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Purification and characterization of a phospholipase A_2 isoenzyme isolated from *Lachesis muta* snake venom

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Abstract

A new phospholipase A_2 (PLA_2) isoenzyme was isolated from *Lachesis muta* crude venom, and was named LM-PLA_2-II. This enzyme was purified by gel filtration on a Sephacryl S-200 HR column followed by reverse-phase chromatography on a C_2/C_{18} column. LM-PLA_2-II consists of a single polypeptide chain with an apparent molecular mass of 18 kDa and an isoelectric point at pH 5.4. The amino terminal sequence of the enzyme revealed a high degree of homology with other PLA_2s from several sources. LM-PLA_2-II has a high indirect hemolytic activity and a potent inhibitory effect on platelet aggregation induced by ADP and collagen. It also produces a significant paw edema reaction in rats. The edematous response in rats was abolished by pretreatment with either indomethacin or dexamethasone, suggesting the involvement of cyclo-oxygenase. Pretreatment of LM-PLA_2-II with *p*-bromophenacyl bromide abolished all of these actions, clearly indicating that the biological activities, including the edematogenic effect, are dependent entirely on its enzymatic activity.

Keywords: *Lachesis muta* venom; Purification of phospholipase A_2; Platelet aggregation; Edema; Myotoxicity; Lysophosphatidylcholine

1. Introduction

PLA_2 enzymes (EC 3.1.1.4), small proteins that are widespread in nature, are found in many biological sources, including snake venoms. These enzymes can induce several pharmacological actions, such as neurotoxicity, myotoxicity, and the edematogenic response. They also affect platelet reactions (aggregation and secretion) and blood coagulation [1,2], and are able to hydrolyze phospholipids, producing a variety of free fatty acids and lysophospholipids. Some free fatty acids thus produced may act as second messengers, or they can be involved in other reactions as precursors of biologically active eicosanoids [3]. Meanwhile the lysophospholipids formed, mainly lysophosphatidylcholine (lyso-pc), can generate intracellular messengers able to affect cellular physiology [4,5].

The ability of a specific PLA_2 to induce edema and other biological activities does not always occur in parallel with its enzymatic activity [6–8]. In general, PLA_2s displaying high enzymatic activity can also produce a pronounced edematous effect, whereas those PLA_2s with poor enzymatic action have little or no inflammatory effect. In contrast, some basic PLA_2s with low enzymatic activity are still able to induce an effective edema reaction [9]. Some PLA_2s are known to selectively affect platelets, either inducing or inhibiting platelet aggregation, whereas other enzymes present a biphasic effect on platelets [10]. Several PLA_2s from snake venoms that affect platelet aggregation have been purified and characterized. However, for these enzymes, there is no clear correlation between the enzymatic activity and the inhibitory effect on platelet reaction [10]. We purified a PLA_2 from *Lachesis muta* snake venom and described its physicochemical and biological properties [11,12]. This enzyme, named LM-PLA_2-I, demonstrated a high enzymatic activity, which correlated well with other important pharmacological properties [12]. We now report on the purification and

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Abbreviations: FPLC, fast protein liquid chromatography; LM-PLA_2-I, *Lachesis muta* phospholipase A_2-I; LM-PLA_2-II, *Lachesis muta* phospholipase A_2-II; *p*-BPB, *p*-bromophenacyl bromide; pI, isoelectric point; PLA_2, phospholipase A_2; PPP, platelet-poor plasma; PRP, platelet-rich plasma; WRP, washed rabbit platelets.

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characterization of another enzyme, a PLA_2 isoform, isolated from the same snake venom. The new enzyme, named LM-PLA_2-II, displayed high enzymatic (indirect hemolytic) activity, was edematogenic, and inhibited platelet aggregation induced by the physiological agonists ADP and collagen. Several properties correlating LM-PLA_2-I and LM-PLA_2-II catalytic activities as well as their pharmacological effects were compared and are presented here.

2. Materials and methods

The reagents ADP, ampholines, BSA, 2-mercaptoethanol, protein molecular mass markers, *p*-BPB, and egg-yolk phosphatidylcholine were obtained from the Sigma Chemical Co. Type I collagen from bovine tendon was purchased from the Chrono Log Corp., and bovine thrombin from Roche. Lyophilized *L. muta* venom was obtained from either Sigma or Fundação Ezequiel Dias (FUNED). All other reagents were of the best grade available.

2.1. Purification of PLA_2 enzymes

Homogeneous LM-PLA_2-I and LM-PLA_2-II were obtained by gel filtration of crude venom on a Sephacryl S-200 HR column, followed by reverse-phase chromatography on a C_2/C_{18} column using a FPLC (Pharmacia) system.

