



AN AQUEOUS ENDPOINT ASSAY OF SNAKE VENOM PHOSPHOLIPASE A₂

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M. Holzer and S. P. Mackesy: An aqueous endpoint assay of snake venom phospholipase A₂. *Toxicon* 34, 1149-1155, 1996.—Phospholipase A₂ (PLA₂), an enzyme found in most snake venoms, catalyzes the hydrolysis of phospholipids in biological membranes, and some have presynaptic neurotoxic activity. A synthetic substrate, 4-nitro-2-(p-nitrophenyl)acetylphosphatidylcholine, and

minimizing waste disposal concerns. Whole venoms, partially purified enzyme isolated from *Crotalus mitchelli pyrrhus* venom, tissue extracts and commercial preparations were employed as sources of PLA₂. Results show that this method is convenient and specific assay for PLA₂ from several sources and is particularly suited for assaying large numbers of fractions generated during purification procedures. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Phosphatidate 2-acylhydrolase [E.C. 3.1.1.4.; trivial name phospholipase A₂ (PLA₂)] is a common and abundant enzyme found in most snake venoms (Rosenberg, 1990; Davidson

Slotta and Fraenkel-Conrat, 1938; Fraenkel-Conrat *et al.*, 1980; Middlebrook and Kaiser, 1989) and several Australian elapid snakes, and one of these, notexin, is among the most toxic of known venom components (Cull-Candy *et al.*, 1976). *In vivo*, PLA₂s have a variety of activities, including presynaptic neurotoxicity, platelet aggregation activity (Landucci *et al.*, 1994; Huang and Chiang, 1994) and nephrotoxicity (Sitprija *et al.*, 1971). Products of hydrolysis (commonly arachidonic acid) can serve as precursors for pain mediators such as leukotrienes and prostaglandins, and released lysophospholipid may enter acetylation *et al.*, 1993; Zimmerman *et al.*, 1992).

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The extensive literature on PLA₂s indicates the importance of these enzymes to many areas of research in biochemistry, molecular biology, structural biology, toxicology, immunology, and medicine. Because PLA₂ liberates bioactive components with a variety of effects, monitoring its activity and developing inhibitory drugs is of significant interest. Numerous assays for PLA₂ activity utilizing a variety of substrates have been developed (Reynolds *et al.*, 1991, 1992; Washburn and Dennis, 1990; Farooqui *et al.*, 1984; Donne-Op den Kelder *et al.*, 1982; Cohen *et al.*, 1976; Wells and Hanahan, 1969); however, each of these methods either requires materials not commercially available or involves lengthy procedures. In the present study we describe an endpoint assay which is rapid and inexpensive and requires a minimum of specialized equipment. This method is particularly well suited for use during the purification of PLA₂ from rich sources such as snake venoms.

MATERIALS AND METHODS

Reagents and venoms

2-Hydroxy-4-nitrobenzoic acid was purchased from Aldrich Chemical Company. Bio-Gel B-100 (medium) was obtained from Bio-Rad. Carboxymethyl Sephadex A-50 ion-exchange resin was purchased from LKB-Pharmacia. Electrophoretic supplies were purchased from Novel Experimental. Venoms from *Crotalus mitchelli pyrrhus*, *C. molossus*, *C. scutulatus* and *Bitis gabonica* were extracted from adult snakes using standard techniques (Mackessy, 1988). Venoms from adult *C. atrox*, *C. durissus terrificus* and *Naja melanoleuca* were a gift from Mr Barney Tomberlin. All other venoms, enzymes and reagents (analytical grade or better) were obtained from Sigma Chemical Company.

An aqueous extract of fresh *C. atrox* pancreas was obtained by homogenizing approximately 2 g wet tissue in 5.0 ml Millipore-filtered distilled water with a Virtis Virtishear tissue homogenizer for 5 min at the highest setting. The supernatant obtained after centrifugation at 4000 × *g* for 10 min was then lyophilized and stored at -20°C until used.

