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Antimicrobial and antiborrelial characterization of *Dermacentor marginatus* defensin

RNDr. Thesis

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Annotation:

Ticks, as blood sucking arthropods, are able to transmit various pathogens. Their immune system involves many antimicrobial molecules to fight against them. Among these antimicrobials, defensins, 5.3 kDa peptides, play an important role in rapid immune answer.

In this study we examined the antimicrobial spectrum of *Dermacentor marginatus* defensin (def DM) with respect to the fact that *Dermacentor variabilis* ticks are not able to successfully maintain and transmit *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease.

Expression of the def DM gene was detected in hemolymph, midgut and salivary glands. Defensin was isolated from hemolymph using RP-HPLC and its sequence was determined by mass spectrometry and Edman degradation. Synthetic mature peptide def DM revealed an anti-Gram-positive bacterial role as well as borreliacidal activity, with concentration dependent influence. These results suggest a possible role in the clearing of borrelia spirochetes ingested by *D. marginatus* ticks.

Declaration [in Czech]:

Prohlašuji, že svoji rigorózní práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své rigorózní práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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Expression of personal work on the publication:

I carried out all the molecular biology experiments, such as mRNA isolation and Reverse Transcription – Polymerase Chain Reaction of defDM gene transcripts in the tick organs. Tick organs were dissected by Gabriela Loosová. With her help, I also collected hemolymph for Reversed Phase-High Performance Liquid Chromatography, and for determining defDM sequence by mass spectrometry and Edman degradation which was performed by Václav Čeřovský in the institute of Organic Chemistry and Biochemistry (Academy of Science of the Czech Republic). The antimicrobial testing, hemolytic activities as well as antiviral testing of synthetic defDM were handled by myself (establishing of minimal inhibition concentration I did with Jiřina Slaninová). Borreliacidal activity was performed by Ryan Rego.

The manuscript was written by me in close communication with all authors, the chapters Methods/Results were written by all authors according to their contribution.

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Short Communication

Defensin from the ornate sheep tick *Dermacentor marginatus* and its effect on Lyme borreliosis spirochetes

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ABSTRACT

Expression of the previously reported defensin of the tick *Dermacentor marginatus* (defDM) was analysed in different organs by RT-PCR. mRNA of the *defDM* gene was detected in the hemolymph, midgut and salivary glands. Moreover defDM was isolated from the tick hemolymph using RP-HPLC and its sequence was determined by mass spectrometry and Edman degradation. Synthetic peptide was used for determining biological activities. The results showed an anti-Gram-positive bacterial role for the defensin. As *D. marginatus* ticks appear not to be vectors of the Lyme disease agent of the complex *Borrelia burgdorferi* sensu lato, we tested the influence of defDM on *Borrelia afzelii*. There is a very clear borrelicidal activity of the defensin, which is concentration dependent and suggests a possible role in the clearing of *Borrelia* ingested by *D. marginatus* ticks.

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1. Introduction

Ticks are generally known as obligate blood sucking ectoparasites. Their impact on men and animals may be different: from unpleasant itching to severe anaemia or transmission of various pathogens. The European ornate sheep tick *Dermacentor marginatus* is a common vector of *Ricketsiia slovaca*, the causative agent of tick-borne lymphadenopathy (TIBOLA) in southern Europe (Masala et al., 2012). *D. marginatus* may also be playing a role as a vector of tick-borne encephalitis virus (TBEV), *Coxiella burnetii*, and the protozoan parasite *Babesia canis* (Kocianová et al., 2001; Nosek and Kožuch, 1985; Pietrobelli et al., 2007).

Ticks of the genus *Dermacentor* are found throughout various geographical regions that overlap areas where there is an incidence of human Lyme disease (LD). Studies revealed, that in contrast to *Ixodes scapularis* ticks, which are vectors of LD in North America, *Dermacentor variabilis* ticks are not able to successfully maintain and transmit *Borrelia burgdorferi* sensu lato, the causative agent of LD (Piesman and Sinsky, 1988). Many tick species of the genera *Dermacentor* and *Amblyomma* were examined to prove their incompatibility to maintain or transmit *B. burgdorferi* (Piesman and Happ, 1997; Piesman and Sinsky, 1988; Ryder et al., 1992). *D. variabilis* was shown to possess a 5.3 kDa peptide (defensin) with borrelicidal activity within the tick hemolymph (Johns et al., 2001a,b). Also salivary gland (SGE) and midgut extracts (MGE) from the tick *Dermacentor reticulatus* had an impact on *Borellia garinii in vitro*. Both SGE and MGE affected the spirochaetal motility; moreover the SGE had changed borrelial morphology (Rudolf and Hubálek, 2003).

