

A HIGH EFFICIENCY METHOD FOR PURIFICATION AND ASSAY OF BEE VENOM PHOSPHOLIPASE A₂

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Summary

Phospholipase A₂ (PLA₂) is the main allergen of hymenopteran venoms. We describe a highly efficient reverse phase high performance liquid chromatographic method (HPLC) for isolating PLA₂ from crude bee venom. This method removes all detectable contaminants such as melittin from PLA₂ while preserving the hemolytic action of PLA₂. In addition we describe a simple functional assay of PLA₂ based on its propensity to cause hemolysis of guinea pig red blood cells. These techniques are particularly well suited to the isolation and assessment of PLA₂ of venoms which are available in limited quantities.

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INTRODUCTION

Phospholipases A₂ (PLA₂) are a family of small (MW 19000) water soluble enzymes produced by both vertebrates and invertebrates. There are multiple isoforms of PLA₂ in nature, differing both between tissues and between species. PLA₂ is a major component of hymenopteran and elapid venoms^{1,2} and its numerous functions include membrane metabolism, hemostasis and prostaglandin production. Its name derives from its catalysis of the hydrolysis of the 2-ester acyl group of sn-3-phosphoglycerides. Although PLA₂ enzymes from different sources share the same EC number (EC 3.1.1.4), they are antigenically quite distinct.³ Although cross reactivity between different forms of PLA₂ has been noted,⁴ immunoreactive methods developed for one particular PLA₂ form may not be applicable to other types of PLA₂.

Crude venoms are complex mixtures of enzymes, peptides and inorganic compounds. Phospholipase A₂ is the dominant antigen in venoms and most patients who are allergic to hymenopteran species have high titres of IgE antibodies against it.⁴ Several methods have been described to purify PLA₂ from crude venom, based largely on column chromatographic techniques. With the advent of high efficiency techniques such as high performance liquid chromatography (HPLC), there is the potential for improved separation of PLA₂ from potential contaminants such as melittin (which constitutes up to 50% of the dry weight of bee venom).

We are currently interested in the cellular regulation of IgG₄ responses to PLA₂ in beekeepers who are free of allergic reactions to venoms. As IgG₄ antibodies are directed against both PLA₂ and melittin, purified PLA₂ is required for antigen stimulation studies. Our studies have also demonstrated that a commercial preparation of PLA₂ was contaminated by substantial amounts of

melittin and as such was unsuitable for antigen-specific studies. To overcome this problem, we have developed a reverse phase HPLC method of purification of PLA₂ from crude bee venom. In addition to assessing the purity of our PLA₂ preparation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), we also describe a simple functional assay for purified bee venom PLA₂. This measures the hemolytic action of PLA₂ on guinea pig red cells. With the removal of contaminants which can potentially cause hemolysis, this assay is well suited to measure PLA₂ function.

MATERIALS AND METHODS

Acetonitrile (Chromar HPLC) was supplied by Mallinkrodt Specialty Chemicals Company, USA. Agarose (Ultrapure) of electrophoresis grade was obtained from Bethesda Research Laboratories, USA. Sodium chloride, sodium barbitone, barbituric acid and calcium chloride were all obtained from Aldrich Chemical Company, Wisconsin, USA. Phospholipase A₂ (*Apis mellifera*), crude bee venom (*Apis mellifera*), bovine serum albumin, trifluoroacetic acid (TFA) were obtained from Sigma Chemical Company, St Louis Mo, USA.

Reagents and gel apparatus for polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting including molecular weight markers were purchased from Bio-Rad, USA. The fraction collector and UV monitor were obtained from LKB Instruments, Uppsala, Sweden. NCCLS Type I water was used in the preparation of all reagents. The HPLC equipment was supplied by the Waters division of Millipore Ltd and included M6000 and M440 pumps. Also included were the U6K injector, M680 automated gradient controller and M440 UV monitor.

Reverse phase HPLC purification of crude bee venom

Initially 50 mg of crude bee venom was solubilised in 500 µL of Type I water and filtered with a 0.2 µm filter to remove large particulate matter. The solubilised venom was then purified using reverse phase HPLC. All 500 µL of solubilised bee venom was injected into a Brownlee Aquapore RP 300 CB column equilibrated with 0.1% TFA running at 1 mL/min. The column was eluted with a gradient of acetonitrile from 0 to 60% over 45 mins at 1 mL/min. 1 mL fractions were collected and absorbance measured at 280 nm. The purity of the collected fractions was assessed by SDS PAGE.⁵ PLA₂ Fractions containing detectable melittin on SDS PAGE were lyophilised and repurified with reverse phase HPLC. All fractions containing pure PLA₂ were pooled and lyophilized. The protein content of the pooled fractions was measured by spectrophotometry at 280 nm.

