KARADENİZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MOLECULAR BIOTECHNOLOGY PROGRAM

THE EFFECTS OF HELICOVERPA ARMIGERA SINGLE NUCLEOPOLYHEDROVIRUS ORIGINATED CHITINASE AND CATHEPSIN PROTEINS ON THE VIRULANCE OF CHILO IRIDESCENT VIRUS

MASTER'S THESIS

Kevser AKYÜZ

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TRABZON

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PREFACE

The study entitled ‘‘The Effect of Helicoverpa armigera Single Nucleopolihedrovirus Originated Chitinase and Cathepsin Proteins on The Virulence of Chilo Iridescent Virus (CIV)’’ was prepared as a ‘‘Master's thesis’’ in the Molecular Biotechnology Program at Institute of Natural and Applied Sciences, Karadeniz Technical University. The effects of baculovirus chitinase and cathepsin genes on oral infectivity of CIV were investigated in this study.

I would like to thank my advisor, Prof. Dr. Zihni DEMİRBAĞ, for his help and support from planning to conclusion of the thesis. I would like to thank Prof. Dr. Remziye NALÇACIOĞLU for assisting in guiding the thesis. I would like to thank Dr. Gözde Büşra EROĞLU and Dr. Aydın YEŞİLYURT for their generous helps during my studies in the laboratory. I also would like to thank my colleagues in the Microbiology Laboratory and friends in Department of Biology and Molecular Biotechnology.

I would like to express my endless thanks to my family who always supported me and was by my side.

Kevser AKYÜZ
Trabzon, 2022

THESIS ETHICS STATEMENT

The study titled "Helicoverpa armigera Single Nucleopolyhedrovirus Originated Chitinase and Cathepsin Proteins Effects on The Virulence of Chilo Iridescent Virus" was conducted as my Master's Thesis. I declare that I completed under the responsibility of Prof. Dr. Zihni DEMİRBAĞ, that I collected the data myself, that I carried out the experiments in the relevant laboratories, that I showed the information I received from other sources completely in the text and in the bibliography, that I acted in accordance with scientific research and ethical rules during the work process, and that I accept any legal consequences in the event that the opposite occurs. 17/03/2022

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Danışman: Prof. Dr. Zihni DEMİRBAĞ
2022, 66 Sayfa

Chilo iridescent virüs (CIV) tarımda zararlı bazı böcekleri enfekte ettiği için ekonomik ve ekolojik olarak özel bir öneme sahiptir. Fakat, CIV enfekte ettiği konaklarda düşük virülans oluşturmaktadır. Bu durumun nedenleri arasında birçok böcek virüsünün sahip olduğu ancak CIV'de olmayan kitinaz ve katepsin genlerinin bulunmaması gösterilebilir. Bakülovirüslerde bulunan kitinaz, virüs replikasyonunun geç fazında katepsin ile birlikte ifade edilir. Katepsinin peritrofik membranın yapısını bozarak, kitinazın ise kitin tabakasını tahrip ederek böcek üzerinde etkili olduğu bilinmektedir. Bu bilgiler ışığında, bu çalışmada bakülovirüslere ait kitinaz ve katepsin proteinleri kullanılarak CIV'nin virülansının arttırılabilmesi hedeflenmiştir. Bu doğrultuda, ilk önce kitinaz ve katepsin proteinlerinin bakteriyal ifade sisteminde üretilmeleri ve elde edilen proteinlerin böceğe yedirildikten sonra CIV virülansına etkilerinin araştırılması hedeflenmektedir. Bakülovirüs genomundan kitinaz ve katepsin genleri uygun primerler kullanılarak polimeraz zincir reaksiyonuyla (PCR) çoğaltıldı. Çoğaltılan genler pJET1.2 vektörüne klonlandı. Gerekli doğrulamalar yapıldıktan sonra, her iki gen ayrı ayrı pET28a+ ifade vektörüne aktarılarak *Escherichia coli* BL21(DE3) hücresinde ifadeleri sağlandı. Kitinaz (64 kDa) ve katepsin (43 kDa) proteinleri SDS-PAGE ve western blot analiz yöntemleriyle belirlendikten sonra, nikel afinite kromatografisi ile saflaştırıldılar. Elde edilen proteinlerden 500 ng, 1000 ng, 1500 ng, 2000 ng, 2500 ng, 5000 ng konsantrasyonlarında *Galleria mellonella* larvalarına yedirildikten sonra CIV ile enfekte edildiler. Bu çalışma neticesinde, bakülovirüse ait kitinaz ve katepsin proteinlerinin CIV'nin virülansını % 23 arttırdığı tespit edildi. Buna göre bakulovirüs kitinaz ve katepsin proteinlerinin CIV'nin virülansını artırmak amacıyla kullanılabileceği belirlendi.

Anahtar Kelimeler: Chilo iridescent virüs, kitinaz, katepsin, gen ifadesi, mikrobiyal control

Master Thesis

SUMMARY

THE EFFECTS OF HELICOVERPA ARMIGERA SINGLE NUCLEOPOLYHEDROVIRUS ORIGIN OF CHITINASE AND CATEPSIN PROTEINS ON VIRULANCE OF CHILO IRIDESCENT VIRUS

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2022, 66 Pages

Chilo iridescent virus (CIV) is of particular economic and ecological importance as it infects some pests in agriculture. However, it causes low virulence in CIV-infected hosts. Accordingly, it was determined that baculovirus chitinase and cathepsin proteins could be used to increase the virulence of CIV. Chitinase in baculoviruses is expressed together with cathepsin in the late phase of virus replication. It is known that cathepsin is effective on the insect by destroying the structure of the peritrophic membrane, and chitinase is effective on the insect by destroying the chitin layer. In the light of this information, in this study, it was aimed to increase the virulence of CIV by using chitinase and cathepsin proteins belonging to baculoviruses. Accordingly, it is primarily aimed to produce chitinase and cathepsin proteins in the bacterial expression system and to investigate the effects of the obtained proteins on CIV virulence after ingestion of the insect. Chitinase and cathepsin genes from the baculovirus genome were amplified by polymerase chain reaction (PCR) using appropriate primers. The amplified genes were cloned into the pJET1.2 vector. After the necessary validations were made, both genes were transferred separately into the pET28a+ expression vector to ensure their expression in *Escherichia coli* BL21(DE3) cell. After the chitinase (64 kDa) and cathepsin (43 kDa) proteins were determined by SDS-PAGE and western blot analysis methods, they were purified by nickel affinity chromatography. They were infected with CIV after feeding to *Galleria mellonella* larvae at concentration of 500 ng, 1000 ng, 1500 ng, 2000 ng, 2500 ng, 5000 ng from the obtained proteins. As a result of this study, it was determined that the chitinase and cathepsin proteins of baculovirus increased the virulence of CIV by 23%. Accordingly, it was determined that baculovirus chitinase and cathepsin proteins could be used to increase the virulence of CIV.

Key Words: Chilo Iridescent Virus, chitinase, cathepsin, gene expression, microbial control

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ABBREVIATIONS

HearSNPV	: Helicoverpa Armigera Single Nucleopolihedro Virus
bp	: Base pair
CIV	: Chilo iridescent virus
chit	: Chitinase
cath	: Cathepsin
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleoside triphosphate
EDTA	: Etilendiamin Tetraasetik Asit
kDa	: Kilo dalton
PAGE	: Polyacrylamide Gel Electrophoresis
PCR	: Polymerase Chain Reaction
SDS	: Sodium Dodecyl Sulfate

1. GENERAL INFORMATION

1.1. Introduction

Insects cause significant damage in forest and agricultural fields. Chemical pesticides have been used for years to eliminate or reduce the harmful effects of insects. However, these chemical ipesticides are harmful not only to harmful insects, but also to beneficial insects and organisms that are harmless or even non-targets. Considering these disadvantages, biological contral, which is an environmentally friendly method, is preferred.

Reducing the pest populations and therefore the damage of insects by using living organisms and their products is called biological control. Among the biological materials used for this purpose, microorganisms that cause disease and death of insects are of great importance. These organisms (bacteria, viruses, fungi, nematodes or protozoa), also called entomopathogens, are isolated from diseased or dead insects in nature.

Viruses are one of the most promising biological control agent that have been used for many decades (Flexner and Belnavis 2000, Harrison and Bonning 2000, Sezen and Demirbağ 2005). Insect viruses have several advantages over other entomopathogens. Having a narrow host range, not causing disease in mammals, not easily degrading, and controlling insect outbreaks that cause many viruses to become infected are just some of these advantages. So far, several insect viruses have been isolated and identified from many pests. More than several hundreds baculoviruses have been isolated from these insects (Murphy et al. 1995). Insect viruses are also important experimental materials in which research is designed to understand basic biological mechanisms (Bilimoria 1991, Demir 2004). They have long been used in biotechnology as gene expression vectors that produce a variety of medically, economically and industrially important proteins (King and Possee 1992, Demirbağ et al. 1998, Demir et al.2000, Beljelarskaya 2002). Also, studies on

the use of these viruses as gene transfer vector to transfer foregin genes to mammalian cell systems have intensified in recent years (Ghosh et al. 2002, Hu 2006). Almost all invertebrate viruses are used for biotechnological purposes (Nalçacıoğlu 2003, Sezen 2004). However, baculovirus is the best one to be used for biological control prupose.

1.2. Insect Viruses as Biocontrol Agent

Insects and their larvae cause serious damages in agriculture and forestry. For many decade chemical pesticides have been used to reduce or eliminate the harmful effects of insects. These chemical insecticides effect not only target pest, but also harmless and even beneficial insects and organisms, they also cause serious environmental pollution. Baculoviruses are the most preferred microbial control agent among viruses (Orlovskaya 1998; Fuxa et al. 2002; Toprak et al. 2005). Since baculoviruses are host specific and highly infectious particles, they attract so much attention to be used as pest control agent. They infect only certain insect groups. So far, the molecular genetics of these viruses have been studied in detail (Herniou et al. 2001). In addition, these studies have allowed the genomes of baculoviruses to be modified for the expression of foreign genes and the improvement of their insecticidal properties. In nature, baculoviruses cause numerical declines in susceptible insect populations.

Table 1. Some baculoviruses used for biological control

Virus used	Harmful insects	Product
Granulozis viruses (GV) *Cydia pomonella* GV	Fruit worms	Apple, pear, walnut etc.
Agrotis segetum GV	Bozkurt	Corn, lettuce, root crops, cotton etc.
Nükleopolihedrovirüses (NPV) *Agrotis segetum* NPV	Bozkurt	Corn, lettuce, root crops etc.
Mamestra brassicae NPV	Cabbage worm, Potato tumor founded Green worm American cone worm	Cabbage, tomato, peas, sugar beet, cotton, vegetables etc.
Spodoptera littoralis NPV	Cotton leafworm	Cotton, tobacco, banana, corn, tomato
Helicoverpa (Heliothis) armigera NPV	*Green worm*	*Cotton, tobacco, sunflower,* chickpeas, beans, vegetables etc
Spodoptera exigua NPV	Beet worm	Vegetables, greenhouse plants
Anagrapha falcifera NPV	Celery worm	Vegetables
Autographa californica NPV	Clover caterpillar	Clover, oats etc.
Neodiprion setifer NPV	Reddish yellow bush antennae leaf bee	Pine wood products
Orgyia psuedotsugata NPV	Bush moth	Forest products, timber
Lymantria dispar NPV	Shredded	Forest products, timber

GV: Granulovirus, NPV: Nucleopolyhedrovirus

The use of these viruses as biological control material was first suggested by Reiff in 1911. Today, baculoviruses are considered as natural agents in the biological control of harmful insects (Hunter-Fujita et al. 1998). Baculoviruses used for this purpose are shown in Table 2. Another well studied insect virus is iridovirus. However, little is known about the factors determining the virulence of iridoviruses; but it is thought that the non-fatal infection allows vertical transmission of the virus and has a significant effect fitness of the insect population (Williams et al., 2005; Williams & Ward, 2010; Ince, 2012). The insect iridovirus is considered to be a potential biological control agent because insect iridescent virus-6 (IIV-6) causes infections in important pest species including bollworm, rootworm and cotton aphids (Henderson et al., 2001). However, the virulance of CIV is low. Unlike baculoviruses, it has been determined that there are no chitinase and cathepsin genes in the genome of CIV (Ince et al. 2010). In the light of this information, the absence of chitinase and cathepsin genes in the CIV genome may be the reason for low virulance.

1.3. Iridoviruses

The first iridovirus was discovered in England (Xerox 1954). The word *iridoviridae* is used because of the blue-green color produced in insects infected by the invertebrate iridoviruses. The blue-green mixture forms a color that shines as the light hits it. Therefore, this family was named "*Iridoviridae*", based on the English word "iridescence", which means a rainbow-like color. In vertebrate iridoviruses, such color generation is out of the question (Williams, 1996). Figure 1 shows the Costelytra zealandica larva, the hindgut area turning blue as a result of CIV infection.

Figure 1. *Iridovirus* infection in *Costelytra zealandica* larva. The last part of intestinal region has turned to iridescent color. (Williams, 1996).

Iridoviruses have icosahedral symmetry. The viral particle consists of an inner DNA/protein core surrounded first by a lipid membrane and then by a viral capsid (Figure 2). Capsid protein is around 50 kDa and constitutes 45% of the total proteins of the virus.

