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Quantification of Phosphatidic Acid in Foodstuffs Using a Thin-Layer-Chromatography-Imaging Technique

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ABSTRACT: Apical application of lysophosphatidic acid (LPA), a growth-factor-like phospholipid, was shown to prevent or restore gastrointestinal (GI) disorders, such as diarrhea and stomach ulcer, in experimental animals. Because LPA is formed from phosphatidic acid (PA) by the activity of digestive phospholipase A_2, PA is a potential component for dietary treatment of such GI disorders. Here, we quantified PA contained in 38 foodstuffs and 3 herbs by a thin-layer-chromatography-imaging technique. Vegetables belonging to Brassicaceae, such as cabbage leaves (700 nmol/g of wet weight) and Japanese radish leaves (570 nmol/g), contained higher amounts of PA than other foodstuffs. Amounts of PA in fruits, cereals, and starchy root vegetables were below 300 nmol/g. Animal foodstuffs contained low amounts of PA (<60 nmol/g). Interestingly, leaves of *Mallotus japonicas*, a Japanese edible herb used for treatment of stomach ulcer, had the highest PA (1410 nmol/g) among those examined. The data shown here will be useful for the development of dietary treatment for a damaged GI tract.

KEYWORDS: *Phosphatidic acid, lysophosphatidic acid, cruciferous vegetables, TLC-imaging, antiulcer*

INTRODUCTION

Recent investigations revealed that supplementation of phospholipids has a protective effect on the gastrointestinal (GI) tract.[1] One of mechanisms of the effects is strengthening the barrier capability of mucous gel, where phospholipids form a hydrophobic lining on the luminal surface of the GI tract.[1] Other mechanisms involve cytoprotective or wound-healing actions of phospholipid via its specific receptors.[2] Lysophosphatidic acid (LPA) is a growth-factor-like phospholipid that may help to maintain epithelial integrity of the digestive tract.[3,4] Orally administrated LPA is known to protect against radiation-induced apoptosis in mice jejunal epithelial cells[4] and inhibit secretory diarrhea by modulating the chloride channel activity of mice intestinal epithelial cells in a receptor-dependent manner.[5] Sturm et al. have shown that rectal application of LPA significantly ameliorates intestinal epithelial injury in trinitrobenzenesulfonic-acid-induced colitis in rats.[6] Our recent study showed that oral administration of LPA prevents stress-induced stomach ulcer in rats.[7] Several reports[8,9] and a database (The Human Protein Atlas; http://www.proteinatlas.org/) showed that at least types 1, 2, 3, and 5 of LPA-specific receptors moderately or strongly express in the GI tract. Interestingly, the type 2 LPA receptor was demonstrated to localize on the apical surface of intestinal epithelial cells,[5,10] suggesting that luminal epithelia can respond to LPA contained in ingested foods.

Ingested phospholipids are converted to lysophospholipids by digestive phospholipase A_2 (PLA_2). A well-known source of PLA_2 in the digestive tract is the pancreas. However, the pancreatic-type PLA_2 has been reported to be abundantly present in the stomach.[11] Previously, we examined amounts of LPA in several foodstuffs and found that raw cabbage leaves are the richest source of LPA.[12] We also demonstrated that LPA is formed from abundant PA during incubation of cabbage homogenates with pancreatic juice.[12] Considering our previous results and LPA action in the GI tract, it may be possible to consider that PA-rich foods exert a beneficial effect on certain kinds of GI disorders through the supplementation of LPA. However, the study on PA contents in foodstuffs is limited[13] and has not yet been systematically examined.

PA is an intermediate in the synthesis of glycerolipids in both animals and plants. They do not accumulate much PA at a normal condition. However, in plants, a substantial amount of PA rapidly emerges in response to various stresses, such as wounding of the tissue.[14] In fact, it is known that grinding triggers the activation of phospholipase D (PLD) in cabbage leaves and leads to the production of abundant PA from endogenous phospholipids.[15] In this regards, we demonstrated that mastication of raw cabbage leaves produce PA.[12] Thus, we expected that there might be other foodstuffs that increase their PA levels during cooking or mastication.

