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

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Abstract

Glycosylinositol phosphoceramide (GIPC) is a sphingophospholipid in plants. Recently, we identified that GIPC is hydrolyzed to phytoceramide 1-phosphate (PC1P) by an uncharacterized phospholipase D activity following homogenization of certain plant tissues. We now developed methods for isolation of GIPC and PC1P from plant tissues and characterized their chemical stabilities. Hydrophilic solvents, namely a lower layer of a mixed solvent system consisting of isopropanol/hexane/water (55:20:25, v/v/v) was efficient solvent for extraction and eluent in column chromatography. GIPC was isolated by Sephadex column chromatography followed by TLC. A conventional method, such as the Bligh and Dyer method, was applicable for PC1P extraction. Specifically, PC1P was isolated by TLC following mild alkali treatment of lipid extracts of plants. The yields of GIPC and PC1P in our methods were both around 50-70%. We found that PC1P is tolerant against heat (up to 125 °C), strong acid (up to 10 M HCl), and mild alkali (0.1 M KOH). In contrast, significant degradation of GIPC occurred at 100 °C and 1.0 M HCl treatment, suggesting the instability of the inositol glycan moiety in these conditions. These data will be useful for further biochemical and nutritional studies on these sphingolipids.

Keywords

Sphingolipid; Phytoceramide 1-phosphate; Glycosylinositol phosphoceramide; TLC; Sephadex column chromatography; Plants

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. 1). 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.

2. Materials and Methods

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.

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.

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.

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.

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.

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].

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.

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.

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.

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.

3. Results and Discussion

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. 2A). 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. 2B).

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. 3A). The elution profile of the Sephadex column chromatography (Fig. 3B) 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. 3D). 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. 3C). 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. 4A).

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. 4B).

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. 5A) and Series B GIPC from rice bran (Fig. 5B) 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.

As shown in Fig. 6, 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].

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. 7A 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. 7B left). Similarly, the isolation step for PC1P includes alkali treatments with KOH. As shown in Fig. 7B 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. 7C 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.

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.

Declaration of Competing Interest

All authors have no conflict of interest to declare

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Figure captions

Fig. 1. 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.

Fig. 2. 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.

Fig. 3. 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 (D). GIPC and sticky materials that hamper migration of developing solvent are indicated by arrows.

Fig. 4. 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.

Fig. 5. 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.

Fig. 6. 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).

Fig. 7. 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.

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

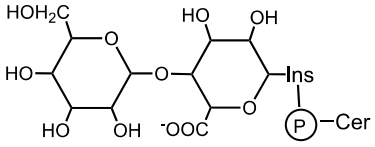
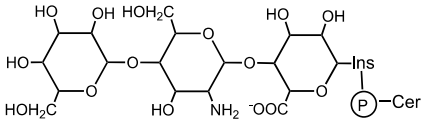
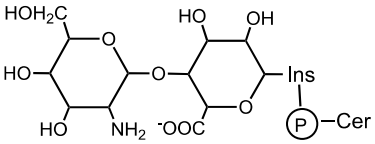
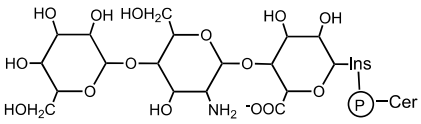
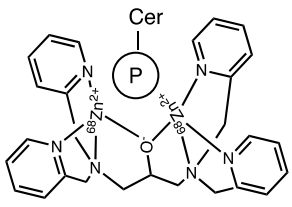
Plants	Structure of GIPC	*m/z*	Possible assignments of ceramide moieties	Ref.
Radish root		1148.5	*t*18:1/h16:0	[16,17]
		1232.7	*t*18:1/h22:0	
		1246.8	*t*18:1/h23:0	
		1258.7	*t*18:1/h24:1	
		1260.7	*t*18:1/h24:0	
	Series A	1282.7	*t*18:1/h24:0 + Na	
Rice bran		1407.9	*t*18:1/h23:0	[21]
		1423.9	*t*18:0/h24:0	
		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	

Fig. 1.

