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Determination of enzymatic activity and properties of secretory phospholipase A_2 by capillary electrophoresis

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Abstract

A capillary electrophoretic (CE) system coupled with a diode array UV detector was used for the assay of secretory phospholipase A_2 ($sPLA_2$) activity. This method is based on monitoring both the breakdown of substrates and the formation of products simultaneously using micellar electrokinetic chromatographic techniques. Under our developed separation conditions, we analyzed the substrates and products quantitatively, and investigated enzyme activity as a function of reaction time and presence of enzyme activator or inhibitor. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was also utilized to confirm the phosphatidylcholine, a substrate of $sPLA_2$. In order to test the feasibility of the developed method for measurement of enzymatic activity, we compared it to the conventional radioactive assay method for $sPLA_2$. On the basis of our results, the conventional method can be complemented, or even replaced, by this new CE method which possesses the advantages of short analysis time, use of non-radiolabeled and inexpensive substrates, simple measurement of enzymatic activity, and exact quantitation of substrate and product.

Keywords: Phospholipases; Enzymes

1. Introduction

The secretory phospholipase A_2($sPLA_2$) family is composed of small, water-soluble proteins of M_r 13 000–18 000 consisting of a single polypeptide chain containing five to eight disulfide bonds. They are found in snake and bee venoms and in mammalian pancreas [1–3]. These extracellular enzymes were originally thought to play a role in digestion, but the recent discovery of cell surface receptors for this enzyme family suggests that they may also have a role in signal transduction [4]. Therefore, $sPLA_2$ enzymes have been the subject of considerable research, and the development of new $sPLA_2$ assay methods has become increasingly important, not only for the study of biological processes but also for clinical implications.

The most sensitive and widely used phospholipase assay requires the use of synthetic, radiolabeled phospholipids, many of which can be quite expensive. The radioactive assay follows hydrolysis by directly measuring the liberation of one of the hydrolysis products. The assay is discontinuous and requires the separation of the radioactive substrate from the labeled products by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), solvent extraction, or centrifugation.

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These separations are laborious and time consuming [5].

The micellar electrokinetic chromatography (MEKC) mode of capillary electrophoresis (CE) has been developed into a very powerful tool [6–8]. The development of MEKC was a major advancement in CE since it provided a method for the separation of charged or uncharged compounds [6–8] and electrically neutral compounds. This mode of CE is based on the partitioning of solutes between micelles and the run buffer [9–11]. Solutes that have a comparable absorption spectrum can be separated based on their different partition coefficients between the micellar and the aqueous phases. Using MEKC, extremely hydrophobic compounds have been separated [12]. In addition to separating electrically neutral compounds, MEKC has been used to separate ionized compounds [13] and large peptides and phosphopeptides [8]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) also has been a versatile and sensitive technique for the analysis of a variety of large biomolecules including mixtures of high molecular mass proteins [14,15], enzymatic protein digests [14,16–19], oligosaccharides [20], nucleic acids [21,22], and some biomolecules in crude biological fluids [14]. In addition, application of MALDI-TOF-MS has been extended to analyzing phospholipids with molecular masses $<10^3$ [23]. MALDI-TOF-MS is the fundamental technique in the field of biological MS.

We previously combined the high resolution of CE and the precise identification of MALDI-TOF-MS for the phosphatidylinositol-specific phospholipase C (PI-PLC) assay, which led to the very useful PI-PLC assay method [24]. Using MEKC and MALDI-TOF-MS, we have demonstrated that both the substrate and product of the enzyme can be separated exactly and conveniently with high sensitivity and reproducibility. In this study, the MEKC mode of CE was employed for characterization of $sPLA_2$. This method is based on monitoring both the breakdown of substrates and formation of product. To obtain the best conditions for separation of substrate and product, we varied the pH of the electrolyte buffer, the concentrations of borate and deoxycholic acid, and the voltage applied to the system. Under the developed conditions, the breakdown of phosphatidylcholine (PC), the substrate of $sPLA_2$, and formation of arachidonic acid (AA), the product of the enzyme, were simultaneously observed on a CE electropherogram. This assay method was used for confirming the well-known properties of $sPLA_2$. MALDI-TOF-MS was also utilized to identify the molecular mass of PC.

