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Research paper

Purification, characterization and bactericidal activities of basic phospholipase A_2 from the venom of *Agkistrodon halys* (Chinese pallas)

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Abstract

Agkistrodon snake venoms contain a variety of phospholipases (PLA_2), some of which are myotoxic. In this study, we used reverse-phase HPLC to purify PLA_2 from the venom of *Agkistrodon halys*. The enzyme named as AgkTx-II, a basic Asp49 PLA_2, has a molecular masses of 13,869.05. The amino acid sequence and molecular mass of AgkTx-II was identical to those of an Asp49 basic myotoxic PLA_2 previously isolated from this venom. Antibacterial activities were tested by susceptibility and broth-dilution assays. AgkTx-II exerted a potent antibacterial activity against *Staphylococcus aureus*, *Proteus vulgaris*, *Proteus mirabilis*, and *Burkholderia pseudomallei*. The MIC values of AgkTx-II ranged between 85 and 2.76 μM and was most effective against *S. aureus*, *P. vulgaris*, *P. mirabilis* (MIC of 21.25 μM) and *B. pseudomallei* (MIC of 10.25 μM). This AgkTx-II rapidly killed *S. aureus*, *P. vulgaris* and *B. pseudomallei* in a dose-dependent manner. The effect of the AgkTx-II on bacterial membranes was evaluated by scanning and transmission electron microscopy. AgkTx-II caused morphological alterations apparent on their cellular surfaces, suggesting a killing mechanism based on membrane permeabilization and damage. Cytotoxicity was measured by XTT tetrazolium (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) and lactate dehydrogenase (LDH) assays using U-937 cells (monocytes). The AgkTx-II did not affect cell viability up to 500 μM concentrations but cell death was evident at 1000 μM concentration after 24 and 48 h. Furthermore, the repeated exposure of AgkTx-II (2–14 μM) treated mice showed different tissue alterations, mainly at the brain and kidney; the toxicological potential of AgkTx-II remains to be elucidated. The AgkTx-II exhibits no hemolytic action even at high doses (10–100 μM) in human erythrocytes. However, the AgkTx-II is believed to exert its bactericidal effect by permeabilizing the bacterial membrane by forming pores. In addition, the basic PLA_2 AgkTx-II displays a bactericidal effect, which may be either dependent or independent of catalysis.

Keywords: Bactericidal; Myotoxin; Phospholipase A_2; Amino acid sequence; *Agkistrodon halys*; Cytotoxicity

1. Introduction

Secreted phospholipases A_2 (PLA_2) constitute a distinct group of enzymes that are abundant in animal venoms. Snake venom PLA_2s display varied pharmacological properties which include myotoxicity [1,2], edema formation [3], presynaptic [4] plus post-synaptic neurotoxicity [5], cardiotoxicity [6] and platelet aggregation [7,8]. In addition to their catalytic activity of hydrolyzing the *sn*-2 ester bond of glycerophospholipids, $sPLA_2$s display an array of biological actions which may be either dependent or independent of catalysis [9,10]. The group IIA $sPLA_2$ of mammalian origin has been

Abbreviations: AgkTx-II, *Agkistrodon halys* toxin; LPS, lipopolysaccharide; TFA, trifluoroacetic acid; PLA_2, phospholipases A_2; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MIC, minimum inhibitory concentration; MH, Mueller Hinton; TS, Tryptic Soy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; RPMI, Roswell Park Memorial Institute; XTT, tetrazolium salts; LDH, lactate dehydrogenase; FBS, fetal bovine serum; TBS, Tris-buffered saline.

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shown to exert potent bactericidal activity [11,12]. The bactericidal activity of this protein involves both the recognition of anionic sites, and the enzymatic degradation of phospholipids in the target membranes, preferentially Gram-positive species. On the other hand, killing of Gram-positive species by this enzyme requires a synergistic action with bactericidal permeability-increasing protein, but is equally dependent on enzymatic membrane degradation [13]. In contrast to the mammalian group IIA $sPLA_2$, isolated from *Bothrops asper* (also classified within group IIA) snake venom, was shown to directly kill both Gram-positive and Gram-negative bacteria [14]. Neurotoxic activities of two myotoxic phospholipases A_2 from *Bothrops neuwiedi pauloensis* snake venom showed bactericidal activity against *Escherichia coli* and *Staphylococcus aureus* [15]. Several authors reported that the basic phospholipase A_2 isolated from *Agkistrodon halys* showed hemolytic and anticoagulant activities [16]. Basic PLA_2 of *A. halys* showed Ca^{2+} binding properties [17]. Furthermore, in common with other snake venom PLA_2s, Lys49-PLA_2s also possess combined myotoxic and cytolytic activities [2]. Membrane leakage is induced by the synergistic action of Lys-49 and Asp-49 type PLA_2s purified from *Agkistrodon piscivorus piscivorus* [18]. Previous studies showed that this protein interacts with lipopolysaccharide (LPS) and lipid A from different Gram-negative bacteria or with lipoteichoic acid from *Staphylococcus aureus*, and relies on a membrane-permeabilizing mechanism to exert its bactericidal effects [18]. LPS is a complex molecule composed of a fatty acid (lipid A), an *O*-polysaccharide chain, and a core sugar inserted into the outer membrane of Gram-negative bacteria.

