Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Expression and characterization of defensins from the hard tick *Ixodes ricinus*

Bachelor Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens

INSTITUTE OF PARASITOLOGY

Biology Center, ASCR

Julie Krátká

Supervisor: MSc. Ryan O. M. Rego, Ph.D.
Co-supervisor: MSc. Atul Kumar, Ph.D.

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Annotation

From *Ixodes ricinus* was extracted cDNA containing gene with a defensin. The defensin was cloned and expressed in modified *E. coli* bacteria. Results were analyzed via Western blot technique. Peptides used for testing of antibacterial activity were obtained from *I. ricinus* and added into *borrelia* cultures to observe their bactericidal effect against *borrelia*.

Affirmation

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 14.12. 2021

Julie Krátká

Abstract

Defensins are types of antimicrobial peptides found in different kinds of organisms, which are examined due to their antibacterial properties against wide scale of pathogens. In this work was cloned and expressed Preprodefensin from *Ixodes ricinus* via modified *E. coli* cells. The results were visualized via Western blot technique. Another aim of the work was to examine antibacterial properties of five defensins from *I. ricinus* against two types of *borrelia*.

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Abbreviations

AA	amino acid(s)
AGE	agarose gel electrophoresis
AMP	antimicrobial peptide
BME	β-merkapto ethanol
bp	base pair(s)
BSK	Barbour-Stonenner Kelly medium
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
MKP	modified Kelly-Pettenkofer medium
PCR	Polymerase chain reaction
RF	relapsing fever (*borrelia*)
SDS-Page	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TAE	tris-acetate-EDTA

1. Introduction

1.1. Ticks

Ticks are ectoparasitic animals that belong to arthropods. Their body compose of two main parts – an anterior gnathosoma carrying a mouth, a pair of pedipalps, and a pair of chelicerae. The second part called posterior idiosoma, which is found behind the gnathosoma, bears tick legs (Basu & Charles, 2017). Their life cycle is hemimetabolous, i.e. they hatch from eggs as larvae and subsequently develop into nymphs, and in the last stage, they grow into adults. (Ribeiro & Valenzuela, 2011).

They are divided into two main groups called hard ticks (*Ixodidae*) and soft ticks (*Argasidae*). Over 75% of tick species belong to the *Ixodidae* family (Fogaça et al., 2021). Hard ticks can be found among woods or grass fields, they feed continuously on their host. In contrast to *Ixodidae*, soft ticks feed intermittently. The integument of *Ixodidae* is hard and compose of a chitinous shield or scutum. Soft ticks lack dorsal shield or scutum, their integument is tough and leathery (Basu & Charles, 2017; Britannica 2021).

Ticks are ectoparasites feeding on the blood of their hosts, thus serving as vectors of viruses, bacteria, or protozoa causing a wide scale of diseases (Bell-Sakyi et al., 2007). They feed on the blood for several days, unusually compared to other arthropods. Some tick species even change the type of the host in a different phase of their development. If tick feeds on a host infected with a pathogen, the pathogen first gets into the tick gut from where it traverses via hemolymph or the hemocoel to its salivary glands which produce saliva, thus at next feeding, the microorganism can be transmitted to another host.

The most common tick species in Europe belongs to the *Ixodidae* family and is called *Ixodes ricinus* (Crippa et al., 2002). It is a vector for pathogens like *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, tick-borne encephalitis virus, louping ill virus, and *Babesia spp* (Biernat et al., 2014; Hudson et at., 1995; Rizzoli et al., 2014; Tonk et al., 2015).

1.2. Immune System of Ticks

Vertebrates possess highly-developed adaptive system immunity which is absent in ticks (Tonk et al., 2015). Ticks have only a primitive innate immune system which is composed of cellular and humoral responses and keep the level of pathogens in their body on a sustainable

level (Tonk et al., 2015; Hajdusek et al., 2013). The cellular response is based on hemolymph. Hemolymph prevents microbes to spread to the tick's body and its major component are hemocytes which play an important role in the tick's defense by performing nodulation, phagocytosis, and encapsulation. (Borovičková & Hypša 2005; Aguilar-Díaz & Cossío-Bayúgar 2018).

