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# Phosphatidic Acid Production in the Processing of Cabbage Leaves

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**ABSTRACT:** Lysophosphatidic acid (LPA) is a lipid mediator involved in various physiological responses, including wound healing. Evidence of the antiulcer activity of LPA has been reported, and soybean LPA at a concentration of 10 $\mu$M is effective in reducing stress-induced gastric ulcer. Because LPA can be formed from phosphatidic acid (PA) by digestive phospholipase $A_2$, dietary PA can be considered a potential antiulcer phospholipid. In this study, PA production in cut processing of cabbage leaves was examined. The amounts of PA in sliced, minced, and homogenized cabbage leaves were 107 ± 5, 134 ± 19, and 286 ± 29 nmol PA/g (wet weight), respectively, all being significantly higher than the amount of PA found in intact leaves. Mixing mayonnaise with sliced cabbage dramatically increased the PA content (1586 ± 393 nmol/3 g), indicating phospholipase D activity leaked raw cabbage produced PA. These results indicate that fine cutting raw cabbage leaves and mixing them with foods rich in phospholipids resulted in an abundant production of PA.

**KEYWORDS:** *cabbage, lecithin, food ingredient, phospholipase D, phosphatidic acid, lysophosphatidic acid, antiulcer effect*

## INTRODUCTION

Lysophosphatidic acid (LPA) is a phospholipid mediator that induces various cellular responses, including cell proliferation and migration. In the intestine, it was reported that LPA modulates epithelial wound healing through its effect on cell migration.[1] Orally administered LPA was found to protect jejunal epithelial cells of mice against radiation-induced apoptosis [2] and also inhibit secretory diarrhea in mice by modulating the chloride channel activity of intestinal epithelial cells in a receptor-dependent manner.[3] Our previous study demonstrated that cabbage lipids and synthetic 1-oleoyl LPA induced fibroblast proliferation and promoted migration of gastric cells.[4] Recently, we showed evidence of the protective effect of LPA against stress-induced gastric ulcers in rats.[5] It is thus thought that the daily intake of LPA-rich food, such as cabbage, is beneficial to the epithelial integrity of the gastrointestinal tract. However, the LPA content in foods[4] is not sufficient to produce an effect, and the presence of LPA is controlled by decomposing enzymes such as phosphatases and lysophospholipases.[6]

Phosphatidic acid (PA) is formed by phospholipase D (PLD) action. PA is also produced with diacylglycerol kinase (DGK) or hydrolysis of PI-PLC followed by DGK. In plants, the formation of PA is triggered in response to various biotic and abiotic stressors, including pathogen infection, drought, salinity, wounding, and cold.[7] The enzyme responsible for PA production is PLD, which is released from the cells by tissue disruption. Brassicaceae vegetables have high PLD activity, so a large amount of PA is formed by various treatments such as tissue cutting and homogenization. Among the vegetables examined so far, cabbage is the richest source of PA and PLD activity.[8−10] Secretory phospholipase $A_2$ is active in the digestive tract. Hence, when cabbage is chewed and swallowed, the PA formed is further hydrolyzed to LPA.[4] PA ingestion may thus enhance the LPA effect on the gastrointestinal tract. In the present paper, we examine ways to obtain an abundant supply of PA from cabbage, and examine the mechanism of PA generation with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

## MATERIALS AND METHODS

**Materials.** Phosphatidylcholines (PC) from egg yolk and soybean, phosphatidylserine (PS), phosphatidylinositol (PI) from soybean, synthetic 1,2-dioeoyl-3-phosphatidylethanolamine (PE), 2,4,6-trihydroxyacetophenone (THAP), and 3,5-dihydroxybenzonic acid (DHB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lecithins from egg yolk and soybean were purchased from Wako (Osaka, Japan). Single isotopic $^{68}$Zn-cordinated Phos-tag ($^{68}Zn_2L^{3+}$, 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olatodizinc(II) complex) was a gift from the Nard Institute Ltd. (Amagasaki, Japan) and MANAC Inc. (Fukuyama, Japan). Mayonnaise (Kewpie, Tokyo, Japan) and cabbages were obtained from a local market.

