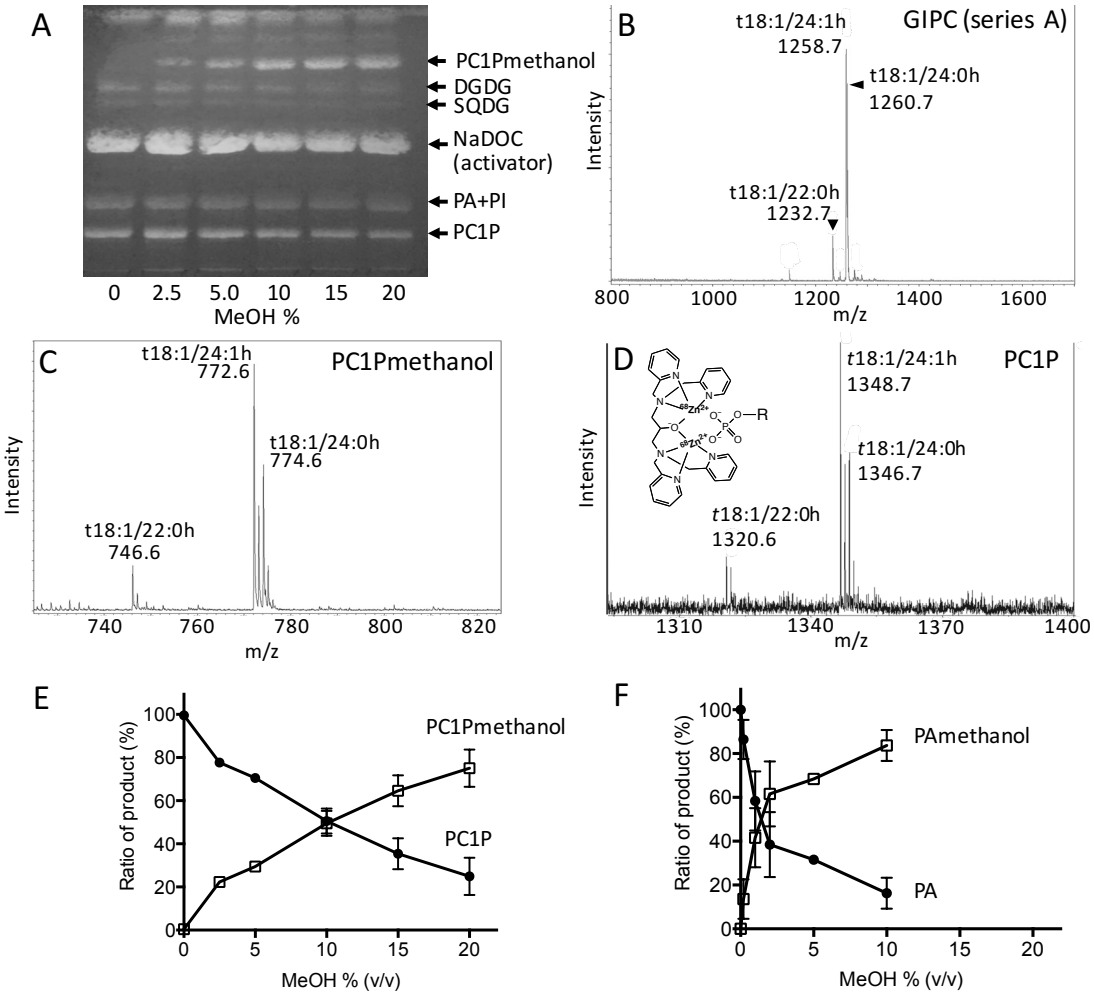


Fig. 10 Methanol concentration–dependent formation of PC1Pmethanol catalyzed by GIPC-PLD

Series A GIPC was treated with GIPC-PLD fraction of cabbage leaves in the presence of indicated concentration (v/v %) of methanol. Lipids recovered from the reaction mixture were separated with TLC (A). Resulting PC1P and PC1Pmethanol were quantified (E). Open squares and closed circles mean PC1Pmethanol and PC1P, respectively. Similar experiments were conducted with PC-PLD fraction of cabbage leaves using soybean PC as substrate (F). Open squares and closed

circles mean PAmethanol and PA, respectively. The molecular species of substrate series A GIPC (B) from cabbage, PC1Pmethanol (C) and PC1P (D) were confirmed by MALDI-TOF/MS. PC1P was detected as Phos-tag complex as inserted in D. Values are presented as mean ± S.D. from three to four independent experiments.