2.2. PLA_2 activity (indirect hemolysis test)

PLA_2 activity was determined by the indirect hemolysis method [11,12], using washed rabbit erythrocytes and hen egg-yolk emulsion as substrate, in a two-step reaction: (a) incubation of enzymes with the egg-yolk emulsion, and (b) measurement of the hemolytic capacity of the released lysolecithin. Briefly, enzyme samples were first incubated with the buffered emulsion prepared by mixing freshly separated egg yolk with 1 vol. of PBS–KCl (150 mM NaCl, 10 mM NaH_2PO_4, 4.2 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) and centrifuged (15,000 *g* for 60 min at 10°) to remove particles. For the assays, 50 μL aliquots of the substrate preparation were added to the reaction medium (250 μL, final volume) containing crude venom or purified enzyme samples and 8 mM $CaCl_2$ (final concentration). After 10 min at 37°, the reactions were stopped by adding 50 μL of 100 mM EDTA. Then the released lysolecithin was measured by adding 3.2 mL of PBS–KCl and 1.3 mL of rabbit erythrocyte suspension [2% (v/v) in PBS–KCl] to each sample. The samples were incubated for 60 min at 37°, and then centrifuged at 380 *g* for 10 min at room temperature. Released hemoglobin was determined at 578 nm. The maximal effect (100% hemolysis) was determined by lysing all erythrocytes with distilled water. One PLA_2 unit was defined as the amount of enzyme that produces 50% hemolysis.

2.3. Preparation of rabbit platelets and platelet aggregation assays

Rabbit PRP and washed platelets were used. Citrated rabbit blood [0.31% (w/v)] was collected, and blood plasma was obtained following the procedure described by Carlini *et al.* [13]. Briefly, PRP was prepared by centrifugation of rabbit citrated blood (380 *g* for 12 min) at room temperature (25°). PRP thus prepared was suspended in Tyrode's solution containing 0.35% (w/v) BSA and 0.1 mM EGTA (final concentration), pH 6.5, in order to have $3–4 \times 10^5$ platelets/μL. WRP were prepared from blood plasma collected in the presence of 5 mM EDTA and prepared as described previously [14,15]. PRP was centrifuged at 1800 *g* for 20 min at room temperature, and the pellet was suspended in Tyrode's solution containing 0.35% (w/v) BSA and 0.1 mM EGTA (final concentration), pH 6.5, and washed twice in this medium to remove plasma proteins. The final pellet was then suspended in Tyrode–BSA solution, pH 7.35, without EGTA, and the suspension was adjusted to give $3–4 \times 10^5$ platelets/μL. Platelet aggregation was measured turbidimetrically (increasing light transmission of the platelet suspension) using a dual-channel Whole Blood Lumni Aggregometer (Chrono Log Corp.). Assays were performed at 37° in siliconized glass cuvettes maintained under stirring, as described [13]. Aggregation was triggered with physiological agonists after preincubation of platelets with PLA_2 test samples. Control experiments were performed in platelets challenged with the agonists (ADP, collagen, and thrombin) alone.

2.4. Rat paw edema

Edema was induced in the paws of Wistar rats. Lightly anesthetized (diethyl ether) adult rats (180–220 g) received a single subplantar injection of 0.05 mL of either crude venom or purified enzymes into one hind-paw. As a control, an equal volume of saline was injected into the other hind-paw. The edematogenic effect produced by test samples was estimated by measuring the increase in hind-paw volume using a glass plethysmometer [16] coupled to a peristaltic pump at the start of the experiment and at different time intervals after injections. Edema formation was expressed as the volume difference between PLA_2- and saline-treated hind-paws. In another series of experiments, rats were pretreated with indomethacin or dexamethasone given orally 1 hr prior to the injection of crude venom, purified PLA_2, or vehicle (in experiments with indomethacin, 5% Arabic gum dissolved in saline was used as the vehicle).

2.5. Chemical modification of PLA_2

Chemical modification of the proteins was performed by incubating protein samples (30–35 μg/mL) with 2 mM

(final concentration) *p*-BPB dissolved in dimethyl sulfoxide. Incubation was carried out overnight at 4° and then followed by reverse-phase chromatography on a C_2/C_{18} column to remove excess reagents. The chemically modified protein was assayed for hemolytic activity, inhibition of platelet aggregation, and edema formation. Control experiments were performed in parallel with dialyzed non-treated enzymes, assayed under the same conditions.

2.6. Electrophoresis and molecular mass determination

Polyacrylamide gel electrophoresis was carried out by using Laemmli's method on 12% acrylamide slab gels containing 0.1% SDS [17]. Migration of LM-PLA_2-I and LM-PLA_2-II was compared to that of standard protein markers: cytochrome *c* (12.4 kDa), soybean trypsin inhibitor (20.1 kDa), ovalbumin (45 kDa), and bovine albumin (66 kDa).