**Extraction and Analysis of Cabbage Lipids.** Cabbage leaves (3 g) were used. Samples were sliced in 1 mm width, minced into 1−2 mm squares, or homogenized, and left at room temperature for 1 h. Cabbage leaves without processing were also left at room temperature for 1 h as intact cabbage leaves. Then, 1.3 mL of distilled water was added, followed by boiling for 10 min to inactivate PLD. After the addition of 5 mL of chloroform and 10 mL of methanol, samples were homogenized with an Ultra-Turrax disperser (IKA, Werke Staufen, Germany). Then, 5 mL each of chloroform and water was added for phase separation by the Bligh and Dyer method.[11] After the addition of 0.2 mL of 6 N HCl for acidification, the mixture was vigorously shaken and centrifuged. The lipid-containing chloroform phase was collected. The extraction was repeated by adding chloroform to the methanol phase, and the chloroform extracts were combined. An aliquot of the extracted lipids was applied to a thin-layer chromatography (TLC) plate for the analysis of phospholipid profiles, as described below. In the experiments with mayonnaise, the sliced cabbage leaves (3 g) were mixed with mayonnaise (0.5 g) in a 50 mL centrifuge tube and left for 1 h at room temperature before lipid extraction.

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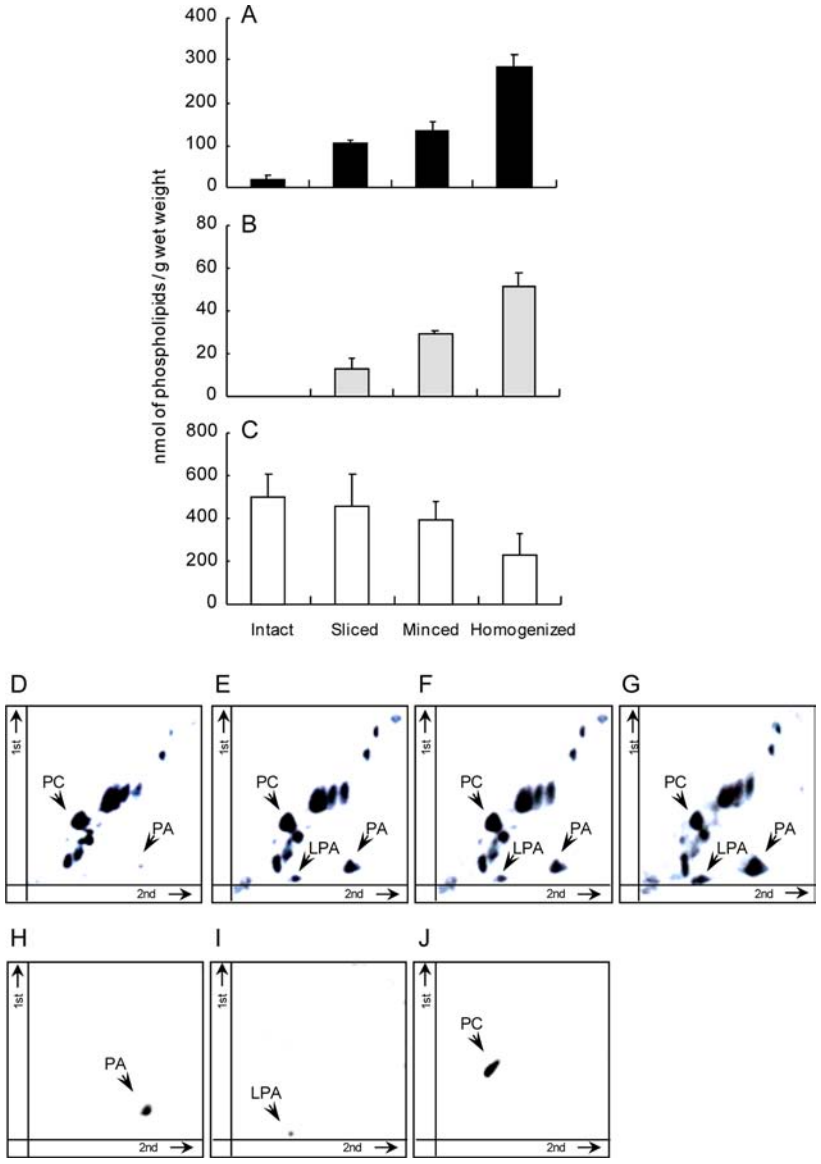


