methods

Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate-capture molecule

Tamotsu Tanaka,[1,*] Hideki Tsutsui,[*] Kaoru Hirano,[*] Tohru Koike,[†] Akira Tokumura,[§] and Kiyoshi Satouchi[*]

Department of Applied Biological Science,* Fukuyama University, Higashimura, Fukuyama, 729-0292, Japan; Department of Functional Molecular Science,† Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima, 734-8551, Japan; and Faculty of Pharmaceutical Sciences,§ University of Tokushima, 1-78-1 Shomachi, Tokushima, 770-8505, Japan

Abstract **Lysophosphatidic acid (LPA) is a lipid mediator that may play an important role in wound healing, embryonic development, and progression of cancer. Here, we report a procedure for the quantification of LPA by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The method is based on a characteristic mass shift with total charge change (from −2 to +1) of the phosphate species due to 1:1 complexation of LPA^{2-} with a dinuclear zinc (II) complex {1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex; Zn_2L^{3+}} at physiological pH. The monocationic complex $[LPA^{2-}\text{-}Zn_2L^{3+}]^+$ was detected in the positive mode, in which no other signal of cation adducts of LPA^{2-} was observed. The detection limit of 18:1 LPA by this method was 0.1 pmol on a sample plate. The intensity ratio of $[LPA^{2-}\text{-}Zn_2L^{3+}]^+$ against an internal standard [17:0 LPA^{2-}-$Zn_2L^{3+}]^+$ increased linearly with their molar ratio. Based on the relative intensities of complex ions, we determined the amounts of LPA homologs in an egg white by this method; the results obtained were in good agreement with those by gas liquid chromatography. This sensitive and convenient procedure for LPA-specific detection is useful for the quantification of LPA homologs occurring in biological materials.—** Tanaka, T., H. Tsutsui, K. Hirano, T. Koike, A. Tokumura, and K. Satouchi. **Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate-capture molecule. *J. Lipid Res.* 2004.** 45: **2145–2150.**

Supplementary key words egg white • matrix-assisted laser desorption/ionization time-of-flight mass spectrometry • serum

Lysophosphatidic acid (LPA) is a lipid mediator that induces diverse biological responses in vitro and in vivo (1, 2). It induces platelet aggregation (3, 4), contraction of smooth muscle cells (5), and proliferation of cells (6, 7). LPA has been shown to be present in several biological fluids, such as fetal calf serum (7), rat plasma (8, 9), human plasma (10–12), and human saliva (13), at significant concentrations. Because of its growth factor-like activity, LPA is considered to play a role in the wound-healing process (13, 14). Furthermore, evidence is accumulating that LPA present in human follicular fluid (15) or hen egg white (16) plays an essential role in embryonic development or egg transport (17). Also of importance are findings that LPA accumulates at high concentrations in plasma from patients with ovarian cancer (10, 11) and that LPA in ascitic fluid from patients with ovarian cancer may act as a mitogen to ovarian carcinoma cells (18, 19). These results suggest that levels of LPA in the body fluids are of clinical importance as a potential biomarker for ovarian cancer progression (10, 11).

Usually, LPA in body fluids consists of several molecular species with different acyl (or alkyl or alkenyl) chains (1, 2). It has been reported that one LPA receptor, LPA_3, discriminates differences in the chain length and number of *cis* double bonds in the acyl residue of LPA as well as the type of linkage and the position of the aliphatic chain at the glycerol backbone of LPA (20). Accordingly, the potency of physiological and pathophysiological responses induced by LPA (e.g., wound-healing activity, carcinoma metastasis activity) is dependent not only on the concentration of LPA but also on the molecular species composition of LPA in the body fluids (21). To this end, a convenient and highly specific method for the quantification of molecular species of LPA would be helpful for better understanding

Manuscript received 14 June 2004 and in revised form 3 August 2004.

Published, JLR Papers in Press, August 16, 2004.
DOI 10.1194/jlr.D400010-JLR200

Abbreviations: DHB, 3,5-dihydroxybenzoic acid; ESI, electrospray ionization; LPA, lysophosphatidic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PC, phosphatidylcholine; THAP, 2,4,6-trihydroxy-acetophenone; Zn_2L^{3+}, 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex.

