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Quantitative analysis of the kinetics of phospholipase A_2 using fast atom bombardment mass spectrometry

Shigeki Isomura, Kazuo Ito, and Mitsumasa Haruna*

Faculty of Pharmacy, Meijo university, 150 Yagotoyama, Tempaku, Nagoya 468, Japan

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Abstract : Fast atom bombardment mass spectrometry that can directly analyze lysophospholipids was used to quantitatively determine the kinetics of phospholipase A_2. This method is 1250 times more sensitive than the colorimetric assay.

Fast atom bombardment (FAB) mass spectrometry has been developed as powerful tool for analyzing structurally significant information from a wide range of materials. This method is well suited to the analysis of intact polar lipid species and has played an important role in the quantitative determination of lysophospholipid in mammalian organs.[1] Lysophospholipids are produced by phospholipase A_2 (PLA_2, EC.3.1.1.4) that plays a vital role as the rate-limiting step in the arachidonate cascade.[2]

Up to now, a number of detection methods have been developed to assay this enzyme activity.[3] Although the radioactive[4a-c] and the fluorescence assays[4d-f] are extremely sensitive detection methods, and the thio,[4g-h] the dye release[4i-j] and the SIBLINKS assays[4k] are useful methods with the spectrophotometer, these assay methods require chemical modification of the substrates. Furthermore, in spite of the fact that the titrametric,[4l-n] the acidimetric[4o] and the CoA-coupled assays[4p-r] can use natural substrates, these methods does not directly detect the hydrolytic product. These methods detect the liberated fatty acid from the phospholipid and the detection limit is 5-20 nmol.[3] In FAB mass spectrometry, lysophosphatidylcholine can be directly analyzed without modification and a slight amount of sample solution. Thus, the FAB mass spectrometry method can be used for the quantitative analysis of the kinetics of PLA_2. We now describe the quantitative analysis of the kinetics of PLA_2 by the application of FAB mass spectrometry.

1-Decanoyl-2-hydroxylphosphatidylcholine **1** ($C_{18}H_{38}NO_7P$ Mw 411) was derived from L-α-1,2-didecanoyl-3-phosphatidylcholine **2** by bovine pancreatic PLA_2[5]. In the FAB mass spectrum, the intensity of the isotope peak of **1** at *m/z* 413 was 23.1792%[6] compared to the molecular ion peak of **1** at *m/z* 412 (MH^+). The isotope labeled **3**[7] as an internal standard that has the same ionic efficiency to **1** was used to confirm the relation of peak intensity and concentration of **1**.

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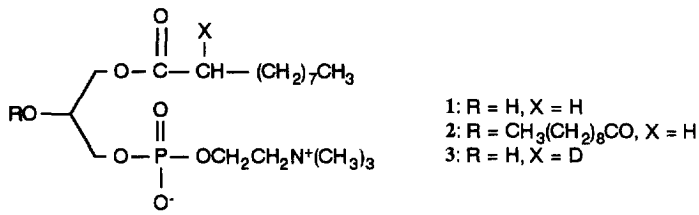

Figure 1. Structures of phospholipid and lysophospholipids

For the quantitative analysis, FAB mass spectrometry was performed as follows.[8] Xenon atoms were used to bombard the target, the ion-gun conditions typically being a 6 kV accelerating potential and 10 mA emission current. All analyses were carried out in the positive mode with the source at 30°C and 10kV accelerating voltage. The sample was measured with a xenon atom at 8×10^{-6} torr and the range from *m/z* 250 to 670 was scanned for 10s. Since the peak intensity ratios of *m/z* 412 and 413 were nearly constant during scans 2 to 15, all mass spectra were analyzed by accumulation for 2 to 5 scans in this study.

