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Molecular cloning of two toxic phospholipases A_2 from the crown-of-thorns starfish *Acanthaster planci* venom

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Abstract

The full-length cDNAs encoding two toxic phospholipases A_2 (AP-PLA_2-I and -II) from the crown-of-thorns starfish *Acanthaster planci* venom were individually cloned by RT-PCR, 3′RACE and 5′RACE. In common with both AP-PLA_2s, the precursor protein is composed of a signal peptide, a propeptide and a mature protein (136 and 135 residues for AP-PLA_2-I and -II, respectively). The four motifs (Ca^{2+}-binding loop, Ca^{2+}-binding site, active site and catalytic network) characteristic of groups I and II PLA_2s are well conserved in both AP-PLA_2s. In addition to this, the presence of the elapid and pancreatic loops and the involvement of a propeptide in the precursors suggested that AP-PLA_2s are highly analogous to the group IB PLA_2s. However, when compared to the amino acid sequence of bovine pancreatic PLA_2, the representative group IB PLA_2, AP-PLA_2s require some amino acid insertions and deletions in the region 76–100, as previously observed for the starfish *Asterina pectinifera* PLA_2s. Furthermore, the phylogenetic tree made clearly demonstrated that AP-PLA_2s and *A. pectinifera* PLA_2s are distinguishable from the group IB PLA_2s as well as other PLA_2s, being classified into a new group.

Keywords: *Acanthaster planci*; cDNA cloning; Crown-of-thorns starfish; Phospholipase A_2; Phylogenetic tree; Primary structure; Secondary structure; Toxin

1. Introduction

The crown-of-thorns starfish *Acanthaster planci* inhabiting tropical and subtropical waters preys on coral polyps, thereby damaging coral reefs. This starfish is also known to have a number of venomous spines on the body surface. When stung by the spines, various pathological symptoms such as severe pain, redness, swelling and protracted vomiting are induced. The crude toxin extracted from the spines exhibits the following diverse biological activities: mouse lethality, hemolytic activity, myonecrotic activity, hemorrhagic activity, capillary permeability-increasing activity, edema-forming activity, phospholipase A_2 (PLA_2) activity (Shiomi et al., 1985), histamine-releasing activity from mast cells (Shiomi et al., 1989), cardio-vascular actions (Yara et al., 1992; Shiroma et al., 1994) and anticoagulant activity (Karasudani et al., 1996). In view of this, the spines seem to contain a variety of toxins. So far, two lethal factors (plancitoxins I and II; Shiomi et al., 2004), two PLA_2s (AP-PLA_2-I and -II; Shiomi et al., 1998) and one anticoagulant factor (plancinin; Karasudani et al., 1996) have been purified as toxic factors from the spines of *A. planci*. Active PLA_2s have also been identified in the venoms of sponges and cnidaria (Nevalainen et al., 2004, 2005).

Plancitoxins of 37 kDa are quite unique not only in having potent hepatotoxicity (Shiomi et al., 1990, 2004) but also in structural resemblance to mammalian deoxyribonucleases II (Shiomi et al., 2004), which are implicated in DNA degradation during apoptosis (programmed cell death) (Counis and Torriglia, 2000) and/or in engulfment-mediated DNA degradation (Evans and Aguilera, 2003). However, they are not responsible for the local inflammation induced upon stinging by the *A. planci* spines, because they exhibit no substantial biological activities other than lethal activity. In addition, it is not fully understood whether plancinin of 7 kDa is associated with the local inflammation. On the other hand, AP-PLA_2-I and -II have hemorrhagic and capillary permeability-increasing activities and hence are considered to be deeply involved in the

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local inflammation (Shiomi et al., 1998). AP-PLA_2-I is a homodimer comprising the same subunit of 15 kDa and AP-PLA_2-II is a monomer of 15 kDa. Both AP-PLA_2s have been clarified only for their N-terminal amino acid sequences (up to the 61st residue).

Elucidation of the primary structures of AP-PLA_2s is the first step toward understanding their toxic actions at the molecular level. The present paper deals with the primary structures of AP-PLA_2s determined by cloning and sequencing of the cDNAs encoding them.