[1] To whom correspondence should be addressed.
e-mail: tamot@fubac.fukuyama-u.ac.jp

of the physiological and pathophysiological roles of LPA and its biosynthetic route.

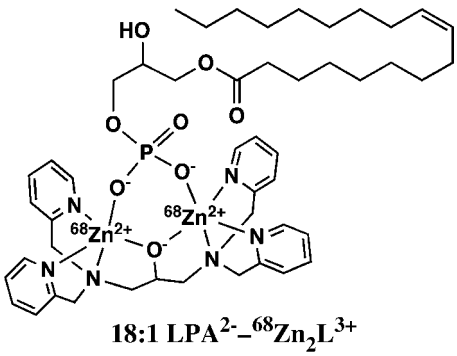
Several methods have been developed for the quantification of LPA (22–27). For the molecular species analysis of LPA, mass spectrometric analyses have been widely performed: GC-MS (electron impact ionization) (13, 24), fast atom bombardment MS (21), electrospray ionization (ESI) MS (11, 12, 26) and matrix-assisted laser desorption ionization (MALDI) MS (27). Among these methods, ESI MS and MALDI MS can detect lysophospholipids with high sensitivity and have the advantage of not requiring derivatization of LPA.

1,3-Bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc (II) complex (Zn_2L^{3+}; Phos-tag®) is a dinuclear zinc (II) complex acting as a phosphate-capture molecule (28) (**Fig. 1**). Takeda et al. (29) have reported that phosphorylated peptides, adenosine monophosphate, acetylphosphate, and LPA can be detected by MALDI-time-of-flight (TOF) MS as a monocationic complex with Zn_2L^{3+}. In the current study, we report a simple and sensitive procedure for the quantitative analysis of LPA homologs in biological samples by MALDI-TOF MS using this novel phosphate-capture molecule.

MATERIALS AND METHODS

Materials

2,4,6-Trihydroxy-acetophenone (THAP) and 3,5-dihydroxybenzoic acid (DHB) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phos-tag® ($^{68}Zn_2L^{3+}$) was obtained from the NARD Institute Ltd. (Hyogo, Japan) and MANAC, Inc. (Hiroshima, Japan). Arachidonic acid, oleic acid, and linoleic acid were purchased from Serdary Research Laboratories (London, Ontario, Canada). Palmitic acid and margaric acid were from Nacalai Tesque, Inc. (Kyoto, Japan). Calf serum was obtained from Gibco BRL and Life Technologies, Inc. (Rockville, MD). Phospholipase A_2 from *Crotalus adamanteus* venom and L-α-glycerophosphorylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). The active fraction of phospholipase D was prepared from cabbage using the method described by Davidson and Long (30). 1-Alkenyl (alkyl)-2-lyso-phosphatidylcholine (PC) was prepared from PC of bovine heart by mild alkaline hydrolysis (31).



18:1 LPA^{2-}–$^{68}Zn_2L^{3+}$

Fig. 1. Structure of the lysophosphatidic acid–1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex (18:1 LPA^{2-}-$^{68}Zn_2L^{3+}$)$^+$.

