Regular paper, Lipid Biochemistry

Glycosylinositol phosphoceramide-specific phospholipase D activity catalyzes transphosphatidylation

Rumana Yesmin Hasi[1], Makoto Miyagi[1], Katsuya Morito[1], Toshiki Ishikawa[2], Maki Kawai-Yamada[2], Hiroyuki Imai[3], Tatsuya Fukuta[1], Kentaro Kogure[1], Kaori Kanemaru[4], Junji Hayashi[4], Ryushi Kawakami[4] and Tamotsu Tanaka[1,4]*

[1]Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8505, Japan
[2]Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan
[3]Graduate School of Natural Science, Konan University, Kobe 658-8501 Japan
[4]Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima 770-8513, Japan

Running title: Transphosphatidylation by GIPC-PLD

Corresponding Author: *Tamotsu Tanaka, Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima 770-8513, Japan.
Tel: +81-88-656-7256; E-mail: tanaka.tamotsu@tokushima-u.ac.jp

Abbreviations: GIPC, glycosylinositol phosphoceramide; IPC, inositol phosphoceramide; LCB, long-chain base; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MIPC, mannosylinositol phosphoceramide; NaDOC, sodium deoxycholate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PC1P, phytoceramide 1-phosphate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; THAP, 2,4,6-trihydroxyacetophenone; PLD, phospholipase D.

Enzyme(s): The enzyme mainly dealing with this manuscript is a kind of phospholipase D (E.C. No. 3.1.4.4). Partially purified enzyme does not hydrolyze glycerophospholipid, but hydrolyzes glycosylinositol phosphoceramide (GIPC). We abbreviated the enzyme activity to GIPC-PLD activity. Sources of this enzyme are plants.

Footnotes: The long-chain bases of ceramide are designated as: sphingosine, *d*18:1; phytosphingosine, *t*18:0; dehydrophytosphingosine, *t*18:1. 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.

Summary

Glycosylinositol phosphoceramide (GIPC) is the most abundant sphingolipid in plants and fungi. Recently, we detected GIPC-specific phospholipase D (GIPC-PLD) activity in plants. Here, we found that GIPC-PLD activity in young cabbage leaves catalyzes transphosphatidylation. The available alcohol for this reaction is a primary alcohol with a chain length below C4. Neither secondary alcohol, tertiary alcohol, choline, serine nor glycerol serves as an acceptor for transphosphatidylation of GIPC-PLD. We also found that cabbage GIPC-PLD prefers GIPC containing two sugars. Neither inositol phosphoceramide, mannosylinositol phosphoceramide nor GIPC with three sugar chains served as substrate. GIPC-PLD will become a useful catalyst for modification of polar head group of sphingophospholipid.

Keywords: Sphingolipid/Phytoceramide 1-phosphate/Glycosylinositol phosphoceramide/ Phospholipase D

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. 1). In general, the backbone of SM in most of mammalian cells is a C18 sphingosine (*d*18:1). On the other hand, a predominant backbone of GIPCs in plants and fungi is a C18 phytosphingosine (*t*18:0) or dehydrophytosphingosine (*t*18:1) (*1*). It has been known that α-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, α-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 SMase2 (*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*

74 hydrolyzed GIPC specifically, but did not hydrolyze phosphatidylcholine (PC),
75 phosphatidylethanolamine (PE), phosphatidylinositol (PI) or SM at substantial levels (*10,11*).
76 Based on these findings, we called this activity as "GIPC-PLD". We also examined the
77 distribution of GIPC-PLD activity in 25 tissues of 10 plants and detected it in roots in most of the
78 plants (*11*). Interestingly, the enzyme activity is higher in growing tissues, but under the detection
79 level in differentiated tissues such as outer aged leaves of cabbage and *Arabidopsis thaliana* (*11*).
80 A well-known characteristic of PLD is a reaction called transphosphatidylation, which
81 catalyzes transference of the phosphatidyl moiety to alcohol (*12*). This PLD-mediated reaction is
82 applicable to synthesis of functional phospholipids with a different head group, such as
83 phosphatidyl ascorbic acid (*13*). It is also available to synthesize PE and phosphatidylglycerol
84 from PC, which is naturally abundant (*12*).
85 In this study, we examined whether the GIPC-PLD activity in plants catalyzes
86 transphosphatidylation. Results showed that GIPC-PLD activities of cabbage young leaves and
87 radish root do it (Fig. 1). We also showed that this reaction proceeds during lipid extraction when
88 alcohol is used. GIPC-PLD will be potential catalyst for enzymatic modification of
89 sphingophospholipids.
90

Materials and Methods

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.

