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Purification and Characterization of a Cytosolic Ca^{2+}-Independent Phospholipase A_2 from Bovine Brain

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The Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) subfamily of enzymes is associated with arachidonic acid (AA) release and the subsequent increase in fatty acid turnover. This phenomenon occurs not only during apoptosis but also during inflammation and lymphocyte proliferation. In this study, we purified and characterized a novel type of $iPLA_2$ from bovine brain. $iPLA_2$ was purified 4,174-fold from the bovine brain by a sequential process involving DEAE-cellulose anion exchange, phenyl-5PW hydrophobic interaction, heparin-Sepharose affinity, Sephacryl S-300 gel filtration, Mono S cation exchange, Mono Q anion exchange, and Superose 12 gel filtration. A single peak of $iPLA_2$ activity was eluted at an apparent molecular mass of 155 kDa during the final Superose 12 gel-filtration step. The purified enzyme had an isoelectric point of 5.3 on two-dimensional gel electrophoresis (2-DE) and was inhibited by arachidonyl trifluoromethyl ketone ($AACOCF_3$), Triton X-100, iron, and Ca^{2+}. However, it was not inhibited by bromoenol lactone (BEL), an inhibitor of $iPLA_2$, and adenosine triphosphate (ATP). The spot with the $iPLA_2$ activity did not match with any known protein sequence, as determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis. Altogether, these data suggest that the purified enzyme is a novel form of cytosolic $iPLA_2$.

INTRODUCTION

Phospholipase A_2 (phosphatide 2-acylhydrolase; PLA_2; EC 3.1.1.4) hydrolyzes the *sn*-2 position of phospholipids to release free fatty acids and lysophospholipids, which are important precursors of bioactive substances including prostaglandins, leukotrienes, lysophosphatidic acid, and platelet-activating factor (PAF) (Schaloske and Dennis, 2006). The PLA_2 family is largely divided on the basis of Ca^{2+} requirement and cellular localization into 3 major subfamilies: the secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$). $sPLA_2$ subfamily are stored in cytosolic granules or synthesized upon stimulation and then secreted extracellularly, whereas $cPLA_2$ and $iPLA_2$ subfamilies comprise intracellular enzymes. To date, this growing family of lipases has been categorized into 12 groups. Groups IIA, IVA, V, and VI have been extensively studied in the central nervous system (Green et al., 2008; Sun et al., 2010). Group VI $iPLA_2$, which does not require Ca^{2+} for catalysis, was purified and cloned in 1996. However, this enzyme was purified from rat brain homogenates by using multiple column chromatographic procedures with a very low yield (Molloy et al., 1998). Reportedly, this enzyme exists in aggregates and occurs in several splice variants (Larsson et al., 1998; Tang et al., 1997). Therefore, a wide variety of $iPLA_2$ activities has been found in different tissues (Tang et al., 1997).

Increased PLA_2 activity and the resultant excessive production of pro-inflammatory lipid mediators potentially lead to neurological disorders, such as ischemia (Bonventre et al., 1997; Tabuchi et al., 2003), Alzheimer's disease (Farooqui et al., 1997; 2006; Stephenson et al., 1996), spinal cord injury (Anderson et al., 1985; Lukacova et al., 1996), Parkinson's disease (Klivenyi et al., 1998; Tariq et al., 2001; Yoshinaga et al., 2000), schizophrenia (Junqueira et al., 2004; Ross et al., 1997; 1999; Yao et al., 2000), and multiple sclerosis (Kalyvas and David, 2004; Kalyvas et al., 2009; Pinto et al., 2003). Besides, lysophosphatidylcholine, a product of PLA_2, leads to demyelination (Dousset et al., 1995). In the brain, the basal expression and activity of $iPLA_2$ are higher than those of $cPLA_2$ or $sPLA_2$ (Farooqui et al., 1999; Molloy et al., 1998). Therefore, inhibition of $iPLA_2$ activity is an attractive approach for designing novel drugs to treat neurodegenerative disease-associated inflammation. However, until now, enzymes of the $iPLA_2$ subfamily have not been fully characterized and identified in nerve tissues. Therefore, identifying a form of PLA_2 in nerve tissues is essential to better understand and efficiently treat neurodegenerative diseases.

In this study, we purified a cytosolic $iPLA_2$ from bovine brain homogenates by using serial column chromatography. We classified this enzyme as a novel form of $iPLA_2$ on the basis of biochemical studies. It had an apparent molecular mass of 155 kDa and an isoelectric point of 5.3 on two-dimensional gel elec-

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trophoresis (2-DE). In addition, the purified $iPLA_2$ functioned at an optimal pH of 9.5 and its activity was inhibited by arachidonyl trifluoromethyl ketone ($AACOCF_3$), Triton X-100, iron ions, and millimolar concentrations of Ca^{2+}. This enzyme differed from the previously reported types of $iPLA_2$ from other tissues (Ackermann et al., 1994; 1995; Hazen and Gross, 1991; Hazen et al., 1990; Wolf and Gross, 1985) in that it was not inhibited by bromoenol lactone (BEL) and adenosine triphosphate (ATP). We expect this novel enzyme to play a crucial role in the production of eicosanoids.

