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Ixodidin, a novel antimicrobial peptide from the hemocytes of the cattle tick *Boophilus microplus* with inhibitory activity against serine proteinases

Andréa C. Fogaça [a,1], *Igor C. Almeida* [a,2], *Marcos N. Eberlin* [b], *Aparecida S. Tanaka* [c], *Philippe Bulet* [d], *Sirlei Daffre* [a,*]

[a] *Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, Zip Code 05508-900 São Paulo, SP, Brazil*

[b] *Laboratório Thomson de Espectrometria de Massas, Instituto de Química, Universidade Estadual de Campinas, Zip Code 13083-970 Campinas, SP, Brazil*

[c] *Departamento de Biofisica, Universidade Federal de São Paulo, Rua 3 de Maio 100, Zip Code 04044-020 São Paulo, SP, Brazil*

[d] *Atheris Laboratories, Case Postale 314, CH-Bernex, Geneva, Switzerland*

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ABSTRACT

The presence of an effective immune response in the hemocoel of arthropods is essential for survival as it prevents the invasion of pathogens throughout the animal body. Antimicrobial peptides (AMPs) play an important role in this response by rapidly killing invading microorganisms. In this study, a novel cysteine-rich AMP has been isolated and characterized from the hemocytes of the cattle tick, *Boophilus microplus*. In addition to growth inhibition of *Escherichia coli* and *Micrococcus luteus*, the newly described AMP, designated ixodidin (derived from the Family Ixodidae), was found to exert proteolytic inhibitory activity against two exogenous serine proteinases, elastase and chymotrypsin. This is the first report of a molecule of an arachnid that has been shown to inhibit bacterial growth and proteinase activity.

1. Introduction

The cuticle and the epithelia of the reproductive, digestive and respiratory systems of arthropods constitute the primary barriers against infection. If the physical barrier is disrupted, an effective immune response in the hemocoel is crucial to prevent the dissemination of pathogens throughout the animal body. Many different compounds of the arthropod hemolymph

* *Corresponding author*. Tel.: +55 11 3091 7272; fax: +55 11 3091 7417.
E-mail address: sidaffre@icb.usp.br (S. Daffre).
[1] Present address: Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748, B12, Zip Code 05508-900 São Paulo, SP, Brazil.
[2] Present address: Department of Biological Sciences, University of Texas at El Paso (UTEP), 500 W. University Avenue, El Paso, TX 79968-0519, USA.
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have been shown to be involved in the recognition and elimination of invading microbes. Among these components, antimicrobial peptides (AMPs) play an important role, rapidly killing a diverse range of pathogenic bacteria, fungi and parasites [5,7,21,35].

The fruit fly *Drosophila melanogaster* is a model system widely used to study the basis of the host–pathogen relationship in arthropods. When injury and/or infection occurs, the production of immune molecules, including seven different AMPs, is upregulated in this insect [26]. The high susceptibility to infection observed in *D. melanogaster* mutants that do not express genes encoding AMPs emphasizes the importance of these molecules [19,27–29]. Furthermore, the constitutive expression of a single AMP in immunodeficient mutant adult flies of the same species can re-establish the wild-type resistance to infection [45]. In contrast to the induced expression of AMPs in holometabolous insects and in two hemimetabolous insects, *Triatoma infestans* [24] and *Rhodnius prolixus* [31], the production of these molecules is constitutive in the hemimetabolous insect *Pseudacanthotermes spiniger* [25], and in other invertebrates, such as shrimps [12], horseshoe crabs [41], spiders [32,33] and mussels [35].

A large number of different AMPs has been described [10], but their precise mechanism of action still remains unknown. The structural features of AMPs (the great majority are cationic and amphypatic) indicate that the microbial membrane is usually their principal target [18,40,43]. Only a few AMPs have been shown to act on other targets, such as microbial proteinases [11,37].

The tick *Boophilus microplus* is the vector of several cattle pathogens, including *Babesia bigemmina*, *B. bovis* and *Anaplasma marginale* [44]. Females of *B. microplus* feed on large volumes of bovine blood thus increasing their body volume by 100-fold. This tick species leads to severe loss in milk, meat production and leather damage and hence depreciates the commercial value of the host. The characterization of an AMP from the midgut content of *B. microplus*, corresponding to the 33–61 segment of the bovine α-hemoglobin has been previously reported [15]. Recently, the NMR-derived 3D structure of this peptide was elucidated, revealing that it exhibits two β-turns, one in the N-terminus and another followed by a α-helical stretch in the C-terminus [39]. Furthermore, it was shown that the peptide is able to disrupt the membrane of the Gram-positive bacterium *Micrococcus luteus*. In addition to the hemoglobin fragment, two cysteine-rich AMPs from the hemolymph of *B. microplus* were also characterized which are synthesized by the tick itself [14]. One of these peptides was isolated from the hemocytes and belongs to the group of invertebrate defensins. The second peptide, designated microplusin, was purified from the cell-free hemolymph and corresponds to a new class of AMPs. The cloned cDNAs of defensin and microplusin showed that they are synthesized as pre-pro-peptide and pre-peptide, respectively. The gene expression of both peptides was detected in the hemocytes, fat body and ovaries, as revealed by semi-quantitative RT-PCR experiments.