Substrate

The substrate 4-nitro-3-(octanoyloxy)benzoic acid was synthesized using a published method (Cho *et al.*, 1988). The substrate is also commercially available from Sigma Chemical Company.

Assays

PLA₂ activity of crude venoms, column fractions, a commercial preparation of PLA₂ and tissue extract were routinely assayed using the following method. One milliliter of buffer (10 mM Tris-HCl, 10 mM CaCl₂, 100 mM NaCl; pH 8.0) was combined with 100 µl venom (4.0 mg/ml in dH₂O) or chromatographic fraction and tubes were placed on ice. One-hundred microliters of substrate [4-nitro-3-(octanoyloxy)benzoic acid, 3.0 mM in acetonitrile] was then added; final concentration of substrate was 0.25 mM. Each tube was vortexed and placed in a water bath (37°C) for 20 min. To stop the reaction, tubes were placed on ice, 100 µl of Triton X-100 (2.5% in dH₂O) was quickly added and tubes were vortexed for 5 sec each. Termination with Triton X-100 did not result in quenching of the chromophore as was observed in earlier experiments using EDTA. Tubes were held at room temperature for 5-10 min and absorbance at 425 nm was recorded. All assays were run in duplicate and values are expressed as averages minus blank controls. Absorbances at 425 nm for controls were typically 0.005 AU after 20 min incubation. A standard curve of absorbance as a function of chromophore (3-hydroxy-4-nitrobenzoic acid) concentration showed that a change in absorbance of 0.10 AU at 425 nm was

Product stability was evaluated by a time-course assay utilizing *C. atrox* venom as a source of PLA₂. Assays were conducted as above and reactions were terminated after 1, 3, 5, 10, 15, 20 and 25 min at 37°C. Absorbances were then recorded immediately and at 5 min intervals for each tube for 60 min. Linearity of the assay with increasing PLA₂ concentration was evaluated using *C. atrox* venom as a source of PLA₂.

Comparison with a titrimetric method

The present method was compared with a titrimetric endpoint assay (Wells and Hanahan, 1969). This method utilizes egg-yolk phosphatidylcholine in ether and is based on the titration of released fatty acids. Activity at 25°C was measured for 10 min using 35 µg crude venom or partially-purified PLA₂ (in 25 µl dH₂O).

Isolation of venom PLA₂

Lyophilized venom from *C. m. pyrrhus* was dissolved in 3.0 ml buffer (10 mM HEPES, 60 mM NaCl, pH 6.8),

Absorbance at 280 nm was used to estimate protein/peptide concentration. Fractions were assayed for PLA₂ activity using the method described above. Venom from *C. atrox* was also subjected to size-exclusion chromatography, but PLA₂ activity was not further purified.

For *C. m. pyrrhus* venom, fractions containing PLA₂ activity (second peak) were combined, dialyzed and lyophilized. This material was redissolved in 5 ml of 10 mM Tris-HCl buffer (pH 6.5) and applied to a carboxymethyl-Sephadex ion-exchange column (1.0 × 10 cm). Bound proteins were eluted using a salt gradient (0–0.4 M NaCl) and the present assay was used to locate PLA₂ activity. Relative purity was estimated electrophoretically using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 14% acrylamide Novex gels.

RESULTS

Using 400 µg of *C. atrox* venom, detectable amounts of chromophore were released within 1 min at 37°C, and absorbance increased linearly for at least 25 min of incubation (Fig. 1). A slight increase in absorbance was sometimes observed during the first 5 min following termination with Triton X-100, and for all later experiments absorbance was recorded at least 5 min after termination and incubation at room temperature. Readings remained stable for at least 60 min after reaction termination, which facilitated simultaneous assay of numerous samples.

