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An unexpected inhibitory activity of Kunitz-type serine proteinase inhibitor derived from *Boophilus microplus* trypsin inhibitor on cathepsin L

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Abstract

Several BPTI-Kunitz-type serine proteinase inhibitors were described in tick *Boophilus microplus* and *Rhipicephalus sanguineus* species. In this work, we present a synthetic gene based on two tick BPTI-Kunitz-type serine proteinase inhibitors, the first domain of *B. microplus* trypsin inhibitor-A (BmTI-A) and the carrapatin, the inhibitors were named BmTIsint and BmTIsint Mut. Our present results showed that BmTIsint and BmTIsint Mut inhibited trypsin (K_i 3.3 and 1.0 nM) and human plasma kallikrein (K_i 16.5 and 35 nM), but in contrast to BmTI-A, the inhibitors did not inhibit human neutrophil elastase. BmTIsint was able to produce immunological response in mice but not in bovines. In addition, it is the first description of a BPTI-Kunitz-type inhibitor as a cysteine proteinase inhibitor, BmTIsint apparent dissociation constant (K_i) for cathepsin L was 108 nM. Our findings open the possibility up to obtain new molecules as potent serine or cysteine proteinase inhibitors using BmTIsint as a model.

Keywords: Cysteine proteinase inhibitor; Serine proteinase inhibitor; Tick; Ectoparasite; *Boophilus microplus*

Ticks are arthropod blood feeders responsible for transmission of infection agents to human and some animals of economic importance, causing important losses to livestock production [1]. Saliva of hematophagous arthropods contains substances that interfere in many of their host's defense response, mainly which could disrupt the feeding process [2]. Among them, several anti-hemostatic molecules presenting the proteinase inhibitor BPTI-Kunitz structure fold have been described, e.g., the tick anticoagulant protein (TAP), a factor Xa inhibitor [3], and ornithodorin [4], a thrombin inhibitor purified from *Ornithodoros moubata*; savignin [5], a thrombin inhibitor, and savignygrin, a platelet aggregation inhibitor that possesses the RGD integrin recognition motif from *O. savignyi* species [6]. Besides them members of the Kunitz-type family with not clear anti-hemostatic function were described in *Boophilus microplus* [7–10] and *Rhipicephalus sanguineus* [11] tick species.

BPTI-Kunitz-type inhibitor family members based on the three-dimensional (3D) structure are classified into two families: canonical Kunitz-type inhibitors including BPTI-like toxins and anticoagulant proteins [12]. The canonical Kunitz-type inhibitor specificities towards serine proteinases are defined by the P1 amino acid (according to the Schechter and Berger nomenclature [13]) and small amino acid sequence differences surrounding the region which interacts with the target enzyme [14]. These small molecules have been already described in bovine pancreas (BPTI) [15], snake venom [16,17], sea anemone [18], snails [19], horseshoe crab [20], insect hemolymph [21–23], fly [24], and ticks [8,10,11]. In 1999, our group partially sequenced BmTI-A (*B. microplus* trypsin inhibitor) and the result allowed classifying it in the BPTI-Kunitz-type serine proteinase inhibitor family. We also demonstrated that BmTI-A is a double-headed inhibitor of trypsin or

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human plasma kallikrein and neutrophil elastase [8]. Proteins containing Kunitz-type motif were already described as canonical serine proteinase inhibitors, ion channel inhibitors, anticoagulant and anti-platelet aggregation but a cysteine proteinase inhibitory activity had not been identified yet. Cysteine proteinases are expressed in organisms from bacteria to humans. In parasites it has been suggested that cysteine proteases are involved in invasion of host tissues and evasion of the host immune system (reviewed in [25,26]). In the hematophagous organisms, cysteine proteinases have been described to be responsible for hemoglobin digestion in the gut of *Schistosoma mansoni* [27] and in the vacuole of the *Plasmodium falciparum* [28]. In the *B. microplus* tick, some cathepsin L-like enzymes have been found in the midgut, which suggests a role in the meal digestion [29,30] and in eggs a role in the vitellin degradation [31]. In the present work, we describe the two *B. microplus* serine proteinase inhibitor (BmTI) synthetic gene construction, the recombinant protein expression, purification, and characterization. In addition, for the first time we presented a Kunitz-type molecule as a cysteine proteinase inhibitor.

