INSTITUTO POLITÉCNICO NACIONAL

CENTRO DE BIOTECNOLOGÍA GENÓMICA

"EXPRESSION, PURIFICATION, PULL-DOWN ANALYSIS AND INSECT BIOASSAY OF IMMUNOSUPPRESSANT PROTEIN, CRV1 OF *COTESIA RUBECULA* (CRPDV) POLYDNAVIRUS"

THESIS

TO OBTAIN THE DEGREE OF

DOCTOR IN BIOTECHNOLOGY

BY

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Expression, purification, pull-down analysis and insect bioassay of immunosuppressant protein, CrV1 of *Cotesia rubecula* polydnavirus (CrPDV)

Presentada por el alumno:

Wei		Lihua
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Con registro: A 1 1 0 6 7 8

aspirante de:

Doctorado en Ciencias en Biotecnología

Después de intercambiar opiniones los miembros de la Comisión manifestaron ***APROBAR LA TESIS***, en virtud de que satisface los requisitos señalados por las disposiciones reglamentarias vigentes.

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Acknowledgements

First I would like to say thanks to my supervisor, Dr. Mario Alberto Rodríguez Pérez, who supported me throughout my thesis and allowed me to work in my own way.

Besides my supervisor, I would like to say thank you to the professors of my PhD committee: Dr Xianwu Guo; Dr Luis Gabriel Brieba de Castro; Dr Juan Manuel Gonzalez Prieto and Dr Raymundo Rosas Quijano. Thanks for the advice.

I would like to thanks to CONACYT and BEIFI/IPN for supporting me the scholarship.

During the daily work in the lab, I would like to say thank you to Dr Erick De Luna-Santillana, Isabel Cristina Rodriguez Luna and Alejandro Sánchez Varela, who help me in ordering the reagents and handling the instruments.

I also wish to say thanks to Dr Ninfa María Rosas García and Jesús Manuel Villegas Mendoza. Thanks for their help in support of insect culture lab and *Spodoptera exigua* larvae.

Next I would like to say thanks to my friends: Miguel, Edgar, Maria, Cristina, Yajuan Fu, Estefany, Alfonso and Wendy.

Finally, I would like to thanks to my parents for their support and encouragement during my studies.

Abbreviations

AcMNPV	*Autographa californica* multinucleopolyhedrosis virus
AD	Activation domain
AgMNPV	*Anticarsia gemmatalis* nucleopolyhedrovirus
Anks	Ankyrins
BAC	Bacterial artificial chromosome
BD	Binding domain
BmNPV	Bombyx mori nuclear polyhedrosis virus
BV	Bracovirus
BVs	Budded virions
CBD	Cellulose-binding domain
CcBV	*Cotesia congregata* bracovirus
CcV1	*Cotesia congregate* virus protein 1
CDNB	1-chloro-2, 4 dinitrobenzene
CkBV	*Cotesia kariyai* bracovirus
Co-IP	Co-immunorecipitation
CpBV	*Cotesia plutellae* bracovirus
CrBV	*Cotesia rubecula* bracovirus
CrV1	*Cotesia rubecula* virus protein 1
CrV2	*Cotesia rubecula* virus protein 2
CrV3	*Cotesia rubecula* virus protein 3
CsIV	*Campoletis sonorensis* ichnovirus
Cys-motif	Cysteine motif containing genes

DAB	3,3′-diaminobenzidine
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
E.coli	*Escherichia.coli*
EDTA	Ethylenediaminetetraacetic acid
Egf-like	Epidermal growth factor-like
EP1	Early-expressed protein1
EPDA	End-point Dilution Assay
ESI	Electrospray Ionization
Far WB	Far western blotting
FBS	Fatal bovine serum
FRET	Fluorescence resonance energy transfer system
G	Gram
GbNV	Gryllus bimaculatus nudivirus
GfBV	*Glyptapanteles flavicoxis* bracovirus
GfIV	*Glypta fumiferanae* ichnovirus
GiBV	*Glyptapanteles indiensis* bracovirus
Glc	Glycosylated central domain proteins
GlcNac	N-Acetylglucosamine
GlmS	Glucosamine-6-phosphate activated ribozyme
GST	Glutathione S-transferase

GVs	Granuloviruses
H	Hours
H4	Histone-4
HA	Hemagglutinin
HaMNPV	*Helicoverpa armigera* nucleopolyhedrovirus
HfIV	*Hyposoter fugitivus* ichnovirus
His	Hexahistidine
HPLC	High-performance liquid chromatography
HzNV	Heliothis zea nudivirus
IMAC	Immobilized metal affinity chromatography
Inex	Innexin genes
IPs	Infectious particles
ITAM	Immunoreceptor tyrosine-based activation motif
IV	Ichnovirus
L	Liter
LB	Luria Bertani
LC_{50}	Median lethal concentration
LT_{50}	Median lethal time
M	Molar per liter
MALDI	Matrix Assisted Laser Desorption Ionization
MBP	Maltose binding protein
MdBV	*Microplitis demolitor* bracovirus
Min	Minutes

mL	Milliliter
mM	Millimolar per liter
MNPV	Multiple nucleocapsids
MOI	Multiplicity of infection
MS	Mass spectroscopy
MS/MS	Tandem MS
Mya	Million years ago
Ng	Nanogram
Ni-NTA	Ni (II)-nitrilotriacetic acid
NPVs	Nucleopolyhedrovirus
NusA	N-Utilization substance
OBs	Occlusion bodies
ODVs	Occlusion derived virus
ORF	Open reading frame
OrNV	Oryctes rhinoceros nudivirus
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Proportional distance
PDV	Polydnaviruses
PFUs	Plaque-forming units
PMF	Peptide mass fingerprinting
PmNV	Penaeus monodon nudivirus
PO	Phenoloxidase

PP	Phosphoprotein
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
S	Seconds
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SeMNPV	*Spodoptera exigua* nucleopolyhedrovirus
Sf21	*Spodoptera frugiperda* 21
Sf9	*Spodoptera frugiperda* 9
SfMNPV	*Spodoptera frugiperda* nucleopolyhedrovirus
SNPV	Single nucleocapsid
SUMO	Small ubiquitin modifier
TAP	Tandem Affinity Purification
TBS	Tris-buffered saline
$TCID_{50}$	Tissue culture infective dose
TEMED	Tetramethylethylenediamine
TEV	Tobacco Etch Virus
TOF	Time-of-flight
TrIV	*Tranosema rostrale* ichnovirus
Trx	Thioredoxin
μL	Microliter

μM	Micromolar per liter
μm	Micrometre

Abstract

Polydnaviruses (PDVs) are symbionts of parasitoid wasps as a natural strategy to overcome the immune defense of the lepidopteran host. PDVs are double-stranded, circular DNA viruses found in some Ichneumonoidea families, being classified as bracovirus (BV) or ichnovirus (IV) according to the host parasitoid and viral morphology. One gene product of the BV of the parasitic wasp *Cotesia rubecula*, CrV1, inhibits the immune system responses of its endoparasitized lepidopteran host through interference with the haematocyte cytoskeletal structure. The mechanism of inactivation is still not clear. In this study, CrV1 was constructed into two transfer vectors, pAcGHLT-B and pAcUW21 and co-transfected to generate two baculoviruses, pAcGHLT-B-CrV1 and pAcUW21-CrV1. GST-CrV1 fusion protein was successfully expressed and purified from pAcGHLT-B-CrV1 baculovirus infected Sf9 cells. GST pull-down assay was carried out to study the interacted proteins of CrV1 in *Pieris rapae* larvae lysate. However, degradation of GST-CrV1 protein was present. This degradation may due to the some kinds of materials existed in the lysate. Another insect larvae lysate, *Spodoptera exigua*, was also used for pull-down assay and the degradation was also detected. The contents of the materials are still unknown and another experiment showed that protease consisting of RNA may involve in the degradation.

Recombinant baculoviruses may be used as biological insecticides by the introduction and expression of exogenous genes such as those coding for proteins interfering with metabolism, metamorphosis (toxins, hormones, and enzymes), and immune system. The CrV1 secreted protein of *Cotesia rubecula* polydnavirus (PDV) is responsible for the actin depolymerization in hemocytes and the abolishment of immune functions such as phagocytosis and cell spreading, thus allowing the successful embryonic development of the parasitoid wasp. In this study,

pAcUW21-CrV1 baculovirus was tested against the insect pests *Spodoptera exigua* and *Pieris rapae*. The recombinant virus expressing CrV1 protein showed significantly lower LC_{50} and LT_{50} as compared with wild-type virus, which indicated that the recombinant baculoviruses expressing only CrV1 gene can improve their virulence.

Key words: Polydnavirus, CrV1 protein, baculoviruses, *Spodoptera exigua*, *Pieris rapae*, pull-down, expression, purification

Resumen

Los polidnavirus (PDVs) son simbiontes de avispas parasitoides como una estrategia natural para evadir la defensa inmune de los lepidopteros hospederos. Los PDVs son virus de ADN circular de hebra, son encontrados en algunas familias de Ichneumonoidea, son clasificados como bracovirus (BV) o ichnovirus (IV) dependiendo el parasitoide hospedero y la morfología viral. Un producto génico de los BV de la avispa parasitoide *Cotesia rubecula* es la proteína CrV1, la cual inhibe la respuesta del sistema imnune del lepidóptero hospedero endoparasitado, a través de la interferencia con la estructura del citoesqueleto del hemocito. El mecanismo de inactivación permanece no claro. En este estudio, el gen CrV1 fue insertado en 2 vectores de transferencia, pAcGHLT-B y pAcUW21 y cotransfectado para generar dos baculovirus recombinantes pAcGHLT-B-CrV1 y pAcUW21-CrV1. La proteína de fusión GST-CrV1 fue expresada exitosamente y purificada de las células Sf9 infectadas con el baculovirus pAcGHLT-B-CrV1. El ensayo "GST pull-down" fue llevado a a cabo para estudiar la interacción de la proteína CrV1 en lisado de larvas de *Pieris rapae*. Sin embargo se observó la degradación de la proteína GST-CrV1, probablemente debida a alguna sustancia presente en el lisado. Un lisado de larvas de *Spodoptera exigua* fue también usado para ensayos "GST pull-down" y la degradación también fue detectada en este caso. El contenido de sustancias en el lisado aun es desconocido, algunos experimentos previos han demostrado que algunas proteasas que consisten en ARN pueden estar involucradas en la degradación.

Los baculovirus recombinantes pueden ser usados como insecticidas biológicos por medio de la introducción y expresión de genes exógenos tales como los que codifican para proteínas que interfieren con el metabolismo, metamorfosis (toxinas, hormonas y enzimas) y con el sistema inmune. La proteína CrV1 secretada por el polidnavirus de *Cotesia rubecula* es responsable de la

depolimerizacion de la actina en los hemocitos y abolición de las funciones del sistema inmune tales como la fagocitosis y la difucion celular, lo cual permite el exitoso desarrollo embrionario de la avispa parasitoide. En este estudio el baculovirus pAcUW21-CrV1 fue probado contra los insectos plaga *Spodoptera exigua* y *Pieris rapae*. El virus recombinante que expresa CrV1 mostró una LC_{50} y una LT_{50} significativamente más bajas comparado con el virus tipo silvestre, lo cual indica que solo la expresión de CrV1 en los baculovirus recombinantes puede mejorar la virulencia.

Palabras Clave: polidnavirus, CrV1, proteína, baculovirus, *Spodoptera exigua*, *Pieris rapae*, *"*pull-down", expresión, purificación.

1 Introduction

1.1 Polydnaviruses

Polydnaviruses ("Poly" referring to the poly-dispersed DNA segments) (PDVs) which occur as proviruses integrated in wasps genomes are the best studied mutualistic viruses (Webb, Strand et al. 2006, Beck, Inman et al. 2007, Roossinck 2011).The study of PDVs started in the late 1960s (Salt 1965). The polydnaviridae was formally recognized as a family of viruses in 1991 (Francki, Fauquet et al. 1991). The selected milestones in the history of polydnavirus research are listed in Table 1.

Observation	**References**
The layer on the surface of parasitoid eggs which can protect them from encapsulation is located in the ovarian calyx.	(Salt 1965)
Viruslike DNA-containing particles are generated in the calyx region, which compose the calyx fluid	(Vinson and Scott 1975)
The ultrastructural investigation of nuclear secretory particles and microorganisms in the calyx cells are reported	(Hess, Benham et al. 1975, Norton, Vinson et al. 1975)
The infection process of virus-like particles is reported	(Stoltz and Vinson 1977, Stoltz and Vinson 1979)
Braconid and ichneumonid particle DNA is double stranded, circular, supercoiled and polydisperse	(Krell and Stoltz 1979, Krell and Stoltz 1980)
Virus in a parasitoid wasp acts to suppress the host's cellular immune response to let the parasitoid egg survive	(Edson, Vinson et al. 1981)
The mRNA transcripts of virus are detected in the infected host	(Fleming, Blissard et al.

	1983)
A new insect virus family, Polydnaviridae, is proposed	(Stoltz, Krell et al. 1984)
The replication of virus DNA does not occur in the host and may be restricted in the wasps.	(Theilmann and Summers 1986)
The DNA splicing in the Polydnaviridae is described	(Blissard, Smith et al. 1987)
Evidence for chromosomal transmission and integration of polydnavirus DNA is provided	(Stoltz 1990, Fleming and Summers 1991)
Polydnaviridae is accepted by the ICTV as the polydnaviruses family name and two genera, bracoviruses and ichnovirus, are established	(Francki, Fauquet et al. 1991)
Evidence for polydnavirus genome segment is provided	(Xu and Stoltz 1993)
A model for the virus DNA integration and excision is proposed	(Gruber, Stettler et al. 1996)
Ultrastructural analysis of the braconid wasp polydnavirus show that only one circular DNA in each nucleocapsid	(Albrecht, Wyler et al. 1994)
The virus DNA amplification is prior to individual circle excision	(Pasquier-Barre, Dupuy et al. 2002)
The polydnavirus-wasp relationship is approximately from a date of origin of 73.7±10 million years ago	(Whitfield 2002)
A polydnavirus DNA segment is integrated in host cells in vitro	(Gundersen-Rindal and Lynn 2003)
RNA interference is used in characterizing the function of PDV genes	(Beck and Strand 2003)
The first complete nucleotide sequence of the bracovirus CcBV is	(Espagne, Dupuy et al.

published	2004)
CsIV and MdBV share a few genes expressed in the hosts and ichneumonid and braconid wasp may evolve in different strategies	(Webb, Strand et al. 2006, Tanaka, Lapointe et al. 2007)
Genome analysis indicates that Baculoviridae derive from ancestral nudivirus	(Bézier, Annaheim et al. 2009)
Genome and proteomics analysis shows that ichnoviruses and bracoviruses particles originated from different viral entities	(Volkoff, Jouan et al. 2010, Heraty, Ronquist et al. 2011)
Sequence data analysis estimates that nudiviruses diverge from baculoviruses 310Mya	(Heraty, Ronquist et al. 2011)
The first experimental evidence that BV segments integrate into the genomes of host insects is described	(Beck, Zhang et al. 2011)
The first experimental insights for the function of some BV genes in virion formation is documented	(Burke, Thomas et al. 2013)
The first comprehensive proteins analysis that wasp introduces into its host	(Burke and Strand 2014)

Table 1. The history of polydnavirus research (Beckage and Drezen 2011, Strand and Burke 2013, Strand and Burke 2015)

Approximately 40000 PDV-carrying wasps belong to families, Braconidae and Ichneumonidae, and the PDVs are subdivided into two genera, Ichnovirus (IV) and Bracovirus (BV) (King, Adams et al. 2012). PDVs are associated with insects named parasitoid wasps. Many parasitoid

wasps lay eggs into the lepidopteran insect host and use PDVs to overcome the immune defense of the lepidopteran host, which facilitate the development of their progeny (Fleming 1992, Turnbull and Webb 2002, Bézier, Annaheim et al. 2009).

1.1.1 The life cycle of polydnavirus

During evolution, PDVs, the parasitoid wasps and insect hosts have formed a complex association. The life cycle of polydnavirus is displayed in Figure 1. PDV persists as an integrated provirus in the germ line and somatic cells of associated parasitoid Hymenopteran wasps and replication specifically occurs in calyx cells of pupal and adult stage female wasp ovaries (Gundersen-Rindal, Dupuy et al. 2013), following excision of the proviral DNA from wasp chromosomes and encapsidated virions accumulate to high concentration in the lumen of the later oviducts (Fleming and Summers 1991, Xu and Stoltz 1991, Gruber, Stettler et al. 1996, Belle, Beckage et al. 2002). The assembled virions contain multiple circular dsDNAs with large aggregate sizes, from 190 to 730 kb. At oviposition, the wasp injects one or more eggs into the lepidopteran host with a quantity of virions and secretions from the venom gland (Schmidt, Theopold et al. 2001). PDV virions rapidly infect the host, express virulence genes and integrate their DNA segments into the genome of infected host cells (Strand and Burke 2014). These gene products have two main functions. First, suppress the host's immune system, which prevents the eggs from encapsulation. Second, regulate the host physiology, which facilitates the wasp offspring development, leads to parasitism success and results in the death of the host (Chevignon, Thézé et al. 2014, Chevignon, Cambier et al. 2015).

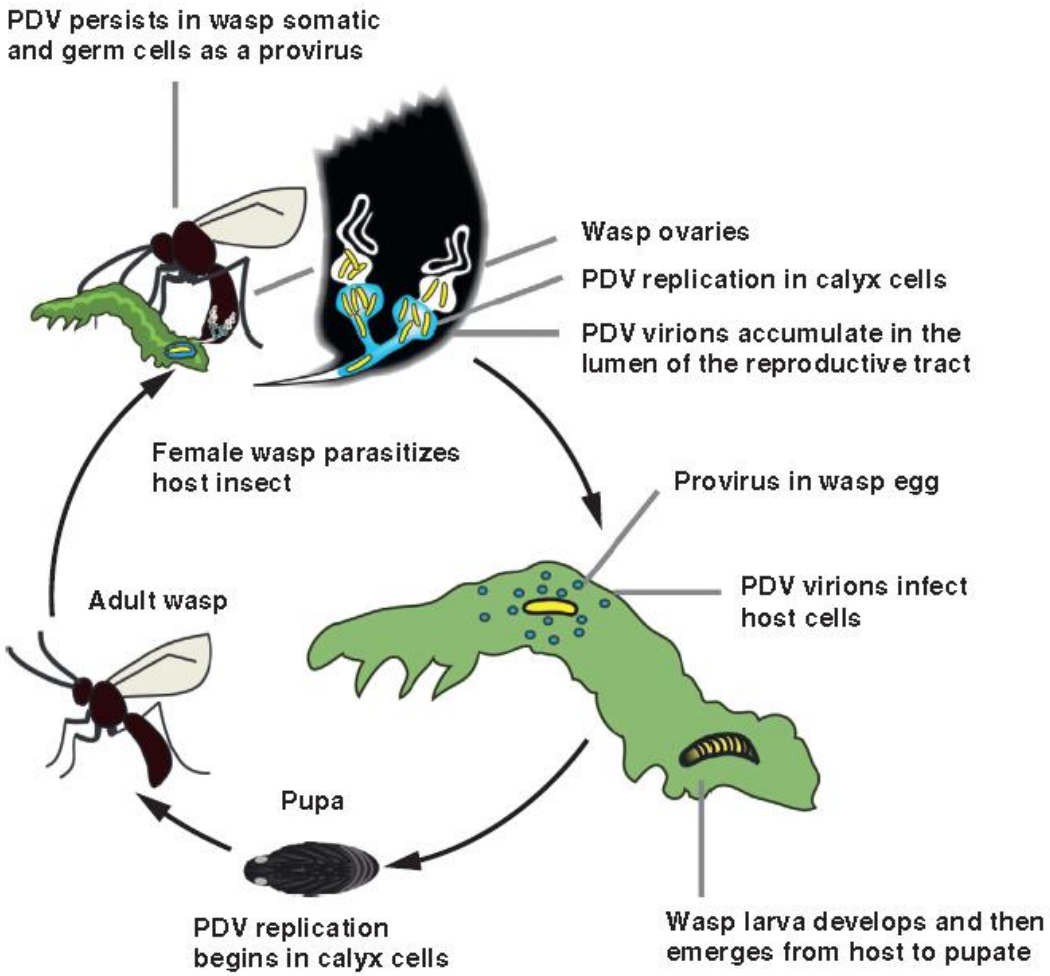

Figure 1. The life cycle of parasitoid wasp and PDVs (Strand and Burke 2013). PDV persists in the germline and all somatic cell of the wasp's body as a provirus. PDV replication only occurs in calyx cells of ovaries of pupal and adult stage female wasps. PDV virions are stored in the lumen of the reproductive tract with wasp eggs. Wasps parasitize the hosts and inject the egg with PDV virions into the hosts. The virions express virulence genes to disable the host's immune system, which allows the wasp larvae to develop and then emerge from the host to pupate.

Unlike viruses in any other family, PDVs cannot replicate in the wasp's host because the genes which are required to produce virus particles are not assembled into virions. Therefore, the PDVs

are only can be vertically transmitted as proviruses between the germline of wasps (Strand and Burke 2013). During evolution, the PDV proviral genomes have divided into two functional units (Figure 2). One is the genes (such as structural genes) with replication function, which are expressed, and form virions in calyx cells, but are not encapsidated into virion and another is multiple DNA domains including virulence genes, which are amplified and assembled into virions (Strand and Burke 2014).

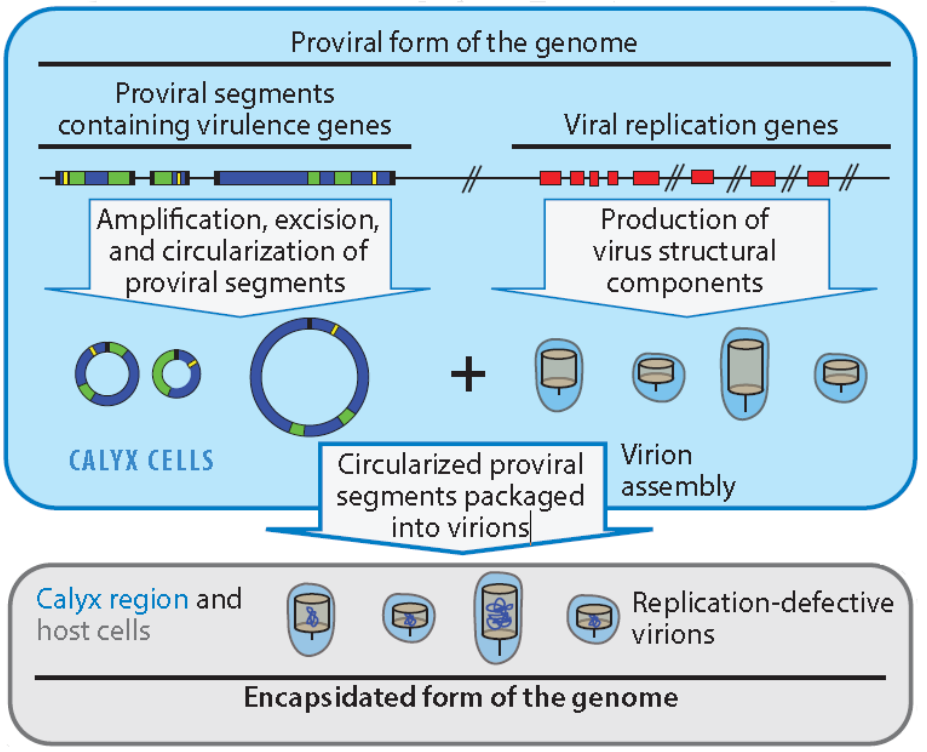

Figure 2. The genome organization of polydnavirus (Strand and Burke 2014). Two parts of the proviral genome are replicated in calyx cells. Viral replication genes are expressed and produce virus structural components. Proviral segments containing virulence genes are amplified, excised, circulated and packaged into virions.

1.1.2 Polydnavirus genes that ineract with the host immune system

The main function of most polydnaviruse genes is preventing the wasp eggs from encapsulation by the host immune system. The host insect's immune system includes cellular and humoral components. The cellular immunity system refers to response to the action of haemocytes, such as nodule formation, encapsulation and phagocytosis (Strand 2008). Haemocytes are composed of plasmatocytes, granulocytes, spherule cells and oenocytoids, which are classified by morphological, functional and molecular markers (Strand 2008, Nakahara, Shimura et al. 2009). Granulocytes take the response of non-self-recognition and phagocytosis, and the plasmatocytes form a capsule around the foreign objects to perform encapsulation response. Oenocytoids can express phenoloxidase and other components of the PO cascade to start melanization, while the function of spherule cells is not clear (Beckage and Drezen 2011). Humoral defenses involve in molecules produced by hemocytes or tissues with ability of killing foreign objects (Kanost, Gorman et al. 2008). Three classes of innate humoral response have been defined, including defensive melanization, sentinel molecule binding and induction of antimicrobial peptides (Gillespie and, Kanost et al. 1997).

The most important immune response of lepidopteran insects toward the eggs of parasitoids is encapsulation (Strand 2008). This response, involved with the action of hemocytes, is mediated by two kinds of recognition receptors. The first kind is cell surface receptors which are expressed on the surface of hemocytes, such as scavenger receptors, integrins and the nimrod superfamily (Kocks, Cho et al. 2005, Moita, Wang-Sattler et al. 2005, Wertheim, Kraaijeveld et al. 2005, Somogyi, Sipos et al. 2008). The second kind is humoral pattern recognition receptors, such as lipopolysaccharide-binding protein, hemolin, soluble peptidoglycan recognition proteins, immunolectins, complement-like thioester-containing proteins and gram-negative bacteria

recognition proteins (Levashina, Moita et al. 2001, Irving, Ubeda et al. 2005, Moita, Wang-Sattler et al. 2005, Dong, Taylor et al. 2006, Ling and Yu 2006, Terenius, Bettencourt et al. 2007). Encapsulation usually starts from 2-6 h after parasitism and is accomplished until 48 h (Strand 2008).

PDV mediated suppression of host immune system is first reported in 1981, which showed that CsIV gene expression is essential for preventing *C.sonorensis* eggs from encapsulated (Edson, Vinson et al. 1981). Subsequently, numerous PDVs genes which interact with immune system of parasitoid have been investigated (Table 2). PDVs infect the hemocytes after being injected into the hemocoel of the host, resulting in morphological alteration (Tanaka 1988), altering spreading behavior apoptosis, inhibiting coagulation and changing actin-cytoskeleton structure in host hemocytes (Strand and Noda 1991, Strand 1994, Lavine and Beckage 1995, Strand and Pech 1995, Asgari, Schmidt et al. 1997, Cui, Soldevila et al. 1997). Viral genes which relate to immune suppression are diverse. It includes proteins with conserved cysteine patterns, cysteine-knot motif-containing proteins, and coiled-coil containing proteins (Cui and Webb 1996, Asgari and Schmidt 2002, Beck and Strand 2003, Dahlman, Rana et al. 2003, Le, Asgari et al. 2003). Many genes have been cloned from infected hemocytes. PDVs genes usually express as early as 2-4 h post-parasitism and may continue throughout the course of parasitization (Blissard, Fleming et al. 1986, Strand, McKenzie et al. 1992). Glatz, *et al* isolated *Cotesia rubecula* bracovirus gene which expressed in the Lepidopteran *Pieris rapae* and found that only four gene products are detected in larval host tissues. Their expression occurred from 4 to 12 h post-parasitization (Glatz, Schmidt et al. 2004).

Genes	PDV species	Function	References
Glc family (2 genes) glc1.8	MdBV	Prevent haemocyte from adhering to foreign surfaces, reducing phagocytosis and disrupting adhesion and phagocytic activity of cells	(Beck and Strand 2003, Beck and Strand 2005, Pruijssers and Strand 2007, Johnson, Bitra et al. 2010)
PTP family (13 genes) ptp-H2		Disable hemocyte-medicated defenses and stimulate apoptosis. PTP-H2 is a tyrosine phosphatase	(Pruijssers and Strand 2007, Suderman, Pruijssers et al. 2008, Eum, Bottjen et al. 2010)
Anks family (12 genes) AnkH4, AnkN4		Inhibit melanization	(Beck and Strand 2007)
Egf family (3 genes) egf1.0, egf1.5		Disrupt Toll and Imd signaling, and AMP expression	(Lu, Beck et al. 2008, Lu, Beck et al. 2010)
CrV1, CrV3	CrBV	Transient alter hemocyte cytoskeletal	(Asgari, Schmidt et al. 1997, Glatz, Schmidt et al. 2004)
CrV2		Alter hemocyte function by changing signaling pathways	(Cooper, Bailey-Hill et al. 2011)
CcV1	CcBV	Disable binding of the pattern	(Labropoulou, Douris

		Recognition protein hemolin to hemocytes	et al. 2008)
PTP gene family		Encode tyrosine phosphatases as inhibitors of cathepsin-like cysteine proteases and may interact with host immune defenses	(Provost, Varricchio et al. 2004, Serbielle, Moreau et al. 2009)
Lectin	CpBV	Disrupt adhesion, spreading and proliferating of the host hemocytes	(Gad and Kim 2008, Kwon and Kim 2008, Lee, Nalini et al. 2008, Nalini and Kim 2009, Kim and Kim 2010, Prasad, Hepat et al. 2014)
Histone (H4)			
EP1-like gene			
CpBV15			
Cys-motif proteins VHv1.1 and VHv1.4	CsIV	Reduce the encapsulation	(Li and Webb 1994, Cui, Soldevila et al. 1997)
IV-encoded ank genes		May act as NF-kappaB inhibitors and inhibit apoptosis	(Fath-Goodin, Kroemer et al. 2009)
Inex genes		Form functional gap junctions in cell-cell communication	(Turnbull, Volkoff et al. 2005)
PDVPTP	GiBV	May disrupt haemocyte activity	(Chen, Taylor et al. 2003)

2.0 kb CkBV cDNA	CkBV	May disrupt haemocyte activity	(Yamanaka, Hayakawa et al. 1996)

Table 2. Summary of polydnavirus genes that interact with the host immune system (Glatz, Asgari et al. 2004, Beckage and Drezen 2011)

1.1.3 *Cotesia rubecula* polydnavirus gene 1 (CrV1)

The CrV1 gene from *Cotesia rubecula* is the best gene characterized so far among the four genes products detected in *Pieris rapae* tissues. This gene was cloned and sequenced by Dr Asgari (Asgari, Hellers et al. 1996). CrV1 gene product is a secreted glycoprotein and expressed in the haemocytes and fat body cells of *Pieris rapae*. That has been implicated in depolymerization of the actin cytoskeleton of host haemocytes and disruption of the capacity of hemocytes to spread onto foreign surfaces (Asgari, Schmidt et al. 1997). In *Cotesia rubecula*, CrV1 is the only gene that expressed in the host hemocyte. The mechanism of inactivation is still not clear. It has been shown that CrV1 is endocytosed or phagocytized by the host haemocytes. Asgari and Schmidt found that a coiled-coil domain containing a putative zipper is required for CrV1 function and this domain affects the binding and uptakes of the CrV1 protein by hemocytes (Asgari and Schmidt 2002). It is known that CrV1 binds to lipophorin forming a complex with the lipid carrier (Asgari and Schmidt 2002, Glatz, Schmidt et al. 2004). It indicates that CrV1 forms a complex with lipophorin to interact with hemocytes, which is then taken up by lipophorin or scavenger receptors as part of a clearance reaction (Schmidt, Glatz et al. 2005).

1.1.4 Polydnavirus evolution

During the study of unique biology of PDVs, one important question is that where did they come from. Early morphological research showed that BV and IV virion were similar to the viruses from the family Baculoviridae and Ascoviridae respectively (Stoltz, Vinson et al. 1976, Federici and Bigot 2003, Bigot, Samain et al. 2008). However, further research noted that the PDVs neither share any significant homologous genes with baculovirus or ascovirus nor share homology with other viral genes which have predicted function in DNA replication, transcription or virion formation (Burke and Strand 2012). Therefore, two models have been suggested for this question. One is that PDVs evolved from a wasp ancestor, during the long evolution, they obtained the ability to produce and package circular DNA which contains wasp genes encoding proteins that withstand the immune responses of the insect host (Whitfield and Asgari 2003, Espagne, Dupuy et al. 2004, Schmidt, Glatz et al. 2005). Another model proposes that PDVs evolved from insect DNA viruses associated with the ancestor of campopleginae and microgastroid wasp and developed a beneficial association. The PDVs integrated into the wasp genome and lost viral replication and structural genes and acquired the virulence genes from the wasp. PDVs are not viruses, but wasp organelles that evolved from virus (Federici and Bigot 2003, Webb and Strand 2005).

Genome analysis is one critical way to answer this question. Currently, nine PDV genomes have been sequenced: *Cotesia congregate* BV (CcBV), *Microplitis demolitor* BV (MdBV), *Cotesia plutellae* BV (CpBV), *Glyptapanteles flavicoxis* BV (GfBV), *Glyptaanteles indiensis* BV (GiBV), *Campoletis sonorensis* IV (CsIV), *Tranosema rostrale* IV (TrIV), *Hyposoter fugitivus* IV (HfIV), and *Glypta fumiferanae* IV (GfIV) (Espagne, Dupuy et al. 2004, Webb, Strand et al. 2006, Lapointe, Tanaka et al. 2007, Tanaka, Lapointe et al. 2007, Desjardins, Gundersen-Rindal

et al. 2008, Chen, Gao et al. 2011). All genome data of PDV viruses share common features, such as large genomes (from 189 to 606 kb), low coding densities (17%-33%), many genes organized into gene families and numerous genes that share homologous genes with genes of wasps or other eukaryotes. BVs and IVs rarely share genes with each other, which indicates that their evolution is independently.

The first breakthrough is the study of transcriptome in ovary DNA libraries of two braconid wasps (*Cotesia congregate* and *Chelonus inanitus*) (Bézier, Annaheim et al. 2009). Complementary genomic and proteomic analysis showed that several genes which expressed in ovaries during replication share homologous genes with nudiviruses (Wang and Jehle 2009). Nudiviruses is a sister taxon to the Baculoviridae, which is an extensively studied insect family, such as *Autographa californica* multinucleopolyhedrosis virus (AcMNPV) (Rohrmann 2014). The divergence of nudiviruses and baculoviruses dated back to 300 Mya, while nudiviruses and bracoviruses diverged approximately 100 Mya (Thézé, Bézier et al. 2011, Burke and Strand 2012). The evolutionary relationships between bracoviruses, nudiviruses and baculoviruses are showed in Figure 3.