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.

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.

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-

157 TOF/MS as described below.

158

159 *MALDI-TOF/MS*

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

175

176

Results

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. 2A). 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. 2C). Because *t*18:1/h22:0, *t*18:1/h24:1 and *t*18:1/h24:0 were the major molecular species of PC1P produced from the GIPC by GIPC-PLD activity (Fig. 2D), the three major ions were assigned to phytoceramide 1-phosphomethanol (PC1Pmethanol) with ceramide structures of *t*18:1/h22:0, *t*18:1/h24:1 and *t*18:1/h24:0, respectively. They were the major molecular species of the series A GIPC of cabbage used as a substrate (Fig. 2B). 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. 2E). 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. 2F). 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 (Supplementary Fig. 1).

Available alcohols for transphosphatidylation of GIPC-PLD

Substrate specificity for transphosphatidylation of GIPC-PLD was characterized using various alcohols (Figs. 3, 4 and Table I). As shown in Fig. 3A, 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. 3B-D. The molecular species compositions of each transphosphatidylation product were essentially the same.

Quantitative analysis of these products revealed that GIPC-PLD prefers primary alcohols with

221 a shorter chain length, up to C4 (Fig. 4). Secondary and tertiary alcohols did not serve as acceptors
222 of transphosphatidylation of GIPC-PLD. In fact, phytoceramide phosphate containing 2-methyl-
223 1-propanol (primary) was detectable, whereas, formations of phytoceramide phosphate
224 containing 2-propanol (secondary) or 2-methyl-2-propanol (tertiary) were not observed. Attempts
225 to produce sphingolipids having polar head groups of glycerophospholipids were unsuccessful.
226 Neither choline, *L*-serine nor *DL*-glycerol was an acceptor of transphosphatidylation of GIPC-
227 PLD. Inositol is one of the structural components of GIPC. However, GIPC-PLD does not
228 catalyze transphosphatidylation of inositol. Tyrosol is an antioxidant alcohol. A sphingolipid
229 having a tyrosol residue was not formed by the treatment of GIPC-PLD even when the high
230 concentration of tyrosol was used (Fig. 4).
231
232 *Formation of PC1Pmethanol during homogenization of plant tissues.*
233 Previously, we demonstrated that PC1P is produced by activated GIPC-PLD during
234 homogenization of the plant tissues. Therefore, PC1P is not so abundantly present in the
235 homogenates if the GIPC-PLD in the leaves is inactivated before homogenization (*10*). We
236 confirmed this using heat-inactivated cabbage leaves. As shown in Fig. 5, the level of PC1P in
237 boiled leaves was very low compared to that of non-treated leaves that were homogenized in
238 water. In both preparations, the PC1Pmethanol level was negligible because GIPC-PLD was heat-
239 inactivated before mixing with methanol for extraction. When non-boiled cabbage leaves were
240 homogenized in the mixed solvent consisting of chloroform/methanol/water (1:2:0.8, v/v), a large
241 amount of PC1Pmethanol was formed, whereas, the amount of PC1P was greatly reduced. These
242 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.

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. 6, each substrate did not contain other analogues of GIPCs, and consisted mainly of *t*18:0/h26:0 (IPC and MIPC), *t*18:1/h24:1 (series A GIPC) and *t*18:0/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).

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 data was obtained using partially purified enzyme. A possibility that enzymes other than GIPC-PLD perform the transphosphatidylation can not 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 *Arabidpsis 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.

309 Primary alcohols below C4 can be attached to the phosphate group of sphingophospholipid by
310 this reaction. Modification of the polar head group of glycerophospholipids by PLDs has been
311 extensively studied, but it is applicable only to glyerophospholipids. Although available alcohol
312 is limited at present, protein engineering will expand the availability of alcohol in the
313 transphosphatidylation of GIPC-PLD. GIPC-PLD will be potential catalyst for enzymatic
314 modification of sphingophospholipids.
315

Acknowledgement

We thank Mr. Kazuki Tsuji and Ms. Mina Fujihara for technical assistance.