Antimicrobial proteins and peptides are cationic in nature, and are believed to exert their bactericidal effect by permeabilizing the bacterial membrane by forming pores [19], thinning the membrane [20], or by destabilizing the membrane bilayer [21]. In addition to membrane permeabilization, antimicrobial proteins and peptides kill bacteria by inhibition of macromolecular biosynthesis [22] and/or interacting with specific vital components inside the bacteria. The *in vitro* antibacterial activity of AgkTx-II has not yet been studied extensively. However, structural evidence for the membrane changes in bacteria induced by AgkTx-II is completely lacking. In this study, we have reported the isolation, characterization, and N-terminal amino acid sequences (primary structure analysis) with special emphasis for the *in vitro* testing of AgkTx-II against a number of clinical isolates, mainly *Burkholderia pseudomallei* (TES and KHW) strains under standard conditions. We have also studied the mode of action as per induced morphological and structural changes on bacteria, with a membrane-dependent mechanism of action for basic myotoxic PLA_2 from *Agkistrodon halys* venom.

2. Materials and methods

2.1. Extraction of venoms

Agkistrodon halys (Chinese pallas) venom was extracted from long-term captive specimens by milking in a sterile manner under strict laboratory conditions. It was centrifuged at 4 °C, frozen and lyophilized within 6 h of extraction. The freeze-dried venom was packed and stored in the dark at −20 °C. Solid venom of *A. halys* was also purchased from commercial sources (Venom Supplies Pte Ltd, Tanunda, South Australia).

2.2. High-performance liquid chromatography

A sample (500 mg) of *Agkistrodon halys* venom was dispersed into 10 ml of 50 mM Tris–hydrochloric acid buffer (pH 7.4), and the suspension was centrifuged at 500 × *g* at 4 °C for 10 min. Aliquots of the yellowish clear supernatant were applied on a Superdex G-75 column (1.6 × 40 cm; Amersham Pharmacia, Sweden) previously equilibrated and eluted with the same buffer. Fractions (2 ml each) were collected at a flow rate of 15 ml/h. Absorbance of all the gel-filtration fractions (AH1–AH6) were monitored at 280 nm. Six fractions (AH1–AH6) were collected from the single pool of venom fractionated on a Superdex column, with aliquots taken for testing biochemical activities and protein measurement. The fraction (AH1) with highest antibacterial and PLA_2 activity was further purified by reverse-phase (RP)-HPLC on C18 and resolved into five fractions (AH-F1, AH-F2, AH-F3, AH-F4, and AH-F5) monitored at 215 nm. The most active AH-F3 was fractionated by a C8 column (250 × 4.60 mm, 5 μm, 100 A, Phenomenexr®) to obtain two proteins AgkTx-I and AgkTx-II in 0.1% aqueous trifluoroacetic acid (TFA), and eluted with a linear gradient of 80% acetonitrile (ACN) in 0.1% TFA. All steps of the purification procedure were carried out at room temperature (25 °C) [23]. The basic myotoxic PLA_2 was named AgkTx-II, lyophilized, as well as used for pharmacological characterization and amino acid sequence determination.

2.3. Biochemical characterization

Electrophoresis materials were SDS, 30% acrylamide/bisacrylamide, bromophenol blue, 2-mercaptoethanol, sodium dodecyl sulfate, Coomassie brilliant blue R-250, ammonium persulfate, glycerol, and TEMED (Sigma, St Louis, MO, USA). Dye reagent for the protein assay was from Bio-Rad, CA, USA, and other reagents were from Sigma. The dye-staining method of Bradford [24] was used for quantification of venom proteins and purified AgkTx-II. Bovine serum albumin (1 mg/ml) was used to establish the standard curve. The crude venom as well as purified AgkTx-II was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [25]. Separating gels containing 15% acrylamide and stacking gels of 4.5% acrylamide were used. The fractions were diluted (1:1) with sample buffer (0.12 M Tris–HCl, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and heated for 5 min in a boiling water bath. Electrophoresis was carried out at a constant current 20 mA for 2.5 h. The gel was fixed with 5% acetic acid overnight and stained for 2 h in 0.1% Coomassie blue R-250 in 5% acetic acid. Destaining was carried