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Purification of a phospholipase A_2 from *Lonomia obliqua* caterpillar bristle extract

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Abstract

Lonomia obliqua caterpillar bristle extract induces both direct and indirect hemolytic activity on human and rat washed erythrocytes, and provokes intravascular hemolysis in Wistar rats. Indirect hemolytic activity is assumed to be caused by a phospholipase A_2 (PLA_2) present in this extract, and this investigation was initiated in order to characterize this enzyme. Phospholipase A_2 activity of crude extract was inhibited by both a PLA_2-specific inhibitor (pBpb) and the metal ion chelator EDTA. *L. obliqua* PLA_2 was purified by liquid chromatography from the crude bristle extract and had a molecular mass of 15 kDa and a p*I* of 5.9; its N-terminal sequence showed high homology to a sequence of a putative PLA_2 obtained from a cDNA library of *L. obliqua* bristles, and it is tentatively placed among Group III phospholipases A_2. This enzyme was stable at 4 °C, sensitive to higher temperatures, and its maximum catalytic activity was at pH 8.0. *L. obliqua* PLA_2 induced hemolysis only when incubated with exogenous lecithin. Thus, the PLA_2 purified herein appears to be responsible for the indirect hemolytic activity of the crude bristle extract.

Keywords: *Lonomia obliqua*; Phospholipase A_2; Indirect hemolysis; Erythrocytes; Intravascular hemolysis; Caterpillar; Lecithin; Chromatography

Envenomation of humans resulting from contact with *Lonomia obliqua* caterpillars has been investigated since the outbreak of several cases in southern Brazil in 1989. Hemorrhagic disturbances are frequently observed and are characterized by a consumptive coagulopathy followed by secondary fibrinolysis [1]. Moreover, hematuria, anuria, oliguria, and renal failure [2–6] have occurred in several cases, and cases of intravascular hemolysis have also been reported during human envenomation [7,8]. Rats envenomed experimentally by *L. obliqua* bristle extract develop hemostatic disturbances and intense intravascular hemolysis [9] similar to those observed in human envenomations [10,11]. In vitro tests showed that direct and indirect hemolytic activity occurred when washed human and rat erythrocytes were incubated with crude *L. obliqua* bristle extract [12]. Intravascular hemolysis and indirect hemolytic activity have been ascribed to the presence of a phospholipase A_2 (PLA_2) in such extract [9,12].

Phospholipase A_2 (EC 3.1.1.4) belongs to a group of enzymes that hydrolyze natural phospholipids, catalyzing the deacylation of 1,2-diacyl-*sn*-3-phosphoglycerides at position 2 and thereby releasing free fatty acids and lysophospholipids, which are potent membrane-active agents. Phospholipase A_2's have been described in vertebrates (mammals, lizards, and many snake venoms) and insects (honeybees and wasps) [13–15]. To distinguish PLA_2's,

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their structures have been organized into groups according to their sources, cellular location (secreted, cytosolic, and intracellular), molecular mass, amino acid sequence, and calcium dependence [13,14,16]. Secreted venom PLA_2s show a wide variety of toxic and pharmacological effects, such as neurotoxicity, cardiotoxicity, impairment of platelet aggregation, myotoxicity, and necrotizing, anticoagulant, hemorrhagic, hypotensive, edema-forming, and hemolytic activity [17–20].

Erythrocyte disturbances in human cases of contact with *L. obliqua* caterpillars may be due to PLA_2 activity, but this relationship has not been demonstrated unequivocally. The current study was undertaken to evaluate the role of *L. obliqua* caterpillar bristle PLA_2 in hemolysis, and we demonstrate for the first time that a specific PLA_2 is present in the bristle extract.

Materials and methods

Crude extract. Lonomia obliqua caterpillars were anesthetized with CO_2 and bristles were removed and maintained on ice. Phosphate-buffered saline (PBS), pH 7.4, at 4 °C was added to achieve a 10% final extract solution. Bristles were homogenized by shaking and then centrifuged to obtain a suspension [21] which was stored at −70 °C. Protein concentration of bristle extracts was determined colorimetrically [22], using bovine serum albumin as a standard.