2. Experimental

2.1. Chemicals and reagents

L-2-Phosphatidylcholine,1-stearoyl,2-arachidonoyl (PC), AA, $sPLA_2$ from porcine pancrease, sodium tetraborate, deoxycholic acid, ethylenediaminetetraacetic acid (EDTA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St. Louis, MO. USA). L-3-Phosphatidycholine, 1-stearoyl-2-[1-^{14}C]-arachidonoyl was from Amersham (Buckinghamshire, UK). All organic solvents were HPLC-grade or better.

2.2. Capillary electrophoresis and sample preparation

The P/ACE 5500 capillary electrophoresis system with a diode array detector and an untreated fused-silica capillary column (47 cm×50 μm I.D., Beckman Instruments, Fullerton, CA, USA) was used for MEKC separations. Samples injections were performed by pressure for 3.0 s at 0.5 p.s.i. (1 p.s.i.= 6894.76 Pa). The detection wavelength was 196 nm, and the applied current was 80 μA. Before each CE run, the capillary was washed with 0.1 *M* sodium hydroxide (3 min), water (3 min) and running buffer (5 min). Data were analyzed using Beckman SYSTEM GOLD software, version 8.1.

2.3. Incubation conditions for the $sPLA_2$ assay in CE

$sPLA_2$ was stored in 3.2 *M* $(NH_4)_2SO_4$ at pH 5.5. The reaction mixture was prepared by mixing 100 μl of PC suspension (2 mg/ml) and 100 μl of enzyme reaction buffer (100 m*M* borate, 5.4 m*M* deoxycholic acid, and 0.2 m*M* $CaCl_2$ at pH 8.5). The reaction was initiated by adding 0.1 units of $sPLA_2$.

Assays were performed at 37°C and reactions were terminated by adding 100 m*M* EDTA solution.

2.4. Incubation conditions for $sPLA_2$ in the radioactive assay

The substrate, L-3-phosphatidylcholine,1-stearoyl-2-[1-^{14}C]arachiodonyl, was dried under N_2, resuspended in absolute ethanol by vigorous mixing, and then diluted in water. The reaction was initiated by adding 1 unit of $sPLA_2$ and incubated at 37°C. The reaction was terminated by adding 100 m*M* EDTA solution.

2.5. MALDI-TOF-MS and sample preparation

MALDI-TOF-MS analysis was performed on a HP G2025A instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 1-m linear type time-of-flight mass spectrometer and a pulsed nitrogen laser (337 nm radiation). This instrument was operated in the positive-ion detection mode. Typically, the spectra from 60–120 laser shots were summed to obtain the final spectrum.

The MALDI-TOF-MS matrix solution was prepared by mixing 100 m*M* 2,5-dihydroxybenzic acid (DHB) with water–methanol (2:1, v/v). The sample–matrix mixture was applied in a sample probe and crystallized with the HP G2024A sample preparation accessory. For the mass range examined in this study, the standard error in mass determination with this instrument is approximately 0.05% based on external calibration.

3. Results and discussion

3.1. Optimal CE conditions for the separation of PC and AA

The optimal conditions for the separation of PC and AA by CE were determined. We investigated the effects of running buffer pH, concentrations of sodium tetraborate and deoxycholic acid and temperature.

Because sodium tetraborate was used in the separation of phosphlipids in our previous study [24], it was chosen again. The influence of borate concentration on resolution of PC and AA is shown in Table 1. We varied the borate concentration from 10 to 50 m*M* and decided that 30 m*M* was the optimal concentration. As shown in Table 1, resolution at 50 m*M* is better than resolution at 30 m*M*. However, the migration time at this concentration is too long for rapid separation.

