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cDNA cloning, expression and fibrin(ogen)olytic activity of two low-molecular weight snake venom metalloproteinases

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ABSTRACT

Two cDNA clones, AplVMP1 and AplVMP2, were isolated from a snake (*Agkistrodon piscivorus leucostoma*) venom gland cDNA library. The full-length cDNA sequence of AplVMP1 with a calculated molecular mass of 46.61 kDa is 1233 bp in length. AplVMP1 encodes PI class metalloproteinase with an open reading frame of 411 amino acid residues that includes signal peptide, pro-domain and metalloproteinase domains. The full-length cDNA of the AplVMP2 (1371 bp) has a calculated molecular mass of 51.16 kDa and encodes PII class metalloproteinase. The open reading frame of AplVMP2 with a 457 amino acid residues is composed of signal peptide, pro-domain, metalloproteinase and disintegrin domains. AplVMP1 and AplVMP2 showed 85% and 93% amino acid identical to PI class enzyme *Agkistrodon contortrix laticinctus* ACLPREF and PII class enzyme *Agkistrodon piscivorus piscivorus* piscivostatin, respectively. When expressed in *Escherichia coli*, most of recombinant proteins of AplVMP1 and AplVMP2 were in insoluble inclusion bodies, with soluble yields of 0.7 mg/l and 0.4 mg/l bacterial culture, respectively. Both affinity purified recombinant proteins show proteolytic activity on fibrinogen, although having an activity lower than that of crude *A. p. leucostoma* venom. Proteolytic activities of AplVMP1 and AplVMP2 were completely abolished after incubation with a final concentration of 100 μM of EDTA or 1,10-phenanthroline. Both AplVMP1 and AplVMP2 were active in a fibrin–agarose plate but devoid of hemorrhagic activity when injected (up to 50 μg) subcutaneously into mice, and had no capacity to inhibit platelet aggregation.

1. Introduction

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, are rich sources of metalloproteinases (Hite et al., 1994; Bjarnason and Fox, 1994; Selistre-de-Araujo et al., 1997; Birkedal-Hansen, 1995). Snake venom metalloproteinases (SVMPs) are a superfamily of zinc-dependent proteases and need zinc or calcium atoms to work properly and degrade capillary basement membranes (Selistre-de-Araujo and Ownby, 1995; Bjarnason and Fox, 1994). Zinc metalloproteinases are widely occurring and participate in a number of important biological, physiological, and pathophysiological processes, such as hemorrhage, fertilization, thrombolysis, cancer metastasis, edema, hypotension, hypovolemia, inflammation and necrosis (Mori et al., 1984; Hooper, 1994; Fox and Serrano, 2005). SVMPs and 'A Disintegrin And Metalloproteinases' (ADAMs) constitute the Reprolysin subfamily (Hite et al., 1994; Bjarnason and Fox, 1994; Fox and Serrano, 2005).

Abbreviations: AplVMP, *Agkistrodon piscivorus leucostoma* snake venom metalloproteinase; cDNA, complementary DNA; GST, glutathione *S*-transferase; ADAMs, A Disintegrin And Metalloproteinases; PCR, polymerase chain reaction; PRP, platelet-rich plasma; PPP, platelet-poor plasma; ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; MMP, matrix metalloproteinase.

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On the bases of cDNA size, the diversity of biological activities and amino acid primary structure features, SVMPs were categorized into four classes (Hite et al., 1994; Bjarnason and Fox, 1994; Fox and Serrano, 2005): (1) PI class enzymes, the smallest reprolysin, contain a pro-domain and a metalloproteinase domain. A wide variety of PI enzymes with various functions have been isolated from different species of snakes, including leucurolysin-a (leuc-a) with fibrinogenolytic activity from *Bothrops leucurus* (Bello et al., 2006), BjussuMP-II with antiplatelet activity from *Bothrops jararacussu* (Marcussi et al., 2007), BlaH1 with hemorrhagic, caseinolytic, fibrinogenolytic, collagenolytic and elastinolytic activities from *Bothrops lanceolatus* (Stroka et al., 2005), BaP1 with hemorrhage, myonecrosis, dermonecrosis, blistering and edema from *Bothrops asper* (Watanabe et al., 2003), BmooMPα-I with fibrinogenolytic activity from *Bothrops moojeni* (Bernardes et al., 2008), as well as many others have been reported in the excellent review (Fox and Serrano, 2005). (2) PII class enzymes, the medium-size enzymes, comprise a pro-domain, a metalloproteinase and a disintegrin domain. To date, a number of PII class enzymes have been isolated from different species of snake such as Atrolysin with hemorrhage activity from *Crotalus atrox* (Hite et al., 1992; Jia et al., 1997), MT-d with proteolytic activity from *Agkistrodon halys brevicaudus* (Jeon and Kim, 1999), Bothrostatin precursor showing high inhibitory activity on collagen-induced platelet aggregation from *Bothrops jararaca* (Fernandez et al., 2005), and Albolatin with inhibiting collagen-induced platelet aggregation from *Trimeresurus albolabris* (Singhamatr and Rojnuckarin, 2007). (3) PIII class enzymes, the reprolysin and the most potent hemorrhagic toxins, have been synthesized with a pro-domain, a metalloproteinase domain, a disintegrin-like domain and an additional cysteine-rich domain. Numerous PIII class enzymes have been identified from different species of snakes, including Bothropasin with hemorrhagic and myonecrotic activities isolated from *B. jararaca* (Assakura et al., 2003), metalloproteinase with proteolytic, edematogenic and myotoxic activities from *Bothrops alternatus* (Gay et al., 2005), BjussuMP-I with hemorrhagic and proteolytic activities from *B. jararacussu* (Mazzi et al., 2004). (4) PIV class enzymes contain the non-processed PIII structure (a pro-domain, a metalloproteinase, a disintegrin-like, and a cysteine-rich domain) and two C-type lectin-like domains in the quaternary structure connected to the main chain of the PIII by disulfide bonds. To our knowledge, four PIV class enzymes have been isolated from different snakes, including RVV-X with an activation of Factor X to Xa from Russell's viper venom (Gowda et al., 1994; Chen et al., 2008), VLFXA, the Factor X activator from *Vipera lebetina* (Siigur et al., 2001, 2004), and VAFXA-I and VAFXA-II with the characteristics of hydrolyzing insulin B-chain, fibrinogen and some components of the extracellular matrix from *Vipera ammodytes ammodytes* (Leonardi et al., 2008). The crystal structure of RVV-X has recently been analyzed by Takeda et al. (2007).