MATERIALS AND METHODS

Materials

1-stearoyl-2-[1-^{14}C] arachidonoyl-*sn*-glycero-3-phosphocholine (2-[1-^{14}C]AA-GPC; 55.3 mCi/mmol), 1-acyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphoethanolamine (2-[1-^{14}C]AA-GPE; 55.1 mCi/mmol), and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]PA-GPC; 55.6 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Adenosine 5′-triphosphate disodium salt and Triton X-100 were purchased from Sigma-Aldrich (USA). Heparin-Sepharose CL-6B gels, Mono Q anion-exchange columns, Mono S cation-exchange columns, and Superose 12 fast protein liquid chromatography (FPLC) gel-filtration columns were purchased from Pharmacia LKB Biotechnology (Sweden). DE52 gel was purchased from Whatman (UK). A phenyl-5PW hydrophobic column was purchased from Tosoh (Japan). Bovine brain tissue was obtained from a local slaughterhouse in Seoul and stored in a -70°C deep freezer. Immobiline DryStrip (pH 4-7, 7 cm), 2-DE reagents, including the IPGphor for first-dimension separation, and the silver staining reagent kit were purchased from Amersham Pharmacia Biotech (Sweden). Second-dimension separation was performed in a Mini Protein System from Bio-Rad (USA).

$iPLA_2$ activity assay

The substrate, 2-[1-^{14}C]AA-GPC, was dried under nitrogen stream and subsequently resuspended in ethanol. The $iPLA_2$ activity assay was performed in 100 μl of the standard reaction buffer [200 mM Tris-HCl (pH 7.5) and 5 mM EDTA] containing $iPLA_2$ preparations and 0.45 nmol of the substrate (~55,000 cpm). Crude or purified $iPLA_2$ samples were incubated with the substrate at 37°C for 15-30 min. The assay was subsequently terminated by adding 560 μl of modified Dole's reagent (*n*-heptane/isopropyl alcohol/1 N H_2SO_4 as 400/190/10 [v/v/v]) and 110 μl of water, vortex mixing, and centrifuging. Then, 150 μl of the upper phase were transferred to a new microtube, to which 800 μl of *n*-heptane and silica gel (~5 mg) was added. The sample was vortex-mixed and centrifuged again. An aliquot (800 μl) of the supernatant was removed and added to 2.5 ml of β-scintillation solution (Insta Gel-XF) and counted for radioactivity with a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., USA). To investigate the optimal pH for $iPLA_2$ activity, the following buffers were prepared: 200 mM Tris-HCl (pH 7.5-8.5) and 200 mM glycine-NaOH (pH 9.0-10.5). The optimal pH for $iPLA_2$ activity was 9.5, but the assay was conducted at pH 7.5 to perform the experiments under physiological conditions. At this pH, $iPLA_2$ activity was 5.7-fold less than that at pH 9.5.

Preparation of enzyme extracts from bovine brain tissues

Frozen bovine brain tissues (1.2 kg) were cut into small pieces and homogenized with 5 volumes of homogenizing buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 3 mM $MgCl_2$, 50 mM KCl, and 10 mM 2-mercaptoethanol). The homogenates were centrifuged at 10,000 × *g* at 4°C for 10 min to remove the cell debris. Subsequently, the supernatants were centrifuged at 10,000 × *g* at 4°C for 1 h, and the pH of the resulting supernatants (S10) was adjusted to pH 5.0 with acetic acid (1:1 dilution in distilled water). These supernatants were again centrifuged at 10,000 × *g* at 4°C for 15 min. The precipitates (P10; pH 5.0) were resuspended in 25 mM Tris (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol, and then applied to a DEAE-cellulose anion-exchange column.

Purification of $iPLA_2$

The resuspension (P10; pH 5.0) was loaded onto a DEAE-cellulose anion-exchange column (DE52; 2.5 × 12.5 cm, 400 ml of bed volume) pre-equilibrated with buffer A (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 10 mM 2-mercaptoethanol) by a peristaltic pump (Eyela, Japan) at a flow rate of 2 ml/min. After the column was washed with buffer A, the washed gel was eluted stepwise with buffer A containing 1.0 M NaCl. Thirty milliliters of the eluate were collected per fraction. Aliquots of each fraction were used for measurement of the $iPLA_2$ activity. The active fraction was pooled. The final KCl concentration was adjusted to 2 M KCl by adding KCl powder and mixing for 2 h at 4°C. The resultant pool was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatants were applied to a Phenyl-5PW hydrophobic column (21.3 mm × 15 cm) pre-equilibrated with 25 mM Tris (pH 7.5) buffer containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1.0 M NaCl. The column was washed with the same buffer and then eluted at a flow rate of 5 ml/min with 150 ml of the same buffer in a linear gradient of buffer B [25 mM glycine-NaOH (pH 9.0), 1 mM EDTA, and 10 mM 2-mercaptoethanol]. Five milliliters of the eluate were collected per fraction. The active fraction was again pooled and the pH was adjusted to pH 6.2 with acetic acid (1:30 dilution in distilled water). The obtained solution was applied to a heparin-Sepharose CL-6B column (5 ml) pre-equilibrated with buffer S [50 mM CH_3COONa (pH 5.6), 1 mM EDTA, and 10 mM 2-mercaptoethanol]. After washing with buffer S, the protein bound to the column was eluted with a linear gradient of buffer A containing 1.0 M NaCl. Three milliliters of the eluate were collected per fraction. The fractions with $iPLA_2$ activity were pooled and concentrated to ~7 ml by using 20-ml Centricon (Vivascience, UK) and applied to a Sephacryl S-300 gel-filtration column (26 mm × 60 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The protein bound to the column was eluted with buffer A containing 0.1 M NaCl at a flow rate of 1 ml/min. Five milliliters of the eluate were collected per fraction. The fractions with $iPLA_2$ activity were pooled and applied to a Mono S cation-exchange FPLC column (5.0 mm × 5.0 cm) pre-equilibrated with buffer S. After washing with buffer S, the protein was eluted with a linear gradient of buffer A containing 1.0 M NaCl at a flow rate of 1 ml/min. One milliliter of the eluate was collected per fraction. The fractions with $iPLA_2$ activity were pooled, diluted with buffer Q [25 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol], and applied to a Mono Q anion-exchange FPLC column (5.0 mm × 5.0 cm) pre-equilibrated with buffer Q. After washing with buffer Q, the protein was eluted at a flow rate of 1 ml/min with 20 ml of the buffer Q in a gradient of 0.0-1.0 M NaCl. The fractions containing $iPLA_2$ activity were pooled and concentrated to ~200 μl by using 4-ml Centricon (Vivascience) and applied to a Superose 12 FPLC gel-filtration column pre-equilibrated with buffer A containing 0.1 M NaCl. The protein was eluted with the buffer A containing 0.1 M NaCl at a flow rate of 0.5 ml/min. Five-hundred microliters of the eluate was collected per fraction. To estimate the apparent molecular

