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Molecular cloning and characterization of a complement-depleting factor from king cobra, *Ophiophagus hannah*

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ABSTRACT

Cobra venom factor (CVF) is an anti-complement factor existing in cobra venom. CVF proteins have been purified from the venoms of *Naja haje*, *Naja siamensis*, *Naja atra*, *Naja kaouthia*, *Naja naja*, *Naja melanoleuca* and *Austrelaps superbus*, but only three full-length cDNA sequences of CVF are available. In the present work, a cobra venom factor termed OVF was purified from the crude venom of *Ophiophagus hannah* by successive gel filtration, ion-exchange and heparin affinity chromatography steps. The purified OVF was homogenous on the SDS-PAGE gel with an apparent molecular weight of 140 kDa under non-reducing conditions. Under reducing conditions, OVF was divided into three bands with apparent molecular weight of 72 kDa (α chain), 45 kDa (β chain) and 32 kDa (γ chain), respectively. OVF consumed complement components with anti-complement activity of 154 units per mg. By using Reverse transcription-PCR and 5′-RACE assay, the open reading frame of OVF was obtained. MALDI-TOF and protein sequencing assays confirmed the cloned cDNA coding for OVF protein. The cDNA sequence of OVF is conservative when aligned with that of other CVFs. Phylogenetic analysis revealed OVF is closer to CVF from *N. kaouthia* than to AVF-1 and AVF-2 from *A. superbus*. Our results demonstrated that OVF has its unique features as following: 1) The N-terminal amino acid sequence of OVF γ chain is different from that of other known CVFs, suggesting that the OVF γ chain might be further processed; 2) Unlike *N. kaouthia* CVF and *A. superbus* AVF-1, which have potential N-linked glycosylation sites located in both α and β chain, OVF only has N-linked glycosylation site in its α chain as revealed by Schiff's reagent staining and protein sequence analysis; 3) In addition to the 27 well conserved cysteine residues in all known CVFs, OVF have an additional cysteine residue in its γ chain. Understanding the importance of above mentioned specific characteristics might provide useful information on structure-function relationship between CVF and complement system.

1. Introduction

The complement system is a major player in innate defense and constitutes one of the key effectors for both antibody-dependent and -independent immunity (Daha, 2010). It can be activated via three initiating pathways including the classical pathway, the lectin pathway and the alternative pathway (Daha, 2010; Ricklin et al., 2010). All of these initiating pathways will ultimately lead to the activation of the central component of complements (C3), and result in many biological functions such as anaphylaxis, chemotaxis, and phagocytosis (Daha, 2010; Ricklin et al., 2010).

CVF is an unusual venom component which exists in the venoms of different cobra species, like *Naja*, *Ophiophagus*,

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and *Hemachatus* (Vogel et al., 1996). It is a structural and functional analog of complement component C3-related molecules (Vogel et al., 1984). Like C3b, CVF binds factor B in the presence of Mg^{2+} ions and the newly formed complex will be cleaved by factor D, then generates a complex CVF,Bb and a peptide Ba (Hensley et al., 1986; Muller-Eberhard and Fjellstrom, 1971; Vogel and Muller-Eberhard, 1982). CVF,Bb is a C3/C5 convertase but exhibits considerable functional differences from other C3/C5 convertases, such as C3b,Bb and C3(H_2O),Bb generated by humans (Vogel et al., 1996). CVF,Bb is more stable than other C3/C5 convertases. Its half-life of decay-dissociation is approximately 7 h, which is far longer than C3b,Bb and C3(H_2O),Bb (Pangburn and Muller-Eberhard, 1986; Vogel and Muller-Eberhard, 1982); CVF,Bb and CVF can escape from the regulation of factors H and I, which can further cleave C3b and C3(H_2O) into inactive form (Lachmann and Halbwachs, 1975; Nagaki et al., 1978). The catalytic efficiency of CVF,Bb enzyme is weaker when compared with that of C3b,Bb and C3(H_2O),Bb (Pangburn and Muller-Eberhard, 1986; Vogel and Muller-Eberhard, 1982); CVF,Bb is a fluid C5 convertase, it does not require additional C3b for C5 cleavage (Vogel et al., 1996). As a complement-depletion factor, CVF has been widely used in a great number of researches concerning the complement's biological function in normal physiology processes and the role in the pathogenesis of autoimmune diseases (Vogel and Fritzinger, 2010). CVF and humanized CVF have even been used for therapeutic complement depletion in preclinical models of human disease, such as myocardial ischemia reperfusion injury (Fritzinger et al., 2008b; Gorsuch et al., 2009; Vogel and Fritzinger, 2010), age-related macular degeneration (Fritzinger et al., 2010), arthritis (Fritzinger et al., 2008b) and paroxysmal nocturnal hemoglobinuria (PNH) (Fritzinger et al., 2008a). Although several CVF proteins have been either fully or partially purified from different venoms from *Naja* genus and *Austrelaps* genus (Eggertsen et al., 1981; Muller-Eberhard and Fjellstrom, 1971; Osipov et al., 2005; Rehana and Manjunatha Kini, 2007; Sharma et al., 2001; Sun et al., 2001; Takahashi and Hayashi, 1982; Vogel and Muller-Eberhard, 1984; von Zabern et al., 1980, 1982), the purified native protein could not provide sufficient resource for relative research and clinic usage, because cobras are extremely endangerous species, and their venom secretions are increasingly difficult to obtain (Vogel et al., 2004). Much effort has been made to produce CVF protein by gene engineering approach (Kock et al., 2004; Kolln et al., 2007; Vogel et al., 2004). Up to now, only a few full sequences of CVF have been reported and all the reported recombinant CVF proteins were expressed based on the cDNA sequence of *Naja kaouthia* CVF. More information on proteins and cDNA sequences of CVF is necessary to reveal the important sites responsible for CVFs functions, thus facilitate the generation of humanized CVF for disease therapy.

