Biochemical and Biophysical Research Communications 449 (2014) 69–73

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RmKK, a tissue kallikrein inhibitor from *Rhipicephalus microplus* eggs

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ARTICLE INFO

Article history:
Received 21 April 2014
Available online 9 May 2014

Keywords:
Rhipicephalus microplus
Eggs
Tissue kallikrein inhibitor
Serine protease inhibitor

ABSTRACT

Rhipicephalus microplus is an important ectoparasite that is responsible for transmission of anaplasmosis and babesiosis to cattle. Tissue kallikrein inhibitors might play an important role in *R. microplus* eggs. In the present work, we purified and characterized, a tissue kallikrein inhibitor presents in *R. microplus* eggs (RmKK), a protein which contains two Kunitz domain in tandem. Purified inhibitor was confirmed by amino terminal determination and its dissociation constant (K_i) for bovine trypsin and porcine pancreatic kallikrein were 0.6 nM and 91.5 nM, respectively. Using a cDNA library from *R. microplus* midgut, we cloned the cDNA fragment encoding mature RmKK and expressed the protein in *Pichia pastoris* system. Recombinant RmKK was purified by ion exchange chromatography and presented molecular mass of 16.3 kDa by MALDI-TOF analysis. Moreover, RmKK showed a tight binding inhibition for serine proteases as bovine trypsin (K_i = 0.2 nM) and porcine pancreatic kallikrein (PPK) (K_i = 300 nM). We performed, for the first time, the characterization of a tissue kallikrein inhibitor presents in *R. microplus* eggs, which the transcript is produced in the adult female gut. BmKK seems to be the strongest PPK inhibitor among all BmTIs present in the eggs and larvae (Andreotti et al., 2001; Sasaki et al., 2004). This data suggests that BmKK may participate in the development of tick egg and larvae phase.

1. Introduction

Rhipicephalus microplus is one of the most important ectoparasites in the world, inflicting economic losses in cattle production. *R. microplus* infestations cause reductions in cattle's weight and milk production [3]. Moreover, this tick species transmits several pathogens, including anaplasmosis [4] and babesiosis parasites, which result in neurological problems and death of cattle [5].

The study of new important molecules for *R. microplus* can help in the development of methods to control infestation by this tick. Ticks are a rich source of inhibitors of serine proteases, particularly those belonging to the family BPTI – Kunitz [2]. Several inhibitors of the Kunitz type have been studied and examples of these are: TAP (tick anticoagulant peptide) [6] ornithodorin [7], BmTI-A [8], RsTI [9], boophilin, chymotrypsin inhibitor [10] and savignin [11], among others. However, the role of some proteins from the Kunitz family remains to be elucidated and it includes the tissue kallikrein inhibitors from tick's presents in the pool of BmTIs [1,2].

It becomes important to study inhibitors of kallikreins, since these enzymes are involved in the main physiologic regulatory systems of animals, i.e. blood coagulation, and fibrinolysis, blood fluidity, vascular growth, and inflammatory process and interferes in the renin–angiotensine system [12]. Therefore, kallikreins are very important molecules in the homeostasis of the animals among them mammals including cattle, which is the host of *R. microplus*.

Furthermore, serine proteases, such as tissue kallikrein, have been related to proteolysis in *R. microplus* eggs [13]. Thus, serine protease inhibitors are important candidates for studying this process in eggs. In attempt to investigate the activity of serine protease inhibitors, we performed the purification and characterization of a Kunitz double-domain tissue kallikrein inhibitor found in *R. microplus* eggs, named RmKK. In addition, it was produced the recombinant RmKK in *Pichia pastoris* system. Finally, we provide an interesting discussion about the possible traffic of RmKK from midgut to the eggs and its role in the *R. microplus* eggs.

2. Materials and methods

2.1. Ticks

R. microplus adults were kindly provided by Dr. Itabajara Silva Vaz Junior from Federal University of Rio Grande do Sul (UFRGS). The adults were maintained at 27 °C to produce eggs. Eggs were used to prepare the crude extract. In addition, adults were dissected to obtain midguts to perform RNA extraction and cDNA preparation.

