Quantitative analysis of glycosylinositol phosphoceramide and phytoceramide 1-phosphate in vegetables

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Summary Previously, we found an unidentified sphingolipid in cabbage, and determined it as phytoceramide 1-phosphate (PC1P). PC1P is found to be produced from glycosylinositol phosphoceramide (GIPC) by the action of phospholipase D (PLD) activity. Although GIPC is abundant sphingolipid, especially in cruciferous vegetables, amount of daily intake, digestibility and nutritional activity of GIPC are not well understood. Here, we investigated amounts of GIPC and PC1P in vegetables. GIPC was found in all vegetables examined (13 kinds) at levels 3-20mg/100g (wet weight). On the other hand, PC1P was present in limited vegetables which show higher GIPC-PLD activity, such as inner cabbage leaves (5.2mg/100g). Because PC1P is formed during homogenization by activated GIPC-PLD, level of PC1P in boiled cabbage leaves was very low. Although digestibility of GIPC is unknown at present, a portion of dietary GIPC is considered to be converted to PC1P during mastication by plant-derived GIPC-PLD activity in some vegetables.

Key Words sphingophospholipid, plant foodstuffs, phospholipase D

Sphingolipids are ubiquitously present in eukaryotes and serve as constituents of biomembrane. Recent investigation revealed that sphingolipids form special architecture in membrane with cholesterol called lipid raft which serves isolated core for the assembly of functional proteins of signaling processes (1). Sphingolipids also serve as sources of signaling molecules. Typical sphingolipid-derived intracellular molecules are ceramide and sphingosine both of which mediate apoptosis and differentiation of mammalian cells and yeast (2). The facts that genetic deletions of metabolic enzymes for sphingolipids induce failure of these cellular processes clearly indicate the importance of the lipids for cellular fate (2).

Ceramide, a backbone of sphingophospholipids and glycosphingolipids, is a main component of the stratum corneum of the epidermis layer of animal skin. It creates water-impermeable structure (3). It is demonstrated that the decline of ceramide contents is associated with decrease in the moisture contents in skin (3,4). Based on this notion, cosmetics or dietary supplements which supply ceramide to the skin are believed to enhance skin beauty, and is creating a big market. However, it is controversial that dietary sphingolipids increase the amount of ceramide in skin. Because digestibility and absorption of dietary sphingolipids are reported to be low (5), and the most parts of dietary sphingolipids absorbed in intestinal epithelia are broken into fatty acids (5,6). On the other hand, a possibility that dietary sphingolipids up-regulate biosynthetetic activity of sphingolipids in our body by supplying components of sphingolipids can not be ruled out. To clarify these points, amounts of sphingolipid taken from diet, digestibility and distribution of ingested sphingolipids and effect of dietary sphingolipids on our sphingolipid biosynthetic activity should be examined in moe detail.

Glycosylinositol phosphoceramide (GIPC), glucosylceramide and ceramide are principal sphingolipids in plants. Relative content of them is reported to be 64%, 34% and 2% respectively, of the total sphingolipids in Arabidopsis thaliana (7). The abundance of GIPC has been reported in other vegetables such as tomato, soybean and rice (7,8). However, available information on GIPC contents in other plant foodstuff is limited. Furthermore, digestibility of GIPC in our gastrointestinal (GI) tract is completely unknown. On the other hand, amounts and digestibility of glucosylceramide in food have been reported by several investigators (9-11).

Previously, we detected an unidentified sphingophospholipid in homogenates of cabbage leaves and determined it as phytoceramide 1-phosphate (PC1P) with ∝-hydroxy fatty acids as the *N*-acyl residues (12).