Defensins are a well studied family of antimicrobial peptides (AMPs) in ticks (Kopáček et al., 2010). Defensins identified within arthropods share sequence similarities and are produced as pre-pro-peptides. The signal sequence is cleaved out after transporting the peptide to the target site. The pro-segment includes a conserved region, the furin motif RVRR that separates the pro- part from the mature peptide. Mature defensins consist of 38–39 amino acid residues including six cysteine residues which are engaged in a characteristic conserved motif of three intramolecular disulfide bridges connected in a Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6 pattern. This specific cysteine pairing forms a common structural element typical for arthropod defensins, known as the cysteine-stabilised αβ (CSαβ) motif that is essential for their antimicrobial activity (Bulet and Stöcklin, 2005). The importance of proper defensin folding was demonstrated by Isogai and colleagues (2011) where the linear form of the tick defensin Persulcatusin exhibited only low antibacterial activity in comparison to the same but well folded, three-dimensional peptide. Besides the previously

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mentioned anti-borrelial activity of *D. variabilis* defensin, tick defensins are known to be active primarily against Gram-positive bacteria with some studies also proving them to have antifungal or antiparasitic effects (Chrudimská et al., 2011; Saito et al., 2009; Tsuji et al., 2007).

In this study, we have focussed on characterizing defensin isolated from the tick *D. marginatus* with the main aim of revealing its anti-microbial properties including anti-borrelial activity.

2. Materials and methods

2.1. Tick samples

D. marginatus tick colony is maintained at the Institute of Parasitology, Biological Centre, Academy of Sciences of the Czech Republic. Uninfected adult females were fed on adult guinea pigs (infection-free animals treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb; ethics approval number 137/2008). Ticks were collected after drop- off following blood feeding (fully fed ticks) or 4–5 days post-attachment (removed forcibly using forceps; semi-fed ticks). Hemolymph was collected from semi-fed females. Organs (midgut, ovaries, malphigian tubes and salivary glands) were dissected under a binocular microscope from fully fed ticks.

2.2. Verifing the 3′end of mature def DM – 3′RACE (Rapid Amplification of cDNA Ends)

To obtain the 3′end of the defDM sequence, total RNA was isolated from two partially engorged adult ticks using TRI reagent (Sigma, USA) according to the manufacturer's recommendation. Single strand cDNA suitable for 3′RACE PCR was obtained by using adaptor primer and Superscript II reverse transcriptase (Invitrogen, USA). 3′-RACE PCR was performed using gene specific primers designed according to the previously published sequence of *D. marginatus* defensin (Chrudimská et al., 2010): defDM forw1: 5′-ATG CGC GGA CTT TGC ATC-3′ and defDM forw2: 5′-GGC TTC GGA TGC CCA CT G-3′, and universal amplification primer on the basis of the manufacturer's protocol (Invitrogen). The amplification conditions were determined experimentally for each set of primers using the MasterGradient (Eppendorf, Germany) thermal cycler. Amplified defensin genes were cloned into pCR®4-TOPO vector (Invitrogen, USA). DNA sequencing was performed using ABI 3130 Sequencer and the Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with M13 forward/reverse primers. Sequences were analyzed with LASERGENE software (DNASTAR, United Kingdom). Similarity searches were carried out of the data available in GenBank, of the National Centre for Biotechnology Information (NCBI, Bethesda, USA) using the BLAST (NCBI) software.

2.3. Semi-quantitative two step RT-PCR analysis

Semi-quantitative two-step reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze *def DM* gene expression in adult female organs. Dissected organs were washed in PBS and total RNA was isolated as described previously (Chrudimská et al., 2011). Single strand cDNA was prepared from total RNA with random primers (200 ng per reaction) using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). Five micrograms of total RNA was used per reaction. The mature defDM was amplified using the specific primer pair (defDM matureF 5′-CAC CGG CTT CGG ATG CCC ACT G-3′ and defDM matureR 5′-TTA ATT CCT GTA GCA GGT GCA GG-3′). As a control, primers amplifying the housekeeping gene actin were used (accession No. HQ645110; Chrudimská et al., 2011). To amplify the target gene, single strand cDNA (150 ng per reaction) was used. Amplification was performed with GoTaq Colorless Master Mix (Promega, USA) in a 20 μl reaction using a fixed number of 31 cycles (96 °C for 25 s, 53 °C (defensin) or 50 °C (actin) for 30 s and 72 °C for 1 min). PCR was performed in a Mastercycler (Eppendorf, Germany) thermal cycler. PCR reaction products were separated by electrophoresis using a 1.8% agarose gel.