Snake venom metalloproteinases play an important role in the digestion of prey tissue, participation in the pathophysiology of envenoming by inducing local and systemic bleeding, as well as other tissue-damaging activities and hemostatic alterations. Thus, these enzymes have been extensively studied, and research has focused on these compounds in the last few years mainly due to their pathological relevance (Gutiérrez and Rucavado, 2000; Rodrigues et al., 2004) and potential applications in therapeutics (Toombs, 2001; Swenson et al., 2004), as well as their potential use as diagnostic, thrombolytic, apoptosis-inducing agents. Therefore, these enzymes merit further investigation. In this study, we isolated two cDNAs clones encoding two different classes of metalloproteinases, AplVMP1 and AplVMP2, from a snake (*Agkistrodon piscivorus leucostoma*) venom gland cDNA library by random sequencing as described by Jia et al. (2008). The nucleotide sequences of AplVMP1 and AplVMP2 have been deposited into GenBank with accession numbers FJ429179 and FJ429180, respectively. Various functions of recombinant proteins of AplVMP1 and AplVMP2 were investigated.

2. Materials and methods

2.1. Plasmid construction

Polymerase chain reaction (PCR) was performed to amplify the full-length cDNAs encoding AplVMP1 and AplVMP2 using the PfuUltra Hotstard DNA Polymerase (Stratagene) and two pairs of primers. (1) AplVMP1F, TAAT*GAATTC*ATGATCCAGGTTCTCTTGGTGA and AplVMP1R, ACT*CTCGAG*TCATCACGCCTCCAAAAGTTCATT. (2) AplVMP2F TAAT*GAATTC*ATGATCCAAGTTCTCTTGGTGA and AplVMP2R ACT*CTCGAG*TCATTAGGCATGGAAGGGATTTCA. Two restriction enzyme sites: EcoR 1 in forward primer and Xho 1 in reverse primer were introduced (italicized sequence). Clones 01E11 accession number FJ429179 for AplVMP1 and 20F10 accession number FJ429180 for AplVMP2 from a cDNA library (Jia et al., 2008) were separately used as PCR templates. PCR was performed using a thermal cycler (Gene Cycler, BIO-RAD Hercules, CA, USA) programmed for an initial denaturation (95 °C for 4 min), followed by 25 cycles for 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min. PCR products were extracted in phenol/chloroform and precipitated using ethanol in −80 °C for 1 h. The pellets were washed in 70% ethanol, dried, dissolved in H_2O and cleaved using EcoR 1 and Xho 1, and then separately subcloned into the EcoR 1–Xho 1 site of pGEX-4T-1 vector (Amersham Biosciences), giving ligation GST–AplVMP1 and GST–AplVMP2. Each ligation was transformed separately into XL-blue competent cells (Invitrogen). Plasmid was extracted using miniprep kit (Sigma-Aldrich, USA), digested with EcoR 1 and Xho 1 for 1.5 h at 37 °C to select plasmids containing inserts of the predicted size for DNA, and further confirmed by sequencing for construction of in-frame.

2.2. Culturing methods and affinity purification

The confirmed plasmids, GST–AplVMP1 and GST–AplVMP2 were separately transformed into the *Escherichia coli* strain BL21 star (Invitrogen) to give strain BL21/GST–AplVMP1 and BL21/GST–AplVMP2. Recombinant strain was first cultured in shaking flasks containing Luria–Bertani (LB) medium overnight. After inoculation of the overnight culture into fresh LB medium, the growth of culture cells