The similar approach was performed with three IV-carrying wasps. However, none of nudivirus or baculovirus-like genes was identified (Volkoff, Jouan et al. 2010). Some domains of the genomes which contain the genes coding for structural proteins in IV virions were found. These genes have the features of virus-like and form clusters in the genome of wasp, which indicate that IVs evolved from a virus, whereas these regions share no homologous genes with other virus families. Therefore whether IVs evolve from an undiscovered taxon or a now-extinct virus taxon is unknown.

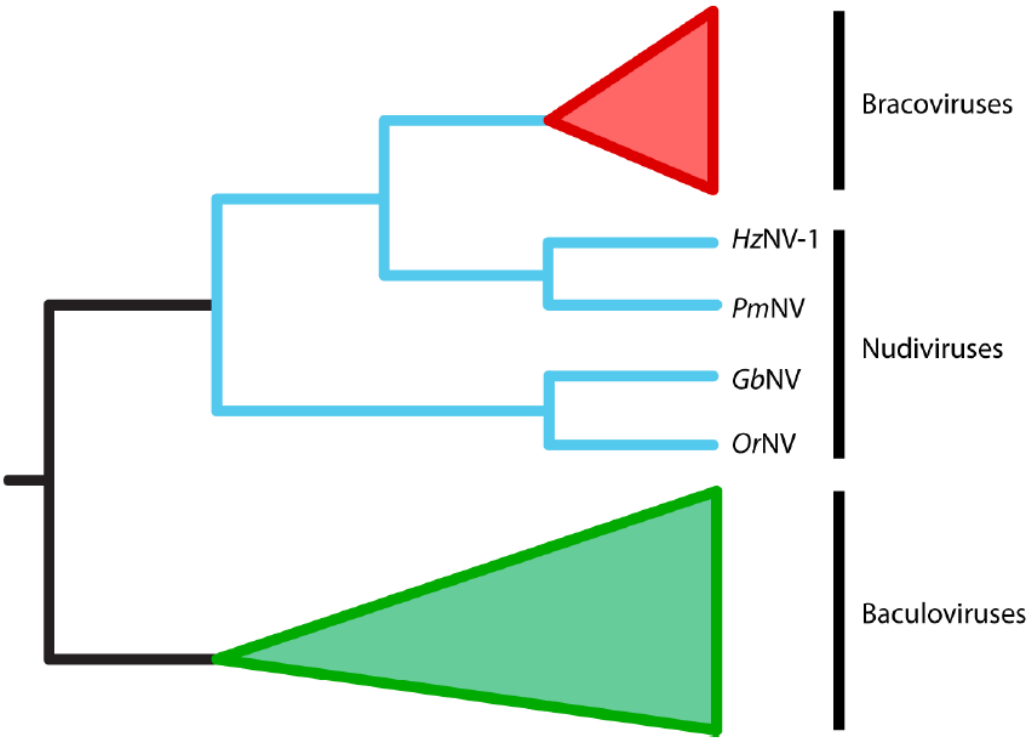

Figure 3. Evolutionary relationships between bracoviruses, nudiviruses and baculoviruses (Burke and Strand 2012). Bracoviruses evolved from within the nudivirus branch. Nudiviruses and baculoviruses are sister taxon of viruses.

1.2 Baculovirus biology

Baculovirus is a large family of inset pathogenic viruses (Baculoviridae) which have enveloped, rod-shaped, double-stranded DNA genomes ranging from 80 to 170 kb in size, depending on the species (Miele, Garavaglia et al. 2011). Baculoviruses as insect pathogens were initially performed as bioinsecticide to regulate insect populations in nature (Szewczyk, Hoyos-Carvajal et al. 2006, Moscardi, de Souza et al. 2011). Heretofore, no less than 56 baculoviruses species and isolates have been published in GenBank. From the genome analyses, we know that about 895 open reading frames encode for structural and nonstructural proteins for crucial functions such as DNA replication and transcription, gene expression, the virion structure and packaging,

the cell cycle arrest, and the proteins involved in primary infection of insect host midgut cells (Miele, Garavaglia et al. 2011, van Oers 2011). Thirty one core genes are conserved in all the sequenced baculovirus genomes (Yang and Zhang 2012).

Baculoviruses have two different virion phenotypes: budded virions (BVs) and occlusion derived virus (ODVs) (Jehle, Blissard et al. 2006) (Figure 4B). BVs bud from the cell membrane and spread the in the infection within the infected host whereas ODVs is assembled in large occlusion bodies (OBs) and spread from insect to insect (Hunter-Fujita, Entwistle et al. 1998). The BVs are taken shape from the infected cell plasma membrane. They consist of a single nucleocapsid which is enveloped with a structure derived from modified host plasma membrane (Hunter-Fujita, Entwistle et al. 1998). Oppositely, the ODVs consist of one or multiple nucleocapsids assembled in an envelope which is different from the BVs envelope (Hunter-Fujita, Entwistle et al. 1998).

On the basis of the phylogenetic and other characteristics evidence, baculoviruses are divided into four genera: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific Granuloviruses), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV) (Jehle, Blissard et al. 2006). Based on the morphologies of the virions, baculoviruses are divided into nucleopolyhedrovirus (NPVs) and granuloviruses (GVs) (Jehle, Blissard et al. 2006, Carstens and Ball 2009, Guarino 2011). Both of them are specific to their larvae hosts (van Oers 2011). The NPV occlusion body contains multiple virions. Depending on the genus, some of the NPVs have a single nucleocapsid (SNPV) in each virion, while others have multiple nucleocapsids (MNPV) (van Oers 2011) (Figure 4A). The diameter of NPV occlusion bodies ranges from 0.15 to 15 μm (Hunter-Fujita, Entwistle et al. 1998). The alphabaculoviruses form two clades, the group I and group II NPVs (Herniou and

Jehle 2007). The main difference between these two groups is the glycoprotein which is necessary for virus to bud from the insect cell and to get the entry into the host cell cytoplasm (Monsma, Oomens et al. 1996). In group I, the GP64 is the protein to operate these processes, while in group II the F protein functions as GP64 (IJkel, Westenberg et al. 2000, Pearson, Groten et al. 2000). The F protein is also detected in Betabaculovirus, Deltabaculovirus and some vertebrate viruses (Pearson, Groten et al. 2000).

The occlusion bodies diameter of GVs is between 0.15 and 0.3 μm and often contain only one enveloped nucleocapsid (Hunter-Fujita, Entwistle et al. 1998). In GVs, the OBs only contain a single enveloped virus particle. Granulin which is similar to polyhedrin is the main GVs matrix protein.

Unlike most other DNA viruses, baculovirus gene expression happens in four phases: immediate-early phase, delayed-early phase, late phase and very late phase (Passarelli and Guarino 2007). In the immediate-early phase, the immediate-early genes which can activate the genes of other phases and involve in infection establishment are transcribed by host encoded RNA polymerase II. In the delayed-early phase, genes which implicate in DNA replication, late gene expression and inhibitors for preventing host defense system are transcribed (Ahrens and Rohrmann 1995, Kool, Ahrens et al. 1995, Todd, Passarelli et al. 1995, Guarino, Xu et al. 1998, Clem 2007). The late phase is associated with virion assembly and virus budding. In the very late phase, the virions become occluded and polyhedrin and P10 proteins are expressed in very large amounts (Rohrmann 1986, Van Oers and Vlak 1997). Polyhedrin forms the matrix of OBs. The P10 is related to the release of OBs from the nucleus of infected host cells at the end of infection (van Oers, Flipsen et al. 1993,Carpentier, Griffiths et al. 2008, Carpentier and King 2009). Viral

proteases lyse the host cells and the occluded virions are dispersed into environment for horizontal transmission.

1.2.1 Theory of the baculovirus expression system

Polyhedrin and P10 proteins expression occur in the very late phase of the virus infection cycle (Figure 4C). However, they are not essential for the production of BVs, the type of virus that is responsible to spread the virus from insect cell to cell. In cell culture, only BV phenotype is needed to continue the replication cycle. Therefore it is possible to express recombinant proteins via deleting the polyhedrin or P10 gene (Smith, Summers et al. 1983, Pennock, Shoemaker et al. 1984) (Figure 4D). This forms the principle of the baculovirus insect cell expression system. As these promoters have a canonical TAAG transcription initiation site, one or more promoters constructed with foreign genes can be added into the baculovirus genome, which hastens protein expression in insect cells. AcMNPV, species of the genus alphabaculovirus, and BmNPV, another alphabculovirus which infects the silk worm, are commonly used for foreign genes expression.

The proteins expressed in the system often have biological activation and immunogenicity. Many viral and parasitic proteins, which have been expressed in this system, have available immunogenicity (Van Oers 2006, Metz and Pijlman 2011). This expression system also makes it possible to research the virion assembling of the virus lack of infectivity and this can be used to develop candidate vaccine on the basis of virion with regular recombinant antigens (Conner, Zarley et al. 1996, Hu, Bentley et al. 1999, Hu, Hsu et al. 2003, Mortola and Roy 2004). Before subunit vaccines, vaccines are made from these parts of a pathogen which is necessary to lead a protective immune response against the disease. In practice, these are usually surface

components, such as the E2 protein from classical swine fever virus and the L1 viral capsid protein of human papillomavirus (Bouma, De Smit et al. 1999, Harper, Franco et al. 2006). The baculovirus expression system also can be used to produce viral vectors for gene therapy (Urabe, Ding et al. 2002).

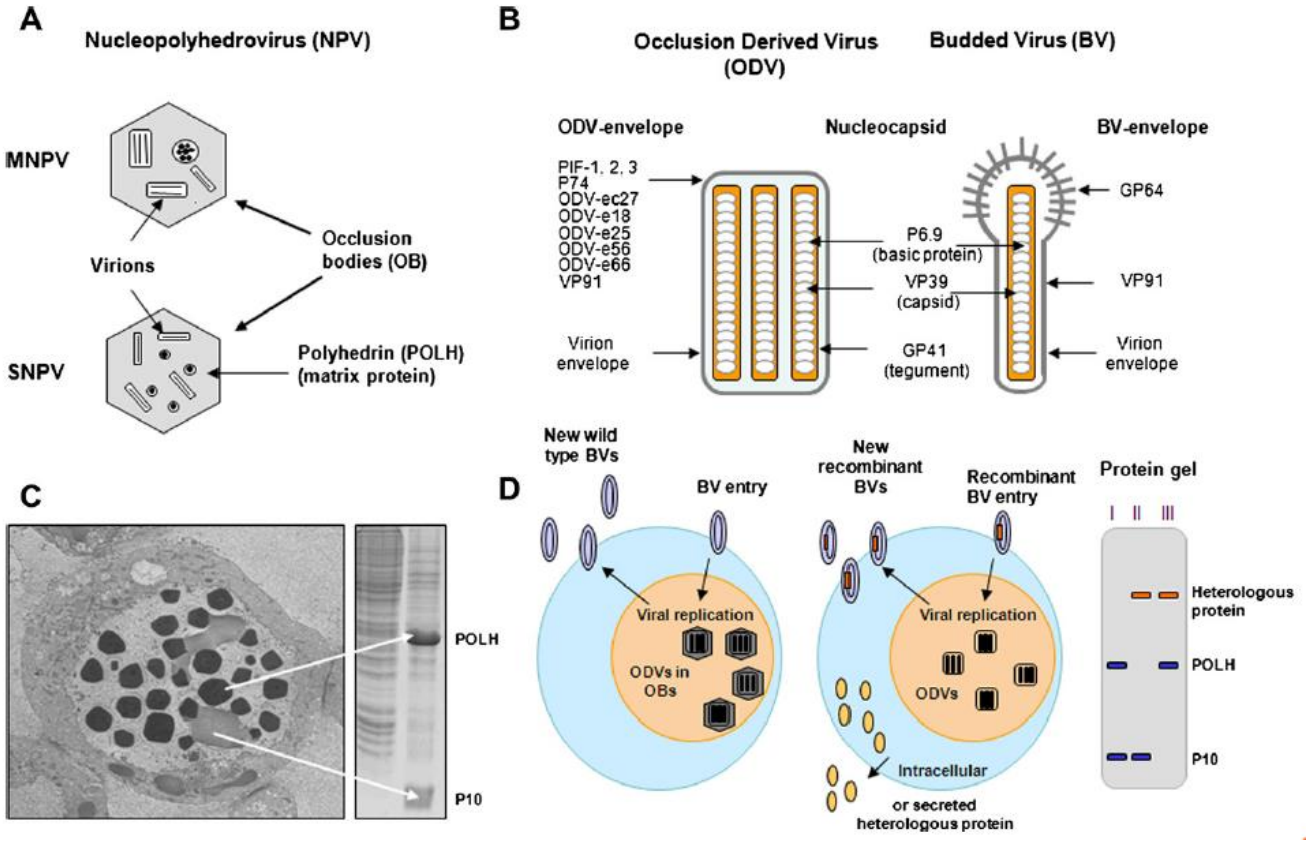

Figure 4. Baculovirus biology and theory of the expression system (van Oers 2011). A: Nucleopolyhedroviruses exist in the surroundings in the form of viral occlusion bodies (OBs). The OBs consist of a polyhedron matrix and the enveloped occlusion derived virions (ODVs) which are embedded in the polyhedron matrix. The ODVs contain a single (SNPV) or multiple (MNPV) nucleocapsids. B: The ODVs spread the infection between insects whereas the OBs spread the infection between cells within the insect body. C: In the very late phase of infection, polyhedrin and P10 proteins are expressed in a large amount. D: Polyhedrin and P10 proteins are

not required to produce BVs, so that their promoters can be used for foreign genes expression. The left panel shows the life cycle of insect cell infected with wild-type BVs. The middle panel shows the life cycle of insect cells infected with a recombinant virus (a foreign gene under polyhedrin promoter). The right panel shows a diagram of a protein gel of wild-type virus-infected (I), polyhedrin (II) and P10 (III) promoters based recombinant baculovirus-infected insect cells.

1.2.2 Production of recombinant baculoviruses

Baculoviruses have large genomes which render direct cloning of foreign genes. The original method for constructing recombinant baculoviruses is by homologous recombination between the circular viral genome and a transfer vector that contains the foreign gene under polyhedrin promoter and is flanked by the sequences of polyhedrin gene region (Smith, Summers et al. 1983, Pennock, Shoemaker et al. 1984) (Figure 5A). This method is inefficient because of the selection and the low success ratio. The selection of recombinant viruses from parental stock is based on the detection of occlusion body negative plaques in the cell culture. These plaques often occur at low ratio. The identification is not easy to perform, which requires skill and conversancy with this system and further screening to confirm the recombinant viruses.

As the identification and purification of the recombinant virus is really a time-consuming procedure, this system was improved by a series of innovations that were propitious to the selection of recombinant viruses and increased the success percentage. One of the methods is using linearized baculovirus genomes at the desired insertion site (Kitts, Ayres et al. 1990, Martens, van Oers et al. 1995) (Figure 5B). This DNA with transfer vector after recombination results in approximately 30% recombinant viruses (Kitts, Ayres et al. 1990). This method was

improved by triple-digested baculovirus genomes (Kitts and Possee 1993). One *Escherichia coli* lacZ coding region was introduced in the polyhedrin gene locus and Bsu36I restriction enzyme sites were added in the upstream of ORF 603 and downstream of ORF 1629 (Kitts and Possee 1993). The protein expressed by ORF 603 is not essential for virus replication, whereas the ORF 1629 is an essential gene which encodes the phosphoprotein PP78/83. PP78/83 is a Wiscott-Aldrich syndrome protein which associates with nuclear actin filament formation during virus infection (Machesky and Insall 2001, Goley, Ohkawa et al. 2006, Wang, Liang et al. 2007, Ohkawa, Volkman et al. 2010). Therefore when the virus DNA is digested, even though it infect into the cells, the infectious virus production cannot be originated. The digested viral DNA with a transfer vector contained a foreign gene co-transfect into the insect cells resulting in over 90% recombinant viruses. Hence the recombinant viruses have to be purified several rounds by plaque assay. This method uses the BacPak6 vector or commercially Baculogold (BD Biosciences) BacN blue vectors (Invitrogen) (Kitts and Possee 1993).

In recent years, another method named bacmid system has been widely applied (Figure 5C). Bacmid is one constructed bacterial artificial chromosome which contains the baculovirus AcMNPV genome. With this bacmid, all the gene operational works to get the recombinant expression vector can be performed in *E.coli* (Luckow, Lee et al. 1993). This system includes three components: a transfer plasmid which contains Tn7 integration signal sequence, a helper plasmid which encodes Tn7 transposase, a bacmid which contains the specific Tn7 target sequence. Foreign genes are inserted into the bacmid by site-specific transposition. And then the recombinant bacmid is isolated and transfected into insect cells. After inside the cells, the early gene of the bacmid is transcribed and then the foreign genes are expressed. The BVs can be collected from the culture supernatant and used to infect more cells. In this system, a novel

transposon Tn7 is exploited. Unlike many other transposons which insert randomly or into a small target sequence in many locations, Tn7 specifically inserts near a highly conserved 36nt sequence at high frequency. Tn7 contains 5 genes and flanking sequences (150 bp Tn7L and 90 bp Tn7R) which are involved in transposition. This system includes a target or attachment site (attTn7) which is 12 C-terminal codons of the bacterial protein glmS that associates with the production of N-acetyl-glucosamine (Gay, Tybulewicz et al. 1986, Mitra, McKenzie et al. 2010). The orientation of insertion is in the same orientation as glmS.

The commercial transfer vectors for the bacmid system are available to express one or two proteins such as pFastBac1 and pFastBacDual from Invitrogen. Tags and signal peptides are also added to facilitate for proteins expression and purification.

Another novel method for producing recombinant baculoviruses is the flashBAC system (Figure 5D). This system combines the vantages of homologous recombination and bacmid technology. Virus DNA has deletions in the C-terminal region of ORF 1629 which encodes a protein associates with associates with nuclear actin assembly during virus infection. The deletions prevent its replication in the insect cells. Meanwhile, a bacterial artificial chromosome (BAC) is added in the polyhedrin locus. Therefore the modified virus DNA can replicate in a circular form in *E.coli* cells. When the modified virus DNA with one transfer vector contained a foreign gene co-transfect into insect cells, homologous recombination which recovers the function of the ORF 1629 and removes the BAC sequence occurs between the transfer vector and modified virus DNA (Possee, Hitchman et al. 2008). In the latest vectors, the chitinase gene, cathepsin gene, p10 are deleted for reducing the proteolysis of recombinant proteins and enhancing the expression of recombinant protein (Hitchman, Possee et al. 2010).

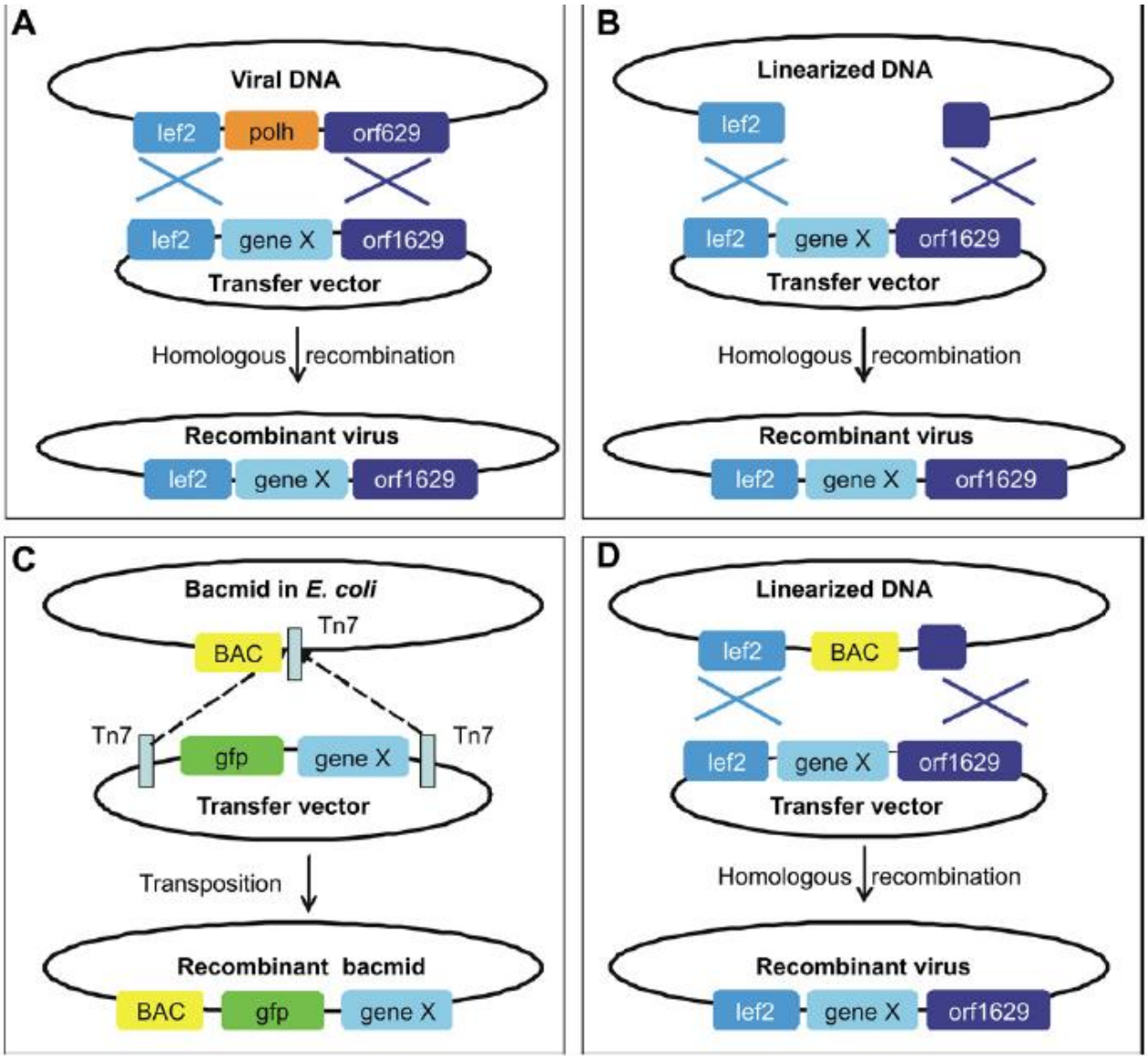

Figure 5. Four methods to obtain recombinant baculoviruses (van Oers 2011). A: the original method for generating the recombinants by homologous recombination between the circular viral genome and a transfer vector. B: linearized viral DNA is used with a transfer vector to get the recombinants via homologous recombination, which can increase the success ratio of recombinants. C: Bacmid system which is based on site-specific transposition to insert a foreign gene into a bacterial artificial chromosome which contains the baculovirus genome .D: FlashBAC system. The baculovirus/ bacmid DNA has a lethal mutation in ORF 1629 which is

essential gene for virus infection and a BAC sequence which can let it replicate in *E.coil*. The recombinant virus is based on repair of the ORF 1629.

1.2.3 Baculovirus transfer vectors

The main function of the baculovirus transfer vectors is inserting foreign genes into the virus genome under appropriate promoters. The transfer vectors include one part of virus genome which contains the sites for insertion of foreign genes. Polyhedrin or p10 genes are usually in this region as both of them are not essential for virus replication in cell culture and insect larvae but are expressed in a large amount (Smith, Summers et al. 1983). A typical baculovirus transfer vector includes a polyhedrin or p10 gene promoter, a transcription terminator, multiple cloning sites, viral gene on both sides of the promoter which is use for homologous recombination, signal peptide or protein tag that convenient for protein secretion and purification.

The first developed transfer vector for baculovirus expression system is on the basis of the polyhedrin gene promoter which needs a virus-encoded α amanitin RNA polymerase to activate (Mans and Knebel-Mörsdorf 1998). It includes a TAAG transcription initiation site flanked by 5' non-coding region that is needed for optimal gene expression. In principle, any baculovirus gene promoter could be used to express foreign genes. In practice, some promoters which are active in the late phase of baculovirus gene expression have been used for expressing recombinant proteins, such as basic protein and p6.9 gene promoters in pAcMP1, pAcMP2 and pAcMP3 that are commercial in BD Biosciences Pharmingen (Hill-Perkins and Possee 1990). The advantage of these transfer vectors is that the recombinant proteins express in the phase of assembling complex structures of virions, which need to produce glycoproteins. So if the recombinant proteins that need post translational modification will be performed better under these promoters.

Some transfer vectors contain selection markers to facilitate the identification of the recombinant virus in plaque assay. Such as the pBlueBac series of transfer vectors from Invitrogen which have the 5'-end of the lacZ gene therefore it recombines with the 3'end of LacZ in the virus DNA, and the pBacgus transfer vectors from Novagen have the β-glucuronidae gene. Moreover the pAcUW21 transfer vector from BD Biosciences Pharmingen has p10 and polyhedrin promoters. When the gene that was constructed under p10 promoter co-transfects with linearized viral DNA, the recombinant plaques are the ones which contain polyhedrin and easy to be identified. This vector is especially used for producing recombinant viruses that express an insecticidal gene as modified biopesticide.

Transfer vectors which can express multiple genes have been exploited. The polyhedrin gene locus has been widely developed for multigene expression. The first double expression vector (pAcVC2) used two polyhedrin promoters (Emery and Bishop 1987). Recently, both p10 and polyhedrin promoters have been used for multigene expression, such as pAcAB4 from BD Biosciences Pharmingen. Most of these vectors have multiple cloning sites at each promoter to allow foreign gene insertion. Some transfer vectors for bacmid system, such as pUCDM and pFBDM which contain multiplication module with polyhedrin and p10 promoters, have been exploited for multiprotein production. The pFBDM vector inserts genes by Cre-LoxP site specific recombination in the chitinase/cathepsin locus and the pUCDM vectors is used the Tn7 transposition to insert the genes at polyhedrin gene locus (RAWLiNGS, PEARL et al. 1992, Hawtin, Arnold et al. 1995).

Transfer vectors which include signal peptide that can direct the recombinant proteins into the endoplasmic reticulum of the virus-infected cells have been produced, such as pBAC-3, pBACgus-6 vectors from Novagen which use the gp64/67 signal peptide. Other commercial

transfer vectors, pMelBacA, B and C from Invitrogen, contain the honeybee melittin secretion signal which can target the proteins to the insect cell surface (Tessier, Thomas et al. 1991). In practice, these vectors can be used to label the living cells, which can prove the display of the expressed genes in the cell surface, as well as immunogold labeling for virions (Kaba, Hemmes et al. 2003). Surface display usually causes immunogenic proteins, so that the surface display BVs can be used for immunization once the DNA is inactivation (Kaba, Salcedo et al. 2004, Mlambo, Kumar et al. 2010, Tang, Lu et al. 2010). Surface display also is used for screen ligands binding to receptors, drug screening and so on (Ernst, Grabherr et al. 1998, Grabherr and Ernst 2001, Makela and Oker-Blom 2008).

1.2.4 Protein glycosylation in the baculovirus expression system

The baculovirus expression system has the ability to perform most of the posttranslational modification similar to eukaryotes. In principle, proteins that are expressed in this system should be folded, modified, assembled well and also have biological activity (Luckow and Summers 1988, O'Reilly, Miller et al. 1994, Jarvis 1997). However, the pathways of proteins expressed in this system still differ from those of higher eukaryotes. One example is the protein glycosylation.

The glycoproteins expressed in the baculovirus expression system only have simple side sugar chains in mannose residues, whereas those produced in mammalian system have complex N-glycans with sialic acid residues at the termini (Harrison and Jarvis 2006, Shi and Jarvis 2007, Geisler and Jarvis 2012) (Figure 6). In insect cells, the primary N-glycan complex is added to asparagine residues in the endoplasmic reticulum or Golgi apparatus, as well as mammalian cells. And then, different carbohydrate molecules are added or removed from this complex by glycosidases, mannosidases and glycosylaminotransferase. Glycosidases and mannosidases

remove glucose and mannose molecules respectively while glycosylaminotransfrase is in charge of addition of a GlcNac residue (Miller, Trimarchi et al. 1983). Although the preliminary reactions are similar between insects and mammalian cells, the following reactions have significantly differences. In the insect cells, the fucosyltransferases and N-acetylglucosaminidase cause one non-fucosylated and two different fucosylated variants, whereas in mammalian cells, they can produce several sialated and non-sialated variants. These differences impact the biological functions and immunogenicity of the proteins compare with those expressed in mammalian cells (Kong, Sheppard et al. 2010).

Many attempts have been performed to improve the baculovirus expression system that can produce the enzymes required for processing the glycosylation patterns in mammalian cells (Jarvis, Weinkauf et al. 1996, Hollister and Jarvis 2001, van Oers 2011, Aumiller, Mabashi-Asazuma et al. 2012). The first modified insect cell line contains a bovine β1, 4-galactosyltransferase gene that can help to produce a foreign protein with β-galactosylated N-glycans termini (Hollister, Shaper et al. 1998). Subsequently, another modified insect cell line which can express bovine β1, 4-galactosyltransferase and rat α2, 6-sialyltransferase that can produce the terminally α2, 6-sialylated N-glycans (Hollister and Jarvis 2001) was exploited. However, some analyses show that these modified insect lines only elongate the lower (α1, 3) branch of the N-glycans. As the glycoproteins in mammalian rarely have monoantennary N-glycans, another insect line, SfSWT series, which can express five mammalian glycosyltransferases was developed (Jarvis, Kawar et al. 1998, Aumiller, Hollister et al. 2003).

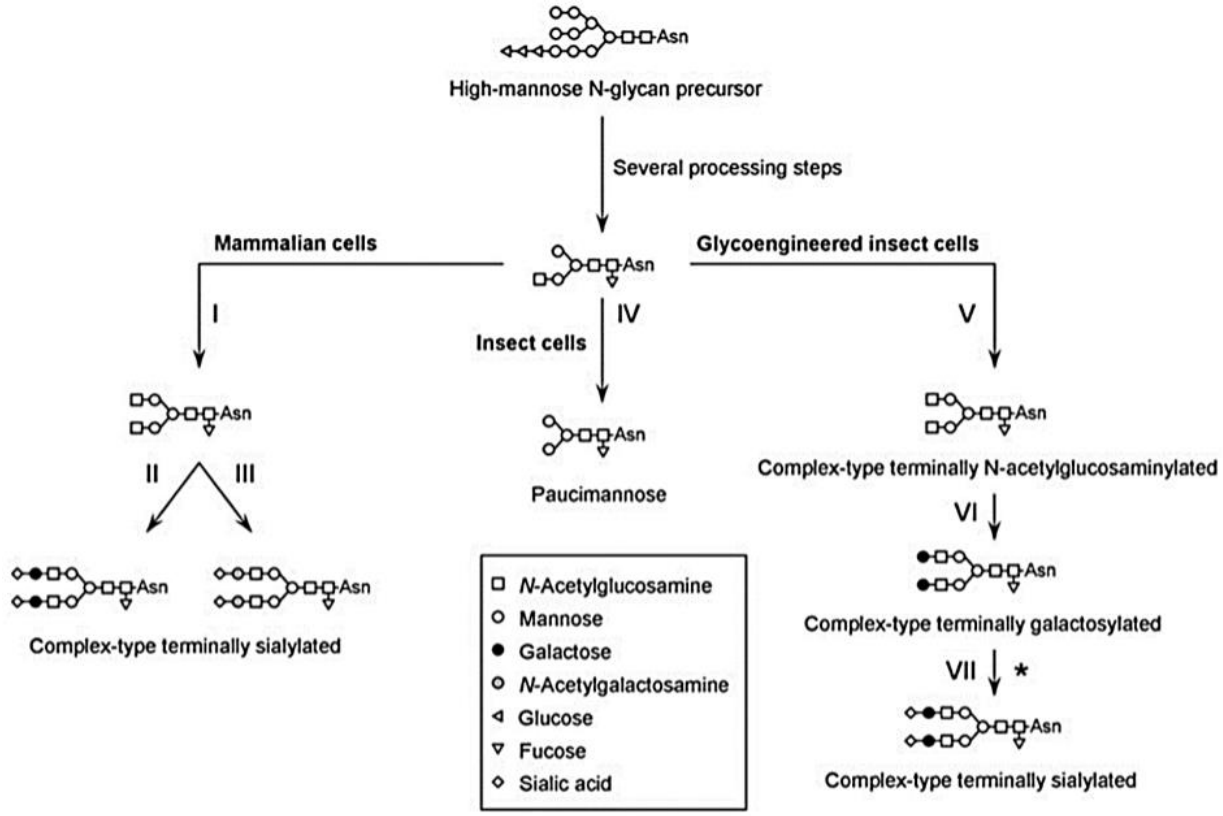

Figure 6. Recombinant protein glycosylation pathways in mammalian and insect cells (Contreras-Gómez, Sánchez-Mirón et al. 2014). In insect and mammalian cells, the N-glycan processing pathways have the same precursor and intermediate. The intermediate forms complex-type terminally sialylated N-glycans in mammalian cells, whereas in insect cells only produce a paucimannose N-glycans. However, the glycoengineered insect lines can produce complex-type terminally sialylated N-glycans. The enzymes used in the processing pathways: (I) N-acetylglucosaminyl transferase 2; (II) galactosyltransferases sialyltransferases; (III) N-acetylgalactosyltransferase sialyltransferases; (IV) N-acetylglucosaminidase; (V) α-1,6-mannosyl-glycoprotein 2-β-N-acetylglucosaminyltransferase; (VI) β-1,4-galactosyltransferase 1; and (VII) β-galactoside α-2,6-sialyltransferase

1.3 Insect cell culture

Insect cells were first isolated and cultured in the late 1950s for insect physiology study and baculoviruses synthesis (Baltz, Demain et al. 2010). Since the first establishment of the insect cell line, over 400 cell lines have been isolated from more than 100 insect species and derived from eggs and adult tissues, and have been utilized for recombinant protein expression and production of baculoviruses, virus-like particles and gene therapy vectors (Maranga, Cruz et al. 2002, G Aucoin, A Mena et al. 2010, Rychlowska, Gromadzka et al. 2011). The cell lines derived from lepidoptera insects have been widely used for recombinant proteins expression by the baculovirus expression system. Three cell lines are used more frequently to produce proteins, Sf21, Sf9 and High-Five™ (Contreras-Gómez, Sánchez-Mirón et al. 2014). Sf21 and Sf9 are from the pupal ovarian tissue of *Spodoptera frugiperda* whereas High-Five™ is from ovarian tissue of *Trichoplusia ni* (Vaughn, Goodwin et al. 1977,Summers and Smith 1987, Wickham, Davis et al. 1992). These three cell lines are susceptible to the infection of AcMNPV.