Table 1
Effect of borate concentration on the resolution of phosphatidylcholine (PC) and arachidonic acid (AA); migration times (t_m) and resolutions (R_S) of PC and AA were investigated as a function of borate concentration[a]

Borate conc. (m*M*)	t_m (min)		R_S[c]
	PC	AA	
10	NS[b]	NS	NS
20	NS	NS	NS
30	14.839	15.432	0.635
40	20.901	21.910	0.625
50	29.239	31.061	0.691

[a] Other conditions of separation solution are borate buffer containing 70 m*M* deoxycholic acid, pH 9.5.
[b] NS, not separated.
[c] R_S, resolution is calculated from $R_S = 1.171 \times (t_2 - t_1)/(W_{1(1/2)} + W_{2(1/2)})$, where t_1 and t_2 are the migration time of the two analytes and $W_{1(1/2)}$ and $W_{2(2/2)}$ are peak width of each peak at half of the peak height.

Since the pH of the electrolyte buffer in CE analysis has a great effect on the migration time of analytes by changing the electrophoretic and electroosmotic flow, we examined the effect of the run buffer pH on separation resolution of the analytes. To study this influence on the migration time and resolution in our analysis system, we varied the pH within the buffer capacity range of sodium tetraborate. As pH was decreased below 9.5, the pK_a of sodium tetraborate, the migration time of the analytes increased due to the reduction of the zeta potential (Table 2). When pH was increased above 9.5, the migration times of the analyte also increased. This increase might be caused by increased electrophoretic flow of analytes in the opposite direction, which was the result of more negative ionization of micelles and analytes. At pH 9.5, fairly good resolution was obtained. We also investigated the influence of pH on the relative migration times (t_R) of PC and AA. t_R values of analytes are shorter at pH 9.5 than at other pH values. Although the use of a pH 10.5 buffer could improve the resolution, the

Table 2
Effect of buffer pH on the resolution of phosphatidylcholine (PC) and arachidonic acid (AA); migration times (t_m) and resolutions (R_S) of PC and AA were investigated as a function of buffer pH[a]

pH	t_m (min)		R_S
	PC	AA	
8.5	16.312	16.987	0.497
9.0	13.120	13.608	0.522
9.5	14.839	15.432	0.635
10.0	25.580	27.143	0.634
10.5	32.899	35.150	0.697

[a] Other conditions of separation solution are 30 m*M* borate buffer containing 70 m*M* deoxycholic acid.

migration time was not adequate because of lengthened migration time. Based on these results, the electrolyte buffer was chosen to be pH 9.5.

Separation of analytes in MEKC mode occurs as a consequence of the difference in partition coefficients of the analytes between micellar and aqueous phases. In this study, we used deoxycholic acid as an additive. Concentration of deoxycholic acid was varied from 50 to 90 m*M* and the resolution at each concentration is compared in Table 3. Because the best resolution was obtained at a concentration of 70 m*M* deoxycholic acid, this concentration was selected.

The effect of temperature of the CE system on resolution of analytes was also investigated. In general, the main purpose of temperature control is to maintain the system at a relatively lower temperature to prevent excessive Joule heating which can cause band broadening. We tested the effects of temperature from 20 to 40°C on theoretical plate number of the analytes. At 25°C, the theoretical plate numbers of PC and AA were higher than at any other temperature (data not shown).

Table 3
Effects of deoxycholic acid concentration on the resolution of phosphatidylcholine (PC) and arachidonic acid (AA); migration times and resolutions of the PC and AA were investigated as a function of deoxycholic acid concentration[a]

Deoxycholic acid conc. (m*M*)	t_m (min)		R_S
	PC	AA	
50	13.119	13.719	0.559
60	13.440	14.011	0.611
70	14.839	15.432	0.635
80	16.876	17.580	0.592
90	18.203	18.896	0.453

[a] Other conditions of separation solution are 30 m*M* borate buffer containing deoxycholic acid, pH 9.5.