Syntheses of LPAs

First, 1-alkenyl (alkyl)-2-acyl-PC was synthesized by the condensation of 1-alkenyl (alkyl)-2-lyso-PC with anhydride of oleic acid (18:1), linoleic acid (18:2), or arachidonic acid (20:4) as described previously (31). The synthesized PC (1.6 μmol) was suspended in 0.6 ml of 0.2 M sodium acetate buffer (pH 5.8) containing 0.1 M $CaCl_2$, and the suspension was mixed with 0.4 ml of diethyl ether. The reaction was started by adding 0.4 ml of the active fraction of phospholipase D from cabbage. The reaction mixture was stirred vigorously at room temperature for 2 h. After evaporation of the ether phase under a stream of N_2 gas, phospholipids including 1-alkenyl (alkyl)-2-acyl-phosphatidic acid (PA) were extracted by the method of Bligh and Dyer (32) from the reaction mixture acidified to pH 2 with 2 M HCl. The phospholipids were then dissolved in 3.8 ml of chloroform-methanol-2.5 M HCl (1:2:0.8, v/v/v) to hydrolyze the 1-alkenyl-2-acyl-PA to 1-lyso-2-acyl-PA. After vigorous shaking for 15 min, phospholipids were extracted by the method of Bligh and Dyer (32) from the reaction mixture and subjected to TLC using chloroform-methanol-28% ammonia hydroxide (65:35:6, v/v/v) for the purification of 1-lyso-2-acyl-PA. The yield of 1-lyso-2-acyl-PA was ~20% of that of 1-alkenyl (alkyl)-2-acyl-PC. Because the acyl residue at the *sn*-2 position of the glycerol backbone of lysophospholipid is liable to migrate to the *sn*-1 position, the 2-acyl LPA was analyzed by MALDI-TOF MS within 24 h after its preparation. 1,2-Dipalmitoyl-PC, 1,2-dimargarinoyl-PC, and 1,2-dioleoyl-PC were synthesized by condensation of L-α-glycerophosphorylcholine with anhydride of palmitic acid, margaric acid, and oleic acid, respectively, as described previously (33). The synthesized PCs were treated with phospholipase A_2 as described (34). The resulting 1-acyl-2-lyso-PC was hydrolyzed by phospholipase D from cabbage, and the product was purified by TLC as described above. The yield of 1-acyl-2-lyso-PA was ~20% of the starting material, 1,2-diacyl-PC. The content of lipid phosphorus in LPA was determined by the method of Bartlett (35).

Extraction and purification of LPA from biological fluids

LPA in calf serum was extracted and purified by the method reported by Nakane et al. (16) with minor modifications. Three nanomoles of 17:0 LPA, 7.5 ml of chloroform, 15 ml of methanol, and 4.2 ml of distilled water were added to 2 ml of calf serum in a glass tube. After vigorous shaking, 7.5 ml of chloroform, 7.5 ml of distilled water, 0.05 ml of 14% ammonia hydroxide, and 0.7 g of KCl were added for phase separation. After centrifugation, the lower phase was discarded and the upper phase was washed with 15 ml of chloroform again. The upper phase was acidified to pH 2 with concentrated HCl and washed twice with 15 ml of chloroform. The lower phases were combined and evaporated to dryness. The residue was dissolved in a small volume of chloroform-methanol (2:1, v/v) and subjected to TLC using chloroform-methanol-28% ammonia hydroxide (65:35:6, v/v/v). The silica gel containing LPA was scraped off the plate, and LPA was extracted by the method of Bligh and Dyer (32) under acidic conditions (adjusting the pH of the upper phase to 2 with HCl). Similar methods for the preparation of LPA were used with 24 g of hen egg white. In this experiment, 17:0 LPA (161 nmol) as an internal standard was added to hen egg white before lipid extraction. The extraction was conducted with 4 g of egg white per glass tube. One-tenth of the LPA fraction obtained from 24 g of hen egg white was subjected to MALDI-TOF MS. The remaining nine-tenths of the LPA fraction of egg white was used for GC analysis. The yield of the egg white LPA prepared by our method was ~25%.

The purity of the LPA fraction prepared from egg white was assessed by TLC with chloroform-methanol-20% ammonia hy-

droxide (60:35:6, v/v/v) as a solvent system that separates lysophosphatidylinositol from LPA. We could not detect lysophosphatidylinositol in this LPA fraction.