On a membrane surface of hemocytes are expressed lectins, which can recognize bacteria by bonding to lipopolysaccharides present on the bacterial surface. After the recognition, hemocyte prevents spreading of the pathogen by forming a sticky mass around the bacterial aggregate (nodules). This process is called nodulation. Lectin found in *I. ricinus* is called Ixoderin A. It can be found in its midgut or on a membrane surface of hemocytes, where it is expressed (Aguilar-Díaz & Cossío-Bayúgar 2018).

One of the first responses which recognize a pathogen in tick's body is known as phagocytosis, a complex mechanism of cellular defense that eliminates parasites by engulfment. It is transferred via hemocytes.

If the pathogen is too large to be destroyed by phagocytosis or nodulation, encapsulation takes its place. It associates melanization process, when quinones and semiquinones are established due to formation of a thick layered capsule around the pathogen created by hemocytes (Sonenshine & Hynes 2008; Marmaras & Lampropoulou 2009).

Humoral immunity response composes of pattern-recognition proteins or effector molecules such as lectins, lysosomes, proteases, proteases inhibitors, etc. The major component of humoral immunity response are antimicrobial peptides (Hajdušek et al., 2013; Tonk, M., 2014).

Another important component of tick immune system is serine proteinase inhibitors. It mediates the melanization and coagulation process of hemolymph and the production of AMPs. Serine proteinase inhibitors help ticks to defend themselves against pathogens due to their antimicrobial properties which consist of inhibiting proteinases of microorganisms that invade the immune system and colonize host tissues (Fogaça et al., 2021).

1.3. Antimicrobial Peptides

Antimicrobial peptides are short proteins with length of 12 to 100 amino acids (Tonk, M., 2014). They can be found among plants, mammals, birds, amphibians, insects, ticks, bacteria, or fungi (Huan et al., 2020, Tonk et al., 2015) and are produced by specific genes

(Lei et al., 2019). AMPs show different kinds of antimicrobial activity against bacteria, fungi, viruses, protozoan parasites, and yeast. Most AMPs with antibacterial properties work against Gram+ and Gram− bacteria (Tonk et al., 2015). Most peptides are cationic or amphipathic (Bahar & Ren, 2013). Cationic peptides neutralize the charge of bacterial cell membrane which leads to the destruction of the bacteria (Lei et al., 2019).

Based on the composition of AA and peptide structures are AMPs divided into two subfamilies. One composes of linear molecules with α-helical structure, with a typical lack of cysteine or abundancy of certain amino acids such as histidine, proline, glycine, tryptophan, and arginine. This group includes for example magainin or cecropin. The other subfamily with β-sheet structure is typical for peptides expressed in insects, consisting of polypeptides with cysteines forming disulfide bridges, which to some extend play an important role in antimicrobial activity of these AMPs. This subfamily is called defensins. (Bahar & Ren, 2013; Lei et al., 2019).

Diversity of AMPs is given by amount of the environmental threats to which the given organism has to face, i.e. organisms who have to defend themselves against more pathogens are expected to have a larger variety of AMPs (Vilcinskas A. et al., 2013; Altincicek B. et al., 2007).

1.4. Defensins

The name “defensin” was for the first time used in 1985 after the identification of peptides extracted from human and rabbit neutrophils (Machado & Ottolini, 2015). Defensins belong to AMPs, their length composes of 3-6 kDa. They can be found among humans, animals, plants, and insects (Raj & Dentino, 2002). Their structure composes of 6-8 cysteine residues which form 3-4 disulfide bridges, and it is folded into alfa helix and β-sheet structure. The disulfide bonds help to maintain the stability and folding of the peptide. The functional part of the defensin is found in γ-core motif, C-terminal β-sheet domain (Aguilar-Díaz & Cossío-Bayúgar 2018; Ganz et al., 2003; Tonk et al., 2014; Tonk, M., 2014). Defensins are produced as prepropeptides, most of them are cationic, but there can be found anionic peptides as well (Tonk et al., 2014).

Some antimicrobial peptides indicate antibacterial and antifungal properties (Ganz et al., 2003). As usual, defensins show antibacterial activity against Gram-positive

bacteria by inducing the formation of membrane-penetrating channels which leads to lysis of the bacterial cell (Gillespie et al., 1997).

Antimicrobial peptides of ticks show activity against Gram-positive and Gram-negative bacteria, intracellular rickettsia, protozoa, and fungi (Tonk et al., 2015). Arthropod defensins differ from the rest of defensins in their structure and sequence (Raj & Dentino, 2002).

