Pergamon

PII: S0041-0101(96)00057-8

Toxicon, Vol. 34, No. 10, pp. 1149–1155, 1996

0041-0101/96 $15.00 + 0.00

AN AQUEOUS ENDPOINT ASSAY OF SNAKE VENOM PHOSPHOLIPASE A_2

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(*Received* 26 *November* 1995; *accepted* 15 *March* 1996)

M. Holzer and S. P. Mackessy. An aqueous endpoint assay of snake venom phospholipase A_2. *Toxicon* **34,** 1149–1155, 1996.—Phospholipase A_2 (PLA_2), 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-3-(octanoyloxy)benzoic acid, was synthesized and purified on a silica gel column using a published method. This substrate was used to develop an endpoint assay which is rapid and requires a minimum of equipment. This aqueous assay system allowed enzyme activity to be examined without the use of radioactive substrates or organic solvents, 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 PLA_2. Results show that this method is a convenient and specific assay for PLA_2 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 A_2 (PLA_2)] is a common and abundant enzyme found in most snake venoms (Rosenberg, 1990; Davidson and Dennis, 1991). In addition to enzymatic activity, PLA_2 is the active component of highly homologous presynaptic neurotoxins found in venoms of rattlesnakes (*Crotalus* sp.; 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*, PLA_2s 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 pathways to form platelet aggregating factor, a potent promoter of inflammation (Venable *et al.*, 1993; Zimmerman *et al.*, 1992).

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The extensive literature on PLA_2s indicates the importance of these enzymes to many areas of research in biochemistry, molecular biology, structural biology, toxinology, toxicology and medicine. Because PLA_2 liberates bioactive components with a variety of effects, monitoring its activity and developing inhibitory drugs is of significant interest. Numerous assays for PLA_2 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 PLA_2 from rich sources such as snake venoms.

MATERIALS AND METHODS

Reagents and venoms

3-Hydroxy-4-nitrobenzoic acid was purchased from Aldrich Chemical Company. BioGel P-100 (medium) was obtained from BioRad. 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

PLA_2 activity of crude venoms, column fractions, a commercial preparation of PLA_2 and tissue extract were routinely assayed using the following method. One milliliter of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl; pH 8.0) was combined with 100 µl venom (4.0 mg/ml in dH_2O) 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 dH_2O) 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 equivalent to 25.8 nmoles of chromophore release. The chromophore has an extinction coefficient of 5039 in this system.

Product stability was evaluated by a time-course assay utilizing *C. atrox* venom as a source of PLA_2. 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 PLA_2 concentration was evaluated using *C. atrox* venom as a source of PLA_2.

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 assayed for 10 min using 25 µg crude venom or partially purified PLA_2 (in 25 µl dH_2O).

Isolation of venom PLA_2

Lyophilized venom from *C. m. pyrrhus* was dissolved in 3.0 ml buffer (10 mM HEPES, 60 mM NaCl, pH 6.8), briefly centrifuged and applied to a 2.8 × 110 cm BioGel P-100 column as described previously (Mackessy, 1993). Absorbance at 280 nm was used to estimate protein/peptide concentration. Fractions were assayed for PLA_2 activity using the method described above. Venom from *C. atrox* was also subjected to size-exclusion chromatography, but PLA_2 activity was not further purified.

For *C. m. pyrrhus* venom, fractions containing PLA_2 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 PLA_2 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

