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Inhibition of Lysophospholipase D Activity by Unsaturated Lysophosphatidic Acids or Seed Extracts Containing 1-Linoleoyl and 1-Oleoyl Lysophosphatidic Acid

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Lysophospholipase D (lysoPLD), generating lipid mediator lysophosphatidic acid (LPA) from lysophosphatidyclcholine (LPC), is known to be inhibited by lysophosphatidic acids. Meanwhile, some plant lipids are known to contain lysophospholipids as minor components. Therefore, it is interesting to test whether edible seed samples, rich in phospholipids, may contain lysophospholipids, which express a strong inhibition of lysoPLD activity. First, the structural importance of fatty acyl group in LPAs was examined by determining the inhibitory effect of various LPAs on bovine lysoPLD activity. The most potent in the inhibition of lysoPLD activity was linoleoyl-LPA (K_i, 0.21 μM), followed by arachidonoyl-LPA (K_i, 0.55 μM), oleoyl-LPA (K_i, 1.2 μM), and palmitoyl-LPA (K_i, 1.4 μM), based on the fluoresecent assay. The same order of inhibitory potency among LPA analogs with different acyl chains was also found in the spectrophotometric assay. Subsequently, the extracts of 12 edible seeds were screened for the inhibition of lysoPLD activity using both spectrophotometric and fluorescent assays. Among seed extracts tested, the extract from soybean seed, sesame seed, or sunflower seed (30 mg seed weight/mL) was found to exhibit a potent inhibition (>80%) of lysoPLD activity. In further study employing ESI-MS/MS analysis, major LPA components in seed extracts were identified to be 1-linoleoyl LPA, 1-oleoyl LPA, and 1-palmitoyl LPA with 1-linoleoyl LPA being more predominant. Thus, the potent inhibition of lysoPLD activity by seed extracts might be ascribed to the presence of LPA with linoleoyl group rather than other acyl chains.

KEYWORDS: Lysophospholipase D; inhibitor; lysophosphatidic acid; polyunsaturated; seed extract

INTRODUCTION

Lysophospholipase D (lysoPLD) activity, responsible for the conversion of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) corresponding to a potent lipid signaling molecule in numerous cellular responses, was first discovered in rat brain microsomes (*1*). Separately, an extracellular lysoPLD was observed to be present in rabbit and human plasma (*2*), and later in many tissues and body fluids (*3*–*5*). Recently, lysoPLD was shown to correspond to autotoxin (ATX), a tumor cell motility-stimulating factor, which belongs to ecto-nucleotide pyrophosphatase /phosphodiesterase family (*6*). Initially, only LPC was considered as a substrate for serum lysoPLD to generate LPA. Later, lysoPLD was found to act on other lysophospholipids, although to a lessor extent (*7*), and to hydrolyze sphingosylphosphorylcholine to produce sphingosine 1-phosphate (S1P) (*8*), another potent lipid signaling molecule.

However, LPA level is relatively low (*9*–*11*) in plasma or freshly isolated blood, where lysoPLD is constitutively active, whereas the level of plasma LPC (*12*, *13*) amounts to ~50 μM, close to the K_m of LPC as substrate for lysoPLD (*14*). Therefore, it has been suggested that lysoPLD may be subjected to feedback inhibition by its hydrolysis products such as LPA or sphingosine 1-phosphate in vivo. In support of this, lysoPLD was found to be strongly inhibited by some LPAs and sphingosine 1-phosphate at relatively low concentrations (*15*). Additionally, cyclic LPAs present in blood were also observed to potently inhibit lysoPLD activity (*16*). Thus, there have been endeavors to find potent inhibitors of lysoPLD, since a selective and potent inhibitor is required to elucidate the physiological or pathological role of lysoPLD activity. Overall, the actual activity of blood lysoPLD seems to depend on the level of LPA (*4*, *7*, *9*, *13*), which is known to be more predominant than cyclic LPA or sphingosine 1-phosphate in blood. Nonetheless, the structural

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importance of acyl chain in the inhibition of lysoPLD activity by LPA has not been fully established.

Meanwhile, an earlier study (*17*) demonstrated that soybean lipid extract contained lysophospholipids such as LPC or LPA. In addition, LPA level in plant sources was suggested to be altered by some enzymes (*18*). Therefore, it is supposed that the intake of some seeds containing a high quantity of LPA may contribute to the increase of LPA level in vivo. Inversely, the physiological level of LPA may be reduced by the intake of LPAs, which can express a strong inhibition of lysoPLD activity. In this respect, it will be interesting to see whether edible seed extracts may contain a sufficient amount of LPAs, which can express a potent inhibition of lysoPLD activity.

