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Expression and purification of fibrinogen-related proteins from the soft tick *Ornithodoros moubata*

Bachelor thesis

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Annotation:

The significance of tick immune proteins is explored, specifically focusing on two fibrinogen-related proteins from the soft tick *Ornithodoros moubata* called Dorin M and OMFREP, which play a role in the tick's innate immune system. During this work, the genes of Dorin M and OMFREP were amplified through molecular cloning, before the proteins were produced in a bacterial expression system for the first time. After optimization of the expression procedure, the proteins were subsequently purified by Ni-NTA chromatography, and results were confirmed via western blotting.

Declaration:

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, 08.05.2024

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Abstract

Tick-borne diseases pose a growing concern for public health, and the development of new strategies to combat these diseases is urgently needed. The tick immune system has emerged as a promising area of research, due to its contribution to the transmission of tick-borne pathogens.

This study focuses on two fibrinogen-related proteins from the soft tick *Ornithodoros moubata* called Dorin M and OMFREP, which are lectins expressed in the salivary glands, and are known to play a role in the tick's innate immune system. This work aimed to express and purify these proteins for the first time, to better understand their function, as well as their potential for therapeutic intervention.

The methods used in this study included molecular cloning for gene amplification, subsequent protein production through bacterial expression systems, followed by protein purification by Ni-NTA chromatography, before results were confirmed via western blot.

Overall, this research contributes to the understanding of tick innate immune proteins and opens possibilities for the development of effective measures against tick-borne disease.

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Abbreviations

AMP	Antimicrobial peptides
ATR	Acquired tick resistance
CCHF	Crimean-Congo hemorrhagic fever
FBG	Fibrinogen-like
FREPs	Fibrinogen-related proteins
GdnHCL	Guanidinium chloride
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBD	Tick-borne disease
TBE	Tick-borne encephalitis
TBP	Tick-borne pathogen
TBRF	Tick-borne relapsing fever
TEMED	Tetramethylethylenediamine

1 Introduction

Ticks are the second most important vector of disease transmission worldwide, with over 70000 cases of tick-borne diseases reported in 2022 in the US alone (CDC DVBD 2022b; Fuente 2008). The relevance of tick-borne diseases (TBDs) is made more pressing by a notable increase in cases associated with environmental changes and greater human-tick interactions. Moreover, ticks pose a great threat to livestock, particularly in economically vulnerable regions such as sub-Saharan Africa. This highlights a critical economic and health concern and the urgent need for targeted research on intervention techniques (Diarra *et al.* 2023; Ghosh *et al.* 2007). The focus of this research was placed on two proteins of the soft tick *Ornithodoros moubata* named Dorin M and OMFREP. Both are lectins expressed in the tick's salivary glands and play an important role in the immune system of ticks (Rego *et al.* 2005, 2006). By examining these proteins more closely, this study aims to contribute to building knowledge that will help the development of effective measures against tick-borne disease.

The expression and purification of Dorin M and OMFREP were performed and optimized during this work. Future research will reveal whether they evoke ATR and their viability for anti-tick vaccines.

In the following chapters, an overview of tick physiology and immune response is given, before the methods and results of the research are discussed.

1.1 Life Cycle of Ticks

All tick species undergo four life phases: egg, larva, nymph, and adult. With both male and female forms, the adult stage exhibits sexual dimorphism. Every mobile life stage has to find a host, attach itself to it, and consume blood. Depending on the species and life stage, a blood meal could take minutes to days and significantly enlarges the tick's abdomen. This provides the nourishment needed for larvae and nymphs to undergo metamorphosis to the next stage, more commonly known as molting (Abreu *et al.* 2018 pp. 1-2; Johnson 2023 pp. 1-2).

Adult females consume blood meals to aid in the maturation of their eggs, whilst males typically skip these meals in favor of locating and mating with females. When a female is fully engorged,

she will separate from the host, find a secure place to process the blood meal, and wait for the eggs to mature. By a process known as oviposition, the female will lay up to thousands of eggs, giving rise to the next generation of ticks (Abreu et al. 2018 pp. 1-2; Johnson 2023 pp. 1-2).

1.2 The Tick Families

Ticks are ectoparasitic Arthropods of the Class Arachnida and represent the Suborder Ixodida (Abreu et al. 2018 p. 25). As with most arthropods, a tick's body is segmented into three distinct regions, the capitulum (mouthparts), the idiosoma (body), and the legs (Sonenshine and Roe 2014 p. 74). The approximately 970 different tick species are divided into three extant families, namely Ixodidae, Argasidae, and Nuttalliellidae (Dantas-Torres and Otranto 2020).

1.2.1 Ixodidae

The biggest and most diverse family is Ixodidae (hard ticks), which comprises ~750 different species (Dantas-Torres and Otranto 2020), with members living in every biogeographical zone (Johnson 2023 p. 12).

Usually, each of the three mobile life stages requires a single blood meal, except for male adults which can consume blood depending on whether the species needs it to form sperm. The adult female typically feeds on one host over several days and can become engorged to several times its initial size. Many females of the Ixodid species can oviposit with just one meal (Johnson 2023 pp. 12-13).

Hard ticks' distinct morphological features include the chitinous scutum or hard shield that, in males, completely covers the dorsal surface but only partially in females, allowing the body to enlarge during engorgement (Abreu *et al.* 2018 pp. 25–27; Johnson 2023 pp. 11-13).

1.2.2 Argasidae

The second largest tick family with ~218 species (Dantas-Torres and Otranto 2020) is Argasidae (soft ticks). They have adapted in several ways to survive in tropical and subtropical areas to withstand extremely high environmental temperatures, including thickened cuticles in immature and adult life stages instead of a dorsal scutum to stop water (Johnson, 2023 p. 13).

To protect themselves from temperature and drought extremes found in more exposed areas, soft ticks are nidicolous, meaning they spend most of their time in the tunnels, nests, and caves of their vertebrate hosts, also including human dwellings, especially in dark, cool places like crawlspaces, between walls or under floorboards (Vial 2009).

In soft ticks, the nymphal stage, which is made up of several instars (immature forms within each life stage), is another distinction seen in the feeding cycle. A blood meal is required for each maturation to the next instar stage, of which there can be more than ten before true metamorphosis. Feeding typically lasts for less than an hour, after which they molt to the following stage. Furthermore, mature females will have blood meals several times daily, ovipositing a comparatively modest clutch of eggs (up to 500) after each feeding. These could all be adaptations to the scarcity of hosts and the harsh weather (Johnson 2023 p. 13).

1.2.3 Nuttalliellidae

Nuttalliellidae is the smallest known extant tick family with only one species. It has challenged the previously established distinction between hard and soft ticks since their main morphological characteristics include aspects of both hard and soft ticks. They have a partly sclerotized pseudoscutum which is usually found in hard ticks, as well as a leathery integument usually found in soft ticks (Johnson 2023 pp. 13-14). According to Mans *et al.* (2011), genetic sequencing revealed that the Nuttalliellidae lineage is basal to Ixodidae and Argasidae, as well as being the most closely related living family to the last common ancestor between the other two families.

1.3 Feeding

Almost all ticks are obligate hematophagous arthropods, meaning that they rely solely on other organisms' blood for sustenance. Most hematophagous arthropods use a needle-like tubular mouthpart that punctures specific capillaries before feeding on the host blood directly from a blood vessel. Ticks have developed a pool-feeding technique, where they lacerate the skin of their host with their mouthparts and then anchor themselves in the wound. They then ingest a mixture of tissue and the blood pooling around them, making them both hematophagic (eating blood) and histiophagic (eating tissue) (Johnson 2023 p. 48).

1.3.1 The Capitulum

The mouthparts of soft and hard ticks are similar, the most notable difference is that the capitulum is positioned ventrally and cannot be seen from a dorsal view in soft ticks. Additionally, many argasid species have flap-like cheeks covering the capitulum and porose regions are missing in argasid females (Sonenshine and Roe 2014 pp. 80-82).

The capitulum is made up of the basis capitul, the hypostome, a pair of chelicerae, and palps. The basis capitul anchors the mouthparts to the body and surrounds the pharynx and the basal regions of the chelicerae. The hypostome, which emerges from the center of the basis capitul, is an immobile plate armed with rows of recurved spine-like denticles, and the dorsal surface has a groove in the middle. In hard ticks, the hypostome is usually lengthier and contains more denticles than in soft ticks. The chelicerae are the cutting and slicing tools that pierce the host's skin deep into the dermis and are made up of an extended muscular base armed with hook-like barbs. They are located dorsally of the hypostome and the ventral surfaces of the chelicerae essentially roof the grooves in the hypostome, creating a tubular channel known as the preoral canal which is designed to direct the flow of food into the tick's oral opening and, alternatively, tick saliva into the host's skin. The palps are linked to the anterior and lateral borders of the basis capitul. They are a pair of sensory structures that are splayed sideways during a blood meal and do not pierce the host's skin. (Johnson 2023 pp. 11-17; Richter *et al.* 2013; Sonenshine and Roe 2014 pp. 80-82; Vancová *et al.* 2020).

1.3.2 The Alimentary System

Hard and soft ticks have evolved different strategies to control blood flow from the preoral canal to the pharynx. Hard ticks utilize a pharyngeal valve, which is a complex structure with movable walls and a wedge that regulates blood flow from the mouth and preoral canal, preventing regurgitation. In soft ticks, the preoral canal is physically divided from the overlying salivarium by an extended, moveable, V-shaped labrum that runs the whole length of the hypostome. It prevents blood and saliva mixing and regurgitation from the pharynx without a separate pharyngeal valve (Sonenshine and Roe 2014 pp. 84-85; Vancová *et al.* 2020).