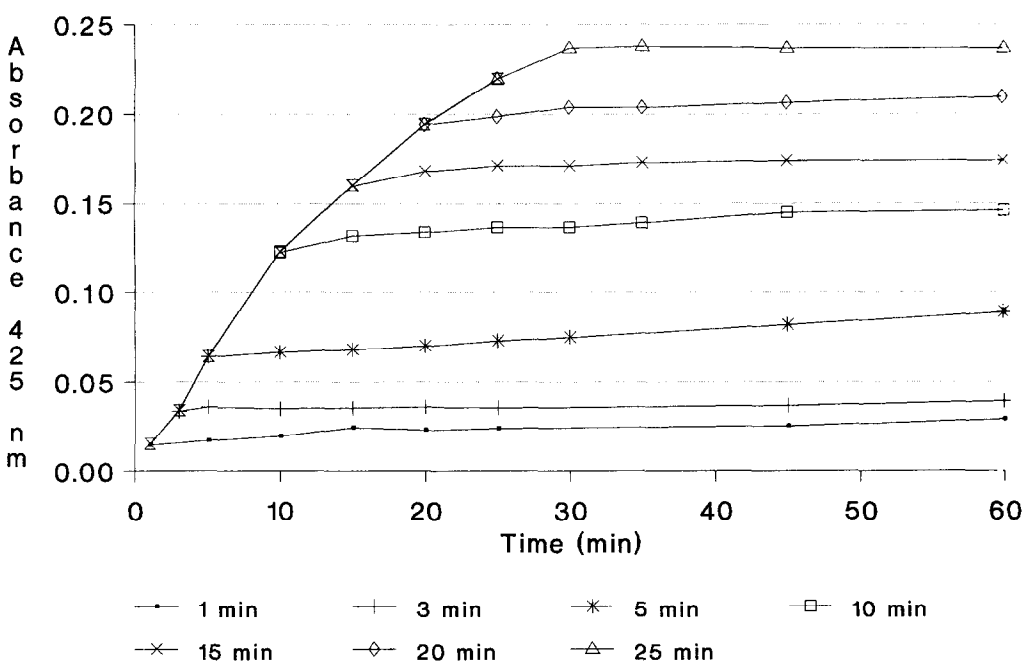

Fig. 1. Stability of product in the presence of Triton X-100.
Substrate and crude venom were incubated for 1–25 min and the reaction was terminated with Triton X-100. Within 5 min after the addition of Triton X-100, apparent activity ceased, and values remained relatively constant for at least 60 min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

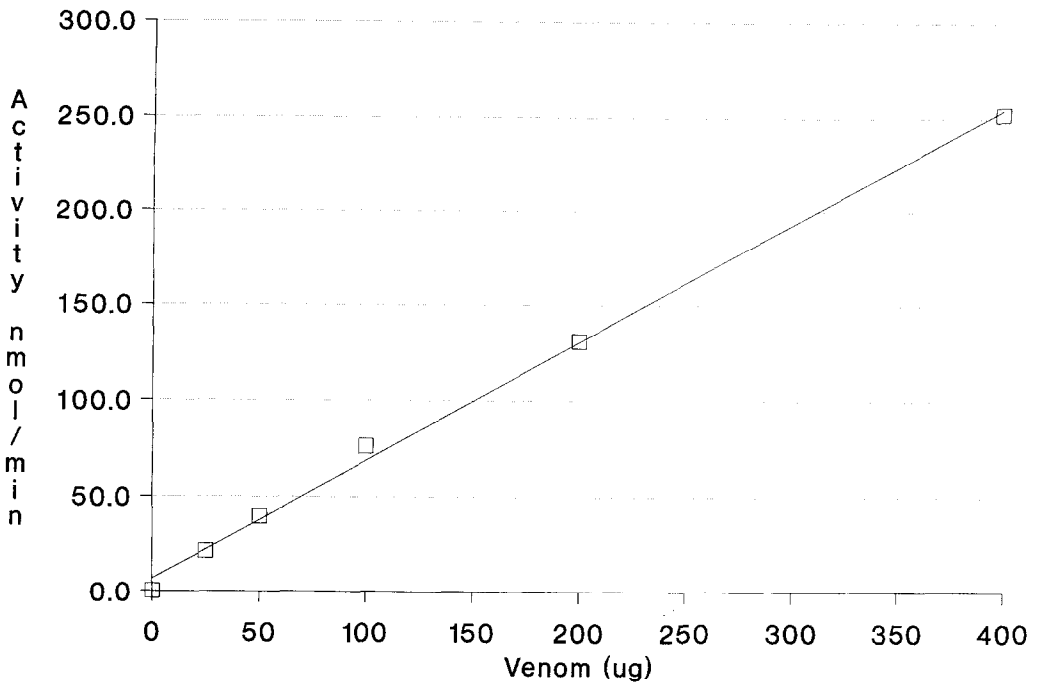

Fig. 2. Dependence of activity on concentration of venom using *C. atrox* venom and 4-nitro-3-(octanoyloxy)benzoic acid as substrate.
Phospholipase A_2 activity of crude venom showed a linear increase with concentration (25–400 μg venom).

(25 μg 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, PLA_2 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 PLA_2 activity. A partially purified PLA_2 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 PLA_2 activity.

For comparative purposes, several PLA_2 sources were assayed for activity toward phosphatidylcholine using a titrimetric method. Both crude venom and partially purified PLA_2 showed considerably higher specific activity toward the native phospholipid substrate (data not shown), but the nitrobenzoate assay was more easily conducted and had a greater level of accuracy of detection.

The substrate proved to be sensitive and specific for PLA_2 when used to assay fractions during the isolation of the enzyme from rattlesnake venom (Fig. 3). Numerous column fractions were rapidly and simultaneously assayed for PLA_2 activity. PLA_2 activity was detected in only one protein peak (peak 2); metalloproteases found in peaks 1 and 3 and amidolytic proteases in peak 3 (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 PLA_2 content of this preparation was > 85%. Further purification steps were not conducted.