weight of $iPLA_2$, β-amylase (200,000 Da), alcohol dehydrogenase (150,000 Da), bovine serum albumin (BSA; 66,000 Da), carbonic anhydrolase (29,000 Da), and cytochrome *c* (12,400 Da) were used as standards and applied under the same experimental conditions.

Protein quantification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To monitor the amount of protein present during the process of $iPLA_2$ purification, absorbance was measured at 280 nm by using an ultraviolet (UV) spectrophotometer. The protein concentration in each sample was determined by Bradford method (Bio-Rad). Enzymatic purity was monitored by SDS-PAGE on 8% gels, as described by Laemmli (1970). The gels were stained by silver staining.

2-DE

The active fractions obtained by Superose 12 FPLC gel-filtration chromatography were changed to a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 0.002% bromophenol blue (BPB), 100 mM dithiothreitol, and 0.5% Pharmalyte (pH 4-7; Amersham Pharmacia Biotech) by using 4-ml Centricon (Vivascience). The dry strips (pH 4-7, non linear, 7 cm of length; Amersham Pharmacia Biotech) were rehydrated with 125 μl of the sample solution for 12 h at 20°C. Electrofocusing of rehydrated gels was carried out by using the following running conditions: 100 μl per strip at 20°C; 150 V for 1 h (step and hold), 500 V for 1 h (step and hold), 1,000 V for 2 h (step and hold), and 8,000 V for 4 h (step and hold). The electrofocused strips were equilibrated by using an equilibration buffer containing 6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl gel buffer (pH 8.8), 2.5% (w/v) acrylamide solution, 20% glycerol, 5 mM tributylphosphine, and a trace amount of BPB for 30 min at room temperature with gently shaking. The equilibrated strips were loaded onto 8% SDS-PAGE gels for separation by size. The strips were embedded with melted 0.5% (w/v) agarose before loading onto the SDS-PAGE gels. Separated proteins were visualized by using a mass spectrometry (MS)-compatible silver staining kit (PlusOne; Amersham Pharmacia Biotech).

Protein identification by peptide mass fingerprinting analysis

Peptide mass fingerprinting analysis was performed as described previously (Scheler et al., 1998). In brief, the 155-kDa spot was stained with silver nitrate, excised from the 2-DE gel, and digested with trypsin. An aliquot of the total digest was used for peptide mass fingerprinting. Masses were measured with a Bruker Reflex IV mass spectrometer (Bruker Daltonik, Germany), equipped with a 337-nm nitrogen laser and operated in positive ion reflector mode. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis was performed using α-cyano-4-hydroxycinnamic acid as the matrix. Spectra were acquired over 750-3000 m/z and calibrated externally using the peptide calibration standard (Bruker Daltonik). Delayed ion extraction resulted in peptide masses with less than 50 ppm of mass accuracy on an average. Proteins were identified by peptide mass finger-printing with the search engine programs ProFound and Mascot.

Statistical analysis

Data are presented as mean values ± standard deviation (SD) of the indicated number of experiments. One-way ANOVA or Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Purification of $iPLA_2$ from bovine brain

