

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

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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.

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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 Loch 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

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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-IITM 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-TATTGCGGCCGCATTATACCTTGAAGATC 3') added of *Sna*BI and *Not*I restriction sites, respectively. The gene was digested with *Sna*BI 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 $\times$ g, 20 min, and 4 °C) and the supernatant containing the inhibitory activity was stored at –20 °C.

2.9. rBmTI-6 (recombinant BmTI-6) purification

The purification of rBmTI-6 expressed in the *P. pastoris* system was carried out using three chromatographic steps: affinity chromatography in a trypsin-Sepharose column; molecular exclusion chromatography using a Superdex 75 column (30 ml) (Amersham Biosciences, Uppsala, Sweden); and reverse-phase chromatography in a Sephasil Peptide C₈ column (Amersham Biosciences, Uppsala, Sweden). The culture supernatant containing rBmTI-6 was applied to a trypsin-Sepharose column previously equilibrated with 50 mM Tris–HCl buffer, pH 8.0 (buffer A). Weakly bound proteins were washed out with buffer A containing 0.2 M NaCl, and the rBmTI-6 was eluted with 0.2 M KCl solution, pH 2.0. The eluted fractions were immediately neutralized using 1 M Tris–HCl buffer, pH 8.0. The fractions containing the rBmTI-6 were pooled, dialyzed, lyophilized, suspended in buffer A and applied to a Superdex 75 column previously equilibrated with buffer A. The protein elution was performed with 35 ml of buffer A and the fractions containing inhibitory activity were divided in two active pools (I and II). The active pool II from Superdex 75 was applied to a Sephasil Peptide C₈ column. The proteins were eluted with a 0–90% acetonitrile linear gradient in 0.1% trifluoroacetic acid (TFA) and the purified rBmTI-6 was stored at –20 °C. The culture supernatant containing rBmTI-6 was also directly applied to Superdex 75 (30 ml) following the same conditions described above.

2.10. NH₂-terminal sequencing

The NH₂-terminal amino acid sequence was determined by Edman degradation (Edman and Begg, 1967) using a PPSQ-23 Model Protein Sequencer from Shimadzu (Tokyo, Japan).

2.11. Serine proteinase inhibition assays

The trypsin inhibitor activity was measured by the remaining amyolytic activity of trypsin on the substrate tosyl-Gly-Pro-Arg-pNA at pH 8.0, after pre-incubation with the inhibitor for 10 min at 37 °C (Erlanger et al., 1961). The concentration of active trypsin was determined by active site titration with *p*-nitrophenyl-*p*'-guanidino-benzoate as described by Chase and Shaw (1969). The equilibrium dissociation constants of complexes formed by rBmTI-6 and bovine trypsin or plasmin were determined using the method described by Bieth (1980). Briefly, the serine proteinases were

incubated with different concentrations of inhibitors in 0.1 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.1% Triton X-100 at 37 °C. The residual enzyme activity was measured after the addition of the chromogenic substrates tosyl-Gly-Pro-Arg-pNA and S2251 (HD-Val-Leu-Lys-pNA) for trypsin and plasmin, respectively. Apparent K_i values were calculated by fitting the steady-state velocities into the equation ($V_i/V_0 = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\} / 2E_t$) for tight-binding inhibitors and using a non-linear regression analysis (Morrison, 1969). The inhibitory activities toward HNE, HuPK, human factor XIIa, human factor Xa, bovine thrombin and chymotrypsin were performed with rBmTI-6 after affinity chromatography (100 nM) in 0.1 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.1% Triton X-100 at 37 °C. The residual enzyme activity was measured after the addition of the following chromogenic substrates: S2484 (Gly-Pro-Val-pNA), S2302 (HD-Pro-Phe-Arg-pNA), S2222 (Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA), S2238 (HD-Phe-Pip-Arg-pNA) and Suc-Ala-Ala-Pro-Phe-pNA for the above mentioned enzymes, respectively.

2.12. SDS-PAGE analysis

SDS-polyacrylamide gel electrophoresis (15%) was carried out as described by Laemmli (1970). After affinity chromatography, 40 µg of rBmTI-6, either treated with endoglycosidase H (endo H) or untreated, was applied to each lane. The protein gel was stained with Coomassie brilliant blue R250 and the gel analyzed for glycosylation was stained with Schiff's reagent.