Silica-gel-based thin-layer chromatography (TLC) is a widely used technique that is efficient for separation of lipids. It requires neither special devices nor special cautions. However, the conventional method for quantification of phospholipids recovered from the silica gel of TLC includes some time-consuming steps, such as oxidative degradation of phospholipids to inorganic phosphate and the formation of the phosphomolybdenum complex for calorimetric assay. In this study, we developed a TLC-imaging method for determination of class composition of phospholipids without the calorimetric assay. Using this method, we determined PA levels in the

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homogenates of 38 foodstuffs and 3 herbs. Information shown here will be useful for the development of the dietary treatment of certain kinds of GI disorders.

MATERIALS AND METHODS

Materials. Foodstuffs used in this study were cabbage (*Brassica oleracea*), Japanese radish (*Raphanus sativus*), green pak choi (*Brassica rapa*), Japanese mustard spinach (*Brassica rapa*; called komatsuna in Japan), and spinach (*Spinacia oleracea*) as leaf vegetables, carrot (*Daucus carota*), Japanese radish (*Raphanus sativus*), edible burdock (*Arctium lappa*), lotus (*Nelumbo nucifera*), and ginger (*Zingiber officinale*) as root vegetables, cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and bell pepper (*Capsicum annuum*) as fruit vegetables, celery (*Apium graveolens*) as a stem vegetable, taro (*Colocasia esculenta*), Japanese yam (*Dioscorea japonica*), sweet potato (*Ipomoea batatas*), and potato (*Solanum tuberosum*) as tuber vegetables, broad beans (*Vicia faba*) and green peas (*Pisum sativum*) as beans, rice (*Oryza sativa*) and corn (*Zea mays*) as cereal, oranges (*Citrus sinensis* and *Citrus sudachi*), kiwifruit (*Actinidia deliciosa*), apple (*Malus domestica*), muskmelon (*Cucumis melo*), strawberry (*Fragaria ananassa*), and banana (*Musa acuminata*) as fruit, wakame seaweed (*Undaria pinnatifida*) as an alga, and pork, chicken, and beef as animal flesh. The minced meat used was a mixture of pork, beef, and poultry. A Japanese littleneck (*Ruditapes philippinarum*) and shrimp (*Penaeus monodon*) were used as typical foodstuffs belonging to shellfish and crustacean, respectively. Mackeral (*Scomber japonicas*) muscle was used as a fish meat. Shiitake mushroom (*Lentinula edodes*), a native mushroom in East Asia, was chosen as an edible fungus. These foodstuffs were purchased from local markets. Japanese mallotus (*Mallotus japonicus*), conandron (*Conandron ramondioides*), and mugwort (*Artemisia indica*) were obtained from the Medicinal Herb Garden of Tokushima University. Egg yolk PA prepared from egg yolk phosphatidylcholine (PC), bovine brain sphingomyelin (SphM), soybean phosphatidylethanolamine (PE), soybean PC, soybean phosphatidylinositol (PI), and egg yolk lysophosphatidylcholine (LPC) were purchased from Sigma-Aldrich (St. Louis, MO). Primuline dye was obtained from Nacalai Tesque (Kyoto, Japan).

Extraction and Analysis of Foodstuff Lipids. Foodstuffs (2.5 g of wet weight) were mashed in a mortar with a pestle for 3 min at room temperature. The homogenate was heated in boiled water for 3 min for inactivation of lipolytic enzymes. Lipids were extracted from the homogenates by the Bligh and Dyer method,[16] with acidification of the water/methanol phase as described previously.[12] Amounts of total phospholipids were quantified by a colorimetrical method based on phosphomolybudenum−malachite green formation.[17]

TLC Imaging. Aliquots of the lipids extracted from foodstuffs were analyzed by two-dimensional (2D) TLC. The solvent systems of the first and second chromatographies were chloroform/methanol/28% aqueous ammonia (60:35:8, v/v/v) and chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v/v/v/v), respectively. The plate was dried for 30 min by blowing air and sprayed with primuline (0.01% in 80% acetone) until the plate became thoroughly wet. After drying the plate overnight at room temperature, an image of the fluorescence spots on the plate was captured by Fuji LAS-4000 imaging system (Fuji Film, Tokyo, Japan). The excitation wavelength and emission filter used were 312 and 605 nm, respectively. The digitized image data were analyzed by NIH image, an imaging software, for the determination of the pixel intensity value (PIV) of each fluorescent spot. Their PIVs were corrected with a background PIV. Phospholipid spots on TLC were detected by spraying Dittmer's reagent, which forms a blue stain when reacting with phospholipids.[18]

