Myotoxic activity of a Gln-49 phospholipase A_2 from *Agkistrodon blomhoffii ussurensis* snake venom*

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Abstract A novel myotoxic protein phospholipase A_2 (PLA_2), denoted as Gln49-PLA_2, has been isolated from snake venom of *Agkistrodon blomhoffii ussurensis*, which has weak lethal effect and apparent anticoagulant activity, but lacks the PLA_2 and hemorrhagenic activity. Gln49-PLA_2 obviously increases of plasma creatine-kinase (CK) upon intramuscular injection in mice, suggesting that it may induce a dose-dependent myonecrosis. Histological studies also reveal morphological changes in mouse skeletal muscles, including extensive myonecrosis, hemorrhage and neutrophil infiltration in the treated animals. The myotoxic ability induced by Gln49-PLA_2 can be partially inhibited by heparin.

Keywords: **snake venom, phospholipase A_2, *Agkistrodon blomhoffii ussurensis*, myotoxic activity.**

The phospholipase A_2 (PLA_2, EC3.1.1.4), a group of enzymes widely spread in many animal tissues, especially in the pancreatic juice of mammals, and venoms from snakes and insects, catalyze the 2-acyl ester bond in 3-sn-phosphoglycerides which produces free fatty acids and lysolphospholipids. They induce edema and inflammatory responses and affect platelet aggregation. Based on their amino acid sequences and cellular locations, snake venom PLA_2s are classified into group I and group II. The latter one can be further divided into two major subgroups, namely, those with an aspartic acid residue at the 49 site (Asp49-PLA_2) having high catalytic activity and those with a lysine at position 49 (Lys49-PLA_2), which have low or no detectable catalytic activity[1]. Naturally occurring PLA_2-homologues in which Asp49 is changed to Ser were also reported[2].

We have recently reported a novel PLA_2-homologue isolated from *Agkistrodon blomhoffii ussurensis* snake venom with Gln at the site 49[3], which demonstrated a higher homology with Asp49-PLA_2 variants (94%—79%) than with Lys49-PLA_2 variants (63%—60%). Besides the change at position 49, some invariant residues in the Asp49-PLA_2 group, such as Asn79 and Arg116 have been found to be replaced by Asp79 and Ile116 in Gln49-PLA_2; and some conserved residues in Lys49-PLA_2 group, as Gln11, Glu12, Gly23, Asn28, Lys53 and Ser74, were not conserved in Gln49-PLA_2, suggesting that Gln49-PLA_2 is a new member of the PLA_2 family.

Snake venom PLA_2 shows marked differences in biological activities in spite of their structural homology. Among these properties, myotoxicity is less understood. The PLA_2 myotoxins may induce muscle necrosis in a specific way[4,5], or cause cytolytic effects[6]. Phospholipase A_2s (PLA_2s) with myotoxic activity belong to type Ⅱ myotoxins[7]. The PLA_2-myotoxins are the main component of myotoxins identified in snake venom from virtually every family and genus examined[8], and divided into three groups[7]: Group Ⅰ includes the presynaptic neurotoxins with PLA_2s hydrolytic activity, such as crotoxin[9]. Group Ⅱ includes the non-neurotoxins with PLA_2s hydrolytic activity, such as myotoxin Ⅰ[10] and a myotoxin from *Vipera russelli* venom[11]. Group Ⅲ includes myotoxin PLA_2s which exhibit very low or no detectable hydrolytic activity, such as a myotoxin from *Bothrops nummifer* venom[12] and myotoxin Ⅱ from *Bothrops moojeni* venom[13]. Myotoxic PLA_2s may also be inhibited by heparin and other polyanions, because they have a high content of lysine residues[14].

Agkistrodon blomhoffii ussurensis is commonly

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found in northeast of China, Korea and Russia. Snake venom from *Agkistrodon blomhoffii ussurensis* contains many kinds of active proteases, such as thrombin-like enzyme, L-amino acid oxidase, and phospholipase A_2. In this study, some biological activities of Gln49-PLA_2 isolated from *Agkistrodon blomhoffii ussurensis* and its myotoxic effects on inducing myonecrosis of mouse skeletal muscles were investigated.

1 Materials and methods

1.1 Purification of Gln49-PLA_2

Purification of Gln49-PLA_2 was performed as described previouly[3].