2.4. Peptide purification from tick hemolymph

The frozen hemolymph stored in extraction solvent (50% aqueous acetonitrile containing 0.5% trifluoroacetic acid (TFA) was thawed, extensively vortexed and sonicated, and then the extract (1.2 ml) was centrifuged. The supernatant was collected and the remaining precipitate was extracted three times with 30% aqueous acetonitrile containing 0.2% TFA; all extracts were combined and ultrafiltered three times in Amicon Ultra-15 centrifugal filter devices with a 100 kDa molecular weight cut-off membrane (Millipore, Carrigtwohill, Co., Cork, Ireland) at 4200 rpm to remove high molecular weight compounds. The filtrate was lyophilized, yielding white stable lyophilisate (5.6 mg) which was dissolved in 30% acetonitrile containing 0.2% TFA (0.4 ml) and again ultra-filtered three times using Amicon Ultra-0.5 centrifugal filter device with a 100 kDa molecular weight cut-off membrane at 10,000 rpm. The filtrate was lyophilized (3 mg) and then fractioned in three consecutive runs by reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac C-18, 250 × 4.6 mm; 5 μm, column (Grace Vydac, Hesperia, USA) at 1 ml/min flow rate using a solvent gradient ranging from 5% to 70% acetonitrile/water/0.1% TFA over 60 min. During the first run, the material of all fractions detected at 220 nm were collected, evaporated using a Speed-Vac, and then tested for the presence of antimicrobial activity against *Micrococcus luteus*. The majority of the activity was detected in the fraction collected at t_R during 23.7–24.1 min (Supplemental Fig. 1). The active materials collected from all three RP-HPLC runs were combined and re-purified by RP-HPLC at 1 ml/min flow rate by using a solvent gradient ranging from 70% of solvent A to 50% of solvent B over 60 min and the same column as used before. Solvent A was 5% acetonitrile/water/0.1% TFA; solvent B was 70% acetonitrile/water/0.1% TFA. The material of the peak eluted at t_R = 7.5 min (Supplemental Fig. 2) showing activity against *M. luteus* was subjected to Edman degradation and analyzed by electrospray ionization mass spectrometry (ESI-MS) (see Supplementary material for Edman degradation and Supplemental Figs. 3 and 4 for mass spectra).

2.5. Peptide synthesis

Crude linear 38-residue peptide – defDM was synthesized on 200 mg of preloaded Fmoc-Asn(Trt)-Wang Resin in an Applied Biosystem 433A peptide synthesizer using the HBTU/HOBt/N,N-diisopropylethylamine activation protocol of Fmoc chemistry. The protected amino acids were coupled in 10-fold excess in N-methyl-2-pyrrolidone as a solvent.

The linear peptide was deprotected and cleaved from the resin using 5 ml of a mixture of trifluoroacetic acid/1,2-ethanedithiol/H_2O/thioanisol/triisopropylsilane (90: 2.5: 2.5: 3: 2) for 3.5 h and precipitated with *tert*-butyl methyl ether, yielding crude products. The crude peptides were purified by preparative HPLC on a Vydac C-18 column (250 × 10 mm) using a gradient ranging from 100% or 80% of solvent A to 100% or 50% of solvent B, respectively, over 60 min at a flow rate 3.0 ml/min (A, 5% acetonitrile/water/0.1% TFA; B, 70% acetonitrile/water/0.1% TFA). A measured molecular mass of the purified peptide was in good agreement with the calculated values.

2.6. Oxidative folding

The lyophilized peptide that was pre-purified by HPLC was dissolved (at a concentration of 1 mg/4 ml) in 0.1 M ammonium acetate buffer, pH 7.8 (prepared by bubbling gaseous NH_3 into 0.1 M acetic acid) and stirred under the open air at room temperature. The time course of the disulfide bonds formation was monitored by HPLC. After 4–6 h of the folding reaction the solvent was removed by lyophilization and the desired folded peptide was further purified by preparative HPLC using a gradient of solvents ranging from 80% of solvent A to 50% of solvent B at a flow rate of 3 ml/min over 60 min. The final product was then lyophilized.