The diameter of the insect cells is between 10 and 20 μm and the shape of the cells is fibroblast like or round (van Oers and Lynn 2010). The cell lines can grow well in monolayer or suspension culture. For Sf21, Sf9 and High-Five™ cells, all of them can work well in monolayer and suspension culture in serum or serum-free medium (Kost, Condreay et al. 2005, Beas-Catena, Sánchez-Mirón et al. 2011, Beas-Catena, Sánchez-Mirón et al. 2013). Under the same cell basis, the High-Five™ cell line can produce more recombinant proteins compare with Sf9 and Sf21 cells while the Sf9 and Sf21 can get higher densities than High-Five™ cells under the same culture conditions (Palomares, Pedroza et al. 2001, Maranga, Cruz et al. 2002, Sander and Harrysson 2007, G Aucoin, A Mena et al. 2010). Except these three cell lines, other less common cell lines also be used to express proteins, such as Bm5, Tn368, Ea88 (Grace 1967,

Hink 1970, Granados and Naughton 1976). Other transgenic cell lines such as Mimic™ Sf9 (Invitrogen) and SfSWT series cell lines are developed to express the complex glycosylated glycans as those produced in mammalian cell (Hollister, Grabenhorst et al. 2002).

The first medium that is used to culture insect cells successfully is from the haemolymph of *Bombyx mori* moth (Wyatt 1956). As large amount of haemolymph is hard to obtain, and some kinds of medium have been designed for the culture of insect cells in vitro (G Aucoin, A Mena et al. 2010), such as TNM-FH, TC-100 and IPL-40. A typical culture medium comprises amino acids, organic acids, carbon source, vitamins, lipids and inorganic salts. In the early period of cell culture, the medium was supplemented with 5 to 20% fatal bovine serum. As the serum have a lot of drawbacks, such as high cost, potential adventitious agents, impact on protein purification and contaminants. Therefore serum-free medium has been used commonly now. Serum-free medium formulation is based on the above medium supplemented with yeastolate, a lipid mixture, Pluronic F-68 and other complements (Grace 1962). Commercial serum-free medium products include BD BaculoGold Max-XP (BD Biosciences), Express Five® and Sf-900™ II and Sf-900™ III (Invitrogen), HyClone SFX-Insect™ (Thermo Scientific) and Insect-XPRESS™ (Cambrex Bio Science). The purpose of the design of the serum-free medium is elimination of animal-derived components, so that they use plant protein hydrolysates to replace serum and other animal-derived ingredients and perform the same function as serum (Deparis, Durrieu et al. 2003).These medium are able to support expression high titer of recombinant protein and high cell densities (Shen, Kiyota et al. 2007, Keil and Tilkins 2013). However, these medium are also expensive, for the proprietary composition and cell line specific (Agathos 2010). Therefore these limits also affect the specific biotechnological applications. Some lab formulated

several generic serum-free medium for insect culture (YPR and YSD) which performed equally with commercial ones and cost much less (Ikononou, Bastin et al. 2001).

The range of growth temperature for insect cells is between 25 ℃ and 30 ℃, but the optimal temperature is 27±0.5 ℃ (van Oers and Lynn 2010). Under this temperature, the insect cells can produce high quality and titer products. The low temperature (< 27 ℃) and high temperature (> 27 ℃) will decrease the amount of the proteins (Reuveny, Kim et al. 1993). For the glycosylation of recombinant proteins, the lower temperature has resulted in more complete glycosylation of the recombinant proteins (Donaldson, Wood et al. 1999). Therefore, glycosylation may be affected by other factors, such as oxygen, agitation rate and so on (Zhang, Saarinen et al. 2002, Joosten and Shuler 2003).

The growth pH values of insect cells is from 6.0 to 6.8, while, for most cell lines, the optimal pH for growing is near 6.2 to 6.4 (Maranga, Cruz et al. 2002). The detail of the pH effects on protein expression is still not so clear. Another factor should be cared during cell culture is dissolved oxygen concentration. In fact, during the growth, the cells are not obviously sensitive to the change of dissolved oxygen concentration (Palomares and Ramirez 1996). However, the cells become sensitive to the dissolved oxygen concentration during infection (Cruz, Peixoto et al. 1998). It has been suggested to use the peak in oxygen consumption rate for determining the best time to collect the culture (Kamen, Bedard et al. 1996). Oxygen limitation post-infection has been found to affect the protein quality and product and glycan processing (Scott, Blanchard et al. 1992, Konz, King et al. 1998, Shao-Hua, Hong-Liang et al. 1998, Zhang, Saarinen et al. 2002). Sufficiently high rate of oxygen transfer is used to avoid this limitation. In addition, during infection, another parameter named the multiplicity of infection (MOI) also affects the productivity of the proteins. MOI is the plaque-forming units (PFUs) or infectious particles (IPs)

added to per cell number (Licari and Bailey 1992). When the cells are infected with a low MOI (much less than 1), only a portion of cells are infected and stop to grow, and uninfected cells continue to grow (G Aucoin, A Mena et al. 2010). The released virus particles infect the remaining cells. The low MOI is widely utilized for baculovirus stock amplification, which can minimize the production of the defective virions that result in the decreased expression ability of the baculovirus after some passages (Pijlman, van Schijndel et al. 2003, G Aucoin, A Mena et al. 2010). When a high MOI (> 5) is used to infect the cells, all the cells cease to growth and simultaneously produce the viruses or proteins. However, high MOI infection not only needs a high titer of baculovirus but also will increase the possibility of production of defective virus particles. An optimal MOI depends upon the baculovirus, the culture medium the cell line and other factors (Wong, Peter et al. 1996, Carinhas, Bernal et al. 2009).

1.4 Protein purification

As hundreds of species genomes have been sequenced, proteomics has become the next great challenge in molecular biology (Shendure and Aiden 2012). For research of the role and biological function of proteins, the first demand is the efficient and effective methods and materials for purification (Marichal-Gallardo and Alvarez 2012). In the protein purification process, the affinity tags acts one important role. The main purpose of affinity tags is to facilitate the purification of the recombinant proteins. Except this, the affinity tags also can increase the yields of recombinant proteins and enhance the solubility of recombinant proteins (Waugh 2005).

1.4.1 Protein affinity tags

The fusion tags can be placed at either the N- or C-terminus of a recombinant protein. N-terminal tags often can improve the productivity of recombinant proteins, which provides a dependable

surrounding for efficient translation initiation. Sequences at N-or C-termini of recombinant proteins can affect the degradation rate (Varshavsky 1997, Hayes, Bose et al. 2002, Flynn, Neher et al. 2003). So that, some affinity tags may improve the yield of recombinant proteins because of rendering them more immunity to intracellular proteolysis (De Marco, Stier et al. 2004). Some tags, such as MBP, Trx and Nus A, are more efficient to the solubility of recombinant proteins when placed at the N-termini (Sachdev and Chirgwin 1998). Tags, whether large or small, have the potential to interfere with the structures and functions of the recombinant proteins. Therefore, it is usually to remove tags after purification. Many cleavage sites can be cloned into the expression constructs and the tags can be removed by specific endoproteases, such as Enterokinase, Thrombin, Factor Xa, PreScission and virus-derived TEV protease (Jenny, Mann et al. 2003, Arnau, Lauritzen et al. 2006).

The size of affinity tags are from a single amino acid to a whole protein (Pina, Batalha et al. 2014). Although many different affinity tags have been developed for production and purification of recombinant proteins, several of them have been widely use in high-throughput protein purification. Here we will review several affinity tags that have been most commonly applied in the protein purification process.

1.4.2 His-tag

The hexahistidine tag (His-tag) is by far the simplest and most commonly used purification tag for high-throughput protein purification. It usually consists of six histidine residues. However, its size can vary from two to ten consecutive histidine residues. These residues can bind with transition metal ions such as Ni^{2+} or Co^{2+} immobilized on a resin or beads for purification. His-tag was first performed to purify recombinant galactose dehydrogenase by immobilized metal

affinity chromatography (IMAC) (LILIUS, PERSSON et al. 1991). Nowdays, IMAC becomes the principal affinity strategy in all large structural genomics centers. Ni (II)-nitrilotriacetic acid (Ni-NTA), which manifests high affinity of histidine residues, is the most commonly matrix for IMAC (Hochuli, Bannwarth et al. 1988, Gaberc-Porekar and Menart 2001). The matrix of IMAC usually exhibits a high binding ability (5-40 mg of His-tagged protein/ mL of matrix), is low cost and able to regenerate multiple cycles. In addition, the His-tag also can work well under denaturing conditions. Since the His-tag binding the matrix does not require a specific protein conformation. As a matter of fact, under denaturing conditions, His-tag binding to matrix is stronger than usual as the His-tag is more exposed. IMAC can work with strong denaturing reagents such as urea, guanidinium-HCl and massive non-ionic detergents.

The tagged proteins binding with IMAC matrix can be eluted with imidazole (100-250 mM), low pH (< 5). In order to minimize nonspecific proteins, a low concentration of imidazole (10-50 mM) should be included in the lysate and wash buffers. When performing the purification process with IMAC, the agents which can reduce the immobilized metal ion should be avoided in all the buffers, such as EDTA, EGTA, DTT, DTE.

As the small size of His-tag, it minimizes the interference to the folding, structure and function of the recombinant proteins. Many proteins have been crystallized with His-tags and the His-tag not only has a little or no impact on the structure of the recombinant protein, but also, in some cases, assists the protein crystal formation (Carson, Johnson et al. 2007, Smits, Mueller et al. 2008). While this tag also can be removed by introducing one protease cleavage site into the expression constructs.

Anti-His antibodies have been developed for the detection of His-tagged proteins by Western Blot. Since the insect and mammalian cells have endogenous histidines, cross-reactivity of primary antibody will occur in Western blot.

1.4.3 GST tag

Recombinant glutathione S-transferase (GST) tag is a 26 kDa eukaryotic protein derived from the parasitic helminth *Schistosoma japonicum*, which, in some cases, exhibits improving solubility and expression as an N-ternini of the recombinant protein (Smith and Johnson 1988). GST tagged proteins can be purified from crude extracts by immobilized sepharose-glutathione matrix and the bounded protein can be eluted under mild conditions via a competitive elution with reduced glutathione (10-40 mM) at neutral pH (Smith and Johnson 1988, LaVallie, Lu et al. 2000). Unlike His-tagged proteins, EDTA and reducing agents can be used in all the buffers to reduce proteolytic damage. The affinity resins used for purification, such as Glutathione-Sepharose beads, are low cost, have high binding capacity (5-10 mg of GST-tagged protein/mL of resin) and can be regenerated multiple times.

The GST tagged proteins are usually expressed in high yields in *E.coli* which can cause the generation of inclusion bodies. However, the glutathione affinity chromatography purification is based on the three-dimension structure of GST tag protein. Therefore the insoluble recombinant proteins must be refolded correctly before purification. Besides this drawback, GST is a homodimer which is not unsuitable for isolation of oligomeric proteins (Kaplan, Erhardt et al. 1997). The four solvent exposed cysteines in each subunit of the GST dimer can be involved in oxidative aggregation (Kaplan, Erhardt et al. 1997). The big size of GST tag not only may affect

the characters of the fusion proteins but also devour more metabolic energy during expression than small tags.

GST fusion proteins can be detected by a colorimetric assay with the substrate 1-chloro-2, 4 dinitrobenzene (CDNB) (Habig, Pabst et al. 1974). Commercial anti-GST antibodies are also performed for detecting these tagged proteins.

GST tag has been successfully used for the detection of protein-protein or protein-DNA interaction (Singh and Asano 2007). In high-throughput proteomics, GST tagged proteins have been immobilized onto protein microarrays (Walls and Loughran 2011). The GST tag is also used with small affinity tags like His-tag in tandem to permit additional purification with IMAC purification system.

1.4.4 MBP tag

Maltose-binding protein (MBP) is a secreted *E.coli* protein involved in the maltose transport system of *E. coli*, which can be expressed at very high levels (Nikaido 1994, Kapust and Waugh 1999). The MBP is usually used to increase the yields or solubility of its fusion partner and also can be used for effective affinity purification. MBP can be placed in either N or C-termini to enhance the expression and folding of recombinant protein (Kapust and Waugh 1999, Riggs 2000, Dyson, Shadbolt et al. 2004). The MBP tagged proteins can be purified with cross-linked amylose resin column and one step affinity chromatography purification can lead to 70-90% purity of a fusion protein (Pattenden and Thomas 2008). The bound fusion protein can be eluted with 10 mM maltose. However, the crude cell extracts can affect the amylase activity of amylose resins and the resins only can be reused three to five times. In addition, amylose affinity

chromatography is unsuitable to denaturing and reducing agents. The MBP tagged proteins can be detected with anti-MBP antibodies.

In practice, MBP tagged proteins often fail to bind to the amylose affinity resin (Routzahn and Waugh 2002, Bell, Engleka et al. 2013). Furthermore, since MBP forms different conformations with or without bound maltose, the maltose will cause heterogeneity in the final product, which can affect the crystal formation (Spurlino, Lu et al. 1991, Sharff, Rodseth et al. 1992, Quiocho, Spurlino et al. 1997). To solve this case, MBP is often used with His tag in tandem for additional purification.

1.4.5 Other tags

At present, a large number of affinity tags have been developed for protein expression and purification (Table 3).

Affinity tag	**Ligand**	**References**
FLAG™	mAb M1, M2	(Einhauer and Jungbauer 2001)
c-myc	mAb 9E10	(Evan, Lewis et al. 1985)
Poly-Arg	Anionic resins	(Sassenfeld and Brewer 1984)
Poly-Asp	Cationic resins	(Stubenrauch, Bachmann et al. 2000)
Poly-Cys	Thiopropyl Sepharose	(Zhao, Ford et al. 1990)
Glu	Cationic resins	(Dalbøge, Dahl et al. 1987)
β-Galactosidase	Thiogalactosidyl Sepharose	(Ullmann 1984)

Chloramphenicol acetyl transferase	Chloramphenicol Sepharose	(DYKES, BOOKLESS et al. 1988)
Staphylococcal protein A	IgG	(Nilsson and Abrahmsén 1990)
Cellulose-binding domain	Cellulose	(Tomme, Boraston et al. 1998)
Starch-binding domain	Starch	(Luojing, Ford et al. 1991)
Exoglucanase CBD	Cellulose	(Ong, Greenwood et al. 1989)
Thioredoxin	Require a purification tag	(Katti, LeMaster et al. 1990)
N-Utilization substance (NusA)	Require a purification tag	(Liu and Hanna 1995)
Small ubiquitin modifier (SUMO)	Require a purification tag	(Marblestone, Edavettal et al. 2006)
Bio tag	Streptavidin/avidin	(Schatz 1993)
Strep-tag	Streptavidin	(Skerra and Schmidt 2000)

Table 3. Other affinity tags for protein expression and purification

1.5 Protein-protein interactions

Protein-protein interactions play essential roles in most cellular processes, including mediating signal transduction, maintaining cellular organization, adjusting the activity of metabolic and signaling enzymes (Braun and Gingras 2012). Over 80% of proteins operate in complexes. Therefore, the analysis of protein-protein interactions is important for understanding the function of proteins which are associated with series of biochemical reactions in vivo. Methods for detecting protein-protein interactions have been developed.

1.5.1 Yeast two-hybrid system

The yeast two-hybrid system is one of the most widely used methods for detection or confirmation protein-protein interactions. This system is based on that many transcription factors have at least two functionally distinct domains, one that mediates transcriptional activation (AD) and another that directs binding to a promoter DNA sequence (BD). The original method was developed by Fields and Song (Fields and Song 1989). The transcription factors employed for this system can activate a reporter gene when its BD and AD are connected. To detect whether two proteins have interactions, each protein is fused to one of these two domains respectively. These two fused proteins are cloned in expression plasmids and then transfected into yeast cells. If these two proteins interact, the transcription of the reporter gene will initiated (Figure 7).

In the original method, the yeast two-hybrid system is GAL4 protein-based. One system derived from LexA protein also has been developed as one alternative (Golemis and Khazak 1997). In this system, an 88-residue acidic *E.coli* peptide acts as a transcriptional activation domain in yeast and binding domain is provided by LexA protein which acts as a transcriptional repressor when bound to LexA operators (Uetz, Titz et al. 2008).

The reporter genes used to indicate a protein interaction are commonly under one artificial promoter construct, which include an upstream activation sequence and a TATA sequence. Most reporter genes are integrated into the yeast genome. So that special yeast strains are needed for this system. The classical yeast host for this system is *Saccharomyces cerevisiae*. β-galactosidase (lacZ) is usually used as one reporter gene. However, lacZ is not so suitable for library screening. Other reporter genes, such as metabolic enzymes which are commonly involved in histidine metabolism (His3), adenine metabolism, leucine metabolism (Leu2) and uracil metabolism (Ura3), have been exploited (Uetz, Titz et al. 2008). These metabolic enzyme reporters which are

deleted or mutated in the yeast strain, allow the cell to grow when the bait and prey proteins interact. An interaction can be easily selected by culturing the modified yeast strain on media without histidine or leucine.

However, this system has some limitations. The interacted fusion proteins are restricted in the nucleus. This limits the study of the trans-membrane proteins which are lack of localization. In addition, the proteins that are transported into the nucleus may not obtain post-translational modification (Kuroda, Kato et al. 2006). Moreover, some proteins of signal transduction pathways which need post-translational modification for their function to interact with other factors require modification enzymes that are not exist in yeast (Guan and Kiss-Toth 2008). To compensate for the limits of the yeast two-hybrid system, many variations of yeast two-hybrid system have been developed.

The three-hybrid system was developed to address the post-modification issue by co-expressing the necessary enzymes in yeast to facilitate the post-modification of the proteins. Protein phosphorylation is the most common modification, which requires particular kinases. This was first attained by Osborne *et al* (Osborne, Dalton et al. 1995). They expressed the tyrosine kinase Lck with an immunoreceptor tyrosine-based activation motif (ITAM)-containing IgE receptor-derived bait and then screened their libraries to identify prey proteins which specifically interact with bait protein. Another use of the three-hybrid system is detection of weak interactions between multiple proteins. Frequently, proteins bind many other proteins and form a multimeric protein complex which contains both weak and strong interactions. To identify new proteins that interact with a protein of interest, co-expression of a previously defined interacting protein might provide a stronger interaction that enable to identify the novel proteins with lower affinity interaction (Tomashek, Sonnenburg et al. 1996, Tirode, Malaguti et al. 1997).

The bacterial two-hybrid system has been established. These systems are based on DNA loop formation, RNA polymerase recruitment, hybrid ToxR activators, dihydrofolate reductase, hybrid transcriptional repressors and the recombination of a cAMP signaling cascade (Kolmar, Hennecke et al. 1995, Dove, Joung et al. 1997, Dimitrova, Younes-Cauet et al. 1998, Karimova, Pidoux et al. 1998, Kornacker, Remsburg et al. 1998, Pelletier, Campbell-Valois et al. 1998). Compare with the yeast system, this system has more advantages, such as higher transformation efficiency, fast growth, nuclear localization not required, conciseness of experimental procedures and fewer indirect interactions by endogenous proteins (Guan and Kiss-Toth 2008). Therefore, the *E. coli* two-hybrid system is more suitable for large-scale protein-protein interaction analysis.

The mammalian two-hybrid system also has been exploited (Dang, Barrett et al. 1991, Vasavada, Ganguly et al. 1991). This system is similar to the yeast two-hybrid system. In the mammalian cells, proteins undergo post-translational modification, including phosphorylation, acylation and glycosylation. Therefore, the protein-protein interactions which base on the post-modifications can be detected.

However, the most serious problem of the two-hybrid system is a large number of false-positives and false-negatives. It has been estimated that maybe approximate 50% of the identified interactions are unreliable (Deane, Salwiński et al. 2002). Therefore, other methods should be used to confirm the interactions identified by the two-hybrid system.

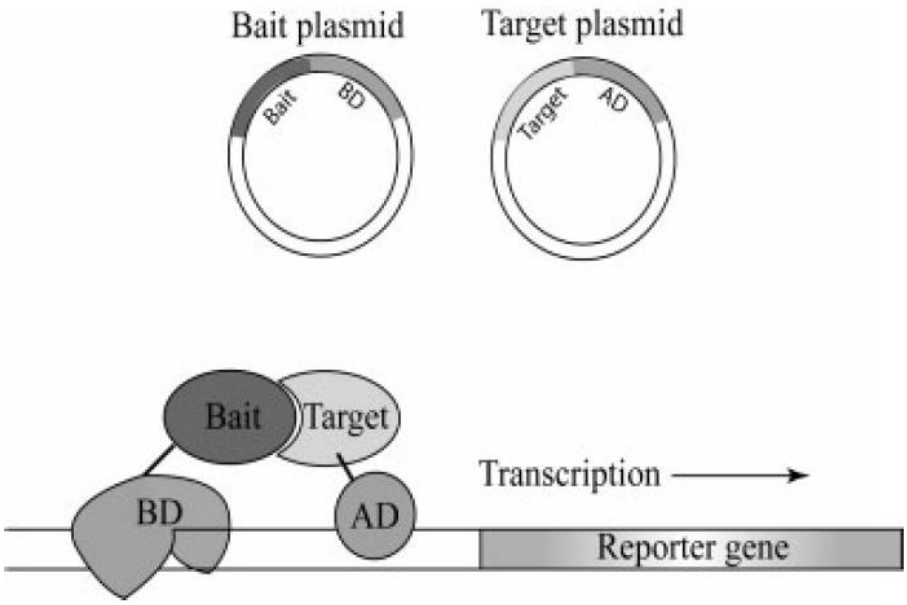

Figure 7. Yeast two-hybrid system (Berggård, Linse et al. 2007). Many transcription factors have at least two functionally distinct domains, one that mediates transcriptional activation (AD) and another that directs binding to a promoter DNA sequence (BD). In the system, two plasmids are constructed, which contain a bait protein fused to a BD and a prey protein fused to an AD respectively. These two constructs are co-expressing in yeast cell. If the bait and prey proteins interact, the transcription of the reporter gene is initiated.

1.5.2 GST pull-down assay

The GST pull-down assay is one widely used method to detect the interactions of two or more proteins in vitro. The GST pull-down is first described in 1991 (Kaelin, Pallas et al. 1991). Traditionally, GST pull-down has been performed to confirm interactions identified from two-hybrid system or other screening methods. This method is based on affinity chromatography. GST fusion proteins can be easily purified from *E.coli* or other hosts by incubating the GST-fused protein lysate with glutathione sepharose beads or resin. The non-specific proteins can be

washed off with neutral washing buffer, such as PBS. Usually, the purified proteins don't require elution and can be incubated with a second purified protein or an extract to detect the interaction. After incubation, the protein interaction complex is washed with washing buffer to remove the non-specific proteins. The final interaction complex can be eluted or boiled directly with SDS loading buffer and the results can be checked by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 8). If the protein concentration is high, it can be stained in the gel. While if the protein is not sufficient for staining, Western blot or mass spectrometry should be used to detect the interact protein. In this assay, a negative control, GST tag alone, should be included to limit the non-specific interactions. Since the GST tag can enhance the solubility and folding of the fused protein, it can produce high quality bait proteins which favor to maintain the original interaction propensity (Singh and Asano 2007).

The radio-labeled prey proteins, such as ^{35}S, which can be obtained from in vitro transcription or translation from a PCR fragment containing a promoter and ORF of the protein of interest, are commonly used (Uetz, Titz et al. 2008). Commercially kits, such as the Promega TnT T7 or S6 RNA polymerase- coupled system, are available.

Except GST tag, other tags also can be used to detect the protein interactions, such as poly-His tag, FLAG tag, C-myc tag and CBP (Uetz, Titz et al. 2008).

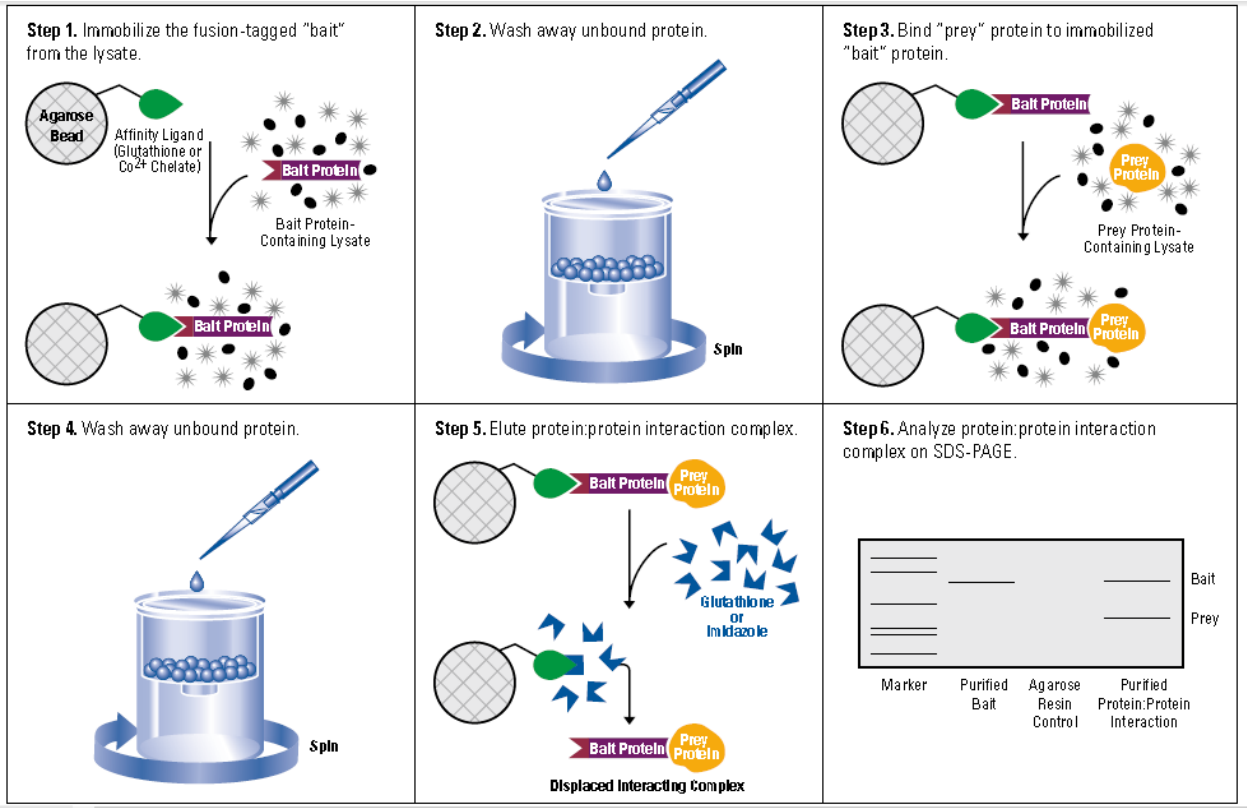

Figure 8. GST pull down assay (Thermo protein interaction technical handbook). The GST-fused bait protein is purified from cell lysate and the unbound proteins are washed off. The bait protein linked with glutathione sepharose or resin is incubated with lysate containing prey protein. The non-specific proteins can be washed off with washing buffer. The interaction complex is eluted or boiled directly and analyzed on SDS-PAGE.

1.5.3 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is one of most commonly methods for confirming protein-protein interactions. Co-IPs usually use sepharose beads coated with protein A which is isolated from *Staphylococcus aureus* (Sjöquist, Meloun et al. 1972). The constant chains of IgG antibodies have high affinity to bind with protein A. Therefore the sepharose-protein A can be coated with specific antibodies. This matrix can be incubated with cell extract. The proteins

which are recognized by the antibody can bind to the column. The non-specific proteins can be washed with washing buffer. The bound proteins can be boiled directly with loading buffer and separated in SDS-PAGE and Western blot.

The proteins also can be identified with mass spectrometry. A major limitation of co-IPs is the requirement of specific antibodies. Furthermore, an epitope-tag fused bait protein which is expressed by one plasmid can be used as an alternative method of specific antibodies (Masters 2004). The epitope-tag antibody can be used in the co-IP experiment. The tags commonly used are hemagglutinin (HA), FLAGT and myc peptides (Singh and Asano 2007). The advantage of this method is that the antibodies against the tags are commercial and specific and the epitope-tagged proteins can be eluted with competing peptides or other molecules. Usually, the specific elution can reduce the protein contamination in the eluate (Berggård, Linse et al. 2007).

1.5.4 Mass spectroscopy

Mass spectroscopy (MS) is a powerful tool for macromolecular interactions research. The principle of MS is using the gas phase ions as one way to characterize molecular structure and separating the ions based on their mass-to-charge ratios to identify polypeptide sequences (Aebersold and Mann 2003, Di Tullio, Reale et al. 2005). At present, two ionization techniques, Matrix Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (ESI), are used to convert peptide molecules from the condensed phase into gas phase ions (Whitehouse, Dreyer et al. 1985, Karas and Hillenkamp 1988, Pieles, Zürcher et al. 1993). Before analysis by MS, proteins should be digested with trypsin to get small peptides for ionization. ESI transfer peptides from a liquid phase to a gas phase ions under atmospheric pressure. In MALDI, the peptides are embedded in a matrix which absorbs laser light to allow ions desorption for analysis

by MS. MALDI is often used with time-of-flight (TOF), which permits more sensitive and accurate measurement of peptides. These peptides are identified by peptide mass fingerprinting (PMF) which can compares the peptide masses obtained from MS to the actual and theoretical peptide masses generated from a protein or DNA database. Except PMF, MS also use tandem MS (MS/MS) to identify proteins (Hunt, Yates et al. 1986).

For identification of interacted proteins, MS is usually used to identify proteins coupled with other protein separation methods, such as affinity purification, one or two-dimensional SDS-PAGE and HPLC. For example, the interaction proteins are separated on a SDS-PAGE and stained with silver or Coomassie blue dye. The visible bands can be cut and identified by peptide mass fingerprinting with MALDI-TOF MS. Alternatively, the protein can be digested directly and separated by online HPLC and analyzed by tandem MS.

1.5.5 Tandem affinity purification tag method

Tandem affinity tag (TAP) is one of the best known methods to purify protein. The TAP tag consists of two affinity tags separated by a cleavage site of tobacco etch virus protease (TEV) (Rigaut, Shevchenko et al. 1999, Puig, Caspary et al. 2001). The application of TEV protease can minimize the chance of cleaving the target protein or other interacted proteins since the cleave sequence of TEV protease is uncommon in the proteome.

A TAP-tagged protein is expressed in a host where it can form interacted complex with other proteins. The TAP-tagged protein complex is purified with a specific column binding the first part of TAP tag. After washing off the non-specific proteins, the TAP-tagged protein complex is cleaved by TEV protease and the first tag is on the column. The second tag of TAP tag still fused with the target protein complex in the eluate. And then, the second column which can bind the

second tag of TAP tag is used for further purification. After washing, the final target protein complex is eluted from the column. The protein complex is separated on a SDS-PAGE gel and analyzed with western blot or MS (Figure 9).

Many tags can be used in TAP tag method, such as his, FLAG, HA, protein A, myc and calmodulin binding peptide (CBP). The original TAP method use protein A and calmodulin binding peptide tags separated by TEV protease. Other protease cleavage sits also can be used, such as Enterokinase and Factor Xa. Inteins which can do self-cleaving tagged protein without protease, have been successfully used (Xu, Paulus et al. 2000).

The advantage of TAP tag method is that two purification steps reduce the non-specific binding compare with other affinity based methods. In addition, the target protein complex is formed in vivo. However, two purification steps maybe result in the loss of transient or weak protein-protein interactions. Another problem is that a large amount of material is needed for the purification and identification of low abundance binding proteins. TAP tag method also generates a lot of false positives.

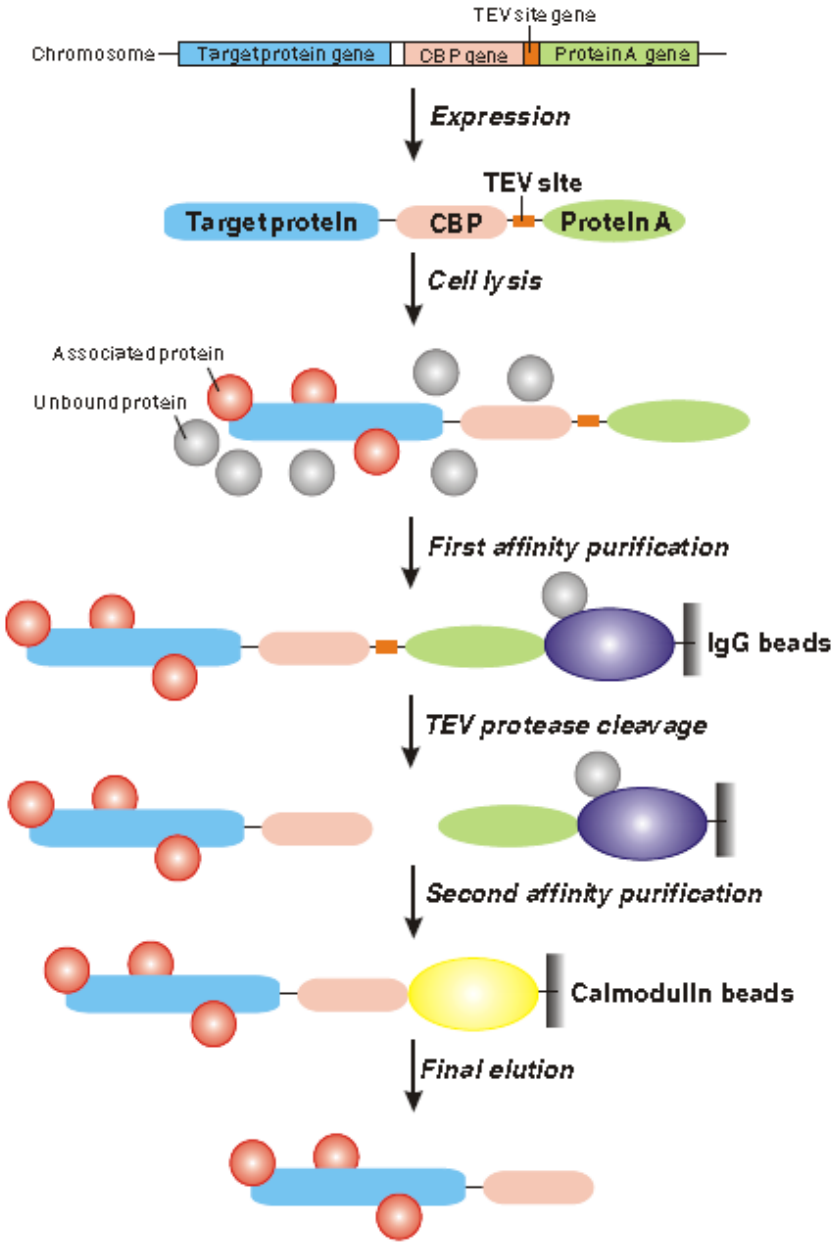

Figure 9. Schematic representation of TAP tag method (Kuroda, Kato et al. 2006). Target protein is fused with TAP tag which consists of protein A, CBP and a TEV protease cleavage site between these two tags. The target protein complex (TAP tagged protein and its associated proteins) is first purified with IgG beads. After washing, the target protein complex is cleaved by TEV protease, followed by the second affinity purification with calmodulin-coated beads. The final target protein complex is eluted from the column and identified with MS.

