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Bothrops jararaca antithrombin: Isolation, characterization and comparison with other animal antithrombins

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ABSTRACT

Antithrombin was purified from *Bothrops jararaca* plasma by affinity chromatography using HiTrap Heparin HP column, and its molecular weight, amino-terminal sequence, carbohydrate content, isoelectric point, inhibition of bovine thrombin, and immunological properties were studied and compared with previously described antithrombins. *B. jararaca* antithrombin is a single-chain glycoprotein with a total carbohydrate content of 18%. The molecular weight from SDS-PAGE was 61 kDa and the inhibitor exhibited an acidic isoelectric point (4.5). The amino-terminal sequence has been determined as His-Glu-Ser-Ser-Val-Gln-Asp-Ile-Ile-Thr, which is highly homologous to the terminal sequences of other animal antithrombins, indicating high amino acid conservation among several animals. Immunological cross-reactivity was observed among fish, frog, chicken, human, non-venomous snake and *B. jararaca* antithrombins. *B. jararaca* antithrombin showed inhibitory activity upon human and *B. jararaca* coagulation and amidolytic substrate S-2238.

1. Introduction

Blood coagulation in vertebrates is a defensive mechanism preventing excessive loss of blood. It is a multi-step process involving the sequential activation of a series of proteolytic enzymes. Blood coagulation proteolytic enzymes circulate in zymogen forms and after their activation one important control mechanism is the complex formation with specific natural inhibitors (Jordan, 1983; Tanaka-Azevedo et al., 2003; Gentry, 2004).

Antithrombin (AT), a serine protease inhibitor (serpin), is an important anticoagulant molecule in mammalian circulatory system (Doolittle, 1993). It is the most effective, well-regulated inhibitor of coagulation proteases, such as thrombin, factors Xa, IXa, XIa, XIIa, and plasma kallikrein; moreover, AT is believed to play a major role in controlling blood coagulation, thereby preventing widespread thrombosis (Roemisch et al., 2002).

The glycosaminoglycan heparin is a positive effector of the reaction, which can enhance AT activity 1000-fold (Rosenberg and Damus, 1973; Barrett and Barret, 1976; Quinsey et al., 2004). AT and heparin thus form an interdependent regulatory mechanism of the coagulation process (Jordan, 1983). Inhibition by AT involves the formation of a stable 1:1 complex between the active domain of the serine protease and the reactive site of AT, which proteases initially recognize as a substrate. During the cleavage of the AT reactive site bond, a conformational change occurs in the inhibitor that traps the protease, making it an irreversible inhibitor (Rosenberg and Damus, 1973).

This inhibitor has been purified from several mammalian plasmas, such as human (Thaler and Schmer, 1975; Koide, 1979), chimpanzee (Jordan, 1983), bovine (Thaler and Schmer, 1975), horse (Thaler and Schmer, 1975), goat (Damus and Wallace, 1974), pig (Koide, 1979), dog (Damus and Wallace, 1974), rabbit (Koide, 1979), guinea pig (Damus and Wallace, 1974; Jordan, 1983), rat (Koide, 1979) and hamster (Mak et al., 1996). Mammalian AT has a fairly narrow range of molecular weights around 60 kDa (Jordan, 1983), with the exception of dog AT, with molecular weight around 77 kDa (Yin et al., 1971; Damus and Wallace, 1974). Besides mammalian, this protein was also isolated from avian plasma like chicken (Koide et al., 1982) and ostrich (Frost et al., 2002), amphibian plasma like bullfrog (Jordan, 1983; Frost et al., 2002) and fish plasma like salmon (Andersen et al., 2000).

By contrast, little information is available about this protein in reptiles, since AT has been isolated exclusively from turtle plasma (Jordan, 1983). In addition, studies concerning this inhibitor in snake plasmas are lacking. Nevertheless, the availability of antithrombin sequences from species so different from humans is of use in examining conserved features of this inhibitor (Gettins, 2002).

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Our group has already isolated a novel thrombin coagulant activity inhibitor from *Bothrops jararaca* (*B. jararaca*) snake previously (BjI) (Tanaka-Azevedo et al., 2003). Thus, this work focuses on the isolation and characterization of one of the most important thrombin inhibitors from *B. jararaca* plasma, AT, and compare it with other described ATs.

2. Materials and methods

2.1. Materials

The Laboratory of Herpetology of Butantan Institute, Sao Paulo (Brazil) supplied specimens of *B. jararaca.* The Animal House of Butantan Institute supplied mice used for immunization. The Institutional Animal Care Committee at the Butantan Institute approved these experimental protocols (CEUAIB protocol no. 156/04).

Serum samples from fish (*Salminus hilarii*) was obtained from the Laboratory of Fish Metabolism and Reproduction, Biosciences Institute, University of Sao Paulo; fresh bullfrog (*Rana catesbeiana*) plasma was obtained from animals supplied by the Laboratory of Pharmacology, Butantan Institute; non-venomous snake (*Oxyrhopus guibei*) plasma was a gift from Dr. Ida Sigueko Sano-Martins; chicken (*Gallus gallus*) plasma was obtained from the Laboratory of Ornithology, Veterinary Medicine School, University of Sao Paulo; rabbit (*Oryctolagus cuniculus*) serum and human (*Homo sapiens sapiens*) plasma were a gift from Dr. Marcelo Larami Santoro.