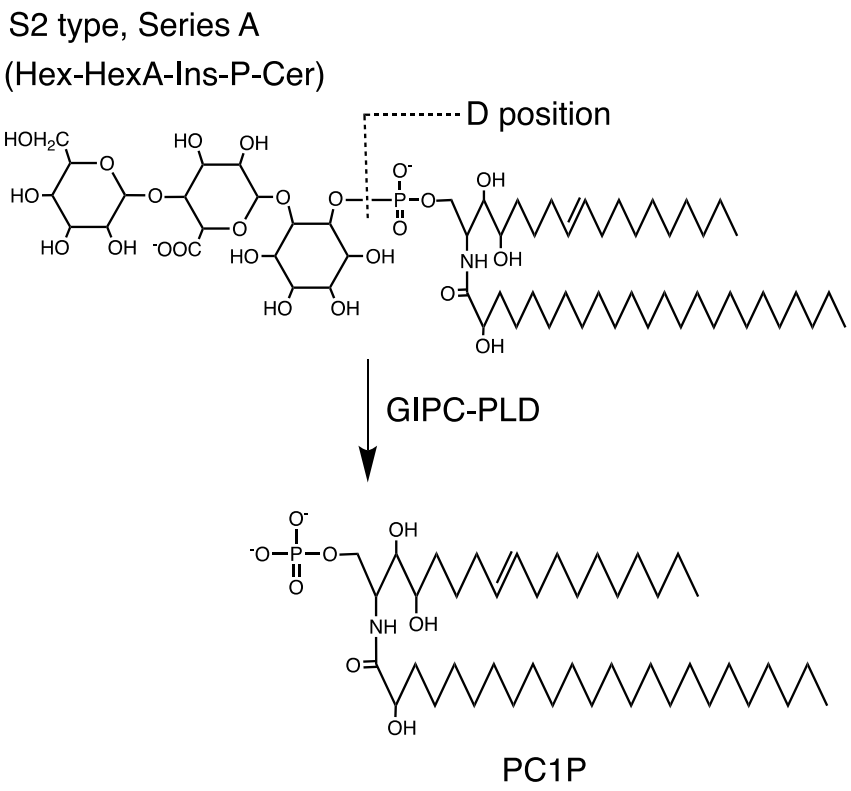


Fig. 2.

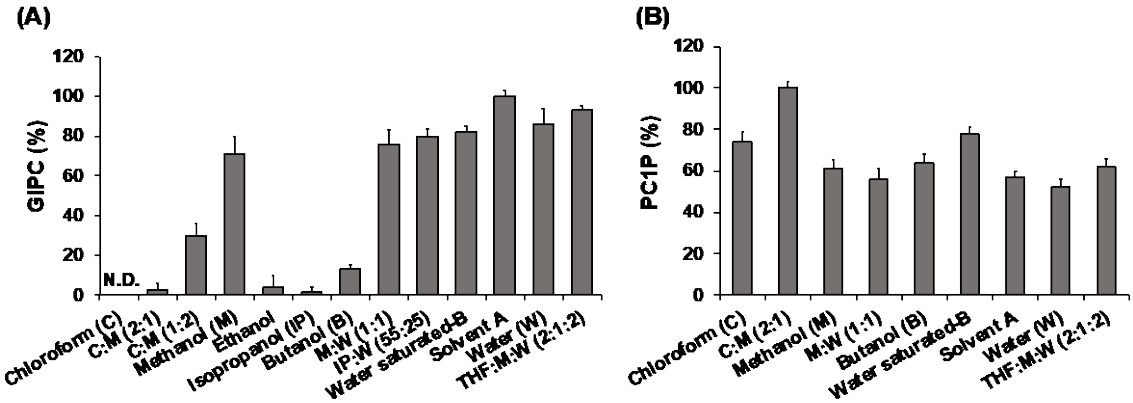

(A)
GIPC (%)
120
100
80
60
40
20
0
N.D.
Chloroform (C)
C:M (2:1)
C:M (1:2)
Methanol (M)
Ethanol
Isopropanol (IP)
Butanol (B)
M:W (1:1)
IP:W (55:25)
Water saturated-B
Solvent A
Water (W)
THF:M:W (2:1:2)
(B)
PC1P (%)
120
100
80
60
40
20
0
Chloroform (C)
C:M (2:1)
Methanol (M)
M:W (1:1)
Butanol (B)
Water saturated-B
Solvent A
Water (W)
THF:M:W (2:1:2)

Fig. 3.