The pharynx is a fusiform structure situated within the basis capituli, which is made up of a delicate cuticular lining encircled by numerous layers of smooth muscle cells. The pharynx narrows to join the esophagus, which enters the synganglion and joins to the midgut. A

proventricular valve prevents the regurgitation of host fluids back into the pharynx (Sonenshine and Roe 2014 pp. 85-86; Vancová *et al.* 2020).

The midgut is the tick's largest organ, and unlike other hematophagous arthropods, the tick's midgut also functions as a storage organ, allowing the tick to slowly digest its contents over months or even years. It comprises a central ventriculus (stomach) from which many caeca (diverticula) arise, reaching almost every section of the body cavity. The midgut wall is made up of an epithelium, which is composed of undifferentiated and digestive cells encased by a thin outer layer of elongated smooth muscle cells. The midgut digests hemoglobin and other nutrients intracellularly, with digestive cells developing from undifferentiated cells during feeding. These cells hypertrophy and ingest hemoglobin, which binds to clathrin-coated pits in the luminal surfaces. Hemoglobin molecules are internalized, incorporated into phagolysosomes, and digested. The distal portions of these cells fill with hematin waste, which is then passed to the exterior via the rectal sac and anus. The midgut usually is connected to the rectal sac by a narrow tubular intestine that transports wastes to the rectal sac, mixing with guanine from the Malpighian tubules. This includes dead or dying digestive cells, cell fragments, hematin, undigested hemoglobin crystals, and fluids from the midgut lumen (Lara *et al.* 2005; Sonenshine and Roe 2014 p. 87).

1.4 The Dangers of Ticks

When a host is bitten by a tick, the resulting physical damage and introduced saliva can cause acute inflammatory reactions at the attachment site. After tick detachment, the lesion left in the skin usually heals in days. However, if a tick is removed incorrectly, parts of the capitulum can break off due to the hypostome's barbs. Left-over mouthpart fragments and lack of proper hygiene at the wound site may lead to infection and granuloma formation (Johnson 2023 p. 56).

1.4.1 Tick as a Vector

The biggest health risk posed by ticks is their potentially infectious bodily fluids which can contain TBPs.

Ticks have been identified as one of the important vectors of human diseases worldwide, second only to mosquitos (Fuente 2008). The incidence rate of TBDs is steadily increasing, in the United

States for example, from 2004 to 2016 reported cases increased by ~218% (Rosenberg *et al.* 2018), and from 2017 to 2019, almost every 2000th emergency department visit in the United States was due to a tick bite (Marx 2021). Ticks do not use humans as a reservoir for infection transmission, making them incidental or dead-end hosts, and further transmission to other humans or ticks is unlikely. Human infections with TBPs are thus unfortunate consequences of a tick's blood meal. Therefore, the rise in human TBDs is mainly accredited to climate change and the shrinking of ticks' natural habitats, forcing them to come into closer contact with humans (Johnson 2023 p. 65).

In both domestic and wild animals, ticks are considered the primary vectors of disease-causing pathogens (Fuente 2008). Over 80% of the world's cattle population is susceptible to TBDs, which pose a significant danger to livestock health, welfare, and production, particularly in sub-Saharan Africa. Tick-borne livestock diseases have a significant economic impact on communities that rely on domestic animals for food and income (Diarra *et al.* 2023; Ghosh *et al.* 2007).

Although there is a great variety of ticks, most TBPs are transmitted by a small number of species that have a broad preference for large vertebrate hosts. Those, however, are extremely versatile disease vectors as they can harbor viruses, bacteria, and protozoa (Johnson 2023 p. 65)

1.4.2 Pathogen Acquisition and Transmission

When it comes to TBP acquisition, the primary pathway is from the vertebrate host to the tick, which is known as horizontal transmission. This is usually facilitated by so-called reservoir hosts, which are animals that may harbor and sustain high levels of infectious pathogens while displaying no symptoms of disease. These reservoir hosts play an important part in the transmission cycle of tick-borne illnesses because they serve as a source of pathogens for ticks in nature (Johnson 2023 pp. 65-67; Ravindran *et al.* 2023; Rocha *et al.* 2022).

Additionally, co-feeding transmission can occur between two ticks feeding on the same uninfected host. When an infected tick and an uninfected tick feed on the same host in close proximity to each other, pathogens can spread between them, resulting in infection (Johnson 2023 pp. 65-67; Rocha *et al.* 2022).

Alternatively, vertical transmission is possible for some pathogens, allowing them to persevere in the tick population regardless of tick-host interactions. The two types of vertical transmission are

transovarial and transstadial transmission. An adult female passing a pathogen to their offspring in the egg stage is known as transovarial transmission. However, not all pathogens can survive into the next generation of ticks and in those that do, transovarial transmission occurs infrequently. Therefore, if a pathogen is not further spread to vertebrate hosts, the pathogen will eventually disappear from tick populations (Johnson 2023 pp. 65-67; Ravindran *et al.* 2023; Rocha *et al.* 2022).

The causative agent of relapsing fever in Africa, transmitted by *O. moubata*, is one of the most well-researched examples of transovarial transmission. The percentage of infected eggs lain by infected females can vary between ~20% to ~70% (Schwan and Raffel 2021).

Another challenge for pathogen persistence is transstadial transmission since TBPs have to survive the molting process ticks undergo to reach the next life stage. Most TBPs can persevere over one or multiple tick life stages without oral infection. This makes transstadial transmission essential for the transmission of TBPs, especially in hard ticks, where the number of vertebrate hosts is limited (Johnson 2023 pp. 65-67; Ravindran *et al.* 2023; Rocha *et al.* 2022).

1.4.3 Tick-borne Diseases

The types of TBD that can be transmitted to humans depend on the type of tick and geographical region it is contracted from. There are a plethora of TBDs, therefore, a short overview of some of the most devastating human diseases associated with TBPs are described below.

Tick-borne encephalitis (TBE) is caused by viruses of the genus *Flavivirus* and family *Flaviviridae* with three main genetic subtypes, named after the regions they occur. The European subtype is transmitted mostly by *I. ricinus,* the Far Eastern and Siberian subtypes are carried by *I. persulcatus*, and *I. ovatus* was found to be a vector of the Far Eastern subtype in Japan. About two-thirds of humans infected with the TBE virus are asymptomatic. TBE presents in two phases, the first phase is characterized by low white blood cell count and low platelet count, while the second phase involves neurological manifestations in the central nervous system such as encephalitis and myelitis (ECDC 2024; Phipps and Johnson 2022).

Lyme disease is mainly caused by the bacteria *Borrelia burgdorferi*, but has also been reportedly caused by several other *Borrelia* species. Lyme disease is spread by a variety of the *Ixodes* genus, mainly by *I. scapularis* and *I. pacificus* in the US, and *I. ricinus* in Europe. Initial symptoms include the well-known bulls-eye rash as well as headache, fever, and fatigue. If left untreated,

the infection can spread throughout the body and cause inflammation in the joints, heart, and nervous system, however, Lyme disease rarely causes death (CDC DVBD 2022a; Radolf *et al.* 2022; Shapiro 2014).

Tick-borne relapsing fever (TBRF) is transmitted by soft ticks of the genus *Ornithodoros* as well as hard ticks of the genus *Ixodes*. TBRF from soft ticks is mainly caused by *Borrelia hermsii,* however, 14 other *Borrelia* species have been identified as well. In hard ticks, the TBRF pathogen has been identified as *B. miyamotoi.* TBRF can be contracted in the Americas, Africa, southern Europe, and Asia. After an incubation time of 4-18 days, up to 12 episodes of recurrent fever can occur, with fever episodes lasting up to 7 days and being separated by afebrile periods of up to 10 days. Accompanying symptoms can range from unspecific manifestations like nausea and headache to neurologic complications. The overall case fatality rate of TBRF has been reported at 6.5% (Jakab *et al.* 2022; WA DOH 2022).

1.5 Tick Immune System

Due to their blood-feeding habit, ticks and pathogens have co-evolved to develop a mutual tolerance. Albeit, ticks need defense mechanisms to keep the levels of pathogens and commensal microbes at a level where it does not diminish their health or maturation (Hajdušek *et al.* 2013).

As in other invertebrates, the immune system of ticks is a lot simpler than in vertebrates, relying solely on innate immune reactions in the form of cellular and humoral responses. Even though it is more rudimentary, its effectiveness is proven by the large number of tick species around the world. Therefore, knowledge about the ticks' immune system is key in understanding TBP and subsequently treating TBDs (Fogaça *et al.* 2021; Hajdušek *et al.* 2013; Sonenshine and Macaluso 2017).

1.5.1 Pathogen Path

During a blood meal on an infected host, the numerous bioactive molecules found in tick saliva regulate immunological and inflammatory responses of the host, promoting pathogen uptake. The ingested blood is accumulated and concentrated in the tick's midgut, making it the primary site for tick-pathogen interplay. Depending on the method of transmission a pathogen employs, it has to not only pass several tissue barriers inside the tick body but also survive the subsequent

molting of the tick to be further transmitted. Pathogens that utilize horizontal transmission must travel to the tick's salivary glands via the hemolymph before being successfully transmitted to another host during the next feeding, and for transovarial transmission, the pathogen has to migrate to the ovaries (Fogaça *et al.* 2021; Hajdušek *et al.* 2013; Sonenshine and Macaluso 2017).

1.5.2 Cellular Responses

Invertebrates have a less complex cell-mediated defense system than vertebrates since antigen-antibody complex and memory cells are absent. Researchers have thoroughly investigated and evaluated the involvement of hemocytes in insect defense systems, however, there has been little research on cell-mediated defense mechanisms and hemocyte involvement in ticks. In insects, hemocytes selectively proliferate during pathogen infection, where they are involved in a crucial part of cellular defense through three processes: phagocytosis, nodule formation, and encapsulation (Gillespie *et al.* 1997; Taylor 2006).