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http://dx.doi.org/10.1016/j.bbrc.2014.04.154
0006-291X/

2.2. Purification of the tissue kallikrein inhibitor from R. microplus eggs

The eggs obtained from adult ticks were homogenized in 0.025 M Tris–HCl buffer pH 8.0 containing 0.02 M NaCl, afterwards the extract was centrifuged during 15 min, at 12,000 rpm, 4 °C and the supernatant was separated. The supernatant was applied to a trypsin–Sepharose column previously equilibrated with 0.050 M Tris–HCl buffer pH 8.0. The fractions eluted with 0.5 M KCl pH 2.0 were immediately, neutralized with 1 M Tris–HCl buffer pH 8.0. Solution containing eluted proteins was dialyzed against 0.05 M sodium acetate buffer pH 5.5. Following, we performed an ion exchange chromatography on a Resource S column connected to an AKTA Purifier System, previously equilibrated with the same buffer. The proteins were eluted by a linear NaCl gradient (0–1 M), with flow rate of 1 mL/min for 60 min. The fractions containing inhibitory activity were pooled. All purification steps were analyzed by SDS–PAGE electrophoresis using 15% polyacrylamide gels [14]. The purified protein was also used for N-terminal amino acid determination by Edman degradation [15] using a PPSQ-23 protein sequencer (Shimadzu).

2.3. Cloning of RmKK DNA fragment into pPIC9 expression vector

Nucleotide sequence encoding RmKK (accession number TC6491) was presented in the *R. microplus* gene index project (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=b_microplus, which was made available by Dr. Felix D. Guerrero from Knipling Bushland US Livestock Insect Research Laboratory – USA. The DNA fragment encoding the inhibitor was amplified by PCR using a cDNA preparation of *R. microplus* midgut as template in 50 μL reaction volume containing 5 pmol of gene-specific primers, 200 μM DNTPs, 1.5 mM $MgCl_2$, and 5 U Taq DNA polymerase (Fermentas). PCR conditions were: 94 °C for 5 min, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s 35 cycles. DNA final extension was carried out at 72 °C for 10 min. The primers used were: RmKKFW 5′-CCGCTCGAGA AAAGAGTCCTTCTCAATGTGACACTG-3′ and RmKKRev 5′-TTTTCCT TTTGCGGCCGCTTAGGCGCGGGGAATGAC-3′. The PCR product was purified from 1% agarose gel using a QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany), followed by digestion with XhoI and NotI restriction enzymes and cloning into pPIC9 vector, which was previously digested with the same enzymes.

2.4. Production of recombinant RmKK (rRmKK)

After cloning, the construction pPIC9-RmKK was linearized with SacI restriction enzyme and *P. pastoris* GS115 yeast strain was transformed with the linearized plasmid using a Gene Pulser II electroporation system (Bio-Rad) according to manufacturer's instructions. Transformed yeasts were randomly selected, screened and the large-scale heterologous protein expressions were performed. *P. pastoris* grown up in BMGY medium [1% yeast extract (p/v), 2% peptone (p/v), 100 mM potassium phosphate, pH 6,0, 1.34% YNB (p/v), 40 μM biotin, 1% glycerol (p/v)]. After that, recombinant RmKK was produced by incubation of the yeast in BMMY medium [1% yeast extract (p/v), 2% peptone (p/v), 100 mM potassium phosphate, pH 6.0, YNB 1,34% (p/v), 40 μM biotin, 0.5% methanol (v/v)]. This procedure was carried out under stirring at 210 rpm at 30 °C for six days and the induction of protein expression was carried out by addition of methanol to a final concentration 0.5% (v/v) at intervals of 24 h.

2.5. Purification of recombinant RmKK

The supernatant of yeast culture containing RmKK was applied to a HiTrap Q column, connected to an AKTA™ purifier system (GE Healthcare) previously equilibrated with 0.05 M Tris–HCl pH 8.0. The protein was eluted with a linear NaCl gradient (0–0.5 M) in 0.05 M Tris–HCl pH 8.0 containing 0.5 M NaCl at a flow rate of 1.5 ml/min for 60 min. The elution profile was monitored by absorbance at 280 nm and fractions were tested regarding inhibitory activity towards bovine trypsin using synthetic substrate. This purification step was analyzed by SDS–PAGE electrophoresis [10].

