

INSECTICIDAL MECHANISMS MEDIATED BY CRY4BA AND CRY11AA
BINDING PROTEINS OF ASIAN TIGER MOSQUITO

by

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Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Graduate Program in Molecular Biology and Genetics
Boğaziçi University

2024



ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my thesis advisor, Assoc. Prof. Necla Birgül, her expertise, patience, and unwavering encouragement have been instrumental throughout this research journey. From the initial conceptualization of the topic to the final stages of writing, their guidance has been a source of inspiration and clarity. Their ability to ask the right questions and provide thoughtful feedback has helped me refine my ideas and approach this study with a critical and innovative mindset.

I am profoundly thankful to my thesis committee members, Prof. Nevin Gül Karagüler, Assist. Prof. Şükrü Anıl Doğan, Prof. Nesrin Özören and Assoc. Prof. Ash Kumbasar for their time, dedication, and invaluable insights. Their constructive critiques and detailed evaluations have pushed me to critically examine my work and strive for excellence. The suggestions provided during the defense have not only enhanced the quality of this thesis but have also broadened my academic perspective. Their expertise in the field has been both a benchmark and a source of motivation.

One of the most significant aspects of this dissertation was the collection of mosquitoes from the natural environment. I am deeply grateful to my colleague, Zeynep Feraye, from the Faculty of Veterinary Medicine, for her invaluable assistance, both in helping me collect larvae and for guiding me to locations where mosquitoes were found in abundance. Her support was instrumental in the successful execution of this critical phase of the study, and I extend my heartfelt thanks for her contributions.

My sincere appreciation also extends to the CSL members and faculty members. I would like to extend my heartfelt gratitude to Davod Khalafkhany, Yusuf Tunahan Abacı, Elif Darbuka, Elif Çelik, Tolga Aslan, Aida Shahraki, Muhammed Fatih Karakaya, Özen Kaya, İlke Süder, Özgecan Arslan, Kübra Kahveci, and Alize Yılmazlar for their invaluable contributions to both the scientific and nonscientific as-

pects of my journey. Their insightful discussions, sense of humor, and vibrant presence have brought joy and creativity to the laboratory, making it an engaging and lively environment. I deeply appreciate their willingness to share their experiences, which have greatly enhanced my knowledge in various areas, and for providing unwavering support throughout this process.

Additionally, I would like to express my sincere thanks to our faculty secretary, Ümit Bayraktar, for her kindness, helpfulness, and cheerful demeanor. Her assistance and warm attitude have made a significant difference, and I am truly grateful for her support.

My deepest gratitude goes to my family for their unwavering love, support, and encouragement throughout my life and academic journey. I wish to extend my heartfelt thanks to my siblings, Duçi and Gülsen, whose presence and support have been a constant source of strength and joy. I am profoundly grateful to my parents, Muhittin Gün and Gülcan Gün, whose sacrifices, guidance, and unconditional love have shaped me into the person I am today. They have not only nurtured and raised me but have also instilled in me the values of perseverance, curiosity, and kindness that have been the foundation of this achievement. Most importantly, I owe an immeasurable debt of gratitude to my wife, Duygu Gün, for her endless patience, understanding, and encouragement. Her belief in my abilities and her steadfast support have been my anchor during the most challenging times of this journey. She has been my confidante, my motivator, and my greatest source of inspiration, making this accomplishment as much hers as it is mine.

This study has been supported by funding from TÜBİTAK (The Scientific and Technological Research Council of Turkey) – 123Z928 and BAP (Boğaziçi University Research Projects) 16464.

ABSTRACT

INSECTICIDAL MECHANISMS MEDIATED BY CRY4BA AND CRY11AA BINDING PROTEINS OF ASIAN TIGER MOSQUITO

The Asian tiger mosquito (*Aedes albopictus*) is a mosquito species native to Southeast Asia that has now spread across Türkiye. Given its vector potential and remarkable ability to colonize various environmental conditions, *Ae. albopictus* is considered one of the most significant invasive mosquito species globally. Under experimental conditions, this mosquito species has demonstrated the potential to transmit four dengue serotypes, yellow fever, chikungunya, Ross River virus, and at least 22 other arboviruses. Consequently, preventing the spread of invasive mosquitoes necessitates the use of environmentally friendly pesticides that are species-specific and have minimal impact on non-target organisms. In this study, we aimed to investigate the larvicidal effects of Cry4Ba and Cry11Aa toxins, which are among the most effective biopesticides for targeting *Aedes* species. Furthermore, we sought to identify the receptors for Cry4Ba and Cry11Aa toxins to elucidate their mechanisms of action in *Ae. albopictus*. Despite the critical role of toxin-receptor interactions in Cry toxin efficacy, the receptors that interact with Cry toxins in *Ae. albopictus* have not yet been characterized. Our findings revealed that two alkaline phosphatases, a vacuolar-type ATPase subunit B, and a maltase-like receptor serve as functional receptors for Cry4Ba and Cry11Aa in *Ae. albopictus* larvae. Additionally, we demonstrated that the susceptibility of mosquito larvae to Cry toxins is influenced by the downregulation of these receptor gene expressions using RNA interference. These results provide new insights into the mechanisms of Cry toxin action and may contribute to developing more effective mosquito management strategies.

ÖZET

CRY4BA VE CRY11AA İLE ETKİLEŞEN ASYA KAPLAN SIVRİSİNEĞİ PROTEİNLERİNİN İNSEKTİSİT MEKANİZMASI

Asya kaplan sivrisineği (*Ae. albopictus*) Güneydoğu Asya kökenli bir sivrisinek türü olup Türkiye geneline yayılım göstermektedir. Çeşitli çevre koşullarında vektör potansiyeli olan ve geniş kolonizasyon kabiliyeti göz önüne alındığında, *Ae. albopictus* en önemli istilacı sivrisinek türlerinden biri olarak görülmektedir. Deneysel koşullar altında, 22 arbovirüs dahil olmak üzere dört tip dang humması serotipi, sarıhumma, chikungunya ve Ross River virüsü için bir vektör olma potansiyeli taşımaktadır. Bu nedenle istilacı sivrisineklerin yayılmasının önlenmesi; çevre dostu, türe özgü, hedef dışı organizmalar üzerinde düşük etkiye sahip pestisitlerin kullanılmasına bağlıdır. Bu nedenle bu çalışmada Aedes türlerinde en etkili biyopestisit olan Cry4Ba ve Cry11Aa toksinlerinin larvisit etkilerinin araştırılması ve bu toksinlerin *Ae. albopictus* üzerindeki etki mekanizmalarının aydınlatılması için bu toksinlerle etkileşen reseptörlerin belirlenmesi amaçlandı. *Ae. albopictus*'ta Cry toksinleriyle etkileşime giren reseptörler henüz tanımlanmamıştır. Bulgularımız, iki alkalın fosfatazın, bir vakuolar tipte ATPaz alt birimi B'nin ve maltaz benzeri reseptörün Asya kaplanı sivrisinek larvalarında Cry4Ba ve Cry11Aa için fonksiyonel reseptörler olduğunu göstermiştir. Ayrıca, RNA interferaz yöntemiyle bu reseptör gen ekspresyonlarının azaltılmasının, sivrisinek larvalarının Cry toksinlerine karşı duyarlılığını değiştirdiği gösterilmiştir. Bu sonuçlar, Cry toksinlerinin etki mekanizmalarına yeni bir bakış açısı sunmakta ve daha etkili sivrisinek kontrol stratejilerinin geliştirilmesine katkı sağlayabilir.

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LIST OF SYMBOLS

g	Gram
l	Liter
M	Molar
m	Meter
n	Nano
p	Pico
V	Volt
U	Unit
α	Alpha
β	Beta
μ	Micro
$\textcircled{R}$	Registered trademark

LIST OF ACRONYMS/ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
Ae	Aedes
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
C α	Carbon Alpha
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CO ₂	Carbon Dioxide
Cry	Crystal toxin
ddH ₂ O	Double Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
FBS	Fetal Bovine Serum
GDP	Guanosine Diphosphate
HCl	Hydrochloric Acid
hr	Hour
kb	Kilobase
LB	Luria-Bertani Broth
MgCl ₂	Magnesium Chloride
MD	Molecular Dynamics
min	Minutes
mRNA	Messenger Ribonucleic Acid

NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
SD	Standard Deviation
SDM	Site-Directed Mutagenesis
sec	Seconds
TAE	Tris-Acetic Acid EDTA
Taq	<i>Thermus aquaticus</i>
UV	Ultraviolet
WGS	Whole Genome Sequencing
WT	Wild Type
v/v	Volume to Volume
w/v	Weight to Volume

1. INTRODUCTION

1.1. Alien Species

Alien species are a major problem when they become invasive. Invasive species can damage biodiversity in different ways: Due to the predominance of invasive species, they replace local species, hybridize with local populations, disrupt the functioning of the local ecosystem by altering ecological factors, and cause parasites and diseases that are not present in local species. Invasive species often have adverse effects on humans in various ways. These can sometimes be allergen effects, and sometimes, they can cause diseases as vectors that carry pathogens to humans and animals. In this context, mosquitoes, in particular, have a significant impact worldwide. Each year, more than one million people die, and more than hundreds of millions of diseases are transmitted [1].

1.2. Mosquito

Invasive mosquito species can colonise new regions and have significant negative impacts on the environment, economy, and public and animal health. Among these, the genus *Aedes* is particularly noteworthy in Europe, with five critical invasive species identified: *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus*, *Ae. atropalpus*, and *Ae. koreicus* [2]. The reasons for the spread of these species are the human population's excessive mobility, commercial goods transport, and the climatic change in Europe [3]. Considering the vector potential and broad colonization ability in various environmental conditions, *Ae. albopictus* and *Ae. aegypti* are the most significant invasive mosquito species in Europe. Under experimental conditions, *Ae. Albopictus* can be a vector for four dengue serotypes: yellow fever, chikungunya and Ross River virus, and 22 arboviruses. *Ae. albopictus* is also a potential vector for *Dirofilaria immitis*, a nematode important in the veterinary field [4].

1.3. *Aedes Albopictus*

Ae. albopictus, originally from Southeast Asia, is naturally zoophilic but has adapted an anthropophilic behavior due to its expansion into new regions. It exhibits hyperaggressive hematophagous activity throughout the day, facilitated by its ability to use artificial environments for breeding [5]. In new areas of spread, it has been observed that people produce excessive allergic reactions after hematophagy. It spread in the Indian and Pacific oceans in the 19th century [6], first reported in Albania [7] in 1979 and later in Texas (1985) [8] and Brazil (1986) [9]. It became an established species throughout the USA, Australia, Europe, and the Pacific islands [10] in the following years. It was first detected in the Thrace region in 2011 in Turkey [11]. As of 2015, populations have been reported in the Eastern Black Sea region (Artvin, Trabzon and Rize) [12] along the Black Sea coast to the Marmara region. Therefore, despite the danger of spreading this species throughout the country, protective measures should be taken, and studies should be conducted to eradicate the species.

1.4. Controlling Mosquitos

Without antiviral therapy or a vaccine, the most effective method of controlling mosquito-borne diseases is the control of vectors. Control of adult mosquitoes generally depends on the use of insecticides, but resistance to insecticides increases over time [13]. In this regard, *Ae. albopictus* in some regions DDT [14], malathion and bendiocarb [15], and pyrethroids [16], such as permethrin and deltamethrin, [15] have been reported to resist or develop resistance to typical insecticides. Nowadays, chemical pesticides dominate mosquito control but contribute to environmental pollution, health risks, and rapid resistance development [17]. Consequently, biological pesticides, particularly microbiological ones like *Bacillus thuringiensis* (Bt), are gaining traction for their specificity, environmental safety, and reduced resistance development [18].

1.5. *Bacillus Thuringiensis*

Bacillus thuringiensis (*Bt*) is a microbiological pesticide widely used worldwide to maintain its viability under long-term storage conditions [19]. It is non-toxic to mammals, non-phytotoxic, and safe for non-target organisms [20]. *Bt* strains can produce toxins such as crystal (Cry) proteins and beta-exotoxin. Examples of these bacillus species are *darmstadiensis*, *gallariae*, *tenebrionis* and *thuringiensis* [21]. Cry genes carried by plasmids encode cry proteins. Approximately 300 *Cry* genes have been identified and divided into 51 groups according to their amino acid sequence similarity [22]. Insecticide-related Cry proteins are divided into four main classes: (i) Lepidoptera specific, (ii) Lepidoptera and Diptera specific, (iii) Diptera specific and (iv) Coleopteran specific. *Bt* subspecies *B. thuringiensis* subsp. *israelensis* (*Bti*) are preferred for the class Lepidoptera, Coleoptera, and Diptera, especially *Aedes*, *Culex*, and *Anopheles*, and they are used to control mosquito species [23]. *Bti* has a pBtoxis megaplasmid encoding Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, Cyt1Ca and Cyt2Ba toxins [24]. *Bti* exerts its effect in combination with sporulation by forming crystal inclusion bodies with at least three Cry proteins (Cry11Aa, Cry4Aa and Cry4Ba) and a Cyt toxin (Cyt1Aa) [25]. There has not been reported resistance to *Bti* toxins in *Ae. albopictus* and it has been reported that the same genus may have different types of resistance. For example, in the studies on *Ae. sticticus* found that 3.5 and 8-fold tolerances have been gained for the Cry4Aa and Cry11Aa toxins, respectively [26]. The separately administered Cry11Aa and Cry4Ba toxins are effective larvicide on *Ae. albopictus* larvae [27].

1.6. Crystal Proteins

Two main modes of action of cry toxins have been proposed. The most accepted model is the activation of Cry toxins with proteases in the digestive tract of the host species and the binding of active toxins to intestinal epithelial cell surface receptors. Binding to the receptor causes a conformational exchange of toxins, resulting in oligomerization from the monomer state, and oligomers form pores on the cell

membrane, causing cell disruption and death of the host organism [28]. Another model is that a signal transduction pathway after the binding of toxins to the receptor may cause an insecticidal effect [30]. Cell death caused by Cry6Aa in *Caenorhabditis elegans* cells is proposed to be due to aspartic protease-1 [31]. In the Cry1Ac-resistant *Plutella xylostella* strain, constitutively active the MAPK signaling pathway protects cell lysis and death by regulating the expression levels of various intestinal receptor genes [32]. This shows that the mechanisms of action of Cry toxins are pretty complex. Nine X-ray crystallography studies of Cry toxins have revealed three protected domains with specific functions. Domain I, with its 7-8 alpha helix, plays a role in pore formation; domain II, beta-prism structure, can bind receptors through its loop regions; and domain III, with beta-sandwich structure, receptor binding, ion channel formation, and insect specificity [34], [35].

1.7. Cry Toxin Resistance

As reported in organophosphate insecticides, toxin and receptor proteins may also be involved in insecticide resistance of insects [17]. Due to similarities in binding sites, the galectin protein binds to the receptor targeted by the Cry5Ba toxin, thereby conferring resistance to this toxin in *Caenorhabditis elegans* [36]. In the *Ae. aegypti* species, galectin-6 protein leads to gain resistance by binding to the Cry receptor (ALP1) binding site of Cry11Aa toxin [37]. Cry toxicity in *Ae. aegypti* was reduced by the interaction of C-type lectin-20 with ALP1 [38]. Mutations in the ABC transporter in *Heliothis virescens* also lead to resistance to the toxin [39].

Recently, ATP binding proteins (ABP) have been reported to play a role in the insects' response to *Bt* toxin. ABP hydrolyzes ATP to provide the energy required for transmembrane transport. A single amino acid mutation in the ABP of *Bombyx mori* caused resistance to *Bt* toxin (Cry1Ab) [40]. Similarly, in *Plutella xylostella*, silencing ABP G via RNA interference (RNAi) decreased sensitivity to Cry toxins [41]. Moreover, ABPs have been found to interact with Cry11Aa in *Culex quinquefasciatus*, enhancing Cry toxicity [42]. These findings suggest that ABPs may influence the

mechanism of action of Cry toxins. However, the full range of proteins involved in Cry protein activation and their interactions with receptors across different insect species remains to be fully elucidated.

1.8. Cry11Aa and Cry4Ba

Competitive binding experiments with Cry11Aa, Cry4Aa, and Cry4Ba in *Ae. aegypti* have revealed distinct patterns of receptor interactions. Cry11Aa and Cry4Aa exhibit competitive binding to similar cadherin-binding regions, whereas Cry4Ba does not share this binding capacity with Cry11Aa, and consequently, it does not induce toxicity via this pathway [43]. This finding suggests a divergence in the mode of action of these toxins, emphasizing the complexity of receptor specificity in mosquito midgut epithelial cells. In *Anopheles albimanus*, studies have identified that the α -amylase protein interacts with Cry4Ba and Cry11Aa, demonstrating toxic effects [44]. Such findings underscore the significant role of midgut proteins in mediating the insecticidal action of *Bacillus thuringiensis israelensis* (*Bti*) toxins. However, specific genetic mutations in receptor proteins have been associated with varying resistance levels to Cry toxins. This resistance, combined with interspecies variations, raises concerns about the potential for widespread resistance development to Bti, particularly given its increasing use as a biopesticide.

Considering invasive mosquito species' ecological and public health significance, it is critical to identify and characterize the host proteins that interact with Cry toxins. This is especially pertinent for *Ae. albopictus*, where the proteins mediating interactions with Cry toxins, such as Cry4Ba and Cry11Aa, remain largely unidentified. Previous studies have shown that Cry4Ba and Cry11Aa exhibit high toxicity against *Aedes* and *Anopheles* species [45], [46], but the specific receptor-ligand interactions in *Ae. albopictus* is yet to be elucidated.

Given the invasive nature of *Ae. albopictus* and its ability to transmit arboviruses such as dengue and chikungunya, understanding the mechanisms of action of Cry tox-

ins is paramount. Identifying interacting receptors could elucidate the mechanisms underlying Cry toxin efficacy and provide critical insights into resistance mechanisms. Recent literature highlights the role of midgut receptors such as cadherins, alkaline phosphatases (ALPs), and aminopeptidases (APNs) as critical mediators of Cry toxin toxicity in related species [47], [48]. Moreover, advanced techniques such as molecular docking and X-ray crystallography have begun to uncover the structural basis of these interactions, identifying key binding domains and their functional implications [49], [50].

This study aims to investigate the larvicidal effects of Cry4Ba and Cry11Aa toxins on *Ae. albopictus* and to identify the specific midgut proteins mediating these interactions. By elucidating receptor-ligand dynamics, this research seeks to contribute to the development of more effective and sustainable mosquito management strategies, mainly targeting invasive species like *Ae. albopictus* in Türkiye. Furthermore, the findings may inform broader efforts to mitigate resistance development and enhance the long-term efficacy of Bti-based biopesticides.

1.9. Cry4Ba Interacted Receptors of *Ae. Aegypti*

Cry4Ba toxin-binding proteins have been identified in the brush border membranes (BBMV) of *Ae. aegypti* midguts [51]. Given the genetic similarity between *Ae. albopictus* and *Ae. aegypti*, receptors are known to bind Cry4Ba in *Ae. aegypti* were selected for investigation in *Ae. albopictus*. These include: aminopeptidase N (XM_029867648, APN, 80.36% peptide sequence identity), ALP-1 like (XM_019684987.2, ALP-like, 93.36% identity), membrane-bound ALP (XM_029879948.1, memALP, 91.81% identity), V-type proton ATPase subunit B (XM_019706260.2, V-ATP 99.80% identity), prohibitin (XM_019677754.2, prohibitin 99.26% identity), maltase 1 like (XM_029859282.1, maltase, 99.25% identity), Flotilin (XM_029870385.1, Flotilin, 99.53% identity) peptid, lachesin (XM_020076957.2, Lachesin, 97.21% identity), calcium-transporting ATPase (XM_029878831.1, Calc, 98.21). While all these receptors are hypothesized to interact with Cry4Ba, the toxicity of these interactions remains to be

fully verified *in vitro*. Some of these receptors have also been reported to interact with Cry11Aa, leading to toxicity. In insects, mALP is found in larval midgut epithelium cells in the BBMV of columnar cells and specifically restricted between the middle and posterior midgut. It is thought to act as a Cry binding receptor in lepidopteran and dipteran larvae [52]. MemALP interacts with Cry11Aa in *Aedes aegypti* [53]. Lachesin has a role in early neuronal differentiation and axon outgrowth and acts as a homophilic cell-adhesion molecule. It is highly expressed in the trachea, hindgut, and foregut glands [54]. This suggests the possibility of lachesin interacting with Cry toxins following *Ae. albopictus* larvae ingestion. V-ATPase is a proton pump that has a role in protein degradation and synthesis, cell growth, and cell autophagy. B subunit of V-ATPase among eight subunits is required for assembly and activity [55]. Additionally, V-ATPase binds directly to actin filaments [56]. Cry1Ac toxin mediates larval toxicity in cotton bollworms by interacting with the V-ATPase B subunit [57]. V-ATP-synthase expression has been altered in Cry1Ac-resistant *Plodia interpunctella* and Cry4Ba- and Cry11Aa-resistant *Ae. aegypti* LiTOX strains. Silencing of V-ATPase B and actin expression via RNA interference (RNAi) leads to hypersensitivity in *Ae. aegypti* larvae to Cry toxins [58]. Prohibitin is a highly conserved and ubiquitously expressed protein across eukaryotic organisms, including various insect orders. In cell lines of *Ae. aegypti* and *Ae. albopictus*, knockdown of prohibitin expression has been shown to reduce viral infection levels [59].

2. PURPOSE

Mosquitoes are vectors of numerous viral diseases, responsible for millions of deaths annually. Using chemical insecticides has long been a cornerstone in efforts to control mosquito populations and reduce the transmission of tropical diseases. However, growing resistance to these chemicals has necessitated alternative approaches. Among these, *Bacillus thuringiensis* subsp. *israelensis* (Bti) has emerged as one of the most promising bio-insecticides due to its specificity, environmental safety, and efficacy against mosquito larvae. Although several proteins in various insect species have been identified as putative binding receptors for Cry toxins derived from Bti, the precise mode of action for individual toxins still needs to be completed. This knowledge gap hinders the full exploitation of Cry toxins for sustainable mosquito control.

The present study aims to investigate the larvicidal effects of Cry4Ba and Cry11Aa toxins using both in vitro and in vivo approaches and to identify their specific binding receptors in *Aedes albopictus*. Understanding the interaction between Cry toxins and their receptors is critical for elucidating the mechanisms underlying toxicity and resistance development. By identifying these receptors, this research explores their functional roles in Cry toxicity and their potential contribution to resistance. Furthermore, this study examines the physiological and molecular responses of *Ae. albopictus* larvae to Cry4Ba and Cry11Aa exposure through gene expression analysis and RNA interference. By silencing candidate receptor genes, we aim to determine their role in larval susceptibility to Cry toxins. The insights gained from this research are expected to deepen our understanding of the larvicidal actions of Cry4Ba and Cry11Aa toxins and their resistance mechanisms. Additionally, the outcomes of this study could inform strategies for improving mosquito management practices, mainly through the targeted use of Bti-based bio-insecticides. This research holds the potential to contribute significantly to the development of innovative and sustainable mosquito control strategies.

3. MATERIALS

3.1. Kits, Enzymes and Reagents

Kits, enzymes, and reagents used in different experiments are listed in Table 3.1.

Table 3.1. List of kits, enzymes, and reagents.

