Biochimie 92 (2010) 1063–1071

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Enzymatic and structural characterization of a basic phospholipase A_2 from the sea anemone *Condylactis gigantea*

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ARTICLE INFO

Article history:
Received 31 March 2010
Accepted 12 May 2010
Available online 26 May 2010

Keywords:
Sea anemones
Condylactis gigantea
Phospholipase A_2
Primary structure
Functional characterization
Molecular modeling
Phylogenetic analysis

ABSTRACT

This work aimed at the isolation and structural/functional characterization of a phospholipase A_2 ($CgPLA_2$) from the extract of the anemone *Condylactis gigantea*. $CgPLA_2$ was isolated with a high purity level through three chromatographic steps, showing pI 8.6 and molecular weights of 14,500 and 29,000 for the monomer and dimer, respectively. $CgPLA_2$ showed a high catalytic activity upon fluorescent phospholipids inducing no direct hemolytic activity. This enzyme, which is Ca^{2+}-dependent, showed a lower stability against temperature and pH variations when compared with snake venom enzymes. The enzymatic activity was significantly reduced or completely abolished after chemical modification of $CgPLA_2$ with BPB. Its cDNA was then obtained, with 357 base pairs which codified for a mature protein of 119 amino acid residues. A comparative analysis of the primary structure of $CgPLA_2$ revealed 84%, 61%, 43% and 42% similarity to the PLA_2s from *Adamsia carciniopados*, *Nematostella vectensis*, *Vipera russelli russelli* and *Bothrops jararacussu*, respectively.

1. Introduction

Anemones are sessile coelenterates that protect themselves against predation through the release of different types of polypeptide toxins. The importance of marine organisms as source of new bioactive agents is growing, since marine species comprise around one half of the global biodiversity, being rich sources for the search of new compounds [1] and of natural molecules to be evaluated regarding their pharmacological activities [2]. Many different types of compounds have been obtained from marine organisms, and they differ in several aspects from those produced by terrestrial organisms.

Venoms isolated from different cnidarian classes, such as sea anemones and corals, contain a complex of cytolytic and cytotoxic proteins. Among these, phospholipases (PLA_2s), hemolysins and pore-forming peptides are well documented [3–9]. In addition to the enzymatic activities induced by toxins isolated from sea anemones [8–11], these toxins can show several other effects, including antimicrobial, antiparasite or action on ion channels [12–15].

PLA_2s (E.C. 3.1.1.4) are enzymes that catalyze the hydrolysis of 2-acyl ester bonds of 3-sn-phospholipids producing fatty acids and lysophospholipids [16,17]. These enzymes are related to several important roles in the dietary lipid catabolism, in cell membrane metabolism and inflammatory diseases, in addition to their pharmacological and pathological effects on living organisms and animal envenomation [16–19].

Until now, *Condylactis gigantea* and several other sea anemone species were little investigated regarding the active principles present in the venom of their tentacles. The present study describes the first report on the cDNA sequence as well as the enzymatic activity of a phospholipase A_2 isolated from *C. gigantea* extract ($CgPLA_2$).

Abbreviations: $CgPLA_2$, *Condylactis gigantea* phospholipase A_2; PLA_2s, phospholipases A_2; BthTX-II, *Bothrops jararacussu* Asp49-PLA_2; BPB, *p*-bromophenacyl bromide.

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doi:10.1016/j.biochi.2010.05.007

2. Material and methods

2.1. Material

The specimens of the giant Caribbean anemone *C. gigantea* used in this work were collected in Havana coast (Cuba) during an one year period. The anemone extract was prepared using the external tissue of the anemone's body column according to Romero et al. [20]. BthTX-II, an Asp49-PLA_2 from *Bothrops jararacussu* snake venom, was isolated by ion-exchange chromatography on CM-Sepharose using 0.05 M ammonium bicarbonate buffer, pH 8.0, as previously described [21]. The fluorescent substrates Acyl 6:0 NBD phospholipids, NBD-phosphatidylcholine (PC), NBD-phosphatidylglycerol (PG), or NBD-phosphatidic acid (PA) were purchased from Avanti Polar Lipids Inc., Alabaster, AL, USA.

2.2. Isolation and biochemical characterization

2.2.1. Isolation of the phospholipase A_2 from *Condylactis gigantea* ($CgPLA_2$)

The dry extract of *C. gigantea* (200 mg) was fractioned by chromatography on a CM-Sepharose column (2 × 20 cm) previously equilibrated with 0.05 M ammonium bicarbonate (AMBIC), pH 8.0. Elution was performed using a continuous concentration gradient up to 1.0 M at a flow rate of 0.5 ml/min. The last fraction, presenting phospholipase but not direct hemolytic activity, was collected, concentrated by ultrafiltration (membrane for MW 10,000) and then applied on a Phenyl-Sepharose CL-4B column (1.5 × 25 cm) previously equilibrated with 0.05 M Tris–HCl, pH 7.4, containing 4 M NaCl. A linear reverse gradient of NaCl (4–0 M) was applied and fractions of 2.5 mL/tube were collected at a flow rate of 0.4 mL/min at 25 °C. The active fraction containing the desired protein was lyophilized (4 mg), dissolved in 150 μL of 5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA), homogenized and centrifuged at 480 × *g* for 2 min, and then submitted to a reverse phase HPLC (Akta Purifier, GE) using a C18 column (2.0 × 25.0 cm). The column was equilibrated in solvent A (5% acetonitrile, 0.1% TFA) and elution proceeded with a concentration gradient from 30 to 100% of solvent B (60% acetonitrile, 0.1% TFA), flow rate = 1 mL/min, during 70 min. Active fraction was ultrafiltrated in an AMICON system using membranes for MW < 10,000. All purification steps were performed at room temperature (26 ± 3 °C). The fractions were monitored in a Beckman DU-640 spectrophotometer at 280 nm, then concentrated, lyophilized and stored at 4°C. The purity level of the $CgPLA_2$ was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the enzyme samples were lyophilized and stored for posterior use in pharmacological and biochemical characterization. Estimation of proteins in solutions followed the microbiuret method, as previously described [22].