Experimental procedures

Materials

Proteinase inhibitors. Aprotinin was obtained from Sigma (St. Louis, MO) and [R15,E52] aprotinin was kindly provided by Dr E. Auerswald [32]. *B. microplus* trypsin inhibitor-A (BmTI-A) from tick larvae was purified as described by Tanaka et al. [8]. Synthetic *B. microplus* trypsin inhibitor (BmTIsint) was prepared in our laboratory based on BmTI-A first domain and the carrapatin sequence from Data Bank (Accession No. P81162).

Enzymes. Bovine trypsin (EC 3.4.21.4) was obtained from Sigma (St Louis, MO). Human neutrophil elastase (EC 3.4.21.37) was from Calbiochem (San Diego, CA). Human plasma kallikrein (EC 3.4.21.34) was prepared as previously described by Sampaio et al. [33]. Human factor XIIa (EC 3.4.21.38) was purchased from Calbiochem (San Diego, CA). Human plasmin (EC 3.4.21.7) was from Boehringer Mannheim GmbH (Germany). Recombinant human cathepsin L (EC 3.4.22.15) was expressed in *Pichia pastoris* as described previously [34] using a plasmid construction kindly provided by Dr. D. Nägler from Department of Clinical Chemistry and Biochemistry—LMU, Munich, Germany. Papain (EC 3.4.22.2) was purchased from Calbiochem (San Diego, CA).

Synthetic substrates. Tosyl-Gly-Pro-Arg-pNA was purchased from Pentapharm (Basel, Sweden). S2484 (Gly-Pro-Val-pNA), S2302 (HD-Pro-Phe-Arg-pNA), and S2251 (HD-Val-Leu-Lys-p-NA) were from Chromogenix (Mölndal, Sweden).

BmTIsint gene construction

The BmTIsint synthetic gene construction was based on the amino acid sequence of BmTI-A first domain [8] (BmTI-d1). The first domain amino acid sequence had unidentified amino acid residues that was filled out with amino acid residues of carrapatin (PDB Accession No. P81162), a Kunitz-type serine proteinase inhibitor from *B. microplus* tick. BmTIsint is a chimera of BmTI-A and carrapatin. Preferential codons to yeast were used to construct the synthetic gene [35].

BmTIsint gene synthetic construction

The BmTIsint gene DNA fragment was defined in 208 bp. The BmTIsint gene nucleotide sequence was divided into 16 oligonucleotides (18 nt to 40 nt in length). The adjacent oligonucleotides of sense and antisense strands have overlaps at both 5′ and 3′ ends. The oligonucleotides (50 nmol) were synthesized by Invitrogen (São Paulo, Brazil). Four-step protocol was used in the gene synthesis to construct the BmTIsint gene. In step 1, all 16 oligonucleotide 5′ ends were phosphorylated using 30 U of T4 Kinase (USB) and 400 pmols ATP (Amersham Pharmacia) by incubation at 37 °C for 30 min, and the enzyme was inactivated heating it to 65 °C for 5 min. In step 2, 200 pmols of each phosphorylated oligonucleotide was mixed and incubated at 94 °C for 5 min and a gradual cooling was performed up to 25 °C to their complementary annealing. The annealed oligonucleotides were purified by phenol–chloroform extraction and isopropanol precipitation (double strand of the synthetic gene are larger than those of the oligonucleotides, therefore they were preferentially purified) following Sambrook et al. [36]. In step 3, double strand adjacent oligonucleotides were ligated by DNA T4 ligase, obtaining the full-length gene product, which in step 4 was amplified by PCR.

BmTIsint Mut gene design

BmTIsint Mut was constructed by substitution of residues Gly_{21} and Leu_{22} for Ala and Arg, respectively. BmTIsint Mut synthetic gene was synthesized following the same method of BmTIsint synthetic gene, mutating the oligonucleotides Bmsint2 (5′ CAGATCAAGGTCCAT GTAGAGCTCG 3′) and 2stBmsint6 (5′ TCTCAAGATGCGAGCTC TACATGGA 3′) to replace the 21st and 22nd amino acid residues of BmTIsint.