Similar to insects, hemocytes of ticks eliminate pathogens from the hemolymph through phagocytosis. Hemocytes in ticks have been shown to phagocytize various microorganisms, including intracoelomally inoculated *Coxiella burnetii*, *Escherichia coli*, and pathogenic yeast cells. Two of the three morphologically different types of hemocytes identified in the tick hemolymph have phagocytic activity, namely plasmatocytes, and granulocytes, while phagocytosis by prohemocytes has been not observed (Inoue *et al.* 2001; Taylor 2006).

When pathogens become trapped in a coagulum of granular cells, this is called nodulation, a typical reaction of hemocytes to large quantities of pathogens. Encapsulation is the process of enclosing nodules or foreign invaders larger than hemocytes in numerous layers of cells to prevent them from entering active circulation (Aguilar-Díaz *et al.* 2021; Taylor 2006).

1.5.3 Humoral responses

Arthropods have a powerful humoral defense, which complements the cell-mediated responses in the fight against foreign invaders. In ticks, the humoral response is made up of pattern-recognition molecules and soluble effector molecules, also known as "humoral factors,". These include defensins, lysozymes, coagulation factors, proteases, and protease inhibitors, together with a variety of common and specified antimicrobial peptides (AMPs) (Aguilar-Díaz *et al.* 2021; Hajdušek *et al.* 2013).

The Toll and IMD pathways are two of the most extensively investigated immunological signaling cascades in arthropod immunology. Both are triggered by specific Pathogen-associated molecular patterns (PAMPs) and induce humoral responses by regulating the production of AMPs. In the tick genome, the majority of the components that form the Toll pathway in insects are preserved, with the dorsal-related immunity factor (DIF) being the only component that has not been discovered yet. Genomic comparison between insects and ticks shows that the IMD network exhibits a significant level of variability including the absence of orthologs of critical components in this pathway. Nevertheless, the tick's IMD pathway is still functional. This implies that there are considerable differences in humoral immunity between insects and ticks. However, the absence of IMD pathway components is not limited to ticks, since they are missing in hemipterans and other arachnids as well (Aguilar-Díaz *et al.* 2021; Chávez *et al.* 2017).

1.6 Antimicrobial peptides

One of the key aspects of tick innate immunity is the fast and transitory generation of AMPs. Antimicrobial peptides (AMPs) are a category of peptides found throughout nature that are widespread contributors to the innate immune response found in mammals, plants, insects, amphibians, and mollusks. AMPs display a variety of inhibitory actions on bacteria, fungi, parasites, and viruses. Various kinds of AMPs share the following characteristics: the number of amino acid residues ranges from 10 to 60, and nearly all AMPs are cationic with an average net charge of 3.32. However, some anionic AMPs also exist, which contain multiple acidic amino acids such as aspartic acid and glutamic acid. (Huan *et al.* 2020; Tonk *et al.* 2014; Wu *et al.* 2022)

Tick defensins have been known to exist in both hard and soft ticks, and have been shown to trigger tick immune responses within the salivary glands, hemolymph, and midgut (Wu *et al.* 2022). Nakajima *et al.* (2001; 2002) first reported the cloning and gene expression of defensins found in the soft tick *O. moubata*. They identified four isoforms of *Ornithodoros* defensins, called A, B, C, and D, all of which are larger than 1.4 kb and are made up of three introns and four exons. All are highly expressed in eggs, and defensins A, B, and C were found in the highest concentrations in the midgut, while defensin D was expressed highest in the fat body (Nakajima *et al.* 2001, 2002).

1.6.1 Lectins

Lectins are (glyco-)proteins of non-immune origin that can recognize as well as reversibly bind to carbohydrates and glycoconjugates. They are involved in effector and regulatory functions of animals, plants, lichen, bacteria, and fungi, and can either be bound to cell surfaces or suspended in humor. During immunological responses and development, lectins are thought to mediate interactions between cellular and cell-substate interactions. They have a possible role as self/nonself-recognition molecules and are thought to play a role in disease transmission. Due to their selective binding to pathogen surface carbohydrate structures, it is hypothesized that arthropod lectins are the functional counterparts of immunoglobulins (Grubhoffer *et al.* 1997; Natori 2001; Santos *et al.* 2014).

1.6.2 Fibrinogen-related Proteins

Fibrinogen is a glycoprotein complex that is enzymatically converted to fibrin, which, in vertebrates, is a critical step in the formation of the supporting protein framework necessary for coagulation of vascular injury (Hanington and Zhang 2011; Herrick *et al.* 1999).

Fibrinogen-related proteins (FREPs) are a type of lectin found in all vertebrates and invertebrates. These proteins have fibrinogen-like (FBG) carbohydrate recognition domains at their C-terminus. The FBG domain contains around 200 amino acid residues and is highly similar to the C-terminal portions of fibrinogen β and γ chains (Hanington and Zhang 2011; Middha and Wang 2008).

FREPs in invertebrates contribute to the defense response against pathogens, however, they have no role in coagulation. Molecules with FBG motifs were found in a wide range of invertebrate animals, with the majority of these molecules being associated with immune responses. Furthermore, recent genome projects on invertebrates have found a remarkable number of FBG loci, indicating that FBG proteins have various essential functions in invertebrates. FREPs have been demonstrated to play a critical role in defensive mechanisms such as agglutination and bacterial defense, as well as self/nonself-recognition and development. Additionally, FREPs can bind to a variety of PAMPs, since can they also function as pattern recognition receptors (Hanington and Zhang 2011; Zou *et al.* 2021).

1.6.3 Dorin M and OMFREP

Dorin M is a FREP from the soft tick *O. moubata* and was the first purified lectin from ticks by Kovář *et al.* (2000). The binding ability was discovered to be unique to sialic acid and its conjugates, and appears unaffected by the presence of Ca^{2+} or a thiol group. Dorin M is mostly expressed in the salivary glands and the cytoplasm of hemocytes. The malphigian tubules showed minimal expression, but the ovary and midgut lacked it. Rego *et al.* (2006) determined its full cDNA sequence with an open reading frame (ORF) of 837 bp encoding a 279 amino-acid protein (GenBank accession no. AY333989). The mature protein has an estimated mass of 29114 Da and three putative N-linked glycosylation sites were observed (Kovář *et al.* 2000; Rego *et al.* 2006).

During their work with Dorin M, Rego *et al.* (2005) found OMFREP (*O. moubata* fibrinogen-related protein) and determined its cDNA sequence has an ORF of 843 bases encoding a 281 amino acid protein (GenBank accession no. AF527411), and the mature protein has an estimated mass of 29 kDa. Dorin M and OMFREP have a high sequence identity of ~65% and also share a similar expression profile with OMFREP being expressed in the hemocytes and salivary glands, but not in the ovaries, malpighian tubules, or midgut (Rego *et al.* 2005, 2006).

Figure 1 shows the superimposition of the predicted 3D structures of Dorin M and OMFREP, visualizing their structural similarities. Especially the core structures are almost identical, only the helical structures show significant differences (Kumar *et al.* 2021).
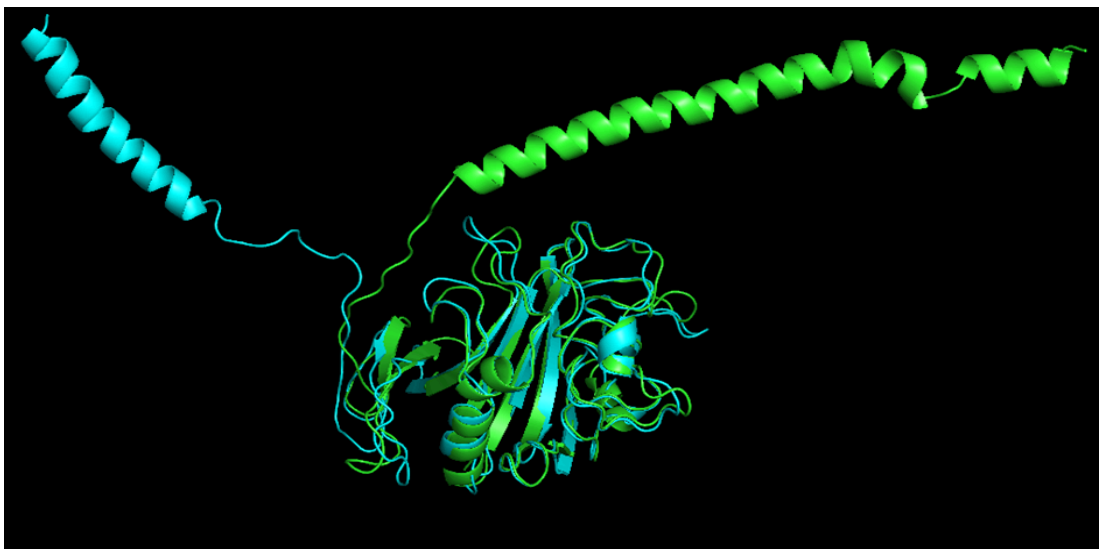

Figure 1: *Predicted homology model of Dorin M (blue) and OMFREP(green) using SWISS-MODEL*

Source: Kumar et al. (2021) Recombinant lectins, Dorin M and OMFREP from Tick O. moubata and their effect on tick development (nymphal molding) and Borrelia infected nymphs. [Unpublished manuscript]. Institute of Parasitology, Biology Center, Czech Academy of Sciences

2 Aims

The main goal of this work was to express for the first time, the FRePs Dorin M and OMFREP from the soft tick *O. moubata*. Starting from the extracted cDNA of *O. moubata*, the genes of interest were to be amplified and inserted into an expression vector. Subsequent protein expression was to be optimized, and the resulting lectins were to be purified.