2.7. High-performance liquid chromatography

RP-HPLC was carried out on an Agilent Technologies instrument equipped with a diode-array detector (Model 1200, Agilent, Santa Clara, USA). The elution was monitored by absorption at 220, 254 and 280 nm. The instrument was controlled and HPLC profiles recorded using ChemStation Software.

2.8. Peptide sequencing by Edman degradation

The N-terminal amino acid sequence of isolated defDM was determined on the Procise Protein Sequencing System (Model 491, Applied Biosystems, Foster City, USA) using the manufacturer's pulse-liquid Edman degradation chemistry cycles.

2.9. Mass spectrometry

The exact mass of purified defDM was determined using an Orbitrap hybrid mass spectrometer (Model LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, USA) equipped with an electrospray ion source. A 1:1 mixture of acetonitrile and water with 0.1% formic acid was continuously delivered to the ion source at a 20 μl/min flow rate. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 5.0 kV, 10 V, 110 V and 300 °C respectively. The mass spectra were internally calibrated using protonated phthalic anhydride as the lock mass.

2.10. Microorganisms

The following microorganisms were used for determination of basic antimicrobial activity profile: *Bacillus subtilis* 168 (*B. s.*) kindly provided by Prof. Yoshikawa (Princeton University, Princeton, NJ, USA); *Escherichia coli* B No. CCM 7372 (*E. c.*) and *M. luteus* No. CCM 144 (*M. l.*) from the Czech Collection of Microorganisms, Brno; *Staphylococcus aureus* (*S. a.*) and *Pseudomonas aeruginosa* (*P. a.*) were obtained as multi-resistant clinical isolates, No. 4231 and 8567, respectively, from Liberec Hospital, Czech Republic; and infectious *Borrelia afzelii* isolate CB43 (*B. a.*) from the Institute of Parasitology, Biology Centre. Yeast, *Candida albicans* F7-39/IDE99 (*C. a.*), kindly provided by the Institute of Organic Chemistry and Biochemistry, was a clinical strain from a collection of fungi at the Institute of Microbiology, Faculty of Medicine and Dentistry, Palacký University Olomouc, Czech Republic. For antiviral activity assay, two TBEV strains, Neudoerfl (Heinz and Kunz, 1981), and Hypr (Pospíšil et al., 1954) were used.

2.11. Determination of antimicrobial activity

2.11.1. Drop-diffusion double-layer technique

Drop-diffusion double-layer technique (Čeřovský et al., 2008) on Petri dishes was used for quick qualitative estimation of antimicrobial properties against *B. s.*, *E. c.*, *M. l.*, *S. a.*, *P. a.* and *C. a.*

2.11.2. Minimum inhibitory concentrations (MICs)

MICs against *M. l.*, *B. s.* and *S. a.* were established by observing bacterial growth in microtitre plates. Different concentrations of tested compound were prepared in 2-fold serial dilutions in fresh LB medium from stock solutions and added to the wells (100 μl). Their final concentrations in the wells after addition of bacterial suspension ranged from 0.1 to 100 μM. Bacteria were grown in LB medium at 37 °C with continuous shaking to mid-log phase. The bacterial suspension was then diluted in LB medium to a final concentration of approximately 1×10^6 CFU/ml and added to the wells (100 μl, final volume in the wells 200 μl). The plates were incubated at 37 °C for 20 h while being continuously shaken in a Bioscreen C instrument (Oy Growth Curves AB Ltd., Helsinki, Finland). The absorbance was measured at 540 nm every 15 min and each peptide was tested at least 3 times in duplicate.