Construction of pPIC9-BmTIsint and pPIC9-BmTIsint-Mut vectors

The BmTIsint gene was amplified by PCR using constructed synthetic gene as template, the sense primer fBmTIsintexp (5′ GGTATCTCTC GAGAAAAGATCTCAACCACA 3′) and anti-sense primer rBmTIsintexp (5′ CCCGTGCGGCCGCTCACTTAGCTTCGTAT 3′) to add restriction sites to *Xho*I and *Not*I enzymes. The synthetic gene was digested with *Xho*I and *Not*I restriction enzymes, the fragment ligated into the plasmid pPIC9 (Invitrogen). The resulting plasmid (pPIC9-BmTIsint) was linearized with *Sac*I restriction enzyme to transform competent *P. pastoris* GS115 yeast strain prepared according to the manufacturer's instructions. Transformed yeast was incubated for 4 days in buffered methanol-complex medium (BMMY). After fermentation, yeast cells were harvested by centrifugation (4000*g*, 20 min, 4 °C) and the supernatant containing the inhibitory activity was stored at −20 °C. The same methods were used for BmTIsint-Mut expression.

BmTIsint and BmTIsint-Mut purification

The purification of BmTIsint expressed in *P. pastoris* system was carried out using three chromatographic steps: affinity chromatography in a trypsin–Sepharose column, hydrophobic interaction chromatography (HIC) using a Source-15PHE column (Amersham Pharmacia), and reverse-phase chromatography in a Sephasil Peptide C_8 column (Amersham Pharmacia). The supernatant of the BmTIsint *P. pastoris* expression was applied to a trypsin–Sepharose column previously equilibrated with 50 mM Tris–HCl buffer, pH 8.0. Weakly bound proteins were washed out with the same buffer, pH 8.0, containing 0.2 M NaCl, and the recombinant BmTIsint was eluted with 0.2 M KCl solution, pH 2.0. The eluted fractions were immediately neutralized with 1 M Tris–HCl buffer, pH 8.0. The fractions containing the BmTIsint were pooled, dialyzed, lyophilized, suspended in buffer A (50 mM sodium phosphate buffer, 1.7 M ammonium sulfate, pH 7.0) of HIC, and applied to a Source-15PHE column previously equilibrated with buffer A. The protein elution was performed with 0–100% linear gradient of buffer B (50 mM sodium phosphate buffer, pH 7.0) and the fractions with inhibitory activity were pooled. In the last step of purification, the pooled BmTIsint was applied to a Sephasil Peptide C_8 column. The proteins were eluted with 0–90% acetonitrile linear gradient in 0.1% trifluoroacetic acid (TFA) and the purified BmTIsint was stored at −20 °C. BmTIsint-Mut expressed in *P. pastoris* was purified following the same procedure for BmTIsint.

NH_2-terminal sequencing

The NH_2-terminal amino acid sequence was determined by Edman degradation [37] using a PPSQ-23 Model Protein Sequencer from Shimadzu (Tokyo, Japan).

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out with an Ettan MALDI-TOF pro (Amersham Biosciences). The purified BmTIsint was dissolved in water and mixed (1:1) with a saturated solution of the matrix (α-cyano-4-hydroxycinnamic acid). Measurements were performed at a positive linear mode and the signal was accumulated and averaged over 200 shots.

Serine proteinase inhibition assays

The trypsin inhibitor activity was measured by the remaining hydrolytic 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 [38]. The concentration of active trypsin was determined by active site titration with *p*-nitrophenyl-*p*′-guanidino-benzoate as described by Chase and Shaw [39]. The equilibrium dissociation constants of complexes between the bovine trypsin and human plasma kallikrein with serine proteinase inhibitors were determined using the method described by Bieth [40]. Briefly, the serine proteinases were incubated with different concentrations of the 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 activities were measured after addition of the chromogenic substrates tosyl-Gly-Pro-Arg-

