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Snake venom glutaminyl cyclases: Purification, cloning, kinetic study, recombinant expression, and comparison with the human enzyme☆

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ABSTRACT

Among various snake venom components, glutaminyl cyclase (vQC) is one of the least understood protein family and none of its members has been purified or characterized. Here we confirmed the presence of vQC activity in a wide spectrum of venom species via enzymatic assay using a synthetic fluorogenic substrate. We have also cloned novel vQC cDNAs from seven species including *Crotalus atrox*. The results revealed more than 96% sequence similarities among vQCs and ~75% sequence identities between vQCs and human secretory QC (hQC). The vQC glycoprotein of 43 kDa was isolated from *C. atrox* venom, and its N-terminal sequence was determined. The optimal pH range for vQC reaction was 7.5–8.0, and the enzymes were stable up to 50 °C. Similar to hQC, vQCs were substantially inactivated by 1 mM 1,10-phenanthroline but slightly affected by 20 mM EDTA, suggestive of a similar zinc-catalytic environment for these enzymes. Although their catalytic residues were highly conserved, vQCs were less susceptible to inhibition by synthetic imidazole derivatives which potently inhibited hQC. The 3D-models revealed that vQC and hQC structures display different surface charge distributions around the active sites, which might affect substrate and inhibitor binding affinities. The recombinant vQCs prepared from *Escherichia coli* displayed weaker substrate binding affinities relative to the native vQCs, possibly due to the lack of glycosylation. The present report offers new structural and functional insights into vQCs and sheds light on the specificity differences between vQCs and hQC.

1. Introduction

As one of the enzymes for protein post-translational modifications, glutaminyl cyclase (QC; glutaminyl-peptide cyclotransferase, EC 2.3.2.5) catalyzes N-terminal pyroglutamate (pGlu) formation on proteins or peptides and the release of ammonia or water molecules (Seifert et al., 2009). This modification appears to be important for structural stability, resistance to aminopeptidase degradation, and interaction of the proteins or peptides with their partners (Stephan et al., 2009). It has been found that the animal QCs are either Golgi-resident or secreted enzymes

Abbreviations: Ah, N-ω-acetylhistamine; Bi, 1-benzylimidazole; PBD150, 1-(3,4-dimethoxyphenyl)-3-[(3-(1*H*-imidazol-1-yl) propyl]thiourea; QC, glutaminyl cyclases; hQC, human secreted glutaminyl cyclase; βNA, β-naphthylamine; PNGase F, peptide *N*-glycosidase F; pGlu, pyroglutamate; PAP, pyroglutamyl aminopeptidase.

☆ Eight nucleotide sequences encoding the vQCs of *Crotalus atrox*, *Micrurus fulvius*, *Cerrophidion godmani*, *Daboia russelii*, *Trimeresurus gracilis*, *Protobothrops mucrosquamatus*, and *Sistrurus catenatus tergeminus* have been deposited in GenBank with the accession numbers JF979132–JF979139JF979132JF979133JF979134JF979135JF979136JF979137JF979138-JF979139, respectively.

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(Schilling et al., 2007). In humans, aberrant expression of hQC is related to certain pathological conditions, such as Alzheimer disease (Jawhar et al., 2011). X-ray crystallography and site-directed mutagenesis studies of recombinant human secreted QC (hQC) have provided much information about the catalytic mechanism of QCs (Huang et al., 2005a, 2008). Notably, hQC inhibitors have been synthesized and developed as drugs to treat the relevant diseases (Buchholz et al., 2009; Schilling et al., 2003a).

Presumably because of the N-terminal pGlu modification, a number of snake venom proteins or toxins were reported to be resistant to Edman-degradation during sequence analysis. The well-known cases include bradykinin-potentiating peptides (Ferreira, 1965; Murayama et al., 1997), metalloproteases (Huang et al., 2002), endogenous metalloprotease inhibitors (Wagstaff et al., 2008), crotoxin A (Chen et al., 2004; Faure et al., 2011), crotalphine (Konno et al., 2008), and dendrotoxin (Harvey and Robertson, 2004). Although several cDNA sequences encoding snake venom QCs (vQCs) could be found in databanks (Pawlak and Kini, 2006), none of the vQCs have been isolated or assayed. Whether vQCs are useful models for the drug development and applications related to hQC also remains to be evaluated and explored.