Here, we show that LPAs with polyunsaturated acyl chains are more potent than those with saturated acyl chains in the inhibition of serum lysoPLD activity. Additionally, we demonstrate that some seed extracts, showing a strong inhibition of lysoPLD activity, contain linoleoyl LPA as major LPA component.

MATERIALS AND METHODS

Materials. DEAE sephacel, concanavalin A-sepaharose, heparin-agarose, phenyl-agarose, choline oxidase, peroxidase (horse radish), 1-palmitoyl-sn-glycerol-3-phosphocholine, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were purchased from Sigma Chemical Co. (St. Louis. MO), and FS-3 and 1-linoleoyl LPA were from Echelon Co. (Salt Lake City, UT). Various LPAs and sphingosine 1-phosphate (S1P) were procured from Avanti Polar Lipids, Inc. (Alabaster, AL).

Purification of LysoPLD from Fetal Bovine Serum. LysoPLD was purified from fetal bovine serum according to a slight modification of the previous reports (*19, 20*) employing ammonium sulfate fractionation (30–60 %), DEAE sephacel chromatography, concanavalin A sepharose affinity chromatography, heparin agarose affinity chromatography, phenyl agarose hydrophobic interaction chromatography, and second DEAE sephacel chromatography. Finally, the enzyme fraction from second DEAE sephacel chromatography was subjected to FPLC employing Superpose 12 column (1 × 28 cm), which was eluted with 0.1 M Tris buffer pH 7.4 containing 0.5 M NaCl at 0.5 mL/min. The purified enzyme possessed a specific activity of approximately 1018 μmole/min/mg protein in the hydrolysis of 1-palmitoyl LPC. For the determination of the purity, the purified enzyme was subjected to 7.5 % acrylamide SDS-PAGE, and the proteins were detected by silver staining (*19*).

Spectrophotometric Assay of LysoPLD Activity. The determination of lysoPLD activity was carried out by measuring the amount of choline released from exogenously added LPC; briefly, LysoPLD was incubated with 1 mM 1-palmitoyl LPC in 100 mM Tris buffer pH 9.0 containing 500 mM NaCl, 5 mM $MgCl_2$, and 0.05 % Triton X-100 for 2 h at 37 °C. Then, to the assay mixture was added choline oxidase (300 unit/mL), horseradish peroxidase (1 unit/mL), and 1 mM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). After 2 min incubation at room temperature, the change of absorbance at 415 nm was determined (*19, 20*).

Fluorometric Assay for LysoPLD Activity. LysoPLD activity was determined using a fluorogenic substrate, FS-3, as described before (*21*); lysoPLD was incubated with FS-3 (1 μM) in 100 μl of 5 mM Tris buffer pH 8.0 containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl, 1 mM $MgCl_2$ and bovine serum albumin (1 mg/mL) for 1 h at room temperature. The change of fluorescent intensity was measured at 520 nm with the excitation at 480 nm.

Inhibition of LysoPLD Activity by LPAs and S1P. LysoPLD (0.2 unit) was incubated with 1-palmitoyl LPC (0.2–1.0 mM) in the presence of each LPA (hexanoyl, myristoyl, palmitoyl, stearoyl, arachidoyl, oleoyl, linoleoyl, or arachidonoyl) or S1P of various concentrations in 100 mM Tris buffer, pH 9.0 containing 500 mM NaCl, 5 mM $MgCl_2$, and 0.05 % Triton X-100 for 2 h at 37 °C. Separately, lysoPLD was incubated with FS-3 (0.5–4 μM) in the presence of each LPA or S1P as described above.

Screening of lysoPLD Inhibitors from Seed Extracts. Twelve plant seeds (wet) were obtained from the local market, Daejeon, Korea. Each seed powder (5 g) was extracted with two volumes of methanol overnight, and the extract, after centifugation (3500 rpm, 20 min), was concentrated under vacuum. The residue was dissolved in ammonium hydroxide (1 mL) to make ammonium salt form, and concentrated to a minimum volume using rotatory evaporator. The final residue was dissolved in water (0.5 mL), and used for the inhibition of lysoPLD. To screen the seed extracts showing strong inhibition of lysoPLD activity, lysoPLD (0.2 unit) was incubated with 1-palmitoyl LPC (0.2 mM) in the presence of each extract for 2 h at 37 °C, and the residual activity was determined as described above. For the fluorescent assay, lysoPLD (0.2 unit) was incubated with FS-3 (1 μM) in the presence of each sample for 1 h at 25 °C. Separately, the solvent extract from soybean powder was subjected to silica gel TLC separation employing solvent mixture (chloroform:acetone:methanol:water = 4.5:2:1:0.5), and each fraction of TLC plate was tested for the inhibition of lysoPLD activity using FS-3 assay.