2.6. Mass spectrometry analysis of rRmKK

The rRmKK was purified by reverse phase chromatography in a C_{18} Sephasil column connected to an AKTA Purifier System. The column was pre-equilibrated in 0.1% trifluoroacetic acid (TFA) and the proteins eluted with a linear acetonitrile gradient (0–90%), with flow rate of 1 mL/min for 60 min. rRmKK molecular mass was determined by MALDI TOF mass spectrometer (Bruker Autoflex®) operating in linear mode and calculated from the *m*/*z* peaks in the charge-distribution profiles of the multiple charged ions.

2.7. Dissociation constant (K_i) determination

Different concentrations of the native and recombinant inhibitor were pre-incubated with bovine trypsin and porcine kallikrein 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 by incubation with chromogenic substrates, Tosyl-Gly-Pro-Arg-pNA and 2266 (D-Val-Leu-Arg-pNA), for bovine trypsin and porcine pancreatic kallikrein, respectively. Finally, dissociation constants were calculated by fitting the steady-state velocities to the Morrison equation ($V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)2 - 4E_tI_t]1/2\}/2et$) for tight-binding inhibitors using a nonlinear regression analysis [16].

2.8. Determination of tissue-specific expression using PCR

PCR reactions were performed using cDNAs preparations from midgut, ovary, hemocyte, salivary gland and fat body of engorged *R. microplus*. The amplicons were analyzed on 1% (p/v) agarose gel.

3. Results

3.1. Purification and characterization of a tissue kallikrein inhibitor from R. microplus eggs

The native RmKK was purified by affinity chromatography using a trypsin–Sepharose column (Fig. 1A) followed by ion exchange chromatography using a Resource S column (Fig. 1B) and reverse phase chromatography in a C_8 Sephasil column (Fig. 1C). The N-terminal sequencing of purified inhibitor provided 10 amino acid residues (VLLNVTLNPV).

3.2. Nucleotide sequence analysis of RmKK

Nucleotide sequence encoding RmKK was found in the *R. microplus* gene index project (see Section 2.3.). Using the nucleotide sequence accession number TC6491 the DNA fragment coding RmKK was cloned and sequenced. Nucleotide sequence encoding RmKK presented 411-bp ORF translated to a Kunitz double domain protein with 137 amino acid residues. The amino acid sequence analysis revealed the presence of 10 cysteine residues and the protein presented a calculated molecular weight of 16.7 kDa and isoelectric point (pI) of 7.54.