Using crude venom, release of chromophore after 20 min of incubation at 37°C showed a linear relation with venom amounts of up to 400 µg (Fig. 2). At the lowest level tested

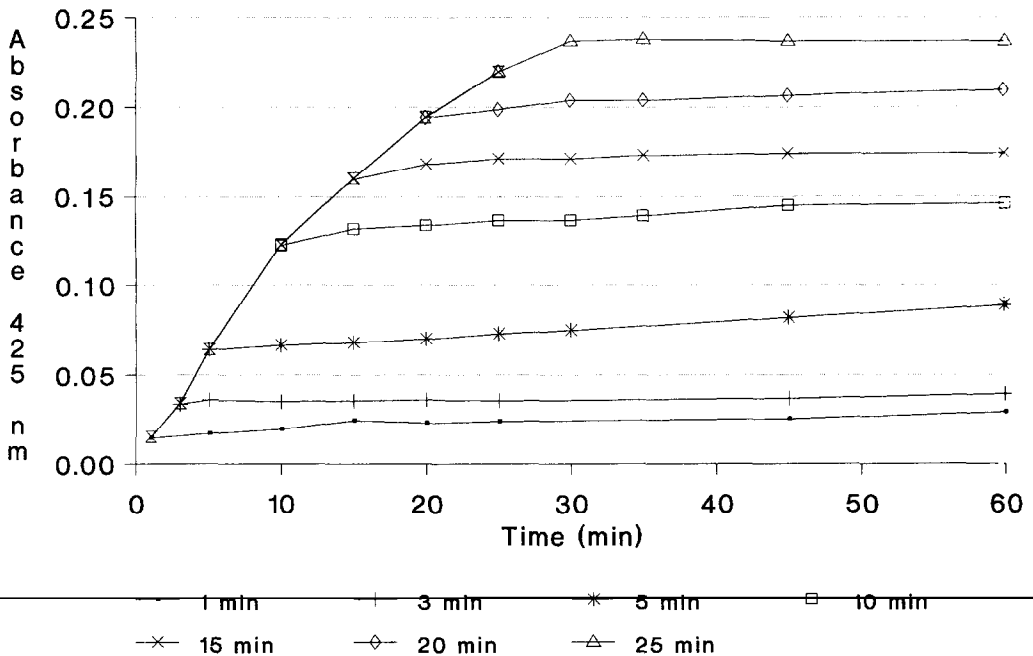
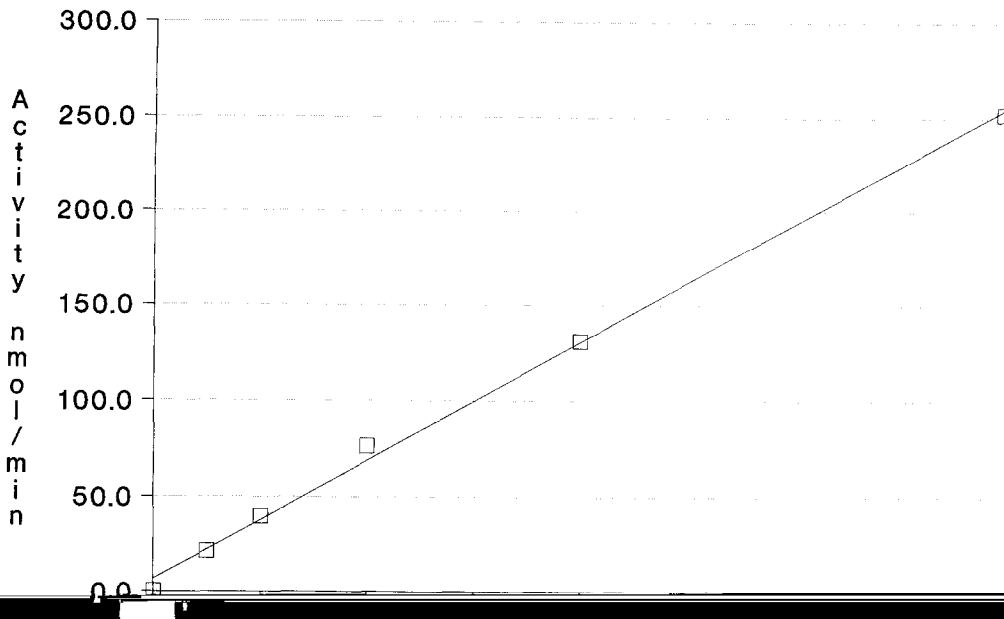