2.7. Isoelectrofocusing

Isoelectrofocusing of purified proteins was carried out according to the method of O'Farrel [18], using Ampholine gels covering different pH ranges. The protein markers were: (a) pH 2.5–6.5, pepsinogen (pI, 2.80), amyloglucosidase (pI, 3.50), glucose oxidase (pI, 4.15), soybean trypsin inhibitor (pI, 4.55), β-lactoalbumin (pI, 5.20), bovine carbonic anhydrase B (pI, 5.85), human carbonic anhydrase B (pI, 6.55); (b) pH 3–10 amyloglucosidase (pI, 3.50), soybean trypsin inhibitor (pI, 4.55), β-lactoalbumin (pI, 5.20), bovine carbonic anhydrase B (pI, 5.85), human carbonic anhydrase B (pI, 6.55), horse myoglobin-acidic band (pI, 6.85), horse myoglobin-basic band (pI, 7.35), lentil lectin-acidic band (pI, 8.15), lentil lectin-middle band (pI, 8.45), lentil lectin-basic band (pI, 8.65), trypsinogen (pI, 9.30); and (c) pH 5–10, amyloglucosidase (pI, 3.50), methyl red (pI, 3.75), soybean trypsin inhibitor (pI, 4.55), β-lactoalbumin (pI, 5.20), bovine carbonic anhydrase B (pI, 5.85), human carbonic anhydrase B (pI, 6.55), horse myoglobin-acidic band (pI, 6.85), horse myoglobin-basic band (pI, 7.35), lentil lectin-acidic band (pI, 8.15), lentil lectin-middle band (pI, 8.45), lentil lectin-basic band (pI, 8.65), trypsinogen (pI, 9.30), and cytochrome *c* (pI, 10.25). Methyl red was used as a dye and does not appear in the final stained gel.

2.8. Protein sequence

Determination of the amino terminal protein sequence of native enzymes was performed on a Porton Automated Protein Sequencer using Edman's degradation method. Phenylthiohydantoin amino acids were detected at 269 nm after separation on a reverse-phase C_{18} column.

3. Results

3.1. Purification and physicochemical characterization of LM-PLA_2-II

Lachesis muta snake venom was fractionated in two chromatographic steps (Fig. 1). The crude venom was first

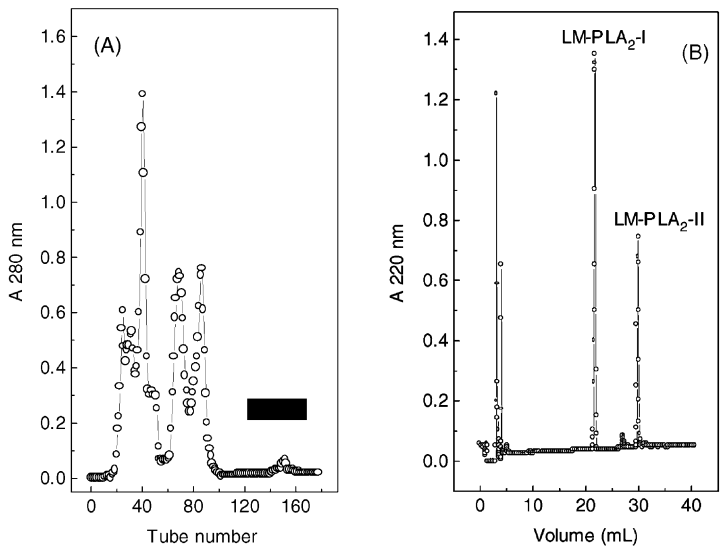

Fig. 1. Purification of a PLA_2 from *L. muta* venom. (A) *L. muta* crude snake venom (75 mg) was fractionated on a Sephacryl S-200 HR column (1.6 cm × 96 cm), equilibrated, and eluted with 20 mM Tris–HCl, 150 mM NaCl, pH 7.5, at a flow rate of 30 mL/hr. Fractions of 1.5 mL were collected and assayed by the indirect hemolytic test. PLA_2 active fractions were pooled (indicated by the solid bar), dialyzed, and further concentrated. (B) The active material from the Sephacryl S-200 HR column was fractionated further by reverse-phase chromatography on a C_2/C_{18} column coupled to a FPLC apparatus. Elution was carried out by a linear gradient (0–100%) of acetonitrile in 0.1% (v/v) trifluoracetic acid, at a flow rate of 0.7 mL/min. Protein elution was monitored at 220 nm, and the fractions were assayed using the indirect hemolytic test. The single peaks that eluted at 37 and 43% acetonitrile (solvent B) correspond to LM-PLA_2-I and LM-PLA_2-II, respectively.

Table 1
Summary of the purification protocol of LM-PLA_2-I and II

Steps	Enzyme	Protein		Specific activity (units)[a]	Total activity (units)[a]	Yield (%)	Purification factor
		(mg)	(%)				
Crude venom	LM-PLA_2-I	75	100	100	7500	100	1
	LM-PLA_2-II	75	100	100	7500	100	1
Gel filtration	LM-PLA_2-I	13	17	526	6842	91	5
	LM-PLA_2-II	0.75	1	476	357	4.76	4.76
Reverse-phase chromatography	LM-PLA_2-I	0.6	0.8	6660	3990	53	66
	LM-PLA_2-II	0.18	0.24	1428	257	3.42	14.28

[a] Units were defined in Section 2.

applied onto a Sephacryl S-200 HR column and the fractions containing PLA_2 activity were pooled (Fig. 1A, solid bar). Combined fractions were further subjected to reverse-phase chromatography on a C_2/C_{18} column (FPLC, Pharmacia System). In this step, two well-separated protein peaks displaying PLA_2 activity were obtained (Fig. 1B). Peak one, corresponding to LM-PLA_2-I, eluted at 37% acetonitrile and peak two, denoting LM-PLA_2-II, eluted at 43% acetonitrile.