**Figure 1.** PA and LPA contents resulting from different treatments of raw cabbage. Raw cabbage was treated in three different ways, and phospholipids were extracted by using the Bligh and Dyer method after leaving the samples at room temperature for 1 h. Panels A, B, and C correspond to PA, LPA, and PC, respectively. Phospholipids from cabbage, intact (D), sliced (E), minced (F), or homogenized (G), were separated by two-dimensional TLC with basic (first dimension) and then with acidic (second dimension) solvents. Standard phospholipids (13 nmol), PA, LPA, and PC, are shown in panels H, I and J. Lipids were detected under UV light after light spraying with primulin. Values are the mean ± SD of three independent measurements.

**Determination of the Phospholipid Composition of Lecithins.** A 10 $\mu$g of phosphorus phospholipid was applied to a TLC plate (10 cm × 10 cm). The plate was first developed with an alkaline solvent composed of chloroform/methanol/28% ammonia (60:35:5, v/v/v) and then developed with an acidic solvent composed of chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v/v/v/v). Lipids were detected under UV light after light spraying of the plate with primurin reagent and recovered from the silica gel in the chloroform phase of the Bligh and Dyer method. Phosphorus quantitation was carried out according to the Bartlett method.[12]

**Preparation of Cabbage Juice.** For the preparation of cabbage juice, raw cabbage leaves were minced and ground using a mortar in a cold room at 4 °C. The paste obtained was wrapped in a double layer of gauze, and the juice was squeezed into a tube kept over ice. From 80 g of cabbage was obtained about 4 mL of juice. Cabbage juices were also prepared using green leaves, white petioles, and stems.

**PLD Assay of Cabbage Juice.** For the determination of the PLD assay, 322 nmol of phospholipid was mixed with 0.5 mL of 0.28 M sodium acetate buffer (pH 5.8), 0.1 mL of 0.1 M $CaCl_2$ and 0.1 mL of cabbage juice. For PLD activity of various cabbage parts, juices from green leaves, white petioles, and stems were used as enzyme sources. For the temperature stability, cabbage juice was preincubated at various temperatures for 30 min. For the pH activity, 0.28 M glycine–HCl buffer (pH 2 or 3) or 0.28 M sodium acetate buffer (pH 4) or 0.28 M phosphate buffer (pH 8) was used. For the substrate specificity, PC, PI, and PS from soybean and dioleoyl PE were used, and each PLD activity was determined at pH 5.8. The mixture was shaken vigorously and incubated at 30 °C with constant shaking. After incubation, the mixtures were heated for 10 min, and their lipids were extracted as previously described. The extract was applied to a TLC plate for analysis of the phospholipid profile, and an aliquot of the extract was applied to MALDI-TOF MS for analysis of the molecular species.

**Transphosphatidylation of PLD.** It is known that PLD generates phosphatidyl alcohol under the presence of alcohol. For the characterization of the cabbage enzyme during a meal with alcohol,

cabbage juice and PC from soybean were incubated in the presence of various amounts of ethanol. The reaction mixture was extracted by using Bligh and Dyer's procedure. The extract was applied to a TLC plate for phospholipid profile, and an aliquot of the isolated spot corresponding to phosphatidyl alcohol was analyzed by MALDI-TOF MS.

**MALDI-TOF MS Analysis.** The extracts were dissolved in 0.1 mL of methanol containing 0.1% aqueous ammonia. Then, 0.05 mL of monoisotopic $^{68}Zn^{2+}$-Phos-tag solution (0.1 mM in water) and 0.02 mL of silica suspension (100 mg/mL in methanol), which has been proved to increase the intensity of the hydrophobic analytes,[13] were added. Silica gel scrapped off the TLC plate (Merck 5721, Darmstadt, Germany) was used for the suspension. After shaking, 0.5 $\mu$L of the mixture was spotted on a sample plate. Immediately, 0.5 $\mu$L of THAP solution (10 mg/mL in acetonitrile) was layered on the mixture as a matrix solution. For the analysis of phosphatidylethanol (PEt), DHB was used as a matrix. The isolated spot was dissolved in 0.1 mL of chloroform/methanol (2:1, v/v); 1 $\mu$L of this solution was mixed with 9 $\mu$L of DHB solution, and 1 $\mu$L of the mixed solution was spotted on a sample plate. MALDI-TOF MS was performed as described in a previous paper.[14] The matrix/analyte cocrystal was analyzed using a Voyager DE STR instrument (Applied Biosystems, Framingham, MA, USA) in the positive mode. The wavelength of the nitrogen-emitting laser, pressure in the ion chamber, and accelerating voltage were 337 nm, 3.7 × $10^{-7}$ Torr, and 20 kV, respectively. To improve reproducibility, 256 single-laser shots were averaged for each mass spectrum.