2.2.2. Biochemical characterization of $CgPLA_2$

SDS-PAGE was performed according to the methodology previously described [23]. Molecular weight standard proteins were purchased from Sigma Chem. Co. (bovine albumin 66,000; ovalbumin 45,000; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle 36,000; bovine carbonic anhydrase 29,000; bovine pancreas trypsinogen 24,000; soybean trypsin inhibitor 20,100 and bovine lactoalbumin 14,200). $CgPLA_2$ p*I* was determined by isoelectric focusing according to the method previously described [24]. The mol. wt of PLA_2 was estimated by mass spectrometry (Quattro II, Micromass). N-terminal sequencing and analysis by mass spectrometry were performed as described before [25,26]. Sequence analysis followed using protein databases (http://www.expasy.org).

2.2.3. Phospholipase A_2 activity

Phospholipase A_2 activity was evaluated using two different methods: a) the indirect hemolysis on agar gel and b) hydrolysis of fluorescent phospholipids. In the indirect hemolytic method, samples containing anemone extracts or $CgPLA_2$ were prepared at different doses (1–50 μg) in PBS (phosphate buffered saline) ($n = 3$) and applied in holes in the agar gels that were kept at 37 °C for 12 h [27]. The other method was performed using the fluorescent phospholipids NBD-PC, NBD-PA, NBD-PG as substrates, according to Rodrigues et al. [28]. The influence of cations was examined in solutions containing 50 mM Tris–HCl, pH 7.5, replacing Ca^{2+} by other divalent ions (final concentration 5 mM). The influence of pH was also evaluated by incubating $CgPLA_2$ in different pH values (3.5–12.5) for 30 min, followed by evaluation of the enzymatic activity, performed as previously described.

The chemical modification of $CgPLA_2$ was performed by incubation with BPB (5 mM, final concentration) dissolved in dimethyl sulfoxide (DMSO). Incubation proceeded for 4 h at 4 °C and then the excess reagent was removed through an ultrafiltration system (AMICON-YM3). Controls were run simultaneously using PLA_2, DMSO or Tris–HCl buffer, pH 7.5.

2.2.4. Edema-inducing activity

Groups of four male Swiss mice (18–22 g) were injected in the subplantar region with various amounts of crude extract or $CgPLA_2$ (in a volume of 50 μL) prepared in PBS, pH 7.2. Then the paw increase was measured at different time intervals (30, 60, 120 and 180 min), subtracting the initial paw measure (time 0 h). The paw edema was measured with the aid of a low-pressure pachymeter (Mitutoyo, Japan).

2.2.5. Myotoxic activity

Groups of four male Swiss mice (18–22 g) were injected in the right gastrocnemius muscle with crude extract or PLA_2 (50 μg/50 μL of PBS) or PBS alone (50 μL). After 3 h, blood was collected from the tail in heparinized capillary tubes and centrifuged for plasma separation. Activity of creatine kinase (CK) was then determined using 4 μL of plasma, which was incubated for 3 min at 37 °C with 1.0 mL of the reagent according to the kinetic CK-UV protocol from Bioclin, Brazil. The activity was expressed in U/L, where one unit corresponds to the production of 1 mmol of NADH per minute.

2.3. Molecular characterization of the phospholipase A_2

2.3.1. cDNA cloning

Tissue samples from the body wall of the anemone *C. gigantea* were collected and macerated in a solution containing 4 M guanidine isotiocyanate (Sigma Chem Co.), 17 mM N-lauryl sarcosine, 25 mM trisodium citrate, and 0.7% β-mercaptoethanol. The total RNA was extracted using the phenol method and guanidine isotiocyanate [29]. Approximately 4 μg of the total RNA were used for synthesis of the first cDNA strand of $CgPLA_2$, in the presence of 5 U of reverse transcriptase (Mu-MLV) and oligo(dT) for 1 h at 37 °C. The second strand of cDNA was synthesized using specific oligonucleotides for amplification of the PLA_2 gene. The primers used were: PLA-F (5′-acnytntggtgyggnatgg-3′), PLA_2-R (5′-catgcaggtcttccttc-3′) and PLA-I (internal: 5′-ggatgvmaydgayhggtgctg-3′) based on the N and C-terminal sequences and the catalytic site domain of PLA_2s from *C. gigantea* (this work), *Rhopilema nomadic* and *Adamsia carciniopados* [30,31]. For the PCR procedure, the conditions were: 95 °C/3 min. (1 cycle), 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s (25 cycles) and 72 °C/10 min (1 cycle). Amplification of the DNA fragment was analyzed on a 1.5% (w/v) agarose gel and purified. The C-terminal was treated with T4 DNA polymerase and, after phosphorylation, the fragment was ligated into the pUC18 vector,