Assay of snake venom phospholipase A2 using scattering mode of a spectrofluorimeter

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When aggregated micelles of phospholipids are hydrolysed by phospholipase A2 (PLA2) in an aqueous dispersion, scattering from the solution is decreased. Hydrolysis of dimyristoyl phosphatidylcholine by PLA2 from Russell’s viper venom has been investigated using a spectrofluorimeter at 650 nm. The rate of decrease in scattering was linearly dependent with venom concentration, while the initial lag at the onset was inversely related to it. Similar dependency was observed with substrate concentration. The reaction was inhibited with venom preincubated with antivenom or withdrawal of Ca2+ by EGTA. Gas-liquid chromatography of the product showed liberation of myristic acid. The amount of fatty acid released by 1 mg of venom was found to be 3470 and 3680 nmol/min, using scattering mode and pH-stat titrimetric assay respectively, that indicated a good correlation between them. The sensitivity of detection by the scattering mode was double that of the titrimetric assay.

Keywords: Dimyristoyl phosphatidylcholine, light scattering, phospholipase A2, Russell’s viper venom, spectrofluorimeter.

PHOSPHOLIPASE A2 (PLA2) is a class of enzymes that hydrolyses the fatty acid ester bonds at position 2 of 1,2 diacyl-sn phosphoglycerides to lysophospholipids and fatty acids. Since the solubility of phospholipids in aqueous phase is exceedingly low, the substrate forms micelles and the enzyme interacts at the water–lipid interface. PLA2 has much higher affinity towards aggregated substrates compared to disperse monomeric ones. X-ray crystallographic structure of this class of enzymes is known at high resolution. PLA2 requires Ca2+ as an essential catalytic cofactor to promote binding to the substrate and for the chemical step of lipolysis. Though PLA2 is required for such essential processes like phospholipid metabolism, signal transduction and other cellular functions, a large number of pathophysiological events are associated with PLA2, particularly from venom.

PLA2 activities are measured by a large number of procedures. In most cases, PLA2 is assayed by pH-stat, where the released fatty acid is quantified by standard alkali at constant pH. Alternately, changes in the chemical or spectroscopic properties of the substrate or the product are followed by titrimetric or radiometric assay coupled with HPLC or TLC, NMR, polarography, spectrophotometry, fluorimetry, ESR, etc. Sometimes the physical state of the substrate or the product is also monitored, e.g., turbidimetric assay. Unfortunately, there is no single procedure that could be applied universally to PLA2 assay with confidence.

Snake-biting being largely a tropical incidence, several laboratories involved in snake venom studies are situated in those regions. It is a hard reality that many of these laboratories are devoid of adequate facilities, including radiometer pH-stat. In comparison, spectrofluorimeters are common. This led us to explore the possibility of PLA2 assay using the scattering mode of a spectrofluorimeter, avoiding time-consuming procurement of expensive fluorescence labelled reagents. When the excitation and emission wavelengths of a spectrofluorimeter are the same and test samples are free from ‘inner filter effect’, the emission 90° (ref. 12) measures Raleigh’s scattering at 90°. There are stray reports of application of 90° scattering at near UV as supportive evidences for PLA2 kinetic studies. Here we report hydrolysis of dimyristoyl phosphatidylcholine (DM-PC) by Russell’s viper venom (RVV), which is a rich source of PLA2, assayed by this scattering mode.