Funding

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (24580185), Sugiyama Sangyou Kagaku Research Foundation and the research program for development of intelligent Tokushima artificial exosome (iTEX) from Tokushima University.

Conflict of interest

None declared for all authors

Author contributions

RYH is primary author of this manuscript, conducted most of experiments, data analysis with aid of TF, K. Kogure and TT. RYH and TT designed this study. MM and KM contributed to mass spectrometric analyses of sphingophospholipids. TI, MKY, HI, JH, RK and K. Kanemaru contributed to preparations of IPC, MIPC and GIPCs. They also critically revised this manuscript. All authors have approved this manuscript.

Legend to figures

Figure. 1. 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, *t*18:1) and an α-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.

Figure. 2. 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.

Figure. 3. 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).

Figure. 4. 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 I.

Figure. 5. 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.

Figure. 6. Substrate specificity of GIPC-PLD of cabbage leaves

382 IPC and MIPCs (series 0 GIPC) were purified from lipids of Saccharomyces cerevisiae. Series A
383 and series B GIPC were obtained from cabbage leaves and rice bran, respectively, as described in
384 materials and methods(A-D). The purified GIPCs were treated with the GIPC-PLD fraction of
385 cabbage leaves. Resulting PC1P were isolated by TLC and quantified (E). Data are expressed as
386 mean ± S.D. from three independent experiments.
387

References

1. Buré, C., Cacas, J-L., Mongrand, S. and Schmitter, J-M. (2014) Characterization of glycosyl inositol phosphoryl ceramides from plants and fungi by mass spectrometry. *Anal. Bioanal. Chem.* **406**, 995-1010

2. Yamashita, R., Tabata, Y., Iga, E., Nakao, M., Sano, S., Kogure, K., Tokumura, A. and Tanaka, T. (2016) Analysis of molecular species profiles of ceramide-1-phosphate and sphingomyelin using MALDI-TOF mass spectrometry. *Lipids* **51**, 263-270

3. Aida, E.C., Felix, M.G. and Kolensnick, R. (2002) Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outocome? *FEBS Lett.* **531**, 47-53

4. Matmati, N. and Hannum, Y.A. (2008) *ISC1* (inositol phosphosphingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases. *J. Lipid Res.* **49**, 922-928

5. Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domae, N. and Hannun, Y.A. (2000) Identification of *ISC1* (*YER019w*) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 39793-39798

6. Henry, J., Guillotte, A., Luberto, C. and Poeta, M.D. (2011) Characterization of inositol phospho-sphingolipid-phospholipase C1 (Isc1) in *Cryptococcus neoformans* reveals uniquebiochemical features. *FEBS Lett.* **585**, 635-640

7. Zhang, O., Wilson, M.C., Xu, W., Hsu, F-F., Turk, J., Kuhlmann, M., Wang, Y.,

Soong, L., Key, P., Beverley, S.M. and Zhang, K. (2009) Degradation of host sphingomyelin is essential for Leishmania Virulence. *PLoS One* **5**, e10000692

8. Selvy, P.E., Lavieri, R.R., Lindsley C.W. and Brown H.A. (2011) Phospholipase D: Enzymology, functionality, and chemical modulation. *Chemical Rev.* **111**, 6064-6119

9. Murakami, M.T., Fernandes-Pedrosa M.F., de Andrade, A.A., Gabdoulhakov, A., Betzel, C., Tambourgi, D.V. and Arni, R.K. (2006) Structural insights into the catalytic mechanism of sphingomielinases D and evolutionary relationship to glycerophosphodiester phosphodiesterases. *Biochem. Biophys. Res. Commun.* **342**, 323-329

10. Tanaka, T., Kida, T., Imai, H., Morishige, J., Yamashita, R., Matsuoka, H., Uozumi, S., Satouchi, K., Nagano, M. and Tokumura, A. (2013) Identification of a sphingolipid-specific phospholipase D activity associated with the generation of phytoceramide-1-phosphate in cabbage leaves. *FEBS J.* **280**, 3797-3809.