2.13. Western blotting analysis

The purified rBmTI-6, after affinity chromatography (200 µg) and treated with endo H, was applied to an SDS-PAGE (15%) and blotted onto a PVDF membrane using the Mini Trans-Blot Cell (BioRad, Hercules, CA) electrophoretic transfer system. The PVDF membrane was cut into 0.5 cm wide strips and blocked with Blocking Buffer (PBS buffer containing 5% skimmed milk) for 1 h at room temperature. The blocked strips were incubated overnight at 4 °C with antiserum (1:50 dilution) from immunized cattle using purified BmTIs from larvae and control cattle (that received only the vehicle). The sera were prepared according to Andreotti et al. (2002). After the washing step with Blocking Buffer, the membrane strips were incubated with anti-bovine IgG conjugated with peroxidase (1:10,000) for 1 h at room temperature. The strips were then revealed

using SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL) and X-ray film (8 in. × 10 in.) (Kodak Biomax MS, NY).

2.14. Preparation of Schiff reagent and gel staining for glycoprotein detection

Fucsin (2 g) was stirred in boiling deionized water (400 ml), for 10 min and cooled down to 50 °C. The solution was filtered through a paper filter, 2 g sodium metabisulfite were added and, after homogenization, it was stored in dark conditions for 12 h. After incubation, 4 g active carbon were added, the mixture was stirred for 20 min, filtered and stored at room temperature.

The SDS-PAGE containing the purified rBmTI-6 was incubated in 7.5% acetic acid solution for 60 min at room temperature. Following this, the gel was incubated in a solution of 0.2% periodic acid solution for 45 min at 4 °C. After the incubation, the gel was stained with Schiff reagent for 45 min at 4 °C and destained using a sodium metabisulfite (0.5%) solution in 0.05N HCl.

2.15. Endoglycosidase treatment

Affinity purified rBmTI-6 (546.3 µg) was incubated with 1 µl of endoglycosidase H (Sigma) in 0.2 M sodium citrate buffer, pH 5.0 for 20 h at 37 °C. The reaction was interrupted by freezing the mixture at −20 °C.

3. Results and discussion

The Kunitz-BPTI inhibitors are widely distributed among arthropods, and mainly described in insects as silkworms (Kurioka et al., 1999), lepidopterans (Saul and Sugumaran, 1986; Nirmala et al., 2001), flies (Azzolini et al., 2004) and beetles (Ueda et al., 2005). These inhibitors may participate in defense processes against entomopathogens by controlling the pro-

phenoloxidase cascade (Aso et al., 1994) or inhibiting proteases from entomopathogenic bacteria and fungi (Nirmala et al., 2001; Azzolini et al., 2004; Ueda et al., 2005).

However, the majority of Kunitz-BPTI inhibitors from ticks have been identified in saliva or extracts of salivary glands and their function speculated to be associated with the prevention of host blood coagulation, facilitating the tick feeding process. Among them, were described inhibitors for the following serine proteinases, FXa, FVIIa/tissue factor, thrombin (Waxman et al., 1990; van de Loch et al., 1996; Nienaber et al., 1999; Lai et al., 2004; Francischetti et al., 2002, 2004; Monteiro et al., 2005) and also one platelet aggregation inhibitor (Mans et al., 2002).

In 1979, Willadsen and Riding studying serine proteinase inhibitors of *B. microplus*, reported the purification and partial characterization of trypsin and chymotrypsin inhibitors from the larval stage. Almost 20 years later, our group determined the primary structure of BmTI-A (*Boophilus microplus* Trypsin Inhibitor A), which classified it as a double-headed Kunitz-BPTI inhibitor (Tanaka et al., 1999). Other members of the Kunitz-BPTI family were further purified from *B. microplus* larvae and eggs (BmTIs) (Andreotti et al., 2001; Sasaki et al., 2004). Although their physiological role is unknown, when partially purified BmTIs were used as antigens (immunogens) for immunization of cattle they elicited an immunological response that interfered with tick development and reduced tick numbers in a subsequent challenge experiment (Andreotti et al., 2002).