RVV was provided by Dipak Mitra, a licensed trophy of Calcutta Snake Park, as desiccated yellowish shining crystals containing approximately 75–80% of protein (w/w). RVV (2 mg/ml) was dissolved in 20 mM K-phosphate, pH 7.4, left overnight at 4°C and centrifuged for 10 min at 6000 rpm in a micro centrifuge to discard cell debris. The whitish clear supernatant was used as the stock. Polyvalent aqueous suspension of antivenom was a product of M/s Haffkin Bio- pharmaceutical Corporation Ltd, Mumbai, India. Its neutralization capability was 0.6 mg of Indian cobra venom and RVV, and 0.45 mg of common krait venom and saw-scaled viper venom/ml. Porcine pancreatic PLA2, Echis carinatus venom, Crotalus atrox venom, phosphatidylcholine, and all its derivatives were from Sigma, USA. Naja kauthia was taken from Calcutta Snake Park. Absolute ethanol was from Bengal Chemicals and Pharmaceuticals Ltd, Kolkata. About 10 mg of DM-PC, stored desiccated at –20°C, was weighed gravimetrically under anhydrous condition and dissolved in 50 μl absolute ethanol. It was diluted to 1 ml by water to yield a uniform suspension used as the stock.

Semi quantitative PLA2 assay was done with egg yolk as substrate. A suspension of 20 ml was made with 9 ml chicken egg yolk, 2.51 ml 34.18 mM NaCl, 1.49 ml 1.34 mM EDTA, 4.44 ml 6.8 mM CaCl2, 2.0 ml 50 mM Tris-HCl, pH 7.5 and 0.56 ml 14.52 mM saline. Venom samples were added to 2 ml egg yolk suspension, mixed well and incubated at 37°C for 1 h. Incubates were placed on a boiling water bath and the time required to coagulate was noted. Pancreatic PLA2 and 14.52 mM saline served as positive and negative controls respectively.

Scattering measurements were done using a Hitachi F 4500 spectrofluorimeter attached with a constant temperature circulating water bath (Polyscience, USA) and a 3 ml quartz
cuvette. Unless mentioned, excitation and emission wave-
lengt hs and slit widths were set at 650 nm and 2.5 nm re-
pectively. General precautions for laboratory practice 
and washing of glassware for scattering measurements were 
followed\(^\text{15}\). For scattering measurement, the lowest recording 
during 5 min was considered. Since the lipid suspension in 
the cuvette survived gravitational settling, no magnetic flea 
was applied. While calculating kinetic parameters, 100% 
completion of the reaction was assumed from the drop of 
scattering that remained constant for at least 10 min.

RVV—PLA\(_2\) was also assayed by measuring decrease in 
turbidity of DM-PC micelle in 20 mM K-phosphate, pH 7.4 
containing 0.2 mM CaCl\(_2\) at 650 nm in a spectrophotome-
ter\(^\text{10}\), where none of the reaction components have absor-
ption. Rate of decrease of absorption per minute for 5 min 
was noted. The released fatty acids formed after hydroly-
sis of lipids were esterified and identified by gas–liquid 
chromatography (GLC) using a 10% DEGS glass 6 mt 
Chromatopak column at a nitrogen flow of 42 ml/min\(^\text{16}\). 
Myristic acid, palmitic acid, stearic acid, arachidonic acid and 
behenic acid served as reference fatty acids.

Release of fatty acids from phospholipids after PLA\(_2\) 
reaction was estimated by a pH-stat model 751 GPD Trtrino 
(Metrohm) at 25°C. Typically, in a 1 ml reaction mixture 
containing 0.29 µmol DM-PC in 5 mM Tris-HCl, pH 7.5 in 
the presence of 0.2 mM CaCl\(_2\), 5–30 µg RVV was added 
and the constancy of pH was maintained after titration with 
0.012 (N) NaOH.

The physical state of the lipid substrates was determined 
by transmission electron microscopy (TEM) using standard 
protocols. In short, a sample volume of 10 µl of 5 mg/ml 
was placed on a plastic-coated, 400-mesh grid for 1 min 
and the unattached substrate was removed using blotting 
paper. To stain the adhered particles, they were treated 
with 1% phosphotungstic acid for 20 s and excess reagent 
was removed using blotting paper. The grid was dried for 
15 min and the sample was served under TEM (JEOL 100 
CX, 20,000X) at an opening voltage of 60 kV.