3 Material and Methods

Unless specifically stated otherwise, the procedure for Dorin M and OMFREP was identical and always performed simultaneously.

3.1 Molecular Cloning

3.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on extracted cDNA from *O. moubata* for amplification. Table 1 displays the forward and reverse primers used, while Table 2 shows the chemicals and their amount used. Two different kinds of DNA Polymerase Master Mix were tested for optimization purposes, namely OneTaq 2x Master Mix (New England Biolabs) and Platinum SuperFi PCR 2x Master Mix (Invitrogen).

Table 1: *Primers used for amplification of Dorin M and OMFREP*

27	DorinM_Xhol_F	5'-CCTCTCGAGATGCTCCGCAACGTGTGTCC-3'
28	DorinM_HindIII_R	5'-ACAAAGCTTTCAAGAGGCTCTGCCATCAGCC-3'
29	OMFREP_Xhol_F	5'-CCTCTCGAGATGCACGTCTTTGTAACTACGC-3'
30	OMFREP_HindIII_R	5'-ACAGGTACCTTATTCAATCCTAGCAACCAACGG-3'

Reagent	Volume [µl]
DNA Polymerase Master Mix	10
DNA Template	0.6
Forward Primer (20 µM)	0.8
Reverse Primer (20 µM)	0.8
mQ H$_2$O	7.8

To check for possible contamination of the primers, a blank control was used, where mQ H$_2$O was used instead of the DNA template. The temperature and duration of the different PCR cycle steps are recorded in Table 3.

Table 3: PCR cycle parameters

	Temperature [°C]	Time [min]	Nb of cycles
Initial denaturation	95	5	1
Denaturation	94	1	
Annealing	57	1	34
Extension	72	1	
Final extension	72	10	1

3.1.2 Agarose gel electrophoresis

To confirm the products from PCR and digestion, Agarose gel electrophoresis (AGE) was employed. For a 1% Agarose gel, 0.7 g of agarose powder was dissolved in 70 ml of 1x TAE by microwaving the mixture for 3 min while swirling in 30-second intervals. After the gel cooled to ~50 °C, 2 µl of GelRed (Biotium) was added before the gel was poured into a tray to set.

All samples, the blanks, and positive controls were stained with 6x DNA Loading Dye before 7 µl of each was loaded into the wells. The prestained GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) was loaded as well. The gel electrophoresis tank was filled with 1x TAE and the electrophoresis was run at 110 V for approximately 1 hour.

After identification of the gene of interest under UV light, the band was cut and gel elution was performed according to the manual included in the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). The gel was solubilized in NTI buffer in a heating block at 50 °C for 10 minutes while vortexing in 30-second intervals. Subsequently, the dissolved gel was loaded into the included column, which was then washed with NT3 buffer twice. The column was dried for 4 minutes at room temperature via centrifugation before being dried on a heating block at 70 °C for another 4 minutes. Lastly, elution was performed with mQ H_2O, which was preheated to 70 °C.

3.1.3 A-Tailing

3 µl of the gel-eluted PCR product were combined with 5 µl OneTaq 2x Master Mix (New England Biolabs) and 2 µl mQ H_2O, before being incubated at 72 °C for 20-30 minutes and briefly spun down.

3.1.4 Ligation into Cloning Vector

The TOPO TA Cloning Kit with pCR2.1-TOPO (Invitrogen) was used for ligating the insert. 4 µl of the A-tailing product was combined with 1 µl of salt solution and 1 µl of TOPO TA vector. The ligation mixture was then incubated at room temperature for 10 minutes before being placed on ice.

3.1.5 Transformation into Cloning Cells

The ligated insert was transformed into thawed cloning cells, *E. coli* DH5α Competent Cells. 1.5 µl of the plasmids were gently combined with 25 µl of cloning cells and kept on ice for 20 minutes. Subsequently, the mixture was heat shocked for 45 seconds at 42 °C and then kept on ice for another 2 minutes. After an addition of 250 µl of room temperature LB medium, the mixture was incubated at 37 °C with agitation for 1 hour.

The cultures were spread on an agarose plate containing carbenicillin, which was previously prepared with 40 µl of 2% X-Gal for blue/white screening. The plates were incubated at 37 °C overnight.

3.1.6 Colony PCR

White colonies were selected off the plates, and cells were transferred to a 2 ml test tube containing 1 ml LB medium and 1 µl carbenicillin, which were incubated at 37 °C with agitation overnight. Cells from the same colonies were also used to perform colony PCR. Table 4 shows the chemicals used and the program from Table 3 was used again.

With 7 µl of the colony PCR product AGE was performed to confirm results.

__Table 4:__ Reagents and their volume used for Colony PCR

Reagent	Volume [µl]
DNA Polymerase Master Mix	7.5
Forward Primer (20 µM)	0.5
Reverse Primer (20 µM)	0.5
mQ H_2O	6.5

3.1.7 Plasmid DNA Purification

The colonies in medium incubated colonies were centrifuged for 5 minutes at 7000 RPM, and the supernatant was discarded before Plasmid DNA purification was performed using the NucleoSpin Plasmid NoLid Mini Kit (Macherey-Nagel). Since the method used differs from the manual included with the kit, it is described below.

For cell lysis, 250 µl of Buffer A1 was used for resuspension of the pellet before adding 250 µl of Buffer A2. After inverting the test tube 8 times, it was kept at room temperature for 5 minutes. Then 300 µl of Buffer A3 were added, and after inversion, the solution, which should turn white at this point, was centrifuged at 11000 RPM for 10 minutes. The supernatant was subsequently added to the column, which was placed in a test tube and centrifuged for 1 minute at 11000 RPM, and the flow-through was discarded. For washing the membrane, 500 µl of Buffer AW, 700 µl of Buffer A4, and 500 µl of Buffer A4 were added to the column and centrifuged off at 11000 RPM for 1 minute, and the flowthrough was discarded. Then the column was centrifuged for another 3 minutes at 11000 RPM before being placed in a heating block set to 70 °C for 3 minutes. The plasmid DNA was eluted twice with ~30 µl of mQ H_2O which was preheated to 70 °C, and subsequently, the concentration was measured with a Nanodrop Spectrophotometer.

3.1.8 Restriction Digestion

The restriction enzyme EcoRI was used for digestion. To a mixture of 1.5 µl EcoRI buffer, 0.6 µl EcoRI enzyme, and 6.9 µl of mQ H_2O, either 10 µl of the plasmids or 5 µl of plasmids and 5 µl of mQ H_2O were added, depending on the plasmid concentration. The samples were kept at 37 °C for 1.5 hours in a heating block. To confirm the results, AGE was performed and the samples were sent for sequencing.

The cells with confirmed inserts were inoculated in 5 ml of LB medium with 5 µl carbenicillin, and incubated at 37 °C with agitation overnight. Thereof, 1 ml was combined with 300 µl of 80% glycerol to create a glycerol stock, which was kept at -80 °C.

3.2 Expression

3.2.1 Double digestion

Double digestion of the TOPO TA plasmid DNA isolate was performed with the restriction enzymes XhoI (New England Biolabs(NEB)) and HindIII (NEB) for Dorin M and XhoI (NEB) and Kpn1-HF (NEB) for OMFREP.

The plasmid DNA samples were diluted to all have the same concentration. 1 µl of each restriction enzyme, 3.5 µl of CutSmart Buffer 10x (New England Biolabs), and 19.5 µl of diluted plasmid DNA were placed in a heating block set to 70 °C for 3 hours.

The expression vectors, namely pET-45b and pBAD/His A, were prepared in the same way.

AGE was performed for confirmation and subsequently, gel elution was performed, and the concentration was measured with a Nanodrop Spectrophotometer.

3.2.2 Ligation into Expression Vector

Both inserts were ligated into the expression vectors with two different concentration ratios. The reaction compositions are displayed in Table 5 and Table 6.

	1:1	2:1
insert	2.5 µl	5 µl
pET vector	0.5 µl	0.5 µl
T4 DNA Ligase 5x buffer (Invitrogen)	1 µl	1.2 µl
T4 DNA Ligase (Invitrogen)	0.5 µl	0.5 µl

For the 1:1 insert-to-vector ratio, final concentrations of 12 ng/µl of Dorin M and 7 ng/µl of OMFREP were achieved, and 16 ng/µl of Dorin M and 9 ng/µl of OMFREP for the 2:1 ratio.

Table 6: *Ligation reaction composition (pBAD)*

	1:1	2:1
insert	2 µl	4 µl
pBAD vector	6 µl	6 µl
T4 DNA Ligase 5x buffer (Invitrogen)	1 µl	1.2 µl
T4 DNA Ligase (Invitrogen)	0.5 µl	0.5 µl

For the 1:1 insert-to-vector ratio, final concentrations of 6 ng/µl of Dorin M and 5 ng/µl of OMFREP were achieved, and 5 ng/µl of Dorin M and 9 ng/µl of OMFREP for the 2:1 ratio.

The mixtures were incubated at 16 °C for 16 hours.

Subsequently, the plasmids were transformed into *E. coli* DH5α Competent Cells, colony PCR was performed as well as plasmid isolation, as described in 3.1.5, 3.1.6, and 3.1.7.

Next, to ensure the gene of interest was still present, double digestion followed as described in 3.2.1, results were confirmed on AGE, and samples were sent for sequencing.

3.2.3 Transformation into Expression Cells

The plasmid isolates were transformed into different *E. coli* strains of competent expression cells, namely BL21 (DE3), C-43 (DE3), and SHuffle Express. The same procedure that was used for the cloning cells was employed again as described in 3.1.5, however, SOC medium was used instead of LB medium. Each culture was grown on two carbenicillin plates and incubated at 37 °C overnight.