A summary of the purification protocol used to obtain the two enzymes is given in Table 1. LM-PLA_2-I and LM-PLA_2-II represent 0.8 and 0.2% of the total protein present in *L. muta* venom; the purified enzymes showed specific activities using the indirect hemolysis assay (see Section 2) of 6660 and 1428 units/mg, respectively (Table 1).

The purified enzymes behaved as homogeneous proteins, showing a single protein band on SDS–PAGE (Fig. 2A), native PAGE (Fig. 2B), and isoelectric focusing electrophoresis (Fig. 2C). The protein homogeneity was confirmed further by N-terminal sequencing analysis in which a single peptide sequence was found for each protein, with a histidine residue at the N-terminal position. From the above experiments, an apparent molecular mass of 18 kDa and a pI at pH 4.7 and 5.4 could be determined for LM-PLA_2-I and LM-PLA_2-II, respectively (Table 2). Fig. 3 shows the N-terminal amino acid sequence (51 residues) of the enzymes. A high degree of homology with other PLA_2s from several sources was seen. This homology included the presence of an aspartic acid residue at position 49, which characterizes D-PLA_2, as well as other conserved residues such as His-48, Tyr-28, and Gly-30, known to be important for the catalytic activity of these enzymes. The sequences of LM-PLA_2-I and LM-PLA_2-II showed, however, several differences in the amino acid residues occupying the same position on each protein (Fig. 3).

3.2. Hemolytic activity

When mixed with washed rabbit erythrocytes, no hemolysis was detected even with a high concentration (50–70 μg/mL) of each enzyme (data not shown). On the other hand, both enzymes were highly active when assayed with washed rabbit erythrocytes (see Section 2) using the indirect hemolysis test (Table 2).

3.3. Effects on platelets

Fig. 4 shows the effect of LM-PLA_2-II on platelets. When preincubated with rabbit PRP, the phospholipase inhibited platelet aggregation induced by both collagen (30 μg/mL) and ADP (10 μM) in a concentration-dependent manner (Fig. 4A). It was observed that collagen- or ADP-induced platelet shape change reactions were not affected by LM-PLA_2-II. As indicated in Fig. 4A, the inhibitory profile was more pronounced with collagen than with ADP, and the IC_{50} values obtained (Table 2) were 0.9

Table 2
A brief comparison between the two PLA_2 isoforms related to physicochemical properties as well as biological activities

Enzyme	Physicochemical properties		Biological activities						
	M_r (kDa)	pI	% Inhibition of platelet aggregation[a]		Hemolytic activity[b] (μg/mL)	Rat paw edema[c]	Myotoxicity	Hemorrhage	Lethality
			(nM)	(μM)					
LM-PLA_2-I	18	4.7	127*	1.39**	0.15	500	+	No effect	No effect
LM-PLA_2-II	18	5.4	900*	3.3**	0.70	375	+	No effect	No effect

[a] Amount of PLA_2 that produces 50% inhibition of platelet aggregation induced by 30 μg/mL (*) of collagen or 10 μM ADP (**).
[b] Amount of PLA_2 that produces 50% hemolysis of rabbit washed erythrocyte suspension as described in Section 2.
[c] Variation in paw volume (μL), due to edema induced by 60 ng/g of PLA_2, measured 60 min after subplantar injection.

