ELSEVIER

Toxicon 50 (2007) 18–31

TOXICON

www.elsevier.com/locate/toxicon

BJ-48, a novel thrombin-like enzyme from the *Bothrops jararacussu* venom with high selectivity for Arg over Lys in P1: Role of *N*-glycosylation in thermostability and active site accessibility

Floriano P. Silva-Junior[a], Herbert L.M. Guedes[a], Laura C. Garvey[c], Aniesse S. Aguiar[d], Saulo C. Bourguignon[b], Enrico Di Cera[c], Salvatore Giovanni-De-Simone[a,b,*]

[a]*Laboratório de Bioquímica de Proteínas e Peptídeos, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045 900 RJ, Brazil*

[b]*Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brazil*

[c]*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, MO 63110, USA*

[d]*Instituto Vital Brazil, Niterói, RJ, Brazil*

Received 11 January 2007; received in revised form 9 February 2007; accepted 19 February 2007
Available online 7 March 2007

Abstract

BJ-48, a serine protease from the venom of *Bothrops jararacussu*, was purified to homogeneity using affinity chromatography on *p*-aminobenzamidine-agarose followed by HPLC gel filtration. BJ-48 presented 52 kDa by SDS–PAGE analysis and 48,036 Da by electron spray mass spectrometry. The enzyme was shown to be highly glycosylated with 42% of N-linked carbohydrates composed of Fuc(1):GalN(4):GlcN(5):Gal(1):Man(2) and a high content of sialic acid residues (8–12%). BJ-48 had optimal esterase activity at pH 7.5 and displayed maximum catalytic rate at 50 °C. Its hydrolytic activity was strongly inhibited by aprotinin and dithiothreitol while *N*-tosyl-L-phenylalanine chloromethyl ketone, 6-aminocaproic acid, E-64 and soybean trypsin inhibitor (SBTI) were ineffective. The kinetics of BJ-48 with chromogenic substrates revealed an unprecedented selectivity (10^4-fold) for Arg over Lys in P1. BJ-48 proved to be a thrombin-like enzyme (TLE) with a specific fibrinogen-clotting activity of 73.4 NIH units/mg. The TLE rapidly digested human fibrinogen Bβ chain, but the Aα chain was cleaved specifically to release fibrinopeptide A with $k_{cat}/K_m = 2.1\,\mu M^{-1}\,s^{-1}$. The TLE showed no activity toward other thrombin substrates like protein C, protease-activated receptor-1 or inhibitors such as hirudin and antithrombin. A non-denaturing procedure using PNGase F and neuraminidase followed by hydrophobic interaction chromatography was employed to obtain active BJ-48 forms with

Abbreviations: TAME, *N*α-Tosyl-L-Arg-methyl ester; BAME, α-*N*-benzoyl-DL-Arg-methyl ester; BApNA, α-*N*-benzoyl-DL-arginine-*p*-Nitroanilide; E-64, L-trans-epoxysuccinyleucyl amido-(4-guanidino) butane; DMTI, *Dimorphandra mollins* trypsin inhibitor; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PMSF, phenylmethysulfonyl fluoride; SBTI, soybean trypsin inhibitor; ATIII, antithrombin III; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLCK, *N*-tosyl-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PAR, protease-activated receptor; ESMS, electron spray mass spectrometry; PNGase F, peptidyl-*N*-glycosidase F

*Corresponding author. Laboratório de Bioquímica de Proteínas e Peptídeos, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045 900 RJ, Brazil. Fax: +55 21 25903495.

E-mail address: dsimone@ioc.fiocruz.br (S. Giovanni-De-Simone).

0041-0101/$ - see front matter
doi:10.1016/j.toxicon.2007.02.018

variable carbohydrate content. Compared to the native enzyme, total or partially deglycosylated BJ-48 forms presented up to 2-fold reduction in their specific activities upon heating at 55/65 °C or treatment with SBTI. These results point out a role for BJ-48 glycosylation in thermostability and controlling the access of some canonical protein inhibitors to the active site.