2.3.2 Available alcohols for transphosphatidylation of GIPC-PLD

Substrate specificity for transphosphatidylation of GIPC-PLD was characterized using various alcohols (Figs. 11, 12 and Table I). As shown in Fig. 11A, transphosphatidylation product were detected when the GIPC-PLD assay was conducted in the presence of methanol, ethanol, propanol and butanol. These products were extracted from the silica gel and analyzed by MALDI-TOF/MS. Ions corresponding to phytoceramide containing 1-phosphorylethanol (PC1Pethanol), 1-phosphorylpropanol (PC1Ppropanol) and 1-phosphorylbutanol (PC1Pbutanol) were detected in the mass range around 800, as shown in Fig. 11B-D. The molecular species compositions of each transphosphatidylation product were essentially the same.

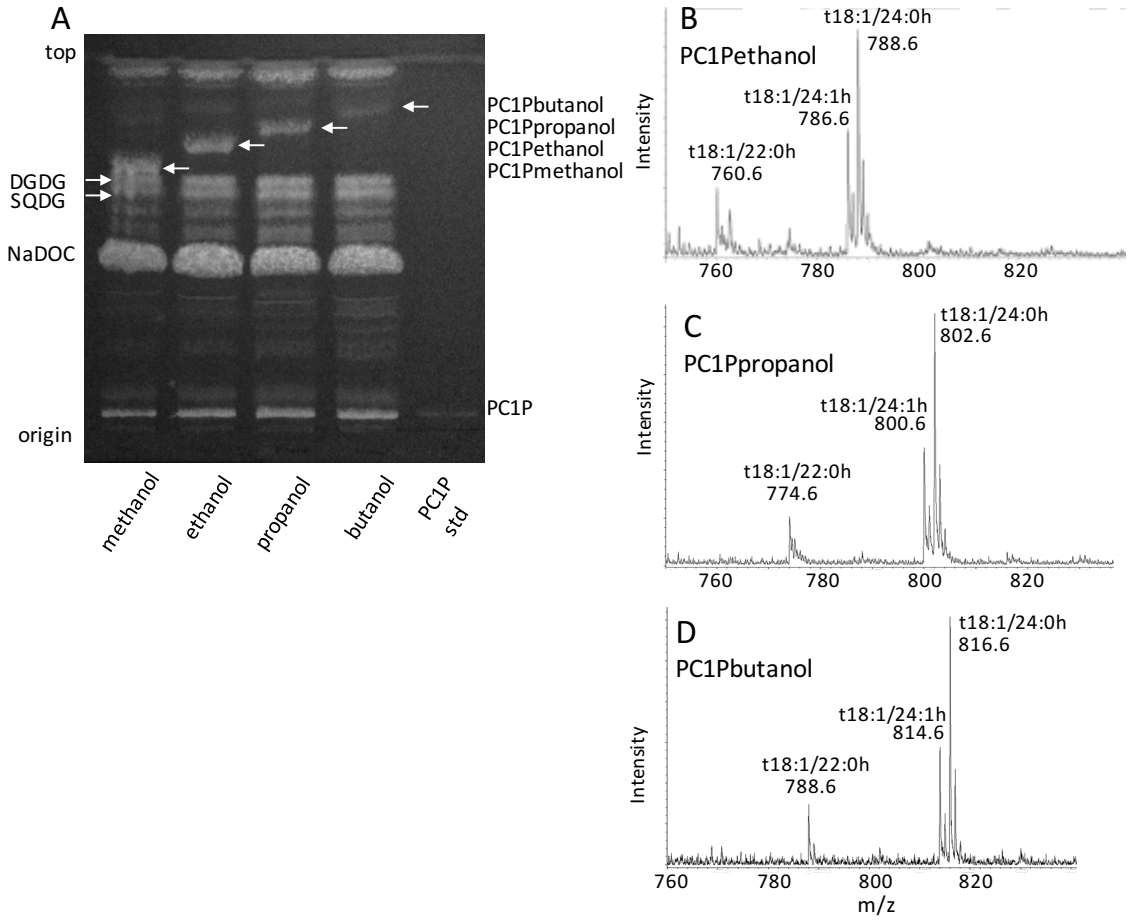


Fig. 11 Production of various phytoceramide 1-phosphoalcohols by transphosphatidylation of GIPC-PLD activity.