2.11.3. Determination of anti-borrelial activity

Activity against *B. a.* was tested using both liquid and solid media. *B. afzelii* was grown at 34 °C, from an initial glycerol stock, in 8 ml of BSKII liquid media. After reaching mid log phase, the number of spirochetes were enumerated using a dark-field microscope and the Petroff-Hausseur chamber. Following dilution in BSKII media, approximately 2×10^3 spirochetes were incubated (total amount 100 μl/tube) with two different concentrations of defensin (400 and 100 μM) as well as with the physiological solution used for dissolving the peptide. As a control *B. a.* was also maintained in BSKII medium alone. At 2, 5 and 24 h post initial incubation, 10 μl from each tube was plated out in duplicate. The plates were then maintained in anaerobic chambers at 34 °C and 3 weeks post-plating the number of colonies on each plate was counted. In addition, after 24 h, 30 μl from each tube was added to tubes containg 8 ml of liquid media and kept at 34 °C. The growth of spirochetes in each tube was enumerated over time using the Petroff Hausseur chamber. A second set of experiments involved the incubation of 10^3 *B. a.* with defDM that had been dissolved in BSKII to a final concentration of 900 μM in a total volume of 55 μl. After 24 h 945 μl of BSKII media was added to the 1.5 ml tubes. The tubes were then placed in an incubator at 34 °C. The growth of *Borrelia* over time, incubated with the defensin or in medium alone, was assayed by enumeration using the Petroff-Hausseur chamber.

2.11.4. Antiviral activity

The viricidal and inhibitory effect of *D. marginatus* defensin was determined as described previously (Chrudimská et al., 2011). The final concentration of defDM used in the experiments was 100 μM.

2.12. Hemolytic assay

EDTA-anticoagulated human venous blood was obtained from young healthy volunteers. Erythrocytes were harvested by centrifugation (2000 rpm, 10 min, and 20 °C) and washed in sterile PBS. A suspension of the erythrocytes (2%; vol/vol) was used for the assay. Stock solution of defDM peptide was diluted in PBS and co-incubated with erythrocytes (37 °C, 2 h) for a final volume of 200 μl and final concentration ranging from 0.75 to 25 μM. After incubation, the suspension was centrifuged (2000 rpm, 10 min, and 20 °C), 100 μl of supernatant was removed, and the absorbance of samples was measured at 405 nm (A_N). The hemolytic activity was calculated in correlation to the negative (A_0, 0% hemolysis in PBS) and the positive (A_{100}, 100% hemolysis obtained by incubation with 0.2% solution of Triton X-100 in PBS) controls (% hemolysis = $(A_N - A_0/A_{100} - A_0) \times 100$).

3. Results

3.1. Identification of defDM at the mRNA level and its expression profile in tick organs using RT-PCR

In the previous study on tick defensin isoforms, we had obtained a *D. marginatus* defensin using degenerative primer pairs (Chrudimská et al., 2010). To verify the 3′end of the mRNA defensin transcript, important for prediction of the mature protein sequence, 3′ RACE was performed. The mRNA defDM sequence (mature part), obtained by this method, was identical to the one previously published (accession number FJ222583; Supplemental Fig. 5).

Two step RT-PCR was used for determination of the expression profile in the main organs of the tick. The mRNA of *defDM* gene was detected in tick midgut, hemolymph and salivary glands. Gene expression was not detected in the ovaries and malphigian tubes (Fig. 1).

Based on the *defDM* expression profile, the tick hemolymph was identified as a good source for native peptide isolation.

3.2. Isolation and identification of defDM at the protein level

Utilizing our experience in the extraction and isolation of defensins from the hemolymph of other arthropods (Čeřovský et al., 2010), we were able to successfully purify defDM from the hemolymph of adult ticks sufficient for the determination of its full-length sequence. We used a strongly acidic aqueous extraction solvent containing a high concentration of organic solvent to protect the peptides of interest against proteolytic degradation while providing optimal conditions for the extraction and solubility of cationic peptides. The ultrafiltration of crude hemolymph extract through 100 kDa molecular weight cut-off membranes resulted in the removal of high molecular weight compounds that would otherwise obstruct HPLC operations. The following RP-HPLC fractionation (Supplemental Fig. 1) resulted in separation of the fraction with apparently high activity against *M. luteus* appearing in the HPLC profile as a tiny peak (arrow in Supplemental Fig. 1). Further purification of the collected RP-HPLC fraction using a second RP-HPLC (Supplemental Fig. 2) revealed prevailing impurities but good separation of the targeted peptide as a single peak at t_R = 7.5 min, which proved to exhibit intensive antimicrobial activity and a purity satisfactory for sequencing by Edman degradation.

