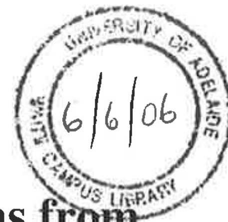
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Isolation and characterization of venom proteins from the endoparasitoid wasp *Cotesia rubecula*

Guangmei Zhang, M. Sc.

Department of Plant Protection

Beijing Agricultural University

A Thesis Submitted for the Degree of

Doctor of Philosophy

Discipline of Plant and Pest Science

The University of Adelaide

August 2004

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Guangmei ZHANG

August 2004

Acknowledgements

I would like to express my sincere gratitude to my supervisors Professor **Otto Schmidt** and Dr **Sassan Asgari** for their guidance, support and encouragement throughout the course of this study.

I wish to thank all my colleagues and my friends in the Insect Molecular Biology Laboratory. I would like to acknowledge Ms **Nicki Featherstone** for her help in my whole experiments. Dr **Dongmei Li**, Dr **Marco Fabbri**, Dr **Sarjan Mohammad**, Dr **Richard Glatz**, 'almost doctors' **Harry Roberts**, **Mehdi Sohani**, **Gang Ma**, **Mahbubur Rahman**, **Zengqi Zhao**, **Judi Bewlati**, **Sobat Bahrami Nejad, Reza Hosseini**, and Miss **Caitlin Byrt** for their help, suggestion and discussion. I'd like to thank all the people in the lab for providing a friendly and active atmosphere.

Many thanks go to Dr **Reza Zareie** from BresaGen Ltd., Dr **Lu Zhi-Qiang** and **Jiang Haobo** from Oklahoma State University for their great help in some experiments.

Special thanks go to Dr **Mike Keller** for his help to set up the population of insects and valuable suggestions. Thanks to our secretary Mrs **Sue Eaton** for her consistent help and friendship, Ms **Heather Fraser**, Dr **Gary Taylor** and all the others in the Discipline of Plant and Pest Science who helped me in one way or another.

My deep thanks go to my wife **Liang Guo** for her endless love, support and understanding. Thanks to my lovely sons, **Guo-Wen Zhang** and **Yang Zhang** for providing much happiness to my life. Specific thanks also go to my parents-in-law for their help in looking after both our little babies.

I am also grateful to my extended family in China, my mum and dad whose endless love and support, and my sisters and brothers for their understanding and support.

Finally I would like to express my sincere gratitude to the University of Adelaide for rewarding me with an Adelaide Postgraduate Scholarship and Overseas Postgraduate Research Scholarship. Without these scholarships, this thesis would have not been possible.

List of publications

1. **Guangmei Zhang,** Otto Schmidt and Sassan Asgari. Factors influencing the entry of polydnaviruses into host hemocytes. Manuscript in preparation.

2. **Guangmei Zhang,** Otto Schmidt and Sassan Asgari. (2004). Is calreticulin from *Cotesia rubecula* venom involved in the immune suppression? Manuscript in preparation.

3. **Guangmei Zhang,** Otto Schmidt and Sassan Asgari. (2004). A novel venom peptide from an endoparasitoid is required for the polydnavirus expression in host cells. *Journal of Biological Chemistry* **(in press)**.

4. **Guangmei Zhang**, Zhi-Qiang Lu, Haobo Jiang, Sassan Asgari. (2004). Negative regulation of prophenoloxidase (proPO) activation by a clip-domain serine proteinase homolog (SPH) from endoparasitoid venom. *Insect Biochemistry and Molecular Biology* **34**, 477-483.

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Abstract

Endoparasitoid wasps oviposit and develop in the hemocoel of their host where they are exposed to the host immune system, including humoral and cellular responses. Endoparasitoids have necessarily evolved effective mechanisms through which they inhibit the immune responses for successful parasitism. Inhibition of insect immune responses may be associated with changes in hemocyte population, down-regulated phenoloxidase activity, and aberrant hemocyte morphologies. To that end, maternal factors are introduced into host together with eggs during oviposition. These factors include symbiotic polydnaviruses, calyx proteins and venom proteins, and may act alone or together with one or more of the other factors to suppress the host immune responses.

Cotesia rubecula (Hym: Braconidae) is an endoparasitoid of *Pieris rapae* (Lepidoptera: Pieridae), a worldwide insect pest of cruciferous crops. During oviposition, *C. rubecula* introduces maternal factors into the host, which facilitate successful parasitisation. In this study, several major venom proteins from *C. rubecula* were isolated and characterized including Vn1.5, Vn4.6, Vn50 and Calreticulin (CRT). Vn1.5 is a small novel peptide containing 14 amino acids with a molecular mass of 1598 Da. No similar sequences were found in protein databases. Although Vn1.5 is not essential for CrBV entry into host cells, it is required for CrBV gene expression in host hemocytes. In the absence of Vn1.5, CrBV transcription was not detected and hemocyte behaviour was not changed.

Vn4.6 has an open reading frame (ORF) of 129 nucleotides encoding 42 amino acids with a molecular mass of 4.6 kDa. The coding region for Vn4.6 is located upstream in opposite direction of a gene coding for a *C. rubecula* PDV-protein, Crp32. Transcripts corresponding to Vn4.6 were detected only in the venom gland and Crp32 only in the ovary (Asgari *et al.*, 1998). Sequence similarity searches using PRINTS blast search showed that Vn4.6 protein has 25.5% identity with the neurotoxin ω-atracotoxin-HV1A from the Australian funnel web spider *Hadronyche versuta*. Vn4.6 interferes with the activation of the host hemolymph prophenoloxidase (proPO), but not completely. This inactivation might have synergistic effects in conjunction with Vn50 (see below) and the

immuno-suppressive action of polydnaviruses introduced by the female into the host hemocoel.

The complete open reading frame of Vn50 contains 1167 nucleotides encoding 388 amino acids with molecular mass of 50 kDa. Vn50 is heavily glycosylated. Sequence homology searches at GenBank showed that Vn50 has high similarity to serine proteinase homologs (SPHs). These proteins, including Vn50, do not have proteolytic activity since the serine at the active site of the proteinase-like domain is changed to glycine. Vn50 sequence contains all the cysteine residues conserved in amino-terminal clip and serine proteinase domains of SPHs. Vn50 is stable in the host hemolymph – it remained intact for at least 72 h after being introduced into the host. Results show that Vn50 did not inhibit active phenoloxidase (PO) or proPO-activating proteinase, but it significantly reduced the proteolysis of proPO. This pathway might be employed by this endoparasitoid to negatively impact the activation in its host to suppress the host immune responses.

C. rubecula calreticulin (CrCRT) has been isolated from both polydnaviral particles and venom gland. The open reading frame of CrCRT contains 1209 nucleotides coding for 403 amino acids. At the end of the C-domain, a conserved HDEL motif was identified. Sequence alignment in GenBank showed a high similarity between the gene product of the isolated cDNA and CRTs. The highest similarities were found with *Galleria mellonella* (73.8%) and *Anopheles gambiae* (69.2%) CRTs. The predicted size for CrCRT is 46.5 kDa with a pI of 4.40, although CRT usually runs atypically at 60 kDa on SDS-PAGE. Slot blot analysis also showed that CrCRT is highly expressed in the venom glands and ovaries compared to the rest of the body. This protein can inhibit hemocyte aggregation and spreading, and this inhibition is dose-dependent. It also protects abiotic objects against host encapsulation response. Since host-specific CRT was identified from the host, this suggests that the soluble parasite-specific CrCRT might function as an antagonist molecule, competing for the binding site(s) with the host CRT.

Moreover, factors influencing the entry of CrBVs into host hemocytes were investigated. The results showed that CrBVs most likely initiate uptake at low pH in a pathway analogous to that of pH-dependent viruses. Venom proteins are not essential

for CrBV entry into host hemocytes, although they are required for CrBV gene expression in host hemocytes. Venom proteins from this wasp do not have antibacterial activity (data not shown).

Abbreviations

Amp	Ampicilline
AMV	Avian Myeloblastosis virus
APS	Ammonium persulfate
p-APMSF	4-Amidinophenylmethanesulfonyl fluoride
BAPNA	4 mM Nα-benzoyl-DL-Arg-p-nitroanilide
BB4	*E. coli* strain
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
CsIV	*Campoletis sonorensis*
cDNA	Complimentary deoxyribonucleic acid
CkBV	*Cotesia karyai* bracovirus
CrBV	*Cotesia rubecula* bracovirus
CrCRT	*Cotesia rubecula* calreticulin
CrHs70	*Cotesia rubecula* heat-shock protein 70
Crp32	*Cotesia rubecula* 32 kDa protein
CrPDVs	*Cotesia rubecula* polydnaviruses
CRT	Calreticulin
CrV1	Encapsidated hemocyte-targeted gene expressed by CrBV
CrV2	Encapsidated gene expressed by CrBV
CrV3	Encapsidated C-type lectin homologue expressed by CrBV
CrV4	Encapsidated gene of unknown function expressed by CrBV
CcPDV	*Cotesia congregata* polydnavirus
Da	Dalton(s)
DAB	3,3'-Diaminobenzidine
dCTP	Deoxycytosine triphosphate
DDC	Dopa decarboxylase
DDW	Double distilled water

DHC	Differential hemocyte count
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
DOPA	Dihydroxyphenylalanine
DT	Dopachrome tautomerase
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
EP1	member of early gene family expressed by CcIV
ER	Endoplasmic reticulum
ERPs	Early-stage encapsulation-relating proteins
et al.	*et alii* (and others)
FITC	Fluorescein isothiocyanate
g	Gram(s)
g	Gravity
GalNac	N-acetyl-D-galactosamine
GRs	Granular hemocytes
HdIV	*Hyposoter didymator* ichnovirus
His	Histidine
HPLC	High performance liquid chromatography
hpp	Hours post-parasitization
i.e.	*id est* (that is)
IgG	Immunoglobuline G
IML2	immunolectin-2
IPTG	Isopropyl-b-D-thiogalactopyranoside
JHE	Juvenile hormone esterase
kb	Kilobase pair(s)
kDa	KiloDalton(s)
LB	Luria-Bertani

LPS	Lipopolysaccharide
Lys	Lysine
M	Molar
M13	Promoter originating from M13 bacteriophage
mA	Milliampere(s)
MdBV	*Microplitis demolitor* bracovirus
mCi	Millicurie
μg	Microgram(s)
mg	Milligram(s)
μl	Microlitre(s)
mJ	Millijoule(s)
ml	Millilitre(s)
min	Minute(s)
μM	Micromolar
mM	Millimolar
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messanger RNA
MW	Molecular weight
NBT	Nitro blue tetrazolium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram(s)
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometre(s)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAP	proPO activating proteinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDVs	Polydnaviruses
PFA	Paraformaldehyde
pfu	Plaque forming unit

PLs	Plasmatocytes
PO	Phenoloxidase
pp	Post-parasitization
PPAE	Prophenoloxidase-activating enzymes
Pro	Proline
proPO	Prophenoloxidase
PTU	Phenylthiourea
r18S	*Pieris rapae* 18 S ribosomal DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
SAPNA	Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Second(s)
Ser	Serine
Sf21	*Spodoptera frugiperda* cell line 21
sp.	Species
SPH	Serine proteinase homolog
TAE	Tris-acetate/EDTA buffer
Taq	*Thermus aquaticus*
TBE	Tris-borate/EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-Tetrametylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris-hydroxymethyl-aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate

U	Unit(s)
UV	Ultraviolet light
V	Volt(s)
v	Volume(s)
VLP	Virus-like particle
Vn1.5	1.5 kDa *C. rubecula* venom peptide
Vn4.6	4.6 kDa *C. rubecula* venom protein
Vn50	50 kDa *C. rubecula* venom protein
v/v	Volume per volume
w/v	Weight per volume
×	Multiplied by or times concentration/volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1: Literature review

Insect immune system and immuno-suppression by insect parasitoids

1.1 Introduction

There are two lines of defence against parasitism in insects including external and internal defences. Insects possess an impressive network of protective immunity against foreign invaders, which starts with the physical barrier of the exoskeleton and is complemented by cellular and humoral components. The external defence systems include structural barriers preventing the penetration of invaders, behavioural adaptations supplying passive defences and secretions of protective substances. The internal defences are the primary immune system of insects towards endo-parasitoids, which are different from those of vertebrates. The understanding of insect immunity has advanced considerably over the past 30 years.

Although insects apparently lack an acquired immune response based on clonal selection of immunoglobulins, insects possess a complex and efficient immune system, defined as innate immunity, to defend against microbial pathogens and parasites. In general, the insect immune system consists of two types of responses that are tightly linked, cellular responses and humoral (cell-free) responses (Hoffmann, 1995). Cellular responses are mainly controlled by plasmatocytes and granulocytes and consist of phagocytosis, nodule formation and encapsulation. Humoral responses include the induction of immune peptides and proteins by fat bodies and hemocytes acting in the hemolymph to fight foreign invaders (Berg *et al.*, 1988). Both responses interact tightly in hemolymph to provide insects with effective protection against foreign agents (Vinson, 1990).

Interestingly, host immune responses are overcome by most insect parasitoids, which are important biological control agents of major insect pests. Parasitoids are able to

develop inside/outside the host bodies successfully. Ectoparasitoid wasps usually deposit their eggs outside the body of their hosts, where they complete their development by feeding on hemolymph. These developing parasitoids are exposed to the hemolymph-derived defence reactions in the gut. Endoparasitoids lay their eggs inside the hosts, directly encountering the host immune responses. To allow them to develop inside their hosts successfully, endoparasitoids must have evolved effective protective mechanisms against host immune responses (Strand & Pech, 1995). At the time of oviposition, endoparasitoids introduce several maternal factors into their hosts, which cause the changes in host physiology and development and suppress host immune responses. These factors include a calyx fluid containing ovarian proteins, symbiotic polydnaviruses (PDVs), virus-like particle (VLPs) and venom proteins (Edson *et al.*, 1981; Schmidt *et al.*, 1993; Jones & Coudron, 1993; Webb & Luckhart, 1994; Asgari *et al.*, 1996; Beckage, 1998). These factors from the two glands may act alone or together with one or more of the other factors.

In endoparasitoids which lack PDVs, venom seems to play a major role in host-parasitoid interaction and host regulation. Some investigations have revealed that venom proteins adversely affect the morphology, viability and immune function of hemocytes of host insects (Richards & Edwards, 1999; Richards & Parkinson, 2000). At sublethal doses, venom proteins have a potent anti-hemocyte action and can damage hemocyte-mediated immune responses (Parkinson & Weaver, 1999). In addition, it was reported that venom proteins from *Aphidius ervi* caused castration of their host (Digilio, *et al.*, 2000).

In endoparasitoids with polydnaviruses, PDVs are considered to play a major role in host physiological regulation and immune suppression (Beckage, 1998), although venom proteins alone can alter the growth and development of their hosts, and regulate host immune systems in some cases (Tanaka, 1987; Tanaka *et al.*, 1992; Nakamatsu *et al.*, 2001; Parkinson *et al.*, 2002b). PDVs have been shown to be essential for successful parasitism and development of the parasitoid inside the host (Edson *et al.*, 1981; Fleming, 1992; Beckage, 1998).

In certain host-parasitoid systems, PDVs are effective only when they are accompanied by venom proteins (Kitano, 1986; Stoltz, 1986). In *Cotesia melanoscela*, it was found that venom proteins might facilitate uncoating of PDVs *in vitro* and their persistence *in vivo* (Stoltz *et al.*, 1988). In *C. glomeratus* and *Apanteles kariyai*, venom proteins are essential for successful parasitism (Kitano, 1986; Tanaka, 1987). It is also proposed that venom proteins perform the rapid short-term immunosuppressive responses before the expression of PDV genes (Webb & Luckhart, 1996; Richards & Parkinson, 2000). It was also found that the venom proteins alone from *Miroplitis croceipes* reduced host larval growth (Gupta & Ferkovich, 1998).

In summary, parasitic hymenopterans lay their eggs into hosts, they also introduce maternal factors to escape or suppress the natural immune responses of hosts. These factors induce a decline in the responsiveness of cellular and humoral components so that parasitoid eggs are not eliminated. They have evolved complex mechanisms for modulating the cellular and humoral immune system of host insects. Here is a brief overview of how hosts defend themselves against parasitoids and reciprocally, how parasitoids suppress natural immunity of hosts. Finally, the factors mediating immune suppression will be discussed.

1.2 Insect immune system

The defence of insects against parasitoids and pathogens depends on the innate capacity to recognize and respond to the foreign invaders. Insects are capable of responding very effectively against foreign invaders, although their immune systems are relatively simple because they lack an antigen-antibody complex. Their immune systems, the primary internal defences of insects towards parasitoids and pathogens, are composed of both cellular (Boman & Hultmark, 1981) and humoral responses (Götz & Boman, 1985; Boman & Hultmark, 1987). Cellular defence responses refer to the activity of hemocytes, which are classified into several types. Each type has different functions in immune responses. Humoral defence responses mainly involve the hemocyte-free

hemolymph proteins. In insects, a number of humoral opsonizing proteins have been reported including lectins, hemolin, hemomucin, LPS-binding proteins ~~and so on~~ (Ladendorf & Kanost, 1991; Matha *et al.*, 1990; Theopold *et al.*, 1996, 1997; Trenzek, 1998). The anti-microbial peptides are important humoral molecules, which are produced by the fat body, hemocytes, midgut and epidermis (Russell & Dunn, 1996; Shen *et al.*, 1999). They can effectively kill bacterial and fungal pathogens. Although it has a cellular basis, the prophenoloxidase (proPO) activating system is also considered to be a humoral response (Söderhäll & Cerenius, 1998).

1.2.1 Recognition molecules

In the early 1950s, Burnet defined immunity as "the distinction by the body between self and non-self' (Cited in Rateliffe, 1989). To allow accurate and effective defence response to take place, organisms have to be able to recognise non-self and distinguish it from self at a molecular level. The recognition process must be very precise, and is considered to be the critical first step in a immune response. Pattern recognition also plays an important role in the initiation of the adaptive immunity in vertebrates (Fearon & Locksley, 1996; Janeway & Medzhitov, 2002).

Since insects lack antibodies, non-self organisms (pathogens and parasites) are detected by pattern recognition receptors, which recognize common chemical features present on the surfaces of non-self organisms (Janeway, 1989; 1992; Trenczek, 1998; Janeway & Medzhitov, 2002). Pattern recognition receptors are comprised of a limited number of molecules including C-type lectins (Kawabata & Iwanaga, 1999; Yu *et al.*, 1999; Yu & Kanost, 2000), β-1,3-glucan and lipopolysaccharide (LPS) recognition proteins (Beschin *et al.*, 1998; Lee *et al.*, 2000; Ma & Kanost, 2000; Ochiai & Ashida, 2000), a peptidoglycan binding protein (Ochiai & Ashida, 1999) and many other proteins (Sun *et al.*, 1990; Theopold *et al.*, 1996, 1997; Clark *et al.*, 1997; Faye & Kanost, 1997). In animals, C-type lectins are calcium-dependent carbohydrate binding proteins, which are involved in pathogen recognition, cellular interactions and innate immunity (Chen *et al.*, 1995; Weis *et al.*, 1998; Vasta *et al.*, 1999; Yu & Kanost 2003).

Several early-stage encapsulation-relating proteins (ERPs) have been isolated and characterized from the coleopteran insect, *Tenebrio molitor* (Cho *et al.*, 1999a, b). When the early stage encapsulated beads were coated with ERP-antibodies and reinjected into larvae, no further encapsulation was observed, which indicated that ERPs might play a crucial role in an early stage encapsulation response (Cho *et al.*, 1999a, b). Recently, a 47 kDa calreticulin was isolated from *Galleria mellonella* and considered to be an ERP as well. It is suggested that calreticulin might be involved in non-self recognition in cellular defence responses (Choi *et al.*, 2002).

When pattern recognition receptors bind to a non-self surface, a variety of innate immune responses is triggered. These responses include blood clotting (Muta *et al.*, 1996), proPO activation (Ratcliffe *et al.*, 1984; Ashima & Brey, 1998; Jiang & Kanost, 2000) and syntheses of antimicrobial peptides (Bulet *et al.*, 1999; Hoffmann *et al.*, 1999). For example, a *Manduca sexta* immulectin-2, binding to bacterial LPS, is involved in the proPO activation (Yu *et al.*, 1999; Yu & Kanost, 2000). However, little is known about the molecular mechanisms, which take place between the recognition processes and proPO activation.

1.2.2 Cellular responses

Cellular defence responses are mainly mediated by the insect blood cells, called hemocytes. In the cellular defence response, hemocytes are able to recognize non-self (Lackie, 1988). In the defence processes, both plasmatocytes (PLs) and granulocytes (GRs) are thought to be the most important hemocytes (Ratcliffe, 1993). Changes in cell morphology, behavior and types of cell composition have been studied with microscopy, monoclonal antibodies and lectins during infection (Mckenzie & Preston, 1992; Strand, 1994; Theopold *et al.*, 1995, 1996). Hemocyte responses to pathogens and parasites can be classified into three different types: phagocytosis, encapsulation and nodule formation depending on the number and size of foreign particles and organisms that intrude into insect hemocoel (Gupta, 1985; Vinson, 1990; Strand & Pech, 1995).

1.2.2.1 Phagocytosis

Phagocytosis was the first to be studied of the host defence responses of animals including invertebrates and vertebrates. It might be regarded as the major cellular response against foreign small particles and organisms such as bacteria, viruses, fungal spores, protozoa and other minute implanted materials. Both the PLs and GRs have been reported to be the predominant hemocytes involved in phagocytosis in insects (Gillespie *et al.*, 1997). The main processes of phagocytosis include recognition, attachment, endocytosis, ingestion and killing (Götz & Boman, 1985; Gupta, 1991). Differences in phagocytosis rates of PLs and GRs have also been observed *in vivo* and *in vitro* (Ehlers *et al.*, 1992).

The biochemistry of phagocytosis in insect hemocytes has not been investigated in detail. It is reported that microbial surface factors, such as glucans, can increase the phagocytosis rate of hemocytes (Huxham & Lackie, 1988). Moreover, phagocytosis may be stimulated by the proPO activating cascade as well (Gillespie *et al.*, 1997). However, there is no direct proof that any component of the proPO activation pathway enhances phagocytosis in insect hemocytes.

1.2.2.2 Encapsulation

In insects, encapsulation is a common and major defensive response against foreign objects that are too large to be phagocytosed by individual hemocytes. These large foreign particles and organisms include nematodes, insect parasitoids, protozoa and fungi (Strand & Pech, 1995; Gillespie *et al.*, 1997). As in phagocytosis, PLs and GRs are the two main hemocyte types involved in this response (Ratcliffe *et al.*, 1984; Strand & Pech, 1995). However, in *Drosophila melanogaster* lamellocytes are the cells forming a capsule around foreign materials, because this insect lacks GRs and PLs (Russo *et al.*, 1996).

Encapsulation processes involve three phases: recognition of the parasitoid as non-self structures, which is mediated by granular hemocytes, strong adhesion between

plasmatocytes when forming a capsule and killing of parasitoids primarily carried out by plasmatocytes (Strand & Pech, 1995; Gillespie *et al.*, 1997). However, in some cases, granulocytes can carry out the capsule formation (Pech & Strand, 1996). The role of granulocytes in this process involves an RGD-dependent cell adhesion mechanism since the addition of RGDs prevents capsule formation (Pech & Strand, 1995, 1996). Typical capsules consist of three cell layers (Ratner & Vinson, 1983). The innermost layers are located closely to the surface of the foreign object, where hemocytes show necrotic and autolytic signs. The intermediate layer is composed of tightly packed hemocytes that are extremely flattened. Outer layer is also composed of tightly packed cells, but still round (Ratner & Vinson, 1983; Strand & Pech, 1995). Very little has been known about the biochemistry of the encapsulation processes.

Encapsulation in insects is divided into two types: cellular encapsulation, mainly described in Lepidoptera as discussed above, and humoral (melanotic) encapsulation more typical for Diptera (Strand & Pech, 1995; Gillespie *et al.*, 1997). In certain dipterans, because there are fewer hemocytes in their hemolymph, humoral encapsulation involving the formation of a melanotic layer, plays an important role in host immune defence against parasitoids and pathogens (Vey, 1993).