PLA_2 activity. PLA_2 activity was determined colorimetrically [23], as modified by Santoro et al. [24], using SWIFT II software for Ultraespec 2100pro spectrophotometer (Amersham Biosciences, Sweden). Fifty microliters of the bristle extract or chromatography fractions was added to 1.5 ml of substrate reagent (100 mM NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.265% soybean lecithin, and 99 μM phenol red, pH 7.6) in a cuvette at room temperature, and the decrease in absorbance (ΔA_{558nm}) was recorded for 5 min to calculate the maximum velocity (U/min) of reaction. One unit of PLA_2 activity was defined as the amount of enzyme necessary to give $\Delta A_{558nm} = 1$/min at 558 nm. PLA_2 activity was initially tested at different concentrations (125–1000 μg/ml) of crude bristle protein extract. To test calcium dependence, 250 μg/ml bristle extract was added to different concentrations of $CaCl_2$ (0–20 mM) in substrate reagent. To test PLA_2 inhibition, 250 μg/ml bristle extract was incubated with 0.1 mM *p*-bromophenacylbromide (pBpb) (Sigma, USA) or Na_2–EDTA (0.2 or 2 mM) (Merck, Darmstadt, Germany) in 25 mM Tris–HCl, pH 7.6, at room temperature for 4 h [17]. Samples were then dialyzed in 10 mM Tris–HCl, pH 7.6, and PLA_2 activity was assayed.

PLA_2 purification from crude bristle extract. Crude extract (5.5 ml, 55.6 mg) was applied to a Sephadex G50 column (110 × 2.6 cm) (Amersham Biosciences, Sweden), previously equilibrated with chromatography buffer (10 mM Tris–HCl, 10 mM NaCl, pH 7.0). The flow rate was set at 12 ml/h and 3 ml fractions were collected, monitored for protein at 280 nm, and assayed as above for PLA_2 activity. Fractions that contained PLA_2 activity were pooled and applied to a Mono-Q HR 16/10 column (Amersham Biosciences, Sweden), equilibrated with 20 mM Tris–HCl, 50 mM NaCl, pH 7.0, at a flow rate of 2 ml/min. The column was washed with five volumes of equilibrium buffer and proteins were eluted with a linear gradient of NaCl (0–0.6 M) in equilibration buffer. Fractions containing PLA_2 activity were pooled and applied to a Superdex 75 column (60 × 2.0 cm), equilibrated with chromatography buffer at a flow rate of 1 ml/min. Fractions of 2 ml were collected and tested for PLA_2 activity.

Homogeneity. Protein concentration was determined as described by Lowry et al. [25], as modified by Markwell et al. [22], using bovine serum albumin as standard. Fractions with PLA_2 activity were reduced with 2-mercaptoethanol and electrophoresed in SDS–PAGE [26]. Gels were silver stained [27].

Effect of storage temperature and pH. The effect of storage temperature on activity was tested by incubating 3 μg of *L. obliqua* PLA_2 at 4, 20, 30, 37, 40 or 50 °C for 30 min and then assaying PLA_2 activity. The effect of different pH on PLA_2 activity was evaluated by incubating 3 μg of lyophilized samples in 10 mM Tris–HCl, 15 mM NaCl buffers, at pH levels ranging from 4.0 to 9.0, at 4 °C for 30 min.

PLA_2 activity on human erythrocytes. Indirect and direct hemolytic activity of *L. obliqua* PLA_2 was determinated on human erythrocytes as described previously [12]. Briefly, indirect hemolytic activity was tested by incubating human erythrocytes with bristle extract (100 μg/ml) or chromatographic fractions (100 μg/ml) and 30 μg/ml egg lecithin (Sigma, USA) in 1.25 mM $CaCl_2$ for 1 h at 37 °C. Absorbances at 412 nm of test tubes were used to calculate the intensity of hemolysis. To test direct hemolytic activity, samples were incubated under the same conditions, but with no lecithin.