The sequencing of the sample by Edman degradation using 38 cycles gave the following N-terminal sequence: Gly-Phe-Gly-X-Pro-Leu-Asn-Gln-Gly-Ala-X-His-Asn-His-X-Arg-Ser-Ile-Arg-Arg-Arg-Gly-Gly-Tyr-X-Ser-Gly-Ile-Ile-Lys-Gln-Thr-X-Thr-X-Tyr-Arg-Asn-OH (accession number FJ222583 in UniProt Knowledgebase at http://www.ebi.ac.uk), assuming that all six undetermined amino acid residues (X) were cysteines (see Supplemental material for all 38 sequencing cycles). The exact molecular mass of DM-defensin measured by ESI-orbitrap MS was manually calculated from the *m*/*z* values of multiply (5×, 6× and 7×) charged molecular ions found in the mass spectra (Fig. 4, in Supplemental material), resulting in a monoisotopic molecular mass of 4222.9. This is in good agreement with the calculated value of 4222.89, based on the sequence determined by Edman degradation and assuming that the six cysteine residues form three disulfide bridges and that the peptide is not C-terminally amidated.

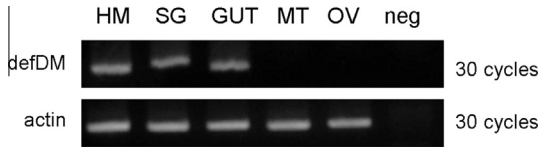

Fig. 1. Differential expression of *defDM* gene in blood fed female *D. marginatus* at the mRNA level using two-step RT-PCR. HM – hemolymph, SG – salivary glands, GUT – midgut, MT – malphigian tubes, OV – ovaries, neg – negative control.

3.3. Synthetic peptide

The defDM (38) was synthesized and purified by HPLC. Then the linear peptide defDM was successfully folded using oxidation under open air conditions and the cyclic peptide isolated by HPLC. The identity of the cyclic properly folded peptide was verified by HPLC analysis and mass spectrometry showing identity to that of native defensin.

3.4. Biological activities

For the determination of biological activities, the defDM, corresponding to the natural mature *D. m*. defensin (both by amino acid sequence and cysteine pairing), was used.

Synthesized peptide was tested against a spectrum of diverse microorganisms – bacteria (*B. afzelii*, *S. aureus*, *B. subtilis*, *M. luteus*, *P. aeruginosa* and *E. coli*), yeast (*C. albicans*) and viruses (two strains of TBEV).

In the drop-diffusion test on Petri dishes, the defDM was active against *B. s.*, *M. l.*, and *S. a.*, and was inactive against all tested Gram-negative bacteria and yeast (*E. c.*, *P. a.* and *C. a.*). The MIC values were thus determined only for tested Gram-positive bacteria and are given in the Table 1a.

The peptide defDM of the tick *D. marginatus*, in which TBEV has been detected (Nosek and Kožuch, 1985), did not interfere with the viruses' life cycle.

In the first set of experiments looking at anti-borrelial activity using the plating method, it is abundantly clear that at 24 h post incubation there is a 50% reduction in the population of *B. afzelii* that was incubated with 400 μM of defensin peptide when compared to the positive controls or the defensin at a lower concentration (Table 1b). This reduction is further amplified with the growth curves that were obtained with the remainder of the population that had been incubated in 8 ml tubes with BSKII media, over time, at 34 °C (data not shown). This borrelicidal activity was evident in the second experimental setup when the defensin was used at 900 μM concentration. No viable spirochetes were detected over time when compared to the positive control of *Borrelia* incubated alone, in BSKII media, under the same conditions.

Table 1
Antimicrobial activities of synthetic defDM.

(A) Establishing of minimal inhibition concentrations (MICs) for sensitive Gram-positive bacteria species.

Bacteria species	MIC [μM]
M. luteus	0.2
B. subtilis	0.2
S. aureus	3

(B) Anti-borrelial activity of defDM.

	Positive control I	Positive control II	DefDM 400 μM	DefDM 100 μM
2 h	134	123	119	119
5 h	151	148	154	No data
24 h	274	279	**121**	284

Number *of B. afzelii* colonies (average of 2 plates) obtained after incubation of spirochetes in the presence or absence of defensin at various time points.
Positive control I indicates incubation of *B. a.* in BSKII only, Positive control II indicates incubation of *B. a.* in BSKII with physiological solution. Decrease in the number of *Borrelia* (colonies) was visible after 24 h of incubation (number in bold).

Hemolytic properties of defDM were studied on human erythrocyte suspension (2%, vol/vol). The highest used defensin concentration was 25 μM and hemolysis did not exceed 5% (Table 1 in Supplementary material).