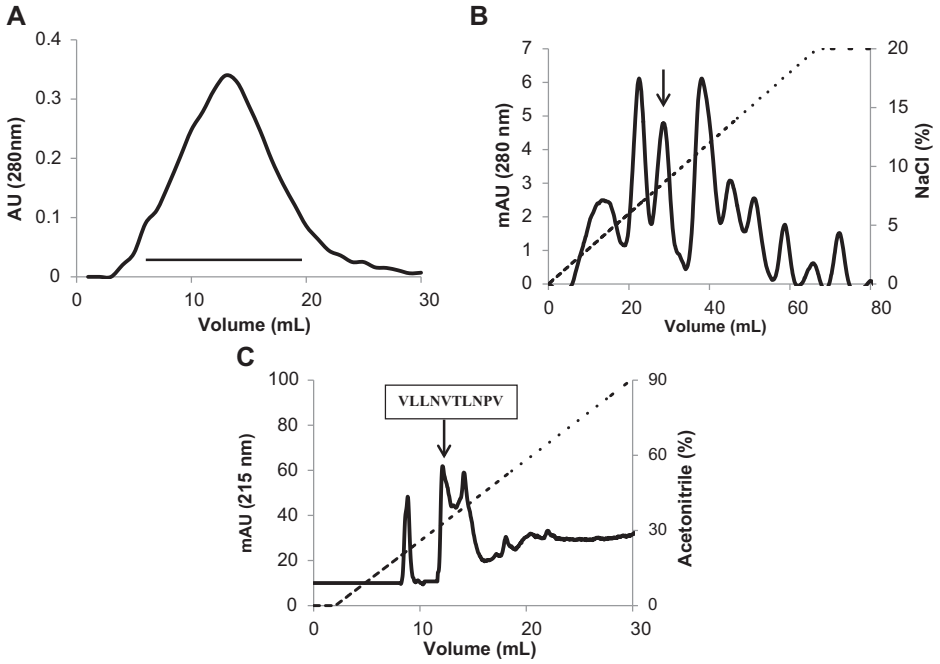

Fig. 1. Native RmKK purification. (A) Affinity chromatography on trypsin–Sepharose column. Eggs extract was applied on a trypsin–Sepharose column and the bound protein was eluted with 0.5 M KCl solution pH 2.0. The fractions with inhibitory activity (indicated by a black bar) were pooled and applied on a Resource S column (B) and the proteins were eluted with linear gradient of NaCl (0–1 M). Finally, a peak from ion exchange chromatography presenting inhibitory activity (indicated by a black arrow) was loaded on a C_8 Sephasil column (C) connected to the AKTA purifier system. Protein elution was with an acetonitrile linear gradient (0–90%). Amino terminal sequence from RmKK was identified in a peak eluted from C_8 Sephasil column (indicated in the reverse-phase chromatography).

3.3. Expression and purification of recombinant RmKK

The cDNA fragment encoding RmKK was cloned in pPIC9 vector and the protein expressed in *P. pastoris* strain. The plasmid construction RmKK/pPIC9 inserted in the yeast *P. pastoris* strain GS115 has provided high level of expression of the recombinant inhibitor (15 mg/L). rRmKK was purified by ion exchange chromatography. The supernatant of culture containing rRmKK was loaded on HiTrap Q column connected to a high-pressure chromatographic system ÄKTA™ Purifier (Fig. 3A). Purified rRmKK showed a molecular weight of 13 kDa by SDS–PAGE analysis (data not shown). Thus, to confirm the molecular mass, we performed MALDI-TOF analysis (Fig. 3B), resulting in three peaks that are related to mass/charge of RmKK (16,331 Da).

3.4. RmKK inhibition profile

To investigate rRmKK specificity towards bovine trypsin and tissue porcine kallikrein was performed (Table 1). Native RmKK was able to inhibit bovine trypsin and porcine kallikrein with K_i of 0.6 nM and 91.5 nM, respectively, whereas recombinant RmKK inhibited the same enzymes in nanomolar order (K_i = 0.2 nM for trypsin and K_i = 300 nM for porcine kallikrein).

Table 1
Dissociation constants (K_i) of native and recombinant RmKK for porcine pancreatic kallikrein and bovine trypsin.

Enzyme	K_i (nM)	
	Native RmKK	rRmKK
Porcine pancreatic kallikrein	91.5	300
Bovine trypsin	0.6	0.2

3.5. RmKK gene expression analysis

RmKK expression profile analysis using different tissues was performed with cDNA preparation from midgut, ovary, hemocytes, fat body, salivary glands and specific primers for RmKK by PCR. RmKK expression was observed only in midgut. Nevertheless, it was absent in other tissues (Fig. 4). Actin gene specific primers were used as an endogenous control.

4. Discussion

The present study describes expression, purification and characterization of a tissue kallikrein inhibitor from the tick *R. microplus*. This is the first tissue kallikrein inhibitor characterized in a tick. We purified the native RmKK from *R. microplus* eggs, its N-terminal amino acid sequence was determined by automatic amino acid sequencing (Fig. 1C). Inhibitory properties of RmKK towards serine proteases confirmed that it is a tight inhibitor of bovine trypsin and porcine pancreatic kallikrein (Table 1).