ESI-MS/MS Analysis. To each seed powder (5 g) was added chloroform (10 mL), methanol (10 mL) containing 0.34 % BHT, and 17% ammonium hydroxide (9 mL), and the mixture was extracted under stirring for 90 min. 1-Arachidoyl LPA (0.6 nmol/mL) as internal standard was included in the mixture. After the storage at 4 °C overnight, the extract was centrifuged (3500 rpm, 20 min), and the supernatant was mixed with 2.5 mL of chloroform and 0.1 mL of Conc. HCl. Finally, the chloroform phase was concentrated under vacuum to provide the residue, which was dissolved in methanol, and used for the ESI-MS/MS analysis as described before (*22*). ESI-MS/MS analysis was carried out on a 4000 QTrap quadruple tandem mass spectrometer equipped with a turbo electrospray ion source (Applied Biosystems, CA). The nebulizer heated at 500 °C was used with a curtain gas 20 psi, nitrogen collision gas (CAD) set to medium, GS1 50 psi and GS2 50 psi. The entrance potential was set to 10 V, and declustering potential (DP) was set to 80 V for all transitions. The sample was diluted in methanol and directly delivered into the ESI source through a PTFE line (0.22 mm I.D.) at 10 μL/min. The data were collected from 300 to 500 *m*/*z*. Multiple reaction monitoring and precursor scan of *m*/*z* 79 were carried out in the negative ionization mode (*22*). Parameter settings for multiple reaction monitoring were as follows; selective ions for Q1 were *m*/*z* 409, *m*/*z* 433, *m*/*z* 435, and *m*/*z* 437, which correspond to molecular masses $[M\text{-}H]^-$ of C16:0, C18:2, C18:1, and C18:0 acyl-LPA, respectively. Selected ion for Q2 was *m*/*z* 79, corresponding to the fragment of phosphoryl moiety.

RESULTS

Previously (*15, 16, 20*), it was reported that lysoPLD activity was inhibited by LPAs. However, the study on the inhibitory action was limited mainly to LPAs with palmitoyl, myristoyl, or oleoyl group, despite the presence of LPAs with various acyl groups in plant or animal sources (*17, 22, 23*). Thus, the structural importance of acyl chain for the inhibitory effect of LPA on lysoPLD activity was not clarified. First, we examined the inhibitory effect of various LPAs, with saturated or unsaturated fatty acyl chains, on bovine lysoPLD activity. For this purpose, lysoPLD was purified from bovine serum as had been described before (*19*), and the purified enzyme was found to be relatively pure in SDS-PAGE analysis (**Figure 1**).

Determination of LysoPLD Inhibition by Spectrophotometric Assay. First, we examined the effect of LPAs on bovine lysoPLD activity using the spectrophotometric assay. In the routine assay, lysoPLD was incubated with 1-palmitoyl LPC (200 μM) in the buffer for 2 h, during which maximal amount of LPA, generated from palmitoyl LPC, was below 20 nmol/mL. Under the above condition, the Km value of 1-palmitoyl LPC was estimated to be approximately 126 μM, which was within the range of plasma LPC level (*8, 13*). Then, when 1-myristoyl LPA was tested for the inhibition of lysoPLD-catalyzed hydrolysis of 1-palmitoyl LPC, it was found to inhibit

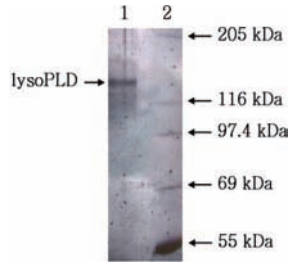

Figure 1. Electrophoretic analysis of purified lysoPLD. The purified lysoPLD was subjected to 7.5% SDS-PAGE, followed by silver staining. Lane 1. purified lysoPLD; Lane 2, protein standards.