The first tick defensin was found in *Ornithodoros moubata* (soft tick from Africa) due to cloning and sequencing of two isoforms from its haemolymph (Nakajima et al., 2001). *O. moubata* defensins showed antibacterial activity against Gram-negative bacteria *E. coli* (Nakajima et al., 2002). Antimicrobial peptides in tick body are expressed mainly in salivary glands, fat body, hemocytes, gut, and ovaries. The action of AMPs differs based on response to a microbial challenge or blood-feeding (Fogaça et al., 2021).

The first two discovered defensins of tick *I. ricinus* were def1 and def2. Further research showed due to sequencing that def1 and def2 are isoforms (Rudenko et al., 2007). There are currently 5 most examined defensins in *I. ricinus*, called defMT2, defMT3, def MT5, def MT6, and defMT7. Their structure composes of α-helix at the N-terminus with an antiparallel β strand at the C-terminus. *I. ricinus* defensins are mostly expressed in midgut, salivary glands, embryo-derived *I. ricinus* cell line (IRE/CTVM19), ovary, haemolymph, or Malpighian tubules, although some of them are ubiquitous (Tonk et al., 2014).

1.4.1. DefMT2

DefMT2 (Figure 1) is tissue-specific, because it is expressed only in salivary glands and embryo-derived cell line of *I. ricinus* (Tonk et al., 2014). In previous *in vitro* research was found strong antiplasmodial activity of defensin MT2, when defMT2 was examined *in vivo* with *plasmodium*, it successfully reduced the concentration of the pathogen in the animal (Couto et al., 2018).

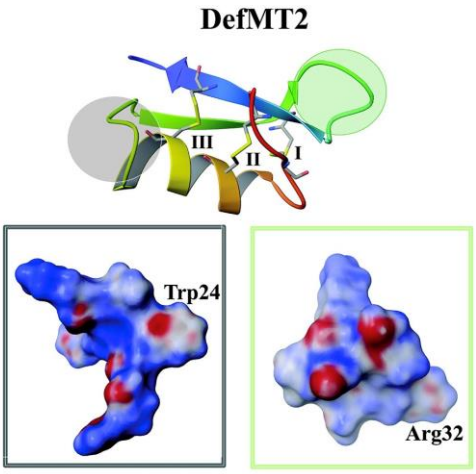

Figure 1: *Structure of defensin MT2 (Tonk et al., 2015)*

1.4.2. DefMT3

Expression of defMT3 (Figure 2) is not tissue-specific, it is produced ubiquitously in the tick body. It was thought there is one more defensin in *I. ricinus* named defMT4, although after further examination was discovered defMT3 and defMT4 are isoforms together with def1 and def1. Both peptides share 98% identical genetic information, and their mature peptide sequence is identical (Tonk et al., 2014; Tonk et al., 2015). Examination of defMT3 proved its antimicrobial activity and its γ-core shows increased bacteriostatic effect. Defensin MT3 evince strong antiplasmodial activity *in vitro*, antifungal properties, and activity against Gram-negative bacteria *P. aeruginosa* and Gram-positive bacteria such as *S. aureus* or *L. fleischmannii* (Couto et al., 2018; Tonk et al., 2015).

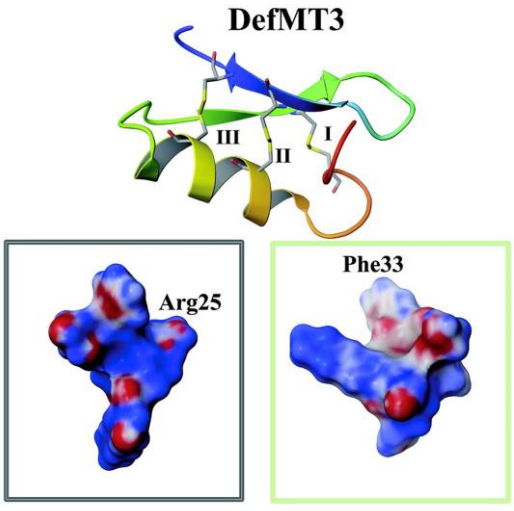

Figure 2: *Structure of defensin MT3 (Tonk et al., 2015)*

1.4.3. DefMT5

DefMT5 (Figure 3) is an anionic kind of defensin. It is a tissue-specific peptide because it is produced only in tick salivary glands. It is the only AMPs of *I. ricinus*, which does not express in the IRE/CTVM19 (Tonk et al., 2014). Observed antimicrobial properties of defMT5 showed inhibition of maximum growth of blood stages of *Plasmodium falciparum* at increased concentration of the defensin (Couto et al., 2018).

