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# A SIMPLE AND VERSATILE AFFINITY COLUMN FOR PHOSPHOLIPASE $A_2$

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**An affinity adsorbent for phospholipase $A_2$ (EC 3.1.1.4) was prepared by reacting 10-*O*-*p*-toluenesulfonyldecane-1-*O*-phosphocholine with AH-Sepharose 4B. Phospholipases $A_2$ bind to the immobilized ligand in the presence of $Ca^{2+}$ and can be eluted with buffers containing EDTA. This principle held not only for soluble phospholipase $A_2$ from porcine pancreas and *Crotalus adamanteus*, but proved also effective in the purification of phospholipases $A_2$ solubilized from the membranes of rat liver mitochondria and rat platelets.**

Phospholipase $A_2$ is a ubiquitous enzyme that occurs in high concentrations in the venom of snakes, bees and scorpions [1]. Among mammalian tissues the pancreas is a rich source of the enzyme [1,2]. In these cases the enzyme is present in soluble form representing from 1–10% of total protein. Numerous reports have indicated the existence of membrane-associated phospholipases $A_2$ in a great variety of mammalian tissues and cells [3]. Research on these membrane-bound phospholipases $A_2$ has been severely hampered because of the well-known difficulties encountered in obtaining membrane-bound enzymes in solubilized form with retention of biological activity. In addition, in those cases in which the purification of these phospholipases $A_2$ to near homogeneity has been achieved [4–10], the enzymes were found to be present in trace amounts representing from 0.01 to 0.1% of the total cellular protein. It is, therefore, obvious that potent purification techniques for such (sub)cellular phospholipases are highly desirable. Affinity chromatography constitutes such a technique. Rock and Snyder [11] have developed an affinity matrix for phospholipase $A_2$ by coupling *rac*-1-(9-carboxy)-nonyl-2-hexadecylglycero-3-phosphocholine to AH-Sepharose 4B via the carboxyl group. This material has been applied successfully in the purification of sheep erythrocyte [8] and human platelet [6] phospholipase $A_2$. On the other hand, the ligand used in this affinity matrix is rather hydrophobic, which could easily give rise to adsorption of other hydrophobic proteins. This phenomenon has been described in the purification of phosphatidylcholine exchange protein on phosphatidylcholine-Sepharose columns [12]. Since hydrophobic proteins can be expected to be predominant in preparations of phospholipase $A_2$ solubilized from biomembranes, an affinity adsorbent was prepared in which the ligand contains only a single aliphatic chain. This paper deals with the synthesis of this material and its use in the purification of phospholipase $A_2$ from rat liver mitochondria.

Part of these results was presented at the 24th International Conference on the Biochemistry of Lipids at Toulouse (France), September 14–16, 1983.

Lyophilized *Crotalus adamanteus* venom was obtained from Sigma, St. Louis, U.S.A. 1,10-Decanediol was a product from Aldrich Europe, Beerse, Belgium. AH-Sepharose 4B and Ultrogel AcA-54 were bought from Pharmacia, Uppsala, Sweden, and LKB, France, respectively. Cytochrome *c* and myoglobin were from Boehringer, Mannheim, F.R.G., and Mann Research Laboratories, U.S.A., respectively.

Protein was determined by the dye binding assay described by Bradford [13] as modified by Vianen and Van den Bosch [14] to increase sensitivity. SDS-polyacrylamide slab gel electrophoresis was done according to Laemmli [15] in a 12.5–25% polyacrylamide gradient gel. The gels were fixed and stained by the silver stain technique described by Oakley et al. [16]. Phospholipase $A_2$ activity assays with 1-acyl-2-[1-$^{14}$C]linoleoylphosphatidylethanolamine as substrate were done as described previously [10].