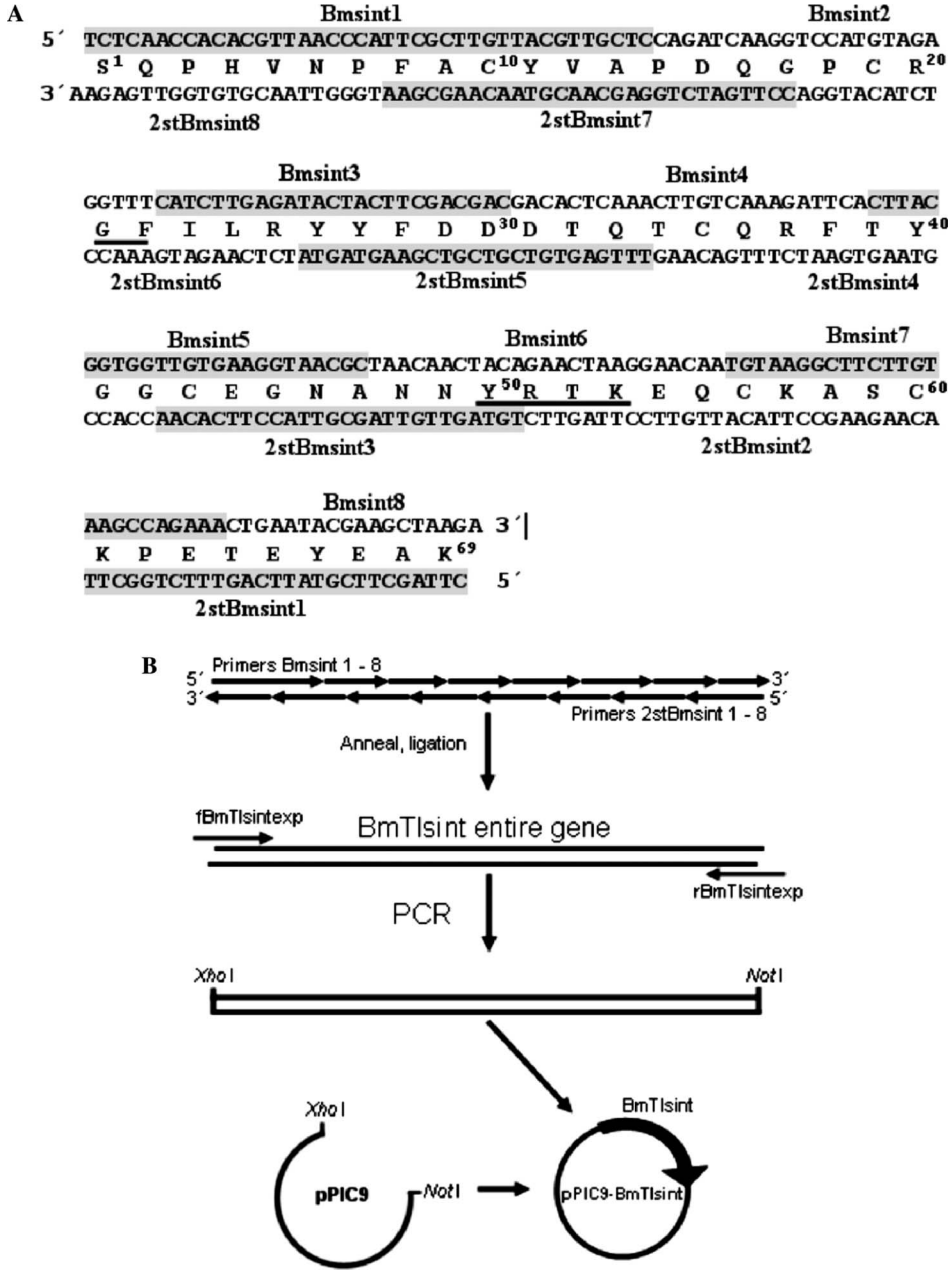

Fig. 1. (A) The complete BmTIsint gene DNA sequence. Each of 16 oligonucleotides is marked with background in gray or white, forward oligonucleotides were named Bmsint 1–8, and reverse oligonucleotides were named 2stBmsint 1–8. The BmTIsint amino acid sequence is shown between the DNA strand sequences. Carrapatin amino acid residues are underlined in black. (B) Construction strategy of synthetic gene followed of BmTIsint DNA amplification by PCR and the expression plasmid pPIC9-BmTIsint construction.

pNA and S2302 (HD-Pro-Phe-Arg-pNa) for trypsin and human plasma kallikrein, respectively. Apparent K_i values were calculated by fitting the steady-state velocities to the equation $(V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t)$ for tight-binding inhibitors using a non-linear regression analysis [41].

The inhibitory activities toward human neutrophil elastase, human factor XIIa, human plasmin, human factor Xa, bovine thrombin, porcine pancreatic kallikrein, and chymotrypsin were performed with BmTIsint and BmTIsint Mut inhibitors (50 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 activities were measured after addition of the following chromogenic substrates: S2484 (Gly-Pro-Val-pNA), S2302 (HD-Pro-Phe-Arg-pNA), S2251 (HD-Val-Leu-Lys-pNa), S2222 (Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA), S2238 (HD-Phe-Pip-Arg-pNA), S2266 (HD-Val-Leu-Arg-pNA), and Suc-Ala-Ala-Pro-Phe-pNA for enzymes given above, respectively. Inhibitory activity of BmTIsint (50 mM) and BmTIsint Mut (50 mM) toward subtilisin-A was performed in 0.1 M Tris–HCl buffer, pH 8.6, containing 0.1% Triton X-100 at 37 °C. The residual enzyme activity was measured after addition of the synthetic substrate Boc-Gly-Gly-Leu-pNA.