Name	Supplier
CellMask	Thermo Fisher Scientific, USA
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, UK
DNA ladder 1kb	GeneOn, Germany
Effectene Transfection Reagent Kit	Qiagen, Germany
Fetal Bovine Serum (FBS)	Gibco, UK
ImProm-II Reverse Transcription System	Promega, USA
MinElute Gel Extraction Kit	Qiagen, Germany
DNA plasmid purification	MACHERY – NAGEL, USA
Penicillin/Streptomycin (10X)	Hyclone, USA
Proteinase K	Thermo Fisher Scientific, USA
Q5 DNA polymerase	NEB, USA
Restriction Endonucleases	Thermo Fisher Scientific, USA
NucleoSpin RNA kit for RNA purification	MACHERY – NAGEL, USA
RNase A Enzyme	Thermo Fisher Scientific, USA
RNase-ZAP	Sigma-Aldrich, Germany
T4 DNA Ligase	NEB, USA
Taq DNA polymerase	Thermo Fisher Scientific, USA
Trypsin-EDTA (0.5 mM EDTA, 0.025% Trypsin)	Hyclone, USA
ZymoPURE Plasmid Midiprep kit	Zymo Research, USA

3.2. Chemicals

Chemicals utilized in different experiments are listed in Table 3.2.

Table 3.2. Chemicals used in this study.

Name	Supplier
Acetic Acid	Merck, USA
Agar	Conda, Spain
Ammonium Persulfate (APS)	Sigma-Aldrich, USA
Ampicillin	BIOCHROM, Germany
β -mercaptoethanol	Merck, USA
Bovine Serum Albumin (BSA)	AppliChem, Germany
Bromophenol Blue	Fluka, USA
Calcium chloride dehydrate	AppliChem, Germany
Cetyltrimethyl Ammonium Bromide (CTAB)	Sigma-Aldrich, USA
Chloroform	Emsure, Germany
Chloroform: Isoamyl alcohol	Emsure, Germany
D-Glucose	Sigma-Aldrich, USA
DMSO	Sigma-Aldrich, USA
EDTA	AppliChem, Germany
Ethanol	Emsure, Germany
Ethidium Bromide	Sigma-Aldrich, USA
EZMix Tryptone	Sigma-Aldrich, USA
Formamide	Emsure, Germany
Glycerol	Sigma-Aldrich, USA
HEPES	Sigma-Aldrich, USA
Isopropanol	Emsure, Germany
Kanamycin	Fluka, USA
Magnesium Chloride (MgCl_2)	Sigma-Aldrich, USA
Continued on next page	

Table 3.2. Chemicals used in this study. (cont.)

Name	Supplier
Methanol	Emsure, Germany
Paraformaldehyde	Sigma-Aldrich, USA
Phenol: Chloroform: Isoamyl alcohol	Emsure, Germany
Phosphate Saline Buffer (PBS)	Gibco, UK
Potassium Chloride (KCl)	Sigma-Aldrich, USA
Sodium Acetate (NaOAc)	Sigma-Aldrich, USA
Sodium Chloride (NaCl)	Thermo Fisher Scientific, USA
Sodium Dodecyl Sulfate (SDS)	AppliChem, Germany
Sodium Hydroxide (NaOH)	Sigma-Aldrich, USA
Tris-Base	AppliChem, Germany
Triton X-100	AppliChem, Germany
Tryptone	Sigma-Aldrich, USA
Yeast Extract	Conda, Spain

3.3. Buffers and Solutions

The buffers and solutions used in the experiments are described below.

- 2X CTAB Extraction Buffer:
 - 100 mM Tris-HCl
 - 1.4 M NaCl
 - 30 mM EDTA
 - 2% (w/v) CTAB
- 50X Tris-Acetic Acid EDTA (TAE):
 - 2 M Tris-acetate
 - 50 mM EDTA
 - pH 8.5
- TE Buffer:

- 10 mM Tris-HCl
 - 1 mM EDTA, pH 8.0
- Ethidium Bromide (EtBr):
 - Stock solution: 10 mg/ml
 - Working solution: 30 ng/ml
- 10X Tris Borate EDTA (TBE):
 - 108 g Tris base
 - 55 g Boric acid
 - 9.3 g EDTA
 - ddH₂O up to 1 L
- 6X Loading Buffer:
 - 0.1 ml 1 M Tris-HCl, pH 7.6
 - 0.3 ml 1% Bromophenol Blue
 - 6 ml 100% glycerol
 - 1.2 ml 0.5 M EDTA
 - ddH₂O up to 10 ml
- Luria-Bertani Medium (LB):
 - 10 g tryptophan
 - 5 g yeast extract
 - 10 g NaCl
 - ddH₂O up to 1 L, autoclaved
- Luria-Bertani Agar:
 - 10 g tryptophan
 - 5 g yeast extract
 - 10 g NaCl
 - 15 g Agar
 - ddH₂O up to 1 L, autoclaved
- Ampicillin Stock:
 - 100 mg/ml in 50% Ethanol
 - Filter-sterilized and stored at -20°C
 - Working concentration: 100 $\mu\text{g/ml}$

- Kanamycin Stock:
 - 50 mg/ml in ddH₂O
 - Filter-sterilized and stored at –20°C

3.4. Equipments

The types of equipment used in this study are listed in Table 3.4.

Table 3.4. Types of equipment used in this study.

Name	Supplier
Agarose Gel Electrophoresis	Mini-sub-Cell GT, BioRad, USA
Autoclave	Midas 55, Prior Clave, UK
Analogue-digital converter	Digidata 1440A, Axon Instruments, USA
Balances	DTBH 210, Sartorius, Germany
Carbon dioxide tank	Genc Karbon, Turkey
Cell culture incubator	Hepa Class 100, Thermo, USA
Centrifuges	Ultracentrifuge J2MC, Beckman Coulter, USA Mini Centrifuge 17307-05, Cole Parmer, USA Centrifuge 5415R, Eppendorf, USA Centrifuge, Allegra X-22, Beckman Coulter, USA
Cold room	Birikim Elektrik Soğutma, Turkey
Deep freezers	-20°C, Arçelik, Turkey -70°C Freezer, Harris, UK -80°C ULT Freezer, Thermo Fisher Scientific, USA -150°C, MDF-1156, Sanyo, Japan
Continued on next page	

Table 3.4. Types of equipment used in this study. (cont.)

Name	Supplier
Documentation System	Gel Doc XR System, Bio-Doc, Italy G-BOX Chemi XX6, Syngene, UK
Freezing Container	Mr. Frosty, Nalgene, Thermo Fisher Scientific, USA
Heat blocks	DRI-Block DB-2A, Techne, UK
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd, UK
Ice Machine	Scotsman Inc., AF20, Italy
Laboratory Bottles	Isolab, Germany
Laminal Flow Cabinet	Labcaire BH18, UK
Magnetic Stirrers	M221 Elektro-mag, Turkey
Micropipettes	Finnpipette, Thermo Fisher Scientific, USA
Microscopes	Axiovert 200 inverted microscope, Zeiss, Germany, with an oil immersion objective (plan-NEOFLUAR 63x/1.25)
Microwave oven	M1733N, Samsung, Malaysia
Plate Reader	Synergy Neo2 plate reader BioTek Instruments, equipped with a monochromator
pH meter	WTW pH330i, Germany
Pipettor	Pipetus-akku, Hirschmann Labogerate, Germany
Power Supply	Biorad, USA
Refrigerators	2082C, Arçelik, Turkey 4030T, Arçelik, Turkey
Shakers	VIB Orbital Shaker, InterMed, Denmark Lab-Line Universal Oscillating Shaker, USA
Continued on next page	

Table 3.4. Types of equipment used in this study. (cont.)

Name	Supplier
Spectrophotometer	NanoDrop 1000, USA
Thermocyclers	Applied Biosystems 2720 Thermal Cyclers BIORAD DNA Engine Peltier Thermal Cyclers
Vacuum pump	KNF Neuberger, USA
Vortex	Vortex mixer VM20, Chiltern Scientific, UK
Water baths	TE-10A, Techne, UK
Water purification system	UTES, Turkey

4. METHODS

4.1. Mosquitoes, Bacterial Strains and Plasmids

The *Aedes albopictus* mosquitoes used in this study were collected from various locations in Türkiye. These specimens were maintained in an environmentally controlled room set at 28°C with a relative humidity of 85%. A photoperiod of 14 hours of light and 10 hours of darkness was employed to mimic natural conditions favorable for their development. Eggs were kept in controlled humidity environments ranging from 80% to 100% at room temperature. Larvae were fed a nutrient-rich medium of yeast extract, peptone, and distilled water, ensuring optimal growth and development.

Bacillus thuringiensis israelensis (Bti) strains HD567, 4Q2-72, and 4Q2-81 (Cry-negative) were utilized for bacterial experimentation. These strains were cultivated in LuriaBertani (LB) liquid medium, which included 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, adjusted to a pH of 7.2. The incubation conditions were maintained at 30°C for *Bti* and 37°C for *Escherichia coli*, with a constant shaking speed of 250 rpm to ensure proper aeration and growth.

Recombinant DNA techniques were applied to facilitate protein expression and functional analysis. *Escherichia coli* DH5-alpha was used for plasmid propagation and cloning, whereas *E. coli* BL21 was selected for protein expression due to its compatibility with toxin production systems. pGEM-T easy plasmid was used for DNA cloning and sequencing. Expression of Cry11Aa and Cry4Ba toxins was conducted using the pET32a and pET30a vectors, which are well-suited for high-yield expression with minimal post-translational modifications.

To assess receptor interactions, receptor genes from *Ae. albopictus* were cloned into the pEGFP-N1 mammalian expression vector, enabling transfection into HEK-293 cells. This system facilitates the visualization and functional study of receptor-toxin

binding, providing insight into the interaction mechanisms. The ability to observe real-time receptor dynamics enhances the reliability of interaction studies. Toxin application experiments were carried out in controlled environments using Falcon tubes to minimize variability and ensure consistent exposure of larvae to toxins. This method ensures precise dosing and exposure conditions, critical for evaluating the larvicidal efficacy of Cry toxins.

4.2. Colony Rearing

Mosquito colonies were maintained in insectaries using standard rearing protocols. Mosquitoes were housed in $30 \times 30 \times 30$ cm cages at 26°C , with 70% relative humidity and a 12:12-hour light:dark photoperiod. These controlled conditions ensured consistent development and reproduction.

4.3. Egg Hatching

Mature eggs of 1 to 2 weeks were hatched by submerging in cooled boiled distilled water filled with water ($\sim 3/4$) overnight. Jars were tightly closed to deoxygenate the water and promote hatching. Alternatively, eggs were hatched by adding 1-2 ml of larval diet solution to distilled water in a container for 3-4 days under growing conditions before adding the egg papers. These methods optimize hatching rates by mimicking natural triggers for egg hatching.

4.4. Larval Rearing

First-instar larvae (L1) were reared at 27°C , and fed with fish food (Tetramin, Tetra GmbH, Melle, Germany). Pupae were transferred to adult cages before emergence, as pupation typically lasted 48 hours depending on ambient temperature.

4.5. Adult Feeding

Adult mosquitoes were fed on bovine blood containing anticoagulants twice weekly using cotton balls. Both male and female mosquitoes were provided with a 5–10% sucrose solution, ensuring sustained energy for oviposition and longevity.

4.6. Egg Collection

Females were allowed to oviposit in a cylindrical black plastic filled 1/3 with deionized water and lined with germination paper. Oviposition containers can be of various sizes depending on cage density. Dark cup color and larval-rearing water were used to promote oviposition in reluctant and newly colonized strains. Egg laying typically began on the third day post-blood feeding and continued for up to two days.

4.7. Egg Development

The embryos are not yet fully developed upon oviposition and require an additional maturation period before hatching. Eggs were kept in an 80 -100% relative humidity environment for proper embryonation for 2-3 days. Oviposition papers were collected and cleaned with deionized water to remove any debris that could be potential sources of mold contamination. Once dried, the papers were used for hatching or dried further for storage in Zipped bags for up to 12 weeks.

4.8. Genomic DNA Isolation from Mosquito

GenomicDNA (gDNA) of the mosquito was extracted from whole body using CTAB method: Cetyltrimethyl ammonium bromide (CTAB) (Sigma-Aldrich, USA) is a cationic detergent. 2X CTAB extraction buffer was prepared. Before adding the tissue sample to the prepared CTAB buffer, 2 U of proteinase K (Thermo Fisher Scientific, USA) was added. Then, carcass sample was mixed in the prepared mixture of CTAB buffer and proteinase K. The mixture was vortexed gently and was incubated for

20 to 30 min at 50 °C in water bath. During the incubation, the mixture was inverted 3 to 5 times each 5 min. The mixture was then transferred to another water bath at 65 °C and incubated for 15 min. Again, inversions were done each 5 min. Then, it was cooled down slightly and 500 µl of cold chloroform: isoamylalcohol (Emsure, Germany) was added on top, then, inverted 2 to 3 times. The mixture was put at shaker and shook gently for 15 min at room temperature. After the incubation, the sample was centrifuged for 10 min at 12,500 rpm and the clear supernatant (400 µl) was transferred to a fresh tube. To precipitate the DNA, ethanol participation was done in which 2X volume of 95% ethanol and 0.1X volume of 3 M sodium acetate was added to the supernatant and the tube was inverted gently. The mixture was incubated at -80 °C overnight and the next day, it was taken out of the deep freezer, thawed briefly, and spun at maximum speed at 4°C. The supernatant was discarded carefully, and the pellet was washed with 1 ml of cold 70% ethanol and spun 5 min at 4 °C. Again, the supernatant was discarded, and the wash step repeated. The pellet was dried and then eluted.

4.9. RNA Elimination and Genomic DNA Purification

Isolated gDNA was incubated with 1 µl RNase A for 30 min at 37 °C. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) (Emsure, Germany) was added and the sample was centrifuged at 14000g for 5 min. The upper phase was taken into a new tube. Then, ethanol precipitation was done. 2X volume of 95% ethanol along with 0.1X volume of ammonium acetate (7.5M) were added to the taken upper phase. The sample was incubated overnight at -80 °C. In the next morning, the sample was thawed briefly and was pelleted by spinning at maximum speed for 10 min at 4 °C. Then the pellet was washed with cold 70% ethanol and spun at full speed for 5 min at full speed. The supernatant was taken carefully, and the wash step was repeated. The pellet was left to dry and then re-suspended in 100 µl of nuclease free water.

4.10. DNA isolation from Bti

Bti cell was grown at 30 °C at 250 rpm for 18 hours in 10 ml LB. Cells were harvested by centrifugation at 5000 g for 5 min. Genomic DNA purifications from cell debris was performed using genomic DNA isolation kit (Zymo Research, USA) according to the manufacturer's protocols. Cell pellets were kept at -20 °C for later usage. Obtained DNA was used for polymerase chain reaction (PCR) that is used to amplify Cry genes.

4.11. RNA Isolation

Larval samples were washed with distilled water and transferred to a clean tube. 1 mL Trizol was added to the sample and the mixture was smashed into finer particles and homogenized in MagnaLyzer. Without touching the pellet, homogenates were taken into another tube. Then, it was incubated at room temperature for 5 min. Chloroform (Emsure, Germany) in a 1:5 Trizol:Chloroform ratio was added to the mixture, and it was vortexed for 20 sec. It was followed with an incubation step at room temperature for 2 min. Centrifugation was applied at 10000 g for 18 min. The supernatant was taken into a microcentrifuge tube and 1 volume of 100% ethanol was added. The sample was inverted 6 times. 700 μ l of it was transferred to a NucleoSpin RNA Column, and the procedure was followed as recommended in the protocol of NucleoSpin RNA kit (Macherey Nagel, Germany).

4.12. Reverse Transcription

Complementary DNA (cDNA) synthesis was done using ImProm-II Reverse Transcription System, Promega (Madison, USA) according to manufacturer's recommendations.

4.13. RT-PCR

Using the cDNA, a PCR reaction was prepared as shown in Table 4.1 and run according to the conditions provided in Table 4.3.

Table 4.1. PCR primers.

GENE	SEQUENCE
Alp-like-Forward	CCCAAGCTTATGTACCTTCTTCGTACGATCGGTG
Alp-like-Reverse	CGGATCCGCTTCTCACCCCTATGCACG
AMN-Forward	GGGGTACCATGGAAGCACGGCTGGTGC
AMN-Reverse	CGGATCCGCTTTGCTAATCATTCCCTCCCTCCGT
Calc-Forward	GCTCGAGATGGAGGACGGCCATAGC
Calc-Reverse	CCAAGCTTGTCCCATTTCTTAATTTGCGCCGTC
Cry11Aa-Forward	ATGGAAGATAGTTCTTTAGAT
Cry11Aa-Reverse	CTACTTTAGTAACGGATT
Cry4Ba-Forward	ATGAATTCAGGCTATCCGTTA
Cry4Ba-Reverse	TCACTCGTTCATGCAAATTAAT
Flotilin-Forward	CCCAAGCTTATGGTGTGGGGCTTTGTGA
Flotilin-Reverse	CGCGGATCCCTACTGCGGCAGCTGGAGCAGT
Lachesin-Forward	CCCAAGCTTATGAGTCGAGTCAGGTTGAATGC
Lachesin-Reverse	CGGATCCAGAAACAGCGCGGCCAATGC
Maltase-Forward	CCCAAGCTTATGAATAATCACCTGTTGCTG
Maltase-Reverse	CGCGGATCCTCACATTTTTTTTCTTCGCTACTA
MemALP-Forward	CCCAAGCTTATGAACGGGTTTCGGTTTAATGTG
MemALP-Reverse	GGGTACCGAGGCGAACACTCGCACAGC
Prohibitin-Forward	CCCAAGCTTATGGCCACACAATTCCTGAA
Prohibitin-Reverse	CGCGGATCCTTACTGCGGCAGCTGGAGCA
V-ATP-Forward	CCGCTCGAGATGTCCGTCAATCGTACCATTTC
V-ATP-Reverse	CCAAGCTTGTGGCGCGAGTCTCGTGGGTA

For cloning, pET30a and pET32a plasmids were used for Cry11Aa and Cry4Ba genes, while pEFGP-N1 was used for receptor cloning as a vector. Primers for PCR reactions were designed as listed in Table 4.1. PCR reactions were prepared as shown in Table 4.2.

Table 4.2. PCR reaction components.

Contents	Amount/Concentration
Forward primer	50 pmol
Reverse primer	50 pmol
gDNA	150 ng
10X taq buffer	1X
MgCl ₂	2.5 mM
dNTP	200 mM
DNA polymerase	0.02 U/ μ L
Nuclease free water	Up to 50 μ L

A thermal cycler was set after the PCR mixture, as shown in Table 4.3.

Table 4.3. PCR conditions.

Steps	Temperature ($^{\circ}$ C)	Time (s)	Cycle
Initial denaturation	95	300	1
Denaturation	95	20	30 cycles
Annealing	52-70	20	
Extension	72	60-90	
Final extension	72	180	1

4.14. Extraction of DNA Samples from Agarose Gel

Samples amplified by RT-PCR were loaded into a 1% agarose gel and run at 90 V for 30 minutes. The desired product size observed in the gel was extracted using the Agarose Gel DNA Extraction Kit (Sigma-Aldrich, Germany) according to the protocol, and the DNA was successfully extracted.

4.15. Digestion of DNA and Ligation

The PCR product and the relevant plasmid were digested with restriction enzymes for each cloning. Inserts and linearized plasmid were ligated using T4 DNA Ligase (NEB, USA) according to the manufacturer's instructions. In brief, 50-100 ng of digested plasmid and inserts were mixed in a 1:1 or 1:3 molar ratio for each 20 μ l of reaction volume. The mixture was incubated at room temperature for 30 minutes. Then, enzyme inactivation was done at 65 °C for 10 minutes.

4.16. Preparation of Chemically Competent *E. Coli* Cells

Glycerol stock of *E. coli* DH5alpha strain aliquoted previously was taken and added to 5 ml LB medium (inoculation). The culture was applied to overnight growth at 37°C while shaking at 200 rpm. The following day, 250 μ l of the culture grown overnight was taken, and 25 ml LB was added. The inoculated medium was applied to further growth in the shaker until optical density (OD) reached a value between 0.4 and 0.6 when measured at 600 nm. At this point, the culture was centrifuged for 10 min at 4000 g, 4°C. The supernatant was discarded, and 2.5 ml ice-cold sterile 50 mM CaCl₂ was added to the pellet, which was resuspended. 10% glycerol was added to the resuspended cells and aliquoted in 100 μ l. Aliquots were immediately transferred to liquid nitrogen and stored at -80°C.

4.17. Transformation of Chemically Competent Bacteria

Previously prepared competent cells were taken out of the freezer, and one vial was used for each cloning. Before adding the ligation mixture, the vial was kept on ice for 15 minutes. After adding the 10-50 ng plasmid of 15-20% ligation mixture to the thawed competent cell, the mixture was incubated on ice for 20-30 min. The heat shock was done by placing tubes at 42°C for 45-60 sec, followed by 2 min of ice incubation. Then, 500 ml LB medium was added, and the mixture was incubated at 37°C for 1 hour while shaking at 200 rpm. After the incubation, 100-200 µl of the cell was taken and spread on relevant antibiotic-containing LB agar plates. The upside-down plates were then incubated overnight at 37°C.

4.18. Colony PCR

Colony PCR was performed to screen the positive colonies, those bearing the desired DNA fragment. Colonies were selected from LB agar plates and mixed in a PCR solution. The PCR mixture was prepared. The mixture was applied to an amplification process in a thermal cycler. Then, the products were loaded into a 1% agarose gel, and electrophoresis was run at 90 V for 30 min.

4.19. Plasmid Purification

Positive colonies identified by colony PCR were grown overnight in LB, to which a suitable antibiotic was added. The next morning, plasmid purifications were performed using the NucleoSpin Plasmid Miniprep kit (Macherey-Nagel, UK) and ZymoPURE Plasmid Midiprep kit (Zymo Research, USA) for mini and midi/maxi isolation scales, respectively, according to the manufacturer's protocols. Plasmids intended to be used in transfections were purified with a midi kit. Spectrophotometric measurements were done to check the quality of the isolated plasmids. OD was measured and those with OD_{260/280} between 1.8-2.00 were considered as good quality.

4.20. Growth and Maintenance of HEK293 Cells

HEK293 cells were grown on a 10 cm cell culture dish. Cell culture medium was prepared using Dulbecco's modified eagle medium (DMEM) (Gibco, UK), to which 10% FBS (Gibco, UK) and 1% Penicillin/Streptomycin (Hyclone, USA) were added. Complete media was stored at 4 °C, and before each use, it was incubated at sterile water bath at 37 °C to reach this temperature. Cells were cultivated in an incubator at 37°C, supplemented with 5% CO₂.

4.21. Passaging

Cells were routinely grown and, before reaching 90% confluency, were passaged to new cell culture dishes. The passaging is started by aspirating the old medium. Then, cells are washed with 1X calcium and phosphate-free PBS. To detach the adherent cells from the dish surface, trypsin (0.025%, ready to use) was added to the cells, and the petri dish was incubated at 37°C for 3-5 min. Then, 2-3 volumes of the added trypsin and complete DMEM were added to the cells to inactivate the enzymatic activity of trypsin. Cells were resuspended, dispersed, and transferred to 50 ml falcon. Then, cells were centrifuged, and the supernatant was discarded. They were resuspended in fresh, complete DMEM and dispersed in a 1:5 ratio.

4.22. Cryopreservation

To preserve the grown cells, when at 90% confluency, they were harvested with trypsin. Then, trypsin was neutralized by adding 10 volumes of growth medium and then precipitated by centrifugation for 3 min at 500 g. Meanwhile, the freezing medium (5% DMSO, 10% FBS and 85% DMEM) was prepared, and 1 ml of it was transferred into 2 ml screw capped-cryotubes. 1-2 million cells were transferred to each vial. The freezing box was filled with isopropanol, and cryo-tubes were placed in it. The box was kept at -80°C overnight. Cell stocks were translocated to the storage tank filled with liquid nitrogen the next day.