Analysis of LPA by TOF MS

LPA was dissolved in 0.1 ml of 10 mM Tris-borate buffer (pH 8.0). Five microliters of $^{68}Zn_2L^{3+}$ solution (0.1–1.0 mM) was mixed with 10 μl of the LPA solution, and 0.5 μl of the mixture was spotted on a sample plate. Immediately, 0.5 μl of THAP solution (10 mg/ml in acetonitrile) was layered on the mixture as a matrix solution. The solution of matrix/analyte on the sample plate was dried for a few minutes. The matrix/analyte cocrystal that formed was subjected to MALDI-TOF MS. In the case of MALDI-TOF MS without the use of $^{68}Zn_2L^{3+}$, LPA was dissolved in 0.1 ml of chloroform-methanol (2:1, v/v). One microliter of LPA solution was mixed with 9 μl of DHB solution in the same solvent (10 mg/ml) as an alternative matrix. One microliter of the mixed solution was spotted on a sample plate. The solution of matrix/analyte on the sample plate was dried for a few minutes. The matrix/analyte cocrystal that formed was subjected to MALDI-TOF MS. In both procedures, MALDI-TOF mass spectra were acquired on a Voyager DE STR (Applied Biosystems, Framingham, MA) in the positive mode. The wavelength of the nitrogen-emitting laser, the pressure in the ion chamber, and the accelerating voltage were 337 nm, 3.7×10^{-7} Torr, and 20 kV, respectively. The detection was conducted in the reflector mode. The low mass gate was set at 400 Da. To enhance the reproducibility, 256 single shots from the laser were averaged for each mass spectrum. For GC analysis of LPA, the fatty acyl moiety of LPA was converted to fatty acid methylesters using 5% methanolic HCl, and the resultant fatty acid methylester was analyzed by GC equipped with a capillary column coated with 0.25 μm film of polar CBP 20, as described previously (34).

RESULTS AND DISCUSSION

Detection of $[LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ by MALDI-TOF MS

Figure 2A shows a MALDI-TOF mass spectrum of $^{68}Zn_2L^{3+}$ adducts of 18:1 LPA^{2-}. An intense ion peak assigned to $[18:1\ LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ was detected at *m/z* 1,023.3, but neither the protonated molecule of 18:1 LPA^{2-} nor the molecular ion of its cation adduct was detected in the mass spectrum, as previously reported by Takeda et al. (29). The detection limit of 18:1 LPA appears to be ~0.1 pmol on a sample plate (Fig. 2B). When 18:1 LPA was analyzed in the absence of $^{68}Zn_2L^{3+}$ in the positive mode using DHB as a matrix, two ions of the cation adduct of 18:1 LPA^{2-} were detected at *m/z* 459.2 $[LPA^{2-}\text{-}2H^+\text{-}Na^+]^+$ and *m/z* 481.2 $[LPA^{2-}\text{-}H^+\text{-}2Na^+]^+$ in the mass spectrum (Fig. 2C), as reported previously (27). In this case, more than 10 pmol of 18:1 LPA on a sample plate was required for its detection under our analytical conditions. Although a single ion assigned to be $[LPA^{2-}\text{-}H^+]^-$ was detected in the negative mode, more than 50 pmol of 18:1 LPA on a sample plate was required for its detection (data not shown). Detection of LPA as forms of multiple adducts is a problem for qualitative and quantitative analysis of LPA in biological samples, in which LPA comprises several molecular species with different acyl chain lengths. In this context, sensitive detection of LPA as a single form of $[LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ is advantageous for molecular species analysis of LPA in biological fluid. Detection of LPA as $[LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ has another advantage: low background noise in a mass range over *m/z* 900, in which ions of the $^{68}Zn_2L^{3+}$ adduct of LPA^{2-} are detected.