Cysteine proteinase inhibition assays

Inhibition assays for cathepsin L were performed in 50 or 100 mM sodium acetate buffer, pH 5.5, in a final volume of 200 μL. Cathepsin L was pre-activated with 1 mM dithiothreitol in sodium acetate buffer, pH 5.5, for 5 min at 37 °C followed by the addition of the synthetic substrate HD-Pro-Phe-Arg-pNA. Five different concentrations of substrate HD-Pro-Phe-Arg-pNA S2302 (0–38.5 nM) were used in the absence or in the presence of BmTIsint to determine the K_i value using Dixon plot [42]. Absorbance was measured continuously at $\lambda = 405$ nm.

Results and discussion

In a previous work, we showed the presence of BPTI-Kunitz-type inhibitor in two tick species, the *R. sanguineus* [11] and *B. microplus* [8,10], which suggested a possible role in hard tick species. The *B. microplus* trypsin inhibitors (BmTIs) showed to be useful tool in the bovine immunization. When larvae BmTIs were used as antigens in a bovine immunization trial, they presented 72.8% efficacy in interfering with tick development [43]. This BmTIs information stimulated us to obtain an inhibitor cDNA fragment. However, in the last years we failed to reach this goal, but considering the immunization results using BmTIs, we decided to construct a synthetic gene in an attempt to develop a recombinant antigen based on one of *B. microplus* trypsin inhibitor sequences. In the present work, we describe the construction of two BmTI synthetic genes based on the first domain of BmTI-A [8], the protein expression, purification, and characterization. The synthetic gene construction strategy is shown in Fig. 1. Sixteen oligonucleotides were designed using the BmTI-Ad1 and carrapatin amino acid sequences which result in the gene named BmTIsint, of a chimera protein. In order to improve the BmTIsint express in yeast, the preferential codons to yeast were used [35]. BmTIsint expression yield using *P. pastoris* expression system was 4.0 mg/L of culture medium. The BmTIsint secreted in the *P. pastoris* supernatant culture was purified using the following three purification steps: affinity, hydrophobic, and reverse-phase chromatographies (Fig. 2A–C). The purified BmTIsint showed the expected molecular mass by SDS–PAGE (Fig. 2D). The correct processing of BmTIsint was confirmed by amino acid sequencing, but mass spectrometry analysis showed a molecular mass of 7780.5 Da (data not shown) in contrast to the expected molecular mass of 7912.7 Da, which could be explained if the lysine residue on the C-terminal sequence of BmTIsint was missed. Isolated recombinant BmTIsint was used in immunization experiments and was able to induce specific serological immune response in Balb-C mice (data not shown). Even though one of the aims of this work had been the production of antigen candidate against bovine tick infestation, the immunological trial using 100 μg/dose of BmTIsint in complete Freund's adjuvant did not produce immunological response. Apparently, an immune response using native BmTIs was more efficient because it was used a pool of one, two or more different Kunitz inhibitor domains [43].

Parallel to this work, several BmTIs were identified and characterized in larvae and eggs of *B. microplus* during their development [10], although BmTIs concentration decreases in the larval stage, the elastase inhibitory activity slightly increases [9]. *B. microplus* larval BmTIs physiological role is still unknown. In order to obtain more information about these molecules, biochemical studies have been done using the protein BmTIsint. The BmTIsint P1′ (glycine) and P2′ (leucine) residues were defined based on the carrapatin sequence. In the case of BmTIsint Mut, these residues were replaced by arginine and leucine. BmTIsint Mut was obtained by the same purification procedures (data not shown). Both inhibitors, BmTIsint and BmTIsint Mut, were analyzed toward different serine proteinases showing strong inhibition for trypsin with K_i of 3.3 and 1.0 nM, respectively, and human plasma kallikrein 16.5 and 35 nM, respectively (Table 1). BmTIsint inhibitory activity data confirmed that BmTI-A inhibition for trypsin and plasma kallikrein occurs by the first domain interaction and that neutrophil elastase inhibition depends on the BmTI-A second domain [8]. The amino acid substitution of BmTI-A d1 by carrapatin amino acid residues did not affect trypsin or plasma kallikrein inhibition activity. In contrast, the chimera molecule showed to be a better plasma kallikrein inhibitor than BmTI-A (Table 1). But, we cannot discard the fact that the second domain can cause steric hindrance for plasma kallikrein inhibition by native BmTI-A. Human plasma kallikrein is involved in several important physiological and patho-physiological pathways including contact activated (intrinsic pathway) coagulation, fibrinolysis, hypotension, and inflammation [44]. Our findings showed BmTIsint as a good tool to develop a specific HuPK inhibitor for a therapeutic candidate, since inappropriate HuPK activity can mediate several diseases.