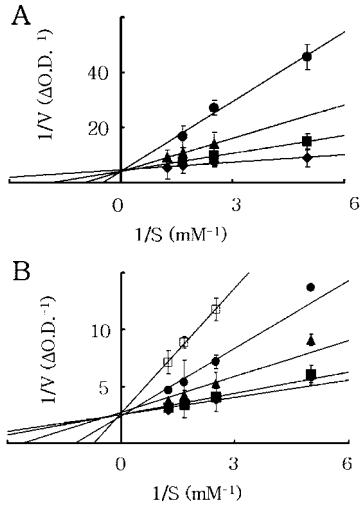

Figure 2. Inhibitory effect of LPAs on lysoPLD-catalyzed hydrolysis of 1-palmitoyl LPC. (**A**) LysoPLD was incubated with 1-palmitoyl LPC of various concentrations (0.2–1 mM) in the presence or absence of 1-myristoyl LPA (3–30 μM) in 100 mM Tris buffer (pH 9.0) containing 500 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100 for 2 h at 37 °C. ♦, Control; ■, 3 μM; ▲, 10 μM; ●, 30 μM. (**B**) LysoPLD was incubated with palmitoyl-LPC of various concentrations (0.2–1 mM) in the presence or absence of 1-linoleoyl LPA (2–16 μM) in 100 mM Tris buffer (pH 9.0) containing 500 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100 for 2 h at 37 °C. ♦, Control; ■, 2 μM; ▲, 4 μM; ●, 8 μM; □, 16 μM. Data were expressed as means ± SD of three experimental sets.

lysoPLD activity remarkably. The double-reciprocal plot analysis indicates that 1-myristoyl LPA displays a competitive inhibition of lysoPLD (**Figure 2A**). A similar competitive inhibition of lysoPLD was also expressed by the other LPAs with different saturated fatty acyl chains. In comparison (**Table 1**), the most potent in inhibiting lysoPLD activity was 1-myristoyl LPA (K_i, 5.0 μM), followed by 1-palmitoyl LPA (K_i, 10.4 μM), 1-stearoyl LPA (K_i, 25.8 μM), and 1-hexanoyl LPA (K_i, > 60 μM), suggesting that the size of acyl chain was crucial for the maximal inhibitory action. It is noteworthy that the inhibitory action of 1-linoleoyl LPA (K_i, 1.8 μM), showing a competitive inhibition (**Figure 2B**), was much greater than that of 1-stearoyl LPA (K_i, 25.8 μM) or 1-oleoyl LPA (K_i, 9.6 μM), which has the same carbon number. In addition, 1-arachidonoyl LPA (K_i, 3.4 μM) was much more inhibitory than 1-arachidoyl LPA (K_i, 9.6 μM), although it was less inhibitory than 1-linoleoyl LPA. Thus, the inhibitory potency of LPAs also appears to be affected by the unsaturation degree. In related study, 1-linoleoyl LPA was subjected to autooxidation or HOCl oxidation and then tested or the inhibition of lysoPLD activity. However, there was no remarkable change in the inhibitory action of 1-linoleoyl LPA under such oxidative conditions (data not shown). In a separate study, where sphingosine 1-phosphate was tested for the inhibition of lysoPLD, it was also found to show a competitive inhibition of lysoPLD with a K_i value of 1.8 μM, similar to that of 1-linoleoyl LPA. Thus, it is suggested that sphingosine 1-phosphate may be also implicated in the negative regulation of blood lysoPLD in vivo.

Table 1. K_i Values of Various LPAs in the Inhibition of LysoPLD Activity[a]

acyl group	spectrophotometric assay K_i (μM)	fluorometric assay K_i (μM)
hexanoyl	>60	12.99 ± 0.47
myristoyl	5.0 ± 2.5	0.65 ± 0.15
palmitoyl	10.4 ± 6.2	1.41 ± 0.34
stearoyl	25.8 ± 6.3	2.09 ± 0.61
oleoyl	9.6 ± 6.1	1.20 ± 0.32
linoleoyl	1.8 ± 0.7	0.21 ± 0.02
arachidoyl	>60	10.31 ± 0.98
arachidonoyl	3.4 ± 1.4	0.55 ± 0.26
SIP	1.8 ± 0.4	0.21 ± 0.06

[a] LysoPLD (0.2 unit) was incubated with 1-palmitoyl LPC (0.2–1.0 mM) in the presence of each LPA as described in the Materials and Methods section. Separately, lysoPLD (0.2 unit) was incubated with FS-3 (0.5–4 μM) in the presence of each LPA. The K_i values were determined as described in **Figure 2**. Data were expressed as means ± SD of three experimental sets.

