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The Protein Journal

ISSN 1572-3887
Volume 33
Number 1

Protein J (2014) 33:61-74
DOI 10.1007/s10930-013-9534-x

Springer

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Isolation, Functional Characterization and Proteomic Identification of CC2-PLA$_2$ from *Cerastes cerastes* Venom: A Basic Platelet-Aggregation-Inhibiting Factor

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Published online: 3 January 2014

Abstract Three-step chromatography and proteomic analysis have been used to purify and characterize a new basic phospholipase A_2 named CC2-PLA$_2$ from the venom of *Cerastes cerastes*. This phospholipase A_2 has been isolated to an extent of about 50-folds and its molecular weight was estimated at 13,534 Da. For CC2-PLA$_2$ identification and LC-MALDI-MS/MS analysis, the protein was reduced, alkylated and double hydrolyzed by lysine-C endopeptidase and trypsin. Tryptic fragments of LC–MS/MS analyzed CC2-PLA$_2$ showed sequence similarities with other snake venom PLA$_2$. This presents only 51 % (61/120 amino acid residues) sequence homology with the first PLA$_2$ (gi |129506|) previously purified from the same venom. The isolated CC2-PLA$_2$ displayed anti-aggregative effect on platelets and induced an inflammatory response characterized by leukocytosis in the peripheral blood. This inflammatory response is accompanied by a release of inflammatory mediators such as IL-6, eosinophil peroxidase and complement system. Obtained results indicate that CC2-PLA$_2$ induced a release of high level of pro-inflammatory (IL-6) cytokine and no effect on the level of anti-inflammatory cytokine (IL-10) in blood sera. Furthermore, eosinophil peroxidase activity and hemolytic complement effect increased in peripheral blood. Mononuclear and neutrophil cells were found predominant in the induced leucocytosis following CC2-PLA$_2$ administration into animals.

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Keywords *Cerastes cerastes* · Basic CC2-PLA$_2$ · LC-MALDI-MS/MS analyses · Anti-platelet aggregation · Inflammatory mediators

Abbreviations

ACN	Acetonitrile
CC2-PLA$_2$	Second *Cerastes cerastes* phospholipase A_2
CHCA	Sinapinic acid
CID	Collision ion dissociation
CM-Sephadex C-50	Carboxymethyl-sephadex C-50
EDTA	Ethylene diamine-tetra-acetic acid
EGTA	Ethylene glycol tetraacetic acid
HPLC	High performance liquid chromatography
IAM	2-Iodoacetamide
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
OPD	O-Phenylene diamine dihydrochloride
PEG	Polyethylene glycol
NaCl	Sodium chloride
NIH	Normal Institute of Health
SV-PLA$_2$	Snake venom PLA$_2$
TFA	Tri fluoroacetic phosphate acid

1 Introduction

Viperidae and *Crotalidae* venoms are a potent bio-source of various bioactive components [3, 26, 41, 42, 52]. These venoms contain abundant hydrolytic enzymes that could affect directly or indirectly the haemostasis process through many mechanisms leading to pro- or anti-coagulant disorders [7–9, 21, 30–33, 36, 45, 46, 49, 58, 66]. Many of these molecules including snake venom

phospholipases ($sPLA_2$) are of biomedical interest as antithrombolytic tools in coagulation research, diagnosis and/or therapeutics. Phospholipases A_2 hydrolyze the *sn*-2 fatty acids of membrane phospholipids and produce a variety of lipid mediators. They play also multiple roles for maintenance of membrane phospholipids' homeostasis [29]. Snake venom PLA_2 are widely found in *Viperidae* venoms where they represent more than 10 % of their dry weight [37]. They are catalytically calcium-dependant and structurally consist of 125–130 amino acid residues cross-linked and stabilized by seven disulfide bonds. Their molecular weights are between 13 and 18 kDa [19, 34]. Some phospholipases A_2 are directly involved in the regulation of the biosynthesis of lipid mediators such as prostaglandins and leukotrienes. These enzymes hydrolyze phospholipids in the *sn*-2 position releasing fatty acids and lysopholipids [37]. Their catalytic activity depends on the presence of Ca^{2+} ions. Ca^{2+} involvement in the binding of PLA_2 to its receptor had been already elucidated [63]. Indeed, $sPLA_2$ receptor consists of five CRD-like domains comprising eight subdomains of carbohydrates. Recognition of PLA_2 is attributed to different subdomains of CRD which have only 30 % of homology between themselves. Structure–function studies indicated that CRD5 domain is involved in the determination of $sPLA_2$ through Ca^{2+}, resulting in the loss of catalytic activity after its binding to receptor [59, 63]. Many other activities are also exhibited by $sPLA_2$. In fact, one molecule could display multiple functions; some of them are beneficial such as, antitumoral, antiplatelet, antimicrobial, bactericidal and anticoagulant activities but others are pathophysiological as haemolytic, convulsive, edematous, neurotoxic, cardiotoxic activities [50, 55]. Anticoagulant phospholipases A_2 act by inhibiting platelet aggregation and/or by blocking plasmatic factor Xa (FXa) which leads to prevent the release of thrombin from prothrombinase complex [23, 24, 43]. SV-PLA_2 hydrolyze also the negatively charged phospholipids which are cofactors for prothrombinase system. Several classifications of $sPLA_2$ have been proposed using several criteria. Indeed, $sPLA_2$ were divided into 11 groups based on their molecular weight, sequence homologies in amino acid residues, the disulfide bridge number and pathophysiological effects. $sPLA_2$ isolated from *Elapidae* and *Hydrophidae* venoms belong to the group IA whereas those isolated from *Crotalidae* and *Viperidae* venoms belong to the group IIA. The $sPLA_2$ group IIA are divided into two sub-groups; the Asp49$sPLA_2$ (D49) having an aspartate residue at position 49 which contributes to the catalytic activity of $sPLA_2$; and the $sPLA_2$ Lys49 (K49) having a lysine residue at position 49 [10]. These $sPLA_2$ revealed low catalytic activity. Indeed, the high catalytic activity of $sPLA_2$ D49 is attributed to their ability to bind Ca^{2+} (required for activity) in residues 26, 34 and 49. Residues Tyr 28, Gly30, Gly32 and Asp49 are required for Ca^{2+} determination with loss of oxygen. These four sites are conserved in the case of $sPLA_2$ D49. Previously, it has been obtained a significant decrease of the catalytic activity of the enzyme when the residue D49 has been substituted by another residue (case of $sPLA_2$ K49) [10]. According to the nature of the residue 49, other subgroups of $sPLA_2$ have been identified: N49$sPLA_2$ where the aspartate residue is substituted by an asparagine residue [60, 61, 64]; Gln49 $sPLA_2$ [2] and Arg49$sPLA_2$ [64]. The $sPLA_2$ also play other cellular and tissue functions, they can be haemolytic, anticoagulant and inhibitors of platelet aggregation [23, 24, 27, 37, 51, 56, 57, 67]. In fact, they present direct action on monocytes and induce also the migration of cancer cells (anti-tumoral activity) [65, 67]. These enzymes also display a pro-inflammatory activity through their ability to hydrolyze membrane phospholipids releasing platelet agonists such as thromboxane A_2 and/or arachidonic acid. In Algeria as well as in the most of northern African countries, *Cerastes cerastes* viper is the most dangerous *Viperidae*. Its venom is a heterogeneous composition of various biomolecules, some of which have been well characterized but others need to be investigated. Most of these components act stepwise on haemostasis, such as PLA_2 [37], RP34 and Afaâcytin [18, 38, 39], anticoagulant protease [13], aggregant serine protease [14], CcH1 (haemorrhagic metalloproteinase) [6], CCSV-MPase (non-haemorrhagic metalloproteinase) [16, 17]. In this study, we report isolation, biochemical/proteomic characterization, biological properties and effects of a second PLA_2 isolated from *C. cerastes* venom on haemostatic and inflammatory processes.