Twelve forms of BmTIs were isolated from larvae and demonstrated to have inhibitory activity for trypsin, HNE and HuPK with dissociation constants (K_{is}) in the nM range (Sasaki et al., 2004). Of the isolated BmTIs, some amino acid sequences were deposited in the SwissProt protein data bank, as the BmTI-6 N-terminal sequence (Fig. 3). Recently, the EST sequence for

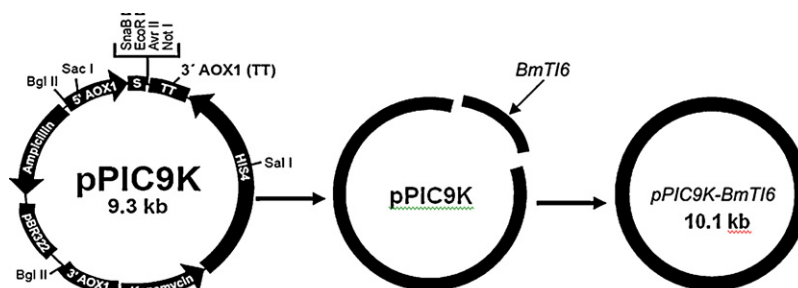


Fig. 1. Construction of the pPIC9K-BmTI6 vector. The plasmid pPIC9K was obtained from Invitrogen (Invitrogen, San Diego, CA). BmTI6 gene fragment was amplified from a *B. microplus* ovary cDNA preparation.