4.23. Thawing

When a new stock of cells was needed, one vial was taken from the nitrogen tank. Before use, it was incubated in a 37°C water bath and thawed immediately. Cells were added to a falcon containing 4 ml of fresh medium and precipitated at 500 g for 3 min. The supernatant was discarded, and resuspension was done in an adequate amount of fresh, complete medium. Then, cells were transferred to a fresh petri dish and incubated in an incubator.

4.24. Production of Cry4Ba and Cry11Aa Protoxins

The recombinant expression vector was transformed into *E. coli* BL21 to express the Cry11Aa and Cry4Aa proteins. Proteins were induced with 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG) when the cells reached an OD₆₀₀ value of 0.3-0.4. Cells were centrifuged for 10 minutes at 5000g, and pellets were sonicated in 50 mM Na₂CO₃ (pH 10.0) buffer. Cell lysates were centrifuged under the same conditions to obtain soluble protoxins. Cry11Aa was further solubilized by 100 mM NaOH treatment at 4°C for 1 hour; then, pH was adjusted to 10.0 with 50 mM Na₂CO₃ buffer. The proteome was applied to the Ni-NTA resin. Purifications and fractions were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.25. Activation of Cry4Ba and Cry11Aa Protoxins

Purified toxins were purified as protoxin form; thus, they are activated by trypsin (1:250) treatment (4µg trypsin for 10µg toxin) for 30 min for Cry11Aa and 1 hour for Cry4Ba at 37°C'. SDS-PAGE was used to visualize them for fragment analysis.

4.26. Larval Toxicity Assay

Bioassays were performed to determine the lethal concentration of toxins by purified toxins and toxin-expressed cells induced by IPTG. *E. coli* BL21 to express the

Cry11Aa and Cry4Aa proteins were applied to 30 larvae as 10^9 cfu/ml, 10^8 cfu /ml, 10^7 cfu/ml and 0 cfu/ml OD₆₀₀ value and 500 ng/ml, 250ng/ml, 100 ng/ml, 500 ng/ml and 0 ng/ml purified toxins. The numbers of dead larvae were counted after 24 hours, and the mortality percentage was calculated. Tests were replicated 3 times.

4.27. Cell Culture and Quantitative Cell Cytotoxicity Assay

Receptors cloned into the pEGFP-N1 vector were transfected with HEK293 cells separately. Lipofectamine 3000 was used with 2 µg plasmids for one well in 6-well plates for transfection. Purified and activated Cry4Ba and Cry11Aa toxins were applied as 4 replicates in 96 well plates in different concentrations (0, 200 and 1000 ng/ml final concentrations) to check their cytotoxicity. pEGFP-N1 transfected cells were used as a negative control. Cytotoxicity was analyzed using an XTT cell proliferation assay (Sigma-Aldrich). In principle, yellow tetrazolium salt is reduced into formazan dye, resulting in a solution measured at 490 nm and 590 nm (as reference) in a plate reader spectrophotometer. 50 µl XTT solution is applied to 100 µl solution (HEK293 and full DMEM) and measured for 1-2 hours. The assay conditions were optimized to ensure consistency across replicates. Careful pipetting techniques were employed to avoid cross-contamination between wells. Additionally, the incubation period was monitored closely to prevent over-reduction of the tetrazolium salt, which could lead to inaccurate absorbance readings.

4.28. Double Strand RNA Synthesis

PCR primers with T7 promoters (Table 4.4) were used to amplify 550-650 bp fragments of the receptor genes from respective cloned receptor plasmids and generate dsRNA molecules.

Table 4.4. dsRNA template primers.

Gene	Primer Sequences
Alp Forward	TAATACGACTCACTATAGGGAGATGACCCCAAGAACCAAGTCGAC
Alp Reverse	TAATACGACTCACTATAGGGAGACTGTTTCGTCCAGCGATTTCGG
MemALP Forward	TAATACGACTCACTATAGGGAGAACGACTTCTCGAGTTACCGCC
MemALP Reverse	TAATACGACTCACTATAGGGAGACCAACGTGTTCTCTCCGACAC
V-ATP Forward	TAATACGACTCACTATAGGGAGATCCGAAGACATGTTGGGTCGCG
V-ATP Reverse	TAATACGACTCACTATAGGGAGACTCACGCAAGGCTTCAGCATACG
AMN-like Forward	TAATACGACTCACTATAGGGAGATCTTCGGAAACCTGGTGACGCC
AMN-like Reverse	TAATACGACTCACTATAGGGAGAGGAAATTTGTGCGGTGCCTTGCG
Prohibitin Forward	TAATACGACTCACTATAGGGAGACCACACAATTCCTGAACCGC
Prohibitin Reverse	TAATACGACTCACTATAGGGAGAGCCTTCTCTACCATGAATCGCG
Flotilin Forward	TAATACGACTCACTATAGGGAGAACGAGGAGGGCTACCTCAAAAG
Flotilin Reverse	TAATACGACTCACTATAGGGAGACGATATTCTCGCCAGGCTTCGG
Lachesin Forward	TAATACGACTCACTATAGGGAGAGTGTTCCGTCCAGTATGCCA
Lachesin Reverse	TAATACGACTCACTATAGGGAGAGCACTCCAGATCCATGTCGT
Glutamyl Forward	TAATACGACTCACTATAGGGAGAATACCTGGAGAGGCTTCGGTAC
Glutamyl Reverse	TAATACGACTCACTATAGGGAGACGTATTCCGTTTCCTGCCCC
Calcium Forward	TAATACGACTCACTATAGGGAGACACTTGCTTGCTCTGGGTACC
Calcium Reverse	TAATACGACTCACTATAGGGAGACTTCTGGGGCACCCTTACAGAAC
Maltase-like Forward	TAATACGACTCACTATAGGGAGATTGGTTTGTGTTGGTTCGGGGA
Maltase-like Reverse	TAATACGACTCACTATAGGGAGATGACCCTTGTCGAACTGGTG

The dsRNA molecules were synthesized *in vitro* from receptor templates containing T7 promoter using the MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. A single PCR with T7 promoter-containing primers (sense and antisense) produced a dsRNA template, which was examined on 1.5% agarose gel. The obtained products were isolated from the agarose gel.

Table 4.5. Transcription reaction components.

Amount	Component
1 μg	Template DNA
2 μl	10X T7 Reaction Buffer
2 μl	ATP Solution
2 μl	CTP Solution
2 μl	GTP Solution
2 μl	UTP Solution
2 μl	T7 Enzyme Mix
Up to 20 μl	Nuclease-free Water

Transcription reactions were incubated at 37°C for 4 hours. All products were treated with DNAase and RNAase to degrade template DNA and single-stranded RNA that did not anneal, for 1 hour at 37°C. Free nucleotides, proteins, and nucleic acid degradation products were removed using a filter cartridge, and the products were eluted with 100 μl nuclease-free water. Elutions were quantified by measuring absorbance at 260 nm and analyzed using agarose gel electrophoresis.

4.29. Bioassay and Quantitative PCR

Larvae at the 4th stage were used in the bioassay as three biological triplicates. Purified Cry4Ba and Cry11Aa toxins (100 ng/ml) were applied for 12 hours, and total RNA was extracted from 5 larvae using TRIzol Reagent (Invitrogen). Subsequently, 1 μg RNA was used to synthesize cDNA with a First Strand cDNA Synthesis Kit (Invitrogen). Primers for quantitative PCR (Q-PCR) are listed in Table 4.6.

Table 4.6. Q-PCR primers.

Gene	Forward Primers	Reverse Primers
Alp-like	TCGGTGTTTGCGTTGTTCTG	CCACGCAGTATGTCTTGACT
MemALP	CAGCTCTCTACGCTCACTCG	ACGGCAGCCTATCGTAACTG
V-ATP	AGATTTGTCGTCAAGCCGGT	TACGTCCTTCAACACGTCCG
APN	GCCTTTGTAGTGTCCGTCCA	TAGTTGGTGTTCGCCGGTAG
Prohibitin	CTCGGCGTTATATAATGTTGATGG	GACCATCTCACGCTGGGTAA
Flotilin	ACGAGGAGGGCTACCTCAAA	TAGTGGCTTCTAGCTCCCGT
Glutamyl aminopeptidase	GTATACTCCGGAAGCCGGG	TCTGGTCTGAATTGCTGCCTT
Lachesin	AACAGGTGGCGCAATCTACA	GTTGATGGCTCGGCAGTAGT
Calcium	CACTTGCTTGGCTCTGGGTA	CGAGGACAATCAGGGCAGTT
MaltaseI	AACGGGAGTGTGGTTCAGTC	TGACCCTTGTCGAACGTGGT

4.30. RNAi Knockdown of Receptor Genes

Groups of 30 1st-instar larvae were treated separately with receptor dsRNAs, and one control group was used without dsRNA treatment as in biological triplicate. Each dsRNA was added directly to water containing 1st-instar larvae to a final concentration of 50 g dsRNA in 500 l water (100 g dsRNA/mL). Five hours after the dsRNA addition, the larvae were fed with the larval diet for 40 hours. 5 larvae were harvested to confirm target knockdown via qRT-PCR. Cry11Aa and Cry4Ba toxins were applied as purified toxins and induced bacteria at 50% lethal doses in 1 ml water for 24 hours. The survival rate was recorded for 48 hours after toxin treatment.

4.31. Structure Prediction of Crystal and Receptor Proteins

Sequences of Cry11Aa, Cry4Ba and their potential receptors, including *Aedes albopictus* aminopeptidase N (AMN), ALP, membrane-bound ALP, V-type proton ATPase subunit B, prohibitin member protein, maltase-like protein were retrieved from NCBI. They were used to predict the 3D structures using rosetta and I tasser soft-

ware. The 3D structure of Cry4Ba reported using X-ray crystallography was used as homology modelling.

4.32. Quality Analysis of Predicted Protein Structures

For the quality analysis and structure validation of all protein structures, Procheck was used, and pyMol was used for the visualization of 3D structures.

4.33. Prediction of Receptor-Ligand Interactions by Protein-Protein Docking

The rigid-body docking program (ZDOCK, PyDOCK and ClusPro) employs a fast Fourier transform (FFT) algorithm to generate receptor-ligand interactions. The top 10 predicted complexes were selected. Complexes showing the most stable interactions and low binding scores were chosen for further analysis, and their bonding interactions were examined.

4.34. Analysis of Docking by PDBsum

All docking predictions were further analyzed using the PDBsum generate. The interaction profiles of all proteins and their receptors were examined. The binding strengths of all receptor-ligand complexes were predicted by explicating the number and types of bonds between proteins and receptors.

4.35. Statistical Analyses

In the XTT viability assay, each experiment was conducted with a minimum of three biological replicates, and the statistical errors were represented as the Standard Error of the Mean (SEM). For the qPCR data, graphs were generated using the standard deviation of biological replicates, and statistical comparisons were performed using the Student's t-test. A p-value of 0.05 or lower was considered statistically significant.

5. RESULTS

5.1. Ovitrap and Anatomical Determination of *Aedes Albopictus*

Larvae of *Ae. Albopictus* from different locations were collected from water samples, and ovitraps were placed where adult mosquito habitats were possible. Ovitrap was prepared from the bottle and covered with black heat and water-resistant bands that attract mosquitos. Stones, hay and cat foods were placed to support larval growth after hatching the eggs. A net was bound to the mouth of the trap to prevent the escape of the adults (Figure 5.1).

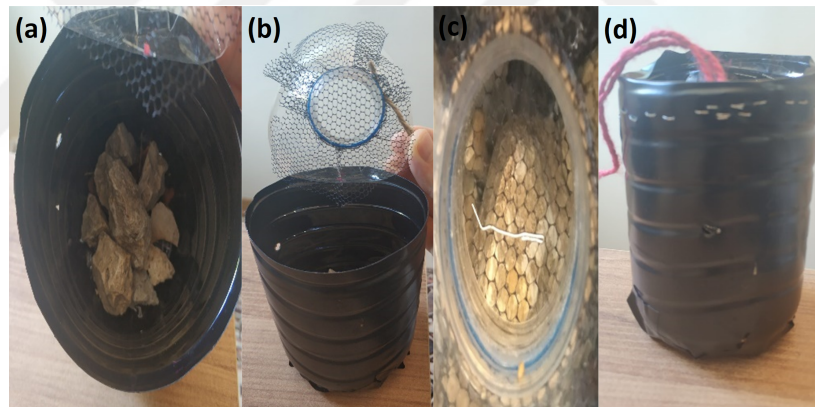


Figure 5.1. Ovitrap preparations for *Ae. albopictus* larva collection (a: inside of the trap, b: Net for entrance, c: Mouth of the trap, d: Prepared ovitrap).

Mature eggs collected from the ovitraps were incubated in deoxygenated water overnight to facilitate hatching. This process involved filling the container with boiled distilled water, which was allowed to cool before immersing the egg-laden papers. De-oxygenation was achieved by tightly sealing the container, creating an environment conducive to hatching. Once hatched, the larvae were carefully collected and analyzed under a light microscope. Detailed anatomical observations were conducted to confirm the identification of *Aedes albopictus* larvae.

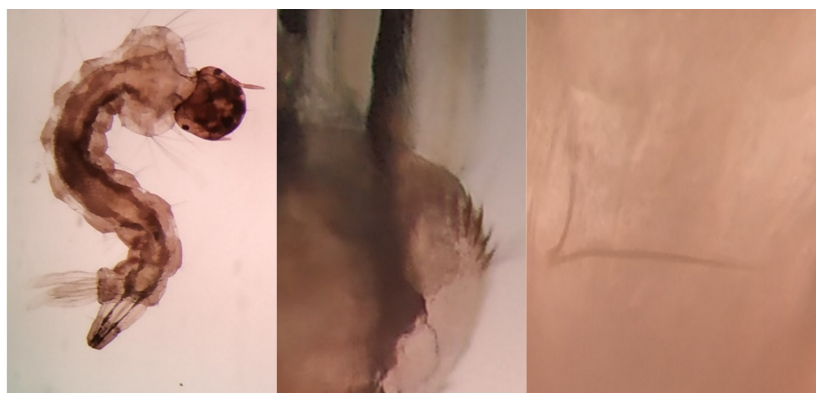


Figure 5.2. Parts of the larval body of *Ae. albopictus* (From left to right: Whole body, VIII abdominal segment and thorax view).

Ae. albopictus larva has Straight, thorn-like comb scales in a row on the VIII abdominal segment and little or no hooks on the side of the thorax (Figure 5.2). Adults obtained from the larvae were fed with 10% sucrose solution as the carbohydrate source. Defibrinated fresh blood was provided to female adult mosquitoes twice weekly after 5 days of emergence. Oviposition was observed after the third or fourth days of blood feeding. Females were allowed to oviposit in a glass cup filled with 2/3 water and covered with germination paper. After oviposition, the papers were collected to prevent premature hatching and loss of larvae. Upon oviposition, eggs were stored in lidded but not air-tight containers in a moist environment (>80% Relative humidity).

5.2. Cloning of Receptors

Receptor gene sequences were obtained from cDNA of a 4th stage of *A. albopictus* larvae. 2.3 µg total RNA was obtained from one larvae and used for cDNA library synthesis and this cDNA pool was used to amplify the receptor genes in PCR as template. To clone receptors in pEGFP-N1 plasmid, PCR products were loaded into agarose gel. The expected length of the PCR products was ALP (1626 bp), memALP (1578 bp), V-ATPase B (1488 bp), AMN (2703 bp), Prohibitin (819 bp), flotilin (1281 bp), lachesin (1074 bp), Calc (3015 bp) and Maltase (822 bp). The products were extracted from agarose gel and purified. The plasmid and PCR products were digested by HindII and

BamHI for ALP and Lachesin; XhoI and HindIII for MemALP, V-ATPase, Prohibitin, Flotilin, Calc and Maltase; XhoI and BamHI for AMN and ligation was performed. The clones were verified with restriction analysis (Figure 5.3) and SANGER sequencing.

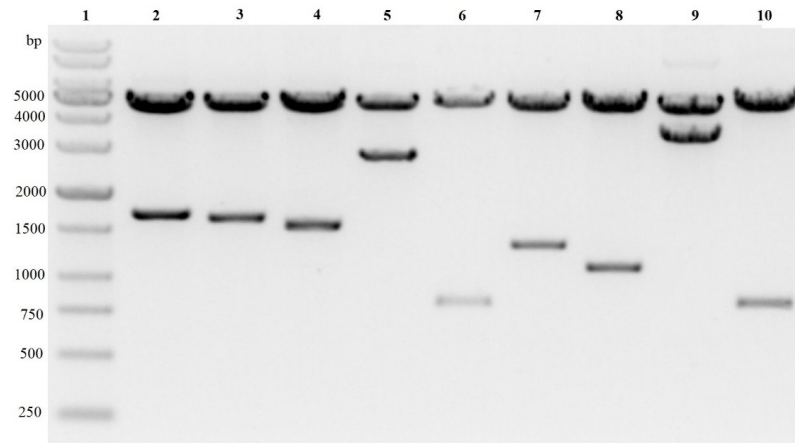


Figure 5.3. Restriction analysis of receptors. (1: 1000 bp ladder, 2: ALP, 3: memALP, 4: V-ATPase, 5: AMN, 6: Prohibitin, 7: Flotilin, 8: Lachesin, 9: Calc and 10: Maltase).

The upper bands from 2 to 10 (Figure 5.3) belong to the pEGFP-N1 backbone, and the lower bands show receptor genes cloned into pEGFP-N1. Verified recombinant vectors were transfected into HEK-293 cells separately and used for cytotoxicity experiments.

5.3. Cloning and Purification of Cry Toxins

Cry4Ba and Cry11Aa genes were obtained from *Bacillus thuringiensis israelensis* plasmid and amplified by PCR. To clone toxins into bacterial expression vectors, pet30a, pet32a plasmids and PCR products were loaded into agarose gel. The products were extracted from agarose gel and purified. The expected length of the PCR products was 3408 bp for Cry4Ba and 1929 bp for Cry11A. The products were extracted from agarose gel and purified. The plasmid and PCR products were digested by HindIII and BamHI, and ligation was performed. Cry4Ba and Cry11Aa were cloned into a pet30a

and pet32a expression vector and analyzed by restriction analysis (Figure 5.4).

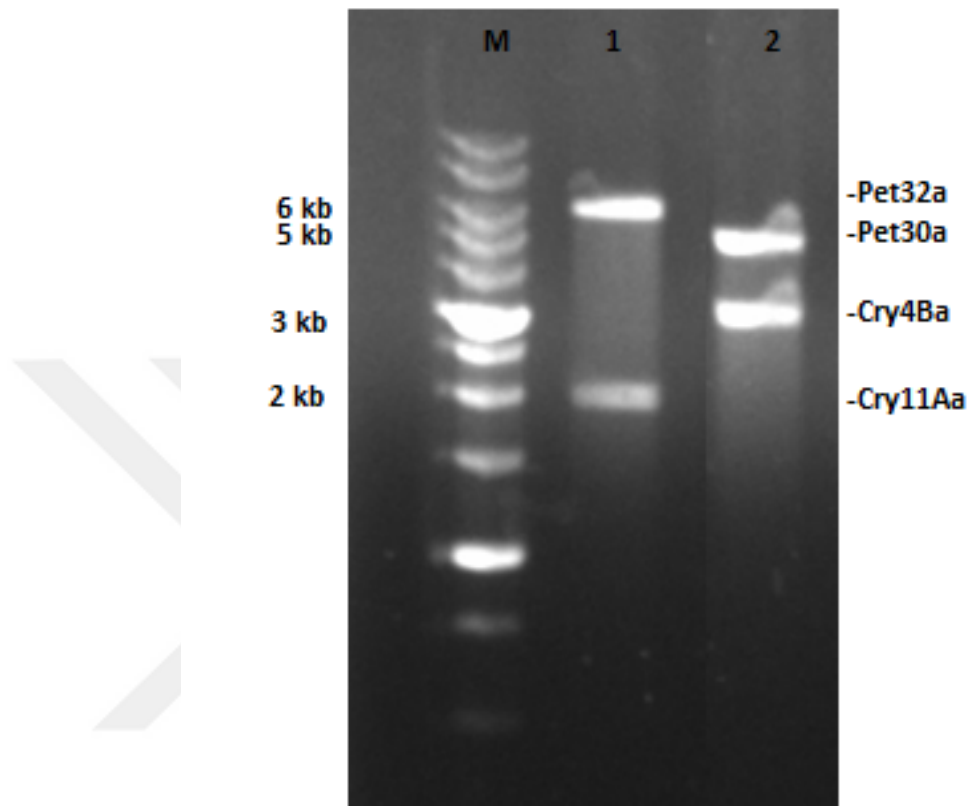


Figure 5.4. Restriction analysis of Cry4Ba and Cry11Aa cloning.

Since Cry11Aa was not solubilized with the pet30a vector, the pet32a vector was used to increase its solubilization by the thioredoxin tag. Band sizes obtained from restriction analysis were 3408 bp for Cry4Ba and 1929 bp for Cry11Aa. The *E. coli* Rosetta strain expressed the Cry4Ba and Cry11Aa toxins, which were induced with 0.5 mM IPTG for 3 hours. Cell pellets were sonicated to obtain the whole proteome. Then, the toxins' induction was analyzed by SDS-PAGE, comparing uninduced and inductions per hour (Figure 5.5). SDS-PAGE visualized whole-cell lysate of Cry4Ba and Cry11Aa-produced cells, and 2 hours of induction time for Cry4Ba and 3 hours for Cry11Aa and 0.5 mM IPTG were determined as optimized conditions (Figure 5.6).

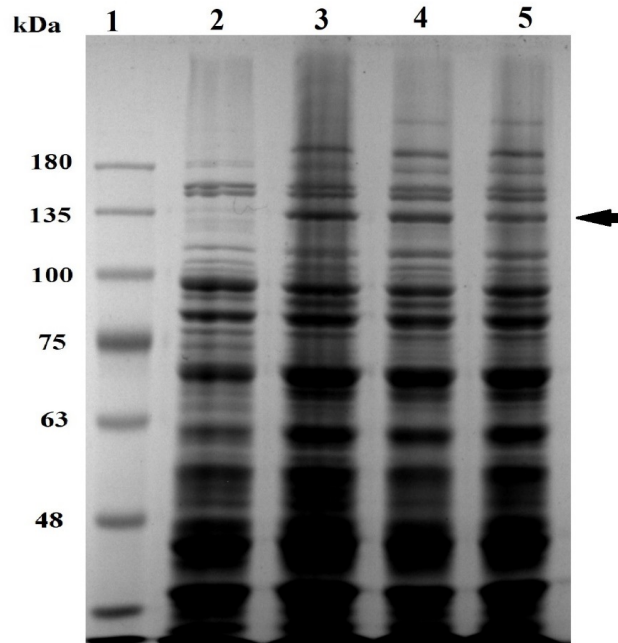


Figure 5.5. IPTG induction of Cry4Ba in *E. coli* Rosetta (1: Marker, 2:Uninduced (OD600: 0.3-0.4) 3:1 hour induction, 4:2 hours induction , 5:3 hours induction, arrow indicates 127 kDa Cry4Ba protein).

