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Purification, cloning and characterization of a metalloproteinase from *Naja atra* venom

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ABSTRACT

The complement system is a very important part of the immune system. Many snake venoms possess activities that influence the complement. A new metalloproteinase (termed atrase B) with anticomplementary activity was purified from *Naja atra* venom. Atrase B is a single chain glycoprotein with a molecular mass of 49.4 kDa and an isoelectric point of 9.7. Its N-terminal sequence shows high homology to those of metalloproteinases from cobra venoms. The cDNA sequence reveals that atrase B is a PIII class metalloproteinase. Atrase B slowly cleaves the Aα chain of fibrinogen. It also exhibits edema-inducing activity, but has no hemorrhagic activity and proteolytic activity against fibrin, azocasein, and N-benzoyl-L-arginine ethyl ester. Interestingly, atrase B inhibits activation of the complement classical and alternative pathways in a dose- and time-dependent manner. Complement components factor B and C6 are major targets for atrase B to cleave. Atrase B is the first identified SVMP that cleaves complement components factor B, C6, C7, and C8.

1. Introduction

The complement system is a part of the innate immune system. More and more evidence revealed that the complement system plays an important role in innate and adaptive immunity (Fearon and Locksley, 1996; Sunyer and Lambris, 1998; Roozendaal and Carroll, 2006). However, abnormalities of the complement system contribute to inflammation and injury in target tissues and associate with a wide variety of diseases (Schmidt and Colten, 2000; Walport, 2001a,b; Holers, 2008). Complement inhibition by pharmacological means has become a potential therapeutic strategy for a wide range of diseases (Holers and Thurman, 2004). Although substantial progress has been made in the development of anticomplement drugs, few complement inhibitors are currently available for clinical.

Many snake venoms possess activities that affecting complement (Eggertsen et al., 1980). Thus snake venoms may probably become an important source for discovery of new or novel anticomplementary proteins. Some anticomplementary proteins have been isolated and characterized from snake venoms, including cobra venom factor (CVF), high molecular weight cobra factor (H-CoF), and complement inhibitor (CI) from cobra venoms (Ballow and Cochrane, 1969; von Zabern et al., 1981); four anticomplement proteinases from *Crotalus atrox* venom (Man and Minta, 1977); a protease purified from *Naja naja siamensis* venom (O'Keefe et al., 1988); two hemorrhagic proteinases (B1 and B2) from *Crotalus basiliscus basiliscus* venom (Molina et al., 1990); a serine proteinase from *Vipera lebetina* venom (Gasmi et al., 1994); a fibrinolytic proteinase M5 from *Crotalus molossus molossus* venom (Chen and Rael, 1997); flavoxobin, a serine proteinase from *Trimeresurus flavoviridis* venom (Yamamoto et al., 2002), and oxiagin, a metalloproteinase isolated from *Naja oxiana* cobra venom (Shoibonov et al., 2005). However, most of them were just

Abbreviations: fB, factor B; fD, factor D; NHS, normal human serum; VBS, veronal buffered saline; GGVB^{2+}, VBS containing gelatin, Ca^{2+}, Mg^{2+} and glucose; EA, antibody-sensitized sheep erythrocytes; GVB, VBS with gelatin; RRBC, rabbit red blood cells; GVB-Mg-EGTA, GVB containing Mg^{2+} and EGTA; MAC, membrane attack complex.

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investigated their effects on hemolytic complement activity or complement components C2, C3, and C4, few studies have been done on their effects on the complement components factor B (fB), factor D (fD), C5, C6, C7, C8, and C9.

In the course of screening anticomplementary protein, we found and purified a new metalloproteinase with anticomplementary activity from *Naja atra* cobra venom, which belongs to PIII snake venom metalloproteinases (SVMPs) and is distinct from all known anticomplementary proteins in cobra venoms. To distinguish it from atrase A, a metalloproteinase we previously purified from the same venom (Sun et al., 2007), it was thus named as atrase B. In this paper, we describe the purification, cloning and characterization of a new metalloproteinase, atrase B, from *N. atra* venom, which cleaves complement components fB, C6, C7, and C8, and thus inhibits hemolytic complement activity.