In this study is reported the isolation, structural characterization and biological properties of a third cysteine-rich AMP from the hemolymph of *B. microplus*. This new 7103 Da AMP (65 residues), named ixodidin (from the Family Ixodidae), was isolated from an acidic extract of tick hemocytes and possesses 10 cysteine residues engaged in 5 internal disulfide bonds. It shows a net positive charge at physiological pH (11 positively charged and 2 negatively charged residues), with calculated pI of 9.53. Databank searches revealed a similarity of ixodidin to a cathepsin G/chymotrypsin inhibitor of *Apis mellifera* [4] and to other serine proteinase inhibitors. Interestingly, besides possessing antimicrobial activity against *Escherichia coli* and *M. luteus*, this newly characterized AMP was also found to possess a strong inhibitory activity against two proteases, namely elastase and chymotrypsin. This is the first report of an arachnid molecule exhibiting bacterial growth inhibition and proteinase inhibitory activities.

2. Material and methods

2.1. Ticks and sample collection

Ticks and hemocytes were obtained as previously described [14].

2.2. Peptide extraction and purification

Hemocytes from 6 mL of hemolymph, obtained from around 2000 ticks, were firstly homogenized in 1.6 M acetic acid using a Dounce homogenizer (maximum, 152 μm; minimum, 76 μm) and secondly by sonication (3 × 30 s) using continuous waves of amplitude 30 (Sonics and Materials Inc., USA; model Vibracell). Extraction was performed for 30 min with gentle shaking in ice-cold water bath. After centrifugation at 8000 × *g* for 30 min at 4 °C, the supernatant was pre-purified by solid-phase extraction using five serially linked Sep-Pak C_{18} cartridges (Waters Corporation, USA). Step-wise elutions were performed with 5, 40 and 80% acetonitrile (ACN) in acidified water. The three eluted fractions were separately loaded onto a reverse-phase semi-preparative C_{18} column (Phenomenex, USA; model Jupiter, 300 Å, 10 mm × 250 mm), previously equilibrated with 2% ACN in acidified water. Elution was performed using a linear gradient of 2–60% ACN (for fractions 5 and 40% ACN) or 20–80% ACN (for fraction 80% ACN) in acidified water over 120 min at a flow rate of 1.5 mL/min. Fractions exhibiting antimicrobial activity were analyzed by electrospray ionisation–mass spectrometry (ESI-MS), as described in Section 2.3.2. The fraction eluted with 27% ACN was further subjected to a final purification step by RP–HPLC, using an analytical C_{18} column (Vydac, USA; 300 Å, 5 μm, 4.6 mm × 250 mm). Following equilibration with 2% ACN in acidified water, peptides were eluted with a linear gradient of 17–27% ACN in acidified water over 60 min at a flow rate of 1 mL/min. Chromatographic analyses were performed using a LC10 equipment (Shimadzu, Japan) and the column effluent was monitored by absorbance at 225 nm.

2.3. Primary structure characterization

2.3.1. Reduction, alkylation and enzymatic treatments

The pure active peptide was suspended in 40 μL 0.5 M Tris–HCl, pH 7.5, containing 2 mM EDTA and 6 M guanidine hydrochloride. After addition of 2 μL 2.2 mM DTT, the mixture

was incubated under oxygen-free conditions for 1 h at 45 °C. After reduction, one aliquot of the sample was alkylated with 4-vinylpyridine, as described earlier [8], while a second aliquot was alkylated with iodoacetamide, using conditions previously detailed [14]. Peptides were desalted by RP–HPLC, employing the same analytical C_{18} column used for the final peptide purification. Elution was performed with a linear gradient of 2–80% ACN in acidified water over 60 min at a flow rate of 1 mL/min.