1.2 SDS—polyacrylamide gel electrophoresis

SDS—PAGE was carried out with gradient gels (4%—10% polyacrylamide) under reducing conditions[15]. Proteins were detected by Coomassie brilliant blue R250 staining.

1.3 Assay of phospholipase A_2 activity

PLA_2 activity was determined by the method of Kawauchi et al.[16] using *sn*-3-phosphatidylcholine as the substrate.

1.4 Hemorrhagic activity analysis

Hemorrhagic activity was estimated by the method of Nikai et al.[17], the hemorrhagic status was observed by opening mice abdomens 24 h after injection of Gln-PLA_2.

1.5 Lethality assay

Groups of 10 mice (18—22 g body weight) were injected with different amounts of Gln49-PLA_2 dissolved in saline, by intraperitoneal route. Controls were injected with saline. Death rate was recorded after 48 h injection and the mean lethal dose (LD_{50}) was estimated by the Spearman-Karber method[18].

1.6 Myotoxic activity analysis

Gln49-PLA_2 from *Agkistrodon Blomhoffii Ussurensis* snake venom (50, 75, 100 and 150 μg), dissolved in 50 μL of PBS buffer, was injected into the left gastrocnemius muscle of the groups of 4 mice after being anesthetized with methoxyflurane. Control group received 50 μL of PBS. After 3 h, blood samples were collected and creatine kinase (CK) levels in plasma were determined at OD_{340} using a detection CK kit. Activity was expressed as U/L, one unit resulting in the phosphorylation of 1 μmol of creatin per minute at 37 ℃.

Meanwhile formalin-fixed muscle tissue samples were obtained and processed for histological examination of muscle damage. Sections of the muscles were stained with hematoxyline and eosin.

1.7 Heparin-binding activity

To investigate the inhibitory effect of heparin on Gln49-PLA_2, three groups of 4 mice were injected with 50 μg heparin, 50 μg heparin + 50 μg Gln49-PLA_2 (incubated for 20 min at 25 ℃) or 50 μg Gln49-PLA_2 (dissolved in 50 μL PBS). Then the myotoxicity assay was carried out as mentioned above.

2 Results

2.1 Biochemical properties of Gln49-PLA_2

Some biochemical properties were determined with the isolated Gln49-PLA_2. Gln49-PLA_2 has a poor lethal effect. The LD_{50} was determined to be 18.2 mg/kg (Fig. 1). No phospholipase A_2 activity was detected on egg yolk phospholipids and no hemorrhagenic activity was observed even with the injection of 4 mg/mouse.

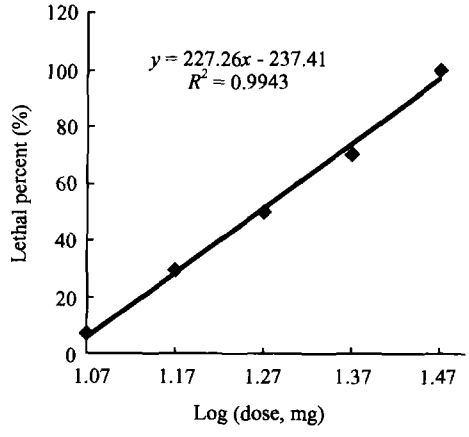

Fig. 1. Lethal dose of of Gln49-PLA_2.

2.2 Myotoxic activity

Purified Gln49-PLA_2 from *Agkistrodon Blomhoffii Ussurensis* venom induced a dose-dependent myonecrosis upon intramascular injection in mice, as evidenced by the significant increase in plasma CK activity (Fig. 2). At the dose of 100 μg/mouse, the plasma CK level showed an increase up to 20 times of the control value. Microscopic observation

also showed obvious morphological changes in skeletal muscle induced by Gln49-PLA_2 (Fig. 3 (a), (b)). Sections of the muscles revealed that there were extensive myonecrosis, hemorrhage and neutrophil infiltration, but the control mice had no these changes.

2.3 Heparin-binding activity

Based on the measurement of plasma CK levels (Fig. 2) and histological examination of tissue samples (Fig. 4), heparin pre-incubated with Gln49-PLA_2 from *Agkistrodon Blomhoffii Ussurensis* snake venom could partially inhibit the myotoxic ability of Gln49-PLA_2 and prevent myonecrosis in gastrocnemius muscle of mice. Results demonstrated that there was a marked decrease of CK activity (58%) in plasma of the mice treated with heparin-treated Gln49-PLA_2, and the same morphological changes were observed in the muscles of the mice exposed to Gln49-PLA_2 and the Gln49-PLA_2 pre-incubated with heparin. The morphotogical changes included myonecrosis and hemorrhage (Fig. 3), while no obvious changes were found in the muscles of the mice treated with heparin only.