Fig. 1. Stability of product in the presence of Triton X-100. Substrate and crude venom were incubated for 1–25 min and the reaction was terminated with Triton X-100. Within 5 min after the addition of Triton X-100, apparent activity ceased, and values remained relatively constant for at least 60 min after termination.



venom).

(25 μ g venom in 1.2 ml total volume), chromophore release was detectable well above control blanks. Enzyme-catalyzed hydrolysis of the substrate was linear with increasing venom concentration.

Activities of various venoms, PLA₂ preparations and pancreatic extract are given in Table 1. Among crotalid snakes, *Crotalus durissus* and *C. scutulatus* venoms showed highest activity toward the substrate; both of these venoms contain large amounts of a presynaptic neurotoxin (crotoxin and mojave toxin, respectively) which also has PLA₂ activity. A partially purified PLA₂ from *C. mitchelli pyrrhus* venom also showed high activity. Venoms from *Vipera russelli* and from the elapids *Bungarus* and *Notechis* showed high levels of enzyme activity, consistent with earlier reports. Bee (*Apis mellifera*) venom showed weak activity toward the substrate, but a crude pancreatic extract from *C. atrox* tissue showed moderate PLA₂ activity.

For comparative purposes, several PLA₂ sources were assayed for activity toward phosphatidylcholine using a titrimetric method. Both crude venom and partially purified PLA₂ showed considerably higher specific activity toward the native phospholipid and a greater level of accuracy of detection.

The substrate proved to be sensitive and specific for PLA₂ when used to assay fractions

fractions were rapidly and simultaneously assayed for PLA₂ activity. PLA₂ activity was detected in only one protein peak (peak 2); metalloproteases found in peaks 1 and 3 and amidolytic proteases in peak 2 (data not shown) did not result in hydrolysis of the

substrate. Activity in *C. m. pyrrhus* venom was also easily detected after a second isolation step (ion-exchange; data not shown). Based on electrophoretic analysis of this material, the PLA₂ content of this preparation was > 85%. Further purification steps were not conducted.

DISCUSSION

PLA₂ is an important regulatory enzyme in many intracellular and extracellular events in vertebrate tissues (Davidson and Dennis, 1991), and the isolation and characterization of these enzymes continues to be an important task. Highly sensitive and specific assays have been developed for PLA₂ and, owing to their high sensitivity, one class of these compounds, termed SIBLINKS (Washburn and Dennis, 1990), is likely to be extremely useful for detailed kinetic analyses and characterization of minute quantities of PLA₂ from

a synthetic substrate in a reaction terminated by Triton X-100, fulfills these criteria, and is particularly useful for following enzyme activity in multiple fractions generated during purification from rich sources such as snake venoms.

It was somewhat puzzling that the addition of Triton X-100 efficiently and rapidly stopped the liberation of chromophore. We hypothesize that termination occurs via formation of mixed micelles, with a concomitant blockage of the substrate's labile bond due to steric hinderance. This is in sharp contrast to the effect of the detergent on native phospholipids. Triton X-100 forms mixed micelles with phosphatidylcholine, and this association promotes phospholipid hydrolysis (Davidson and Dennis, 1991). In the present assay system, 4-nitro-3-(octanoyloxy)benzoic acid may form tighter associations with the micelle, with the labile bond of the chromophore becoming inaccessible. Sequestering of the substrate within micelles seems unlikely, because at the concentration of Triton X-100