Two colonies were picked from each plate and inoculated in 1 ml of LB medium with 1 µl of carbenicillin and incubated at 37 °C with agitation overnight. 100 µl thereof were again inoculated in 5 ml of LB medium with 1 µl carbenicillin and incubated. To confirm the inserts, double-digested plasmid isolates were run on AGE.

For each clone, two glycerol stocks were created by combining 1 ml of inoculate with 300 µl of 80% glycerol, which was kept at -80 °C.

3.2.4 Expression

L-(+) arabinose was used to induce the production of Dorin M and OMFREP. The expression was optimized by varying the temperature and incubation time, as well as the L-(+) arabinose concentration and the medium type. The method which was found to be most successful is described below.

3 ml of LB medium with 3 µl carbenicillin was inoculated from the glycerol stock and incubated at 37 °C with agitation overnight. Thereof, 250 µl were inoculated in 10 ml 2x YT medium with 10 µl carbenicillin and incubated at 37 °C with agitation for 3 hours and 15 minutes. 1 ml of all samples was removed before induction, centrifuged at 13000 RPM for 2 minutes, the supernatant discarded and the cell pellet was resuspended in 20 µl of 4 M Urea and 20 µl of SDS loading dye, before being kept at -20 °C.

The remaining 9 ml were induced with 250 µl of 20% L-(+) arabinose, for a final concentration of 0,5%, and incubated at 25 °C with agitation for 45 minutes. 1 ml of all samples was removed after induction and underwent the same treatment as the before induction samples.

Buffer A was prepared with a composition of 20 mM Tris, 3.3 M NaCl, and 1 mM β-mercaptoethanol, and the pH was adjusted to 7.9.

The rest of the cultures were centrifuged for 10 minutes at 7000 RPM, and the supernatant was discarded. The pellet was washed with 2 ml of Buffer A, then resuspended in 300 µl of Buffer A, and kept at -20 °C until further processing.

3.2.5 Purification

The cells were sonicated 6 times for 45 seconds with 30 seconds rest between each cycle, while being kept on ice.

100 µl of the sonicate was centrifuged at 4 °C for 10 minutes at 7000 RPM. 30 µl of the soluble fraction was combined with 30 µl of SDS loading dye, while the rest was discarded. The pellet was washed four times with 1 ml of Buffer A (centrifuged at 4 °C for 3 minutes at 16000 RPM) before being suspended in 50 µl M Urea and 50 µl of 0.1% SDS. 30 µl of this soluble fraction was mixed with 30 µl of SDS loading dye.

Ni-NTA columns were used for the purification of the sonicated cells, which were prepared in advance by loading each column with 3.5 ml of Protino Ni-NTA Agarose (Macherey-Nagel) and subsequent washing with 100 ml of dH$_2$O.

The remaining sonicate was spun down and washed four times with 1 ml of Buffer A (centrifuged at 4 °C for 15 minutes at 7500 RPM). Then it was suspended in 1 ml of 6 M guanidinium chloride (GdnHCL) in Buffer A before being incubated at 4 °C with agitation overnight.

The next day, the samples were centrifuged for 20 minutes at 16000 RPM, before the supernatant was filtered through a 20 µm syringe filter. The Ni-NTA column was washed with 20 ml of dH$_2$O and 10 ml 6 M GdnHCL in Buffer A, before loading the sample.

The column was washed with 20 ml of 6 M GdnHCL and then with 15 ml of a concentration gradient of Urea in Buffer A (concentration of 6M, 5M, 4M, 3M, and 1M) followed by 15 ml plain Buffer A. the proteins were eluted, with a concentration gradient, with 15 ml of imidazole in Buffer A (concentrations of 25 mM, 100 mM, 500 mM, and 1M).

3.2.6 SDS-Page

Two identical polyacrylamide gels were prepared, the composition of the gels used is described in Tables 7 and 8. The resolver gel was cast and after solidification, the stacking gel was added. 8 µl of a 10-245 kDa ladder was loaded as well as 10 µl of the following samples: before and after induction, soluble and insoluble fraction, the load and wash as well as the elutions of the Ni-NTA column chromatography. The electrophoresis was run at 130 V.

Table 7: *Composition of SDS-PAGE Resolver gel*

Reagent	Volume
Resolver A (Bio-Rad)	2 ml
Resolver B (Bio-Rad)	2 ml
10% APS	20 µl
TEMED	2 µl

Table 8: *Composition of SDS-PAGE Stacking gel*

Reagent	Volume [µl]
30% Acrylamide	170
Stacking buffer (Bio-Rad)	250
mQ H_2O	570
10% APS	10
TEMED	1

3.2.7 Western Blot

While one of the SDS-Page gels was stained using Coomassie Brilliant Blue R-250 (Bio-Rad), the other was used for immunoblotting.

The following buffers were used: blotting buffer (25 mM Tris, 192 mM glycine), blocking buffer (180 ml mQ H_2O, 20 ml TBS, 100 µl Tween with 10 g non-fat skimmed milk powder), and TBS-Tween 20 (900 ml mQ H_2O, 100 ml TBS, 500 µl Tween).

Two pieces of blotting paper and one piece of nitrocellulose membrane were soaked in blotting buffer for 15 minutes and the apparatus was wetted as well. A blotting sandwich with the following arrangement was assembled from bottom to top: blotting paper, membrane, SDS-Page gel, and another blotting paper on top. After the sandwich was smoothed down, the western blot apparatus was set to 25 V for 30 minutes.

The nitrocellulose membrane was shaken in blotting buffer for 2 hours before being incubated at 4 °C in 5 ml of blocking buffer with 1,3 µl His Tag Horseradish Peroxidase-conjugated Antibody (R&D Systems) overnight. After washing the membrane three times in TBS-Tween 20 for 15 minutes, the Pierce ECL Western Blotting Substrate (Thermo Fisher) was used for visualization.

4 Results

4.1 Molecular Cloning

4.1.1 PCR

The first PCR amplification attempt of Dorin M and OMFREP from extracted cDNA (obtained from Dr. Atul Kumar) of *O. moubata* was unsuccessful, but a second try with a different cDNA sample showed bands at the expected weights of 837 bp for Dorin M and 843 bp for OMFREP (Figure 2). Because the bands were rather faint, the amplified genes were gel-eluted and amplified again with another round of PCR, yielding higher concentrations.
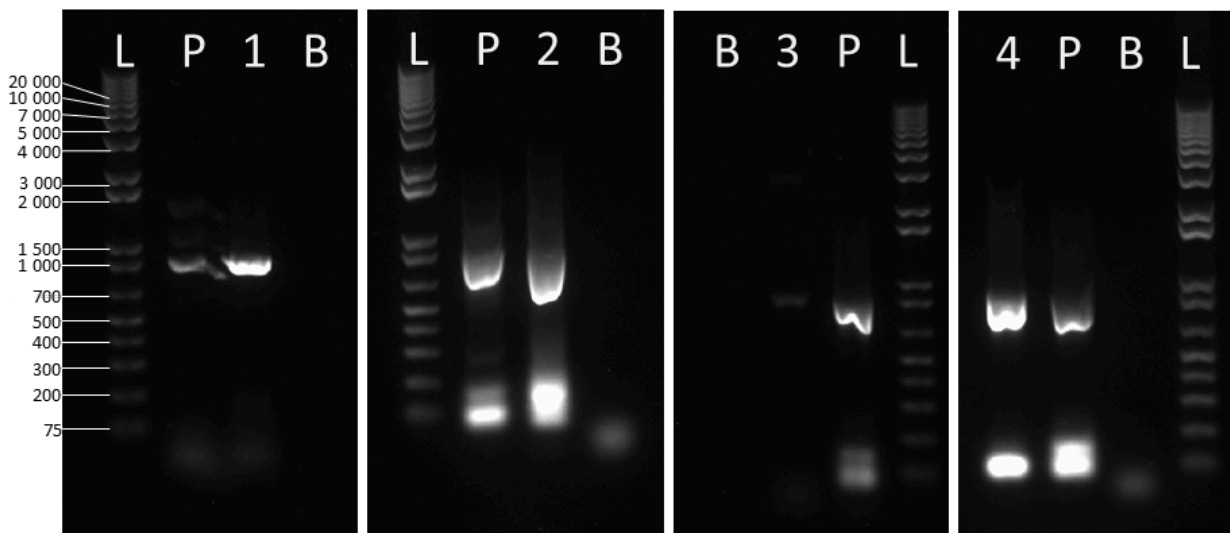

Figure 2: *Gene amplification of Dorin M and OMFREP through PCR. L - 1 kb ladder; P - Positive control; B - buffer blank; 1 - Dorin M after one PCR amplification; 2 - Dorin M after two PCR amplifications; 3 - OMFREP after one PCR amplification; 4 - OMFREP after two PCR amplifications;*