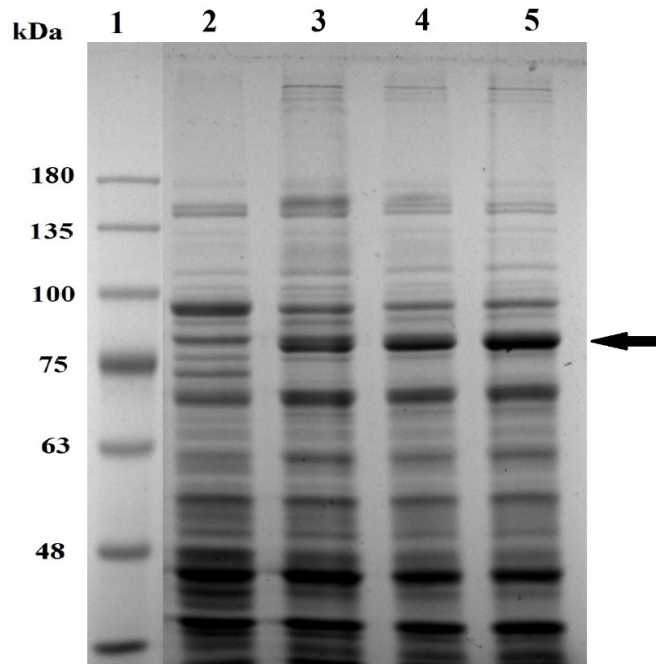


Figure 5.6. IPTG induction of Cry11Aa in *E. coli* Rosetta (1: Marker, 2: Uninduced (OD600: 0.3-0.4) 3:1 hour induction, 4:2 hours induction, 5:3 hours induction, arrow indicates 76 kDa Cry11Aa protein).

Cell pellets were resuspended in Wash buffer and sonicated. Toxins were separated as inclusions, and solubilized fractions were loaded into Co-NTA columns. Toxins were purified by Co-NTA beads, and each purification step was checked by SDS-PAGE (Figure 5.7).

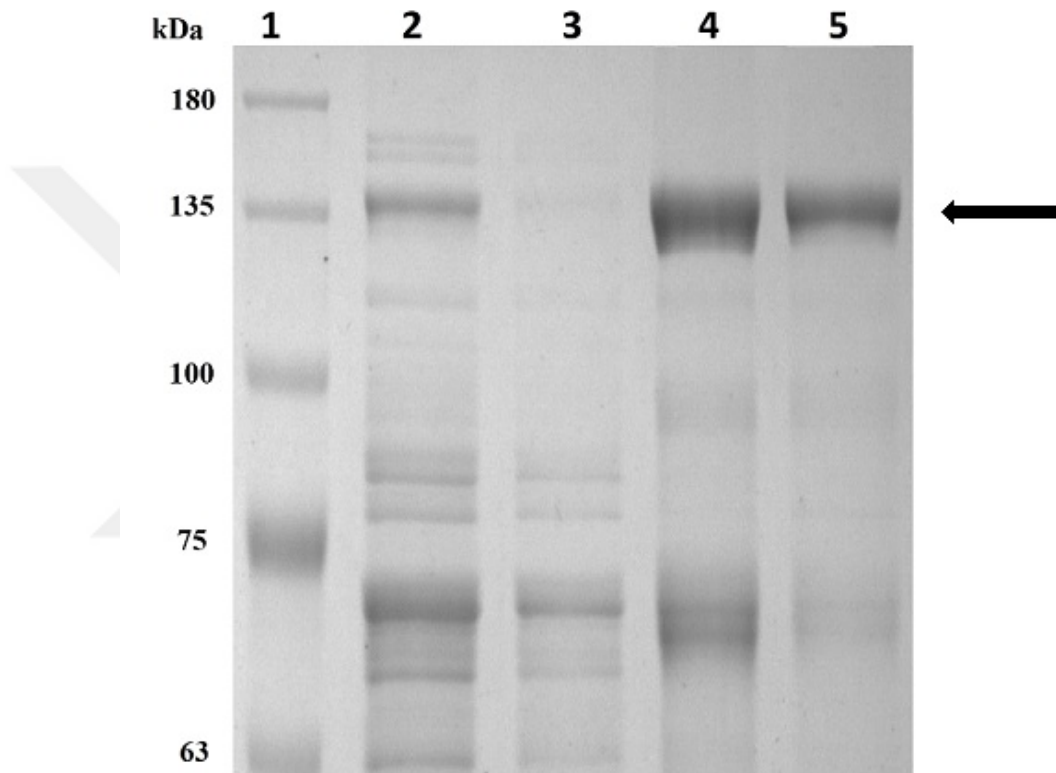


Figure 5.7. Purification steps of Cry4Ba. (1: Ladder, 2: solubilized inclusions, 3: Co-NTA unbound proteins, 4: elution with 8% imidazole, 5: elution with 100% imidazole, Black arrow: Cry4Ba (127 kDa)).

After purifications, the latest fraction was dialyzed against 50 mM Na_2CO_3 (15 mM)/ NaHCO_3 (35 mM), digested with trypsin, and used for XTT cytotoxicity experiments. Since the solubility of Cry11Aa was not enough with Na_2CO_3 incubation, inclusions remained from Na_2CO_3 treatment were further solubilized with 100 mM NaOH for 1 hour at 4°C. Then, the supernatant was mixed with 1:1 w/w Co-NTA wash buffer containing 50 mM Na_2CO_3 at pH 9.0 and loaded into the Co-NTA column (3 liters of bacterial culture products for 1 ml Co-NTA beads). The purification steps

of Cry11Aa were analyzed by SDS-PAGE (Figure 5.8).

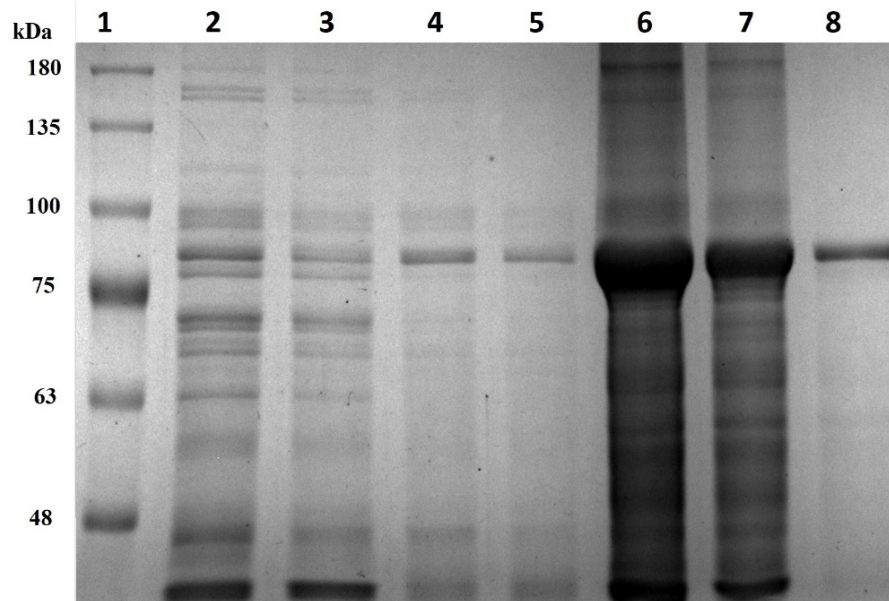


Figure 5.8. Purification steps of Cry11Aa. (1: Ladder, 2: solubilized inclusions, 3: Co—NTA unbound proteins, 4: elution with 8% imidazole, 5: elution with 100% imidazole, 6: NaOH treated inclusions, 7: Co—NTA unbound proteins 8: elution with 8% imidazole, Black arrow: Cry11Aa (76 kDa)).

The whole cell lysate was treated with 50 mM Na_2CO_3 , and the soluble fraction was loaded into the Co-NTA column. Most of the solubilized inclusion of Cry11Aa was lost in the washing step. The pellet obtained from 50 mM Na_2CO_3 treatment was solubilized with 100 mM NaOH and loaded to the column (Lane 6). After purification, the last fraction (Lane 8) was digested with trypsin (Cry11Aa-T) and used for XTT cytotoxicity experiments.



Figure 5.9. Activation of Cry4Ba with trypsin treatment. (Fragments approximately 65, 30 and 20 kDa). (1: 30 min trypsin treatment, 2: 1 hour treatment).

Trypsin treatment of Cry4Ba toxin showed that up to 1-hour incubation at 37°C resulted in 3 different bands as 65, 30 and 20 kDa fragments.

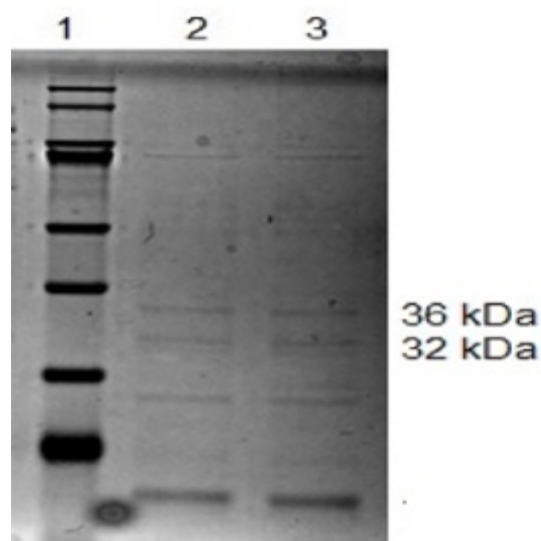


Figure 5.10. Activation of Cry11Aa with trypsin treatment. (Fragments approximately 36 and 32 kDa). (1: Ladder, 2: 30 min trypsin treatment, 3: 1-hour trypsin treatment).

Trypsin treatment of Cry11Aa toxin showed that up to 1-hour of incubation at 37°C resulted in 4 different visible bands. 36 and 32 kDa fragments were obtained from Cry11Aa digestion. Digested toxins were used in XTT cytotoxicity experiments.

5.4. Predicted Interactions of *Ae. Albopictus* Receptors with Cry4Ba

The structure of the active form Cry4Ba was obtained from PDB (1w99.pdb) and minimized to obtain energetically favorable conformation (Figure 5.11).

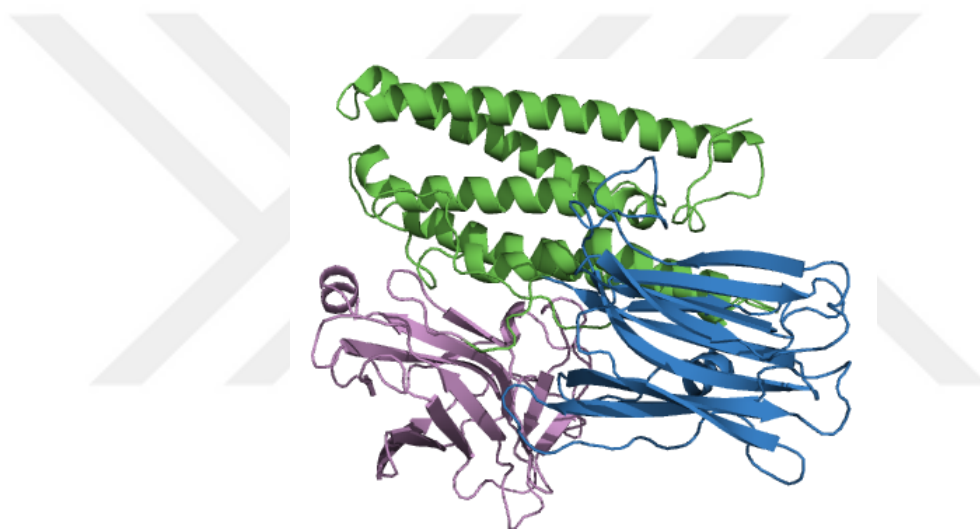


Figure 5.11. Ribbon diagrams of the Cry4Ba structure. Overview with domain I, II, and III colored green, blue, and pink, respectively.

Domain I, made of residues 84–282, is an α -helical bundle of five helices. Domain II is a prism of three antiparallel β -sheets made of residues 290–466. Domain III is a β -sandwich made of 474–641. Molecular docking analysis of Cry4Ba with receptors showed that most of the interactions (hydrogen bonds and salt bridges) of the Cry4Ba domain were made by Domain III (located between 290–466 residues) with receptors (Table 5.1).

Table 5.1. Interacted residues of *A. albopictus* receptors with active Cry4Ba (65 kDa) toxins. (Underlined residues are salt bridges, remaining contacts are hydrogen bonds).

Receptors	Receptor Residues	Cry4Ba-T Residues
ALP-like	Ser19, Tyr17	Ser301, Ser390
MemALP	Thr119, His490, His490, Val501, Thr133, Lys47	Phe364, Ser362, Ile358, Lys342, Tyr332, Thr512
V-ATP	-	-
AMN	Asp638, Ile639, Ala683, Glu678, Glu534, <u>Asp640</u> , <u>Glu678</u> , <u>Glu534</u>	Met353, Met353, Ser362, Lys449, Arg520, <u>Arg323</u> , <u>Lys449</u> , <u>Arg520</u>
Prohibitin	Gly229	Asn375
Flotilin	Asp418, Gly426, Lys406, <u>Lys406</u>	Asn456, Ser361, Tyr332, <u>Asp334</u>
Maltase	Asp248, Arg165, Glu247, <u>Asp157</u>	Ser561, Gln558, Qln613, <u>Arg520</u>

The active structure of Cry4Ba was proposed to interact with ALP-like, MemALP, and Prohibitin by only hydrogen bonding. V-ATPase and Cry4Ba-T showed no interactions. AMN, Flotilin, and Maltase receptor interactions were observed through hydrogen bonds and salt bridges. The highest number of interacted residues resulted from AMN and Cry4Ba-T interactions.

5.5. ALP and Cry4Ba Interactions

The interactions between Cry4Ba and alkaline phosphatase (ALP) were evaluated using the PyDock server, providing insights into the binding energetics and structural characteristics. Docking results revealed an electrostatic energy (Ele) component of -9.596, a desolvation energy (Desolv) component of -31.306, and a Van der Waals energy (VDW) component (weighted by default at 0.1) of 27.388. The calculated total energy score for the first-ranked docked structure was -38.163, indicating a relatively stable

interaction. Visualization and structural analysis of the docking model were performed using PyMOL, providing detailed insights into the binding interface (Figure 5.12 and Figure 5.13).

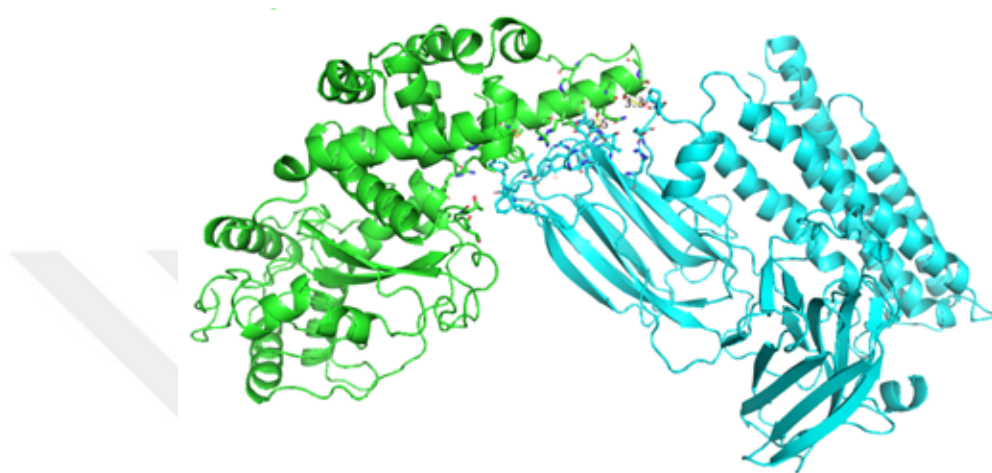


Figure 5.12. Image of docking structure of Cry4Ba (green) and ALP (blue).

Two hydrogen bonds were identified between Cry4Ba and ALP, emphasizing the importance of specific residue interactions in stabilizing the complex.

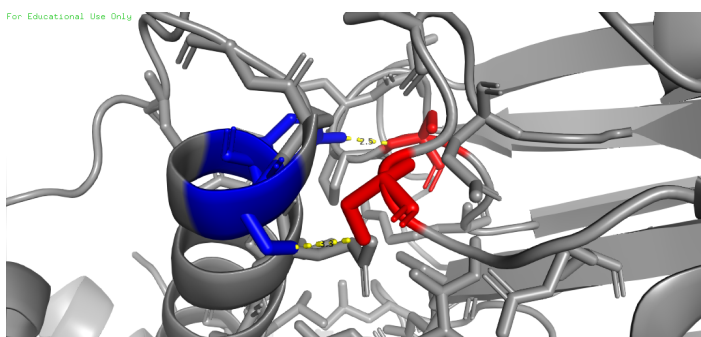


Figure 5.13. Hydrogen bond formation between Cry4Ba (Blue) and ALP (red).

The first hydrogen bond involved Ser19 of ALP and Ser301 of Cry4Ba, while the second bond was formed between Tyr17 of ALP and Ser390 of Cry4Ba. These interactions highlight critical binding sites that may contribute to the stability and

specificity of the Cry4Ba-ALP complex. The results underscore the potential role of ALP as a receptor or co-receptor for Cry4Ba, with the identified interactions likely playing a pivotal role in mediating the toxin's biological activity.

5.6. MemALP and Cry4Ba interactions

The interactions between Cry4Ba and MemALP were analyzed using docking simulations, which revealed detailed binding energy components and structural dynamics. The docking results showed an electrostatic energy (Ele) of -11.380, a desolvation energy (Desolv) of -22.561, and a Van der Waals energy (VDW) of 1.006. The total binding energy was calculated as -33.840, indicating a stable receptor-toxin interaction (Figure 5.14 and Figure 5.15).

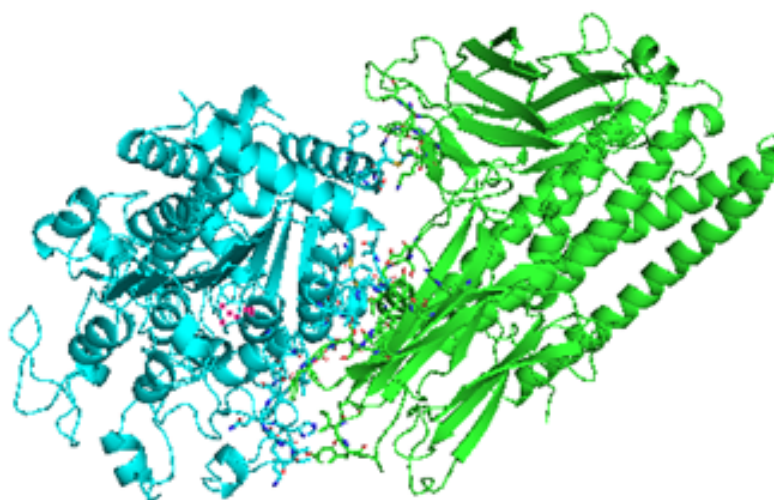


Figure 5.14. Image of docking structure of Cry4Ba (green) and MemALP (blue).

Residue-specific interactions included hydrogen bonds between Thr119 of Mem ALP and Phe364 of Cry4Ba and His490 of MemALP interacting with both Ser362 and Ile358 of Cry4Ba. Additional hydrogen bonds were observed between Lys47 of MemALP and Thr512 of Cry4Ba, and Thr133 of MemALP and Tyr332 of Cry4Ba.

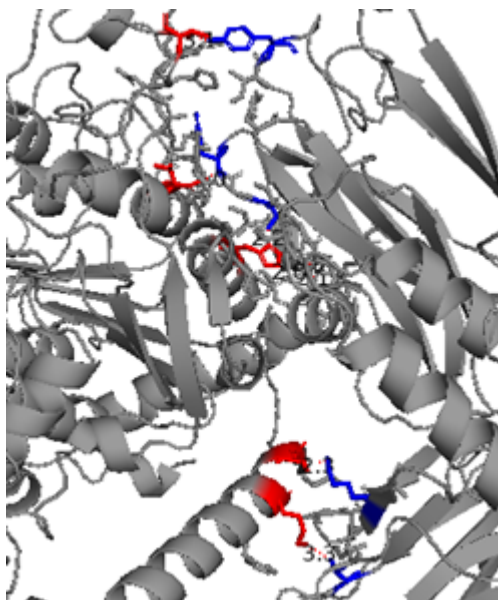


Figure 5.15. Hydrogen bond formation between Cry4Ba (Blue) and MemALP (red).

A salt bridge was identified between Val501 of MemALP and Lys342 of Cry4Ba, as highlighted in red. These interactions highlight the critical roles of hydrogen bonds and salt bridges in stabilizing the MemALP-Cry4Ba complex.

5.7. AMN and Cry4Ba Interactions

The docking analysis of Cry4Ba and AMN provided insights into their binding energies and structural properties. The docking results revealed an electrostatic energy (Ele) of -33.396, a desolvation energy (Desolv) of -16.810, and a Van der Waals energy (VDW) of 38.865. The total binding energy was calculated as -46.319, indicating strong receptor-toxin binding. (Figure 5.16 and Figure 5.17).

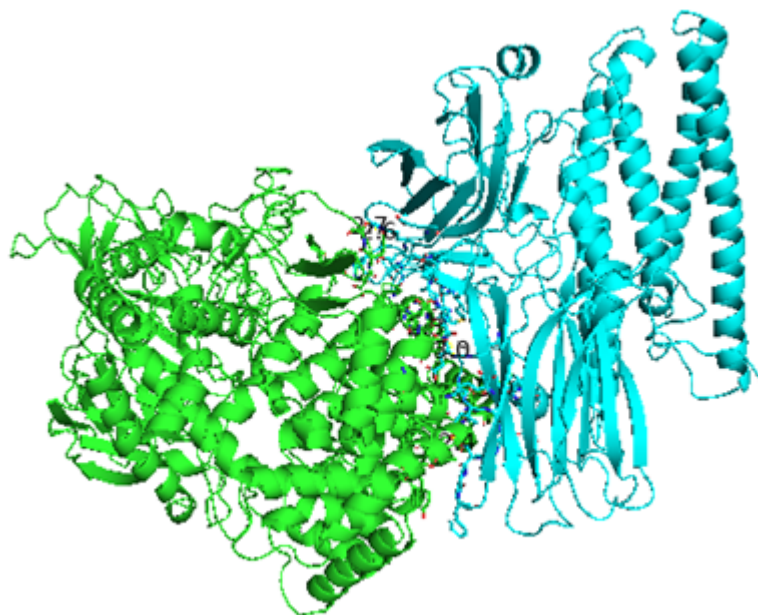


Figure 5.16. Image of docking structure of Cry4Ba (green) and AMN (blue).

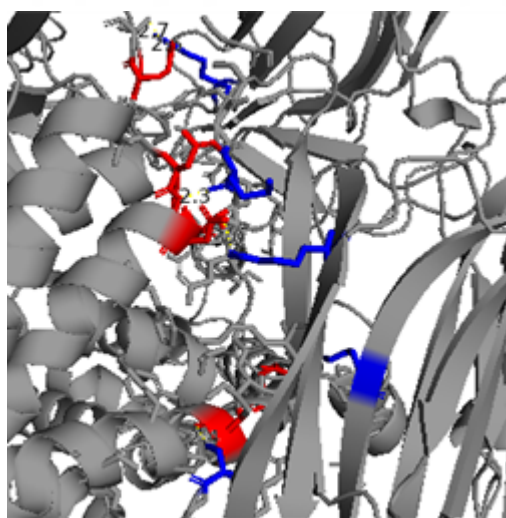


Figure 5.17. Hydrogen bond formation between Cry4Ba (Blue) and AMN (red).

Residue-specific interactions included hydrogen bonds between Asp638 of AMN and Met353 of Cry4Ba, as well as another hydrogen bond involving Ile639 of AMN and Met353 of Cry4Ba. Additional interactions included a hydrogen bond between Ala683 of AMN and Ser362 of Cry4Ba.

Salt bridges were observed between Asp640 of AMN and Arg323, Glu678 of AMN and Lys449, and Glu534 of AMN and Arg520, all highlighted in red.