The S-pyridylethylated peptide was subjected to a digestion using pyroglutamate aminopeptidase, Glu-C or Arg-C endopeptidases. The pyroglutamate aminopeptidase (Roche Molecular Biochemicals) treatment was performed as previously detailed [42]. The digested peptide was purified by RP–HPLC and subjected to sequencing by Edman degradation. Glu-C endopeptidase treatment was carried out in 0.1 M NH_4HCO_3 buffer, pH 8.0, whereas treatment with Arg-C endopeptidase was performed in 100 mM Tris–HCl, pH 8.0, containing 0.01% Tween 20. Both digestions were performed using an enzyme to substrate ratio of 1:50 (w/w). After 16 h at 37 °C, reactions were stopped by acidification with a solution of 0.1% TFA and the resulting products were separated by RP–HPLC, following conditions described above. Peptide fragments generated by the enzymatic treatments were subjected to MALDI-TOF-MS measurement and automated Edman sequencing.

To determine the primary structure of the N-terminus of the peptide, the carboxyamidomethylated molecule was subjected to a treatment with Glu-C endopeptidase, using conditions specified above. Resulting peptides were isolated by RP–HPLC, and analyzed by ESI-MS and ESI-MS/MS for structural characterization.

2.3.2. *Mass spectrometry and amino acid sequencing*

Molecular masses of peptides were determined by either MALDI-TOF-MS or ESI-MS. MALDI-TOF-MS analyses were conducted on a BIFLEX III™ time-of-flight mass spectrometer (Bruker, Germany) and ESI-MS experiments were carried out using a LCQ™ Duo mass spectrometer (ThermoFinnigan, USA), according to procedure already reported [42]. Peptide sequencing was alternatively performed by ESI-MS/MS, using a Q-Tof 1 mass spectrometer (Waters, Micromass, UK) equipped with a nanoelectrospray source. Selected peptide ion was submitted to collision-induced dissociation (CID) with argon, employing a collision energy range of 15–45 eV. Ion spectra were recorded on the positive mode and analyzed manually using the MassLynx software (Waters, Micromass, UK).

N-terminal sequencing of peptides was carried out using automated Edman degradation. Phenylthiohydantoin-derivatives were detected in a pulse liquid automatic sequenator (Applied Biosystems, USA; model 473A).

Resulting sequences were compared to other sequences using the BLAST algorithm and the UniProtKB/Swiss-Prot and UniProtKB/TrEMBL (http://us.expasy.org/sprot/).

2.4. *Biological assays*

2.4.1. *Antimicrobial assays*

M. luteus, *E. coli* and *Candida albicans* strains used in this work were described previously [14]. Antimicrobial activity of samples was monitored using liquid growth inhibition assays [9]. The minimum inhibitory concentration (MIC) was recorded as the *a*–*b* μM range, where *a* corresponds to the highest concentration in which the bacterial growth was observed and *b* corresponds to the lowest concentration of the peptide that caused 100% of inhibition bacterial growth [13].

2.4.2. *Proteinase inhibition assays*

The purified peptide was pre-incubated with optimal concentrations for the following enzymes: chymotrypsin (10^{-8} M), elastase (2×10^{-10} M) or plasmin (5.5×10^{-12} M) in 100 mM Tris–HCl buffer, pH 8.0. The enzyme to peptide ratio was 1:5 (for chymotrypsin and elastase) or 1:200 (for plasmin). After 30 min at 30 °C, specific substrates were added: *Suc*-Ala-Ala-Phe-*MCA* (10^{-5} M) for chymotrypsin (Sigma–Aldrich, USA), *Suc*-Ala-Ala-Pro-Val-*pNA* (10^{-1} M) for elastase and H-D-Val-Leu-Lys-*pNA* (10^{-1} M) for plasmin (Chromogenix, Sweden). The fluorescence intensity of reactions using chymotrysin was monitored every 5 min for 30 min using a spectrofluorometer (ThermoLabsystems, Finland; model Fluorskan Ascent FL). The wavelengths were set at 380 nm for excitation and 460 nm for emission. The absorbance at 405 nm of reactions with elastase and plasmin was determined after 10 min at 30 °C, using a spectrophotometer (ThermoLabsystems, Finland; model iEMS). Control reactions were performed under the same conditions, but in the absence of peptide.

2.4.3. *Determination of the dissociation constant (K_i)*

Chymotrypsin (10^{-7} M) was pre-incubated with the hemocyte AMP at a concentration range of 0–10 nM in 100 mM Tris–HCl buffer, pH 8.0. After 30 min at 37 °C, the specific substrate *Suc*-Ala-Ala-Pro-Phe-*pNA* (10^{-3} M) was added to the reaction, which was monitored spectrophotometrically at 405 nm every 5 min for 30 min. The chymotrypsin apparent K_i value was calculated by fitting the steady-state velocities to the equation for tight-binding inhibitor using a non-linear regression analysis [36].