2 Materials and Methods

2.1 Materials

The venom of *C. cerastes* has been lyophilized and provided from Latoxan (France). All other chemicals used in this work were of analytical grade.

2.2 Animals

Male Wistar rats (150 ± 20 g body mass) were obtained from Laboratory of Cellular and Molecular Biology, Faculty of Biological Sciences in Algiers and were divided into three groups. The control group was injected i.p. with 100 µl of physiological saline solution (0.9 % NaCl) and the treated groups received at different time intervals, an i.p. injection of sublethal dose (150 µg/150 g body weight) of *C. cerastes* venom and CC2-PLA_2 respectively.

2.3 CC2-PLA$_2$ Purification from *C. cerastes* Venom

CC2-PLA$_2$ was purified by three-step chromatography procedure. The first step was operated onto a Sephadex G-75 column (2.5 × 100 cm) by using 1 g of dissolved crude venom in 100 mM ammonium acetate (pH 8.5). After venom centrifugation at 5,000*g* for 10 min at 4 °C, the obtained supernatant was loaded on the column and the separated proteins were eluted with 100 mM ammonium acetate (pH 8.5) at a flow rate of 0.3 ml/min [16]. Among the three collected peaks, only active fraction was lyophilized and loaded onto CM-Sephadex C-50 column (1.5 × 20 cm). Column was washed and equilibrated with ammonium acetate (100 mM pH 8.5) and the proteins were eluted by a continuous ammonium acetate gradient (0.1–0.5 M) followed by an elution with the same buffer at 2 M (pH 8.5). The final step of purification procedure was performed by RP-HPLC on a C8 Beckman column (2.1 mm × 25 cm, equilibrated with 0.1 % TFA in ACN). Purified protein was eluted at a flow rate of 1 ml/min during 80 min with ACN in presence of 0.1 % TFA (linear gradient from 0 to 90 % of ACN). For all purification procedure, the chromatographic profiles were monitored at 280 nm. Homogeneity of the final molecule was assessed by mass spectrometry and SDS-Polyacrylamide gel electrophoresis. As mentioned above, the process by which separation is performed often, creates a need for the sample to be desalted and concentrated to prepare the biomolecule sample for the next step in the purification process. A solution containing the protein of interest often must be further altered before subsequent purification steps are possible. Dialysis is one of the common operations to separate dissolved molecules by passing through a semi-permeable membrane according to their molecular dimensions. In this work, eluted fractions have been dialysed using Cellophane (cellulose acetate). The ammonium acetate buffer used in salting out procedure or eluting proteins has been removed by dialysis "desalting". During dialysis the external fluid (distilled water) has been changed twice within 48 h in order to reach the required composition inside the dialysis bag. Then, desalted fractions have been concentrated using speed vac apparutus leading to lyophilized proteins.

2.4 Phospholipase A$_2$ Activity

PLA$_2$ activity was characterized by method described by Laraba Djebari and Martin-Eauclaire [37]. Samples (venom or purified CC2-PLA$_2$; 0.05 absorbance units) are mixed with lecithin 1 % (v/v) in 0.13 M phosphate buffer, pH 7.4. After incubation for 1 h at 37 °C, 1 ml of diluted 1:20 red blood cells was added and incubated 30 min at 40 °C. Hemolysis activity was revealed after centrifugation of the mixture at 1,000 g for 15 min. Hemoglobin resulting of hemolytic activity of CC2-PLA$_2$ was measured at 540 nm.

2.5 SDS-Polyacrylamide Gel Electrophoresis

Homogeneity of purified CC2-PLA$_2$ was examined by SDS-PAGE with 15 % of acrylamide gel under non-reducing conditions according to the method of Laemmli [40]. Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and Lactalbumin (14.4 kDa) were the standard proteins served as molecular mass markers.

2.6 Liquid Chromatography Coupled Off Line with MALDI-TOF/TOF

2.6.1 Sample Digestion

About 150 µg of purified CC2-PLA$_2$ (protein in 30 µl) were diluted in 100 mM Tris HCl pH 8.5 containing urea (8 M). After protein precipitation with cold acetone (6 volumes) and its re-suspension in 100 mM Tris HCl pH 8.5 containing urea (8 M), CC2-PLA$_2$ was reduced and alkylated with 5 mM TCEP and 10 mM IAM. Then, the protein was firstly incubated for 6 h at 37 °C with Lys-C (1 µg/µl, P-3428, Sigma) and then re-incubated overnight at 37 °C with trypsin (1 µg/µl, trypsin Gold MS grade, V5280 Sigma) in presence of 2 mM $CaCl_2$. A second incubation with trypsin (5 h at 37 °C) was performed to ensure a complete digestion and the obtained digested protein was centrifuged 10 min at 5,000*g*.

2.6.2 Nano Liquid Chromatography

For peptide separation of the digested CC2-PLA$_2$, double columns have been used; a C18 PepMap column (15 cm × 75 µm × 3 µm, 100 Å), (Dionex LC-Packings, Sunnyvale, CA, USA) operated at 40 °C and a trap column (PepMap C18, 5 mm × 300 µm × 5 µm, 100 Å), (Dionex LC-Packings, Sunnyvale, CA, USA) to remove salts. Peptide separation was performed with a 45 min linear gradient of solvent B (0–50 % B, Solvent A was 5 % ACN with 0.1 % TFA and solvent B was 80 % ACN with 0.1 % TFA) followed by a 5 min washing step at 95 % B. Collected fractions were mixed with CHCA matrix (2 mg/ml, 0.8 µl/min) which contained 10 fmoles of Glu-1-fibrinopeptide B (Sigma) for internal calibration of the MS spectra. Then, fractions were directly spotted onto MALDI plate after using the Probot device.

2.6.3 MALDI MS and MS/MS Analyses

4800 MALDI TOF/TOF analyzer (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) was operated in

positive reflector ionization mode (m/z range 800–4,000) for purified CC2-PLA_2 MS analysis. In fact, to ensure good S/N quality for precursor selection, 3,000 laser shots/spot were used. MS spectra calibration was automatically performed using the Glu-1 fibrinopeptide B ($[M + H]^+ = 1{,}570.670$). 2 kV positive CID ON method was chosen for MS/MS experiments and non-redundant ions with S/N >30 were selected as precursors and submitted to CID fragmentation (4,000 laser shots/precursor, until 15 precursors/spot). Calibration was automatically applied to MS/MS spectra.

2.7 Protein Identification

For CC2-PLA_2 identification, a local copy of MASCOT search engine 2.1 (Matrix Science Ltd., UK) embedded into the GPS-Explorer Software 3.5 (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) on the NCBInr database has been used. Protein hits with a MASCOT protein score ≥53 and a protein index ≥95 % were only taken in consideration. Search parameters and validation of protein identification are already given in our previous paper [16]. Parameters were set as follow: 50 ppm mass accuracy for the precursor and 0.3 Da for MS/MS fragments, trypsin (K/P) cleavage, one missed cleavage allowed, carbamidomethylation of cysteines (fixed modification) and oxidation of methionines (variable modification). Validation of protein identifications was carried out with the Scaffold software version 2.01 (Proteome Software, Portland, Oregon, USA). MASCOT files of the identified proteins were re-analyzed by the Scaffold software which used independently a second search engine (X! Tandem) and a workflow including Peptide Prophet (peptide filtering) and Protein Prophet (protein identification filtering). The obtained results with the two search engines were then automatically combined and only proteins identified with a minimum of two distinct peptides (peptide confidence index ≥95) and with a protein confidence index ≥95 % were taken into account.