On the basis of the above results, the optimal separation condition of CE were: 30 m*M* sodium tetraborate (pH 9.5), 70 m*M* deoxycholic acid, and applied current of 80 μA at 25°C. The electropherogram in Fig. 1 clearly show the $sPLA_2$-catalyzed hydrolysis of PC and formation of AA under our separation conditions.

3.2. Confirmation of PC and AA

In order to identify the peaks of PC and AA separated using CE, we first investigated the mass of the first elution peak using the MALDI-TOF-MS (Fig. 2a). Samples were collected several times from repeated CE runs and the collected fractions were added to 2,5-dihydoxybenzoic acid (2,5-DHB) MALDI matrix solution. Then, sample mixed with matrix was analyzed by MALDI-TOF-MS. The molecular ion peak in MALDI-TOF-MS spectrum consists of the $[M+H]^+$ ion. From this fact, we confirmed the exact molecular mass of phosphatidycholine (m/z 810.1). AA was then confirmed by spiking with a standard AA sample (Fig. 2b). After enzyme reaction, an AA standard was added to the reaction mixture. As shown in Fig. 2b, the last peak in the electropherogram was dramatically increased confirming its identity as AA. Based on this mass analysis and peak spiking, we could confirm that the front peak is that of PC and last peak is that of AA.

3.3. Quantitative analysis of phosphatidylcholine

Using the optimized separation conditions, the reproducibility was tested. Good reproducibility for quantitative analysis of PC using CE was obtained, with the relative standard deviations (RSDs) of migration time and peak area obtained at 0.7 and 1.2%, respectively. In the analysis of AA, RSDs of migration time and peak area were 1.7 and 2.0%, respectively.

The detection limit for the PC was calculated from the electropherogram obtained after preparation of standard PC. Taking a signal-to-noise ratio of 3 as a

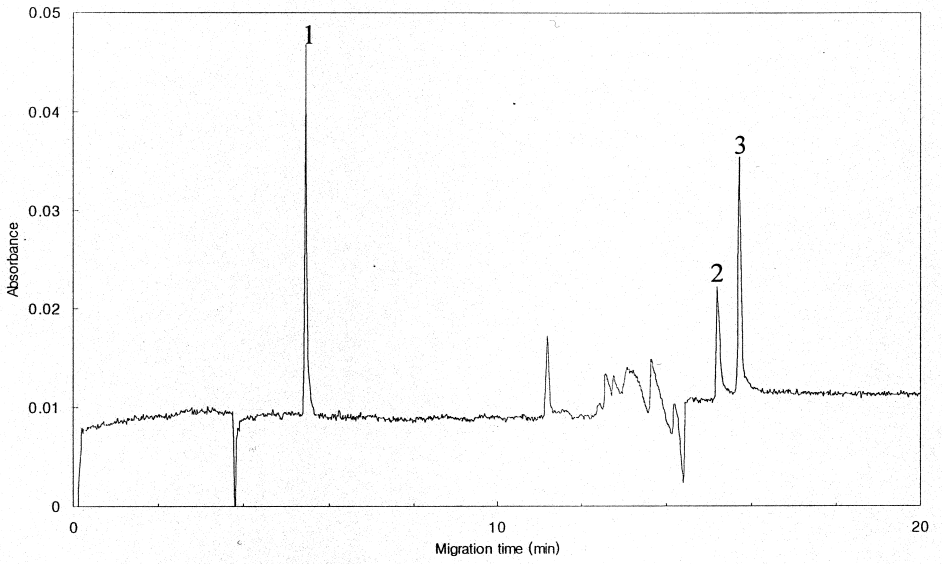

Fig. 1. Electropherogram of the $sPLA_2$-catalyzed hydrolysis of phosphatidylcholine (PC) to arachidonic acid (AA). PC was incubated in 30 m*M* borate buffer containing 3 m*M* deoxycholic acid and 3 m*M* $CaCl_2$, pH 8.5 at 37°C for 20 min in the presence of 1 unit of $sPLA_2$; 1=4-acetoamidophenol, EDF marker, 2=phosphatidylcholine, 3=arachidonic acid.

criteria, the limit of detection in our separation condition found to be 1 μg/ml for PC.