1.5.6 Far western blotting

Far western blotting (Far WB) was originally applied to screen protein expression libraries with ^{32}P-labeled GST-fusion proteins (Blackwood and Eisenman 1991, Kaelin, Krek et al. 1992). Now, Far WB has been used to detect protein-protein interactions, such as screen interacting partners in a library and receptor-ligand interactions (Kaido, Inoue et al. 2007). Compare with regular western blot, the only difference is that, after transferring the first protein (as bait protein) to the membrane, instead of detecting a protein on a membrane with antibodies directly, the membrane is then incubated with another protein (as prey protein) which maybe interacts with the membrane-bound protein. After washing, the membrane is incubated with an antibody against the second protein. If the second protein binds with that first protein, it can be detected.

Far WB is widely used to confirm protein-protein interactions identified by high-throughput screening or other methods and also used to identify interacted proteins of a bait protein (Feldman, Zusman et al. 2005, Gupta, Sharma et al. 2007). Moreover, Far WB can be used to detect the interaction domains. A bait protein is digested with a protease and the fragments are transferred onto a membrane. The membrane is incubated with the prey protein which just binds to the fragments that contain the interaction domain.

In addition, surface plasmon resonance analysis (SPR), fluorescence resonance energy transfer (FRET) system and confocal microscopy are also used to detect the protein-protein interactions (Karlsson, Michaelsson et al. 1991, Jares-Erijman and Jovin 2003, Berggård, Linse et al. 2007).

1.6 Insect bioassay

Since 1975, baculoviruses are commercially available for their use as bio-insecticides (Szewczyk, Rabalski et al. 2009), becoming useful agents in biological control programs against lepidopteran

pests. Baculoviruses have a narrow host range, strong pathogenicity, are harmless to non-target organisms, stable in the environment, and can be applied using simple methods (Szewczyk, Hoyos-Carvajal et al. 2006). Worldwide, several wild-type baculoviruses are used for controlling local pests. For example, *Helicoverpa armigera* nucleopolyhedrovirus (HaMNPV) has been used for large-scale biopesticide production and has been extensively used on cotton fields in China and India since 1993 (Zhang, Sun et al. 1995, Srinivasa, Babu et al. 2008). Another two notable examples are the application of *Spodoptera frugiperda* NPV (SfMNPV) to protect annually about 20,000 hectares of maize in Brazil and the *Anticarsia gemmatalis* NPV (AgMNPV) to control the velvet-bean caterpillar in soybean crop (Moscardi 1999). In Europe and the United States, several formulations of baculoviruses (SPOD-X ™ and Spodopterin ™ containing the *Spodoptera exigua* MNPV and *Spodoptera littoralis* MNPV, respectively) have become important agents for controlling *Spodoptera* pests in cotton, corn, and tomatoes crops (Szewczyk, Rabalski et al. 2009). In addition, formulations of *Spodoptera exigua* NPV (SeMNPV) are being used to protect crops of sweet peppers in Spain against *Spodoptera exigua*. In 2009, the application of this biological insecticide has protected crops grown in 23,000 ha just in Spain (Caballero, Murillo et al. 2009).

However, the application of the baculoviruses is limited by biological or technical reasons. Most baculoviruses have a low speed of kill, therefore, their insect hosts, which continue feeding for days after infection, produce considerable crop damage before succumbing to the viral infection. Higher doses of the baculoviruses are often necessary for adequate control. Baculoviruses are used in early insect development stages as if used in the late stages, several lepidopteran pests are more resistant to infection (Valadez-Lira, Alcocer-Gonzalez et al. 2012). Baculoviruses have

low persistence in very sunny conditions as well. In addition, some cultural practices can affect viral persistence, hiding the viral particles in the soil (Rodriguez, Belaich et al. 2012).

Genetically engineered baculoviruses have been successfully developed to enhance the insecticidal activity of the original wild-type virus. Insertion into baculoviruses with genes encoding for proteins interfering specifically with insect metabolism or metamorphosis such as toxins (Inceoglu, Kamita et al. 2001), hormones (Elvira, Williams et al. 2010), and enzymes (Gramkow, Perecmanis et al. 2010), are more efficient in speed of kill than the wild-type viruses. Incorporation of genes that interfere with insect immune responses into baculovirus genomes, e.g. polydnavirus (PDV) genes, may potentially enhance their efficacy. PDVs are double stranded DNA viruses symbiotically associated with endoparasitoid wasps that are injected along with the endoparasitoid eggs into the Lepidopteran host hemocoel during oviposition, thus enabling endoparasitoids to escape the cellular defense of their habitual host caterpillars (Tanaka, Matsumoto et al. 2002, Gundersen-Rindal and Lynn 2003).

Cotesia rubecula PDV gene (CrV1) is the best characterized gene among four gene products detected in *Pieris rapae* tissues (Asgari, Hellers et al. 1996). CrV1 gene product is a secreted glycoprotein expressed in the hemocytes and fat body cells of *Pieris rapae*. The CrV1 proteins have been implicated in depolymerization of the actin cytoskeleton of host hemocytes disabling the capacity of hemocytes to spread onto foreign surfaces (Asgari, Hellers et al. 1996). In *Cotesia rubecula*, CrV1 is the main gene that is expressed in the host hemocyte. Homologues of this gene have been found in PDVs from other wasps (Le, Asgari et al. 2003).

Pieris rapae (Linnaeus) (Lepidoptera: Pieridae) was first found in Quebec, Canada in 1860 (Harcourt 1963) and it is now widespread in North America. The insect has been also observed in Bermuda, Australia, Hawaii, and other Pacific Islands (Opler and Krizek 1984). *Pieris rapae*

is an important pest on the crucifer and caper families, such as cabbage, cauliflower, broccoli, and collard (Opler and Krizek 1984). Scanty research has been published about the effect of *A. californica* multiple nucleopolyhedrovirus (AcMNPV) infection on *Pieris rapae*. *Spodoptera exigua* is one of the best-known agricultural pest insects with a wide host range that includes asparagus, beans and peas, sugar and table beets, celery, cole crops, lettuce, potato, tomato, cotton, cereals, oilseeds, tobacco, many flowers, and a multitude of weed species; it produces serious economic losses in many areas around the world (Zheng, Cong et al. 2011). *Spodoptera exigua* is susceptible to infection by several MNPVs such as AcMNPV, *S. littoralis* MNPV, SfMNPV, and *Mamestra brassicae* MNPV.

2 Justification

In *Cotesia rubecula*, CrV1 is the only gene that expressed in the host hemocytes and it is sufficient to inactivate hemocytes. However the proteins that interact with CrV1 protein are still unknown. In this study, we will investigate the interacted proteins by GST pull-down assay, which could help to understand the mechanism of immune suppression in parasitoid system. In addition, there is still no published research about insecticidal activity of CrV1 protein. In this work, bioassay will be carried out, which could help to investigate the insecticidal activity of CrV1.

3 Objectives

3.1 General objective

In this study, the interacted protein and insecticidal activity of immunosuppressive CrV1 protein in *Spodoptera exigua* and *Pieris rapae* will be investigated.

3.2 Specific objectives

1. To express the CrV1 protein in the baculovirus expression system for further protein interaction analisys and modify baculorvirus to improve it as bioinsecticide.

2. To investigate the interacted proteins of CrV1 protein in *Pieris rapae* larvae lysate by pull-down.

3. To perform bioassay with pAcUW21-CrV1 baculovirus against two insect pests *Spodoptera exigua* and *Pieris rapae* to check the insecticidal activity of immunosuppressive CrV1 protein in larvae.

4 Materials and methods

4.1 Construction of recombinant transfer vector with CrV1 gene

The maps of two transfer vectors were shown in Figure 10. The clone digrams for construction of two recombinant transfer vectors with CrV1 gene were shown in Figure 11 and 12. All the formulations of solutions used in the experiment were listed in Appendix B.

4.1.1 Competent cells preparation

A glycerol stock of *Escherichia.coli* DH5α stain was streaked onto one LB agar plate and the plate was incubated at 37 ℃ overnight (SHEL LAB Incubator). Next day, a single colony was inoculated into 5 mL LB medium and grown at 37 ℃ overnight with 250 rpm shaking. 1 mL of the culture was inoculated into 100 mL LB medium in a sterile 500 mL flask and grown at 37 ℃ in a shaking incubator (Thermo Scientific MaxQ HP 480 incubator) with 250 rpm until OD_{600} reached 0.6. The culture was chilled on ice for 30 min and then transferred into two 50 mL pre-chilled, sterile centrifuge tubes (Corning®). The tubes were centrifuged at 3800 rpm (Allegra™ 6R centrifuge) at 4 ℃ for 30 min. The supernatant was discarded and each pellet was gently resuspended in 30 mL sterile, ice-cold, 0.1 M $CaCl_2$ solution. The resuspended cells were incubated on ice for 30 min and centrifuged at 3800 rpm at 4 ℃. The supernatant was poured off and each pellet was gently resuspended in 2 mL ice-cold 0.1 M $CaCl_2$ solution which contained 10% glycerol. 100 μL aliquots of resuspended cells were dispensed into ice-cold, sterile, 1.5 mL tubes and stored at -80 ℃ freezer (Thermo Scientific) immediately (Seidman, Struhl et al. 1997).

4.1.2 Primers design

To construct recombinant expression transfer vector, which was used to express and purify CrV1 protein, the pAcGHLT-B transfer vector (BD Biosciences) was selected (Figure 10). The primers were designed for CrV1 gene amplification. The forward primer (5'-ATACTCGAGACATGTCACTCGTCAAAAGTGCGTC-3') contained the *Xho* I restriction site, while the reverse primer (5'-ACTGGTACCGCAAGTTCAGCCTGGTTAAGT-3') contained *Kpn* I restriction site. To construct recombinant AcMNPV baculoviruses expressing the CrV1 protein (AcMNPV-CrV1), the pAcUW21 transfer vector (BD Biosciences) was selected (Figure 10). The primers for amplifying the CrV1 gene were designed to contain the *Bgl* II restriction site in the forward primer (5'-GGCAGATCTATGTCACTCGTCAAAAGTGCGTC-3') and reverse primer (5'-GGCAGATCTGCAAGTTCAGCCTGGTTAAGT-3'). The reverse primer (5'-GGCCGGTACCAAATTGGAAAATATATTCATTTATTC-3') was designed to detect the CrV1 gene transcription in insect cells. The primers which were used to sequence the recombinant constructs with CrV1 gene were also designed (5'-CCAGTATGAATATGAATCTGAAACAT-3' and 5'-CTAGAGGTTGCGGTGAGATT-3'). All the primers were ordered from Eurofins and dissolved in sterile Milli-Q water to 100 μM concentration stocks. The working concentration of the primers was 25 μM.

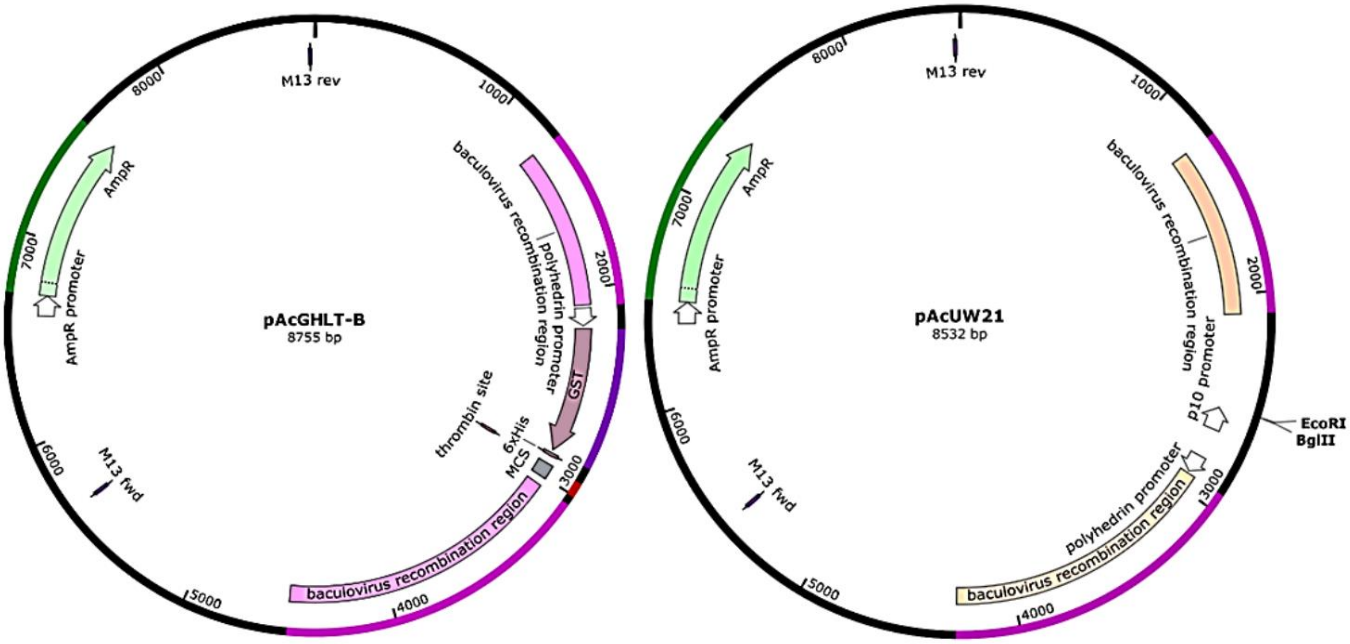

Figure 10. Maps of two transfer vectors-pAcGHLT-B and pAcUW21. The pAcGHLT-B contains a 6×His tag and GST tag upstream of multiple cloning sites (MCS). All the foreign inserts are in frame with the GST open reading frame under polyhedrin promoter. The recombinant protein will be expressed as a GST containing 6×His fusion protein. Therefore, the recombinant proteins can be purified with Ni-NTA agarose and glutathione agarose beads. After purification, the GST and 6×His tags can be removed by thrombin. The transfer vector also has an ampicillin antibiotic resistance marker and baculovirus recombination regions for generation of recombinant baculovirus by homologous recombination. The pAcUW21 transfer vector contains AcMNPV p10 promoter, polyhedrin promoter and SV40 transcription termination signals inserted into upstream of the complete AcMNPV polyhedrin gene. The foreign inserts can be cloned into the *Bgl* II or *EcoR* I site under the p10 promoter. The recombinant virus will be occlusion body-positive. This vector is usually used to research the producing recombinant protein in insect larvae. This transfer vector also contains antibiotic resistance gene and baculovirus recombination regions as pAcGHLT-B.

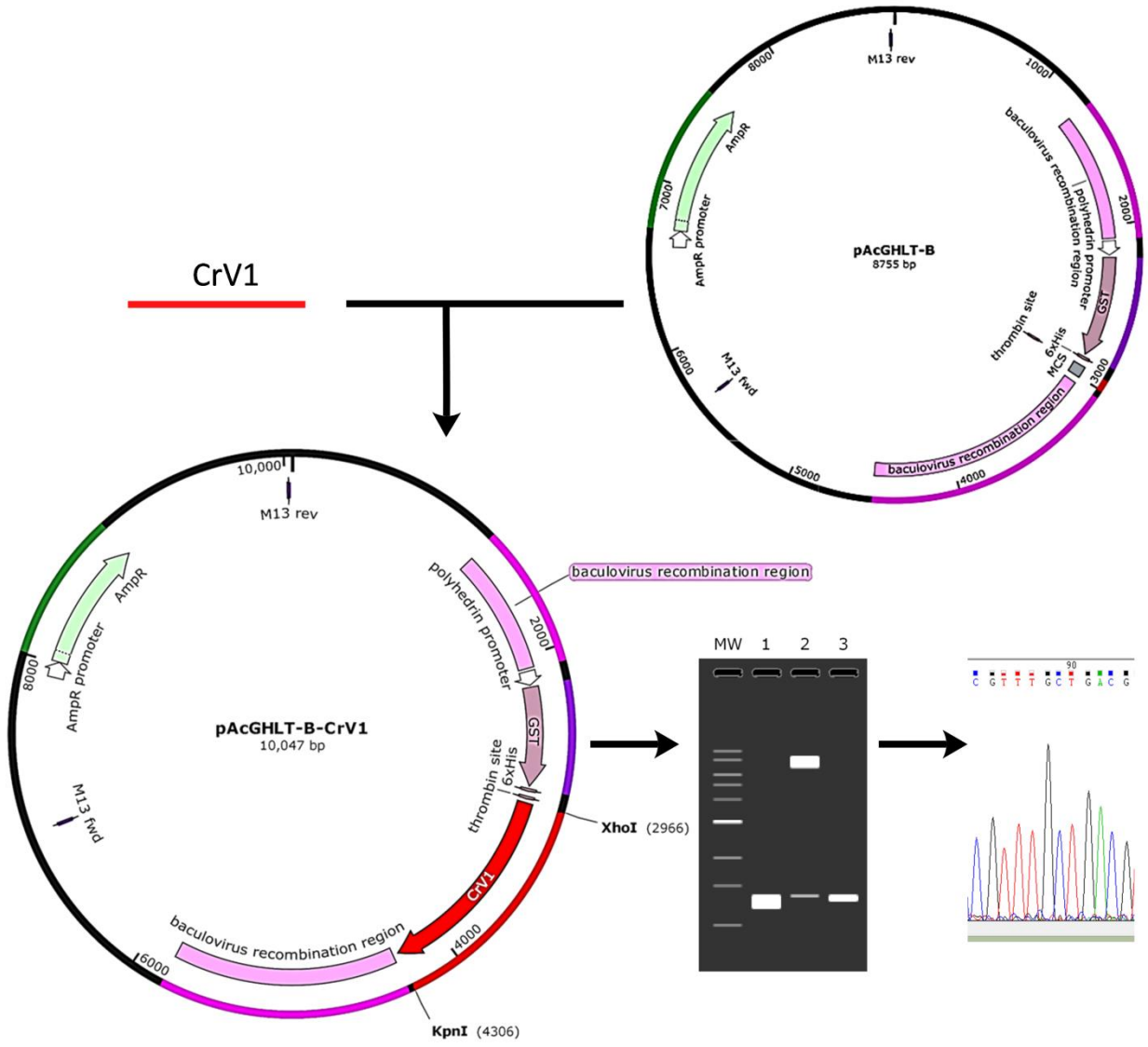

Figure 11. Clone diagram of recombinant transfer vector pAcGHLT-B-CrV1. The CrV1 fragments were amplified from an original plasmid with specific primers containing *Xho* I and *Kpn* I restriction sites. The CrV1 fragments and transfer vector were digested with *Xho* I and *Kpn* I respectively. After ligation and transformation, the recombinant constructs were identified by colony PCR, confirmed by PCR, digestion with *Xho* I and *Kpn* I, and sequencing analysis.

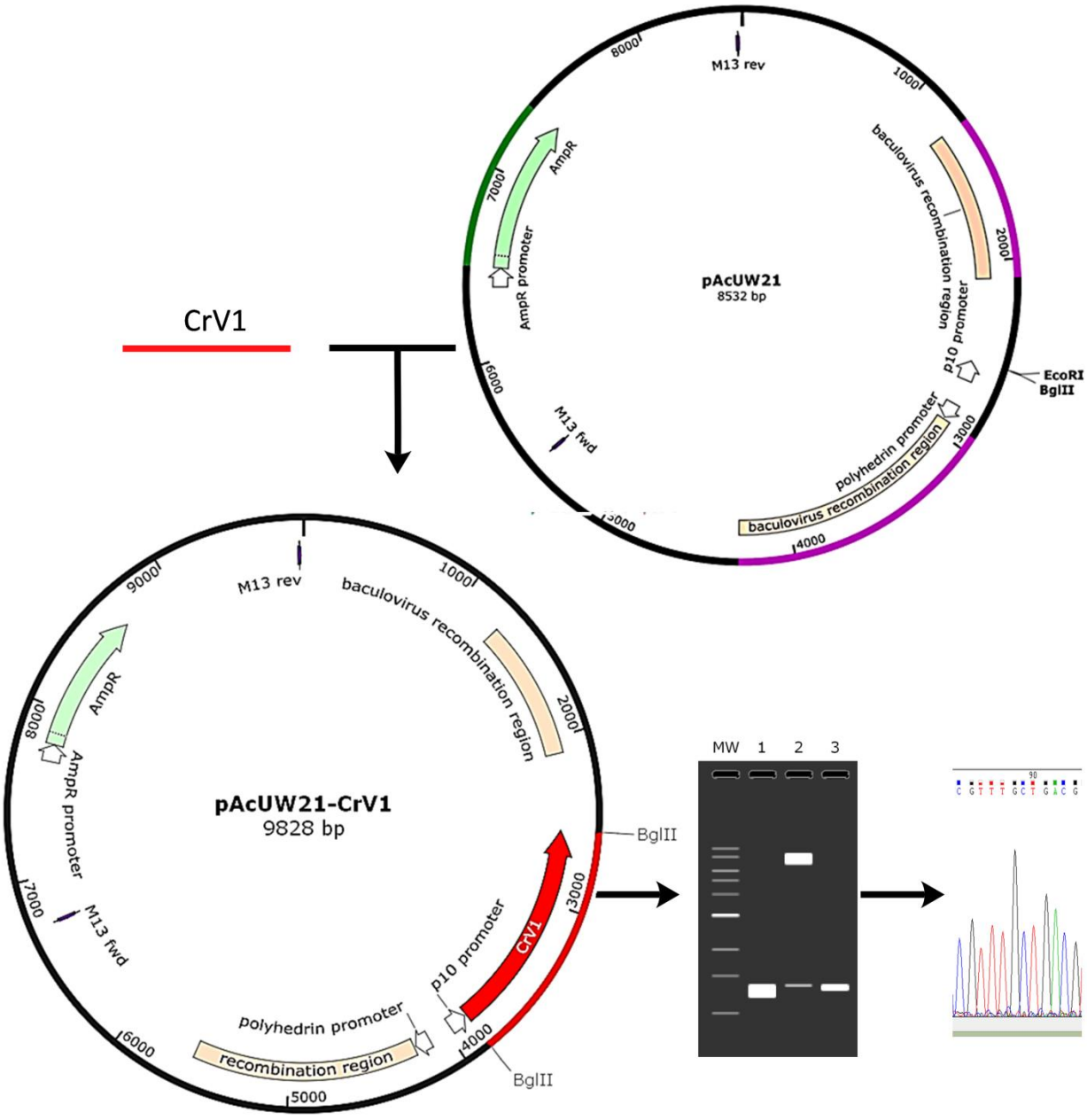

Figure 12. Clone diagram of recombinant transfer vector pAcUW21-CrV1. The CrV1 fragments were amplified from an original plasmid with specific primers containing *Bgl* II restriction sites. The CrV1 fragments and transfer vector were digested with *Bgl* II. After ligation and transformation, the recombinant constructs were identified by colony PCR, confirmed by PCR, digestion with *Bgl* II and sequencing analysis.

4.1.3 Amplification of CrV1 gene by polymerase chain reaction (PCR)

The CrV1 cDNA clone was provided by Dr Sanssan Asgari from the University of Queensland, Australia. To amplify CrV1 gene, pfu DNA polymerase (Prospec) was used. PCR reaction was performed in a final volume of 25 μL, including 0.5 μL DNA template (200-300 ng/ μL), 0.5 μL forward primer (25 μM), 0.5 μL reverse primer (25 μM), 0.5 μL dNTPs (10 mM), 1 μL $MgCl_2$ (50 mM), 2.5 μL reaction buffer (10×), 0.2 μL pfu DNA polymerase and 19.3 μL sterile Milli-Q water. The PCR was carried out with initial denaturation at 95 ℃ for 5 min followed by 25 cycles (95 ℃ for 30 s, 62 ℃ for 30 s, 72 ℃ for 2 min and 10 s) and 72 ℃ for 10 min. PCR products were stored at -20 ℃.

4.1.4 Agarose gel electrophoresis

PCR results were analyzed through agarose gel electrophoresis. 0.8% agarose gel was prepared for separating the DNA fragments. 0.4 g of agarose was added to 50 mL of 1×TAE electrophoresis buffer (40 mM Tris base, 20 mM Acetic acid, 2 mM EDTA, pH 8.2-8.4) and melted in a microwave. When the melted gel was cooled to approximately 55 ℃, the gel was poured into a gel casting platform containing a well comb. After the gel solidified, the comb was removed and the gel was placed in an electrophoresis tank (Bio-rad) which contained sufficient 1× TAE buffer. 5 μL of SYBR® Gold nucleic acid gel stain (Invitrogen) was added to 1 mL of 5× green GoTaq® reaction buffer (Promega), which was used as sample loading buffer. PCR products were mixed with loading buffer and loaded into the wells. The gel was run with 90 volts for 30-60 min and then placed under UV light (Kodak Gel logic 112) to analyze the results.

4.1.5 DNA purification from agarose gel

To recover CrV1 gene from agarose gel for constructing recombinant plasmid, CrV1 fragment was cut from gel and put in a 1.5 mL tube. QIAquick Gel Extraction Kit (Qiagen) (Appendix A) was used to purify the DNA fragment. The quanlity and concentration of purified CrV1 fragment were analyzed through agarose gel electrophoresis.

4.1.6 Isolation of transfer plasmid

A colony contained transfer plasmid (pAcGHLT-B or pAcUW21) was inoculated into 5 mL LB medium supplemented with ampicillin antibiotic and grown overnight at 37 ℃ with 250 rpm shaking (Thermo Scientific MaxQ HP 480 incubator). QIAprep Spin Miniprep Kit (Qiagen) (Appendix A) purified with QIAquick PCR Purification Kit (Qiagen) (Appendix A) was used to extract the plasmid DNA. The quanlity and concentration of plasmid DNA were analyzed by agarose gel electrophoresis.

4.1.7 Digestion of CrV1 fragments and two transfer plasmids with restriction endonucleases

To construct the recombinant transfer vector for expression and purification of CrV1 protein, the purified CrV1 fragments and pAcGHLT-B vector were digested with *Xho* I and *Kpn* I restriction endonucleases respectively. Each reaction was performed in a 60 μL reaction volume, including 15 μL plasmid DNA or CrV1, 6 μL reaction buffer (10×), 3 μL *Xho* I or *Kpn* I, 36 μL sterile Milli-Q water. The reaction was mixed briefly and placed in a 37 ℃ incubator (SHEL LAB Incubator) for 2 h. After the first endonuclease digestion, the digested CrV1 or pAcGHLT-B was purified with QIAquick PCR Purification Kit (Qiagen) (Appendix A). The second enzyme was used for further digestion with the same reaction volume and condition. After the second

purification procedure, the digested CrV1 fragments and pAcGHLT-B vector were saved at -20 ℃ for later ligation. To construct recombinant AcMNPV baculovirus expressing the CrV1 protein for insect bioassay, the purified CrV1 fragments and pAcUW21 vector were digested with *Bgl* II respectively. The procedure was described above. After digestion, the digested pAcUW21 vector was treated with calf intestinal alkaline phosphatase (CIAP) to limit the self-ligation.

4.1.8 Ligation reaction

Two ligation reactions were performed, CrV1 with pAcGHLT-B and CrV1 with pAcUW21. Each ligation reaction was done in 10 μL reaction volume, containing 0.5 μL plasmid DNA, 7.5 μL CrV1 fragment, 1 μL 10× reaction buffer, 1 μL T4 DNA ligase (Promega). The ligation reaction tubes were incubated at 4 ℃ overnight.

4.1.9 Transformation

Competent cells were thawed on ice. 100 μL cells were dispensed into each ligation tube and mixed gently. The tubes were placed on ice for 30 min. The cells were heat shocked for 45 s at 42 ℃, then immediately placed on ice for 2 min. After adding 500 μL LB medium, the tubes were incubated at 37 ℃ in a shaker (Eppendorf thermomixer compact) at 300 rpm for 2 h. Each transformation mixture was centrifuged (Corning® LSE™ High Speed Microcentrifuge). After throwing most of supernatant, the cells were mixed with the left supernatant and spread on LB plates containing 100 ug/mL ampicillin. The plates were incubated at 37 ℃ incubator overnight.

4.1.10 Colony PCR for quick screen for recombinant constructs

In each tube, 15 μL of sterile Milli-Q water was added. A sterile toothpick was used to pick up individual colony and dipped it into each PCR tube. After stirring, 3 μL of the mixture was used

as DNA template in PCR reaction. Other mixture was stored at 4 ℃. The colony PCR reaction volume is 25 μL, including 3 μL DNA template, 0.5 μL forward primer (25 μM), 0.5 μL reverse primer (25 μM), 0.5 μL dNTPs (10 mM), 1 μL $MgCl_2$ (50 mM), 2.5 μL reaction buffer (10×), 0.2 μL Taq DNA polymerase and 16.8 μL sterile Milli-Q water. The PCR was carried out with initial denaturation at 95 ℃ for 5 min followed by 25 cycles (95 ℃ for 30 s, 62 ℃ for 30 s, 72 ℃ for 1 min 30 s) and 72 ℃ for 10 min. The PCR results were analyzed by agarose gel electrophoresis. The positive colony mixtures were inoculated into 5 mL of LB medium with ampicillin and grown at 37 ℃ overnight for extraction of recombinant constructs (Darst, Pardo et al. 2010).

4.1.11 Confirmation of recombinant constructs by restriction endonucleases

Recombinant constructs were extracted using QIAprep Spin Miniprep Kit (Qiagen) (Appendix A) and purified with QIAquick PCR Purification Kit (Qiagen) (Appendix A). After this, recombinant plasmids were digested with restriction endonucleases. The procedure was described above. After digestion, the results were analyzed in one agarose gel. This step was used to confirm the appearance of recombinant constructs. In addition, the extracted plasmids also can be used for sequencing analysis.

4.1.12 Sequencing analysis

BigDye® Terminator v3.1 Cycle Sequencing Kit was used to carry out the sequencing analysis. The reaction volume was 10 μL, including 0.5 μL plasmid DNA (300-400 ng/μL), 2 μL primer (25 μM), 2 μL BigDye v3.1 ready mix, 2 μL reaction buffer (5×), 3.5 μL sterile Milli-Q water. The PCR procedure started with 96 ℃ for 5 min, followed by 25 cycles (96 ℃ for 10 s, 55 ℃ for 5 s, 62 ℃ for 4 min) and 4 ℃ forever. The PCR products were purified with Applied Biosystems BigDye® XTerminator™ purification Kit. For each purification reaction, 45 μL of SAM solution

and 10 μL of XTerminator were mixed with 10 μL of PCR products in a 1.5 mL tube, which was incubated at 25 ℃ for 30 min with maximum mix spend of Eppendorf ThermoMixer. And then, the mixture was centrifuged at 13000 rpm (Corning® LSE™ High Speed Microcentrifuge) for 2 min. 30 μL of the supernatant was transferred into a new PCR tube and sent for sequencing analysis.

4.2 Generation and amplification of recombinant AcMNPV baculoviruses

4.2.1 Initiation of cell culture

One tube of Sf9 cells (Allele Biotechnology) (stored at -80 ℃) was placed in 27.5 ℃ incubator (Eppendorf ThermoMixer) until thawing. Quickly sterilized the outside of the tube with 70% ethanol and placed on ice. After 4 mL of complete TNM-FH culture medium was added to a 25 cm^2 flask, thawed cells were transferred directly into one flask. The flask was placed at 27.5 ℃ (Precision scientific 818 low temperature illuminated incubator) and allowed cells to attach for 30 min. After the cells were attached, the medium was removed and 5 mL of fresh medium was supplied to the cells. After 24 h incubation at 27.5 ℃, the culture medium was changed again and continued to incubate until confluence.

4.2.2 Subculture of Sf9 cells

Confluence is a marker for subculturing cells. The subculture of insect cells includes adherent and suspension culture. In this study, we just used adherent culture. When cells were confluent, medium from flasks was removed and 4 mL of new medium was added to each flask. Cells were resuspended by pipetting medium across monolayer with a 1 mL pipette. The suspended cells

were divided into four new flasks which contained 4 mL fresh medium. All the flasks were incubated at 27.5 ℃ for further use.

4.2.3 The storage of Sf9 cells

Two flasks of confluent cells were suspended by pipetting and transferred into one sterile 15 mL centrifuge tube (Corning®). After centrifugation at 800 rpm (Allegra™ 6R centrifuge) for 10 min, supernatant was discarded and 2.7 mL of fresh medium was put in each tube for resuspending the cells. 300 μL of dimethyl sulfoxide (DMSO) was added to each tube and mixed (10% DMSO as a final concentration). 1 mL of cell suspension was transferred into each sterile 1.5 mL tube and stored at -80 ℃ immediately.

