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Mobile phase effects on membrane protein elution during immobilized artificial membrane chromatography

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Abstract

The eluotropic strength of different mobile phases for eluting membrane proteins from immobilized artificial membrane (IAM) chromatography surfaces was studied. Two protein mixtures containing bovine pancreatic PLA_2 were used in this study. Protein mixture I was PLA_2 obtained from Sigma which contained ~5–10 major protein bands in electrophoretic gels. Protein mixture II was obtained from fresh bovine pancreatic tissue and contained >100 proteins including the target protein, PLA_2. After adsorbing Sigma PLA_2 to IAM columns, the elution conditions common to conventional chromatographic methods were evaluated for their ability to selectively purify PLA_2. Elution conditions tested were (i) detergent gradients, (ii) salt gradients used during ion-exchange chromatography, (iii) salt conditions used during hydrophobic interaction chromatography, (iv) acetonitrile gradients used during reversed-phase chromatography, and (v) a two-step gradient consisting of first a detergent gradient followed by an acetonitrile gradient. Based on silver-stained electrophoretic protein gels, PLA_2 from protein mixture I was purified to electrophoretic homogeneity with 417-fold increase in specific activity in one step using elution condition (v), and PLA_2 from protein mixture II was purified in one step (660-fold increase in specific activity) using elution condition (iv). Total protein recovery from IAM columns is 70–100%.

1. Introduction

The purification of membrane proteins remains an arduous task in the field of separation science. No general methods exist for purifying membrane proteins. Our laboratory has been developing immobilized artificial membrane (IAM) chromatography surfaces specifically for the purpose of purifying membrane proteins. IAM chromatographic surfaces are modeled after cell membranes and are prepared by immobilizing membrane lipids at a monolayer density on a rigid matrix [1–3]. Currently we have immobilized analogs of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidic acid (PA). We have prepared IAMs that are either zwitterionic (^{ether}IAM-PC$^{C10/C3}$ and ^{ether}IAM-PE$^{C10/C3}$) or negatively charged (^{ether}IAM-PA$^{C10/C3}$, ^{ether}IAM-PG$^{C10/C3}$ and ^{ether}IAM-PS$^{C10/C3}$) [1,2].

Chromatographic separations have a mobile-phase/stationary-phase combination that effects the purification of the target compound. How-

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ever, identifying efficient stationary-phase/mobile-phase combinations is very difficult for membrane proteins, particularly because many proteins require detergents in the mobile phase to maintain functional activity. Our previous efforts have focused on the surface chemistry of preparing IAM stationary phases for purifying membrane proteins. This report focuses on identifying mobile phase conditions that may be used to selectively elute proteins from IAM surfaces. Conventional mobile phases used for reversed-phase chromatography, hydrophobic interaction chromatography, and ion-exchange chromatography were evaluated for their ability to elute proteins from IAM surfaces.

To evaluate the effect of these mobile phases on protein elution, phospholipase A_2 (PLA_2) was used as the target enzyme. Many biochemical and pharmacological studies require the isolation of PLA_2s from several different cell and tissue sources. PLA_2s have been purified from bovine intestine [4], rabbit and rat inflammatory exudate [5,6], rat, sheep and human platelet [7,8], rat and human spleen [7,8], rat liver [9], canine myocardium [10] and synovial fluid [11,12]. Elution conditions for purifying bovine pancreatic PLA_2 by IAM chromatography in one step were identified in this report. Traditional PLA_2 purification procedures require several steps (e.g., ammonium sulfate precipitation, followed by several chromatographic steps) and frequently only 20–30% of pure PLA_2 is recovered. For some PLA_2 purifications, the recovery of PLA_2 is as low as 4–8% (e.g., [11,12]). In this report we demonstrate that mobile-phase conditions selectively elute proteins from IAMs and that bovine pancreatic PLA_2 can be purified to homogeneity in one step using ^{ether}IAM-$PC^{C10/C3}$ and ^{ether}IAM-$PE^{C10/C3}$ columns.

IAM surfaces have been also used to immobilize enzymes [13], facilitate covalent coupling between polar and non-polar reactants [14–16], predict drug transport across human skin and other biological barriers [14], and predict the pathophysiological effects of bile salts [17]. IAM surfaces have also been used to purify cytochrome P450, oxidoreductase [18,19], cholesterol binding protein [20] as well as a membrane bound N-acylphosphatidylethanolamine synthase [21].

2. Experimental

2.1. Materials

Electrophoretic reagents SDS, BIS, acrylamide, APS, TEMED and molecular-mass markers for gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA). CHAPS, Triton X-100, sodium octanesulfonate (SOS) were from Aldrich (Milwaukee, WI, USA). Acetic acid, sodium phosphate, ethylene glycol (EG), silver nitrate, 37% formaldehyde solution, organic solvent compatible scintillation fluid (ScintVerse II) and the 96-well microtiter plate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sodium carbonate, sodium thiosulfate, calcium chloride and sodium chloride were from J.T. Baker (Philipsburg, NJ, USA). Glycine, sodium deoxycholate (DOC), trichloroacetic acid (TCA), trypsin type I and Tris base were ordered from Sigma (St. Louis, MO, USA). Micro and Macro BCA protein assay reagent kit was from Pierce (Rockford, IL, USA). Methanol was obtained from Mallincrodt (Paris, KY, USA). Absolute ethanol was obtained from McCormick Distilling Co. (Pekin, IL, USA). Mega-8 and Mega-9 were purchased from Calbiochem (La Jolla, CA, USA). Silica gel 60 (230–400 mesh) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY, USA). [^{14}C]DPPC (112 mCi/mmol) was purchased from Amersham Life Science (Arlington Heights, IL, USA). DPPC and DCPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Bovine pancreatic PLA_2 was either purchased from Sigma (P8913) or obtained from crude pancreatic tissue preparations as described below.

2.2. IAM chromatography

^{ether}IAM-$PC^{C10/C3}$ (zwitterionic), ^{ether}IAM-$PE^{C10/C3}$ (zwitterionic), ^{ether}IAM-$PG^{C10/C3}$ (nega-

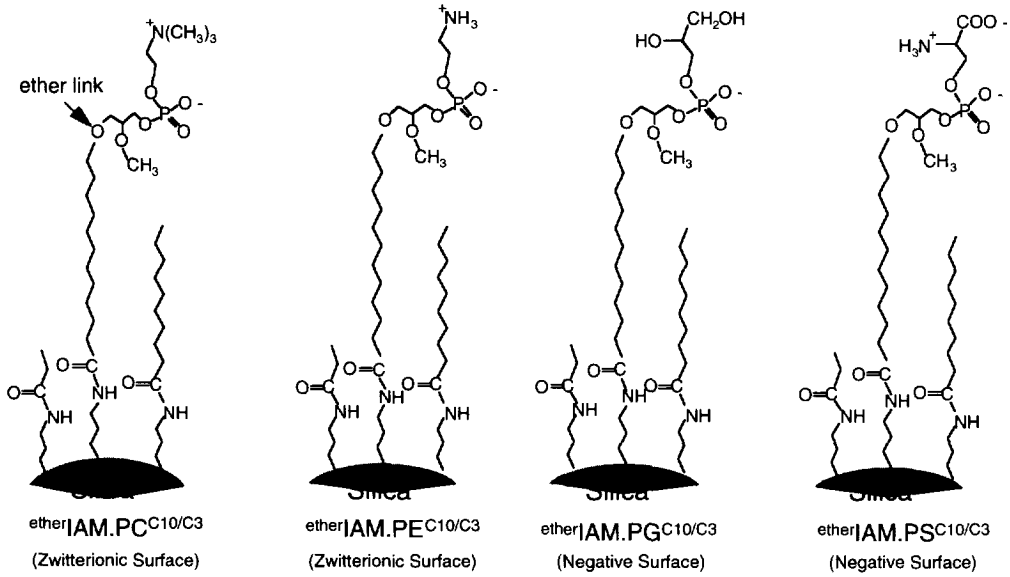

Fig. 1. IAM chromatography surfaces used in this study.

tively charged), and ^{ether}IAM-PS$^{C10/C3}$ (negatively charged) surfaces were synthesized in our laboratory [1,2] and are shown in Fig. 1. The superscript "ether" denotes an ether linkage between the alkyl chain and the glycerol backbone of the phospholipid. IAMs are prepared by a 3-1 step bonding process: step (i) phospholipid immobilization, step (ii) end capping with decanoic (C_{10}) symmetric anhydrides, and step (iii) end capping with proprionic (C_3) symmetric anhydrides. The C_{10} and C_3 alkyl chains from the end capping reactions are shown in Fig. 1 for each IAM surface. Phospholipids were bonded to chromatographically efficient 12-μm particles with 300-Å pores. High-performance liquid chromatography columns were packed at Regis Company (Skokie, IL, USA). Pilot-size columns were 3×0.46 cm and analytical size columns were 10×0.46 cm, or 15×0.46 cm. The IAM surfaces shown in Fig. 1 are very stable from pH 2 to 7.5; no phospholipid leaching occurs during perfusion of 50 000 column volumes through the IAM column [3].