Compared with phagocytosis, encapsulation is more complex in several ways. Firstly, encapsulation requires intercellular signaling for recruitment and cooperation of several types of hemocytes. Secondly, encapsulation requires that the first cells adhere to the surface of foreign invaders, the following cells then adhere to other cells, which probably involves other adhesion molecules. Finally, how is encapsulation regulated to keep the reaction localized and to stop the growth of capsules remains to be elucidated (Gillespie *et al.*, 1997)?

1.2.2.3 Nodule formation

When a number of small foreign substances such as bacteria, fungi spores and protozoa are too large to be removed from circulation by phagocytosis, nodule formation occurs (Ratcliffe, 1993). In the process of nodule formation, a coagulum is produced by the degenerating granular cells that are centrally melanized. Particulate and non-particulate materials are entrapped in the coagulum around the centre and surrounded by a sheath of hemocytes (Ratcliffe & Rowley, 1979). Finally, large multicellular hemolytic aggregates or nodules are formed. Nodule formation can be induced by surface components such as lipopolysaccharide (LPS), zymosan, laminarin and some glycoproteins (Lackie, 1988; Gillespie *et al.*, 1997). The hemocytes involved in the mechanisms of nodule formation are probably similar to encapsulation.

Substantial progress in understanding nodule formation and trapping of bacteria has been achieved in *Ceratitis capitata* (Marmaras & Charalambidis, 1992; Marmaras *et al.*, 1994; Charalambidis *et al.*, 1994, 1996). After being stimulated by LPS, *C. capitata* hemocytes secrete a 47 kDa protein, which caused *Escherichia coli* cells to aggregate (Marmaras & Charalambidis, 1992) and which might also be involved in the adhesion to hemocyte membranes. However, no role for this protein was detected in phagocytosis and encapsulation (Marmaras *et al.*, 1994).

1.2.3 Humoral responses

When bacteria are injected into the hemocoel of insects, they induce the synthesis of a number of antibacterial peptides and proteins (Boman, 1991). Based on antibacterial peptides and proteins that are either present constitutively or induced (Boman & Hultmark, 1987; Berg *et al.*, 1988), humoral immune responses of insects include synthesis of antimicrobial peptides and proteins, lectins, and cell adhesion molecules (Hultmark, 1993; Hoffmann, 1995; Karp, 1996; Vasta *et al.*, 1996; Gillespie *et al.*, 1997). Since the first insect antibacterial peptides, lysozyme and cecropin were discovered in 1968, more than 170 antibacterial peptides and proteins have been identified, which are grouped into 25 families based on sequence similarities (Gillespie

et al., 1997; Trenczek, 1998; Bulet *et al.*, 1999; Lowenberger, 2001). These proteins are classified into five groups and one miscellaneous group (Hoffmann, 1995). Each group of antibacterial proteins may have a specific mode of action. The induction of antibacterial and antifungal proteins is controlled by different mechanisms as discussed below (Lemaitre *et al.*, 1996; Lowenberger, 2001).

Nornally, antibacterial peptides are released into the hemolymph and found in concentrations ranging from 1 μM to 10 μM, depending on the molecular types (Hetru *et al.*, 1994). Fat body is the major tissue responsible for synthesis of inducible immune compounds (Lowenberger, 2001). Hemocytes, pericardial cells, Malphigian tubules and midgut also make smaller contributions to the synthesis of peptides and proteins (Mulnix & Dunn, 1994; Russell & Dunn, 1996; Gillespie *et al.*, 1997). Moreover, antibacterial peptides can be induced by epidermal cells in response to wounding and infection in the cuticle (Brey *et al.*, 1993; Lowenberger, 2001).

Antibacterial peptides cause harm to both gram-positive and gram-negative bacteria (and liposomes) but little or no harm to eukaryotic cells (defensins and magainins are slightly cytotoxic) (Boman, 1991). About the antifungal proteins, only one family of proteins from insect hemolymph has recently been discovered (Iijima *et al.*, 1993). Investigations of antifungal proteins would be of great interest.

Besides synthesis of antimicrobial peptides (Bulet *et al.*, 1999; Hoffmann *et al.*, 1999), humoral immune responses also include the hemolymph clotting (Muta & Iwanaga, 1996) and the prophenoloxidase activation system (Ashida & Brey, 1998). Hemolymph clotting is one of the important pathways against microbial infection by insects and wound response. Although many studies have been done in other arthropods, little is known about the clotting response of insect hemolymph. In insects, melanogenesis and encapsulation are considered as two independent events in immune responses (Rizki & Rizki, 1990; Marmaras *et al.*, 1996), but encapsulation following melanization is an effective immune response against parasites and pathogens (Nappi *et al.*, 1995; Strand

& Pech, 1995; Beerntsen *et al.*, 2000). Activation of the proPO system has been linked to the stimulstion of cellular responses (Johanson & Söderhäll, 1996).

Prophenoloxidase, the zymogen of PO, is probably activated by a cascade of serine proteinase, prophenoloxidase-activating proteinase (PAP) or prophenoloxidase-activating enzymes (PPAE), which exist in insect hemolymph as an inactive zymogen (proPAP) (Ashida & Brey, 1998). So far, it is unknown how many proteinases are involved in the cascade. Fig.1-1 shows the proPO activating system. In insects, melanin synthesis and production of toxic quinones around pathogens and parasites help to kill these invaders (Marmaras *et al.*, 1996), but the spread of these components may be harmful to hosts. Melanin, in nature, is limited in a small area such as invading sites, surface of nodules and surface of encapsulation objects.

1.3 Immune evasion by parasitoids

Unlike other life stages of insects, eggs lack a functional immune system until the later stages of embryogenesis (Abrams *et al.*, 1993; Strand & Pech, 1995). To successfully develop on or inside a host body, parasitoids have to evolve mechanisms to overcome host cellular and humoral immune responses. Parasitoids may evade the immune responses from their hosts in two ways. One is to evolve or acquire surface characteristics that are identical to or very similar to the surfaces within the host so that the latter fails to respond to foreign surface. This has been referred to as molecular mimicry or passive immune evasion. Another approach is to inject maternal factors that suppress the immune responses in their hosts. These factors include mutualistic viruses, venom proteins and ovarian proteins. The inactivation of the humoral and cellular capacity to respond to intruders is referred to as active immune suppression.

1.3.1 Passive mechanisms of immune evasion

Passive mechanisms of immune evasion includc developing in locations that protect the parasitoid from the host's immune response or possessing surface features that prevent

the parasitoids from being recognized as non-self. These factors could arise from the production of epitopes shared by the host, or the presence of surface factors that do not elicit a response. For example, a complex of glycoproteins and proteoglycans, which was found to often coat the eggs of microgatrine braconids and campoplegine ichneumonids, can prevent the eggs from encapsulation (Salt, 1964; Rotheram, 1967, 1973; Brewer *et al.*, 1973; Davies & Vinson, 1986; Asgari & Schmidt, 1994; Asgari *et al.*, 1998).

The only evidence that parasitoids might share homologous surface molecules with their hosts stems from the investigations of *Venturia canescens* and its host *Ephestia kuehniella*. Virus-like particles, lacking nucleic acid core, can protect parasitoid eggs from the host immune responses (Rotheram, 1967, 1973; Schmidt & Schuchmann-Feddersen, 1989; Schmidt & Theopold, 1990; Schmidt *et al.*, 1990; Hellers *et al.*, 1996). The study of Li (2002) showed that VLP1 had no enzymatic function but provided a possible passive protection role against host immune responses by interacting with membrane phospholipids.

In addition, mucinous components, which have been found to cover the egg surface in some cases, can also protect the eggs from the host immune responses in a number of parasitoid-host systems (Rotheram, 1973; Osman & Fuhrer, 1979; Davies & Vinson, 1986). Hemomucin, a novel insect mucin, was found to participate in hemolymph coagulation by attracting the pro-coagulant, lipophorin (Theopold *et al.*, 1996; Theopold & Schmidt, 1997). Li (2002) found that a hemomucin homologue in *Venturia* potentially protected the eggs from host cellular attacks.

1.3.2 Active mechanisms of immune evasion

Many endoparasitoids actively suppress the cellular immune responses of their hosts, assuring the successful development of their eggs. In most cases, the factors responsible for these alterations are produced by the adult female parasitoids and introduced into the hosts at oviposition. One of the most potent weapons used in suppressing their host

encapsulation response by parasitoids is a group of infective particles, called polydnaviruses, PDVs (Schmidt & Theopold, 1991; Summer & Dib-Haji, 1995; Beckage, 1995, 1998). Suppression of encapsulation by a PDV was first reported in 1981 (Edson *et al.*, 1981). In most instances, injection of purified PDVs into hosts results in suppression of encapsulation in a manner similar to natural parasitism. Usually, either eggs or any other foreign materials introduced into hemocoel of an immuno-suppressed larvae are not encapsulated (Lavine & Beckage, 1995; Strand & Pech, 1995; Hayakawa & Yazaki, 1997). In addition, there are some other factors involved in suppressing immune responses from hosts, including ovarian proteins, venom proteins and other proteins (Kitano, 1986; Dahlman, 1991; Hayakawa *et al.*, 1994; Luckhart & Webb, 1996, Hayakawa & Yazaki, 1997).

1.4 Factors mediating immune suppression

Parasitoids are able to escape or suppress the immune reactions of their hosts (Vinson, 1990). Several parasitoid-associated factors appear to be involved in mediating immune suppression. They include ovarian proteins (Luckhart & Webb, 1996; Webb & Luckhart, 1996), PDVs (Lavine and Beckage, 1995; Strand & Pech, 1995), venom proteins (Kitano, 1986; Tanaka, 1987), proteins covering the egg surface (Davies & Vinson, 1986) and teratocytes (Dahlman, 1991). Effects of parasitoid-associated factors encompass modification of the hemocytes and differential hemocyte count (DHC), as well as alteration of hemocyte morphology and behaviour. These factors might act alone or in connection with one or more other factors (Webb & Luckhart, 1996; Hayakawa & Yazaki, 1997).

1.4.1 Ovarian proteins

Ovarian proteins usually derive from the calyx tissue. They are abundant in the oviduct lumen and are introduced into host insects together with eggs during oviposition (Shelby & Webb, 1999). In *Campoletis sonorensis*, a 29 kDa ovarian glycoprotein was found to be synthesised in calyx cells and encoded by a single copy parasitoid gene

(Luckhart & Webb, 1996). Ovarian proteins from *C. sonorensis* could be detected in host hemolymph for four days but lost their protective role in the second day after parasitization (Shelby & Webb, 1999). These proteins bind to both GRs and PLs, and are taken up by endocytosis and in the process disrupt the cytoskeleton, thus preventing hemocyte encapsulation (Webb & Luckhart, 1996; Shelby & Webb, 1999). Digilio *et al.* (1998) reported that the ovarian fluid from *Aphidius ervi* arrested its host's development. SDS-PAGE analysis of hemolymph samples obtained from the aphids suggested that the active component involved in this function is an ovarian protein (Digilio *et al.*, 1998).

It has also been proposed that ovarian proteins mediated the rapid and short-term immuno-suppressive reactions before the expression of PDV genes begins (Asgari & Schmidt, 1994; Webb & Luckhart, 1994, 1996). In *Heliothis virescens* larvae parasitised by *C. sonorensis*, CsPDV-encoded proteins could not be detected until 6 h after parasitisation. It is suggested that before the CsPDV gene expression, ovarian proteins complement the rapid and short-term suppression of immune reactions (Webb & Luckhart, 1994, 1996). Interestingly, ovarian proteins were first identified due to high antigenic similarity with viral structural proteins (Webb & Summers, 1990).

1.4.2 Polydnaviruses

PDVs are symbiotic viruses associated with certain hymenopteran parasitoids belonging to the Braconidae and Ichneumonidae families (Krell *et al.*, 1982; Brown, 1986). They comprise the viral family Polydnaviridae with a polydisperse genome structure, which are organized as a series of different circular DNAs. PDVs are a unique group of double-stranded DNA viruses. PDV DNAs are integrated into the genome of its parasitoid wasp (Flemming & Summers, 1991; Xu & Stoltz, 1991). Until now only a handful of PDV genomes have been studied in some detail (Webb, 1998) and shown to be vertically transmitted in each parasitoid species in Mendelian fashion (Stoltz *et al.*, 1986; Stoltz, 1990).

PDV genes are expressed with various temporal patterns in host tissues and cells. The expression of *Cotesia rubecula* bracovirus (CrBV) genes was shown to be strongly transient with expression taking place in a period of 4 to 8 h pp (Asgari *et al.*, 1996; Glatz, 2003). The infected hemocytes could recover from disruption after several days (Asgari *et al.*, 1996). CrBV genes were abundantly expressed in host hemocytes at 6 h pp. *C. karyai* bracovirus (CkBV) genes were also found to be abundantly expressed in host hemocytes up to 6 h pp (Yamanaka *et al.*, 1996). By contrast, in most other parasitoids, PDV transcripts could be detected for many days. For example, EP1 could be detected after 6 days (Harwood & Beckage, 1994), and *Hyposoter didymator* ichnovirus (HdIV) after 10 days (Volkoff *et al.*, 1999). Between them, there are some intermediate cases, for example, *Microplitis demolitor* bracovirus (MdBV) genes were highly expressed at 12 h pp, then down at 24 h and almost undetectable at 96 h (Strand & Pech, 1995).

PDVs are replicated in the female reproductive tract, released into the oviduct lumen and injected into the host insects during oviposition, together with eggs (Webb, 1998). Inside the host, PDV particles infect different tissues and virus genes are expressed in the presence of viral DNA but no viral replication has been observed in host tissues (Fleming *et al.*, 1983; Theilmann & Summers, 1988). PDVs play an important role in the disruption of both cellular and humoral immune responses (Edson *et al.*, 1981; Stoltz & Guzo, 1986; Summer & Dib-Haji, 1995; Asgari *et al.*, 1996, 1997; Beckage, 1998; Shelby & Webb, 1999). In addition, PDV-expressed products lead to developmental arrest of the hosts, reduction of host feeding and growth, pigmentation anomalies and precocious metamorphosis (Beckage, 1998; Shelby & Webb, 1999; Glatz *et al.*, 2004). The effects of PDVs on the host physiology and immune responses are listed in Table 1-1.

Table 1-1: Effects of polydnaviruses on host physiology and development

Effects	References
Development arrest, larval growth and pupation	Dalhmann & Vinson (1977); Vinson *et al.* (1979); Thompson (1983); Beckage *et al.* (1990); Hayakawa (1991); Tanaka (1992); Dushay & Backage (1993); Hayakawa & Yasuhara (1993); Fathpour & Dahlmann (1995); Latine & Beckage (1996); Gupta & Ferkovich (1998); Pennacchio *et al.* (1998); Nakamatsu *et al.* (2001)
Reduction of food consumption and utilization	Guillet & Vinson (1973); Nakamatsu *et al.* (2001)
Detrimental effects on host reproduction	Junnikkala (1985); Yagi & Tanaka (1992); Tanaka *et al.* (1994); Brow & Friedländer (1995); Jones (1996); Reed & Beckage (1997); Brown & Reed (1997); Tagashira & Tanaka (1998)
Changes in hemolymph proteins	Greene & Dahlmann (1973); Frokovich *et al.* (1983); Cook *et al.* (1984); Beckage *et al.* (1987); Thompson *et al.* (1990); Beckage & Kanost (1993); Beckage *et al.* (1994); Hawood *et al.* (1994)
Alteration of fat body metabolites and protein storage	Tanaka (1986); Thompson *et al.* (1990)
Changes in hemocyte number, morphology and behaviour	Wago & Tanaka (1985); Stoltz & Guzo (1986); Guzo & Stoltz (1987); Davies *et al.* (1987); Prevost *et al.* (1990); Latine & Beckage (1996); Asgari *et al.* (1997)
Suppression of cellular immune responses	Edson *et al.* (1981); Stoltz & Guzo (1986); Guzo & Stoltz (1987); Dushay & Beckage (1993); Lavine & Beckage (1995; 1996); Summers & Dib-Hajj (1995); Asgari *et al.* (1996, 1997); Beckage (1998); Glatz *et al.*, (2004b)
Suppression of humoral immune responses	Stoltz & Cook (1983); Stoltz & Guzo (1986); Beckage *et al.* (1990); Latine & Beckage (1996); Shelby et al. (2000)

1.4.2.1 Polydnavirus-mediated changes in cellular responses

In endoparasitoids, PDVs protect the developing larvae from cellular immune reactions by the host insects. Edson *et al.* (1981) demonstrated that *C. sonorensis* eggs were encapsulated and melanised in the absence of CsPDV and that host defences were abrogated by the introduction of CsPDV. It was also found that a *C. kariyai* PDV-associated gene was probably secreted and heavily expressed at a time period corresponding with disruption of encapsulation (Hayakawa *et al.*, 1994; Yamanaka *et al.*, 1996; Hayakawa & Yazaki, 1997). A protein from *C. rubecula* PDV (CrV1) is able to block hemocyte encapsulation by disrupting the cytoskeleton of hemocytes (Asgari *et al.*, 1996, 1997). Strand *et al.* (1999) found that four to six hours after being infected by *Microplitis demolitor* PDVs, host plasmatocytes lost the ability to adhere to surfaces of foreign objects.

1.4.2.2 Polydnavirus-mediated changes in humoral responses

It was found that CsPDV infection leads to reduction in larval plasma melanisation (Stoltz & Cook, 1983), which indicates that polydnaviral infection may compromise humoral elements of the host immune responses. CsPDV infection reduces activities of host hemolymph phenoloxidase (PO), DOPA decarboxylase (DDC) and dopachrome tautomerase (DT), enzymes involved in melanisation and eumelanin formation (Shelby & Webb, 1999). Decreases in the enzymes' activity appear to be attributable to post-translational down-regulation, a means by which other viruses have been shown to regulate cell physiology (Pleogh, 1998; Shelby & Webb, 1999). However, the specific mode of action in this regard is still unknown.

When parasitized or injected with viruses, the activity of the antibacterial proteins, cecropin and lysozyme, was decreased in three permissive hosts, but not in a non-permissive host (Shelby *et al.*, 1998). Further study found that the lysozyme mRNA in infected tissues was shown to be normally induced, which indicates that the reduction of lysozyme activity might be post-translational. After parasitization by *C. sonorensis*, the induction of a LPS-induced hemolymph protein, phospholipase C, in the host *H.*

virescens was inhibited, which potentially leads to the decrease in resistance against bacterial infection (Shelby & Webb, 1999).

1.4.2.3 Polydnavirus-mediated changes in host development

Apart from the effects on cellular and humoral responses discussed above, PDV infection has also been correlated with altered host metabolism, leading to developmental arrest of the host insect, reduced host feeding and growth, pigmentation anomalies and precocious metamorphosis (Beckage, 1998; Shelby & Webb, 1999). The developmental arrests of host *M. sexta* by *C. congregata* parasitism have been studied in some detail (Dushay & Beckage, 1993; Beckage & de Buron, 1993; Beckage *et al.*, 1994; Alleyne & Beckage, 1997). However, no PDV genes have been directly linked to these functions.

CsPDV produces several pronounced and persistent effects on host larvae (Shelby & Webb, 1999). After parasitization, concentrations of amino acids and trehalose in plasma are increased, while overall protein concentration is decreased (Vinson, 1990). Further studies indicated that the reduction of amino acids and proteins in the plasma was due to post-translational down-regulation in fat body synthesis of storage proteins, arylphorin and riboflavin-binding protein (Shelby & Webb, 1994, 1997). Parasitized larvae could not complete their moult although they might still develop and moult after infection (Shelby & Webb, 1999)

Dover *et al.* (1988) demonstrated that CsPDV alone was responsible for developmental arrest in *H. viriscens* larvae and that the percentage of larvae arrested was proportional to the infective dosage. After being injected with virus suspension, larvae were found to be temporarily arrested at the stage injected and then recovered to develop normally. Synthesis of juvenile hormone esterase (JHE) is normally detected in *H. viriscens* on the third day of the fifth instar (Shelby & Webb, 1999), but it was inhibited by CsPDV infection (Shelby & Webb, 1997). It is thought that the absence of plasma JHE leads to high plasma juvenile hormone concentrations and thus the host pupal development was abrogated (Shelby & Webb, 1999).

1.4.3 Venom proteins

Venom components consist of different molecular weight proteins, polypeptides, polyamine-like nucleic acids and inorganic salts. Two-dimensional SDS-PAGE analysis of venom proteins of *Chelonine* wasps detected the presence of about 40 different kinds of proteins, most of which were acidic, although several were found to be quite basic (Jones & Leluk, 1990). Venom components are secreted by the venom gland and stored in a venom reservoir. They are introduced into the host body together with eggs during oviposition. It was observed that most venom components entered the host during oviposition, ahead of the entry of the parasitoid eggs (Leluk & Jones, 1989). Venom proteins can disrupt host development, disable hemocytes and mediate the pattern recognition of host to foreign organisms. In addition, venom proteins have a rapid, short-term effect suppressing the immune response, a function that is subsequently taken over by the PDVs (Webb & Luckhart, 1996).

1.4.3.1 Ectoparasitoid venom proteins

In general, ectoparasitoids have long been thought not to contend directly with the host immune system (Schmidt *et al.*, 2001), since they are not directly exposed. In fact, ectoparasitoids acquire nutrients from host body through a wound made in the host cuticle, and the damage of host cuticle by wasps should certainly stimulate specific components of the insect immune responses (St. Leger, 1991; Brey *et al.*, 1993). Exposure of hemolymph components at the gut lumen of the developing wasp larvae may cause defence-related damage. It is speculated that ectoparasitoids may purposefully deposit maternal factors, such as venom, into the wound site to inhibit specific aspects of host humoral and cellular immune responses (Strand & Pech, 1995).

With regard to ectoparasitoid wasps, it is known that maternal venom proteins induce host paralysis by paralytic factors, preventing attack of the wasp eggs and larvae by biting. They also regulate physiological states of the hosts to ensure the development of eggs and larvae (Uematsu & Sakanoshita, 1987; Coudron & Puttler, 1988; Coudron *et al.*, 1990, 1994; Quistad *et al.*, 1994; Coudron & Brandt, 1996; Weaver *et al.*, 1997;

River *et al.*, 2002). Table 1-2 shows the effect of ectoparasitoid venom on the physiological state and immune responses of the host. Paralysis may be transient, occurring immediately or complete and permanent (irreversible). Many toxins have been identified from ectoparasitic braconids, including high molecular weight proteins and low molecular weight peptides (Rivers & Denlinger, 1994; Coudron *et al.*, 1997; Nakamatsu & Tanaka, 2003; Beckage & Gelman, 2004).

Uematsu & Sakanoshita (1987) reported that venom of *Euplectrus kuwanae* resulted in developmental arrest of its host *Argyrogramma albostriata* and the arrest was dose-dependent. A venom dose of 0.1 to 0.05 female equivalents caused head-capsule slippage or partially tanning of integument. Nakamatsu & Tanaka (2003) observed a similar effect. Several venom proteins with different molecular weights have been isolated from several ectoparasitoid and confirmed to induce developmental arrest (Coudron & Brandt, 1996; Coudron *et al.*, 2000; Nakamatsu & Tanaka, 2003). Venom proteins also affect the protein and lipid composition of host hemolymph. It is found that the proteins and lipid in both hemolymph and fat body of host increased after parasitization (Rivers & Denlinger, 1994; Coudron *et al.*, 1997; Nakamatsu & Tanaka, 2003). It is suggested that enhancement of lipid and protein concentrations in the host hemolymph may be a benefit to the growth and development of the developing parasitized larvae.

After *Lacanobia oleracea* was parasitized by the ectoparasitoid wasp, *Eulophus pennicornis*, hemocyte-mediated recognition of foreign materials and phagocytosis were suppressed (Richards & Edwards, 2000). These hemocyte perturbations are not due to non-specific effects of nutritional deprivation, but rather may be specific factors; their source and nature are currently unknown (Richards & Edwards, 2000). Venom from *Nasonia vitripennis* led to an increase in Na^+ and activities of phospholipase C and A2 in cultured insect cells (Rivers *et al.*, 2002). Venom proteins led to a drastic reduction of circulating plasmatocytes due to cell death as well as inhibited the hemocyte spread (Rivers *et al.*, 2002). However, Richards & Edwards (1999) reported that venom proteins did not lead to hemocyte damage, changes of hemocyte viability and number.