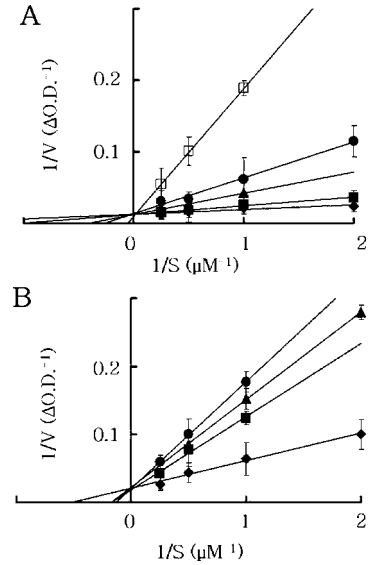

Figure 3. Inhibitory effect of LPAs on lysoPLD-catalyzed hydrolysis of FS-3. (**A**) LysoPLD was incubated with FS-3 of various concentrations (0.5–4 μM) in the presence or absence of myristoyl-LPA (0.3–10 μM) in 5 mM Tris buffer (pH 8.0) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ for 1 h at 25 °C. ♦, Control; ■, 0.3 μM; ▲, 1 μM; ●, 3 μM; □, 10 μM. (**B**) LysoPLD was incubated with FS-3 of various concentrations (0.5–4 μM) in the presence or absence of lineoyl-LPA (0.1–1 μM) in 5 mM Tris buffer (pH 8.0) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ for 1 h at 25 °C. ♦, Control; ■, 0.1 μM; ▲, 0.3 μM; ●, 1 μM. Data were expressed as means ± SD of three experimental sets.

Nonetheless, the assessment of lysoPLD inhibition may be obscured by limitations of the spectrophotometric assay, since LPA, generated from the incubation of lysoPLD with LPC, may interfere with the apparent inhibition of lysoPLD by added LPA. To avoid such a complication in the end-point LPC hydrolysis assays, we took advantage of another assay employing FS-3 as nonlipid substrate of lysoPLD (*21*).

Evaluation of LysoPLD Inhibition Using Fluorometric Assay. As shown in **Figure 3A** and **3B**, the hydrolysis of FS-3 by bovine lysoPLD exhibited Michaelis–Menten kinetics with a Km of 2 μM, much lower than the Km value of 1-palmitoyl

Table 2. Inhibition of lysoPLD Activity by Seed Extracts[a]

seed	residual activity (%)	
	spectrophotometric assay	fluorometric Assay
control	100	100
pumpkin	58.7 ± 16.5	60.6 ± 0.9
walnut, brown	15.6 ± 7.6	47.7 ± 0.2
pecan	10.6 ± 9.2	13.9 ± 0.3
cashew	91.4 ± 5.8	39.5 ± 0.2
sunflower	10.0 ± 4.4	6.7 ± 0.5
walnut, white	53.0 ± 11.2	19.7 ± 2.5
kidney bean	10.6 ± 9.3	15.0 ± 0.4
sesame	16.6 ± 0.8	7.5 ± 0.1
acorn	50.1 ± 10.4	24.8 ± 0.9
peanut	56.2 ± 4.9	47.1 ± 0.7
pinenut	61.7 ± 20.9	16.5 ± 1.3
soybean	8.4 ± 0.8	2.7 ± 0.4

[a] LysoPLD (0.2 unit) was incubated with 1-palmitoyl LPC (0.2 mM) in the presence of each seed extract (50 mg seed weight /mL) as described in the Materials and Methods section. Separately, lysoPLD was incubated with FS-3 (1 μM) in the presence of each seed extract (30 mg seed weight/mL). The residual activity was expressed as a percentile value of control activity (100%). Data were expressed as means ± SD of three experimental sets.

LPC in the spectrophotometric assay. When 1-myristoyl LPA and 1-linoleoyl LPA were tested for the inhibition of lysoPLD in the fluorometric assay, it was found that lysoPLD-catalyzed hydrolysis of FS-3 was competitively inhibited by 1-myristoyl LPA or 1-linoleoyl LPA at relatively low concentrations (**Figure 3A** and **3B**). From the double-reciprocal plot analysis, the K_i values of 1-myristoyl LPA and 1-linoleoyl LPA were estimated to be below 1 μM (**Table 1**). In comparison, the most potent in inhibiting lysoPLD was 1-linoleoyl LPA with a K_i value of 0.21 μM, followed by 1-arachidonoyl LPA (K_i, 0.55 μM), 1-myristoyl LPA (K_i, 0.65 μM), 1-oleoyl LPA (K_i, 1.20 μM), 1-palmitoyl LPA (K_i, 1.41 μM), and 1-stearoyl LPA (K_i, 2.1 μM). Again, 1-linoleoyl LPA was the most potent in inhibiting lysoPLD in FS-3 hydrolysis assay. Thus, the inhibitory action of LPAs was strongly dependent on the length of acyl chain as well as unsaturation degree. In further study, sphingosine 1-phosphate also showed a competitive inhibition of lysoPLD with a strong inhibition with a K_i value of 0.21 μM. Taken together, it is suggested that LPAs and sphingosine 1-phosphate may be implicated in the inhibition of lysoPLD in vivo.