2.8 Assay for Platelet Aggregation

CC2-PLA_2 effect on platelet-rich plasma (PRP) aggregation was carried out using human plasma from volunteer donors who had not taken any medication prior to sampling for at least 2 weeks. PRP was obtained after centrifugation of diluted blood (1:10) with 3.8 % sodium citrate at 200*g* for 5 min at 4 °C and PPP (platelet-poor plasma) was obtained after centrifugation at 2,000*g* for 20 min at 4 °C. Using PPP, platelet count was adjusted to $250 \times 10^3/\mu l$ before experiment. Induced platelets with 10 µM ADP or 0.05 U/ml of thrombin were used to evaluate platelet-aggregation-Inhibitory activity of purified CC2-PLA_2. Platelet aggregation was recorded for 10 min at 37 °C using a Chrono-log aggregometer (ServiBIO 540 VS, USA).

2.9 CC2-PLA_2 Effect on Blood Cell Counts

Collected blood samples after i.p. venom injection or CC2-PLA_2 administration were centrifuged at 1,500*g* for 15 min to obtain leukocyte cells. The diverse cell populations were counted in cytocentrifuge after Giemsa stained, whereas total cell counts were performed in a hemocytometer (Hematek 2000, Bayer Corporation, USA) [40]. The results represent the mean ± standard deviation (SD) per percentage of cell suspension.

2.10 IL6 and IL10 Cytokines Analysis

According to the manufacturer's instructions, quantification of cytokine production (IL-6 and IL-10) was assessed by a double-ligand ELISA kit (Amersham Bioscience, USA), in serum of envenomed or injected rats with CC2-PLA_2 at 3 and 24 h. Standard curves have been used for deducing IL-6 and IL-10 concentrations which are expressed as pg/ml of cytokines in the serum. Sensitivity for detection of IL-6 and IL-10 levels was respectively 5 and 10 pg/ml.

2.11 Hemolytic Complement Assay

Sera of control and envenomed rats were obtained from 3 h to 1 week after i.p. injection. For the injected rat group with CC2-PLA_2, sera were obtained 3 and 24 h after i.p. molecule administration. Washed sheep erythrocytes with barbital buffer (pH 7.4) and anti-sheep erythrocyte antibody (hemolysin, Biomérieux, France) were incubated together for 1 h at 37 °C for obtaining sensitized erythrocyte suspension. Hemolytic assay was performed with 900 µl of diluted sera (1:30) with barbital buffer mixed and incubated (37 °C for 1 h) with sensitized erythrocyte suspension (100 µl). After centrifugation at 1,000*g* for 5 min, the absorbance of the supernatants was monitored at 540 nm. Cells incubated in barbital buffer pH 7.4 represented negative control (0 % lysis control) and cell blood from controls were lysed in PBS buffer pH 2 (100 % lysis control). Results were expressed as a percentage of hemolytic activity [4].

2.12 Eosinophil Peroxidase Activity Assessment

Eosinophil peroxidase activity is assayed in lung homogenate of envenomed rats or injected with CC2-PLA_2. Lungs were homogenized with Tris–HCl (0.06 M, pH 8, 2 ml) containing 1 % Triton x 100 and then centrifuged at

3,000 g for 15 min. Supernatant (50 µl) of each sample was placed in a microplate (96 wells) in the same buffer (100 µl), 10 µl of H_2O_2 (0.06 %) and 20 mg of the chromogen OPD (O-Phenylene diamine Dihydrochloride). Absorbance was performed at 490 nm.

2.13 Statistical Analysis

For statistical result validation, a Student *t* test was used to compare differences between groups. Results were expressed as the Mean ± SD. At p values <0.05, data were considered statistically significant.

3 Results

3.1 CC2-PLA$_2$ Purification

Cerastes cerastes venom (1 g) fractionation on the Sephadex G-75 gel filtration column has separated the venom components into three fractions (Fig. 1a) as described in our previous paper [16]. Fractions I and II showed both approximately the highest phospholipase activity but the fraction I was selected for further separation. This was pooled, concentrated, desalted and re-separated with cation exchange chromatography on CM-Sephadex C-50. In this step, fraction I was resolved into three main peaks among them only peak II displayed a high phospholipase activity (Fig. 1b). RP-HLPC on a C8 column was carried out on the pooled, concentrated and desalted peak I. Two peaks were obtained; the first peak represents the non-hydrophobic material not retained on the column, whereas, the second peak presents phospholipase activity which was called CC2-PLA$_2$ (Fig. 1c, d). Purified CC2-PLA$_2$ seems to be a basic protein, as it was eluted at the middle of gradient of ammonium acetate (0.1 M, pH 8.5) from CM-Sephadex C-50 column. Moreover, the theoretical pI of CC2-PLA$_2$ calculated thank to its amino acids sequence is up to seven. The purified CC2-PLA$_2$ represented 0.118 % of the whole venom and retained about 0.06 % of its total phospholipase activity (Table 1). Purified CC2-PLA$_2$ with a final yield of 0.95 % (protein by weight), has been isolated to an extend of about 50-fold, it exhibits a specific activity of 51.5 units/min/mg. CC2-PLA$_2$ seems to be homogeneous on 15 % SDS-PAGE analysis (Data not shown) with an apparent molecular mass of around 14 kDa.

3.2 Mass Spectrometry Analysis of CC2-PLA$_2$

Fractions (312) collected after off-line nano liquid chromatographies of digested CC2-PLA$_2$ were analyzed by LC-MALDI-MS/MS. Overall; 1,207 MS/MS spectra were obtained. Protein identification was carried out in two independent experiments leading to many isolated snake phospholipases. These proteins were identified with at least two unique peptides and shared some sequence homologies with the purified CC2-PLA$_2$ (Table 2; Fig. 2). LC/MS and MS/MS analyses of tryptic fragments of purified CC2-PLA$_2$ showed sequence similarities with other PLA$_2$ isolated from other venoms. LC-MALDI-MS/MS analysis has allowed the determination of the amino acid sequence of purified CC2-PLA$_2$ by alignment with sequences of other venom phospholipases (Fig. 3). This amino acid sequence was validated with Scaffold software version 2.01 (Proteome Software, Portland, Oregon, USA). The obtained molecular weight of the purified CC2-PLA$_2$ using LC-MALDI-MS/MS was 13,534 Da compared to the first PLA$_2$ previously isolated from the same venom of *C. cerastes*. Indeed, there are 61/120 amino acid residues in common between the both phospholipases with 51 % as coverage.

3.3 Inhibitory Effect of CC2-PLA$_2$ on Platelet Aggregation

Platelet agonists were used as controls (thrombin and ADP). Inhibitory activity of platelet aggregation was assessed using 3-min pre-incubation platelets with CC2-PLA$_2$ before adding ADP or thrombin. The obtained result revealed that CC2-PLA$_2$ inhibited significatively the ADP- or thrombin-induced platelet aggregation (Fig. 4a, b). Therefore, thrombin (0.05 U/ml) is able to induce platelet aggregation, whereas CC2-PLA$_2$ revealed a platelet aggregation-inhibition activity with significant reduction (95–100 %). This antagonist effect to thrombin is of biological and pharmacological interest since this anti-aggregative effect could be a useful tool in reducing blood viscosity which is often necessary in the treatment of blood-clotting dysfunction [16, 17].