5.8. Maltase and Cry4Ba Interactions

The docking simulations of Cry4Ba and Maltase revealed detailed binding energy components and residue-specific interactions. The docking results revealed an electrostatic energy (Ele) of -35.789, a desolvation energy (Desolv) of -4.278, and a Van der Waals energy (VDW) of 2.744. The total binding energy was calculated as -39.793, indicating a moderately stable interaction. (Figure 5.18 and Figure 5.19).

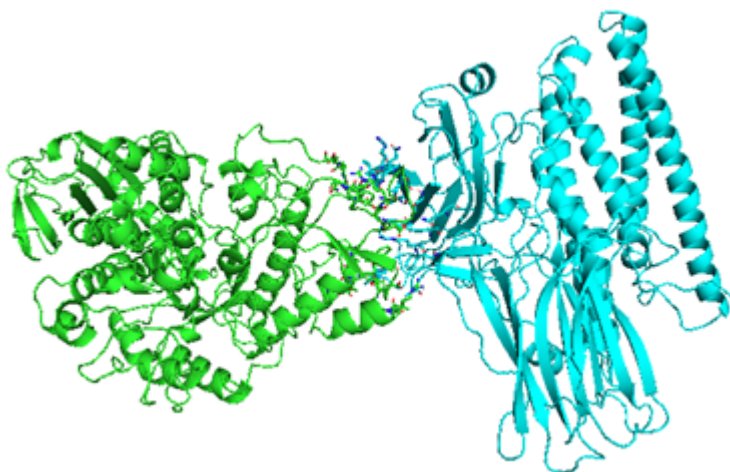


Figure 5.18. Image of docking structure of Cry4Ba (green) and Maltase (blue).

Residue-specific interactions were observed, with one involving a hydrogen bond between Asp248 of Maltase and Ser561 of Cry4Ba, highlighting the critical role of these residues in stabilizing the interaction. Similarly, another hydrogen bond was identified between Glu247 of Maltase and Gln613 of Cry4Ba, further emphasizing the importance of specific residue interactions in mediating the binding affinity. In addition to the hydrogen bonds, two salt bridges were also identified, further contributing to the stability of the interactions.

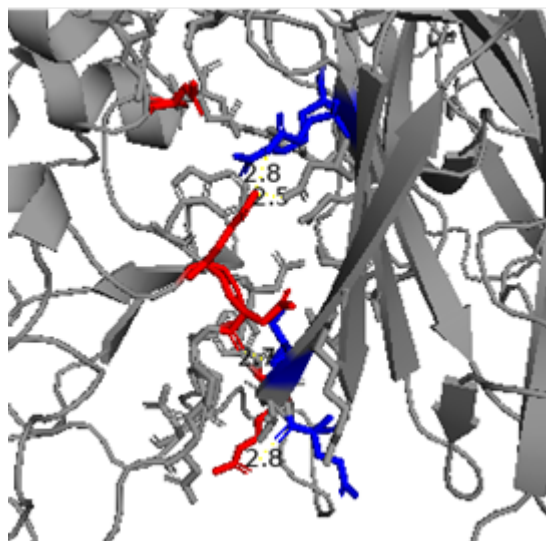


Figure 5.19. Hydrogen bond formation between Asn375 of Cry4Ba (Blue) and Maltase (red).

Salt bridges were identified between Asp157 of Maltase and Arg520, and between Arg165 of Maltase and Gln558, both highlighted in red. These interactions demonstrate the combined roles of hydrogen bonds and ionic interactions in stabilizing the Maltase-Cry4Ba complex.

5.9. Prohibitin and Cry4Ba Interactions

The interactions between Cry4Ba and prohibitin were analyzed using the PyDock server, yielding insights into their binding energies and structural features. The docking results revealed an electrostatic energy (Ele) component of -19.494, a desolvation energy (Desolv) component of -32.938, and a Van der Waals energy (VDW) component (weighted by default at 0.1) of 79.714. The total energy score for the first-ranked docked structure was calculated as -44.460, which suggests a moderately stable interaction. The top-ranked docking model was subsequently visualized and analyzed using PyMOL (Figure 5.20 and Figure 5.21).

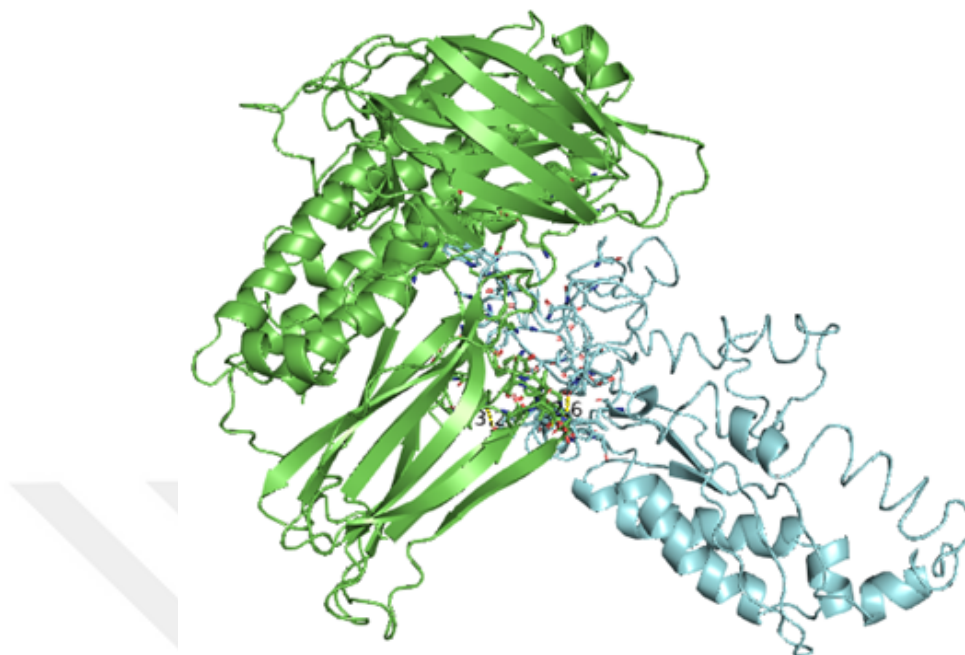


Figure 5.20. Image of docking structure of Cry4Ba (green) and prohibitin (blue).

A single hydrogen bond was identified between Cry4Ba and prohibitin. This interaction involved Asn375 in domain III of Cry4Ba and Gly229 of prohibitin, highlighting a specific interaction site that could contribute to the stability and specificity of the binding. Docking analysis predicted one primary binding site, with Asn375 of Cry4Ba playing a critical role. This residue is situated in domain III, which has been previously implicated as the receptor-binding cavity of Cry toxins.

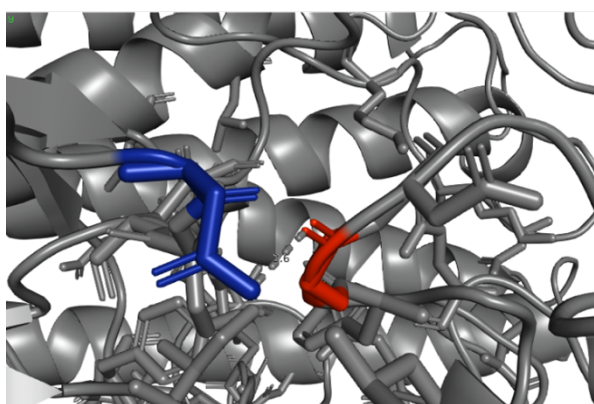


Figure 5.21. Hydrogen bond formation between Cry4Ba (Blue) and prohibitin (red).

5.10. Flotilin and Cry4Ba Interactions

Cry4Ba and flotilin interactions were obtained with docking by PyDock server. As a result, Ele: -19.617, Desolv: -27.174, VDW: 56.872 values were obtained, and the first ranked docked structure was used with -41.104 total energy. The structure was visualized and measured by pymol (Figure 5.22 and Figure 5.23).

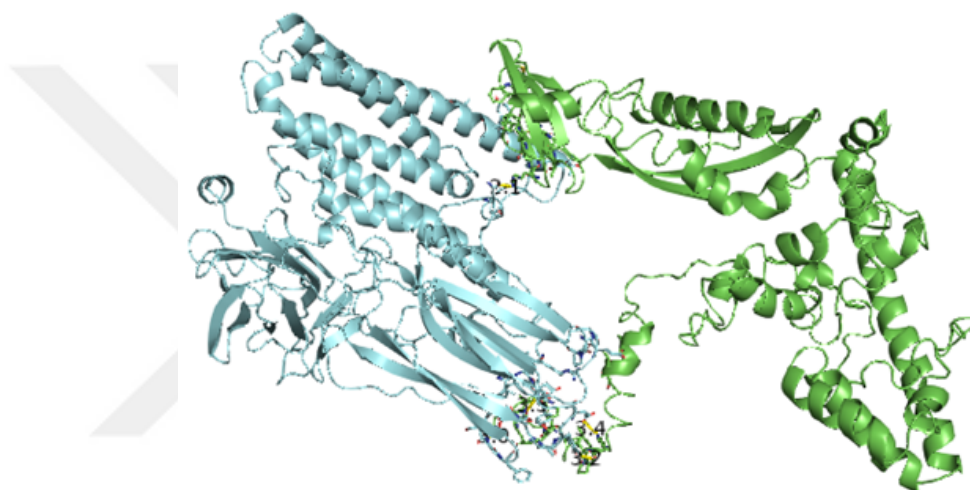


Figure 5.22. Image of docking structure of Cry4Ba and flotillin (Whole view).

Three hydrogen bonds and one salt bridges were predicted as Cry4Ba and flotillin interactions. Then the residues were further analyzed by Pymol to check their positions and distances. Four binding sites were predicted by docking analysis. Asp418, Gly426 and Lys 406 of flotilin were predicted to interact with Asn 456, Ser361 and Tyr332 of Cry4Ba by hydrogen bonds, respectively. These interactions suggest a specific binding mechanism that facilitates the functional engagement of Cry4Ba with flotillin. Moreover, the predicted distances between interacting residues were within the optimal range for stable molecular interactions, highlighting the structural compatibility of the binding sites.

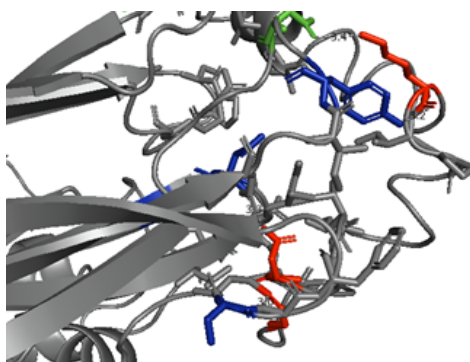


Figure 5.23. Hydrogen bond formation between Cry4Ba residues (Blue), flotillin residues (red) and Salt bridges (Green).

Lys406 and Asp334 residues of receptor and toxin were interacted with salt bridges, respectively.

5.11. Predicted Interactions of *Ae. Albopictus* Receptors with Cry11Aa

The structure of full-length Cry11Aa (72 kD) was created by the Robetta homology modelling tool and minimized to obtain energetically favorable conformation (Figure 5.24).

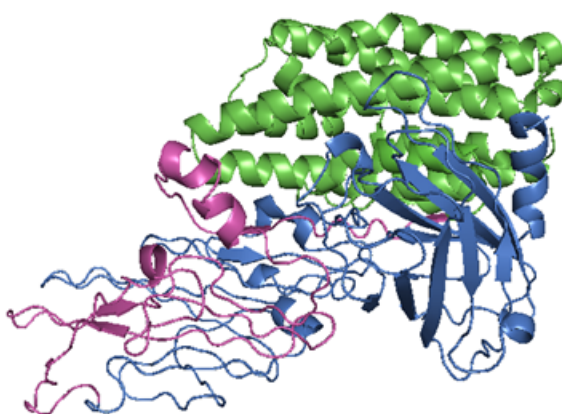


Figure 5.24. Ribbon diagrams of the Cry11Aa structure.

Overview with predicted domains I, II and III colored green, blue, and pink, respectively. Domain I, made of residues 1–246, is an α -helical bundle of five helices.

Domain II is a prism of three antiparallel b-sheets made of residues 247-360 Domain III is a b-sandwich made of 361-645. Full-length Cry11Aa was digested with trypsin and obtained two fragments. 32 kDa fragment was modelled and used for docking structures according to trypsin activation. Full length molecule was first cleaved between Leu9 and Ser10 and between Phe348 and Tyr349, generating a 36-kDa C-terminal fragment and a 40-kDa N-terminal fragment. C-terminal fragment was further processed into 32-kDa fragment and 40-kDa fragment was processed into 34 kDa fragment.

Table 5.2. Interacted residues of *A. albopictus* receptors with active Cry11Aa (32 kDa) toxins. (Underlined residues are salt bridges, remaining contacts are hydrogen bonds).

Receptors	Receptor Residues	Cry11Aa-T Residues
ALP	Glu361, Ser20, Met1, <u>Asp23</u> , <u>Arg54</u> , <u>Glu361</u>	Lys388, Asp513, Asp446, <u>Arg552</u> , <u>Asp446</u> , <u>Lys388</u>
memALP	His366, Asn2, Asn7	Asp446, Asp510, Tyr443
V-ATP	Glu328, Thr315, Tyr314, Gln27	Gln640, Asp513, Glu586, Leu447, Asp446
AMN	Thr706, Val748	Ser491, Leu644
Prohibitin	Arg41	Ala500
Flotilin	Gln42, Ala32	Asn367, Val387
Maltase	Trp9, Ser663, Ser663, His634, His634, Gly637, <u>Arg10</u> , <u>His634</u>	Ala442, Asp513, Glu586, Asn582, Thr58, Leu644, <u>Asp446</u> , <u>Asp589</u>

5.12. ALP and Cry11Aa Interactions

The interactions between Cry11Aa and ALP were examined using docking simulations, providing insights into their binding dynamics and structural properties. The docking results revealed an electrostatic energy (Ele) of -25.071, a desolvation energy (Desolv) of -44.044, and a Van der Waals energy (VDW) of 83.365. The total binding

energy was calculated as -60.779, indicating a highly stable interaction.

Multiple hydrogen bonds and salt bridges were residue-specific interactions, highlighting the stability of the ALP-Cry11Aa complex (Figure 5.25 and Figure 5.26).

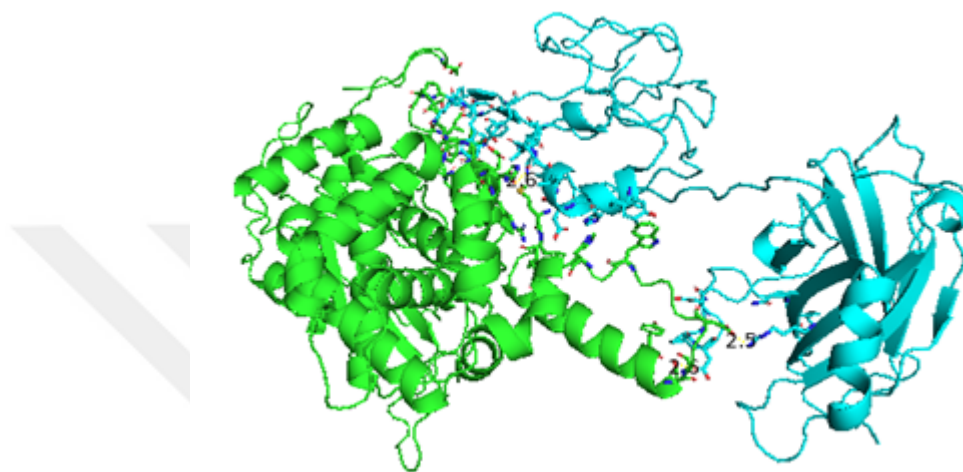


Figure 5.25. Image of docking structure of Cry11Aa (green) and ALP (blue).

Hydrogen bonds were observed between SER20 of ALP and ASP513 of Cry11Aa, as well as between MET1 of ALP and ASP446 of Cry11Aa. Furthermore, salt bridges were identified between ASP23 of ALP and ARG552, and between ARG54 of ALP and ASP446, both highlighted in red. An additional salt bridge was noted between Glu361 of ALP and Lys388 of Cry11Aa, further emphasizing the significant contribution of ionic interactions to the overall binding stability.

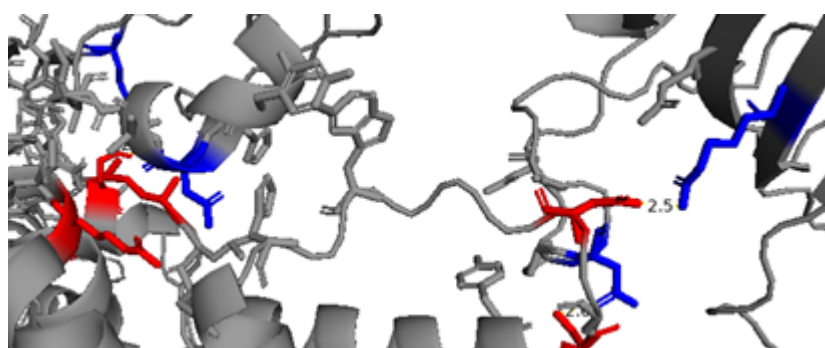


Figure 5.26. Hydrogen bond formation between Cry4Ba (Blue) and ALP (red).

These interactions demonstrate a complex interplay of hydrogen bonds and salt bridges, contributing to a highly stable receptor-toxin interface. The involvement of critical residues such as Glu361 and Lys388 in forming ionic interactions underscores their importance in maintaining the specificity and strength of the binding. This level of molecular detail provides valuable insights into the structural basis for the strong affinity between Cry11Aa and ALP (Figure 5.26).

5.13. MemALP and Cry11Aa Interactions

The docking simulations for Cry11Aa and MemALP revealed detailed residue-specific binding energy components. The docking results showed an electrostatic energy (Ele) of -21.987, a desolvation energy (Desolv) of -37.265, and a Van der Waals energy (VDW) of 21.185. The total binding energy was calculated as -57.134, indicating a strong receptor-toxin interaction (Figure 5.27 and Figure 5.28)

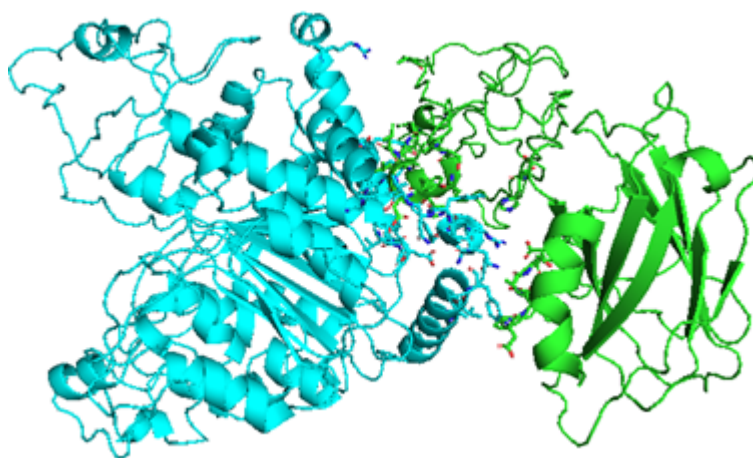


Figure 5.27. Image of docking structure of Cry11Aa (green) and MemALP (blue).

Residue-specific interactions included multiple hydrogen bonds that contribute significantly to the stability of the MemALP-Cry11Aa complex. Hydrogen bonds were identified between His366 of MemALP and Asp446 of Cry11Aa. Moreover, Asn2 of MemALP formed a hydrogen bond with Asp510 of Cry11Aa, while Asn7 of MemALP

established a bond with Tyr443 of Cry11Aa.

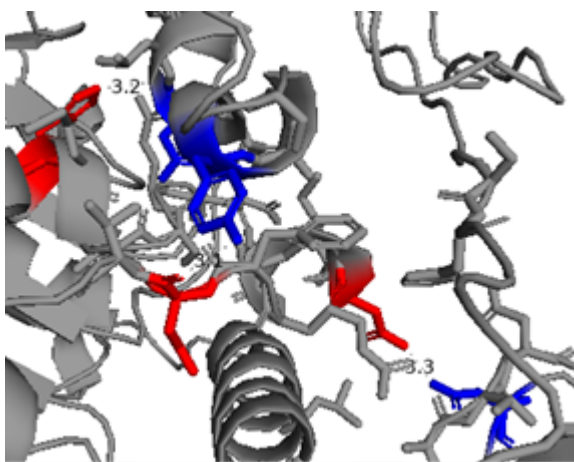


Figure 5.28. Hydrogen bond formation between Cry4Ba (Blue) and MemALP (red).

These detailed residue-level interactions emphasize the critical role of specific amino acids in maintaining the strength and specificity of the binding. The involvement of multiple hydrogen bonds across diverse residues demonstrates the complex nature of this interaction, highlighting its structural and functional stability.

5.14. V-ATPase and Cry11Aa Interactions

The docking simulations of Cry11Aa and VATP revealed detailed residue-specific binding dynamics. The docking results showed an electrostatic energy (Ele) of -16.306, a desolvation energy (Desolv) of -39.909, and a Van der Waals energy (VDW) of -1.412. The total binding energy for the top-ranked docking model was calculated as -56.356, indicating a moderately strong interaction between VATP and Cry11Aa. (Figure 5.29 and Figure 5.30) Residue-specific interactions involved multiple hydrogen bonds, which are critical in stabilizing the VATP-Cry11Aa complex. A key hydrogen bond was observed between Glu328 of VATP and Gln640 of Cry11Aa. Additional interactions included hydrogen bonds between Thr315 of VATP and Asp513, as well as Thr315 of VATP and Glu586 of Cry11Aa. Another interaction involved Tyr314 of VATP forming a hydrogen bond with Glu586 of Cry11Aa. Furthermore, Gln27 of

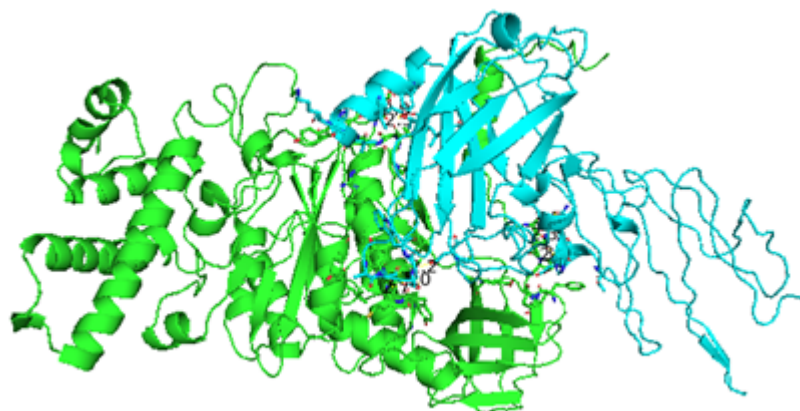


Figure 5.29. Image of docking structure of Cry11Aa (green) and V-ATPase (blue).

VATP established bonds with both Leu447 and Asp446 of Cry11Aa. These extensive residue-level interactions underscore the critical role of VATP in Cry11Aa binding.

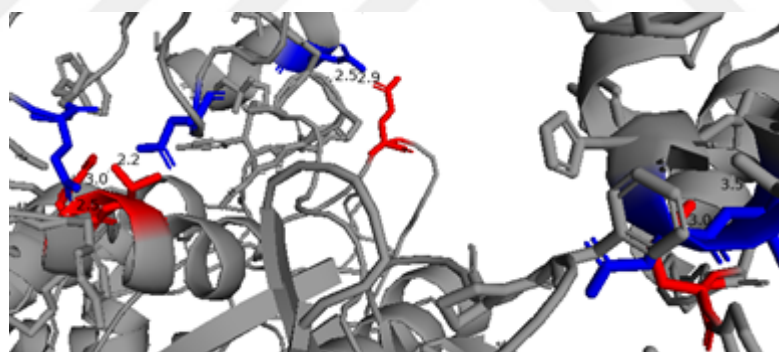


Figure 5.30. Hydrogen bond formation between Cry4Ba (Blue) and V-ATPase (red).