PLD Assay of Foodstuff Juice. Shredded foodstuffs (5 g of wet weight) were added to 15 mL of water and then homogenized with an ultradisperser (LK-21, Yamato Scientific, Tokyo, Japan) for 1 min at 4 °C. The homogenates were centrifuged at 1300*g* for 5 min, and the supernatant was used as a foodstuff juice for the experiments. The reaction mixture consisted of 167 nmol of egg yolk PC, 0.5 mL of acetate buffer (0.2 M, pH 5.6), 0.1 mL of calcium chloride (0.1 M), and 0.4 mL of the foodstuff juice. The reaction mixture was vigorously shaken for 15 min at 30 °C and heated in boiling water for 3 min to inactivate lipolytic enzymes. Then, 2.2 mL of chloroform/methanol (1:1, v/v) and 0.12 mL of 2 M HCl were added for acidification. After centrifugation, the chloroform layer was withdrawn and the remaining water/methanol phase was mixed with 1.1 mL of chloroform. After vigorous shaking and centrifugation, the chloroform phase was combined to the first chloroform phase and the mixtures were evaporated to dryness. Resulting PA was isolated by 2D TLC and quantified by the TLC-imaging method. A reaction mixture without egg yolk PC was prepared as a blank. The amount of PA formed by hydrolysis of exogenous PC was calculated by subtracting the amount of PA in the blank sample.

RESULTS

Quantification of Phospholipids on the TLC Plate. Several methods have been developed for quantification of phospholipids on the TLC plate.[19−22] In this study, we employed a method reported by White et al., in which lipids stained with primuline were quantified by measuring their fluorescent intensity under ultraviolet (UV) light.[21] According to the report, the lipid structure influenced the fluorescent intensity, and therefore, different phospholipid classes had different detection efficiencies.[21] Thus, first we made standard curves with standard phospholipids under our analytical conditions. As shown in Figure 1A, the fluorescent intensity of the stained PA increased dose-dependently. The integrated pixel intensity values (PIVs) of the spots nearly linearly correlated with the amounts of PA up to 193 nmol (r^2 = 0.908) (Figure 1B). The slopes of standard curves of other phospholipids were 0.104 (SphM), 0.097 (PC), 0.067 (PE), 0.065 (PI), 0.040 (LPC), and 0.037 (PA) (panels B and C of Figure 1). In our experience, the fluorescent intensity of primuline was influenced by moisture and the time passed after primuline spray, even though the amount of primuline sprayed on TLC was carefully controlled. In contrast, the relative intensity of each spot seemed stable, regardless of the analytical conditions. Therefore, we quantified PA based on its percentage in total phospholipid. The procedure that we employed for the quantification of PA was as follows: (1) measure PIVs of phospholipid spots on the primuline-sprayed plate, (2) correct each PIV using values from the standard curves, (3) calculate total PIVs of phospholipid spots and the percentage of PA in total phospholipids, and (4) determine the amounts of PA (nmol/g of wet weight) based on phospholipid contents in foodstuffs. Although many standard curves for different phospholipid classes need to be accurate, for convenience, we applied averaged detection efficiency (slope of 0.068) to correct PIVs of minor phospholipids. It should be noted that PIVs tended to saturate beyond 200 nmol/spot. Thus, underestimation occurs when a large amount of phospholipids present as a condensed spot on the plate. Therefore, the amount of sample loading on a plate should be limited to avoid condensed spots in this imaging analysis.

To validate our quantification method, we determined phospholipid class composition of raw cabbage leaves. As a result, the composition determined by the TLC imaging was in good agreement with that obtained by a conventional method, which involves extraction of phospholipids from silica gel, degradation of the phospholipids, and formation of phosphomolybdenum−malachite green (Figure 2). To evaluate the accuracy of our method, a standard PA (160 nmol) was mixed with phospholipids prepared from mouse brain (160 nmol), which does not contain detectable PA, and the mixture was subjected to our TLC-imaging method. As a result, the amount of the PA