To detect $iPLA_2$ activity in brain, we utilized bovine brain homogenates and traced the enzyme activity in Tris buffer containing 5 mM EDTA. Initially, to remove most contaminant proteins before proceeding to column chromatography, we used an isoelectric point precipitation method. When we precipitated the proteins present in the S10 supernatants under various pH ranges, we could maximally recover 71.8% of the total $iPLA_2$ activity in pH 5.0 precipitates, suggesting that brain-derived $iPLA_2$ may have a pI value of ~5.0. $iPLA_2$ was then purified from the pH 5.0 precipitates (P10) by the sequential process of anion exchange, hydrophobic interaction, heparin affinity, size exclusion, cation-exchange FPLC, anion-exchange FPLC, and gel-filtration FPLC. First, P10 were applied to a DE52 anion-exchange column. This step did not yield a high purification factor (1.3-fold), but many redundant proteins were eliminated in the pass-through fractions (Fig. 1A, Table 1). Next, the active fractions were salted out by the addition of a 2 M KCl solution and mixed for 2 h at 4°C, after which the pool was centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting supernatant, which was free from larger amount of contaminants but retained the full $iPLA_2$ activity, was applied to a Phenyl-5PW hydrophobic column (Fig. 1B). At this step, although only 10% of the total applied $iPLA_2$ activity could be recovered in the active fractions of the eluate, the $iPLA_2$ activity-containing fraction was eluted as a single peak slightly earlier than the main protein peak. Therefore, the specific activity was drastically increased from 0.012 to 0.349 $nmol \cdot min^{-1} \cdot mg^{-1}$ (Table 1). A pool of the active fractions was subsequently applied to a heparin-Sepharose affinity column (Fig. 1C). The proteins were eluted at two major protein peaks, but $iPLA_2$ activity was obtained as ~65% of the total applied activity in earlier fractions than the main protein peaks. Therefore, this step was also effective for $iPLA_2$ purification. Next, the active fractions of the heparin-Sepharose column were concentrated to ~2 ml and applied to a Sephacryl S-300 gel-filtration column (Fig. 1D). Although the majority of the proteins were eluted in void volume with no activity, 73% of the loaded $iPLA_2$ activity was recovered in a single active peak at ~160 kDa when we estimated the molecular mass by applying standard proteins under the same conditions (data not shown). Therefore, this step raised the specific activity by ~3-fold. The Sephacryl S-300 active fractions were pooled and applied to a Mono S FPLC column (Fig. 1E). At this step, the proteins were separated as two major peaks, with 88% of the applied $iPLA_2$ activity being eluted in the smallest one. Then, the active fractions of the Mono S column were pooled and loaded to a Mono Q FPLC column (Fig. 1F). This step resulted in a slight increase in the specific activity. Besides, we could reduce the volume of the active eluate from 13 ml to 5 ml. The active fractions obtained from the Mono Q column were further concentrated to ~200 μl and injected into a Superose 12 gel-filtration column (Fig. 1G). At this step, the total activity dropped to 20%, probably because of the concentration step in which proteins might be denatured and lose their activity, but the specific activity increased by 3.15-fold. As shown in the inset of Fig. 1G, $iPLA_2$ activity was observed as a single peak with an estimated molecular mass of ~160 kDa. The chromatographic processes resulted in a 4,174-fold purification of $iPLA_2$ and yielded ~0.11% of the S10 fraction, as summarized in Table 1.

MALDI-TOF analysis of the brain-derived $iPLA_2$

To identify the purified $iPLA_2$ form, we separated proteins of the active fractions from the final purification step by SDS-PAGE