Advances in the method for QC activity assays (Schilling et al., 2002) and the discovery of new hQC inhibitors (Buchholz et al., 2009) have rendered better tools to explore vQCs. In the present study, we have assayed the QC activities in more than 18 venom species with a fluorogenic substrate. One of the vQC has been isolated and its N-terminal sequence was determined for the first time. Furthermore, we successfully cloned and sequenced the vQC cDNAs from seven representative species and compared the sequences with those of human and other animal QCs (Huang et al., 2008). We also examined the thermal stability, optimal pH, and the effects of metal ions, metal chelators, and synthetic QC inhibitors on the representative vQCs. Because the vQC sequences indicated three N-glycosylation sites, we investigated the effect of glycan-removal on the vQC activities. Furthermore, recombinant vQCs were prepared using *Escherichia coli* and their kinetic properties were studied. Finally, the structure homology models of vQCs were generated and compared. The results provide new insights into the occurrence, enzymology, mechanism and phylogenetics of this venom protein family.

2. Materials and methods

2.1. Snake venoms and venom glands

The pooled venom samples of *Bothrops atrox*, *Crotalus atrox*, *Crotalus horridus atricaudatus*, *Crotalus durissus terrificus*, *Daboia russelii*, *Echis pyramidium*, *Micrurus fulvius* and *Naja haje* were purchased from Kentucky Reptile Zoo (KY, USA). *Cerrophidion godmani*, *Causus rhombeatus*, *Cerastes vipera* and *Dendroaspis angusticeps* venoms were obtained from Miami Serpentarium Laboratories (FL, USA). *Protobothrops elegans* venom was purchased from La Toxan (Valence, France). *Pseudechis australis* venom was acquired from Venom Supplies Ltd. (Tanunda, South Australia). *Laticauda semifasciata* venom was obtained from Sigma Chemical (MO, USA). Venom and venom glands of *Deinagkistrodon acutus* and *Protobothrops mucrosquamatus* were obtained from antivenin manufacture group of Disease Control Center, Taiwan. A healthy specimen of *M. fulvius* was obtained from Glades Herp Co. (Fort Myers, Florida, USA). After anesthesia of the snake, the venom glands were dissected for the preparation of mRNA and cDNA. Previously, mRNA was extracted from the fresh venom glands and the cDNAs have been prepared for *C. atrox* (Tsai et al., 2001), *C. godmani*, *S. c.tergeminu*, *D. russelii* (Tsai et al., 2007), *P. mucrosquamatus* (Tsai et al., 1995), and *Trimeresurus gracilis* (Tsai et al., 2012).

2.2. Biochemicals and other reagents

Gln-β-naphthylamide (Gln-βNA) and recombinant pyroglutamyl aminopeptidase (PAP) from *Bacillus amyloliquefaciens* were purchased from Bachem (Bubendorf, Switzerland) and Qiagen (Hilden, Germany), respectively. Peptide *N*-glycosidase F (PNGase F) was obtained from New England Biolabs. (Ipswich, UK). Restriction enzymes and the pGEM-T easy vector were acquired from Promega Corp. (WI, USA). Buffers and other chemicals were purchased from Merck Co. (Darmstadt, Germany) or Sigma–Aldrich Co. (MO, USA). The synthetic inhibitors, 1-(3,4-dimethoxyphenyl) -3-[(3-(1*H*-imidazol-1-yl) propyl]thiourea (PBD150), N-ω-acetylhistamine (Ah), 1-benzylimidazole (Bi), and hQC were gifts from our colleague, Prof. A. H.-J. Wang (Huang et al., 2011).

2.3. Protein determination and vQC assay

Crude venoms were dissolved in reagent grade water and quantified using a BCA protein assay kit (Pierce Chemical Co., IL, USA) with bovine serum albumin as a standard. The vQC activities were assayed using the fluorogenic substrate Gln-βNA in the presence of an accessory enzyme pyroglutamyl aminopeptidase (PAP) (Huang et al., 2011). A 96-well microplate was incubated at 30 °C, and each well contained 0.025 units PAP in 170 μl 50 mM Tris–HCl (pH8.0), which was sufficient to hydrolyze pGlu-βNA instantaneously. A small aliquot (10 μl) of the enzyme sample was added to each well and mixed, and the reaction was initiated by adding Gln-βNA to a final concentration of 0.50 mM (i.e., $[S] >> K_m$). Using a SpectraMax M2 fluorescent spectrometer reader (Molecular Devices Corp. CA, USA) with excitation and emission wavelengths at 320 and 405 nm, respectively, the fluorescence increase was monitored for 10–20 min. For kinetic analyses, the substrate concentrations varied over a 20-fold range and the reaction rates were determined in triplicate. The initial rates were calibrated by a standard curve of the fluorescence of 2.5–50 μM βNA. The specific activity was determined based on the initial rate and the protein concentration.

2.4. The pH profile and the thermal stability

The venom solution was mixed with various buffers (final concentration 50 mM), including: acetate (pH 3.5, 4.0, 4.5 and 5.0), MES (pH 5.5, 6.0 and 6.5), HEPES (pH 7.0 and