4.1.2 Colony PCR

After ligation into the TOPO TA cloning vector and transformation into DH5α cloning cells, colonies were grown on agarose plates. 14 white colonies each of Dorin M and OMFREP clones were selected and colony PCR was performed for the screening of successful gene insertion (Figures 3 & 4).
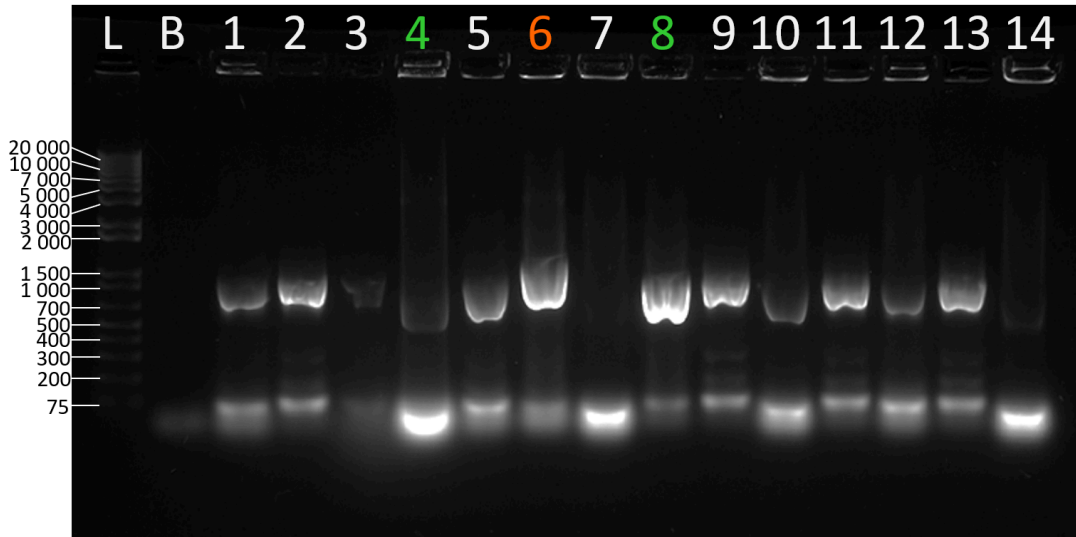

Figure 3: *AGE of Colony PCR for the screening of TOPO TA Dorin M clones. L - 1 kb ladder; B - buffer blank; Samples from Colony PCR product of Dorin M clones labeled 1 - 14;*
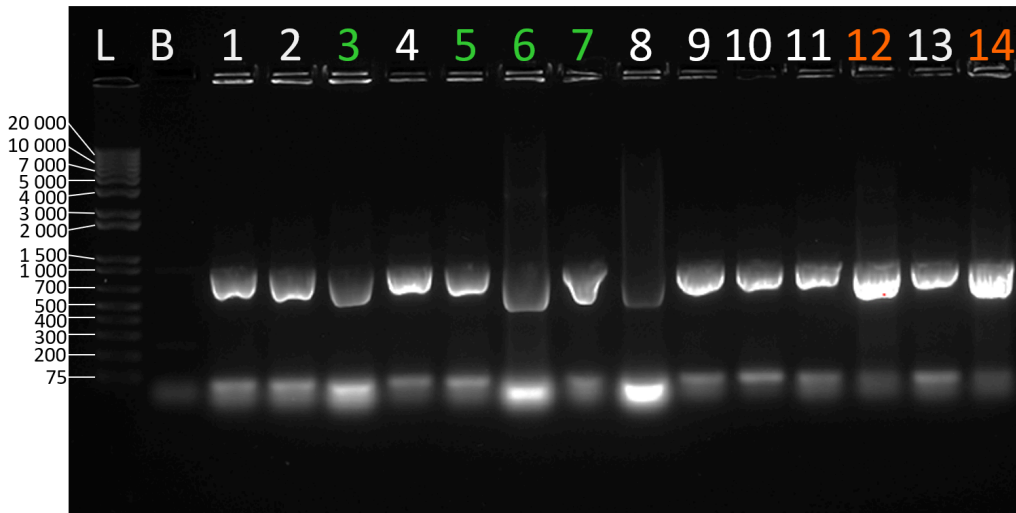

Figure 4: *AGE of Colony PCR for the screening of TOPO TA OMFREP clones. L - 1 kb ladder; B - buffer blank; Samples from Colony PCR product of OMFREP clones labeled 1 - 14;*

4.1.3 Restriction Digestion

To ensure that the genes of interest were still present, restriction digestion was performed after plasmid isolation. Initially, three colonies were selected for both, namely clones 4, 6, and 8 for Dorin M and clones 6, 12, and 14 for OMFREP.

Dorin M samples number 4 and 6 showed the expected band at around 837 bp (Figure 5). Only OMFREP sample number 6 showed the expected band at around 843 bp. To ensure that no mutations had occurred, those three samples were sent for sequencing and all came back positive.
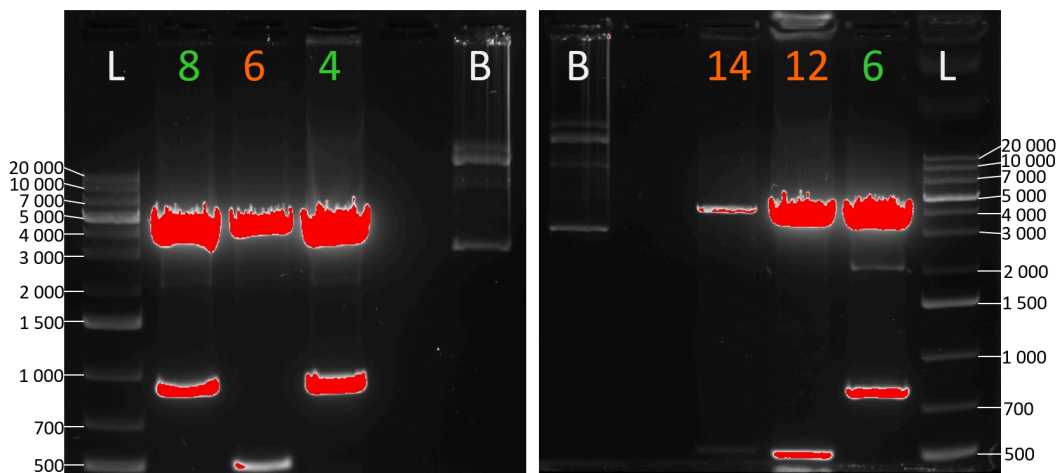

Figure 5: AGE after restriction digestion to confirm gene insertion. L - 1 kb ladder; B - buffer blank; Samples from restriction digestion product of Dorin M on left gel labeled 8, 6, and 4; Samples from restriction digestion product of OMFREP on right gel labeled 14, 12, and 6;

Because only one of three OMFREP samples was positive, plasmid isolation and restriction digestion were repeated for clones 3, 5, and 7. Again only OMFREP sample 6 showed the expected band, however, samples 3, 5, and 7 all showed two bands at around 500 bp and 400 bp.

However, the cleavage site of the EcoRI restriction enzyme is GAATTC (Nevinsky 2021), which is a motive that can be found in the ORF of OMFREP starting at position 373 (GenBank accession no. AF527411). Therefore, two fragments are to be expected for the digestion of OMFREP with EcoRI, creating two fragments of 470 bp and 373 bp, which can be seen in Figure 6.
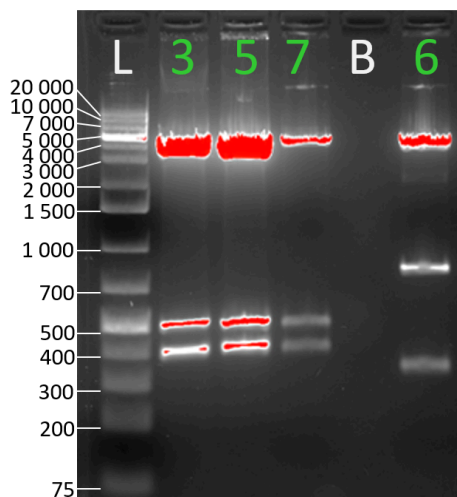
Figure 6: AGE after repeated restriction digestion to confirm gene insertion. L - 1 kb ladder; B - buffer blank; Samples from restriction digestion product of OMFREP labeled 3, 5, and 7; Sample from previous restriction digestion product of OMFREP was labeled 6;

4.2 Expression

The clones Dorin 8 and OMFREP 7 were used for further experiments.

4.2.1 pET vector

Even though the double digestion of the vectors and inserts looked promising (Figure 7), the ligation was not successful using the pET-45b vector system (Figure 8).

The double digestion of Dorin M, OMFREP, and the pET vector showed bands as expected at around 840 bp for the FREPs and 5300 bp for the vector.
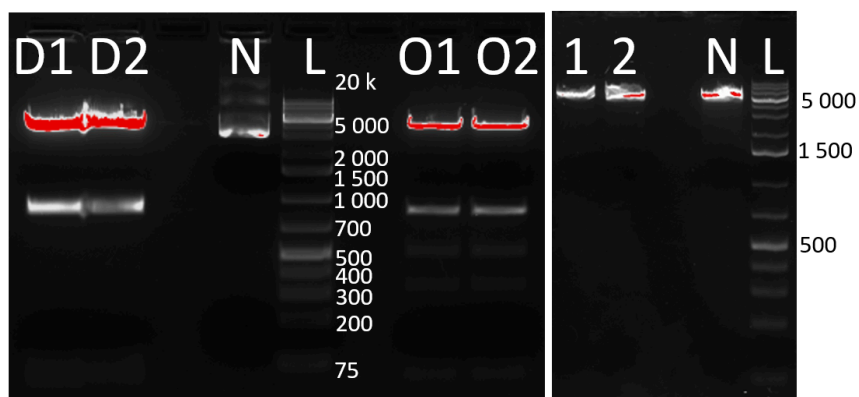

Figure 7: AGE of double digestion product from inserts and pET vectors. L - 1 kb ladder; N - negative control; Samples from double digestion product of Dorin M labeled D1 and D2; Samples from double digestion product of OMFREP labeled O1 and O2; Samples from double digestion product of pET vector labeled 1 and 2;

After transformation into DH5α, plasmid isolation, and double digestion, AGE of Dorin M samples showed the expected bands, but the ligation product of OMFREP did not.

Therefore, the double digestion and ligation reaction was repeated for OMFREP, however, still no bands could be detected, as seen on the right side of Figure 8.