Series A GIPC was treated with the GIPC-PLD fraction of cabbage leaves in the presence of 10% methanol, ethanol, propanol and butanol. Resulting reaction products were separated by TLC (A). The PC1Palcohols formed in the presence of various alcohols were isolated. The molecular structures of the resulting products were confirmed by MALDI-TOF/MS (B-D).

Quantitative analysis of these products revealed that GIPC-PLD prefers primary alcohols with a shorter chain length, up to C4 (Fig. 12). Secondary and tertiary alcohols did not serve as acceptors of transphosphatidylation of GIPC-PLD. In fact, phytoceramide phosphate containing 2-methyl-1-propanol (primary) was detectable, whereas formations of phytoceramide phosphate containing 2-propanol (secondary) or 2-methyl-2-propanol (tertiary) were not observed. Attempts to produce

sphingolipids having polar head groups of glycerophospholipids were unsuccessful. Neither choline, L-serine nor DL-glycerol was an acceptor of transphosphatidylation of GIPC-PLD. Inositol is one of the structural components of GIPC. However, GIPC-PLD does not catalyze transphosphatidylation of inositol. Tyrosol is an antioxidant alcohol. A sphingolipid having a tyrosol residue was not formed by the treatment of GIPC-PLD even when the high concentration of tyrosol was used (Fig. 12).

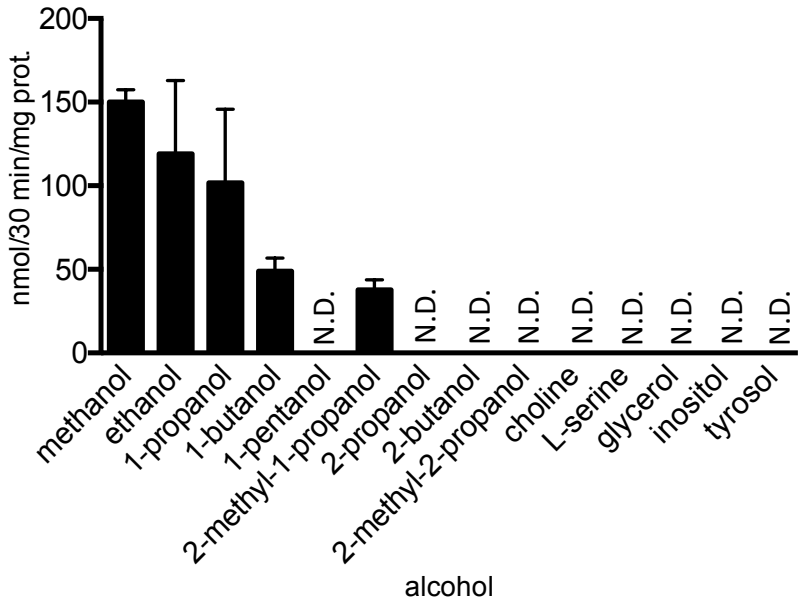
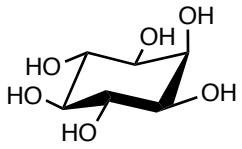
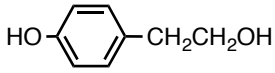


Fig. 12 Available alcohols for transphosphatidylation of GIPC-PLD

GIPC was treated with the GIPC-PLD fraction of cabbage leaves in the presence of various alcohols (10%). Resulting reaction products were separated by TLC and quantified. Values are presented as mean ± S.D. from three independent experiments.

N.D.: not detectable. The names and structures of OH-containing compounds are shown in Table 2.

Table 2
Availability of alcohol for GIPC-PLD-catalyzed transphosphatidylation

Type	Name	Structure	Availability
primary	methanol	CH_3OH	yes
	ethanol	CH_3CH_2OH	yes
	1-propanol	$CH_3CH_2CH_2OH$	yes
	1-butanol	$CH_3CH_2CH_2CH_2OH$	yes
	1-pentanol	$CH_3CH_2CH_2CH_2CH_2OH$	no
	2-methyl-1-propanol	$CH_3CH(CH_3)CH_2OH$	yes
secondary	2-propanol	$CH_3CH(OH)CH_3$	no
	2-butanol	$CH_3CH_2CH(OH)CH_3$	no
tertiary	2-methyl-2-propanol	$CH_3C(CH_3)(OH)CH_3$	no
functional group of glycero-phospholipid	choline	$HOCH_2CH_2N^+(CH_3)_3$	no
	L-serine	$HOCH_2CH(NH_2)COOH$	no
	glycerol	$HOCH_2CH(OH)CH_2OH$	no
	myo-inositol		no
functional alcohol	2-(4-hydroxyphenyl) ethanol (tyrosol)		no