For quantitative analysis of PC, an internal standard method was employed. Various concentrations of PC ranging from 0.5 to 4.0 mg/ml were analyzed and the corresponding peak areas to the area of internal standard versus the concentration of the PC (data not shown) plotted. In this linear calibration curve, good linear relationship was obtained over the whole range of concentration ($R^2=0.9998$). Using this calibration curve, we could determine the precise amount of PC and monitor the enzymatic activity by comparing the amount of PC between the control group without enzyme and the experimental group. Therefore, the good linearity achieved in this study suggests not only the validity of the method for quantitative analysis of PC but also the feasibility of the method for monitoring the activity of enzyme.

3.4. Measurement of activity and properties of $sPLA_2$ in CE

Under the optimal separation conditions, we studied the enzymatic activity of $sPLA_2$. The electropherogram in Fig. 3 shows the breakdown of substrate, PC and formation of product, AA, as a function of reaction time. During the first 10 min, more than 50% of the total PC was consumed, and after 40 min, most of the substrate had been hydrolyzed to form AA. The amount of PC hydrolyzed by $sPLA_2$ was calculated using the standard calibration curve (data not shown). In this quantitative analysis, the initial rate of linearity was decreased. Perhaps the loss of initial rate linearity in this assay method was due to the relatively rapid depletion of substrate caused from our use of excessive amounts of $sPLA_2$.

The developed method was also used to study the effect of divalent metal ions on the enzymatic reaction of $sPLA_2$. Other investigators have shown that the activity of $sPLA_2$ is calcium-dependent, and divalent metal ions, such as Mg^{2+}, have an inhibitory effect on enzyme activity. Fig. 4 was constructed using the relative *V*, which represents the normalized value of phosphatidylcholine consumption in the enzyme reaction on the concentration of $CaCl_2$ or $MgCl_2$. As the concentration of Ca^{2+} increased, activity of the enzyme increased. Therefore, we confirmed that $sPLA_2$ is a calcium-dependent enzyme. In contrast, magnesium ions inhibited the activity of enzyme, as shown in Fig. 4. This