The involvement of residues such as Gln27, Thr315, and Tyr314 highlights the complexity of the binding mechanism, where specific amino acid interactions contribute to the structural stability and specificity of the receptor-toxin interface.

5.15. AMN and Cry11Aa Interactions

The docking analysis of Cry11Aa and AMN revealed detailed residue-level interactions and binding dynamics. The docking results showed an electrostatic energy

(Ele) of -16.264, a desolvation energy (Desolv) of -33.650, and a Van der Waals energy (VDW) of 12.066. The total binding energy was calculated as -48.708, indicating moderately strong binding.(Figure 5.31 and Figure 5.32).

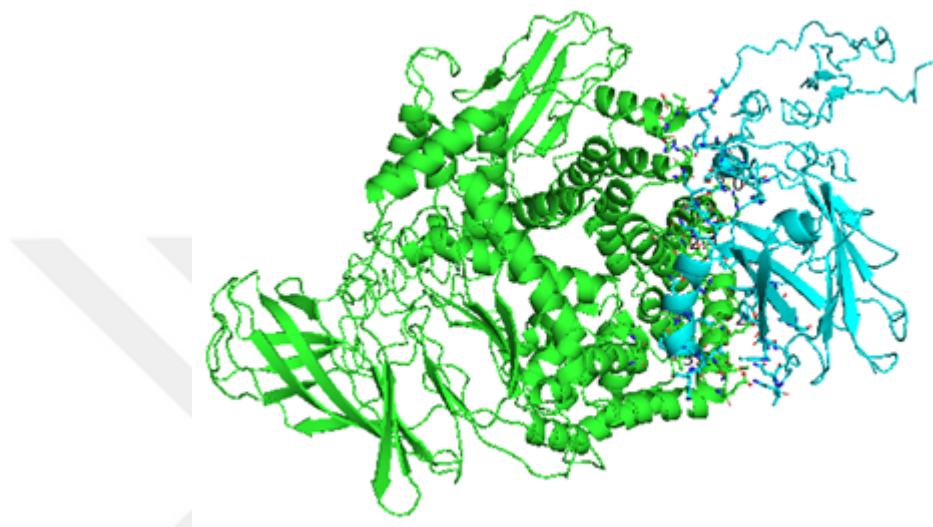


Figure 5.31. Image of docking structure of Cry11Aa (green) and prohibitin (blue).

Residue-specific interactions, including key hydrogen bonds and salt bridges, contributed to the stability of the AMN-Cry11Aa complex.

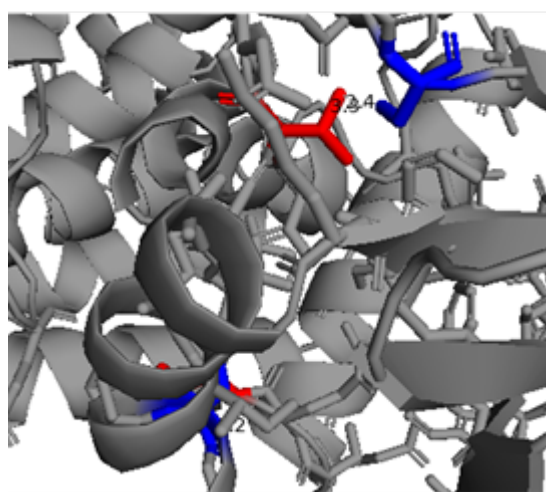


Figure 5.32. Hydrogen bond formation between Cry4Ba (Blue) and AMN (red).

Hydrogen bonds were observed between Thr706 of AMN and Ser491 of Cry11Aa, as well as between Val748 of AMN and Leu644 of Cry11Aa. These interactions highlight the role of specific residues in ensuring the structural stability of the complex. The diversity of residue-level interactions underscores the robust nature of the binding between AMN and Cry11Aa.

5.16. Maltase and Cry11Aa Interactions

The docking simulations of Cry11Aa and Maltase revealed intricate residue-specific interactions that contribute to binding stability. The docking results showed an electrostatic energy (Ele) of -32.195, a desolvation energy (Desolv) of -17.242, and a Van der Waals energy (VDW) of -2.584. The total binding energy was calculated as -49.696, indicating a stable receptor-toxin interaction (Figure 5.33 and Figure 5.34).



Figure 5.33. Image of docking structure of Cry11Aa (green) and Maltase (blue).

Residue-specific interactions included multiple hydrogen bonds and salt bridges that stabilize the Maltase-Cry11Aa complex. Hydrogen bonds were identified between Trp9 of Maltase and Ala442 of Cry11Aa, as well as between Ser663 of Maltase and both Asp513 and Glu586 of Cry11Aa. Additionally, His634 of Maltase formed hydrogen bonds with Asn582 and Thr58 of Cry11Aa, while Gly637 of Maltase interacted with Leu644 of Cry11Aa. Two salt bridges were also observed: one between Arg10 of Maltase and Asp446 of Cry11Aa, and another between His634 of Maltase and Asp589

of Cry11Aa, both highlighted in red.

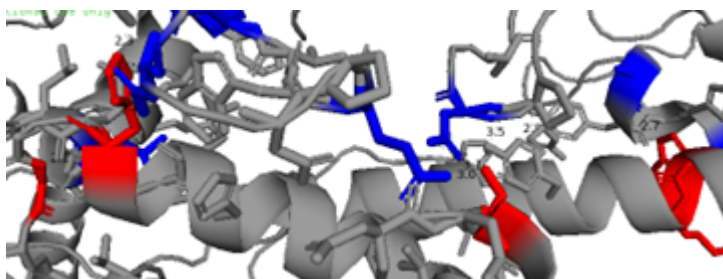


Figure 5.34. Hydrogen bond formation between Cry4Ba (Blue) and Maltase (red).

5.17. Prohibitin and Cry11Aa Interactions

The docking simulations of Cry11Aa and prohibitin revealed detailed residue-specific binding dynamics. The docking results showed an electrostatic energy (Ele) of -10.524, a desolvation energy (Desolv) of -47.178, and a Van der Waals energy (VDW) of 11.779. The total binding energy for the top-ranked docking model was calculated as -56.524, indicating a strong interaction between Cry11Aa and prohibitin (Figure 5.35 and Figure 5.36). Specific interactions were crucial in stabilizing the Cry11Aa-prohibitin complex.

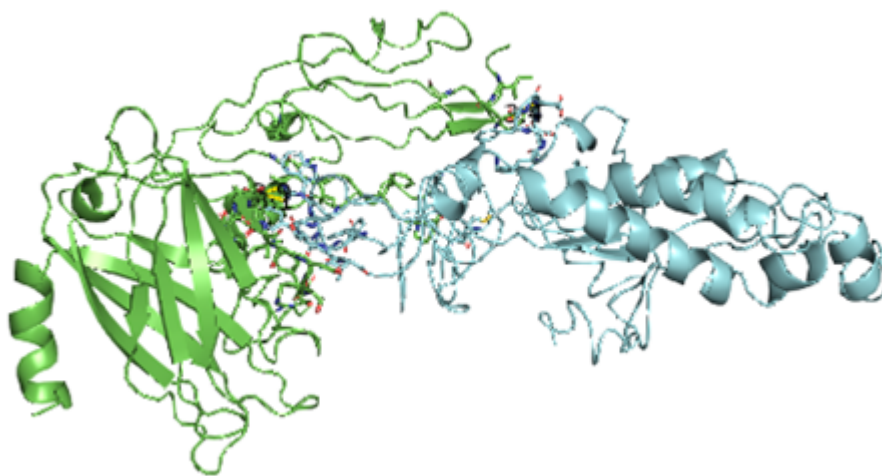


Figure 5.35. Image of docking structure of Cry11Aa (green) and prohibitin (blue).

Notably, a hydrogen bond was formed between Arg41 of prohibitin and Ala500 of Cry11Aa, which plays a key role in maintaining the interaction's structural integrity.

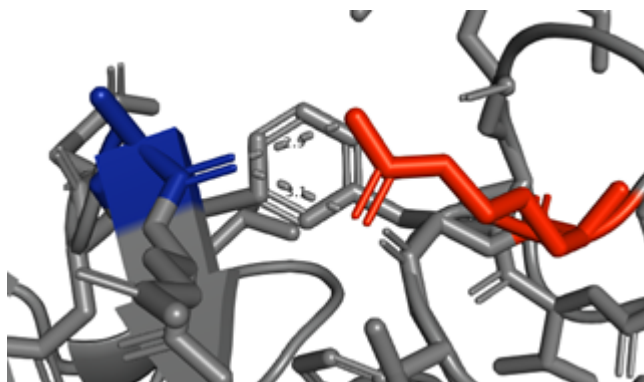


Figure 5.36. Hydrogen bond formation between Cry4Ba (Blue) and prohibitin (red).

These results highlight the importance of specific amino acid interactions in the binding and stability of the Cry11Aa-prohibitin complex.

5.18. Flotillin and Cry11Aa Interactions

The docking simulations of Cry11Aa and flotillin revealed detailed residue-specific binding dynamics. The docking results showed an electrostatic energy (Ele) of -7.860, a desolvation energy (Desolv) of -55.033, and a Van der Waals energy (VDW) of 54.424. The total binding energy for the top-ranked docking model was calculated as -57.451, indicating a strong interaction between Cry11Aa and flotillin (Figure 5.37 and Figure 5.38). Residue-specific interactions were crucial in stabilizing the Cry11Aa-flotillin complex. Notably, a hydrogen bond was formed between Gln42 of flotillin and Asn367 of Cry11Aa, while another hydrogen bond was observed between Ala32 of flotillin and Val387 of Cry11Aa. These interactions significantly contribute to maintaining the structural stability of the complex. These findings underscore the importance of specific amino acid interactions in the binding and stability of the Cry11Aa-flotillin complex. These findings underscore the importance of specific amino acid interactions in the binding and stability of the Cry11Aa-flotillin complex. Such interactions provide insights into the molecular basis of protein-protein recognition. Understanding these

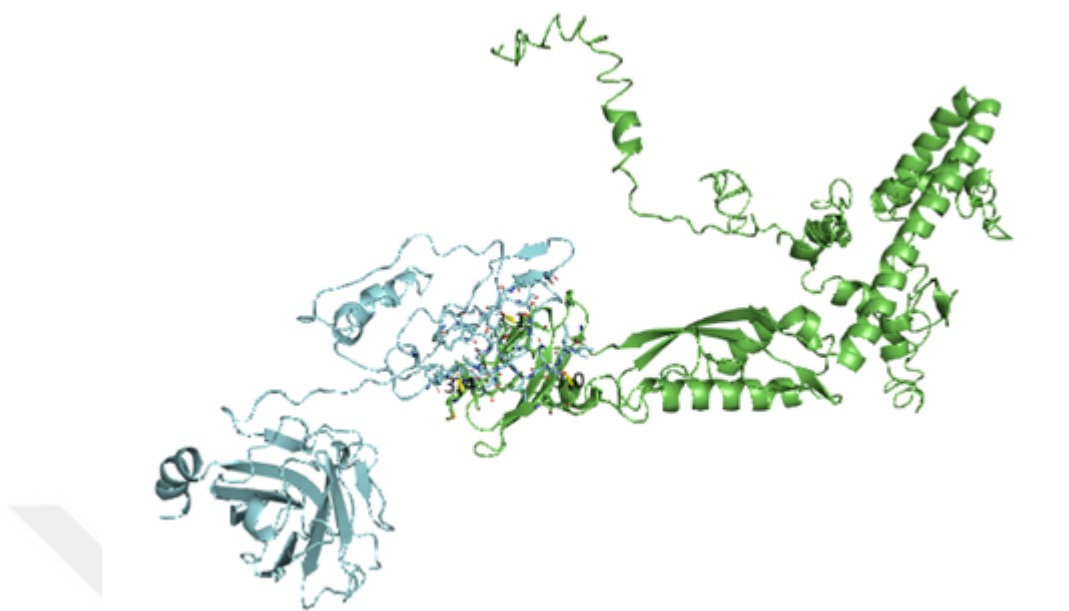


Figure 5.37. Image of docking structure of Cry11Aa (blue) and prohibitin (green).

stabilizing factors can aid in the design of targeted interventions or protein engineering efforts.

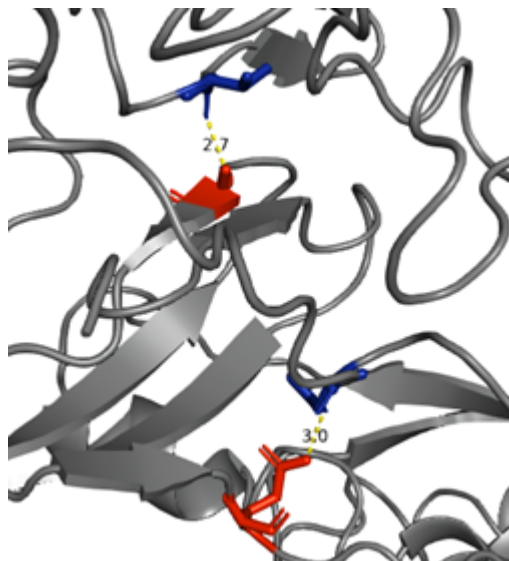


Figure 5.38. Hydrogen bond formation between Cry11Aa (Blue) and flotilin (red).

Table 5.3. Number of interactions between receptors and toxins obtained using PDBsum-generate from docking structures.

Receptors	Predicted Interactions with Cry4Ba			Predicted Interactions with Activated Cry11Aa		
	Salt Bridges	H-Bonds	Other Contacts	Salt Bridges	H-Bonds	Other Contacts
ALP-like	-	2	127	3	3	229
MemALP	-	8	172	-	3	181
V-ATP	-	-	204	-	6	200
AMP	3	5	262	-	2	269
Prohibitin	-	1	193	-	2	136
Flotilin	1	3	207	-	2	178
Maltase	1	3	158	2	6	240

5.19. Cytotoxicity Assay

Cytotoxicity assay of the receptors was performed separately by using activated Cry4Ba and Cry11Aa. The receptors of *A. albopictus* were expressed in HEK-293 cells separately and XTT test was performed on the HEK-293 cell line treated with 100 ng/ml Cry4Ba-T or Cry11Aa-T (Figure 43 and Figure 44). Alkaline phosphatase (ALP), membrane-bound ALP (memALP), and V-ATPase subunit B (V-ATP) expressed cell viabilities were decreased by Cry4Ba and Cry11Aa treatments separately. Toxin treatments did not significantly affect AMN, prohibitin, flotillin, lachesin, maltase and Calcium transport ATPase (Calc) receptor-expressed cells. Negative control (only pEGFP-N1 expressed cells) showed no toxicity for 100 ng/ml Cry4Ba-T and Cry11Aa-T treatments.

The lowest cell viability was obtained with V-ATP receptor expressions, at 66.32% viability with Cry4Ba-T treatment. ALP and memALP-expressed cells also had lower cell viability than the pEGFP-N1-expressed cell control (78.34% and 75.42%, respectively). AMN, Proh, Flot, Calc, and Malt expressions resulted in 91.02%, 89.46, 94.23%, 88.92%, and 92.32%, respectively. However, these changes were not significant. when compared to pEGFP-N1 expressed control cells ($p < 0.05$) (Figure 5.39).

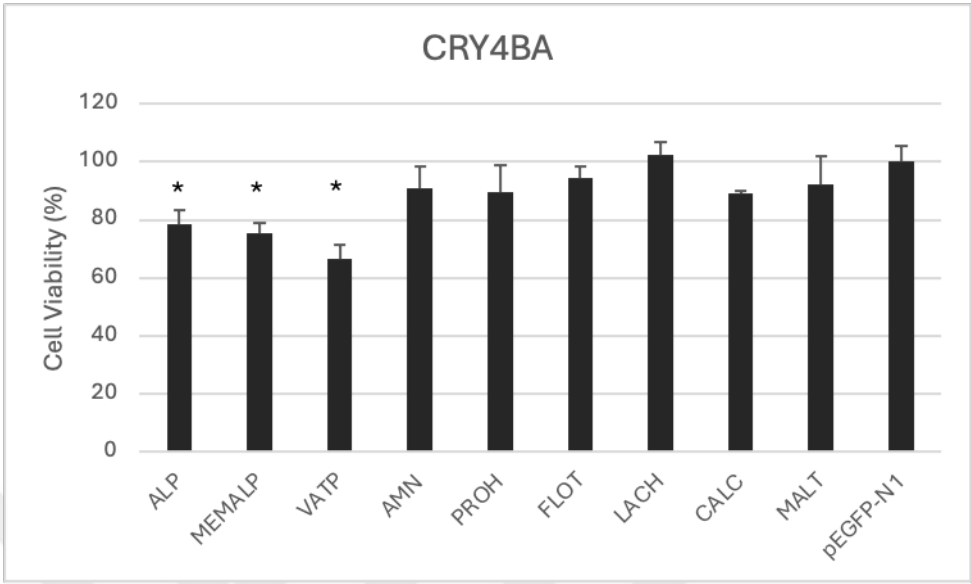


Figure 5.39. XTT cytotoxicity assay of activated Cry4Ba toxin on HEK-293 cells expressed receptors of *A. albopictus* separately. Statistically significant changes are indicated by an asterisk ($P<0.05$).

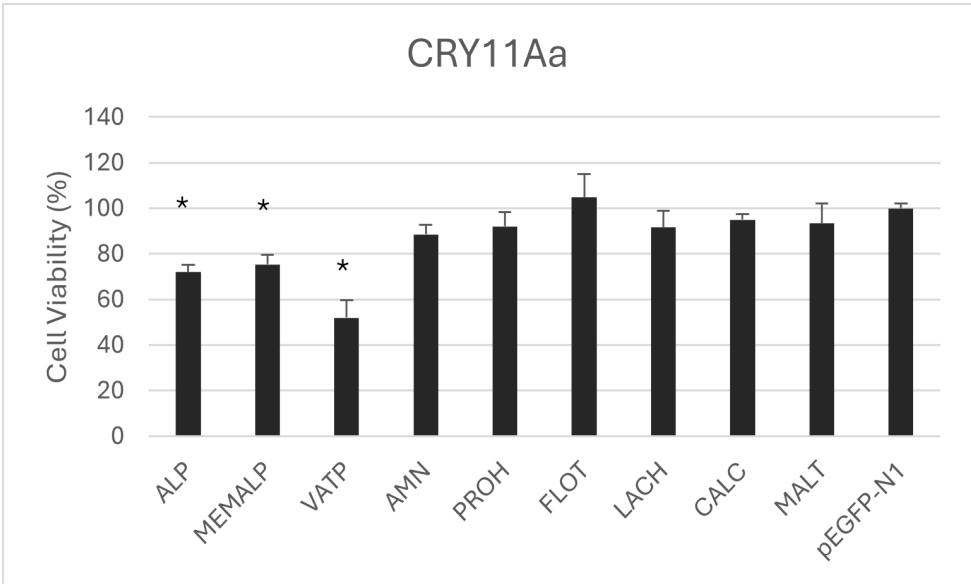


Figure 5.40. XTT cytotoxicity assay of activated Cry11Aa toxin on HEK-293 cells expressed receptors of *A. albopictus* separately. Statistically significant changes are indicated by an asterisk ($P<0.05$).

Cry11Aa treatment resulted in the lowest cell viability with V-ATPase subunit B expressed cells at 52,04% viability ($p < 0.01$). Cell viabilities of ALP and memALP expressed cells resulted decrease in cell viabilities at 72,09% and 75,44%, respectively ($p < 0.05$). AMN, Proh, Flot, Calc, and Malt expressions resulted in 88,53%, 92,06, 104,87%, 95% and 93,44% respectively. However, these changes were not significant when compared to pEGFP-N1 expressed control cells ($p < 0.05$) (Figure 5.40). The XTT cytotoxicity assay was performed in triplicate for biological replicates and in quadruplicate for technical replicates to ensure reliable results.

5.20. Larval Bioassay with Toxins and Quantitative PCR

Purified Cry4Ba protoxin was applied to 4th-star larvae in protoxin form for 24 hours. The receptor expression levels were analyzed by Q-PCR (Figure 5.41).

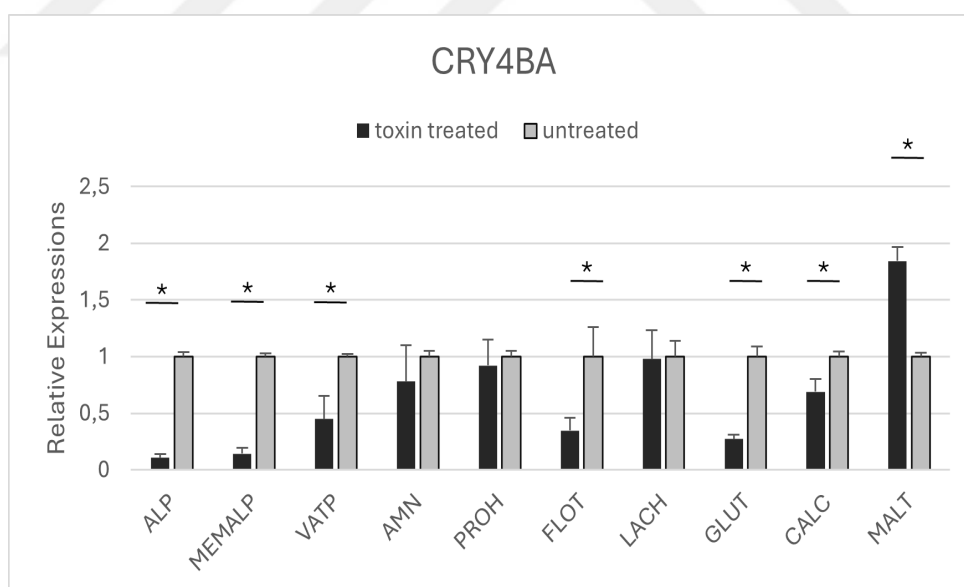


Figure 5.41. Relative expression levels of receptors treated with Cry4Ba toxins.

Statistically significant changes are indicated by an asterisk ($P < 0.05$).

AMN, Prohibitin and Lachesin gene expression levels were not affected by Cry4Ba treatment. ALP, memALP, V-ATP, flotilin, glutamyl aminopeptidase and Calc expression levels were decreased with Cry4Ba treatment (0.11, 0.14, 0.45, 0.34, 0.27 and 0.69

fold decrease, respectively). Only the Maltase receptor expression level was increased with Cry4Ba treatment by 1.84 fold ($p < 0.05$) (Figure 5.41). EF-1 gene expression level was used as a housekeeping gene, and relative expression levels of receptor genes were compared with the toxin untreated control group.

Purified Cry11Aa protoxin was applied to 4thstar larvae, and expression levels of the receptors were analyzed by Q-PCR (Figure 5.42).