1.3.3 Formation of PC1Pmethanol during homogenization of plant tissues.

Previously, we demonstrated that PC1P is produced by activated GIPC-PLD during homogenization of the plant tissues. Therefore, PC1P is not so abundantly present in the homogenates if the GIPC-PLD in the leaves is inactivated before homogenization (10). We confirmed this using heat-inactivated cabbage leaves. As shown in Fig. 13, the level of PC1P in boiled leaves was very low compared to that of non-treated leaves that were homogenized in water. In both preparations, the PC1Pmethanol level was negligible because GIPC-PLD was heat-inactivated before mixing with methanol for extraction. When non-boiled cabbage leaves were homogenized in the mixed solvent consisting of chloroform/methanol/water (1:2:0.8, v/v), a large amount of PC1Pmethanol was formed, whereas the amount of PC1P was greatly reduced. These results indicated that the accumulation of PC1P in homogenates of plant tissues varies depending on the protocol of lipid extraction. Therefore, special care is needed to extract PC1P from plant

samples or reaction mixtures that contain active GIPC-PLD. In other words, alcohols used for extraction must be added after inactivation of GIPC-PLD.

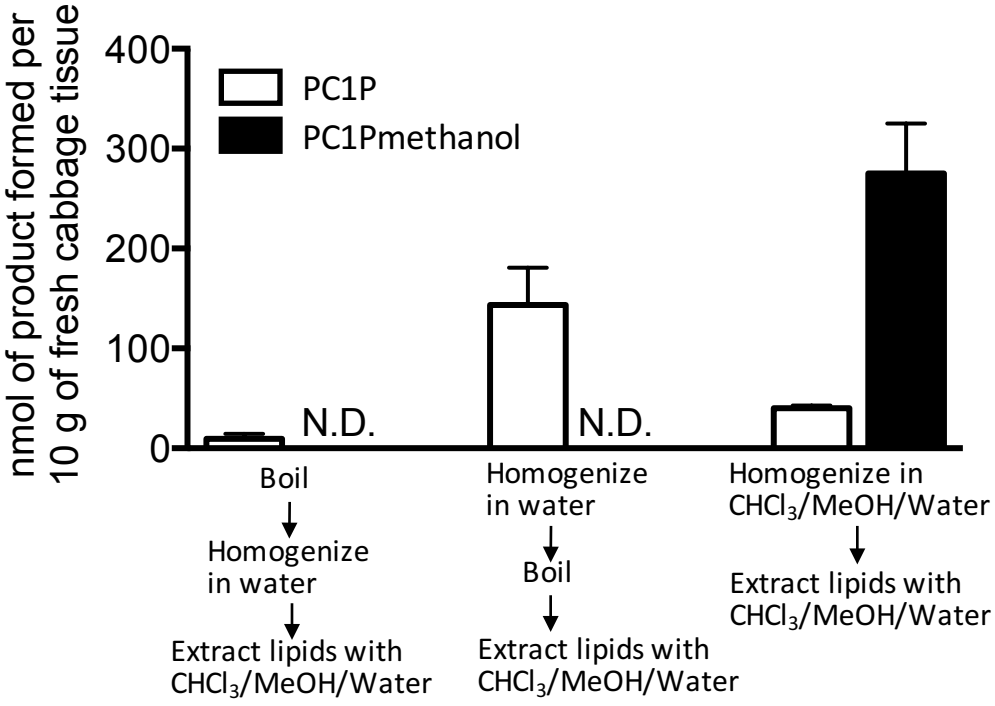


Fig. 13 PC1P and PC1Pmethanol formation during process of lipid extraction

Young cabbage leaves were boiled, homogenized in water, then subjected to extraction with chloroform/methanol/water, (1:2:0.8, v/v) (left). Young cabbage leaves were homogenized in water, boiled, and then subjected to extraction with chloroform/methanol/water (1:2:0.8, v/v) (middle). Young cabbage leaves were homogenized in chloroform/methanol/water (1:2:0.8, v/v), and then subjected to extraction (right). PC1P and PC1Pmethanol were isolated by TLC from the lipid extract and quantified. Values are presented as mean ± S.D. from three independent experiments. N.D.: not detectable.