11. Kida, T., Itoh, A., Kimura, A., Matsuoka, H., Imai, H., Kogure, K., Tokumura A. and Tanaka, T. (2017) Distribution of glycosylinositol phosphoceramide-specific phospholipase D activity in plants. *J. Biochem.* **162**, 449-458

12. Damnjanović J. and Iwasaki, Y. (2013) Phospholipase D as catalyst: Application in phospholipid synthesis, molecular structure and protein engineering. *J. Biosci. Bioeng.* **116**, 271-280

13. Nagao, A., Ishida, N. and Terao, J. (1991) Synthesis of 6-phosphatidyl-L-ascorbic acid by phospholipase D. *Lipids* **26**, 390-394

14. Glick B.S. and Pon L.A. (1995) Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods in Enzymol.* **260**, 213-223

15. Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917

16. Markham, J.E., Li, J., Cahoon, E.B. and Jaworski, J.G. (2006) Separation and identification of major plant sphingolipid classes from leaves. *J. Biol. Chem.* **281**, 22684-22694

17. Chalvardjian, A. and Rudnicki, E. (1970) Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.* **36**, 225-230

18. Davidson, F.M. and Long, C. (1958) The structure of the naturally occurring phosphoglycerides, 4. Action of cabbage-leaf phospholipase D on ovolecithin and related substances. *Biochem. J.* **69**, 458-466

19. Yang, S.F. and Freer, S. and Benson, A.A. (1967) Transphosphatidylation by phospholipase D. *J. Biol. Chem.* **242**, 477-484

20. Lipmann, F. and Tuttle, L.C. (1950) Lipase-catalysed condensation of fatty acids with hydroxylamine. *Biochim. Biophys. Acta* **4**, 301-309

21. Hechtberger, P., Zinser, E., Saf, R., Hummel, K., Paltauf, F. and Daum, G. (1994) Characterization, quantification and subcellular localization of inositol-containing sphingolipids of the yeast, *Saccharamyces cerevisiae*. *Eur. J. Biochem.* **225**, 641-649

22. Ishikawa, T., Ito, Y. and Kawai-Yamada, M. (2016) Molecular characterization and targeted quantitative profiling of the sphingolipidome on rice. *The Plant J.*

88, 681-693

23. Yang, H. and Roberts, M.F. (2003) Phosphohydrolase and transphosphatidylation reactions of two Streptomyces phospholipase D enzymes: Covalent versus noncovalent catalysis. *Protein Sci.* **12**, 2087-2098

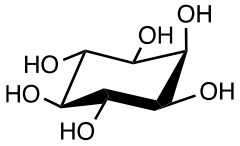
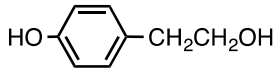
24. Cacas, J-L., Buré, C., Furt F., Maalouf J-P., Badoc, A., Cluzet, S., Schmitter, J-M., Antajan, E. and Mongrand, S. (2013) Biochemical survey of the polar head of plant glycosylinositolphosphoceramides unravels broad diversity. *Phytochem.* **96**, 191-200

25. Nina, B. and Humpf H-U. (2013) Structural profiling and quantitation of glycosyl inositol phosphoceramides in plants with fourier transform mass spectrometry. *J. Agric. Food Chem*. **61**, 4257-4269

26. Smith, C.K. and Fry, S.C. (1999) Biosynthetic origin and longevity in vivo of □-D-mannopyranosyl-(1→4)-□-D-glucuronopyranosyl-(1→2)-*myo*-inositol, an unusual extracellular oligosaccharide produced by cultured rose cells. *Planta* **210**, 150-156

470 **Table 1**

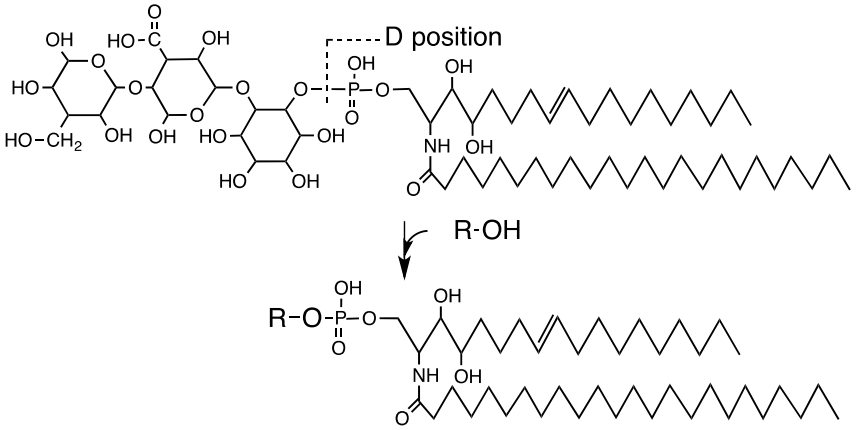
471 **Availability of alcohol for GIPC-PLD-catalyzed transphosphatidylation**