SDS-PAGE and western blotting

The purity of both PLA₂ preparations (the Sigma preparation and the HPLC purified PLA₂ described above) was assessed by SDS PAGE under reducing conditions as described.⁵ 5 µg protein was loaded per lane and the 15% gel run for 40 mins at 200 volts. The gel was stained with Coomassie blue dye and destained with methanol: acetic acid: water. The purity of samples were further assessed by silver staining of PAGEs.

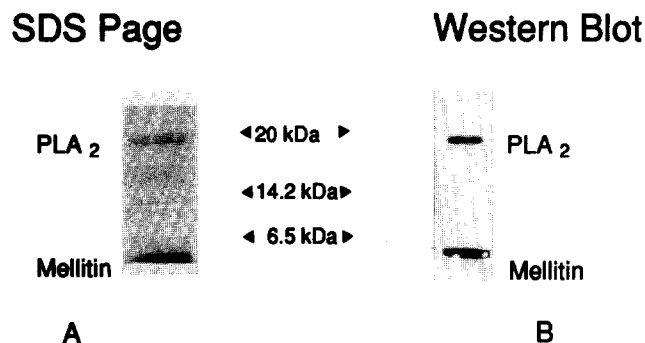


Fig. 1 SDS PAGE and western blotting under reducing conditions, which shows the presence of melittin in the Sigma PLA₂ preparation. The western blot confirms the immunogenicity of both PLA₂ and melittin. See text for details of methodology.

Western blotting was performed by electrophoretically transferring the SDS-PAGE separated proteins onto a nylon membrane as previously described.⁵ After a blocking step, the membrane was incubated with a beekeeper's serum. IgG4 anti-PLA₂ antibodies were detected with biotin-labelled anti-IgG4 antibodies (HP 6025, Sigma Chemical Company). The membrane was then incubated with streptavidin-peroxidase and developed with 4-chloronaphthol (0.6 mg/mL) and hydrogen peroxide (0.1% w/v). The reaction was stopped with distilled water.

Radial hemolysis assay for bee venom PLA₂

Guinea pig erythrocytes were obtained by bleeding guinea pigs into a tube containing heparin to prevent clotting. The cell suspension was stored at 4°C for up to 7 days before use. At the time of plate preparation, the cells were washed 3 times in 0.9% saline and centrifuged at 725 g for 2 mins in a tabletop centrifuge. The final guinea pig cell concentration was approximately 2×10^{10} cells/mL.

Purified lyophilized PLA₂ from the HPLC procedure was reconstituted in Type I water to a concentration of 25 mg/mL. A series of 7 standards with PLA₂ concentrations from 1.0 to 0.001 mg/mL were reconstituted in veronal buffered saline (73 mmol/L NaCl, 8.22 mmol/L sodium barbitone, 22 mmol/L barbituric acid, 0.75 mmol/L CaCl₂, 2.5 mmol/L MgCl₂, pH 7.3) and stored at 4°C.

330 μ L of guinea pig cell concentrate was added to 10 mL veronal buffered saline at 37°C. After mixing with 10 mL of 1% agarose in veronal buffered saline at 56°C, a plate of dimensions 17 cm $\times$ 10 cm $\times$ 1 mm was poured vertically. After 30 mins at room temperature, a series of wells (4 mm diameter) was made in the agarose plate and 6 μ L of standard or sample added to each well. Each standard and sample was assayed in duplicate. After incubation for 15 hrs at 37°C in a humidity chamber, the diameter of the circular zone of hemolysis around each application well was measured for each specimen pair and the mean diameter recorded for each specimen. Radial diameters were measured in increments of 0.5 mm units. Non specific lysis was assessed by measurement of hemolysis from samples of veronal buffered saline alone.

RESULTS

Electrophoretic studies

The results of SDS-PAGE and western blotting under reducing conditions of the PLA₂ obtained from Sigma Chemical Company is shown on Fig. 1. A significant amount of melittin was present in this preparation. The western blotting studies (Fig. 1) show that the patient has IgG4 antibodies which react against melittin. This confirms that melittin is an important antigen in bee venom which must be removed for antigen specific studies.