Absorption spectra of RVV and the substrates were 
scanned between 200 and 800 nm with Analytical Jena 
Specord 200 (Germany) spectrophotometer. The absorption 
spectra of 10 mg/ml of RVV in 20 mM K-phosphate, pH 7.4, 
between 240–800 nm showed a peak at 280.2 nm charac-
teristics of proteins, but no absorption was detected above 
320 nm (<0.001). DM-PC (10 mg/ml) in spectral-grade etha-
ol showed absorption of <0.001 between 400 and 
800 nm. This confirmed the absence of ‘inner filter effect’ 
in these experiments\(^\text{17}\). Protein estimation was done with 
Bio-Rad Protein Assay Reagent (Catalog no. 10044), having 
BSA as reference.

A uniform distribution of the size of DM-PC in the 
aqueous phase was ensured after repeated dispersion of the 
stock in water with 100-fold dilution in 20 mM K-phosphate, 
ph 7.4, followed by measuring turbidity (spectrophotometri-
cally) or scattering (spectrofluorimetrically) at 650 nm. It 
revealed variation by ±5% (n = 5). Once dispersed in the 
buffer, scattering remained constant for at least 30 min 
justifying avoidance of magnetic flea in the cuvette. A linear 
dependence of scattering intensity within 400–9000 (arbitrary 
units) by 0–40 mM of DM-PC was observed (\(R^2 = 0.9841,\) 
where R is the regression coefficient; result not shown). Also, 
scattering intensity of 0–40 mM of DM-PC was found to be 
variably related with the fourth power of wavelength be-
tween 400 and 700 nm as per classical Raleigh’s equation\(^\text{17}\).

This excluded association–dissociation-like phenomenon of 
substrates. Further, when RVV (0.1 mg/ml) was incubated 
with buffer at 25°C, no change in scattering intensity was 
observed for 1 h, indicating absence of proteolysis to alter 
scattering intensity.

The reactivity of PLA\(_2\) is highly dependent on the physical 
state of its substrate\(^\text{15}\). Therefore, the state of DM-PC under 
conditions of its hydrolysis was determined by TEM. It 
showed that the particles formed aggregated micelles hav-
ing size in the range of 265–440 nm. Electron micrograph of 
two such micelles has been shown in Figure 1, which corre-
sponded to 400 and 440 nm.

PLA\(_{2,5}\) are sub-classified depending on molecular weight, 
Ca\(^{2+}\) ion dependency and mechanism of action\(^\text{2}\). Also the size 
and polarity of the head group and length of fatty acyl chain at 
\(S_n\)-1 or \(S_n\)-2 position are important factors in determining 
the overall catalytic turnover at the interface\(^\text{13,14}\). Since RVV is 
known to contain multiple species of PLA\(_2\), suitability of 
several phospholipids as substrate was tested on the basis 
of change in scattering intensity. The rate of hydrolysis 
expressed as change of scattering intensity/s using 0.29 µmol 
phospholipids and 5–50 µg RVV was as follows: DM-PC, 
0.80; phosphatidylycholine, 0.02; phosphatidyl serine, < 0.1; 
phosphatidyl ethanolamine, < 0.10 and phosphatidylcholine 
dipalmityl, < 0.10. Thus among the five phospholipids 
tested, DM-PC was the best choice for further studies.

When DM-PC was incubated with RVV, its scattering 
intensity at 650 nm was reduced by approximately 2.5-fold 
(Figure 2a). The reduced scattering remained stable for 10–15 min. The rate of hydrolysis was measured from the 
time zone where the change of scattering intensity with time

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**Figure 1.** Electron micrograph of DM-PC prepared under conditions 
as they were applied in scattering assay (x = 40,000).
was linear. The rates were found to be linearly dependent on RVV concentration between 5 and 80 µg/ml holding DM-PC concentration at 0.59 µmol ($R^2 = 0.9961$). Decrease in scattering was initiated after experiencing a lag. The duration of lag (50–200 s) was also found to follow an inverse linear relation with RVV concentration ($R^2 = 0.9930$; Figure 2a, inset). In all cases, decrease in scattering was associated with drop in turbidity from 0.351 ± 0.025 to 0.042 ± 0.004 as measured spectrophotometrically at 650 nm; thus indicating reduction in size of the particles.