The alignment of BmTIsint and BmTIsint Mut with other BPTI-Kunitz-type amino acid sequences using Clustal-X software confirmed its inclusion in this family even if they are chimera molecules (Fig. 3).

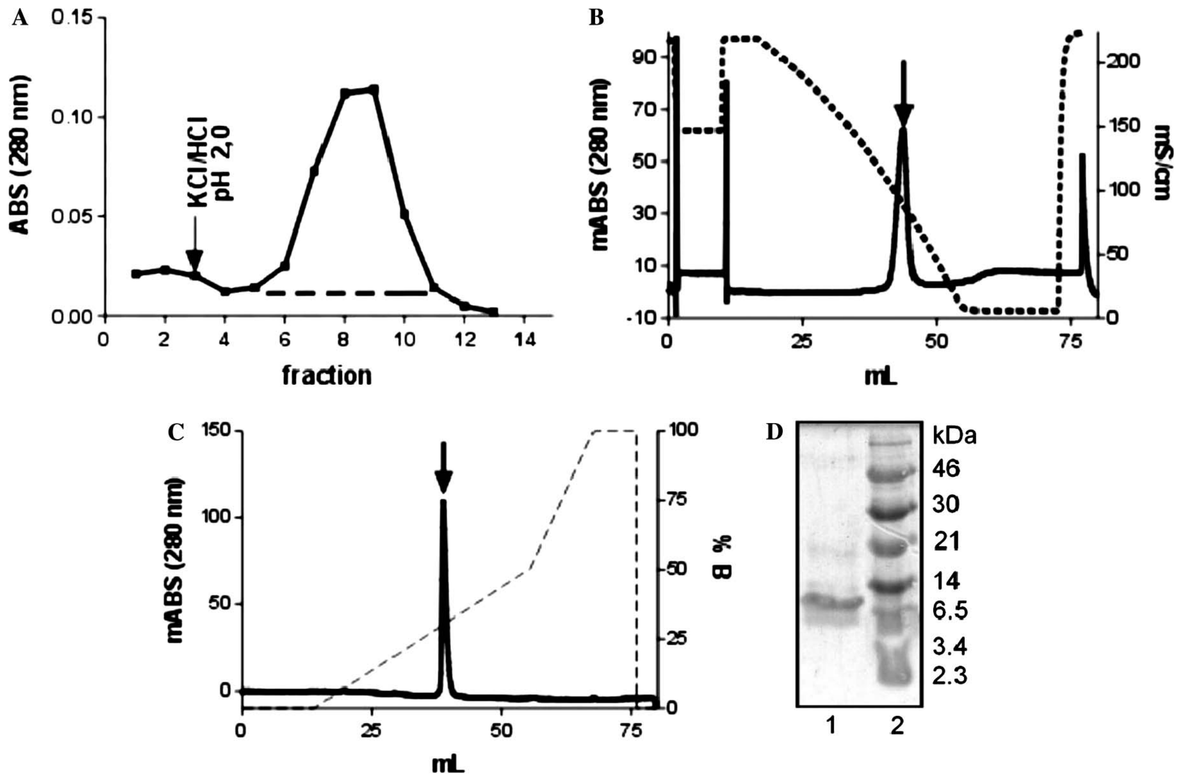

Fig. 2. BmTIsint purification. The culture supernatant of *Pichia pastoris* containing BmTIsint was applied on a trypsin–Sepharose column (A), active fractions toward trypsin (dotted line) were pooled and applied on Source-15PHE column (B), and the active peak was isolated on reverse-phase C_8 column (C). Arrows are showing active peaks of hydrophobic and reverse-phase chromatographies. (D) SDS–PAGE (15%) of BmTIsint purified by reverse-phase chromatography on C_8 column.