The bands from D1 and D2 were sent for sequencing, and the results were negative for the correct Dorin M sequence.
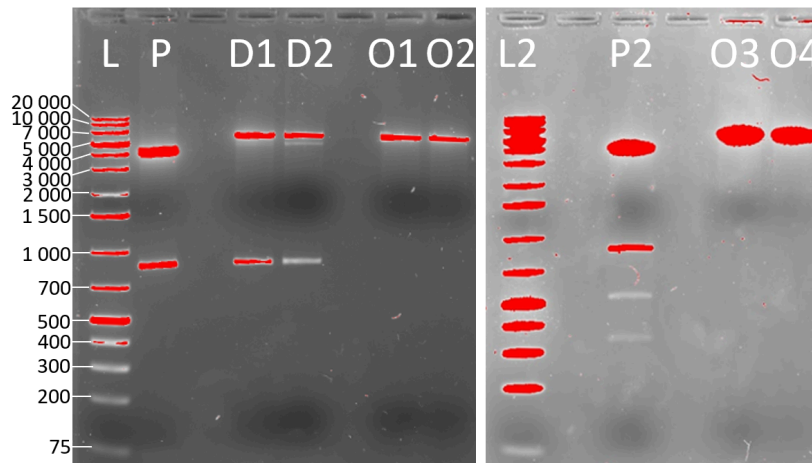

Figure 8: _AGE after double digestion to confirm Dorin M / OMFREP pET vector ligation. L & L2 - 1 kb ladder; P & P2 - positive control; Samples from double digestion product of Dorin M labeled D1 and D2; Samples from double digestion product of OMFREP labeled O1, O2, O3, and O4;_

4.2.2 pBAD vector

Ligation was successful with the pBAD/His A vector system, and after transformation into DH5α, the presence of the genes of interest was confirmed through AGE (Figure 9).
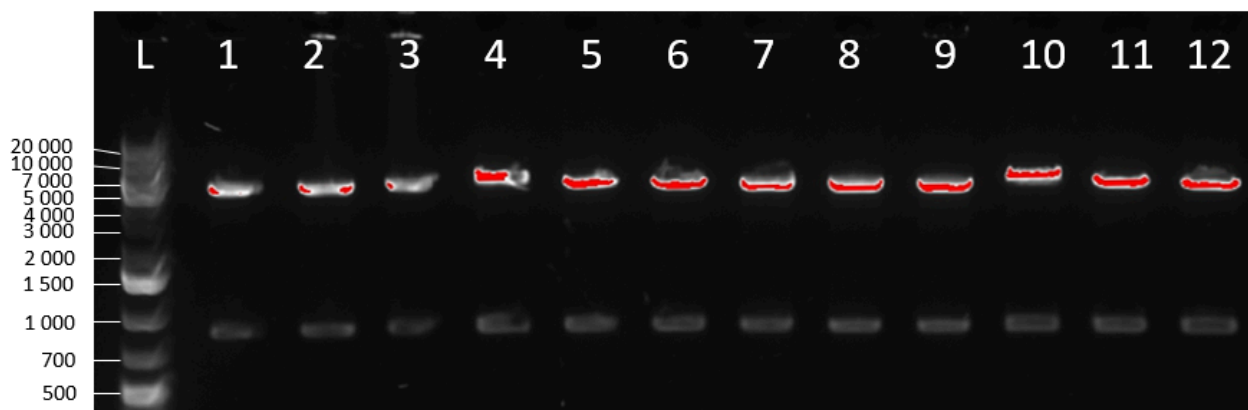

Figure 9: _AGE of double digestion product for confirmation of Dorin M / OMFREP pBAD DH5α clones. L - 1 kb ladder; Samples from double digestion of Dorin M pBAD DH5α labeled 1 - 6; Samples from double digestion of OMFREP pBAD DH5α labeled 7 - 12;_

The results of the colony PCR performed were not conclusive, therefore, plasmid isolation and double digestion were performed. 6 clones were chosen each for Dorin M and OMFREP, and all 12 samples showed the expected band at around 840 bp, as seen in Figure 9.

Two samples each (Dorin M 2 & 3 and OMFREP 8 & 9) were selected for gel elution and sequencing confirmed that the genes of interest were still present in all.

4.2.3 Production and Purification

Initially, production optimization was attempted using the pBAD BL21 system by varying temperatures (Table 9).

Table 9: *Influence of Temperature Variation on Cell Growth of Dorin M / OMFREP pBAD DH5α clones*

	OD600 at 17 °C	OD600 at 37 °C
Dorin M before induction	0.59	0.59
Dorin M after induction	0.58	0.72
OMFREP before induction	0.57	0.57
OMFREP after induction	0.57	1.22
Positive control before induction	ND	0.60
Positive control after induction	ND	2.20

SDS-Page was performed with the samples grown at 37 °C, as seen in Figure 10.
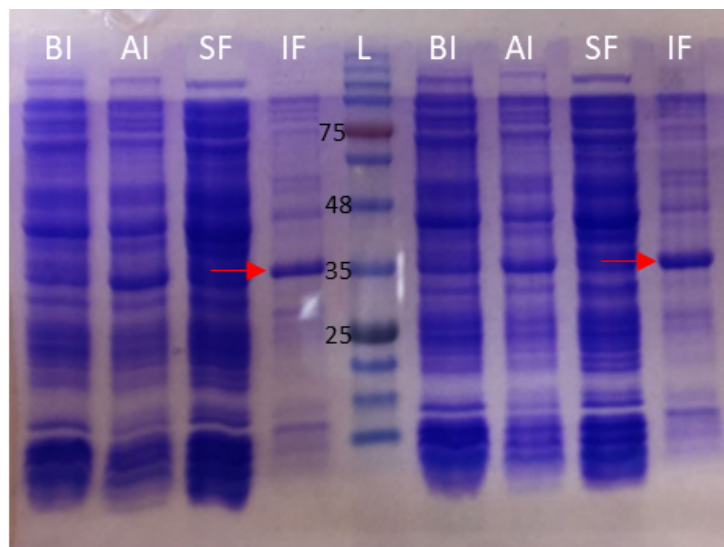

Figure 10: *SDS-Page of Dorin M / OMFREP in pBAD - BL21 system. L - 10-245 kDa ladder; left side of ladder - Dorin M samples; right side of ladder - OMFREP samples; BI - before induction; AI - after induction; SF - soluble fraction; IF - insoluble fraction;*

The insoluble fractions of both Dorin M and OMFREP showed a prominent band near the expected value of around 37 kDa (Kovář *et al.* 2000).

However, after purification of the insoluble fraction using Ni-NTA column chromatography, no band could be observed in the expected region.

Using the pBAD Shuffle expression system, purification of both Dorin M and OMFREP using the Ni-NTA column was successful (Figures 11 & 12).
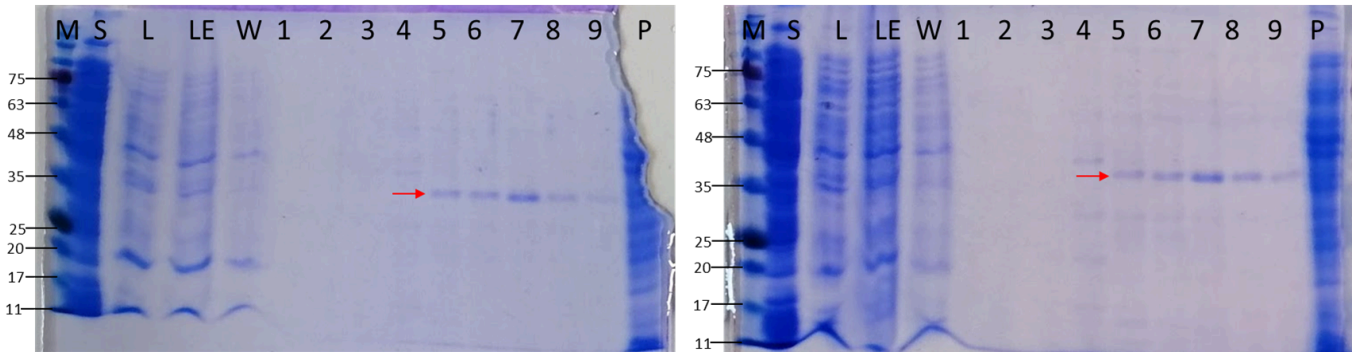

Figure 11: *SDS-Page of Dorin M / OMFREP purification in pBAD - Shuffle system. left gel - Dorin M; right gel - OMFREP; M - 10-245 kDa ladder; S - sonicate; L - sample loaded into Ni-NTA column; LE - flowthrough of L; W - wash; P - positive control; samples 1 to 9 are elutions: 1 - 6 M Urea, 2 - 5 M Urea, 3 & 4 - 25 mM Imidazole, 5 & 6 - 100 mM Imidazole, 7 & 8 - 500 mM Imidazole, 9 - 1 M Imidazole;*

The successful purification of Dorin M and OMFREP was confirmed via western blot using anti-his antibodies (Figure 12).