3.4 Peripheral Blood Cell Counts

Mononuclear and neutrophil cells were predominant in the peripheral blood in envenomed animals compared to the control (Fig. 5a). Results revealed significant time-dependent increase of monocytes and neutrophils with maximal values after 24 h. Indeed, total leukocytes increased from 5.6×10^3 to 7×10^3 cells/µL at 3 and 48 h in envenomed animals compared to controls (3.7×10^3 cells/µL). Indeed, results showed that pulmonary tissue is significantly altered by the components of the venom with a massive presence of eosinophils. When injected into rats by i.p. route CC2-PLA$_2$, also induces an increase in total leukocyte mainly lymphocytes, monocytes and neutrophils (Table 3; Fig. 5b).

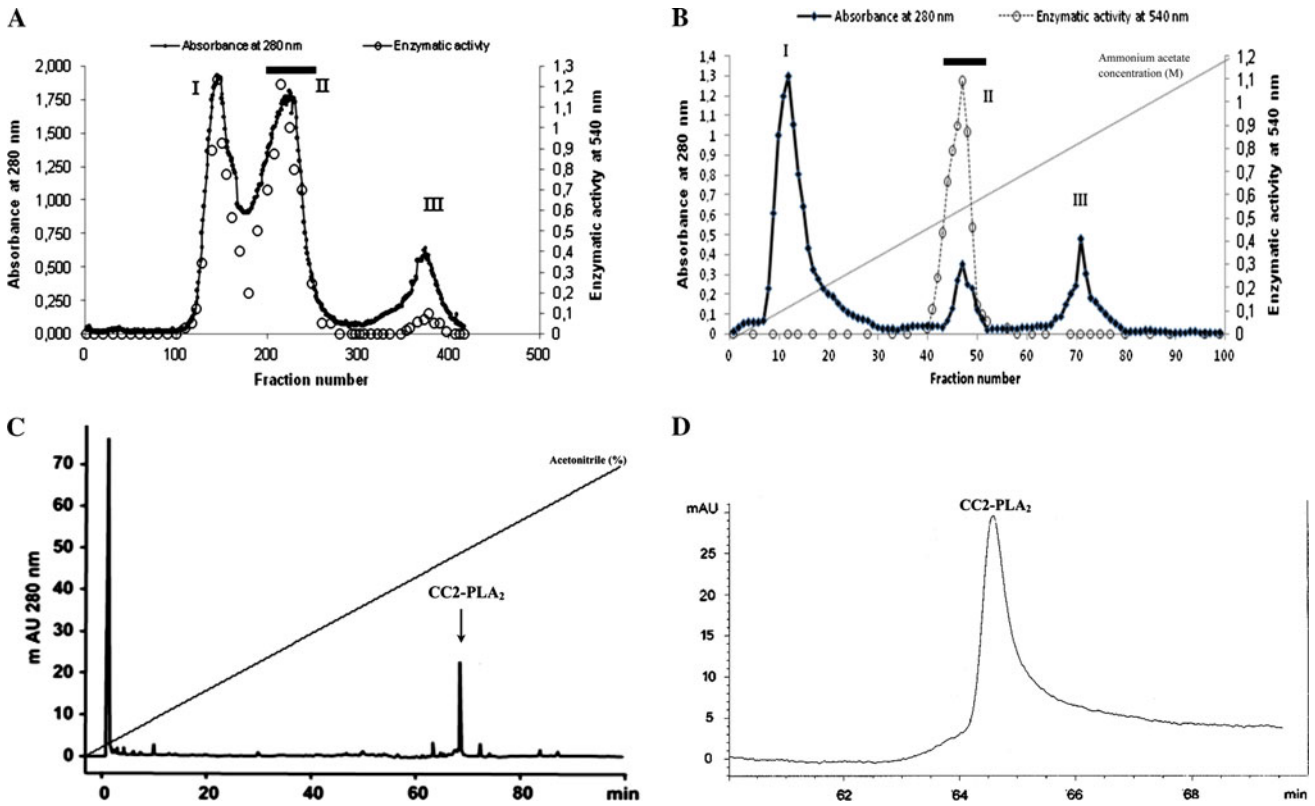

Fig. 1 Purification of CC2-PLA_2 from *C. cerastes*. Chromatographic profile of venom fractionation on G-75 Sephadex (column, 2.5 × 100 cm). Protein elution was carried out with 100 mM ammonium acetate (pH 8.5), fractions were collected at a flow rate of 0.3 ml/min. Relative phospholipase activity corresponding to hemoglobin release at a wavelength of 540 nm (**a**). CM-Sephadex C-50 chromatography for active fraction with phospholipase activity on column (1.5 × 20 cm). Subfractions were eluted stepwise using a continuous ammonium acetate gradient (0.1–0.5 mM) followed by an elution with the same buffer at 2 M (pH 8.5) (**b**). Collected fractions showing phospholipase activity was re-chromatographied on RP-HPLC using Beckman C8 column (0.21 × 25 cm; 2.1 mm × 25 cm, equilibrated with 0.1 % TFA in ACN). Purified protein was eluted at a flow rate of 1 ml/min during 80 min with acetonitrile in presence of 0.1 % TFA (linear gradient from 0 to 90 % of acetonitrile). For all purification procedure, the chromatographic profiles were monitored at 280 nm (**c** and **d**)

Table 1 Purification of CC2-PLA_2 from *C. cerastes* venom

Purification step	Protein recovery (mg)	Protein recovery (%)	Phospholipase activity (units/mg/min) × 10^{-1}	Total activity (units)	Activity yield (%)	Fold purity
Crude venom	1,000	100	10.30	1,030	100	1
Sephadex G-75 chromatography	81.35	8.135	16.67	83.35	8.10	8.092
CM-sephadex C-50 chromatography	1.18	0.118	515	60.77	0.060	50

3.5 Effect of CC2-PLA_2 on Cytokine Levels

Purified CC2-PLA_2 as well as the venom of *C. cerastes*, induces an inflammatory reaction leading to the pro-inflammatory and anti-inflammatory cytokines production (IL6 and IL10; Fig. 6). Injected animals with saline solution presented undetectable cytokine levels. CC2-PLA_2 induces an inflammatory response leading to the release of cytokines (IL6 and IL10). Indeed, CC2-PLA_2 revealed a release of high level of interleukin IL6 after 24 h (178 ± 16 pg/mL, $p < 0.05$; Fig. 6). A maximum concentration of IL-6 is obtained at 24 h after envenomation (243 ± 24 pg/mL, $p < 0.01$). IL6 release following the inoculation of venom, was marked with a high concentration (198 ± 21 pg/mL, $p < 0.01$) at 3 h after envenomation, leading to a synthesis of early cytokines (IL1-β and TNF-α). These two cytokines have a synergistic role mainly in the initiation of the inflammatory reaction and are themselves involved in the production of IL-6. *C. cerastes* venom causes also a significant increase in anti-inflammatory interleukin levels (IL10) in the vascular compartment with a maximal concentration after 24 h (178 ± 24 pg/mL, $p < 0.01$; Fig. 6), however,

Table 2 Some identified proteins by LC-MALDI-MS/MS sharing sequence homology with CC2-PLA$_2$

Protein name	Accession number	Species	Molecular mass (kDa)	Common peptide number	Scaffold protein confidence index
Ammodytin I2 (A)	gi |50874464 |	*Vipera berus berus*	14	3	100
Ammodytin I2 (A) isoform	gi | 50874456|	*Vipera ammodytes ruffoi*	13.5	2	99.8
Phospholipase A$_2$	gi|6967298|	*Vipera ammodytes*	13.5	3	100
Ammodytin I2 (C) isoform	gi|50874498|	*Vipera ammodytes meridionalis*	13.7	3	100