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	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$	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

472

Fig. 1

GIPC (series A)
Hex-HexA-Ins-P-Cer

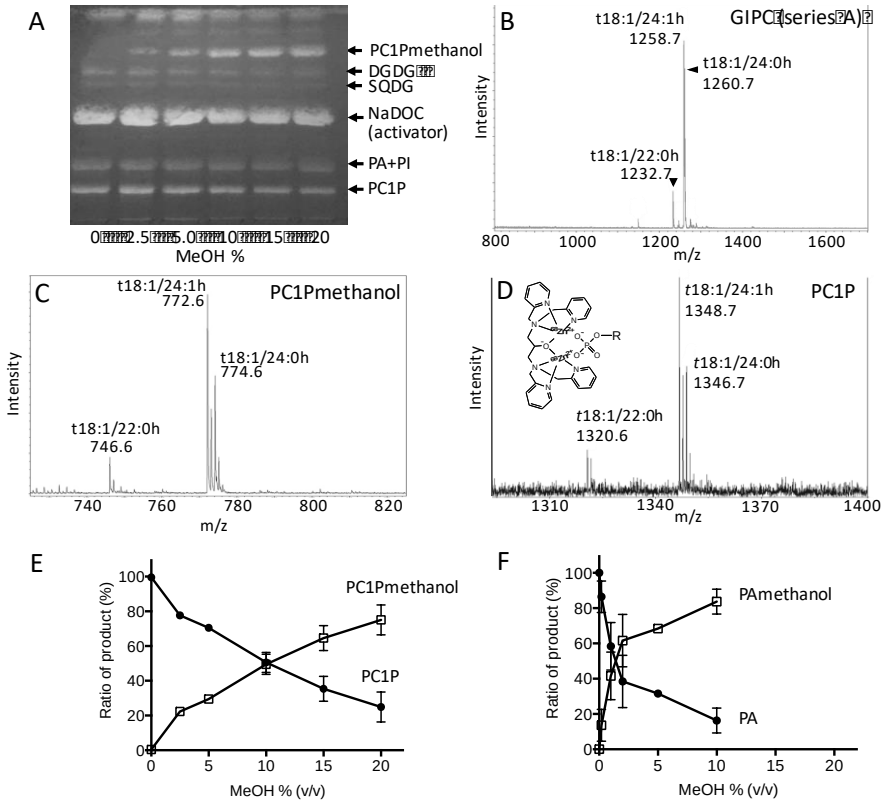


PC1Palcohol
Phytoceramide 1-phosphoalcohol

473

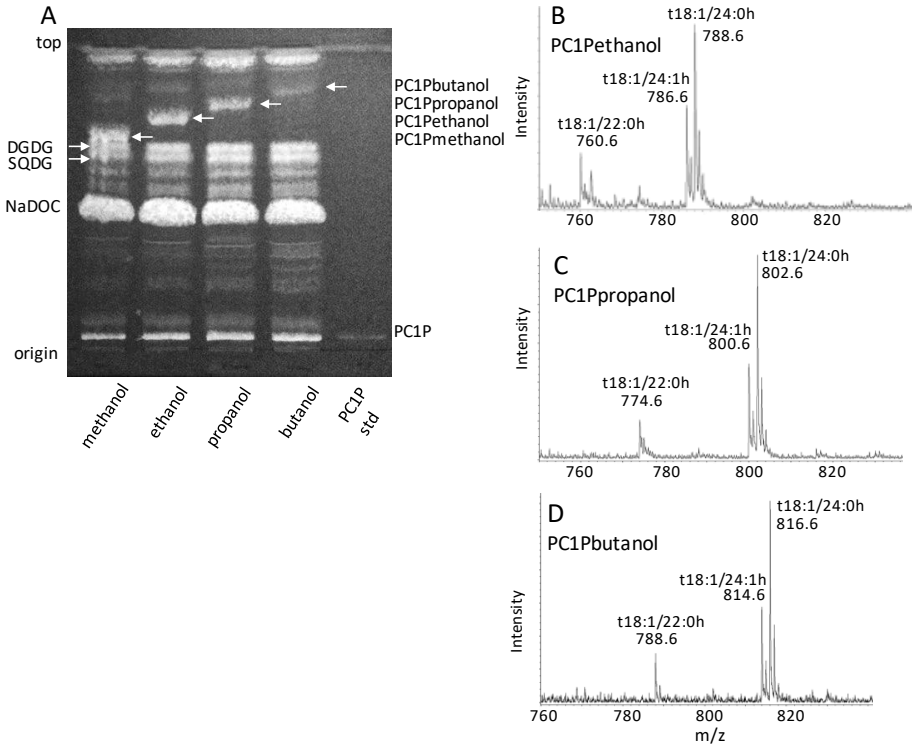
474

Fig. 2


A
PC1Pmethanol
DGDG
SQDG
NaDOC
(activator)
PA+PI
PC1P
MeOH %
B
GIPC (series A)
t18:1/24:1h
1258.7
t18:1/24:0h
1260.7
t18:1/22:0h
1232.7
Intensity
m/z
C
PC1Pmethanol
t18:1/24:1h
772.6
t18:1/24:0h
774.6
t18:1/22:0h
746.6