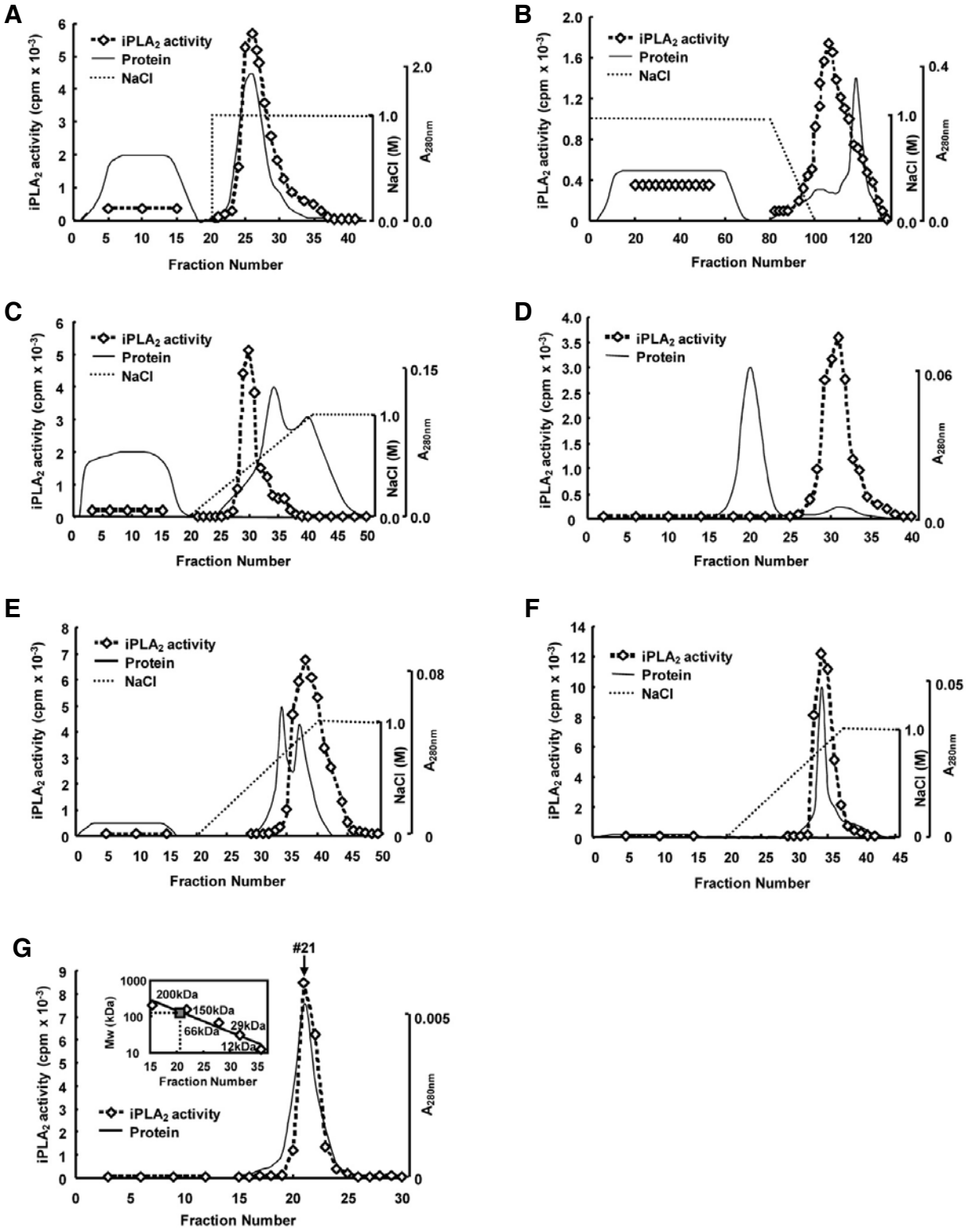

Fig. 1. Column chromatography profiles of $iPLA_2$ from bovine brain. $iPLA_2$ was prepared from bovine brain homogenates by precipitation at pH 5.0 with acetic acid followed by a series of chromatography steps. DEAE-cellulose anion-exchange chromatography (A), Phenyl-5PW hydrophobic chromatography (B), heparin-Sepharose CL-6B affinity chromatography (C), Sephacryl S-300 gel-filtration chromatography (D), Mono S cation-exchange FPLC (E), Mono Q anion-exchange FPLC (F), and Superose 12 FPLC (G). The inset in (G) shows the calibration curve for estimation the apparent molecular mass of $iPLA_2$. Molecular mass standard proteins were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Data is representative of at least ten independent experiments.

Table 1. Purification of a cytosolic $iPLA_2$ from the bovine brain

Step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)	Yield (%)
S10	36,000	74.6	0.002	1.0	100
pH 5.0-extract	5,750	53.6	0.009	4.5	71.8
DE 52	1,386	16.7	0.012	5.9	22.4
Phenyl-5PW	5	1.7	0.349	169.8	2.34
Heparin-Sepharose	2.3	1.1	0.48	229.3	1.42
Sephacryl S-300	0.5	0.8	1.61	780.6	1.08
Mono S	0.26	0.7	2.71	1,320	0.95
Mono Q	0.20	0.5	2.72	1,325	0.73
Superose 12	0.01	0.1	8.58	4,174	0.11

Data is representative of at least ten independent experiments.

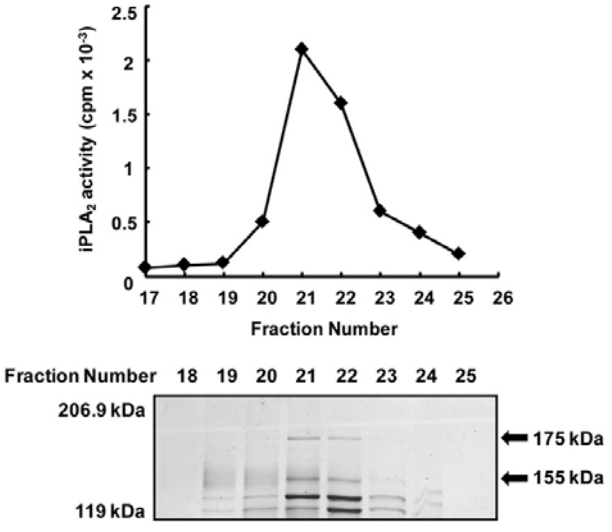

Fig. 2. Superose 12 FPLC of $iPLA_2$ from the active eluate obtained from Mono Q FPLC. Equal volumes of the fractions from the Superose 12 FPLC were assayed for $iPLA_2$ activity, and separated by SDS-PAGE, and visualized by silver staining, as described in the "Materials and Methods". These data are representative of at least five independent experiments.

(Fig. 2). Several proteins were observed. The enzymatic activity paralleled the intensity of the 155- and 175-kDa bands, in close agreement with the apparent molecular mass determined by Superose 12 gel-filtration (Fig. 1G). The proteins were further evaluated by 2-DE. The same samples were electrofocused on IPG dry strips (pH 4-7, nonlinear) and separated by SDS-PAGE (Fig. 3). The 155- and 175-kDa bands were each separated into two spots. Whereas the intensities of the two spots at 175 kDa and of one spot at 155 kDa did not correlate with the enzymatic activity, the spot at 155 kDa (which we named "spot A") correlated well. The identification of 155-kDa $iPLA_2$, separated by 2-DE SDS-PAGE, was achieved by peptide mass fingerprint using MALDI-TOF MS. MALDI-TOF MS analysis of the purified enzyme revealed no homology to any known protein (data not shown).

Characterization of the purified $iPLA_2$

To characterize the purified $iPLA_2$, the active fractions obtained from the final Superose 12 gel-filtration column step were used. First, we examined Ca^{2+} dependency. The activity was not induced by Ca^{2+} addition, but instead significantly decreased at millimolar concentrations of Ca^{2+} (Fig. 4A). We then examined the effects of several metal ions on the $iPLA_2$ activity, including Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+} (Fig. 4B). The enzymatic activity was reduced to less than 15% at 0.1 mM of Fe^{2+} or Fe^{3+} and less than 40% at 0.5 mM of Mn^{2+}. However, similar concentrations of Cu^{2+} and Mg^{2+} had little effect on $iPLA_2$ activity.

