Comparative Biochemistry and Physiology, Part C 179 (2016) 107–115

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Purification and characterization of tenerplasminin-1, a serine peptidase inhibitor with antiplasmin activity from the coral snake (*Micrurus tener tener*) venom

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ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form 14 September 2015
Accepted 19 September 2015
Available online 28 September 2015

Keywords:
Fibrinolytic system
Hemostasis
Micrurus tener tener
Plasmin inhibitor
Snake venom

ABSTRACT

A plasmin inhibitor, named tenerplasminin-1 (TP1), was isolated from *Micrurus tener tener* (*Mtt*) venom. It showed a molecular mass of 6542 Da, similarly to Kunitz-type serine peptidase inhibitors. The amidolytic activity of plasmin (0.5 nM) on synthetic substrate S-2251 was inhibited by 91% following the incubation with TP1 (1 nM). Aprotinin (2 nM) used as the positive control of inhibition, reduced the plasmin amidolytic activity by 71%. Plasmin fibrinolytic activity (0.05 nM) was inhibited by 67% following incubation with TP1 (0.1 nM). The degradation of fibrinogen chains induced by plasmin, trypsin or elastase was inhibited by TP1 at a 1:2, 1:4 and 1:20 enzyme:inhibitor ratio, respectively. On the other hand, the proteolytic activity of crude *Mtt* venom on fibrinogen chains, previously attributed to metallopeptidases, was not abolished by TP1. The tPA-clot lysis assay showed that TP1 (0.2 nM) acts like aprotinin (0.4 nM) inducing a delay in lysis time and lysis rate which may be associated with the inhibition of plasmin generated from the endogenous plasminogen activation. TP1 is the first serine protease plasmin-like inhibitor isolated from *Mtt* snake venom which has been characterized in relation to its mechanism of action, formation of a plasmin:TP1 complex and therapeutic potential as anti-fibrinolytic agent, a biological characteristic of great interest in the field of biomedical research. They could be used to regulate the fibrinolytic system in pathologies such as metastatic cancer, parasitic infections, hemophilia and other hemorrhagic syndromes, in which an intense fibrinolytic activity is observed.

1. Introduction

Hemostasis is a complex physiological process that, under normal conditions, balances the formation and dissolution of fibrin clots to prevent hemorrhagic or thrombotic clinical disorders (Colman et al., 2006; van Geffen and van Heerde, 2012). The fibrinolytic system removes fibrin from the circulation in a controlled way and, therefore, prevents excessive fibrin accumulation. This system constitutes a tissue repair mechanism composed of plasminogen, activators, inhibitors, receptors and modulators, which may be activated at the site of fibrin formation and is involved in several processes, such as hemostatic balance, tissue remodeling, tumor invasion, angiogenesis and reproduction. Excessive local or systemic fibrinolysis activity can result in bleeding, as the weakened plug is dissolved. Plasmin has trypsin-like specificity *in vitro*, cleaving peptides on the C-terminal side of lysine and arginine residues; it plays a major role in the modulation of hemostasis, thrombosis, fibrinolysis, immune response, inflammation, apoptosis, and complement cascade (Lijnen, 2001; Hoover-Plow, 2010; Tsurupa et al., 2010; Jennewein et al., 2011). The main physiological inhibitor of plasmin is α2-antiplasmin. However, other plasma peptidase inhibitors, such as α2-macroglobulin, also show antiplasmin activity (Stassen et al., 2004; Colman et al., 2006).

In several venomous animals, there have been described hemostatically active molecules. (e.g., snakes, leeches, scorpions, caterpillars, ticks and spiders). They may show procoagulant (thrombin-like enzymes and prothrombin or factor X activators) or anticoagulant activities (protein C activators, serine peptidase inhibitors, anti-factor Xa, anti-thrombin), platelet-activating or anti-platelet function (metallopeptidases, disintegrins, C-type lectin-like proteins), fibrinolytic or hemorrhagic activities (Marsh, 1994; Markland, 1998; Sajevic et al., 2011). Several inhibitors of serine peptidases have also been identified in a number of animal venoms. They modulate the enzymatic activity of serine peptidases involved in hemostasis, such as plasmin, and show high pharmaceutical potential (Masci et al., 2000; Flight et al., 2005, 2009; Qiu et al., 2013; Wan et al., 2013).