Our HPLC system uses a Rainin Rabbit-HP pump and a Knauer variable-wavelength UV monitor set at 280 nm for detection. Typically, the detector was set to a range of 0.08 and a response time of 1.0 s. The flow-rate was 0.2 ml/min for the adsorption step (i.e., the protein loading step). Protein loading typically required ~8 min for the analytical size column and 3 min for the pilot column. After protein loading, the flow-rate increased over 2 min from 0.2 ml/min to 0.5 ml/min; this 0.5 ml/min was continuously maintained after protein loading. Mobile-phase conditions for column equilibration, and protein elution are given in each figure legend. Ethylene glycol (EG) was included in the mobile phase as a precaution to preserve enzymatic activity during protein purification [22] and NaN_3 was used to inhibit bacterial growth. All chromatography experiments were performed at room temperature.

For PLA_2 specific activity measurements, protein content and PLA_2 activity were measured from the same chromatographic run, whereas for total protein recovery study, an independent injection was made with different amounts of sample loading. The elution was performed with a steeper gradient at 1 ml/min, and consequently the shorter elution times diminish the total volume of solvents needed.

2.3. PLA_2 sample preparations

Dry powders containing PLA_2 were solubilized in buffer (25 m*M* $CaCl_2$, 50 m*M* Tris, pH 7.6), and then filtered through a microcentrifuge filter (0.2-μm cellulose acetate membrane). These clarified protein mixtures were injected onto IAM columns that had been pre-equilibrated with mobile phase A as described in each figure legend. After each chromatographic experiment, columns were washed with 50 ml of isopropanol followed by ~50 ml of water prior to re-equilibrating the IAM column with aqueous mobile-phase buffers.

Protein mixtures containing PLA_2 from bovine pancreatic tissue were prepared according to the method of Dutilh et al. with one modification [23]. The original procedure used a 50–72% saturation $(NH_4)_2SO_4$ precipitation cut, whereas we used a 60%S $(NH_4)_2SO_4$ precipitation. Fig. 2 shows the protein composition of the PLA_2 samples used for this study. For Sigma-derived PLA_2, PLA_2 is one of the major proteins, whereas for the tissue PLA_2 the 60%S $(NH_4)_2SO_4$ protein fraction contains PLA_2 as a minor protein in the complex mixture. The tissue 50–72%S $(NH_4)_2SO_4$ precipitation cut contains less contaminating proteins (Fig. 2) than the 60% cut, but the 50–72% cut was not used for this study because one of our objectives was to evaluate the ability of mobile phases to selectively elute PLA_2 from very complex protein mixtures.

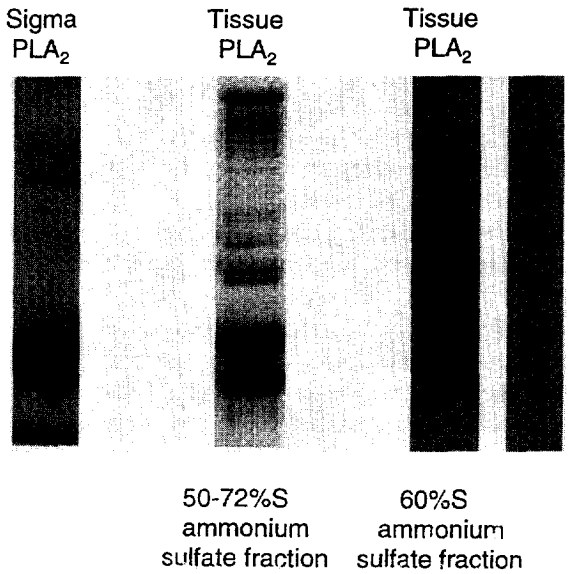

Fig. 2. Protein distribution in bovine pancreatic Sigma PLA_2 compared to bovine pancreatic tissue PLA_2.

The general method for preparing the pancreatic tissue sample was as follows. The tissue (300 g) was homogenized in 300 ml of 0.1 *M* NaCl using a Waring blender for 30 s. After homogenization, the tissue preparation was adjusted to pH 4 with concentrated HCl, heated to 70°C for 2–3 min and cooled down in an ice water bath before readjusting the pH back to 7 with concentrated NH_4OH. The sample was centrifuged at 3500 *g* for 5 min and the supernatant (~250 ml) was brought to 60%S $(NH_4)_2SO_4$ and precipitated in an ice bath for 1 h. Precipitated protein was collected by pelleting in a centrifuge (5000 *g* for 10 min). The pellet was dissolved in 2.5 ml of doubled distilled H_2O and 50 μl of a 0.1 *M* solution of phenylmethylsulfonylfluoride (PMSF) in isopropanol was added, and the sample incubated on ice for 1 h. Lyophilization gave 863 mg of solid containing approximately 10% protein. Prior to use, the lyophilized material was activated by addition of 5% trypsin relative to the total protein. Trypsin converts the inactive PLA_2 zymogen to its active form by selectively cleaving an N-terminal octapeptide [23].

2.4. SDS-PAGE

The chromatography fractions were subjected to TCA precipitation to concentrate the proteins prior to gel electrophoresis. The TCA precipitation method involved adding 0.1 ml of 0.15% DOC to the ~1 ml chromatography fractions, incubating at room temperature for 10 min, and then adding 0.1 ml of 72% TCA [24]. The sample was cooled on ice for 1 h and then centrifuged at 14 000 rpm using an Eppendorf Centrifuge (Model 5415) for 15 min. After centrifugation, the supernatant was discarded, the protein pellet was solubilized with 15 μl tank buffer (25 m*M* Tris, 0.2 *M* glycine, 0.1% SDS, pH 8.3) and if necessary, adjusted to pH ~8 by a trace amount of dry Tris base powder. The 15 μl of solubilized protein mixture was mixed with 15 μl of twice-concentrated gel electrophoresis sam-

ple buffer, boiled for 3 min. After brief vortexing, 10 μl of the sample was loaded onto a 15% polyacrylamide gel. Gels were run at a constant current of 20 mA/gel for about 1 h. The gels were then silver stained using the method of Merril [25]. If organic solvent was present in the sample it was evaporated at 60°C for 2 h and the volume reconstituted to 1 ml aqueous solution prior to protein precipitation with TCA.

2.5. Protein content measurements

Chromatography fractions always contain EG, EDTA, Tris, and detergents that contribute to the absorbance during protein assays. Because of these interfering chemical substances protein content cannot be directly measured. For this reason, TCA precipitation was performed prior to the MicroBCA assay. Briefly, 200 μl of 100% TCA was added to each chromatography fraction, and the samples were vortexed and incubated in ice for 1 h. Precipitated proteins were pelleted by centrifugation at 14 000 rpm for 8 min. The supernatant was decanted and the pellet was washed gently with 50 μl of a saturated NaCl solution. After the pellet was solubilized with 1 ml of 0.3 *M* phosphate buffer (pH 8.0), BCA microassay reagent (1 ml) was added to each sample and the samples were then incubated at 60°C for 60 min. The absorbance at 562 nm was measured on a Beckman-7 spectrophotometer. The protein concentration was calculated from a BSA standard curve. To generate the BSA standard curve, known concentrations of BSA solutions were subjected to the same TCA precipitation method prior to analysis by the MicroBCA assay. The standard curve had a correlation coefficient $r^2 > 0.999$ for protein concentrations in the range 0.5–20 μg/ml.

2.6. PLA_2 activity assays

Two PLA_2 assays were performed: a quick qualitative assay for monitoring the presence of PLA_2 in numerous chromatography fractions, and a radioactive method for measuring PLA_2 specific activity. To monitor chromatography fractions for the presence of PLA_2, the method of Araujo and Radvanyi was used [26]. On a microtiter plate, 200 μl of substrate solution (3.5 m*M* DCPC, 7 m*M* Triton X-100, 5.5 m*M* Phenol Red, 0.1 *M* NaCl, 10 m*M* $CaCl_2$) was mixed with 10 μl of the HPLC fraction and incubated at room temperature. The presence of PLA_2 was visually confirmed when the color changed from dark orange to light yellow.

For quantitating the specific activity of PLA_2 in chromatography fractions, a radioactive method was used. PLA_2 activity was assayed by measuring the release of radioactive fatty acid from the *sn*-2 position of radiolabeled phospholipids. The released fatty acid can be conveniently extracted by a modified Dole extraction procedure. The assay solution was prepared according to Kramer et al. with a little modification [27]. The assay substrate solution contains 0.1 *M* Tris, 0.3 *M* NaCl, 1 m*M* Triton X-100, 20 m*M* $CaCl_2$, 1 m*M* DPPC, pH 8.0 and [^{14}C]DPPC at a specific activity of $1.1 \cdot 10^5$ dpm/μmol. The analysis involved mixing aliquots of the chromatographic fraction with the stock solution containing [^{14}C]DPPC to a final volume of 0.5 ml. The mixture was incubated at 40°C for 60 min with occasional hand-shaking. The enzymatic reaction was terminated by adding 2.5 ml of Dole's extraction solution (2-propanol–heptane–1 *M* H_2SO_4, 20:5:1, v/v) [28]. Immediately after adding about 0.1 g of silica gel, the solution was vortexed and then 1.5 ml of heptane and 1.5 ml of deionized water were added followed by vortexing for at least 10 s. Thus, the palmitic acid released was extracted into the heptane layer for analysis by scintillation counting. One milliliter of the upper heptane layer was removed, mixed with 5 ml of scintillation fluid, and the radioactivity measured using a Beckman LS 1801 scintillation counter. Control solutions that did not contain enzyme were assayed to correct for non-enzymatic DPPC hydrolysis and also for the incomplete extraction of the palmitic acid [29].