This was the first report to show evidence of natural occurring ceramide 1-phosphate (C1P) class sphingolipid in plants. PC1P accounted for 5% of total phospholipids in homogenates of cabbage leaves. We also found enzyme activity that produces PC1P by hydrolysis of the D position of GIPC in cabbage leaves. Importantly, a partially purified GIPC-PLD fraction from cabbage leaves did not hydrolyze phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SM) at substantial levels. Conversely, PLD fractions prepared from cabbage leaves did not hydrolyze GIPC (12). Other properties, such as calcium requirements and pH dependency, were also distinct. These observations indicated the presence of an unidentified enzyme that specifically hydrolyzes GIPC to PC1P in cabbage leaves. Based on this findings, we called this enzymatic activity in cabbage leaves to "GIPC-PLD". Recently, we found that GIPC-PLD activity is detected in roots or young tissues in plants and activated during homogenization of the raw tissues (13). Thus, we always ingest PC1P from some plant foodstudds. However, there are no reports on PC1P content in diets.

Sphingomyelin (SM) is one of the major sphingolipids in animals. Sphingosine (*d*18:1), sphinganine (dihydrosphingosine, *d*18:0) are frequently found as a sphingoid backbone in mammalian cells (14). On the other hand, the major sphingoid backbone of GIPCs in plants and fungi is phytosphingosine and phytosphinganine (*t*18:1 and *t*18:0 respectively). It has been known that α-hydroxy fatty acid with long (such as C16) or very long chain (such as C24) is linked as N-acyl residue in GIPC (7,12). In contrast, α-hydroxy fatty acid is rarely detected in SM except for skin.

In the present study, we examined amounts of GIPC and PC1P in 13 vegetables.

2. Materials and Methods

2-1. Plant materials

Vegetables used in this study were cabbage (Brassica oleracea L. var. capitata), broccoli (Brassica oler- acea L. var. italica), radish (Raphanus sativus L.), Japanese mustard spinach called komatsuna in Japan (Brassica rapa L) and carrot (Daucus carota L.). These vegetables were obtained from a local farmer. Cucumber (Cucumis sativus L), burdock (Arctium lappa L.), welsh onion (Allium fistulosum L.), rice flour and wheat flour were purchased from local markets. Seeds of mung bean (Vigna radiata) were obtained from a local nursery. As animal food ingredients, salmon, chicken eggs, pork were purchased from a local market.

2-1. Isolation of GIPC:

Lipids were extracted from the foodstuffs by the modified procedure reported by Markham et al (7). First, foodstuffs were heated in hot water at 80℃ for 5 min to inactivate the lipolytic enzymes. The boiled tissues were homogenized in the lower layer of a mixed solvent consisting of isopropanol/water/hexane (55:25:20, v/v/v) (solvent A) using an ultradisperser followed by centrifugation (1,100 x g, 4 ℃, 10 min). After centrifugation, the supernatant was collected and evaporated to dry. The lipid extracts were incubated at 50℃ for 1h in 40% methylamine/ethanol (7:5, v/v) to remove glycerolipids. After drying, the extracted lipids were dissolved in a small volume of solvent A and subjected to the thin-layer chromatgraphy (TLC) using a solvent system consisting of chloroform/methanol/4 M ammonia (45:35:10, v/v/v). The obtained GIPC was quantified by colorimetric method on the basis of phosphomolybdenum-malachite green formation (15).

2-2. Isolation of PC1P

Plant materials were chopped into very small pieces and homogenized using an ultradisperser. The homogenized plant tissues were heated in boiled water for 5 min to inactivate the lipolytic enzymes. Then, lipids of vegetables were extracted according to Bligh & Dyer method with acidification of water/ methanol phase as described previously (16). The obtained lipids were treated with 0.1 M KOH in 95% methanol at 65℃ for 15 min. After cooling, the alkali lysates were extracted by Bligh & Dyer method under acidic condition. The lipid extracts were subjected to TLC and developed in chloroform/ methanol/ 28% aqueous ammonia (60:35:8, v/v/v). After development, the PC1P was extracted from silica gel by Bligh & Dyer method under acidic condition. The isolated PC1P was determined by colorimetric method on the basis of phosphomolybdenum-malachite green formation (15).