Table 1-2: Effects of ectoparasitoid venom on the host physiology and development

Effects	References
Permanent paralysis and death	Visser *et al.* (1976, 1983); Bocchino & Sullivan (1981); Petters & Stefanelli (1983); Rivers & Denlinger (1995a); Rivers *et al.* (1999b); Weaver *et al.* (2001)
Development arrest, larval growth and pupation	Shaw (1981); Uematsu & Sakanosshita (1987); Kelly & Coudron (1990); Mackauer & Sequeira (1993); Rivers *et al.* (1993); Marris & Edwards (1995); Coudron & Brandt (1996); Gelman *et al.* (1997); Coudron *et al.* (1997, 1998, 2000); Richards & Edwards (1999); Nakamatsu & Tanaka (2003, 2004)
Metabolic regulation	Hayakawa (1987); Rivers & Denlinger (1994, 1995a,b); Morales-Ramos *et al.* (1995); Doury *et al.* (1995, 1997); Baker & Fabrick (2000); Nakamatsu & Tanaka (2003)
Changes in hemocyte viability, morphology and behaviour	Richards & Edwards (2002); Rivers & Denlinger (1995a); Rivers *et al.* (1993, 2002)
Suppression of cellular immune responses	Richards & Edwards (1999, 2000a, b, 2001, 2002a, b)

1.4.3.2 Endoparasitoid venom proteins

In endoparasitoids without PDVs, venom seems to play a major role in host immune suppression and host regulation. For example, in *Pimpla hypochondriaca* (Braconidae), venom adversely affects the morphology, viability, and immune function of hemocytes of the tomato moth, *L. oleracea* (Richards & Edwards, 1999, Richards & Parkinson, 2000). Digilio *et al.* (1998) found that venom proteins from *Aphidius ervi* (Braconidae) arrest its host development. In addition, venom from *A. ervi* caused castration of female *Acyrthosiphon pisum* aphids (Digilio *et al.*, 2000). Venom proteins from this endoparasitoid produced deleterious effects on the host reproductive system by 24 h following parasitization. After performing micro-injections in the non-parasitised host aphids, cellular alterations of the apical germaria of ovarioles were observed. The active components were two venom proteins with an approximate molecular mass of 21 kDa and 36 kDa (Digilio *et al.*, 2000).

In vitro studies have revealed that venom components of endoparasitoids adversely affected hemocyte morphology, viability and their phagocytic and encapsulation capability (Tanaka, 1987; Richards & Parkinson, 2000). It has been reported that venom produced a variety of effects on cellular morphology, including blebbing of the plasma membrane, degranulation, and the formation of cytoplasmic vacuoles (Richards & Parkinson, 2000). Venom could reduce cell viability up to 92 %, confirming that venom components were cytotoxic to hemocytes. In addition, venom reduced the capacity of hemocytes to phagocytose *E. coli* cells by 85 % (Richards & Parkinson, 2000). Together, the above results indicate that venom proteins have potent anti-hemocyte action and are able to impair hemocyte-mediated immune responses (Richards & Parkinson, 2000).

In endoparasitoids with PDVs, the functions of venom proteins include disabling hemocytes, arresting host development, mediating short-term immune suppression including cellular and humoral defence responses and enhancing PDV effects (Kitano, 1986; Tanaka, 1987; Webb & Summers, 1990; Webb & Luckhart, 1996; Nakamatsu *et al.*, 2001; Dani *et al.*, 2003, see Table 1-3). In *G. mellonella*, venom from *M. croceipes*

reduced larval growth (Gupta & Ferkovich, 1998). Nakamatsu *et al.* (2001) reported that calyx and venom proteins from *C. kariyai* synergically inhibited the growth and metabolic efficiency of *Pseudaletia separata* larvae. Junnikkala (1985) first reported in detail the detrimental effects on host reproduction by an endoparasitoid and speculated that a virus might be responsible for the effect. Tanaka *et al.* (1994) described that combination of PDVs and venom caused a reduction in testicular volume.

However, the role of endoparasitoid venom in suppressing host immune defence responses has not been clearly determined in most cases. In some braconid species, venom may contribute to inhibition of encapsulation by acting synergistically with calyx fluid or PDVs (Kitano, 1986; Tanaka, 1987, Wago & Tanaka, 1989). In such cases, the mechanism of action of venom is not clear, although it has been suggested that the supportive role of some venom proteins may entail facilitating the entry of PDVs into host cells (Stoltz *et al.*, 1988).

Table 1-3: Effects of endoparasitoid venom with PDVs on host physiology and development

Effects	References
Development arrest, larval growth and pupation	Tanaka (1987); Tanaka *et al.* (1992); Dushay & Beckage (1993); Allyne & Beckage (1997); Reed & Beckage (1997); Nakamatsu *et al.* (2001)
Reduction of food consumption and utilization	Guillet & Vinson (1973); Tanaka *et al.* (1992); Nakamatsu *et al.* (2001)
Detrimental effects on host reproduction	Yagi & Tanaka (1992); Tanaka *et al.* (1992, 1994); Reed & Beckage (1997); Tagashira & Tanaka (1998); Nakamatsu *et al.* (2001)
Alteration of fat body metabolites and protein storage	Tanaka (1986); Thompson *et al.* (1990); Nakamatsu *et al.* (2001)
Changes in hemocyte number, morphology and behaviour	Fitton *et al.* (1988)
Suppression of cellular immune responses	Tanaka (1987); Fitton *et al.* (1988); Leluk & Jones (1989); Jones & Leluk (1990); Dushay & Beckage (1993); Webb & Luckhart (1994); Morries *et al.* (1999)
Suppression of humoral immune responses	Dani *et al.* (2003); Asgari *et al.* (2003a, b)
Synergistic role with PDVs	Kitano (1986) Guzo & Stoltz (1987); Tanaka (1987); Wago & Tanata (1989); Strand & Dover (1991); Strand & Noda (1991); Strand *et al.* (1996)

1.5 The endoparasitoid wasp *C. rubecula*

C. rubecula (Hymenoptera: Braconidae) is a solitary endoparasitoid of white cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae), a serious worldwide insect pest of cruciferous crops. *C. rubecula* is considered to be an important biological control agent of *P. rapae* and has been introduced into many countries, e.g. Australia in the 1940s (New, 1994). There are four stages in its life cycle including egg, larva, pupa and adult, which are shown in Fig. 1-2.

Whilst considered to be specific to *P. rapae*, *C. rubecula* is recovered from the closely related species, *P. brassicae*, but the adults were significantly smaller, and presumably less fit (Harvey *et al.*, 1999). Although the mechanism is unknown, it is found that growth of parasitised larvae by *C. rubecula* was markedly inhibited in both host species. In both hosts, *C. rubecula* survived better, completed development faster and grew larger in earlier instar larvae than in later instar larvae. Adult wasps emerging from *P. rapae* were significantly larger than those from *P. brassicae*. At the early stage after parasitization, larval growth of *P. brassicae* was arrested (Harvey *et al.*, 1999). These results have shown that *P. rapae* was considered to be a more suitable host than *P. brassicae* for the development of *C. rubecula* (Harvey *et al.*, 1999).

Like other endoparasitoids, *C. rubecula* also introduces maternal secretions into host body together with eggs at oviposition to successfully protect the eggs and larvae inside host hemocoel. These secretions include Crp32, *C. rubecula* bracoviruses (CrBVs) and venom proteins (Asgari & Schmidt, 1994; Asgari *et al.*, 1996, 1997, 1998, 2003a, c; Glatz *et al.*, 2003). Crp32 and CrBVs manipulate the host immune system by a combination of two different mechanisms involving passive evasion (Asgari & Schmidt, 1994; Asgari *et al.*, 1998) and active suppression of the cellular responses (Asgari *et al.*, 1996, 1997; Glatz *et al.*, 2003). Both mechanisms are essential for the completion of wasp development. Before the expression of CrBV genes, Crp32 plays an important role to protect the egg against the immediate attack by host hemocytes (Asgari & Schmidt, 1994; Asgari *et al.*, 1998).

1.5.1 Crp32

Asgari & Schmidt (1994) discovered a layer of glycoprotein on the surface of *C. rubecula* egg, designated Crp32. The DNA sequence revealed that Crp32 is composed of an open reading frame of 765 bps coding a deduced protein of 253 amino acids with 32 kDa molecular weight (Asgari *et al.*, 1998). A transmembrane domain was found at the N-terminus. Interestingly, it was found that Crp32 could be a viral structural protein or a parasitoid-derived protein released into the calyx lumen together with virus particles (Asgari & Schmidt, 1994). This protein is produced in the calyx cells of the female wasp ovaries and attaches to the surface of the parasitoid egg and exterior of CrBV virions (Asgari & Schmidt, 1994; Asgari *et al.*, 1998).

Like many other braconid wasps (Davies & Vinson, 1986; Hayakawa & Yazaki, 1997), Crp32 was found to provide passive protection to eggs from encapsulation by host hemocytes in the time period before the CrBV gene expression (Fig. 1-3 A, B, C, D), which provide active suppression (Asgari & Schmidt, 1994; Asgari *et al.*, 1998). Crp32 might mimick host tissues and prevent non-self recognition and subsequent immune activation, because Crp32 has antigenic similarities with a 34 kDa protein from its host *P. rapae* (Asgari *et al.*, 1998).

1.5.2 CrBVs

Only four genes (Fig. 1-4) have been found from *C. rubecula* bracoviruses to be expressed in host tissues, which are designated as *CrV*1, *CrV*2, *CrV*3 and *CrV*4 (Asgari *et al.*, 1996; Glatz, 2003; Glatz *et al.*, 2003, 2004a). Like other PDVs, they only replicate in the wasp ovary and are abundantly expressed in different host tissues including hemocytes and fat body cells in a transient fashion between 4 to 12 h post parasitization (Asgari *et al.*, 1996; Glatz *et al.*, 2003; 2004a,b). In the host, they might act alone or together to provide active protection by suppressing host cellular responses (Asgari *et al.*, 1996, 1997; Glatz *et al.*, 2003).

*CrV*1 is a single copy gene containing an open reading frame (ORF) of 912 bps coding for a deduced protein of 304 amino acids with 45 kDa molecular weight (Asgari *et al.*, 1996). The deduced protein contains a hydrophobic signal peptide at the N-terminus with a possible cleavage site. *CrV*1 is mainly expressed in the host hemocytes and fat body cells and secreted into the extracellular space from infected cells. This protein inactivates host hemocytes by destabilizing the cytoskeleton (Asgari *et al.*, 1996, 1997, see Fig. 1-5). Recombinant CrV1 caused similar effects on host hemocytes in the presence of hemolymph, but not in hemolymph-free hemocytes, which indicates that CrV1 may undergo extracellular modifications or interact with plasma proteins (Asgari *et al.*, 1997). A coiled-coil domain containing a putative leucine zipper is thought to be important for lipophorin binding and uptake of CrV1 by host hemocytes (Asgari & Schmidt, 2002). In contrast to other systems (Le *et al.*, 2003), no cell death and apoptosis of hemocytes were observed in *P. rapae* caterpillars. CrV1-mediated inactivation of hemocytes is reversible.

CrV2 like CrV1 is also a glycoprotein with a deduced protein of 320 amino acids (Glatz *et al.*, 2004a). *CrV*2 transcripts were detected in both fat body cells and hemocytes, and expressed CrV2 protein was detected in fat body and hemocytes with a large amount in cell-free hemolymph, which indicates that CrV2 is secreted into hemolymph from infected cells as well (Glatz *et al.*, 2004a).

CrV3 is a C-type lectin homologue of about 17 kDa coded by a gene with an ORF of 480 bps producing a deduced protein of 159 amino acids (Glatz 2003; Glatz *et al.*, 2003). CrV3 is also produced in infected host hemocytes and fat body cells and subsequently secreted into host hemolymph. Preliminary *in vitro* experiments suggested that CrV3 may inhibit the spreading of host hemocytes and cause the agglutination of host hemocytes at high concentrations. It is possible that CrV3 might interact with the components in host hemolymph, resulting in the suppression of host immune responses (Glatz, 2003; Glatz *et al.*, 2003).

1.5.3 Venom proteins

Venom proteins are produced by a pair of specialized glands associated with the female reproductive tract and serected into venom reservoir (Fig. 1-6). In an earlier analysis, venom proteins from this endoparasitoid wasp were analysed using 2D-gel (two-dimensional) electrophoresis (Fig. 1-7) and reverse phase high-pressure liquid chromatography (rpHPLC)) (Fig. 1-8). By using 2D-gel electrophoresis, six major proteins were detected in the venom fluid, which were present in relatively large quantities (Fig. 1-7). However, rpHPLC showed that there are more proteins in the venom fluid (Fig. 1-8). The nature and possible role of these venom proteins in host regulation are still unknown.

1.6 Summary and program aims

For a successful parasitism, endoparasitoids have to circumvent the host immune responses using passive and/or active mechanisms. Eggs can use molecular disguise to prevent hosts from recognising them as non-self or be covered by immune factors that locally or systemically suppress host immune responses (Davies & Vinson, 1986; Asgari & Schmidt, 1994; Asgari *et al.*, 1998). During oviposition, endoparasitoid wasps introduce maternal factors into the host body, which are involved in host physiological regulation and host immune suppression. These factors include virus-like particles, symbiotic PDVs, ovarian and venom proteins (Edson *et al.*, 1981; Schmidt *et al.*, 1993; Jones & Coudron, 1993; Webb & Luckhart, 1994; Asgari *et al.*, 1996).

In endoparasitoids lacking symbiotic PDVs, venom proteins might play a major role in host-parasitoid interaction and host immune regulation. Some investigations have revealed that venom proteins adversely affect the morphology, viability and behaviour of host hemocytes and interfere with hemocyte-mediated immune responses (Richards & Edwards, 1999; Richards & Parkinson, 2000). In PDV-producing endoparasitoids, venom proteins alone play an important role in host regulation. Venom alone can disable host hemocytes, arrest host development, mediate short-term immune suppression including cellular and humoral defence responses and enhance PDV effects

to meet their own nutritional and developmental requirements (Kitano, 1986; Tanaka, 1987; Webb & Summers, 1990; Webb & Luckhart, 1996; Richards & Parkinson, 2000; Nakamatsu *et al.*, 2001; Dani *et al.*, 2003). It is also proposed that venom proteins complement the rapid short-term immunosuppressive responses before the expression of PDV genes (Webb & Luckhart, 1996; Richards & Parkinson, 2000).

In some instances, PDVs are effective only when they are accompanied by venom proteins (Kitano, 1986; Stoltz, 1987). In *C. glomeratus* and *A. kariyai*, venom proteins enhance the effects of PDVs and are essential for a successful parasitism (Kitano, 1986; Tanaka, 1987). Some venom proteins might provide protection for the eggs before the expression of PDV genes (Webb & Luckhard, 1994). In *C. melanoscela*, venom proteins are probably involved in the uncoating of PDVs *in vitro* and virus persistence *in vivo* (Stoltz *et al.*, 1988). However, the mode of action of venom proteins is not clear in such cases.

The PDVs from *C. rubecula* wasp have been studied in detail through the past few years (Asgari & Schmidt, 1994; Asgari *et al.*, 1996, 1997, 1998; Glatz 2003; Glatz *et al.*, 2003, 2004a). Four CrBV genes have been isolated and characterized (Asgari *et al.*, 1996; Asgari & Schmidt, 2002; Glatz 2003). Their functions in host regulation have been investigated in detail (Asgari *et al.*, 1996, 1997; Glatz 2003). However, there is no report about the venom components from this wasp and no information on their function in host regulation and immune suppression. Since the PDV functions from this wasp have now been characterized at the molecular level, the possible roles of venom proteins from *C. rubecula* in host regulation and the interaction of venom proteins with PDVs can be approached.

This study aims to isolate and characterise the proteins and genes from *C. rubecula* venom. Then, their functions in host regulation and interaction with PDVs will be studied. Increased knowledge of molecular interaction in *C. rubecula*/venom/*P. rapae* system may allow us to understand insect host manipulation with some venom components. This study will provide an experimental basis to study the functional roles in host regulations. In addition, some of the venom components might be useful for genetic improvement of microbial control agents of insect pests.

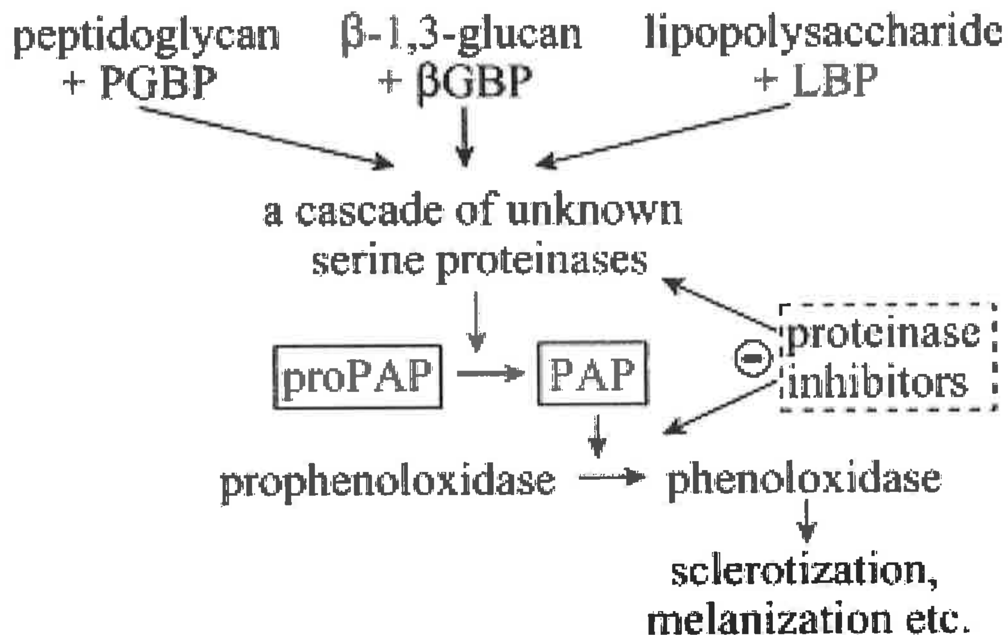

Fig. 1-1: The prophenoloxidase activating system in insect hemolymph (from Jiang & Kanost, 2000). Peptidoglycan from Gram-positive bacteria, lipopolysaccharide from Gram-negative bacteria, and β-1,3-glucan from fungi are recognized by specific binding proteins in hemolymph: peptidoglycan-binding protein (PGBP), lipopolysaccharide-binding protein (LBP), and β-1,3 glucan-binding protein (βGBP). Formation of recognition complexes somehow triggers a cascade of unknown serine proteinases. At the end of the proposed pathway, prophenoloxidase activating proteinase (PAP) is activated through limited proteolysis. Activated PAP cleaves prophenoloxidase to generate phenoloxidase. Prophenoloxidase activating proteinases contain one or two amino-terminal clip domains. Phenoloxidase catalyzes the formation of quinones that are precursors for melanization and sclerotization. The proteinases in the cascade may be regulated by serpins or other types of proteinase inhibitors in hemolymph.

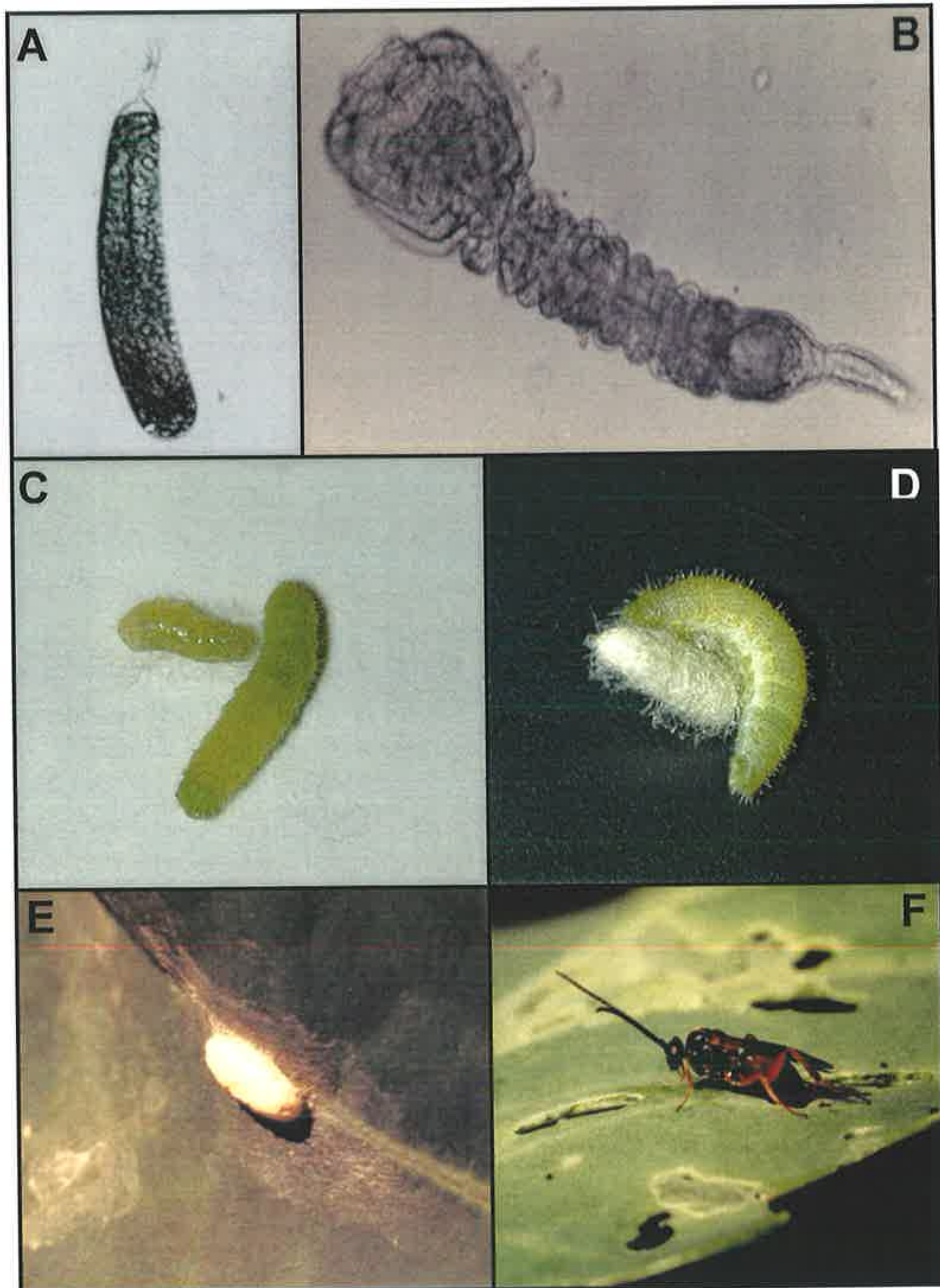

Fig. 1-2: Life stages of endoparasitoid, *Cotesia rubecula* (Braconidae: Microgastrinae). A, egg. B, first instar larva. C, last instar larva (left) after emerging from *Pieris rapae* larva (right). D, initial cocoon construction by last instar larva. E, mature cocoon of parasitoid containing the developing pupa. F, adult. (Photographs by Sassan Asgari and Mike Keller).