Table 1. Phospholipase A₂ activities of venoms and pancreatic extract

	Activity*
Family Crotalidae	
<i>Crotalus atrox</i>	7.7
<i>Crotalus durissus terrificus</i>	22.3
<i>Crotalus mitchelli pyrrhus</i>	19.6
<i>Crotalus molossus</i>	19.3
<i>Crotalus scutulatus</i>	30.2
Family Viperidae	
<i>Bitis gabonica gabonica</i>	2.2
<i>Vipera russelli</i>	41.4
Family Elapidae	
<i>Bungarus caeruleus</i>	46.7
<i>Naja melanoleuca</i>	4.7
<i>Notechis ater</i>	35.7
Family Hydrophiidae	
<i>Enhydrina schistosa</i>	5.4
<i>Laticauda semifasciata</i>	8.6
Bee (<i>Apis mellifera</i>) venom PLA ₂	0.3
<i>Crotalus atrox</i> pancreas extract	9.5
Partially purified <i>C. m. pyrrhus</i> PLA ₂	122.8

*nmoles product/min/mg protein.

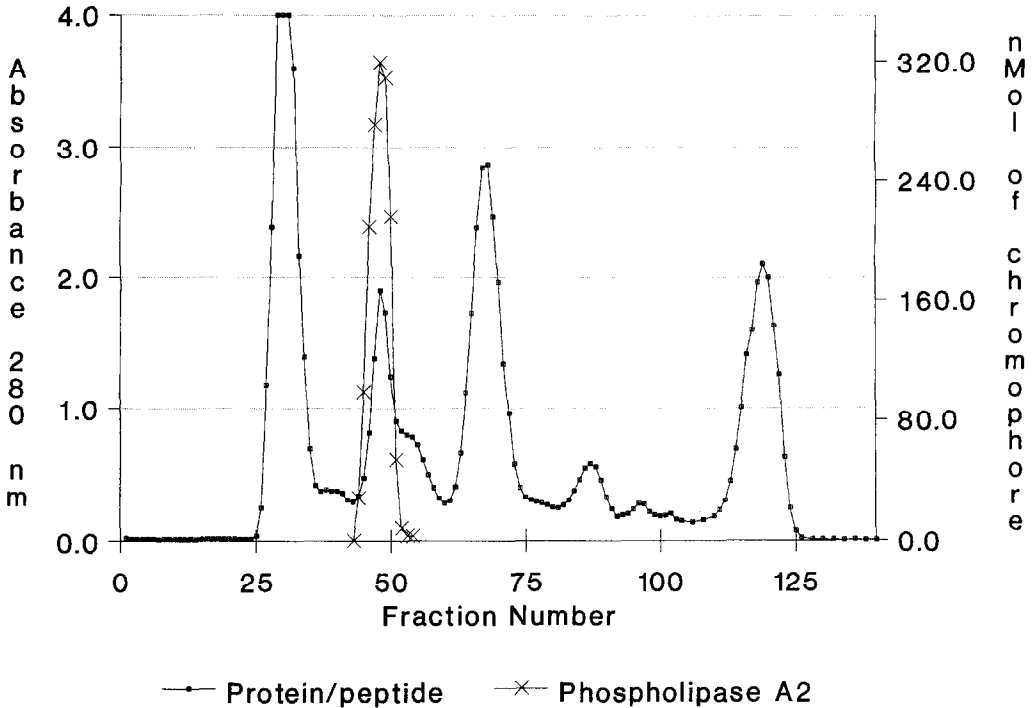


Fig. 3. Detection of phospholipase A₂ activity during purification. Assay of fractions obtained from size-exclusion chromatography of *C. mitchelli pyrrius* venom on BioGel P-100; activity was located in the second peak.

observed at higher detergent concentrations or after the addition of EDTA, as determined by preliminary experiments. Alternatively, the addition of Triton X-100 may change the aggregation state of the substrate (not determined), making it inaccessible to the enzyme.