Molecular mass and isoelectric point. To confirm the molecular mass and determine the p*I* of the purified *L. obliqua* PLA_2, a sample from ion exchange chromatography was further purified on a Sephasil Peptide C18 column (Äkta System, Amersham Biosciences, Sweden). Proteins were eluted with a linear gradient of acetonitrile (0–60%) in 0.1% (v/v) trifluoroacetic acid. Fractions that contained PLA_2 activity were rechromatographed using a linear gradient between 36% and 37.2% acetonitrile. Molecular mass of *L. obliqua* PLA_2 was obtained by tricine–SDS–PAGE (16.5% resolving gel) [28]. Isoelectric focusing was performed at room temperature using precast polyacrylamide microgels (Phast Gel IEF 3-10) in a PhastSystem Apparatus (Amersham Biosciences, Sweden). A sample of PLA_2 (0.5 μg protein) was applied onto the gel using a 4-μl sample applicator. Calibration standards were between pH 3.5 and 9.3, and gels were silver stained [27].

N-terminal sequencing. In order to obtain the aminoterminal sequence of *L. obliqua* PLA_2, a sample from Mono-Q chromatography was electrophoresed as above, blotted onto PVDF membrane, and sequenced on an Applied Biosystems Procise protein sequencer.

Results

A dose–response curve was observed for PLA_2 activity in crude *L. obliqua* bristle extract, using soybean lecithin as substrate (Fig. 1A). This activity was augmented prominently by increasing $CaCl_2$ concentration in medium (Fig. 1B). PLA_2 activity in crude *L. obliqua* caterpillar bristle extract was inhibited 72% by 0.1 mM pBpb, 40% by 0.2 mM Na_2–EDTA, and 67% by 2.0 mM Na_2–EDTA (Fig. 2).

PLA_2 was purified by three chromatographic steps (Fig. 3). PLA_2 activity was detected in the first peak obtained from a Sephadex G50 column (Fig. 3A). Fractions containing PLA_2 activity were pooled and applied to a Mono-Q column (Fig. 3B), and then eluted with 0.4 M NaCl. Fractions with PLA_2 activity were applied in a Superdex 75 column (Fig. 3C); such preparation of PLA_2 was 75% pure as determined by densitometric analysis of SDS–PAGE electrophoresis (Fig. 3D).

A summary of the purification steps of *L. obliqua* PLA_2 is shown in Table 1. A purification of 15-fold was achieved with recovery of 3% of total PLA_2 activity. Thus, from 55.6 mg of crude extract protein, only 2 mg of PLA_2 was obtained. An increase in 40% total activity occurred after gel filtration in Sephadex G50, probably due to the dissociation of an inhibitor of this enzyme present in crude extract. When compared with the crude extract, a decrease in 50% of total PLA_2 activity was noted after chromatography on

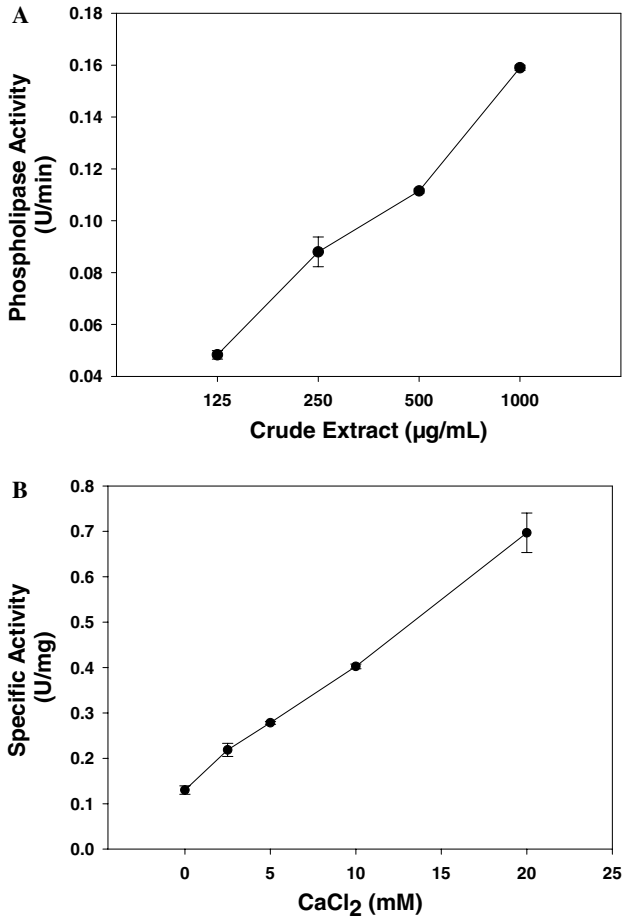

Fig. 1. PLA_2 activity of crude bristle extract of *L. obliqua*. Dependence of activity on (A) extract protein concentration and (B) Ca^{2+} concentration in assay medium. Data are expressed as means ± standard error of mean ($N = 3$). (B) A concentration of 250 µg extract protein/ml was used for all assays.

