

Myotoxic activity of a Gln-49 phospholipase A₂ from *Agkistrodon blomhoffii ussurensis* snake venom*

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Abstract A novel myotoxic protein phospholipase A₂ (PLA₂), denoted as Gln49-PLA₂, has been isolated from snake venom of *Agkistrodon blomhoffii ussurensis*, which has weak lethal effect and apparent anticoagulant activity, but lacks the PLA₂ and hemorrhagic activity. Gln49-PLA₂ 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-PLA₂ can be partially inhibited by heparin.

Keywords: snake venom, phospholipase A₂, *Agkistrodon blomhoffii ussurensis*, myotoxic activity.

The phospholipase A₂ (PLA₂, 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 lysolipids. They induce edema and inflammatory responses and affect platelet aggregation. Based on their amino acid sequences and cellular locations, snake venom PLA₂s 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-PLA₂) having high catalytic activity and those with a lysine at position 49 (Lys49-PLA₂), which have low or no detectable catalytic activity^[1]. Naturally occurring PLA₂-homologues in which Asp49 is changed to Ser were also reported^[2].

We have recently reported a novel PLA₂-homologue isolated from *Agkistrodon blomhoffii ussurensis* snake venom with Gln at the site 49^[3], which demonstrated a higher homology with Asp49-PLA₂ variants (94%—79%) than with Lys49-PLA₂ variants (63%—60%). Besides the change at position 49, some invariant residues in the Asp49-PLA₂ group, such as Asn79 and Arg116 have been found to be replaced by Asp79 and Ile116 in Gln49-PLA₂; and some conserved residues in Lys49-PLA₂ group, as

Gln11, Glu12, Gly23, Asn28, Lys53 and Ser74, were not conserved in Gln49-PLA₂, suggesting that Gln49-PLA₂ is a new member of the PLA₂ family.

Snake venom PLA₂ shows marked differences in biological activities in spite of their structural homology. Among these properties, myotoxicity is less understood. The PLA₂ myotoxins may induce muscle necrosis in a specific way^[4,5], or cause cytolytic effects^[6]. Phospholipase A₂s (PLA₂s) with myotoxic activity belong to type II myotoxins^[7]. The PLA₂-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 PLA₂s hydrolytic activity, such as crotoxin^[9]. Group II includes the non-neurotoxins with PLA₂s hydrolytic activity, such as myotoxin I^[10] and a myotoxin from *Vipera russelli* venom^[11]. Group III includes myotoxin PLA₂s 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 PLA₂s 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 A₂. In this study, some biological activities of Gln49-PLA₂ 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-PLA₂

Purification of Gln49-PLA₂ was performed as described previously^[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 A₂ activity

PLA₂ 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-PLA₂.

1.5 Lethality assay

Groups of 10 mice (18—22 g body weight) were injected with different amounts of Gln49-PLA₂ dissolved in saline, by intraperitoneal route. Controls were injected with saline. Death rate was recorded after 48 h injection and the mean lethal dose (LD₅₀) was estimated by the Spearman-Kärber method^[18].

1.6 Myotoxic activity analysis

Gln49-PLA₂ 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 OD₃₄₀ 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 °C.

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-PLA₂, three groups of 4 mice were injected with 50 μg heparin, 50 μg heparin + 50 μg Gln49-PLA₂ (incubated for 20 min at 25 °C) or 50 μg Gln49-PLA₂ (dissolved in 50 μL PBS). Then the myotoxicity assay was carried out as mentioned above.

2 Results

2.1 Biochemical properties of Gln49-PLA₂

Some biochemical properties were determined with the isolated Gln49-PLA₂. Gln49-PLA₂ has a poor lethal effect. The LD₅₀ was determined to be 18.2 mg/kg (Fig. 1). No phospholipase A₂ activity was detected on egg yolk phospholipids and no hemorrhagic activity was observed even with the injection of 4 mg/mouse.