Some iridoviruses have an envelope surrounding the capsid. This envelope is usually acquired when the virus is relased from the cell by budding method in iridoviruses that reproduce in cell culture (Williams, 1998). Members of the *Iridoviridae* family are called as Iridovirid.

Figure 2. Capsid symmetry and morphology of iridoviruses.
A) capsid symmetry, B) detailed structure (URL-2, 2000).

As a result of infection by iridovirides, a large virus aggregation site is seen in the cytoplasm. This part (virogenic stroma) is the region where virus proteins and DNAs are the most intense, virus particles gather together, in other words, virus assembly takes place. Icosahedral particles are lined up in the cytoplasm, mostly in an array of 120-200 nm in diameter. This sequence is called the "paracrystalline array" and is an important feature that distinguishes iridoviruses from other DNA viruses. However, iridovirides differ from other eukaryotic DNA viruses by their genomic organization too.

The iridovirid genome is produced as large concatamers as a result of rolling circle replication. Since the iridovirid genome is packaged into the capsid by a volume-dependent (headfull) mechanism, it is circularly permuted and terminally redundant at the ends of the genome. Terminal repeats add 10-30% excess to the genome (Williams et al., 2005). The phylogenetic relationship of *Iridoviridae* family with other nucleocytoplasmic double strand DNA viruses is shown in Figure 3.

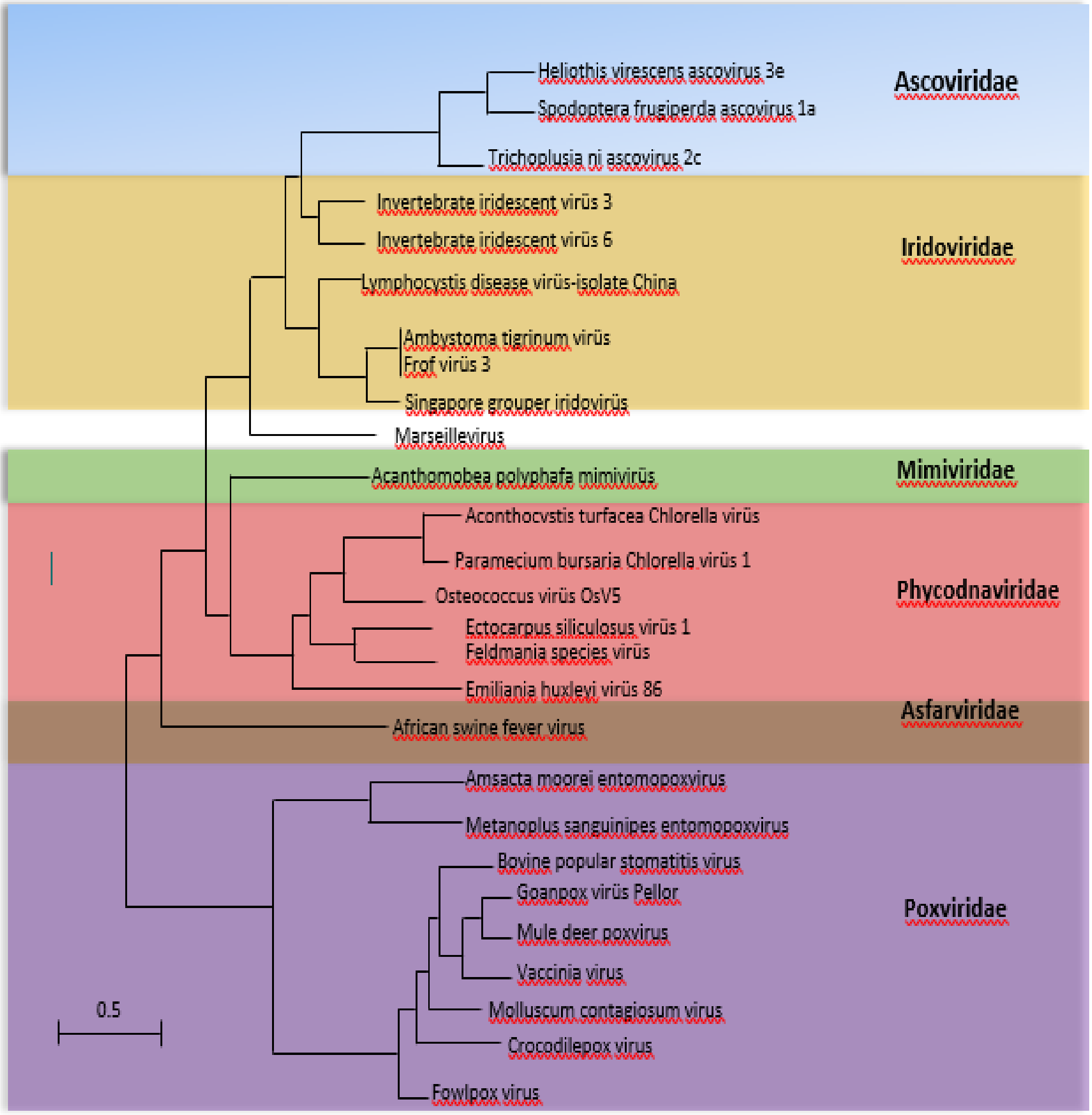


Figure 3. Phylogenetic relationship of *Iridoviridae* family with nucleocytoplasmic double strand DNA viruses.

Members of the *Iridoviridae* family have gained importance especially after 1980 due to the high amounts of fatal infections they have created in wild and cultured animals such as fish, frogs and grasshoppers (Williams et al., 2005). Members of the Iridovirus genus include important species that can be used as biological control material (D'Costa et al., 2012). At the same time, the iridescent color formation and capsid structure formed by the members of the Iridovirus genus are potentiall biotechnological models (Radloff et al., 2005; Hartschuh et al., 2008).

1.3.1. *Iridovirus* Genus

Iridovirus genus is the largest genus of the *Iridoviridae* family and includes species that infect invertebrates. It includes two species that have been accepted by the International Virus Taxonomy Committee (ICTV) and 8 species that have not yet been accepted (Table 2) (V. Gregory Chinchar et.al., 2021).

Oral infection of these viruses is very slow. The first proliferation area in the insect body is the cytoplasm of the adipose tissue cells. They can reproduce so much that they constitute 25% of the dry weight of the host insect they infect. They do not cause epidemic infections. The disease is fatal if the larva becomes infected in its early development stages. This type of infection is known as open infection, and the larval body turns a blue-green mixture (Figure 4). In mild infections, the cream-colored larva body takes on a light color close to white. These types of infections are also known as latent infections and are not fatal. Insects infected in this way appear healthy and can grow and reproduce until adulthood.

Interest in these viruses has been limited as they are thought to have low potential as biological control agents against pests. However, awareness of the potential impact of below-lethal iridovirus disease on the viability of insect populations is increasing day by day (Williams, 2008). Iridoviruses can reproduce *in vitro* in many insect cells and even in reptile cells. The majority of iridovirus strains have a fairly wide host range when injected into the hemolymph of the insect compared to when the virus is infected by oral transmission (Williams, 2008).

Figure 4. *Galleria mellonella* larva infected with iridovirus. Larvae turn clearly blue from healthy one due to severe iridovirus infection (URL-1).

Table 2. Taxonomy of Irıdoviridae

Subfamily *Alphairidovirinae*						
Genus	**Species**	**Isolate**	**Genom (bp)**	**ORFs**	**%GC**	**Accession number**
Lymphocystivirus	*Lymphocystis disease virus 1*	lymphocystis disease virus 1 (LCDV1)	102,653	108	29	L63545
	Lymphocystis disease virus 2	lymphocystis disease virus-China (LCDV-C)	186,250	176	27	AY380826
	Lymphocystis disease virus 3	lymphocystis disease virus-*Sparus aurata* (LCDV-Sa)	208,501	183	33	KX643370
Ranavirus	*Frog virus 3*	Frog virus 3 (FV3)	105,903	97	55	AY548484
	Ambystoma tigrinum virus	Ambystoma tigrinum virus (ATV)	106,332	92	54	AY150217
	Epizootic haematopoietic necrosis virus	Epizootic hematopoietic necrosis virus (EHNV)	127,011	100	54	FJ433873
	Common midwife toad virus	Common midwife toad virus (CMTV)	106,878	104	55	JQ231222

[a] ND: not determined. The genome sequences of large mouth bass virus, doctor fish virus and guppy virus 6 are unpublished, but the nucleotide sequences of their 26 core protein genes have been determined and aligned with those of other members of the family; all three isolates are placed in the species *Santee-Cooper ranavirus*. The accession number listed is for the major capsid protein gene of the largemouth bass virus isolate (V. Gregory Chinchar et. al., 2021).

The continuation of the Table 2.

	Santee-Cooper ranavirus	largemouth bass virus (LMBV)	ND[a]	ND	ND	DQ159940
	Singapore grouper iridovirus	Singapore grouper iridovirus (SGIV)	140,131	139	49	AY521625
Megalocytivirus	*Infectious spleen and kidney necrosis virus*	Infectious spleen and kidney necrosis virus (ISKNV)	111,362	117	55	AF371960
	Scale drop disease virus	Scale drop disease virus (SDDV)	124,244	129	37	KR139659
Subfamily *Betairidovirinae*						
Genus	**Species**	**Isolate**	**Genom(bp)**	**ORFs**	**%GC**	**Accession number**
Iridovirus	*Invertebrate iridescent virus 6*	invertebrate iridescent virus 6 (IIV6)	212,482	211	29	AF303741
	Invertebrate iridescent virus 31	invertebrate iridescent virus 31 (IIV31)	220,222	203	35	HF920637
Chloriridovirus	*Invertebrate iridescent virus 3*	invertebrate iridescent virus 3 (IIV3)	191,132	126	48	DQ643392
	Invertebrate iridescent virus 9	invertebrate iridescent virus 9 (IIV9)	206,791	191	31	GQ918152
	Invertebrate iridescent virus 22	invertebrate iridescent virus 22 (IIV22)	197,693	167	29	HF920633
	Invertebrate iridescent virus 25	invertebrate iridescent virus 25 (IIV25)	204,815	177	30	HF920635

The continuation of the Table 2.

	Anopheles minimus iridovirus	*Anopheles minimus iridovirus (AMIV)*	163,023	148	ND	KF938901
Decapod-iridovirus	*Decapod iridescent virus 1*	*Shrimp hemocyte iridescent virus (SHIV) - 20141215*	165,809	170	35	MF599468

1.3.2. Genome Organization and Replication in Iridoviruses

Iridovirids have a dsDNA genome. Putative open reading frames (ORFs) are located on the both strand of the genome of virus. The gene density in the genome is high, intergenic regions are generally short, and they contain repetitive sequences. Ökaryotlardan farklı olarak, iridoviruslerin genlerinde intronlar bulunmaz, virüs mRNA'ları poli [A] hedeflerinden görünüşlerdir (Ince et al., 2017). However, chitinase and cathepsin genes, whose products play role at host liquefaction, are not found in the iridovirids genus. Iridoviruses are also called nucleocytoplasmic viruses, since their genome duplications begin in the nucleus and end in the cytoplasm. The first step of viral infection is to attach to the host cell surface in a receptor-dependent pathway. After binding to the host cell, enveloped virions enter the cell by receptor-mediated endocytosis. On the other hand, non-enveloped virions enter the host cell with the fusion that occurs between the cell membrane and the virus (Braunwald et al., 1985). After iridoviruses enter the cell, their DNA genomes are transported to the cell nucleus. In the nucleus, viral transcripts belonging to the early (IE) and delayed early (DE) classes are expressed using virion DNA as tamplate (Williams et al., 2005). Early transcripts encode proteins needed for DNA replication and expression of late genes. The newly synthesized DNA passes into the cytoplasm and forms concatamers (Willis et al., 1984). Late (L) viral mRNA synthesis occurs in the cytoplasm of cells after DNA replication. Late viral mRNA synthesis is accomplished by virus-modified cellular RNA polymerase or viral RNA polymerase II enzyme. Late viral transcripts encode viral structural proteins involved in virion formation. The concatemeric viral DNA packaged into virions via a “headful” mechanism. Separation of virions from the cell occurs either by budding or by cell disruption. A brief summary of the *iridoviridae* replication strategy is shown in Figure 5 (Chinchar et al., 2017).

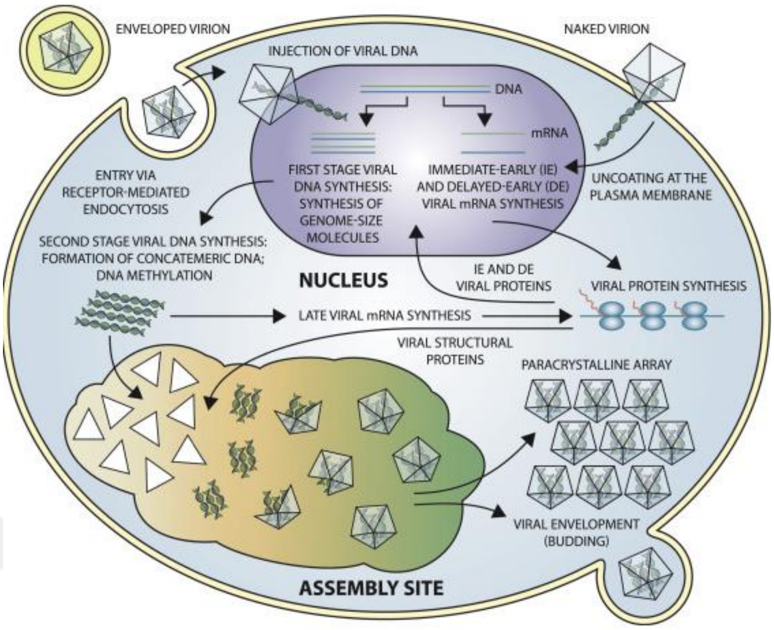


Figure 5. Schematic representation of the iridovirid replication strategy(Chinchar et al., 2017).