The method described here has been particularly useful for assaying large numbers of fractions generated during low-pressure column chromatographic isolation of large amounts of PLA₂. Enzyme activity was easily and specifically followed during gel filtration and ion exchange chromatography. It should also be useful for the detection of PLA₂ during HPLC isolation procedures, and the method is amenable to further automation via microtiter plate-reading systems. However, since these systems may not be as generally available, the present method was developed to provide reasonably sensitive and rapid detection of PLA₂ activity during purification procedures. It should be stressed that other

specificity, but for assaying multiple samples simultaneously this method has proved quite

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- Cun-Candy, S. G., Jomman, J., Gustavsson, D., Lunaman-Ranch, K. and Thesich, S. (1976) The effects of taipoxin and notexin on the function and fine structure of the murine neuromuscular junction. *J. Neurosci.* **1**, 175-180.
- Davidson, F. F. and Dennis, E. A. (1991) Structure, function and mode of action of snake venom and other phospholipases A₂. In: *Handbook of Natural Toxins*, Vol. 5, *Reptile Venoms and Toxins*, pp. 107-145 (Tu, A. T., Ed.). New York: Marcel Dekker.

sequence of crotoxin B. In: *Natural Toxins*, p. 561 (Eaker, D. and Wadstrom, T., Eds). Oxford: Pergamon Press.

- Huang, T. F. and Chiang, H. S. (1994) Effect on human platelet aggregation of phospholipase A₂ purified from *Heloderma horridum* venom. *Biochim. biophys. Acta* **1211**, 61-68.
- Landucci, E., Condino-Neto, A., Perez, A. C., Hyslop, S., Corrado, A. P., Novello, J. C., Marangoni, S., Oliveira, B., Antunes, E. and de Nucci, G. (1994) Crotoxin induces aggregation of human washed platelets. *Toxicon* **32**, 217-226.
- Mackessy, S. P. (1988) Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* **1988**, 92-101.
- Mackessy, S. P. (1993) Fibrinogenolytic proteases from the venoms of juvenile and adult northern Pacific rattlesnakes (*Crotalus viridis oreganus*). *Comp. Biochem. Physiol.* **106B**, 181-189.
- Middlebrook, J. L. and Kaiser, I. I. (1989) Immunological relationships of phospholipase A₂ neurotoxins from

phospholipases. *Mem. Enzymol.* **191**, 3-25.

- Reynolds, L. J., Hughes, L. L. and Dennis, E. A. (1992) Analysis of human synovial fluid phospholipase A₂ on short-chain phosphatidylcholine mixed micelles: development of a spectrophotometric assay suitable for a microplate reader. *Analyt. Biochem.* **204**, 190-197.
- Rosenberg, P. (1990) Phospholipases. In: *Handbook of Toxicology*, pp. 67-277 (Shier, W. T. and Mebs, D., Eds). New York: Marcel Dekker.
- Sitarin, V., Saibikhedh, B. and Demisici, G. (1971) Human platelet phospholipase A₂. *Biochem. Biophys. Res. Commun.* **43**, 216.
- Slotta, K. H. and Frankele-Corrot, H. (1978) Two active proteins from rattlesnake venom. *Nature* **272**, 142.

- Washburn, W. N. and Dennis, E. A. (1990) Novel general approach for the assay and inhibition of hydrolytic enzymes utilizing suicide-inhibitory bifunctionally linked substrates (SIBLINKS) exemplified by a phospholipase A₂ assay. *J. Am. chem. Soc.* **112**, 2040-2041.
- Wells, M. A. and Hanahan, D. J. (1969) Studies on phospholipase A₂. I. Isolation and characterization of two enzymes from *Crotalus adamanteus* venom. *Biochemistry* **8**, 414-424.
- Zimmerman, G. A., Prescott, S. M. and McIntyre, T. M. (1992) Platelet activating factor: a fluid-phase and cell-associated mediator of inflammation. In: *Inflammation: Basic Principles and Clinical Correlates*, Vol. 2, pp. 149-176 (Gallin, J. I. and Goldstein, I. M., Eds). New York: Raven Press.