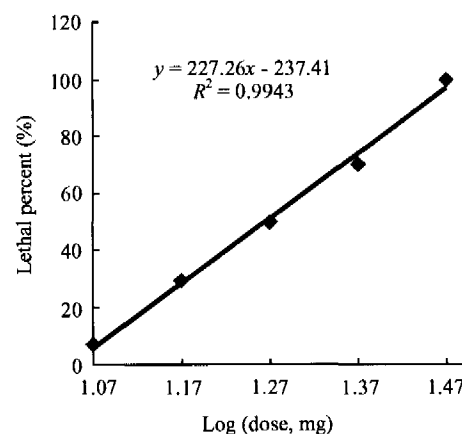


Fig. 1. Lethal dose of of Gln49-PLA₂.

2.2 Myotoxic activity

Purified Gln49-PLA₂ from *Agkistrodon Blomhoffii Ussurensis* venom induced a dose-dependent myonecrosis upon intramuscular 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-PLA₂ (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-PLA₂ from *Agkistrodon Blomhoffii Ussurensis* snake venom could partially inhibit the myotoxic ability of Gln49-PLA₂ 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-PLA₂, and the same morphological changes were observed in the muscles of the mice exposed to Gln49-

PLA₂ and the Gln49-PLA₂ pre-incubated with heparin. The morphological changes included myonecrosis and hemorrhage (Fig. 3), while no obvious changes were found in the muscles of the mice treated with heparin only.

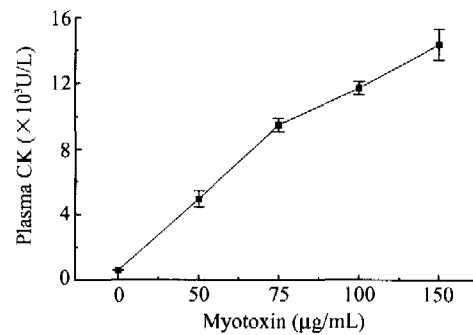


Fig. 2. Myotoxic activity of Gln49-PLA₂ homologue from *Agkistrodon Blomhoffii Ussurensis* venom. Plasma CK level was determined 3 h after the injection of the myotoxin in the gastrocnemius of mice. Each point represents the mean ± SD of four animals.