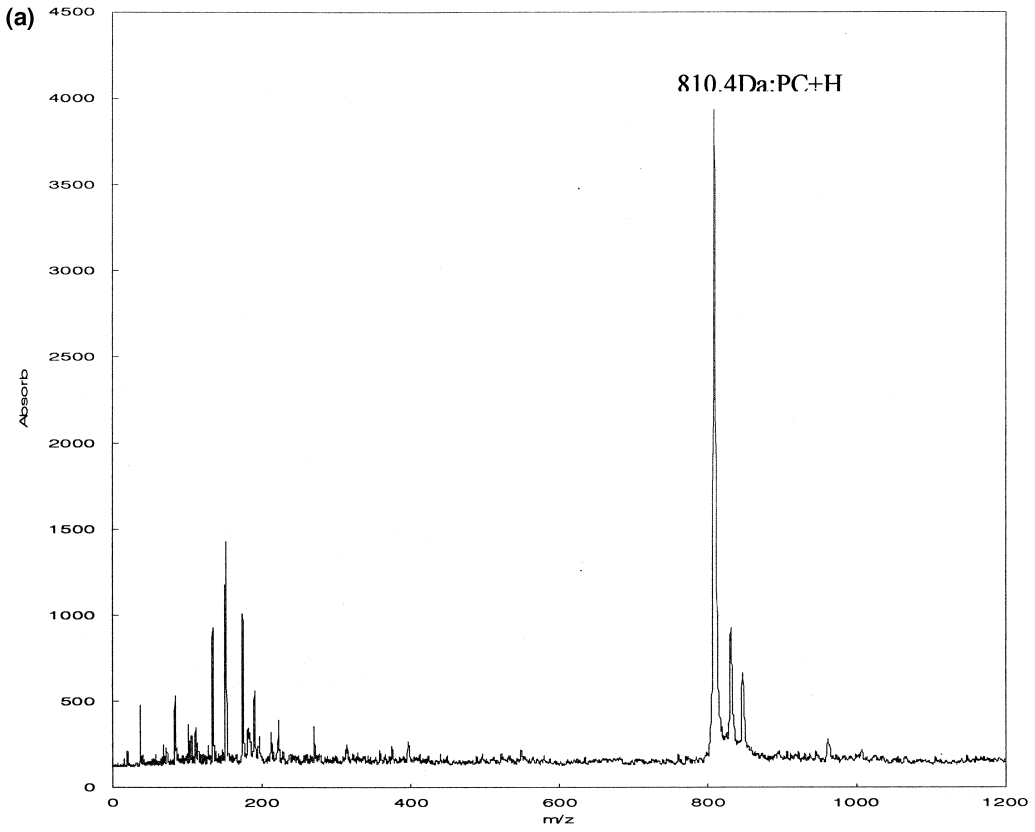

Fig. 2. (a) MALDI-TOF mass spectra of phosphatidylcholine (PC). The matrix solution of 2,5-dihydroxybenzoic acid (DHB) with water–methanol (2:1. v/v) was used. The ion accelerating potential was +28 kV and the length of flight tube was 1 m. (b) Electropherogram of spiked arachidonic acid (AA). Standard AA was added to sPLA$_2$-catalyzed PC sample; 1=4-acetoamidophenol, 2=phosphatidylcholine, 3=arachidonic acid.

inhibitory effect of Mg^{2+} is caused by the fact that divalent metal ions, such as Mg^{2+}, interact with phospholipid.

3.5. Comparison of the MEKC method to the radioactive assay method

In order to present the feasibility of the MEKC method for measurement of the enzymatic activity, we compared this method to the conventional radioactive assay method. In general, typical specific activities for commercially available phospholipids are 50–100 Ci/mmol for ^{3}H and 50–100 Ci/mmol for ^{14}C, which correspond to detection limits of about 1 fmol and 1 pmol, respectively. The radioactive assay also requires the separation of radioactive substrate from the labeled products by a laborious separation method, such as TLC. The radioactive assay was performed three times in our experiment. The cpm values of each assay for the product in our assay conditions were 11 709, 13 071 and 10 922 and dpm values were 12 684, 14 118 and 11 805, respectively. We also determined the detection limit of radiolabeled AA, in our radioactive assay to be about 1 pmol.

Compared to the conventional radioactive assay method, the MEKC method does not require radiolabeled substrates, which are expensive, dangerous, and generally not commercially available. Therefore, the MEKC method has many advantages in this respect. CE requires a short analysis time in comparison to TLC or HPLC allowing many analyses to