2.7. Protein sequencing analysis

Proteins were transferred from unstained 15% SDS-PAGE to PVDF (polyvinylidene difluoride) membranes (0.45 μm, Millipore, Immobilon P) in a buffer containing 192 m*M* glycine, 25 m*M*

Tris (pH 8.3) and 20% MeOH. Electrotransfer was performed at room temperature for 2 h at 300 mA after which the protein bound to PVDF was visualized using Commassie Blue. The protein band was cut and sequenced at the Purdue University Laboratory for macromolecular structure.

3. Results

To date, all membrane proteins that were purified by IAM chromatography were purified using detergent gradients [19,20]. Based on these earlier purifications, we initially added detergents in the mobile phase to selectively purify Sigma PLA_2. Fig. 3 shows the elution profile after injecting Sigma PLA_2 on a 3×0.46 cm $^{ether}IAM\text{-}PC^{C10/C3}$ column. A steep 2-min detergent gradient (0% to 1% CHAPS) followed by isocratic elution at 1% CHAPS for approximately 15 min was used. Activity of PLA_2 eluted from the IAM column is shown in Fig. 3. The chromatogram shows a broad "pass through" peak (fractions 1 and 2) and a narrow "retained" peak (fraction 6). Gel lanes 1 and 2 show that many contaminating proteins were removed from the PLA_2 sample in the "pass through" peak. Gel lanes 3, 4 and 5 indicate that 2 low-molecular-mass proteins were removed from PLA_2 during the 7 to 15 min time interval of the CHAPS gradient. Furthermore, virtually all of the PLA_2 activity eluted at 16 min (lane 6, Fig. 3). As shown in Table 1, PLA_2 was purified 45-fold during this chromatographic step indicating that ~50% of the contaminating proteins

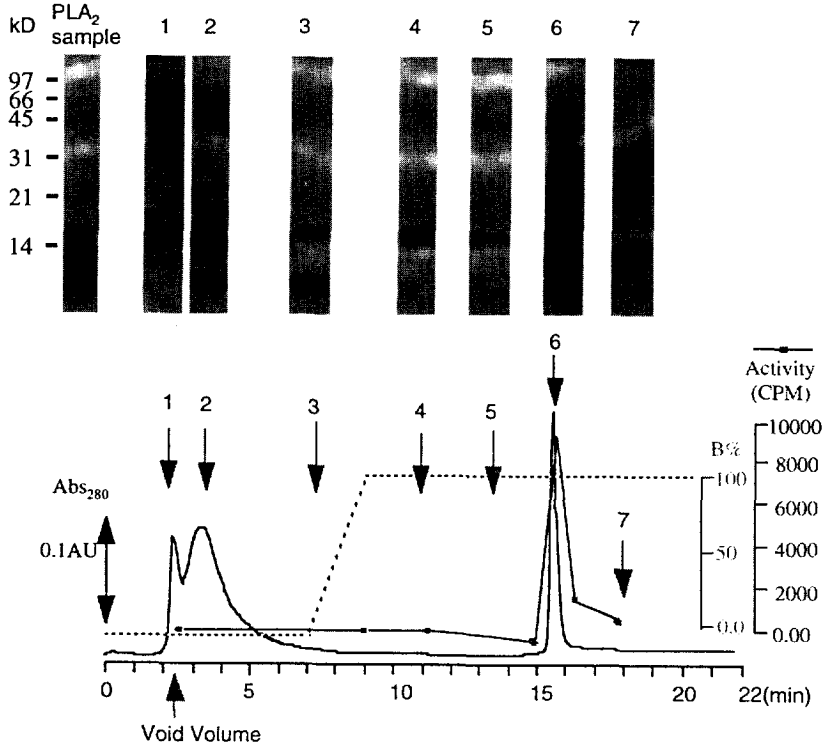

Fig. 3. Elution of PLA_2 from $^{ether}IAM\text{-}PC^{C10/C3}$ pilot columns using a CHAPS gradient. The column was loaded with 45 μg of Sigma PLA_2. The injection volume was ~50 μl. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG, 0.05% NaN_3. Mobile phase B contained 1% CHAPS dissolved in mobile phase A. During protein loading the flow-rate was 0.2 ml/min and after protein loading the flow-rate was 0.5 ml/min. SDS-PAGE results corresponding to seven typical fractions are given. The dotted line represents the CHAPS gradient and solid squares represent the PLA_2 activity.

Table 1
Purification of bovine pancreatic PLA_2 on $^{ether}IAM\text{-}PC^{C10/C3}$ and $^{ether}IAM\text{-}PC^{C10/C3}$ columns

Chromatography (Fig., elution)	Activity[a] (cpm)	Protein content[b] (μg)	Relative sp. activity[c] (cpm/mg/min)	Purification (-fold)
Sigma PLA_2 [d]	14 922	44.75	$5.56 \cdot 10^3$	–
$^{ether}IAM\text{-}PC^{C10/C3}$ (Fig. 3, CHAPS)	8681	0.578	$2.50 \cdot 10^5$	45
$^{ether}IAM\text{-}PC^{C10/C3}$ (Fig. 6, SOS/ACN)	9213	0.066	$2.32 \cdot 10^6$	417
$^{ether}IAM\text{-}PE^{C10/C3}$ (CHAPS)	7636	0.104	$3.97 \cdot 10^5$	71
$^{ether}IAM\text{-}PE^{C10/C3}$ (Fig. 7, SOS/ACN)	16 654	0.090	$1.70 \cdot 10^6$	305
Tissue PLA_2 [e]	12 251	342.0	$5.97 \cdot 10^2$	–
$^{ether}IAM\text{-}PC^{C10/C3}$ (Fig. 8, ACN)	1963	0.083	$3.94 \cdot 10^5$	660

[a] PLA_2 activity was measured as the enzymatic release of the radiolabeled fatty acid from DPPC.
[b] Protein content was measured by the Pierce MicroBCA method.
[c] Relative specific (Sp.) activity was calculated by normalizing the PLA_2 activity over a 60-min incubation by the amount of total protein in the sample.
[d] PLA_2 was purchased from Sigma.
[e] PLA_2 was obtained as a crude tissue extract from fresh bovine pancreatic tissue.

were removed. For this experiment 22.4 μg of protein was injected on to the IAM column and 27.7 μg of protein was recovered (Table 2). Within the experimental error, quantitative recovery of the injected protein was achieved using the 1% CHAPS gradient on IAM surfaces. This elution condition was evaluated for all of the IAM columns shown in Fig. 1, and no significant differences were observed regarding the chromatogram tracing or the gel pattern of the proteins eluting from the IAM columns.

The selective elution of PLA_2 using the steep 2-min CHAPS gradient was not satisfactory since many proteins contaminate PLA_2 (Fig. 3, lane 6). When a shallow 20-min gradient (0% to 1% CHAPS) was used, most of the proteins eluted with 1% CHAPS; thus, virtually no protein resolution occurred by changing from a steep to

Table 2
Protein recovery from the IAM columns

Column	Chromatography[a]	Protein injected (μg)	Protein recovered (μg)	Total volume[b] (ml)	Protein recovery (%)
$^{ether}IAM\text{-}PC^{C10/C3}$	CHAPS detergent elution	22.4	27.7	8.85	100
	50% ACN elution	508	356	21.0	70
$^{ether}IAM\text{-}PE^{C10/C3}$	CHAPS detergent elution	15	17.3	25.1	100
	50% ACN elution	17	13.1	11.7	76

[a] For the total protein recovery study, an independent injection was made with different amount of sample loading other than the purification study. Also, the elution was made in sharper gradient and shorter elution time to eliminate the total volumes.
[b] Total volume collected with the mobile conditions used. One milliliter of each fraction (~4–5 ml) was assayed as described under "protein content measurements".

a shallow detergent gradient. In addition, when the plateau CHAPS concentration is ≤0.5% CHAPS, the eluotropic strength of the mobile phase decreased to the point where adsorbed proteins would not elute from the IAM column.