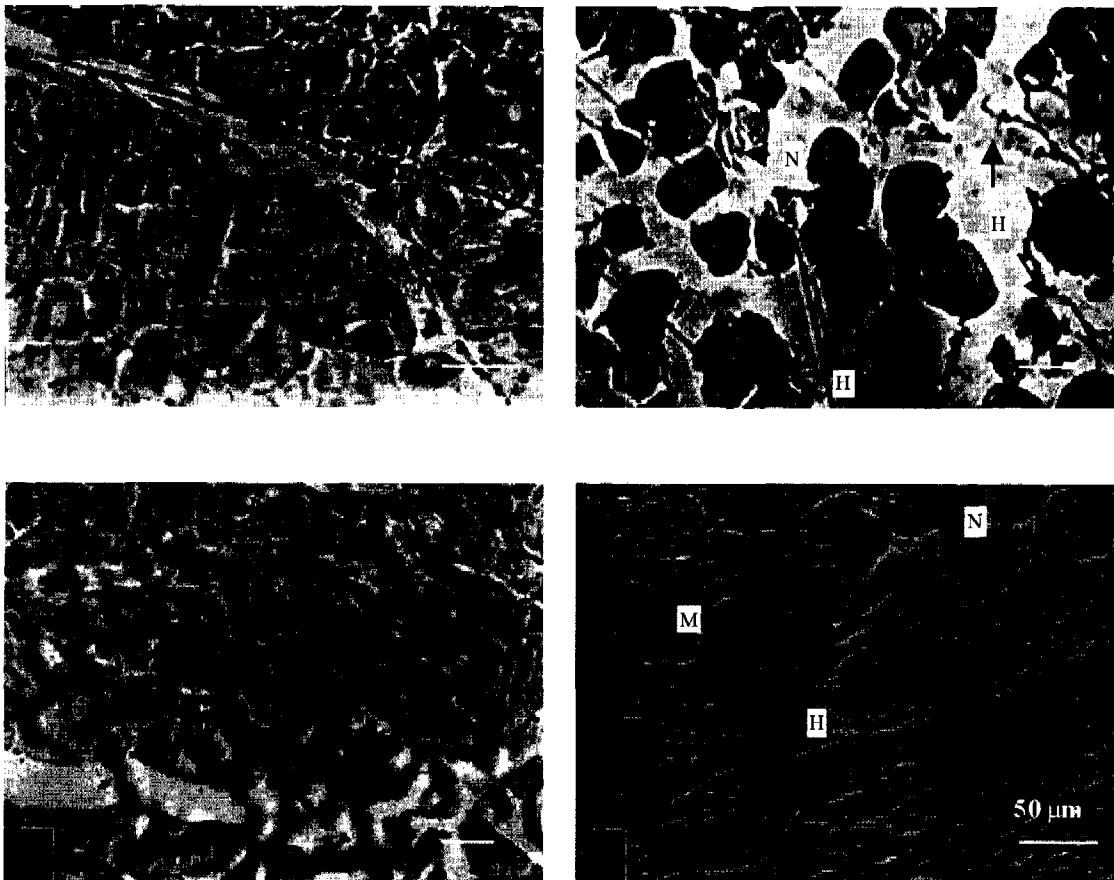


Fig. 3. Micrographs of mouse skeletal muscles. Micrographs of mouse gastrocnemius muscles taken 3 h after the injection of (a) 50 µL PBS, (b) Gln49-PLA₂ (50 µg) from *Agkistrodon Blomhoffii Ussurensis* venom, (c) heparin (50 µg) alone and (d) phospholipase A₂ homologue homologue (50 µg) pre-incubated 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).

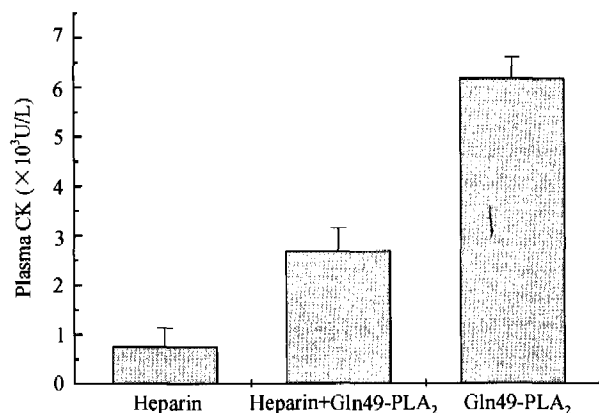


Fig. 4. The effect of heparin on Gln49-PLA₂ homologue from *Agkistrodon Blomhoffii Ussurensis* venom. Plasma CK level was determined 3 h after the injection of heparin (50 μg), phospholipase A₂ homologue pre-incubated (50 μg) with heparin (50 μg) or Gln49-PLA₂ homologue (50 μg). Each point represents the mean ± SD of four animals.

3 Discussion

Phospholipase A₂ can specifically catalyze the 2-acyl ester bond in 3-sn-phosphoglyceride. Calcium is an essential cofactor for catalysis, and Asp49 is essential for Ca²⁺ binding. Naturally occurring PLA₂-homologues in which Asp49 is changed to Lys^[19], Ser^[2] or Ala, are therefore catalytically inactive. Crystal structures of Lys49-PLA₂ homologues reveal that the N (ε-NH₂) atom of Lys49 occupies the position of the calcium ion in the catalytically active Asp49-PLA₂^[20,21]. The results of Gln49-PLA₂, with a Gln instead of Asp at position 49, do not show any phospholipase A₂ activity, which is probably due to loss of its ability to bind the co-factor Ca²⁺.

In previous reports, Lys49 and Asp49 PLA₂s 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 PLA₂ catalytic activity is not necessary for myotoxicity^[23]. The novel Gln49-PLA₂ we purified from *Agkistrodon Blomhoffii Ussurensis* snake venom shows none of the phospholipase A₂ catalytic activity, but with an obvious myotoxic activity, suggesting that its myotoxic activity is independent of PLA₂ catalytic activity. The relationship between the enzymatic activity and myotoxic activity should be further studied by measuring the site mutant product of Gln49-PLA₂ gene.

Usually, the interactions of PLA₂ with heparin are electrostatic in nature, originating from the highly negative charge density of heparin. But increasing facts show that heparin can bind to PLA₂ 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 PLA₂-myotoxin is helpful to understanding the mechanism of action of myotoxic PLA₂ and PLA₂ homologues.

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