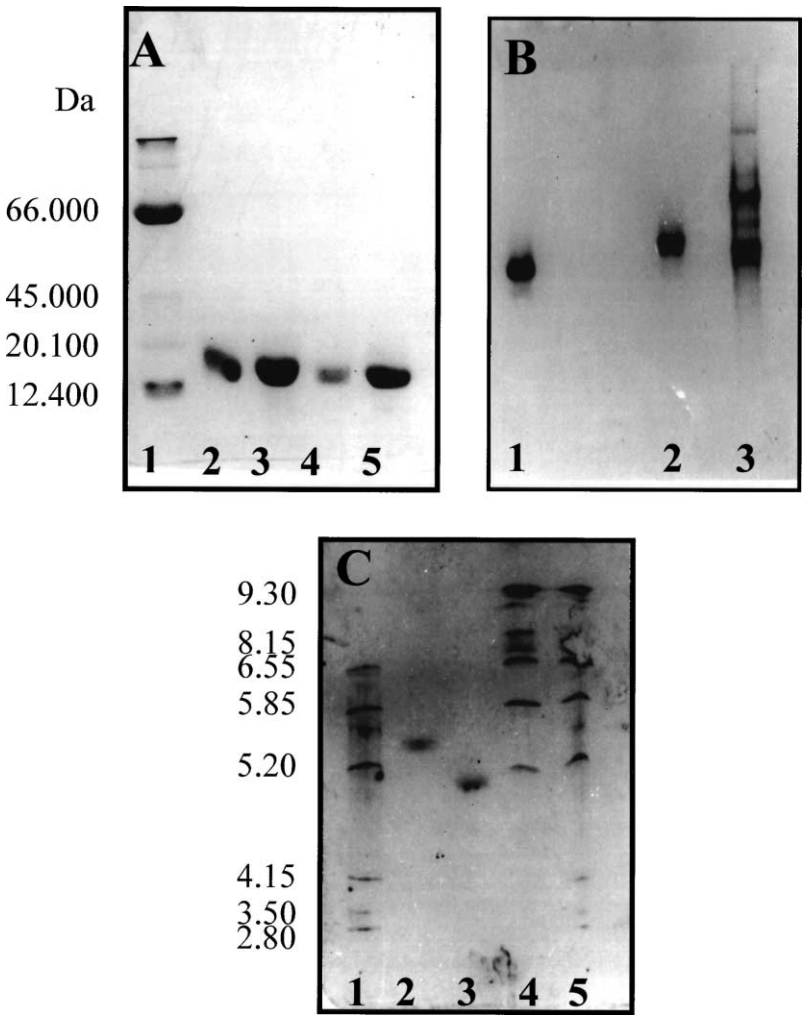

Fig. 2. Electrophoresis of the LM-PLA_2s isolated from *L. muta* venom. (A) SDS–PAGE on a 12% polyacrylamide gel, containing 0.1% (w/v) SDS. Proteins were denatured at 100° for 5 min and then were subjected to electrophoresis. Lane 1: standard proteins: bovine albumin (66 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (20.1 kDa), and cytochrome *c* (12.4 kDa); lane 2: LM-PLA_2-II; lane 3: 2-mercaptoethanol-treated LM-PLA_2-II; lane 4: LM-PLA_2-I; lane 5: 2-mercaptoethanol-treated LM-PLA_2-I. (B) Non-denaturing polyacrylamide gel (12%) electrophoresis of native proteins. Lane 1: LM-PLA_2-I; lane 2: LM-PLA_2-II; lane 3: *L. muta* crude venom. (C) Isoelectric focusing analysis of the proteins at different pH ranges. Lanes 1, 4, and 5 are standard protein mixtures at the following pH ranges: pH 2.5–6.5; pH 5–10, and pH 3–10, respectively; lane 2: LM-PLA_2-II; lane 3: LM-PLA_2-I.

and 3.3 μM for collagen and ADP, respectively. When the PRP was exposed to LM-PLA_2-II and then challenged with collagen, a complete blockage of the platelet's response to the agonist was seen with as little as 1 μg protein/assay (3 μg/mL). Contrary to this, when LM-PLA_2-II (20 μg/mL) was preincubated with WRP, no inhibition of either collagen- (Fig. 4B) or thrombin-induced platelet aggregation was observed, even at much higher concentrations

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                    **  *     ***            *
a) LM-PLA2-II   HLLQFEQLIRKIAGRGFR-YYGFYGCYCGLGGQGRPQDATDRCCFVHDCC
b) LM-PLA2-I    HLLQFGDLIDKIAGRSGFWYYGFYGCYCGLGGRGRPQDATDRCCFVHDCC
c) BJPLA2       DLWQFGQMMNDVMREYVVFNYLYYGCYCGWGGIGKPRDATDRCCFVHDCC
d) BOVINE PLA2  ALWQFNGMIKCKIPSSEPLLDFNYGCYCGLGGSGTPVDALDRCCQTHDNC
e) OVH-PLA2     HLIQFGNMIQCTVPGFLSIKYADYGCYCGAGGSGTPVDKLDRCCQVHDNC
f) AHP-PLA2     SLIQFETLIMKVAKKSGMFWYSNYGCYCGWGGQGRPQDATDRCCFVHDCC
g) AHB-PLA2     SLMQFETLIMKIAGRSGIWYYGSYGCYCGAGGQGRPQDASDRCCFVHDCC
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Fig. 3. Amino terminal protein sequence of LM-PLA_2-II and comparison with LM-PLA_2-I and with PLA_2s from various sources. The N-terminal sequence of LM-PLA_2-II as determined in this work (see Section 2) is compared to that of other PLA_2s. The sequences were taken from: (a) LM-PLA_2-II, present work; (b) LM-PLA_2-I, Fuly *et al*. [11]; (c) PLA_2 from *Bothrops jararaca* (BJPLA_2), Serrano *et al*. [19]; (d) PLA_2 from bovine pancreas (BOVINEPLA_2), Fleer *et al*. [20]; (e) *Ophiophagus hannah* PLA_2 (OVHPLA_2), Huang *et al*. [21]; (f) *Agkistrodon halys pallas* PLA_2 (AHPPLA_2), Chen *et al*. [22]; and (g) *Agkistrodon halys blomhoffii* (AHBPLA_2), Tomoo *et al*. [23]. Amino acid residues in boldface indicate total homology among the PLA_2s, and an asterisk (∗) indicates differences in the amino acid residue sequence between LM-PLA_2-I and LM-PLA_2-II.