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                                                1         2↓                 3       4                5   6
 1 BmTIsint                01---------SQPHVNPFACYVAPDQGPCRGFILRYYFDDDTQTCQRFTYGGCEGNANNYRTKEQCKASCKPETEYEAK------
 2 BmTIsint Mut            01---------SQPHVNPFACYVAPDQGPCRARILRYYFDDDTQTCQRFTYGGCEGNANNYRTKEQCKASCKPETEYEAK------
 3 BmTI-A                  01---------SQPHVNPFACYVAPDQGPCRXXILRYYFDDDTQTCQRFTYGGCEGNANNXXXXEQCKASCKPETEYEAK------
 4 Lacunin               2013-----------------ACNLPKVKGACLGYNIRWYYDAEQEQCSQFIYGGCLGNANNYASLQLCQEQCQPE------------
 5 Ixolaris                98---------------KPPCSLEVDYGVGRANIPRWYYDTNNATCEMFTYGGITGNKNNFESEEECKETCKGFSLLKKVNVTIN-
 6 Kun-5                   21----------LVKNGQERCTEVVDEGPCRALIPRYFYNMETEKCEEFDYGGCYGNNNNFFNESSCTSTCKGVSKMDMCEL----
 7 BPTI                    01--------------RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGAIGPWENL-----
 8 [R-15,G-52]Aprotinin    01-------------MRPDFCLEPPYTGPCRARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCERTCGGA------------
 9 AAM68494.2             493--VNPIDCHINAEQAKDICVQAPDPGPCRGTYMRYAYDPQNQHCYSFTYGGCRGNRNNFLTENDCLNTC---------------
10 S12957                  01--------------RPRFCELAPSAGSCFGFVSSYYYNRYSNTCHSFTYSGCGKNANRFRTIDECNRTCVV-------------
11 Pp5                     23-----DASQAPPGNNAEICLLPPDDGPCRARIPSYYYDRYTQSCREFMYGGCEGNANNFETLEACNEACWKIEKV---------
12 Textilin                25------------KDRPELCELPPDTGPCRVRFPSFYYNPDEQKCLEFIYGGCEGNANNFITKEECESTCAA-------------
13 AAY66790.1              20-------ATDAKKSSGERCYMKPKTGPCKAKYERFHYDRKDLTCKPFIYGGCGGNENNFKTKEECQKACK--------------
14 AAM93648.1              21--------VDAKRSPGERCTLPKKTGPCKASFSRFYFDNKDGQCKSFTYGGCGGNGNNFKTLKKCQRTCK--------------
15 Boophilin               16-------------QRNGFCRLPADEGICKALIPRFYFNTETGKCTMFSYGGCGGNENNFETIEECQKACGAPERVNDFESA---
16 S30332                  01----------------SICSEPKKVGRCKGYFPRFYFDSETGKCTPFIYGGCGGNGNNFETLHQCRAICRA-------------
17 CSTI_BOMMO              01---------------NPDCLLPIKTGPCKGSFPRYAYDSSEDKCVEFIYGGCQANANNFETIEECEAACL--------------
18 Bitilisin-3             01-PLRPGTEKHTFPFPAEFCNLPADLGPCKNYTGRFYYDSASNKCEVFIYGGCPGNANNFKTREECRKTCVE-------------
19 Carrapatin              01-----------AVDFDKQCVPTADPGPCKGFMPMWWYNIFTSQCEEFIYGGCQGNDNRYRTKEECDKTCAEASATWDVNA----
20 TFPI                    41-----DTELPPLKLMHSFCAFKADDGPCKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTRDNANRIIKT----
21 HiTI                    01 ------------FDKADCSLPKEVGPCRKSDLSYYYDTESKACQEFFYGGCHGNNNRFSSKEQCEQSCM--------------
22 Bikunin                148 --------------EAACRLPMDAGPCKAFVDLWAFDSSSGKCLSLKYGGCKGNGNRFYSKKECDEYCGAMMRNGDDEFLSVN
23 Amblin                  16 -----------QRVPGYCKKKPAVGPCKALIEKWYFDYSTQSCKTFYYGGCGGNGNKFSSRKKCREACLPKRP----------
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Fig. 3. Comparison among BmTIsint and BmTIsint Mut primary sequences and Kunitz-type serine proteinase inhibitors from different animal species. 1, BmTIsint; 2, BmTIsint Mut; 3, BmTI-A (P83609, *B. microplus* [8]); 4, lacunin (AAF04457, *Manduca sexta* [46]); 5, ixolaris (AAK83022.1, *Ixodes scapularis* [47]); 6, KUN-5 (AAT92116.1, *Ixodes pacificus* [48]); 7, BPTI (*Bos taurus*); 8, [Arg-15,Glu-52]aprotinin (AAA72784.1 [32]); 9, AAM68494.2 (*Drosophila melanogaster*, [49]); 10, S12957 (venom animal Kunitz-type chymotrypsin inhibitor, *Naja naja naja* [16]); 11, Pp5 (AAO84035.1, *Bos taurus*); 12, textilinin (AAK95520.1, *Pseudonaja textilis textilis* [50]); 13, AAY66790.1 (*Ixodes scapularis* [51]); 14, AAM93648.1 (*Ixodes scapularis* [47]); 15, Boophilin (CAC82583.1, *B. microplus*); 16, S30332 (*Stichodactyla helianthus* [52]); 17, CSTI (P81902, *Bombyx mori* [53]); 18, bitisilin-3 (AAR19275.1, *Bitis gabonica*); 19, carrapatin (P81162, *B. microplus*); 20, TFPI protein (AAH15514.1, *Homo sapiens*); 21, HiTi (AAL87009.1, BPTI-like serine protease inhibitor, *Haematobia irritans irritans* [24]); 22, bikunin precursor (AAH55598.1, *Danio rerio*); 23, amblin (AAR97367.1, *Amblyomma hebraeum* [54]) The black arrow shows the P1 residue of BmTIsint and BmTIsint Mut by similarity. Identity of BmTIsint and BmTIsint Mut, and other sequences is marked in gray. Kunitz-type conserved cysteines are indicated by numbers. Multiple alignments were performed using CLUSTAL W (1.82).