4.2.4 Co-transfection

BD BaculoGold™ Transfection Kit (BD Biosciences) was used for co-transfection. In this study, four co-transfection reactions were performed, including pVL1392-XylE control vector DNA (positive control), recombinant pAcGHLT-B transfer vector, recombinant pAcUW21 transfer vector and pAcGHLT-B transfer vector with linearized baculovirus DNA respectively. Recombinant pAcGHLT-B transfer vector will be used as an example to describe the co-transfection procedure.

Sf9 cells (3×10^6) were seeded into one 25 cm^2 flask. After Sf9 cells attached firmly to the flask, culture medium was removed and 1 mL of transfection buffer A was added to Sf9 cell monolayer. Let all areas of the flask were covered with transfection buffer A. 2 ug of recombinant transfer vector pAcGHLT-B containing CrV1 cDNA and 0.5 ug of BD BaculoGold baculovirus DNA were mixed in a sterile 1.5 mL tube. After 5 min, 1 mL of transfection buffer B was added to the

mixture and mixed well. The mixture was added to Sf9 cells monolayer drop by drop, and after every three drops gently rocked the flask back to forth to mix well with the transfection buffer A. The flask was incubated at 27.5 ℃ for 4 h. After that, the transfection solution was removed from the flask and 5 mL of TNM-FH medium was added to the flask. After rocking the flask back and forth several times, the medium was discarded and another 5 mL of fresh TNM-FH medium was added. The co-transfection flask was incubated in a 27.5 ℃ incubator for at least 5 days. After incubation, cells and medium were collected to a sterile 15 mL centrifuge tube. After centrifugation at 800 rpm (Allegra™ 6R centrifuge) for 10 min at 4 ℃, the supernatant was transferred to another sterile 15 mL centrifuge tube covered with aluminum foil and saved in a 4 ℃ refrigerator for further amplification.

4.2.5 Virus amplification

Co-transfection supernatant contains viruses which are referred as P0 stock virus. In this study, three recombinant baculoviruses were amplified three times to get the high titer virus stock for further study (Figure 13).

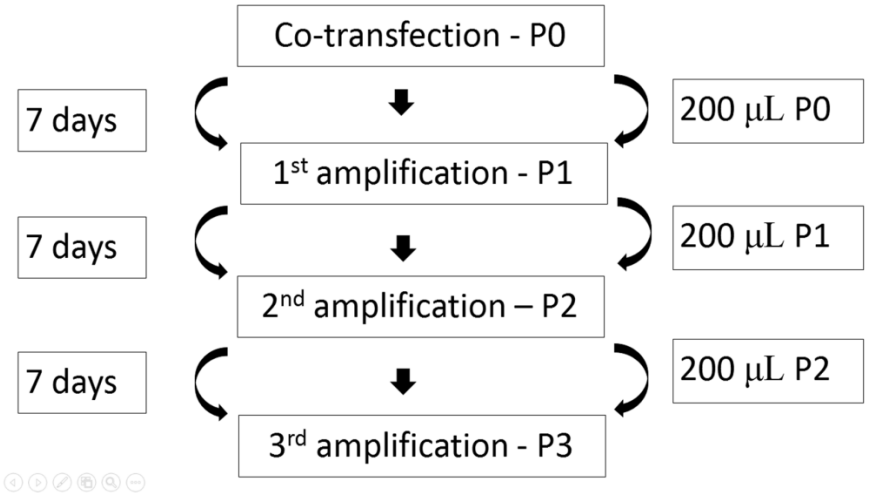

Figure 13. Illustration of virus amplification. The detail description is as follow.

For P1 amplification, 4×10^6 Sf9 cells were seeded in a 25 cm^2 flask containing 5 mL of TNM-FH medium. After Sf9 cells attached to the flask for 15 min, 200 μL of P0 virus stock was added to the medium and the flask was incubated at 27.5 ℃ for 7 days. The medium containing viruses was collected in a sterile 15 mL centrifuge tube and centrifuged at 800 rpm (Allegra™ 6R centrifuge) for 10 min at 4 ℃. The supernatant was transferred into another sterile 15 mL centrifuge tube (Corning®) which was covered with aluminum foil and stored in a 4 ℃ refrigerator.

For P2 virus stock amplification, 1.2×10^7 Sf9 cells were seeded in a 75 cm^2 flask with 15 mL TNM-FH medium. 200 μL of P1 virus stock was added into the medium. Incubated the flask at 27 ℃ for 7 days, collected medium, centrifuged at 800 rpm for 10 min at 4 ℃, and kept the P2 viral stock at 4 ℃.

For P3 virus stock amplification, 3×10^7 Sf9 cells were seeded into a 182 cm^2 flask containing 30 mL of TNM-FH medium. 200 μL of viruses from P2 virus stock was added into the medium. After 7 days incubation, medium containing viruses was collected, centrifuged and stored at 4 ℃ (Unger and Peleg 2012).

4.2.6 Determination of virus titer by End-point Dilution Assay (EPDA)

One 25 cm^2 flask of exponential growth phase Sf9 cells was collected and centrifuged. The supernatant was removed and 3 mL of fresh medium was added. After mixing well, concentration of cell suspension was determined by hemocytometer measurement. Cell suspension was diluted to 1×10^5 /mL. 100 μL of diluted cell suspension was dispensed to each well of a 96-well plate and attached for 1 h. During the cells were attaching, a serial of dilutions of one virus stock were prepared. 12 sterile 1.5 mL tubes were arrayed in a rack. 950 μL of

medium was added to each tube. 50 μL of the virus stock to be determined was added to the tube 1. After mixing well, 50 μL of mixture from tube 1 was transferred to tube 2. Repeated the last step for tubes 3 to 11 and left the medium in the last tube. 50 μL of tube 2 was added the each well of column 1 of the plate. 50 μL of tube 3 was dispensed to each well of column 2 of the plate. The same procedure was repeated for the left tubes. The last column of the plate was left as negative control. After gently mixing, the plate was incubated at 27.5 ℃. After 5 days incubation, the signs of infection of each well were scored. Wells which received at least one productive virus particle infection were recorded positive, whereas wells which received no productive virus particles infection were recorded negative. All the results were listed under each dilution. The tissue culture infective dose ($TCID_{50}$) is the dose which can infect the 50% of the wells. To calculate the $TCID_{50,}$ the proportional distance (PD) should first be calculated as follow:

$$DP = \frac{(\text{Positive percent above } 50\%) - 50\%}{(\text{Positive percent above } 50\%) - (\textit{Positive percent below } 50\%)}$$

The product of DP * logarithm of dilution factor was added to the logarithm of the lower dilution below 50% mortality, and the result was the logarithm of endpoint. The antilog of the logarithm of endpoint is the dilution capable of infect 50% of the wells (Reed and Muench 1938).

4.3 Transcription of CrV1 in the infected Sf9 cells

To confirm transcription of CrV1 gene in the infected Sf9 cells, reverse transcription polymerase chain reaction (RT-PCR) was performed.

4.3.1 Infection of Sf9 cells with recombinant baculoviruses

Sf9 cells (4×10^6) were seeded in a 25 cm^2 flask with 5 mL of medium. After attaching to the flask for 15 min, 30 μL of P2 virus stock was added to the flask. The flask was incubated at 27.5 ℃

for 3 days. After incubation, infected cells were collected in a 15 mL centrifuge tube (Corning®) and centrifuged at 800 rpm for 10 min (Allegra™ 6R centrifuge) at 4 ℃. Supernatant was discarded and 5 mL of 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) was added to the tube. After mixing well, cell suspension was divided into five 1.5 mL tubes. Tubes were centrifuged at 1500 rpm for 1 min (Corning® LSE™ High Speed Microcentrifuge). Supernatant was removed and pellets were store at -20 ℃ for the extraction of RNA.

4.3.2 RNA isolation from virus infected Sf9 cells

One milliliter of TRI reagent (Molecular Research Center) was added into the tube which contained pellet of virus infected Sf9 cell. Cells were lysed in TRI reagent by pipetting. After that, 100 μL of chloroform was supplemented into the tube. Shake the tube vigorously for 15 s and let it still at room temperature for 15 min. The tube was centrifuged at 12000 rpm for 15 min at 4 ℃ (Hettich Mikro 22R centrifuge). The upper aqueous was transferred into another 1.5 mL tube which contained 0.5 mL of isopropanol. After mixing well, the sample was stored at room temperature for 10 min and then centrifuged at 12000 rpm for 15 min at 4 ℃. Supernatant was discarded and RNA pellet was washed with 1 mL 75% ethanol which was prepared with DEPC water. The tube was centrifuged at 12000 rpm at 4 ℃ for 5 min and ethanol was removed. Let RNA pellet dry 5 min at room temperature and then added 50 μL of nuclease free water into the tube. RNA was dissolved by incubating the tube at 55 ℃ for 10 min. The quanlity and concentration of RNA were analyzed via agarose gel electrophoresis. RNA was stored at -20 ℃ for further reverse transcription.

4.3.3 RNA extraction from infected *Spodoptera exigua* larvae

Infected *Spodoptera exigua* larvae (80 mg) were added into each 1.5 mL tube. 1 mL of TRI reagent was added to the tube followed by homogenizing larvae with polypropylene pestles. Other steps were described as above.

4.3.4 Elimination DNA from RNA sample

Deoxyribonuclease I (DNase I) (Invitrogen) was used to digest DNA from RNA sample prior to RT-PCR. Reaction volume was 10 μL, including 4 μL (1 ug) RNA sample, 1 μL 10× DNase I reaction buffer, 1 μL DNase I (1 u/μL), 4 μL nuclease free water (Promega). After mixing well, the reaction tube was incubated for 15 min at room temperature. DNase I was inactivated by adding 1 μL of 25 mM EDTA solution to the reaction mixture and heated for 10 min at 65 ℃. Treated RNA sample was ready for reverse transcription.

4.3.5 Reverse transcription for infected Sf9 cells

Reverse transcription system from Promega was used in the reaction. The volume of the reaction was 20 μL, containing 0.5 μL recombinant RNasin® ribonuclease inhibitor, 4 μL $MgCl_2$ (25 mM), 2 μL 10× reverse transcription buffer, 2 μL dNTP mixture (10 mM), 1 μL random primers (0.5 ug/μL), 9.5 μL RNA sample (1 ug), 1 μL AMV reverse transcriptase (15 u/μL). After mixing, the reaction was incubated at room temperature for 10 min then incubated at 42 ℃ for 15 min. After sample was heated at 95 ℃ for 5 min then incubated on ice for 5 min. CDNA was ready for PCR amplification and stored at -20 ℃.

4.3.6 Reverse transcription for infected *Spodoptera exigua* larvae

For CrV1 gene transcription in *Spodoptera exigua* larvae, M-MLV reverse transcriptase (promega) was used. The reaction volume was 25 μL, including 5 μL 5× reaction buffer, 1 μL RNasin® ribonuclease inhibitor, 2 μL dNTP mixture (10 mM), 1 μL random primers (0.5 ug/μL), 1 μL M-MLV reverse transcriptase (200 u/μL), 4 μL RNA (2 ug), 11 μL nuclease free water. After mixing by flicking the tube, they were incubated at 37 ℃ for 1 hour. CDNA was ready for PCR amplification and stored at -20 ℃.

4.3.7 PCR amplification

CDNA was diluted to 100 μL with nuclease free water. 5 μL of diluted cDNA was used as template. The PCR reaction volume was 25 μL, containing 5 μL cDNA, 0.5 μL dNTP mixture (10 mM), $MgCl_2$ 1.9 μL (25 mM), 2.5 μL 10× reverse transcription buffer, 0.5 μL forward primer (25 mM), 0.5 μL reverse primer (25 mM), 0.2 μL Taq DNA polymerase, 13.9 μL nuclease free water. PCR was carried out with denaturation at 95 ℃ for 5 min followed by 30 cycles (95 ℃ for 30 s, 62 ℃ for 30 s, 72 ℃ for 1 min 20 s) and 72 ℃ for 10 min. PCR results were analyzed by agarose gel electrophoresis.

4.3.8 DNA extraction from pAcUW21-CrV1 polyhedra

For DNA extraction, 200 μL of 0.5 M Na_2CO_3 was added to 300 μL of purified pAcUW21-CrV1 polyhedra suspension at 5×10^8 polyhedra/ml. After incubating at 60 ℃ for 10 min, the tubes were centrifuged at 3800×g for 5 min to remove undissolved polyhedra. The supernatant was treated with 15 μL of proteinase K (50 mg/ml) at 50 ℃ for 15 min. Viral DNA was purified with chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm (Corning® LSE™ High Speed Microcentrifuge) for 15 min. 0.5 mL isopropanol was added to the supernatant in an new 1.5 mL

tube. After mixing well, the tube was put in the room temperature for 10 min followed by spinning at 13000 rpm for 15 min. Supernatant was discarded and pellet was washed with 600 μL of 75% ethanol. Pellet was dissolved in 50 μL TE buffer and incubated at 60 ℃ for 10 min. The quanlity of virus DNA was analyzed by gel electrophoresis. The left DNA was stored at -20 ℃ for further use.

4.4 Expression of CrV1 protein in the infected Sf9 cells

In this study, the expression of CrV1 protein from pAcGHLT-B-CrV1 recombinant baculovirus was detected.

4.4.1 Infection of Sf9 cells with different MOI

Sf9 cells (4×10^6) were seeded into each 25 cm^2 flask. 5 flasks were prepared. The titer of pAcGHLT-B-CrV1 recombinant baculovirus was determined by EPDA. Five different MOIs (1, 2, 5, 10, 15, 20) were tested. The corresponding dose of viruses of five different MOIs was inoculated into 5 flasks respectively. The flasks were incubated at 27.5 ℃ for 3 days. Floating and adherent cells of 5 flasks were collected into 15 mL centrifuge tubes (Corning®) respectively. The tubes were Centrifuged (Allegra™ 6R centrifuge) at 800 rpm for 10 min at 4 ℃. The supernatant was removed and 5 mL of cold 1× PBS buffer was added to each tube to wash the cells. After washing two times, 500 μL of PBS buffer containing protease inhibitor cocktail (Sigma) was added to each pellet tube and mixed well. Cells were lysed by twice freeze and thaw cycle at -80 ℃. After centrifuging the tubes at 12000 rpm at 4 ℃ for 20 min, the supernatant from each tube was transferred into a new 1.5 mL tube respectively. The supernatant was scored at -20 ℃ for further analysis by SDS-PAGE.

4.4.2 SDS-PAGE

CrV1 protein expression was detected by SDS-PAGE. This gel includes two parts, separating gel and stacking gel. The SDS-PAGE electrophoresis apparatuses were ordered from Bio-Rad. Before gel preparation, the two clean and dry glasses were locked to the casting stand. 10% gel was prepared for analysis of CrV1 protein expression. For one mini SDS-PAGE gel, 5 mL of mixture was prepared, including 1.3 mL Tris-HCl (1.5 M, pH 8.9), 1.275 mL 40% acrylamide/bisacrylamide, 50 μL 10% SDS, 50 μL 10% ammonium persulfate, 2 μL TEMED, 2.325 mL Milli-Q water. The TEMED and 10% ammonium persulfate were added at last. After mixing, the mixture was poured into the glasses prepared at beginning until reached two-thirds of the short glass. 400 μL of isopropanol was added to the top of the separating gel. After 30 min polymerization, isopropanol was poured off and the top of gel was washed with Milli-Q water. For stacking gel, 3 mL of mixture was required for one mini gel, containing, 0.38 mL Tris-HCl (1 M, pH 6.9), 0.375 mL 40% acrylamide/bisacrylamide , 30 μL 10% SDS , 30 μL 10% ammonium persulfate, 3 μL TEMED, 2.225 mL Milli-Q water. The TEMED and 10% ammonium persulfate were added at last. After mixing, the solution was poured to the top of separating gel and then one comb was inserted into the layer of stacking gel solution. While the stacking gel polymerized, samples to be analyzed were prepared.

Each protein sample was diluted 4:1 (v/v) with 5×SDS PAGE loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 500 mM DTT) and heated for 8 min at 98 ℃ in a 1.5 mL tube respectively. After centrifuging at 12000 rpm for 5 min (Corning® LSE™ High Speed Microcentrifuge), samples were ready to be loaded.

Comb was carefully removed from the gel and gel was assembled into one buffer tank which was filled with 1×SDS electrophoresis buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

Protein samples were loaded into wells. Stacking gel was run at 80 volts while separating gel was run at 120 volts (Gallagher 2006).

4.4.3 Staining SDS-PAGE gel by blue silver

Blue silver is a sensitive colloidal Coomassie G-250 staining method. Dye solution was prepared as follow. For 1 L of staining solution, 100 mL of phosphoric acid was added to 100 mL of Milli-Q water and mixed. 100 g of ammonium sulfate was dissolved in the solution and then 1.2 g of Coomassie blue G-250 was added to the mixture. When all the solids had dissolved, Milli-Q water was added to 80% of the final volume. At last, 200 mL of methanol was added to the solution. The dye was stored at room temperature.

After the bromphenol blue reached the bottom of separating gel, gel was removed from the glasses and placed in fixing solution (60% methanol, 10% acetic acid) for 30 min. Gel was washed twice with Milli-Q water for 15 min at room temperature and then placed into staining dye overnight. Next day, gel was rinsed with Milli-Q water and stored at 4 ℃ for further use (Candiano, Bruschi et al. 2004).

4.5 Purification of CrV1 protein

4.5.1 Infection of Sf9 cells with pAcGHLT-B-CrV1 recombinant baculovirus

For expression of CrV1 protein, 2×10^7 cells were seeded into each 75 cm^2 flask. 4 flasks of cells were seeded. Let cells attached for 15 min. The optimal dose of viruses was inoculated into each flask. All the flasks were incubated at 27.5 ℃ for 3 days. After incubation, cells were collected into a 50 mL centrifuge tube (Corning®) and centrifuged at 1000 rpm for 15 min at 4 ℃

(Allegra™ 6R centrifuge). Cell pellets were washed twice with cold 1×PBS buffer and stored at -20 ℃ for further purification.

4.5.2 Lysis of infected Sf9 cells

Sf9 cell pellets were resuspended in 4 mL of cold lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.1% IGEPAL® CA-630, protease inhibitor cocktail, pH 7.5). Froze the pellet at -80 ℃ for 20 min and thawed at room temperature. The freeze and thaw cycle was repeated twice and the lysis was incubated on ice for 30 min. After centrifuging at 12000 rpm at 4 ℃ for 20 min (Hettich Mikro 22R centrifuge), the supernatant was transferred into a pre-chilled tube.

4.5.3 Purification of recombinant GST-fusion CrV1 protein

To purify recombinant protein, 100 µL of glutathione sepharose 4B beads (GE Healthcare Life Sciences) which was completely resuspended by gently shaking the bottle was transferred into a column. The glutathione sepharose 4B beads were washed with 10× bed volumes of washing buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.1% IGEPAL® CA-630, 5% glycerol, pH 7.5). After washing, cell lysate was applied to the equilibrated column and the column was incubated for 2 h on ice with end-over end mixing. After binding step was complete, the column was washed with 20× bed volumes of washing buffer. The fusion protein was eluted with 5 mL of elution buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 5% glycerol, 10 mM reduced glutathione, pH 8.0). The eluted fusion protein was concentrated with an Amicon® Ultra filter device (Millipore) and analyzed by SDS-PAGE. Left purified protein was scored at -20 ℃ for further use.

4.5.4 Ammonium sulfate precipitation

To optimize the purification condition, ammonium sulfate precipitation was utilized. 100 mL of 100% ammonium sulfate saturated solution was prepared. Saturated solution was stored at 4 ℃ overnight prior to use. A precipitation test was performed to determine the optimal ammonium sulfate precipitation condition for the purification of the recombinant protein, GST-CrV1. Six 1.5 mL tubes were arrayed in a rack and each tube contained 500 μL of lysate from 1.2×10^7 cells of pAcGHLT-B-CrV1 baculovirus infected Sf9 cell. The calculated amount of 100% ammonium sulfate solution was added to each tubes to give 20%, 30%, 40%, 50%, 60%, 70% saturation, respectively. After mixing, tubes were incubated on ice for 1 h and centrifuged at 12000 rpm for 20 min at 4 ℃ (Hettich Mikro 22R centrifuge). The supernatant of each tube was transferred into another tube and each pellet was dissolved in 100 μL of lysis buffer for analyzing on SDS-PAGE gel later. The calculated amount of 100% ammonium sulfate solution was added to each supernatant to give the 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70% ammonium sulfate cuts (the proteins that are just soluble at 20% saturation but precipitate at 30% saturation are referred as the 20%-30% ammonium sulfate cut) respectively and mixed well. After incubating the tubes for 1 h on ice, tubes were centrifuged at 12000 rpm for 20 min at 4 ℃. The supernatant of each tube was removed and each pellet was dissolved in 100 μL of lysis buffer for analyzing on SDS-PAGE.

The equation for calculation of the volume (A) of saturated ammonium sulfate solution to be added to 500 μL of cell lysate to increase saturation from S_1 to S_2 is as follow (Burgess and Deutscher 2009).

$$500 \times S_1 + A \times 1.0 = (500+A) \times S_2$$

$A\ (\mu L) = (500 \times (S_2 - S_1)) / (1 - S_2)$

4.6 GST pull-down assay

In this study, pull-down assay was performed to identify protein-protein interactions between CrV1 protein and proteins of *Pieris rapae* and *Spodoptera exigua* tissue lysate.

4.6.1 Preparation of insect lysates

One gram of insect larvae was homogenized with a polypropylene pestle in a centrifuge tube containing 5 mL lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 5% glycerol, 0.1% IGEPAL® CA-630, pH 7.5). The insect lysate was incubated on ice and placed on a shaker (Lab-Line 3520 Adjustable Speed Orbital Shaker) with 200 rpm for 30 min. After the lysate was centrifuged at 16000 rpm for 30 min at 4 ℃ (Hettich Mikro 22R centrifuge), the supernatant was transferred to a clean cold tube for further pre-clearing step.

Pre-clearing was used to remove the endogenous GSTs from the insect lysate. 50 μL of glutathione sepharose beads was added to insect lysate and incubated on ice for 30 min with 200 rpm shaking. After centrifugation at 800 rpm (Allegra™ 6R centrifuge) for 3 min at 4 ℃, the supernatant was transferred to a new tube and repeated this pre-clearing one time more.

4.6.2 GST pull-down

Pre-cleared insect lysate was added to one tube which contained 100 μL glutathione sepharose beads with 5 ug of GST-CrV1 protein. The tube was incubated on ice for 2 h with end-over-end mixing and then centrifuged at 2000 rpm for 1 min at 4 ℃ (Hettich Mikro 22R centrifuge). The supernatant was removed and the beads were washed four times with ice-cold washing buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 5% glycerol, 0.5% IGEPAL® CA-

630, pH 7.5). Centrifuged the tube and discarded the supernatant. The protein interaction complex was eluted by boiling the beads with 100 μL of 1×SDS loading buffer and heated at 98 ℃ for 5 min. The tube was centrifuged and the supernatant was analyzed by SDS-PAGE.

One GST tag protein was used as one control. GST tag protein was purified from pAcGHLT-B recombinant baculovirus. The infection, expression, purification and pull-down procedures of GST tag protein were the same as GST fusion CrV1 protein (Brymora, Valova et al. 2004).

4.6.3 Western blot

GST antibody (GenScript) was used to confirm the expression of GST fusion CrV1 protein. The procedure was described as follow. The samples needed to be identified were separated on a SDS-PAGE gel as described above. Stacking gel was cut off and separating gel was placed in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol). PVDF membrane was soaked in methanol for 30 s and then put in transfer buffer. Other material, including two fiber pads and two Whatman papers, were also placed in transfer buffer. After 15 min, transfer sandwich was prepared on the black panel of transfer apparatus gel cassettes in a tray filled with transfer buffer. The order was that, one fiber pad, one Whatman paper, SDS-PAGE gel, PVDF membrane, one Whatman paper, one fiber pad. The sandwich was covered with clear panel and gel cassette was inserted into one electrode module with black panel facing to black cathode electrode panel. Buffer chamber was filled with transfer buffer and incubated on ice. The process of transfer lasted 1.5 h at 100 volts.

After transfer, the membrane was incubated with blocking buffer (3% BSA, dissolved in TBS) on a shaker for 2 h at room temperature. After blocking, the membrane was washed twice with TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and incubated with the primary antibody, GST

antibody, which was diluted with antibody dilution buffer (3% BSA, dissolved in TBST) (1:5000), at 4 ℃ overnight. Next day, the primary antibody solution was collected and saved at 4℃, which can be reused several times. The membrane was washed six times with TBST (0.05% Tween 20 in TBS) on a shaker (Lab-Line 3520 Adjustable Speed Orbital Shaker) for 6 min each time. After washing, the membrane was incubated with the second antibody, HRP conjugated IgG antibody, which was diluted with antibody dilution buffer (1:10000), on a shaker for 1.5 h at room temperature. The membrane was washed six times with TBST on a shaker for 6 min each time. The membrane was stained with DAB solution (30 mg DAB, 60 μL 30% H_2O_2, 50 mL TBS).

4.7 Bioassy

4.7.1 Insects rearing

Spodoptera exigua larvae were reared in 30 mL plastic cups with 5 mL of an artificial diet capped with cardboard. Larvae were incubated under controlled conditions 26 ± 1 ℃ with a photoperiod of 14/10 h light/dark and 65 ±5% RH until pupation. Pupae were placed in 4 L plastic containers used as emergency chambers. Chambers were coated inside with paper towels and covered with a piece of cheese cloth hold with a rubber band until adult emergency. After oviposition, eggs were collected during 5 days, and second instar larvae were used for all tests. The emerged caterpillars were reared on a soy flour-based artificial diet (Mihm 1984) and growth in cups at 26 ℃ under 10L:14D photoperiod, and 70% ±1% humidity. Adults were fed on 10% sugar water solution using similar insectary conditions. *Pieris rapae* were bought from Carolina Company. The larvae were reared on a wheat germ-based artificial diet (Bio-Serv) and

growth in cups at 24 °C under 16L: 8D photoperiod. The butterflies were fed on 10% sugar water solution using similar insectary conditions.

4.7.2 Virus harvesting

Recombinant baculoviruses (pAcUW21-CrV1) and AcMNPV wild-type viruses were produced using Sf9 cells. The infected Sf9 cells were collected 7 days post-infection and centrifuged at 800 rpm for 10 min at 4°C (Allegra™ 6R centrifuge). The supernatant was drained and lysis buffer (50 mM Tris-HCL pH 8.0, 0.4% SDS, 10 mM EDTA pH 8.0) was added to the pellets to release polyhedra. Polyhedra were washed with Milli-Q water and counted using a hemocytometer.

4.7.3 Bioassays

The diet for bioassay was prepared and divided 5 mL in each 1-oz plastic cup. Recombinant virus pAcUW21-CrV1 and AcMNPV wild-type polyhedra were diluted in serial concentrations. For *S. exigua*, nine polyhedra concentrations were prepared (12, 59, 588, 5882, 2.5×10^4, 5.9×10^4, 1.2×10^5, 3.5×10^5, and 7.1×10^5 polyhedra/cm^2). For *Pieris rapae*, six polyhedra concentrations were prepared (5882, 2.5×10^4, 5.9×10^4, 1.2×10^5, 3.5×10^5, and 7.1×10^5 polyhedra/cm^2). Polyhedra dilutions were spread over the entire surface of the diet and allowed to air dry. For each treatment, 30 larvae were used and the experiment replicated twice. Dead larvae were counted and recorded every day until 10 days.

4.7.4 Data analysis

LC_{50} and LT_{50} were calculated using the POLO-PC software (Finney 1971, Russell, Robertson et al. 1977, Software 1987). LT_{50} was also calculated using the same software and the

concentration of 5882 polyhedra for *Spodoptera exigua* and of 3.5×10^5 polyhedra for *Pieris rapae*. For each pest, LC_{50} and LT_{50} values between the recombinant virus pAcUW21-CrV1 and the wild-type AcMNPV were examined for significant differences at $\alpha = 0.05$ indicated by no overlapping of the 95%-confidence intervals.

5 Results

5.1 Construction of recombinant transfer vectors containing CrV1 gene

5.1.1 Construction of recombinant transfer vector-pAcGHLT-B with gene CrV1 (pAcGHLT-B-CrV1)

The coding sequence of CrV1 was amplified from a cDNA clone (pBluescript SK) with specific primers which contained *Xho* I and *Kpn* I respectively (Figure 14). Purified CrV1 fragments and pAcGHLT-B transfer vector were digested with *Xho* I and *Kpn* I respectively. After ligation and transformation, recombinant plasmids were confirmed by restriction endonucleases digestion and PCR reaction. The results were analyzed by agarose gel electrophoresis (Figure 15).

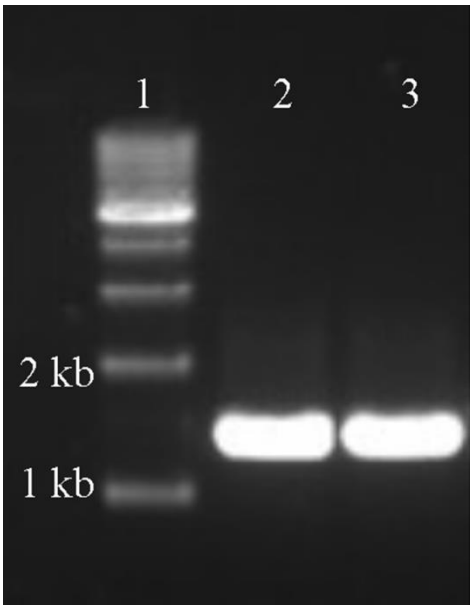

Figure 14. CrV1 PCR products amplified from pBluescript SK vector. Lane 1: 1 kb DNA marker (Promega); lane 2 and 3: CrV1 PCR results.

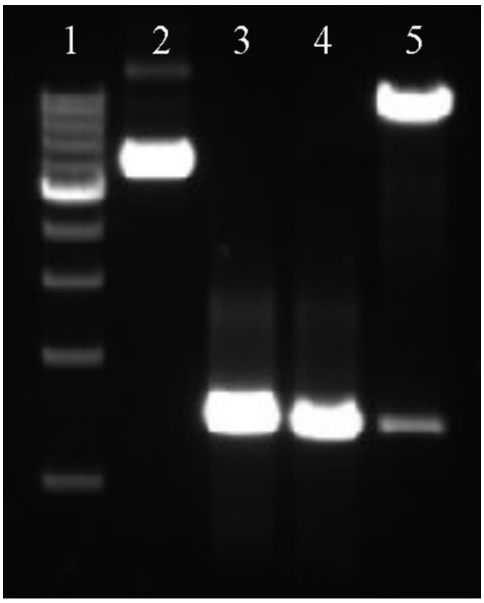

Figure 15. Recombinant transfer vector pAcGHLT-B-CrV1. Lane 1: 1 kb DNA Marker (Promega); lane 2: Recombinant plasmid; lane 3: CrV1 gene (positive control); lane 4: PCR results amplified from recombinant construct; lane 5: Recombinant vector digested by restriction endonuclease *Xho* I and *Kpn* I

The sequencing result was examined by comparison to the GenBank by using BLAST (NCBI) and 100% of identity with *Cotesia rubecula* virus CrV1 protein mRNA was obtained (Figure 16). In addition, the sequencing results also showed that the CrV1 was inserted in frame with the GST ORF under polyhedrin promoter. Hence CrV1 protein was fused with GST and 6×His tags. Therefore, recombinant transfer vector pAcGHLT-B-CrV1 which was used for expression and purification was successfully constructed.

Figure 16. Sequencing analysis of recombinant transfer vector pAcGHLT-B-CrV1. A: Multiple cloning regions of pAcGHLT-B vector; B: Sequencing results of recombinant construct pAcGHLT-B-CrV1. The green, brown and blue color sequences correspond to *Xho* I, *Kpn* I restriction sites and thrombin cut site respectively. The red and black color sequences correspond to CrV1 and vector sequences respectively.

5.1.2 Construction of recombinant transfer vector-pAcUW21 with gene CrV1 (pAcUW21-CrV1)

The coding sequence of CrV1 was amplified from a cDNA clone (pBluescript SK) with specific primers which contained *Bgl* II respectively. Purified CrV1 fragments and pAcUW21 transfer vector were digested with *Bgl* II respectively. After ligation and transformation, recombinant plasmids were confirmed by restriction endonucleases digestion and PCR reaction. The results were analyzed by agarose gel electrophoresis (Figure 17).

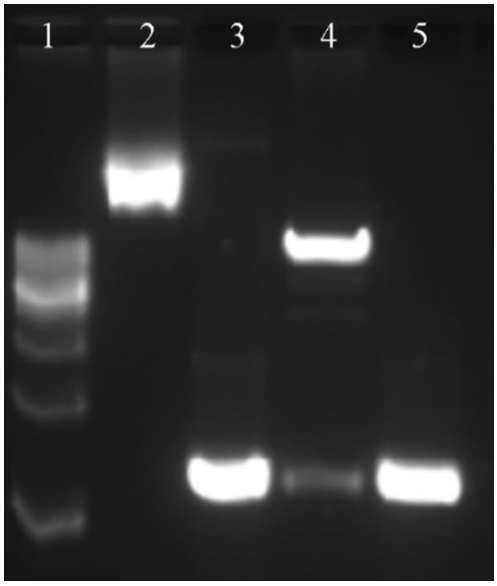

Figure 17. Recombinant transfer vector pAcUW21-CrV1. Lane 1: 1 kb DNA Marker (Promega); lane 2: Recombinant plasmid; lane 3: PCR results amplified from pAcUW21-CrV1; lane 4: Recombinant vector digested by restriction endonuclease *Bgl* II; lane 5: CrV1 gene (positive control)

The sequencing result was examined by comparison to the GenBank by using BLAST (NCBI) and 100% of identity with *Cotesia rubecula* virus CrV1 protein mRNA was obtained (Figure 18). In addition, sequencing results also showed that CrV1 was inserted into the *Bgl* II site in the

downstream of p10 promoter. From the sequencing result, recombinant transfer vector pAcUW21-CrV1 which was used to produce recombinant protein in the insect larvae was successfully constructed.