2. Materials and methods

2.1. Purification of AP-PLA_2s

AP-PLA_2-I and -II were purified from the spines of *A. planci* by a combination of hydrophobic chromatography, gel filtration and reverse phase high-performance liquid chromatography (HPLC), as reported previously (Shiomi et al., 1998). Proteins were quantified by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.2. Reduction and pyridylethylation or carboxymethylation

AP-PLA_2-I (300 μg) and II (250 μg) were individually dissolved in 300 μl of 0.5 M Tris–HCl buffer (pH 8.5) containing 6 M guanidine hydrochloride and 2.5 mM EDTA and reduced with 2 mg of dithiothreitol in the dark at room temperature for 1 h. Then, the AP-PLA_2-I solution was added with 2 μl of 4-vinylpyridine and the AP-PLA_2-II solution with 40 mg of iodoacetamide. Each solution was further kept in the dark at room temperature for 1 h. Pyridylethylated AP-PLA_2-I thus produced was purified by reverse phase HPLC on a TSKgel Phenyl 5PW-RP column (0.46 × 7.5 cm; Tosoh, Tokyo, Japan), which was eluted by a linear gradient of acetonitrile (0–70% in 60 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. For the purification of carboxymethylated AP-PLA_2-II, reverse phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh) was adopted. The column was eluted by a linear gradient of acetonitrile (0–80% in 30 min) in 0.1% TFA at a flow rate of 1 mL/min.

2.3. Enzymatic digestion

Pyridylethylated AP-PLA_2-I (150 μg) was dissolved in 100 μl of 0.1 M ammonium hydrogencarbonate containing 8 M urea. After incubation at 37 °C for 1 h, the solution was diluted with 300 μl of 0.1 M ammonium hydrogencarbonate, added with 12 μg of TPKC treated trypsin (Worthington Biochemical, Lakewood, NJ, USA) and digested at 37 °C for 22 h. Carboxymethylated AP-PLA_2-II (75 μg) was dissolved in 200 μl of 0.1 M ammonium hydrogencarbonate solution and digested with 2 μg of V8 protease (Wako, Tokyo, Japan) at 37 °C for 20 h. To isolate peptide fragments, both digests were separately applied to reverse phase HPLC on a TSKgel ODS-120T column. The column was eluted by a linear gradient of acetonitrile (0–42% in 80 min for the digest of pyridylethylated AP-PLA_2-I and 24.5–49% in 60 min for the digest of carboxymethylated AP-PLA_2-II) in 0.1% TFA at a flow rate of 1 mL/min. Some isolated peptides were randomly selected and analyzed for their amino acid sequences by the Edman degradation method with a gas-phase protein sequencer (LF-3400D TriCart with high sensitivity; Beckman Coulter, Fullerton, CA, USA).

2.4. Cloning experiments

A part (10 g) of the arm portion was obtained from an *A. planci* sample stored at −80 °C and ground to a fine powder in liquid nitrogen. Total RNA was extracted from the powdered sample with the TRIzol reagent (Lifetech Oriental, Tokyo, Japan). First strand cDNA was synthesized from 5 μg of total RNA using a 3′RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Designations and nucleotide sequences of the primers used in the following cloning experiments are summarized in Table 1. A fragment of cDNA encoding either AP-PLA_2-I or -II was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using degenerate primers (I-1f, I-1r, I-2f and I-2r for AP-PLA_2-I and II-1f, II-1r, II-2f and II-2r for AP-PLA_2-II) designed from the determined partial amino acid sequences. Amplification was performed using Ex Taq Polymerase (Takara, Otsu, Japan) under the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 48 (first PCR) or 55 °C (nested PCR) for 30 s and 72 °C for 1 min; and 72 °C for 7 min. The nested PCR products were subcloned into the pT7Blue T-vector (Novagen, Darmstadt, Germany). Nucleotide sequences of the PCR products were determined using a Thermo Sequence Cy5 Dye Terminator kit (Amersham Biosciences, Piscataway, NJ, USA) and a Long-Read Tower DNA sequencer (Amersham).

Based on the partial nucleotide sequences determined by RT-PCR, the remaining sequences were analyzed by 3′ and 5′ rapid amplification of cDNAs ends (RACE). 3′RACE was performed using a 5′/3′RACE Kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. The 3′-terminal cDNA fragments were amplified by the first 3′RACE, followed by the nested 3′RACE, using the gene-specific primers (I-3 and I-3n for AP-PLA_2-I and II-3 and II-3n for AP-PLA_2-II) and the oligo dT-anchor primer under the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; and 72 °C for 7 min. For 5′RACE, the 5′RACE System for Amplification of cDNA Ends kit (Invitrogen) was used. First strand cDNA was synthesized from 4.5 μg of total RNA using the gene specific primers (I-5s for AP-PLA_2-I and II-5s for AP-PLA_2-II) and used as a template. The 5′RACE reactions were completed using the gene-specific primers (I-5 and I-5n for AP-PLA_2-I and II-5 and II-5n for AP-PLA_2-II) and the adaptor primer (for the first 5′RACE) or universal amplification primer (for the nested 5′RACE). Amplification was carried out under the same conditions as adopted for 3′RACE.