Since a gradient of zwitterionic CHAPS does not purify PLA_2 to homogeneity using any of the four IAM columns shown in Fig. 1, other detergents were evaluated. Mega-8, Mega-9, deoxycholate, Lubrol PX, Triton X-100, and SDS were evaluated. SDS was found to denature PLA_2 and therefore it was not used. Deoxycholate, Lubrol PX and Triton X-100 gave similar chromatograms but interfered with gel electrophoresis after TCA protein precipitation. Consequently, we did not further evaluate these detergents. Mega-8 and Mega-9 are homologous nonionic detergents with CMC values of 48 m*M* and 18 m*M*, respectively. Under identical mobile phase conditions shown in Fig. 3, Mega 9 gave virtually identical chromatograms and gel electrophoresis patterns for chromatography using all of the IAM surfaces (data not shown). Thus, a 1% Mega-9 mobile phase has an eluotropic strength capable of eluting PLA_2 from all IAM columns, but contaminating proteins co-elute. Under identical elution conditions shown in Fig. 3, Mega-8 eluted PLA_2 from all IAM columns except the ^{ether}IAM-$PS^{C10/C3}$ column. This indicates that PLA_2 has higher affinity for immobilized PS compared to immobilized PG, PE and PC.

Since elution conditions containing detergent gradients were not successful in selectively eluting PLA_2 from IAM surfaces, we used elution conditions common to ion-exchange chromatography (IEC). A low salt (10 m*M*) to high salt (2.0 *M*) gradient was used to elicit protein desorption from chromatography surfaces. PLA_2 would not elute with increasing ionic strength from either the zwitterionic ^{ether}IAM-$PC^{C10/C3}$ surface or the anionic ^{ether}IAM-$PS^{C10/C3}$ surface (data not shown). Considering that the high salt concentration routinely used during its purification does not affect the activity of bovine pancreatic PLA_2 (e.g., [23]), lack of elution of PLA_2 activity from the IAM columns indicates that ionic interactions do not dominate the binding interaction between PLA_2 and IAM surfaces.

Membrane proteins are routinely purified by multiple chromatographic steps and frequently hydrophobic-interaction chromatography (HIC) is one of the chromatographic steps. For HIC, protein loading is performed under aqueous high salt conditions such that the proteins are essentially "salted out" on the column surface. After protein loading, proteins are typically eluted using a solution of low ionic strength. Lowering the salt concentration allows the proteins to be re-dissolved into the mobile phase. We evaluated loading and elution conditions common to HIC. HIC chromatographic conditions were not able to elute PLA_2 from the ^{ether}IAM-$PC^{C10/C3}$ or ^{ether}IAM-$PS^{C10/C3}$ columns but a protein with M_r 16 000 was purified to homogeneity based on silver stained gels (Fig. 4). Since PLA_2 did not elute, HIC elution conditions were not further evaluated. However, the ability of HIC elution conditions to selectively elute proteins from IAM surfaces indicates that this may be useful for other protein samples. Complete details of the HIC conditions of loading and elution of PLA_2 are given in Fig. 4.

Usually membrane proteins are not purified by reversed-phase (RP) conditions because proteins tend to denature when adsorbed to hydrophobic surfaces and proteins also denature in the presence of organic solvents in the mobile phase. However, since PLA_2 was stable in the presence of up to 50% acetonitrile (ACN) [30], we explored the RP conditions in the PLA_2 purification. More than 50% acetonitrile is usually needed to elute membrane proteins from RP columns, we initially investigated acetonitrile gradients that ranged from 0 to 50%. Preliminary studies demonstrated that PLA_2 and all of the injected proteins eluted from the ^{ether}IAM-$PC^{C10/C3}$ column at less than 20% acetonitrile. Thus we carefully evaluated low acetonitrile gradients for their ability to purify PLA_2 and found that 4% ACN was sufficient to elute Sigma PLA_2 from ^{ether}IAM-$PC^{C10/C3}$ columns.

Fig. 5 shows that a shallow 25-min gradient of 0% to 4% ACN was successful in separating the proteins in the Sigma PLA_2 sample on an

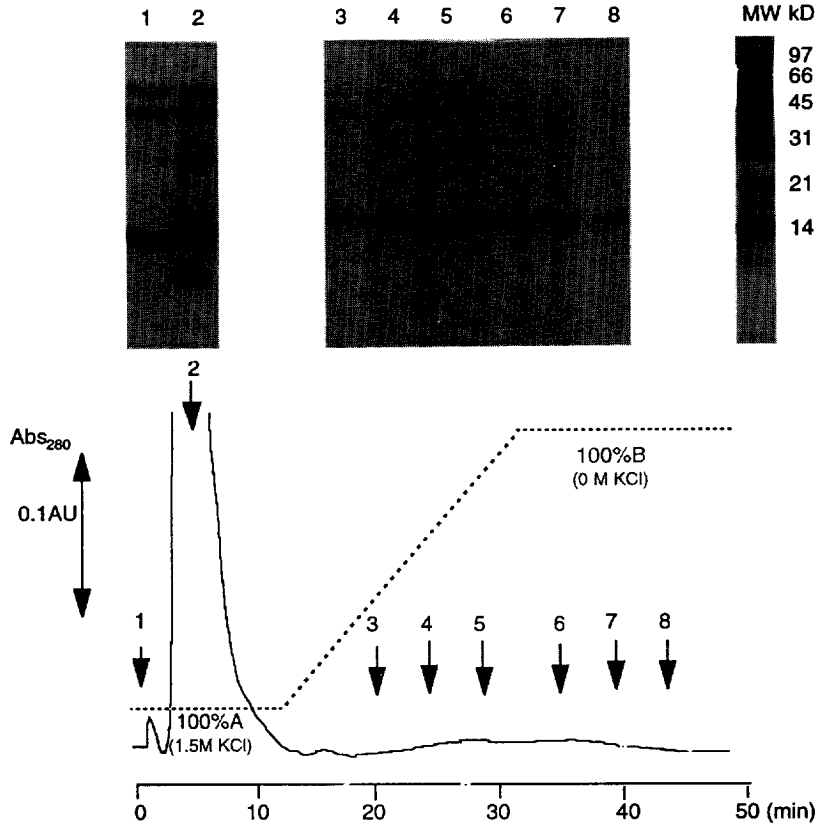

Fig. 4. Elution of proteins in the Sigma PLA_2 sample using elution conditions common to HIC. The same IAM column, flow-rates, and detection wavelength, sample loading, and injection volumes used to generate the data in Fig. 3 were used for this experiment. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 1.5 *M* KCl, 20% EG, and 0.05% NaN_3. Mobile phase B contained 0.1 *M* Tris (pH 7.2), 20% EG, and 0.05% NaN_3. The flow-rate was 0.2 ml/min during protein loading and then 0.5 ml/min for the remaining elution time. The dotted line represents the chromatography gradient that involved a high-salt to low-salt profile over 20 min.

^{ether}IAM-PC$^{C10/C3}$ column. The elution profile contains two "retained" peaks eluting at ~26 min and 39 min. Although all of the injected proteins eluted from the IAM column with this acetonitrile gradient, based on gel electrophoresis very little protein-selectivity was observed. The second "retained" peak eluting at ~39 min contains PLA_2 activity as monitored by the microtiter plate method, but as shown in gel lanes 10–15, PLA_2 is not pure. Pancreatic PLA_2 has a molecular mass of 14 000, and therefore the upper band in Fig. 5 is assigned to be PLA_2.

In order to remove the low-molecular-mass contaminating protein from PLA_2 as shown in Fig. 5, short-chain sulfonates were tested for their ability to selectively elute PLA_2. Sodium butanesulfonate (SBS) and sodium octanesulfonate (SOS) differ by four methylene units and these compounds were added to the mobile phase. When a shallow 10-min SBS gradient (0–1%) was used, no proteins eluted from the ^{ether}IAM-PC$^{C10/C3}$ column. However, as shown in Fig. 6, a shallow 10-min gradient of 0% to 1% SOS eluted many of the contaminating proteins, but 1% SOS was not able to elute PLA_2 even after ~60 min. A step gradient of ACN (0% to 4%) immediately eluted pure PLA_2 (Fig. 6, gel lanes 4–6). Based on the specific activity of

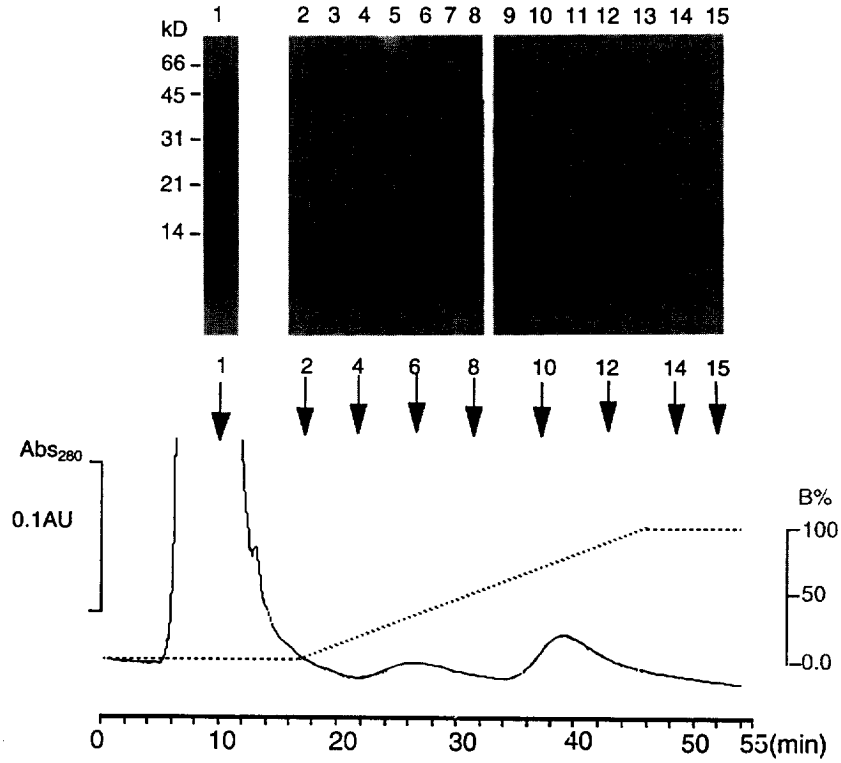

Fig. 5. Elution of proteins in the Sigma PLA_2 sample using elution conditions common to RPC. The same IAM column, flow-rates, detection wavelength, protein sample loading, and injection volume used to generate the data in Fig. 3 were used for this experiment. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG, 0.05% NaN_3. Mobile phase B contained 4% ACN in mobile phase A. The dotted line represents the chromatography gradient.

injected and recovered PLA_2, a 417-fold purification (Table 1) and quantitative protein recovery (Table 2) was obtained.