DISCUSSION

PLA_2 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 PLA_2 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 PLA_2 from intracellular sources. However, there is a continuing need for an easily conducted assay of PLA_2 which is inexpensive, commercially available and requires a minimum of specialized equipment. The present assay, utilizing 4-nitro-3-(octanoyloxy)benzoic acid as 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 used, significant color quenching was not observed. Quenching of the chromophore was

Table 1. Phospholipase A_2 activities of venoms and pancreatic extract

	Activity*
Family Crotalidae	
Crotalus atrox	7.7
Crotalus durissus terrificus	22.3
Crotalus mitchelli pyrrhus	19.6
Crotalus molossus	19.3
Crotalus scutulatus	30.2
Family Viperidae	
Bitis gabonica gabonica	2.2
Vipera russelli	41.4
Family Elapidae	
Bungarus caeruleus	46.7
Naja melanoleuca	4.7
Notechis ater	35.7
Family Hydrophiidae	
Enhydrina schistosa	5.4
Laticauda semifasciata	8.6
Bee (*Apis mellifera*) venom PLA_2	0.3
Crotalus atrox pancreas extract	9.5
Partially purified *C. m. pyrrhus* PLA_2	122.8

*nmoles product/min/mg protein.

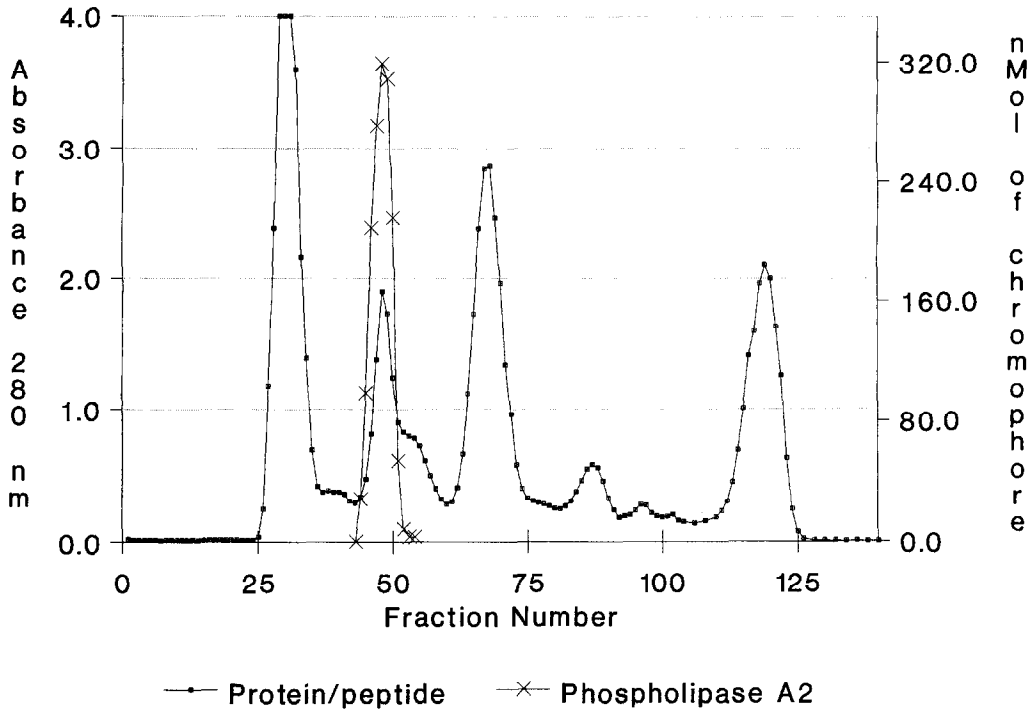

Fig. 3. Detection of phospholipase A_2 activity during purification.
Assay of fractions obtained from size-exclusion chromatography of *C. mitchelli pyrrhus* venom on BioGel P-100; activity was located in the second peak.

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 PLA_2. Enzyme activity was easily and specifically followed during gel filtration and ion-exchange chromatography. It should also be useful for the detection of PLA_2 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 PLA_2 activity during purification procedures. It should be stressed that other assay systems are available for the determination of kinetic parameters and substrate specificity, but for assaying multiple samples simultaneously this method has proved quite effective. The method is also useful for the comparison of enzyme levels present in venoms and other tissues.

Acknowledgements—Support for this work was provided by a grant from the University of Northern Colorado Faculty Research and Publications Board. We would like to thank Dr Jennifer A. Clarke for reviewing the manuscript and for helpful comments, Dr Richard M. Hyslop for helpful suggestions and Mr Alan E. Wang for his assistance during PLA_2 isolation.

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