1.3.3. *Chilo* Iridescent Virus (CIV)

Chilo iridescent virus (CIV), which is the type species of the *Iridovirus* genus, is also known as Insect Iridescent Virus Type-6 (IIV-6). CIV was isolated from the root borer *Chilo suppressalis* (rice weevil) (Fukaya and Nasu, 1966) belonging to the lepidopter group. Regarding its biological significance, CIV is of particular economic and ecological importance, as it infects a few herbivore insects causing major damage to agriculture, such as rice cultivation and stone fruit (Smith, 1976). As a result of inoculation of CIV into organisms by injection, the host spectrum was investigated and it was found that it infects more than 100 insect species belonging to 6 orders (Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera and Orthoptera) (Williams & Ward, 2010).

The awareness of the potential impact of sublethal iridovirus disease on the viability of insect populations is increasing day by day (Williams, 2008). Iridoviruses can replicate *in vitro* in many insect cells and even reptile cells. Most of them have a fairly wide host range when injected compared to the oral administration of the virus (Williams, 2008). This insect iridovirus can be considered a potential agent for biological control, as these viruses cause infections in important pest insect species (Kleespies et al. 1999, Hernandez et al. 2000). CIV also infects a large number of non-insect arthropods (Ohba and Aizawa, 1979).

It has been determined that the base composition of the viral genomic DNA sequence is 71.37% A + T and 28.63% G + C, consistent with the values previously reported (Jakob et al., 2001). There are many short linear, reverse and palindrome repetitive DNA sequences in the genome. In addition, it was determined that the gene regions related to replication, transcription, protein modification, nucleotide metabolism and virus-host interaction show significant homology with other large DNA viruses. As a result of the computer-aided analysis of the CIV genome, the existence of 468 potential open reading frames (ORFs) with a polypeptide coding capacity ranging from 40 to 2432 amino acids were revealed. Information on the molecular biology of CIV is limited to a few studies in genomics, transcriptomics and proteomics (Bigot et al., 2013; Ince et al., 2013; Dizman et al., 2012; Ince et al., 2010; Nalcacioglu et al., 2007; 2003; D'Costa et al., 2004; 2001). There are also some studies on apoptosis (Chitnis et al., 2008; 2011; Ince et al., 2008; Paul et al., 2007). The first proteomic analysis study on invertebrate iridoviruses was carried out in CIV, and a total of 46 viral proteins were identified in the virion structure based on the detection of 2 or more distinct peptides, an additional 8 protein were detected based on a single peptide (Ince et al., 2010). The most interesting point was the absence of cathepsin and chitinase genes in CIV genome. It was thought that the absence of chitinase and cathepsin proteins in the CIV genome could result in very low virulance of the virus.

1.4. General Properties of Chitinase and Cathepsin Proteins

The main component of the peritrophic membrane, that protects the cuticle in insects and the intestines of most insects, is a polysaccharide called chitin (Figure 6). Pathogens that infect the insect gut must first cross the barrier rich in the chitin (Shen and Jacobs-Lorena, 1997; Sampson and Gooday, 1998; Hoell et al., 2005).

Splitting chitin into disaccharides or larger oligosaccharides is done extracellularly by means of chitinase enzyme (Citrit et al., 1995).

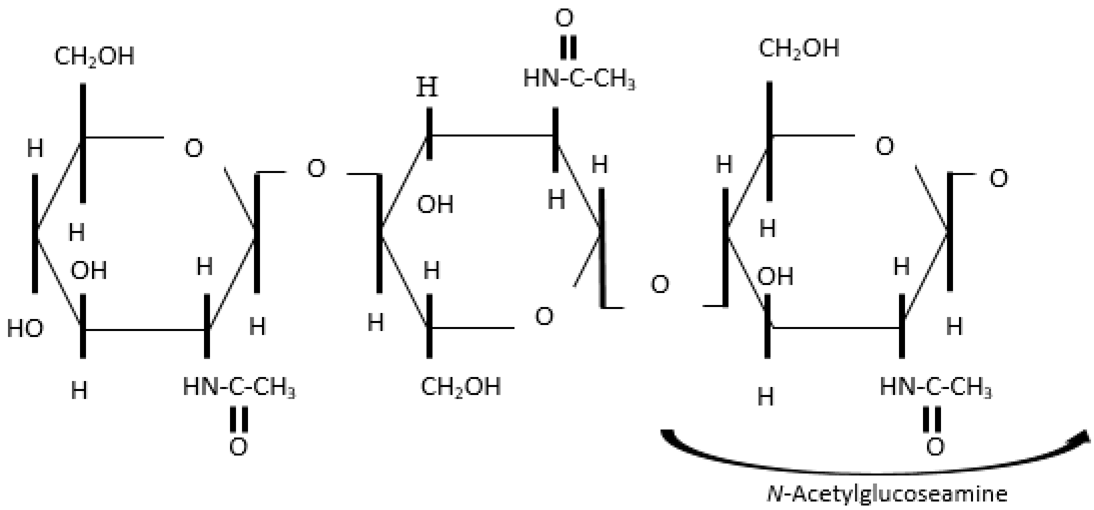


Figure 6. Molecular structure of the chitin

1.5. The Roles of Chitin and Chitinase in Insect Digestion System

The insect gut (or alimentary canal) has three main regions, and the sphincter muscles (valves) control fluid movement between the food-regions (Figure 7) (Gullan ve Cranstons, 2012). The insect's foregut (stomodeum) is involved in the uptake, storage, grinding and then transport of nutrients to the midgut (mesenteron). Absorption of digestive enzymes produced and secreted in the midgut takes place. Material remaining in the intestinal lumen with urine from the Malpighian tubules then enters the hindgut (proctodeum). Before the stool is expelled from the anus, many molecules needed are absorbed in the hindgut. The intestinal epithelium is a thick layer of cells along the canal and rests on a basement membrane surrounded by a variably developed muscle layer. There is a cuticular lining in both the foregut and the hindgut, while it is absent in the midgut. The generalized middle intestine has two main areas - the tubular ventricle and a blunt-ended lateral diverticulum called the caeca (ceca).

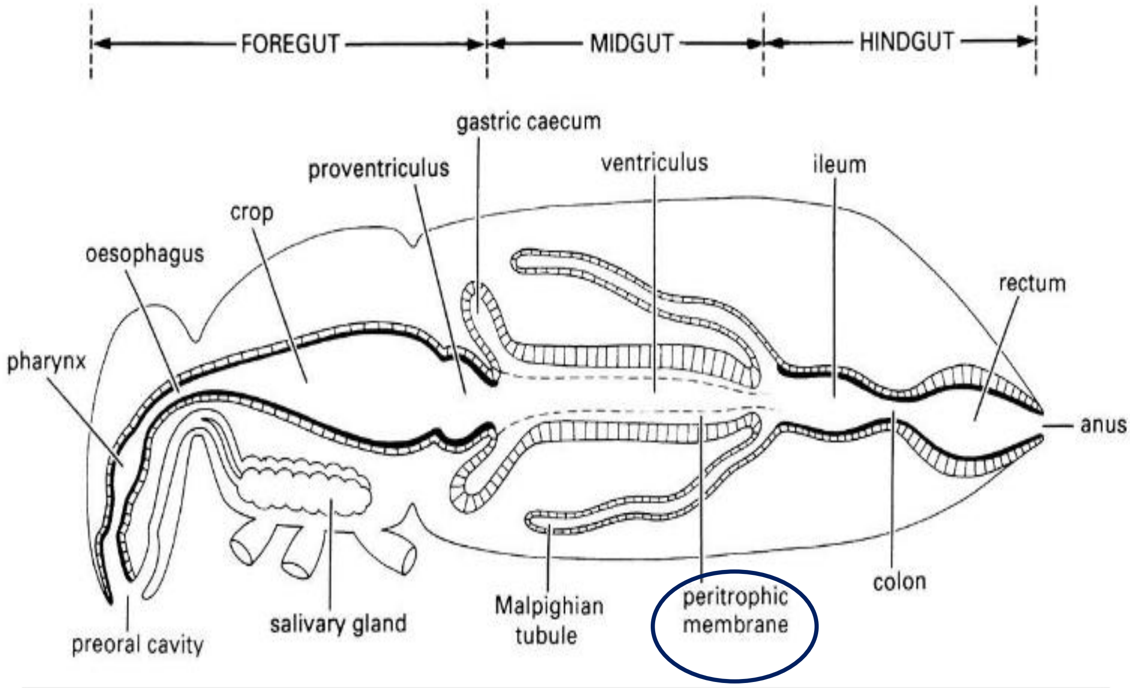


Figure 7. Generalized feeding channel of insects (Gullan ve Cranstons, 2012)

Most midgut cells have finger-like protrusions of microvilli that resemble each other. The foregut and midgut are differentiated in terms of epithelial thickness in histological sections. A thin layer of midgut epithelium called the peritrophic membrane is separated from food by a thin sheath of protein-glycoprotein structure. These chitin-binding proteins, called peritrophins, may have evolved from gastrointestinal mucus proteins. Accordingly, chitinase and cathepsin proteins are effective in damaging the peritrophic membrane.

Chitinases are glycosyl hydrolases that catalyze the hydrolytic degradation of chitin (Kurita, 2001). In recent years, chitinases have gained increasing importance due to their wide application area. Cathepsin were discovered in the cell lysosome, a group of protease enzymes with several members that are expressed ubiquitously in the organism. The expression and localization profiles of these enzymes vary in different cells and tissues. Baculoviruses contain the chitinase and cathepsin genes, among which the Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) *chiA* is well characterized. AcMNPV chiA has both exo- and endo-chitinase activities (Hawtin et al., 1997) and accumulates in a C-terminal region and localizes in the endoplasmic reticulum (ER) of infected cells (Thomas et al., 1998; Saville et al., 2002). The chitinolytic activity of AcMNPV chiA is required to cause host liquefaction, and deletion of the C-terminal region causes it to be secreted from infected cells (Thomas et al., 1998; Saville et al., 2002). Another known function of AcMNPV chiA is to aid proper folding of the virus-encoded cysteine protease, cathepsin (v-Cath) (Thomas et al., 2000). V-Caths are required to cause terminal liquefaction of infected larvae after death. Baculovirus chitinase and cathepsin must function harmoniously and timely to effectively disrupt host tissues. If the timing of their enzymatic activity is altered, host tissues can be degraded too early during virus infection, compromising host loss and thus limiting virus replication.

The exoskeleton of late-stage Tricopulasia ni larvae infected with AcMNPV becomes brittle and liquefied 1-2 days after the insect's death. During this process, occlusion bodies are released into the external environment to initiate a new cycle of infection. In the absence of chitinase or cathepsin activity, insect tissues are resistant to degradation long after the insect has been infected (Daimon et al., 2007; Hawtin et al.1997, Katsuma et al., 2009; Ohkawa et al., 1994; Slack et al., 1995; Thomas et al., 2000). Chitinase and cathepsin expression and activity, which are carefully choreographed during virus replication, are very important for these enzymes to work together. Chitinase is expressed in the late phase of virus replication and with v-cathepsin, promoting liquefaction of the host during the final stages of infection (Hawtin et al., 1997). Chitinase converts to a secretory protein that suppresses the protein translocation mechanism and competes strongly with recombinant secretory proteins on its way to the secretory pathway (Thomas et al., 1998; Possee et al., 1999). v-cathepsin accumulates in the endoplasmatic reticulum (ER) of infected cells and is synthesized as an inactive proenzyme (Hom et al., 2002). Chitinase can function as a chaperone for the smooth folding of the inactive v-cathepsin in the ER (Hom and Volkman, 2000). Chaotropic agents such as sodium dodecyl-sulfate trigger the conversion of the inactive v-cathepsin to the active v-cathepsin, which may result in the degradation of recombinant proteins (Hom and Volkman, 1998).

1.6. Purpose of the Study

Since CIV infects some hazardous insects in agriculture, it has a special economic and ecological importance (Smith, 1976). However, due to their broad host range, limited mortality and low oral infectivity IIVs are of little interest as biocontrol agent. It is considered that the virulance of CIV can be increased by using baculovirus proteins. In this thesis, it was investigated whether chitinase and cathepsin proteins of Helicoverpa armigera SNPV (HearSNPV), belongs to Baculovirus genus, increase the virulance of *Chilo* iridescent virus (CIV), a member of the Iridovirus genus. For this purpose, chitinase and cathepsin proteins belonging to HearSNPV were expressed in the bacterial system and their additive effect on CIV virulence was tested on *Galleria mellonella* larvae.