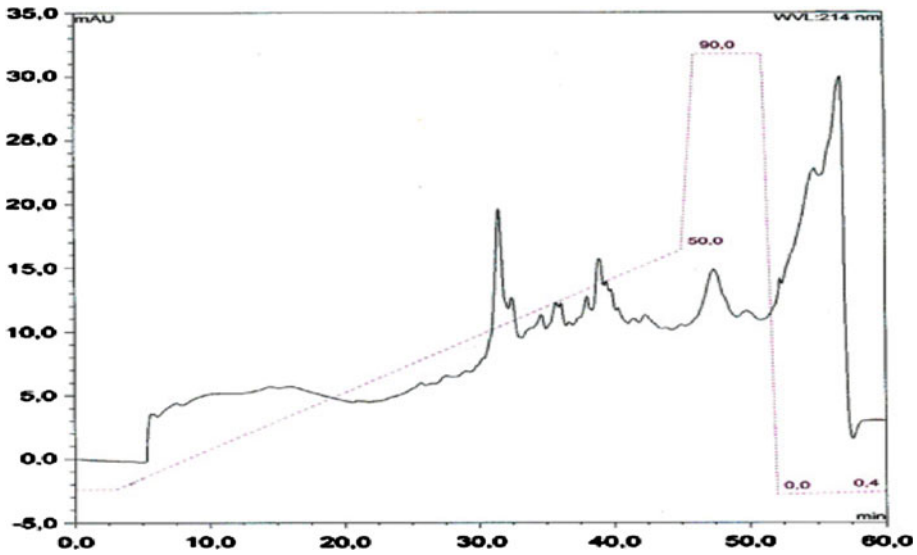

Fig. 2 Liquid chromatography of the digested CC2-PLA$_2$. PepMap C18 column (15 cm × 75 µm × 3 µm, 100 Å) combined with a pre-column (PepMap C18, 5 mm × 300 µm × 5 µm, 100 Å) were used for peptide separation. After a period of 5 min of washing, Peptide separation was performed with a 45 min linear gradient of solvent B (0–50 % B, Solvent A was 5 % ACN with 0.1 % TFA and solvent B was 80 % ACN with 0.1 % TFA) followed by a 5 min washing step at 95 % solvent B

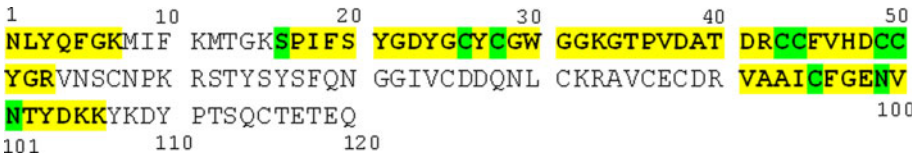

Fig. 3 Amino acids sequence of CC2-PLA$_2$ deduced from the previously purified PLA$_2$ from *C. cerastes* venom. The complete sequence of amino acids (1–120) corresponds to the first purified phospholipase A$_2$ from *C. cerastes* venom [37]. The common amino acid residues between the first PLA$_2$ (gi \|129506\|) [37] and CC$_2$-PLA$_2$ (this work) are colored in *yellow*; it is a partial sequence of 61 residues amino acid i.e. 51 % sequence homology between the two phospholipases (51 % as coverage). Amino acids in *green* are residues that have undergone chemical changes during different treatments required for proteomic analysis (Color figure online)

IL10 concentration at 3 h of envenomation is similar to that obtained with control (26 ± 4 pg/mL, $p < 0.01$).

3.6 Effect of CC2-PLA$_2$ on Complement System and Eosinophil Peroxidase Activity

Experimental envenomation induced a significant high hemolytic activity reaching its maximum after 24 h and returned to control levels at 72 h (Fig. 7a). Sera from animals injected with purified CC2-PLA$_2$ and sacrificed at 3 and 24 h revealed a lytic activity which varies according to the sacrifice time of animal (Fig. 7b). Indeed, CC2-PLA$_2$ induced a significant increase of hemolytic activity (43.34 ± 2.58 %) compared to that induced by the crude venom. Maximal hemolytic activities were observed at 24 h. These increased concentrations of complement components could be the result of tissue damage or directly provoked by the venom itself. However, the second peak obtained at 24 h is probably due to an increased synthesis of complement components because of the inflammatory reaction induced by the venom of *C. cerastes* or by its purified CC2-PLA$_2$. Our results are in agreement with those previously obtained in study of rats envenomed by *Tityus serrulatus* [4]. Ability of *C. cerastes* venom and purified CC2-PLA$_2$ to activate eosinophil peroxidase release revealed maximal activities observed at 3 and 24 h after envenoming and is therefore biphasic (Fig. 8a). As shown in figure, animals injected with purified CC2-PLA$_2$ and sacrificed after 3 and 24 h present varying levels of enzymatic activity (Fig. 8b). Indeed, the results showed increased levels of eosinophil peroxidase at 3 and 24 h when the animals are treated with CC2-PLA$_2$.