Bovine thrombin, bovine serum albumin, peroxidase-conjugated antibody (anti-mouse IgG), trifluoromethanesulfonic acid and bicinchoninic acid were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Polyvinylidene (PVDF) and nitrocellulose membranes were obtained from Millipore (Bradford, MA, USA) and chromogenic substrate S-2238 from Chromogenix (Milano, Italy). HiTrap Heparin HP column (1 mL), precast polyacrylamide gels (PhastGel IEF 3-10) and calibration standards were purchased from GE Healthcare (Uppsala, Sweden). Marcol 52 was obtained from Esso Standard Oil Co and Montanide 888 from SEPPIC (Paris, France). Microplates were acquired from Nalge Nunc International (New York, NY, USA). *B. jararaca* fibrinogen was purified according to Vieira et al. (2008). All other reagents were of analytical grade or better.

2.2. Methods

2.2.1. Blood collection

Adult snakes (*B. jararaca*) (n=2) were anesthetized with pentobarbital (30 mg/kg) before being bled by puncture of the exposed aorta. Samples of snake blood were collected in 9:1 of blood and 3.8% sodium citrate solution. The plasmas were obtained by centrifugation for 15 min at 1200 ×g at room temperature, and stored at −20 °C.

2.2.2. Purification of B. jararaca AT

Seven mL of *B. jararaca* plasma was diluted in 3.5 mL of 0.1 M Tris, 0.01 M sodium citrate and 0.25 M NaCl pH 7.4 buffer. This solution was applied to a HiTrap Heparin HP column previously equilibrated with the same buffer using a FPLC (Äkta design system) (GE Healthcare, Sweden), at a flow rate of 1 mL/min. The elution was performed by stepwise gradient with an increasing NaCl concentration (2 M) in the same buffer. Protein concentration was monitored by measuring the absorbance at 280 nm (Stoscheck, 1990). Fractions of 1 mL were collected and the AT activity was measured by inhibition of the thrombin activity using S-2238. Fractions containing AT activity were pooled and submitted to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE).

2.2.3. Measurement of protein

The protein concentration of samples was measured using bicinchoninic acid (BCA), using bovine serum albumin (BSA) as standard (Smith et al., 1985).

2.2.4. Determination of AT activity

AT inhibitory activity was measured using S-2238, after addition of an excess of heparin and thrombin, according to the manufacturer's recommendations (Chromogenix). Briefly, 100 μL of sample (prediluted 60 fold in buffer 50 mM Tris, 7.5 mM EDTA, 3 U heparin and 175 mM NaCl pH 8.4) were incubated at 37 °C for 5 min, followed by the addition of 25 μL of bovine thrombin (2 U/mL) and incubation at 37 °C for 30 s. Then 15 μL of chromogenic substrate S-2238 (4 mM) was added. After incubation at 37 °C for 5 min the absorbance was measured photometrically at 405 nm.

2.2.5. Thrombin clotting time

AT activity in thrombin clotting time was evaluated by incubating 50 μL of bovine thrombin (5 U/mL), 50 μL of heparin (1 U/mL) and 50 μL of *B. jararaca* AT at 37 °C. After 1 min of incubation, 100 μL of this mixture was added to 100 μL of *B. jararaca* or bovine fibrinogen (1 mg/mL). The solution was mixed and, immediately, the clotting time recorded. The test was also performed without AT and/or heparin as control.

2.2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970) either in the presence or absence of reducing agent (β-mercaptoethanol). The proteins were silver stained according to Blum et al. (1987). The gels were analyzed by densitometry analysis using Total Lab software.

2.2.7. Polyacrylamide gel isoelectric focusing (PAG-IEF)

The isoelectric focusing (pI) of AT was determined by performing focusing in a PhastSystem Apparatus (GE Healthcare, Sweden) with precast polyacrylamide gels (PhastGel IEF 3-10). Calibration standards were between pH 3 and 9. The proteins were silver stained (Blum, 1987).

2.2.8. B. jararaca AT antiserum production

Antibodies were raised in mice (Balb C) by the intramuscular injection of 50 μg of purified *B. jararaca* AT emulsified with 0.5 mL Marcol-Montanide adjuvant in mice. Three additional boosters (30, 60 and 90 days after the first immunization) were administered, repeating the same procedure. Blood was collected by puncture of the ocular plexus, and serum was obtained after centrifugation at 1500 ×g. Serum titers were determined by ELISA assays (Santoro et al., 1999). For titration of antibodies, 96-well polystyrene plates were coated overnight at 4 °C with 100 μL of purified AT (10 μL/mL).The plates were then blocked with 200 μL of Tris-buffered-saline containing 5% fat free milk (TBS-milk) for 1 h at 37 °C, and incubated with different dilutions of anti-*B. jararaca* AT serum in TBS-milk for 1 h at 37 °C. The plates were washed four times with 0.1% Tween 20 (TBST-milk) and then incubated with peroxidase-conjugated second antibody (anti-mouse IgG) diluted in TBS-milk (1:10 000). After 1 h at 37 °C, the plates were washed five times with TBST-milk. The reaction was developed with o-phenylenediamine and H_2O_2 according to the manufacturer's recommendations (Sigma).

2.2.9. Western blotting

Several animal plasma or serum samples and purified AT from *B. jararaca* separated by SDS-PAGE were electrotransferred at 20 V for 90 min onto nitrocellulose membranes. Thereafter, membranes were blocked with TBS-milk overnight at 4 °C. The membrane was incubated with anti-*B. jararaca* AT antibody (1:1000) for 2 h at room temperature. After the washing step using TBS-milk containing 0.1% Tween 20, the membranes were exposed to peroxidase-labelled anti-mouse IgG (1:10 000) for 2 h at room temperature. The reaction was revealed using a chromogenic substrate (diaminobenzidine and H_2O_2) according to the manufacturer's recommendations (Sigma) (Harlow and Lane, 1988).