3. Results

We have previously reported the purification and characterization of a defensin from the hemocytes of the tick *B. microplus* [14]. In this study, we observed a partial inhibitory activity against *M. luteus* in a fraction eluted shortly before the defensin peptide. To obtain sufficient material for structural elucidation, we have doubled the volume of hemolymph in the current study.

3.1. *Peptide purification*

The three fractions obtained (5, 40 and 80% ACN) were separately loaded onto a reverse-phase semi-preparative C_{18} column and the eluted fractions were analyzed by the growth inhibition assay against *M. luteus*, *E. coli* and *Candida albicans*. Only the fractions from the 40% ACN chromatography were shown to have any antimicrobial activity against *M. luteus* (see Fig. 1, labeled BMH0 and BMH1–BMH4), whereas none of the fractions were active against either *E. coli* or *C. albicans*. The analysis of fractions by ESI-MS revealed that BMH0 consisted of a mixture of peptides with different molecular masses, whereas BMH1–BMH4 mainly contained *B. microplus* defensin,

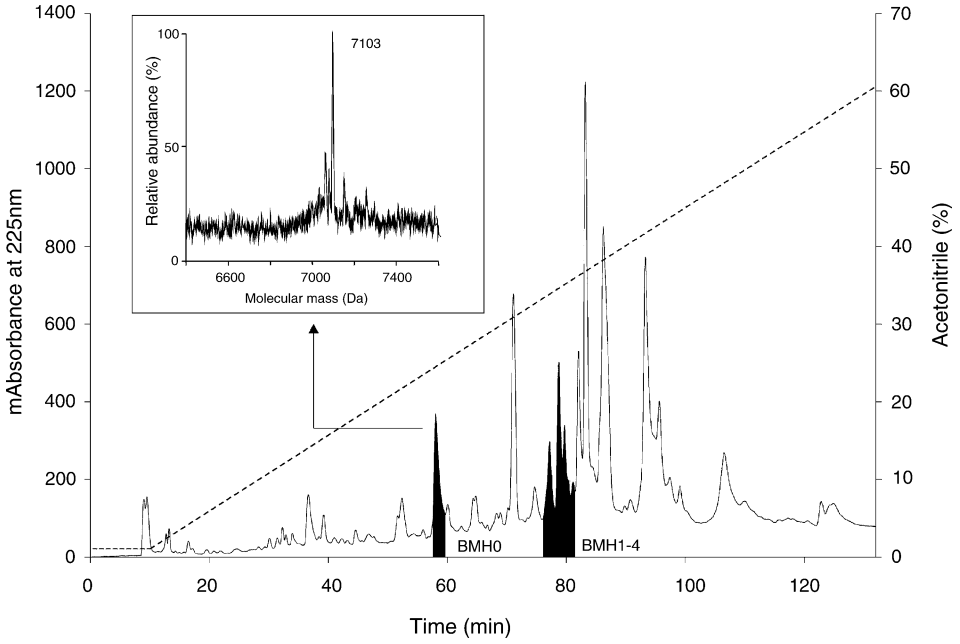

Fig. 1 – Purification by RP–HPLC of a novel AMP from the tick hemocytes. The fraction eluted with 40% ACN, resulting from the pre-purification of the acidic extract of hemocytes by solid-phase extraction chromatography, was subjected to RP–HPLC using a semi-preparative C_{18} column. Peptides were eluted using a linear gradient of 2–60% ACN in acidified water for 120 min. Five anti-*M. luteus* fractions (black areas, labeled BMH0 and BMH1–BMH4) were detected. Fraction BMH0 (arrow), eluted with 27% ACN, was subsequently loaded on an analytical C_{18} column and the active fraction was analyzed by ESI-MS (inset). A single molecular mass at 7103 Da was obtained by deconvolution of the MS-spectra.

as previously reported [14]. To isolate the active compound contained in BMH0, this fraction was submitted to a final purification step using an analytical reverse-phase column (data not shown). ESI-MS analysis of the active fraction, eluted with 18% ACN, showed a peptide with a molecular mass of 7103 Da that was pure enough for protein sequencing (Fig. 1, inset).