In the preparation of calibration curve, various concentrations of **1** from 3.125μM to 200μM were prepared by 0.25mg/mL PLA_2 in 50mM Tris HCl, pH 8.0. The FAB mass spectra of 1.3μL of the above solutions were measured with 1μL of constant concentration of **3** in 30% methanol glycerol as the standard matrix. The value of "p" that corresponds to the molar concentration ratio of **1** to **3** was calculated from the obtained I_{412}/I_{413} using equation 1 (Fig. 2).

$$p = \frac{I_{412} / I_{413}}{1\text{-}0.231792 \times I_{412} / I_{413}} \times f \qquad \text{eq.1}$$

"I_{412}/I_{413}" and "f" are the peak intensity ratio at *m/z* 412 to 413 and dilution factor, respectively. The calibration curve was obtained from concentrations of **1** (shown q in eq.2) and p values from equation 1 as the first order equation with a correlation coefficient >0.9995.

$$p = 0.052545 \times q + 0.002669 \quad r^2 > 0.9995 \qquad \text{eq.2} \quad \text{(Fig. 3)}$$

Thus, the amount of **1** in the reaction mixture of PLA_2 can be measured by the analysis of the FAB mass spectra and the catalytic activity of PLA_2 was analyzed using equation 1 and 2.

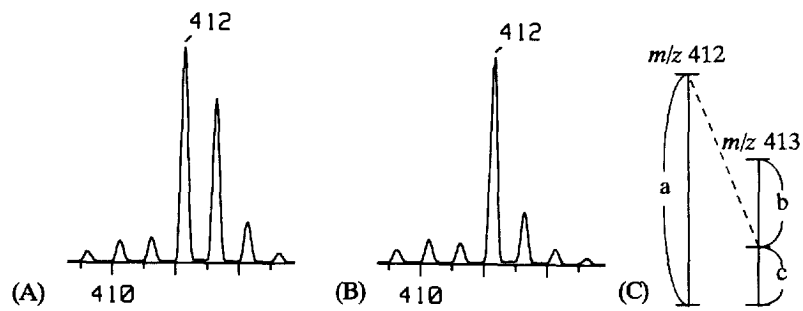

Figure 2. FAB mass spectra of lysophospholipid **1** with (A) or without (B) internal standard **3** and principle of equation 1 (C); these peak intensities correspond with these concentrations. a: peak intensity of **1**, b: peak intensity of **3**, c: isotope peak intensity of **1** (23.1792%).

The hydrolytic reaction of **2** by PLA_2 with Triton X-100[9] was carried out in the 2mM sodium deoxycholate,[10] 4mM $CaCl_2$ and 50mM Tris HCl, pH 8.0 at 25°C. After 5 minutes, this reaction was stopped by adding 12mM EDTA. To efficiently measure the produced **1**, the reaction mixture was 1/10 diluted[11] by the addition of 50mM Tris HCl, and the catalytic activities of PLA_2 were calculated using equation 1 and 2. The concentrations of the substrate and hydrolytic rates were analyzed by double reciprocal plots using average of three times reactions, and the Michaelis-Menten kinetics afforded a k_{cat} of 1.03 μmol/min/mg and K_m of 2.3mM (Fig. 4).

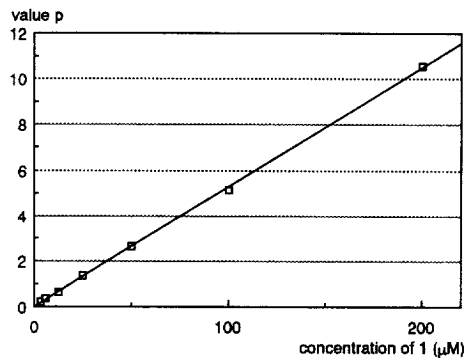

Figure 3. The calibration curve was prepared from various concentrations of lysophospholipid **1** using equation 1. This line showed a correlation coefficient above 0.9995 .