Screening of Seed Extracts Expressing a Strong Inhibition of LysoPLD Activity. After establishing the inhibition of lysoPLD by various LPAs, the extracts from edible plant seeds were tested for the inhibition of lysoPLD activity. For this purpose, plant seed powders were extracted with methanol, and the ammonium hydroxide-extractable fraction from methanol extract was tested for the inhibition of lysoPLD, based on the hydrolysis of 1-palmitoyl LPC or FS-3 by lysoPLD. As demonstrated in **Table 2**, the inhibitory action of seed extract differed according to the seed type and assay method. In comparison, the fluorometric assay is favored over the spectrophotometric assay since the former method requires a smaller amount of inhibitor than the latter method. In this regard, the fluorometric assay may be favorable for samples contaminated with compounds, which may directly interfere with the assay. Therefore, the selection of seed extracts, used for the identification of inhibitory LPAs, was based on the findings obtained with the fluorometric assay. When the samples from 12 edible plant seeds were tested for the inhibition of lysoPLD, it was found that extracts from three seed samples, soybean, sunflower, and sesame, showed a remarkable inhibition (>80 %) of lysoPLD at a concentration as low as 30 mg seed weight per mL (**Table 2**).

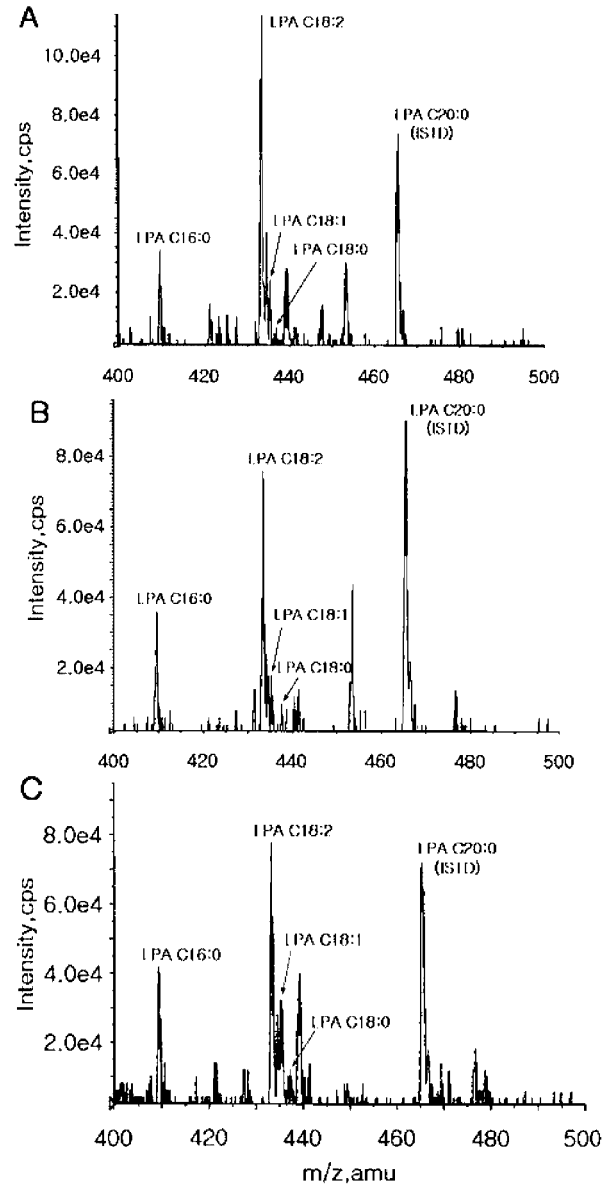

Figure 4. ESI-MS/MS spectrum of major LPAs from seed lipid. Lipid fraction, extracted from each seed powder, was subjected to ESI-MS/MS employing precursor scan at *m/z* 79 (phosphoryl group) as described in the Materials and Methods section. ISTD, internal standard.