4. Discussion

Like most other arthropods, the tick immune system consists of only innate immune pathways and is able to utilize them effectively as a defence against certain pathogens. In the last two decades, the tick hemolymph as well as tick hemocytes have become a rich source for the identification and characterization of many antimicrobial proteins/peptides (AMPs). These include defensins, histidine rich AMPs, lysozymes and pattern recognition molecules (Chrudimská et al., 2011; Fogaça et al., 2004; Lai et al., 2004; Pichu et al., 2009; Rego et al., 2006; Simser et al., 2004).

Defensins are expressed in many important tick organs, including the salivary glands, midgut, hemocytes, fat body and malphigian tubes and serve as the primary antimicrobial defence mechanism. Generally, their production is at a basal level during the life of the tick with an increase in expression after blood feeding, during moulting (where there is a potential for infection) as well as after infection or injury (Chrudimská et al., 2011; Nakajima et al., 2002). In this work we have tested only defDM mRNA expression after blood feeding in adult female organs. The test revealed high amounts of defensin mRNA in hemocytes, salivary glands and midgut tissues. These results suggest that the blood meal may be an important avenue for infection, with the AMPs ready to detect and destroy potential microbial agents that may affect the tick. We can assume, based on the *I. scapularis* Genome Project and the recently published sequences of *Ixodes ricinus* (Tonk et al., 2014) defensin isoforms, that the possibility exists for such isoforms to be present in *D. marginatus*, functioning as part of the defense mechanism in various organs (Hill and Wikel, 2005; Wang and Zhu, 2011). This assumption is supported by several defensin studies, where each defensin isoform has a different expression profile within the same set of tick samples (Chrudimská et al., 2011; Nakajima et al., 2002).

Antimicrobial activities of tick defensins are generally known to function against species of Gram-positive bacteria (Chrudimská et al., 2011; Isogai et al., 2009). This was confirmed in this study using the *D. marginatus* defensin. We have successfully purified an amount of defDM from the hemolymph of adult *D. marginatus* ticks sufficient for the determination of its full-length sequence. The peptide was prepared synthetically using SPPS and oxidative folding. DefDM revealed an antibacterial influence only on Gram-positive bacteria at very low concentrations.

Research oriented at discovering borrelicidal peptides/molecules in non-vector ticks emerged in the year 2000. The inoculation of LD spirochetes directly into the *D. variabilis* hemocel resulted in an increase of hemocyte number and hemolymph protein content. After 24 h no viable bacteria were observed and no culturable spirochetes were present in any tissue. When plasma from *D. variabilis* was incubated with *B. burgdorferi* a strong anti-borrelial activity was determined, which rapidly decreased the survival of co-cultured spirochetes (Johns et al., 2000, 2001a). The evidence that *D. variabilis* hemolymph possess a borrelicidal protein/peptide resulted in the discovery of small 5.3 kDa protein (Johns et al., 2000). The protein was identified as a defensin and showed inhibitory activity within 24 h. Its effect on *Borrelia* was enhanced by chicken lysozyme – the synergistic effect of these two proteins on spirochetes was observed within one hour (Johns et al., 2001b).

We demonstrate here, that another tick from the genus *Dermacentor*, *D. marginatus* possesses a defensin with anti-borrelial activity, even though the activity was evident at high concentrations (400–900 μM). These results do not have any pharmacological relevance, but support the view that non-vector ticks are able to destroy *Borrelia* inside the tick body. We have to take into account that studies done *in vitro* are only models of the processes that take place *in vivo*. We do not know anything concerning the real concentrations of defensin within the tick cells or body fluids. However, we may assume that within the tick, the naturally occurring defensin may function under optimal conditions and, due to compartmentalization and the effect of molecular crowding, clear the ticks of pathogens. It would be ideal to undertake possible defensin studies using RNAi in *D. marginatus* to identify if it has a role in the clearance of *Borrelia* that are ingested by the ticks when feeding on infected animals.

5. Conclusions

In conclusion, a recently identified defensin from the hard tick *D. marginatus* was detected in different tick organs, purified from the tick hemolymph, synthesized and biologically characterized. Its low hemolytic activity as well as effective anti-Gram-positive bacterial properties may indicate a potential for future research. Anti-borrelial activities were confirmed suggesting a possible role in clearance of ingested *Borrelia* from a blood meal. It provides another layer in our understanding of an innate immune protein that could be playing a role in what essentially makes a tick become or not become a vector of the Lyme disease pathogen.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2014.04.005.

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