## RESULTS

**PA Production According to Treatment of Cabbage Samples.** The amount of PA in raw intact cabbage leaves was 16 ± 11 nmol/g (wet weight). In contrast, 107 ± 5, 134 ± 19, and 286 ± 29 nmol/g of PA were detected in sliced, minced, and homogenized leaves, respectively (Figure 1A), which represents a significant increase in PA content compared with the intact leaves. In the same way, these treatments resulted in an increase in the amount of LPA (Figure 1B). On the contrary, PC levels decreased with the extent of leaf processing (Figure 1C).

The levels of PA and LPA in the mixture of sliced cabbage and mayonnaise were 1586 ± 393 and 234 ± 106 nmol, respectively, being about 4 times higher than those of sliced cabbage alone (Figure 2). No PA production was detected in the mixture of boiled cabbage and mayonnaise (data not shown). These results suggest that PLD released from the disrupted cabbage tissues generated PA by the hydrolysis of endogenous and exogenous phospholipids.

**PA Production by Incubation of Lecithin with Cabbage Juice.** PLD activity in cabbage juice was characterized. First, differences in the PLD activity of various cabbage parts were examined by using green leaves, white petioles, and stems. We found that the PLD activities of the juices obtained from these cabbage parts were 168, 177, and 201 nmol/10 min/0.1 mL, respectively. Therefore, juices were prepared from the whole cabbage. The optimum temperature was 30 °C, and the activity decreased slightly at 25 and 37 °C. PLD activity was completely lost at 70 °C. The optimum pH of the juice was 5.8, and about half of the activity was retained at an acidic pH of 4.0. PC and PE were the preferred substrates, with PLD activities of 127 ± 41 and 159 ± 26 nmol/10 min/0.1 mL for soy PC and dioleoyl PE, respectively. In contrast, the PA activities formed from PS and PI were only 30 ± 19 and 28 ± 3 nmol/10 min/0.1 mL, respectively.

PA production from two typical emulsifiers, egg yolk lecithin and soy lecithin, was examined using cabbage juice.

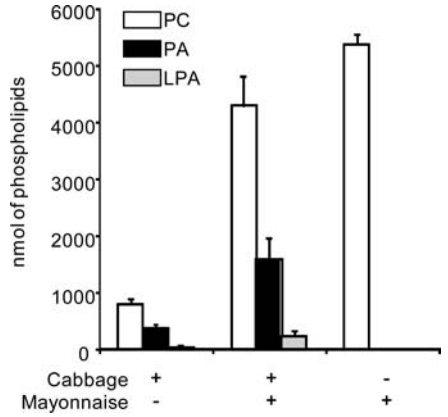


**Figure 2.** PA and LPA contents in sliced cabbage mixed with mayonnaise. Sliced cabbage (3 g) and mayonnaise (0.5 g) were mixed and left at room temperature. After 1 h, lipids were extracted by using the Bligh and Dyer method, and phospholipids were separated by two-dimensional TLC. Phosphorus quantitation was carried out according to the Bartlett method. Values are the mean ± SD of three independent measurements.