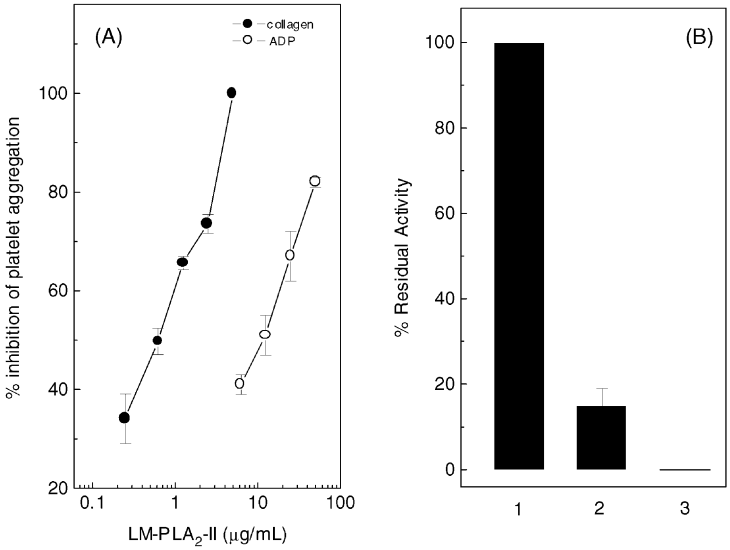

Fig. 4. Effect of LM-PLA_2-II on platelet aggregation. (A) Rabbit PRP was preincubated for 2 min at 37° with vehicle or various concentrations of the test protein, with stirring. Platelet aggregation was then triggered with either collagen (30 μg/mL) or ADP (10 μM). Maximal aggregation was obtained 6 min after the addition of the inducer. Data are expressed as means ± SEM (N = 4). (B) Rabbit WRP were preincubated for 2 min at 37° with 20 μg/mL of LM-PLA_2-II (column 1), 5% (v/v) PPP (column 2), or 10 μg/mL of phosphatidylcholine (PC, column 3). Then collagen (30 μg/mL) was added to the medium to trigger platelet aggregation. In control experiments, PC or PPP was incubated with WRP under the same conditions in the absence of LM-PLA_2-II before challenging with collagen, and 100% of residual activity was obtained for all of them. Data are expressed as means ± SEM (N = 3).

of LM-PLA_2-II (up to 50 μg/mL, data not shown). It was found, however, that the inhibitory effect produced by LM-PLA_2-II on collagen-induced platelet aggregation could be fully restored in the WRP preparation upon the addition of rabbit PPP [5% (v/v)] or exogenous phosphatidylcholine (10 μg/mL final concentration) (Fig. 4B).

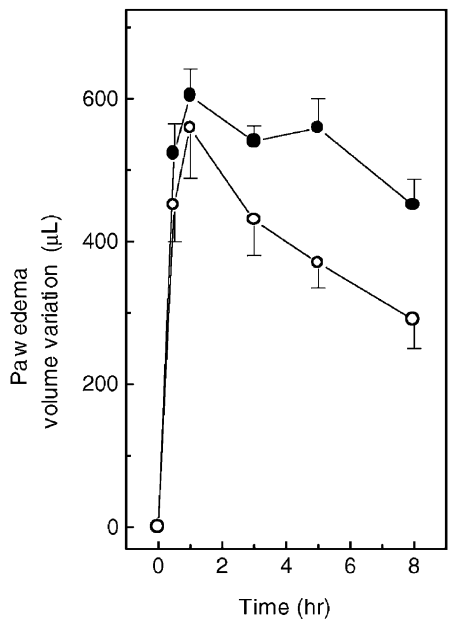

Fig. 5. Edematogenic effect induced by *L. muta* crude venom and by LM-PLA_2-II on the rat hind-paw model. *L. muta* crude venom (76 ng/g) (●) or LM-PLA_2-II (62 ng/g) (○) were injected in the rat subplantar paw, and the edematogenic effect was determined as described in Section 2. Data are expressed as means ± SEM (N = 6).

3.4. Paw edema formation, hemorrhage, and inflammatory reaction

As depicted in Fig. 5, subplantar injection of either *L. muta* crude venom or purified LM-PLA_2-II caused a rapid increase in the rat hind-paw volume within 30 min, reaching a maximum at 60 min after injection. Higher doses of LM-PLA_2-II (150–200 ng/g) induced a sustained edema reaction that lasted for more than 10 hr (data not shown). No hemorrhage was seen in experiments with LM-PLA_2-II. In contrast, higher doses of *L. muta* crude venom, besides inducing a pronounced edema, also produced a macroscopic hemorrhage that did not disappear for up to 24 hr thereafter (data not shown). To elucidate the mechanism by which LM-PLA_2-II could be producing this inflammatory response, rats were pretreated with two anti-inflammatory drugs: indomethacin (10 mg/kg) and dexamethasone (2 mg/kg), given orally 1 hr before injection of the protein. As shown in Fig. 6A and B, the marked edematogenic effect produced by LM-PLA_2-II was reduced markedly in indomethacin- and dexamethasone-treated rats as compared with animals that received the enzyme plus vehicle (see Section 2).

