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Biochemical and biological activities of the venom of the Chinese pitviper *Zhaoermia mangshanensis*, with the complete amino acid sequence and phylogenetic analysis of a novel Arg49 phospholipase A_2 myotoxin

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Abstract

Zhaoermia mangshanensis (formerly *Trimeresurus mangshanensis*, *Ermia mangshanensis*) represents a monotypic genus of pitviper known only from Mt Mang in China's Hunan Province, and is among the largest and most spectacular of Asian venomous snakes. The venom of *Zhaoermia* exhibits high coagulant activity on bovine and human fibrinogen and human plasma, high phosphodiesterase and arginine ester hydrolytic activity, and moderate to low L-amino acid oxidase, kallikrein, caseinolytic, phospholipase A_2 (PLA_2), haemorrhagic and myotoxic activities. The approximate i.p. LD_{50} of the venom in mice was estimated to be 4 mg/kg. We purified the major toxin of *Zhaoermia* venom by gel-filtration, cation-exchange chromatography and HPLC. The toxin, a homodimer with an experimental monomeric mass of 13,972 Da, induced edema and myonecrosis in mice, but was devoid of detectable PLA_2 catalytic activity. Its complete amino acid sequence is composed of 121 amino acid residues cross-linked by seven disulfide bridges, and shows more than 80% identity to two Lys49-PLA_2s from distantly related Asian pitvipers, *Protobothrops mucrosquamatus* and *Calloselasma rhodostoma*. Phylogenetic analysis of the novel toxin, zhaoermiatoxin, confirmed that it is rooted within a comprehensive sample of Lys49-PLA_2s despite having an arginine residue in position 49, suggesting a secondary Lys49 → Arg substitution which did not alter the catalytic inactivity of the molecule.

Keywords: Squamata; Viperidae; Crotalinae; *Zhaoermia mangshanensis*; *Ermia*; *Trimeresurus*; *Protobothrops flavoviridis*; Snake venom; Coagulation; Edema; Enzymatic activity; Fibrinogen; Arg49-phospholipase A_2; Asp49-phospholipase A_2; Lys49-phospholipase A_2; Phylogeny; Myotoxin; Toxicity; China

1. Introduction

The Mt Mang Viper, *Zhaoermia mangshanensis* (Zhao in Zhao and Chen, 1990), is one of the most recently discovered members of the *Trimeresurus* complex, a major evolutionary radiation of pitvipers in Asia that

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contains more than 50 species (Gumprecht et al., 2004). As a consequence of progress in reconstructing their evolutionary relationships, these species are currently arranged in several genera (Malhotra and Thorpe, 2004; also reviewed in Gumprecht et al., 2004). In line with these developments, the Mt Mang Viper that had originally been described as a member of *Trimeresurus* was soon referred to the new monotypic genus *Ermia* Zhang (1993) based on osteological features (Zhang, 1998). This concept and the unique phylogenetic position of *E. mangshanensis* were also supported by recent molecular studies (Herrmann et al., 2004; Malhotra and Thorpe, 2004). Subsequently, Gumprecht and Tillack (2004) found that the name *Ermia* was preoccupied, having been proposed previously for a genus of locusts. In such cases, the rules of zoological nomenclature (ICZN, 1999) require replacement of the junior synonym by another available name. The name *Zhaoermia* chosen by Gumprecht and Tillack (2004) satisfies this requirement while supporting nomenclatural transparency by using the previous name as part of the replacement name.

Zhaoermia mangshanensis was first discovered on Mt Mang (=Mang Shan), Pingkeng District, Yizhang County, Hunan Province, People's Republic of China, where it has been found in forests at elevations from 700 to 1300 m (Zhao and Chen, 1990; Chen in David and Tong, 1997). To date, the known range of these snakes is restricted to this mountain range, where they are believed to occur in an area spanning only a few tens of square kilometres (Chen in David and Tong, 1997), rendering this species vulnerable to habitat destruction or overcollecting.