Phospholipids of egg yolk lecithin were exclusively composed of PC and PE (Figure 3A). Phospholipids of soy lecithin were composed of PC, PE, PI, and PA (Figure 3B). The incubation

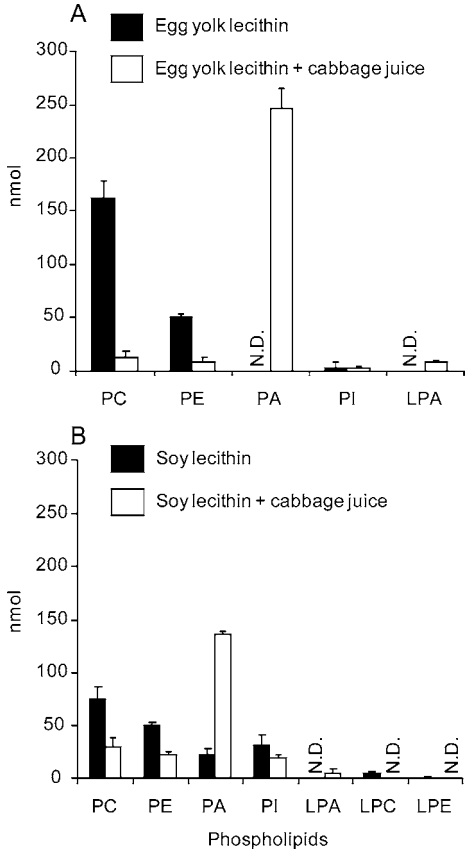


**Figure 3.** PA production after lecithin incubation with cabbage juice. Egg yolk (A) or soy (B) lecithin containing 10 $\mu$g of phosphorus was incubated with cabbage juice. After 30 min, lipids were extracted by using the Bligh and Dyer method, and phospholipids were separated by two-dimensional TLC. Phosphorus quantitation was carried out according to the Bartlett method. Values are the mean ± SD of three independent measurements.


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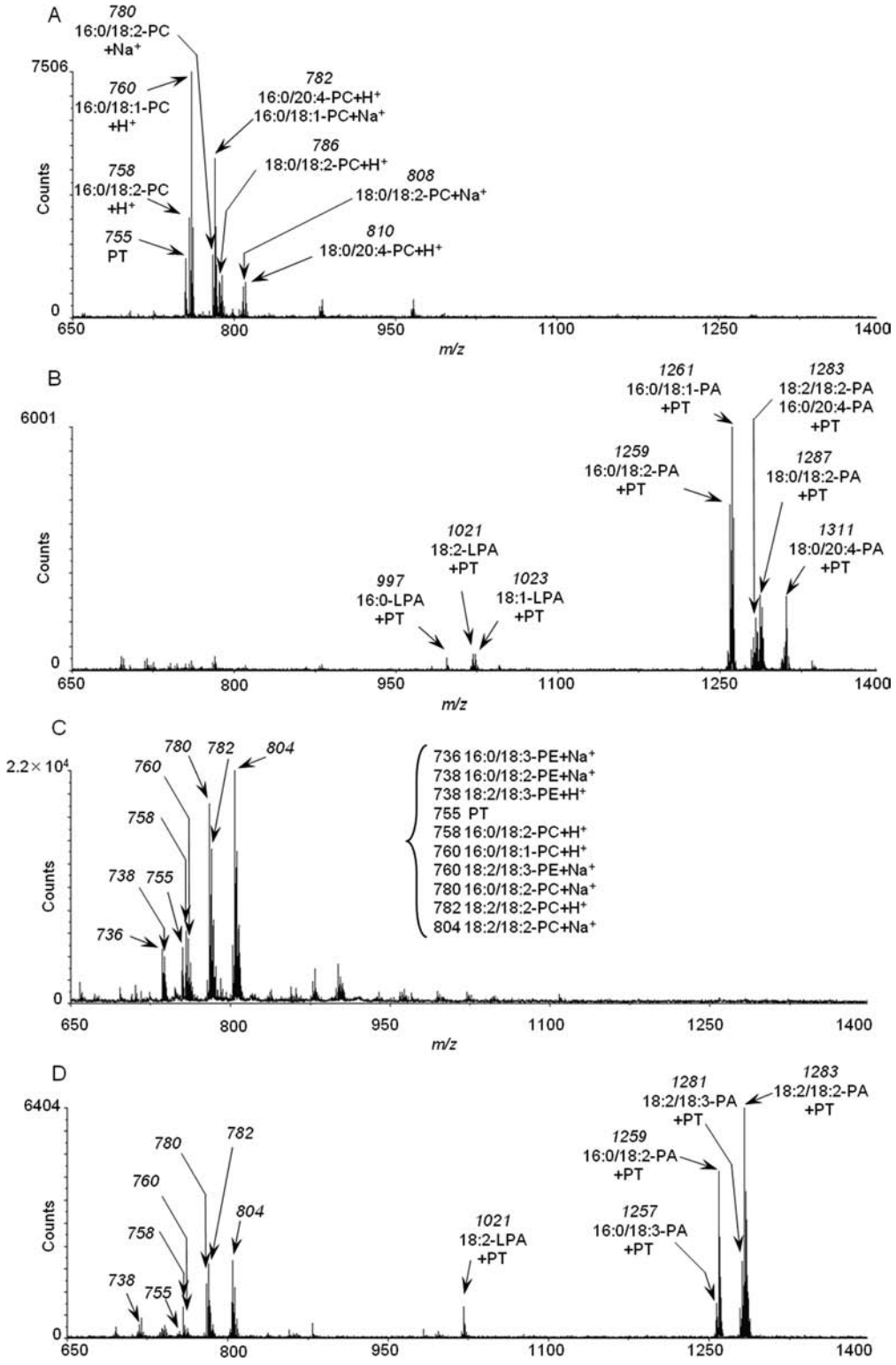