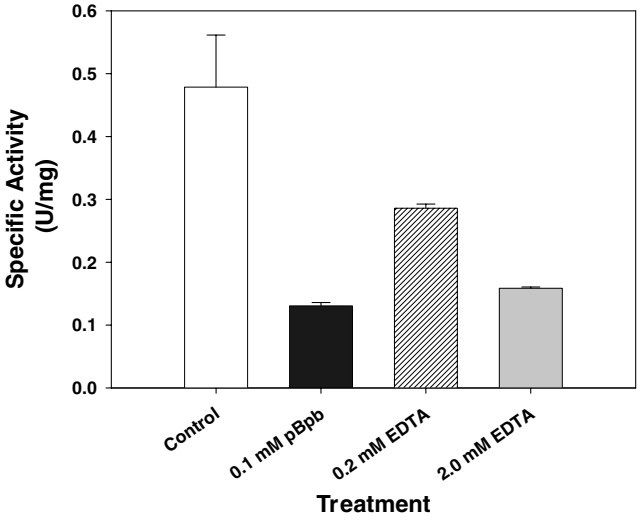

Fig. 2. PLA_2 activity present in crude bristle extract of *L. obliqua* was inhibited by 0.1 mM *p*-bromophenacylbromide (pBpb), 0.2 mM Na_2–EDTA, and 2.0 mM Na_2–EDTA after incubation at room temperature for 4 h. Data are expressed as means ± standard error of mean ($N = 3$).

Superdex 75, but a 15-fold increase in specific activity was observed.

Lonomia obliqua PLA_2 is a thermolabile enzyme, and its activity decreases as temperature is raised. The optimal temperature and pH to maintain maximum activity of this enzyme were 4 °C and 8.0, respectively (Figs. 4A and B).

Indirect hemolytic activity was induced by crude bristle extract preparations and PLA_2 chromatographic fractions. Total hemolysis occurred exclusively in PLA_2 fractions obtained from gel filtration in Superdex 75 (Table 2).

To confirm the nature of the purified protein, fractions with PLA_2 activity obtained from ion Mono-Q HR 16/10 were pooled and subjected to reverse-phase chromatography on a C18 column (Fig. 5). Thus, 0.15% of total PLA_2 activity was recovered, with a purity grade of greater than 95% in tricine–SDS–PAGE (Fig. 5C). The molecular mass and isoelectric point of *L. obliqua* PLA_2 were 15 kDa and 5.9, respectively. The N-terminal sequence obtained from this protein was: FMMFPGTKWCGPD TTAXKXE, which is aligned with part of a putative PLA_2 predicted from a cDNA library obtained from *L. obliqua* bristles and tegument (GenBank Accession No. AY829845) [29] (Fig. 6).

Discussion

Secreted PLA_2s are important for digestion and immobilization of prey, and these enzymes are responsible for some of the physiological disturbances observed in humans following bee, wasp, spider, and snake envenomations [14,16,30–32]. PLA_2 activity has been reported in the crude *Euproctis* caterpillar bristle extract [33] and, more recently, in the crude caterpillar bristle extract of *L. obliqua* [12]. In the present study, a secreted PLA_2 was purified to homogeneity from the crude bristle extract of *L. obliqua*.

PLA_2 activity present in crude *L. obliqua* caterpillar bristle extract was dependent on a phospholipid substrate (lecithin) and Ca^{2+}. Similar to other PLA_2 [14], calcium ions play an essential role in the catalytic mechanism of *L. obliqua* PLA_2, and Na_2–EDTA inhibited its PLA_2 activity, presumably by chelating Ca^{2+}. pBpb also significantly decreased *L. obliqua* PLA_2 activity, further indicating a specific PLA_2. The effects of pBpb on *L. obliqua* PLA_2 strongly suggest that histidine is conserved in the active site of this enzyme [13]. Inhibition of enzymatic activity by pBpb is frequently observed in PLA_2s that possess a well-conserved active site, composed of a histidine and typically followed by an aspartic acid [31,34].