3.2. *Primary structure elucidation*

To estimate its cysteine content, the 7103 Da peptide was reduced and alkylated with 4-vinylpyridine, desalted and subjected to MALDI-TOF-MS analysis. The difference between the molecular masses of the non-treated (7103 Da) and the S-pyridylethylated (8165 Da) molecules indicated the presence of 10 cysteine residues engaged in the formation of five internal disulfide bridges (S-pyridylethylation confers an increase of 106 Da per half-cysteine residue). No sequence was obtained by Edman degradation analysis, suggesting that the N-terminal residue was blocked. This indicated that the blocking group could be a pyroglutamic acid. To prove such a hypothesis, an aliquot of the fraction of the S-pyridylethylated peptide was subjected to a pyroglutamate aminopeptidase treatment. Following purification and MALDI-TOF-MS analysis, a decrease of 111 Da in the molecular mass of the peptide was recorded, which is the exact mass of a pyroglutamic acid residue (data not shown). The treated peptide was subjected to Edman sequencing and a partial sequence was obtained (Fig. 2A, Pyro). Nevertheless, the first residue following the pyroglutamic acid could not be identified. To investigate the primary structure, the rest of the S-pyridylethylated peptide was subjected to Glu-C or Arg-C endopeptidase treatment. After digestion with Glu-C endopeptidase, three fragments were isolated by RP–HPLC, analyzed by MALDI-TOF-MS and subjected to Edman sequencing (Fig. 2A, Glu-C). The first seven N-terminal residues of the largest fragment, with a molecular mass at 5795 Da, overlapped with the C-terminal sequence of the pyroglutamate aminopeptidase treated peptide (Fig. 2A, Pyro). The second fragment, with a molecular mass at 931 Da, did not exhibit a glutamic acid residue at the C-terminal, strongly suggesting that it corresponded to the C-terminal region of the peptide. No sequence was generated by the analysis of the third fragment, with molecular mass at 1476 Da, indicating that it corresponded to the N-terminal blocked portion of the peptide. Additionally, the S-pyridylethylated peptide was treated with Arg-C and the five resulting fragments, with molecular masses at 1940, 2725, 507, 836 and 776 Da, were isolated and analyzed as described above (Fig. 2A, Arg-C). The amino acid sequences of all the five Arg-C fragments matched to the sequences previously obtained (see Fig. 2A). To identify the residue downstream from the pyroglutamic acid, the peptide was reduced and alkylated with iodoacetamide (instead of the 4-vinylpyridine treatment as performed above), and subsequently, treated with Glu-C endopeptidase. The purified N-terminal blocked fragment was subjected to tandem mass spectrometry analysis (Fig. 2B, ESI-MS/MS). Analysis of spectra generated after CID of the ion with *m/z* 1427.44, corresponding to the N-terminal fragment with one carboxyamidomethylated half-cysteine residue, lead to identification of arginine as the