**Figure 4.** Molecular PA species obtained by the incubation of lecithin with cabbage juice. Egg yolk or soy lecithin was incubated with cabbage juice, and the lipids were extracted. The extracts were mixed with $^{68}$Zn Phos-tag (molecular weight 589) and analyzed by MALDI-TOF MS in the positive mode: (A, B) egg yolk lecithin before and after incubation; (C, D) soy lecithin before and after incubation. The ion at $m/z$ 755 corresponded to an unidentified adduct of $^{68}$Zn Phos-tag.

of egg yolk lecithin with cabbage juice produced 246 ± 16 nmol/30 min PA with a concomitant decrease of PC and PE (Figure 3A). For soy lecithin, the amount of PA formed during incubation was 136 ± 3 nmol/30 min, which was 6 times the amount of PA before incubation (Figure 3B). Small amounts of LPA were detected after the incubation of both soy and egg yolk lecithin with cabbage juice.

The incubation products were analyzed by MALDI-TOF MS in the positive ion mode. For egg yolk lecithin, peaks at $m/z$ 760 and 782 were prominent (Figure 4A). These ions were

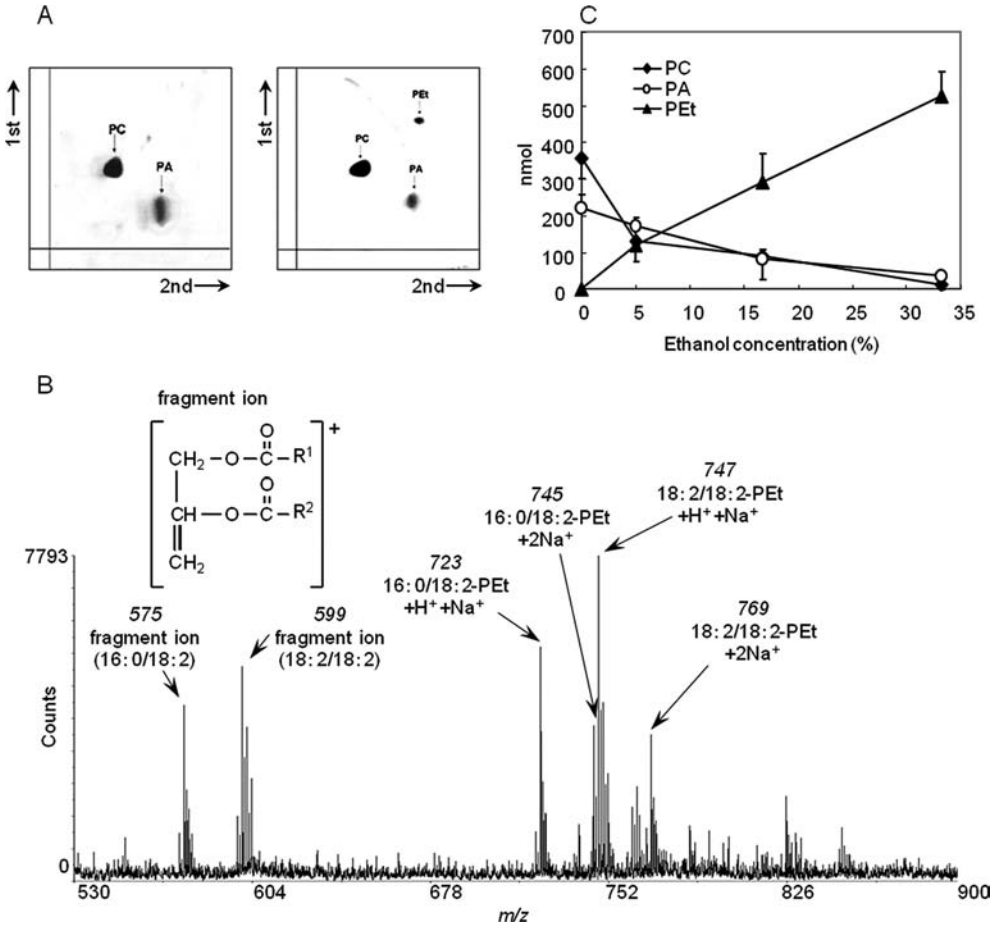


**Figure 5.** Products from the transphosphatidylation reaction of cabbage juice. PC from soybean (644 nmol) was incubated with cabbage juice in the presence of ethanol concentrations: (A) lipid extracts were separated by two-dimensional TLC with basic (first dimension) and then with acidic (second dimension) solvents, sprayed with primuline, and visualized under UV light (ethanol concentration = 5%; products of PLD in the absence (left) or presence (right) of ethanol); (B) mass spectrum of the isolated first migrating spot; (C) dose dependency of transphosphatidylation on ethanol. Values are the mean ± SD of three independent measurements.