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http://dx.doi.org/10.1016/j.cbpc.2015.09.009
1532-0456/

Several elapid snake venoms with neurotoxic activity (e.g., cobra venom) also contain proteins that activate or inhibit the hemostatic system (Utkin and Osipov, 2007; Osipov et al., 2010; McCleary and Kini, 2013). Elapid venom from Australia and India presents several components which are active on platelets, thrombin and fibrinogen (Gerads et al., 1992; Jagadeesha et al., 2002; Rao et al., 2004; Banerjee et al., 2005; Osipov et al., 2010; Skejić and Hodgson, 2013). Numerous components with myotoxic, cardiotoxic, hemolytic and edematogenic activities have been isolated from *Micrurus* snake venoms (Weis and McIsaac, 1971; Aird and da Silva, 1991; Tan and Ponnudurai, 1992; Alape-Girón et al., 1994; Barros et al., 1994). Recently, Salazar et al. (2011) described fibrinolytic and anti-fibrinolytic activities in *Micrurus tener tener* venom. In this study the isolation and characterization of a component with anti-fibrinolytic activity present in *M. tener tener* venom was described, which could have potential applications in the control of bleeding disorders associated with hyper-fibrinolysis syndromes.

2. Materials and methods

2.1. Reagents

Molecular exclusion column Superdex-200 (10 × 300 mm) was purchased from GE Healthcare (USA). Reverse phase chromatography column C18 Vydac (250 × 4.6 mm) from Alltech Grade Division (USA). Chromogenic substrates (S-2251, S-2222, S-2238 and S-2288) and plasmin from Chromogenix AB (Italy). Human fibrinogen, factor Xa and double chain tPA (tcu-PA) from American Diagnostic (Greenwich, USA). ADP from Chrono-log (USA). Molecular mass markers for SDS-PAGE from Invitrogen Corporation (USA). Trifluoroacetic acid from Riedel-de Haën (Germany). Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific, USA). Ammonium acetate, hydrochloric acid and acetic acid from Merck (Germany). Ethylenediaminetetraacetic acid (EDTA), ε-aminocaproic acid (EACA), bovine thrombin, dithiothreitol (DTT), rabbit anti plasmin, goat anti-rabbit IgG peroxidase-conjugated and other reagents were purchased from Sigma (Sigma Chemical Co., USA).

2.2. Venom

Lyophilized venom of *M. tener tener* snakes was purchased from the National Natural Toxins Research Center, Texas A&M University-Kingsville, Texas, USA. The venom was stored at −80 °C until used.

2.3. Plasma

Platelet-rich plasma (PRP) was obtained from healthy blood donors with their previous consent. All selected donors had not used any drugs known to interfere with platelet function during the previous 14 days, as approved by the IVIC Bioethics Committee. The blood was mixed with 3.8% sodium citrate in a 9:1 volume ratio, followed by centrifugation at 190 ×*g* for 20 min at 24 °C. The platelet-poor plasma (PPP) was obtained from the remaining blood by re-centrifuging at 2000 ×*g* for 15 min at 4 °C.

2.4. Protein concentration determination

Protein concentration was spectrophotometrically determined assuming that 1 unit of absorbance at 280 nm corresponds to 1 mg protein/mL (Simonian and Smith, 2006).

2.5. Polyacrylamide–SDS–tricine gel electrophoresis

Protein samples were run on 7, 8, 9 and 10% polyacrylamide–SDS–tricine gels using the Schägger and von Jagow (1987) method.