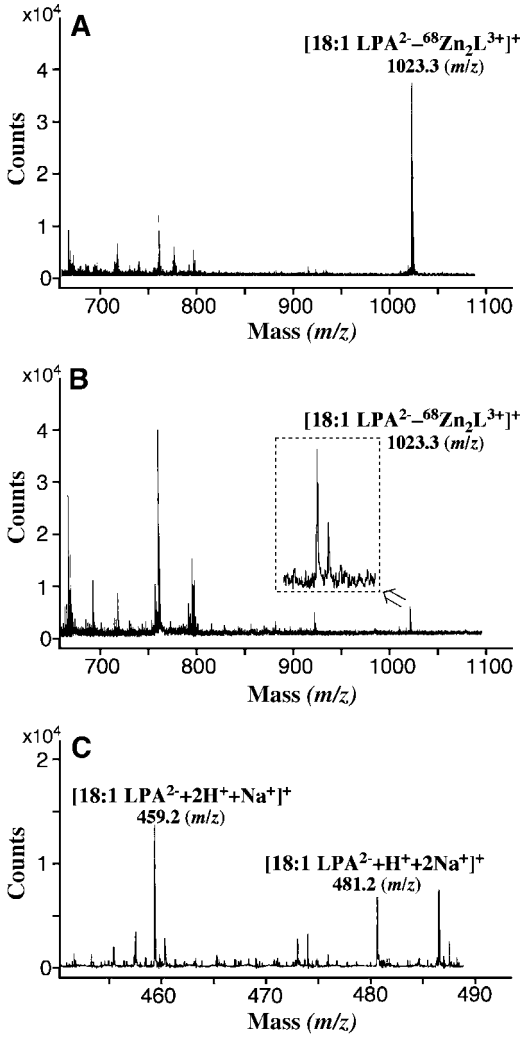


Fig. 2. Positive ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of the $^{68}Zn_2L^{3+}$ adduct of 1-18:1 LPA^{2-} (A and B) and the proton-sodium adduct of LPA^{2-} (C). A and B: The $LPA^{2-}\text{-}^{68}Zn_2L^{3+}$ solution (0.5 μl) was applied to the sample plate followed by the same volume of 2,4,6-trihydroxyacetophenone (THAP) solution (10 mg/ml in acetonitrile). The amounts of 1-18:1 LPA^{2-} and $^{68}Zn_2L^{3+}$ on the sample plate were 10 pmol and 17 pmol (A) and 0.1 pmol and 1.7 pmol (B), respectively. C: 1-18:1 LPA in a mixture of chloroform-methanol (2:1, v/v) was mixed with 9 μl of 3,5-dihydroxybenzoic acid solution (10 mg/ml in the same solvent). One microliter of the mixed solution was spotted on a sample plate. The amount of 1-18:1 LPA on the sample plate was 500 pmol.

Quantitative analysis of molecular species of LPA

First, we checked the difference in the detection efficiency between the positional isomers of LPA. Based on the relative intensity of ions of the $^{68}Zn_2L^{3+}$ adduct of 1- or 2-18:1 LPA^{2-} to that of 17:0 LPA^{2-}, detection efficiencies of these positional isomers of LPA were compared. Results showed that there was no difference in the detec-

tion efficiency between the $^{68}Zn_2L^{3+}$ adduct of 1-18:1 LPA^{2-} and that of 2-18:1 LPA^{2-} (data not shown). In the course of the experiments, we noticed that the $^{68}Zn_2L^{3+}$ adduct of LPA^{2-} with an unsaturated acyl chain was detected at a higher efficiency than that of LPA^{2-} with a saturated acyl chain. To determine the detection efficiencies of LPA homologs, samples containing different amounts of 16:0-, 18:1-, 18:2-, or 20:4-LPA (0.8–17 pmol on the sample plate) and a fixed amount of 17:0 LPA (17 pmol on the sample plate) as an internal standard were analyzed by MALDI-TOF MS with $^{68}Zn_2L^{3+}$. When a mixture of 16:0 LPA and 17:0 LPA was analyzed, the intensity ratio of the $^{68}Zn_2L^{3+}$ adduct of LPA^{2-} corresponded to its molar ratio. The slope of the straight line was ~1, suggesting that the detection efficiency of 16:0 LPA was equal to that of 17:0 LPA (**Fig. 3A**). On the other hand, the intensity ratio of the $^{68}Zn_2L^{3+}$ adduct of 18:1 LPA^{2-} to that of 17:0 LPA^{2-} was approximately two times the corresponding molar ratio, indicating a more effective ionization of the $^{68}Zn_2L^{3+}$ adduct of 18:1 LPA^{2-} than of 17:0 LPA^{2-} (Fig. 3B). We conducted similar experiments for 2-18:2 LPA and 2-20:4 LPA and found that their detection efficiencies were also approximately two times that of 17:0 LPA (Fig. 3C, D). It should be mentioned that the intensity ratio was not proportional to the molar ratio when the molar ratio of unsaturated LPA to 17:0 LPA was greater than 2. Therefore, an excess amount of 17:0 LPA over that of endogenously occurring unsaturated LPAs should be added to a sample. The presence of ions containing stable isotopes detected for each monoisotopic (M) ion should be taken into consideration in some cases. For example, the (M + 2) ion of 18:2 LPA and the M ion of 18:1 LPA appear at the same mass (*m/z* 1,023.3). Therefore, compensation of the intensity is necessary in such cases based on the fact that the intensity of the (M + 2) ion is ~15% of that of the corresponding M ion.