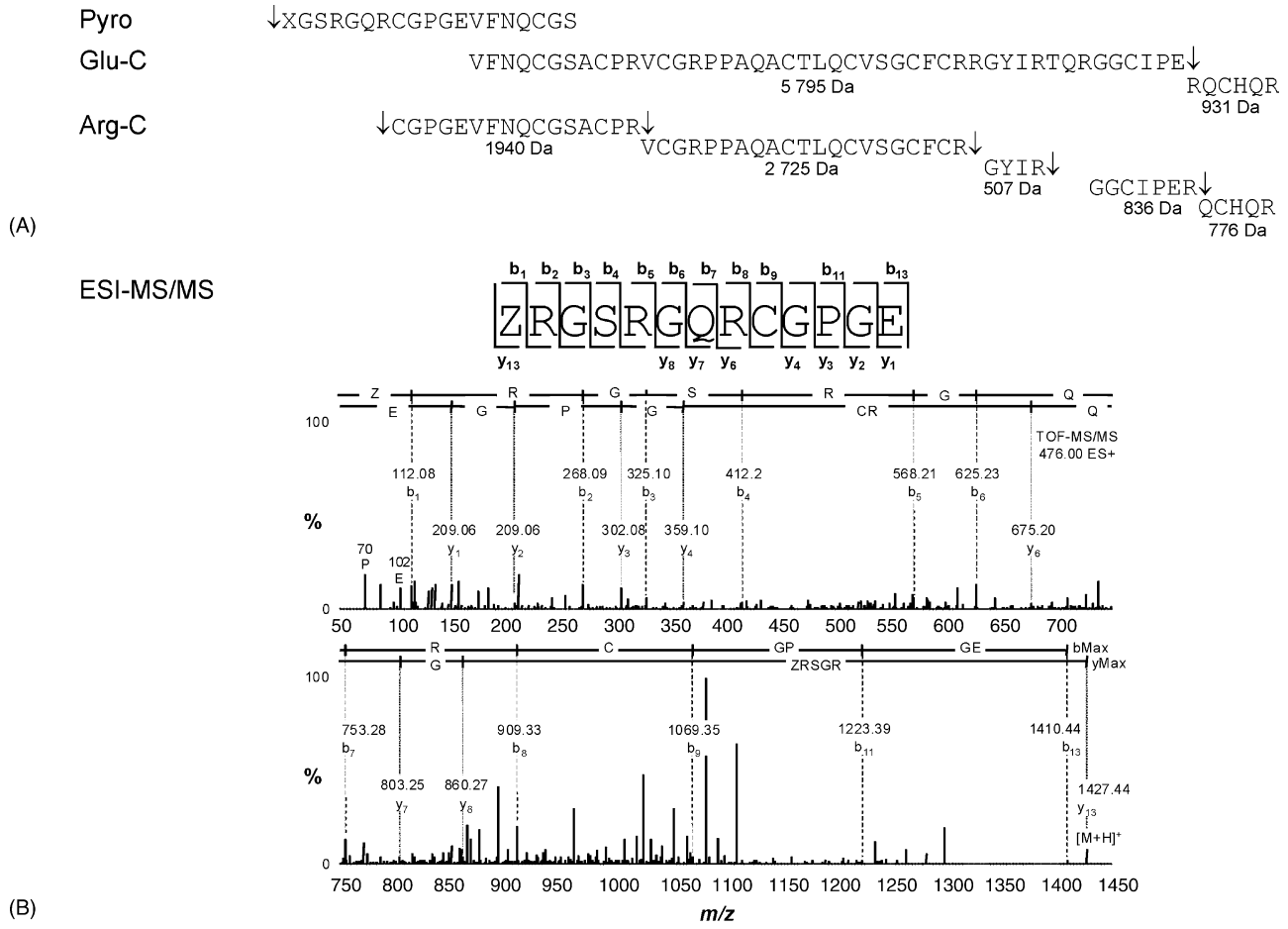

Fig. 2 – Primary structure of the 7103 Da peptide. After S-pyridylethylation, the peptide was treated with pyroglutamate aminopeptidase and analyzed by Edman degradation (Pyro). Purified peptide fragments generated by the treatment of the S-pyridylethylated molecule with either Glu-C or Arg-C endopeptidases were also sequenced by Edman degradation (Glu-C and Arg-C, respectively) (A). Following caboxyamidomethylation, cleavage with Glu-C endopeptidase and RP–HPLC purification, the fragment corresponding to the N-terminal blocked region was further analyzed by tandem mass spectrometry (ESI-MS/MS) (B). Combining data, the primary structure of the active peptide was obtained (full sequence) (C). Arrows mark the cleavage sites, X corresponds to the formerly unidentified residue (later identified as arginine) and Z stands for a pyroglutamic acid residue.

unique unknown residue of this peptide. Combining all the sequence information, the full primary structure of the peptide was obtained (Fig. 2C, full sequence). The sequence data of this 7103 Da peptide is in the Swiss-Prot and TrEMBL databases under the accession number P83516. This novel hemocyte AMP of *B. microplus* was named ixodidin.

Databank searches revealed similarities of this peptide to a cathepsin G/chymotrypsin inhibitor of *A. mellifera* (Am) [4] to three serine proteinase inhibitors from *Ascaris* spp. (C/E1, ATI [20] and C/E4 [3]), to the 292–348 fragment of the human von Willebrand factor (vWF) [46] and to a trypsin inhibitor from *Bombina bombina* (BSTI) [34] (Fig. 3).

3.3. *Biological properties of ixodidin*

Prior to this investigation of the biological properties of ixodidin, the quantity of the peptide was calculated from repetitive and initial yields of the Edman sequencing, in comparison with a well-quantified control molecule (polypeptide β-lacto globulin).

3.3.1. *Antibacterial activity*

The antibacterial properties of ixodidin were investigated by evaluating its activity against *M. luteus* and *E. coli*. The MIC of ixodidin against *M. luteus* was 0.12–0.24 μM. *E. coli* was less susceptible and showed only 20% growth inhibition at 1 μM, the highest concentration obtainable.