2.6. Isolation of TP1

Plasmin inhibitor was isolated from *M. tener tener* venom after fractionation on a high performance liquid chromatography system. Five milligrams of crude venom was diluted in 50 mM ammonium acetate, pH 6.9 (equilibrium buffer) and then applied to a Superdex-200 (10 × 300 mm) column, equilibrated with the same buffer at room temperature. Protein elution was performed at 0.5 mL/min under isocratic conditions. The fraction showing antiplasmin amidolytic activity was applied to a C-18 column (250 × 4.6 mm, Vydac) equilibrated with 0.12% trifluoroacetic acid (TFA) in water at 1 mL/min flow rate. Protein elution was performed at 1 mL/min with a 0–50% acetonitrile gradient in 0.12% TFA over 30 min. The active fraction was re-chromatographed on the same column and the elution was performed at 1 mL/min using the same acetonitrile gradient over 60 min. The absorbance was monitored at 280 nm. Active fraction named tenerplasminin 1 (TP1) was lyophilized and stored at −80 °C before further biochemical and biological characterization.

2.7. Mass analysis (MALDI-TOF-MS) of TP1

The molecular mass of TP1 was evaluated by MALDI-TOF MS on the AB SCIEX TOF/TOF™ 5800 system in positive linear mode, described by Magalhães et al. (2013). Briefly, TP1 was resuspended in 0.1% TFA and further spotted (0.3 μL) on the target MALDI plate, followed by immediate addition of an equal volume of a saturated matrix solution (10 mg/mL of α-cyano-4-hydroxycinnamic acid, in 50% acetonitrile/0.1% TFA). External calibration was performed with aprotinin (6.5 kDa).

2.8. Effect of TP1 on platelet aggregation

Platelet aggregation was determined by turbidimetry using a dual channel Chrono-log model 560 CA aggregometer (USA). To evaluate the effect on platelet aggregation, 10 μL of TP1 (150 nM final concentration), crude venom (10 μg) or Tyrode's buffer (aggregation control) were added to 490 μL of PRP (platelet count was adjusted to 3.0×10^5 platelets/mL with platelet-poor plasma). The mixtures were incubated for 4 min at 37 °C in silicone-treated glass cuvettes containing a stir bar. Aggregation was induced by 5 μL of ADP (10 μM final concentration) and changes in light transmittance were continuously registered for 8 min. The aggregation response induced by ADP/Tyrode's buffer was used as a reference of 100% aggregation. The percentage of inhibition induced by crude venom or TP1 was calculated by comparing the light transmittance of ADP-induced aggregation in the presence or in the absence of venom or TP1 (Born and Cross, 1963). The results were expressed as % inhibition of platelet aggregation.

2.9. Effect of TP1 on the amidolytic activity of thrombin, factor Xa and tPA

The effect of TP1 on the amidolytic activity of thrombin, factor Xa (FXa) and tissue plasminogen activator (tPA) was assayed upon the chromogenic substrates S-2238 (6 mM), S-2222 (4 mM) and S-2288 (12 mM), respectively. Thrombin (0.1 IU/mL), FXa (0.05 IU/mL) or tPA (0.1 ng/μL) were incubated for 30 min at 37 °C with crude venom (0.5 μg), with the chromatographic fractions (10 μg) or with TP1 (2 nM) in 10 μL 0.05 M Tris–HCl buffer, pH 7.4. Briefly, the samples were mixed on polystyrene plates of 96 wells with 10 μL of the enzymes for 30 min at 37 °C, followed by the incubation with 10 μL of the chromogenic substrate and 70 μL of buffer solution, as recommended by the manufacturer. After incubation at 37 °C for 15 min, the absorbance at 405 nm was determined. Enzymes incubated with only Tris–HCl buffer were used as positive controls. Results were expressed as inhibition percentage, considering the enzyme activity in Tris–HCl buffer as 100% activity. Soybean trypsin inhibitor (SBTI) (50 μg/mL) and hirudin (0.1 IU/mL) were used as inhibition controls of FXa and thrombin, respectively. Benzamidine (10 nM) was used as inhibition control of tPA.