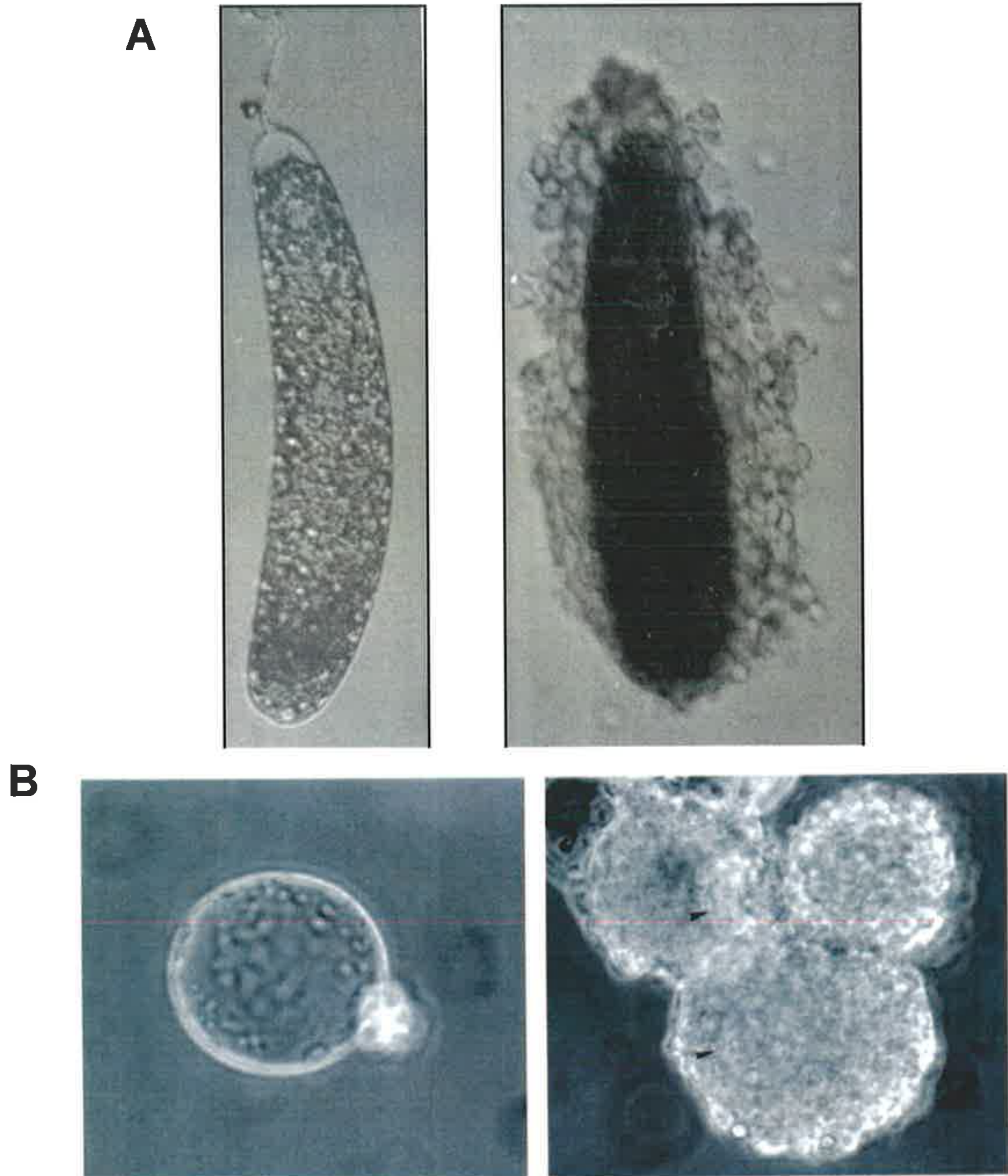

Fig. 1-3: (A) *In vitro* and *in vivo* encapsulation results (from Asgari *et al*., 1998). Beads coated with Crp 32 (*Left*) and beads covered with proteins from bacteria containing the expression vector (*Right*), were incubated with *P. rapae* hemocytes. In p32-covered beads no sign of hemocyte attachment was detected, whereas control beads were encapsulated. (B) Beads covered with Crp32 and masked with anti-CrV antiserum were incubated with *P. rapae* hemocytes (*Left*). *In vivo* encapsulation assay using beads covered with an unrelated plant virus protein were injected into caterpillars and dissected 24 h later (*Right*).

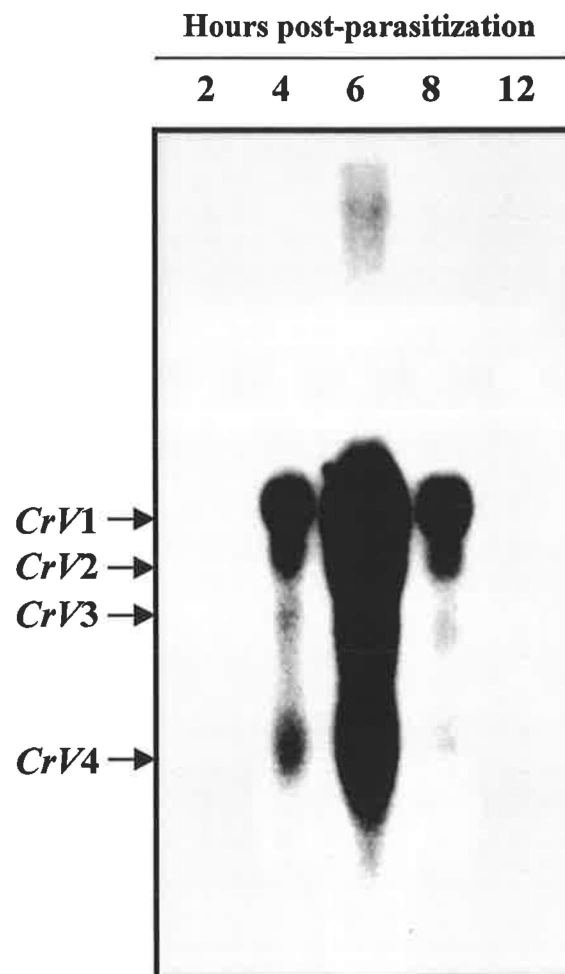

Fig. 1-4: Temporal expression of *C. rubecula* bracovirus genes in larvae of the host, *P. rapae* (from Asgari *et al.*, 1996). Four different sized transcripts were detected by Northern blot analysis of RNA extracted from host larvae, at various times post-parasitization, probed with total CrBV DNA. Transcript were designated *CrV1-CrV4* by decreasing size.

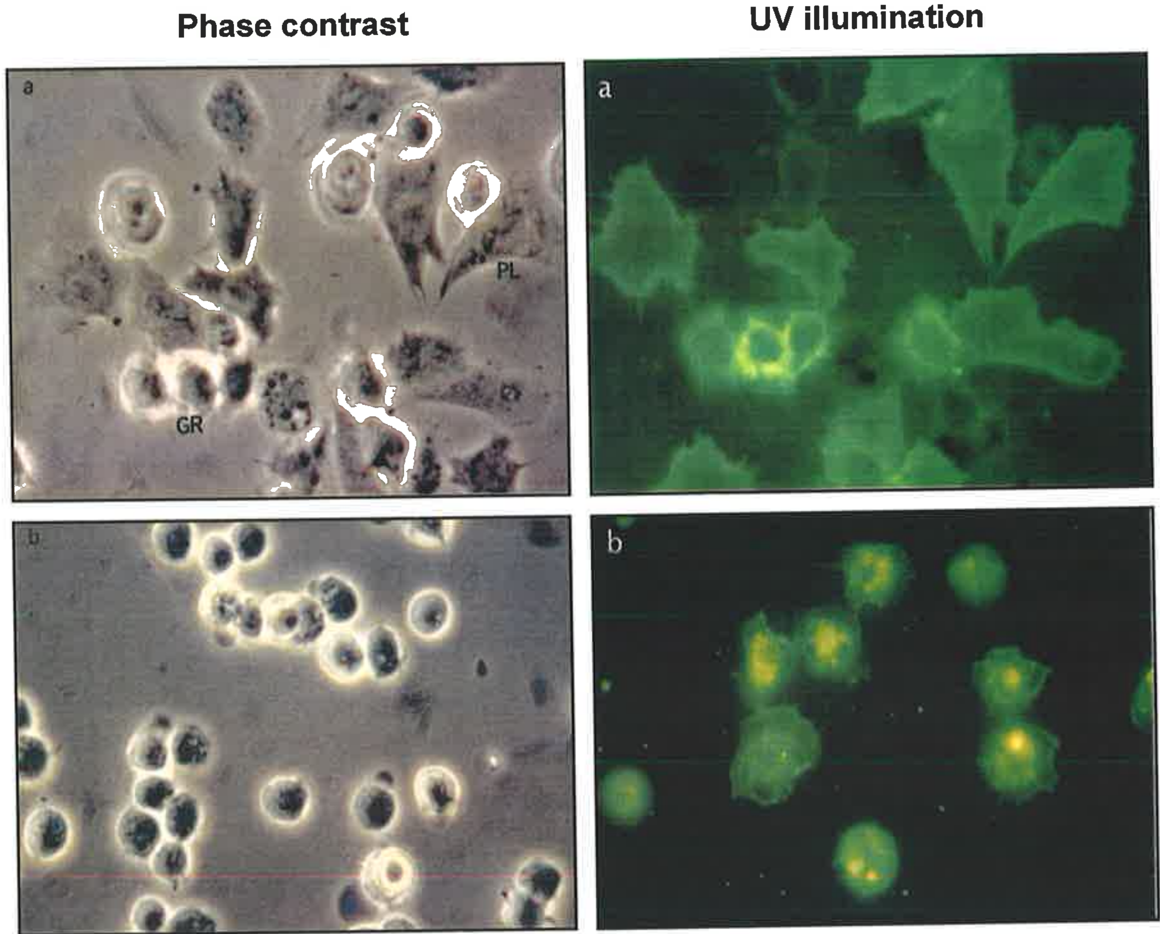

Fig. 1-5: Effect of CrV1 on CrBV-infected hemocytes (from Asgari *et al.*, 1996). Plasmatocytes (PL) and granulocytes (GR) were isolated from naive (upper) and parasitized (down) *P. rapae* larvae, before being staining with FITC-labelled phallodin, in order to visualise the hemocyte cytoskeleton under UV illumination. The cytoskeleton of infected cells is depolymerised and formed conglomerates in the cytoplasm whereas healthy cytoskeleton is distributed evenly on the cell surface.

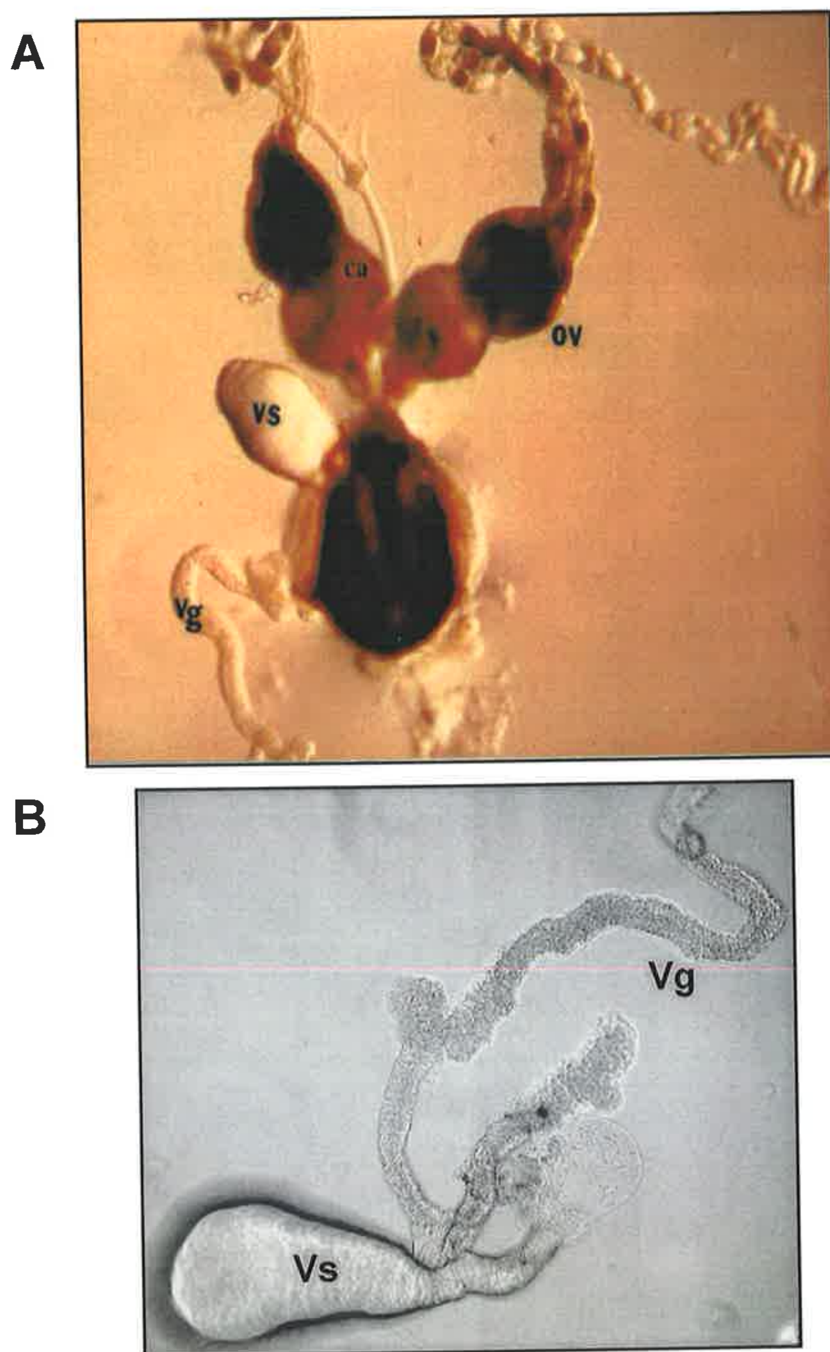

Fig. 1-6: A, Female reproductive tract removed from endoparasitoid, *C. rubecula*. Eggs are produced in the ovary (*Ov*) before passing into the calyx lumen (*Ca*), a neck-like region at the ovaries base. Wasp venom proteins are produced in the venom gland (*Vg*) and stored in the venom sack (*Vs*) before release into common oviduct (*Co*) lumen. B, the structure of separated venom glands (*Vg*) and venom sack (*Vs*). (Photographs by Sassan Asgari and Guangmei Zhang).

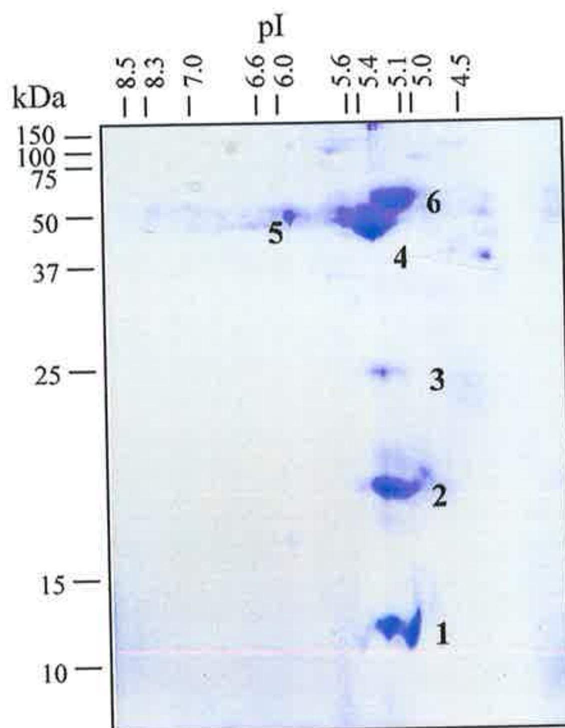

Fig. 1-7: 2-dimentional (2D) gel electrophoresis analysis of *Cotesia rubecula* venom proteins (from Sassan Asgari). Venom fluid from 10 female wasps was isolated and analysed on a 2D gel. Six major proteins were detected that are present in relative large quantities.

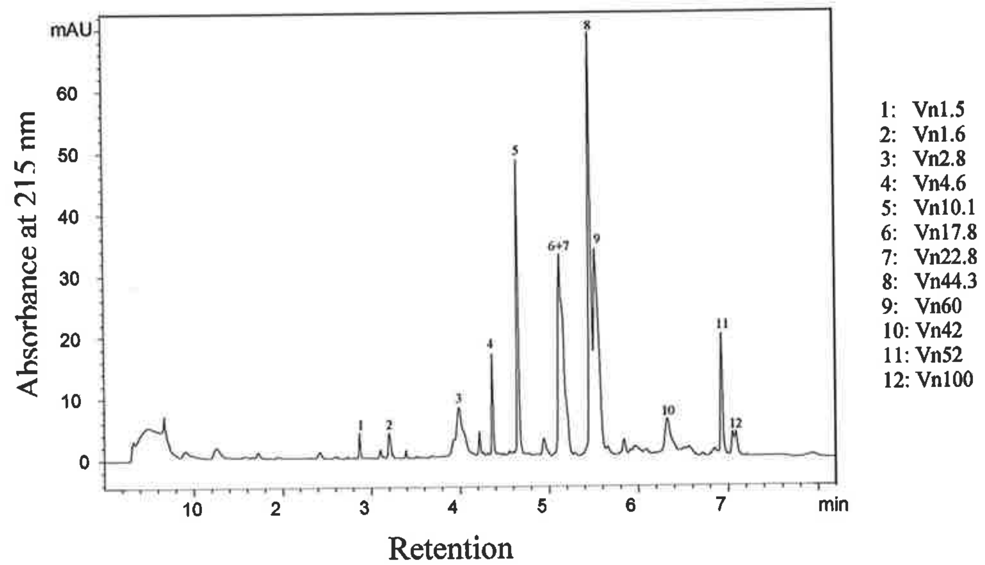

Fig. 1-8: Venom protein separation by rpHPLC. Crude *C. rubecula* venom proteins isolated from 50 female wasps were separated by reverse phase high pressure liquid chromatography (rpHPLC). Twelve main proteins and peptides were collected for further bioassays.

Chapter 2: General materials and methods

2.1 Introduction

In this chapter, the materials and methods often used in various experiments are described. Specific materials and methods are described in corresponding chapters. The solutions and media often used in the described protocols and experiments are also listed at the end of this chapter.

2.2 Insect culture

2.2.1 *Pieris rapae* culture

P. rapae was reared at about 25 °C, under a 14 h : 10 h (light : dark) photoperiod. Caterpillars were fed on cabbage plants until pupation. Then pupae were collected and put in small cages for emergence. Adults were fed on a 20 % honey-water solution in large flight cages for collecting eggs. One or two plants were introduced into this cage to collect eggs every week.

2.2.2 *Cotesia rubecula* culture

The wasps were maintained in the laboratory at 25 °C under a 14 h : 10 h (light : dark) photoperiod. Females and males were kept together for at least half a day to allow mating. Then 2-10 mated females were introduced into the cages with a number of 2nd instar caterpillars. Several drops of honey were added to the cage wall. Cocoons were collected and placed into a new cage for emergence.

To obtain parasitised larvae, *P. rapae* caterpillars were introduced in small containers along with female wasps. Oviposition was observed. Once the caterpillars were parasitised, they were removed and reared on cabbage leaves. The hemolymph and hymocytes are collected at indicated times.

2.3 *P. rapae* cell line culture

A *P. rapae* cell line, which was originally established from embryonic cells, was used in *in vitro* CrBV infection studies. Preliminary studies found that these cells supported CrBVs transcription and that transcripts were similar to those observed *in vivo*. Adherent cell line was maintained at 25 °C in modified insect cell culture medium TC 199-MK (Sigma).

2.4 Isolation of fat body, hemocytes and hemolymph

Fourth instar *P. rapae* larvae were surface sterilized in 70 % ethanol and bled into cold PBS (saturated with phenyl-thiuorea to prevent melanization) by removing one of the prolegs. Hemocytes were collected by centrifuging the hemolymph at 750 × *g* for 5 min at room temperature. Serum was removed and cells were resuspended in PBS or HyQ-SFX insect cell culture medium (HyClone) for further culture.

Gut and head capsule were removed, and fat body was washed with PBS. Sample was homogenized with a pestle in an eppendoff tube and centrifuged at 9300 × *g* for 10 min. The supernatant (fat body proteins) was collected and stored at – 20 °C.

2.5 Isolation of polydnaviruses

C. rubecula bracoviruses (CrBVs) were purified from newly-emerged female wasps as described previously (Beckage *et al*., 1994). Briefly, isolated ovaries were macerated in PBS by using micro-scissors. The suspension was centrifuged at 750 × *g* for 5 min to exclude large cellular debris. The homogenate was then passed through a 0.45 μm syringe filter (Ministart®) and centrifuged at 15800 × *g* for 20 min at room temprature. Pelleted viral particles were resuspended in PBS for use.

2.6 Extraction of DNA from tissues or cells

Procedure:

1) Tissues were ground by using a pestle or cultured cells were collected by centrifugation, then suspended in 400 μl of homogenization buffer (4 μl of Tris-HCl 1M pH 8.0, 8 μl of 0.5 M EDTA pH 8.0, 40 μl of 10 % SDS and 340 μl of H_2O) and homogenized.

2) After adding 3 μl of proteinase K (20 μg/ml), the sample was incubated at 40 °C for 2 h. To remove RNA, 0.4 μl of RNase A (10 mg/ml) was added, and the tube was incubated at 37 °C for 30 min.

3) After 400 μl of phenol was added, sample was gently mixed by inversion and left at room temperature (RT) for 5 min. Then the tube was centrifuged at 12500 × *g* at RT for 5 min, the upper phase was collected carefully avoiding the interphase, and transferred to a fresh tube.

4) To remove phenol residues, 400 μl of chloroform was added, mixed gently and then left at RT for 5 min.

5) The mixture was centrifuged again at 12500 × *g* for 5 min. The upper phase was removed and transferred to a new tube. To 200 μl of the upper phase, 400 μl of 100 % ethanol and 10 μl of 5 M NaCl were added. The tube was inverted carefully several times and placed on ice for 20 min.

6) To precipitate DNA, the sample was spun at 12500 × *g* for 20 min at room temperature, then the pellet was washed with 200 μl of 70 % ethanol and centrifuged at full speed for an additional 5 min.

7) Afterwards the supernatant was discarded and the pellet was dried at 65 °C for about 5 min in a heating block with the lid open.

8) Finally, the DNA pellet was dissolved in 30 μl of sterile double distilled water and stored at 4 °C for up to several weeks.

9) OD_{260} and OD_{280} were measured in the spectrophotometer to determine the quality and quantity of the DNA.

2.7 Preparation of plasmid DNA

BRESApure Plasmid Kit (Bresatec Ltd., Thebarton, SA, Australia) and Qiaprep Mini kits (QIAGEN Pty Ltd, Clifon Hill, Victoria, Australia) were used according to the supplier's protocols.

2.8 Extraction of DNA from gel

The Wizard™ PCR Preps DNA Purification kit (Promega) and Geneclean kit (BIO 101, California, USA) were used to isolate and purify DNA fragments from PCR or DNA restriction fragments out of agarose gels according to the manufacturer's instructions.

2.9 Extraction of total RNA

It was extremely important that all components were RNase free, so treatments were carried out before total RNA extraction. All the solutions were made using diethylpyrocarbonate (DEPC)-treated water (except Tris containing solution). The glassware was washed thoroughly with DEPC-H_2O and autoclaved.

For RNA extraction, acid guanidinium thiocyanate phenol chloroform method was used as following:

Procedure:

1) Insect tissues or cells were ground in 0.5 ml of solution D using a pestle in an Eppendeff tube.

2) Then following solutions were added sequentially: 50 μl 2 M NaAc, pH 4.0; 0.5 ml of phenol pH 4.3; 100 μl of chloroform (acid-saturated). The mixture was shaken vigorously on Vortexer for 10 sec and cooled on ice for 15 min.

3) The mixture was centrifuged at 15800 × *g* at 4 °C for 20 min. The upper water phase was collected in a new tube.

4) One volume of isopropanol was added to precipitate RNA at – 20 °C for at least 30 min or overnight. The mixture was centrifuged at 15800 × *g* at 4 °C for 20 min.

5) Pellet was dissolved in 0.3 ml of solution D and one volume of isopropanol to precipitate at – 20 °C for 30 min, followed by 20 min centrifugation at 4 °C.

6) The pellet was suspended in 300 μl of cold 70 % ethanol and centrifuged at 4 °C for 15 min. Pellet was dried at 65 °C for 5 min.

7) Resuspended pellet in 50 μl DEPC H_2O to dissolve the pellet at 65 °C for 15 min and concentration of RNA was determined by spectrophotometer.

Stock solution: dissolve 2.5 g guanidium thiocyanate in 3 ml of DEPC-H_2O, add 235 μl of 0.75 M sodium citrate pH 7.0 and 66 μl of 40 % sarcosyl at 65 °C.

Solution D: Mix 3.5 ml of stock solution with 25 μl of 2-mercaptoethanol.