assigned to proton and sodium adducts of the molecular ion of PC 34:1 (16:0/18:1). PC 34:2 (16:0/18:2) was assigned by the molecular ions of proton and sodium adducts at *m/z* 758 and 780. After 30 min of incubation, egg yolk lecithin was almost fully hydrolyzed and the PA formed was detected as single ions of Phos-tag adducts at *m/z* 1259 and 1261 (Figure 4B). They were assigned as PA 34:2 (16:0/18:2) and PA 34:1 (16:0/18:1). In Figure 4C, PC and PE were found in soy lecithin, and the major species were 36:4 (18:2/18:2) and 34:2 (16:0/18:2) for PC and 36:5 (18:2/18:3) and 34:3 (16:0/18:3) for PE. After 30 min, PA 36:4 (18:2/18:2) and PA 34:2 (16:0/18:2) were generated as the major species, although substantial amounts of PC and PE remained (Figure 4D). Small amounts of 16:0, 18:1 and 18:2 LPA in egg yolk lecithin and 18:2 LPA in soy lecithin were detected after incubation with cabbage juice.

**Transphosphatidylation of PLD.** In the presence of an appropriate alcohol, PLD transfers the phosphatidyl moiety to an acceptor alcohol. The TLC pattern of PLD products in the absence or presence of ethanol is shown in Figure 5A. The fast migrating spot in both acidic and basic developments was detected only in the presence of ethanol. The mass spectrum of the spot in Figure 5B showed the typical fragmentation pattern of acidic phospholipids.[15] Peaks at *m/z* 575 and 599 corresponded to the fragmented diglyceride ion. Peaks at *m/z* 723 and 745 were proton and sodium adducts and disodium adduct molecular ions, indicating PEt 34:2 (16:0/18:2). Peaks at *m/z* 747 and 769 were proton and sodium adducts and disodium adduct molecular ions, indicating PEt 36:4 (18:2/18:2) (Figure 5B). In the absence of ethanol, 221 nmol of PA was generated by incubation with cabbage juice. The amount of PA decreased by the addition of alcohol in a dose-dependent manner, whereas the amount of PEt increased (Figure 5C).