3. Results

We determined the amount of GIPC in 13 different kinds of vegetables (Fig.2). GIPC content of leaf vegetables (cabbage, komatsuna and lettus) was estimated to be 10-20 mg. On the other hand, in root vegetables (radish, carrot, burdock), it was 7-9 mg/100g. The fruit vegetables (cucumber and tomato) and tuber vegetable such as potato contained less than 5 mg/100g of GIPC. The amount of GIPC in bulb vegetables (onion and leek) was 6-8 mg/100g. The GIPC was not detected in wheat and rice flours. Foodstuffs from animal sources, such as pork, salmon and egg, did not contain detectable GIPC. As we demonstrated previously [13], amount of PC1P was depending on the GIPC-PLD activity in the materials. In fact, the highest PC1P content was found in raw cabbage leaves (5 mg/100g), whereas, the boiled cabbage leaves contained 1/5 of that of the raw materials (Fig. 3). The raw radish roots contained 2 mg/100g of PC1P. The contents of PC1P in boiled radish and mung bean sprouts were less than 1 mg/100g. On the other hand, PC1P was not found

in komatsuna leaves, lettuce leaves, radish leaves, carrot roots, rice flour and white flour. Pork, salmon meat and egg yolk did not contain PC1P.

4. Discussion

Our daily intakes of sphingomyelin and glucosylceramide are reported to be around 300 mg/day (17) and 26-77 mg/day (18), respectively. Although GIPC is reported to be the most abundant sphingolipids in plants, the GIPC content in vegetables has not been extensively studied. In this study, we found that contents of GIPC in 13 kinds of vegetables were around 3-20 mg/100g. The average of GIPC amount in vegetables was 10 mg/100g. According to the national health and nutrition survey, Japanese people ingests 283.1 g of vegetables per day, so daily GIPC intake is calculated to be about 28 mg/day. This value is comparable to that of glucosylceramide. It is reported that dietary sphingomyelin is hydrolyzed to ceramide and phospholcholine by alkaline sphingomyelinase in the small intestine (19-20). The resulting ceramide is converted to sphingosine and fatty acid (triglycerides) by acid ceramidase (19-20). Although all subtypes of sphingomyelinases are present in the intestinal tract (20), alkaline sphingomyelinase is known to be localized abundantly in jejunum and little in ilium, duodenum, stomach and colon (19). Acidic ceramidase activity is found in intestinal mucosa and lumen. Sphigosine is thought to be absorbed in the intestinal enterocytes. After absorption, most of sphingosine is metabolized to palmitic acid, and a small portion is resynthesized to complex sphingolipids (21). The digestibility and absorption of plant-derived glucosylceramide are similar to that of SM except for first enzyme attacking to glucosylceramide. The glucosylceramide is firstly degraded to phytoceramide and glucose by glucosylceramidase, followed by conversion of phytoceramide to phytosphingosine and free fatty acid (6). Not only sphingosine (*d*18:1) base but also sphinganine (*d*18:0) and sphingadienine (*d*18:2) bases are present in plant sphingolipids (22). It has been reported that these plant origin sphingoid bases have beneficial effects in the skin. (14).

At present, we do not know the digestibility of GIPC in our GI tract and absorption. Possible digestive process of GIPC is the following two routes. 1) GIPC to phytoceramide and glycosylinositol phosphate. This enzyme activity, namely, GIPC-PLC has been reported to be present in yeast (23-26), but not in plants and animals to our best knowledge. However, it does not mean digestive inability of GIPC in animals. Many phosphodiesterase (PDE) genes are identified in human [27]. At present, 11 PDE genes (glycerophosphodiesterase (GDE) family genes) have been identified so far. But, they have not been fully characterized yet. It is interesting to investigate whether GIPC can be hydrolyzed by such potential enzymes. It is also interesting to investigate whether microbial flora in animal gut digest GIPC at C or D position. 2) GIPC to PC1P and glycosylinositol. The enzyme, namely GIPC-PLD, has been found by us [12]. It is widely distributed in plants. As mentioned above, enzymes classified to GDE family are potential candidates to perform this process. It is also interesting to investigate whether animals or microbial flora living in animal gut hydrolyze at D position of GIPC.