2.3.4 Substrate specificity of GIPC-PLD activity

Substrate specificities of GIPC-PLD toward GIPC with different sugar chains were characterized by determination of the PC1P formed. The partially purified enzyme used in this assay was prepared from young cabbage leaves. Substrates used were IPC, MIPC and GIPCs containing two sugars (series A GIPC) or three sugars (series B GIPC). They were prepared from yeast (IPC and MIPC), cabbage leaves (series A GIPC) and rice bran (series B GIPC) by TLC. As shown in Fig. 14, each substrate did not contain other analogues of GIPCs, and consisted mainly of t18:0/h26:0 (IPC and MIPC), t18:1/h24:1 (series A GIPC) and t18:0/h24:0 (series B GIPC). These structural assignments were based on earlier reports from other investigators (21) and our group (22). Results showed that cabbage GIPC-PLD activity prefers series A GIPC from cabbage. Neither MIPC nor series B GIPC served as a substrate of cabbage GIPC-PLD. We observed formation of PC1P from IPC, but the amount was marginal. We also found that PC1Pmethanol was formed only from series A GIPC but not from IPC, MIPC and series B GIPC when assay was conducted in the presence of 10% methanol (data not shown). These results indicate that cabbage GIPC-PLD recognizes sugar chains of GIPC. Similar experiments were conducted with GIPC-PLD from radish root. We found that the radish root enzyme also prefers GIPC with two sugars (data not shown).