The method described above was used for the quantification of molecular species of LPA in hen egg white. As shown in **Fig. 4**, ions of the $^{68}Zn_2L^{3+}$ adduct of LPA^{2-} were detected at *m/z* 995.3, 997.3, 1,011.3, 1,021.3, 1,023.3, 1,045.3, and 1,069.3. These ions were assigned to be LPA^{2-} having 16:1, 16:0, 17:0, 18:2, 18:1, 20:4, and 22:6, respectively. Based on the intensity ratio of $[LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ against $[17{:}0\ LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$ of the internal standard, amounts of LPA homologs were determined. We also determined the amounts of LPA homologs of the same sample by GC as fatty acid methylesters. The results obtained by MALDI-TOF MS were similar to those obtained by GC (**Table 1**), showing that unsaturated LPAs were abundant in egg white (16). From these experiments, analysis of LPA by MALDI-TOF MS using $^{68}Zn_2L^{3+}$ is applicable to the quantification of LPA in biological samples.

Using this novel method, we determined the amounts of LPA homologs in 2 ml of calf serum. As shown in **Fig. 5**, several molecular species of LPA were detected as $[LPA^{2-}\text{-}^{68}Zn_2L^{3+}]^+$, and their intensities were measured against that of 17:0 LPA (**Table 2**). The sum of LPA homologs in calf serum (2.1 nmol/ml) is slightly greater than that of freshly prepared human plasma (0.5–1.9 μM) (11) and one-thirteenth that of fetal calf serum (28.4 nmol/ml) (7). The abundance in LPAs having 16:0, 18:1, and 18:2 in calf serum is compatible with the data obtained from human plasma (44.4–99.5 μM) (12). One of the reasons for the large differences in total LPA contents in these blood