Ophiophagus hannah is a monotypic genera of Elapidae family. It is one of the most dangerous and feared snakes in Asia (http://www.enotes.com/topic/King_Cobra). Many components of *O. hannah* have been purified and characterized, such as L-amino acid oxidase (Jin et al., 2007; Li et al., 1994; Tan and Saifuddin, 1989, 1991), phospholipase A_2 enzymes (Tan and Saifuddin, 1990), alpha-neurotoxins (Chang et al., 1993; He et al., 2004), beta-cardiotoxin (Rajagopalan et al., 2007), ohanin (Pung et al., 2005), metalloproteinases (Guo et al., 2007), OhS1 (Zhang et al., 1994).

Here, we report the purification, characterization and molecular cloning of CVF cDNA from king cobra, *O. hannah*.

2. Materials and methods

2.1. Materials

Living *O. hannah* snakes were collected in the area of Guangxi province, south of China. Lyophilized crude venom of *O. hannah* were the stock of our laboratory. Sephadex G-100 gel (superfine), Resource Q column (1 ml) and Hitrap Heparin HP column (1 ml) were the products of Amersham Bioscieces of GE-Healthcare. Schiff's reagent was obtained from sigma. Guinea pig serum was separated from fresh blood obtained by cardiac puncture. Fresh blood of sheep was obtained from Animal Center of Kunming Medical College (Kunming, China). Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the Ethics Committee of Kunming Institute of Zoology, The Chinese Academy of Sciences. Rabbit anti-sheep red blood cell hemolysin was purchased from Baiji Biotechnology Co. Ltd. (Zhengzhou, China). Total RNA isolation kit, 5′-RACE kit and first-strand cDNA synthesis kit were purchased from Invitrogen. LA-Taq DNA polymerase, DNA fragment extraction kit and pMD19-T vector were from TaKaRa Biotechnology Co. Ltd (Dalian, China). All other reagents used were analytical grade.

2.2. Purification of CVF from O. hannah venom

The lyophilized crude venom (500 mg) was dissolved in 3 ml of buffer A (50 mM Tris–HCl, pH 7.8) over night and centrifuged at 3000 g for 10 min. Then the supernatant was loaded onto a Sephadex G-100 (superfine) column which was pre-equilibrated with buffer A containing 100 mM NaCl, collecting fractions of 3.5 ml/tube, 20 min/tube. Fractions with CVF activity were pooled, dialyzed against buffer A and applied to a Resource Q column equilibrated with the same buffer on AKTA Explorer 100 FPLC system (Amersham Biosciences of GE Healthcare). After washing the unbound proteins with 10 ml buffer A, a linear salt gradient elution was achieved with buffer B (50 mM Tris–HCl, pH 7.8, containing 0.8 M NaCl). Fractions with CVF activity were pooled and dialyzed against buffer C (20 mM PBS, pH 6.5) and then loaded onto a Hitrap Heparin HP column pre-equilibrated with buffer C. After extensive washing with buffer C, a linear salt gradient elution was achieved with buffer D (20 mM PBS, pH 6.5, containing 0.5 M NaCl). During the isolation processes, absorption at 280 nm was monitored.

2.3. SDS-PAGE analysis and protein quantitation

SDS-PAGE was performed in 12% acrylamide gel according to the methods described (Laemmli, 1970). The