GTAAATTACATTTTATTTACAATCACAGATCTATGTCACTCGTCAAAAGTGCGTCTGTGCTGCTCTCAGC
TCTCCTGAGTCAATCATGTACGCAAGCTTATCCATCCGAATACCAATGGGAAAGAATTCAAAATATTCCA
TTTGCTGACGTTGCTTCCCTTAGTCCGCTGACCGATTATTTCTCTCCAAAATCAGATTCTACAGCCGAAA
GACTTGCGCCATCAACAAACGACTTCGATGAATCTGATATGAACAATAGAGAATTTTTGATGATTCCGAA
GTCTTATTCTGAGGCACCTCGGAAGCATTTTGATGTTGAATATTTTCTTAAGCATCACCCTCATATCAAG
CCTGGACACAGTACAAGACATAGACCTGCGTTCGAAATAAGACCTTTCGTAGGACCTATTAGTGAATTAT
CCAGTGGAAGTAGCGGTATCGGTCAACTTGGATCGAGCGTTGCAGATGAAATATTTTCCGGACTTAAAA
ATTTCATTGGAAATGTGAAATTTACTCAACCTGAATTATTTGACACATCCAAAACCCACCAGTATGAATAT
GAATCTGAAACATCTGAAAAACGCCACATTACCCGTGAAGATTTGCTTTCTGAACTACATGCAATAAAAG
AGGCACTTCAAAATCTAAAATCTGCTGTCATTCGGGTAGAAAATGAGATCAGCTTTAACAAAGAAGGAG
TTACTTACCCCTCTGTGGCAATCTCACCGCAACCTCTAGTTCTGATTAATCCTAACGGAGCATCTGAACA
GGGACCCATAGAAGCTATTACCGAAGAAGCAGTTACTCATCCCACTGTGACAAACTCATCGCAACCTCC
AGTTCTGATTAATCCTAACGGAGCATCTGAACAGGGACCCATAGAAGCTAATAACGAAGAAGGAGTTAC
TTATCCCAGTGATACAACCCCATCGCAAACTTTTTTTTGATCCCTATATGTAGCATCTGAACTTGAACTCA
GTAGAGTCTACTTAAGAAAGTATGTATAAAAATACTGCTACAAATGAAGCCATATGATAATCGACCCCTAG
AGAACACACTATCTAAAACTTATTTCCCATAATTTTTTTAGGAGAAAGAACAAGCGTTCTTAAAGTACTG
AACATATCGAATTTCTTGTCATCGTCAAATATGTATATAATAACTTAGAAAACCTTGCAATCTAAATGTTCT
AGCTTTAAGTAAGTTTTGATCGAAGAAAAACGCCAATTTTTATAGTGTAAACAGTTTTGTATTTTGATGA
ATAAATGAATATATTTTCCAATTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CGCTTGGACTTAA
CCAGGCTGAACTTGCAGATCTGAATTCCA

Figure 18. Sequencing analysis of recombinant transfer vector pAcUW21-CrV1

The green, red and black color sequences correspond to *Bgl* II restriction sites, CrV1 and vector sequences respectively.

5.2 Generation of recombinant baculoviruses by co-transfection

In this step, three co-transfection reactions (pAcGHLT-B-CrV1, pAcGHLT-B, pAcUW21-CrV1 vectors with BD BaculoGold DNA respectively) were performed and one positive control (co-transfection of BD BaculoGold DNA with pVL1392-XylE control vector DNA) and one negative control (Sf9 cells) were set. The results were as follows (Figure 19).

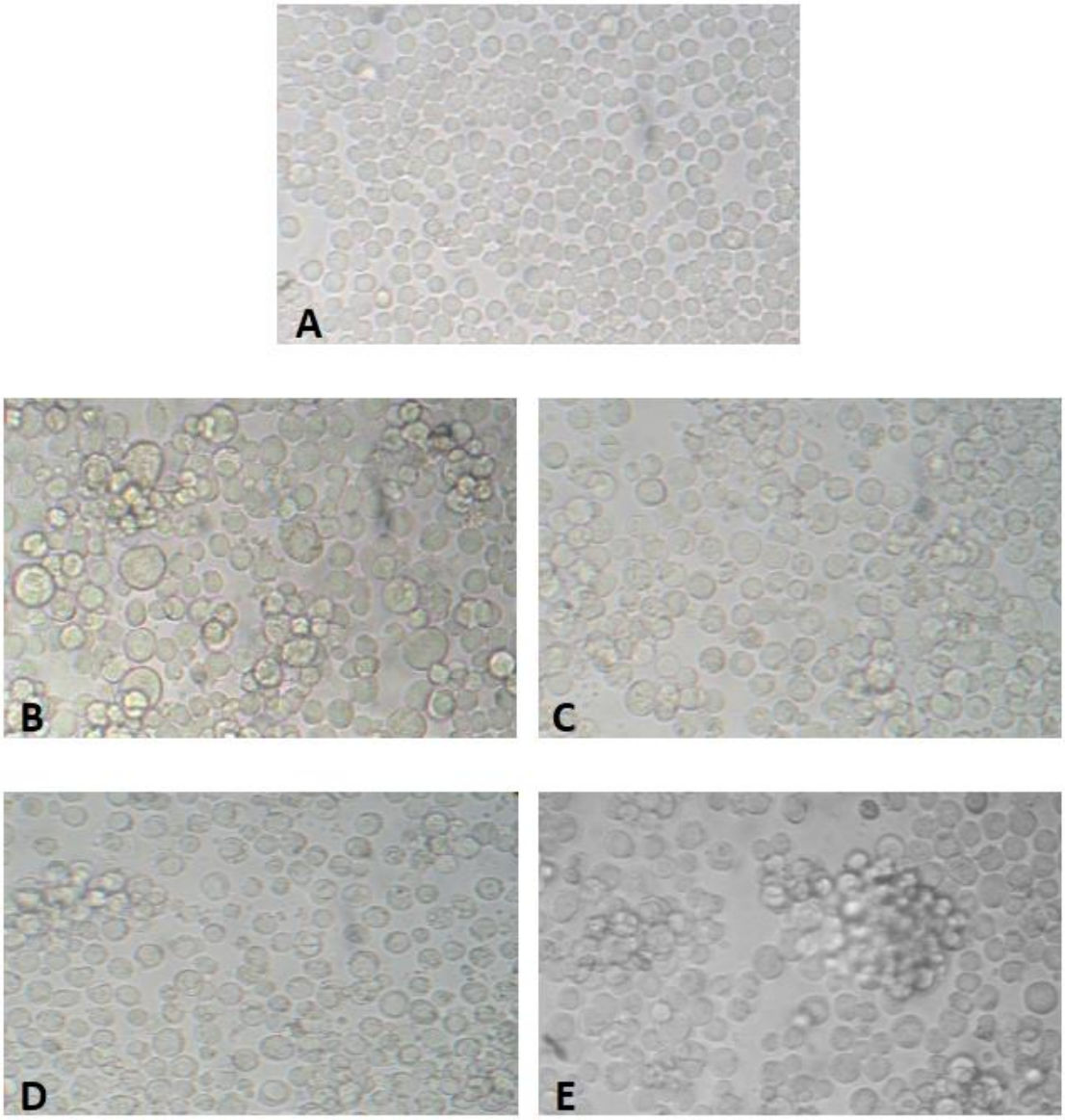

Figure 19. Co-transfection results. A: Negative control Sf9 cells; B: Positive control; C: pAcGHLT-B-CrV1 co-transfection; D: pAcUW21-CrV1 co-transfection; E: pAcGHLT-B transfer vector co-transfection

When Sf9 cells are infected, they will become bigger than normal cells, will have enlarged nuclei, will stop diving and will float in the medium. From the co-transfection results, those

morphological changes were obvious. Negative control continued to proliferate. Recombinant viruses were successfully generated and needed to be amplified.

5.3 Amplification of recombinant baculoviruses

Three recombinant baculoviruses (pAcGHLT-B-CrV1, pAcUW21-CrV1 and pAcGHLT-B baculoviruses) were amplified. The results were as follows (Figure 20).

After three rounds amplification, the titer of P3 virus stock was determined by EPDA (Figure 21). For pAcUW21-CrV1 baculovirus, occlusion bodies can be seen from P2 and P3 amplification.

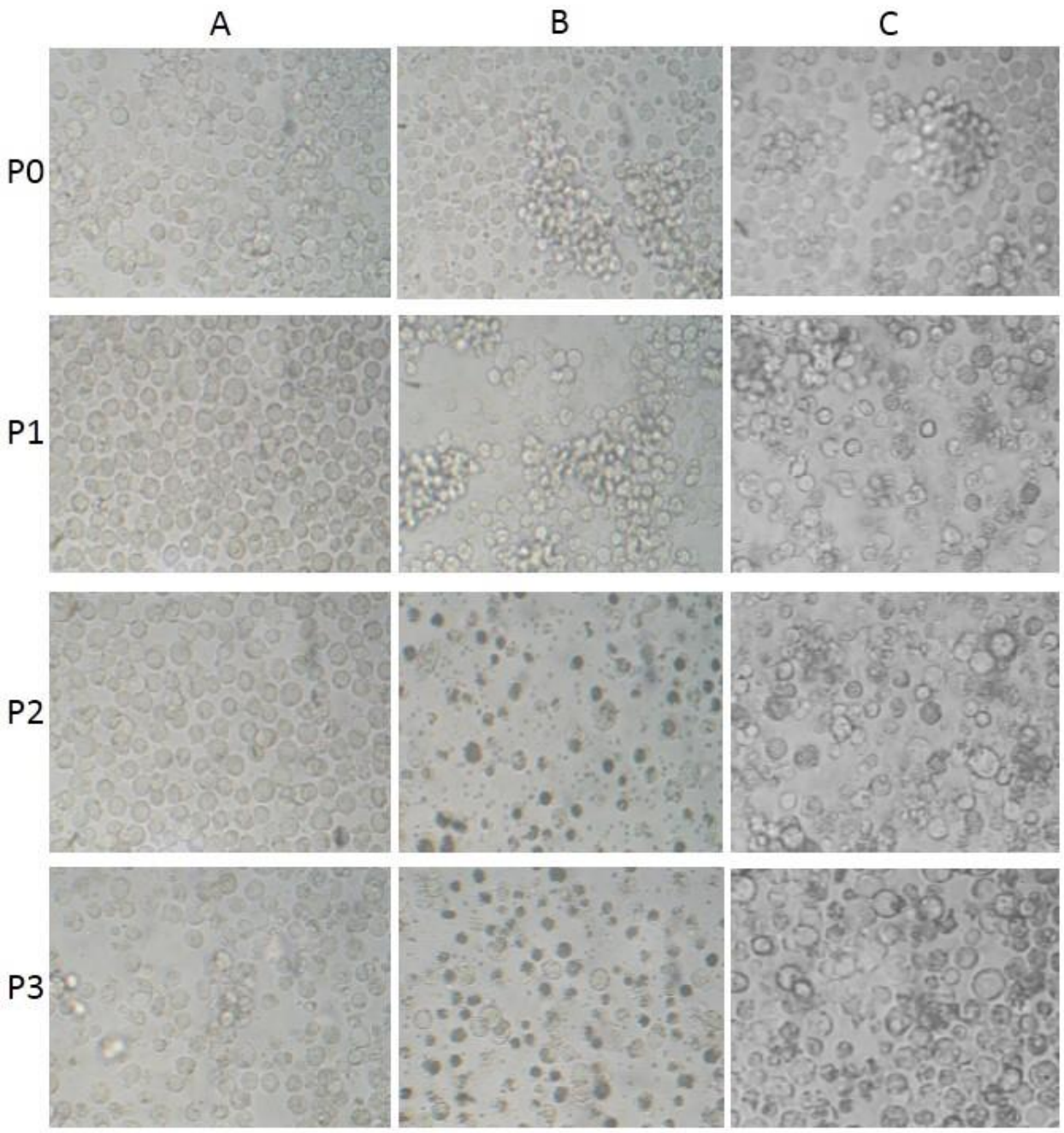

Figure 20. Amplification of recombinant baculoviruses. Three rounds amplification results of pAcGHLT-B-CrV1 baculovirus (A), pAcUW21-CrV1 baculovirus (B) and pAcGHLT-B baculovirus (C)

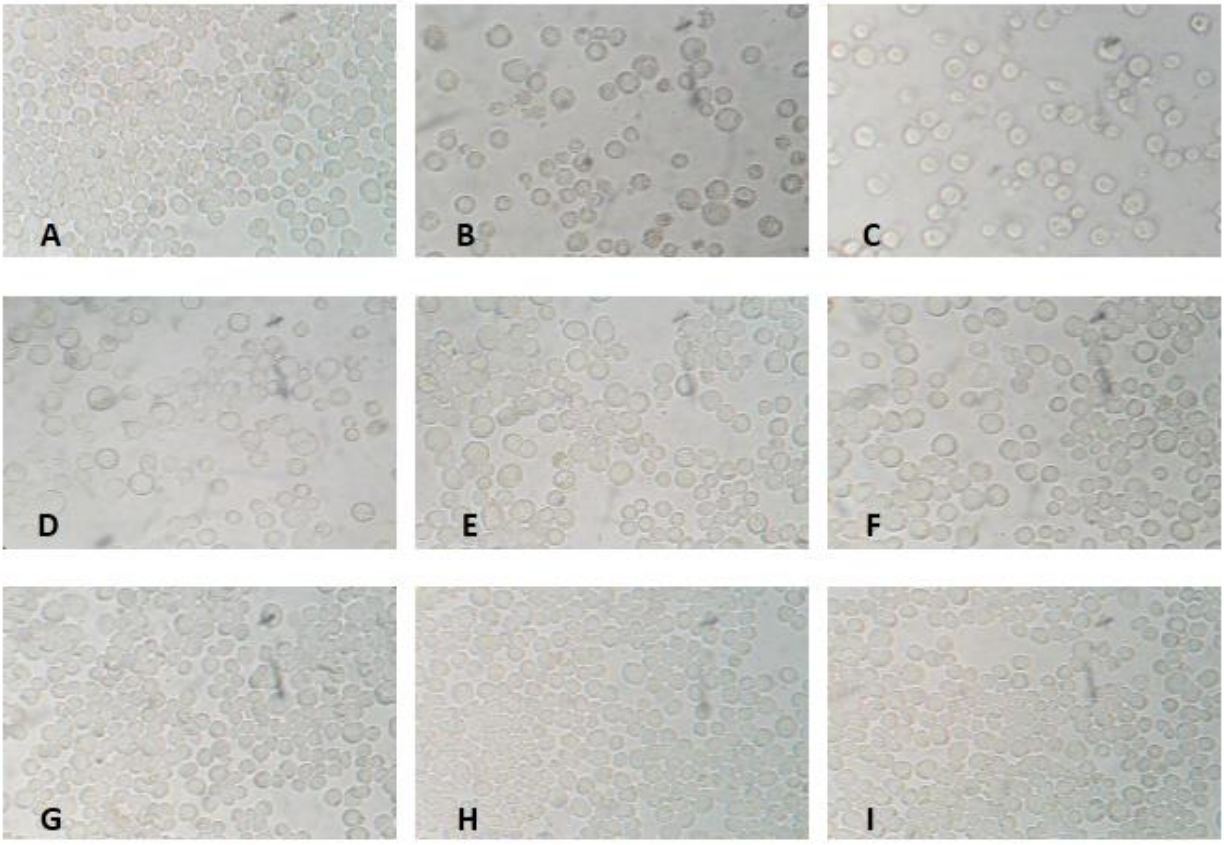

Figure 21. One example of virus titer determination. A: Negative control Sf9 cells; B to I: the first dilution to 8th dilution.

As dilution was increasing, the infectivity of virus was decreasing (Figure 21). The pAcGHLT-B-CrV1 baculovirus titer was 3×10^9 pfu/mL. The titer of pAcUW21-CrV1 baculovirus was 6×10^9 pfu/ mL.

5.4 Transcription of CrV1 in infected Sf9 cells

To verify the transcription of CrV1 in the recombinant baculoviruses infected Sf9 cells, RT-PCR was performed. RNA from healthy Sf9 cells and recombinant baculoviruses infected Sf9 cells were extracted using TRI reagent respectively (Figure 22). The quanlity and concentration of the

RNA were analyzed in an agarose gel. RT-PCR was performed with reverse transcription kit. The results were as follows (Figure 23, 24).

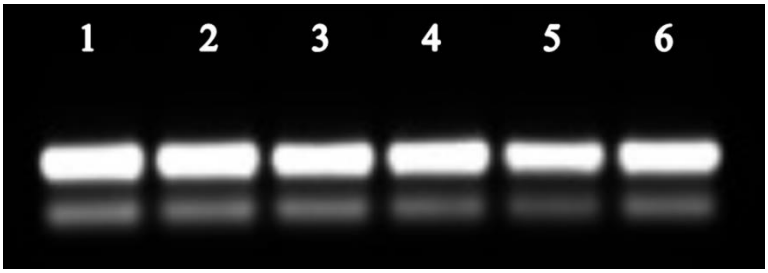

Figure 22. Extraction of RNA from infected Sf9 cells. Lane 1 and 2: RNA of healthy Sf9 cells; lane 3 and 4: RNA from pAcUW21-CrV1 baculovirus infected Sf9 cells; lane 5 and 6: RNA from pAcGHLT-B-CrV1 baculovirus infected Sf9 cells

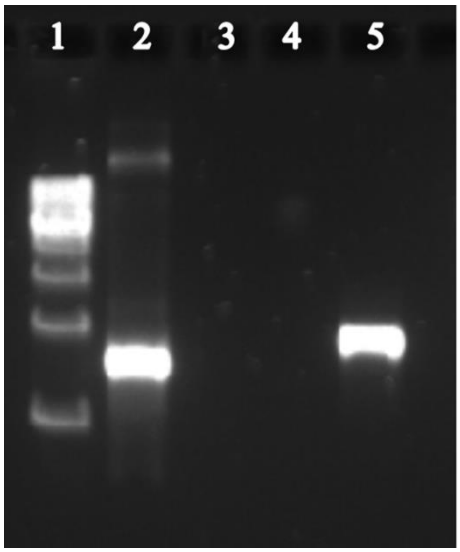

Figure 23. RT-PCR results of pAcGHLT-B-CrV1 baculovirus. Lane 1: 1 kb DNA marker; lane 2: CrV1 PCR result from pAcGHLT-B-CrV1 vector as positive control; lane 3: PCR result from the RNA of pAcGHLT-B-CrV1 infected Sf9 cells; lane 4: PCR result from the cDNA of healthy Sf9 cells; lane 5: PCR result from the cDNA of pAcGHLT-B-CrV1 baculovirus infected Sf9 cells

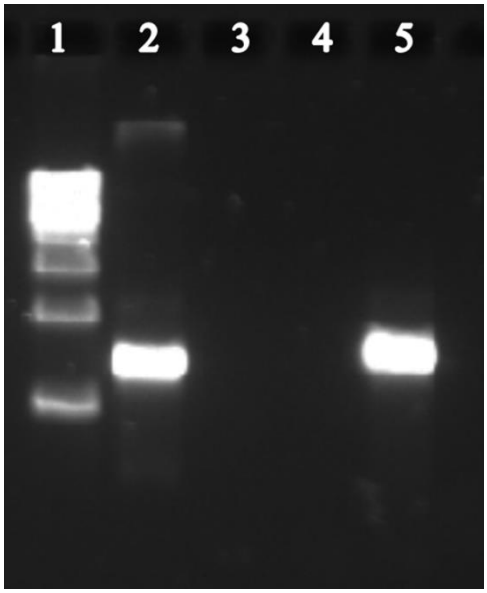

Figure 24. RT-PCR results of pAcUW21-CrV1 baculovirus. Lane 1: 1 kb DNA marker; lane 2: CrV1 PCR result from pAcGHLT-B-CrV1 vector as positive control; lane 3: PCR result from the RNA of pAcUW21 -CrV1 infected Sf9 cells; lane 4: PCR result from the cDNA of healthy Sf9 cells; lane 5: PCR result from the cDNA of pAcUW21 -CrV1 baculovirus infected Sf9 cells

In RT-PCR step, CrV1 fragment amplified from original plasmid was set as positive control. Two negative controls were set. One is PCR results from DNAase I treated RNA of each baculovirus infected Sf9 cells, which is used as confirmation of DNA elimination in RNA samples. Another is PCR results from the cDNA of healthy Sf9 cells. RT-PCR results showed that CrV1 mRNA was only detected in pAcGHLT-B-CrV1 and pAcUW21-CrV1 baculoviruses infected Sf9 cell (Figure 23, 24). These two recombinant baculoviruses can successfully transcribe CrV1 gene in infected Sf9 cells.

5.5 Transcription of CrV1 in infected *Spodoptera exigua* larvae

To verify transcription of CrV1 in the *Spodoptera exigua* larvae, RT-PCR was carried out. RNA was isolated from wild-type or recombinant baculoviruses infected larvae (Figure 25). In RT-

PCR results, CrV1 gene was only detected in pAcUW21-CrV1 baculoviruses infected *Spodoptera exigua* larvae, which indicated that CrV1 gene can be successfully transcribed in the infected *Spodoptera exigua* larvae (Figure 26).

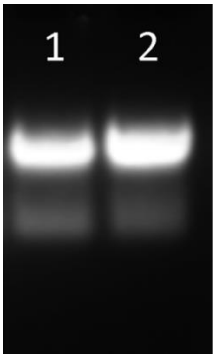

Figure 25. Extraction of RNA from infected *Spodoptera exigua* larvae. Lane 1: RNA from wild-type virus infected *Spodoptera exigua* larvae; lane 2: RNA from pAcUW21-CrV1 recombinant virus infected *Spodoptera exigua* larvae.

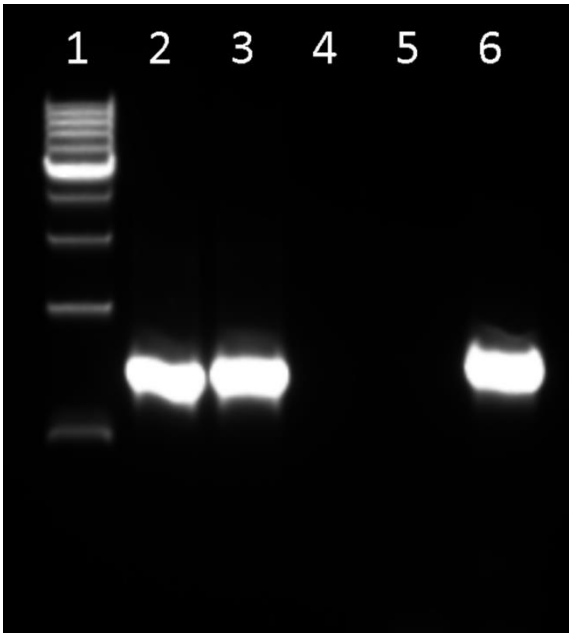

Figure 26. RT-PCR analysis for confirming the transcription of CrV1 gene of infected *Spodoptera exigua* larvae. Lane 1): Weight marker (Promega 1 kb DNA ladder G694A); 2)

CrV1 gene from recombinant pAcUW21 transfer vector, used as positive control; 3) AcMNPV-CrV1 polyhedra DNA; 4) cDNA from wild-type baculovirus infected *S. exigua* larvae; 5) RNA from recombinant virus infected *S. exigua* larvae treated by DNAse I; 6) cDNA from AcMNPV-CrV1 recombinant baculovirus infected *S. exigua* larvae.

5.6 Expression of CrV1 protein in infected Sf9 cells

Expression of CrV1 protein from pAcGHLT-B-CrV1 baculovirus infected Sf9 cell was investigated. In order to optimize the expression conditions of CrV1 protein, a range of MOI infection was performed. GST tag is a 26 kDa protein and the predicted molecular weight of CrV1 protein is 46 kDa. Therefore, the predicted size of GST-CrV1 fusion protein is 72 kDa. In Figure 27, compared with healthy Sf9 cells, one expressed protein band migrated between 66 kDa and 97 kDa was detected. Along with the growth of MOI infection, the protein expression was also increasing. When the range of MOI, from 1 to 10, was used to infect Sf9 cells, the expression level of fusion protein was dramatically increasing, while MOI was 10 or more, no significant difference was observed in the fusion protein expression level. Therefore, MOI 10 was selected for GST-CrV1 fusion protein expression.

To test the solubility of the expressed GST-CrV1 fusion protein, freeze-thaw cycle was utilized as the cell lysis method and PBS buffer which contained protease inhibitor was used as lysis buffer. The samples were analyzed by SDS-PAGE. In Figure 28, recombinant protein was detected, near 66 kDa. Hence recombinant protein was soluble in lysis buffer.

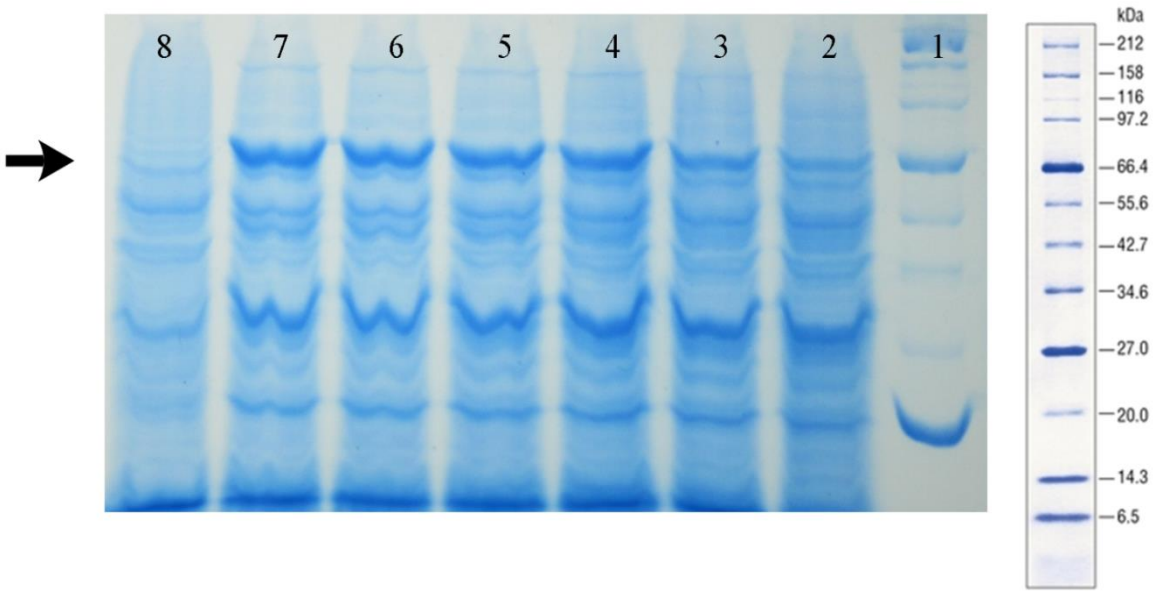

Figure 27. Whole cellular protein of a range of MOI infected Sf9 cells. Lane 1: Protein marker (Biolab); lane 2-7: Whole cellular protein of a range of MOI infected cells (MOI= 1, 2, 5, 10, 15, 20); lane 8: Cellular protein of healthy Sf9 cells

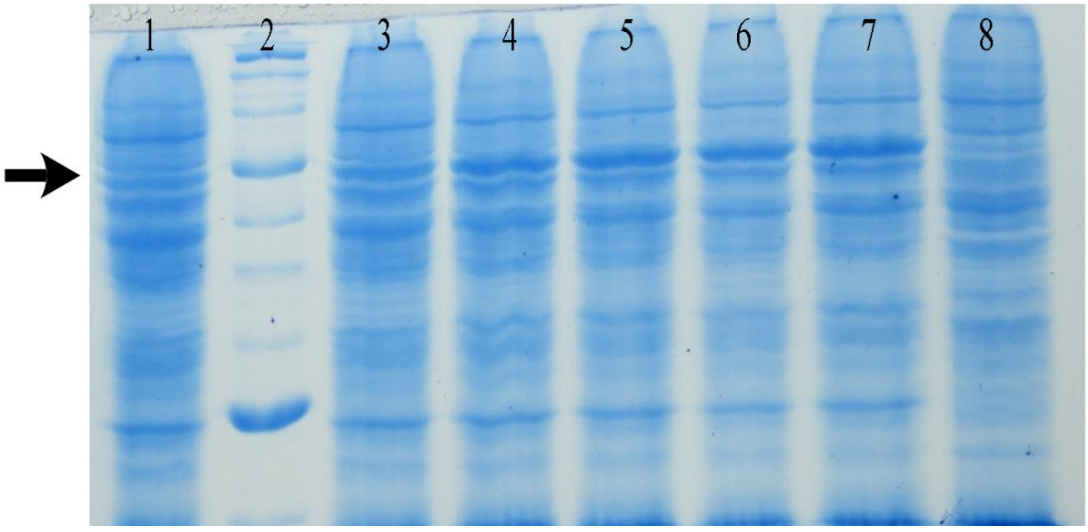

Figure 28. Soluble cellular protein of a range of MOI infected Sf9 cells. Lane 2: Protein marker (Biolab); lane 1, 3-7: Soluble cellular protein of a range of MOI infected cells (MOI= 1, 2, 5, 10, 15, 20); lane 8: Soluble protein of healthy Sf9 cells

5.7 Purification of recombinant GST-CrV1

Recombinant GST-CrV1 protein was purified with glutathione sepharose 4B beads. Purification result showed that recombinant protein can be purified by glutathione beads, while endogenous GSTs from Sf9 cells and other non-specific proteins were also co-purified with recombinant GST-CrV1 protein (Figure 29). Therefore optimizing conditions of purification was required.

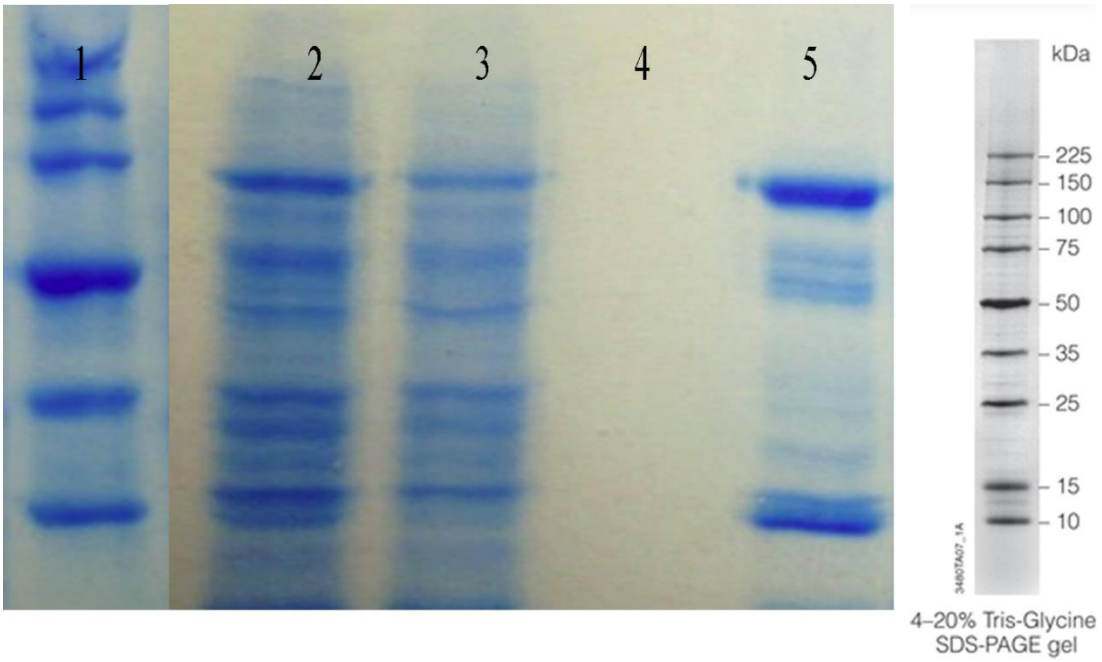

Figure 29. Purification of the recombinant GST-CrV1 protein. Lane 1: Protein marker (Promega); lane 2: Cell lysate of the pAcGHLT-B-CrV1 baculovirus infected Sf9 cell; lane 3: Unbound protein; lane 4: Flow through; lane 5: Purified recombinant GST-CrV1 protein.

5.7.1 Optimization of purification conditions

To remove endogenous GSTs, ammonium sulfate precipitation was applied. The supernatant of infected Sf9 cell lysate was precipitated by a serial of ammonium sulfate saturations. The results were as follows (Figure 30).

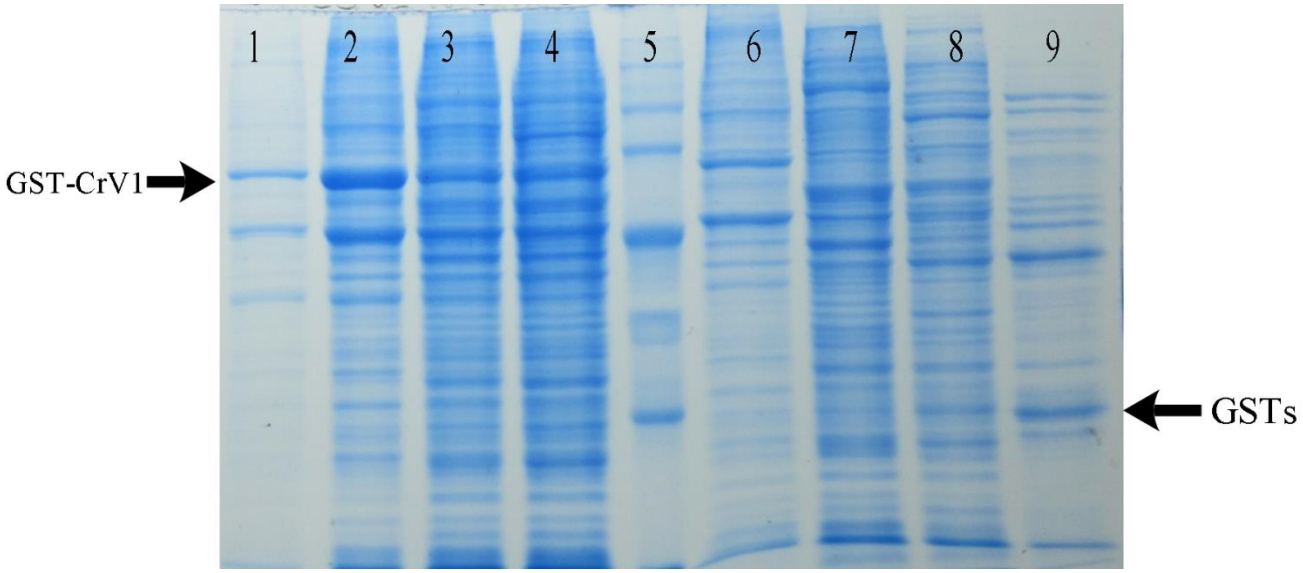

Figure 30. Ammonium sulfate precipitation. Lane 1 to 4: The pellets from 20%, 30%, 40%, 50% saturated ammonium sulfate respectively; lane 5: Protein marker (Promega); lane 6 to 9: The pellets from 20%-30%, 30%-40%, 40%-50%, 50%-60% ammonium sulfate cuts respectively.