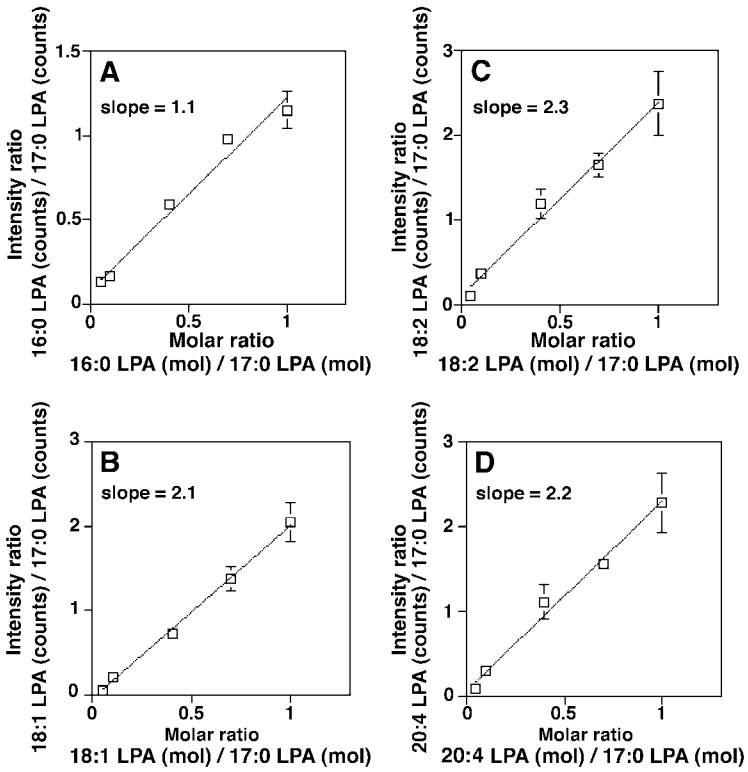


Fig. 3. Detection efficiencies of various molecular species of LPA. Different amounts (0.25–5 nmol) of 1-16:0 LPA (A), 1-18:1 LPA (B), 2-18:2 LPA (C), or 2-20:4 LPA (D) were mixed with a fixed amount of 17:0 LPA (5 nmol) in 0.1 ml of 10 mM Tris-borate buffer. Ten microliters of the mixed LPA solution was mixed with 5 μl of 0.2 mM $^{68}Zn_2L^{3+}$ solution. The mixed solution (0.5 μl) was applied to a sample plate and mixed with 0.5 μl of 10 mg/ml THAP solution in acetonitrile. The amount of 17:0 LPA on the sample plate was 17 pmol. Values are the means ± SD of three independent experiments.

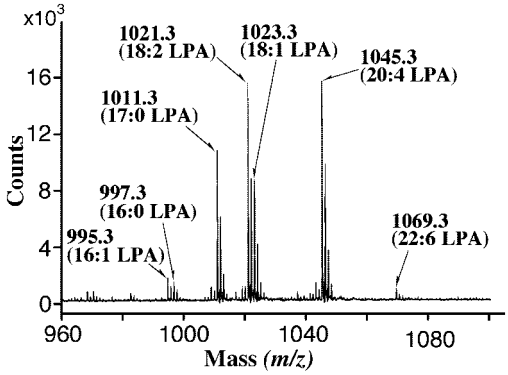


Fig. 4. Quantitative analysis of LPA homologs in hen egg white. The LPA fraction was prepared from hen egg white (24 g) that was mixed with 17:0 LPA (161 nmol) as an internal standard before lipid extraction. One-tenth of the LPA fraction was dissolved in 0.1 ml of 10 mM Tris-borate buffer, and 10 μl of the LPA solution was mixed with 5 μl of 1 mM $^{68}Zn_2L^{3+}$ solution. The solution of LPA^{2-}-$^{68}Zn_2L^{3+}$ was applied to a sample plate as a 0.5 μl droplet and mixed with 0.5 μl of 10 mg/ml THAP solution in acetonitrile. MALDI-TOF MS was conducted in the positive mode.

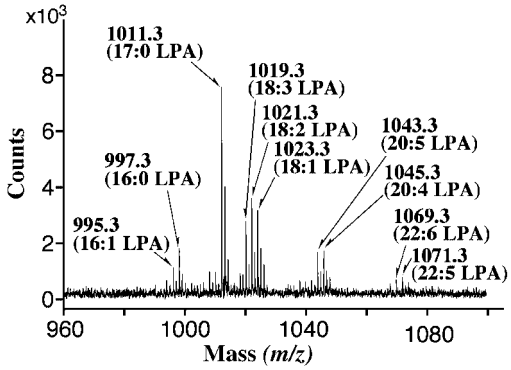


Fig. 5. MALDI-TOF mass spectrum of LPAs extracted from 2 ml of calf serum. The LPA fraction was prepared from 2 ml of calf serum that was mixed with 17:0 LPA (3 nmol) as an internal standard before lipid extraction. The TLC-purified LPA fraction was dissolved in 0.1 ml of 10 mM Tris-borate buffer. Ten microliters of the LPA solution was mixed with 5 μl of 0.1 mM $^{68}Zn_2L^{3+}$ solution. The solution of LPA^{2-}-$^{68}Zn_2L^{3+}$ was then applied to a sample plate (0.5 μl droplet) and mixed with 0.5 μl of 10 mg/ml THAP solution in acetonitrile. MALDI-TOF MS was conducted in the positive mode.

samples might be ascribable to the lysophospholipase D that produced LPA from lysophosphatidylcholine. The enzyme which exists in the serum or plasma has been shown to increase the LPA content in blood samples by incubation (8). This suggests the possibility that LPA contents in blood samples vary depending on the handling of the blood sample.