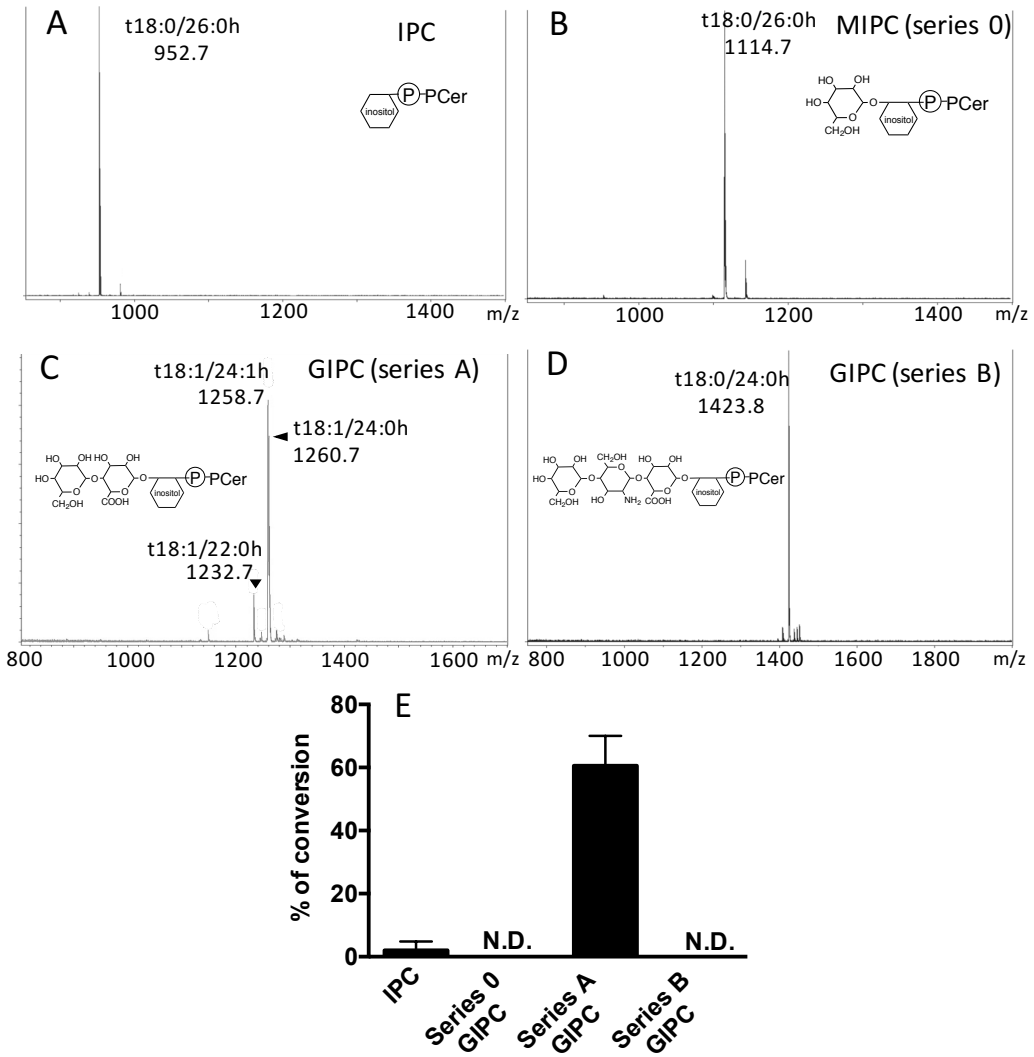


Fig. 14 Substrate specificity of GIPC-PLD of cabbage leaves

IPC and MIPCs (series 0 GIPC) were purified from lipids of Saccharomyces cerevisiae. Series A and series B GIPC were obtained from cabbage leaves and rice bran, respectively, as described in materials and methods(A-D). The purified GIPCs were treated with the GIPC-PLD fraction of cabbage leaves. Resulting PC1P were isolated by TLC and quantified (E). Data are expressed as mean ± S.D. from three independent experiments.

2.4 Discussion

Previously, we reported the presence of PC1P in cabbage lipids [10]. The PC1P is not a trace component. In fact, it accounted for 5% of total phospholipids in homogenates of cabbage leaves. We also found that PC1P is formed by hydrolysis at the D position of GIPC, and that this enzyme activity hydrolyzes GIPC specifically suggesting the existence of an uncharacterized GIPC-specific PLD in the preparation [10]. Here, we showed that GIPC-PLD activity in cabbage catalyzes transphosphatidylation. The alcohol available for this reaction is limited to primary alcohols with a short chain below C4. Neither choline, serine nor glycerol serves as an acceptor for transphosphatidylation of GIPC-PLD. In this regard, glycerol has been reported to be a good acceptor in transphosphatidylation catalyzed by PC-PLD in cabbage [19]. This result suggests that structures of active sites in these PLDs are different from each other.

PLD can be found widely in organisms such as bacteria, fungi, plants and animals [1]. Most of the enzymes have a conserved amino acid sequence, HxKxxxxDx6GSxN motif (HKD motif). PLD containing this motif is named HKD PLD after this catalytic domain. On the other hand, PLDs that do not contain the HKD motif exist. A typical non-HKD PLD is *Streptomyces chromofuscus* PLD (scPLD). A characteristic of transphosphatidylation of scPLD is requirement of a higher concentration of alcohol (8-10 M, 26-32%) [23]. This is distinct from PLD of the HKD type, which requires a low concentration (0.2-0.6 M, 0.6-1.9%) of alcohol [8, 23]. Here, we found that the maximum level of transphosphatidylation of GIPC-PLD was attained in the presence of 15-20% of alcohol (7 M). This concentration was higher than that observed in the conventional PC-PLD in cabbage as shown here. Corynebacterium PLD, Arcanobacterium PLD and Loxosceles PLD are also non-HKD PLDs. They are PLDs that hydrolyze the D position of SM. It should be emphasized that GIPC-PLD exclusively hydrolyzes GIPC, a major sphingophospholipid in plants [10-11]. Considering these facts, it seems likely that GIPC-PLD is a non-HKD type. However, presented these data was obtained using partially purified enzyme. A possibility that enzymes other than GIPC-PLD perform the transphosphatidylation cannot be ruled out. Purification of GIPC-PLD is now conducting in our laboratory.

Our recent research showed that PC1P can be detectable only in immature tissues, such as young leaves and roots, and that a large part of PC1P is produced during homogenization of the tissues [11]. In this study, we found that a large amount of PC1Pmethanol is formed when methanol is contained in a solvent for homogenization. Thus, alcohols should not be used for solvents of homogenization for detection of PC1P. Limited occurrence and limited extraction protocols would be reasons that the occurrence of PC1P had not identified until recently.