Here we investigated 13 vegetables. PC1P was detected in cabbage leaves, radish roots, mung bean sprouts. This result reflects GIPC-PLD activity in the plant tissues (13). The amount of PC1P in these vegetables is around 2.3 mg/100g. Our recent findings showed that PC1P is dephosphorylated by the intestinal enzyme and is digested into phytoceramide. So, phytoceramide derived from PC1P may follow the glycosylceramide degradation pathway. It remains unknown whether PC1P is incorporated into intestinal cells without hydrolysis.

Effects of dietary sphingolipids on skin beauty (14) or prevention of colon cancer (28) are now attracting much attention. At present, physiological relevance of dietary GIPC and PC1P is largely unknown, despite that they are major sphingolipids in plant foodstuff. To know this, in vivo and in vitro experiments on digestibility of GIPC and PC1P are needed.

COI: The authors have no conflict of interest associated with this manuscript.

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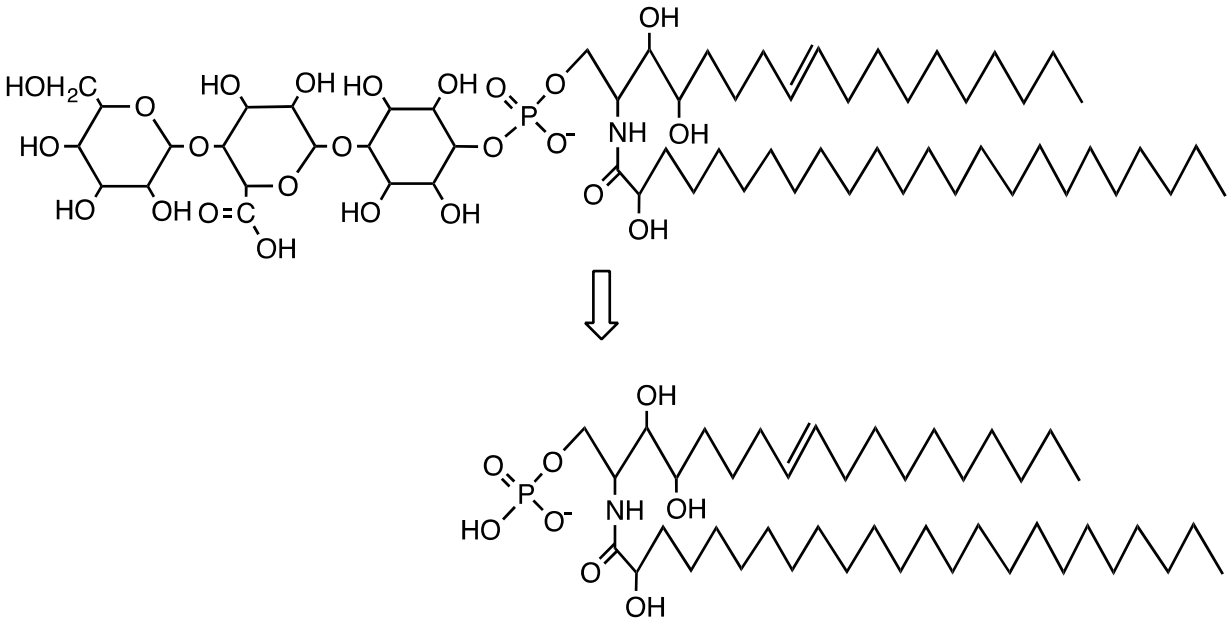
Legend to Figures

Fig. 1. Structure of glycosylinositol phosphoceramide (GIPC) and phytoceramide 1-phosphate (PC1P), and action of GIPC-specific phospholipase D

Fig. 2. Amounts of glycosylinositol phosphoceramide (GIPC) in foodstuffs

Fig. 3. Amount of phytoceramide 1-phosphate (PC1P) in foodstuffs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HO
OH
O
HO
OH
HO
OH
HO
OH
O=C
OH
HO
OH
O
P
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NH
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OH
O
OH

Fig. 1.