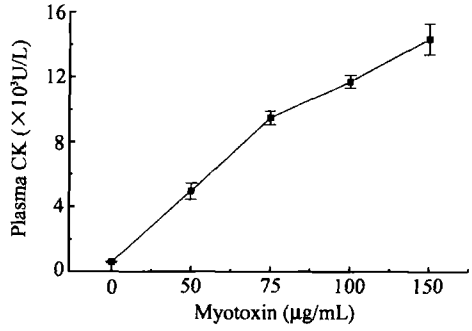

Fig. 2. Myotoxic activity of Gln49-PLA_2 homologue from *Agkistrodon Blomhoffii Ussurensis* venom. Plasma CK level was determined 3 h after the injection of the myotoxin in the gastrocnemius of mice. Each point represents the mean ± SD of four animals.

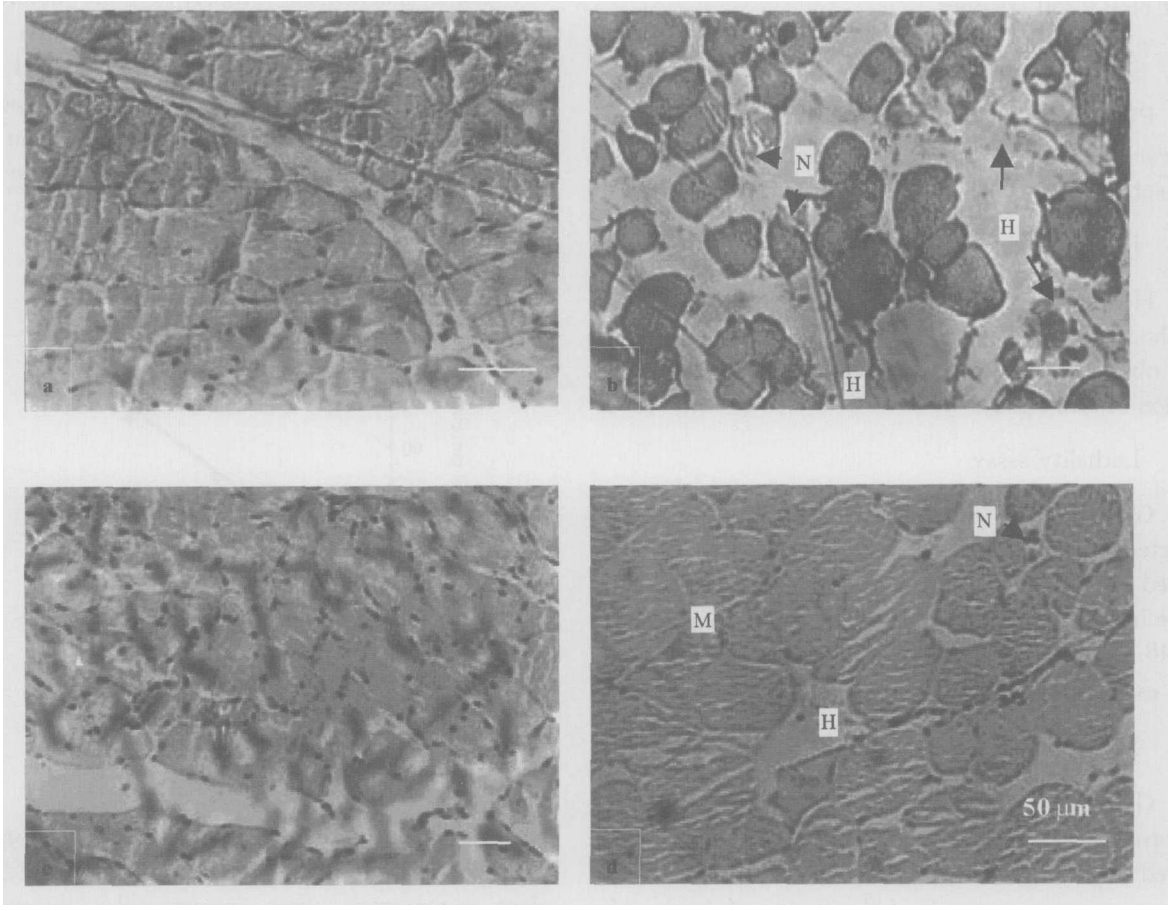

Fig. 3. Micrographs of mouse skeletal muscles. Micrographs of mouse gastrocnemius muscles taken 3 h after the injection of (a) 50 μL PBS, (b) Gln49-PLA_2 (50 μg) from *Agkistrodon Blomhoffii Ussurensis* venom, (c) heparin (50 μg) alone and (d) phospholipase A_2 homologue homologue (50 μg) pre-incubated with heparin (50 μg). M, normal muscle cells; N, necrotic muscle cells; H, hemorrhage. The myofibrils appeared normal in (a) and (c). There are extensive hemorrhage (H) and necrotic muscle cells in (b) whereas myonecrosis and hemorrhage were significantly reduced in (d).

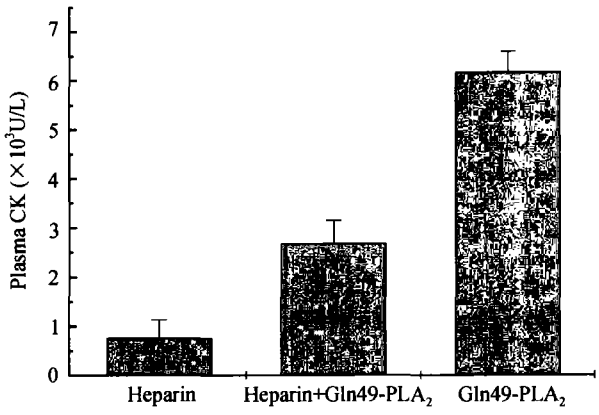

Fig. 4. The effect of heparin on Gln49-PLA_2 homologue from *Agkistrodon Blomhoffii Ussurensis* venom. Plasma CK level was determined 3 h after the injection of heparin (50 μg), phospholipase A_2 homologue pre-incubated (50 μg) with heparin (50 μg) or Gln49-PLA_2 homologue (50 μg). Each point represents the mean ± SD of four animals.

3 Discussion

Phospholipase A_2 can specifically catalyze the 2-acyl ester bond in 3-sn-phosphoglyceride. Calcium is an essential cofactor for catalysis, and Asp49 is essential for Ca^{2+} binding. Naturally occurring PLA_2-homologues in which Asp49 is changed to Lys[19], Ser[2] or Ala, are therefore catalytically inactive. Crystal structures of Lys49-PLA_2 homologues reveal that the N (ε-NH_2) atom of Lys49 