Identification of Inhibitory LPA by ESI-MS/MS Analyses. To isolate the inhibitory compounds from three seed extracts, the respective powder of wet seed samples was extracted with the solvent mixture (chloroform + methanol + ammonia), and the extract was tested for the inhibition of lysoPLD activity; most of the inhibitory action was found to be extracted with the solvent mixture. When the extract was subjected to TLC separation (data not shown), the greater part of lysoPLD-inhibitory activity was found to migrate with standard LPA region (Rf, 0.35–0.45). Finally, the solvent extract from soybean seed, after acidification with HCl, was subjected to ESI-MS/MS analyses using precursor scan of *m/z* 79, which corresponds to the fragment of phosphoryl group (*22*). When the selected ions corresponding to molecular masses $[M-H]^-$ of natural LPAs were searched, it was found that $C_{18:2}$ acyl-LPA (*m/z* 433), $C_{18:1}$ acyl-LPA (*m/z* 435) and $C_{16:0}$ acyl-LPA (*m/z* 409) appeared as major LPA components (**Figure 4A**). Similarly, when the active fractions from the extracts of sunflower seed or sesame seed were subjected to ESI-MS/MS analysis, the ions corresponding

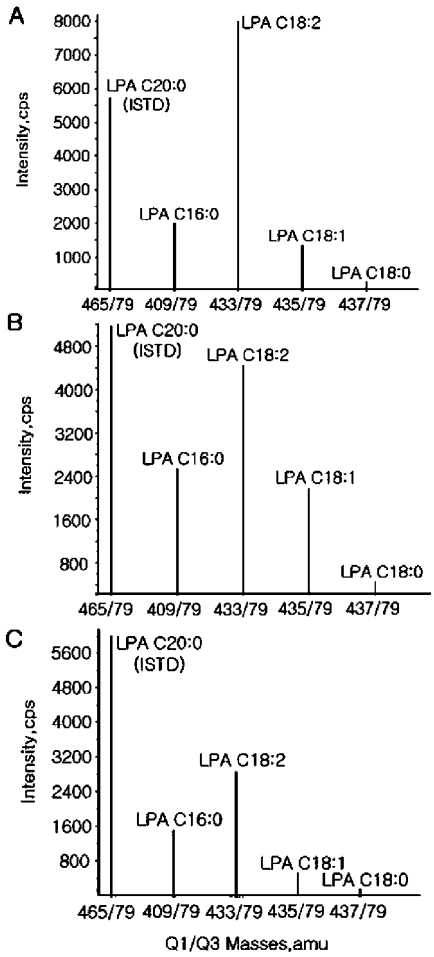

Figure 5. MRM spectrum of major LPAs from seed lipid. Lipid fraction, extracted from each seed powder, was subjected to ESI-MS/MS, and multiple reaction monitoring was carried out as described in the Materials and Methods section. ISTD, internal standard.

Table 3. Amount (nmole/g wet wt) of LPAs in Seeds[a]

	LPA C16:0	LPA C18:0	LPA C18:1	LPA C18:2
soybean	1.96 ± 0.24	<0.45	0.92 ± 0.23	6.00 ± 2.07
sunflower	2.20 ± 0.49	<0.45	1.84 ± 0.46	4.84 ± 1.38
sesame	0.98 ± 0.49	<0.45	0.46 ± 0.23	3.00 ± 0.46

[a] Amount of LPAs in each seed sample was calculated by measuring the height of each LPA relative to that of internal standard (1-arachidoyl LPA) as described previously (*22*). Data were expressed as means ± SD of three determinations.

to molecular masses of $[M-H]^-$ of $C_{18:2}$ acyl-LPA, $C_{18:1}$ acyl-LPA and $C_{16:0}$ acyl-LPA also appeared as major ones (**Figure 4B** and **4C**). However, the amount of other LPAs or sphingosine 1-phosphate was negligible. From these results, it is suggested that the inhibition of lysoPLD activity by three seed extracts may be mainly due to presence of 1-linoleoyl LPA, 1-oleoyl LPA, and 1-palmitoyl LPA. In a separate experiment, the amount of each LPA in seed extracts was quantified using 1-arachidoyl LPA (internal standard) as presented in multiple reaction monitoring (MRM) spectrum (**Figure 5A**, **B**, and **C**). As shown in **Table 3**, it was commonly observed that the most predominant was 1-linoleoyl LPA, followed by 1-palmitoyl LPA and 1-oleoyl LPA.

DISCUSSION

Previously, lysoPLD activity was reported to be inhibited by lysophosphatidic acids (LPAs) such as 1-oleoyl LPA or 1-myristoyl LPA, and cyclic LPAs (*15*, *16*, *20*). However, there has been no report on the inhibition of lysoPLD by LPAs containing polyunsaturated fatty acyl chains. Our present study indicates that LPAs with polyunsaturated fatty acyl chains, such as 1-linoleoyl LPA or 1-arachidonoyl LPA, are more potent than those with monounsaturated or saturated acyl chains in inhibiting lysoPLD-catalyzed hydrolysis of 1-palmitoyl LPC or FS-3; 1-linoleoyl LPA > 1-arachidonoyl LPA > 1-myristoyl LPA >1-oleoyl LPA > 1-palmitoyl LPA > 1-stearoyl LPA > 1-arachidoyl LPA. Among LPAs containing saturated fatty acyl moieties (C14-C20), those with shorter fatty acyl chains are more potent than those with longer ones. However, 1-hexanoyl LPA was a poor inhibitor, indicating that there may be a minimal size of acyl chain for the inhibitory action. Taken together, it is suggested that the inhibitory action of LPA may be determined by the degree of unsaturation as well as the length of acyl chain.