Lonomia obliqua PLA_2 is an extracellular enzyme, has a low molecular mass (15 kDa), and requires Ca^{2+} for its catalytic activity. Such characteristics may be used to classify it among Groups I, II or III of PLA_2 [14]. With limited sequence data, it is difficult to assign it to a specific group unequivocally. Nevertheless, since the N-terminal sequence of *L. obliqua* PLA_2 showed high homology to a predicted PLA_2 from *L. obliqua* bristle extract [29] and with other Group III PLA_2s [*Apis mellifera* (AAL30844), *Heloderma*

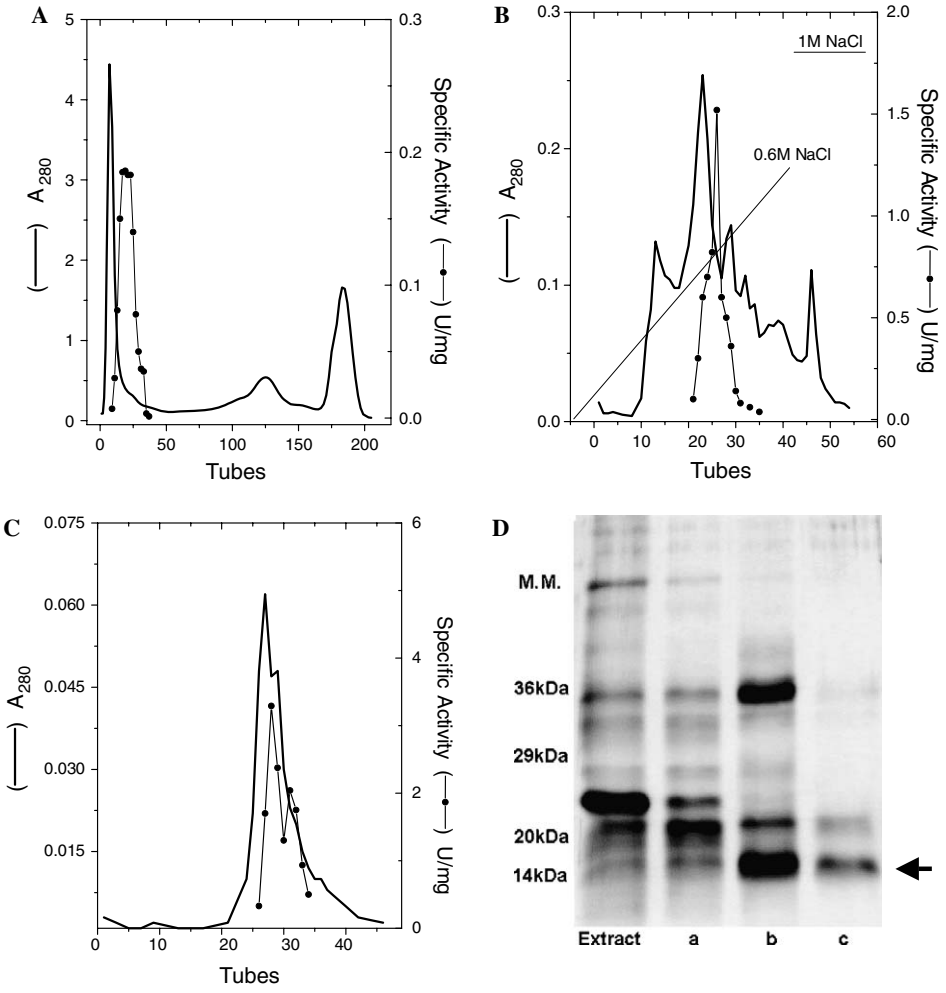

Fig. 3. Isolation of PLA_2 from *L. obliqua* caterpillar bristle extract. (A) Fractionation on a Sephadex G50 column (110 cm × 2.6 cm). (B) Sephadex fractions containing PLA_2 activity were applied to a Mono-Q HR 16/10 column. (C) Active fractions were applied to a Superdex 75 column (60 cm × 2.0 cm). (D) Protein profiles of reduced samples of crude bristle extract (Extract) and pooled PLA_2-containing fractions [Sephadex G50 (a), Mono-Q (b), and Superdex 75 (c)] following SDS–PAGE (12% resolving gel). MM, molecular mass standards. The arrow indicates the position of PLA_2.