2. MATERIALS AND METHODS

2.1. Viral DNA Isolation

DNA of Helicoverpa armigera SNPV (HearSNPV) was extracted from pure PIB suspension of HearNPV-TR (Eroğlu et al.,2020) using DNA isolation kit (Qiagen). For this purpose, 50 µl 0.1 M NaOH (pH: 12.5) was added to dissolve the polyhedrin protein on 500 µl PIB suspension and the suspension was incubated for 15 min at 37 ℃. Then, 50 µl of 0.1 M Tris-HCl (pH 8) was added to neutralize the pH. Subsequently, 180 µl ATL buffer and 20 µg/µl proteinase K were added onto the virions whose polyhedrin protein was dissolved and incubated at 55 ℃ for 2 hours. Additionally, 200 µl AL buffer was added and vortexed for 10 seconds. Suspension was incubated at 70 ℃ for 10 minutes. Ethanol (200 µl) was added on suspension and inverted. The mixture was filtered through columns by centrifuging at 8000 rpm for 1 minute. To wash the columns, 500 µl AW1 buffer was added and centrifuged for 1 min at 8000 rpm. Later, 500 µl of AW2 buffer was added and centrifuged at 14000 rpm for 3 minutes. The column was placed in a fresh eppendorf tube, 100 µl AE buffer was added, incubated for 1 minute at room temperature, and then centrifuged at 8000 rpm for 1 minute. DNA amount and purity were measured in nanodrop, and the presence of DNA was monitored under ultraviolet light by running at 80 V for 30 minutes in a 0.7% agarose gel (Nakai, and Kunimi, 1997).

2.1.1. Amplification of Target Genes from Viral Genome

DNA sequences and protein active regions of chitinase and cathepsin genes of HearSNPV were determined using NCBI Blast and PROSITE programs (Worley et al. 1995), and the regions of the signal sequence and transmembrane domain were determined using SignalP (Nielsen et al 1997) and TMHMM - 2.0 program. Sequences of chitinase and cathepsin gene regions belonging to *H. armigera* SNPV in the gene bank were used to design primers (Table 2). Restriction endonuclease sites were added to the ends of the primers for cloning.

Table 3. Primer sequences of chitinase and cathepsin genes of HearSNPV

Primer	Sequence (5- 3')	Tm
Chit-Fw:	5'- CG**GGATCC**GCTCCACCCGGCGTTCCCA -3' (*Bam*HI)	65.8 ℃
Chit-Rv:	5'- C**CAAGCTT**GTGAAACGACATTGGCCAAAATCA -3 (*Hind*III)	62 ℃
Cath-Fw:	5'- CG**GGATCC**GATGAAATATCTTTGCATACGTCGTC -3'(*Bam*HI)	54.3 ℃
Cath-Rv:	5'- C**CAAGCTT**TTATTGAATTACCGACGACGCG -3' (*Hind*III)	55.4 ℃

*Bam*HI and *Hind*III are shown in underlined letters, Chit: Chitinase, Cath: Cathepsin.

Chitinase and cathepsin genes were amplified from H. armigera SNPV (HearSNPV) genomic DNA. Reaction was prepared 1 µl of 10 ng/µl template DNA, 10 µl 5X PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM sterile KC1), 1.5 mM $MgCl_2$, 1U *Taq* DNA Polymerase, 1 µl of 1 mM forward primer, 1 µl of 1 mM reverse primer, 170 mM d ATP, 170 mM dCTP, 170 mM dGTP and 170 mM dTTP and the volume was adjusted to 50 µl with sterile distilled water. Amplification was carried out in 200 µl tubes using thermal cycler ("Biometra's T-Personal). DNA amplification was performed under the following condition: 2 minutes at 95 ℃ for initial denaturation, 35 cycles consisting of 1 minute at 94 ℃ for denaturation, 1 minute at 56 ℃ for annealing and 2 minutes at 72 ℃ for extension. The PCR product was run on a 1% agarose gel, visualized on the "BioDoc Analyze" system.

2.1.2. Cloning the Target Genes into Transfer Vector

Amplified DNA fragments excised from the gel were cloned into pJET1.2 vector (Thermofisher) at the desired vector:insert ratio of 1:3. Reactions were carried out separately both for chitinase and cathepsin DNA fragments in a volume of 20 µl and incubated at 25 ℃ for 30 minutes. After 30 minutes ligation reaction was ready to transform to compotent cells.

2.1.2.1. Competent Cell Preperation

A single colony was transferred from *E. coli* DH10β cells into 3 ml of LB (Luria Bertani) broth medium without antibiotics with the help of a sterile toothpick and grown at 37 ℃ overnight (16 hours optimum time) on a shaker. The bacteria culture was measured in the spectrophotometer at 600 nm wavelength. Thirty milliliter of LB medium was inoculated with this culture at an optical density (OD) of 0.1. It was incubated at 37 ℃ for 1 hour for the concentration reachs between 0.45-0.55. Then, when it reached the desired density, it was transferred to a 50 ml flask and centrifuged at 40 ℃, 4500 rpm for 5 minutes. The supernatant was discarded. The pellet was dissolved by adding 10 ml of cold 100mM $CaCl_2$ and kept on ice for 30 minutes. It was centrifuged again at +4 ℃, 4500 rpm for 5 minutes. The liquid part was discarded, and the pellet was dissolved in 2 ml of cold $CaCl_2$ and stored at +4 ℃.

2.1.2.2. Transformation

The ligation products (5 µl) were left on 200 µl competent *E. coli* DH10β cells and incubated at 45 ℃ for 2 minutes and subsequently transferred immediately on ice. The reactions were added on 1 ml LB in glass tubes and incubated at 37 ℃ for 2 hours. Then, the samples in tubes were transferred to 1 ml microcentrifuge tube and centrifuged at 5000 rpm for 3 minutes. Liquid in the upper part was discarded. The remaining 50 µl of liquid and the pellet were mixed and spread on LB agar plates containing 50 µg / ml ampicillin with a glass rod. Plates were incubated overnight at 37 ℃. Next day, bacterial colonies were observed on plates. Only bacterial cells with recombinant plasmids are able to form colonies. These colonies were checked for the presence of target genes. For this aim, overnight cultures were prepared.

Fast miniprep method (Yie et al.,1993) was applied for the isolation of plasmid DNAs. Overnight cultures were precipitated by centrifugation at 13,000 rpm for 2 minutes. The precipitate, with remaining 50 µl supernatant, was dissolved by vortexing. TENS buffer (300 µl) (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.1 N NaOH, 0.5% SDS) was added on it. Subsequently, 150 µl of 3 M sodium acetate (pH 5.2) was added and mixed again 5-6 times by turning. Tubes were centrifuged at 13,000 rpm for 3 minutes. Supernatants were transfered to a new microcentrifuge tube, and 900 µl of 100% ethanol was added and centrifuged at 13,000 rpm for 2 minutes. The precipitates were washed again by centrifugation at 13,000 rpm for 2 minutes with 70% ethanol and left to dry. Dried precipitates were dissolved in 25 µl dH_2O. The plasmid DNAs were treated with restriction enzymes that the sequences were added to the ends of the primers of the genes in order to confirm cloning of the target fragments. For this, 10 µl DNA, 0.5 µl *Bam*HI (10U/µl), 0.5 µl *Hind*III (10U/µl), 2 µl 10X reaction buffers of each enzyme and 5 µl H_2O were prepared in 20 µl volumes and incubated at 37 ℃ for 3 hours. Then, the samples were electrophoresed in 1% agarose gel. Clones containing chitinase and cathepsin genes were identified.

Sequence analysis was performed to confirm the sequences of the chitinase and cathepsin genes. Plasmid DNAs were isolated from the clones containing these genes using plasmid DNA isolation with Wizard Plus SV Minipreps DNA Purification System" kit (Promega). DNA's were sent to Macrogen Company (Netherlands) for sequence analysis. The nucleotide sequences (Table 4-5) obtained as a result of the analysis were compared with the sequences of the genes in the gene bank using the BLAST program in the NCBI database.

The plasmid DNAs, containing confirmed chitinase and cathepsin genes and confirmed about their sequences, were further digested with the related restriction enzymes to obtain high amount of chitinase and cathepsin gene fragments. These gel purified fragments are used at subsequent cloning to expression vectors.

2.1.3 Cloning the Target Genes into the Expression Vector

E. coli cells including the expression vector pET-28a (+) was inoculated into 5 ml of LB medium containing kanamycin and incubated at 37 ℃ for overnight. After centrifugation at 13,000 rpm for 3 minutes, plasmid DNA was isolated using the "Wizard Plus SV Minipreps DNA Purification System" kit (Promega). Plasmid DNA was digested with *Bam*HI and *Hind*III restriction enzymes. Reaction was prepared with 1 µl of 10 ng/µl of plasmid DNA, 3 µl of 10X Tango buffer, 1 µl of *Bam*HI (10U/µl), 1 µl of *Hind*III (10U/µl) and 15 µl of sterile dH_2O in a tube to achieve a final volume of 30 µl, and incubated at 37 ℃ for 3 hours. After incubation, the product was electrophoresed on 1% agarose gel.

The linearized pET-28a (+) vector was cleaned from the gel using a DNA cleaning kit (Macherey-Nagel). Gel purified chitinase and cathepsin genes cloned into the pET-28a (+) vector in a reaction including 2 µl of 10X T4 DNA ligase buffer (Promega), 1 µl of T4 DNA ligase enzyme (Promega), 1 µl of pET-28a (+) vector, 3 µl of insert DNA and 13 µl of dH_2O. The reaction was incubated at +4 ℃ for overnight.

The recombinant plasmids transformed to the component *E. coli(*DH10β) cells as described under title 2.1.2. and their gene content was confirmed by restriction endonuclease digestions. These recombinant plasmids purified from the cells and used at transformation process to the *E. coli* BL21(DE3) cells in order to obtain protein expression. For this, a colony of *E.coli* BL21(DE3) cell was inoculated into 3 ml of LB medium and incubated at 37 °C at 200 rpm overnight. Again, the method with $CaCl_2$ was used to generate compotent. Recombinant pET28a vectors containing chitinase genes were transformed into prepared compotent cells. For this, 3 µl of recombinant plasmid DNA was taken and mixed with 200 µl of cells. The mixture was incubated on ice for 30 minutes and then at 45 °C for 2 minutes. Then, the reaction mixture was poured into a glass tube containing 1 ml of LB medium and shaken for 1 hour at 200 rpm at 37 °C. At the end of this period, the culture taken into Eppendorf tubes was centrifuged at 6000×g for 3 minutes. The supernatant was poured so that 100 µl of medium remained on the precipitated cells, and the cells were dissolved in the remaining liquid. Then, smears were made on LB agar plates containing 50 µg/ml kanamycin and incubated overnight at 37 °C. By the same method, the pET-28a(+) vector without gene cloned was transferred into *E.coli* BL21(DE3) cell itself. To ensure that cloning processes were present, plasmid isolation and then re-cutting with restriction enzymes were validated.

2.1.4 Expression and Detection of Chitinase and Cathepsin Proteins

One of the colonies, formed above for each genes, was transferred with a toothpick to 5 ml LB medium containing kanamycin. These tubes were incubated at 230 rpm at 37 ℃ overnight. The resulting overnight culture was added to 250 ml of LB medium containing kanamycin. When OD_{600} = 0.5-0.6, 250 µl IPTG (Stock concentration 240 mg/ml) was added to the medium to induce gene expression and incubated at 37 ℃ approximately 4 hours. Later the cultures were transferred to 50 ml falcon tubes and centrifuged at 6000 rpm for 10 minutes at 4 ℃. Supernatant parts were removed and precipitates were placed on ice. These samples are further used for determining the presence of expressed proteins.

For detecting the expressed proteins, polyacrylamide gel electrophoresis and western analysis was performed. For this aim, 5ml of PBS (1X, pH = 7.2) was added to each of the pellets and dissolved by gentle vortexing. Later, 1 ml of lysis buffer (10 ml 1XPBS buffer

+ 1 ml Glycerol + 50 µl Triton X-100) was added, and then it was treated in the sonicator for 3 times as 30 sec amplitude on 30 sec off for 1min.

Samples taken from the sonicator were centrifuged at 4 ℃ for 5 min at 5000 rpm. The supernatant was taken and transferred to another microtube. After taking 20 µl from the samples, 20 µl of SDS treatment buffer was added on them. Then, it was incubated at 95 ℃ for 5 minutes and taken back to ice for 1 minute later centrifuged for 1 minute at the highest speed. It was taken back and kept on ice. It was made ready to be loaded onto the gel. Proteins were then uploaded to the 12% SDS-PAGE (Laemmli.,1970). Electrophoresis was performed at 80V current.