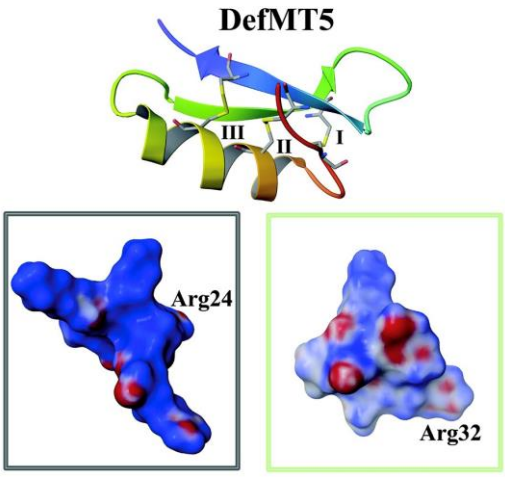

Figure 3: *Structure of defensin MT5 (Tonk et al., 2015)*

1.4.4. DefMT6

Defensin MT6 (Figure 4) is only expressed in salivary glands, Malpighian tubules, and ovaries (Tonk et al., 2014). Investigation of this peptide indicated its antibacterial activity against Gram-negative and Gram-positive bacteria and revealed bacteriostatic activity against *E. coli*. Its γ-core shows bacteriostatic activity against Gram-positive bacteria *S. epidermidis* and *L. grayi*. Defensin MT6 shows in addition to antibacterial properties also antifungal activity against *F. culmorum* and *F. graminearum* (Tonk et al., 2015) and strong antiplasmodial activity (Couto et al., 2018).

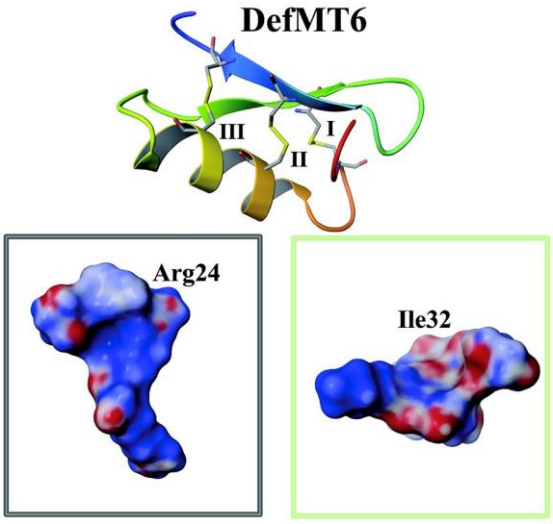

Figure 4: *Structure of defensin MT6 (Tonk et al., 2015)*

1.4.5. DefMT7

The only non-cationic defensin is defMT7 (Figure 5). Due to this reason this defensin might differ in antimicrobial activity from the other's peptides found in *I. ricinus,* because Gram-negative bacteria have higher resistance against cationic defensins. DefMT7 differs also in its structure from the other AMPs found in *I. ricinus*, it is missing the characteristic β strand at the C-terminus and it is intronless. The presence of this defensin in tick's body is ubiquitous (Tonk et al., 2014).

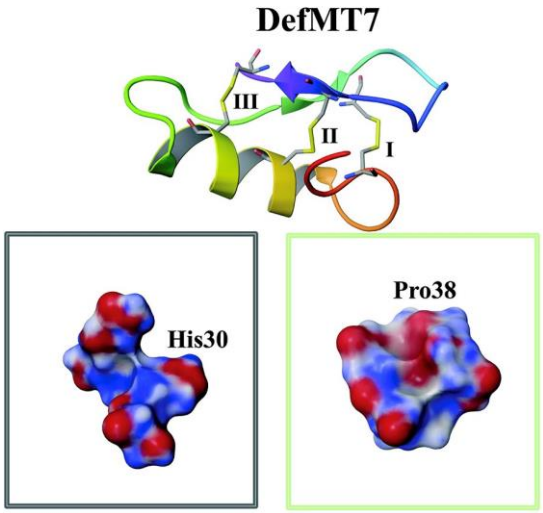

Figure 5: *Structure of defensin MT7 (Tonk et al., 2015)*

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