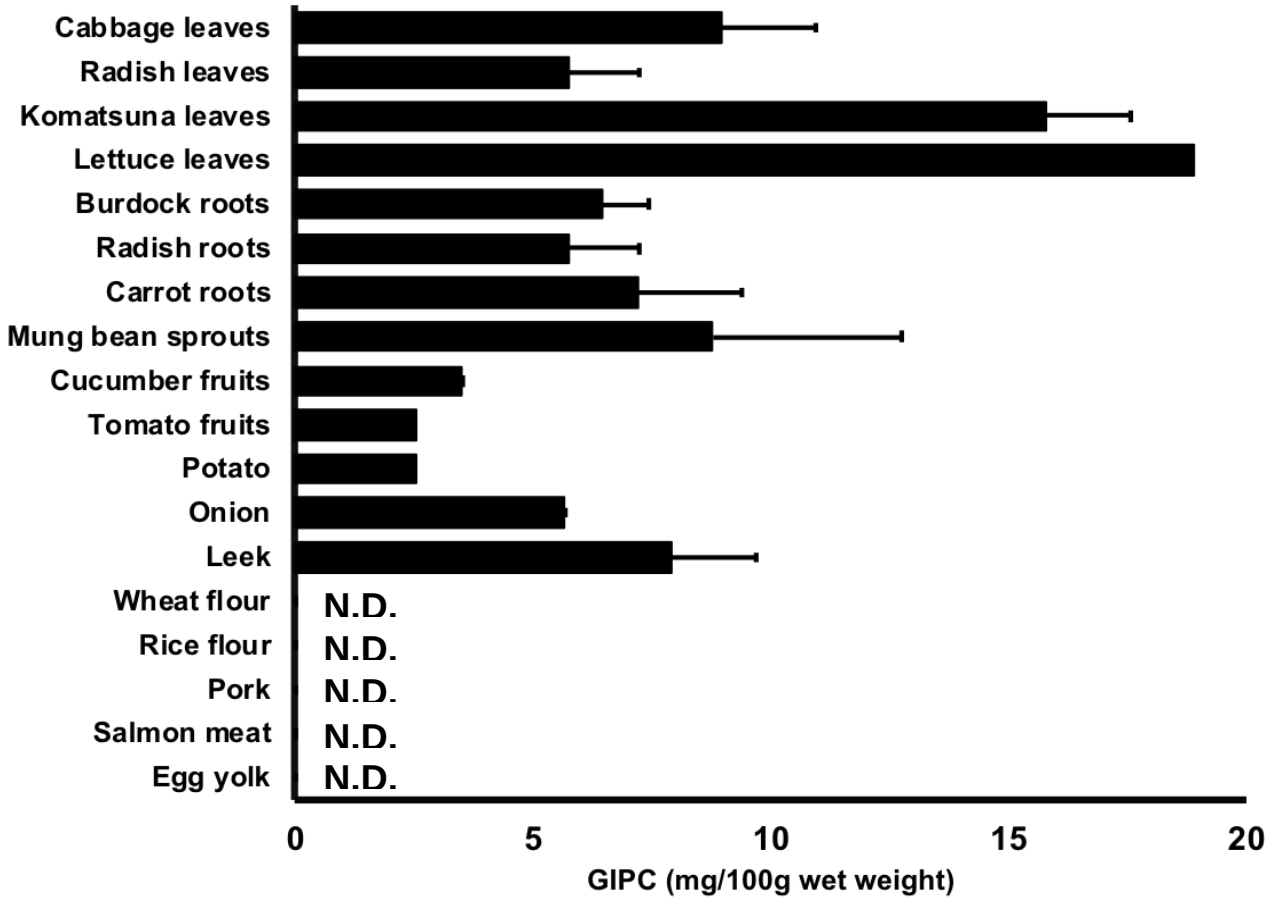
Cabbage leaves
Radish leaves
Komatsuna leaves
Lettuce leaves
Burdock roots
Radish roots
Carrot roots
Mung bean sprouts
Cucumber fruits
Tomato fruits
Potato
Onion
Leek
Wheat flour
Rice flour
Pork
Salmon meat
Egg yolk
N.D.
N.D.
N.D.
N.D.
N.D.
0
5
10
15
20
GIPC (mg/100g wet weight)