After the process was completed, western blot hybridization analysis was performed using histidine antibody specific for proteins to see whether the chitinase and cathepsin proteins were expressed. Proteins in polyacrylamide gel were transferred to nitrocellulose membrane using a semi-dry blotting system. For this, nitrocellulose membrane and Whatman papers were cut in gel size. After soaking in the electroblotting buffer, 2 Whatman papers, 1 nitrocellulose membrane, gel and 2 Whatman papers were placed in the device in order from bottom to top. The device was operated at 40 microamper for 2 hours. The membrane was transferred to a 50 ml vial, 5 ml of the washing buffer and 1µl anti-poly Histidine antibody (1: 1000 dilution) (Sigma, H-1029) (1st Antibody) added and incubated for 2 hours. The membrane was then washed 3 times with washing buffer for 10 minutes each. Then it was treated with 1µl Anti-Rabbit (2 antibodies) for 2 hours. It was washed again 3 times with PBS wash buffer. Subsequently, washing was carried out in Alkaline Phosphatase (AP) (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5, 1X) buffer. Finally, the membrane was treated with 100 µl of NBT (nitroblue tetrazolium) and BCIP (bromochloroindolyl phosphate) (NBT / BCIP stock solution, Roche, 11681451001) imaging dye in 10 ml AP buffer, and the hybridisations were visualized. After visualizing the bands, the reaction was stopped by pouring the solution and adding plenty of dH_2O on it.

2.1.5 Purification of Chitinase and Cathepsin Proteins

When genes were cloned into the pET-28a (+) expression vector, proteins were expressed with histidine tail. Taking advantage of this situation, purification was performed using the MagneHis Protein Purification System (Promega) kit according to the manufacturer's instructions. Dialysis was performed to purify the proteins from chemicals in the buffer in which they were dissolved. In the dialysis process, the membrane was prepared. 10 g $NaHCO_3$ and 1 ml 0.5M EDTA were added to 500 ml distilled water, and after this mixture boiled, the membrane was left in it, and the membrane was allowed to boil for 10 minutes. The membrane was then transferred into boiling distilled water and boiled for 10 minutes. After boiling, the membrane was allowed to cool. The lower part of the cooled membrane was closed, and the upper part was closed after the proteins was left inside. Protein-containing membranes were spun in 1xPBS buffer for 24 hours. During this time, the PBS solution was changed three times. Later protein concentration was determined by Bradford (1976) method. First, the standard curve was prepared using bovine serum albumin (BSA). For this, BSA solutions at different concentrations (0, 5, 10, 25, 50, 100, 150, 200, 250, 300 µg) were prepared. 10 µl of chitinase and cathepsin proteins and BSA solutions were mixed with 200 µl dye solution (Protein Reagent, Sigma). These mixtures were dropped into the wells of the 96-well plate in 3 repetitions. After 15 minutes of incubation, measurements were made at 595 nm with the SpectraMax M2 (Molecular Devices) device. To control the purity of the proteins a polyacyrlamide gel was run and treated with Coomassie Brillant Blue (0.125% Coomassie Brillant Blue R-250, 50% methanol, 10% acetic acid) dye for 30 minutes. It was then washed in wash solution (36% Methanol, 9% Acetic Acid) for 30 minutes. Gel image was transferred to computer.

2.2. Rearing *Galleria mellonella* Larvae

In this study, *G. mellonella* larvae, belonging to the order Lepidoptera, was used. The larvae at third instar were included in the study. The larvae were reared in an incubator at 28 ± 2 ° C and 65 ± 5% relative humidity. Artificial diet (200gr bran, 1.5 honeycomb, 250 ml glycerol, 100 ml flower honey, 100 ml distilled water) was used to feed the larvae. The mixture was prepared for G. mellonella larvae. After the food was prepared, about 20 pupae and crushed black honeycomb were placed in a jar. It was covered with two layers of American cloth and fixed with rubber. As the honeycomb decreased, the nutritional supplement was added and the jar was cleaned every two days. Third stage larvae were used in the study. The continuity of the stock culture was ensured by the opening of the eggs laid by the females as a result of mating of *G. mellonella* adult female and male insects left in food jars.

2.2.1 Infectivity Tests

Five different concentrations of chitinase and cathepsin proteins were prepared to feed the insects. The concentrations to be applied were determined as 500 ng, 1000 ng, 1500 ng, 2000 ng, 2500 ng, 5000 ng. In addition, chitinase and cathepsin proteins, each at equal amount, were given together to the insects at 2500 ng and 5000 ng treatments. Proteins were prepared the specified concentrations in PBS. For infection, 3rd instar *G. mellonella* larvae were used. The larvae starved for 8 hours before infection. Chitinase and cathepsin proteins at different concentrations were given orally to the larvae together with food and the larvae were kept in single pots. The larvae that finished the foods contaminated with chitinase and cathepsin proteins were given new food without chitinase and cathepsin proteins. These larvae were left in the normal laboratory conditions for 14 days and the dead larvae were counted by checking every day. Thirty larvae were used for each concentration and experiments were done in triplicate. PBS was used as negative control. Insecticidal activity results were interpreted using the Abbott formula (Abbott, 1925). The LC_{50} and LT_{50} values of the groups were evaluated using excel probit analysis. In order to evaluate the additive effects of chitinase and cathepsin proteins to the virulance of CIV, insects were fed with proteins and virus together. The larvae were first feed with proteins. Later twenty-for hours 15 µl of CIV at 100 ng/µl concentration was given to the insects. Insect groups, fed with only virus and proteins, were used as negative controls.

3. RESULTS

The study performed for increasing the virulence of Chilo iridescent virus (CIV) using baculovirus chitinase and v-cathepsin proteins is summarized below in Figure 8.

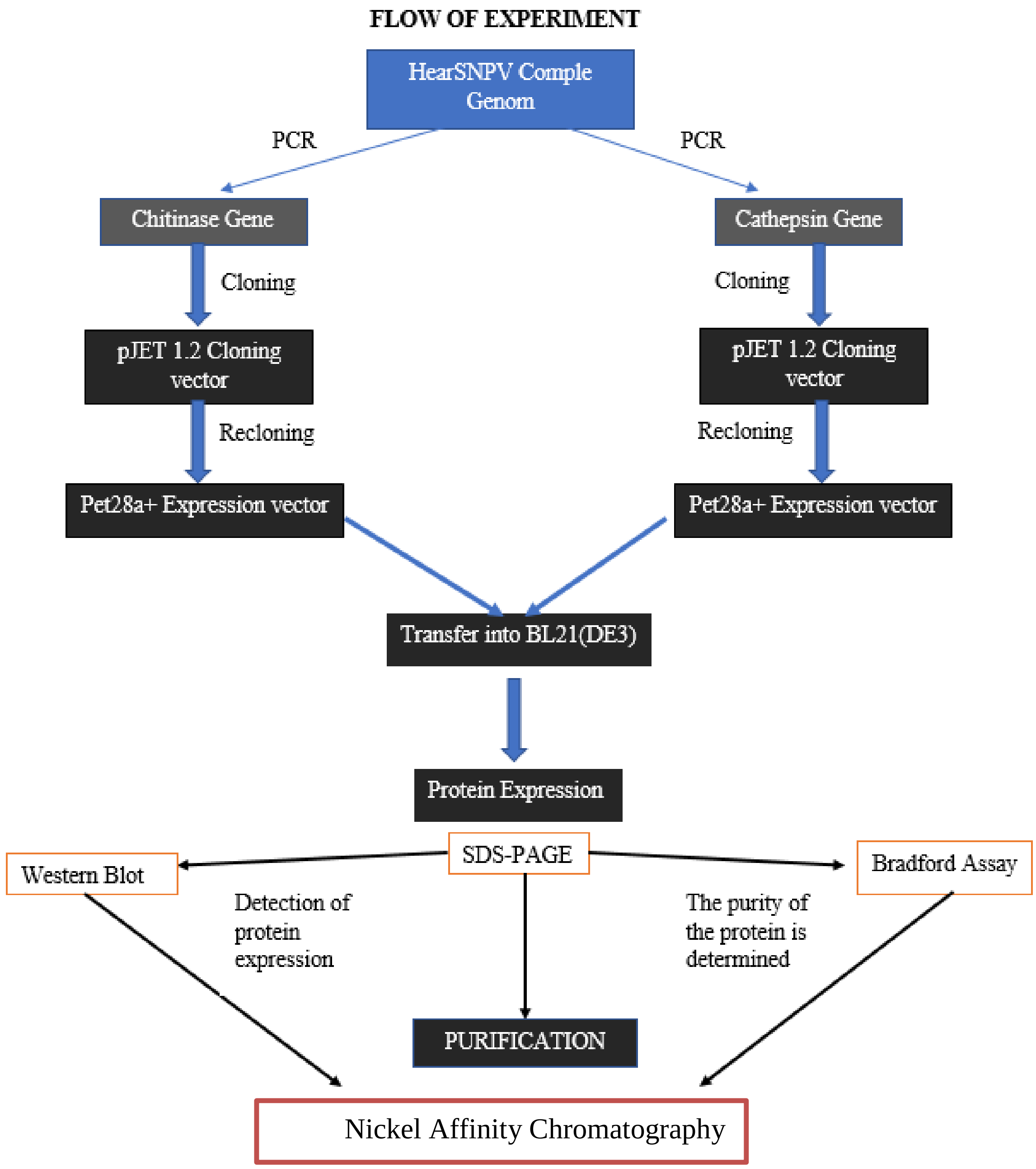
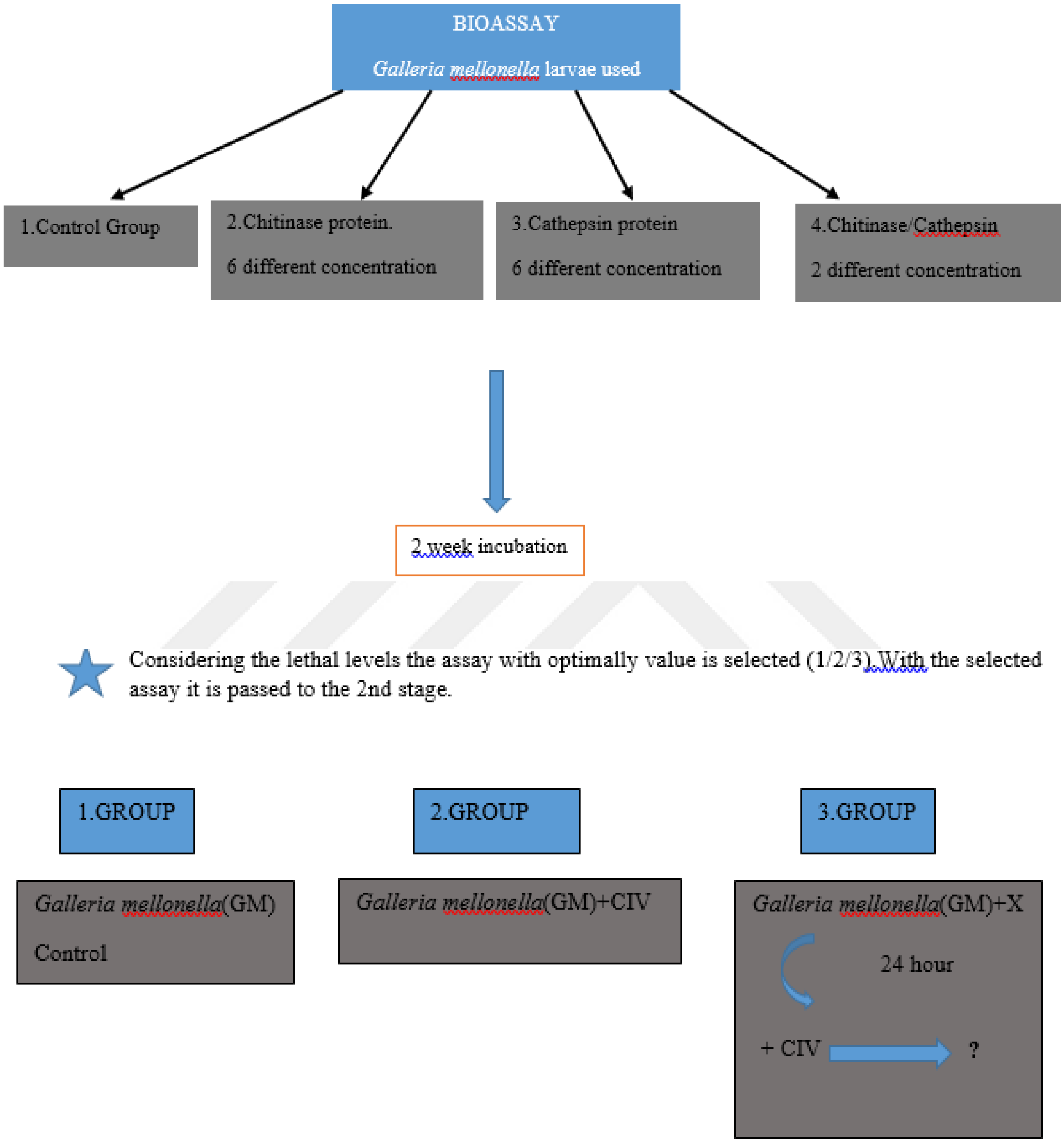


Figure 8. Experimental study to the virulence of CIV

The continuation of the Figure 8



3.1. Expression of Chitinase and Cathepsin Proteins

3.1.1. Obtaining Chitinase and Cathepsin Genes

Chitinase and Cathepsin genes were amplified from viral DNA. DNA was isolated from *Helicoverpa armigera* larvae infected with HearSNPV-TR. The concentration of DNA was determined as 82 ng / µl, and the quality was determined as 1.81 at 260/280 wavelength. The DNA was electrophoresed in a 0.7% agarose gel at 80 volts for 30 minutes (Figure 9).