The native RmKK N-terminal sequence allowed identifying the DNA fragment sequence encoding for a Kunitz type multi domains inhibitor in the *R. microplus* gene index project. The RmKK amino acid sequence corresponded to the two last domains into the transcript. Thus, we decided to perform the cloning of cDNA fragment encoding for RmKK and production the recombinant RmKK. The putative amino acid sequence of the double domain protein shows that this inhibitor belongs to Kunitz type inhibitor family (Fig. 2), which each RmKK domain includes three highly conserved disulfide bridges, basic character and low molecular weight [1,8,10]. Expression of Kunitz-type inhibitors in yeast *P. pastoris* is an important tool for the production of these proteins. Indeed, several inhibitors of this family, especially those produced by ticks, were successfully expressed at high levels [17–20].

```
1     GAGAAAAGAGTCCTTCTCAATGTGACACTGAACCCTGTGTGCAACAAAACAAAGTTCCCA
1      E  K  R  V  L  L  N* V  T  L  N  P  V  C  N  K  T  K  F  P

61    GGACCTTGCAGAGGCTATTTCCCTCGTTATTACTTCGACAATGTGACCAAGACTTGCGAG
21     G  P  C  R  G  Y  F  P  R  Y  Y  F  D  N  V  T  K  T  C  E

121   AAGTTCATCTACGGAGGATGTCAGAGCAACGGCAACAACTTCGTCACTATCGAAGAGTGC
41     K  F  I  Y  G  G  C  Q  S  N  G  N  N  F  V  T  I  E  E  C

181   AAGAAAACCTGCTGGGTGTCTCTGGACCAGAATGCTGTTGAGGAGATCGGTGCCTTCGAA
61     K  K  T  C  W  V  S  L  D  Q  N  A  V  E  E  I  G  A  F  E

241   GTTCCCTTCCATCCATTTGCGCCACCTGAAGTCTGTACCTACCCGGCCGACTCGGGACCC
81     V  P  F  H  P  F  A  P  P  E  V  C  T  Y  P  A  D  S  G  P

301   TGCAAGGCATACATGCCTCGCTTTTTTTTACGTCTGTACCTACCCGGCCGACTCGGGACCC
101    C  K  A  Y  M  P  R  F  F  Y  V  C  T  Y  P  A  D  S  G  P

361   TGCAAGGCATACATGCCTCGCTTTTTTTTACAACTTCCTCACCTTCGATGCCTGCGAGAAG
121    C  K  A  Y  M  P  R  F  F  Y  N  F  L  T  F  D  A  C  E  K

421   AAGTGTCTGAAGGTTTTCGGCGTCATTCCCCGCGCCTAA
141    K  C  L  K  V  F  G  V  I  P  R  A  *
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Fig. 2. Analysis of amino acid sequence from RmKK cloned in pPIC9 vector. Predicted amino acid sequence of RmKK shows aino acid sequence obtained from the native protein (underlined) and the stop codon is represented by an asterisk. The sequences in gray represent fragments of pPIC9 vector obtained in RmKK sequencing.

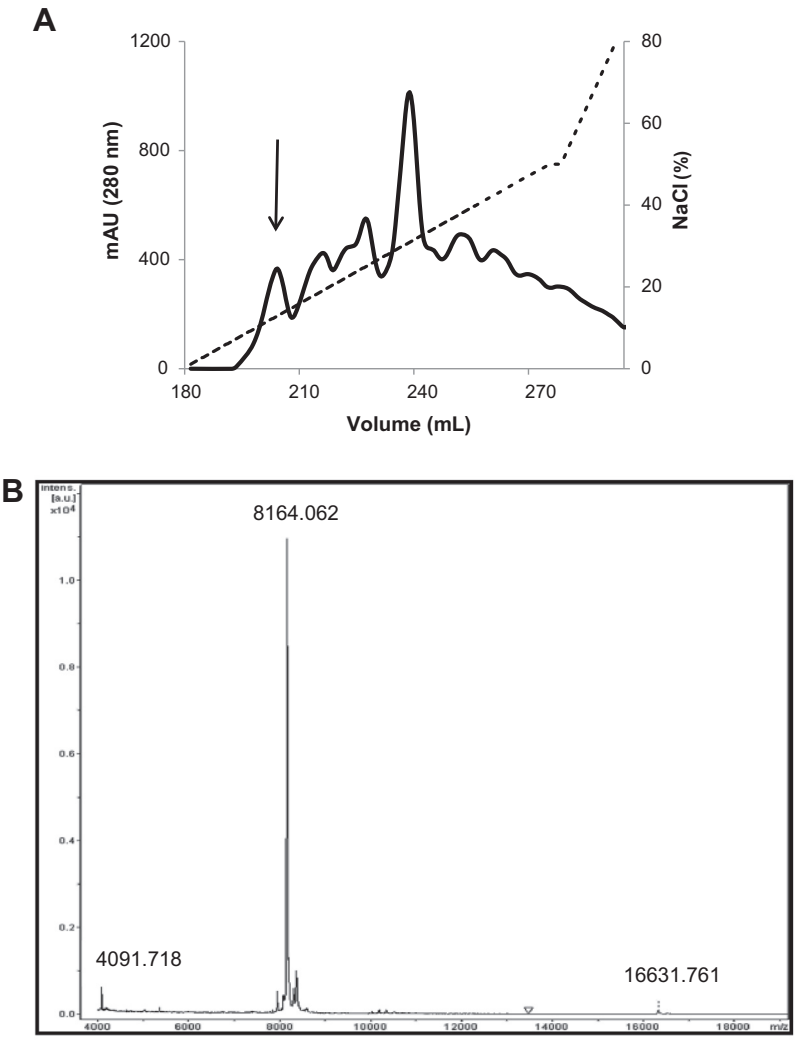

Fig. 3. rRmKK purification. (A) The supernatant of the *Pichia pastoris* strain containing RmKK (15 mg) was applied on HiTrap Q column and proteins were eluted with a linear gradient of NaCl (0–0.5 M). The peak presenting inhibitory activity is indicated by a black arrow. (B) RmKK obtained from ion Exchange chromatography was submitted to mass spectrometry to determine molecular weight.

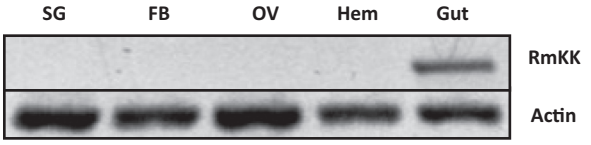