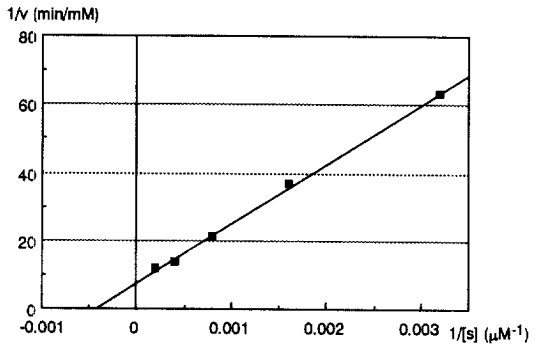

Figure 4. Hydrolytic rate of PLA_2 was calculated using equation 1 and 2 from FAB mass spectrum and double reciprocal plot of concentration of substrates and hydrolytic rates are shown. Closed square is average of three hydrolytic reactions to **2**.

From the colorimetric assay,[4q-r] the kinetic parameters of PLA_2 were analyzed under the same assay conditions and the Michaelis-Menten kinetics for the hydrolysis of **2** by PLA_2 afforded a k_{cat} of 1.06 μmol/min/mg and a K_m of 2.4mM.

In conclusion, the kinetics data from the FAB mass spectrum corresponds with that of the general quantitative analysis method. The major advantage of the FAB mass spectrometry method is 1250 times[12] more sensitive than the colorimetric assay. In addition, the obtained structural information is useful to analyze regioselectivity of the hydrolytic activity,[13] when the phospholipid has a different side chain at the C_1 and C_2 positions and are hydrolyzed by either phospholipase A_1 or A_2. Thus, the quantitative mass spectrometric analysis of the lysophospholipid can be used to determine the kinetics on a small scale without chemical modification. Furthermore, this methods can provide important structural information.

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5. PLA_2, the lyophilized powder containing approximate 20% protein, was purchased from SIGMA.
6. The ratio of isotope peak intensity of **1** was calculated from average data of mass spectrum at 10 times the practice value.
7. 1-(2'-Deuterodecanoyl)-2-hydroxylphosphatidylcholine (**3**, $C_{18}H_{37}DNO_7P$ Mw 412) was synthesized as the internal standard according to Eibl's Method using 2-deuterodecanoic acid derived from decanoic acid. **3**: ^{1}H-NMR (CD_3OD) δ: 0.89 (t, 3H, J = 6.73 Hz, -$\underline{CH_3}$), 1.29 (s, 12H, -$\underline{CH_2}$-), 1.59 (m, 2H, -$\underline{CH_2}$-), 2.34 (dt, J = 6.40, 7.39 Hz, -CO$\underline{CHD}$-), 3.22 (s, 9H, -$N^+(\underline{CH_3})_3$), 3.63 (m, 2H, -$\underline{CH_2}$N-), 3.89 (m, 2H, 1-$\underline{CH_2}$O-), 3.96 (m, 1H, 2-$\underline{CH}$OH), 4.10 (dd, 1H, J = 11.42, 6.05 Hz, 3-$\underline{CH_2}$O-), 4.16 (dd, 1H, J = 11.42, 4.70 Hz, 3-$\underline{CH_2}$O-), 4.28 (m, 2H, PO$\underline{CH_2}\underline{CH_2}$-). FABMS(+) m/z : 813 $(MH)^+$. Eibl, H.; arnold, D.; Weltzien, H. U.; Westphal, O. *Liebigs Ann. Chem.* **1967**, *709*, 226. The standard **3** contains the 69.8148% of the labeled compound.The peak intensities of labeled (I_{413}) and non-labeled (I_{412}) were corrected to the amount of non-labeled **3**.
9. The molar ratio of Triton X-100 to substrate is two to one. Dennis, E. A. *J. Lipid Res.* **1973**, *14*, 152.
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11. FAB mass spectrometry is very sensitive and a large quantity of lysophospholipid produces a saturated peak intensity.
12. The detection limits in this method and the colorimetric assay are 4 pmol (3.125 μM x 1.3 μL) and 5 nmol,[3] respectively.
13. 1-Octanoyl-2-decanoylphosphatidylcholine and 1-decanoyl-2-octanoylphosphatidylcholine, which consist of different side chain lengths were used for to the hydrolysis of PLA_2 and reactive products were measured by FAB mass spectrometry. The molecular ion peaks are observed at m/z 384 and m/z 412, respectively.