Similar experiments were performed with DM-PC between 0.147 and 0.885 µmol, holding RVV concentration at 40 µg/ml (Figure 2b). In each set, a linear change in scattering intensity was observed at the middle of the reaction time zone. Further, the rate of change in scattering intensity was found to be linearly dependent on substrate concentration ($R^2 = 0.9949$), while the kinetic lag observed at the onset of the reaction was found to follow inverse linear relation on it ($R^2 = 0.9952$; Figure 2b, inset).

Crude RVV, dialysed overnight against buffer failed to abolish the kinetic lag. The lag appeared to be associated with the reaction mechanism and was not an artifact. Supplementing the assay buffer with 0.1 and 0.2 M salts inhibited the rate by 32 and 75% respectively. Similarly, supplementing by 5, 7.5 and 10% methanol inhibited the reaction rate by 46, 59 and 66% respectively. Thus alteration of solvent polarity negatively affected the reaction, similar to earlier reports.

Hydrolysis of DM-PC by RVV leading to destabilization of micelles was completely prevented when the venom was heat-treated at 100°C for 10 min or after treatment with antivenom (two-fold excess w/w, 30 min at 25°C). In the presence of EDTA or EGTA (10 mM), hydrolysis of DM-PC by RVV was similarly arrested (result not shown). This observation indicated the necessity of Ca$^{2+}$ ions for PLA$_2$ activity.

Semi-quantitative assay of PLA$_2$ using 5–50 µg/ml venom has been done with egg yolk as substrate, to correlate coagulation time with scattering assay. The observed coagulation time varied from 30 to 490 s and was found to be linearly dependent on venom concentration ($R^2 = 0.9906$). With higher concentration of venom at 100 µg/ml, coagulation time was more than 30 min. Coagulation time was similar to the control without venom, i.e. 30 ± 2 s after heating RVV at 100°C for 5 min or preincubation with two-fold molar excess of antivenom at 25°C for 10 min (Table 1).

The GLC profiles of DM-PC, RVV and RVV treated DM-PC are shown in Figure 3. While DM-PC yields only one major peak of retention time ($R_t = 2.54$ min, RVV yields a number of volatile products, including one of the said retention time. RVV-treated DM-PC yielded an additional peak of $R_t = 1.82$ min, which corresponded closely to the myristic acid derivative of $R_t = 1.78$ min. RVV-treated DM-PC, being a multi-components system, possibly suffered minor alteration of retention time of myristic acid derivative by 0.04 min compared to the reference.
Titrmetric assay for PLA₂ is one of the standard protocols, where the released fatty acids are estimated against standard alkali. PLA₂ assay by scattering mode as described was correlated with pH-stat (titrimetric) method. In pH-stat method, PLA₂ activity of RVV was found to be linearly dependent with the enzyme concentration between 5 and 30 µg (R² = 0.9748). The amount of fatty acid released by 1 mg venom was found to be 3470 and 3680 nmol/min using scattering mode and pH-stat titrimetric assay respectively. The comparable figures thus validate the scattering mode with respect to titrimetric assay.

So far results have been presented with crude RVV as a source of PLA₂. To check whether the same scattering mode of assay is sensitive to pure enzymes, the same experiment (Figure 2) was done using porcine pancreatic PLA₂ in the presence of 37.19 µM Na-taurocholate. Pancreatic PLA₂ activity was also found to be linearly dependent on enzyme concentration and inversely related to kinetic lag at the initiation of reaction, as observed in case of crude RVV–PLA₂ (Figure 4).