The results showed that most of GST-CrV1 recombinant protein was precipitated by 30% saturated ammonium sulfate, whereas endogenous GSTs were precipitated by 40%-50% and 50%-60% ammonium sulfate cuts (Figure 30). Therefore, the supernatant of cell lysate was first precipitated by 30% saturated ammonium sulfate prior to binding with glutathione sepharose beads. After centrifugation, supernatant was removed and pellet was dissolved in lysis buffer, subsequently with purification procedure.

To minimize the protein degradation, protease inhibitors cocktail was added to lysis buffer to avoid the proteolysis. 5 mM of EDTA and 10 mM of DTT were added to reduce oxidation damage. 5% glycerol was added to the washing buffer and elution buffer to stabilize protein and reduce the non-specific binding.

After optimization of purification conditions, non-specific bands were removed from the recombinant GST-CrV1 protein purification (Figure 31). GST tag protein was also purified from pAcGHLT-B baculovirus infected Sf9 cells with the same purification procedure (Figure 31). Western blot was performed with GST antibody, which was used to confirm the expression of the recombinant protein (Figure 32).

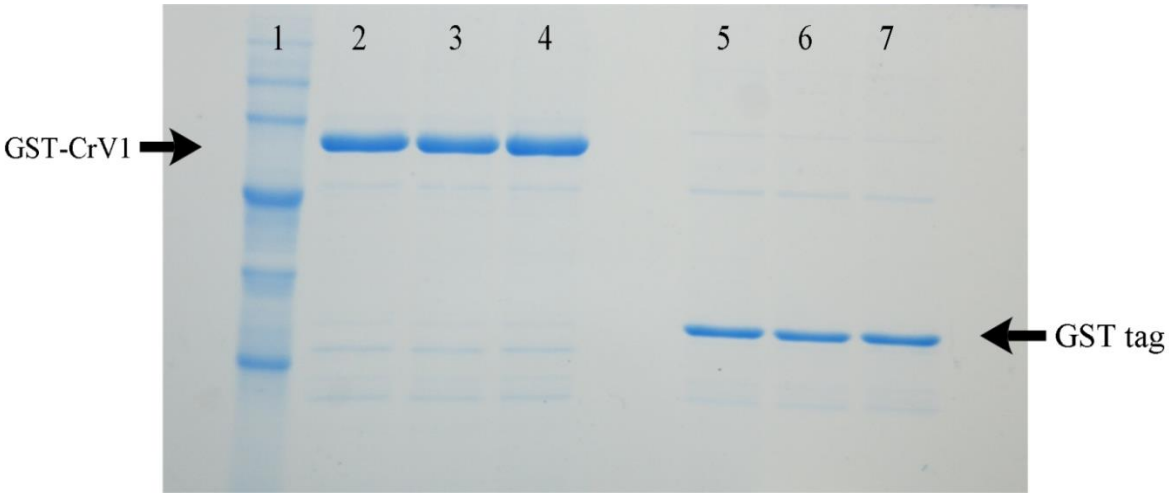

Figure 31. Purification results after optimizing conditions. Lane 1: Protein marker (Promega); lane 2 to 4: Purified recombinant GST-CrV1 protein; lane 5 to 7: Purified GST tag protein from pAcGHLT-B baculovirus infected Sf9 cells.

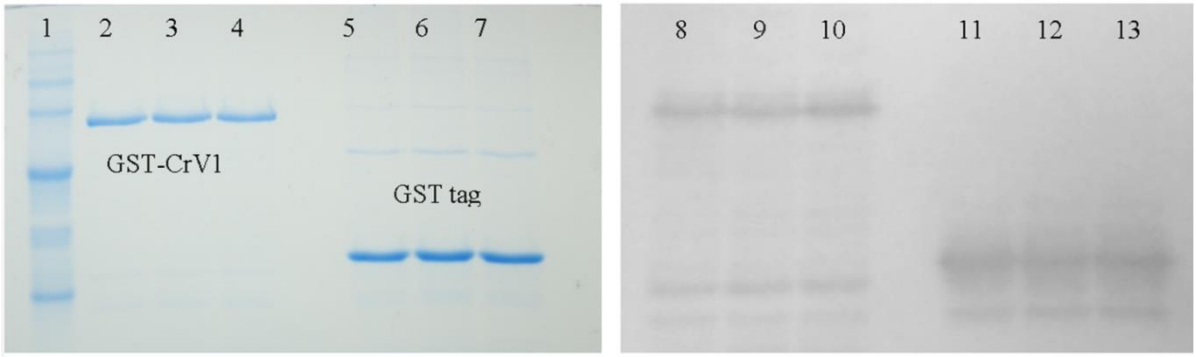

Figure 32. Western blot results of GST and GST-CrV1 protein with GST antibody. Lane 1: Protein marker (Promega); lane 2 to 4: GST-CrV1 recombinant protein; lane 5 to 7: GST tag

protein; lane 8 to 10: Western blot results of GST-CrV1 recombinant protein; lane 11 to 13: Western blot results of GST tag protein

Western blot analysis was performed by running purified GST-CrV1 and GST tag proteins on a SDS-PAGE gel and transferring them to a PVDF membrane. The results showed that the anti GST bounded to the GST-CrV1 protein and GST tag protein, which confirmed that GST-CrV1 and GST tag protein were successfully expressed and purified from viruses infected Sf9 cells (Figure 32).

5.8 GST pull-down assay

CrV1 protein is involved in suppressing the immune system of wasps' host, *Pieris rapae*. To study the protein-protein interactions between CrV1 and proteins from *Pieris rapae*, GST pull-down assay was carried out (Figure 33).

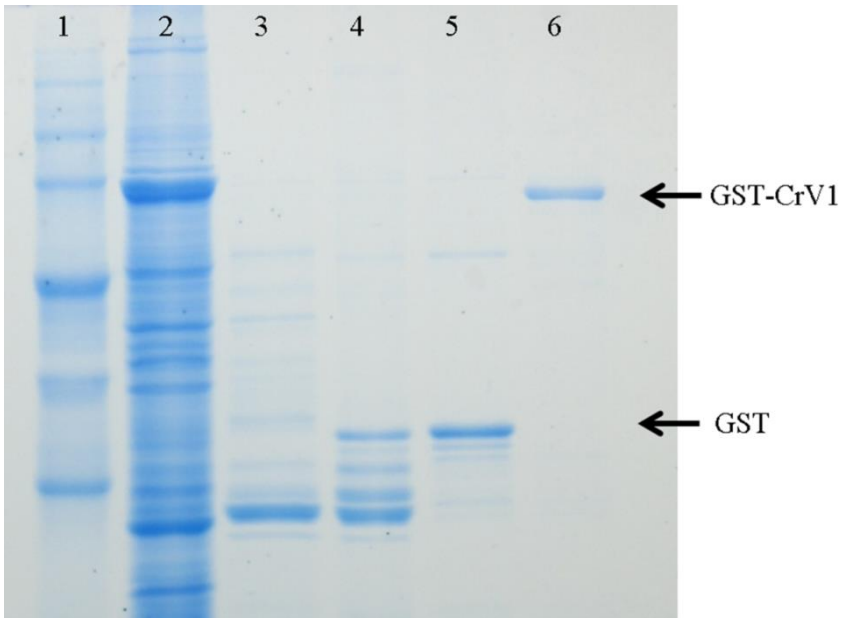

Figure 33. GST pull-down assay from *Pieris rapae* larvae lysate. Lane 1: Protein marker (Promega); lane 2: *Pieris rapae* larvae lysate; lane 3: Pull-down assay of GST-CrV1 + *Pieris*

rapae larvae lysate; lane 4: Pull-down assay of GST + *Pieris rapae* larvae lysate; lane 5: GST tag protein; lane 6: GST-CrV1 protein.

In pull-down assay (Figure 33), two controls were set, including GST tag protein and glutathione sepharose beads. Both of them were incubated concurrently in pull-down assay with *Pieris rapae* larvae lysate. The results showed that GST tag protein (lane 4) and beads alone (data not show) interacted only with endogenous GSTs from the insect lysate. However, the GST-CrV1 protein, after incubating with the lysate, was disappeared from gel and just left some smaller size of protein bands in lane 3. It was not clear that whether the protein bands were from *Pieris rapae* lysate or the degradation of GST-CrV1 protein.

GST-CrV1 protein which was used to do pull-down assay was also loaded in SDS-PAGE gel with the same amount (lane 6) as a control, which was a conformation of the presence of the GST-CrV1 protein in pull-down assay. To determine the disappearance phase of GST-CrV1 protein, another try was done. After the beads with GST-CrV1 protein was incubated with lysate for 2 h, the beads was boiled directly before washing step and loaded to the SDS-PAGE gel. The results were as follows (Figure 34).

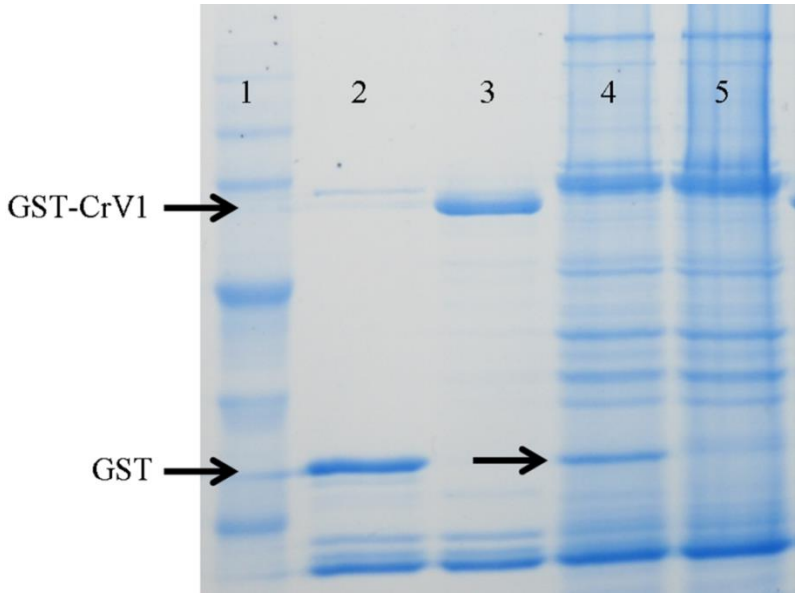

Figure 34. Determination of the fusion protein disappearance phase. Lane 1: Protein marker (Promega); lane 2: GST tag protein; lane 3: GST-CrV1 fusion protein; lane 4: The pull-down result of GST protein + lysate before washing step; lane 5: The pull-down result of GST-CrV1 fusion protein + lysate before washing step.

The results showed that GST tag protein, after incubation, still bound the beads, while GST-CrV1 fusion protein was disappeared. It indicated that GST-CrV1 fusion vanished during incubation step.

In the incubation phase, incubation time was next condition to be investigated, which was used to study the phenomenon of disappearance. 0.5, 1, 1.5, 2 h were chosen as incubation time respectively. As GST tag protein, except interaction with endogenous GSTs, has no other interactions, so that GST tag protein was not included in subsequent pull-down assay.

The results showed that, during increasing of incubation time, the degradation of GST-CrV1 fusion protein was also raising (Figure 35). Even the lysate passed the gravity flow column quickly (lane 7), the fusion protein was still degraded compare with control protein (lane 6). In

Figure 35, a number of small size protein bands below GST-CrV1 fusion protein appeared on SDS-PAGE gel. Where do the smaller size protein bands come from? Whether from insect lysate or GST-CrV1 protein? To confirm it, western blot assay with GST antibody was carried out.

In Figure 36, GST antibody did not bind to any protein from *Pieris rapae* larvae lysate. When the incubation time was increasing, the amount of GST-CrV1 fusion protein was decreasing. The amount of the protein bands below fusion protein in lane 6 were less compared with lane 2 to 5, which suggested that during the increasing of the incubation time, the amount of smaller protein bands below fusion protein was increasing. Hence the results showed that the small bands below GST-CrV1 fusion protein were from fusion protein degradation not from insect lysate.

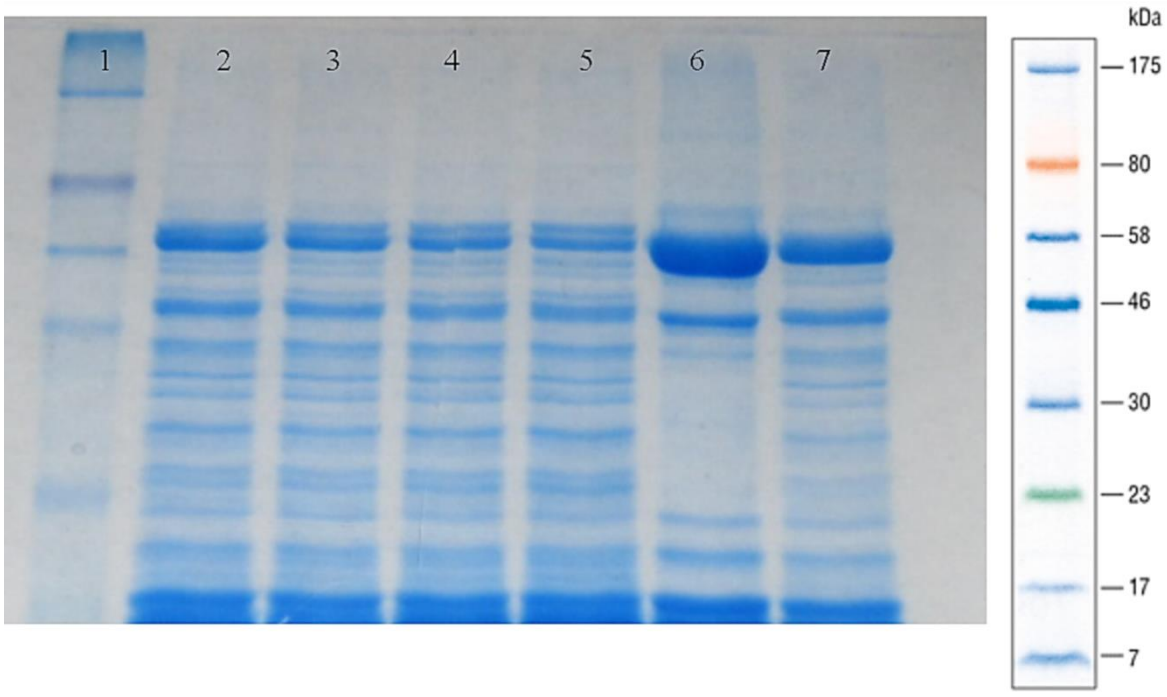

Figure 35. Pull-down assay of a serial of incubation time with *Pieris rapae* larvae lysate. Lane 1: Protein marker (Biolab); lane 2 to 5: 0.5, 1, 1.5, 2 h incubation respectively; lane 6: GST-CrV1 fusion protein; lane 7: The pull-down results of the lysate passing the gravity flow column which contained beads with GST-CrV1 fusion protein.

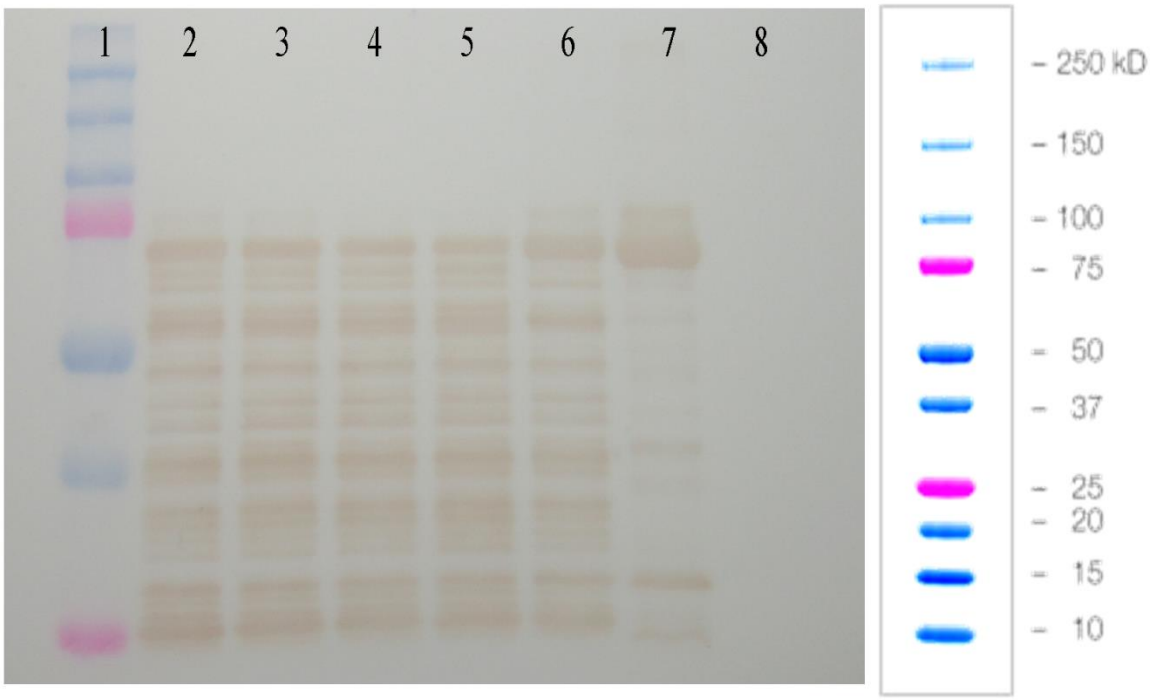

Figure 36. Western blot results of pull-down assay of different incubation time. Lane 1: Protein marker (Bio-rad); lane 2 to 5: 0.5, 1, 1.5, 2 h incubation respectively; lane 6: The pull-down results of the lysate passing the gravity flow column which contained beads with GST-CrV1 fusion protein; lane 7: GST-CrV1 fusion protein; lane 8: *Pieris rapae* larvae lysate.

Another question had arisen, which kind of material from the *Pieris rapae* lysate results in the degradation of the GST-CrV1 fusion protein. To answer this question, another experiment was set, which was used to investigate the role of proteases in the degradation process. Proteases consist of protein or RNA. In this pull-down assay, protease inhibitor cocktail was added to the insect lysis buffer, which indicated that the proteases which consist of protein did not work in the pull-down assay. Another kind of proteases which consist of RNA should be studied.

The results showed that, with or without RNase A, GST-CrV1 protein was still degraded (Figure 37). The interesting discovery was that, with RNase A in insect lysis buffer, one more protein

band was found in lane 4 (arrow) compared with lane 3. It suggested that some proteases which consist of RNA involved in the degradation of GST-CrV1 fusion protein.

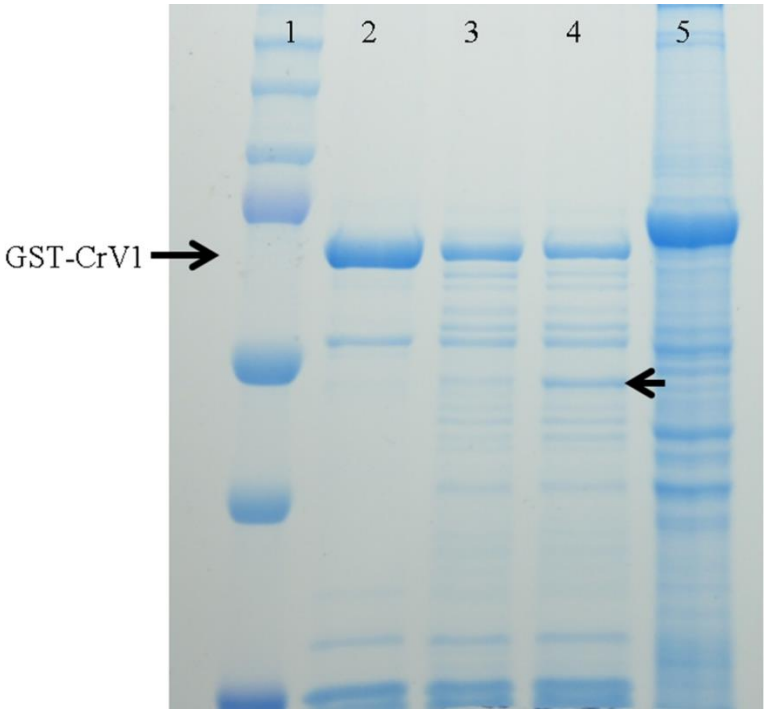

Figure 37. Pull-down assay with RNase A in insect lysis buffer. Lane 1: Protein marker (Bio-rad); lane 2: GST-CrV1 fusion protein; lane 3: Pull-down result of insect lysis buffer without RNase A; lane 4: Pull-down result of insect lysis buffer with RNase A; lane 5: *Pieris rapae* larvae lysate

All the results above showed that some kinds of unknown material in *Pieris rapae* larvae lysate can result in the degradation of GST-CrV1 fusion proten. However, does the degradation only occur in *Pieris rapae* larvae lysate? To confirm this, the GST pull-down assay was also performed in other insect, *Spodoptera exigua*.

The pull-down assay in *Spodoptera exigua* larvae lysate obtained similar result as *in Pieris rapae* larvae lysate (Figure 38). Compared with the pull-down assay in *Pieris rapae* larvae lysate, the

degradation of the fusion protein was faster in *Spodoptera exigua* larvae lysate. Western blot using GST antibody was also carried out. In Figure 39, the degradation of GST-CrV1 fusion protein was increasing during the increasing of incubation time.

The results showed that the materials which can cause the degradation of the GST-CrV1 fusion protein dose not only exist in the original host.

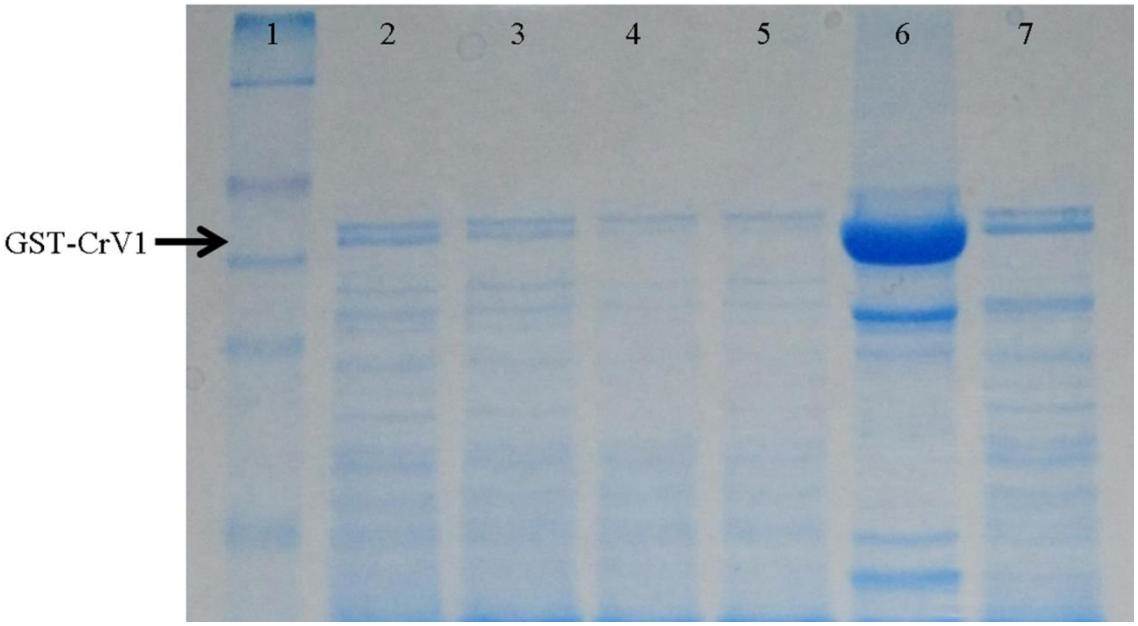

Figure 38. Pull-down assay of a serial of incubation time with *Spodoptera exigua* larvae lysate. Lane 1: Protein marker (Biolab); lane 2 to 5: 0.5, 1, 1.5, 2 h incubation respectively; lane 6: GST-CrV1 fusion protein; lane 7: The pull-down results of the lysate passing the gravity flow column which contained beads with GST-CrV1 fusion protein.

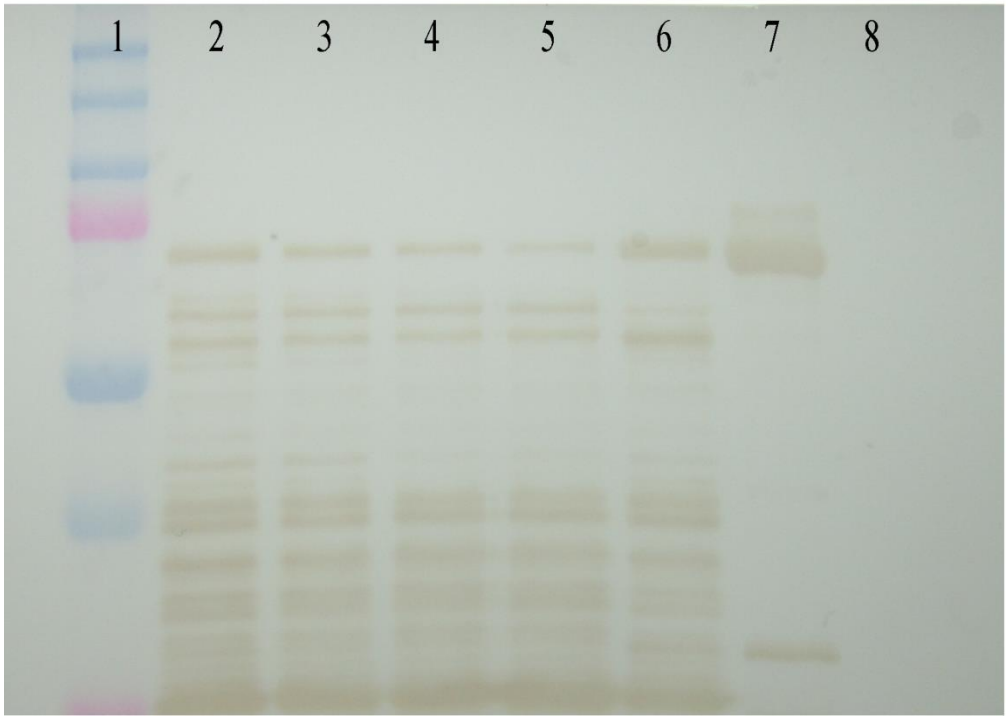

Figure 39. Western blot results of pull-down assay of different incubation time with *Spodoptera exigua* larvae lysate. Lane 1: Protein marker (Bio-rad); lane 2 to 5: 0.5, 1, 1.5, 2 h incubation respectively; lane 6: The pull-down results of the lysate passing the gravity flow column which contained beads with GST-CrV1 fusion protein; lane 7: GST-CrV1 fusion protein; lane 8: *Spodoptera exigua* larvae lysate

5.9 Bioassay

The ability of pAcUW21-CrV1 to infect and kill *Pieris rapae* and *Spodoptera exigua* larvae was evaluated and compared with that of wild-type baculoviruses. LC_{50} and LT_{50} values showed statistically significant difference for *Pieris rapae* because it is accepted that both parameters are no similar at $\alpha = 0.05$ because that of AcMNPV wild-type (17.9 and 125, respectively) lies outside 0.8-5.2, and 66-72, respectively the confidence interval estimated for pAcUW21-CrV1. For *Spodoptera exigua*, there were also not overlaps of 95% confidential limits.

The LC_{50} of pAcUW21-CrV1 against *Pieris rapae* (2.6×10^4) and *Spodoptera exigua* (1×10^3) was lower than that using the wild-type AcMNPV (1.79×10^5 and 1×10^4, respectively; Table 4). As expected, the viral-infection symptoms were similar for the pAcUW21-CrV1 and AcMNPV wild-type baculoviruses, *i.e*. we noted that the larvae stopped feeding, presented dark spots in their skin, and there was a liquefaction of their internal organs after death (Figure 41).

The LT_{50} of *Spodoptera exigua* and *Pieris rapae* was determined using 5582 and 3.5×10^5 polyhedra/cm^2 respectively of either pAcUW21-CrV1 or AcMNPV. In both pests, the LT_{50} was lower using the pAcUW21-CrV1 virus compared with that of the wild-type, as shown in Table 1. All bioassays produced significant non-zero slopes with t-ratios ranging from 13.5 to 16.0. Al t-ratio values of the slopes were significant at the at the P= 0.05 level, because all exceeded 1.96 (Jacobson, Foster et al. 2009).

	Pieris rapae				***Spodoptera exigua***			
	LC_{50}	LT_{50}	Slope ±SE	t-ratio	LC_{50}	LT_{50}	Slope ±SE	t-ratio
AcMNPV	17.9 (10.8-30.9)	125 (120-130)	2.334 ± 0.17	13.558	1 (0.3-2.3)	214 (204-226)	1.196 ± 0.075	16.0
pAcUW21-CrV1	2.6 (0.8-5.2)	69 (66-72)	2.534 ± 0.20	12.496	0.1 (0.06-0.2)	139 (136-142)	1.570 ± 0.111	14.15

Table 4. Probit analysis results from dose-mortality assays of wild (AcMNPV) and recombinant (pAcUW21-CrV1) baculovirus against *Pieris rapae* and *Spodoptera exigua*. LC_{50} was calculated using polyhedra $\times10^4$ (95%-CI) in both pest species. LT_{50} was in Hours (95%-CI)

The comparison between mortality rates of recombinant baculoviruses and wild-type baculovirues is summarized in Figure 40. The first larva dead was observed in the second day post-infection with the pAcUW21-CrV1. When the mortality rate was about 50% and 100%

using the pAcUW21-CrV1, the mortality rate was less than 2%, and 40%, using the wild-type baculovirus, respectively (Figure 40.C). In the first 4 days, using either pAcUW21-CrV1 or the AcMNPV wild-type baculovirus, none larvae had died until the end of the fourth day. When the mortality rate was about 50%, and 100% using the pAcUW21-CrV1 the mortality rate was less than 10%, and 22%, using the AcMNPV wild-type baculovirus, respectively (Figure 40.D).