2. Materials and methods

2.1. Materials

Lyophilized crude venoms of *N. atra* and *Trimeresurus mucrosquamatus* were obtained from Yuanling snake farm in Hunan Province of China. SP Sephadex C-25, HiTrap Heparin HP column (5 ml), HiTrap Q HP column (1 ml), HiTrap SP HP column (1 ml), Ampholine (pH 3.5–9.5) for isoelectric focusing (IEF), low molecular weight calibration kit for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and low molecular weight gel filtration calibration kit were purchased from Amersham Biosciences (Uppsala, Sweden). Total RNA isolation reagent and pMD19-T vector were from TaKaRa (Dalian, China). M-MLV reverse transcriptase was from Promega (Madison, USA). Taq DNA polymerase and DH5α competent cells were from Tiangen (Beijing, China). Complement C3, C5, C6, C8, C9, factor B (fB), and factor D (fD) were from Calbiochem (San Diego, CA, USA), and C7 was from Quidel (San Diego, CA, USA). Hemolysin, Bovine fibrinogen, human thrombin, soybean trypsin inhibitor (SBTI), ethylene glycol-N,N,N′,N′-tetraacetic acid (EGTA), azocasein, N-benzoyl-L-arginine ethyl ester (BAEE), and reagents for SDS-PAGE were obtained from Sigma (St Louis, MO, USA). BCA protein assay kit was purchased from Beyotime Biotechnology Institute (Haimen, China). All other reagents used were of analytic grade from commercial sources.

Normal human serum (NHS) was prepared from blood taken from healthy donors. *N. atra* snake was obtained from Yuanling snake farm in Hunan Province of China. Laboratory animals and sheep erythrocytes used in this work were purchased from Laboratory Animal Center, Guiyang Medical College (Guiyang, China). All animals were housed in a climate-controlled environment and allowed free access to food and water. Procedures that involved animals were conducted in accordance with guidelines for the care and use of laboratory animals of the key Laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences.

2.2. Purification

2.0 g of lyophilized *N. atra* venom was dissolved in 12 ml of 20 mM sodium acetate buffer (pH 5.8). After centrifuged, the solution was applied to an SP Sephadex C-25 column (2.6 × 40 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.8). The column was eluted at a flow rate of 30 ml/h, and collected 6 ml per tube. After elution of unbound material, a linear gradient of 0–1 M NaCl in 20 mM sodium acetate buffer (pH 5.8) was developed. The fractions exhibiting anticomplementary activity were pooled and exchanged the buffer to buffer A (10 mM sodium phosphate buffer, pH 7.0) by using an Amicon Stirred Cells 8050 ultrafiltration system with Amicon YM-10 membrane (Millipore, Bedford, MA, USA). The pooled fractions were then loaded on a HiTrap Heparin HP column (5 ml) equilibrated with buffer A. After the column was thoroughly washed, elution was carried out by a linear gradient to 60% buffer B (10 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl) at a flow rate of 2.0 ml/min. The active fractions were pooled, and were exchanged the buffer to 25 mM Tris–HCl buffer, pH 8.9. Then the fractions were applied to a HiTrap Q HP column (1 ml) equilibrated with 25 mM Tris–HCl buffer, pH 8.9. The column was eluted at a flow rate of 1 ml/min using a NaCl gradient (0–0.5 M) in 25 mM Tris–HCl buffer (pH 8.9). The active fractions were pooled and exchanged the buffer to buffer A. Then the fractions were loaded on a HiTrap SP HP column (1 ml) equilibrated with buffer A. The following elution protocol was employed at a flow rate of 1 ml/min: (a) a linear gradient to 20% buffer B, 30 ml; (b) 20% buffer B, 20 ml; (c) a linear gradient of 20–30% buffer B, 10 ml. The fractions containing anticomplementary activity were collected and the highly purified target protein was termed as atrase B. Purification procedures were performed at the temperatures ranged from refrigerator temperature to room temperature. Protein concentration of the final preparation was determined by BCA protein assay kit.

2.3. Assay for anticomplementary activity of column fractions

Anticomplementary activity in column fractions was detected by measuring consumption of hemolytic activity of the complement classical pathway. 100 μl of fractions in veronal buffered saline (VBS) was mixed with 100 μl of NHS diluted 1:45 in VBS containing gelatin, Ca^{2+}, Mg^{2+} and glucose ($GGVB^{2+}$). 100 μl of antibody-sensitized sheep erythrocytes (EA) (5×10^8 cells/ml) was then added. The mixture was incubated for 30 min at 37 °C. After incubation, the mixture was diluted with 1 ml of cold saline, centrifuged, and the erythrocyte lysis was determined at 405 nm. Decreased lysis in the presence of tested fractions indicated anticomplementary activity.

2.4. Molecular mass determination

SDS-PAGE was performed according to the method of Laemmli (1970) on 12% gels. Gel filtration was performed to determine the apparent molecular weight of atrase B on a Sephacryl S-200 column (1.0 × 60 cm) under non-denaturing conditions. Mass spectrometry was performed on Ultraflex III TOF/TOF spectrometer (Bruker Daltonics, Germany) in a linear mode using sinapinic acid as a matrix.