Purification of LPA by TLC appears to be necessary for its sensitive detection, since we could not detect $[LPA^{2-}$-$^{68}Zn_2L^{3+}]^+$ on direct measurement of crude lipid extract mixed with $^{68}Zn_2L^{3+}$. Because the purification of LPA by TLC is a time-consuming process, we are now developing a convenient method for LPA purification using Zn_2L^{3+} linked to agarose. In addition, we have confirmed that the method described here is applicable for the detection of sphingosine-1-phosphate, another bioactive phospholipid having a phosphate monoester. Both LPA and sphingosine-1-phosphate will be effectively purified and analyzed using Zn_2L^{3+} as a tool.

In the analysis of the mixture of LPA homologs, relative intensities of ions of LPA homologs did not vary much with the laser target point of the crystal. This observation suggests that homogeneous matrix/analyte cocrystal is formed under our procedure. As a matrix, THAP rather than DHB is better for the detection of $[LPA^{2-}$-$^{68}Zn_2L^{3+}]^+$. Possibly, DHB decreases the pH of the sample solution and thus prevents the formation of $[LPA^{2-}$-$^{68}Zn_2L^{3+}]^+$ (29).

Because MALDI-TOF MS cannot detect fragment ions

TABLE 1. Quantification of molecular species of LPA in egg white

Molecular Species	Amount	
	TOF MS	GC
	nmol/g	
16:0	0.5 ± 0.2 (5%)	0.8 ± 0.6 (9%)
16:1	0.5 ± 0.1 (5%)	0.5 ± 0.4 (6%)
18:0	N.D.	N.D.
18:1	1.8 ± 0.3 (17%)	1.6 ± 0 (18%)
18:2	4.4 ± 1.8 (40%)	3.6 ± 0.8 (40%)
20:4	3.3 ± 0.9 (30%)	2.0 ± 0.4 (23%)
22:6	0.4 ± 0.2 (4%)	0.4 ± 0.2 (5%)

17:0 lysophosphatidic acid (LPA; 161 nmol) was added to hen egg white (24 g) before lipid extraction, and the LPA fraction was prepared by TLC. One-tenth of the LPA fraction was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and analyzed with 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) ($^{68}Zn_2L^{3+}$) as described in the legend to Fig. 4. A half-value of the intensity was used for the calculation of unsaturated LPA. Because of the presence of (M + 2) ions, the actual intensities of 16:0 LPA and 18:1 LPA were calculated by subtracting the intensities of (M + 2) ions of 16:1 LPA and 18:2 LPA, respectively. The remaining nine-tenths of the LPA fraction of egg white was used for GC analysis. Values shown are means ± SD of three different hen eggs. N.D., not detectable.

TABLE 2. Quantification of molecular species of LPA in calf serum

Molecular Species	Amount
	nmol/ml
16:0	0.4 ± 0.1 (19%)
16:1	0.1 ± 0 (5%)
18:0	N.D.
18:1	0.3 ± 0.1 (14%)
18:2	0.4 ± 0.1 (19%)
18:3	0.3 ± 0.1 (14%)
20:4	0.2 ± 0 (10%)
20:5	0.2 ± 0 (10%)
22:5	0.1 ± 0 (5%)
22:6	0.1 ± 0 (5%)

The LPA fraction was prepared from 2 ml of calf serum that was mixed with 17:0 LPA (3 nmol) as an internal standard before lipid extraction. The TLC-purified LPA fraction was analyzed by MALDI-TOF MS using $^{68}Zn_2L^{3+}$ as described in the legend to Fig. 5. A half-value of the intensity was used for the calculation of unsaturated LPA. Because of the presence of (M + 2) ions, the actual intensities of 16:0, 18:1, 18:2, 20:4, and 22:5 LPA were calculated by subtracting the intensities of (M + 2) ions of 16:1, 18:2, 18:3, 20:5, and 22:6 LPA, respectively. Values shown are means ± SD of three independent experiments.

from the parent ion, information on the structure of LPA, such as the position of the acyl group at the glycerol backbone, is limited. The detailed structural analysis can be achieved using the ESI tandem MS system (11).

MALDI-TOF MS is used by many scientists for structural analysis of biological materials and is becoming commonly available not only for analyses of proteins but also for lipids, such as triglyceride, phospholipids, and glycosphingolipid (36, 37). The sensitive and convenient procedure for LPA-specific detection is useful for the quantification of molecular species of LPA that occur in small volumes of biological materials.

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