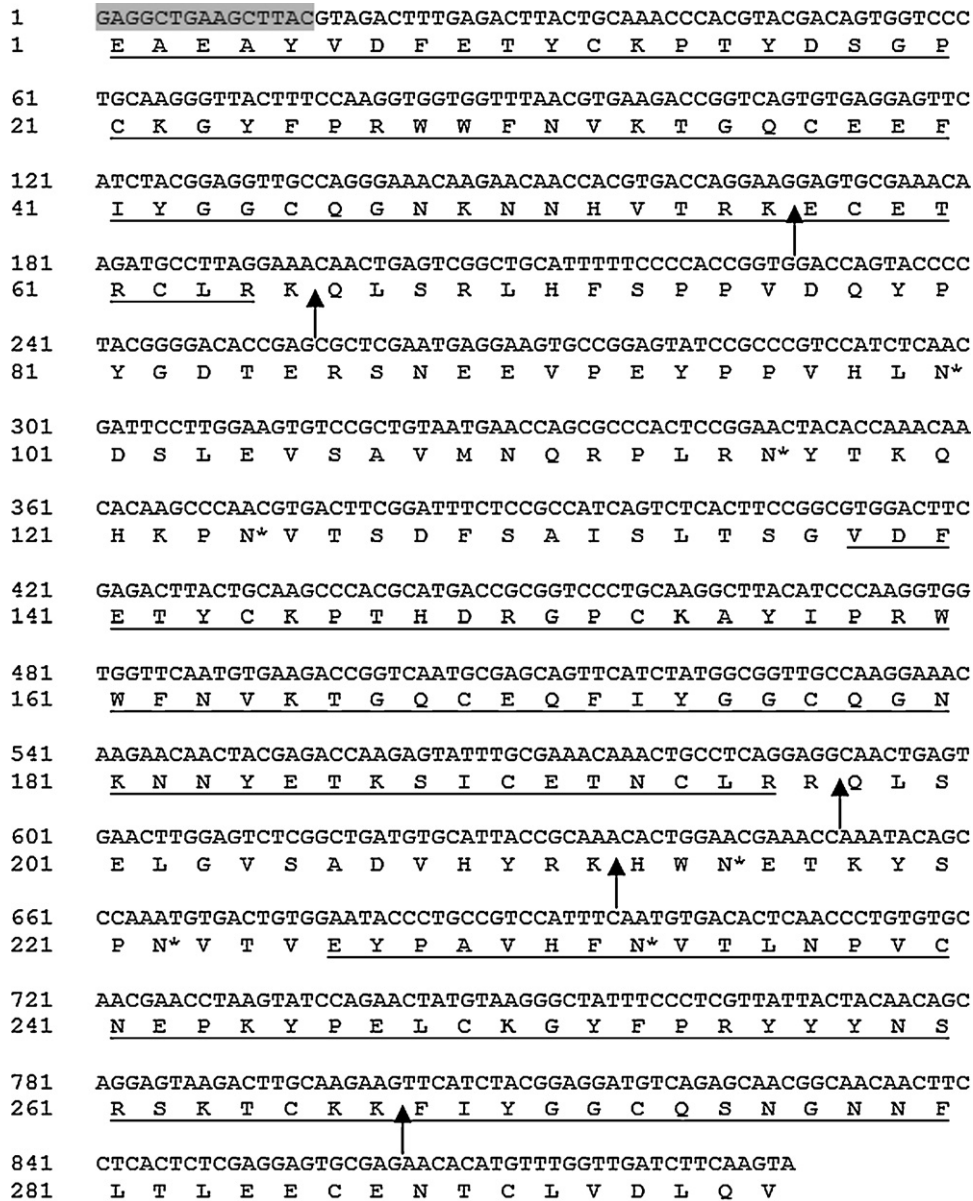


Fig. 2. Nucleotide sequence and deduced amino acid sequence of *BmTI6* gene fragment amplified from ovarian tissue. Nucleotide sequence added to the constructed pPIC9K-*BmTI6* vector in grey. Deduced Kunitz-BPTI domains are underlined. Putative glycosylated asparagines are indicated by asterisks. KEX2 protease possible processing sites are indicated by arrows.

BmTI-6 was deposited in GeneBank with accession number CK186726 (Guerrero et al., 2005); we named it *BmTI6* gene fragment. In this work we amplified one *BmTI6* gene fragment (873 bp) sequence, from *Boophilus microplus* ovarian cDNA, that translates into a protein composed by three Kunitz-BPTI domains (Fig. 2) containing 291 amino acids and a calculated molecular mass of 33857.7 Da.

BmTI-6 presented 94.3% of identity with rBmTI6-d1 (the first domain of rBmTI-6), probably due to the

exchange of phenylalanine for lysine in the automatic protein sequencing. The comparison of rBmTI-6's three putative domains shows a high identity between rBmTI6-1d and rBmTI6-2d (the second domain of rBmTI-6) (81.7%). In contrast, the identity between rBmTI6-d1 and rBmTI6-d3 (the third domain of rBmTI-6) or rBmTI6-d1 and BPTI was only 43.3% and 32.8%, respectively (Fig. 3). All three domains of rBmTI-6 presented lysine in the P1 reactive site position (Schechter and Berger, 1967), confirming their activity

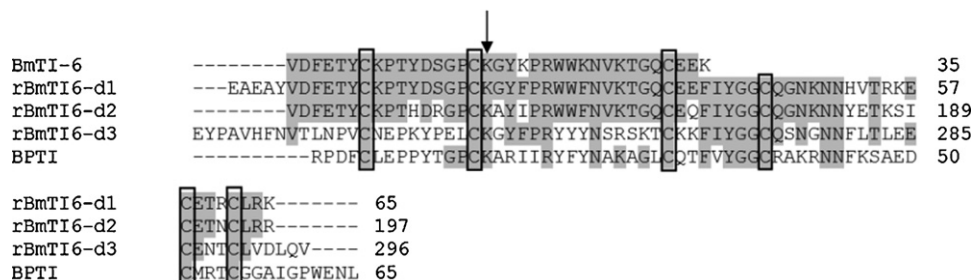


Fig. 3. Alignment of BmTI-6 Kunitz-BPTI domains and BPTI amino acid sequences. BmTI-6: amino terminal sequence of natural inhibitor from *B. microplus* larvae (access number: P83606). rBmTI6-d1, rBmTI6-d2, rBmTI6-3d: first, second and third domains of rBmTI-6. BPTI: bovine pancreatic trypsin inhibitor. Conserved cysteines are boxed. Identity of rBmTI6-d1 with other Kunitz-BPTI sequences marked in grey. Amino acid residues at P1 of reactive sites are indicated by black arrow.

for trypsin and plasmin. *BmTI6* gene fragment was amplified from ovarian cDNA; however, the gene fragment was also transcribed in *B. microplus* fat body (data not shown). The rBmTI-6 amino acid sequence has five amino acids more (EAEAY) on the N-terminal portion which was added by means of cloning procedures using the pPIC9K vector to insert the *Sna*BI restriction site (Fig. 3).

The yeast culture medium containing rBmTI-6 exhibited inhibitory activity for trypsin, the sample was then centrifuged and the supernatant collected. The supernatant titration using trypsin presented an expression level of 20.0 mg/L of culture medium. The rBmTI-6 was purified by affinity chromatography as the first step, following the methodology previously described by Tanaka et al. (1999) and Sasaki et al. (2004). Purified rBmTI-6 analyzed by SDS-PAGE showed an unexpected protein band profile (Fig. 4); we thus decided to test for glycosylation and the affects of enzymatic processing during rBmTI-6 expression in *Pichia pastoris*.