3.3.2. *Proteinase inhibitory activity*

Databank searches revealed that the primary structure of ixodidin had structural similarities to different serine proteinase inhibitors (see Fig. 3). To determine whether ixodidin exhibited inhibitory activity against some selected proteinases, proteinase inhibition assays were carried out. Ixodidin

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          1        10        20        30        40        50        60
Bm        ZRGSRGQRCGPGEVFNQC-GSACPRVCGRPP-AQACTLQCVS-GCFCRRG---YIRTQRGGCI-PERQCHQR
Am              EECGPNEVFNTC-GSACAPTCAQPK-TRICTMQCRI-GCQCQEG---FLRNGEGACVLPEN-C
C/E1          GQESCGPNEVWTECTG--CEMKCGPDENTP-CPLMCRRPSCECSPGRG-MRRTNDGKCIPA-SQCPEH
C/E4          R-KPCGKNEVWTECTG--CELKCGQDENTP-CALMCRPPSCECSPGRG-MRRTHDGKCVPV-SECPRKM
ATI             EECGPNEVFNTC-GSACAPTCAQPK-TRICTMQCRI-GCQCQEG---FLRNGEGACVLPEN-C
vWf           S--PVCPAGMEYRQCV-SPCARTCQSLHINEHCQERCVD-GCSCPEGQ-EGL------CV-ESTEC
BSTI            NF-VCPPGQTFQTCA-SSCPKTCETRNKLVLCDKKCNQR-CGCISGTVL--------CVH-PSKC
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Fig. 3 – Multisequence alignment of ixodidin with other serine proteinase inhibitors. Ixodidin amino acid sequence (Bm) was aligned with: cathepsin G/chymotripsin inhibitor from *Apis mellifera* (Am); chymotrypsin/elastase inhibitor from *Ascaris suum* (C/E1); chymotrypsin/elastase from *Ascaris lumbricoides* (C/E4); trypsin inhibitor from *A. lumbricoides* (ATI); the 292–348 fragment of the von Willebrand factor from human (vWf); and the trypsin inhibitor from *Bombina bombina* (BSTI). Sequences were aligned according to the cysteine residues (boxes). Gaps (–) were introduced to optimize alignment. Identical residues are in bold letters. Numbering refers to ixodidin amino acid residues.

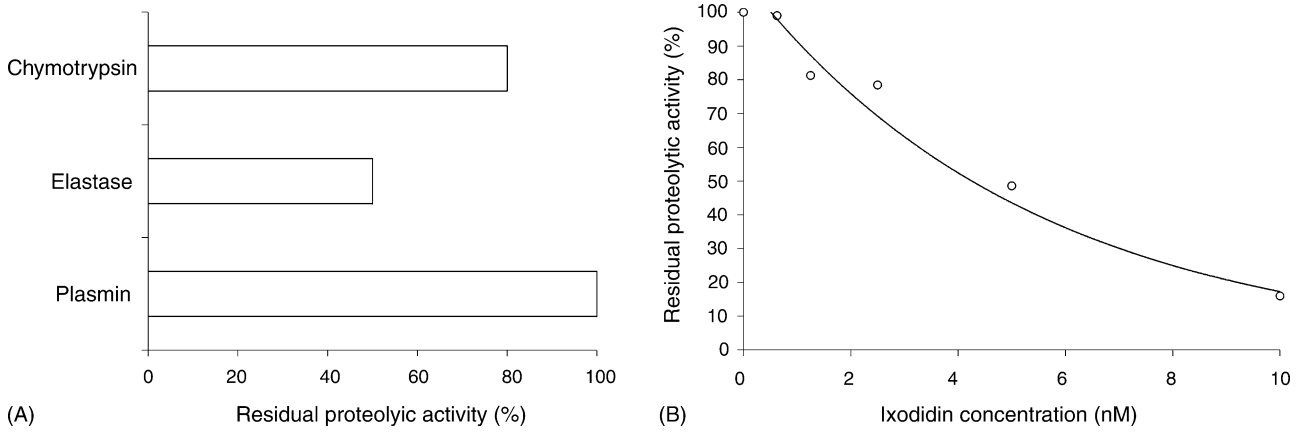

Fig. 4 – Proteolytic inhibitory activity of ixodidin. Ixodidin was pre-incubated with either chymotrypsin, elastase or plasmin. After 30 min at 30 °C, specific substrates for these enzymes were added. Fluorescence intensity of chymotrysin reaction was monitored with wavelengths set at 380 nm for excitation and 460 nm for emission. Absorbance of reactions with either elastase or plasmin were monitored at 405 nm. Control reactions were performed under the same conditions, but in the absence of ixodidin. Residual proteolytic activity (%) was determined comparatively, attributing the value of 100% of activity to control reactions (A). The constant of dissociation (K_i) was estimated using the residual activity of chymotrypsin in presence of ixodidin at a concentration range of 0–10 nM (B).

was found to inhibit about 20 and 50% of chymotrypsin and elastase activities, respectively. Nonetheless, the peptide was unable to inhibit plasmin (Fig. 4A). The apparent K_i of ixodidin against chymotrypsin was 3.8 nM (Fig. 4B).