Table 1
Purification of PLA_2 from *L. obliqua* caterpillar bristle extract

	Protein		PLA_2 activity			Yield (fold)
	Volume (ml)	Total protein (mg)	Activity (U/min)	Total (U)	Specific (U/mg)	
Extract	5.5	55.6	1.20	6.6	0.12	1
Sephadex G50	80	17.6	0.13	10.5	0.60	5
Ion exchange	28	4.2	0.17	4.8	1.14	10
Superdex 75	15	1.9	0.23	3.4	1.78	15

suspectum (P16354), and *Rhopilema nomadica* (P43318)], the enzyme purified herein is classified provisionally as a Group III PLA_2. In addition, an N-terminal motif which is highly conserved in Group III PLA_2s, PGTLWCG (residues 5–11), is also present in this *L. obliqua* PLA_2; several of these residues (W9, G11) are also involved in coordination of the Ca^{2+} cation in bee venom PLA_2 [35]. However, *L. obliqua* PLA_2 significantly differed in mass from PLA_2s found in other venomous insects, such as those of *Euproctis* caterpillars (100 kDa) and *Polybia paulista* wasps (115–132 kDa) [33,36].

Unlike *Apis mellifera* Group III PLA_2, which is a basic enzyme (p*I* 10.0), *L. obliqua* PLA_2 is acidic, with an isoelectric point of 5.9. Similar to *L. obliqua* PLA_2, the Group III PLA_2 isolated form *Heloderma horridum horridum* is also an acidic protein (p*I* 4.5) [37].

The purified PLA_2 showed a pH optimum of 8.0 which was stable when maintained at low temperature (4 °C). Most enzymatically active secretory PLA_2s show optimal activity at basic pH [19,38,39]. However, when stored at higher temperatures, enzymatic activity was gradually lost, indicating enzyme instability. This is in strong contrast to most secreted PLA_2s, which typically are very stable upon heating, and it suggests that the three-dimensional structure of *L. obliqua* PLA_2 is much less constrained than those of Group I and II PLA_2s, which are stabilized by seven disulfide bridges.

Lonomia obliqua PLA_2 induced exclusively indirect hemolytic activity, similar to activities observed for PLA_2s present in bristle extracts of *Euproctis chrysorrhoea* and

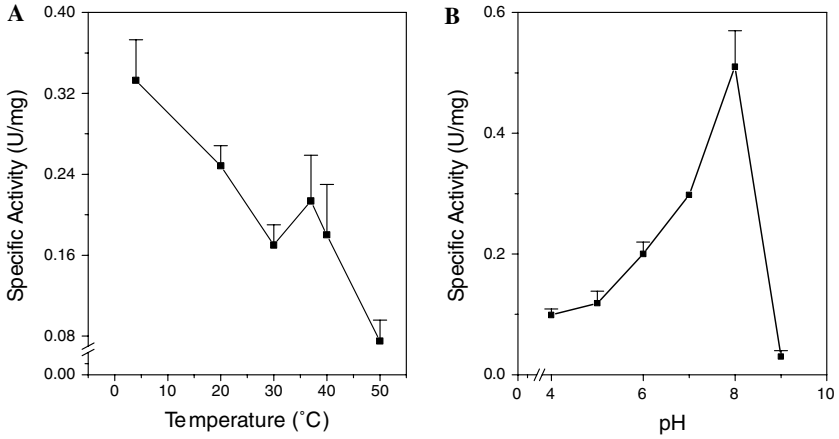

Fig. 4. Specific activity of *L. obliqua* PLA_2 following different conditions of incubation temperature (A) and pH (B). (A) Enzyme (3 μg) was incubated at specified temperatures for 30 min and then assayed for activity. (B) Enzyme (3 μg) was incubated in buffer of the appropriate pH for 30 min at 4 °C and then assayed. Data are expressed as means± standard error of mean ($N = 3$).

Table 2
Hemolytic activity of *L. obliqua* caterpillar bristle extract and PLA_2 purified fractions

Samples (100 μg/ml)	Hemolysis (%)
Crude extract	52.8 ± 11.94
Sephadex G50	87.7 ± 0.82
Ion-exchange	95.3 ± 3.72
Superdex 75	100.0 ± 0.00

Data are expressed as means ± standard error of mean ($N = 3$).