KEX2 protease is the processing protease of *P. pastoris* responsible for recombinant protein secretion (Laroche et al., 1994), and has specificity for two basic amino acids at the P1 and P2 positions (Holyoak et al., 2004). Five possible cleavage sites for *KEX2* protease were identified within the rBmTI-6 expressed fragment (Fig. 2). Our data suggest that rBmTI-6 was cleaved by *KEX2* protease in at least one site during expression. Affinity purified rBmTI-6 and yeast culture supernatant containing rBmTI-6 were chromatographed on Superdex 75 (Fig. 5A). Those proteins were separated into 4 fractions (1, 2, 3, 4). Those fractions were then pooled into pool 1 (fractions 1 and 2) and pool 2 (fractions 3 and 4) and trypsin inhibitory activity was found in both pools as a result of contributions made by original fractions 1 and 3 (Fig. 5A). Results of SDS-PAGE suggest that pool 1 contained protein ranging from 158 (fraction 1) to 21 kDa (fraction 2) and that pool 2 contained one band at 6.7 kDa and the majority of the protein banded at 9.0 kDa (Fig. 5B). Further purification

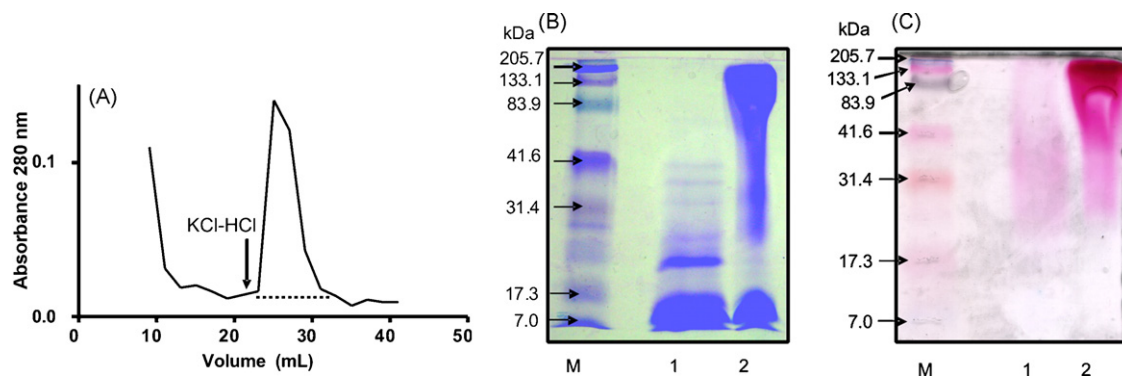


Fig. 4. rBmTI-6, affinity chromatography and SDS-PAGE analysis. A: affinity chromatography; culture supernatant (20 ml) was applied to a trypsin-Sepharose column (5 ml). The arrow shows the protein elution starting point, using KCl solution, pH 2.0. The protein elution was monitored by absorbance at 280 nm. The dotted bar shows the fraction containing trypsin inhibitory activity. B and C: SDS-PAGE (12%) of purified rBmTI-6 by affinity chromatography. 1: Inhibitor treated with endoglycosidase H; 2: inhibitor untreated with endoglycosidase H; M: protein molecular mass marker (Kaleidoscope, BIO-RAD). The gel was stained using Coomassie brilliant blue R 250 (B) or Schiff reagent (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

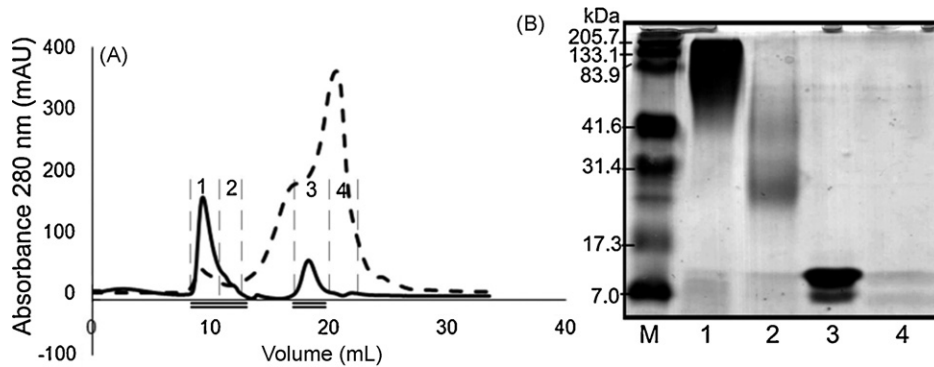


Fig. 5. rBmTI-6 gel filtration chromatography and SDS-PAGE analysis. A: The purified rBmTI-6 by affinity chromatography (continuous line) and the yeast culture supernatant containing rBmTI-6 (segmented line) were chromatographed on a Superdex 75 column. Double black lines show the region with trypsin inhibitory activity on both chromatographies. 1, 2, 3 and 4-pools of proteins collected from gel filtration chromatography of rBmTI-6 from affinity chromatography. B: The pools 1, 2, 3 and 4 from gel filtration chromatography were analysed by SDS-PAGE (15%).