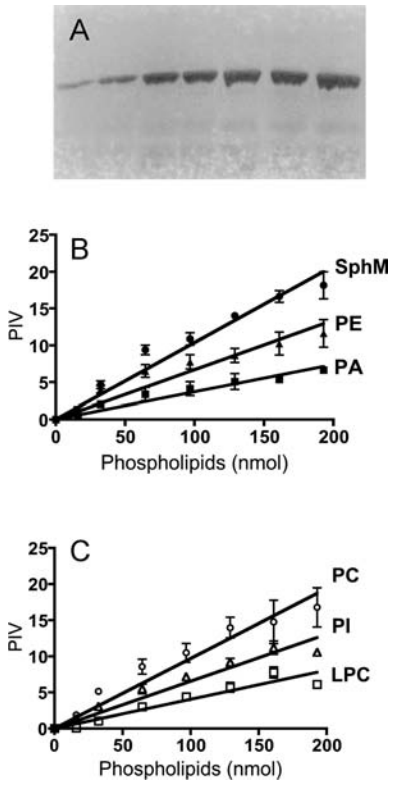

Figure 1. Standard curves for fluorescent intensities of phospholipids stained with primuline. (A) Different amounts of PA (16, 32, 65, 97, 129, 161, and 193 nmol) prepared from egg yolk PC were spotted on a TLC plate and developed. After spraying the primuline reagent, fluorescence of lipid spots was detected under UV light and captured by the imaging analyzer. (B and C) Same experiments as panel A were conducted with SphM from bovine brain, PE from soybean, PC from soybean, PI from soybean, and LPC from egg yolk. The pixel intensity value of each spot on TLC was calculated with NIH image software. Values are the mean ± standard deviation (SD), from experiments performed in three independent experiments. Correlation coefficients of these calibration curves are 0.935 (SphM), 0.822 (PE), 0.908 (PA), 0.828 (PC), 0.942 (PI), and 0.871 (LPA). Slope values are indicated in the text.

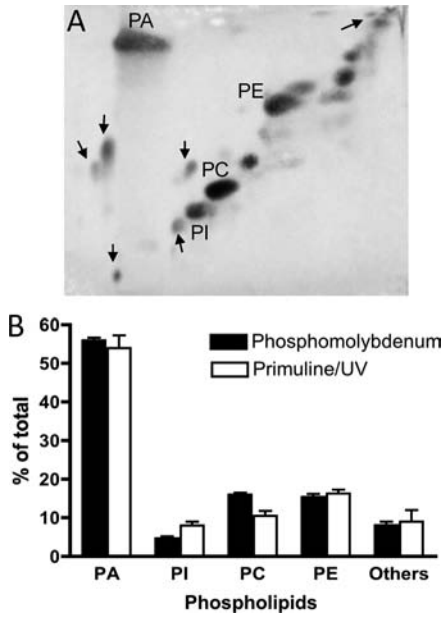

Figure 2. Phospholipid class compositions of cabbage leaves determined by the TLC-imaging method and the conventional phosphomolybudenum method. (A) Lipids extract from raw cabbage leaves were separated by 2D TLC, sprayed with primuline, and visualized under UV light. Spots indicated by arrows were reactive to Dittmer–Lester's reagent. Pixel intensity values of these phospholipid spots were summed and expressed as "others" in panel B. (B) Percentage of each phospholipid was calculated on the basis of the pixel intensity value of each spot on TLC (white bars) or the quantification of isolated phospholipid by the phosphomolybdenum–malachite green method (black bars). Values are the mean ± SD, from three independent chromatograms.

was determined to be 180 ± 2 nmol (n = 3). From these results, we employed the TLC-imaging method for the determination of PA amounts in various foodstuffs and herbs.

PA Amounts in Foodstuffs. Foodstuffs that we chose were leafy vegetables (cabbage, Japanese radish leaf, Green pak choi, Japanese mustard spinach, and spinach), root vegetables (carrot, Japanese radish root, edible burdock root, lotus root, and ginger root), fruit vegetables (cucumber, tomato, eggplant, and bell pepper), stem vegetables (celery), tuber vegetables (taro, Japanese yam, sweet potato, and potato), beans (broad been and green pea), cereals (rice and corn), fruits (orange, *Citrus sudachi*, kiwifruit, apple, muskmelon, strawberry, and banana), alga (wakame seaweed), animal fleshes (pork, chicken, and beef), shellfish (Japanese littleneck), crustacean (shrimp), and fish (mackerel). Shiitake mushroom (*Lentinula edodes*), a native mushroom in east Asia, was chosen as an edible fungus. We also examined three kinds of edible herbs, which are believed to have a beneficial effect on GI damages in Japan. Japanese mallotus (*Mallotus japonicus*) and conandron (*Conandron ramondioides*) are known to have antiulcer activity, and mugwort (*Artemisia indica*) is known to have antidiarrheal action in addition to stomachic action.