Fig. 4. Tissue specific expression profile of RmKK. PCR analysis of RmKK expression in different tissues of *R. microplus*. Electrophoresis on agarose gel (1%) of PCR products using cDNA preparations of: (1) fat body; (2) salivary glands; (3) ovary; (4) hemocytes; (5) midgut. Actin specific primers were used as endogenous control in all cDNAs tested.

Purified recombinant RmKK showed a protein band of approximately 13 kDa on SDS–PAGE (data not shown), which did not correspond to the theoretical size expected of about 16 kDa, according to the amino acid sequence analysis. This difference in molecular weight might occur due to low resolution of SDS–PAGE. In an attempt to solve this problem, the molecular weight of the rBmKK (16.3 kDa) was confirmed by mass spectrometry (Fig. 3B), which confirmed the expected theoretical molecular mass.

Tissue specific expression showed the presence of RmKK transcript only in midgut of *R. microplus* (Fig. 4). However, the native protein was obtained from eggs, suggesting that the protein might be expressed in midgut of this ectoparasite and taken up by ovaries with incorporation into the eggs. It has been shown that during tick oogenesis and eggs maturation, some proteins from midgut can be incorporated in ovaries by receptor-mediated endocytosis [21,22]. This process seems to be common to other tick inhibitors, such as, the *R. microplus* trypsin inhibitor, BmTI-6 [18].

RmKK can be important for the egg stage of the life cycle, since inhibitors of kallikreins have been suggested as protective molecules against undesired proteolysis in eggs [13]. Actually serine proteases are related to vitellin degradation in eggs [23]. Therefore, RmKK could be controlling kallikreins in the eggs to avoid non-specific proteolytic action. We cannot discard that those inhibitors are maintaining during the larvae stage and they play a role during the host's infestation. However, the role of kallikrein inhibitors in eggs and/or larvae remains to be elucidated as the route of this inhibitor from gut to the eggs.

In conclusion, native and recombinant kallikrein inhibitor RmKK showed inhibitory activity for trypsin and tissue kallikrein. RmKK transcript was expressed only in midgut, but the protein was extracted from eggs, indicating a mechanism of RmKK translocation in *R. microplus*. From our results, we can also suggest that RmKK might be involved in control of undesired proteolysis of kallikrein-like enzymes. Our future studies can be helpful to increase the knowledge about proteases–inhibitors role in eggs and/or larvae.

Acknowledgments

We thank Dr. Izaura Y. Hirata from the Department of Biophysics, UNIFESP, for performing the amino acid sequencing, and Marina Z. Hayashida and Flávia S. Bonfim for performing some of the BmKK purification experiments. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Proc. no. 2005/03514-9 and 2012/03657-8), Conselho Nacional de Desenvolvimento Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and INCT – Entomologia Molecular.

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