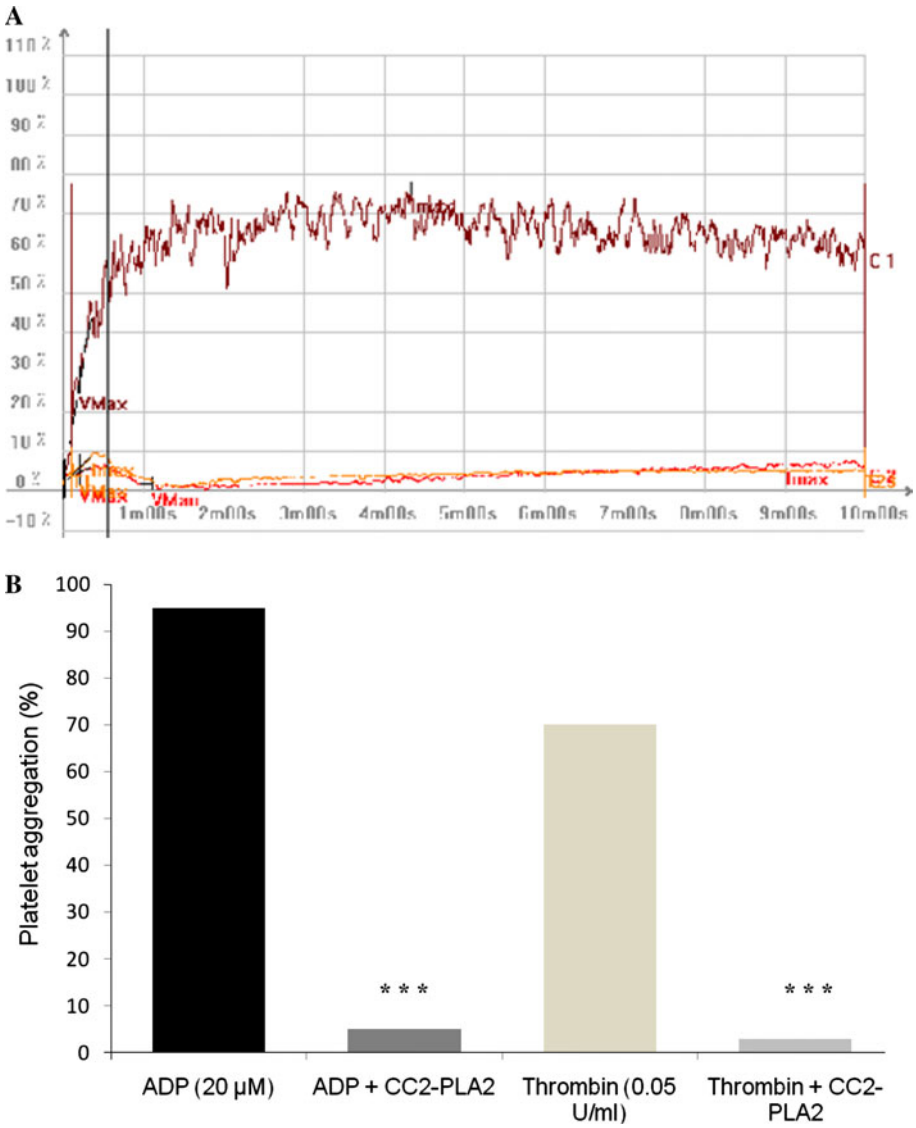

Fig. 4 Anti-aggregative role of CC2-PLA_2 on ADP or thrombin induced platelets. The experiment was carried out using human PRP (platelet-rich plasma) from volunteer donors who had not taken any medication prior to sampling for at least 2 weeks. Induced platelets with 10 µM ADP or 0.05 U/ml of thrombin were used to evaluate platelet-aggregation-Inhibitory activity of purified CC2-PLA_2. Platelet aggregation was recorded for 10 min at 37 °C using a Chrono-log aggregometer (ServiBIO 540 VS, USA). Values are expressed as mean ± SD ($n = 5$). ***$p < 0.001$ compared to the control

4 Discussion

Isolated phospholipases A_2 from snake venoms can be involved in many pathophysiological process. Among them, these proteins may act at different steps on the haemostatic system. Therefore, they can exhibit beneficial antagonistic roles as pro-coagulants, anti-coagulants and pro- or anti-platelet aggregative factors [18]. Some of these biomolecules, particularly from *Viperidae* venoms, could be used in diagnosis or treatment of thrombotic and ischemic heart diseases. Phospholipases A_2 are widely found in *Viperidae* venoms where they represent more than 10 % of their dry weight [37]. In this study, CC2-PLA_2, a new anti-aggregative phospholipase A_2 with pro-inflammatory effect, was purified and functionally characterized from the venom of *C. cerastes* by three-step chromatography procedure on Sephadex G-75, CM-Sephadex C-50 cation exchange column and RP-HPLC on C8 column. 50-folds purified CC2-PLA_2 revealed a final yield of 0.95 % (protein by weight) and a specific activity of 0.515×10^{-2} units/min/mg. In the whole venom, CC2-PLA_2 is a minor component since it found be only represented by 0.118 % and retained about 0.06 % of its total phospholipase activity. Characterization of CC2-PLA_2 by SDS-PAGE analysis (Data not shown) showed that the molecule seems to be homogeneous with an apparent molecular mass of around 14 kDa. The partial peptide sequence (about 51 % of the total sequence; 61 over 120 amino acid residues) of CC2-PLA_2 was obtained by alignment with sequences of other venom PLA_2s. The obtained results revealed a molecular weight of 13,534 Da compared to the first PLA_2 previously isolated from the same venom of *C. cerastes* (gi |129506|) [37]. Furthermore, some snake venom phospholipases were identified sharing sequence similarities with CC2-PLA_2, three of these proteins corresponded to *Vipera ammodytes* venom and one to

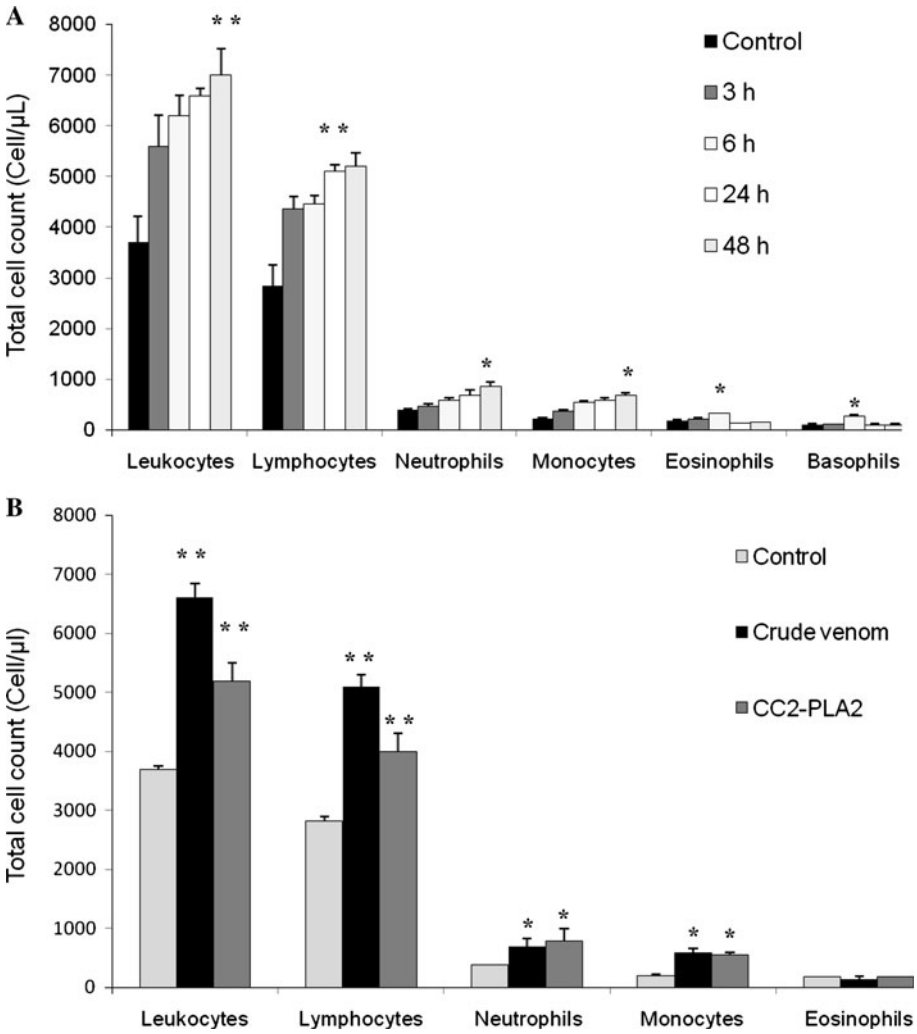

Fig. 5 Kinetics of leukocyte migration into peripheral blood. Group of five rats were injected with venom (150 µg/150 g body weight) (**a**) or CC2-PLA$_2$ (**b**). Leukocyte migration was evaluated at varying times. Values are expressed as mean ± SD ($n = 5$). *$p < 0.05$, **$p < 0.01$ compared to the control group

Table 3 Leukocyte number in peripheral blood 24 h after envenomation (i.p. route) with *C. cerastes* venom or purified CC2-PLA$_2$

Cell population	Control	Crude venom	CC2-PLA$_2$
Leukocytes (×10^3/µL)	3.7 ± 0.51	6.6 ± 0.13**	5.2 ± 0.8***
Neutrophils	382 ± 35	681 ± 93**	797 ± 21***
Monocytes	207 ± 25	589 ± 38**	554 ± 13**

Values are expressed as mean ± SD (n = 5); **$p < 0.01$ and ***$p < 0.001$ compared to the controls