Intensity
m/z
D
PC1P
t18:1/24:1h
1348.7
t18:1/24:0h
1346.7
t18:1/22:0h
1320.6
Intensity
m/z
E
PC1Pmethanol
PC1P
Ratio of product (%)
MeOH % (v/v)
F
PAmethanol
PA
Ratio of product (%)
MeOH % (v/v)

475
476

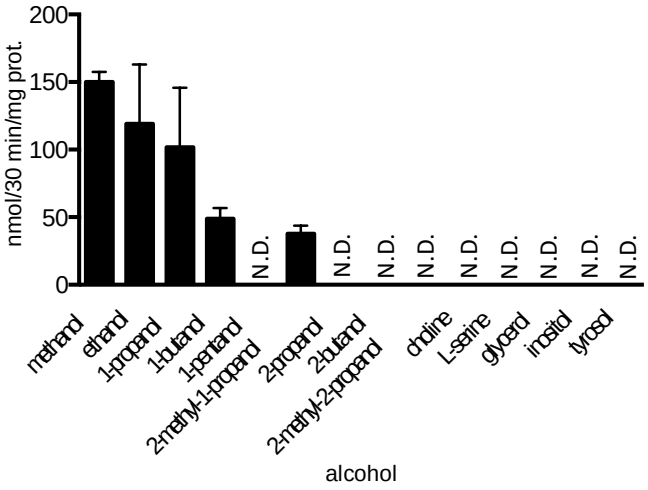
Fig.3 revise



477

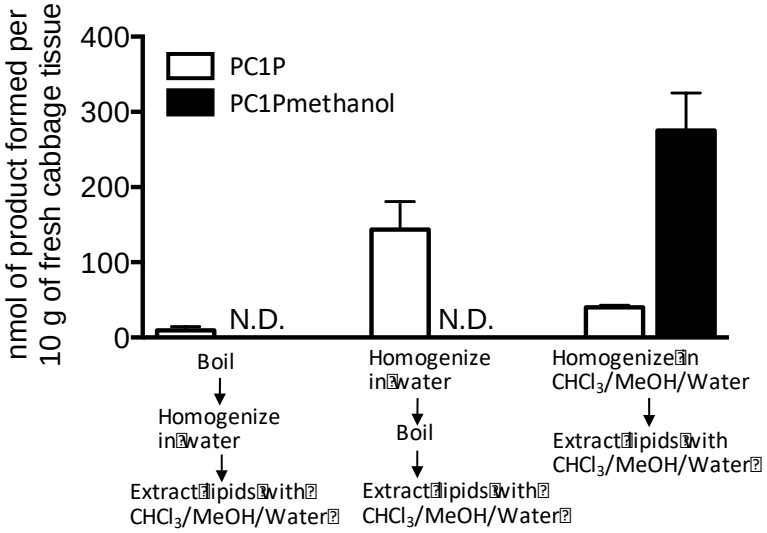
478

Fig.4


200
150
100
50
0
nmol/30 min/mg prot.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
methanol
ethanol
1-propanol
1-butanol
1-pentanol
2-methyl-1-propanol
2-propanol
2-butanol
2-methyl-2-propanol
choline
L-serine
glycerol
inositol
tyrosol
alcohol