Reverse phase HPLC purification studies

The results of HPLC and gel electrophoresis of the Sigma crude bee venom is shown in Figs. 2 and 3. Two clear peaks are visible in the chromatogram. Identification of

1.11.91 HPLC Purification of Crude Bee venom

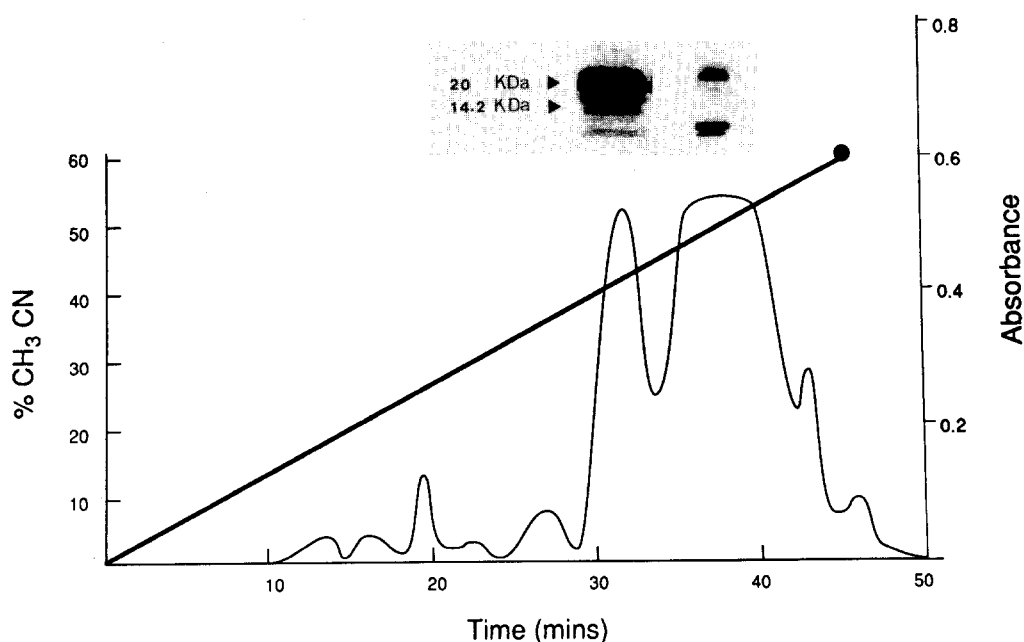


Fig. 2 Chromatogram of the Sigma crude bee venom preparation. Absorbance measured at 280 nm. HPLC peaks: PLA₂ 31 mins, melittin 38 mins. PAGE: Upper bands PLA₂, lower bands melittin. The fractions for repurification are indicated by a bold line on the chromatogram.

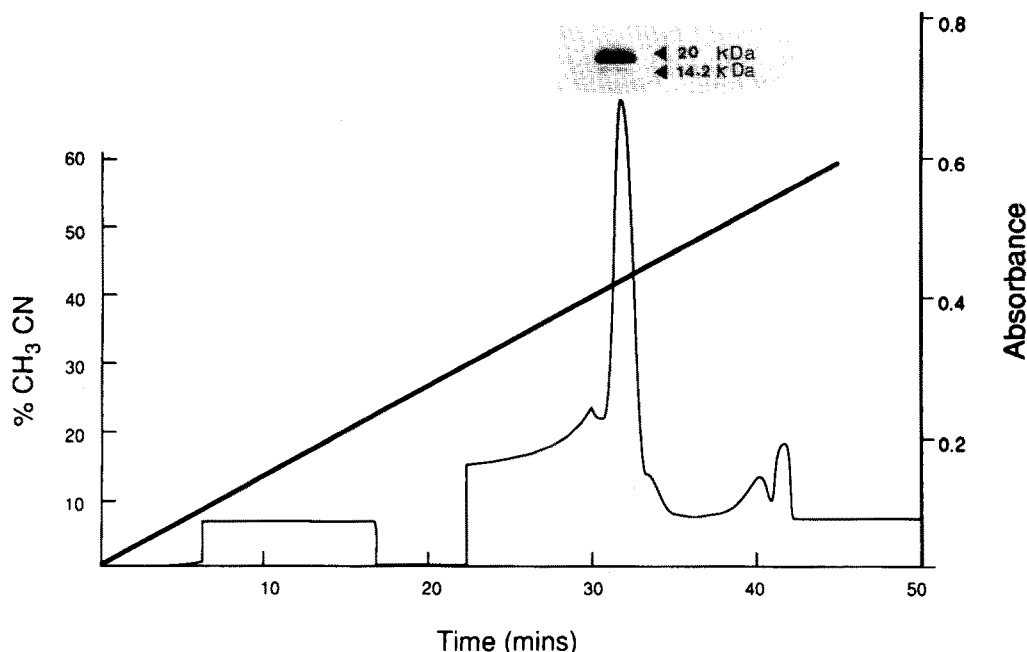
5.11.91 Purification of PLA₂

Fig. 3 Chromatogram of the second reverse phase HPLC purification. Absorbance measured at 280 nm. HPLC peak: PLA₂ 31 mins. PAGE: bands identified only as PLA₂.

the peaks as melittin and PLA₂ was achieved by correlation with PAGEs of eluate fractions run with MW markers (MW marker tracks not shown). This allowed identification of the first peak as PLA₂ (upper bands on electrophoresis) and the second peak as melittin (lower bands on electrophoresis). It is clear from the PAGE that there is a considerable amount of melittin contamination after the first HPLC purification step.