Vipera berus venom but all of them belonged to the phospholipases class; this obviously could prove the purity of CC2-PLA$_2$. Biological characterization of CC2-PA$_2$ revealed that it exhibited a significant inhibiting effect of ADP- or thrombin-induced platelet aggregation. Similar results have been reported with two isolated phospholipases from the venom of *C. cerastes* [67]. However, the first one showed no effect on platelet aggregation [37]. EGTA markedly abolished this effect on platelet aggregation, suggesting that Ca^{2+} ion is required for its platelet inhibitory effect (Data not shown). *C. cerastes* venom contains also other molecules which have antagonist activities of anti-platelet CC2-PLA$_2$. Indeed, Afaâcytin (a serine proteinase) was able to induce irreversible aggregation of human platelets, but no effect on rabbit platelets [18, 39]. A new acidic phospholipase A$_2$ called BpirPLA$_2$-I isolated and characterized from *Bothrops pirajai* venom, is also able to induce hypotension in vivo. The natural BpirPLA$_2$-I or its synthetic peptide of BpirPLA$_2$-I, containing only residues of the C-terminal region, displayed the same inhibitory effect on platelet aggregation and phospholipase activity upon some artificial substrates [59]. Many studies have already demonstrated the involvement of snake venom phospholipases A$_2$ in local and systemic inflammation. In fact, leukocytosis appears to be the consequence of induction of pro-inflammatory cytokines TNFα and mainly IL-6 and anaphylatoxin C3a generated after complement activation on bone marrow. Decrease of eosinophil and basophil number after 6 h of envenomation is probably due to the migration of cells from the blood into tissue compartments such as lungs, which is in agreement with

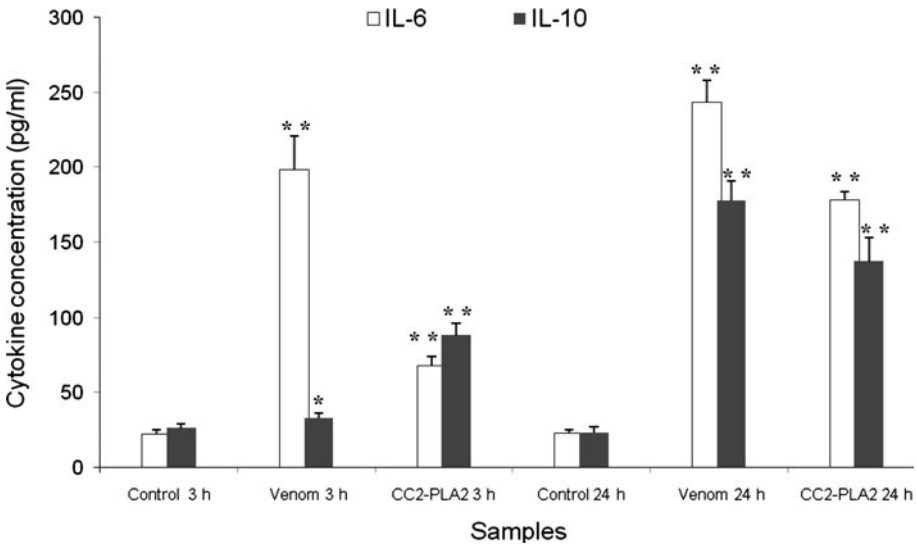

Fig. 6 Effects of i.p. injections of *C. cerastes* venom (150 µg/150 g body weight) or purified CC2-PLA_2 on circulating levels of IL-6 and IL-10 in rats. Each point represents the Mean ± SD of five animals. $*p < 0.05$, $**p < 0.01$ relative to control

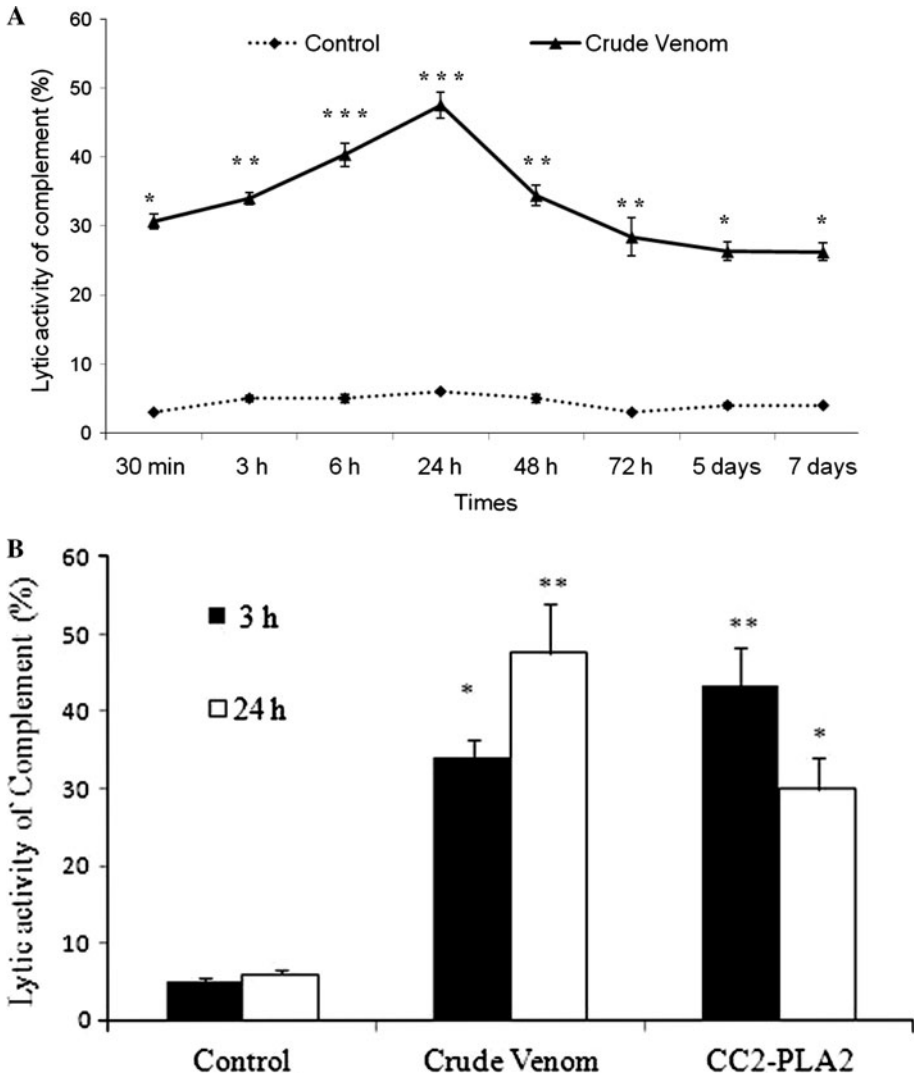

Fig. 7 Kinetics of lytic activity of complement system after *C. cerastes* envenomation (150 µg/150 g body weight) (**a**) or administration of purified CC2-PLA_2 (**b**). Values expressed a percentage of their respective controls (100 %). $*p < 0.05$, $**p < 0.01$ relative to control. Mean ± SD ($n = 6$)