Fig. 2

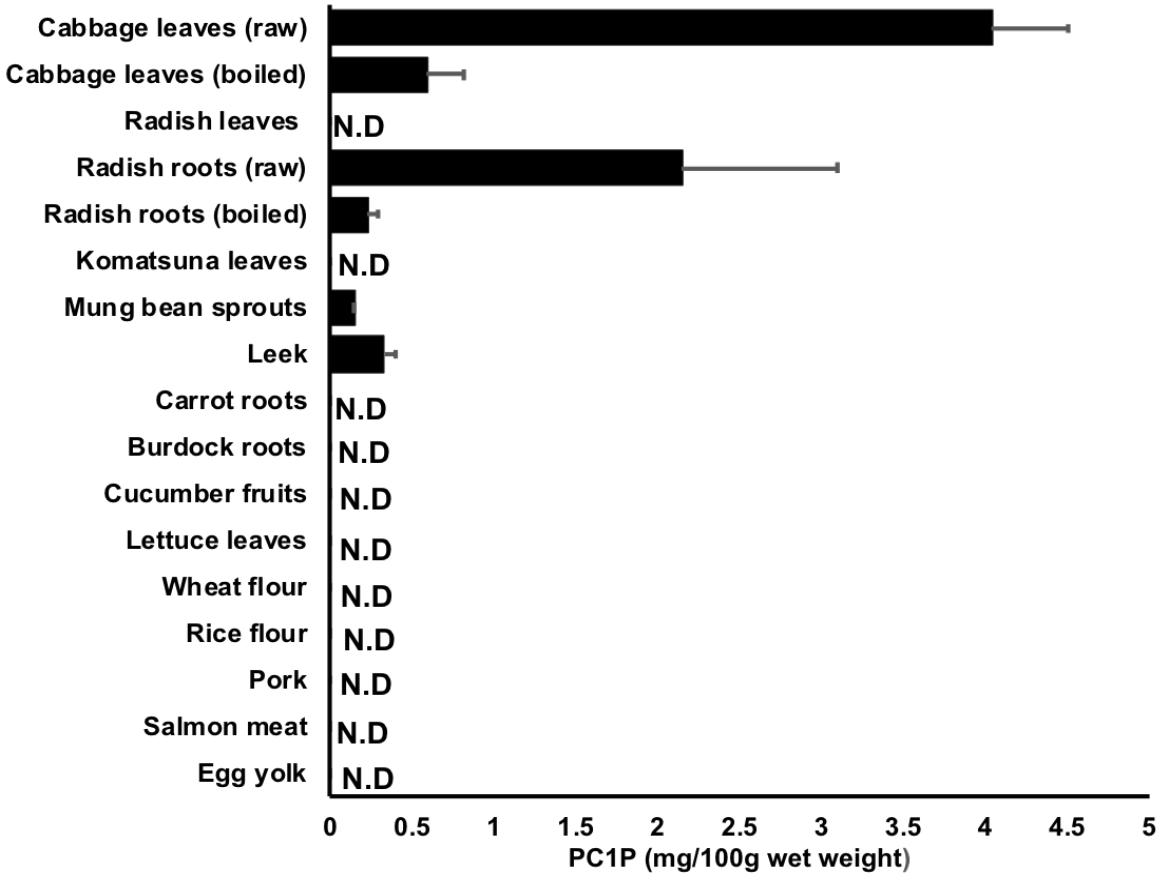
Cabbage leaves (raw)
Cabbage leaves (boiled)
Radish leaves
Radish roots (raw)
Radish roots (boiled)
Komatsuna leaves
Mung bean sprouts
Leek
Carrot roots
Burdock roots
Cucumber fruits
Lettuce leaves
Wheat flour
Rice flour
Pork
Salmon meat
Egg yolk
N.D
N.D
N.D
N.D
N.D
N.D
N.D
N.D
N.D
N.D
N.D
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
PC1P (mg/100g wet weight)

Fig. 3