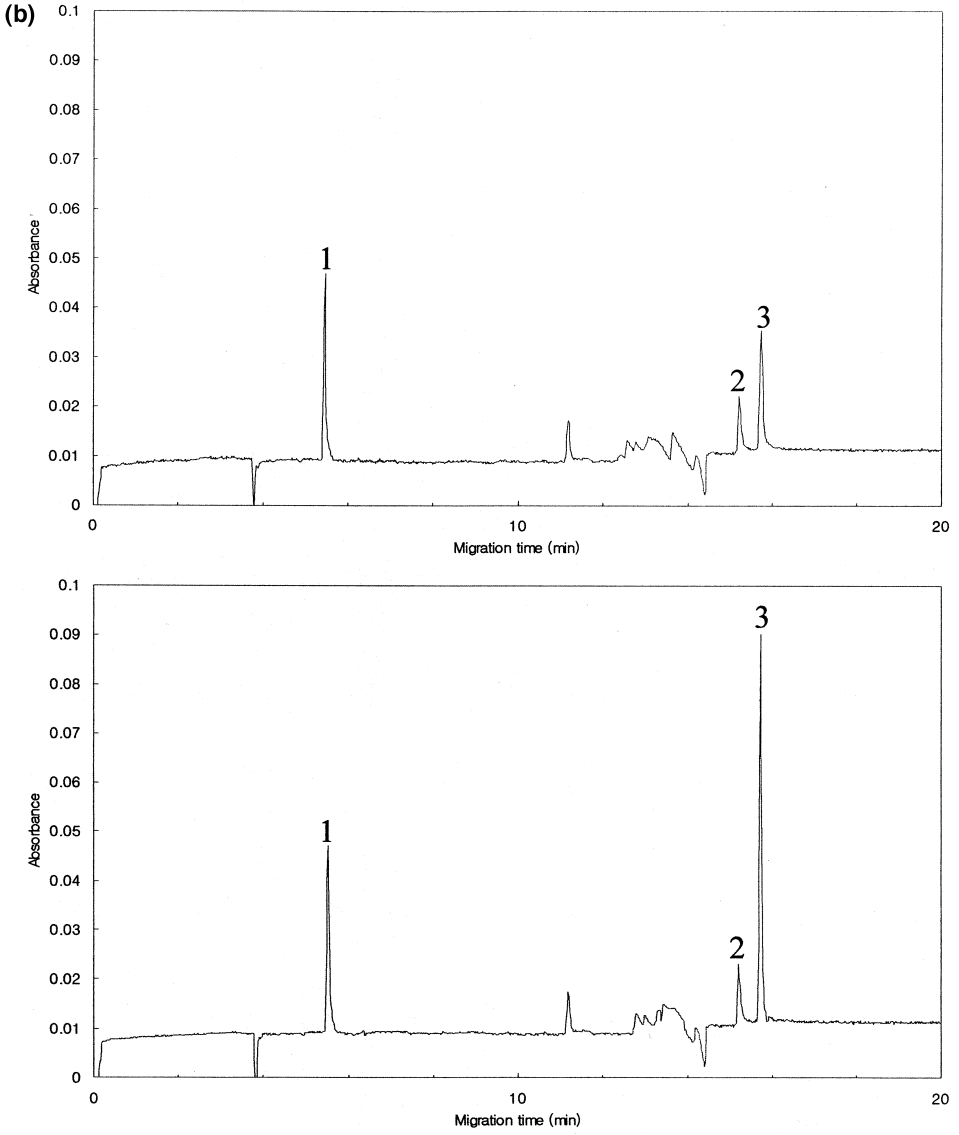

Fig. 2. (*continued*)

be performed in a short time. The detection limit of the MEKC method is lower than the conventional method using radioisotope-labeled substrates and TLC.

Based on these results, the conventional method can be complemented or even replaced by this new CE method. Compared to other separation techniques, MEKC is relatively rapid, reproducible and requires minimal amounts of sample. Moreover, the progression of the enzyme reaction can be visualized in an electropherogram with high sensitivity and reproducibility.

4. Conclusion

We report here a new approach for the enzymatic assay of $sPLA_2$. Under our developed conditions, we

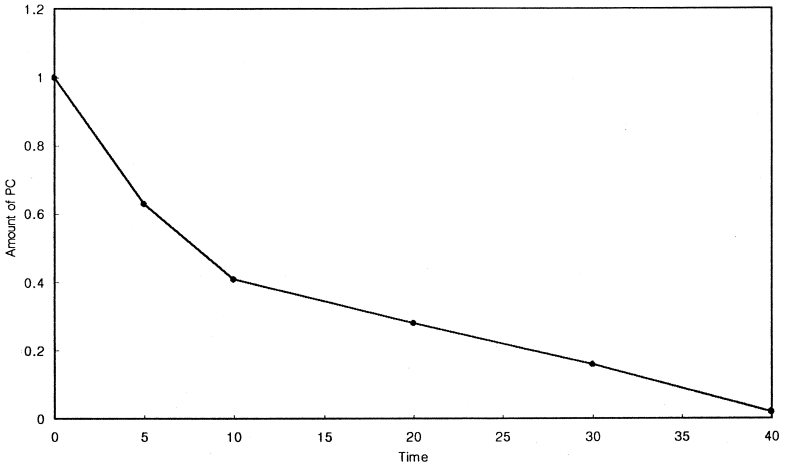

Fig. 3. Amount of time-course phosphatidylcholine (PC) hydrolyzed by $sPLA_2$. PC was incubated in 30 m*M* borate buffer containing 3 m*M* deoxycholic acid and 3 m*M* $CaCl_2$, pH 8.5 at 37°C for 20 min in the presence of 1 unit of $sPLA_2$. Amount of PC was calculated using the linear calibration curve. *x*-axis in min; *y*-axis in mg/ml.