2.10 Construction of a *C. rubecula* ovary/venom cDNA library

A *C. rubecula* ovary/venom gland expression cDNA library was constructed using SMART cDNA library construction kit (Clontech), according to the manufacturer's instructions. Briefly described as follows.

2.10.1 Total ovary/venom RNA extraction by acid guanidinium thiocyanate phenol chloroform method

Fifty female wasps were dissected to collect their ovaries and venoms. Total RNA was extracted according to the methods described as above.

2.10.2 SMART cDNA synthesis by primer extension

Procedure:

1) First-strand systhesis. 1.6 μg of total RNA was used. For the first-strand synthesis and primer extension step, all components and reaction vessels were pre-chilled on ice.

2) ds cDNA systhesis by primer extension. Cycles were determined by the amount of total RNA. 5 μl of reaction product was analyzed on a 1.1 % agarose/EtBr gel.

3) Proteinase K digestion.

4) *Sfi* I digestion.

5) cDNA size fractionation by CHROMA SPIN™-400 column. The single drop fractions were collected according to manufacturer's instructions. The profile of the fractions was checked on a 1.1 % agarose/EtBr gel. Collected the fractions containing desired sized distribution.

2.10.3 Construction of cDNA library

Procedure:

1) Ligation of cDNA to λTripIEx2 vector. An optimized protocol was used for ligation of SMART cDNA to the λTripIEx2 DNA provided by supplier.

2) Titration of the unamplified library and determining the percentage of recombinant clones by using CLONTECH's Advantage® 2 cDNA PCR kit.

3) Determining the percentage of recombinant clones.

4) Library amplification and titration (a successfully amplified library will have a very high titer ~10^{10} pfu/ml).

2.11 Screening of libraries with anti-venom antibodies

2.11.1 Screening library to isolate single positives

Procedure:

1) XL1-Blue was used. Bacteria were cultured at 37 °C overnight. Library was diluted and plated on 150 mm plates (about 2.5×10^4 plaques on one plate). Plates were incubated at 37 °C or 42 °C for about 5 h until plaques reached expected size.

2) Plaques formed on plates were induced for protein expression by overlaying plates with nitrocellulose membranes soaked in 10 mM IPTG and dried. Induction was carried out at 37 °C for 4-6 h.

3) Plates were placed at 4 °C for 30 min, membranes were then removed from plates and washed in TBST for 5 min to remove debris.

4) Membranes were blocked with milk blocking buffer (I) for 1 h at room temperature, then incubated with anti-venom antibodies (1:4000) at room temperature for 18 h.

5) After 4 times of wash with TBST, each for 15 min, membranes were incubated in blocking buffer (II) and alkaline phosphatase-conjugated secondary anti-rabbit antibody (1:5000) for 1 h (not over 2 h).

6) The membranes were rinsed 4 times with TBST, and stained with NBT and BCIP as described by Sambrook *et al.* (1989). Positive plaques were collected in SM buffer. If necessary, the re-screenings were done as above until single positive plaques were isolated. Single plaques were stored in SM.

2.11.2 Convert λTriplEx2 to plamid TriplEx2

The bacterial BM25.8 strain was used to convert λTriplEx2 to plamid TriplEx2 according to the manufacturer's instructions (Clontech). Briefly, BM25.8 was cultured at 31°C overnight. 200 μl of the culture was combined with 150 μl of a single positive plaque suspension and incubated at 31 °C for 30 min without shaking. 400 μl of LB medium was added and incubated at 31 °C with shaking for an additional 1 h.

LB/Ampicillin plates were prepared. About 10-20 μl of infected cell suspension was spread on each plate and incubated at 37 °C overnight to obtain isolated colonies containing TripIEx2 plasmids.

PCR with sequencing primers (5'-sequencing primer and 3'-sequencing primer) was performed to check the plasmid DNA. Plasmid DNA of selected clones was purified and inserts were sequenced in both directions.

2.12 Screening of libraries with DNA probes

The bacterial BM25.8 strain was used. To screen λTripIEx2 containing ovarian and venom gland-specific cDNA libraries, library suspension was diluted and plated on 150 mm plates (about 2.5×10^4 plaques on a plate). Plates were incubated at 37 °C or 42 °C for about 5 h until plaques reached expected size. After plates were placed at 4 °C for 30 min, DNA was transferred and fixed onto membranes by placing membrane on the plates for 10 min as described (Sambrook, 1989).

Probes were prepared as described (Ready-To-Go^{TM} DNA labelling beads-Amersham), with hybrisation at 65 °C overnight. Then filters were washed twice in 2 × SSC, 0.1 % SDS at 65 °C, each for 15 min. Washing was then continued at 65 °C with 0.2 × SSC 0.1 % SDS twice, each for an additional 15 min. The membrane was exposed to a film at – 70 °C for 24 h and aligned to the plates. Positive plaques were collected in SM. If necessary, the re-screenings were repeated until single positive plaques were isolated as above. Sequences were done as above in 2.12.

2.13 Determination of DNA and RNA concentration

The concentrations of purified DNA and RNA were measured using absorption spectrophotometry (Varian DMS 100S UV visible spectrophotometer) whereby DNA and RNA were diluted in H_2O and optical absorbance was measured at 260 nm (ABS_{260}). If absorbance was larger than 1, samples were diluted further and ABS_{260}

remeasured. Concentration was calculated using the following formula, where the nucleic acid constant is 50 for double strand DNA and 40 for RNA:

$$[\text{Nucleic acid}]\ (\mu g/\mu l) = \frac{ABS_{260} \times \text{dilution factor} \times \text{nucleic acid constant}}{1000}$$

2.14 DNA sequencing and sequence analysis

Sequencing reaction was set up as follows:

Big dye reaction mix	4 μl
Template DNA (1 μg)	x μl
Primer (3.2 pmol	2 μl
d H_2O	x μl
Total volume 20 μl	

In the reaction water's volume was determined by template DNA's volume.

To purify reaction products for sequencing, a fresh batch of 75 % isopropanol was made. 20 μl of sequencing reaction and 80 μl of 75 % isopropanol was added to a 1.5 ml tube. Tube was vortexed and left at room temperature for 15 min and then centrifuged at 15,800 × g for additional 20 min. Supernatant was discarded and 50 μl of 75 % isopropanol was added to wash the pellet. The tube was centrifuged for an additional 5 min and pellet was dried 5 min at 65 °C.

DNA sequencing was performed by Institute of Medical and Veterinary Sciences, Adelaide. Oligonucleotide Geneworks (Adelaide, South Australia).

Raw sequence data were refined and translated into the deduced amino acid sequence by using SeqEd software (1.0.3; Applied Biosystems). This software was used to align

various sequences. Mapdraw software (3.0.9; DNAstar Inc.) was used to identify restriction sites with these genes. Sequence similarities were compared with those that existed in the GenBank database by BLAST search accessed via NCBI wibsite (http://www.ncbi.nlm.nih.gov/blast).

2.15 Southern blot analysis

DNA samples (ideally 20 μg DNA) were run on a 1 % agarose gel in TAE buffer containing ethidium bromide as described (Sambrook *et al.*, 1989). Gels were photographed against a ruler to allow relative positioning of hybridized regions on the gels. To denature DNA, the gels were incubated in 1.5 M NaCl, 0.5 M NaOH for 45 min and then rinsed 4 times with deionised water and soaked in 1 M Tris (pH 7.4), 1.5 M NaCl for 30 min to neutralize.

2.15.1 Transfer of DNA from gel to membrane

DNA was transferred onto a nylon membrane (Amersham Biosciences) as described (Sambrook *et al.*, 1989). Briefly, 2 pieces of 3 mm Whatman paper were placed on a glass stand in a tray, 0.4 M sodium hydroxide was poured onto the paper so that the papers were completely wet and tray contained 1-2 cm sodium hydroxide. The gel was placed on the paper and rolled flat to remove air bubbles. A membrane was placed above the gel and 3 pieces of Whatman paper on the top. A stack of tissue papers and a weight were put on the top overnight to allow the suction of the buffer from the gel through the membrane. The transferred DNA was then cross-linked to the membrane by exposing to UV for 50 sec (BiRad UV cross-linker).

2.15.2 Pre-hybridisation and hybridization

The membrane was placed in a hybridization tube along with prehybrisation solution and incubated for at least 1 h. The hybridization was prepared as described (Ready-To-Go™ DNA labelling beads-Amersham). Briefly, 45 μl of probe specific nucleic acid

(25-50 ng) was boiled 5 min and placed immediately onto ice for 5 min to prevent reannealing. 45 µl of the probe and 5 µl of ^{32}P-labelled cytosine were added into a tube (Provided with kit) containing other nucleotides, random hexamer primers and the klenow fragment. The mixture was incubated at 37 °C for 30 min to allow production of labeled probe fragments. A spin column (provided with the kit) was vortexed and spun at 3000 rpm for 1 min to remove the buffer and labeled nucleotides. The mixture was then added to the column and the tube was spun for an additional 2 min to elute the probe. Pre-hybridised membrane was incubated with the eluted ^{32}P-labelled probe at 65 °C overnight with agitation.

2.15.3 Washing and autoradiography

The hybridization solution was collected into another tube and stored at – 20 °C for further use. The membrane was rinsed twice in 2 × SSC, 0.1 % SDS at 65 °C with rotation for 20 min. The washing was then continued at 65 °C with 0.2 × SSC, 0.1 % SDS for an additional 20 min. The membrane was wraped in a plastic bag and exposed to a film (Kodak) for 24 h or indicated time.

2.16 Northern blot analysis

RNA samples were run on a denaturing paraformaldehyde 1.2 % agarose gel containing ethidium bromide in 1 × MOPS buffer as described (Ausubel *et al.*, 1993; Sambrook *et al.*, 1989). Each sample was prepared as follows:

5 µl	10 × MOPS
10 µl	RNA suspension (20 µg RNA)
8.5 µl	37 % paraformaldehyde
25 µl	formamide

Sample was then heated at 65 °C for 15 min beforc addition of 10 µl of loading buffer. The transferof RNA from gel to membrane and hybridization were carried out as for

Southern blotting above except that denaturation and transfer were performed by using 10 × SSC.

2.17 DNA and RNA slot blots

DNA and RNA samples were blotted onto a nylon membrane (Amersham Biosciences) under denaturing conditions according to the manufacturer's instruction (BioRad). Probes were prepared as described (Ready-To-Go™ DNA labelling beads-Amersham). The blot was hybridized with ^{32}P-labelled probe at 65 °C overnight. Following overnight hybridization at 65 °C, membrane was rinsed twice in 2 × SSC, 0.1 % SDS at 65 °C, each for 15 min. The washing was then continued at 65 °C with 0.2 × SSC, 0.1 % SDS twice, each for an additional 15 min. The membrane was exposed to a film for 24 h. A *P. rapae* 18S ribosomal DNA fragment probe (Glatz, 2004) was used as a control for RNA slot blot.

2.18 Removal of hybridized probe from membrane to allow reprobing

Used membranes were washed in 0.4 M NaOH at 45 °C for half an hour. The membrane was then washed 3 times in probe-removal solution (0.1 × SSC, 0.1 % SDS, 0.2 M Tris-HCl pH 8.0) at 45 °C, each for 10 min. The washed membrane was examined with X-ray film and wrapped in plastic and stored at – 20 °C for later reuse.

2.19 Reverse Transcription-PCR (RT-PCT)

Total RNA was treated with RQ1 RNase-free DNase prior to RT-PCR according to the technical bulletin (Promega). RNA sample was afterwards purified with a standard phenol:chloroform extraction followed by an ethanol precipitation. For reverse transcription reactions, 1-2 μg RNA samples and 1 μl reverse primer (0.1 μg/μl) were added to DEPC-treated water (final volume 10.7 μl) and heated to 95 °C for 5 min and immediately placed on ice for 5 min. The following solutions were added to the

reaction: 3 µl 5 × AMV buffer, 0.5 µl AMV reverse transcriptase (Promega, 10 U/µl), 0.3 µl RNasin (Promega, 30 U/µl), 0.5 µl dNTPs (15 mM). The reaction was incubated at 42 °C for 1 h followed by 5 min at 95 °C to inactivate the enzymes.

A PCR reaction was performed by adding the following solutions to the RT reaction: 1 µl reverse primer (0.1 µg/µl), 1 µl forward primer (0.1 µg/µl), 0.5 µl dNTPs (15 mM), 3 µl 10 × PCR buffer, 0.4 µl Taq DNA polymerase (Promega, 5 U/µl) and water to the final total volume of 50 µl. The reaction, eletrophoretic separation and visualization protocols were as for standard PCR.

2.20 Preparation of transformation-competent cells

Bacterial cells were plated out on LB-plates containing appropriate antibiotics and incubated overnight at 37 °C. A single colony was picked and used to inoculate a starter culture of 5 ml LB medium (with antibiotics). The culture was grown at 37 °C with shaking overnight. Overnight culture was used to inoculate 100 ml of sterile LB medium (with antibiotics) in a 250 ml flask and cells were allowed to proliferate at 37 °C with shaking to a density, whereby $ABS_{600} \approx 0.5$ (Varian DMS 100S UV visible spectrophotometer). The culture was allowed to stand on ice for 30 min and then centrifuged at 750 × *g* for 5 min at 4 °C. From this step onwards the cells were always kept on ice. The pellet was gently resuspended in cold (4 °C) 30 ml TFB1 buffer and incubated on ice for 90 min. Then, the solution was centrifuged at 750 × *g* for 5 min at 4 °C. The supernatant was carefully discarded and the pellet gently dissolved in ice-cold 4 ml TFB2 buffer. Finally, 100 µl aliquots were prepared and snap frozen in liquid nitrogen. The competent cells were stored at – 80 °C.

2.21 Heat-shock transformation of competent *E. Coli* Cells

Frozen competent *E. coli* cells (100 µl) were removed from – 80 °C and thawed for about 5 min on ice and gently mixed before use. For each transformation, 50 µl cells were added to the DNA in a pre-chilled microfuge tube. The transformation mix was

gently mixed by flicking the tube and placed immediately on ice for 20 min. The cells were heat-shocked by placing them in a 42 °C water bath for 90 sec without shaking and then immediately placed on ice for 2-5 min. 450 μl LB medium was added to the transformation mix and incubated at 37°C for 90 min with shaking. If the bacteria are supposed to be tested for a-complementation (white/blue screening), 200 μl of IPTG (20 mg/ml) and 20 μl of X-gal (50 mg/ml) were spread onto LB plates supplemented with the appropriate antibiotic (e.g. 100 μg/ml ampicillin). The solutions were allowed to dry for at least 30 min at 37 °C. Several volumes of transformed *E. coli* cells (e.g. 10 μl, 100 μl and 400 μl, respectively) were plated out onto the pre-prepared antibiotic plates and incubated overnight at 37 °C. Individual colonies (white ones in case of a blue/white screening) were picked for PCR to confirm the correct DNA-insert.

2.22 Reverse phase high pressure liquid chromatography (rpHPLC) and mass spectrometry

Venom reservoirs from 50 female *C. rubecula* wasps were dissected and disrupted in water by using micro-scissors to release venom proteins into H_2O. Venom proteins were collected by centrifigation at 10,000 × *g* for 1 min for exclusion of tissue debris. High pressure liquid chromatography (HPLC) of samples was carried out in a Hewlett Packard 1090 Liquid Chromatograph. The crude venom sample was loaded onto a Vydac reverse phase C18 column and eluted at the flow rate of 0.2 ml per minute using a gradient of 5 to 100 % of buffer B (0.04 % trifluoroacetic acid, TFA, in 70 % acetonitrile) against buffer A (0.05 % TFA in water) over 82 min. Collected fractions were detected by absorbance at 214 nm and protein fractions were collected manually. Individual fractions were vacuum-dried and dissolved in sterile water.

To determine the molecular mass of the proteins, electrospray ionisation mass spectrometry was carried out using a PE Sciex API-100. Generally, an amount equivalent to ca. 7 picomoles of purified protein was directly infused and the results were analyzed using the BioMultiView 3.1.1 software.

2.23 SDS-polyacrylamide gel eletrophoresis (SDS-PAGE)

SDS polyacrylamide-gel electrophoresis was performed essentially according to Laemmli (1970). SDS, an ionic detergent, denatures the proteins as they migrate through the gel due to electric current. The gel consists of a stacking and a separating gel. Proteins were dissolved in loading buffer and heated 10 min in boiling water. Gel was run at 40 mA for about 1.5 h on Mini protein II Dual Slab Cell until dye was out of the gel. The pre-stained protein marker SeeBlue was used according to the supplier's instructions (Novex, San Diego, CA, USA).

2.24 Coomassie staining and destaining of polyacrylamide gel

The gel was immersed in Coomassie stain (0.25 g Coomassie Brilliant Blue R-250 in 90 ml of 50 methanol and 10 ml glacial acetic acid) and incubated at room remperature for about 30 min with gentle agitation. The Coomassie Stain was poured back for reuse and destain solution (10 % glacial acetic acid, 30 % methanol) was added to cover the gel and changed several times. The gel was incubated overnight in destain solution with tissue paper to absorb the colour. The gel was then removed and placed on wet Whatman paper and wrapped in plastic wrap for preservation.

2.25 Western blotting

The transfer of separated proteins from a gel onto a nitrocellulose membrane (Amersham) was carried out in a Mini Trans-Blot cell (Bio-Rad) applying 200 mA/70 V (or 220 mA/100 V) for about 1 h by using transfer buffer containing 192 mM glycine, 25 mM Tris-base and 20 % (v/v) methanol (Towbin *et al.*, 1979). The basis is to transfer negatively charged proteins from the gel to solid support (membrane) by applying an electric field.

Membranes were washed in PBS for 10 min. After pre-incubation of the filters in the first blocking solution (8 % w/v non-fat milk powder and 0.02 % sodium azide in

PBS) for 1 h at RT with gentle agitation, the primary antiserum was added to a specific final concentration (1:4,000). And the incubation was continued for an additional 2 h to overnight. Then, the filters were washed 4 times with moderate shaking, 15 min each in TBST. The alkaline phosphatase-conjugated secondary anti-rabbit antibody (Pierce) diluted 1:5,000 in the second blocking solution (5 % w/v non-fat milk powder, 150 mM NaCl, 50 mM Tris-HCl pH 7.5) was added and filters were incubated for 1-2 h at room temperature. The filters were washed 4 times, each for 15 min in TBST with moderate shaking. Finally, for the visualisation of the protein bands, the filters were stained in developing solution with NBT and BCIP as described by Sambrook *et al* (1989).

2.26 Expression and purification of fusion proteins in *E. coli*

For expression of recombinant proteins, the QIAexpress system (QIAGEN) was used. The regions coding for different venom proteins were amplified by PCR and subcloned in frame into type IV constructs (pQE-30, -31, -32). Subsequently, the recombinant protein was purified through the 6 × His tag.

PCR reactions were carried out by using different pairs of specific primers for different genes (see in different chapters). Resulting PCR fragments were digested with different enzymes, subcloned into the correct digested pQE30 expression vector. Afterwards, M15 cells were transformed and proteins were induced by adding 0.5 mM IPTG after incubation for 2 h. *E. coli* cells transformed with different plasmids were found to produce significantly different amounts of recombinant proteins. The induced fusion proteins were mainly detected in the insoluble fraction. Native protein was purified with Ni-resin column and eluted according to manufacturer's instructions.

2.27 Preparation of antibodies

For the preparation of a specific antiserum, recombinant proteins were purified on Ni-beads or further purified on a preparative polyacrylamide gel and used directly for

immunisation. The proteins were mixed with an equal volume of complete Freund's adjuvant for first injection and incomplete Freund's adjuvant for further boosters. Immunisations were performed according to Harlow and Lane (1988) and conducted by the Animal Care Centre, Waite Campus, Adelaide University, Australia.

2.28 Elution of antibodies

To confirm the identity of venom genes, antibodies were eluted from the positive clones. Western blots were performed with venom proteins using the eluted antibodies as probes. The elution of antibodies was done as follows:

After staining in developing solution with NBT and BCIP, the stained membrane was cut down and placed in washing solution. Three tubes containing 100 μl of elution buffer (0.05 M glycine-HCl pH 2.3, 0.05 M NaCl, 0.5 % Tween 20, 100 μg BSA) and 0.5 M Na_2HPO_4 neutralizing solution were prepared. The membranes were submerged in the first tube for 30 sec and immediately transferred to second tube. Neutralizing buffer was immediately added to a final concentration of 50 mM. The procedure was repeated for the second and third tube. Individual aliquots were pooled together and stored at 4 °C for reuse.

2.29 Hemolymph prophenoloxidase enzyme activity assay

The assay was carried out as described previously with minor modifications (Beck *et al.*, 2000). Hemolymph was collected from individual *P. rapae* larvae by surface sterilizing in 70 % ethanol and bleeding from a proleg into 100 μl ice-cold PBS. To obtain a cell-free serum, collected hemolymph was centrifuged at 750 × *g* for 5 min. Cell-free hemolymph was added to 900 μl fresh substrate solution (20 mM DL-3,4-dihydroxyphenylalanine in PBS, DL-DOPA) and mixed immediately. Absorbance at 490 nm was monitored at room temperature for 120 min using a DMS100 spectrophotometer (Varian Techtron). For inhibition assays, total venom proteins in PBS (10 μl), different fractions in PBS purified on rpHPLC (10 μl) or fusion

recombinant proteins were added to the above mixture and absorbance was measured as above. The experiments were repeated three times.

2.30 Concentrations of proteins using microassays with microtiter plates

To make 1 mg/ml BSA stock solution, 0.001g BSA was added to 1 ml TBS buffer. Then 0, 1, 5, 10, 15 and 20 μg/ml dilutions were made by addition of 0, 0.5, 2.5, 5, 7.5 and 10 μl of the stock BSA solution to TBS to a final volume of 500 μl. 160 μl of each dilution was used in each well and 40 μl of the Bio-RAD dye was added to each dilution and mixed by pippetting up and down. Then 160 μl from the dialysed samples and 160 μl of a 10 × diluted sample separated and purified from rpHPLC were used to measure the concentration. The plate was placed and read for about 20 min after mixing the dye with the samples.

The standard curve was made from data using the prepared BSA concentrations. The sample concentrations were determined by comparing the data of measured proteins with the standard curve.

2.31 Vectors, markers, antisera and proteins

AMV reverse transcripase	Promega Corp., Madison, WI, USA
Bovine serum albumin	Sigma Chemical Co., St. Louis, MI, USA
DNA marker (GeneRuler 100 bp)	MBI Fermentas, Vilnius, Lithuania
Goat anti-rabbit IgG (Fc), alkaline phosphatase-conjugated	Pierce, Rockfood, IL, USA
Goat anti-rabbit IgG, FITC-	Sigma Chemical Co., St. Louis, MI, USA

conjugated	
pGEM-T easy vector system	Promega Corp., Madison, WI, USA
pQE vectors	QIAGEN Inc., Chatsworth, CA, USA
Protein markers, SeeBlue & Mark12	Novex, San Diego, CA, USA
Proteinase K	Sigma Chemical Co., St. Louis, MI, USA
Restriction endonucleases	Promega Corp., Madison, WI, USA
RNase A	Boehringer Mannheim, Mannheim, Germany
Taq DNA polymerase (5 U/μl)	Promega Corp., Madison, WI, USA
T4 DNA ligase (3 U/μl)	Promega Corp., Madison, WI, USA

2.32 Solutions and media

Generally used solution and media are listed below, some solutions were also described in the corresponding method chapter.