To determine substrate preference, $iPLA_2$ activity was assayed by analyzing the reaction products by thin layer chromatography using various phospholipids as described previously (Kim and Bonventre, 1993). We found $iPLA_2$ activity that preferred 2-[1-^{14}C]AA-GPC and 2-[1-^{14}C]AA-GPE by 28- and 81-fold, respectively, to 2-[1-^{14}C]PA-GPC (Table 2). These data suggested the high selectivity of $iPLA_2$ for phospholipids containing arachidonic acid (AA).

We examined the effect of pH on $iPLA_2$ activity. $iPLA_2$ had optimal activity at an alkaline pH (Fig. 4C). This pH profile seems to be unique; enzymes of group IV and group VI PLA_2 are known to have optimal activity at pH 7.5 (Hiraoka et al., 2002), and group XV lysosomal PLA_2 has optimal activity at pH 4.5 (Hiraoka et al., 2002), suggesting that this brain-derived type of $iPLA_2$ may be different from other $iPLA_2$ types. Next, we examined the effect of PLA_2 inhibitors on the $iPLA_2$ activity (Fig. 4D). The activity was not decreased by 100 μM of BEL, a selective inhibitor of group VI Ca^{2+}-independent PLA_2s with IC_{50} of 0.5-5.0 μM (Hooks and Cummings, 2008), but was significantly inhibited by 10 μM of $AACOCF_3$, which is known to inhibit enzymes of group IV and group VI PLA_2 with IC_{50} of 1.5 and 6.0 μM, respectively (Farooqui et al., 2006; Riendeau et al., 1994). In addition, the purified $iPLA_2$ activity was not significantly influenced by ATP (Fig. 4E), which is known to stabilize and activate group VI PLA_2 (Lio and Dennis, 1998). On the other hand, Triton X-100 is required to form micelles that allow the enzyme to be efficiently accessible to the substrate. Therefore, Triton X-100 should be added to the assay buffer at a concentration of at least 400 μM to favor the activities of enzymes of group IV and group VI PLA_2 (Lucas and Dennis, 2005). However, unexpectedly, the purified $iPLA_2$ activity was very sensitive to Triton X-100; the activity was almost completely inhibited even in the presence of 0.01% Triton X-100 (~170 μM; Fig. 4E). These data suggested that this brain-derived form of $iPLA_2$ might be distinct from enzymes of group IV and group VI PLA_2.

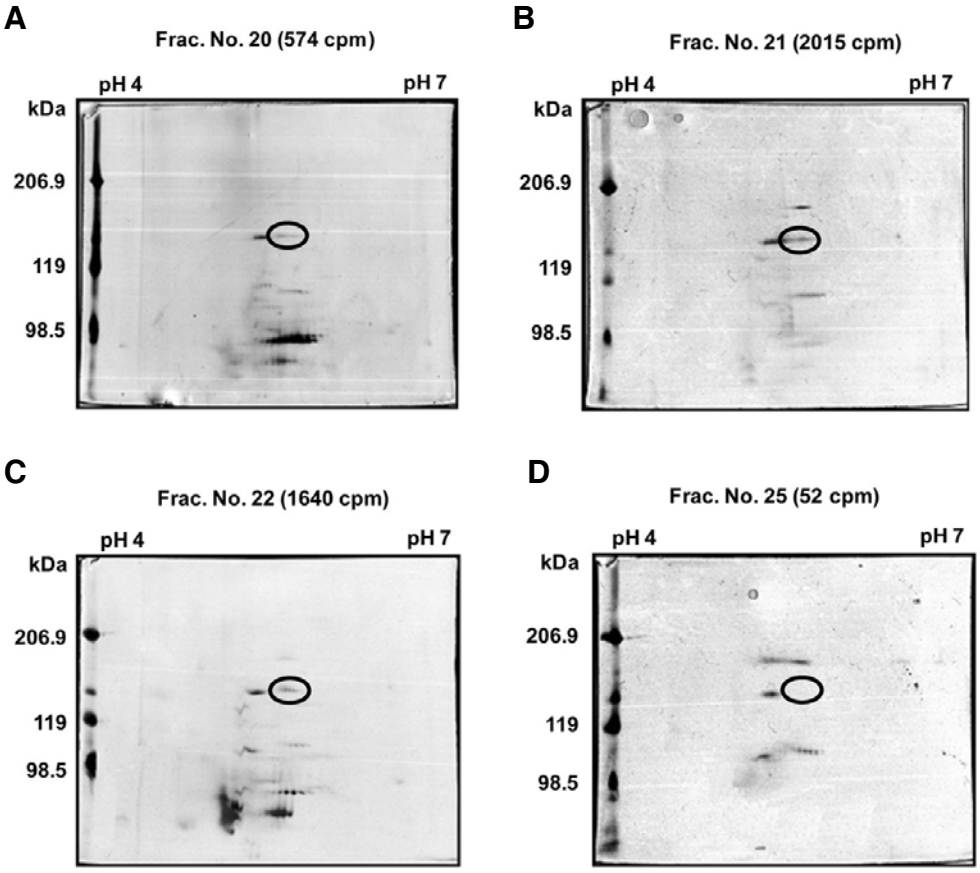

Fig. 3. 2-DE analysis of Superose 12 FPLC fractions. The fractions [20 (A), 21 (B), 22 (C), and 25 (D)] obtained from Superose 12 FPLC were analyzed by 2-DE, as described in the "Materials and Methods". The positions of external marker proteins are shown on the left side of the gels. The encircled areas indicate spot A.