of pool 2 proteins by reverse-phase chromatography followed by N-terminal sequencing of the major peak protein (9 kDa) demonstrated that the polypeptide was consistent with domains 1 of the expressed rBmTI-6 fragment suggesting that domain 1 had been cleaved from domains 2 and 3. Treatment of affinity purified rBmTI-6 with endoglycosidase H revealed eight polypeptide bands (81.0, 42.0, 39.0, 32.0, 22.5, 16.7, 9.0, 6.7 kDa) on SDS-PAGE (Fig. 4B) while the glycosylated rBmTI-6 sample revealed the pattern of 21–158 kDa bands as seen in Figs. 4C and 5B. Fig. 4C clearly demonstrates that the cleaved domain 1 of rBmTI-6 was not glycosylated during expression while the other 2 domains were. Also apparent is the affect of glycosylation on the electrophoretic mobility of the Kunitz domains 2 and 3 fragment. rBmTI-6 presents six *N*-glycosylation sites (Fig. 2), located mainly in the loops between the Kunitz-BPTI domains: three putative glycosylated asparagines located in the first loop (between the first and second domains of rBmTI-6) and two others in the second loop (between the second and third domains of rBmTI-6); finally, one possible glycosylated asparagine is located in the third domain of rBmTI-6 (Fig. 2).

In brief, during the expression procedure, rBmTI-6 was split in two parts; the first domain (rBmTI6-d1) was not glycosylated, but the portion formed by the two other domains (rBmTI6-d2 and rBmTI6-d3) was glycosylated. The rBmTI-6 glycosylation, apparently, did not interfere in the inhibitory activity, as it was still able to inhibit trypsin (data not shown).

After affinity chromatography, the rBmTI-6 sample presented inhibitory activity toward trypsin (K_i 1.7 nM) and plasmin (K_i 20 nM) but did not inhibit HuPK, HNE, chymotrypsin, FXa, FXIIa or thrombin.

Finally, in an attempt to show rBmTI-6's immunological potential, we performed Western-blotting experiments using purified rBmTI-6, after affinity chromatography, treated with endoglycosidase H, and antiserum from bovines immunized with natural BmTIs (Andreotti et al., 2002). Purified rBmTI-6 was recognized by the antiserum from bovines immunized with natural BmTIs (Fig. 6), demonstrating that antibodies bound to the rBmTI-6 fragments and suggesting a possible cross reaction among the epitopes of natural BmTIs and rBmTI-6. However, one cattle immunization trial using the rBmTI-6 is necessary to

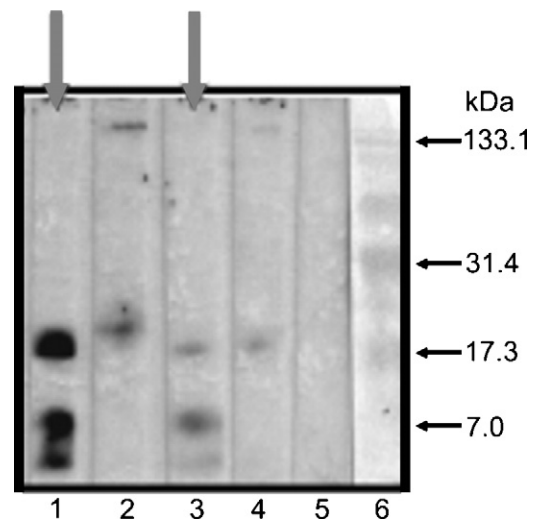


Fig. 6. Western-blotting analysis of the purified rBmTI-6 (affinity chromatography) using the antiserum from bovines immunized with natural BmTIs. Each strip of PVDF membrane was incubated with a different antiserum. 1 and 3: Antiserum from BmTIs immunized bovines; 2 and 4: Antiserum from control animals; 5: without antiserum; 6: protein molecular mass marker (Kaleidoscope, Bio-Rad).

confirm the rBmTI-6 protective potential against *Boophilus microplus* infestation.

Acknowledgments

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