10-*O*-*p*-Toluenesulfonyldecane-1-ol was synthesized by adding *p*-toluenesulfonylchloride (19.1 g, 100 mmol) in pyridine (20 ml) dropwise to a solution of 1,10-decanediol (17.4 g, 100 mmol) in 40 ml pyridine at 0°C. After 1 h at 0°C and 3 h at room temperature the reaction was stopped by addition of ice-cold 1 N HCl and the products were extracted with 400 ml of diethyl ether. The ether layer was washed successively with 300 ml each of a 5% (w/v) $NaHCO_3$ solution and water. After drying ($Na_2SO_4$), the ether layer was concentrated in vacuo and the product was purified on a column ($4 \times 50$ cm$^2$) of Kieselgel 60 using petroleum ether/diethylether (3 : 7, v/v) as eluent. The monotosyl derivative of 1,10-decanediol was obtained in a yield of about 70%. $R_F$ values on TLC (Schleicher and Schüll F 1500/LS 254) in $CHCl_3$ were 0.07, 0.27 and 0.65 for 1,10-decanediol, monotosyl- and ditosyl derivatives, respectively. Tosylated derivatives were ultraviolet-positive (256 nm). $^1$H-NMR ($C^2HCl_3$/$TMS_{int}$) of the purified monotosyl product gave: $\delta$-1.1–1.7 (c, 16H, $-(CH_2)_8-$); 2.0 (s, 1H, $-CH_2OH$); 2.4 (s, 3H, $\phi$ $-CH_3$); 3.56 (c, 2H, $-CH_2-OH$); 3.96 (t, 2H, $-CH_2OSO_2-$); 7.32 and 7.76 (two doublets, 4H, $-C_6H_4$).

10-*O*-*p*-Toluenesulfonyldecane-1-*O*-phosphocholine was prepared by adding a solution of the monotosyl compound (1.71 g, 5.2 mmol) and pyridine (12.5 mmol) in 30 ml dry dichloromethane slowly to a stirred solution of $POCl_3$ (843 mg, 5.5 mmol) in 20 ml of dry dichloromethane a 0°C. Stirring was continued for 1 h at room tem perature. TLC revealed the complete disappearance of the monotosyl derivative of decanediol. Pyridine (1 ml, 12.5 mmol) and choline toluenesulfonate (1.9 g, 6.9 mmol) were added and stirring was continued for 16 h [17]. After addition of 1 ml each of pyridine and water and stirring for 2 h the reaction was worked up by addition of 50 ml water and 100 ml methanol. The pH was adjusted to 3 with 1 N HCl. The product was extracted twice with 50 ml $CHCl_3$ and percolated over a mixed ion-exchange column (Amberlite IRA-45 and IRC-50) in chloroform/methanol/water (5 : 4 : 1, v/v). The product was further purified on Kieselgel 60 by elution with chloroform-methanol mixtures. The purified compound showed an $R_F$ value of 0.33 on TLC (see above) with chlorom/methanol/$H_2O$ (60 : 40 : 10, v/v) as solvent system. For comparison, egg phosphatidylcholine and its lyso-derivative have $R_F$ values of 0.44 and 0.33, respectively, in this system. On TLC 10-*O*-*p*-toluenesulfonyldecane-1-*O*-phosphocholine is ultraviolet-, phosphor- and choline-positive. Phosphor determinations [18], gave a purity of 93% on weight basis. $^1$H-NMR of the purified compound ($C^2HCl_3$/$TMS_{int}$) gave $\delta$ = 1.1–1.7 (c, 16H, $-(CH_2)_8-$); 2.45 (s, 3H, $\phi$ $-CH_3$); 3.35 (s, 9H, $-N(CH_3)_3$); 3.6–4.4 (c, 8H $-CH_2-O-PO_2-CH_2CH_2N$, $-CH_2OSO_2-$); 7.35 and 7.80 (two doublets, 4H, $-C_6H_4$).

Freeze-dried AH Sepharose 4B (2 g) was swollen and washed as recommended by the supplier. Ligand was coupled according to the procedure of Nilsson and Mosbach [19]. Briefly, the ligand (245 mg, 0.5 mmol) was allowed to react with AH