Mt Mang Vipers are spectacular snakes. Comparable in many ways to the bushmasters (*Lachesis* spp.) of the Neotropics, they may be the biggest of Asian pitvipers, reaching total lengths in excess of 2.1 m and considerable girth, with body weights of more than 5 kg on record (Chen in David and Tong, 1997; Gumprecht et al., 2002). Their broad and massive heads are shaped in a heart-like form, and palpation suggests that adult specimens harbour large venom glands with considerable lumen. The fangs of *Zhaoermia* are also very long, which along with hissing as part of their defensive behaviour and the availability of large quantities of venom may contribute to the observed ability of these snakes to 'spit' venom over a distance when striking at enemies (Chen in David and Tong, 1997; Gumprecht et al., 2002). In captivity, juveniles of this oviparous, rodent-eating pitviper are irritable, whereas adult specimens have appeared relatively mild-tempered. However, when cornered and fully aroused, the size and striking range of these snakes alone suffice to render them very redoubtable adversaries.

While several *Protobothrops* species attaining comparable total lengths are widely recognized for their medical importance and consequently have received due attention from toxinologists, nothing seems to be known about cases of envenoming by *Z. mangshanensis*, or general properties of its venom. Tsai et al. (2004a) isolated and characterized seven phospholipases A_2 (PLA_2; EC 3.1.1.4) apparently from a venom sample of this species (under the name of *Protobothrops mangshanensis*) and analyzed their N-terminal sequences, documenting the presence of a remarkably diverse set of subtypes of these important venom components. Here, we report on biochemical and biological activities of *Zhaoermia* venom, its toxicity for mice, and the isolation and characterization of its major toxin, a novel edema-inducing basic PLA_2 myotoxin named zhaoermiatoxin.

2. Materials and methods

2.1. Snake venoms

Zhaoermia mangshanensis venom was collected from an adult male snake (ca. 2 m total length, 2350 g body weight) maintained in A.G.'s live collection. The snake was manually restrained and venom was collected by placing an opened 1.5 ml cryovial over one half-erected fang. Approximately, 1.5 ml of yellowish liquid venom was obtained and lyophilized. The dry weight of the venom sample, obtained from only one fang, was more than 960 mg. The venom of *Protobothrops flavoviridis* (WHO standard venom for *Trimeresurus flavoviridis*, i.e., lyophilized pooled venom of Habu snakes from Okinawa Island, Japan) was used for comparison. All dry venoms were stored at 4 °C.

2.2. Enzyme assays

Assays of snake venom enzymatic activities followed Mebs (1970). For all enzyme assays, aliquots of the tested snake venoms were dissolved in saline. Assays were carried out in triplicate, and 1 U of activity was defined as the quantity of enzyme that hydrolysed 1 μmol of substrate per minute. Proteinase activity was assayed by photometrical determination of casein hydrolysis, where 1 U was defined as the amount of enzyme that in 20 min at 37 °C caused an increase of 0.001 in absorbance (at 280 nm) of trichloroacetic acid supernatant. Arginine ester hydrolysis (Schwert and Takenaka, 1955) was assayed photometrically (at 253 nm, 25 °C) using the synthetic substrate benzoyl-arginine ethyl ester (BAEE, Serva). Phospholipase A_2 activity was assayed using egg yolk suspension as substrate (Marinetti, 1965); a decrease in absorbance (at 900 nm) of 0.01 in 10 min at 37 °C was defined as 1 U of enzymatic activity. Phospholipase A_2 activity of venom fractions was assayed after reconstituting venom protein in 1 ml 0.1 M ammonium acetate (pH 4.5), and incubating aliquots of 10, 20 and 100 μl with 5 ml egg yolk suspension for 4 h. Phosphodiesterase activity was assayed using the synthetic substrate bis(*p*-nitrophenyl)phosphate (Sigma) and measuring the increase in absorbance (at 405 nm, 25 °C). L-amino