Blocking buffer (I)	8 g non-fat dry milk, 0.02 % sodium azide, 100 ml PBS pH 8.0. Store at 4 °C
Blocking buffer (II)	5 g non-fat dry milk, 15 ml 1 M NaCl, 5 ml 1 M Tris-HCl pH7.5, 80 ml H_2O, Store at 4 °C

DEPC water	0.2 ml diethylpyrocarbonate, 100 ml H_2O, autoclaved to inactivate remaining DEPC
Denhardt's reagent (50 ×)	Per litre: 10 g Ficoll (type 400), 10 g poly-vinylpyrrolidone, 10 g BSA. Store at –20 °C
DNA extraction buffer	10 mM Tris-Cl pH 8, 10 mM EDTA pH 8, 1% SDS
DNA loading buffer (6 ×)	40% (w/v) sucrose in water, 0.25% BPB
LB medium (1 L)	10 g tryptone, 5 g yeast extract, 5 g NaCl; adjusted to pH 7 with 0.2 ml NaOH
LB plates (1 L)	15 g bacto-agar is added to LB medium. Autoclaved, allow to cool down to 50 °C, add antibiotics if neccesary
NZCYM broth	Sigma Chemical Co., St. Louis, MI, USA
PBS	1.47 mM KH_2PO_4, 7.3 mM NaH_2PO_4, 138 mM NaCl, 2.7 mM KCl; pH 7.5
RNA extraction buffer	100 mM NaCl, 50 mM EDTA, 1 % SDS
SDS gel-loading buffer (3 ×)	100 mM Tris-HCl pH 6.8, 20 % glycerol, 10 % (v/v) β-Me, 4 % SDS, 0.2 % BPB
SM buffer (1L)	5.8 g NaCl, 2 g $MgSO_4 \cdot 7 H_2O$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml 2 % gelatin

	solution; autoclaved for 20 min at 15 lb/sq. in fluids cycle
SSC (20 ×)	Per litre: 175.3 g NaCl, 88.2 g sodium citrate. Adjusted to pH 7 with NaOH. Autoclaved
TAE	40 mM Tris-acetate, 1 mM EDTA
TBE	45 mM Tris-borate, 1 mM EDTA
TBST	10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20
TE	10 mM Tris-HCl pH 8, 1 mM EDTA pH
TFB1	100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15 % glycerol; pH 5.8 Sterile-filtered and adjusted with diluted acetic acid
TFB2	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerol; pH 8.0 Autoclaved and adjusted with diluted NaOH
Top-agar/agarose	To 1 litre of LB medium, 7 g alternatively agarose was added. Autoclaved
Wash buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol; pH adjusted to 6.0 with NaOH

Chapter 3: Isolation and characterization of a novel venom protein, Vn4.6 from an endoparasitoid, *Cotesia rubecula* (Hym: Braconidae)

3.1 Introduction

Endoparasitoid wasps manipulate host physiology by transmitting maternal factors into the body of their host upon parasitization/oviposition. These factors include calyx fluid containing symbiotic polydnaviruses (PDVs) and venom proteins. PDVs are replicated in the ovaries of certain hymenopteran parasitoid wasps that belong to the families Ichneumonidae and Braconidae (Stoltz & Vinson, 1979). These wasps use PDVs to actively overcome host immune defences, which is essential for successful parasitism and development of the parasitoid inside the host (Edson *et al.*, 1981; Fleming & Summers, 1991; Lavine & Beckage, 1995; Beckage, 1998; Shelby & Webb, 1999). When parasitoid eggs devoid of PDVs are artificially injected into the host hemocoel, they are encapsulated by a cellular immune response displayed by insects against large objects (Edson *et al.*, 1981).

In certain host-parasitoid systems, PDVs are only effective when they are complemented by venom proteins. This is the case in some braconid parasitoids (Stoltz, 1986). For example, in *C. melanoscela*, it was reported that venom facilitates the uncoating of PDVs *in vitro* and virus persistence *in vivo* (Stoltz *et al.*, 1988). In the absence of venom, viruses could not be released into the cytoplasm or attach to nuclear pores (Stoltz *et al.*, 1988). In *C. glomeratus* (Kitano, 1986) and *Apanteles kariyai* (Tanaka, 1987), venom is an essential requirement for successful parasitism. It is also proposed that during the period between oviposition and the expression of PDV genes, venom proteins might have a rapid, short-term effect to suppress the host immune response (Webb & Dahlman, 1985; Webb & Luckhart, 1994).

In endoparasitoids which lack PDVs, venom seems to play a major role in host immune suppression and host regulation. For example, in *P. hypochondriaca* (Braconidae), venom adversely affects the morphology, viability, and immune function of hemocytes of the tomato moth, *L. oleracea* (Richards & Edwards, 1999, Richards & Parkinson, 2000). These authors have shown that at sublethal doses venom has a potent anti-hemocyte action and can impair hemocyte-mediated immune responses. In endoparasitoids with PDVs, venom proteins alone cause developmental effects in the host organism as well (Jones, 1996; Gupta & Ferkovich, 1998; Richards & Edwards, 1999). In *G. mellonella*, venom from *M. croceipes* reduced larval growth (Gupta & Ferkovich, 1998). Venom from *A. ervi* (Braconidae) causes castration of female *A. pisum* aphids (Digilio *et al.*, 2000). Preliminary investigations have indicated that the venom contains a prophenoloxidase, a prophenoloxidase inhibitor and a cytotoxic factor (Parkinson & Weaver, 1999).

Here, the purification and characterization of a venom protein (Vn4.6) from the endoparasitoid *C. rubecula* is described which seems to interfere with melanin formation. Interestingly, the coding region for Vn4.6 is located in the opposite direction upstream of a gene coding for a *C. rubecula* PDV structural protein (Crp32) described previously (Asgari *et al.*, 1998). The data presented in this chapter were obtained by a team effort involving S. Asgari, R. Zareie and J. Lahnstein.

3.2 Methods and Materials

3.2.1 Reverse phase HPLC and mass spectrometry

Venom sacs from 20 female *C. rubecula* wasps were dissected and disrupted in PBS by micro-scissors to release venom proteins into the buffer solution. High pressure liquid chromatography (HPLC) of samples was carried out in a Hewlett Packard 1090 Liquid Chromatograph as described in Chapter 2.

To determine the molecular mass of the proteins, electrospray ionisation mass spectrometry was carried out using a PE Sciex API-100. Generally, an amount equivalent to ca. 7 picomoles of purified protein was directly infused and the results were analyzed using the BioMultiView 3.1.1 software.

3.2.2 Peptide sequencing

Approximately 100 picomol of the purified protein from rpHPLC was freeze-dried, reconstituted in 8 M urea containing 0.1 M NH_4HCO_3 and 4 mM DTT (DL-Dithiothreitol) and finally alkylated by addition of sodium iodoacetate to the final concentration of 10 mM. The sample was acidified with TFA to stop the reaction. Peptide sequencing was carried out using a Hewlet Packard G1000A Protein Sequencer.

3.2.3 RNA isolation and RT-PCR

Ovaries and venom glands were dissected from female *C. rubecula* wasps. Total RNA was isolated from the tissues as described in Chapter 2 (Ausubel *et al.*, 1993). Specific anti-sense primers were used in reverse transcription reactions using AMV (Avian Myeloblastosis virus) reverse transcriptase. The reactions were performed as described previously in Chapter 2.

Vn4.6-R: (5'-TTATACTAAAAGGCCAATTG-3')
Vn4.6-F: (5'-ATGATTGATGCTCCATGTAAAG-3')

Crp32-R: (5'-GCCTTTTTTTGGCAGTCC-3'
Crp32-F: (5'-ATGGATAAGAAGATAATA-3').

PCR amplification followed the cDNA synthesis using corresponding specific forward primers and Reactions were carried out in 50 µl by an initial heating at 96 °C for 5 min

followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. A standard PCR reaction was set up as following:

Template DNA (cDNA)	15 μl
dNTP's (15 mM)	0.5 μl
Forwards primer (0.1 μg/ml)	1 μl
Reverse primer (0.1 μg/ml)	1 μl
10 × reaction buffer	5 μl
Taq polymerase (5 U/μl)	0.4 μl
H_2O	31.6 μl

3.2.4 Screening a venom-specific cDNA library

The PCR amplified cDNA coding for Vn4.6 (see above) was labeled by [^{32}P]-dCTP (Ready-To-Go, Pharmacia) and used as a probe to screen 10^6 plaques from a cDNA library made from mRNA isolated from *C. rubecula* venom glands. Positive plaques were re-screened and the insert from one was sequenced from both directions. The method was described previously in Chapter 2.

3.2.5 Enzymatic assays

Trypsin and α-chymotrypsin assays were carried out in the absence and presence of Vn4.6 protein to determine its possible enzyme inhibitory activities. Enzyme assays were carried out as described previously with minor modifications (Muharsini *et al.*, 2001). Briefly, 4 mM Nα-benzoyl-DL-Arg-p-nitroanilide (BAPNA, Sigma) and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA, Sigma) were prepared in 100 mM Tris, pH 8.0, containing 20 mM $CaCl_2$ as substrate solutions for trypsin and α-chymotrypsin enzymes, respectively.

Reactions were carried out in microtitre plates in a final volume of 100 μl containing 40 μl substrate solution, 10 μl enzyme (100 U/ml trypsin and 15 U/ml chymotrypsin, Sigma) and 5 μl Vn4.6 (5 wasp-equivalents). After incubation at 37 °C for 2 h, absorption was measured at 405 nm in a BioRAD microplate reader.

3.2.6 Hemolymph phenoloxidase enzyme activity

The assay was carried out as described previously in chapter 2 with minor modifications (Beck *et al*., 2000). For inhibition assays, total venom from one wasp in PBS (10 μl) or one wasp-equivalent Vn4.6 in PBS purified on rpHPLC (10 μl) was added to the above mixture and absorbency was measured as above. The experiments were repeated three times.

3.3 Results and Discussion

3.3.1 Purification of venom proteins

An immunosuppressive gene (*CrV1*) from *C. rubecula* polydnaviruses (CrPDVs) is involved in the breakdown of the host hemocyte cytoskeleton in *P. rapae* and provides active protection of the endoparasitoid eggs against host cellular response (Asgari *et al*., 1996, Asgari *et al*., 1997). In addition, a calyx protein (Crp32) is on the CrPDV's particle surface as well as on the egg's surface, providing passive protection of the parasitoid egg against host encapsulation reactions (Asgari & Schmidt, 1994; Asgari *et al*., 1998). However, there is no report about venom proteins from the endoparasitoid.

Venom proteins from parasitoid wasps are known to have a protective function and can be obtained in a concentrated form by dissection of the venom gland storage sac. When crude venom preparations, isolated from female *C. rubecula* endoparasitoid wasps, were fractionated on a rpHPLC column, several proteins were found (Fig. 3-1). The size of the proteins eluted from the column was determined by mass spectrometry. One of the proteins

eluted at 28.7 % acetonitrile was identified as a 4655 dalton protein (average mass, Vn4.6) and analyzed further (Fig. 3-1).

3.3.2 Sequence analysis of Vn4.6

The rpHPLC fraction containing Vn4.6 was N-terminally sequenced and 36 amino acids were obtained (Fig. 3-2 A, underlined). When the peptide sequence was analyzed against the existing genes in the GenBank at NCBI using translate option, the peptide showed 100 % similarity to the 5'-untranslated region of Crp32 cDNA described previously (Asgari *et al.*, 1998). However, the open reading frame (ORF) is located in the opposite direction to the Crp32 ORF (Fig. 3-2 B). Primers were designed to amplify part of the coding region for Vn4.6 (Fig. 3-2 B) by RT-PCR (Fig. 3-4). The amplified fragment (135 bps) was used as a probe to screen a venom gland-specific cDNA library. A positive clone containing the full-length cDNA coding for Vn4.6 was obtained and sequenced in both directions (Fig. 3-2 A). A methionine at position 1 was identified as the putative initiation site (Cavener & Ray, 1991). The cDNA consists of 277 bps containing an open reading frame of 129 nucleotides coding for 42 amino acids (Fig. 3-2 A). A polyadenylation signal was identified downstream of the stop codon. A putative cleavage site was detected using PSORTII (http://psort.nibb.ac.jp/form2.html) between amino acids 23 and 24 consistent with the N-terminal sequence obtained for the secreted protein (Fig. 3-2 A). The estimated molecular weight for the secreted protein is 4658.11 dalton, which is very close to the actual size of the secreted protein measured by mass spectrometry (see above). The estimated isoelectric point is 3.99.

The Vn4.6 cDNA sequence from nucleotide 19 until just before the polyA tail is present in the upstream 5'-untranslated region of cDNA coding for Crp32 (Fig. 3-2 B; also see Asgari *et al.* 1998). The actual initiation site for Vn4.6 might lie further upstream from the remaining of the gene on the chromosome. When specific DNA probes for Vn4.6 and Crp32 were used in Southern blot analyses containing *Pst*I-digested genomic DNA isolated

from female *C. rubecula* wasps, both probes hybridized to the same DNA fragment indicating that the genes are located at the same position as single copy genes (Fig. 3-3). Whether the promoters are located between the two initiation codons or extend into the open reading frame(s) remains to be established.

3.3.3 Expression of Vn4.6

Since the ORF for Vn4.6 was identified upstream of Crp32, expression of both genes was investigated in venom gland and ovaries of female *C. rubecula* by RT-PCR, using specific primers to the ORFs. Interestingly, transcripts corresponding to Vn4.6 were detected only in the venom gland and Crp32 only in the ovaries (Fig. 3-4). This indicates that there are differential transcription mechanisms controlling the mutually exclusive expression of the two genes in different tissues. Alternatively, expression of Crp32 in the ovaries might inhibit expression of Vn4.6, and vice versa in the venom gland.

3.3.4 Enzyme inhibition assays

Sequence similarity searches using PRINTS blast search (protein fingerprint database, http://www.bioinf.man.ac.uk/dbbrowser/PRINTS) showed that Vn4.6 protein has 25.5 % identity with ω-atracotoxin-HV1A from the Australian funnel web spider *Hadronyche versuta* (Fig. 3-5). The ω-atracotoxins are small peptide neurotoxins (36 to 37 residues) that specifically block insect voltage-gated calcium channels with insecticidal activities (Wang *et al.*, 1999). Since no paralyzing effect is observed in *P. rapae* larvae parasitized by *C. rubecula*, Vn4.6 probably does not affect the host in the same way as ω-atracotoxins do. Since phylogenetic studies suggest the evolution of endoparasitoids from ectoparasitoids, Vn4.6 might be reminiscent to paralyzing proteins present in ancestral ectoparasites, which adapted to a different function in endoparasitoids.

In addition, sequence analysis, using 3D-PSSM that recognizes folds (Kelley *et al.*, 2000), showed similarities of Vn4.6 to small Kunitz-type protease inhibitors, although not very significant (data not shown). The Kunitz family of serine protease inhibitors are short (~50 residue) proteins with few secondary structures involving disulfide bridges. The family includes snake venom basic proteases, mammalian inter-alpha-trypsin inhibitors, trypstatin, etc. The presence of four cysteine residues in the deduced Vn4.6 sequence (Fig. 3-2 A) suggests the formation of disulfide bridges in this protein which could resemble trypsin inhibitors. To investigate a possible protease inhibitor activity of Vn4.6 in immune activation processes, a prophenoloxidase inhibition assay was carried out. When cell-free hemolymph from *P. rapae* larvae was mixed with DL-DOPA, a substrate for PO, extensive melanin formation was detected when spectrophotometrically measured at 490 nm (Fig. 3-6, hemolymph). When one wasp-equivalent of purified Vn4.6 protein was added to the assay, melanin formation was significantly reduced (Fig. 3-6, hemolymph+Vn4.6). However, when total venom fluid from one wasp was added to the assay, melanin formation was almost completely inhibited. This indicates that additional factors or inhibitors may exist in the venom fluid (see chapter 5, Vn50).

To further investigate the protease inhibitory function of Vn4.6, standard enzyme assays using artificial substrates were carried out. When five wasp-equivalents of purified Vn4.6 were tested in trypsin and α-chymotrypsin assays using BAPNA and SAPNA as substrates, respectively, no inhibition was detected (Fig. 3-7 A & B). However, Vn4.6 itself was found to be highly resistant to trypsin digestion (data not shown). Although the protein may lack Kunitz-type protease inhibitors properties, the proPO inhibition assays suggest that Vn4.6 may interfere with specific components of the prophenoloxidase-activating system, by competitive binding to the zymogen and thereby interrupting the immune-activation.

In conclusion, we have isolated and characterized a small venom protein from *C. rubecula* that inhibits proteases from the host involved in the activation of proPO in the hemolymph. This inhibition might have synergistic effects in conjunction with the immunosuppressive

action of polydnaviruses introduced by the female into the host hemocoel. An interaction with calyx fluid is also suggested by the gene structure and expression data. The ORF coding for Vn4.6 is located in the opposite direction upstream to a gene coding for an immune-protective protein (Asgari *et al.*, 1998) causing the two genes to be expressed in calyx and venom tissues in a mutually exclusive fashion.

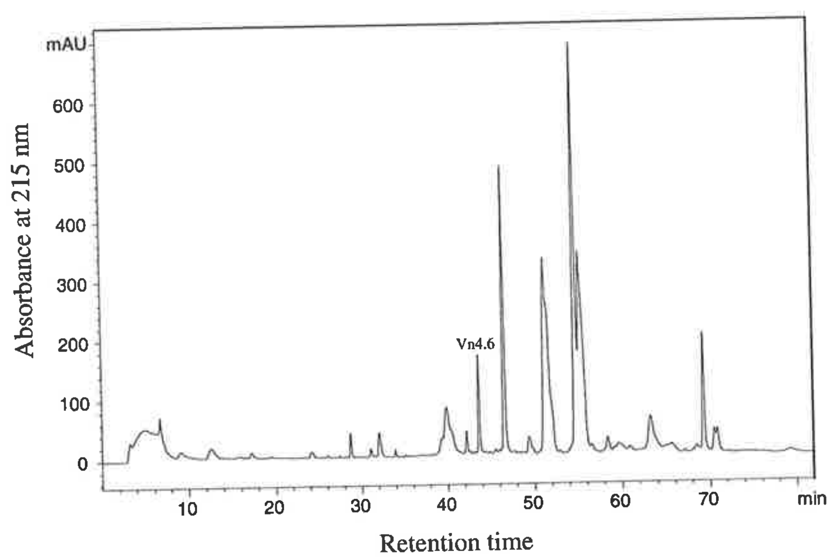

Fig. 3-1: Venom protein separation by rpHPLC. Separation of crude *C. rubecula* venom proteins extract using reverse phase high pressure liquid chromatography (rpHPLC). Several components are present in the venom including Vn4.6, which was eluted at 28.7 % acetonitrile.

(A)

TAAACAAA CATACAACA ATTTACAAA GATCAATAA ATCGCAAGA **ATG**TCGAAA ATAATTTTG 18

M S K I I L 6

GCTATTTTT TTAATCGTC CTGTGTGGG CTCATTTTT GTTACTGTG GACGCAATG ATTGATGCT 81

A I F L I V L C G L I F V T V D A M I D A 27

CCATGTAAA GATAATGAC GATTGTGAT CGATTCACC GAATACTGC GCAATTTAT GCTGATGAA 144

P C K D N D D C D R F T E Y C A I Y A D E 48

AATGGAAAT GAGGCAGGA AAAAGATGT GAAGATGCA ATTGGCCTT TTAGTATAA GTCGGCAGT 207

N G N E A G K R C E D A I G L L V * 65

ATAATGTGG TCATAATAA ACTCATCTG ATTTAATAT GATCAAAAA AAAAAAAAA AAAAAAAAA 270

AAAAAAA 277

(B)

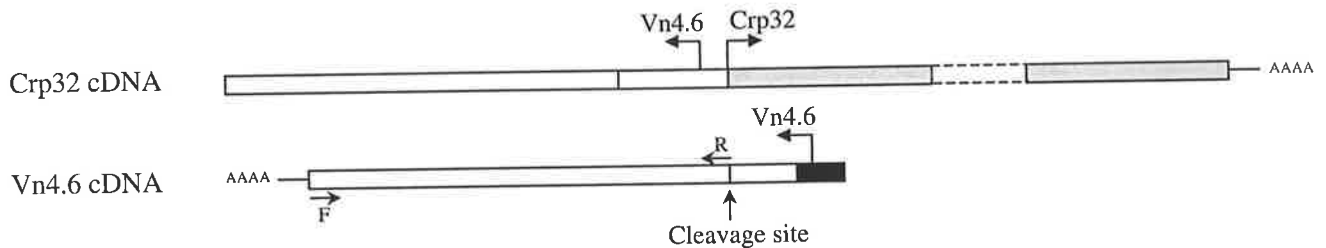

Fig. 3-2: Analyses of Vn4.6 sequence. (A) Nucleotide sequence and deduced amino acid sequence of a cDNA coding for Vn4.6. The numbers of nucleotides and amino acid residues are shown at the end of each line. Amino acid sequences obtained from N-terminal microsequencing are underlined. A putative cleavage site is indicated by an arrowhead. A putative polyadenylation site is underlined. The putative start codon (ATG) is shown in bold. Star shows the termination codon. (B) Schematic representation of cDNAs for Crp32 and Vn4.6. Most of the open reading frame for Vn4.6 (open box) is found in the 5'-untranslated region of Crp32 cDNA in an opposite direction. The locations of the PCR primers used in RT-PCR reactions (see Fig. 3-4) are shown by arrows (F & R). The black boxed region indicates a few sequences that differ in the two cDNAs.

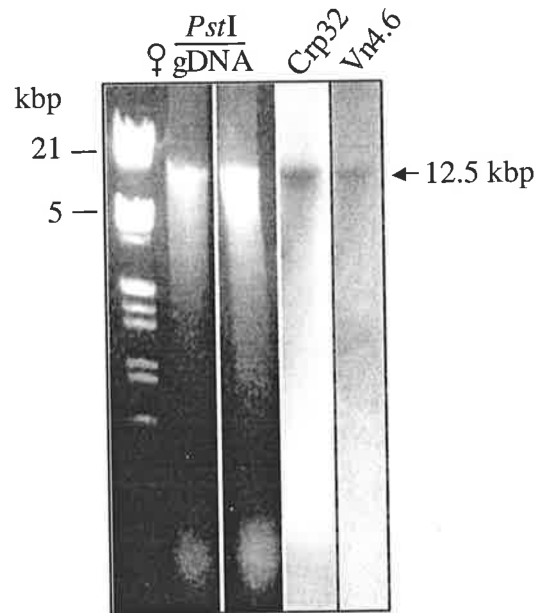

Fig. 3-3: Southern blot analyses of *C. rubecula* genomic DNA. 20 µg *Pst*I-digested genomic DNA isolated from female wasps was run on a 1% agarose gel and transferred onto a nylon membrane. The membrane was cut into two pieces, one probed with [^{32}P]-labelled cDNA fragment from Vn4.6 and the other from Crp32 (see Fig. 3-4). Both probes hybridized to the same DNA fragment in the genomic DNA indicating that they are located at the same position in the chromosome as single copy genes.

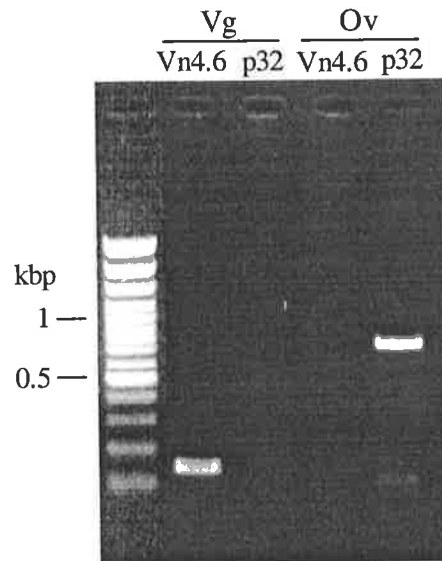

Fig. 3-4: RT-PCR analysis of venom gland and ovaries from *C. rubecula*. 2 μg total RNA from venom gland (vg) and ovaries (ov) was reverse transcribed using specific reverse primers to Crp32 and Vn4.6. Using specific primers to Vn4.6 and Crp32 (p32) genes, PCR reactions were carried out to detect expression of the genes in the tissues. A 135 bp fragment corresponding to the Vn4.6 ORF was amplified only in the venom gland and a 768 bp fragment corresponding to the Crp32 ORF was detected only in the ovaries. The molecular weight marker is a 100 bp stepladder.

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MIDA.....PCKDNDDCDRFTEYCAIYADENGNEAGKRCEDAIGLLV   Vn4.6
SPTCIPSGQPCPYNENC..CSQSCTFKENENGNTV.KRCD.......   ω-ACTX
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Fig. 3-5: Vn4.6 sequence alignment. Sequence of Vn4.6 was aligned with ω-atracotoxin-HV1A from *Hadronyche versuta* snake. The identical and similar residues are boxed black and gray, respectively. Vn4.6 has 25.5 % identity with ω-atracotoxin-HV1A.