Euproctis subflava caterpillars [33]. However, the PLA_2 of *Agelaia pallipes pallipes* wasp venom (agelotoxin) also induced direct hemolytic activity [38].

PLA_2 activity was also detected in caterpillar hemolymph (data not shown), suggesting that this enzyme may also have an important function in homeostasis of *L. obliqua*. However, in the bristles, this enzyme can induce both local reactions—such as pain and inflammation observed in humans and animals [10,40,41]—and systemic

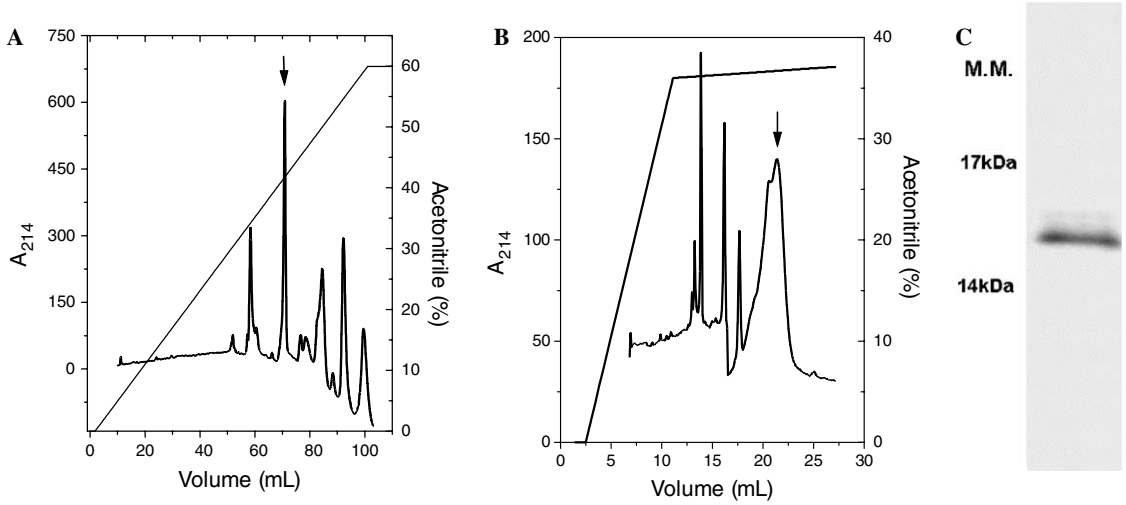

Fig. 5. Reversed phase (C_{18} column) HPLC purification of PLA_2 obtained from Mono-Q HR 16/10 chromatography. (A) Elution was carried out using a linear gradient of acetonitrile (0–60%) in 0.1% (v/v) trifluoroacetic acid. (B) The fraction containing PLA_2 activity was rechromatographed using a linear gradient of 36–37.2% acetonitrile. (C) Tricine–SDS–PAGE of the material obtained from reversed-phase chromatography (B). MM, molecular mass standards.

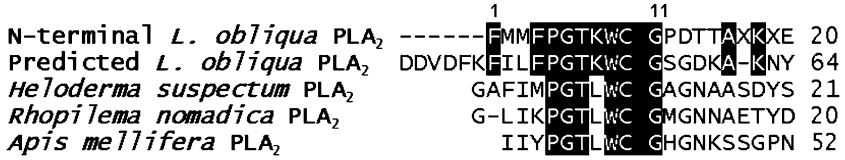

Fig. 6. Alignment of the N-terminal sequence of the PLA_2 purified from *L. obliqua* bristle extract with a predicted PLA_2 from a cDNA library from *L. obliqua* bristles [29] and several other Group III PLA_2s (*Heloderma suspectum* [42], *Rhopilema nomadica* [43], and *Apis mellifera* [44]).

manifestations, such as intravascular hemolysis [7–9]. Redirection of regulatory/homeostatic proteins, from a regulatory role to a role in homeostasis disruption, is a motif commonly seen among snake venom components, and it appears to be present in insect venoms as well. To evaluate the role of this enzyme in the etiopathology of hemolysis that occurs in *L. obliqua* envenomations, we are currently investigating its activity on lipids and glycoproteins of erythrocyte cell membrane.

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