Foodstuffs from animal sources, such as meats of flesh, shellfish, crustacean, and fish, did not contain a substantial amount of PA. An alga (wakame seaweed) was also a poor source of PA. On the other hand, plants and a fungus (shiitake mushroom) contained a substantial amount of PA (20–1400 nmol/g of wet weight). The richest source of PA was cabbage (700 nmol/g), a plant belonging to Brassicaceae (cruciferous vegetables). Japanese radish (570 nmol/g), green pak choi (430 nmol/g), and Japanese mustard spinach (390 nmol/g) were also PA-rich leafy vegetables belonging to Brassicaceae. Tomato, taro, cucumber, celery, carrot, and edible burdock contained relatively high PA. What is common to all of the plant foodstuffs mentioned above is that percentages of PA in total phospholipid were relatively high, as shown in Table 1. Although the percentage of PA was not so high, green peas were PA-rich foodstuffs (270 nmol/g). On the other hand, amounts of PA in cereals and fruits were relatively low. The percentages of PA in total phospholipid in fruit and cereal were less than 10%, except for oranges. Interestingly, both amounts and percentages of PA in total phospholipid of Japanese traditional edible herbs were very high (Figure 3 and Table 1). The amounts of PA in corn, potato, sweet potato, and apples were similar levels to those reported by Weihrauch and Son,[13] who summarized the published data on phospholipids in foodstuffs. On the other hand, amounts of PA in the carrot and cucumber were 10 and 50 times higher than those in the reported data, respectively.

Effect of Boiling on PA Contents in Foodstuffs. Amounts of PA in boiled foodstuffs were quite lower than those in raw foodstuffs. In fact, PA contents of boiled corns, boiled cabbage

Table 1. Amounts of Phospholipids in Foodstuffs and Percentages of PA in Total Phospholipid[a]

foodstuff	phospholipids (μmol/g of wet weight)	PA (% in total phospholipid)
cabbage	2.43 ± 0.70	30.1 ± 10.5
Japanese radish leaf	1.65 ± 0.08	33.4 ± 3.0
green pak choi	1.32 ± 0.92	37.6 ± 10.8
Japanese mustard spinach	0.89 ± 0.17	43.6 ± 3.6
tomato	0.94 ± 0.37	35.5 ± 13.4
taro (eddoe)	1.02 ± 0.17	31.7 ± 15.3
green pea	3.02 ± 0.38	9.3 ± 2.7
cucumber	0.99 ± 0.01	27.2 ± 4.8
celery	0.87 ± 0.27	29.6 ± 2.5
carrot	0.79 ± 0.07	32.3 ± 16.7
edible burdock	0.79 ± 0.07	29.9 ± 5.4
shiitake mushroom	2.15 ± 0.44	10.3 ± 4.0
orange	0.72 ± 0.07	25.3 ± 7.9
corn	2.56 ± 0.33	6.2 ± 4.8
Japanese radish root	0.51 ± 0.11	25.3 ± 2.7
broad bean	3.47 ± 0.26	3.3 ± 2.7
Citrus sudachi	0.46 ± 0.14	23.1 ± 11.4
ginger	0.47	20.0
kiwifruits	0.87	8.8
Japanese yam	0.55	13.5
lotus root	0.55	9.8
sweet potato	0.78	6.8
bell pepper	0.58	8.9
rice	2.02	1.4
apple	0.37	7.2
muskmelon	0.58	4.5
eggplant	0.16	16.3
potato	0.17	14.7
strawberry	0.73	3.3
seaweed	1.52	0.6
banana	0.51	0.3
spinach	1.18	0.1
pork	5.61	ND[b]
mackerel	3.32	ND
Japanese littleneck	4.97	ND
shrimp	3.13	ND
chicken	5.61	ND
beef	5.19	ND
Japanese malllotus leaf	3.57 ± 1.26	42.0 ± 10.2
mugwort	1.54 ± 0.14	27.3 ± 0.2
conandron leaf	0.58 ± 0.14	60.7 ± 8.4
Japanese mallotus leaf (boil)	4.65 ± 0.28	2.0 ± 1.8
corn (boil)	2.31 ± 0.41	3.7 ± 3.5
cabbage (boil)	0.90 ± 0.37	5.0 ± 3.3
rice (boil)	3.34	1.0