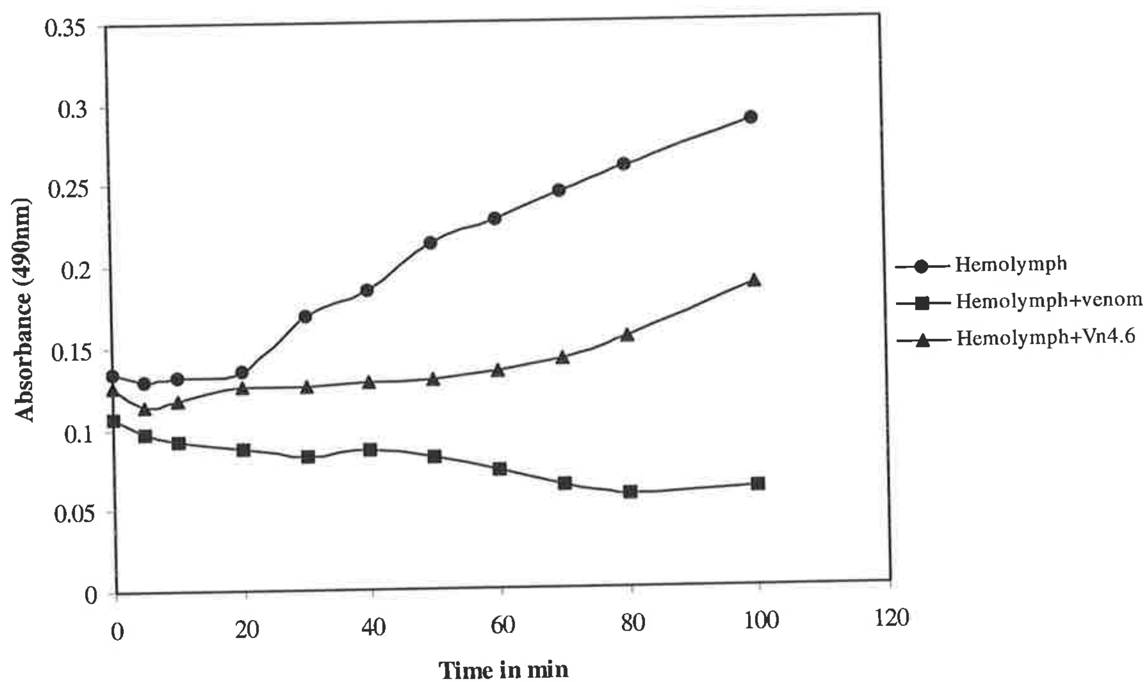
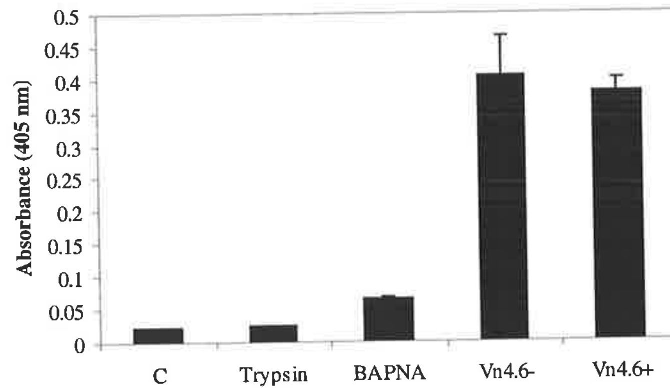


Fig. 3-6: Inhibition of prophenoloxidase (proPO) activity by Vn4.6. Cell-free hemolymph from a *P. rapae* larva was mixed with 900 µl 20 mM DOPA substrate and absorbance was monitored at 490 nm for 2 h (hemolymph). In other treatments, total venom from one wasp (hemolymph+venom) and Vn4.6 (one wasp-equivalent; hemolymph+Vn4.6) were added to the reaction. Total venom completely abolished proPO activity, whereas Vn4.6 had a milder effect on proPO, although the difference was significant compared to the control. The experiment was repeated three times with similar results.

(A)



(B)

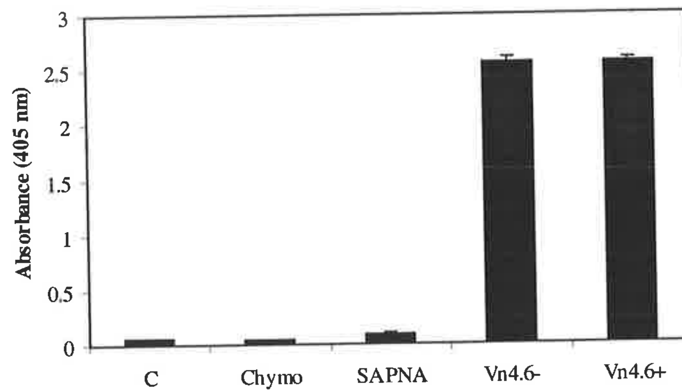


Fig. 3-7: Protease inhibitory activity of Vn4.6 using synthetic substrates BAPNA (A) and SAPNA (B) for trypsin and α-chymotrypsin (chymo) enzymes, respectively. Hydrolyzed chromogenic substrates were detected spectrophotometrically at 405 nm after 2 h. C, 50 mM Tris, pH 8.0 and 10 mM $CaCl_2$ (buffer); Trypsin/Chymo, 1 U/0.15 U enzyme plus buffer; BAPNA/SAPNA, 4 mM substrate in buffer; Vn4.6-, enzyme and substrate in buffer without Vn4.6; Vn4.6+, enzyme reaction including 5 wasp-equivalents purified Vn4.6. No significant difference was observed when Vn4.6 was added to the enzyme reactions. Bars indicate standard errors of the means.

Chapter 4: A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits activation of the host hemolymph prophenoloxidase

4.1 Introduction

In insects defence against parasites and pathogens involves both cellular and humoral immune responses (Gillespie *et al.*, 1997; Trenczek, 1998; Hoffmann *et al.*, 1999). Hemocytes participate in phagocytosis, encapsulation and nodule formation. Humoral responses include synthesis of antimicrobial peptides (Hoffmann *et al.*, 1999), the blood clotting system (Muta & Iwanaga, 1996) and prophenoloxidase activation system (Ashida & Brey, 1998; Söderhäll & Cerenius, 1998). Phenoloxidase is thought to be one of the key enzymes in insect defence against infection and parasitization. Activation of phenoloxidase involves a serine proteinase cascade that can be triggered by microbial polysaccharides (Ashida & Brey, 1998), which leads to the generation of melanin and other toxic phenolic compounds. This reaction is considered as a vital defence mechanism mounted against intruding organisms (Ashida & Brey, 1998; Vass & Nappi, 2000). Although melanin formation and encapsulation are considered as two independent events in insect immune responses (Rizki & Rizki, 1990; Marmaras *et al.*, 1996), melanotic encapsulation is a common form of immune response against parasites and pathogens (Nappi & Vass, 1993; Strand & Pech, 1995; Beerntsen *et al.*, 2000).

Inhibition of melanization following parasitism has been reported from several systems (reviewed in Lavine & Backage, 1995). However, the molecular mechanisms and components involved behind this suppression are unknown. Introduction of maternal factors into the body cavity of their host insects is a common strategy evolved in endoparasitic wasps to manipulate host physiology for their own benefits. These include calyx fluid containing viruses or virus-like particles, such as polydnaviruses, and protein secretions from venom glands. In most host-parasitoid systems, PDVs are only effective

when accompanied by venom proteins. In *C. melanoscela*, it was reported that venom facilitates the uncoating of PDVs *in vitro* and virus persistence *in vivo* (Stoltz *et al.*, 1988). In *C. glomeratus* (Kitano, 1986) and *A. kariyai* (Tanaka, 1987), venom is an essential requirement for successful parasitism. It is also proposed that during the period between oviposition and the expression of PDV genes, venom proteins might protect the egg from the host immune reaction (Webb & Dahlman, 1985; Webb & Luckhart, 1994). In addition to their synergistic effects together with PDVs, venom components affect host physiology and development (Digilio *et al.*, 2000; Gupta & Ferkovich, 1998). In other endoparasitoids that do not produce PDVs, venom becomes the only factor in suppression or regulation of the host immune system (Richards & Parkinson, 2000). In this parasitoid/host system venom contains a prophenoloxidase, a prophenoloxidase inhibitor and a cytotoxic factor (Parkinson & Weaver, 1999).

This chapter will focus on isolation and characterization of a 50 kDa protein (Vn50) from the venom of *C. rubecula*, which blocks hemolymph melanization of its host, *P. rapae* (Asgari *et al.*, 2003c). The protein is similar in sequence and domain structure to SPHs (serine proteinase homolog) from various insects (Kwon *et al.*, 1999; Lee *et al.*, 2002; Yu *et al.*, 2003). Vn50 is not cleaved after introduction into the host hemolymph and is stable for a long period of time after parasitization. The recombinant Vn50 specifically interacts with proPO and PAP in host *P. rapae* hemolymph. Some data presented in this chapter were obtained by a team effort involving S. Asgari, R. Zareie and J. Lahnstein.

4.2 Methods and Materials

4.2.1 Insects and isolation of hemolymph and hemocytes

The parasitoid *C. rubecula* and its host *P. rapae* were maintained as described in Chapter 2. Hemolymph (ca. 80 μl) was collected from five 4th instar *P. rapae* larvae as described in

Chapter 2. To obtain the plasma, hemolymph was centrifuged at 750 × *g* for 5 min and the supernatant was transferred into a fresh tube.

4.2.2 Reverse phase HPLC (rpHPLC)

Venom sacs from 20 female *C. rubecula* wasps were dissected and disrupted in sterile water by micro-scissors to release venom proteins followed by centrifugation for 1 min at 16,000 × *g* to remove membrane debris. rpHPLC of the sample was carried out as described in chapter 2. Proteins were detected by absorbance at 214 nm and protein peaks were collected manually.

4.2.3 N-terminal and peptide sequencing

To obtain the N–terminal sequence for Vn50, approximately 100 picomol of the purified protein from rpHPLC was freeze-dried, reconstituted in 8 M urea containing 0.1 M NH_4HCO_3 and 4 mM DTT and finally alkylated by addition of sodium iodoacetate to the final concentration of 10 mM. The sample was acidified with TFA to stop the reaction. The protein was sequenced using a Hewlet Packard G1000A Protein Sequencer.

In order to obtain more peptide sequence data to design degenerate primers to Vn50, the protein was excised from a SDS-PAGE gel and digested in gel using trypsin. Digested and eluted peptides were separated on a rpHPLC from which one was sequenced as above.

4.2.4 RNA isolation and RT-PCR

Venom glands were dissected from female *C. rubecula* wasps from which total RNA was isolated as described in Chapter 2. To construct cDNA molecules, a poly-dT (17 mer) primer was used in a reverse transcription reaction using AMV reverse transcriptase as described in Chapter 2

Degenerate primers:

Vn50-F: 5'-CCNCCNCARCARGCNGCNCC-3'

Vn50-R: 5'-AANGCYTCNARYTGYTGRTC-3'

Amplification followed the cDNA synthesis using degenerate primers designed based on Vn50 peptide sequences. The reaction was carried out in 50 µl (see Chapter 3) by an initial heating at 96 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 48 °C for 70 sec, 72 °C for 90 sec and a final extension at 72 °C for 10 min. The PCR product was excised from an agarose gel, purified using Wizard PCR Purification kit (Promega) and cloned into pGEM-T-Easy vector (Promega).

4.2.5 Screening a cDNA library

To obtain a full-length cDNA coding for Vn50, a cDNA library made from venom gland and ovaries of *C. rubecula* wasps was screened using the partial sequence obtained from RT-PCR using degenerate primers as a probe (see above). Positive clones were re-screened and the longest cDNA was sequenced in both directions. The detail procedure for screening was described in Chapter 2.

4.2.6 Production and purification of Vn50 expressed in *E. coli*

A partial Vn50 protein was expressed in bacteria containing most part of the protein from amino acid 28 to 385. The coding region was amplified by designing specific primers containing restriction sites (in box, underlined) to facilitate direct cloning into the expression vector. The PCR amplified product was digested with the restriction enzymes and cloned into the corresponding sites in pQE30 expression vector (Qiagen). The protein expressed in this system is a fusion protein containing 6 histidine residues. Transformed bacteria were induced with 1mM IPTG for 2 h and analyzed on a 12 % SDS-PAGE gel as

described in Chapter 2. The identity of the protein was confirmed by Western blotting using a monoclonal anti-polyhistidine alkaline phosphatase conjugate (1:5000, Sigma) and a polyclonal antiserum raised against total venom proteins (1:5000). Alkaline phosphatase-conjugated anti-rabbit IgG antibodies were used as secondary antibodies (1:5000, Sigma).

Specific primers for Vn50 production:

Vn50-*Bam*HI-F: 5'-GCGCGGATCCCCCCAGCAAGCCGCTCCG-3'

Vn50-*Kpn*I-R: 5'-GCGCGGTACCTTACGCCTCTAGTTGCTGGTC-3'

Since the expressed protein was found in the insoluble fraction, it was purified under denaturing conditions according to the manufacturer's instructions (Qiagen). The fusion protein was purified by affinity chromatography using Ni-NTA technology (Qiagen) and eluted with 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris at pH 4.5. To refold the protein, it was first dialyzed against 1 liter 1 M urea, 150 mM NaCl, 10 mM Tris (pH 7.5) and then 150 mM NaCl, 10 mM Tris (pH 7.5), 30 h each at 4 °C.

4.2.7 Production of anti-Vn50 antibodies

Purified recombinant Vn50 expressed in *E. coli* was run on preparative 10 % SDS-PAGE gels, stained in 0.05 % Coomassie blue R-250 prepared in water and destained by several changes of water. Vn50 corresponding band was excised from the gels and squashed into fine pieces in PBS. The protein was injected into a rabbit as described in Chapter 2 (ca. 5 μg/injection). Two subsequent booster injections were carried out in 2-week intervals, four weeks after the initial injection. The first and booster injections contained complete and incomplete Freud's adjuvants, respectively. Serum was obtained one week after the last injection. Production of anti-Vn50 antibodies was confirmed by Western blots containing

the recombinant Vn50 and the total venom from *C. rubecula* using 1:5000 dilution of the antiserum.

4.2.8 Detection of glycosylation

Venom proteins from two wasps were run on a 12 % SDS-PAGE gel, transferred onto a nitrocellulose membrane. To detect carbohydrate portions of venom proteins, ECL Glycoprotein Detection Module was used according to the manufacturer's instructions (Amersham).

4.2.9 Enzymatic assays

To investigate possible protease activity of Vn50, trypsin and α-chymotrypsin assays were carried out. Enzyme assays were performed as described previously with minor modifications (Muharsini *et al.*, 2001). Briefly, 4 mM Nα-benzoyl-DL-Arg-p-nitroanilide (BAPNA, Sigma) and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA, Sigma) were prepared in 100 mM Tris, pH 8.0, containing 20 mM $CaCl_2$ as substrate solutions for trypsin and α-chymotrypsin enzymes, respectively. Reactions were carried out in microtitre plates in a final volume of 100 µl containing 40 µl substrate solution, 10 µl enzyme (100 U/ml trypsin and 15 U/ml chymotrypsin, Sigma) or 5 µl Vn50 (4 wasps equivalent, ca. 0.5 µg). After incubation at 37 °C for 2 h, absorption was measured at 405 nm in a BioRAD microplate reader.

4.2.10 Hemolymph phenoloxidase enzyme activity

The assay was carried out as described previously in Chapter 2 with minor modifications. For inhibition assays, total venom from four wasps in PBS (10 µl), four wasps equivalent Vn50 (ca. 0.5 µg) in PBS purified on HPLC, or 0.5 µg purified recombinant Vn50 was

added to the above mixture and absorbency was measured as above. The assays were repeated three times for each treatment.

4.2.11 Stability of *C. rubecula* Vn50 in *P. rapae* hemolymph

Hemolymph was isolated from third instar *P. rapae* larvae (ca. 20 μl) at various times following parasitization by *C. rubecula,* as described above. Samples were analyzed on a Western blot using anti-Vn50 antibodies.

4.2.12 Identification of hemolymph proteins bound to Vn50

Binding of *P. rapae* hemolymph proteins to Vn50 was examined as described previously with minor modifications (Yu *et al.*, 2003). Briefly, washed Ni-NTA agarose beads (Qiagen, 0.1 ml) were coated with renatured 6xHis-tagged recombinant Vn50 or bovine serum albumin (BSA) in Tris buffered saline (TBS) for 60 min at room temperature. Beads were washed 3 times with TBS to remove unbound proteins and incubated with 0.5 ml plasma-PBS containing PTU from four *P. rapae* larvae. The incubation was carried out at room temperature for 1 h with shaking. After washing with TBS, nonspecifically bound proteins were eluted with 1.0 M NaCl. SDS sample buffer was then added to the resuspended beads in TBS, treated at 95 °C for 5 min, and subjected to 12 % SDS-PAGE (Laemmli, 1970) and immunoblot analysis. Blots were probed with various antiserum against *C. rubecula* Vn50 (1:5000 dilution), *M. sexta* PAP-1 (1:2000 dilution) (Wang *et al.*, 2001), or *Tenebrio molitor* proPO (1:2000 dilution) (a kind gift from Prof. Bok Lee at Pusan National University, Korea). Alkaline phosphatase-conjugated anti-rabbit IgGs (Sigma) were used as secondary antibodies.

4.2.13 Quantification of Vn50 injected into *P. rapae* larvae

Comparative Western blot analysis was used to determine the amount of Vn50 injected into a host larva at various times after parasitization. Three larvae were used for each time point. Recombinant Vn50 was produced in bacteria and purified as described. The recombinant protein concentration was measured using Bio-Rad Protein Assay based on the method of Bradford. Cell-free hemolymph (see above) was isolated from parasitized larvae and a fixed volume of 20 µl of each sample was run on Western blots together with known concentrations of recombinant Vn50. Intensity of bands was compared to estimate the amount of Vn50 injected into the caterpillars.

4.3 Results

4.3.1 Isolation and characterization of Vn50

When crude venom preparations are separated on a reverse phase high pressure liquid chromatography (rpHPLC) one of the major proteins elutes at 55 sec (Fig. 4-1 A). When the eluted protein was analyzed on a 12 % SDS-PAGE gel, a 50 kDa protein was detected (Vn50) with Coomassie staining as the only major protein (Fig. 4-1 B). N-terminal sequencing of the eluted fraction from rpHPLC resulted in 21 amino acids (NSDVXPPPQQAAPVXTXTNXL). The protein was also digested with trypsin and fractionated on rpHPLC. One of the peptides was sequenced containing 10 amino acids (EWIDQQLEAF).

Degenerate primers were designed based on N-terminal and peptide digest sequences (Fig. 4-2). Total RNA isolated from venom gland of *C. rubecula* wasps was reversed transcribed using an oligo dT primer followed by a PCR reaction using the degenerate primers. A ca. 1000 bp fragment was obtained which was cloned and sequenced in both directions (Fig. 4-2). To obtain a complete cDNA, a venom/ovary cDNA library was screened using the amplified RT-PCR product as a probe. The longest clone was sequenced in both directions.

A methionine at position 1 was identified as the putative initiation codon (ATG; Cavener & Ray, 1991). The complete open reading frame (ORF) contained 388 amino acids. Several putative N- and O-glycosylation sites were found in the sequence (Hansen *et al.*, 1988).

To confirm that the protein is glycosylated, a glycosylation detection kit was used and was shown that Vn50 is indeed heavily glycosylated (Fig. 4-1 C). In addition to Vn50, other venom proteins were also found to be glycosylated. Using SignalP V1.1 software, a putative cleavage site was detected between amino acids 19 and 20 (Fig. 4-2; Nielsen *et al.*, 1997), consistent with the N-terminal sequence of the secreted protein from the venom storage sac (see above).

Sequence homology searches at GenBank showed that Vn50 has similarity to serine proteinase homologs (SPH) from *M. sexta* SPH1 (44 %), *Tenebrio molitor* (50 %), *Drosophila melanogaster* (48 %), and *Limulus* factor D (34 %). Vn50 sequence contains all the cysteine residues conserved in amino-terminal clip and serine proteinase domains of SPHs (Fig. 4-3; Jiang & Kanost, 2000). However, SPHs including Vn50 do not have proteolytic activity since serine at the active site of the proteinase-like domain is changed to glycine (Fig. 4-3, arrow head).

4.3.2 Enzymatic assays

To experimentally show that Vn50 does not have proteolytic activity, synthetic peptides were used as substrates. When four wasp equivalents of purified Vn50 (ca. 0.5 μg) were tested in trypsin and α-chymotrypsin assays using BAPNA and SAPNA as substrates, respectively, no activity was detected (Fig. 4-4 A and B). In both controls, trypsin and α-chymotrypsin cleaved the corresponding substrates. This indicates that Vn50 lacks enzymatic activity similar to other SPHs.

4.3.3 Inhibition of prophenoloxidase activation

Serine proteinase homologs isolated so far from various insects have been shown to be necessary for activation of proPO zymogen (e.g. Yu *et al.*, 2003). To determine whether Vn50 from *C. rubecula* venom has a similar effect on the host *P. rapae* hemolymph, proPO activation assays were carried out. When hemolymph alone was tested in the presence of DL-DOPA, a substrate for the PO enzyme, melanization was detected at 490 nm wavelength (Fig. 4-5; Hem). However, when total venom (four wasp equivalents) or purified Vn50 (four wasp equivalents, ca. 0.5 μg) were added to the reaction, melanization was inhibited (Fig. 4-5; Hem+Ven and Hem+Vn50). Furthermore, purified Vn50 expressed in bacteria also inhibited melanization (Fig. 4-5; Hem+Rec). The reduced inhibition of melanization observed with recombinant Vn50, although still significant compared to the control, could be due to the fact that protein renaturation is usually less efficient after purification of the protein under denaturing conditions and dialysis, whereas active Vn50 from venom is more effective in inhibiting host hemolymph melanization.

To further confirm the inhibition of hemolymph melanization and investigate the plasma proteins bound to Vn50 using recombinant protein (see 4.3.5 below), the coding region for the secreted Vn50 was expressed in *E. coli*, in which the expression of the protein was subsequently induced. Analysis by Western blot, containing proteins from induced (T2 in Fig. 4-6 A) and non-induced (T0 in Fig. 4-6 A) recombinant bacterial cells, showed the presence of a ca. 42 kDa protein (Fig. 4-6 A) in induced cells, which is close to the predicted size (40.7 kDa). The fusion protein was purified by affinity chromatography using Ni-NTA technology (Qiagen) and refolded. The identity of recombinant protein was confirmed by its cross reaction with anti-venom antibodies (Fig. 4-6 B). When thc recombinant Vn50 was used in melanization assays, it also inhibited melanization (Fig. 4-5, Hem+Rec). The inhibition of melanization by recombinant Vn50 was markedly reduced, although still significant compared to the control. The reduction could be due to post-translational modifications or the fact that protein renaturation is usually less efficient after

purification of the protein under denaturing conditions and dialysis, whereas active Vn50 from venom is more effective in inhibiting host hemolymph melanization.

Dose-dependent inhibition of hemolymph melanization by total venom proteins and purified Vn50 by rpHPLC was investigated. The results show that melanin formation was completely inhibited when total venom or purified Vn50 protein equivalent of four wasps was included in the melanization assays, whereas one wasp equivalent had no effect and two wasp equivalents showed intermediate melanization (Fig. 4-7).

4.3.4 Status of Vn50 in the host hemolymph following parasitization

The clip-domain serine proteinases are produced as zymogens and cleaved at a specific location between the clip domain and the proteolytic domain (Jiang and Kanost, 2000). The cleavage is necessary for activation of these enzymes. Clip-domain SPHs, with a similar domain structure, were also cleaved (Yu *et al.*, 2003, Lee *et al.*, 2002). Although cleaved, their clip and proteinase-like domains remain attached by an interchain disulfide bond. Assuming that the cleavage is also necessary for SPHs to exert their function as enhancers/mediators for proPO activation, we investigated the status of Vn50 after it was injected into the host hemolymph to block melanization. At 2 h after parasitization with *C. rubecula*, we analysed the plasma sample from *P. rapae* larvae by SDS-PAGE and Western blotting under reducing and non-reducing conditions. A 50 kDa immunoreactive band was detected in the parasitized hemolymph and positive control of venom, but not in the non-parasitized hemolymph (Fig. 4-8). There was only a small size difference in Vn50 under the two conditions. As a control, venom fluid was analysed under the same conditions and found to be identical to the samples from the cell-free hemolymph in terms of the sizes of the immunoreactive bands (Fig. 4-8).