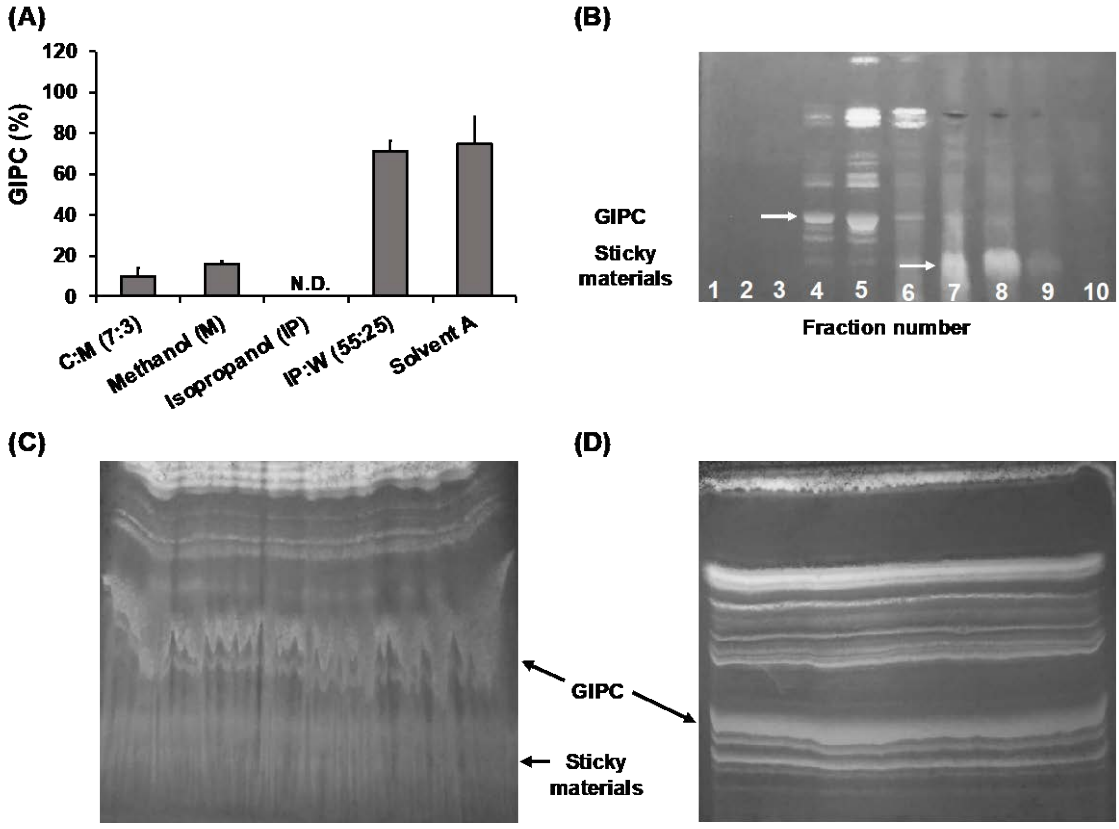

(A)
GIPC (%)
120
100
80
60
40
20
0
N.D.
C:M (7:3)
Methanol (M)
Isopropanol (IP)
IP:W (55:25)
Solvent A
(B)
GIPC
Sticky materials
1 2 3 4 5 6 7 8 9 10
Fraction number
(C)
(D)
GIPC
Sticky materials

Fig. 4.