[a]Values are the mean ± SD from experiments performed in three independent experiments. Values of foodstuffs with low PA content (below approximately 100 nmol/g of wet weight) were single determination. [b]ND = not detectable (below 1.0% of total phospholipids).

leaves, and boiled Japanese mallotus leaves were $^1/_2$, $^1/_{15}$, and $^1/_{14}$ of those in raw foodstuffs, respectively (Figure 3). This is consistent with a well-known fact that PA was not formed from endogenous phospholipids in heat-treated cabbage leaves because of inactivation of enzymes.[15]

PLD Activity in Foodstuff Juices. We examined the PLD activity in juices made from several foodstuffs (Figure 4). As expected, the juice of cabbage leaves showed the highest PLD activity among those tested. In contrast, the PLD activities in the juices of spinach and minced meats were low. The ascending order of PLD activity in those foodstuff juices partially reflected that of the amount of PA (Figure 3).

DISCUSSION

It has been known that PLD is abundantly contained in cabbage leaves[23] and that repeated grinding of raw cabbage leaves activates the PLD to form PA from endogenous phospholipids.[15] Because other plants, such as radish and tomato, had substantially high PLD activity in original condition (Figure 4), hydrolysis of endogenous phospholipids by PLD activity during homogenization was expected to occur in these raw vegetables and possibly most raw plants. Accordingly, it is considered that PA contents in boiled plants may reflect those in native plants and that PA contents obtained from the raw plants may include additionally produced PA. However, this does not mean that cabbage is not a "PA-rich food", because PA is abundantly produced possibly by mastication. In fact, masticated cabbage leaves contained a similar PA level to that contained in mechanically homogenized cabbage leaves.[12] Thus, it is safe to say that the most effective and popular foodstuff for taking PA is raw cabbage leaves. Because cabbage juice produces PA from exogenously added PC (Figure 4), additional PA production occurs when raw cabbage leaves are masticated with other PC-rich food, such as egg or soybean. In this regard, an appropriate combination of foods, such as raw vegetables and dressing or mayonnaise containing lecithin, is also considered to increase the amount of PA during mastication.

In this study, we found that homogenized cabbage leaves contain PA (700 nmol/g of wet weight). This is 32 times as much as LPA in cabbage homogenates (22 nmol/g of wet weight).[12] Because PA is effectively converted to LPA by pancreatic PLA_2,[12] a large portion of LPA supplied to the GI tract upon ingestion of foods may be attributable to PA hydrolysis. Concerning the region where PA hydrolysis takes place, PLA_2 activity with a neutral pH optimum has been reported to be abundant in stomach mucosa of guinea pig and rat.[11,24] Previously, we showed that orally administrated LPA prevents stress-induced stomach ulcer.[7] The minimum effective dose of LPA was found to be around 0.01 mM (80 nmol/kg of body weight). It corresponds to 5 μmol of LPA for a human weighing 60 kg. About 7 g of cabbage leaves are enough for taking that amount of LPA if all ingested PA is converted to LPA in the stomach. To evaluate the effectiveness of PA on the prevention of stomach ulcer, we are now conducting experiments using an animal model.

Other interesting findings are that edible herbs known to be effective on GI disorders contained PA at high levels and that foodstuffs from animal sources did not contain substantial amounts of PA. There are many kinds of vegetables that are believed to be effective on stomach ulcer or diarrhea, such as cabbage leaves, radish root, and Japanese mustard spinach leaves. On the other hand, there are few animal foodstuffs that are believed to have such effects. The existence of PA-rich foodstuffs used for empirical therapy may be evidence that PA is effective on the restoration or prevention of certain kinds of GI disorders.

In conclusion, vegetables belonging to *Brassica* and some herbs produce high amounts of PA when they are mashed.