The two-step mobile phase elution shown in Fig. 6 was tested for PLA_2 purification on ^{ether}IAM-$PS^{C10/C3}$ and ^{ether}IAM-$PE^{C10/C3}$ surfaces. As shown in Fig. 7, the neutral ^{ether}IAM-$PE^{C10/C3}$ column performed virtually identical to the neutral ^{ether}IAM-$PC^{C10/C3}$ surface regarding the purification of PLA_2. The protein peak eluting at 66 min gives the maximum PLA_2 activity and elutes with 4% ACN in the mobile phase. Tables 1 and 2 demonstrate that PLA_2 was purified 305 fold and virtually all of the injected protein eluted from the ^{ether}IAM-$PE^{C10/C3}$ column. For Sigma PLA_2, 400 μg total protein could be loaded on an analytical size (10×0.46 cm) ^{ether}IAM-$PC^{C10/C3}$ column without PLA_2 activity eluting in the pass-through peak. Sigma PLA_2 protein loading may be higher but it was not tested. Using the two-step gradient shown in Figs. 6 and 7 on the ^{ether}IAM-$PS^{C10/C3}$ column, PLA_2 would not elute even up to 50% ACN. This further supports the hypothesis that PLA_2 has higher affinity for immobilized PS compared to immobilized PC or PE. Even with its high affinity for PLA_2, ^{ether}IAM-$PS^{C10/C3}$ is not very useful for PLA_2 isolations because strong eluotropic strength mobile-phase conditions needed to elute PLA_2 from this surface eliminates protein resolution during chromatography. For example, 1% CHAPS and Mega-9 can elute PLA_2 from ^{ether}IAM-$PS^{C10/C3}$ columns, however, numerous contaminating proteins prohibit this mobile-phase/stationary-phase combination to be useful for the purification of

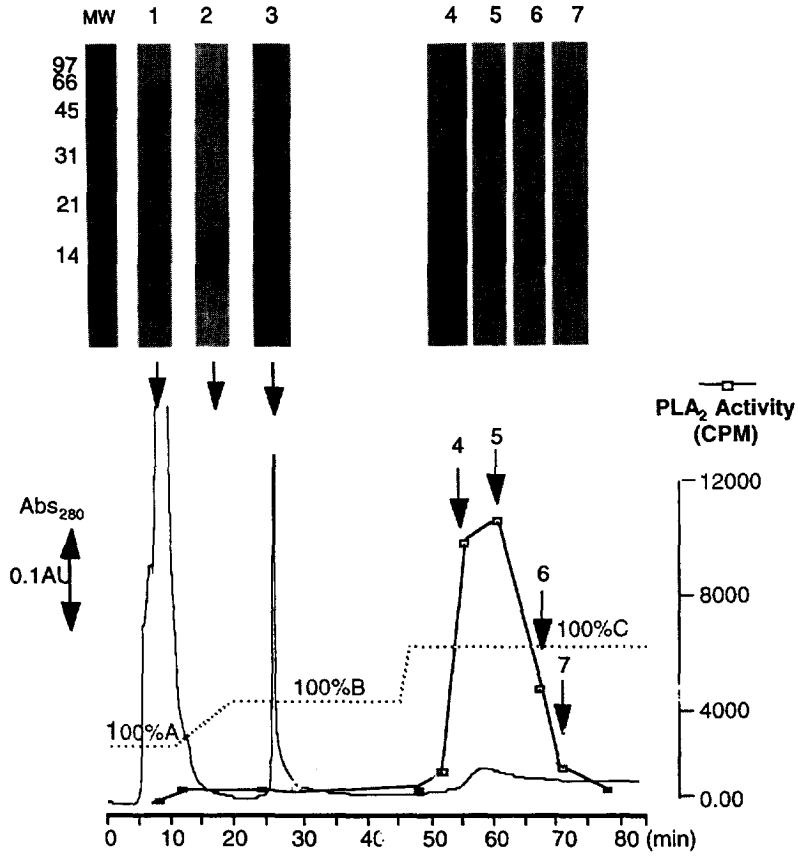

Fig. 6. Elution of proteins in the Sigma PLA_2 on using SOS and ACN gradients. The same flow rates, detection wavelength, used to generate the data in Fig. 3 were used for this experiment. However, the protein loading was 200 μg, the injection volume was ~200 μl, and the ^{ether}IAM-$PC^{C10/C3}$ column was 10 × 0.46 cm. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG, and 0.05% NaN_3. Mobile phase B contained 1% SOS in mobile phase A. Mobile phase C contained 4% ACN in mobile phase B. The dotted line represents the chromatography gradient. (□) PLA_2 activity. Each square represents a 1-ml chromatographic fraction assayed for PLA_2 activity.

PLA_2. In summary, it appears that detergents cannot be used to purify PLA_2 from negatively-charged PS surface.

The results shown in Figs. 3–7 utilized Sigma PLA_2 which is a partially purified PLA_2 membrane preparation (ca. ten proteins present in the sample). However, most tissue samples have much more contaminating proteins and therefore we tested the ability of IAMs to purify PLA_2 in tissue samples without any prior partial purification. Fig. 8 shows the elution conditions that were found to purify PLA_2 in a single step using an ^{ether}IAM-$PC^{C10/C3}$ analytical-size column. The IAM column was loaded with 555 μg protein derived from the 60%S $(NH_4)_2SO_4$ cut of the pancreatic tissue. Most of the injected proteins eluted in the "pass-through" peak, but there were two "retained" peaks in the chromatogram. The first retained peak eluting at ~85 min corresponds to 7% acetonitrile and the second retained peak eluting at 105 min corresponds to 50% acetonitrile. Silver-stained gels corresponding to the peak eluting at ~85 min show a single homogeneous protein band (lanes 4 and 5 in Fig. 8) and this corresponds to virtually all of the PLA_2 activity injected onto

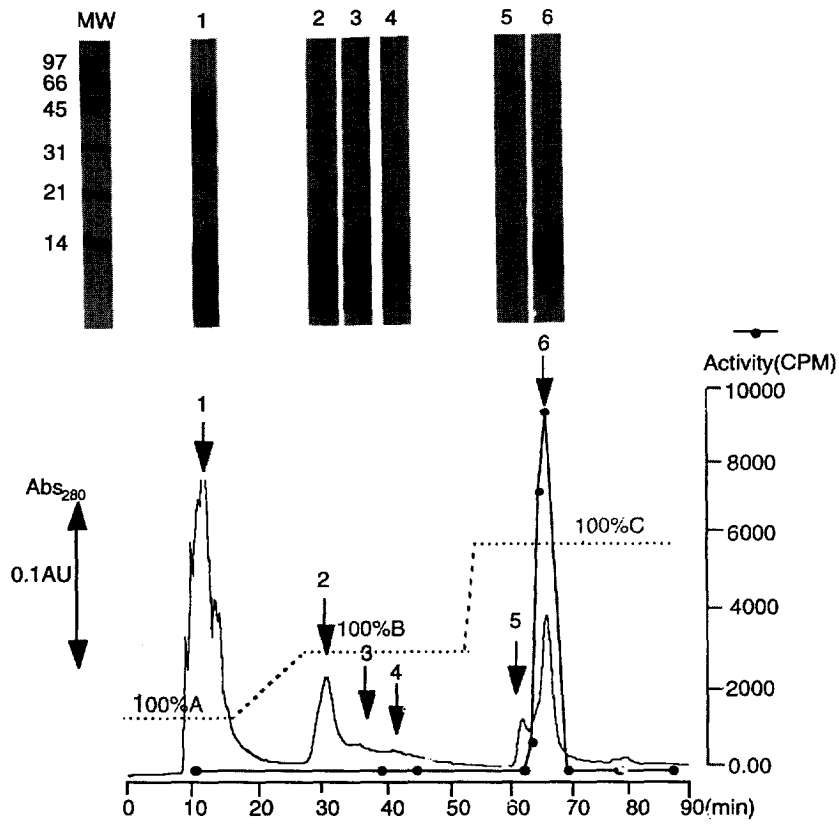

Fig. 7. Single-step purification of PLA_2 from Sigma PLA_2 samples on ^{ether}IAM-$PE^{C10/C3}$ columns. The column was 15 × 0.46 cm, detection was at 280 nm, and the flow-rates were 0.2 ml/min during protein loading followed by 0.5 ml/min during the remaining elution time. The column was loaded with 200 μg of protein and the injection volume was ~200 μl. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG, 0.05% NaN_3. Mobile phase B contained 1% SOS dissolved in mobile phase A. Mobile phase C contained 25% ACN dissolved in mobile phase A. The dotted line represents the elution gradient. (●) PLA_2 activity. Each circle represents a 1-ml chromatographic fraction assayed for PLA_2 activity.