The order of inhibitory potency of LPAs in the hydrolysis of FS-3 by bovine serum lysoLPD is somewhat different from the previous finding (*15*) in the hydrolysis of CPF-4, a fluorescent substrate, by human lysoPLD; the most potent in inhibiting human lysoPLD activity was 1-oleoyl LPA, followed by 1-palmitoyl LPA and 1-myristoyl LPA. Morover, the K_i values of LPAs in the hydrolysis of FS-3 by bovine lysoPLD are relatively higher than those of LPAs in human lysoPLD-catalyzed hydrolysis of CPF-4, but smaller than those in human lysoPLD-catalyzed hydrolysis of p-nitrophenyl-5′-thymidine monophosphate (*15*). Thus, the inhibitory property of LPAs seems to differ according to the source of lysoPLD or the type of substrate. In the meantime, the higher K_i values of LPAs in the hydrolysis of 1-palmitoyl LPC, compared to FS-3, is likely explained by the notion that LPA produced during the incubation of lysoPLD with 1-palmitoyl LPC may interfere with the true inhibition of lysoPLD by added LPA under the same assay condition. This might be one of reasons why fluorescent assay is preferred to spectrophotometric assay. Further, the combinational use of fluorescent assay and spectrophotometric assay would allow more credible evaluation in the extensive screening of lysoPLD-inhibitory compounds.

Previous investigation indicates that the plasma contains a high level (~0.1 mM) of LPC, a large part of which consists of LPAs with palmitoyl or linoleoyl group (*13*). Then, it is possible that 1-palmitoyl LPA and 1-linoleoyl LPA may exist as predominant hydrolysis products in sera containing lysoPLD. A support for this may come from earlier report that the level (1.29–1.93 μM) of 1-palmitoyl LPA or 1-linoleoyl LPA (*23*) is greater than that (0.36–0.65 μM) of 1-oleoyl LPA, 1-stearoyl LPA or 1-arachidonoyl LPA in EDTA-treated plasma. Since the inhibitory action of 1-linoleoyl LPA is at least 5-fold greater than that of 1-palmitoyl LPA, it is more likely that 1-linoleoyl LPA may play an important role in the negative regulation of lysoPLD activity in blood. Furthermore, the negative control of lysoPLD activity would be more remarkable in some disease states (*24*, *25*), which the level of LPA in blood increased considerably.

Previously, the lipid preparation from soybean seed was reported to contain 1-linoleoyl LPA (*17*). Noteworthy, present data indicate that the seed extracts, showing potent inhibition of lysoPLD activity, commonly contain 1-linoleoyl LPA, 1-oleoy LPA and 1-palmitoyl LPA as major LPA components. Moreover, the amount of 1-linoleoyl LPA, showing greater inhibitory action, is higher than that of 1-oleoy LPA or 1-palmitoyl LPA in these seed extracts. These may support the notion that 1-linoleoyl LPA may play a major role in the strong inhibition of lysoPLD by these seed extracts. In this regard, it

is conceivable that the excessive intake of the plant seeds, containing 1-linoleoyl LPA as a major LPA component, may affect the LPA level in gastrointestinal tract by regulating lysoPLD activity negatively. However, it is not excluded that the intake of extraneous LPA can contribute to the increase of total LPA level in vivo. This discrepancy could be clarified by future study using animals administered with dietary LPA.

Here, it is shown that fluorometric assay, in combination with spectrophotometric assay, can be utilized as reliable method to screen inhibitors of lysoPLD present in food sources. Both assays indicate that LPAs with polyunsaturated fatty acyl chains are more potent than those with monounsaturated or saturated acyl chains in inhibiting lysoPLD activity. It is of note that the seed extracts, expressing a strong inhibition of lysoPLD activity, contain 1-linoleoyl LPA as major LPA component. Future study to establish the relationship between dietary consumption of plant LPAs and serum LPA levels would divulge the physiological effect of dietary LPA.

ABBREVIATIONS USED

LysoPLD, lysophospholipase D; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; ATX, autotoxin; S1P, sphingosine 1-phosphate.

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