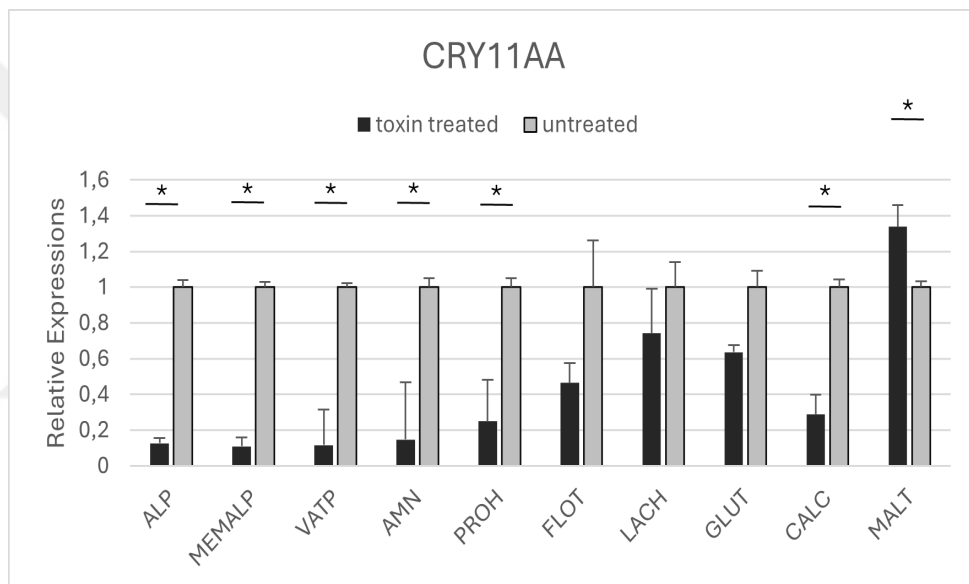


Figure 5.42. Relative expression levels of receptors treated with Cry11Aa toxins.

Statistically significant changes are indicated by an asterisk ($P < 0.05$).

ALP, memALP, V-ATP, AMN, Prohibitin, and Calc expressions were decreased with Cry11Aa treatment (0.12, 0.11, 0.12, 0.15, 0.25, 0.29 fold decrease, respectively). ($p < 0.05$). Only the Maltase receptor expression level was increased with Cry11Aa treatment by 1.34 fold (Figure 5.42). EF-1 gene expression level was used as a housekeeping gene, and relative expression levels of receptor genes were compared with those of the untreated control group of toxins. For the Q-PCR experiments, each test group consisted of 30 larvae, and a minimum of three biological replicates were performed. The cDNA samples obtained from each group were analyzed with 4–5 technical replicates to ensure accuracy and reproducibility.

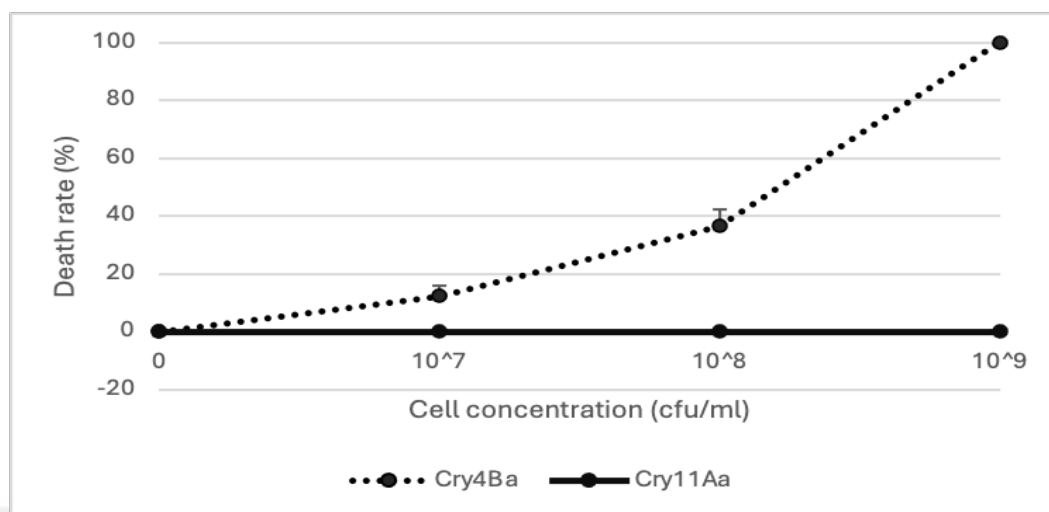


Figure 5.43. Larval toxicity with Cry4Ba and Cry11Aa expressed BL21 cells.

5.21. RNAi Knockdown of Receptor Genes

Larval cytotoxicity values of Cry4Ba and Cry11Aa-expressed BL21 cells were applied to 3rd-instar larvae. The death rate was obtained from 10⁸ cfu/ml Cry4Ba-expressed cells, a 36.6% death rate. This concentration was used for RNAi knockdown experiments on 3rd-instar larvae. Cry11Aa-expressed cells did not result in any larval death at all cell concentrations (Figure 5.43).

Purified Cry4Ba and Cry11Aa toxins were applied to 3rd instar larvae at different concentrations. For knockdown experiments, a death rate close to 50% was used. 100 ng/ml Cry4Ba and Cry11Aa protoxin treatments resulted in a death rate of 72.1% and 69.7%, respectively (Figure 5.44). PCR primers containing the T7 promoter region on sense and antisense DNA produced templates for dsRNA reactions. PCR products at the expected size were excised and purified from an agarose gel (Figure 5.45).

The expected product size from lane 1 to lane 10: 626 bp, 628 bp, 561 bp, 641 bp, 600 bp, 580 bp, 604bp, 611 bp, 583 bp, 612 bp, respectively). 1 µg of purified dsRNA templates were used for *in vitro* dsRNA synthesis. Expected dsRNA bands (650-750 bp) were visualised by 1.5% agarose gel (Figure 5.46), and 50 µg/ml concentrations were used for RNAi knockdown experiments. dsRNA products contained two bands,

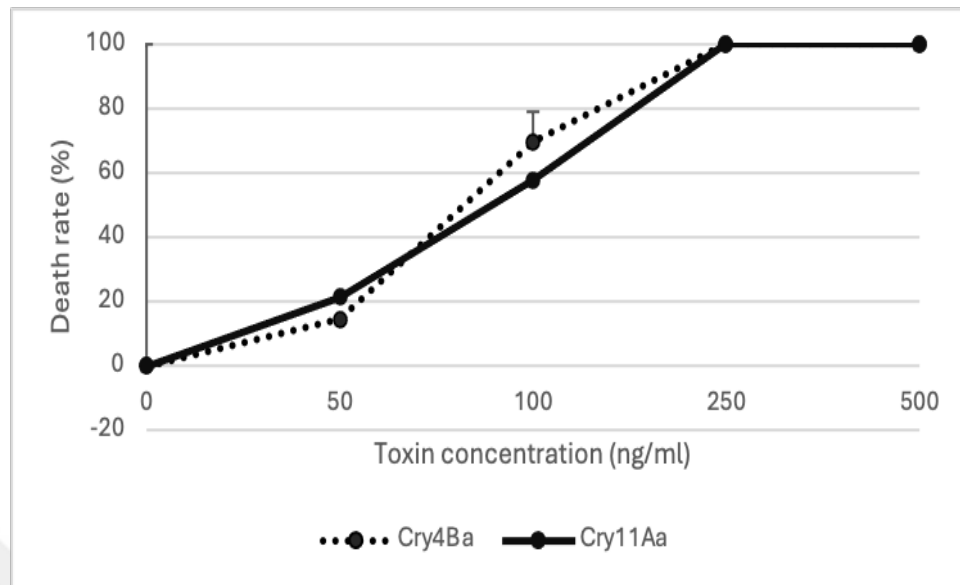


Figure 5.44. Larval toxicity with purified Cry4Ba and Cry11Aa protoxins.

faint or strong, around 400 bp, proportional to the dsRNA product size (Figure 5.46). Each receptor dsRNA was applied to 1st instar larvae for 5 hours separately.

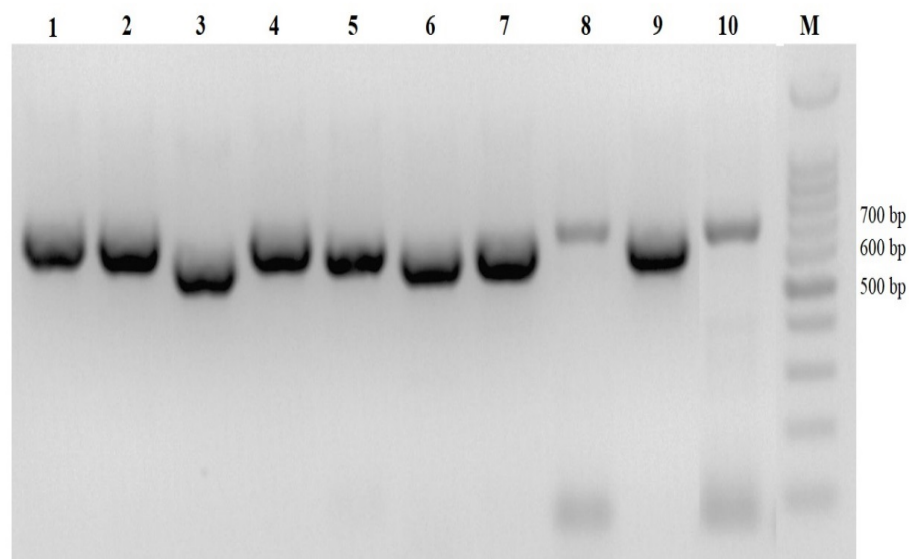


Figure 5.45. PCR templates produced with T7 promotor for *A. albopictus* receptors. (1: ALP, 2: memALP, 3: V-ATPase, 4: AMN, 5: Prohibitin, 6: Flotilin, 7: Lachesin, 8: Glut, 9: Calc and 10: Maltase, M: 100 bp ladder).

After 40 hours, larvae from each treated group were collected, and gene expression levels were analyzed by Q-PCR.

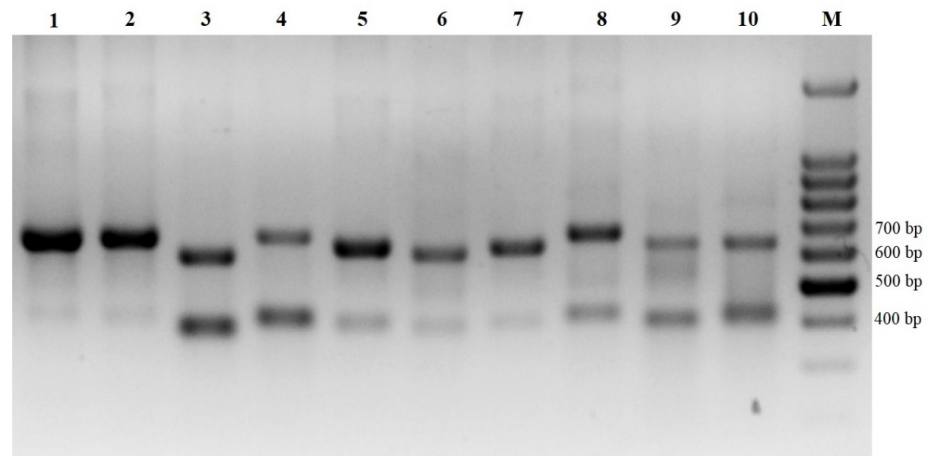


Figure 5.46. dsRNA products obtained by in vitro dsRNA synthesis. (1: ALP, 2: memALP, 3: V-ATPase, 4: AMN, 5: Prohibitin, 6: Flotilin, 7: Lachesin, 8: Glut, 9: Calc and 9: Maltase, M: 100 bp ladder).

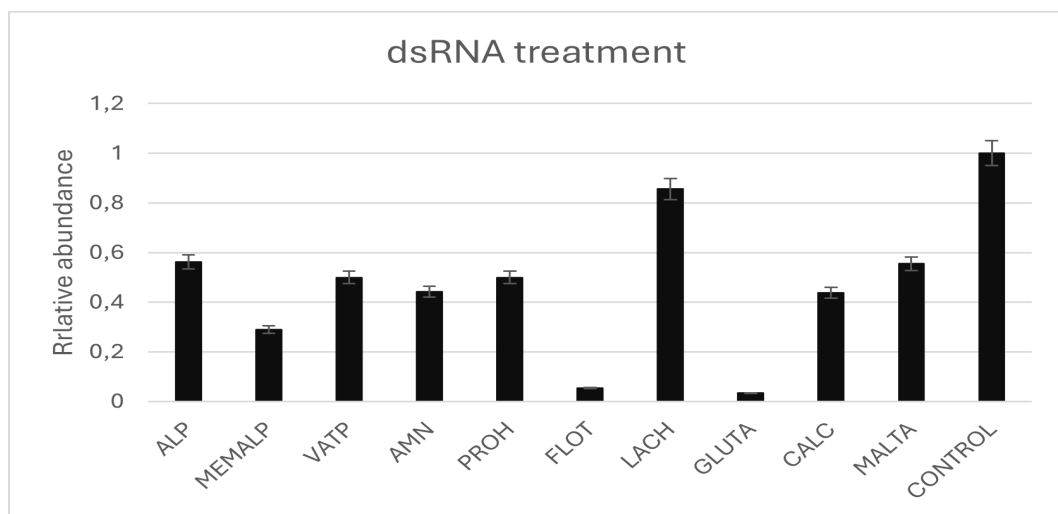


Figure 5.47. Relative expression levels of receptors treated with dsRNA.

Gene expression levels for each receptor dsRNA-treated group were normalized against EF-1 gene expression levels, and a negative control group was incubated with water (Figure 5.47). All receptor genes were silenced by at least a twofold decrease

except Lachesin (0.86 fold decrease). Cry4BA/Cry11Aa toxins were applied to the dsRNA-treated larvae for 24-48 hours, and the larval death percentage was calculated.

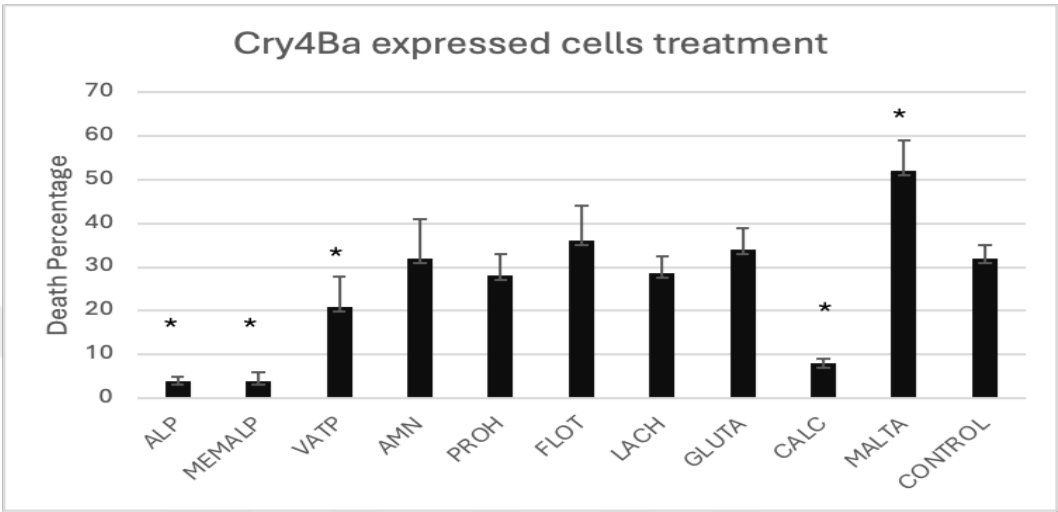


Figure 5.48. Survival rate of knockdown larvae after Cry4Ba treatment. Statistically significant changes are indicated by an asterisk ($P<0.05$).

The larval survival rate was calculated by counting dead larvae after 93 hours from the first larval stage (after 48 hours from Cry4Ba toxin-expressed cell treatment).

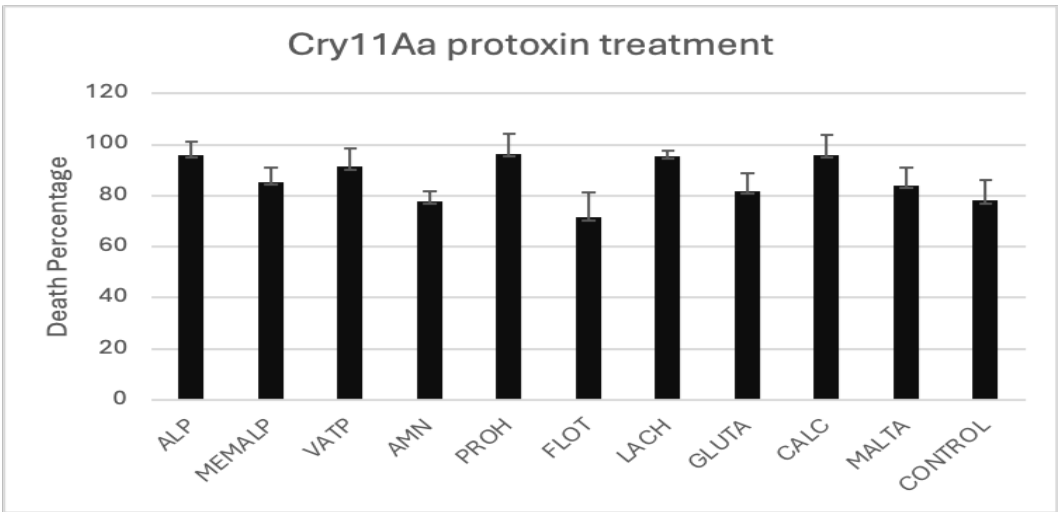


Figure 5.49. Cell viability after knockdown Cry11Aa. Statistically significant changes are indicated by an asterisk ($P<0.05$).

The ALP, memALP, V-ATP, and Calc dsRNA-treated groups had lower larval death rates than the non-dsRNA-treated group (4%, 4%, 21%, and 8%, respectively). On the other hand, the Maltase dsRNA-treated group had higher larval death rates than the control group, with a 52% death rate (Figure 5.48). Since Cry11Aa expressed *E. coli* Rosetta cells did not lead to larval death (Figure 5.43) at 4th instar larvae, Cry11Aa protoxin was applied at 100 ng/ml for 24 hours. The death rate of control groups and dsRNA-treated groups did not result in significant changes ($P \geq 0.05$) (Figure 5.49). For dsDNA applications, each receptor and control group was designed to include 30 larvae with three biological replicates. Relative expression measurements were performed with four technical replicates for each biological replicate to ensure precision and reliability.

6. DISCUSSION

The primary mode of action of *Bacillus thuringiensis israelensis* (Bti) toxins involves interaction with specific membrane receptors located on the epithelial cells of the insect midgut. This interaction facilitates the formation of pores in the apical microvilli membrane, ultimately leading to cell lysis and insect mortality. While this phenomenon is well-characterized in Lepidopteran species, the precise mechanisms in mosquitoes remain poorly understood [60]. This knowledge gap is particularly critical as mosquito populations continue developing resistance to synthetic insecticides, highlighting the importance of environmentally friendly alternatives such as mosquitocidal Bti toxins. These toxins, comprising a mixture of Cry (crystal) and Cyt (cytolytic) proteins, demonstrate high specificity, limited impact on non-target organisms, and a reduced propensity for resistance development [61].

In this study, Cry4Ba and Cry11Aa proteins were studied among other mosquitocidal *bti* toxins since they have comparatively high toxicity against *Aedes* species [62]; their complete 3D structures were elucidated [63], [64] that provide to study interacting ligands *in silico in vitro*, and these dipteran-specific toxins can bind similar receptors as *bti* toxins in lepidoptera [65]. Insecticidal mechanisms of Cry toxins have not been yet fully understood. Three models have been proposed based on existing information to explain Cry toxicity: the classical killing model, sequential binding model and signaling pathway model [66]. The classical model describes Cry protoxins as being ingested as crystalline inclusions and solubilized in the alkaline conditions of the insect midgut. Subsequent proteolytic activation yields smaller, protease-resistant toxin fragments that bind to specific midgut membrane receptors. This binding induces pore formation, creating non-selective ion channels that disrupt nutrient uptake and osmotic balance. Ultimately, these disruptions lead to epithelial cell lysis, midgut disarrangement, and insect death [67]. While this model is widely accepted, evidence suggests that it may not fully account for Cry toxicity, as some studies have reported larvicidal activity even in the absence of pore formation [68]. The sequential binding

model suggests that activated Cry toxins by midgut proteases bind to a transmembrane glycoprotein (e.g. cadherin-like protein) as a receptor. This interaction favors conformational changes, and removal of the -1 helix from domain I of activated toxin facilitates oligomeric pre-pore structure. This structure binds to other receptors, such as alkaline phosphatase, that facilitate localization of pre-pore form into the midgut membrane, which leads to larval death [69]. This model highlights the importance of receptor cooperation and toxin conformational flexibility in mediating toxicity. On the other hand, the signaling-pathway model proposes that toxicity is caused by binding to a cadherin receptor, leading to a signaling pathway involving stimulation of a G protein and adenylyl cyclase/protein kinase A, increased cAMP levels resulting in membrane blebbing, cell swelling, and necrotic cell death [30]. While several potential receptors have been proposed as candidates for Cry toxin binding [70], their actions' precise roles or modes for individual toxins are yet unclear.

This dissertation research aims to identify Cry4Ba and Cry11Aa toxin interacting receptors of *Aedes albopictus* that lead to larval toxicity. Understanding these receptor interactions will provide valuable insights into mosquitocidal Bti toxins' relationship with their target hosts. Candidate receptors were selected based on their demonstrated binding potential or toxicity association with other mosquito species, mainly *Aedes aegypti*, due to the high sequence similarity between these two species. Co-precipitation experiments have confirmed toxin binding in various species, but the toxic properties of these interactions in *Ae. albopictus* remains unexplored. Thus, the receptors that may have toxicity potential have been selected for this study as membrane-bound alkaline phosphatase (XM_019684987.2) (ALP), membrane-bound alkaline phosphatase-like (XM_029879948.1) (memALP), V-type proton ATPase subunit B (XM_019706260.2) (V-ATP), aminopeptidase N-like (XM_029867648.1) (AMN), protein l(2)37Cc (XM_019677754.2) (prohibitin), flotillin-1 (XM_029870385.1) (flotillin), lachesin (XM_020076957.2) (lachesin), glutamyl aminopeptidase-like (XM_029874353.1) (Glutamyl), calcium-transporting ATPase (XM_029878831.1) (XM_029878831.1) and maltase 1-like (XM_029859282.1) (maltase).

Several membrane proteins, such as aminopeptidase, membrane-bound alkaline phosphatases and cadherin, have been identified as Cry receptors. These are first identified in lepidopteran species as Cry1A binding receptors. Similar receptors have been described in mosquito larvae, such as cadherin-Cry4Ba binding in *Anopheles gambiae* and cadherin-Cry11Aa in *Ae. aegypti*, alkaline phosphatases-Cry11Aa interaction caused toxicity in *Anopheles quadrimaculatus*, *An. gambiae*, and *Ae. aegypti* [18]. However, little is known about *Ae. albopictus* midgut proteins that bind to Cry4Ba and Cry11Aa toxins. In this study, these candidate receptors were identified and characterized.