the IAM column. Based on specific activity, the purity of PLA_2 increased 660 fold from this one chromatographic step (Table 1). In addition, we noted that the tissue sample contained many proteins with higher affinity for the ^{ether}IAM-$PC^{C10/C3}$ surface than PLA_2 (Fig. 8, lane 6). These high-affinity proteins required 50% acetonitrile for elution and are not present in the Sigma PLA_2 sample. For tissue PLA_2, 560 μg of protein resulted in a loss of ~10% of the PLA_2 activity in the pass-through peak using an analytical-size IAM column. The SOS/ACN two-component gradient (shown in Fig. 7) that successfully purified Sigma PLA_2 was not needed for purifying tissue PLA_2 because the low-molecular-mass protein contaminating the Sigma PLA_2 is not always present in the tissue sample.

During these studies of mobile-phase elution conditions, we frequently encountered a purified protein in chromatographic fractions that eluted prior to the elution of the active PLA_2. The electrophoretic mobility of this protein was identical to that of PLA_2, but no PLA_2 activity was found in these fractions (Fig. 3, lane 4; Fig. 4, lanes 4–7). In order to determine the identity of this protein and to see if the fraction containing active PLA_2 was contaminated with this unknown protein, we sequenced the first six amino acids of both proteins (Fig. 9, arrows). The sequences of both proteins matched exactly

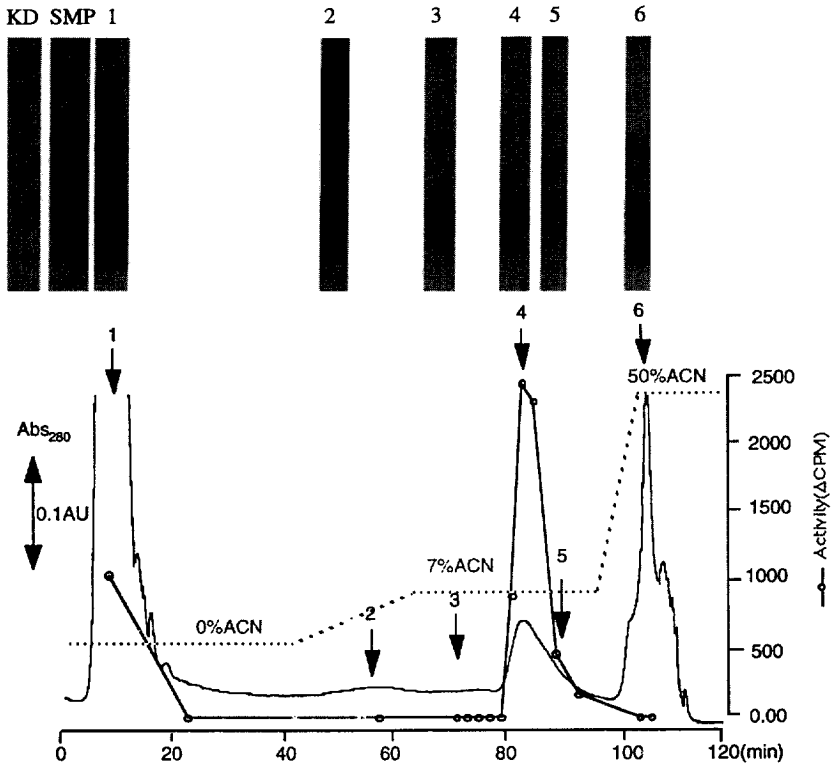

Fig. 8. Single-step purification of tissue PLA_2 on ^{ether}IAM-$PC^{C10/C3}$ using an ACN gradient. The same flow-rates, column size, and detection wavelength used to generate the data in Fig. 6 were used. The column was loaded with 555 μg protein and the injection volume was ~555 μl. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG, 0.05% NaN_3. Mobile phase B contained 50% ACN dissolved in mobile phase A. The dotted line represents the ACN gradient and the ACN concentration at each plateau is given. (○) PLA_2 activity. Each circle represents a 1-ml chromatographic fraction assayed for PLA_2 activity.

the reported sequence of bovine pancreatic PLA_2 [31]. This indicates that inactive PLA_2 has less affinity for the IAM surface. IAM is able to resolve active from inactive PLA_2.

4. Discussion

The most common chromatographic methods used to purify proteins include RPC, HIC and IEC. IAM surfaces have both hydrophobic alkyl groups and polar head groups and thus IAM surfaces resemble, in part, the traditional RPC, HIC, and IEC chromatographic surfaces. Furthermore, the hydrophobic/hydrophilic IAM surface generates physicochemical surface properties that elicit mixed-mode protein retention mechanisms. This unavoidable mixed-mode property of IAM surfaces indicates that for some proteins, IEC elution conditions might be successful, whereas, for other proteins RPC elution conditions might be acceptable, and for still other proteins, HIC elution conditions might be preferred. One of the main goals of this work was to document the elutropic strength of the mobile phases used for HIC, RPC, and IEC regarding IAM chromatography.

PLA_2 has been purified by several laboratories by several different methods. The most common method involves gel filtration followed by ion-exchange chromatography. For bovine pancreatic PLA_2, the purification also includes an ammonium sulfate precipitation step prior to chromatography on both DEAE and CM cellulose [23]. A specific affinity column for PLA_2 was prepared by immobilizing a low density of

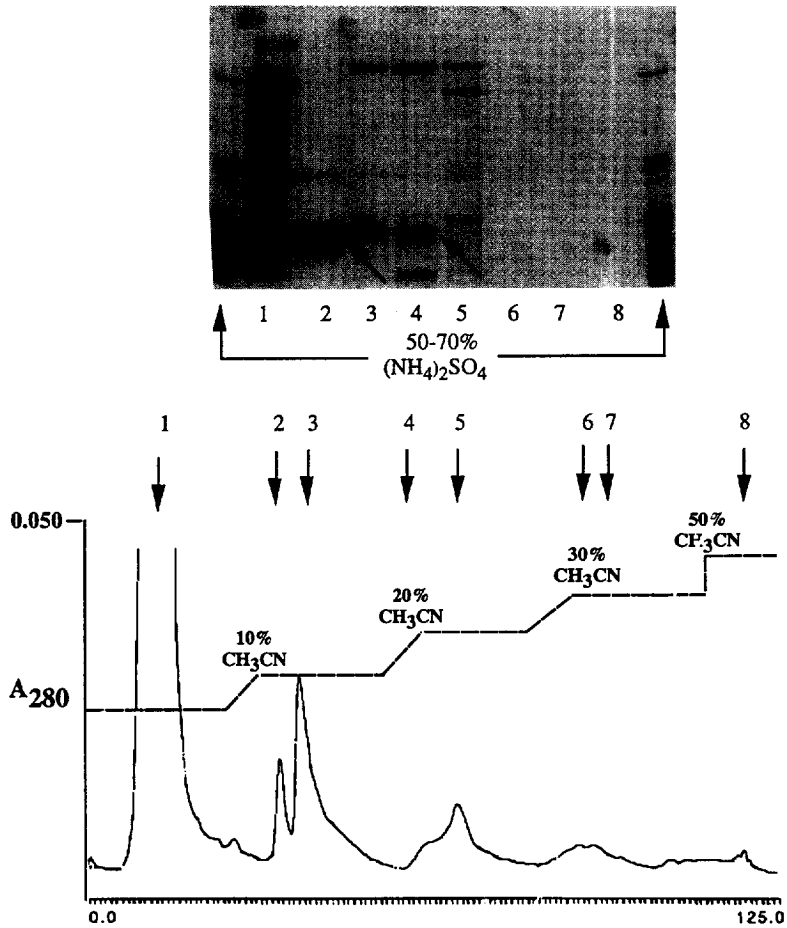

Fig. 9. Separation of active and inactive pancreatic tissue PLA_2 on a 6.5 × 1.0 cm ^{ether}IAM-$PC^{C10/C3}$ using an CH_3CN gradient. Mobile phase A contained 0.1 *M* Tris (pH 7.2), 0.2 *M* KCl, 20% EG and 0.05% NaN_3. The CH_3CN gradient is shown. Proteins marked with an arrow were electroblotted and sequenced. Sample loading was 2.3 mg/400 μl of total protein. Fraction 2 contained virtually no PLA_2 activity whereas fraction 4 contained all of the injected PLA_2 activity.

phosphatidylcholine (PC) on Sepharose with the PC head group protruding from the chromatographic surface [32]. PLA_2 was partially purified on this affinity column. Aarsman et al. immobilized antibodies that have avidity for PLA_2 to purify rat liver PLA_2 [9]. Ligand affinity chromatography using glycosylation sites of honeybee venom PLA_2 was highly successful at purifying PLA_2 [33]. In this report we demonstrate that intrinsic membrane binding properties of PLA_2s allow IAM surfaces to function as an affinity surface for this group of proteins.