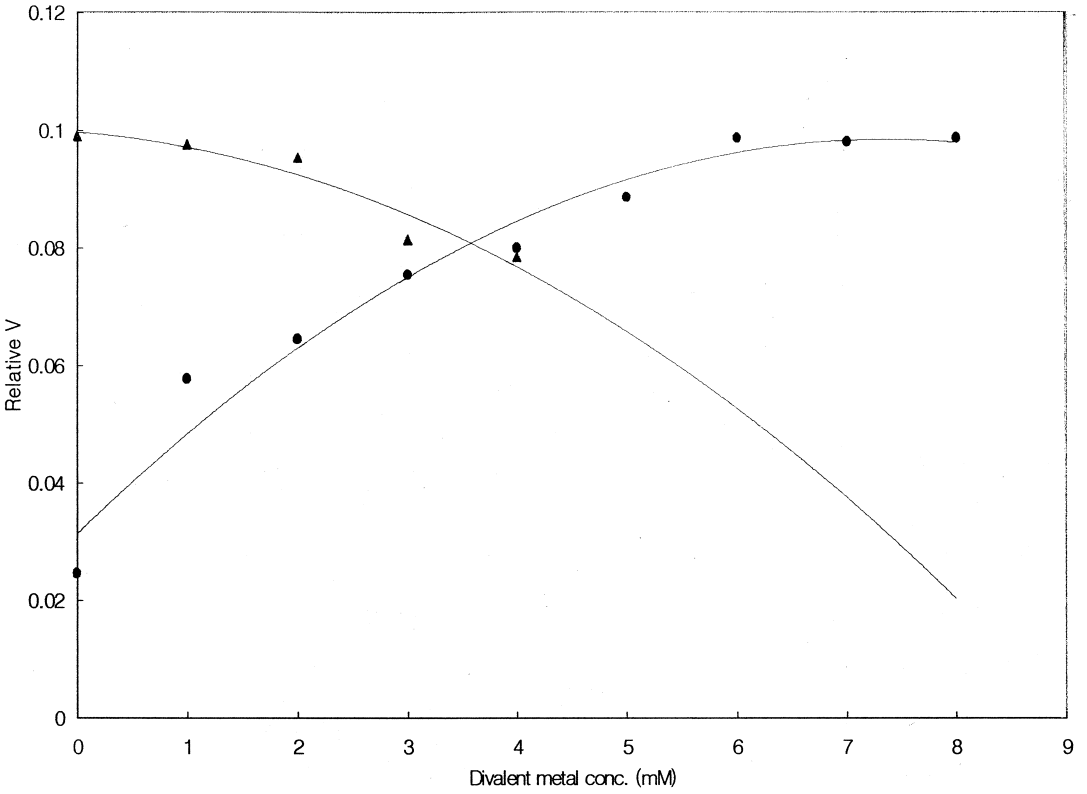

Fig. 4. Influence of $CaCl_2$ and $MgCl_2$ on PC hydrolysis by $sPLA_2$ under standard assay conditions. PC was incubated in 30 m*M* borate buffer containing 3 m*M* deoxycholic acid and various concentration of $CaCl_2$ or $MgCl_2$ at 37°C for 20 min with 1 unit of $sPLA_2$.

obtained high separation resolution for PC and AA. The loss of substrate and formation of product in the reaction of $sPLA_2$ could be clearly observed simultaneously and conveniently. Compared to the conventional radioactive enzyme assay, this new assay method is relatively rapid and reproducible, and has many advantages.

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