<p>| Table 1. PLA₂ activity of RVV under modified conditions as measured by scattering mode and egg yolk coagulation time |</p>
<table>
<thead>
<tr>
<th>Coagulation time (s)</th>
<th>Rate of hydrolysis*</th>
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<tbody>
<tr>
<td>Control</td>
<td>30</td>
</tr>
<tr>
<td>RVV</td>
<td>1800</td>
</tr>
<tr>
<td>EGTA/EDTA-incubated RVV</td>
<td>30</td>
</tr>
<tr>
<td>Heat-denatured RVV</td>
<td>30</td>
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<tr>
<td>AVS-treated RVV</td>
<td>30</td>
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*Rate of hydrolysis was expressed as change in scattering intensity/s. Control refers to a reaction without RVV. In each experiment, 20 µg/ml RVV and 200 µg/ml DM-PC were used.

Thus we report the assay of PLA₂ using Raleigh’s scattering from a spectrofluorimeter at 650 nm using crude RVV and DM-PC. Since snake venom is a composite mixture of PLA₂ with varying substrate specificity, and venom composition depends on the zoogeographical location¹⁹, twenty, the animal species was confined to eastern India origin. The rate of hydrolysis was found to be dependent on both enzyme and substrate concentration (Figure 2a and b). The observed kinetic lag was a common feature of PLA₂ hydrolysis²¹. Further, actual release of myristic acid from DM-PC after hydrolysis was confirmed by GLC analysis (Figure 3).

An advantage of spectrofluorimetric measurements is increasing sensitivity to several orders by altering slit width. With wider slit width or measuring emission below 650 nm, the concentration of DM-PC could be sufficiently reduced within the limit of detection. However, the reduced reaction rate was unfit for reliable measurements. Therefore, it is the rate constant and not the scattering intensity that limits the assay. This mode has been validated by pH-stat titrimetric assay. The amount of fatty acid released by 1 mg of venom as measured by the two methods was 3680 and 3470 nmol/min. Further, in the pH-stat, 24–144 µg of crude venom could be assayed yielding 80–50 nmol fatty acid/min. In case of scattering mode, these figures stand at 12–60 µg and 40–220 nmol/min respectively, indicating the scattering mode to be over two-fold more sensitive. Applicability of this assay with venom from Russell’s viper (western India, Haffkin Institute, Mumbai), N. kauthia (eastern India, Calcutta Snake Park), Crotalus atrox (Western diamond rattlesnake, Sigma), Echis carinatus (Saw-scaled viper, Sigma) and porcine pancreatic PLA₂ (Sigma, in the presence of 0.02% sodium taurocholate) was tested. The dependencies were similar to that seen in Figure 1.

The turbidometric assay of RVV–PLA₂ with DM-PC at 650 nm was found to be inconsistent, though the reaction was completely arrested in the absence of Ca²⁺ ions, after
heat or AVS treatment. This asserts the notion that there is hardly any PLA₂ assay which could be applied universally. The presented procedure has been applied successfully to some plant aqueous extracts popularly known as ‘antivenom’ and was found to be completely inhibiting hydrolysis. Thus it may serve as an empirical guide for screening similar plants. This is in relation to our long-term interest to address better snake-bite management in the Indian sub-continent.


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Germination improvement in Swertia angustifolia: a high value medicinal plant of Himalaya

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The present communication deals with improvement in seed germination of Swertia angustifolia via various hormonal treatments (GA₃, IAA and KNO₃). Germination of the species under controlled conditions is found to be low (<32.0%). GA₃ is found to be the best with respect to germination (96.0%) and reducing mean germination time (7.6 days) followed by KNO₃ (81.3%; 8.4 days) and IAA (66.0%; 16.6 days). A high degree of variation with regard to the germination percentage and mean germination time in different populations and treatments is recorded. The possible reasons for such variations are discussed.

Keywords: Conservation, endangered, gibberellic acid, Himalaya, Swertia angustifolia.

Swertia angustifolia Ham. ex D. Don (family Gentiana-ceae), an endangered medicinal plant of west Himalaya, is listed among medicinal plants prioritized for conserva-

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