Using immobilized PC ligands as an affinity surface, PLA_2 activity was bound in the presence of mobile phase Ca^{2+} and eluted using EDTA to chelate the Ca^{2+} [32]. This Sepharose-PC affinity surface contains "isolated" PCs tethered to Sepharose and the binding site for PLA_2 is actually the enzyme cavity that is responsible for the natural productive hydrolysis of phospholipids. In another words, the surface of the PLA_2 protein is not participating in the affinity of PLA_2 of the Sepharose-PC affinity surface. As shown in this report, IAM surfaces also have affinity for PLA_2 enzymes. However, in contrast to the Sepharose-PC affinity surface, the binding site for PLA_2 proteins on IAMs involves the PLA_2 protein surface and not the PLA_2 en-

zymatic cavity. This is based on the observation that PLA_2 does not hydrolyze immobilized diacylated PC ester ligands on IAM surfaces; we believe that this indicates that the PLA_2 enzyme pocket is not occupied when PLA_2 is adsorbed on the IAM surface. Thus PLA_2 affinity adsorption to the IAM surface is a membrane–protein interaction that involves the surface of the protein and the surface of the IAM. Although the Sepharose-PC affinity column is very efficient for PLA_2 purification, neither the affinity ligand nor the columns are commercially available; this prohibits the method from being utilized as general purification strategy for PLA_2.

In the broadest sense, IAM chromatography surfaces are highly selective affinity surfaces regarding protein adsorption. In other words, for any given membrane preparation, only a small fraction of the injected proteins adsorb to the IAM surface. The advantage of this selective affinity is that when protein affinity does occur on the IAM surface, then usually 50–80% of the contaminating proteins elute in the pass-through peak. Consequently, the target protein eluted in the retained peak is highly purified. This report demonstrates that IAMs are affinity surfaces for PLA_2 enzymes and that 70–80% of the contaminating proteins are removed in the pass-through peak. Once a group of proteins has been identified as having affinity for IAMs, general purification methods rapidly evolve. For instance, cytochrome P450 was shown to have high affinity for IAMs and this resulted in the rapid purification of cytochrome P450 from rat liver, rat renal, rat adrenal, rat kidney, and other sources [19]. Other proteins that have been purified using IAMs are cholesterol-binding protein [20], an intestinal peptide transporter (C. Pidgeon, unpublished) and a N-acylphosphatidylethanolamine synthase [21]. We note that SOS (a short-chain sulfonate analog of SDS) selectively elutes proteins from IAM surfaces without loss of protein functional activity. SOS may be considered as a weak detergent or weak mobile-phase modifier regarding the eluotropic strength of the mobile phase. Since it is very difficult to identify detergents that selectively elute proteins from any column, the observation that SOS selectively elutes proteins from IAMs may have practical significance.

Detergents undoubtedly partition into the IAM interfacial region and convert the immobilized membrane surface into a detergent enriched surface. Microviscosity is an important factor controlling the vertical displacement of membrane proteins from the membrane surface [34]. It is reasonable that detergents adsorbing to the IAM surface cause a non-specific increase or decreases in microviscosity that results in non-selective desorption of proteins, i.e. no selectivity during protein elution. However, since ACN was able to selectively elute proteins from the IAM column, organic solvents solvating the IAM surfaces may exhibit a concentration dependent change in the motional properties of the immobilized lipids; our lab is currently investigating these motional properties using ^{31}P NMR spectroscopy [35].

Although PLA_2 binds with affinity to IAMs, detergents do not provide the selective elution or resolution of proteins as they elute from the IAM column. For this reason, we explored elution conditions that have been established for other types of chromatography. IEC relies on a salt gradient of increasing ionic strength to elute proteins that have been adsorbed to the chromatographic surfaces through ionic interactions. Since an increasing salt gradient did not elute PLA_2 from IAMs, electrostatic interactions between the IAM polar head group surface and the PLA_2 surface do not seem to be the dominating mechanism of retention of PLA_2 on the IAM surface. Although electrostatic interactions may play a role in PLA_2 binding to IAMs other more important binding mechanism exists. This is true for both neutral IAMs (^{ether}IAM-PC$^{C10/C3}$ and ^{ether}IAM-PE$^{C10/C3}$) and negatively-charged IAMs (^{ether}IAM-PS$^{C10/C3}$ and ^{ether}IAM-PG$^{C10/C3}$).

Hydrophobic-interaction chromatography is a common technique for purifying membrane proteins. The elution conditions for this method involve a gradient of high salt to low salt. Proteins are "salted out" during the protein adsorption step and selectively "salted in" as the

salt gradient is lowered. Although PLA_2 adsorbed to the IAM columns with high salt loading conditions, lowering the ionic strength did not elute PLA_2 from the IAM column. However, the more important observation is that a homogeneously purified protein eluted from the IAM column when the salt concentration was lowered (Fig. 4). This indicates that some proteins may be purified from IAM surfaces using elution conditions common to hydrophobic-interaction chromatography.

We note that salts are usually ordered according to their ability to precipitate proteins on to HIC surfaces as phosphates > sulfates > acetates > chlorides > nitrates > thiocyanates, and this order of anions is frequently referred to as the Hofmeister series. The phosphates, sulfates, and acetates are referred to as polar kosmotropes that cause water structuring and therefore are strong protein precipitants. The salt used to obtain the protein elution profile shown in Fig. 4 was KCl which does not precipitate proteins on to surfaces as well as the polar kosmotropes. However, in addition to KCl, both phosphate and sulfate salt gradients were evaluated but similar results were obtained. Thus these preliminary experiments suggest that the salt type is not critical for the elution. An interesting observation was that high salt caused all of the injected proteins to bind to the IAM surface (i.e., there was no pass-through peak) except when 20% EG was present in the mobile phase. Apparently mobile phase EG increases protein solubility in the presence of high salt. The starting point for any chromatographic separation involves selectively adsorbing proteins to a surface. If the type of proteins adsorbed to IAM surfaces depends on both salt and EG concentrations, then selective adsorption of proteins on IAMs may be possible. Selective adsorption of proteins on IAMs may allow many more types of proteins to be purified using IAM chromatography.

RPC is a common method to purify peptides and proteins but frequently this method is not used for membrane protein purification because membrane proteins unfold when bound to hydrophobic surface and in the presence of organic solvent in the mobile phase. However, IAM surfaces are significantly less hydrophobic compared to RPC surfaces and protein elution frequently requires much less organic solvents. Our earlier studies showed that the first generation of IAM-PC columns performed chromatographically similar to C_3 RPC columns [36]. Thus it is not surprising that for ^{ether}IAM-PC$^{C10/C3}$ only ~4% ACN is required to elute PLA_2. Using C_8 RPC columns, PLA_2 requires 35% ACN for elution [37].

PLA_2 had high affinity for all of the IAMs shown in Fig. 1. The elution of PLA_2 from these neutral- and negatively-charged IAM surfaces is in agreement with the known affinity of PLA_2 for neutral- or negatively-charged fluid membrane surfaces. Mega-8 is a weak eluotropic strength mobile-phase modifier with a CMC of 48 m*M* and can elute PLA_2 from neutral ^{ether}IAM-PC$^{C10/C3}$ and ^{ether}IAM-PE$^{C10/C3}$ surfaces but not from ^{ether}IAM-PS$^{C10/C3}$ surfaces. Mega-8 cannot desorb PLA_2 bound to immobilized PS but it can desorb PLA_2 bound to neutral IAMs. This is consistent with the observation that PLA_2 has high affinity for fluid membranes containing phosphatidylserine [38]. The elution of PLA_2 using ACN gradients also supports the hypothesis that PLA_2 has higher affinity for immobilized PS compared to the other lipids that are immobilized. Even with 50% ACN in the mobile phase PLA_2 would not elute from ^{ether}IAM-PS$^{C10/C3}$ surfaces, whereas, only 4–7% ACN elutes PLA_2 from ^{ether}IAM-PC$^{C10/C3}$ columns and 25% ACN elutes PLA_2 from ^{ether}IAM-PE$^{C10/C3}$ columns.

The loading capacity of PLA_2 protein preparations on IAM columns is about 0.5 mg of total protein for an analytical-size (10 × 0.46 cm) IAM column. Based on gel electrophoresis, Sigma PLA_2 is ~10% of the total protein which indicates that ~50–100 μg of PLA_2 binds to the analytical-size column. This analytical-size IAM column contains ~0.9 g of IAM packing material and 105 mg of immobilized phosphatidylcholine (PC). Under this loading condition the molar ratio of PLA_2 protein to immobilized PL ligand is roughly 1:30.