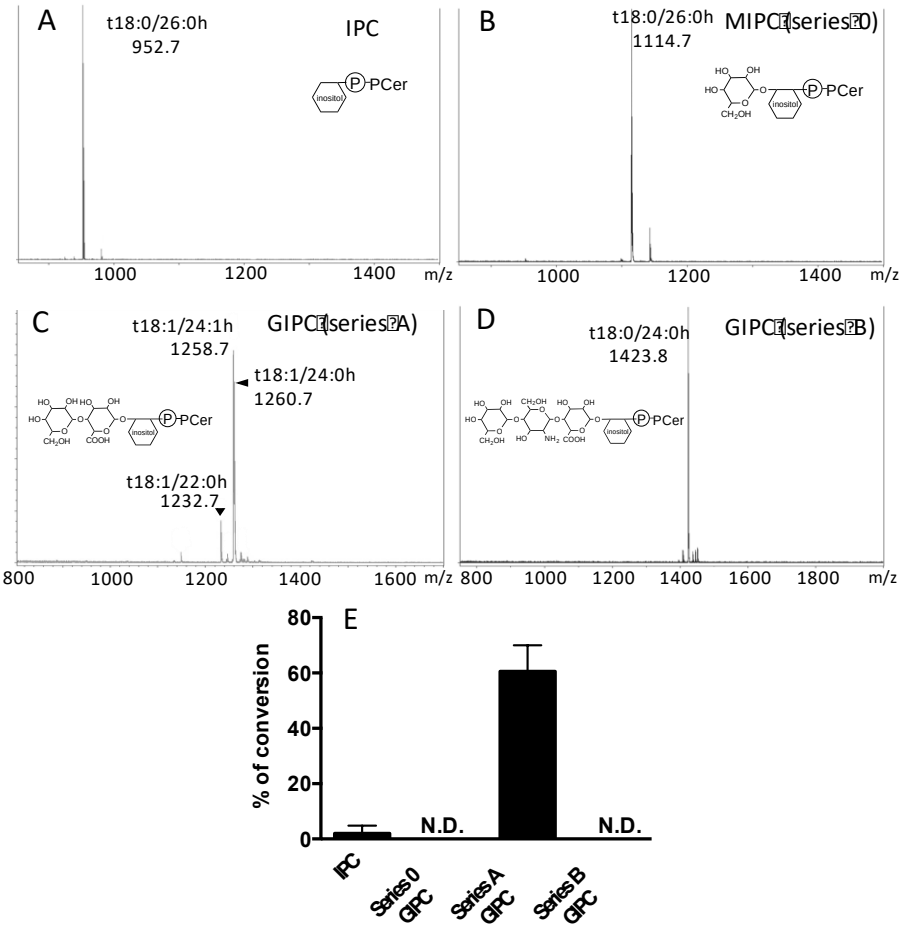
479
480

Fig. 5


nmol of product formed per 10 g of fresh cabbage tissue
400
300
200
100
0
PC1P
PC1Pmethanol
N.D.
N.D.
Boil
Homogenize in water
Extract lipids with CHCl3/MeOH/Water
Homogenize in water
Boil
Extract lipids with CHCl3/MeOH/Water
Homogenize in CHCl3/MeOH/Water
Extract lipids with CHCl3/MeOH/Water

481
482

Fig. 6


A
t18:0/26:0h
952.7
IPC
PCer
inositol
1000
1200
1400
m/z
B
t18:0/26:0h
1114.7
MIPC (series 0)
C
t18:1/24:1h
1258.7
GIPC (series A)
t18:1/24:0h
1260.7
t18:1/22:0h
1232.7
800
1600
D
t18:0/24:0h
1423.8
GIPC (series B)
1800
E
% of conversion
80
60
40
20
0
N.D.
IPC
Series 0 GIPC
Series A GIPC
Series B GIPC

483