3.5. Chemical modification of LM-PLA_2-II

As summarized in Table 2, all biological activities expressed by LM-PLA_2-II as reported here, namely the hemolytic activity, the inhibitory effect upon collagen- and ADP-induced platelet aggregation, and the edematogenic effect, were totally abolished in the *p*-BPB-treated protein (data not shown).

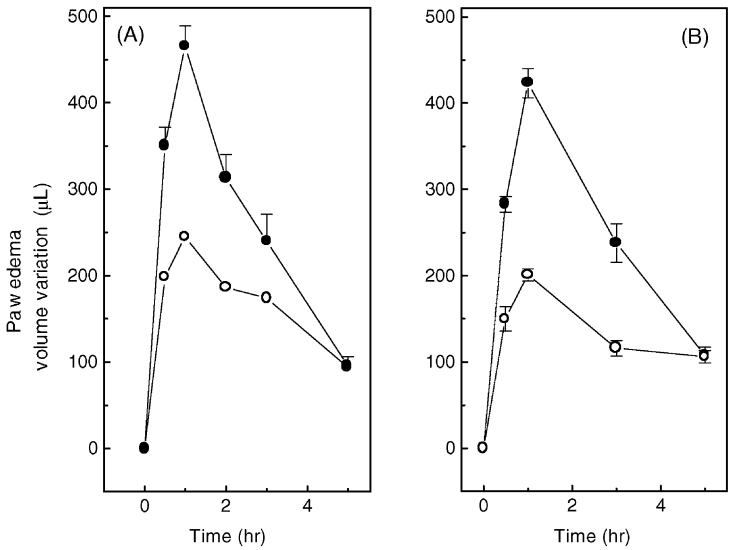

Fig. 6. Inhibition by indomethacin and dexamethasone of paw edema induced by LM-PLA_2-II. (A) Edematogenic effect induced by LM-PLA_2-II (12 ng/g) plus vehicle in control rats (●); rats pretreated (see Section 2) with indomethacin (10 mg/kg) (○). (B) Rats treated with vehicle before injection of LM-PLA_2-II (12 ng/g) (●); rats treated with dexamethasone (2 mg/kg) 1 hr before subplantar injection of LM-PLA_2-II (12 ng/g) (○). Data are expressed as means ± SEM (N = 4).

4. Discussion

The presence of PLA_2 isoforms in *Crotalinae* snake venoms has been demonstrated by several authors and discussed by Valiente *et al*. [24]. In the venom of *L. muta* we have shown previously [25] the occurrence of a complex pattern of proteins, eluted at different ionic strength from a DEAE-cellulose column, some of them displaying indirect hemolytic activity. Recently, an acidic PLA_2 (LM-PLA_2-I) was purified to homogeneity and further characterized by Fuly *et al*. [11,12]. With a different approach, Fortes-Dias *et al*. [26], using the inhibitory pattern produced by a PLA_2 inhibitor isolated from the plasma of another snake, revealed the presence of five isoforms of PLA_2 in *L. muta muta* venom, corresponding to four acidic PLA_2s and one basic isoform. In this work, we describe the isolation of two PLA_2 isoenzymes in *L. muta* snake venom, by using a previously described procedure [11,12]. The enzymes were then characterized through some physicochemical properties and biological effects. The protocol used to isolate the isoforms included as the first step, fractionation of *L. muta* crude venom proteins by gel filtration on a Sephacryl S-200 HR column. The enriched-PLA_2 active material thus obtained, when subjected to reverse-phase chromatography on a C_2/C_{18} column (the second step), systematically yielded a reproducible (several runs) elution profile with two distinct, well separated active peaks. These fractions were named LM-PLA_2-I and LM-PLA_2-II, respectively. The enzymes were shown to be homogeneous preparations by several analytical criteria: (a) a single and well defined peak for each enzyme on the reverse-phase chromatogram; (b) one single band representing each enzyme in both native and SDS–PAGE; (c) on isoelectrofocusing, one single band focused at pH 4.7 (LM-PLA_2-I) and another at pH 5.4 (LM-PLA_2-II); (d) a single polypeptide chain for each enzyme with a histidine residue at the amino terminus; and (e) a distinct amino acid sequence for each isoform. These studies indicated that LM-PLA_2-II belongs to the acidic PLA_2 group (pI value at pH 5.4), with an apparent molecular mass of 18 kDa. The N-terminal amino acid sequence of the protein showed a high homology with other enzymatically active PLA_2s, including the characteristic Asp-49 residue of the known D-49 PLA_2s. Elucidation of the complete amino acid sequence of LM-PLA_2-II as well as LM-PLA_2-I is in progress. It will be an important step towards the understanding of structure–function relationships of these PLA_2s. We also investigated the spectrum of biological and pharmacological effects of LM-PLA_2-II. Despite the potent indirect hemolytic activity observed, the enzyme was unable to produce a direct hemolytic effect when incubated with rabbit erythrocytes, suggesting its inability to hydrolyze phospholipids present in the membrane of the erythrocyte [8]. On the other hand, LM-PLA_2-II strongly inhibited platelet aggregation induced by collagen or ADP in rabbit PRP. As indicated by the IC_{50} values obtained with both agonists (0.9 and 3.3 μM, respectively), LM-PLA_2-II was *ca*. 4-fold more effective as an inhibitor of collagen- than of ADP-induced platelet aggregation. With LM-PLA_2-I (see [11]), a similar pattern was seen, with enzyme IC_{50} values of 127 nM and 1.39 μM for the same agonists (Table 2). Since collagen and ADP interact with different receptors on the membrane of the platelet, it seems clear that the enzymes may act through an indirect mechanism affecting a common cellular pathway mediating the responses observed with rabbit PRP. While aggregation of WRP was not affected by any of the enzymes, the inhibitory effect of both LM-PLA_2 isoforms