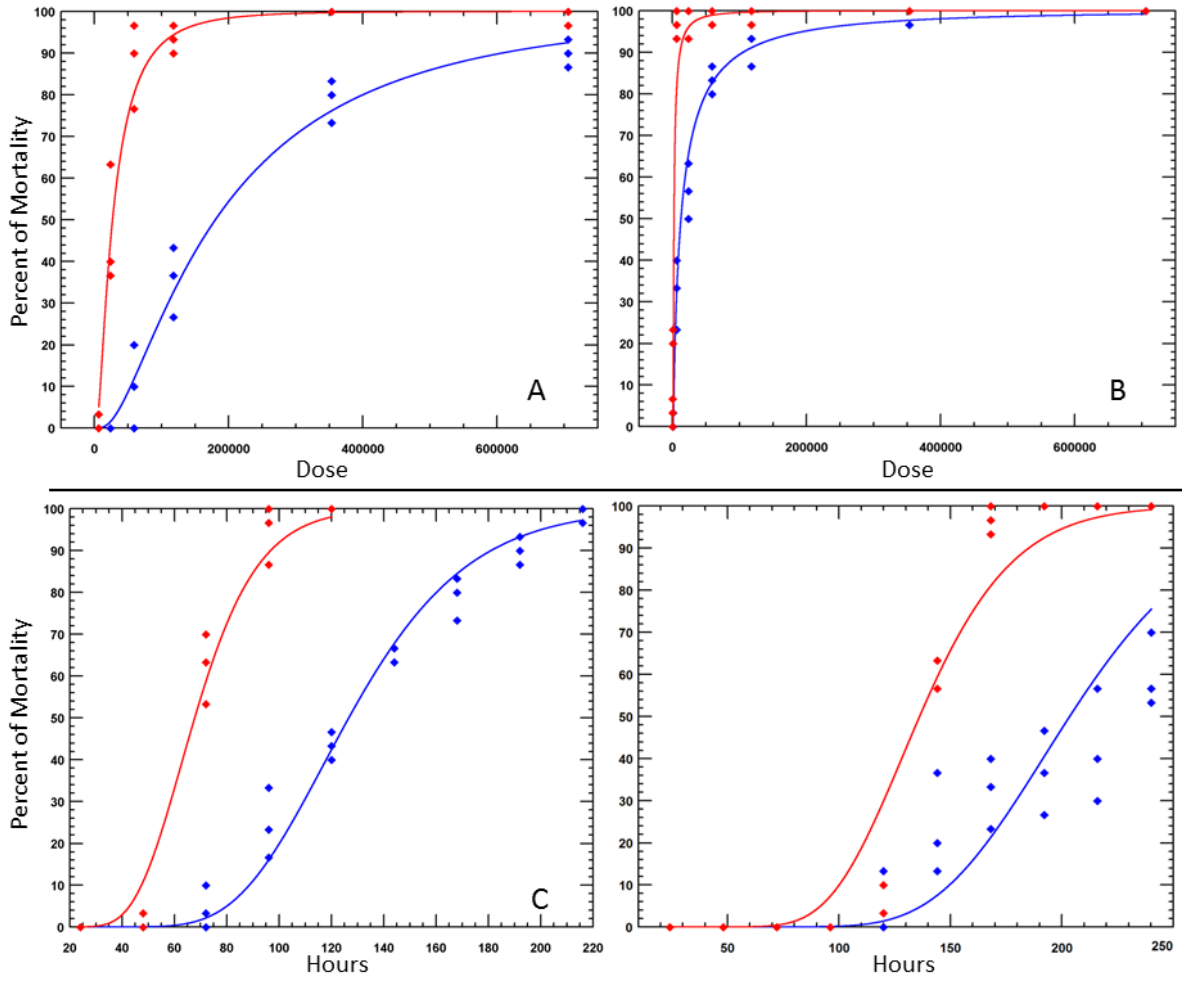

Figure 40. The relationship between recombinant or wild-type baculoviruses dose with *Pieris rapae* and *Spodoptera exigua* mortality. Panel A) Blue and red lines correspond, respectively, to AcMNPV and pAcUW21-CrV1 baculoviruses against *Pieris rapae*. Panel B) Blue and red lines correspond, respectively, to AcMNPV and pAcUW21-CrV1 baculoviruses

against *Spodoptera exigua*. Panels C) and D), mortality of *Pieris rapae* and *Spodoptera exigua* produced throughout the time (hours) by AcMNPV (blue line) and pAcUW21-CrV1 (red line) using 3.5×10^6 polyhedra/cm^2 and 5,582 polyhedra/cm^2, respectively

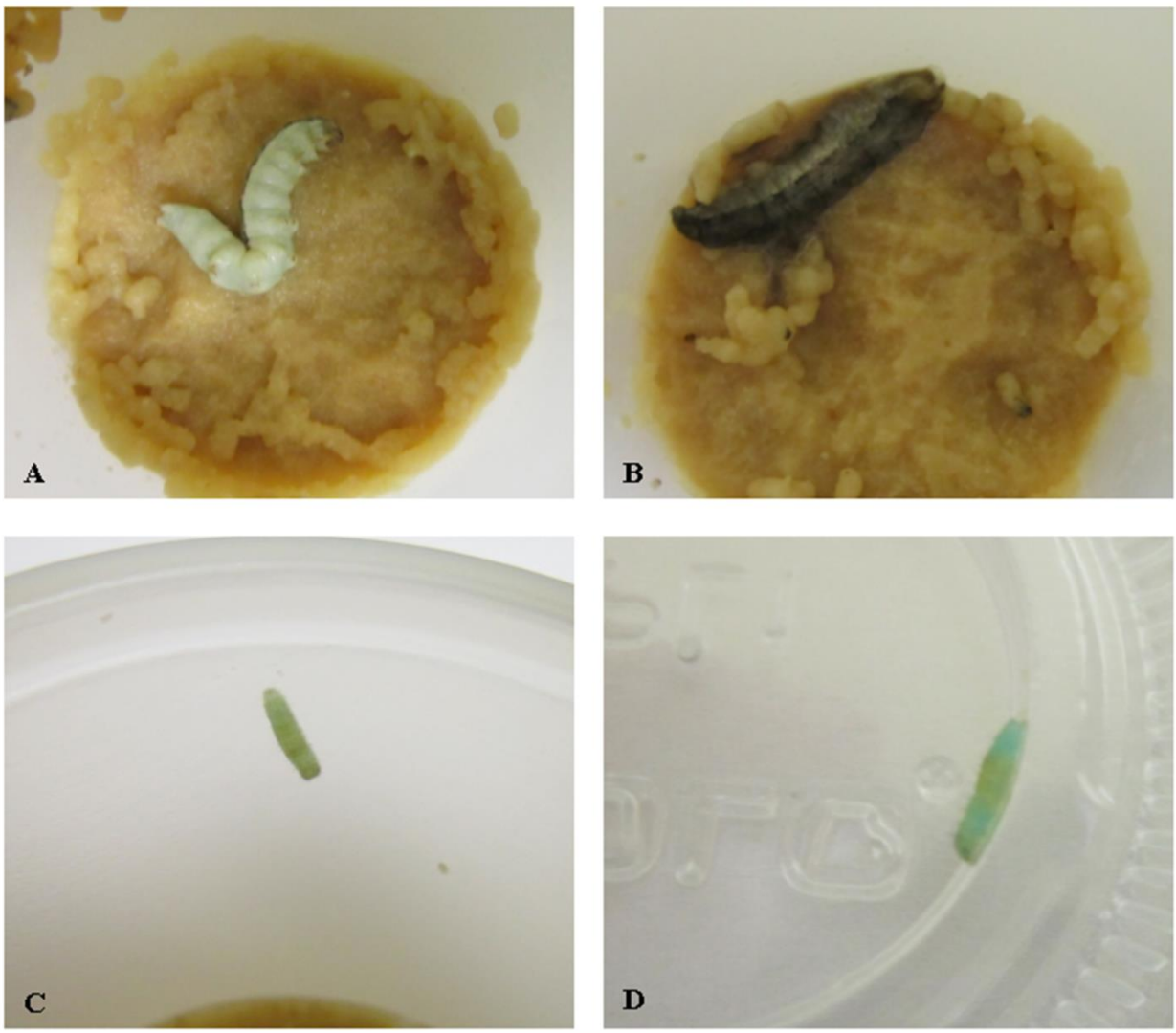

Figure 41. Recombinant or wild-type baculoviruses infected *Pieris rapae* and *Spodoptera exigua* larvae. A: Wild-type baculovirus infected *Spodoptera exigua* larvae; B: Recombinant baculovirus infected *Spodoptera exigua* larvae; C: Wild-type baculovirus infected *Pieris rapae* larvae; D: Recombinant baculovirus infected *Pieris rapae* larvae

6 Discussion

6.1 The baculovirus expression system

In this study, baculovirus expression system was used for expression GST-CrV1 fusion protein. This system includes insect cell culture, generation of recombinant baculoviruses, amplification of recombinant baculoviruses and expression of recombinant proteins or production of infective recombinant virus particles. Sf9 cell line was chosen for this project as Sf9 cells grow well in monolayer and suspension culture and are adaptable to serum-free medium. When the cell culture is performed, several notes should be considered. While the culture started from frozen stocks, frozen cells should be thawed at 27 ℃ and put on ice immediately to minimize the damage to cells by long DMSO exposure in high temperature. When thawed cells are added to one flask with fresh medium, let cells attach the flask for less than half h at 27 ℃. Long time incubation will cause harm to Sf9 cells because of DMSO. Sf9 cell line was maintained in TNM-FH culture medium which contained 10% fetal bovine serum (FBS), and incubated at 27±1 ℃. Subculture was performed by pipetting to new flasks when cell line was confluent. Scraper also can be used for subculture, but will increase the cost of this system compared with pipette tips. In addition, pipette tips are not only easy to be sterilized but also have no damage to the bottom of the flasks, which facilitates to the reuse of culture flasks. However, pipette tubes are only convenient for 25 or 75 cm^2 flask, and for other large size flasks such as 182 cm^2, larger size of scrapers could be better for culture.

The medium with FBS has desirable effects such as promote the growth of the cells, provide shear force protection and contribute cellular attachment factors, while for recombinant protein expression, FBS may decrease product yields. Therefore, how to adapt cell line to serum-free

culture medium is another task before large scale recombinant protein expression. In this study, sequential adaptation (Invitrogen) was chosen. When the serum-free media was more than 75% in the culture medium, insect cells became large, float and slow growth (data not show), and while changed to 100% serum free media, the phenomena described above became more serious. By this time, go on and go back are two choice. For this study, going on was chosen and the later success confirmed that it was a good decision. This adaption underwent approximately two months and cells grew well in the serum free medium.

During culture, the redundant cells can be stored for further use. DMSO is a preferred reagent for the storage. However, in our culture, glycerol and DMSO both were used to store cells and no big difference was observed after culture. DMSO is hazardous and caution should be taken when handing it. The best way for storing cells is to place them in liquid nitrogen. Nevertheless, maintaining the cells in liquid nitrogen, for many labs, is impossible.

Based on our experience, Sf9 cells can be stored at -80 ℃ at least one year and the cells can be thawed and recovered. The more secure way is that save cells every two weeks or one month.

The flasks for culture are another large cost in this system. In our experience, the flasks after use can be washed with sterilize Milli-Q water several times and stored at a clean room for further use and the flasks can be reused many times following this protocol.

The recombinant baculovirus was generated via homologous recombination between transfer vector and linearized baculovirus genome DNA. The important step is confirmation of the sterilization of recombinant transfer vector DNA. The preferred way is that, a few microliters of extracted transfer plasmid DNA which was sterilized by filtration were incubated with insect cells and check whether the culture is contaminated or not after 3 or 4 days prior to co-

transformation performance. From the co-transfection results (Figure 19), it is difficult to verify whether it is successful from the phenotype of cells, even recombinant virus is occlusion body positive, due to the fact of the low concentration of viruses. In most cases, viruses should be amplified for further confirmation. During the course of amplification, the matter of MOI which is used for amplification should be concerned as the high MOI increases the amount of virus with extensive mutations in their genome (O'Reilly, Miller et al. 1994). For the first two rounds of amplification, it is not significant, while prior to the third round infection, the titer of P2 virus stock is essential to determine. In addition, the P2 virus stock can be used to confirm the transcription and expression of insert genes. The extensive passaging of the recombinant baculovirus results in dramatic drop in production (van Oers, Pijlman et al. 2015), therefore, in the process of our study, the P3 virus stock was used as the inoculation for production of recombinant baculovirus and fusion protein. All the viruses can be stored at 4 ℃ for long time, but after some months the titer of the viruses will decrease. Therefore, if the viruses are stored long time at 4 ℃, it is better to determine the titer of the viruses prior to use. For optimal protein production, the MOI is usually between 3 and 10 (O'Reilly, Miller et al. 1994). In this study, different MOI was tested to determine the optimum (Figure 27) and MOI 10 was used to express the fusion protein.

6.2 Purification of CrV1 protein

The gene of interested, CrV1, in this study was fused with GST tag under polyhedrin promoter. CrV1 is a secreted protein, so the protein can be purified from the culture medium. At the beginning, the fusion protein was purified from the supernatant of culture medium. Nevertheless, the amount of purified protein was a little. Subsequently, the protein was tried to purify from infected Sf9 cells and the quantity of the protein was more than that from culture medium.

Therefore, the secreted fusion protein for further use was initiated to purify from infected cells. For the purification of the fusion protein from infected cells, the time of infection should be cared. In the event that the infection time is excessive, the cells will lyse, while the time is insufficient, the production will reduce. Based on our experience, 3 days was chosen to harvest of infect cells for expression of the GST-CrV1 protein. The purification result was shown in Figure 29. The fusion protein was purified but the endogenous GSTs were co-purified with the fusion protein. Chromatography, such as gel filtration, was considered to solve this problem. However it was not carried out due to the small scale purification of GST-CrV1 protein in this study. Afterward ammonium sulfate precipitation was selected (Figure 30) and endogenous GSTs were removed from the purified fusion protein (Figure 31). For ammonium sulfate precipitation, solid ammonium sulfate or saturated ammonium sulfate solution can be utilized. The solid ammonium was commonly used in large volume protein lysate for the reason that the solid ammonium doesn't increase a lot of the final volume which facilitate the later centrifugation step, while the saturated ammonium sulfate solution was usually used in small volume protein lysate due to simple manipulation. In this study, the saturated ammonium sulfate was used due to the small scale protein purification. For the purification of GST tag protein which was used as one control in pull-down assay, the ammonium sulfate precipitation was also used , which was precipitated by 35% saturated ammonium sulfate (date not show). In this study, another occlusion body positive recombinant baculovirus pAcUW21-CrV1 was also constructed under p10 promoter. However, CrV1 protein was not shown in SDS-PAGE gel maybe due to the fact of low level expression of CrV1 protein. As CrV1 antibody was absent in the lab, CrV1 protein expression was just verified by CrV1 gene transcription in infected Sf9 cells (Figure 24). Another recombinant baculovirus pVL1392-CrV1 was also constructed under polyhedrin

promoter and CrV1 expression also can't be observed in SDS-PAGE gel but can be confirmed by transcription (data not shown). The low expression of CrV1 protein of these two recombinant baculoviruses indicated that GST tag in pAcGHLT-B transfer vector increased the expression level of CrV1 protein drastically.

In addition, western blot was carried out after GST-CrV1 purification and pull-assay with GST antibody. Based on our experience, in the process of western blot, several crucial points should be cared. It is better to load fewer microliter samples to each well in comparison with normal SDS-PAGE assay, which can favor to get clear separated bands. The antibody can be diluted in the range of antibody dilution of the product introduction. The most critical step is the washing. The washing step presents two times in whole process. The first time is after incubation of the membrane with the first antibody and the second time is after the incubation of the membrane with the second antibody. Numerous washing buffer formulas are used, such as PBS, TBS. In this study, TBS was used. TBS also has different formulas and the difference between these formulas is the concentration of NaCl, from 150 mM to 500 mM, which represents the stringent condition. In this work, the more stringent washing condition, 500mM NaCl, was chosen. Another important point in the washing step is the speed of shaker which also concerns the washing stringency. In this experiment, 150 rpm (Lab-Line 3520 Adjustable Speed Orbital Shaker) was used. More times of washing and less of washing time are propitious to good washing results. In this work 6 times and 7 min each time were chosen. Moreover, the stain time should be cared to avoid the overstain.

6.3 GST-CrV1 pull-down assay

CrV1, which is from the parasitoid species *Cotesia rubecula* PDV, involves in immune-suppression by disrupting the cytoskeleton of the host hemocytes to inactivate hemocytes (Asgari, Schmidt et al. 1997). Previous work showed that polydnavirus-infected larvae and larvae were injected with recombinant CrV1 manifested similar haemocyte alterations which indicated that CrV1 alone is sufficient to lead to haemocyte inactivation (Asgari, Schmidt et al. 1997). The expression of CrV1 protein is in a transient fashion inside the haemocytes, starting from 4 to 8 h post parasitization (Asgari, Hellers et al. 1996, Glatz, Schmidt et al. 2004). The reason for the quick shut-off CrV1 gene expression is still unknown. Two opinions were proposed. One is that regulatory events initiated by polydnavirus gene expression result in a remarkable change which may cause the temporary inactivation of infected cells and another is that the breakdown of the cell cytoskeleton induced by CrV1 expression may cause the inactivation of the gene expression machinery of the PDV-infected cells. CrV1 does not bind to hemocytes in default of hemolymph plasma, which suggested that CrV1 may interact with hemolymph proteins (Asgari and Schmidt 2002). One coiled-coil region has been reported, which may be essential for CrV1 uptake or function (Asgari and Schmidt 2002, Kumar and Kim 2014).

In this study, GST pull-down was performed, which aimed to investigate the interacted proteins with CrV1 protein. The results showed that CrV1 protein was disappeared during the pull-down process (Figure 33) and a number of small size protein bands were observed in SDS-PAGE gel, which indicated that some kinds of materials existed in *Pieris rapae* larvae lysate may digest CrV1 protein. Different incubating time of pull-down assay was also carried out and the same results were obtained (Figure 35). Western blot results with GST antibody shown in Figure 36 illustrated that the small size protein bands were from the digestion of GST-CrV1 fusion protein,

which confirmed that GST-CrV1 fusion protein was digested during the course of pull-down assay. The pull-down assay was also carried out in another insect (*Spodoptera exigua*) and the similar results were obtained (Figure 38). The results above indicated that the material which can digest CrV1 protein did not only exist in the original host *Pieris rapae*. What are the components of the material? This question is far to be answered. The protease inhibitor and RNase A were added into insect lysis buffer, which was used to identify whether the material was protease. The interesting discovery was that some kinds of RNA or proteases which consist of RNA can cause the digestion of GST-CrV1 fusion protein. Why GST-CrV1 fusion protein was digested during pull-down assay. Several possibilities will be discussed subsequently.

Previous works showed that glycosylated CrV1 protein expressed from infected Sf21 cells was incubated with cell-free hemolymph and analyzed on a western blot probed with anti-CrV1 antiserum, and the degradation of CrV1 protein was not observed (Asgari, Schmidt et al. 1997). Compare with our pull-down assay, some conditions are different, such as protein glycosylation, GST tag, lysate (not only hemolymph) and cell line.

In this study, fusion protein GST-CrV1 was expressed under polyhedrin promoter which started in the very late phase of baculovirus gene expression. Therefore glycoprotein CrV1 may not obtain enough post-modification. Another report showed that non-glycosylated CrV1 protein can be taken up by hemocytes which indicated that the binding and uptake of CrV1 protein don't depend on the sugar modification (Asgari and Schmidt 2002). In addition, it means that non-glycosylated CrV1 protein can interact with the proteins in the hemolymph plasma. The binding of bacterial- expressed CrV1 protein to hemolymph proteins was visualized by ligand blot experiment with anti-CrV1 antibodies and no degradation was observed (Asgari and Schmidt 2002). Hence, glycosylation is not the reason of CrV1 degradation. The function of glycosylation

may involve in the activity inside the hemocytes. Another difference compare with previous work is that our protein has a GST tag which facilitates the purification of CrV1 protein. GST tag is a 26 kDa protein. Some report showed that GST tag can cause misfolding of target protein which may lead to the false positive GST pull-down result (Wissmueller, Font et al. 2011). Proteins with misfolding can be recognized and selectively degraded in eukaryotic cells (Goldberg 2003). Regardless of the fact that there is no information that GST fusion misfolded target protein will result in the degradation in the course of pull-down assay, this still is a possibility that misfolded CrV1 protein is easily be digested by the proteins from *Pieris rapae* larvae lysate. Next different condition is the lysate we used for pull-down assay. In the previous work, only hemocytes and hemolymph were reported to use to incubate with CrV1 protein. However, in our study, the whole *Pieris rapae* larvae lysate was used to incubate with GST-CrV1 fusion protein for pull-assay. The degradation was not observed when CrV1 protein incubated with hemocytes or hemolymph. Therefore, the degradation of our fusion protein may due to the lysate except the hemocytes and hemolymph. In this study, due to the fact that it is hard to get enough hemolymph for pull-down assay, so we chose the whole lysate of the larvae, which may result in the degradation of our fusion protein. The last difference is the cell line used for protein expression, in the previous work above, Sf21 cell line was used to express the CrV1 protein and in our study Sf9 cell line was chosen to express GST-CrV1 protein. However Sf9 is a substrain of these cells that isolated from Sf21, so these two cell lines have no significant difference. Therefore there is scarce possibility that the different cell line cause the degradation of GST-CrV1 protein during pull-down assay.

The expression of CrV1 was detected in haemocytes and fat body (Asgari, Hellers et al. 1996). From our pull-down assay results, in the event that the degradation of CrV1 was due to the lysate,

it means that, besides the haemobytes and fat body, the other tissue of *Pieris rapae* larvae exist materials, such as protease, which can digest CrV1 protein.

6.4 Bioassay

The present study focused on the construction and evaluation of a recombinant baculoviruses expressing an immunosuppressor gene from *Cotesia rubecula* PDV, CrV1 protein (pAcUW21-CrV1). In Sf9 insect cell culture, infection showed clear differences between infected and healthy Sf9 cells. The cells inoculated with the baculoviruses had infection-like phenomena as described in the section or results. Increased of nuclear volume was observed in *Spodoptera exigua* and *S. frugiperda* cells 72 h pi with *A. californica* and *Spodoptera exigua* wild-type (AcMNPV and SeMNPV) baculoviruses similarly to that reported by(Yanase, Hashimoto et al. 1998). We also noticed that the infection with wild-type baculoviruses stopped the growth of 84% of cells cultured in phase G2 / M 18 within 24 hpi (Braunagel, Parr et al. 1998). The infection with wild-type baculoviruses also resulted in cell lysis and death 5-6 dpi as demonstrated elsewhere (Clem 2001, Hu 2005). Confirmation of infected Sf9 cells with the CrV1 gene in AcMNPV was performed by PCR indicating that the *in vitro* production process of the pAcUW21-CrV1 recombinant baculovirus was successful.

Although the production of recombinant baculoviruses may be more expensive, this could be overcome with a higher biological product demand to control the insect pests *Spodoptera exigua* and *Pieris rapae,* which represent a significant threat to the agriculture and greenhouse crops. For example, tomato growing in greenhouse or tunneling is most susceptible to the damage by *Spodoptera exigua* as compared with open field crop, especially near to fruit maturity in North America (south of USA, in addition to Mexico and Central America) (Zalom, Wilson et al. 1986).

Furthermore, *Pieris rapae* is one of the most abundant butterflies in Northern and Central Europe, and it has recently also become a pest in North America (Wittstock, Agerbirk et al. 2004), where it damages several crucifer crops (Chen, Zhao et al. 2008, Zhang, Cheng et al. 2012).

It has been reported that LC_{50} of AcMNPV against *Spodoptera exigua* was of 9,141 polyhedra/cm^2 (Shapiro 2000), which is consistent with our findings of 10,000 polyhedra/cm^2 for the same species. In the same study, a LT_{50} of 151 h using 4,300 polyhedra/cm^2 was determined. Here, we used 5,882 polyhedra/cm^2, resulting in a LT_{50} of 214 h (Table 4). Previous studies have shown that AcMNPV can infect *Spodoptera exigua* larvae providing a decent control of this beet armyworm (Bianchi, Joosten et al. 2000, Bianchi, Snoeijing et al. 2000); however, pAcUW21-CrV1 resulted in a lower LC_{50} (10 fold reduction) and LT_{50} (24 h faster) compared with the wild-type AcMNPV to control *Spodoptera exigua* under insectary conditions. Previously, the effect of one recombinant virus expressing the immunosuppressive CsIV gene P-vank-1 against fourth-instar *S. littoralis* larvae injected budded virus was reported, where the recombinant virus showed a 10-fold lower LD_{50} compared with the wild-type AcMNPV (Rivkin, Kroemer et al. 2006). The baculovirus life cycle implicates the entrance of virus particles through the digestive track (Cory and Myers 2003), and their subsequent spread through insect tracheal system (Engelhard, Kam-Morgan et al. 1994, Flipsen, Martens et al. 1995). In several hosts, such as *Spodoptera exigua*, *Trichoplusia ni* and *Heliothis virescens*, just one tracheolar cell infection is enough to give rise to a nonreversible and mortal infection. In our bioassays, the CrV1 protein likely contributed to produce the 10 fold decrease in virus concentration because the CrV1 protein effect on the *Spodoptera exigua* actin cytoskeleton, as previously reported by *Pieris rapae* (Asgari, Hellers et al. 1996).

Equal slopes from Probit analysis indicated that the mortality rate is similar along changes in concentration units (Software 1987). In our bioassay, dose-mortality relationship showed similar slopes between AcMNPV and pAcUW21-CrV1, suggesting that the insertion of CrV1 in the AcMNPV genome did not interfere with the usual pathogenic infection process, but it has added a new pathogenic factor, the CrV1 protein. To the best of our knowledge, there are not reports about the *Pieris rapae* susceptibility to AcMNPV. Some authors concur that *Pieris rapae* are not susceptible to AcMNPV infection (Chen, Leisy et al. 1996) but some studies on *Pieris rapae* cell lines indicate that AcMNPV cause cell lysis within 24 h, since the virus do not replicate after the primary infection (Dwyer, Webb et al. 1988). It has also been shown that lower AcMNPV virus titers are produced from *Pieris rapae* cell line infection; therefore, those cell lines were considered semi-permissive (Mcintosh, Grasela et al. 2005). In our bioassays using *Pieris rapae*, the LC_{50} of pAcUW21-CrV1 was seven fold lower compared with the LC_{50} of the wild-type while the LT_{50} was reduced two fold. In *Pieris rapae*, the LC_{50} of pAcUW21-CrV1 was similar to that showed by wild-type against the highly susceptible *Spodoptera exigua* indicating the improvement of the recombinant within the host range.

The approach for producing a more effective recombinant baculovirus than wild-type was because we used an immune-suppressor gene (CrV1) of PDV. The CrV1 protein suppresses the insect innate immune cellular response of *Pieris rapae* larvae (Asgari, Hellers et al. 1996). In both pests, this recombinant showed higher toxicity to the insect host because the synergism between CrV1 and others AcMNPV proteins. It has been shown that *Manduca sexta* larvae infected with both *C. congregata* PDV and AcMNPV die faster than larvae injected orally with baculovirus alone; hence, the PDV acts synergistically with baculoviruses to cause rapid insect death (Washburn, Haas-Stapleton et al. 2000).

Different attempts have been made to improve the baculovirus speed of kill using hormone and toxin genes inserted into the baculovirus genome (Inceoglu, Kamita et al. 2001). The foreign protein and the recombinant virus became more toxic and reduced the speed of kill, hence, the recombinant virus had the original virulence factors from the wild-type baculovirus and the new feature. This effect has also been noted in our experiments, the CrV1 gene inserted in the AcMNPV genome had resulted in a more toxic virus for the two economically important pests. CrV1 effects on *Pieris rapae* haemolymph included lower viscosity and changes in cell surface composition, adhesive properties of hemocytes, and cell spreading due to the destabilization of the cell cytoskeleton (Asgari, Schmidt et al. 1997, Asgari and Schmidt 2001). Hence, the highest toxicity and speed of kill noted were likely due to the expression of the CrV1 protein that destabilized the actin cytoskeleton.

In addition, we observed that *Spodoptera exigua* larvae stopped feeding the diet contaminated with the recombinants and there was a decreasing activity of the larvae after infection with both the recombinant and wild-type viruses. The larvae infected with recombinant viruses also produced only few feeding marks on the diet (data not shown). These features are similar to those documented as polyhedrosis disease (Inceoglu, Kamita et al. 2001), which results in liquefaction of the insect tissues and release of millions of bodies of occlusion for those polyhedrin-positive baculoviruses that may be transmitted to new hosts (Washburn, Trudeau et al. 2003, Haas-Stapleton, Washburn et al. 2005). However, this does not happen in a polyhedron-negative baculovirus unless it is co-occluded in a wild-type baculovirus.

A review of *pros* and *cons* of wild-type and recombinant baculoviruses as biopesticides was given by Rodriguez et al. (2012). This includes the safety of genetically modified baculoviruses which showed no side effects on non-target organisms as well as their superior advantages over

the wild-type. The study concluded that bioinsectides based on both types of viruses are environmentally friendly with much lower risk than conventional chemical insecticides. Herein, we presented a PDV-baculovirus construct with enhanced virulence for two Lepidopteran larval pests which increased the speed of action and extended the host range. However, its safety to non-target organisms has not been evaluated. A recent work reported a recombinant baculovirus which was highly effective and environmentally friendly (Shim, Choi et al. 2013). Bioassays with the developed recombinant and other constructions are currently underway on *Sesamia nonagrioides*, a polyphagous species with a fairly wide range of host plants, the pink bollworm *Pectinophora gossypiella* (pest of cotton), *Heliothis virescens* (pest of a variety of crops), and *H. zea* (pest of maize), which is normally a non-permissive host of AcMNPV due to mobilization of a cellular immune response (Simón, Williams et al. 2004). The enhanced virulence of the recombinant baculovirus may result in an expansion of the host range of the virus as well as facilitate rapid death of those insects treated with the virus. Hence, they could become potential agents for their use in biological control programs. Further research on PDV will also lead to the identification of new genes and specific PDVs promoters which could be expressed in baculovirus as demonstrated in the present work or using such PDV genes and promoters in other biological expression systems in order to evaluate its efficiency as biopesticides.

7 Conclusion

In this study, two recombinant baculoviruses were constructed. One of them was used to expression and purification of CrV1 protein and another was used to do bioassay. The GST-CrV1 protein was successfully expressed and purified from the infected Sf9 cells for pull-down assay. During the course of pull-down assay, the GST-CrV1 protein was degraded, suggesting that the lysate may contain some materials which cause the degradation. The contents of the materials are still unknown and another experiment showed that protease consisting of RNA may involve in the degradation. In the bioassay study, the recombinant virus was then tested against the insect pests *Spodoptera exigua* and *Pieris rapae*. The recombinant virus expressing CrV1 protein showed significantly lower LC_{50} and LT_{50} as compared with the wild-type virus, which indicated that the recombinant baculoviruses expressing only CrV1 gene improve their virulence.

Glossary

Affinity chromatography

A method of separating biochemical components based on specific interactions between the components of the biochemical mixture and other molecules (e.g., antigen-antibody or receptor-ligand).

Cell culture

The treatment of cells removed from an organism, and sustained in an artificial environment that simulates the condition of the tissue the cells came from; the proces of maintaining or multiplying cells in a nutrient solution, and at an optimal temperature, under incubation.

Electrophoresis

Electrophoresis is a technique for separating macromolecules (proteins, RNA, DNA) on a gel using an electric field.

Expression vector

Cloning vector containing DNA sequences such as a promoter, a ribosome-binding site, and transcription initiation and termination sites that allow DNA fragments inserted into the vector to be transcribed and translated.

Gel electrophoresis

Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA, or proteins according to their sizes

Homologous recombination

When a manipulated gene is introduced into a cell, it can be incorporated into the genome either randomly or at a specific locus. By incorporating sequences that normally flank the desired locus, a manipulated gene can be specifically (albeit rarely) introduced into the genome. Selection for

this unlikely event can be enhanced by introduction of the herpes thymidine kinase (TK) gene into the original targeting construct. Should the construct be randomly incorporated into the genome, the TK gene will also be introduced, rendering the cell sensitive to gancyclovir. If homologous recombination occurs, the TK gene will be eliminated, as there are no homologous sequences at the specific genetic locus of interest and the resultant cell will be resistant to the antibiotic.

PCR (polymerase chain reaction)

This technique finds use in several arenas of recombinant DNA technology. It is based on the ability of sense and antisense DNA primers to hybridize to a cDNA of interest. Following extension from the primers on the cDNA template by DNA polymerase, the reaction is heat-denatured and allowed to anneal with the primers once again. Another round of extension leads to a multiplicative increase in DNA products. Therefore, a minute amount of cDNA can be efficiently amplified in an exponential fashion to result in easily manipulable amounts of cDNA.

Promoter

DNA sequence to which the transcription apparatus binds so as to initiate transcription; indicates the direction of transcription, which of the two DNA strands is to be read as the template, and the starting point of transcription.

Provirus

DNA copy of viral DNA or viral RNA; integrated into the host chromosome and replicated along with the host chromosome.

Restriction endonuclease

Technical term for a restriction enzyme, which recognizes particular base sequences in DNA and makes double-stranded cuts nearby

Reverse transcriptase PCR

A polymerase chain reaction that produces multiple copies of a selected RNA sequence rather than a DNA sequence; compare to polymerase chain reaction.

Taq polymerase

DNA polymerase commonly used in PCR reactions. Isolated from the bacterium Thermus aquaticus, the enzyme is stable at high temperatures, and so it is not denatured during the strand-separation step of the cycle.

Vector

A piece of DNA that ferries a foreign seqence of DNA into a cell or organism; together with the foreign DNA, the vector forms recombinant DNA. In disease transmission, the organism that carries an infectious agent (virus, parasite) from one host to another.

Western blotting

This technique is designed to detect specific protein present in a heterogenous sample. Proteins are denatured and size-fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose or other synthetic membranes, and then probed with an antibody to the protein of interest. The immune complexes present on the blot are then detected using a labeled second antibody (for example, a 125I-labeled or biotinylated goat anti-rabbit IgG). As the original gel electrophoresis was done under denaturing and reducing conditions, the precise size of the target protein can be determined.

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Appendix A

Protocols

Gel Extraction Kit protocol

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 μL).
3. Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 mL collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Add 0.5 mL of Buffer QG to QIAquick column and centrifuge for 1 min.
10. Add 0.75 mL of Buffer PE to QIAquick column and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at > 10,000 × g (~13,000 rpm).
12. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
13. To elute DNA, add 50 μL of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 μL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

QIAprep® Spin Miniprep Kit protocol

1. Pellet 1-5 mL bacterial overnight culture by centrifugation at > 8000 rpm (6800 × g) for 3 min at room temperature (15-25°C).
2. Resuspend pelleted bacterial cells in 250 μL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 μL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min.
4. Add 350 μL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 × g) in a table-top microcentrifuge.
6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting.
7. Wash the QIAprep spin column by adding 0.5 mL Buffer PB.
8. Centrifuge for 30-60 s and discard the flow-through.
9. Wash the QIAprep spin column by adding 0.75 mL Buffer PE. Centrifuge for 30-60 s and discard the flow-through.
10. Centrifuge for 1 min to remove residual wash buffer.
11. Place the QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 μL Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

QIAquick® PCR Purification Kit protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a provided 2 mL collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 mL Buffer PE to the QIAquick column centrifuge for 30-60 s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add 50 μL Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 μL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Appendix B

Buffers and solutions

50×TAE	2 M Tris base
	1 M acetic acid
	100 mM EDTA
	pH 8.2-8.4
5×SDS-PAGE loading buffer	250 mM Tris-HCl pH 6.8
	10% SDS
	0.5% bromophenol blue
	50% glycerol
	500 mM DTT
1×SDS electrophoresis buffer	25 mM Tris base
	250 mM glycine
	0.1% SDS
Blue silver staining dye	10% phosphoric acid
	10% ammonium sulfate
	0.12% coomassie blue G-250

	20% methanol
Fixing solution	60% methanol
	10% acetic acid
1×PBS buffer	137 mM NaCl
	2.7 mM KCl
	10 mM NaH_2PO_4
	2 mM KH_2PO_4
	pH 7.4
Cell lysis buffer	20 mM Tris-HCl
	300 mM NaCl
	5 mM EDTA, 10mM DTT
	0.1% IGEPAL CA-630
	protease inhibitor cocktail
	pH 7.5
Washing buffer of purification	20 mM Tris-HCl
	150 mM NaCl
	5 mM EDTA

	10 mM DTT
	0.1% IGEPAL® CA-630
	5% glycerol
	pH 7.5
Elution buffer	20 mM Tris-HCl
	150 mM NaCl
	5 mM EDTA
	10 mM DTT
	5% glycerol
	10 mM reduced glutathione
	pH 8.0
Insect lysis buffer	20 mM Tris-HCl
	150 mM NaCl
	5 mM EDTA
	10 mM DTT
	5% glycerol
	0.1% IGEPAL® CA-630

	pH 7.5
Washing buffer of pull-down	20 mM Tris-HCl
	150 mM NaCl
	5 mM EDTA
	10 mM DTT
	5% glycerol
	0.5% IGEPAL® CA-630
	pH 7.5
Transfer buffer	25 mM Tris base
	190 mM glycine
	20% methanol
Blocking buffer	3% BSA, dissolved in TBS
TBS buffer	20 mM Tris
	500 mM NaCl
	pH 7.5
Antibody dilution buffer	3% BSA, dissolved in TBST
Washing buffer of Western blot	0.05% Tween 20 in TBS

DAB solution	30 mg DAB
	60 μL 30% H_2O_2
	50 mL TBS
Lysis buffer for getting polyhedral	50 mM Tris-HCL pH 8.0
	0.4% SDS
	10 mM EDTA pH 8.0