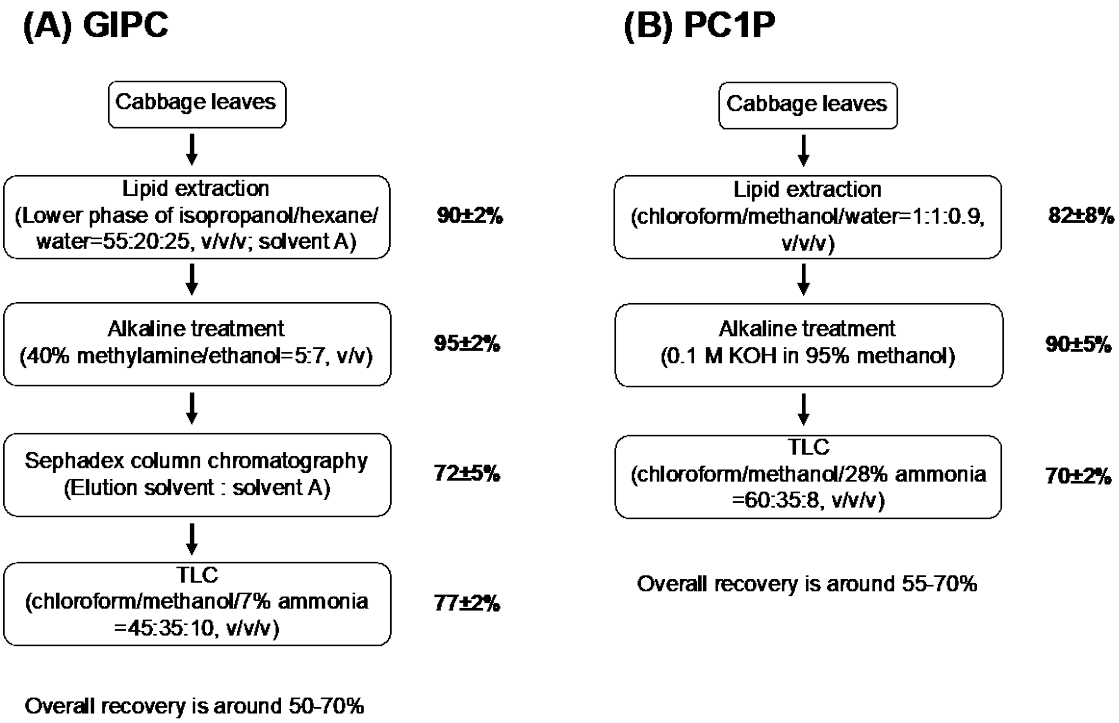

(A) GIPC
Cabbage leaves
Lipid extraction
(Lower phase of isopropanol/hexane/
water=55:20:25, v/v/v; solvent A)
90±2%
Alkaline treatment
(40% methylamine/ethanol=5:7, v/v)
95±2%
Sephadex column chromatography
(Elution solvent : solvent A)
72±5%
TLC
(chloroform/methanol/7% ammonia
=45:35:10, v/v/v)
77±2%
Overall recovery is around 50-70%
(B) PC1P
Cabbage leaves
Lipid extraction
(chloroform/methanol/water=1:1:0.9,
v/v/v)
82±8%
Alkaline treatment
(0.1 M KOH in 95% methanol)
90±5%
TLC
(chloroform/methanol/28% ammonia
=60:35:8, v/v/v)
70±2%
Overall recovery is around 55-70%

Fig. 5.