4. Discussion

An effective immune response in the hemocoel of arthropods is essential to control infections, assuring survival of the animal. Antimicrobial peptides play a central role in this immune response, acting rapidly and directly against invading pathogens. We have previously reported the isolation of three different AMPs from the cattle tick *B. microplus*, namely a bovine hemoglobin fragment from the gut content [15], microplusin from the cell-free hemolymph and defensin from the hemocytes [14].

In this study, the isolation of a fourth peptide from *B. microplus* with activity against *M. luteus* (MIC 0.12–0.24 μM) and *E. coli* (20% growth inhibition at 1 μM) is reported. This novel peptide, named ixodidin, was isolated from an acidic extract of hemocytes, as well as the previously reported defensin. Ixodidin is a 65 residues peptide with five internal disulfide bridges and an N-terminal pyroglutamic acid. Databank searches revealed similarities of this peptide to some serine protease inhibitors, such as a cathepsin G/chymotrypsin inhibitor of *A. mellifera* (46%) [4] and a chymotrypsin elastase inhibitor of *Ascaris suum* (40%) [20] (see Fig. 3). The study of the molecular structure of the chymotrypsin elastase inhibitor of *A. suum*, named C/E1, revealed that the disulfide pattern of this molecule is Cys1–Cys5, Cys2–Cys7, Cys3–Cys6, Cys4–Cys10 and Cys8–Cys9 [20]. It has been proposed that proteins that present the same location of cysteine residues also present similar disulfide arrays [4,6,38]. The relative conservative positions of the cysteine residues of ixodidin in comparison to the amino acid sequence of C/E1 suggests that this peptide shares a similar disulfide pattern, as postulated for the cathepsin G/chymotrypsin inhibitor of *A. mellifera*. By comparison to the inhibitory

site P1-P1′ reported for C/E1 (Leu31-Met32) and for the inhibitor of *A. mellifera* (Met30-Gln31), it can be extrapolated that the inhibitory site in ixodidin may be the dipeptide (Leu31-Gln32). In addition, ixodidin, besides showing structural similarities to the honeybee inhibitor, also exhibits a similar spectrum of proteinase inhibitory activity, inhibiting the enzymatic activity of both chymotrypsin and elastase.

It is well known that serine proteinase inhibitors are involved in several mechanisms of the immune system of arthropods. For example, they mediate the coagulation and melanization of the hemolymph and the production of AMPs [17,23]. Moreover, the presence of proteinase inhibitors may block the invasion and proliferation of pathogens that use proteinases for: (i) invasion of the host tissues; (ii) acquisition of nutrients; (iii) evasion of the host immune system [2]. Besides showing proteinase inhibitory activity against chymotrypsin and elastase, ixodidin also presents growth inhibitory properties against *M. luteus* and *E. coli*. Peptides capable of inhibiting both proteinase activities and microbial were previously described in the horseshoe crab *Tachypleus tridendatus* [1] and in flowers of *Helianthus annuus* [16], but have not previously been reported in arachnids. It has also been noted that synthetic analogues of indolicidin, an AMP of bovine neutrophils [30], and cystatin-C, a proteinase inhibitor of human extracellular fluids [22], both exhibit inhibitory activity against cysteine proteinases and microbial growth. Nevertheless, it remains unclear if the antimicrobial activity is due to the inhibition of proteinase activity or to a direct effect of the peptide on the bacterial membrane. Due to the low recovery of the native ixodidin, it was not possible to extend this study to the mechanisms of action of such a peptide. To overcome this problem, we are attempting to express this peptide in *Saccharomyces cerevisae*.

In conclusion, the current report presents the isolation, structural characterization and biological properties of ixodidin, a 65-residue cysteine-rich AMP from the hemocytes of *B. microplus*. Interestingly, in addition to inhibiting the growth of the two representative strains of Gram-positive and Gram-negative bacteria, *M. luteus* and *E. coli*, respectively, ixodidin shows in vitro inhibitory properties against two serine proteases, elastase and chymotrypsin. Hence, ixodidin might exert different activities in the tick hemolymph, acting directly against invading microorganisms, mediating other immune responses in *B. microplus* hemocoel, and preserving its own integrity, as well as the integrity of other AMPs of the tick hemolymph.

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