occupies the position of the calcium ion in the catalytically active Asp49-PLA_2[20,21]. The results of Gln49-PLA_2, with a Gln instead of Asp at position 49, do not show any phospholipase A_2 activity, which is probably due to loss of its ability to bind the co-factor Ca^{2+}.

In previous reports, Lys49 and Asp49 PLA_2s might exert their myotoxic activity by different mechanisms: the former may utilize their C-terminal regions as main membrane-destabilizing elements (which combine more cationic and hydrophobic amino acids)[22]; and the latter probably involve their catalytic activity as a relevant step[14]. But some evidence showed that the PLA_2 catalytic activity is not necessary for myotoxicity[23]. The novel Gln49-PLA_2 we purified from *Agkistrodon Blomhoffii Ussurensis* snake venom shows none of the phospholipase A_2 catalytic activity, but with an obvious myotoxic activity, suggesting that its myotoxic activity is independent of PLA_2 catalytic activity. The relationship between the enzymatic activity and myotoxic activity should be further studied by measuring the site mutant product of Gln49-PLA_2 gene.

Usually, the interactions of PLA_2 with heparin are electrostatic in nature, originating from the highly negative charge density of heparin. But increasing facts show that heparin can bind to PLA_2 via both electrostatic interaction and non-electrostatic interaction, and this binding is quite specific to the sequence of the amino acids near the carboxy-terminus[24]. The investigation of heparin-binding activity on PLA_2-myotoxin is helpful to understanding the mechanism of action of myotoxic PLA_2 and PLA_2 homologues.

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