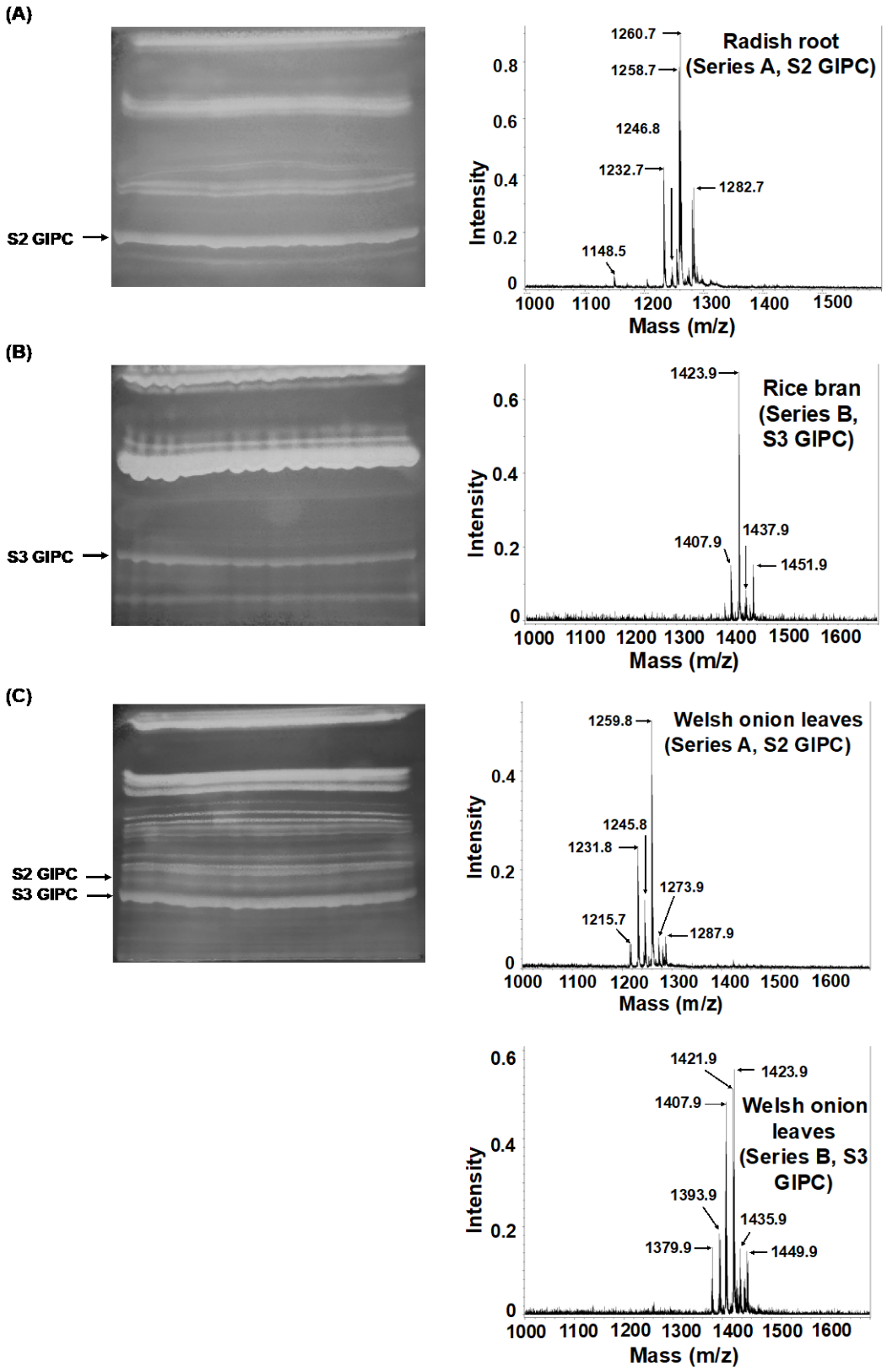

(A)
S2 GIPC
1260.7
1258.7
1246.8
1232.7
1282.7
1148.5
Radish root
(Series A, S2 GIPC)
Intensity
Mass (m/z)
(B)
S3 GIPC
1423.9
1437.9
1407.9
1451.9
Rice bran
(Series B,
S3 GIPC)
Intensity
Mass (m/z)
(C)
S2 GIPC
S3 GIPC
1259.8
1245.8
1231.8
1273.9
1215.7
1287.9
Welsh onion leaves
(Series A, S2 GIPC)
Intensity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