Keywords: Toxin; Snake venom; Serine protease; Carbohydrate; Clotting

1. Introduction

Death is an unusual outcome of *Bothrops* envenoming (0.3–0.5% of cases) and is caused by acute renal and respiratory failure, hemorrhage, shock and/or sepsis (Benvenuti et al., 2003). Coagulation disorders, and especially consumption coagulopathy due to fibrinogen depletion, are associated with many of these clinical conditions. Venom components involved in this process are known to be proteolytic enzymes pertaining to the serine and metalloprotease classes (Markland, 1998). These fibrinogen-degrading serine proteases are part of a larger group of snake venom serine proteases (SVSP) able to interfere with the prey hemostatic system. These enzymes exert their toxic effects by cleaving specific bonds in one or more of the following hemostatic system components: kininogen (kalikrein-like activity); coagulation factors V, VII, VIII or X; fibrin/fibrinogen [fibrin(ogen-o)lytic activity] and plasminogen (plasminogen activators) (Serrano and Maroun, 2005). Not uncommonly, fibrinogenolytic SVSPs are referred to as thrombin-like enzymes (Castro et al., 2004), although this term should be reserved to enzymes that clot fibrinogen *in vitro*.

At present, numerous snake venom thrombin-like enzymes (SVTLEs) have been described from members of the Crotalinae and Viperidae families (Pirkle, 1998). SVTLEs are single-chain enzymes able to release fibrinopeptides A and/or B, from fibrinogen Aα and Bβ chains, respectively. All of them present trypsin-like specificity cleaving after Arg or Lys in susceptible protein substrates. Morevoer, the great majority of SVTLEs display esterolytic and amidolytic activities over small synthetic substrates with Arg at P1 (Schechter and Berger, 1967). Accordingly, SVTLEs hydrolytic activity can be impaired by inhibitors of trypsin-like serine proteases such as benzamidine and TLCK or by more general serine protease inhibitors as PMSF and DFP. Another common characteristic of these enzymes is the high thermostability of their arginine esterase or amidase activities, e.g. (Smolka et al., 1998; Zaganelli et al., 1996). The structural origin of this property remains elusive.

A survey of the literature indicates the striking biochemical diversity of SVTLEs. In particular, the reported molecular weights (often from SDS–PAGE analysis) of SVTLEs range from 25 to 28 kDa to values as high as 65–70 kDa, although primary structures of many SVTLEs indicate a molecular weight of <28 kDa. This discrepancy suggests a major role for post-translational modifications (PTMs) in the molecular diversity of SVTLEs, among which glycosylation could be the most prominent, e.g. (Koh et al., 2001; Serrano et al., 2000; Huang et al., 1999; Aguiar et al., 1996).

Our group has previously studied the ability of benzamidine-based affinity sorbents to selectively purify serine proteases from venoms of different snake species (De-Simone et al., 2005). It was demonstrated that a commercial resin based on *p*-aminobenzamidine, specifically produced through CNBr coupling of a glycyl–glycine spacer arm to an agarose support, represent an efficient chromatographic medium to isolate a 48–52 kDa species from *Bothrops jararacussu* venom. Sequencing the first 15 N-terminal residues indicated that this protein (denoted as BJ-48) is a SVSP. Here we report extensive biochemical characterization showing that BJ-48 is a novel clotting SVSP with unprecedented selectivity for Arg over Lys at the P1 position of substrate. Our results also provide evidence that N-linked carbohydrate chains are implicated in its high thermostability and in the low susceptibility to inhibition by SBTI.

2. Materials and methods

2.1. Materials and B. jararacussu venom

Sequence reagents, PTEE (polytetrafluoroethylene) membrane, polybrene and 6-aminocaproic acid were from Wako Pure Chemicals (Richmond, CA, USA). Shinpack-Diol 150 and CLC-ODS