Table 2. Substrate preference of the purified $iPLA_2$

Substrate	Activity (nmol/min/mg)
2-[1-^{14}C]AA-GPE	24.2
2-[1-^{14}C]AA-GPC	8.5
2-[1-^{14}C]PA-GPC	0.3

The active fraction obtained from the Superose 12 gel-filtration column was assayed with the indicated phospholipids using standard assay conditions.

DISCUSSION

The activation of PLA_2 boosts the inflammatory response by catalyzing the release of AA and subsequent production of its metabolites, including prostaglandins and leukotrienes. Because the lipid metabolites mediate many neurodegenerative diseases including cerebral ischemia, Alzheimer's disease, and neuronal injury (Takemiya et al., 2007), PLA_2 is considered to plays important pathophysiological roles in the nervous system (Shinzawa et al., 2008; Strokin et al., 2007; Sun et al., 2004). Therefore, identifying a form of PLA_2 specific to nervous tissues would be of great importance for understanding neurodegenerative diseases and developing efficient therapies.

In the present study, we purified a form of $iPLA_2$ from the bovine brain by using sequential column chromatographic steps. We found that this $iPLA_2$ has several unique biochemical properties when compared with the previously reported forms of $iPLA_2$, including group IVC cytosolic, group VI Ca^{2+}-independent, and group XV lysosomal PLA_2 forms, and group VII and group VIII PAF acetylhydrolases (Burke and Dennis, 2009). First, the purified $iPLA_2$ was insensitive to micromolar concentrations of Ca^{2+} but was inhibited by millimolar concentrations of Ca^{2+} (Fig. 4A). Interestingly, the enzymatic activity was also almost completely inhibited by millimolar concentrations of Fe^{2+}, Fe^{3+}, and Zn^{2+} and was reduced to ~40% by 0.5 mM of Mn^{2+} but was unaffected by Mg^{2+} or Cu^{2+} (Fig. 4B). Second, the enzyme was purified without any detergent. Enzymes of group IVC and group VI PLA_2 reportedly require 400-500 μM of Triton X-100 for optimal activity (Lucas and Dennis, 2005). However, this $iPLA_2$ was completely inhibited even in the presence of 0.01% Triton X-100 (~170 μM; Fig. 4E). It is difficult to distinguish between the effect of Triton X-100 on the enzyme and a structural conformation of the substrate. However, considering the sensitivity of the inhibitory effect, we hypothesize that Triton X-100 might mask a lipophilic active site, as in the case of the mammalian NADH-ubiquinone oxidoreductase (Ushakova et al., 1999). Third, the enzymatic activity-containing fraction was eluted as a peak at a molecular mass of ~160 kDa with a Superose 12 FPLC gel-filtration column (Fig. 1G). Although several protein bands were separated by SDS-PAGE, the intensity of the bands corresponding to 155- and 175-kDa proteins paralleled the enzymatic activity (Fig. 2). When the proteins were further separated by 2-DE, two spots at 175 kDa and one spot at 155 kDa were not correlated with the enzyme activity. However, we found that the enzyme activity was well correlated with the spot at 155 kDa, which we named "spot A" (Fig. 3). Some reports have suggested that the high apparent molecular mass of some $iPLA_2$ forms is due to their association with other proteins (Ackermann et al., 1994; Larsson et al., 1998; Ramanad-