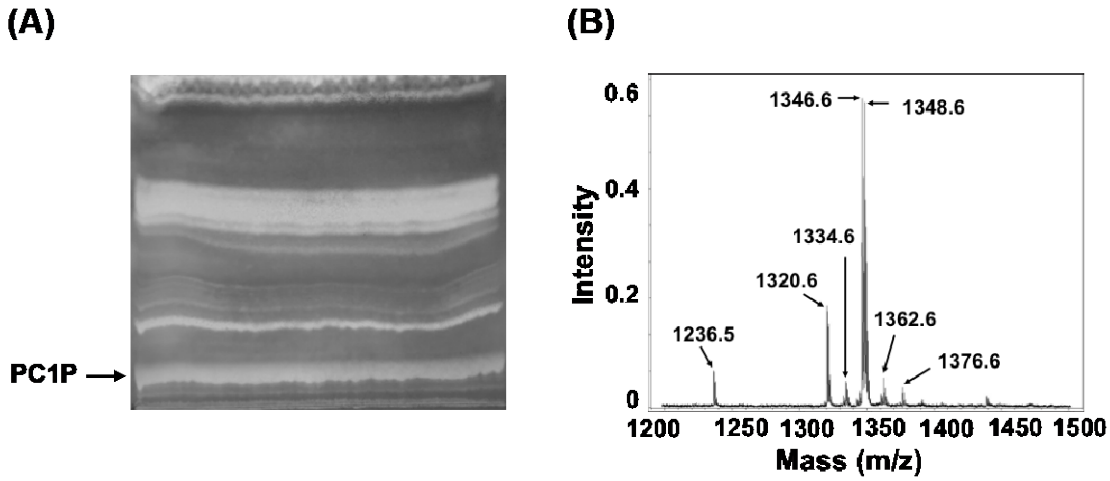
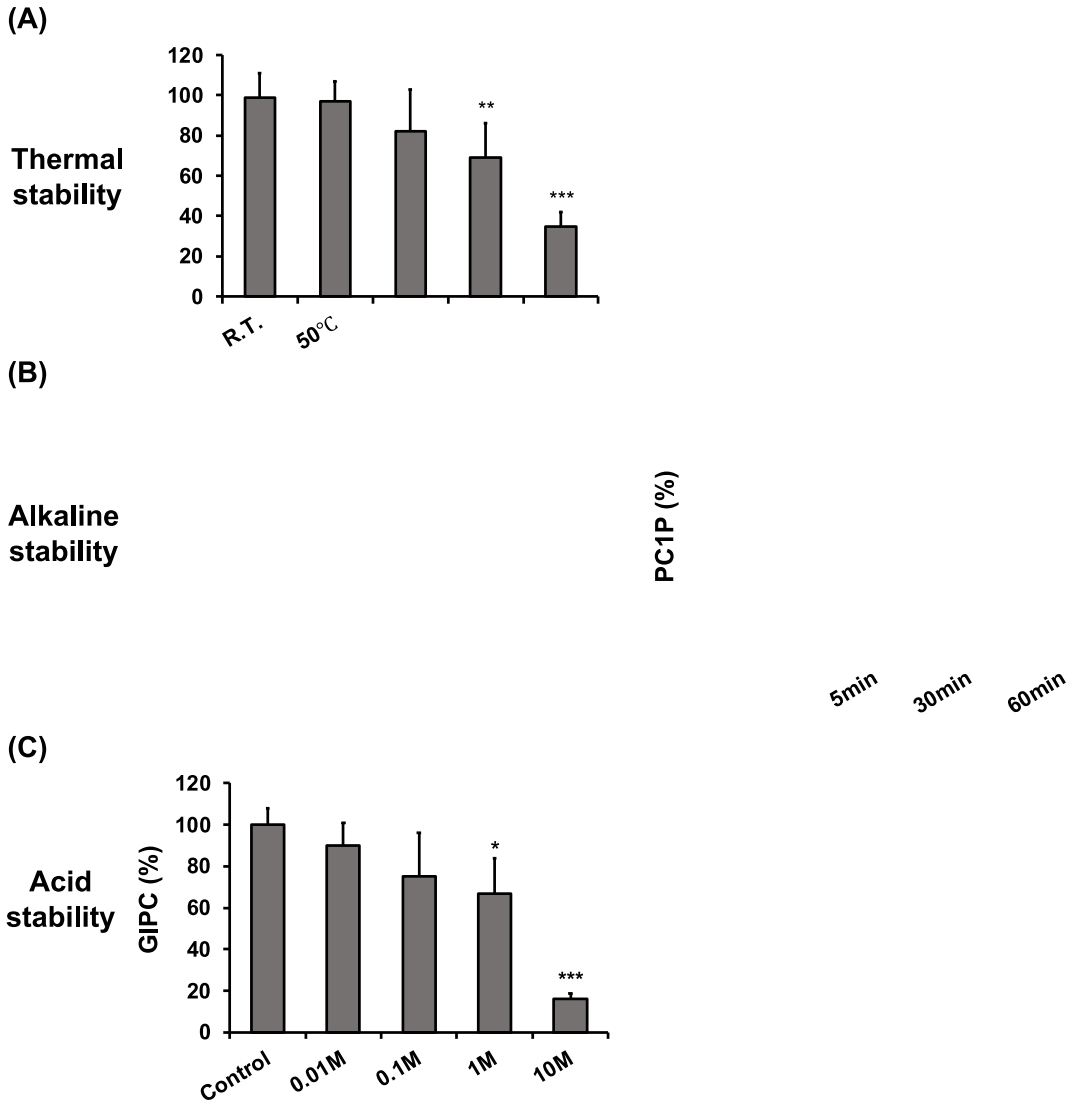
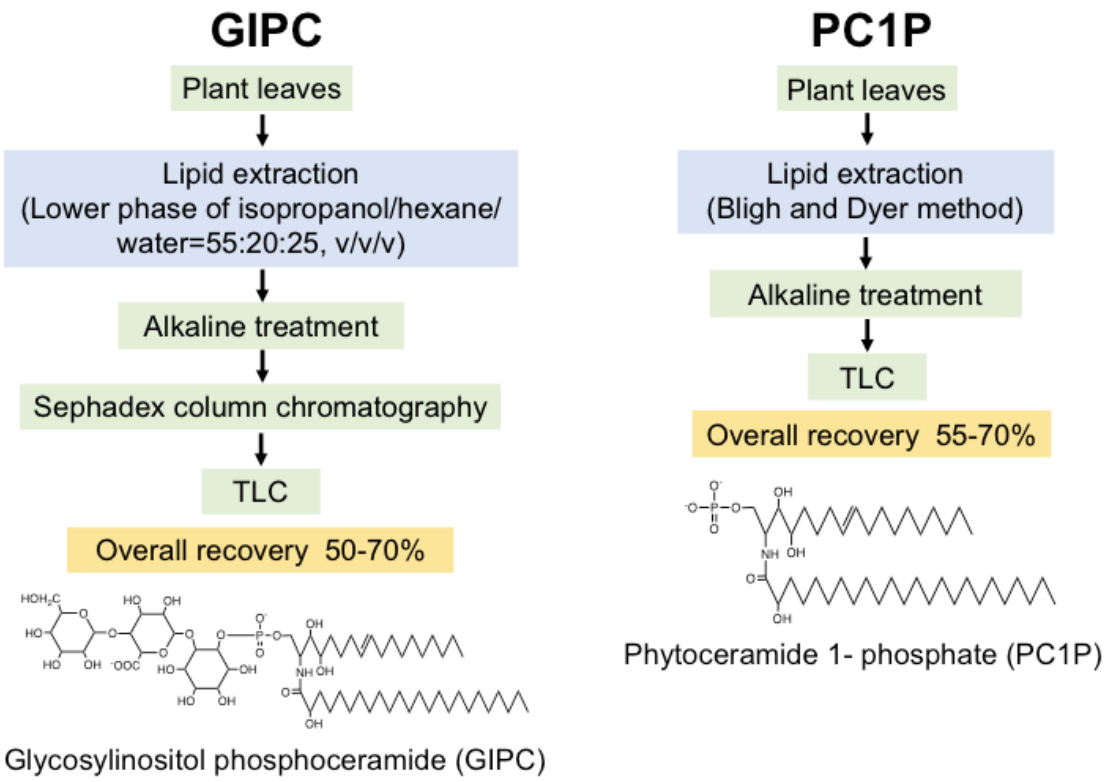

(A)
PC1P
(B)
Intensity
0.6
0.4
0.2
0
1346.6
1348.6
1334.6
1320.6
1362.6
1376.6
1236.5
1200
1250
1300
1350
1400
1450
1500
Mass (m/z)

Fig. 7.


(A)
Thermal stability
120
100
80
60
40
20
0
**
***
R.T.
50℃
(B)
Alkaline stability
PC1P (%)
5min
30min
60min
(C)
Acid stability
GIPC (%)
120
100
80
60
40
20
0
*
***
Control
0.01M
0.1M
1M
10M

Graphical abstract



Abbreviations: GIPC, glycosylinositol phosphoceramide; LCB, long-chain base; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PC1P, phytoceramide 1-phosphate; SM, sphingomyelin; THAP, 2,4,6-trihydroxyacetophenone; PLD, phospholipase D.

Footnotes: The long-chain bases of ceramide are designated as: phytosphingosine, t18:0; dehydrophytosphingosine, t18:1. The *N*-acyl residues of ceramide are designated as: α-hydroxy behenoyl, h22:0; α-hydroxy lignoceroyl, h24:0; α-hydroxy nervonoyl, h24:1; α-hydroxy cerotoyl, h26:0.