The role of alkaline phosphatase (ALP) as a functional receptor for Cry toxins, including Cry4Ba and Cry11Aa, has been extensively documented across multiple insect species. In *Aedes aegypti*, ALP is crucial for toxin-mediated cell lysis, as shown by Jiménez et al. (2012), where downregulation of ALP led to a decrease in mortality when exposed to Cry4Ba. In a study, *A. aegypti* larvae were exposed to Bti toxins, resulting in a 30% mortality rate. This exposure led to a decrease in ALP expression levels. Subsequent dsRNA-mediated silencing of the ALP gene resulted in a 14-fold reduction in transcription. When these ALP-silenced larvae were exposed to Cry4Aa toxin, mortality decreased from 48% to 37%, indicating that reduced ALP expression enhances larval survival against Cry toxins[71]. Our study aligns with this observation, as dsRNA-mediated silencing of ALP and memALP in *Aedes albopictus* larvae significantly increased survival rates (96%) upon Cry4Ba exposure compared to the control group (68%). This suggests that ALP functions as a receptor for Cry toxins in *Ae. albopictus* larvae and their downregulation can confer increased resistance to these toxins. In our study, ALP and memALP expression levels in *Ae. albopictus* decreased by 1.78-fold and 3.4-fold, respectively, following exposure to Cry4Ba. Despite these associations, not all ALPs were consistently differentially expressed upon Cry toxin exposure [71]. For instance, ALPs (AAEL003298 and AAEL003313) were downregulated in resistant strains, aligning with their role in toxin susceptibility. Still, an ALP (AAEL009077) was unexpectedly over-transcribed in the susceptible strain exposed to Cry toxins. This suggests that overexpression does not always correlate with increased

toxicity, emphasizing the complexity of ALP interactions [71]. These findings underline that while certain ALPs serve as critical toxin receptors, their transcriptional regulation under toxin exposure is not uniform across all strains or toxins. The present study and others highlight complex transcriptional responses of ALPs to toxin exposure. Non-toxin-binding ALPs also exhibited altered constitutive expression, consistent with findings in other Cry11Aa-resistant strains. The over-transcription of non-receptor ALPs might reflect a broader stress or compensatory response to toxin exposure, as evidenced in Cry11Aa-resistant *A. aegypti* [72]. This dynamic transcriptional regulation indicates that ALP responses to Cry toxins involve direct receptor-mediated interactions and indirect physiological adjustments to toxin exposure. This adaptive mechanism appears conserved across Diptera, likely limiting receptor availability and mitigating toxicity. In Lepidopteran species such as *Helicoverpa armigera*, ALP expression was downregulated when larvae were exposed to Cry1Ac [73]. However, in *Plutella xylostella*, ALP levels remained unaffected, highlighting potential evolutionary differences in receptor regulation among insect orders [74]. Therefore, our study's findings support the hypothesis of a conserved but species-specific regulatory mechanism of ALP in response to Cry toxins. Our cytotoxicity assays with HEK-293 cells expressing ALP and memALP confirmed their involvement in mediating Cry toxin action. Exposure to Cry4Ba and Cry11Aa resulted in 72–78% cell viability, consistent with earlier studies in *Ae. aegypti*, where ALP silencing reduced Cry4Ba-mediated cytotoxicity [46]. These findings highlight ALP's role as a receptor across species and emphasize the variability in toxin efficacy. Notably, Cry4Ba caused significant larval mortality in *Ae. albopictus* even at low concentrations, while Cry11Aa required denaturation and renaturation to achieve comparable activity. This discrepancy aligns with observations in *Culex pipiens*, where Cry11Aa's solubility and activation were similarly hindered [75]. Such findings highlight the importance of toxin structure and midgut conditions in mediating efficacy. Cry toxins often exhibit synergistic effects when combined. In *Helicoverpa armigera*, ALP fragments enhanced Cry1Ac efficacy, reducing the lethal dose by more than 50% [75]. Our findings suggest a potential for similar synergistic interactions in *Ae. albopictus*, particularly between Cry4Ba and Cry11Aa, though further studies are needed to validate this hypothesis. The specificity of ALP interactions with Cry toxins

also varies across species. For example, in *Anopheles gambiae*, ALP binds Cry11Ba more effectively than Cry4Ba, reflecting receptor isoform differences [76]. Such variations highlight the need for tailored approaches to optimize toxin formulations for specific mosquito species. The observed downregulation of ALP and memALP in response to Cry toxins poses challenges for long-term efficacy. In *Spodoptera littoralis*, similar patterns of ALP downregulation were associated with reduced susceptibility to Cry1C [77]. Combining Cry toxins with complementary modes of action, such as those targeting aminopeptidase-N (APN) or cadherins, could mitigate resistance development. Our findings provide a basis for exploring such combinatorial strategies in *Ae. albopictus*.

In addition to ALP, other midgut receptors, including APN, cadherins, and V-type proton ATPases, play roles in Cry toxin binding. For instance, cadherin silencing in *Helicoverpa armigera* reduced Cry1Ac toxicity, similar to the protective effects observed with ALP silencing in our study [78]. In conclusion, ALP and memALP might be receptors for Cry toxins in *Ae. albopictus*, revealing conserved yet species-specific mechanisms. In contrast to the consistent role of certain ALPs (e.g., AAEL000931, AAEL009077) in Cry4Ba and Cry11Aa binding, their transcriptional responses to toxins vary between resistant and susceptible strains, suggesting a role for secondary resistance mechanisms beyond receptor interaction. The variability in ALP transcriptional responses offers opportunities to develop resistance-breaking strategies. Targeting ALP overexpression or functional disruption could reduce Cry toxin efficacy and improve the longevity of Bti-based insecticides. Comparative analyses with other species highlight the complexity of receptor dynamics and the potential for tailored mosquito control strategies. The findings underscore the importance of integrating toxin-receptor interaction studies with field applications to enhance the efficacy and sustainability of Cry toxin-based interventions.

Vacuolar ATPase subunit B (V-ATP) plays a central role in insect midgut physiology by maintaining the highly alkaline pH necessary for Cry toxin activation and mediating ion transport [79]. This study explored the functional role of V-ATP as a

receptor for Cry4Ba and Cry11Aa toxins in *Aedes albopictus*, demonstrating significant cytotoxicity in V-ATP-expressing HEK293 cells. Cry4Ba and Cry11Aa reduced cell viability to 66.32% and 52.34%, respectively, confirming V-ATP's receptor function in facilitating toxin-induced cell death. These findings align with studies in *Helicoverpa armigera*, where overexpression of V-ATPase B in sf9 cells enhanced Cry1Ac cytotoxicity, while resistant larvae exhibited reduced V-ATP expression [80]. Similarly, in *Spodoptera exigua*, RNAi knockdown of V-ATPase reduced larval susceptibility to Cry2Aa, further corroborating its role as a receptor for diverse Cry toxins [81]. Purified Cry4Ba and Cry11Aa were applied to 4th instar larvae for 12 hours, resulting in significant downregulation of V-ATP expression by 2.22-fold and 8.33-fold, respectively (Figure 45 and Figure 46). While no larval mortality was observed, the pronounced reduction in V-ATP expression suggests a regulatory feedback mechanism to enhance larval survival. Such receptor downregulation has been observed in other species; for example, *Helicoverpa armigera* larvae exhibited a ~3-fold reduction in V-ATP expression after exposure to Cry1Ac [80]. In *Anopheles gambiae*, exposure to Cry11Aa led to a 2-3 fold reduction in ALP expression, reflecting a conserved mechanism of receptor downregulation to mitigate toxicity [76]. These findings mirror our results in *Ae. albopictus* and support the hypothesis that receptor regulation is a widespread adaptive response in insects exposed to Cry toxins. To elucidate the functional importance of V-ATP, we employed dsRNA-mediated gene silencing in 1st instar larvae. A two-fold reduction in V-ATP expression resulted in a 12% decrease in larval mortality (32% to 20%) when exposed to Cry toxins (Figure 51 and Figure 52). This protective effect shows V-ATP's role as a critical receptor mediating Cry toxin susceptibility in *Ae. albopictus*. Similar trends have been reported in *Spodoptera exigua*, where RNAi knockdown of V-ATP decreased susceptibility to Cry2Aa by ~15% [81]. In *Helicoverpa armigera*, silencing V-ATP similarly reduced Cry1Ac toxicity, further reinforcing the receptor's functional significance [80]. Although both Cry4Ba and Cry11Aa demonstrated cytotoxicity in V-ATP-expressing cells, their larvicidal efficacy in *Ae. albopictus* differed significantly. Cry4Ba induced substantial mortality, whereas Cry11Aa showed no toxic effects despite significant V-ATP downregulation. Such differences highlight the importance of toxin structure and midgut-specific conditions in determining efficacy. In

Spodoptera littoralis, V-ATPase enhances Cry1Ca binding and toxicity by maintaining optimal midgut conditions, highlighting its conserved function as a receptor across Lepidoptera and Diptera [77]. These findings parallel our observations in *Ae. albopictus*, where V-ATP downregulation reduced Cry toxin efficacy, demonstrating a conserved but context-dependent role in mediating susceptibility. The conclusions of this study have significant implications for resistance management. The observed downregulation of V-ATP in response to Cry toxins suggests that larvae actively modulate receptor availability to mitigate toxicity. Combining Cry toxins targeting multiple receptors, such as ALP and V-ATP, could enhance efficacy and delay resistance development. RNAi-based approaches to silence specific receptors could also serve as a novel resistance management strategy, reducing receptor availability and limiting susceptibility to Cry toxins. In conclusion, V-ATP can be a receptor for Cry4Ba and Cry11Aa in *Ae. albopictus*, demonstrating its role in mediating cytotoxicity and larval mortality. Comparative analyses with Lepidopteran species highlight V-ATP-mediated Cry toxin susceptibility's conserved but species-specific nature.

The maltase-like receptor, an -glucosidase, has evolved to serve dual functions: its primary enzymatic role in carbohydrate metabolism and its secondary function as an insect toxin receptor. This receptor's involvement in hemozoin nucleation, as observed in the bloodsucking bug *Rhodnius prolixus* [82], [83], highlights its adaptability across diverse insect taxa. Additionally, its role as a toxin receptor in mosquitoes such as *Culex pipiens*, *Culex quinquefasciatus*, and *Anopheles gambiae* underlines its functional diversification [83]. In our study, Cry4Ba and Cry11Aa treatments in *Aedes albopictus* did not induce cell death in HEK293 cells expressing the maltase receptor. These results suggest that while maltase-like receptors may interact with Cry toxins, the interaction does not follow the classical pore formation pathway. Instead, maltase likely modulates susceptibility through indirect or compensatory mechanisms. In this study, Cry4Ba and Cry11Aa treatments resulted in significant upregulation of maltase receptor expression in 4th instar larvae. RNAi-mediated silencing reduced maltase expression by ~2-fold in 1st instar larvae (Figure 5.47). This marked an increase in larval mortality from 32% to 52% (Figure 5.48). These results contrast with other receptors,

such as ALP and V-ATP, where silencing reduces susceptibility to Cry toxins [46], [80]. The increased mortality observed here suggests that maltase silencing may induce compensatory upregulation of other midgut receptors, enhancing Cry4Ba efficacy.

Comparable findings were reported in *Aedes aegypti*, where erythritol-mediated inhibition of maltase increased larval mortality when combined with *Bacillus thuringiensis israelensis* (Bti). Specifically, erythritol and Bti combinations resulted in 85% mortality compared to 24% with boric acid and Bti [84]. These studies collectively highlight maltase's indirect role in modulating Cry toxin susceptibility. In *Culex quinquefasciatus*, the maltase receptor facilitates Cry toxin binding but lacks the pore formation capability observed in receptors such as ALP and V-ATP [83]. Similarly, our findings in *Ae. albopictus* indicates that while maltase interacts with Cry4Ba and Cry11Aa, it does not induce cytotoxicity *in vitro*. Our results suggest that Cry4Ba and Cry11Aa toxicity in *Ae. albopictus* does not primarily rely on maltase-mediated pore formation. Instead, compensatory mechanisms may play a pivotal role.

For example, in *Spodoptera exigua*, Cry2Aa susceptibility was enhanced by RNAi-mediated silencing of non-essential receptors, possibly due to the upregulation of primary receptors such as cadherins [81]. Similar mechanisms may underlie the increased mortality observed in maltase-silenced *Ae. albopictus* larvae. The synergistic effects of maltase inhibition and Cry toxins are well-documented. In *Aedes aegypti*, the combination of erythritol and Bti significantly increased larval mortality compared to either treatment alone [84]. This aligns with our findings, where maltase silencing enhanced Cry4Ba efficacy, further emphasizing maltase's modulatory role in Cry toxin pathways. The classical model posits that toxin-receptor interactions result in pore formation and cell death. However, our findings challenge this paradigm for maltase-like receptors. Despite the observed upregulation of maltase expression following Cry toxin exposure, RNAi-mediated silencing increased rather than decreased larval mortality. This suggests that maltase interactions with Cry4Ba and Cry11Aa may facilitate alternative pathways distinct from pore formation. These results highlight the complex role of maltase-like receptors in Cry toxin mechanisms. While Cry4Ba and Cry11Aa inter-

actions with maltase do not induce direct cytotoxicity, receptor silencing significantly enhances susceptibility, suggesting an indirect regulatory function.

Calcium-transporting ATPase (Calc) is vital in maintaining calcium ion homeostasis within cells, critical for various cellular processes, including signaling, ion transport, and stress responses [85]. Calc has been implicated in Cry toxin interactions in insects, serving as a candidate receptor or modulator of toxin efficacy [86]. In this study, RNAi-mediated suppression of Calc expression in 1st instar *Aedes albopictus* larvae resulted in a 2.3-fold decrease in gene expression, correlating with an 8% larval death rate compared to 32% in controls (Figure 52). These findings suggest Calc downregulation enhances larval survival under Cry toxin stress, similar to observations in *Aedes aegypti* [87]. Cry4Ba and Cry11Aa exposure significantly reduced Calc expression by 1.4-fold and 3.4-fold in 4th instar larvae. These results align with studies in *Aedes aegypti*, where Bti-resistant strains exhibited a 4.2-fold reduction in Calc expression, suggesting downregulation as an adaptive mechanism to mitigate toxin effects [87]. In Lepidopteran pests like *Helicoverpa armigera*, Calc expression decreased by 2.5-fold under Cry1Ac exposure, correlating with reduced susceptibility [80]. Similarly, in *Culex quinquefasciatus*, suppression of Calc led to a marked increase in survival rates under Cry48Aa and Cry49Aa stress, supporting its role as a modulator of Cry toxin interactions [88]. In our study, Cry4Ba and Cry11Aa treatments did not induce cytotoxicity in Calc-expressing HEK293 cells. This contrasts with results for ALP and V-ATP, which showed significant cell death upon Cry toxin exposure [46], [77]. The involvement of Calcium-transporting ATPase in calcium ion homeostasis makes it a plausible candidate for mediating these pathways. Similar mechanisms have been proposed in *Myzus persicae*, where Calc interacts with Cry41-related toxins to modulate calcium-dependent apoptosis [89]. Its role in Cry toxin susceptibility differs significantly from ALP and V-ATP. While ALP mediates pore formation and V-ATP regulates pH balance to enhance toxin activity, it appears to act indirectly, potentially modulating other receptor pathways or cellular stress responses [46]. RNAi-mediated Calc silencing in *Ae. albopictus* enhanced larval survival, similar to ALP silencing in *Aedes aegypti* [71]. However, Calc's role seems more nuanced, involving compensatory mechanisms rather

than direct toxin-receptor interactions. Its role appears conserved across species, but its specific interactions vary. In *Culex pipiens*, Calc regulates ion homeostasis under insecticide stress but does not directly mediate cytotoxicity [90]. The downregulation of Calc in response to Cry toxin exposure suggests it plays a protective role in larvae. Combining RNAi-mediated Calc suppression with Cry toxin treatments could enhance larval mortality, as demonstrated in this study. Targeting Calc in resistant strains may also mitigate resistance development by disrupting compensatory mechanisms. Combining Calc-targeting strategies with other receptor-modulating treatments, such as ALP inhibition or erythritol supplementation, could further enhance Cry toxin efficacy. In conclusion, Calcium-transporting ATPase plays a significant but indirect role in Cry toxin mechanisms in *Ae. albopictus*. Its downregulation under Cry toxin stress enhances larval survival, suggesting an adaptive function. While it does not mediate direct cytotoxicity, its involvement in ion homeostasis and signal transduction pathways highlights its potential as a modulator of Cry toxin susceptibility.

Prohibitin, flotillin and aminopeptidase receptors have garnered interest as potential Cry toxin interaction candidates in insect species due to their roles in cellular signaling, transport, and structural integrity [80], [88], [91]. In our study, the expression of these receptors in *Aedes albopictus* was significantly downregulated upon exposure to Cry4Ba and Cry11Aa toxins. However, gene silencing of these receptors via RNA interference (RNAi) did not influence larval survival, suggesting a non-essential role in direct Cry toxin-mediated toxicity. Further, receptor expression in HEK293 cells and subsequent Cry toxin treatment failed to induce cytotoxicity, pointing to the possibility of indirect or non-receptor-mediated interactions (Figure 5.47). Exposure to Cry4Ba and Cry11Aa caused differential downregulation in receptor expression levels. Prohibitin showed a 1.5-fold decrease, flotillin a 2.2-fold decrease, aminopeptidase a 3.0-fold decrease, and glutamyl transferase a 1.8-fold decrease (Figure 5.42). These findings align with studies in *Aedes aegypti*, where flotillin and prohibitin expression levels decreased by 1.7-fold and 2.0-fold, respectively, in response to Bti exposure [87]. Aminopeptidase and glutamyl transferase, however, demonstrated variable patterns in other species, suggesting species-specific regulatory mechanisms. In *Helicoverpa*

armigera, aminopeptidase N (APN) has been shown to bind Cry1Ac directly, forming toxin-receptor complexes that mediate pore formation and cell death [92]. Our study's absence of similar interactions indicates that such mechanisms may not be conserved in *Ae. albopictus* or require additional cofactors. Prohibitin, known for its role in mitochondrial integrity and signaling, has been implicated in Cry toxin susceptibility in *Plutella xylostella*. Here, RNAi-mediated prohibitin silencing reduced larval mortality under Cry1Ac stress by 20%, suggesting a protective role [93]. In contrast, our findings show no significant effect of prohibitin silencing on larval survival in *Ae. albopictus* (Figure 52), highlighting potential differences in prohibitin's role across species. Flotillin is a scaffolding protein that aids membrane domain organization and receptor clustering [94]. Flotillin expression in HEK293 cells in our study did not result in cytotoxicity upon Cry toxin treatment, suggesting that flotillin alone is insufficient to mediate Cry4Ba or Cry11Aa toxicity in *Ae. albopictus*. While APN has been widely established as a Cry toxin receptor in Lepidopteran pests, its role in Dipteran species remains unclear. RNAi silencing of APN in *Ae. albopictus* did not affect larval survival, and APN expression in cell culture failed to elicit cytotoxicity under Cry toxin exposure. This contrasts with findings in *Helicoverpa armigera*, where APN knockdown reduced Cry1Ac and Cry2A toxicity [91]. Such discrepancies may reflect differences in receptor-ligand specificity between species. Our results, showing no cytotoxicity or survival effects, suggest that glutamyl transferase may require additional co-receptors or environmental factors to facilitate toxin activity in *Ae. albopictus*. The absence of cytotoxicity and survival effects in our experiments suggests that Cry4Ba and Cry11Aa may require secondary receptors or accessory molecules for effective toxicity. In *Aedes aegypti*, cadherin and ALP are established Cry toxin receptors that cooperate with APN to mediate pore formation and cell death [95] investigating similar interactions in *Ae. albopictus* may uncover additional layers of receptor complexity. The lack of conserved receptor functionality across species highlights the need for tailored approaches in Cry toxin-based pest management. Comparative analyses of receptor expression and functionality in *Ae. albopictus*, *Aedes aegypti*, and other Dipteran species will provide insights into the evolutionary pressures shaping Cry toxin susceptibility. In conclusion, prohibitin, flotillin, aminopeptidase, and glutamyl transferase do not directly mediate

Cry4Ba or Cry11Aa toxicity in *Ae. albopictus*. The absence of cytotoxic effects and the lack of influence on larval survival highlights the complexity of Cry toxin-receptor interactions. These findings contrast with studies in other insect species, suggesting that *Ae. albopictus* employs alternative mechanisms to resist Cry toxin stress.

Cry toxins, a large family of proteins derived from *Bacillus thuringiensis* (Bt), are well-known for their lethal effects on various insect orders, including Lepidoptera, Diptera, and Coleoptera. These toxins exhibit a wide range of receptor interactions across insect species, contributing to their versatility and efficacy in pest control. Despite the diversity in their amino acid sequences, Cry toxins share a conserved three-domain structure, suggesting a common mechanism of action rooted in this structural homology. This conserved structure facilitates their ability to bind to specific midgut receptors in target insects, initiating a cascade of events that ultimately leads to cell lysis and insect death. Previous studies have extensively documented the interaction of Cry toxins with various receptor proteins, such as alkaline phosphatase (ALP), aminopeptidase N (APN), cadherins, and ATP-binding cassette (ABC) transporters, across multiple insect species. However, significant variability exists in the specific receptors and binding affinities among different species. This variability can be attributed to evolutionary adaptations that influence receptor structure and expression. In the context of mosquito species, Cry4Ba and Cry11Aa are highly effective mosquitocidal toxins, binding to specific midgut receptors to exert their toxic effects.

However, the exact mechanisms underlying Cry toxin activity, particularly in *Aedes albopictus*, remain poorly understood. This study sought to bridge this knowledge gap by identifying novel candidate receptors in *Ae. albopictus* and comparing these findings with Cry-interacting proteins in other insect species. Our study identified two alkaline phosphatases (ALP and memALP) and a vacuolar-type ATPase subunit B (V-ATP) as potential receptors for Cry4Ba and Cry11Aa in *Ae. albopictus*. The identification of these receptors in *Ae. albopictus* suggests that these proteins may serve conserved functions across mosquito species, highlighting the evolutionary significance of these receptor interactions. ALPs are widely recognized as critical mediators

of Cry toxin binding and toxicity in mosquitoes. Our study showed that ALP and memALP in *Ae.*

albopictus binds Cry4Ba and Cry11Aa and modulates larval responses to these toxins. V-ATP interacts with Cry4Ba and Cry11Aa in *Ae. albopictus*, consistent with its role as a functional receptor in other insect species, such as *Helicoverpa armigera* [80]. Interestingly, our study also highlighted a potential interaction between Cry toxins and maltase-like receptors in *Ae. albopictus*. While maltase does not directly mediate toxicity through pore formation, its silencing enhanced larval mortality under Cry toxin exposure. This finding suggests that maltase may influence resistance mechanisms by modulating the expression or availability of other midgut receptors. This study contributes to the growing knowledge of Cry toxin mechanisms by identifying new candidate receptors in *Ae. albopictus* and comparing these interactions with those documented in other insect species. The conserved roles of ALP and V-ATP across mosquito species highlight their potential as universal targets for enhancing Cry toxin efficacy. Additionally, the involvement of maltase-like receptors in resistance mechanisms showed the complexity of Cry toxin action and the need for further research into secondary receptors and accessory proteins.

7. CONCLUSION

In conclusion, this study provides significant insights into the molecular mechanisms underlying Cry toxin action in *Aedes albopictus*, focusing on Cry11Aa and Cry4Ba binding receptors in the mosquito midgut. This research has identified key candidate receptors that mediate Cry toxin susceptibility through a comprehensive approach involving purified Cry protoxins, cytotoxicity assays, RNAi-mediated gene silencing, and larval bioassays. The identified receptors, alkaline phosphatase (ALP), membrane-bound alkaline phosphatase (memALP), vacuolar-type ATPase subunit B (V-ATP), and maltase-like receptor, demonstrate distinct and overlapping roles in toxin-receptor interactions. The results of this study clearly showed that Cry4Ba and Cry11Aa show their toxicity on mosquito larvae by binding more than one larval midgut cell receptor. *Ae. albopictus* tended to decrease gene expression levels of the candidate receptors of Cry11Aa and Cry4Ba. These receptors (ALP, memALP and V-ATP) also had cell cytotoxicity with the toxin interactions. The results of maltase receptor and toxin interaction experiments that lead to increased toxin susceptibility of larva with down-regulation of maltase receptor may provide invaluable insight into mosquito resistance mechanisms against Cry toxins. Overall, this study advances our understanding of Cry toxin mechanisms in *Aedes albopictus* and provides a robust framework for developing targeted and sustainable mosquito control strategies.

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