By comparison, the PAGE and elution profile of the HPLC double purification of the bee venom is shown in Fig. 3. Clear separation of the PLA₂ peak and melittin was achieved. The beginning of the peak is however seen in the figure. No contamination of PLA₂ peak eluate fractions with melittin is seen on PAGE. The amount of PLA₂ obtained from this procedure was 8mg protein, which represents a yield of 16% of the crude bee venom. Experiments performed to further assess the purity of the PLA₂ preparation showed no melittin bands on silver staining (not shown).

Radial hemolysis assay

An example of the radial hemolytic assay for PLA₂ is shown in Fig. 4. This Fahey plot shows a logarithmic relationship between the concentration of PLA₂ and diameter of hemolysis. It is necessary to read hemolytic diameters at a fixed time (15 hrs) as gel hemolysis continues unabated for 24-48 hrs, finally consuming all red cells in the gel without achievement of endpoint. By interpolation of the Fahey plot, the unknown concentration of the PLA₂ in the sample could be estimated. The interbatch coefficient of variation of duplicate analyses at a PLA₂ level of 0.05 mg/mL and 0.001 mg/mL were 0.26 and 0.12 respectively.

DISCUSSION

Published methods for venom PLA₂ purification are multi-process chromatographic methods which yield low quantities of PLA₂.⁶ The Sigma company, for example purifies PLA₂ by extensive dialysis followed by size exclusion chromatography and ion exchange chromatography. In contrast, our method is quick and results in high recovery of purified PLA₂ (16%). Purification factors are difficult to estimate accurately after the second HPLC procedure because even with silver staining there is no detectable melittin. Although our technique is more efficient than conventional methods, it has the disadvantage of requiring HPLC equipment and appropriate expertise. The technique we have described may be particularly suited for venoms which are available only in limited quantities such as arachnid venoms.

In the past, enzyme assay techniques have used the hemolytic property of PLA₂.⁷ A major disadvantage of these early assays was their lack of specificity. Erythrocyte lysis can be caused by multiple mechanisms. Apart from direct action of PLA₂ on erythrocyte membranes,⁸ basic peptides such as melittin and lysophospholipids generated from exogenous phospholipids by PLA₂ can each cause hemolysis. There appears to be a synergistic hemolytic action between PLA₂ and melittin. Hemolytic assays of venoms containing both PLA₂ and melittin may thus overestimate the amount of PLA₂ present. Given the absence of detectable contaminants on silver staining, the hemolysis seen is likely to reflect only the activity of PLA₂ in this assay.

Assay systems with greater specificity for PLA₂ activity, such as titrimetric or GLC procedures, have been developed but are either laborious or require specialised

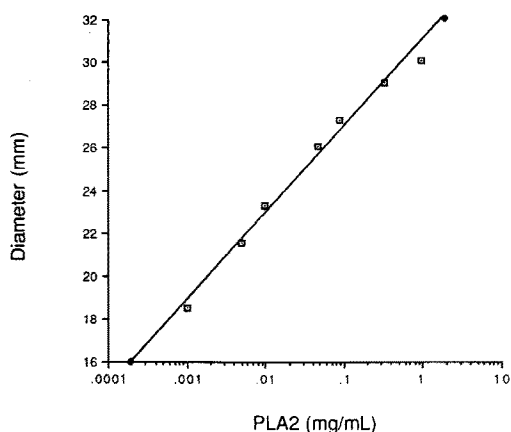


Fig. 4 Radial hemolytic assay for PLA₂ activity using HPLC-purified PLA₂.

equipment. In contrast, our radial hemolysis assay is simple and easy to perform, requiring a minimum of laboratory equipment. A possible drawback is the imprecision of the assay (CV up to 0.30) at higher PLA₂ concentrations. This probably reflects both the wide dynamic range of the assay (1000 fold) and the relatively large unit of hemolysis diameter (0.5 mm). Improved precision could be achieved by optimising guinea pig red cell concentration for a smaller dynamic range and by measuring hemolysis diameters in smaller increments. Despite poor precision at the higher end of the range, the present assay exhibits acceptable linearity. In contrast to previous hemolytic assays,⁹ this assay uses a small sample volume, which may be useful when measuring PLA₂ activity in precious samples such as arachnid venoms.

The reverse phase HPLC purification technique and radial hemolysis assay represent useful innovations in venom PLA₂ assay technology. These techniques should be applicable to other venoms and allow separation of

PLA₂ from potential contaminants. There is increasing interest in examining antigen specific responses *in vitro*. The techniques described in this paper will be useful in providing purified antigens which are essential for such studies.

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