## ■ DISCUSSION

LPA is a lipid mediator that induces various biological activities through its specific binding to G protein-coupled receptors.[16] Among these activities, a lot of attention has been paid to its potent wound-healing effects in the digestive tract.[17] Recently, GPR92/93 (LPA5), one of the recently identified G protein-coupled receptors, was reported to express in enterocytes and induce mucosal cell proliferation by interaction with LPA.[18] Thus, it is thought that the supply of LPA from food could contribute to wound-healing in the digestive tract. PA would be hydrolyzed to LPA in the lower gastrointestinal tract, where phospholipase $A_2$ is active. Thus, abundant PA supplied from food would be hydrolyzed to LPA in the lower tract. Among the vegetables so far examined, cabbage has the PLD with the highest activity.[9] However, cabbage PLD is labile at temperatures >50 °C, so only raw cabbage can be a source of PLD for PA production. Thus, we chose to cut the raw cabbage leaves

and found that finer tissue cutting resulted in a higher PA content (Figure 1). To get higher levels of PA, it is recommended that a combination of foods rich in phospholipids, especially PC, which is the most susceptible to hydrolysis, be eaten. We found that a mixture of mayonnaise and sliced cabbage had PA levels 4 times higher than those of sliced cabbage alone (Figure 2). To characterize in detail, two emulsifiers, lecithin from egg yolk and soybean, are mixed with the compressed cabbage juice. Reflecting the substrate specificity of cabbage PLD, egg yolk lecithin is more susceptible than soy lecithin. Although in minute amounts, LPA was detected after the cabbage leaves had been sliced and was further increased by mixing the sliced cabbage with some other ingredients (Figures 1−3), which can be explained by the lysophospholipase D (lysoPLD)[19] activity present in the leaves.

ESI LC-MS is generally used for phospholipid analysis. However, PA is an anionic phospholipid and interacts with cationic compound such as metals, making purification by conventional HPLC difficult.[20] Another concern about PA is multimolecular ion production, as was observed for phosphatidylcholines. Even in the negative ion mode, molecular anions of alkali metals and proton adducts are generated, causing confusion during the assignment of molecular species. Phos-tag binds to the phosphoric anion and gives a monocationic complex with no production of other cation adducts (Figure 4).[21] The positions and types of fatty acids esterified to glycerol could not be assigned from the MALDI-TOF MS data. However, such information was obtained from the intramolecular analysis using $PLA_2$.[22] PAs from egg yolk lecithin were 1-saturated-2-unsaturated species, whereas those from soybean lecithin were 1,2-unsaturated spices and 1-saturated-2-unsaturated species. Pancreatic-type $PLA_2$ is known to be present in the stomach,[23] and 1,2-dilinoleonoyl PA, which is major species produced by the hydrolysis of soy lecithin, would form 1-linoleonoyl LPA by $PLA_2$ hydrolysis in the stomach. In our previous experiment, 1-linoleoyl or 1-linolenoyl LPA proved to be more effective against gastric ulcers than 1-oleoyl species, and the effective concentration of LPA from soybean was <0.01 mM.[5] This evidence suggests that a combination of foods containing soy lecithin with raw cabbage would be a choice for the production of linoleoyl LPA. In addition to its hydrolytic activity, PLD is able to transfer the phosphatidyl moiety to an acceptor alcohol.[24] Both the hydrolysis and transphosphatidylation occurred in the presence of ethanol, and the formation of PEt was proportional to the concentration of ethanol. In contrast, PA content decreased with an increase in ethanol concentration (Figure 5). It was reported that lysoPEt, which is produced by $PLA_2$ hydrolysis, is not as active as LPA.[25] It is thus suggested that drinking alcohol during meals is thus not favorable for the generation of PA.

Finely cut raw cabbage together with mayonnaise produced abundant PA. Previously, we confirmed that PA was efficiently hydrolyzed by pancreatic $PLA_2$.[4] Recently, the formation of LPA from PA by stomach wash and isolated stomach and the protection against aspirin-induced ulcer by administration of PA in the stomach have been proved.[26] Taken together, it is concluded that the daily intake of raw cabbage together with foods rich in phospholipids would help to maintain a healthy gut.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

PA, phosphatidic acid; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLD, phospholipase D; $PLA_2$, phospholipase $A_2$; PEt, phosphatidylethanol; TLC, thin-layer chromatography; PC 34:1, phosphatidylcholine (PC) molecular species with a chain length of 34 fatty acids and 1 unsaturation.

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