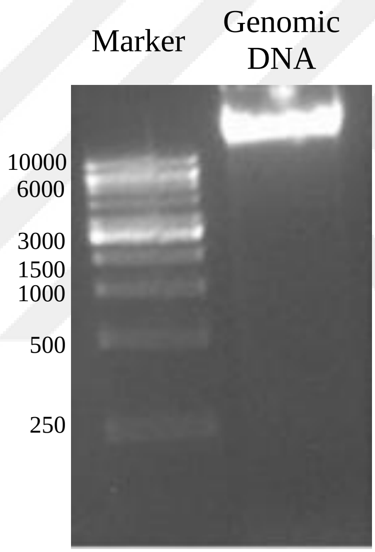


Figure 9. Genomic DNA of HearSNPV-TR

After the HearSNPVgenom sequence analysis was revealed, the chitinase and cathepsin genes were analyzed in the NCBI Blast program. Chitinase (1728 bp) and cathepsin (1104 bp) genes of the HearSNPV-TR were amplified by PCR from genomic DNA using gene specific primers. The expected sizes of PCR products were visualized in a 1% agarose gel containing ethidium bromide (Figure 10).

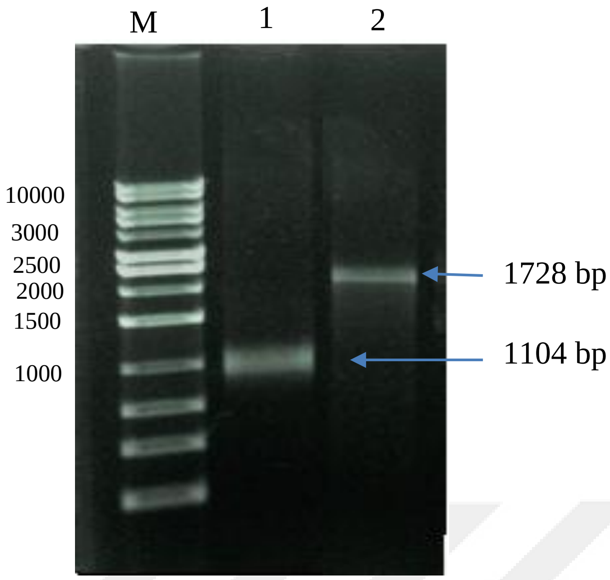


Figure 10. PCR amplified chitinase and cathepsin genes of HearSNPV. M: Marker; 1: cathepsin and 2: chitinase genes

3.1.2. Cloning the Target Genes into the Transfer Vector

After the PCR amplification, products obtained with blunt ends were cleared from the gel, cloned into the pJET1.2 cloning vector and then transformed into *E. coli* DH10β cells. Plasmid DNA was isolated from the resulting colonies using DNA Purification kit (Promega) and treated with restriction enzymes to detect the presence of the cloned genes. The results of digestion were shown on the gel (Figure 11). Digestion result produced bands in the right places, showing that the cloning was successful.

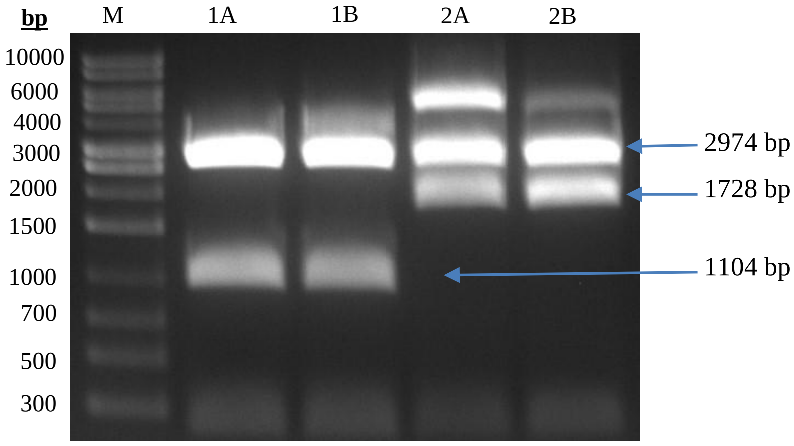


Figure 11. Verification of the chitinase and cathepsin genes in the pJET1.2 cloning vector. M: marker (1 kb); 2A and 2B pJET1.2v chitinase plasmid (digested by *Bam*HI and *Hind*III); 1A and 1B: pJET1.2v-cathepsin plasmid (digested by *Bam*HI and *HindIII*).

3.1.3. Cloning the Chitinase and Cathepsin Genes into the Expression Vector

After sequence verification, plasmid DNAs containing each gene was isolated from one of the available clones. The pJET1.2 vectors, containing chitinase and cathepsin genes, were digested with *Bam*HI and *Hind*III restriction enzymes and subjected to agarose gel electrophoresis (Figure 12). Meanwhile, the pET-28a (+) expression vector (5369 bp) was also digested and linearized with the same enzymes. The genes and the linearized vector are ligated and transformed to *E. coli* DH10β cells. The presence of recombinant plasmids in these cells was demonstrated by plasmid DNA isolation followed by digestion with restriction enzymes. Visualization of expression vectors and gene regions in the gel as expected at the end of the digestion was confirmed (Figure 12). As a result, expression vector gave a band of 5369 bp, chitinase 1728 bp and cathepsin 1104bp as expected.

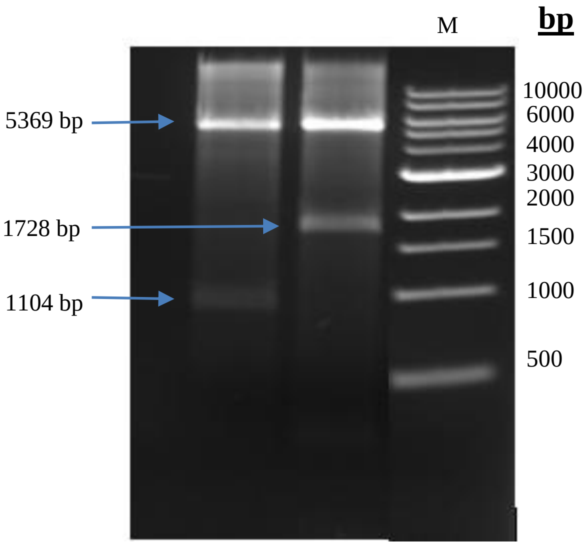


Figure 12. Agarose gel electrophoresis of pET-28a chitinase and pET-28a cathepsin digested by *Bam*HI and *Hind*III restriction enzymes. (M): 1kb DNA Ladder, 1: pET-28a (+) *Bam*HI and *Hind*III digestion result of cathepsin, 2: *Bam*HI and *Hind*III digestion result of pET-28a (+) v-chitinase.

Table 4. The amplificate sequence of chitinase

ATGAATAATTATAGTTTGTATTTGTTTGCTTTTAGTATTTTAATATTACATAGCTACGCTC**GGA**
TCCCTACAATTCGTTATGAATCAATCTACTGTTTGTACCTAGGCCGTTGCGATTTGTTTTCATC
AAGCCAACATGCATAGCGTTGAGCAAATCGCCGTTGTCCGCATCGATTTCCCATGCGAATAA
TCCGCCGAGTTTACGCTTAAGCACGTATTCGCCTTTGGCCAGTACCGATCTTTCGCTGTCGTA
CGATATTAAGTCGCCAGAGGCGCGGTCGAATACGTACGCAGCTTTGGCCACGTCGTCGAATG
CGTATTCGTAACGCGAAATGTTTTTAGCAATCTGTCTGTAGTCGACCACGCCGTTTTCCCATG
TACCCGTGATCGGTCCGACGGCTACGCCACTGAATGGATTGTCACTGTCGTAATTGTGGACTC
CCGTCCAACCGCGGCCGTACATTGCTACGCCCAACACGAGTTTTTTGGATTCACTCGTTGCG
CGAGCAAAGCGTCCACGGCCACGTTGGCGGTGTACGGTTCGTTAGGTTTCCACGCGGAACCG
TACAGTGCCGTCTGATGACCGAGATCGGTATTAGACCAAGCTCCTTTGAAATCATAACTCATC
ACGAAAATTTTATCTAAATACTGTTGTGCTCGGTCGTAATTAACCGCGGCGATCTTGTCTATG
CCAGCGCTAATCGCTGTGGTGAGTTCTAAAGTACGATTCGTTTGTATTTGAACTTGATCGAGC
ATGGCGCGCAATTCGCTTAACAAAGCAATATACGTGTTATTGTCGCGTTCCACATCGCCAACG
TTTGGATTGGCGCCTTTGCCGCCCGGAAATTCCCAATCAATATCGATGCCGTCGAAAAATTTC
CATGTCAACACGAATTCACGCACGGATTCGACAAAAATTTGTCGCGTTCGCGCATCGTGCAT
ATGATAGAAGGGATCGGACAGTGTCCAACCGCCGATTGATGCCAACACTTTTAGATTGGGAT
TGGCCAATTTTGCTGCCATTAATTGACCAAAATTGCCTTTGTAGGGTTCGTTCCATGCGCTAA
CGCCTGTTTGTGGTTTTTGGAGCGCCGCCCACGGATCGTGTATGGAAACTTTAAAATTGTCCC
TGCCAGCGCACGATCTTTGCAACGCTTCAAAACTCCCCGTGATAGATTTTAAACTGTCGTTTA
TACCGTCACCGCCGCAAATCGGTATAAATCCGTATAAAATGTGCGAAAGGTTCGGCGTGGGC
ACTTTGTCCACGGGAAAAGATCGACCGTAAACGCCCCATTCGACAAAATAGGCCGCTACGGT
GTGATCAGTGTTGTACGTATACGGTTTATTGTTTTCTTGCCACGTGTATTGTAACGGTTTTAAA
TGTTTACCATCGGTGTCTGCGATCACGACTTCTACGGTTTGACTAGCCGAACAGCCGTCGGCA
TTGCACAGTTTTACGTACATCGAATAACGACCGCTCGTATTGTAATCAAAAGTAGCAAAACG
ATCTTGCGTCGGACCGGTCCAAACGTTGATTACTTTGCTGTCTAGTTTATTTTCTAGATACACT
TGAGCGATTTCGCCTTGTTCGCCCGACCATACGCTCCATGATACTTTGATAGTTACAAACGGT
TGAGAGTTTACTAATGATTCGTAAGAGGTGGCTTGATGATTTATTTGCACTAAAGCGTAACTG
TGATCGGCCCAGTCTAATGTGGGAACGCCGGGTGGAGCAGCGTAGCTATGTAATATTAAAAT
ACTAAAAGCAAACAAATACAAACAATAATTATTCATTGATTTTGGCCAATGTCGTTTCAC**CA**
AGCTT

The chitinase gene sequence is between the *Bam*HI(**GGATCC)** and *Hind*III(**CAAGCTT)** restriction sites. *Bam*HI and *Hind*III are shown in bold and ATG start codon is underlined.

Table 5. The amplificate sequence of cathepsin

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATAT
GGCTAGCATGACTGGTGGACAGCAAATGGGTC**GGATCC**GATGAAATATCTTGCATACGTCG
TCGTCGCCACCGCCATTGTCTTCGCCAGTGCCAGTGCTGTATTATAATTTAGATCAATCCGAA
ATTTATTTCAAACATTTCCTACAGCAATACAACAAAAGCTACGACGATCCCAAAGAATACCA
ATACCGTTACAATGTGTTCAAAGACAATTTGAACAAAATTAATTCTCAAAATCGAGAAAATC
TGTTGAATAACAAAAACAATAACGATTCGCTTTCTACATCGGCTCAATTTGGTGTGAACAAA
TTCAGTGACAAGACCCCAAACGAAGTGTTACACTCGAACACTGGTTTTTTTTAAATCTTAGC
CAGCACTACACATTATGCGAAAATAGAATAGTTAAAGGCGCGCCCGACATACGTTTGCCCGA
TTTTTATGATTGGCGCGACACCAATAAAGTGACTCCCATAAAAGATCAAGGAGTTTGCGGAT
CGTGTTGGGCTTTCGTAGCAATAGGCAATATTGAAAGTCAATATGCCATACGGCACAACAAA
TTAATAGATCTGTCCGAACAGCAACTGTTAGATTGCGATGAAGTTGATTTAGGTTGTAATGG
TGGTTTGATGCATTTAGCGTTTCAAGAACTATTGCTGATGGGCGGTGTTGAAACGGAAGCAG
ATTATCCCTATCAGGGCAGTGAACAAATGTGCACTTTAGATAATCGCAAAATAGCTGTCAAA
TTGAACTCTTGCTTTAAATACGACATACGTGACGAGAATAAATTGAAAGAATTGGTGTACAC
TACTGGACCTGTGGCGATAGCAGTCGACGCCATGGACATTATTAATTATCGCAGAGGAATAT
TGAATCAATGTCACATTTATGATTTGAATCATGCCGTTTTGCTTATCGGTTGGGGTATCGAAA
ACAATGTACCCTATTGGATTATAAAAAATTCATGGGGGAGAAGATTGGGGCGAAAACGGCT
ATTTGCGAGTGCGGCGCAACGTCAACGCCTGCGGATTACTCAACGAATTCGGCGCGTCGTCG
GTAATTCAATAA**CAAGCTT**

The cathepsin gene sequence is between the *Bam*HI(**GGATCC)** and *Hind*III(**CAAGCTT)** restriction sites. *Bam*HI and *Hind*III are shown in bold and ATG start codon is underlined.