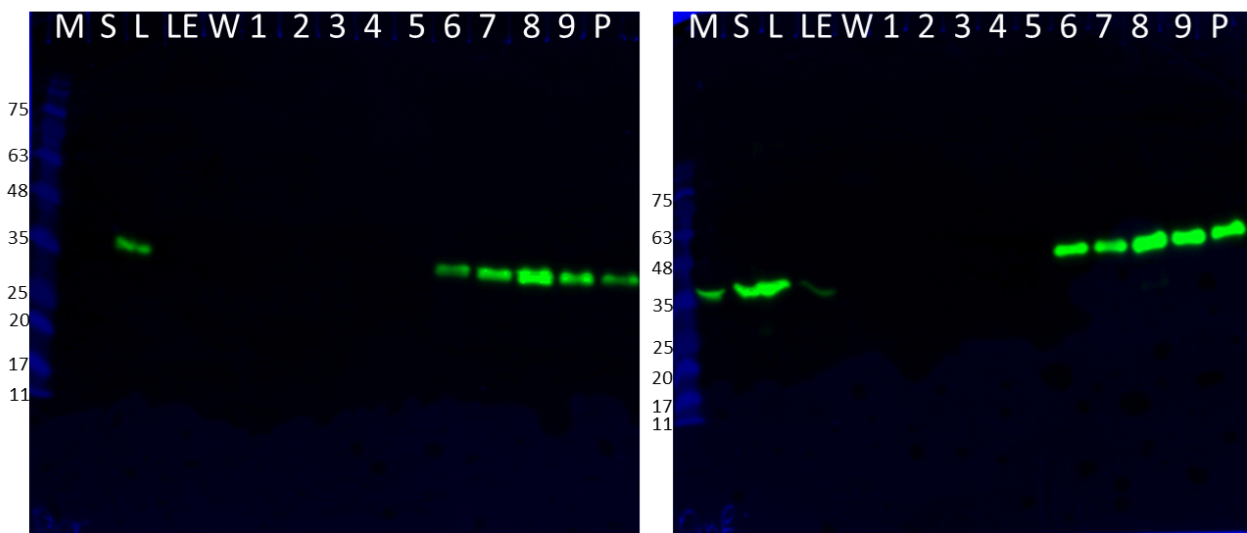***Figure 12:*** *Western blot of Dorin M / OMFREP in pBAD - Shuffle system. Left membrane - Dorin M; Right membrane - OMFREP; M - 10-245 kDa ladder; S - sonicate; L - sample loaded into Ni-NTA column; LE - flowthrough of L; W - wash; P - positive control; samples 1 to 9 are elutions: 1 - 6 M Urea, 2 - 5 M Urea, 3 & 4 - 25 mM Imidazole, 5 & 6 - 100 mM Imidazole, 7 & 8 - 500 mM Imidazole, 9 - 1 M Imidazole;*

5 Discussion

In previous studies, FREPs have been shown to play diverse roles in the immune system of arthropods. Some of the best-characterized FREPs in arthropods include a group of FREPs from the horseshoe crab *Tachypleus tridentatus* called tachylectins which have been established to function as nonself-recognizing molecules with the ability to agglutinate Gram-positive and Gram-negative bacteria as well as all types of human erythroid cells (Gokudan *et al.* 1999). Two FREPs from the razor clam *Sinonovacula constricta*, named ScFREPs, also exhibit agglutination of Gram-positive and Gram-negative bacteria, however, their activity is Ca^{2+} dependent. Additionally, the expression levels of ScFREPs are greatly upregulated in the hemolymph and liver after microbial infection (Wu *et al.* 2021).

FREPs from *Anopheles* mosquitoes also interact with Gram-positive and Gram-negative bacteria, and gene expression is up-regulated after infection. Gene silencing of different anopheline FREP genes showed not only a significant decrease in survival rates after microbial infection but also a significant increase in overall Plasmodium concentration after gene silencing. Gene silencing of FREPs whose expression increases in response to microbial challenge showed a significant rise in mortality rate and bacterial concentration even in the absence of immune challenge. Therefore, anopheline FREPs also play an important role in immune homeostasis and defense against opportunistic microbes, which is crucial for survival due to the hematophagous lifestyle of mosquitoes (Dong and Dimopoulos 2009).

In the hard tick *Ixodes ricinus*, two FREPs called Ixoderin A and Ixoderin B were identified, and their full cDNA sequence was determined. Both Ixoderins exhibit a strong homology with other C-terminal FReD proteins. Ixoderin A has an ORF of 831 bp, and the conservation of 4 cysteine residues is observed at the same position in the FBG domain of Dorin M and OMFREP, suggesting a similar secondary structure and carbohydrate-binding activity. Ixoderin B has an ORF of 858 bp and a substitution of one of the cysteine residues with proline was shown (Rego *et al.* 2005).

Screening of the *Ixodes scapularis* genome database revealed 27 genes encoding proteins including a single FBG domain, which can be categorized into three groups. The first group displays great similarity to Ixoderin A from *I. ricinus*, and was therefore called Ixo-a group. Ixo-a FREPs show the highest similarity to Dorin M, OMFREP, and Tachylectin 5, and are expressed in the hemocytes and malpighian tubules, with expression being upregulated after feeding. The

second group, Ixo-b, shows high similarities with anopheline FREPs, and is only expressed in the salivary glands, while the third group, Ixo-c is expressed throughout the entire body, with increased transcription in the trachea and gut (Honig Mondekova *et al.* 2017).

Several putative FREPs were found in the hard ticks *Dermacentor marginatus, Rhipicephalus appendiculatus, R. pulchellus, and R. sanguineus*. Four proteins with molecular weights of 36 kDa, 79 kDa, 80 kDa, and 290 kDa were detected in the hemolymph of *D. marginatus*, which all showed reactivity with not only antibodies directed against hemagglutination activity but also against anti-human ficolin 1 antibodies. They were demonstrated to be expressed in the midgut, hemocytes, and salivary glands. In all three Rhipicephalus species, three possible FREPs were recognized by antibodies directed against hemagglutination activity (58 kDa, 75 kDa, and ~290 kDa), while only the 75 kDa and 290 kDa proteins showed reactivity with anti-human ficolin 1 antibodies (Sterba *et al.* 2011).

The focus of this research lies on two FREPs of the soft tick *O. moubata* named Dorin M and OMFREP, which are expressed in the tick's salivary glands and play an important role in the immune system of ticks. This study aimed to contribute to building knowledge that will help the development of anti-tick vaccines, by creating an optimized production protocol for both proteins. To be a viable option for vaccines, they have to be able to be successfully produced in large quantities, which was achieved during this work.

The results of this study directly point to the antimicrobial properties in Dorin M and OMFREP, since the transformation was unsuccessful using the pET vector but worked with the pBAD vector, where expression is more tightly controlled due to the promoter systems used. Especially for OMFREP, where no gene insertion was observed at all, it indicates high levels of antimicrobial activity of the protein, since it could possibly cause the death of the cloning cells at the very low basal expression level exhibited by the pET vector system.

The FREPs Dorin M and OMFREP, which were successfully expressed and purified in the course of this work, were subsequently used for immunizing rabbits, and the successful generation of antibodies raised against both the proteins in the blood of the immunized rabbits was confirmed. Additionally, cross-reactivity was observed between both antibodies and both FREPs (Kumar *et al.* 2021).

To investigate the viability of Dorin M and OMFREP as a possible active component of an anti-tick vaccine, Kumar *et al.* (2021) observed the death and molting percentage of second stage

nymphal *O. moubata*, fed on the Dorin M / OMFREP immunized rabbits, as well as a control animal. The ticks that fed on the rabbits with Dorin M antibodies showed no deviation from the control group. For ticks fed on the rabbit with OMFREP antibodies, however, death in the first week after the blood meal had increased by 10%, and molting had gone down by 24% (Kumar *et al.* 2021). These results indicate that antibodies against OMFREP were able to elicit at least some degree of ATR in rabbits injected with the purified proteins, however, Dorin M did not.

Similar results of cross-reactivity were observed by Sterba *et al.* (2011), who used antibodies raised against Dorin M, which were isolated directly from the tick hemolymph, to detect the presence of FREPs in the hemolymph of the hard ticks *D. marginatus* and *R. appendiculatus*. They were able to find 4 proteins from *D. marginatus* which reacted with the Dorin M antibodies, and 3 proteins from the hemolymph of *R. appendiculatus*. None of these proteins could be definitively identified as FREPs, however, the biggest protein identified in both hard ticks was determined to be hemelipoglycoprotein (HLGP). Even though HLGP is a carbohydrate binding protein, it does not have a FBG domain. Nevertheless, Dupejová (2010), who also observed this cross-reaction, hypothesized that it is due to a similarity in the tertiary structure of Dorin M and HLGP (Dupejová 2010; Sterba *et al.* 2011).

A high degree of cross-reactivity between different tick species, a high degree of antimicrobial activity, and the ability to elicit ATR are among the most important factors for choosing an active compound for anti-tick vaccines (Johnson 2023 p. 59, pp. 206–208; Rego *et al.* 2019). Dorin M and OMFREP both seem like promising candidates.

While previous research has either focused on the purification of tick FREPs directly from the hemolymph (Kovář *et al.* 2000; Man *et al.* 2008; Rego *et al.* 2006) or has taken a bioinformatics approach (Rego *et al.* 2005), this research was able to perform the first successful expression and subsequent purification of the Dorin M and OMFREP genes using an *E. coli* expression system. By optimizing the procedure, large-scale experiments can be carried out in the future using Dorin M and OMFREP.

Avenues for future research include a large-scale experiment determining the extent of ATR which can be induced by Dorin M and OMFREP antibodies raised in different host animal models. Additionally, the effect of Dorin M and OMFREP antibodies on different tick species other than *O. moubata* should be analyzed.

6 Conclusion

With tick-borne diseases on the rise, the search for a viable solution against TBPs continues. In recent years, the focus has shifted from studying the numerous diseases transmitted by ticks to proteins found in tick saliva, which can elicit immune responses from the host, to prevent tick attachment in the first place.

This research was able to successfully express and purify Dorin M and OMFREP, two FREPs expressed in various organs including salivary glands of *O. moubata,* and possibly plays an important role in the tick immune system. By producing and purifying these two tick lectins, this research has laid the groundwork for their potential use as a transmission-blocking vaccine. Of course, their effects and side effects on mammals and ticks still need to be extensively examined before an anti-tick vaccine can be developed.

7 Literature

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