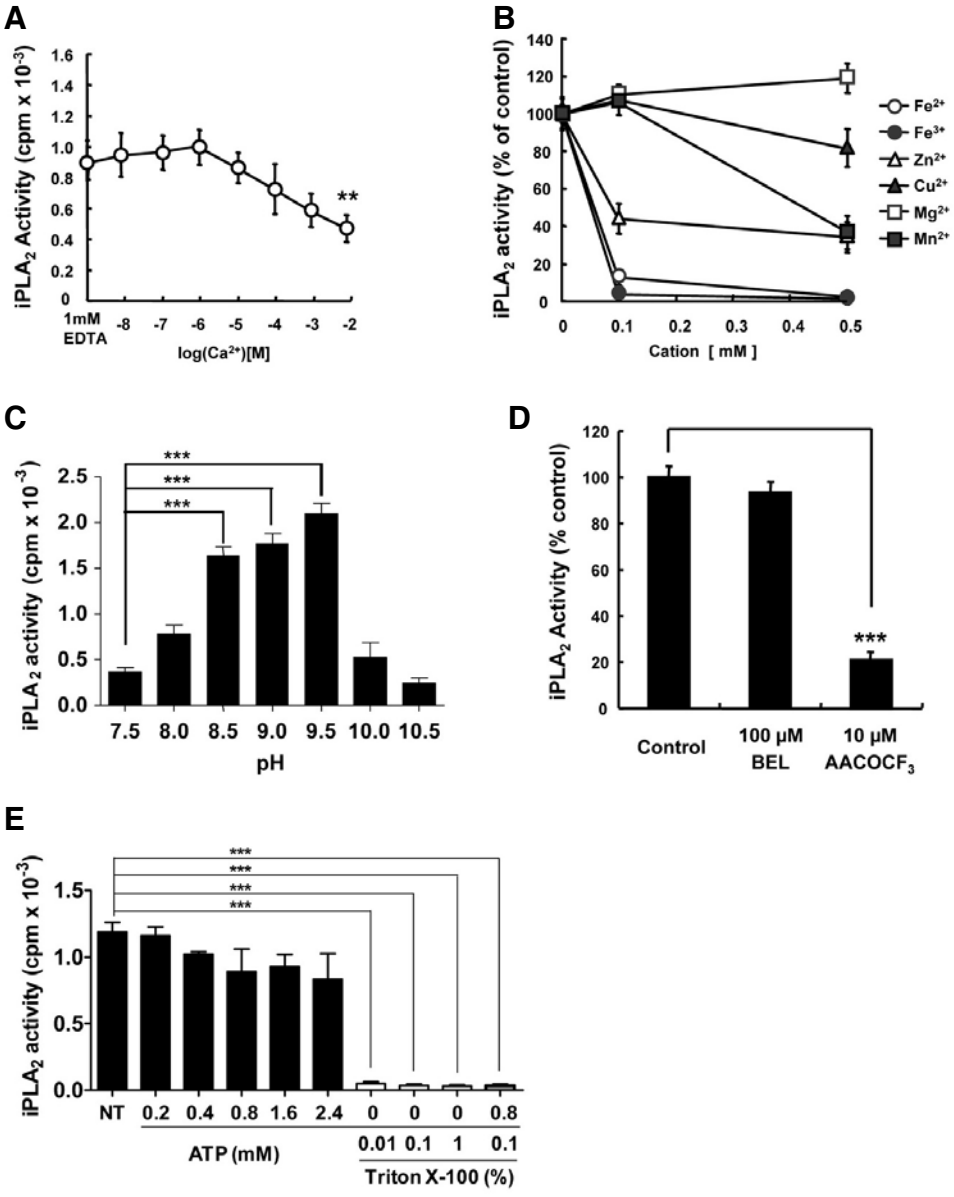

Fig. 4. Characterization of the purified $iPLA_2$. Purified $iPLA_2$ was pre-incubated with the indicated concentrations of Ca^{2+} (A) or metal ions (B) for 10 min at 37°C and then incubated with 2-[1-^{14}C]AA-GPC for 30 min at 37°C. Each data point represents the mean ± SD of at least five independent experiments. (C) The purified $iPLA_2$ activity was assayed in buffers at pH 4-11 by incubation with 2-[1-^{14}C]AA-GPC for 30 min at 37°C. Each data point represents the mean ± SD of at least three independent experiments. (D) Purified $iPLA_2$ was pre-incubated with the indicated concentration of BEL or $AACOCF_3$ for 10 min at 37°C, followed by incubation with 2-[1-^{14}C]AA-GPC for 30 min. (E) Purified $iPLA_2$ was pre-incubated with the indicated concentrations of ATP and Triton X-100 for 10 min at 37°C, and then incubated with 2-[1-^{14}C]AA-GPC for 30 min at 37°C. Statistical significance was assessed by one-way ANOVA in (A, C, and E) and Student's *t*-test in (D). *** $P < 0.001$; ** $P < 0.01$.

ham et al., 1996). However, our purified 155-kDa $iPLA_2$ did not form aggregates with other proteins, because PLA_2 activity was obtained as a single peak at a molecular mass of ~160 kDa from the Superose 12 FPLC gel-filtration column.

Fourth, the purified $iPLA_2$ activity was inhibited by $AACOCF_3$, which is also known to inhibit group IV PLA_2 through a slow- and tight-binding mechanism (Street et al., 1993; Trimble et al., 1993). However, the enzymatic activity was not influenced by BEL (Fig. 4D), which is known to specifically inhibit group VI PLA_2 activity as a mechanism-based suicidal inhibitor (Hooks and Cummings, 2008). Fifth, ATP, which seems to bind and stabilize group VIA PLA_2 (Lio and Dennis, 1998), had no influence on the brain-derived $iPLA_2$ activity (Fig. 4E). Sixth, the purified brain form of $iPLA_2$ exhibited head group preference for ethanolamine rather than for choline as the substrate. In addition, it showed remarkable selectivity toward phospholipids containing AA at the *sn*-2 position (Table 2). This substrate preference of the purified enzyme is different from the previously identified PLA_2 forms. For instance, enzymes of group IVC and group VI PLA_2 show very little specificity for AA-containing phospholipids (Burke and Dennis, 2009; Pickard et al., 1999); group VII and group VIII PAF acetylhydrolases primarily remove the acetyl group from the *sn*-2 position of PAF; group XV lysosomal PLA_2 seems to preferentially have the activity of 1-*O*-acylceramide synthase rather than PLA_2 (Hiraoka et al., 2002). Finally, group XV lysosomal PLA_2 has optimal activity at pH 4.5 (Hiraoka et al., 2002), but the purified $iPLA_2$ was optimally activated at pH 9.5 (Fig. 4C).

In summary, we purified a novel form of $iPLA_2$ from cytosolic fractions of the bovine brain by using acidic pH precipitation and sequential column chromatographic methods with ~0.11% yield and 4,174-fold purification. This enzyme has several different biochemical characteristics from the previously identified $iPLA_2$ forms, including insensitivity to BEL and ATP, complete inactivation by Triton X-100, and pH dependency. The protein spot with $iPLA_2$ activity did not match any annotated protein se-

quence, as determined by 2-DE and MALDI-TOF. Given these data, the purified enzyme is a novel type of cytosolic $iPLA_2$. In the future, the primary structure of this novel enzyme should be resolved and its underlying molecular mechanisms comprehended. Such knowledge would provide insight into the phathophysiology of neuronal disease.

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