on collagen-induced platelet aggregation could be restored when exogenous rabbit PPP or phosphatidylcholine was added to a WRP suspension. These findings strongly suggest that a plasma phospholipid, being a specific substrate, mediates the inhibitory effect of LM-PLA_2s on the platelet response to the agonists. Since phosphatidylcholine can be substituted for normal plasma to restore the enzyme-induced platelet aggregation and since platelets were not lysed upon incubation, it is concluded that the lyso-pc released by *L. muta* PLA_2 may lead to the production of intracellular messengers that interfere with platelet functions [11,27,28]. By itself, this finding indicates that *L. muta* PLA_2s can be used as a tool for cellular signaling studies in platelets.

Like the more abundant acidic isoform LM-PLA_2-I [12], the more basic LM-PLA_2-II produced a dose-dependent edematous response in the rat hind-paw. The edematous response was detectable within 5 min, reached a maximum within 60 min, and vanished thereafter. This edematous pattern is consistent with the edema response produced by *L. muta* crude venom injected by the same route, suggesting that the edema response, as well as other clinical symptoms of envenomation, could be due, at least in part, to the action of the PLA_2s present in this snake venom. Pretreatment of rats with indomethacin or dexamethasone markedly reduced the edema response induced by LM-PLA_2s, thus indicating participation of both the cyclooxygenase pathway and arachidonic acid metabolism in this reaction. PLA_2s from other snake venoms [29,30] produced similar results. This is seen, for instance, with *L. muta rhombeata* crude venom, which induces a significant edema reaction when injected into the hind-paw of the mouse [29].

To investigate the contribution of the enzymatic activity of LM-PLA_2-II on its pharmacological effects, the protein was chemically modified with *p*-BPB. The ability to induce indirect hemolysis, its anti-platelet activity, and the edematous effect were all totally abolished in the *p*-BPP-treated protein, thus confirming that all these effects are associated with the enzymatic activity of the protein, and the presence of an intact His residue in its catalytic site. The same findings were reported previously for the other PLA_2 isoform, LM-PLA_2-I, isolated from the same venom [11,12].

Table 2 summarizes a comparison of some characteristics of LM-PLA_2-I and LM-PLA_2-II isoforms. Both acidic proteins are D-49-dependent enzymes, having a similar apparent molecular mass (18 kDa). The potency of the isoforms varies depending on the biological activity studied. Except for the ability to induce paw edema in rats, for which the isoenzymes had a closer potency, LM-PLA_2-II, in general, was less potent than LM-PLA_2-I. These differences could result from the more acidic character presented by LM-PLA_2-I (pI, 4.7) compared with the more basic character of LM-PLA_2-II (pI, 5.4). There are also several differences in the amino acid residues occupying equivalent positions throughout the protein sequences on both isoenzymes.

In general, acidic PLA_2s are enzymatically more active than basic PLA_2s, but their catalytic properties are not well correlated with their pIs [8,31].

Information on PLA_2 structures is generally available, but their mechanisms of action are still mostly obscure. Two possibilities are being presently explored to explain the structure–function relationship of these enzymes: (a) the presence of a specific receptor PLA_2 on the plasma membrane of target cells, and (b) the existence of two separate protein sites, one responsible for the binding step and the other responsible for the catalytic activity of the enzyme. Thus, other studies are necessary to further advance our knowledge of the properties of different PLA_2s. One approach is to search for the amino acid residues specifically involved in their actions. The 3-D structure of PLA_2s may lead to new insights to establish a better correlation between structure–function relationship on the various biological effects of these enzymes.

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