Table 1
Apparent dissociation constant (K_i) of BPTI-Kunitz-type inhibitors for different serine proteinases

	K_i (nM)			
	Trypsin	Neutrophil elastase	HuPK	Plasmin
BmTIsint	3.3	n.i.	16.5	—
BmTIsint Mut	1.0	n.i.	35.0	—
BmTI-A	3.0[b]	1.4[b]	120[b]	590[b]
Aprotinin	0.25	3600[a]	30[a]	1.0[a]

n.i., not inhibited.
[a] Fritz and Wuderer [55].
[b] Tanaka et al. [8].

Table 2
Apparent dissociation constant (K_i) BPTI-Kunitz-type inhibitors for cathepsin L and the inhibitor reactive site amino acid sequences

Serine proteinase inhibitor	Reactive site region P4–P5′[a]	Cathepsin L K_i (nM)
BmTIsint	GPCRGFILR	108
BmTIsint Mut	GPCRARILR	n.d.
BmTI-A d1	GPCR–ILR	n.d.
BPTI	GPCKKARIIR	500

n.d., not determined.
[a] Schechter and Berger [13].

Kunitz-type motif includes several molecules with different functions such as canonical serine proteinase inhibitors, ion channel inhibitors, and anticoagulant, and anti-platelet aggregation. In addition, the information that it has much less serine proteinases than cysteine proteinases described in ticks, e.g., in *B. microplus* tick, cathepsin L-like enzymes has been found in the midgut [29,30] and in eggs [31]. For this reason, we analyzed BmTIsint toward cysteine proteinases, which result in a cathepsin L inhibitor with K_i of 108 nM (Table 2). Cathepsin L was also inhibited by the other Kunitz-type inhibitor such as BPTI with K_i of 500 nM (Table 2). Similar result was described for papain and the plant Kunitz trypsin inhibitor from *Prosopis juliflora* (PTPKI), which presents a higher molecular mass, 20 kDa and different three-dimensional structure of BPTI-Kunitz inhibitors. The papain inhibition seems to be worse than cathepsin L inhibition, but the dissociation constant was not determined [45]. If we consider the amino acid in the reactive sites of BmTIsint (Table 2) and the PTPKI that interact to papain [45], the BmTIsint residues at P1, P3, and P3′ positions Arg, Pro, and Ile, respectively, seem to be important primary structural features for cysteine proteinase inhibitors. However, the BmTIsint important structural regions for cysteine proteinase inhibition will be only confirmed by BmTIsint and cathepsin L complex three-dimensional structure determination.

In conclusion, we described the synthetic gene strategy to construct a recombinant protease inhibitor from two BPTI-Kunitz-type inhibitors of *B. microplus* tick, which open up the possibility to obtain new molecules as potent serine and cysteine protease inhibitors and also different mutants to be used as antigen. This is the first description of cysteine proteinase inhibition by BPTI-Kunitz-type inhibitor, suggesting a possible role of BmTIs in the tick endogenous enzyme control.

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