In this study, we found that cabbage GIPC-PLD prefers GIPC with two sugars. Neither GIPC with no (IPC), one nor three sugars served as substrate of this enzyme. It has been reported that the GIPC in cabbage and *Arabidopsis thaliana* consists mainly of GIPC with the two-sugar type [24-25], whereas GIPC in rice root consists of GIPCs with two and three sugars [22]. At present, we do not know the physiological significance of sugar chain recognition of the enzyme. The physiological significance of GIPC-PLD itself may answer this question. In this regard, Smith and Fry have demonstrated that mannopyranosyl-glucuronopyranosyl-inositol (MGI), another hydrolysate of series A GIPC by GIPC-PLD, accumulated in the spent medium of cell-suspension cultures of rose during the period of rapid cell growth [26]. They hypothesized a signaling role for released MGI and suggested GIPC as a source of the MGI 20 years ago [26].

In conclusion, GIPC-PLD activity in cabbage leaves catalyzes transphosphatidylation. Primary alcohols below C4 can be attached to the phosphate group of sphingophospholipid by this reaction. Modification of the polar head group of glycerophospholipids by PLDs has been extensively studied, but it is applicable only to glyerophospholipids. Although available alcohol is limited at present, protein engineering will expand the availability of alcohol in the transphosphatidylation of GIPC-PLD. GIPC-PLD will be potential catalyst for enzymatic modification of sphingophospholipids.

2.5 References

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Conclusion

Conclusion

GIPC has been considered as the most abundant sphingolipids on earth [1]. However, little is known about the metabolism and physiological functions of GIPC in plants. Human ingests much amount of GIPC from vegetables. However, digestibility and nutritional relevance of GIPC in human are remained to be unknown. GIPC is not soluble in organic solvents due to the presence of hydrophilic sugar chains in its polar head. The fact that popular lipid extraction methods are not applicable for GIPC was one of major obstacles for lipid researches to perform biochemical experiments on GIPC. Previously, our group identified PC1P as hydrolysate of GIPC in cabbage leaves. As far as we know, that was the first report showing natural occurrence of PC1P and its producing activity from GIPC in plants [2]. In this study, I established methods for isolation of GIPC and PC1P, and examined their chemical stabilities in chapter 1. In chapter 2, I characterized the GIPC-PLD activity in terms of transphosphatidylation reaction of the enzyme activity.

Firstly, I confirmed that GIPC is extractable from plant tissues using solvent A (lower layer of isopropanol: hexane: water, 55:20:25, v/v/v; solvent A) as reported by other investigators [3]. The extract was found to contain hydrophilic materials that hinder the migration of GIPC during TLC. I found that these hydrophilic materials co-existing with GIPC are successfully removed from the extract by Sephadex column chromatography using solvent A as eluate. As a result, GIPC was easily isolated by TLC with high purity. The yield of GIPC from cabbage leaves by the method was around 50-70%. The method developed here has advantage on the purity of GIPC compared to the method using water/butanol partition. S2 and S3 GIPCs in welsh onion were also successfully isolated by the method followed by TLC.

I also examined chemical stability of these lipids. Results revealed that GIPC, but not PC1P degraded at high temperature (over 125 ℃) and high concentration of acid (over 1.0 M HCl), indicating the susceptibility of inositol glycan moiety of GIPC under these conditions. I also found that both GIPC and PC1P are stable against mild alkali treatment. These knowledges will be useful for biochemical studies or industrial application of GIPC and PC1P.

PLD-catalyzed transphosphatidylation is a useful and eco-friendly technique for modification of polar head group of phospholipids [4]. In this study, we found that GIPC-PLD has an ability to

perform transphosphatidylation. The concentrations of acceptor alcohols required for this reaction were 10-15%. The alcohol preferred for this enzyme is primary with short chain below C4. The PLD-catalyzed transphosphatidylation has been well documented using plant- and bacteria-derived PLD [5]. However, most of reactions are limited to glycerophospholipids. The GIPC-PLD activity which modifies polar head of sphingolipids has high value.

I established a method for isolation of GIPC. However, the method has several points to be improved. Firstly, the yields of GIPC in the method was not so high (50-70%). Secondly, organic solvent and alkali reagent used for isolation of GIPC is not applicable for food industry. Thirdly, evaporation of water containing solvents and Sephadex column chromatography are time consuming. Now I am trying to isolate GIPC by using safer solvents for foods with high recovery. I am also conducting experiments on GIPC-PLD purification to identify its amino acid sequences and genes.

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