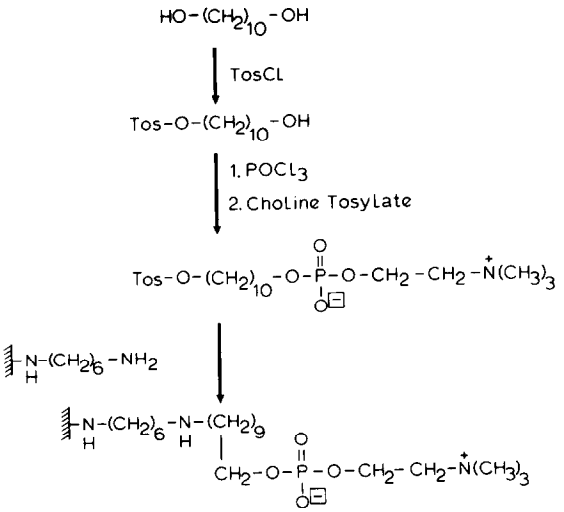


Fig. 1. Schematic representation of the synthesis of affinity adsorbent for phospholipase $A_2$.

Sepharose 4B in 10 ml of 0.2 M $NaHCO_3$ (pH 10.7) for 20 h at 37°C with gentle agitation. The gel was washed successively with 150-ml portions of water, 0.1 M acetate buffer (pH 4.0), water, 0.5 M NaCl, water, 0.2 M bicarbonate and water. Phosphor determinations on destructed gel [18] showed the presence of 1.0–3.2 μmol ligand per ml gel for various preparations.

Fig. 1 depicts the synthesis of the affinity adsorbent as described above. The leading thought in the synthesis of this affinity resin has been that phospholipase $A_2$ is able to hydrolyze glycolphosphatidylcholines [2] and to bind *n*-alkylphosphocholines [20]. Preliminary experiments with purified porcine pancreatic phospholipase $A_2$ showed complete retention of enzymatic activity when applied to the column in buffer containing 10 mM $Ca^{2+}$, even in the presence of 0.2% bovine serum albumin. However, in the presence of 1% bovine serum albumin phospholipase $A_2$ was only partially bound. Bound enzyme could be eluted with buffer containing 50 mM EDTA (results not shown).

Fig. 2 represents a purification of phospholipase $A_2$ from crude *C. adamanteus* venom. Virtually all protein but no phospholipase $A_2$ activity elutes from the column in the presence of $Ca^{2+}$. Subsequent elution with EDTA releases the enzyme from the column. As can be seen in Fig. 3

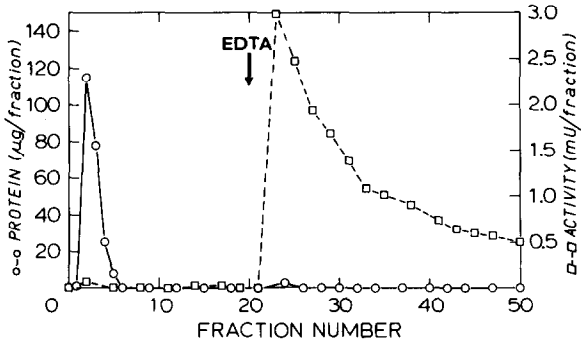


Fig. 2. Affinity purification of *Crotalus adamanteus* phospholipase $A_2$. Crude venom (300 μg protein) in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 10 mM $CaCl_2$ and 0.2 M NaCl was applied to an affinity column (0.5×3.5 cm) and eluted with this buffer. Enzyme elution was started as indicated with a buffer in which the 10 mM $CaCl_2$ was replaced by 50 mM EDTA. Fractions of 0.67 ml were collected at a flow-rate of 8 ml/h. Recoveries: protein, 79%; phospholipase $A_2$ activity: 80%.