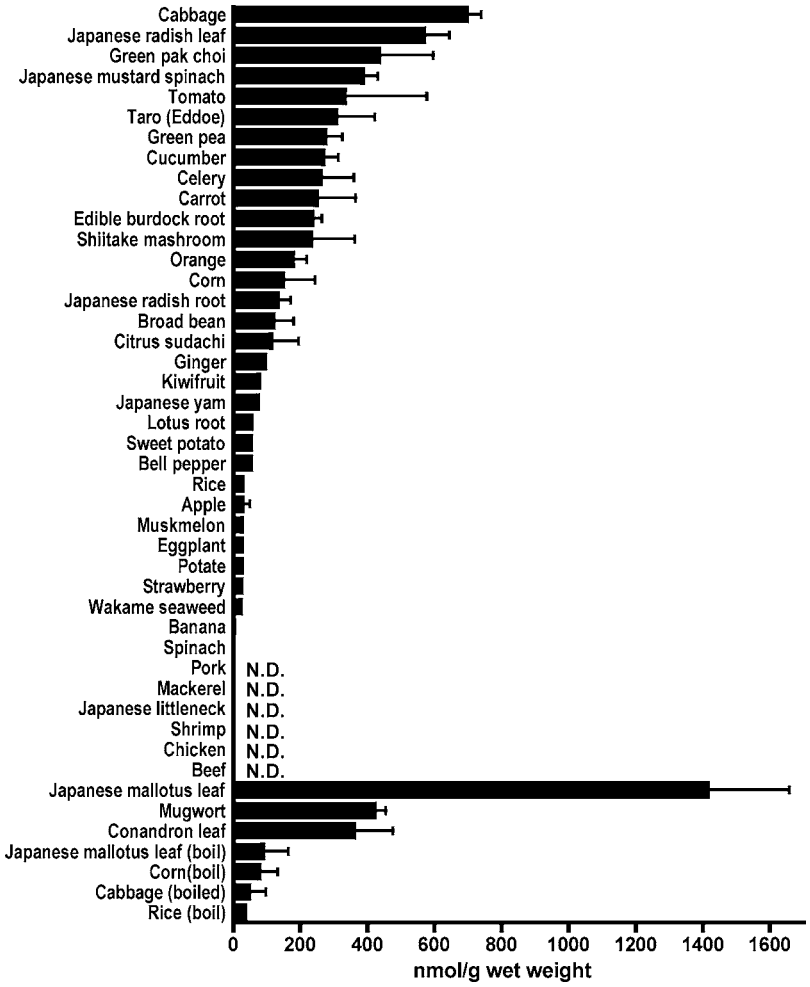

Figure 3. Amounts of PA in foodstuffs and herbs. Amounts of total phospholipids in foodstuffs and herbs were determined by a conventional molybudenum/blue method. The percentage of PA to the total phospholipids was determined by the TLC-imaging technique. Amounts of PA were calculated from these two values. Values are the mean ± SD from experiments performed in three independent experiments, except for foodstuffs containing PA approximately below 100 nmol/g of wet weight. Values of foodstuffs with low PA contents were obtained from single determination.

The information shown here will be useful knowledge for the consideration of the dietary treatment of certain kinds of GI disorders.

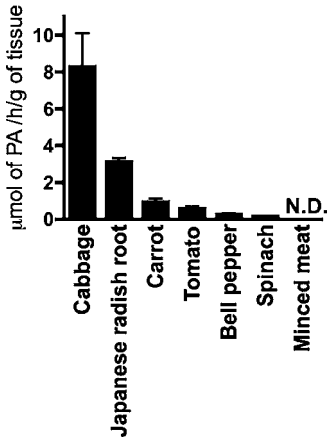

Figure 4. PLD activities in juices of raw foodstuffs. The supernatant fraction of the foodstuff homogenates was used for the determination of PLD activity using egg yolk PC as the substrate. Data were expressed as nanomoles of hydrolyzed PC per hour per gram of tissue (wet weight).

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Notes

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ABBREVIATIONS USED

GI, gastrointestinal; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIV, pixcel intensity value; SphM, sphingo-

myelin; TLC, thin-layer chromatography; PLA_2, phospholipase A_2; PLD, phospholipase D

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