3.1.4. Expression and Isolation of Chitinase and Cathepsin Protein

The pET-28a (+) vectors, including chitinase and cathepsin genes were transformed into *E. coli* BL21(DE3) cells. Protein expression was induced with 1 mM IPTG over 4 hours. The prepared proteins were analyzed in 12% SDS-PAGE. Western blot hybridization was performed after SDS-PAGE analysis. Protein bands of 64 kDa for chitinase and 43 kDa for cathepsin were detected (Figure 13).

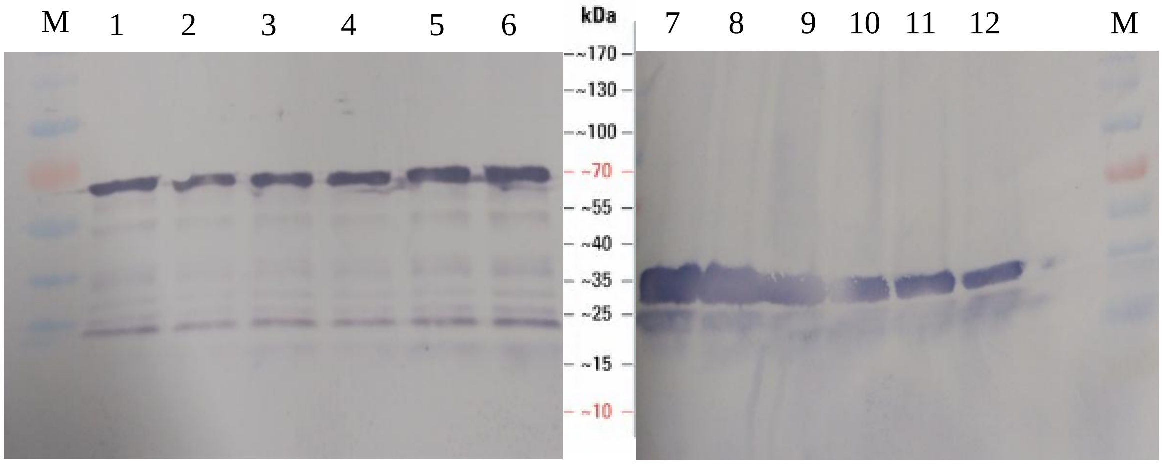


Figure 13. Western blot analysis of chitinase and chatepsin proteins of HearSNPV-TR. M: marker (prestained), 1, 2, 3, 4, 5, 6: proteins encoded by chitinase gene, 7, 8, 9, 10, 11, 12 proteins encoded by cathepsin gene.

Proteins were purified using the Magne-His Protein Purification System (Promega) kit and subsequently dialysed. Purified and dialysed proteins were subjected to SDS polyacrylamide gel electrophoresis. In order to control the purity of the proteins, the gel was treated with Coomassie Brillant Blue staining (Figure 14). As expected, the proteins gave bands of the expected sizes.

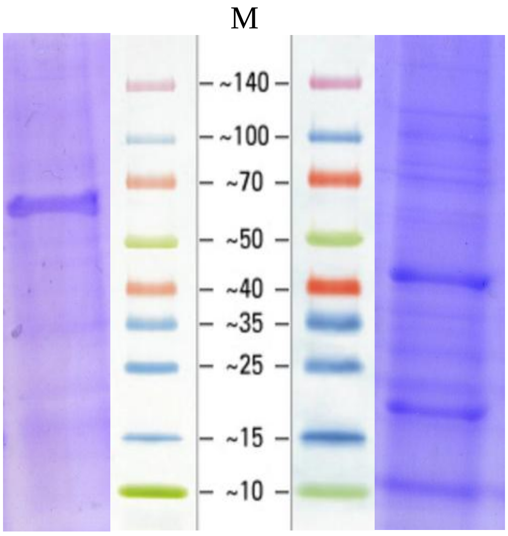


Figure 14. Coomassie staining of chitinase and cathepsin genes expressed in *E. coli* BL21 cells. 1: IPTG-induced pET-28a /chitinase in *E. coli* BL21, 2: IPTG-induced pET-28a /cathepsin in *E. coli* BL21(DE3), M: Protein marker (Promega).

3.2. Effects of Chitinase and Cathepsin Proteins on *Galleria mellonella*

In this study, 3rd instar larvae of *G. mellonella* insect belonging to Lepidoptera group were used as test organisms. The effects of viral chitinase and cathepsin on larvae were observed for 14 days and the results were compared. The data obtained were interpreted using the Abbott formula (Abbott, 1925). Insecticidal activity results showed that the effects of viral chitinase and cathepsin proteins have effects on larvae at similar levels. However, the combined effects of two proteins on larve is higher (Figure 15).

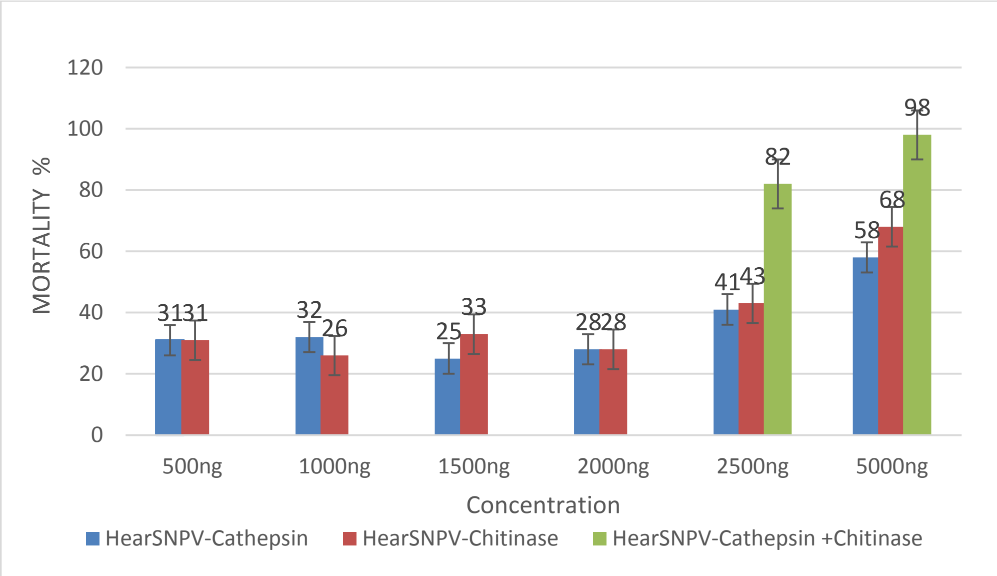


Figure 15. Insecticidal effects of purified HearSNPV-Chitinase, HearSNPV-Cathepsin and HearSNPV-(Chitinase+Cathepsin) proteins at different concentrations on *G. mellonella* larvae.

Using the insecticidal activity results, protein concentrations (LC_{50}) required to kill 50% of the larvae were calculated (Table 6). In line with these data, using 2500 ng concentration, dead larvae rates were determined depending on time and a graph was created (Figure 16). Also, using these results, the time required to kill 50% of the larvae (LT_{50}) at 2500 ng concentrationwas calculated (Table 7).

Table 6. LC_{50} values of HearSNPV-Chit, HearSNPV-Cath and HearSNPV- (Chit + Cath) proteins

PROTEIN	LC_{50}	Slope±SE	df	X^2
HearSNPV-Chit	3981ng	0,65±0,292	5	0,937
HearSNPV-Cath	6310 ng	0,93±0,252	5	0,651
HearSNPV- Chit +Cath	1585 ng	3,75±0,01	1	3,753

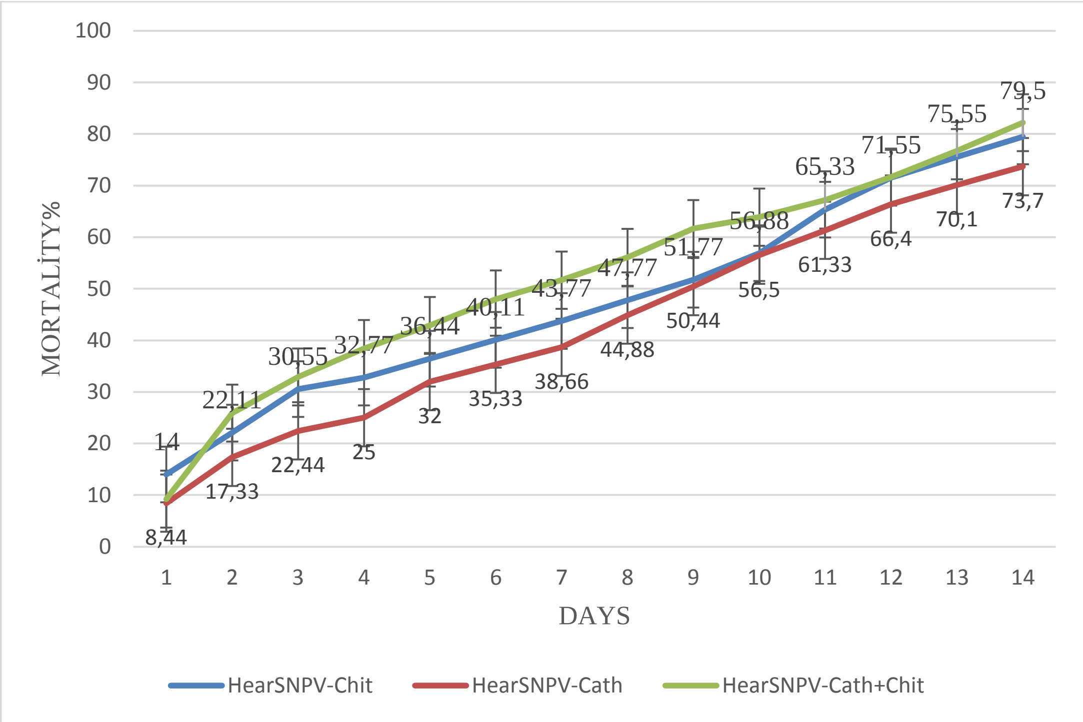


Figure 16. Mortality rates of purified HearSNPV-Chit, HearSNPV-Cath and HearSNPV-(Chit+Cath) proteins on *G. mellonella* larvae depending on time.

Tablo 7. LT_{50} values of HearSNPV-Chit, HearSNPV-Cath and HearSNPV-(Chit+Cath) proteins

PROTEIN	LT_{50}(Day)	Slope±SE
HearSNPV-Chit	6,93	4,7856±0,210
HearSNPV-Cath	7,89	4,9513±0,205
HearSNPV- Chit + Cath	6,07	4,8565±0,224

3.3. Determination of the Insecticidal Effects of Chitinase and Cathepsin on CIV

The chitinase and cathepsin proteins at 2500 ng/µl concentration was used to test the insecticidal effect of these proteins with CIV on *G. mellonella* larvae. The results obtained showed that HearSNPV-(Chit + Cath) protein produced more effective results when applied with the virus and increased the virulance of CIV (Figure 17).

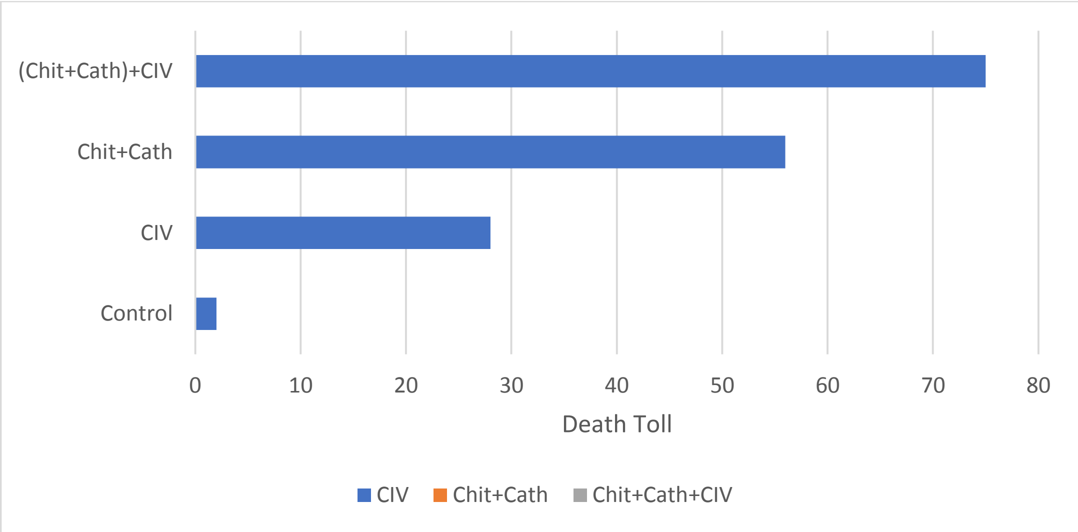


Figure 17. Insecticidal effect of purified HearSNPV-(Chit + Cath) proteins and CIV on *G. mellonella*. Control: Larvae fed only with food and water, CIV: Larvae fed only with virus, Chit + Cath: Larvae fed only with chitinase and cathepsin protein, (Chit + Cath) + CIV: Larvae fed first with chitinase and cathepsin proteins and CIV at the end of 24 hours.