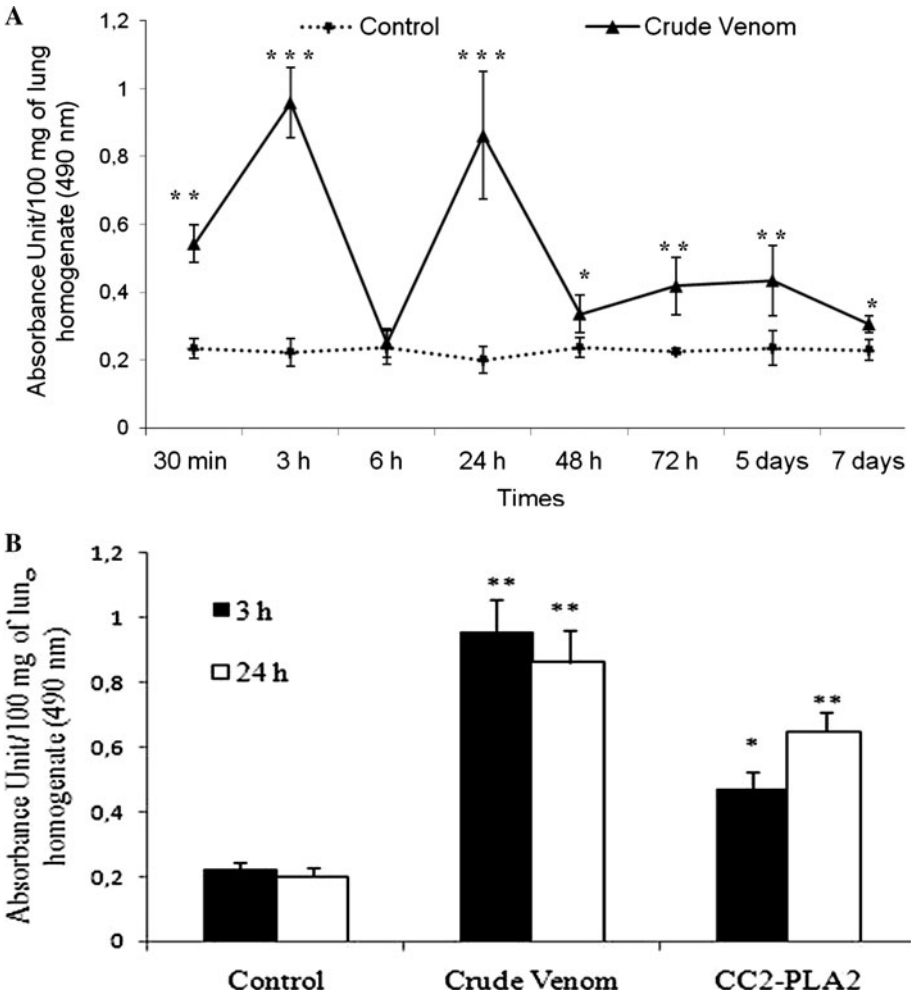

Fig. 8 Kinetics of eosinophil peroxidase activity after treatment with *C. cerastes* venom (150 μg/150 g body weight) (**a**) or purified CC2-PLA$_2$ (**b**). Values expressed a percentage of their respective controls (100 %). *$p < 0.05$, **$p < 0.01$ relative to control. Mean ± SD ($n = 6$)

our previous results [12, 14]. These results indicate that purified CC2-PLA$_2$ is mainly involved in the induction of leukocytosis. It has been demonstrated that the recruitment of leukocytes at the site of injury is an important event in the host's defense against offending agents in inflammatory response [53]. In this study, obtained results indicate also that purified CC2-PLA$_2$ as well as the crude venom rapidly induced neutrophil and mononuclear cell migration into peripheral blood. Many studies have already demonstrated that neutrophil cells predominate in an immediate inflammatory response because they are usually the first cell population to reach the injury site. Indeed, independently of type of venoms; snake or scorpion (*Bothrops* spp. or *Tityus* spp. venoms), an identical leukocyte infiltration was observed in envenomed animals [5, 25, 44]. Many cytokines are involved in the inflammatory process triggered during ophidian envenomation [20, 44]. The determination of serum cytokines leads to better understand the inflammatory process after snake envenomation. IL-10 increase can be considered as a restoration of homeostasis, regulating the processes induced by pro-inflammatory cytokines and chemical mediators. Similar results were obtained with one LD50 of *Bothrops asper* venom injected by i.p. route, which induces two peaks of pro-inflammatory cytokines at 4 and 18 h after envenomation. The pro-inflammatory cytokines are also involved in the activation of mediators which are responsible for the systemic inflammatory response [22, 48]. IL6 and IL1β elevation in muscle was observed after injection of *B. asper* venom [11]. However, a rapid increase in IL-6 against a later onset of IL1 and TNF was observed after *B. asper* venom injected by s.c. route [44]. Promutoxine (R49 sPLA$_2$) isolated from *Probothrops muscrosquamatus* venom induces a release of IL12, TNF, IL1β and IL6 cytokines from a culture of human monocytes and IL2, IL6 and TNF cytokine from human T lymphocytes [64]. Promutoxine is catalytically inactive due to the presence of an arginine residue instead of the aspartate at position 49. According to Fukahara et al. [28], high plasmatic concentrations of both cytokines IL-1β and TNF-α lead to IL-6 release and some pro inflammatory factors production (nitric oxide (NO), platelet activating factor (PAF), leukotrienes and prostaglandins). All of these

pro-inflammatory mediators are involved in the systemic inflammatory response syndrome (SIRS) [20, 47, 48]. Furthermore, IL-6 plays a key role in the synthesis of acute-phase proteins in the liver [1, 35]. Moreover, in the case of accidental envenoming, the plasmatic release of IL-6 with high level has been often correlated with the severity of envenomation [28]. In this work, the ability of CC2-PLA_2 and the whole venom of *C. cerastes* to activate the proteins of complement was also investigated. Previous studies have already demonstrated that tissue damage and pulmonary edema were the main effects observed during envenomation with *Viperidae* venoms; therefore, complement system activation may contribute to those tissue alterations [5]. Otherwise, complement system activation may contribute to chemotactic migration of leukocytes, to degranulation of phagocytic cells and also to an increased vascular permeability [5]. Eosinophil peroxidase is a hemoprotein heterodimeric strongly cationic with 71–77 kDa. It is released by activated eosinophils by mediators of the inflammatory cascade. The assessment of eosinophil sequestration in pulmonary tissue by eosinophil peroxidase assay showed this enzyme in azurophilic granules of eosinophils [12]. Eosinophil peroxidase can generate entire free radicals exerting cytotoxic effects. These results indicate that CC2-PLA_2 is able to induce inflammationless pronounced when compared to that of injected animal with crude venom. On the other hand, high activity was observed with CC3-SPase, a serine proteinase purified from the *C. cerastes* venom [15].

In conclusion of this work, a novel basic platelet-aggregation-inhibiting factor denominated CC2-PLA_2 was purified from *C. cerastes* venom. It shared sequence homology with other PLA_2 purified from other snake venoms and therefore; 51 % of its amino acid sequence has been identified. CC2-PLA2 appears to be a multi-functional molecule since it exhibited indirect hemolytic and platelet aggregation-inhibition activities. In addition, CC2-PLA_2 induced a marked elevation of plasmatic levels of IL-6 and IL-10, eosinophil peroxidase and complement lytic activities and also it provoked a drastic increase of leukocytes in peripheral blood accompanied by a rapid intense migration of neutrophils to the peritoneal cavity. Obtained result revealed also a significant increase in total amount of leukocytes mainly in polynuclear neutrophils followed by mononuclear cell recruitment. This study contributes to better understanding how a single molecule can be endowed with several bioactivities. Thus, here the functional mechanisms of one of anti-aggregative phospholipase A_2 have been studied since this molecule might have potential clinical applications.

Acknowledgments This work was partially supported by ATRSS, Agence Thematique de la Recherche Scientifique en Santé (Thematic Agency of Scientific Resarech on Health, Oran, Algeria). The authors want to thank; Jean-Claude Rousselle and Benayad Tahar (Institut Pasteur, Plate-Forme de Protéomique, CNRS URA 2185, 75724 Paris cedex15, France and Laboratory of Food Security and the Environment, Scientific Police, Algiers, Algeria) for proteomic analysis and technical assistance.

Conflict of interest Authors have no potential conflicts of interest.

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