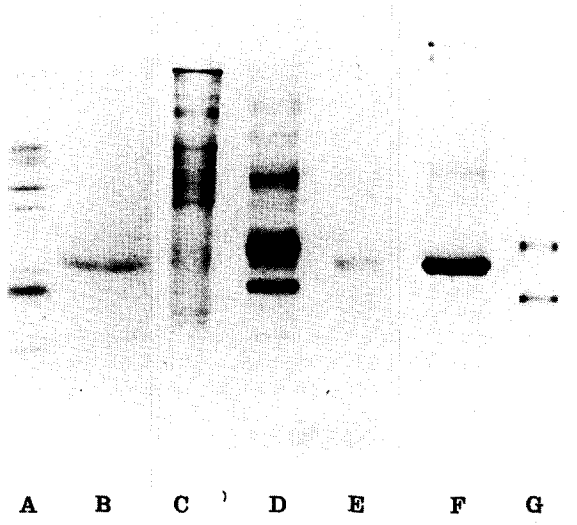


Fig. 3. SDS-polyacrylamide slab gel electrophoresis. Lane A, total *Crotalus adamanteus* venom (10 μg protein); lane B, broad band of the phospholipase $A_2$ peak eluted from the affinity column (1 μg protein); lane C, extract from delipidated mitochondrial membranes (20 μg protein); lane D, mitochondrial phospholipase $A_2$ after AcA-54 chromatography (40 μg protein); lane E, affinity purified mitochondrial phospholipase $A_2$ (0.5 μg protein). The latter fraction was applied in 20-fold higher concentration on lane F along with a mixture of 0.5 μg each of myoglobin and cytochrome *c* as marker proteins (lane G).

(lanes A and B), this single step yields pure phospholipase $A_2$.

We have previously reported on the purification of a membrane-associated phospholipase $A_2$ from rat liver mitochondria by conventional purification techniques such as Ultrogel AcA-54, hydroxyapatite and Matrex gel Blue A column chromatography [10]. Solubilization of mitochondrial phospholipase $A_2$ was achieved by ammoniacal acetone delipidation of mitochondria followed by extraction of delipidated proteins with buffers containing 1 M KCl. When such extracts were chromatographed over affinity resin in the presence of $Ca^{2+}$, part of the phospholipase $A_2$ activity was not retarded. However, when the mitochondrial extract was first chromatographed over an AcA-54 column to give a 7-fol purification of the enzyme, successful application of the affinity column became possible. Fig. 3 (lanes C and D) shows, as expected, that the combined AcA-54 fractions are

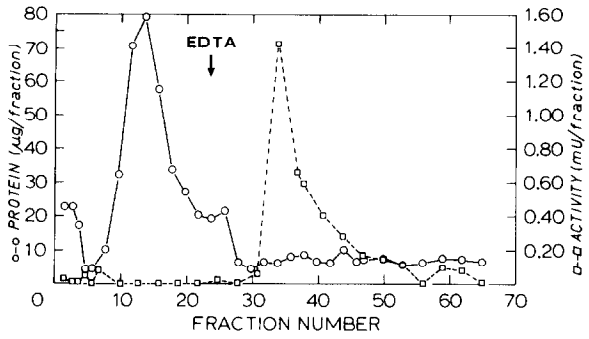


Fig. 4. Affinity purification of mitochondrial phospholipase $A_2$. AcA-54 fractions were concentrated with Aquacide, dialyzed against 50 mM Tris-HCl (pH 7.4) containing 0.2 M KCl, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM $CaCl_2$ and 1 mM EDTA and applied and eluted with this buffer. At fraction 24 elution was started with the above buffer in which 0.2 M KCl and 10 mM $CaCl_2$ were replaced by 0.5 M KCl and 50 mM EDTA, respectively. Fractions of 0.67 ml were collected at a flow-rate of 8 ml/h. Recoveries: protein, 67%; phospholipase $A_2$ activity, 127%.

relatively rich in low molecular weight proteins, including a weak band of phospholipase $A_2$ in between vast amounts of other proteins. As can be seen in Figs. 4 and 3 (lanes E and F), these are effectively removed in a single chromatographic step using the affinity column. Phospholipase $A_2$ solubilized from rat platelets by extraction with 1 M KCl [5] and partially purified by AcA-54 gel filtration gave results upon chromatography over the affinity resin similar to those shown in Fig. 4 for mitochondrial phospholipase $A_2$. Recently, Natori et al. [21] have prepared an affinity resin for phospholipase $A_2$ by coupling oxidized phosphatidylcholine (PC) to AH-Sepharose to give diacyl PC-Sepharose. Although mitochondrial phospholipase $A_2$ was bound to this column, elution with EDTA and 0.01% Triton X-100 to release the phospholipase $A_2$ released other proteins as well. Presumably the latter were unspecifically bound by hydrophobic interaction, as was observed in earlier experiments using PC-Sepharose columns [12].

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