4. DISCUSSION

In recent years, the increasing use of chemical pesticides and the effects of these chemicals negatively affect the entire ecosystem. Because of these negative effects, as an alternative to chemical pesticides, more protective substances and systems are needed. Intensive research have been carried out on these issues by several scientists. The number of scientific study on biopesticide production is increasing day by day. Biopesticides are classified as microbial biopesticides, plant pesticides and biochemical pesticides (Yarsan and Çevik, 2007). Microbial biopesticides may include living microorganisms such as viruses, bacteria, fungus, protozoa or nematodes themselves or their products. Microorganism originated products include mainly *Bacillus thuringiensis* crystal (cry) δ-endotoxin proteins, vegetative insecticidal proteins (vip) and cytolytic proteins (cyt), chitinase proteins of bacterial, viral or fungal origin, and various proteases (Harrison and Bonning, 2010).

Baculoviruses and entomopathogenic bacteria are microorganism groups that frequently cause epidemics in insects and are used in biological control (Francesca et al., 2018). Viruses are natural enemies of insects, and since baculoviruses cause diseases only in insects, they are used as safe biological control materials. So far, no resistance to baculoviruses has been found, and the molecular biology of these viruses has been studied in detail.

Microbial enzymes or metabolites, which are the products of living organisms themselves, are considered as an alternative way to combat harmful insects (Huang and Chen, 2008; Karasuda et al., 2003). Baculovirus and some of the entomopathogenic bacteria produce the chitinase enzyme that breaks down the chitin polymer and the cathepsin protein that chaperones it. In insects, the intestine and exoskeleton are the main parts targeted by the chitinase enzyme, due to the chitin polymer they contain. Therefore, chitinase enzyme encoded by microorganisms are potential microbial agents that can be used in combating harmful insects. Chitinase and cathepsin proteins of baculoviruses are expressed during virus replication, breaking down the chitin layer of the host cell and promoting the fluidization of the host. In this context, chitinase and cathepsin proteins have been considered as potential microbial agents that can be used to combat pests (Hawtin et al., 1997).

In this thesis, insecticidal effects of chitinase and cathepsin proteins of HearSNPV were tested on *G. mellonella* and compared with each other. In addition, the contribution of the combined use of chitinase and cathepsin enzymes to the oral infectivity of CIV has been also investigated. Chitinase and cathepsin proteins of HearSNPV expressed in *E. coli* BL21 (DE3) cells were purified and tested at different concentrations on *G. mellonella*. Although the insecticidal effects of the chitinase and cathepsins used are similar, it has been found that the chitinase and cathepsin proteins of HearSNPV are more effective when used together than the use of chitinase and cathepsin proteins alone. Additionally, although the insecticidal activity is the highest when chitinase and cathepsin proteins are given together, the insecticidal activity of chitinase is a little higher than and cathepsin protein.

Kramer et al. (1993) and You et al. (2003), showed that chitinase has increased the insecticidal activity of ticks by hydrolyzing chitin and altering the permeability of the peritrophic membrane. Their study showed that recombinant baculovirus chitinase can kill ticks. In addition, there was a synergistic effect in the rate of killing when chitinase was used in conjunction with the recombinant virus. It has therefore been suggested that the recombinant AcMNPV-CHT1 can be used as an alternative or a supplement to a biological tick control agent. The mortality was changed depending on the concentration of the chitinase used. It was determined that the higher the concentration, the higher the mortality rate. In a study by Kutsukake et al. (2008), soldier-caste nymphs of *Tuberaphis styraci* social aphid species produce a toxic cathepsin B protease in their intestines. When aphid predators threaten galls produced by breeding aphids, soldier nymphs introduce piercing mouthparts (styles) to intruders and release this cathepsin B orally, causing paralysis or death of intruders. Purified recombinant *T. styraci* cathepsin B was found to kill the larvae of wax moth *G. mellonella* within 2-4 hours when injected into the hemocele.

In a study by Hawtin et al. (1997), it was shown that larvae treated with AcNPV chitinase A protein had softening and liquefaction in their bodies. Also, in this thesis, it was observed that the dead larvae had softening after the chitinase and cathepsin protein was applied. In a study by Raw et al. (2004), it has been suggested that the AcNPV chitinase A (ChiA) protein that was expressed in *E. coli* cells. Causes liquefaction of the body by breaking down insect tissues, and they suggested that this chitinase may be a potential new tool for insect control. In the study in question, the AcNPV ChiA gene was expressed in *E.*

coli cells. Subsequently, the recombinant protein purified by affinity chromatography showed both exo- and endo-chitinase activities and insecticidal activity was investigated on *Bombyx mori* larvae. Depending on the concentration of the chitinase used, it was determined that the higher the concentration, the higher the mortality rate. Lysosomal catepsins are powerful enzymes; regulation of the proteolytic activity of these enzymes is essential to prevent damage to the biosynthetic mechanism of the cell, and activation of cathepsins only in acidic vesicles is one way to protect cellular processes.

Most cathepsins are unstable and are only weakly active at neutral pH (if any); therefore they are optimized to function inside the acidic cell. Finally, the cytoplasm and extracellular spaces are equipped with natural inhibitors of these enzymes (Chapman et al., 1997), which allows cathepsins to be active in the stomach of the insect, thereby entering the mid-intestine in the insect's stomach and triggering the insect's liquefaction with chitinase. V-cathepsin, a protease, is believed to be responsible for the degradation of the proteinaceous components of cadavers, while chitinase is essential in liquefying infected cadavers. Genes encoding chitinases are present in most of the fully sequenced baculoviral genomes so far, except for Plutella xylostella granulovirus (PxGV; Hashimoto et al., 2000). In a study conducted by Ozgen et al. (2013), *S. marcescens*' chitinase B and C proteins were transferred to *Bacillus thuringien*sis containing crystal proteins and the insecticidal activity of the resulting recombinant bacterium on *G. mellonella* was tested. Mortality rates have been recorded between 45% and 55%.

In this study, the chitinase protein of HearSNPV was tested on G. mellonella and resulted in an insecticidal effect of approximately 52% at a concentration of 2500 ng. Subsequently, the cathepsin protein of HearSNPV had approximately 36% insecticidal activity at the 2500ng concentration, and the chitinase and cathepsin proteins of HearSNPV together at the 2500ng concentration had approximately 80% insecticidal activity. Considering the studies conducted with nucleopolyhedrovirus (NPV) and *Granulavirus* (GV) belonging to two genes of the baculovirus family, an increase in the mortality rates of larvae with low instar were observed in the tests resulting in the addition of GV and application in combination according to the tests in which NPV was used alone (Shigeyuki and Chie, 2007; Martin, 2000).

Members of the Iridovirus genus infect invertebrates, mainly insects and terrestrial isopods, in humid and aquatic habitats, and both are known as invertebrate iridescent viruses (IIVs) because of the iridescent hues observed in heavily infected hosts. Such patent infections are almost always fatal, but there is now increasing evidence that latent lethal infections may be common in some host species (Williams 1993, Tonka & Weiser 2000). Although latent infection has been associated with long development time, reduced adult body size, and decreased fertility and longevity, hosts with such latent infections can survive and reproduce into adulthood (Marina et al., 1999, 2003).

Chilo iridescent virus (CIV), type species of the Iridovirus genus, replicates in many insect cell lines (Cerutti M et.al., 1981; Constantino M et al. 2001; Kelly D C and Tinsley T W. 1974; Ohba M. 1975) including cells from *Bemisia tabaci* (Funk CJ et al.2001), *Diaprepes rootworm* (Hunter WB and Lapointe S L. 2003) and boll weevil (D'Souza S, Henderson C et al.1997). (Cerutti et al. 1981, Constantino M et al. 2001; Kelly DC and Tinsley T W. 1974; Ohba M. 1975) can proliferate and even infect reptile cells (McIntosh AH and Kimura M. 1974). However, CIV does not reproduce *in vivo* in frogs (Ohba M and Aizawa K. 1982).

The results obtained from the studies conducted throughout this thesis are consistent with the studies in the literature and encourage the acceleration of the use of viral chitinase and cathepsin in biological control. As a result, the purification and use of chitinase and cathepsin enzymes isolated from virus genomes and expressed in *E. coli* may enable new applications in biological control and allow the production of new biopesticides. In this context, cathepsin and chitinase proteins of baculovirus can be purified and used as new biopesticides to increase oral infection of CIV. The results obtained from the studies conducted throughout this thesis are consistent with the studies in the literature and encourage the acceleration of viral chitinase and cathepsin use in biological control. Consequently, the purification and use of chitinase and cathepsin enzymes isolated from virus genomes and expressed in *E. coli* could enable new applications in biological control and allow the production of new biopesticides. In this regard, chitinase and cathepsin proteins of the baculovirus can be purified and used as new biopesticides to increase oral infection of CIV. In a recent study, the cell line AFKM-On-H, from hemocytes of the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae) was also studies for its ability to productively sustain CIV infection (Belloncik S et al. 2007). Several ultrathin sections demonstrated internalization of virus particles in cytoplasm of cells without clear signs of CIV replication.

However, suspicions were raised concerning the toxicity of the virus because cells were inoculated with high concentrations of CIV and since CIV readily infects *O. nubilalis* and replicates abundantly in different tissues and cells of larvae including the fat tissue and hemocytes (Belloncik et al. 1988).

In this tesis, the insecticidal effects of chitinase and cathepsin proteins of HearSNPV were tested on *G. mellonella* and compared with each other. In addition, the contribution of the combined use of chitinase and cathepsin enzymes to oral infectivity of CIV has been investigated. Chitinase and cathepsin proteins of HearSNPV expressed in *E. coli* BL21 (DE3) cells were purified and tested at different concentrations on *G. mellonella*. Although the insecticidal effects of chitinase and cathepsins used are close to each other, it has been observed that the use of HearSNPV chitinase and cathepsin proteins together causes more effects than they are used separately. The results obtained from the studies conducted throughout this thesis are consistent with the studies in the literature and encourage the acceleration of viral chitinase and cathepsin use in biological control. Consequently, the purification and use of chitinase and cathepsin enzymes isolated from virus genomes and expressed in *E. coli* could enable new applications in biological control and allow the production of new biopesticides. In this regard, cathepsin and chitinase proteins of the baculovirus can be purified and used as new biopesticides to increase oral infection of CIV.

5. CONCLUSIONS

In this study, the cloning of chitinase and cathepsin genes of Helicoverpa armigera singlenuleopolyhedrovirus (HearSNPV) belonging to the *Baculoviridae* family and their expression in the *E. coli* host were carried out. The expressed proteins were purified and their insecticidal effects were investigated on *G. mellonella*. As the second part of the thesis, it was investigated whether chitinase and cathepsin proteins increase the oral infectivity of Chilo iridescent virus. Based on the results of this study we conclude that;

1-The insecticidal effects of chitinase and cathepsin proteins on G. mellonella were found to be close to each other. However, but if we sort by insecticidal activity, HearSNPV-chitinase and HearSNPV-cathepsin proteins together showed higher insecticidal effects, followed by HearSNPV-chitinase protein and HearSNPV-cathepsin protein.

2-The lowest LC50 value was listed as HearSNPV- (chitinase + cathepsin), followed by HearSNPV-chitinase protein, followed by HearSNPV-cathepsin protein in proportion to insecticidal activity. HearSNPV- (chitinase + cathepsin) ranked first for the lowest LT50 value, followed by HearSNPV-chitinase protein, followed by HearSNPV-cathepsin protein. This proved that the chitinase and cathepsin proteins work together to increase activation.

3-Insecticidal activity test was performed on *G. mellonella* to determine whether chitinase and cathepsin proteins of HearSNPV contribute to oral infection of CIV.

4-According to these results; It was observed that the oral infection of chitinase and cathepsin proteins of HearSNPV on G. mellonella was quite high. Additionally; The chitinase and cathepsin proteins of HearSNPV were found to increase oral infection of CIV by 23%.

5-In the light of these studies, it was concluded that chitinase and cathepsin proteins of HearSNPV have potential to be used as biopesticides, additionally they are effective on oral infection of CIV and that these proteins together can be used as a biopesticide to provide oral infection of CIV.

6. RECOMMENDATION

Biopesticides can be used as an alternative to chemical pesticides in the pest management systems. In this context, the most used microorganism groups for biological control are bacterial and viral agents. In this study, microbial products of viral origin were tested on *G. mellonella* larvae.

Testing the chitinase and cathepsin proteins in this study on different insect species will provide more comprehensive information on the comparison of the effects of chitinases and cathepsins of bacteria and viral origin. Likewise, increasing the number of varieties of chitinase and cathepsin proteins that can be obtained from different living things will provide richer information for comparison results. In this study, the effect of chitinase and cathepsin protein combined with virus was investigated.

In other studies, insecticidal activity can be investigated by combining with chitinase or with cathepsin or with other enzymes or proteins known to be effective on insects. In addition, chitinase or cathepsin genes can be added to the CIV genome, thereby obtaining viral agents that are more resistant to natural conditions. Finally, purified chitinases and cathepsins can be used together with other entomopathogens and insecticidal activity trials can be performed.

In addition, the effect of cathepsin and chitinase on CIV has been determined within the scope of this thesis, and the lowest effective dose can be tested with an additional study, so that when these proteins are used together, at least how much dose they are effective on CIV can be tested.

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