An interesting discovery in this study was the

ability of IAM to resolve active and inactive PLA_2s (Fig. 9, lanes 2 and 4). During development of optimal elution conditions for the purification of PLA_2, we usually found an inactive protein with identical electrophoretic mobility on fractions prior to the elution of the active PLA_2 (Fig. 3, lane 4). Sequencing (Fig. 9, arrows) showed that the first six N-terminal amino acids of PLA_2 and the unknown protein of the same molecular mass were the same and furthermore, the sequence exactly matched the reported sequence of bovine pancreatic PLA_2. The inactive PLA_2 elutes first as a pure band (Fig. 9, lane 2) and active protein elutes with mobile phase of stronger eluotropic strength (lane 4). This suggests that the strength of the binding interaction between the IAM surface and PLA_2 strongly depends on the tertiary structure of the protein.

The inactive form of PLA_2, we believe, originates from trypsin activation instead of due to the denaturation caused by IAM column. During IAM elution studies using prepurified Sigma PLA_2, the activity of the protein was never lost. Extensive mobile-phase conditions were assayed, and loss of PLA_2 activity only took place with trypsin-activated tissue samples. It is possible that the presence of residual trypsin from the activation step could cause inactivation of the PLA_2 on the IAM surface if the protein column adsorption step lasts 30 min or more. It is most likely that prepurified Sigma PLA_2 has no residual trypsin and a stable amount of active PLA_2 forms is present.

5. Conclusions

Mobile-phase detergents tend to non-specifically elute proteins adsorbed to IAMs with little resolution of the eluting proteins. Elution conditions common to hydrophobic-interaction chromatography show promise in selectively eluting proteins from IAMs. Very low mobile-phase acetonitrile concentrations show selectivity regarding protein elution from IAM surfaces. Low acetonitrile concentrations are non-denaturing to PLA_2 and perhaps many other proteins. Short-chain analogs of SDS show great promise in functioning as mobile-phase modifiers that do not denature proteins but selectively elute proteins from IAM columns. Selective adsorption of proteins to IAM surfaces may be possible with mixtures of ethylene glycol and salt. IAM chromatography has high affinity for PLA_2 enzymes and is a very efficient chromatographic method for purifying this group of proteins.

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List of abbreviations

IAM	Immobilized artificial membrane
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PG	Phosphatidylglycerol
IAM-PC	IAM column immobilized with PC ligand
PLA_2	Phospholipase A_2
SDS	Sodium dodecyl sulfate
SOS	Sodium octanesulfonate
CHAPS	3-[3-(Chloromaidopropyl)-dimethylamminio]-1-propanesulfonate
Triton X-100	*tert*-Octylphenoxypoly-ethoxyethanol
DOC	Deoxycholate
TCA	Trichloroacetic acid
EG	Ethylene glycol
DCPC	Dicaproyl phosphatidylcholine
DPPC	Dipalmitoyl phosphatidylcholine
CMC	Critical micelle concentration
ACN	Acetonitrile
IEC	Ion-exchange chromatography
HIC	Hydrophobic interaction chromatography
RPC	Reversed-phase chromatography

References

[1] X. Qiu, S. Ong, C. Bernal, D. Rhee and C. Pidgeon, J. Org. Chem., 59 (1994) 537.
[2] S. Ong, S.J. Cai, C. Bernal, D. Rhee, X. Qiu and C. Pidgeon, Anal. Chem., 66 (1994) 782.
[3] D. Rhee, R.J. Markovich, W.G. Chae, X.X. Qiu and C. Pidgeon, Anal. Chim. Acta, (1994) in press.
[4] R. Verger, F. Ferrato, C.M. Mansbach and G. Pieroni, Biochem., 21 (1982) 6883.
[5] S. Forst, J. Weiss, P. Elsbach, J.M. Maraganore and R.L. Heinrikson, Biochemistry, 25 (1986) 8381.
[6] H.W. Chang, I. Kudo, M. Tomita and K. Inoue, J. Biochem. (Tokyo), 102 (1987) 147.
[7] T. Ono, H. Tojo, S. Kuramitsu, H. Kagamijama and M. Okamoto, J. Biol. Chem., 263 (1988) 5732.
[8] A. Kanda, T. Ono, N. Yoshida, H. Tojo and M. Okamoto, Biochem. Biophys. Res. Commun., 163 (1989) 42.
[9] A.J. Aarsman, J.G.N. de Jong, E. Arnoldussen, F.W. Neys, P.D. van Wassenaar and H. van den Bosch, J. Biol. Chem., 264 (1989) 10008.
[10] S.L. Hazen, R.J. Stuppy and R.W. Gross, J. Biol. Chem., 265 (1990) 10622.
[11] E. Stenfanski, W. Pruzanski, B. Sternby and P. Vadas, J. Biochem., 100 (1986) 1297.
[12] S. Hara, I. Kudo, H.W. Chang, K. Matsuda, T. Mayamoto and K. Inoue, J. Biochem. (Tokyo), 105 (1989) 395.
[13] X.-M. Zhang and I.W. Wainer, Tetrahedron Lett., 34 (1993) 4731.
[14] F.M. Alvarez, C.B. Bottom, P. Cahickale and C. Pidgeon, in T. Ngo (Ed.), Molecular Interactions in Bioseparations, Plenum Press, New York, 1993, p. 151.
[15] C. Pidgeon, S. Ong, H. Liu, X. Qiu, M. Pidgeon, A.H. Dantzig, J. Monroe, W.J. Hornback, J.S. Kasher, L. Glunz and T. Sc zerba, J. Med. Chem., 38 (1995) 590.
[16] S. Ong, H. Liu, X. Qiu, G. Bhat and C. Pidgeon, Anal. Chem., 34 (1995) 755.
[17] D.E. Cohen, M.R. Leonard, A.R. Leonard, Donovan and M.C. Caray, Gastroenterology, 104 (1993) A889.
[18] S. Otto, C. Marcus, C. Pidgeon and C. Jefcoate, Endocrinology, 129 (1991) 970.
[19] C. Pidgeon, J. Stevens, S. Otto, C. Jefcoate and C. Marcus, Anal. Biochem., 194 (1991) 163.
[20] H. Thurnhofer, J. Schnabel, M. Betz, G. Lipka, C. Pidgeon and H. Hauser, Biochim. Biophys. Acta, 1064 (1991) 275.
[21] S. Cai, R. McAndrew, B. Leonard, K. Chapman and C. Pidgeon, J. Chromatogr. A, 696 (1995) 49.
[22] M.P. Deutscher, in M.P. Deutscher (Editor), Methods in Enzymology, Vol. 182, Academic Press, San Diego, CA, 1990, p. 83.
[23] C.E. Dutilh, P.J. Van Doran, F.E.A.M. Verheul and G.H. de Haas, Eur. J. Biochem., 53 (1975) 91.
[24] G.L. Peterson, Anal. Biochem., 83 (1977) 346.
[25] C.R. Merril, in M.P. Deutscher (Editor), Methods in Enzymology, Vol. 182, Academic Press, San Diego, CA, 1990, p. 477.
[26] A.L. Araujo and F. Radvanyi, Toxicon, 25 (1987) 1181.
[27] R.M. Kramer, C. Wuthrich, C. Bollier, P.R. Allegrini and P. Zahler, Biochem. Biophys. Acta, 507 (1978) 381.
[28] V.P. Dole, J. Clin. Invest., 35 (1956) 150.
[29] L. Reynolds, W.N. Washburn, R.A. Deems and E.A. Dennis, in E.A. Dennis (Editor), Methods in Enzymology, Vol. 197, Academic Press, San Diego, CA, 1991, p. 3.
[30] H. Tojo, T. Teramoto, T.O. Yamano and M. Okamoto, Anal. Biochem., 137 (1984) 533.
[31] T. Ono, H. Tojo, K. Inoue, H. Kagamiyama, T. Yamano and M. Okamoto, J. Biochem., 96 (1984) 785.
[32] C.O. Rock and F. Snyder, J. Biol. Chem., 250 (1975) 6564.
[33] V.I. Gritsuk, E.A. Meshcheryakova, V.V. Okhanov, E.S. Efrmov and A.I. Miroshnikov, Bioorgh. Khim., 5 (1979) 1222.
[34] H. Borochov and M. Shinitzky, Proc. Nat. Acad. Sci., 73 (1976) 4526.
[35] X. Qiu and C. Pidgeon, J. Phy. Chem., 97 (1993) 12399.
[36] C. Pidgeon and U.V. Vaenkataram, Anal. Biochem., 176 (1989) 36.
[37] R.M. Kramer, C. Hession, B. Johansen, H.G.P. McGray, E.P. Chow, R. Tizard and R.B. Pepinski, J. Biol. Chem., 264 (1989) 5768.
[38] H.M. Verheij, A.J. Slotboom and G.H. de Haas, Rev. Phys. Biochem. Pharmacol., 91 (1981) 91.