Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 155 (2008) 133–141
veterinary parasitology
www.elsevier.com/locate/vetpar

rBmTI-6, a Kunitz-BPTI domain protease inhibitor from the tick *Boophilus microplus*, its cloning, expression and biochemical characterization

Sergio D. Sasaki [a], Aparecida S. Tanaka [b,*]

[a] *Centro de Ciências Naturais e Humanas, UFABC, Rua Catequese 242, 09090-400 Santo André, SP, Brazil*
[b] *Departamento de Bioquímica, UNIFESP-EPM, Rua 3 de Maio 100, 04044-020 São Paulo, SP, Brazil*

Received 10 October 2007; received in revised form 5 March 2008; accepted 20 March 2008

Abstract

Boophilus microplus is a rich source of trypsin inhibitors, numerous Kunitz-BPTI (bovine pancreatic trypsin inhibitor) inhibitors have been described from larvae and eggs, named BmTIs. Among them, were characterized inhibitors for trypsin, human neutrophil elastase, human plasma kallikrein and plasmin. BmTIs elicited a protective immunological response against *B. microplus* infestation in cattle. However, only a small amount of purified natural BmTIs can be obtained from larvae and eggs by chromatographic methods, thus if BmTIs are to be used as vaccine antigens (immunogens) the production of recombinant BmTIs (rBmTIs) is essential. In this work we describe the cloning, expression, purification and characterization of rBmTI-6. rBmTI-6 is a three-headed Kunitz-BPTI inhibitor, expressed in the *Pichia pastoris* system. Although rBmTI-6 was processed by proteases and glycosylated during the expression process, these post-translational modifications did not alter the ability of rBmTI-6 to inhibit protease activity. Purified rBmTI-6 inhibited trypsin and plasmin.

Keywords: Tick; *Boophilus microplus*; Protease inhibitor; Trypsin inhibitor; Kunitz

1. Introduction

Boophilus microplus is an ectoparasite responsible for the transmission of infectious diseases such as babesiosis and anaplasmosis, which cause important losses in livestock production (Sauer et al., 1995). The control of this parasite is performed mainly by acaricides; however, alternative control methods like vaccines (Willadsen and Kemp, 1988; Rodriguez et al., 1995) have been developed and used, thus avoiding the excessive use of chemical products. Thereby, the study of *B. microplus* molecules can help in the development of control methods for tick infestations, for example, selecting proteins that can be used as vaccine antigens. Ticks are rich sources of serine proteinase inhibitors, mainly belonging to the BPTI-Kunitz family. Among them, the following molecules have been well studied: the tick anticoagulant peptide (TAP) (Waxman et al., 1990), a blood coagulation factor Xa inhibitor, purified from *Ornithodoros moubata*; ornithodorin (van de Locht et al., 1996) and savignin (Mans et al., 2002), two thrombin inhibitors from the *O. moubata* and *O. savignyi* species, respectively. Ixolaris (Francischetti et al., 2002) and Penthalaris (Francischetti et al., 2004) are factor Xa and FVIIa/tissue factor inhibitors

* Corresponding author. Tel.: +55 1155764444; fax: +55 1155723006.
E-mail address: tanaka.bioq@epm.br (A.S. Tanaka).

0304-4017/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2008.03.031

containing two and five Kunitz-BPTI domains, respectively, from *Ixodes scapularis*. Several *B. microplus* Trypsin Inhibitors (BmTIs) with inhibitory activity toward Human Plasma Kallikrein (HuPK), Human Neutrophil Elastase (HNE) and plasmin present in larvae and eggs have been described (Tanaka et al., 1999; Andreotti et al., 2001 and Sasaki et al., 2004). Purified natural BmTIs have been used as antigens in an immunization/challenge experiment and elicited a protective efficacy of 72.8% in interrupting tick development and a reduction in tick number of 69.7% (Andreotti et al., 2002). However, due the small amount of purified BmTIs obtained from eggs or larvae, it was impossible to use the natural inhibitors as antigens in a vaccination experiment. Thus, a strategy to obtain one or more rBmTIs as vaccine antigens is required.

In the present work, we describe the cloning, expression, purification and characterization of rBmTI-6, a recombinant *B. microplus* trypsin inhibitor containing three BPTI-Kunitz domains, In addition, antibody in the serum of calves immunized with natural BmTIs bound to the rBmTI-6.

2. Materials and methods

2.1. Engorged

Boophilus microplus female ticks were provided by Dr. Itabajara da Silva Vaz Junior from Universidade Federal do Rio Grande do Sul, Brazil.

2.2. Pichia pastoris

(GS115 strain) and vector pPIC9K were purchased from Invitrogen (San Diego, CA).

2.3. Proteinase inhibitors

BPTI, the bovine pancreatic trypsin inhibitor, was purchased from Sigma (St. Louis, MO).

2.4. Enzymes

Bovine trypsin (EC 3.4.21.4) was purchased from Sigma (St Louis, MO). Human neutrophil elastase (EC 3.4.21.37), human factor XIIa (EC 3.4.21.38) and human factor Xa (EC 3.4.21.6) were purchased from Calbiochem (San Diego, CA). Human plasma kallikrein (EC 3.4.21.34) was prepared as previously described by Sampaio (Sampaio et al., 1974). Human plasmin (EC 3.4.21.7) was obtained from Boehringer Mannheim GmbH (Germany).

2.5. Synthetic substrates

Tosyl-Gly-Pro-Arg-pNA was purchased from Pentapharm (Basel, Switzerland). S2484 (Gly-Pro-Val-pNA), S2302 (HD-Pro-Phe-Arg-pNA), S2222 (Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA) and S2251 (HD-Val-Leu-Lys-pNA) were acquired from Chromogenix (Mölndal, Sweden). Suc-Ala-Ala-Pro-Phe-pNa was purchased from Sigma (St. Louis, USA).

2.6. Boophilus microplus ovary cDNA

The total RNA of the *B. microplus* ovaries (200 mg) was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed into cDNA using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI).

2.7. BmTI6 cDNA analysis

The cDNA sequence which codifies for a native BmTI-6 inhibitor, purified from larva extract (Sasaki et al., 2004), GenBank accession number: P83606, was identified in GenBank (accession number: CK186726). The sequences analysis was performed using BLAST algorithm tools (Altschul et al., 1997). The alignment of protein sequences was performed with the ClustalW program, version 1.83 (Thompson et al., 1994). The putative *N*-glycosylated residues were determined using a *NetNGlyc 1.0 Server* (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.8. Construction of pPIC9K-BmTI6

The BmTI-6 gene was amplified by PCR using the *B. microplus* ovary cDNA as a template, the sense primer BmTI6f (5′ GAAGCTTACGTAGACTTTGAGAC 3′) and the anti-sense primer BmTI6r (5′ AGTT-TATTGCGGCCGCATTATACTTGAAGATC 3′) added of *SnaB*I and *Not*I restriction sites, respectively. The gene was digested with *SnaB*I and *Not*I restriction enzymes, the generated fragment was ligated into the plasmid pPIC9K (Invitrogen, San Diego, CA). The resulting plasmid (pPIC9K-BmTI6) (Fig. 1), linearized with *Sac*I restriction enzyme, was used in the transformation of competent cells of the *P. pastoris* GS115 yeast strain, prepared according to the manufacturer's instructions. The transformed yeast was then incubated for 5 days in a buffered methanol-complex medium (BMMY). After cultivation, the yeast cells were harvested by centrifugation (4000 × *g*, 20 min, and 4 °C) and the supernatant containing the inhibitory activity was stored at −20 °C.