We demonstrated that intact Vn50 suppresses the melanization reaction in the host hemolymph (see above). To maintain this status, Vn50 must remain at a certain level in the circulation as an uncleaved proSPH until the host immune system is suppressed by other

maternal components such as the PDVs. Therefore, we analysed the persistence of Vn50 in the host hemolymph after parasitization. In our analysis in which we analysed plasma samples from *P. rapae* larvae up to 96 h after parasitization under reducing condition, the intact protein was still detected at a significant level at 72 h following parasitization (Fig. 4-9). The low turnover indicates that Vn50 could be resistant to degradation by host proteinases. Under this condition, the developing parasitoid eggs are protected from melanotic encapsulation for a prolonged period of time.

4.3.5 Identification of plasma proteins bound to Vn50

In a model that was recently proposed based on experimental evidence, SPHs mediate proPO activation by directly interacting with proPO, PAP-1, and immulectin-2 (Yu *et al.*, 2003). Although the exact mechanism is not understood, two scenarios were envisaged: 1) SPHs bring proPO into a correct spatial orientation or 2) the interaction among the proteins might confer a conformational change in proPO to facilitate its activation by the PAP. To find out whether proPO and PAP in *P. rapae* hemolymph interact with Vn50, recombinant 6×His-tagged Vn50 was used to coat nickel agarose beads. After incubation with the plasma, washing, and elution with high salt buffer, proteins bound to the beads were separated by SDS-PAGE and subjected to Western blot analysis. Two immunoreactive bands, recognized by the antibodies against *M. sexta* PAP-1, may represent *P. rapae* proPAP (46 kDa) and its catalytic domain (35 kDa) (Fig. 4-11). Similarly, the antibodies against *T. molitor* proPO reacted with two Vn50-binding proteins at 90 and 62 kDa. Based on the typical size of insect proPO, we suggest that they may correspond to zymogen and a cleaved form of proPO in *P. rapae* hemolymph.

4.3.6 Quantification of Vn50 in *P. rapae* hemolymph following parasitization

Due to the effect of Vn50 on proPO activation, which was found to be concentration-dependent (see 4.3.3; Asgari *et al.*, 2003c), we measured the amount of Vn50 injected by *C. rubecula* into *P. rapae* larvae at different times after parasitization by comparative western blot analysis using recombinant Vn50 protein as reference. The results (Fig. 4-10) showed that the concentration of Vn50 in the host hemolymph at 20 min after parasitization was much higher than what is needed for reducing 50 % of proPO activation *in vitro* (Zhang *et al.*, 2004a). While the concentration gradually decreased, Vn50 remained at a significant level up until 72 h after parasitization.

4.4 Discussion

Maternal factors introduced by hymenopteran endoparasitoids into host insects interfere with host physiology and development. One of the major effects of these factors is subversion of the host immune system. Those parasitoids that do not produce virus-like particles (such as PDVs) mainly rely on the venom fluid for host regulation including suppression of the host immune defence, whereas those with particles use a combination of factors, where venom usually plays a synergistic role with particles (e.g. Tanaka, 1986; Stoltz *et al.*, 1988; Wago & Tanaka, 1989). Factors introduced by the wasps affect cellular (eg. prevention of encapsulation) as well as humoral immunity (e.g. inhibition of melanization) of the host (Beckage, 1998). Calyx fluid containing PDVs has been shown to be involved in inactivation of host hemocytes (Strand, 1994; Asgari *et al.*, 1996; Webb & Luckhart, 1996). Inhibition of melanization after parasitism has been reported from several systems (Stoltz & Cook, 1983; Beckage *et al.*, 1990; Beck *et al.*, 2000; Shelby *et al.*, 2000), although the exact mechanism(s) is not well understood.

In insects, proPO is activated upon injury or invasion, which results in localized melanization of the wound or invading microorganisms and parasites in modules/capsules.

This involves activation of a series of serine proteinases in a proPO activation cascade (Ashida & Brey, 1998). Serine proteinase homologs (SPH) present in the plasma of several insects function as co-factors for proPO-activating proteinase (PAP). In a recent study, Yu *et al.* (2003) showed that SPHs from *M. sexta* in conjunction with other components from the hemolymph mediate activation of proPO (Yu *et al.*, 2003). In the proposed model (Fig. 4-12), immunolectin-2 (IML2), a pattern recognition molecule, initiates protein-protein interactions with SPH and proPO after binding to carbohydrates on the foreign surface. Then, SPH mediates recruitment of other plasma proteins such as PAP, which leads to the activation of proPO and melanization. The mediation effect of SPH is not well understood at the molecular level. It is assumed that SPHs might bring proPO into a correct position for proteolysis by PAP or cause conformational changes in proPO to facilitate its cleavage by PAP (Yu *et al.*, 2003).

We have isolated a venom protein from C. *rubecula* (Vn50) with similarity to SPHs, which inhibits melanization of the host hemolymph *P. rapae*. When total venom, purified or recombinant Vn50 were added to proPO activation assays, melanization was completely inhibited (Fig. 4-5). Similar to other SPHs, Vn50 contains the two domains, a carboxyl-terminal serine proteinase domain and an amino-terminal "clip" domain. However, in SPHs and in Vn50 the serine proteinase domain is not functional since they lack a serine at the conserved site, precluding enzymatic activity. Although Vn50 resembles SPHs at the structural level, it appears that Vn50 differs from SPHs at the functional level. Whereas SPHs interact with proPO to activate the enzyme complex (Yu *et al.*, 2003), Vn50 inhibits proPO activation. One explanation for this discrepancy is the unique functional context of parasitoid-host interactions, where evolutionary adaptations of regulatory proteins are exploited for host manipulations. A possible assumption for the mechanism by which Vn50 inhibits melanization is that having structural and sequence similarities to SPHs, the protein might function as an antagonist molecule competing with host SPHs for binding sites of immunolectins and proPO, instead of activating the complex. To elucidate the exact mechanism(s) by which Vn50 inhibits this reaction, further information is required

regarding the mode of action of SPHs to be able to examine differences with Vn50 activities.

The predicted molecular mass for secreted Vn50, based on its deduced amino acid sequence, is 40.6 kDa. The difference between the calculated and observed sizes has been shown to be due to glycosylation. Based on the other SPHs which were characterized biochemically, we predict that the conserved cleavage site in Vn50 would be located after Arg^{127} residue. In other words, if the protein is cleaved, it should result in an 11.8 kDa clip domain and a 28.9 kDa proteinase-like domain under the reducing conditions. Our observation, however, indicates that Vn50 remained intact even in the presence of β-mercaptoethanol. In fact, the protein ran slightly slower under this condition than under the non-reducing conditions. The change in electrophoretic mobility is likely caused by the breakdown of intramolecular disulfide bonds (Fig. 4-8).

While Vn50 is maintained in an active form to block melanization, it is not clear how this proSPH exerts its function. Detection of Vn50-binding proteins in the host hemolymph could provide some useful cues on its mode of action. In our investigation, it was found that Vn50 might interact with proPO and PAP in *P. rapae* hemolymph (Fig. 4-11). The interaction among the proteins might confer a conformational change in proPO to facilitate its activation by the PAP (Yu *et al.*, 2003). The antibodies recognized the respective proteins in *P. rapae* hemolymph, confirming that proteins bound to the beads came from the larval hemolymph. Similar observations were made in *M. sexta* SPH-1, whose proteinase-like domain binds to proPO, PAP-1, and immulectin-2 (Yu *et al.*, 2003). In the negative control, none of these proteins bound to BSA-coated nickel agarose beads (Fig. 4-11). These results suggest that Vn50 may interact with one or more of proteins involved in melanization. Further experiments are needed to examine which components bound to Vn50 directly and which ones were pulled down simply because of their association with the Vn50-interacting proteins.

Complementary studies (Zhang *et al.*, 2004a) carried out by our collaborators using purified *M. sexta* proPO, PAP-1, and SPHs, as an *in vitro* system to test the possible role of Vn50 in regulating the proPO activation reaction, indicated that Vn50 efficiently down-regulated proPO activation mediated by the proteins. Vn50 did not inhibit active phenoloxidase (PO) or PAP-1, but it significantly reduced the proteolysis of proPO (Zhang *et al.*, 2004a). While molecular details are still lacking, our results strongly suggest that interactions among the substrate, proteinase, and cofactor were impaired by Vn50. To our best knowledge, this is the first report indicating that the proPO activation step is affected by a venom protein from endoparasitoid wasps.

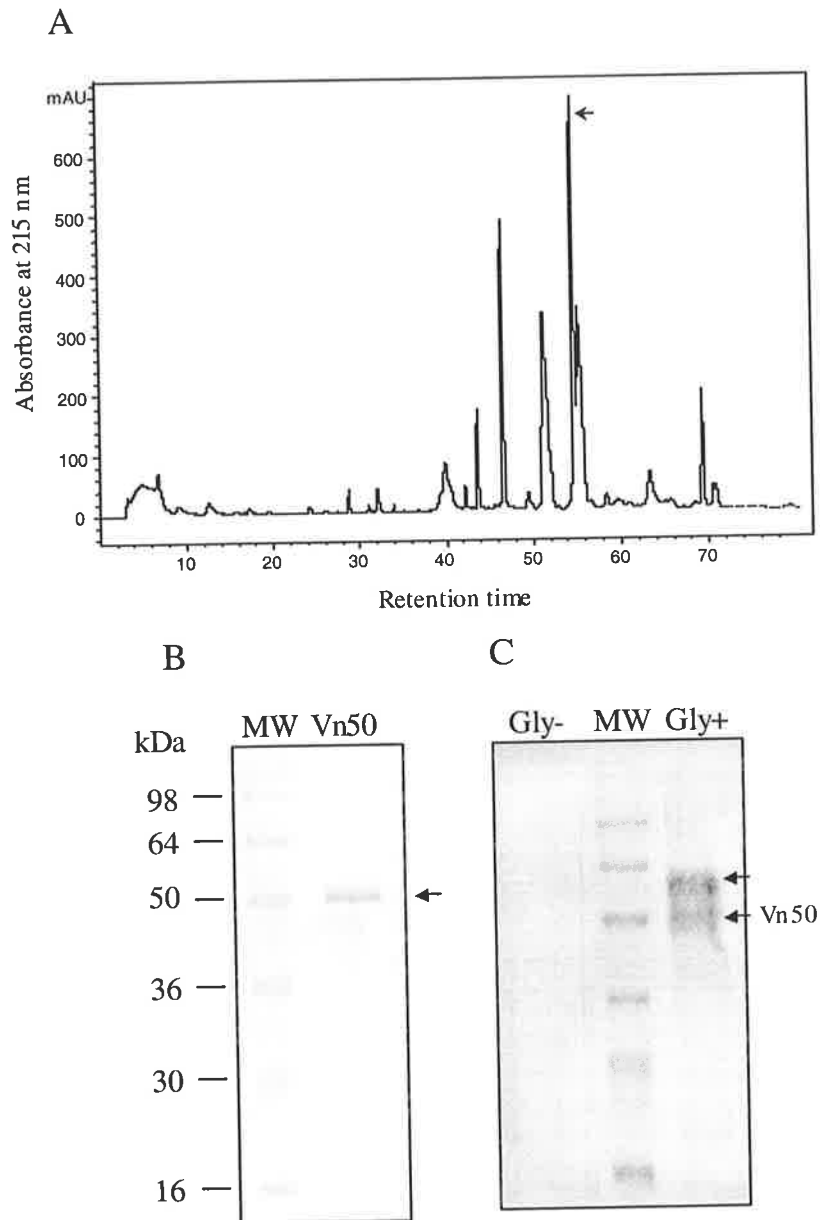


Fig. 4-1: A) rpHPLC of crude *C. rubecula* venom. Several components are present in the venom. The most abundant protein was eluted at 28.7 % acetonitrile (arrow). **B)** SDS-PAGE analysis (12 %) of the most abundant polypeptide in the venom from (A). **C)** Detection of glycoproteins in the venom. Venom proteins from two wasps were run on a 12 % SDS-PAGE gel, transferred onto a nitrocellulose membrane. Glycoproteins were detected using ECL Glycoprotein Detection Module. Two major glycoproteins were detected in the venom fraction including Vn50 (arrow).

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                                                              M  K             2
GTCAGCTTCTGGTCTTCGTTATTTGCGCATCGAAAAAACAAATTAACTTTTTAGATTATCATGAAG          6
W  K  I  F  S  I  I  F  L  M  G  L  S  S  S  Q  S▼ E  N  S  D  V             24
TGGAAAATATTTAGTATAATATTTCTAATGGGATTGTCATCTTCACAATCTGAAAATTCTGATGTA         72
T  P  P  P  Q  Q  A  A  P  V  P  T  T  T  N  R  L  S  T  T  G  I             46
ACACCACCACCACAACAAGCAGCTCCGGTTCCAACTACAACAAACCGCTTGTCGACGACGGGAATT        138
N  I  N  T  Q  E  N  N  G  C  E  C  V  N  Y  Y  L  C  S  N  G  T             68
AATATTAACACTCAAGAAAATAATGGTTGCGAATGCGTTAATTATTATTTGTGTTCCAACGGCACA        204
S  N  N  G  G  V  G  V  I  N  I  R  L  G  E  N  T  C  D  N  Y  L             90
AGTAATAATGGTGGTGTTGGAGTAATTAATATCAGACTTGGAGAAAATACCTGTGACAACTACTTA        270
H  V  C  C  S  P  S  N  I  L  D  R  D  K  R  F  T  P  K  P  K  V            112
CACGTTTGCTGTTCACCTTCAAACATCCTGGACCGCGACAAGCGTTTTACACCAAAGCCAAAGGTC        336
H  R  R  C  G  H  R  N  F  N  G  V  G  F  R  I  S  G  A  E  N  S            134
CATCGTCGATGTGGACATCGTAACTTCAATGGTGTAGGTTTTAGAATCAGCGGAGCTGAAAATAGT        402
E  A  E  F  G  E  F  P  W  M  V  A  V  L  V  S  N  E  T  S  V  N            156
GAAGCTGAGTTTGGTGAATTTCCTTGGATGGTTGCTGTTTTAGTTTCGAATGAAACTTCGGTGAAT        468
T  Q  E  E  K  K  Y  H  Y  K  C  G  G  S  L  I  H  L  R  A  V  L            178
ACTCAAGAAGAAAAAAAATACCATTATAAGTGTGGAGGTTCTTTAATTCATCTTCGAGCTGTTTTG        534
T  A  A  H  C  L  K  E  L  N  S  S  N  L  L  V  R  A  G  E  W  D            200
ACAGCTGCGCACTGTCTTAAAGAATTAAACTCTAGCAATTTACTCGTAAGAGCCGGTGAATGGGAC        600
T  Q  T  Q  S  E  P  L  P  H  Q  D  R  S  V  S  R  I  I  R  H  L            222
ACTCAAACACAAAGTGAACCACTTCCACATCAAGATCGAAGTGTTTCAAGGATTATTAGGCACCTT        666
E  F  Q  T  R  T  G  Q  Y  D  L  A  I  L  I  L  S  E  A  M  E  Q            244
GAGTTCCAGACTCGAACTGGACAGTATGACCTTGCTATATTAATTTTATCTGAAGCGATGGAACAA        732
A  E  N  V  D  I  V  C  L  P  N  K  N  E  I  F  D  N  T  R  C  V            266
GCTGAGAATGTTGATATTGTTTGTCTGCCGAATAAAAATGAAATTTTTGACAATACGAGGTGCGTT        798
A  S  G  W  G  K  N  V  F  G  N  E  G  E  Y  Q  V  I  L  K  R  V            288
GCAAGTGGCTGGGGAAAAAATGTCTTTGGTAACGAAGGAGAATACCAAGTTATTTTGAAACGAGTA        864
D  L  R  V  V  D  H  A  A  C  Q  N  A  I  R  K  T  R  I  G  E  H            310
GACTTGCGAGTAGTTGATCATGCAGCCTGTCAAAATGCTATACGTAAAACTAGAATTGGAGAACAT        930
F  K  L  E  E  S  S  I  C  A  G  G  E  P  G  I  D  T  C  K  G  D            332
TTCAAATTAGAAGAAAGTTCAATTTGCGCTGGAGGTGAACCTGGAATAGACACTTGCAAGGGCGAT        996
G  G  S  P  L  V  C  P  L  R  N  D  P  T  R  Y  S  Q  A  G  I  V            354
GGCGGCAGTCCACTAGTCTGCCCACTCCGCAATGATCCAACTCGATACTCACAAGCTGGAATAGTT       1062
S  W  G  V  D  C  G  E  D  G  V  P  A  I  Y  S  N  V  A  Y  A  R            376
TCTTGGGGCGTCGATTGTGGCGAAGACGGAGTTCCAGCTATCTATTCAAATGTCGCTTATGCACGT       1128
E  W  I  D  Q  Q  L  E  A  F  A  D  *                                       388
GAATGGATTGACCAACAACTAGAAGCATTTGCTGACTAATTTCCACCAATTTTTCTTACAATAAAT       1194
AAAATAATGTTTTACAATATATAAATAATATTAATTAATTATTATGACACTGAAGTTGGCAGACGT       1260
CTAAAAATTTTTGATTTTTTTTCTAATTAAATTACAACTAAAAAGATATTTATGAAAAAATGCACT       1326
TATAGTTTTTCGAGTTTTTAACATGTGATATTTTTTTTTTAATTGTTTAATTGAAATTTTTTCTAA       1392
AAATTTTGAAATGTCAGCTAACTTAATTTTCATTAATTATTACAAAATGTTAAACAAAAAGCAAAA       1458
AAAAAAAAAAAAAAAAAA                                                       1476
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Fig. 4-2: Nucleotide sequence and deduced amino acid sequence of a cDNA coding for Vn50. The numbers of nucleotides and amino acid residues are shown at the end of each line. Amino acid sequences obtained from N-terminal and peptide microsequencing are underlined. A putative polyadenylation site is underlined. The location of degenerate primers used for initial isolation of the gene is dot underlined.

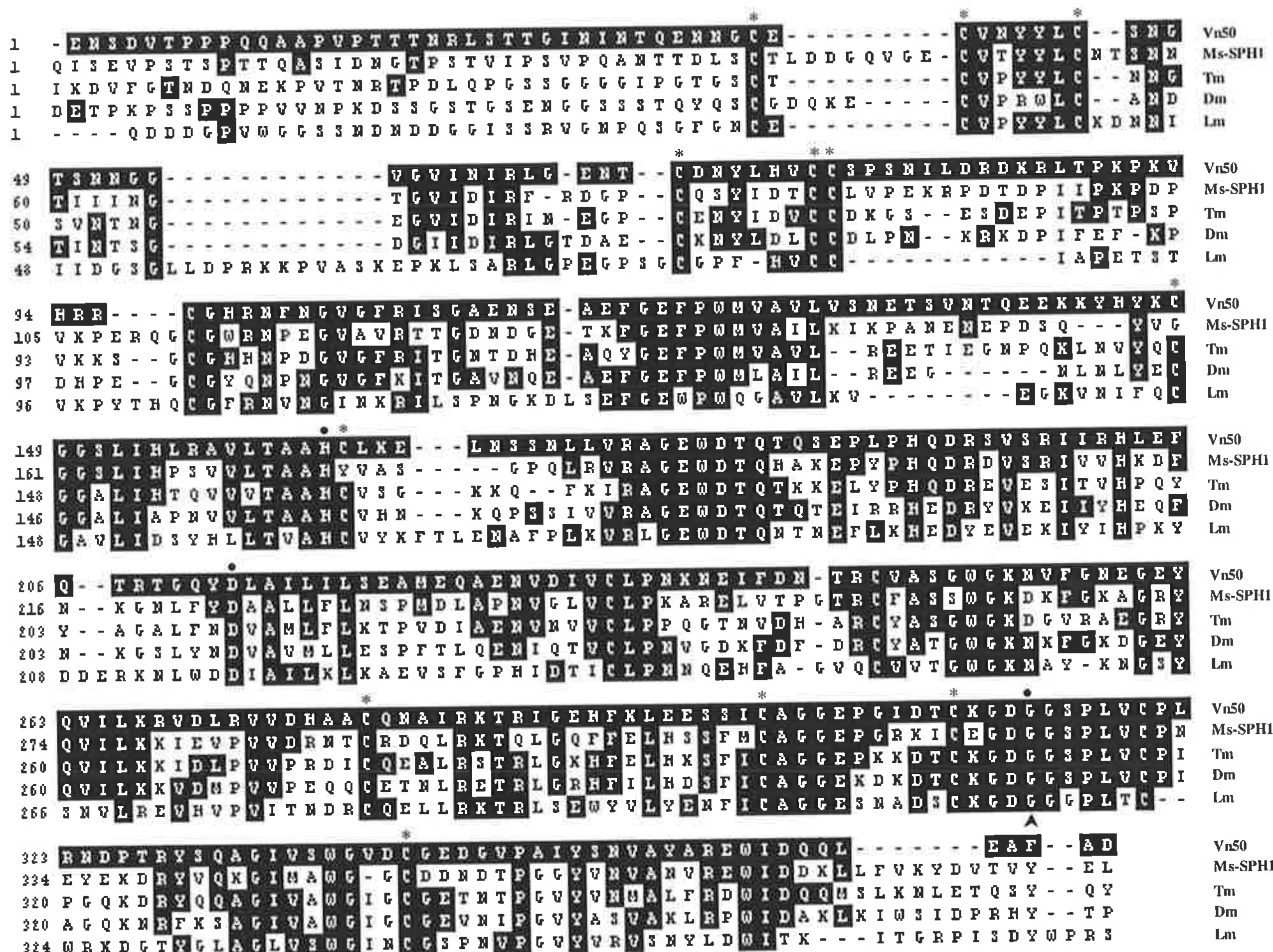
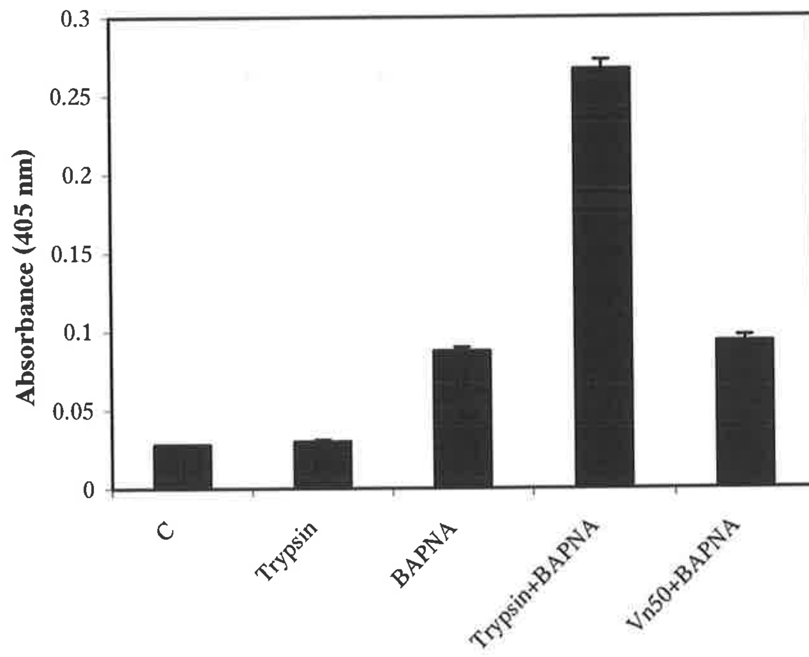


Fig. 4-3: Sequence alignment of *C. rubecula* Vn50 with serine proteinase homologs (SPH). The mature polypeptide sequences of Vn50 are aligned with SPH from *M. sexta* (Ms-SPH1, AAM69352), *T. molitor* (Tm, CAC12696), *D. melanogaster* (Dm, AAF52904), and *Limulus* factor D (Lm, BAA13312). GenBank accession numbers are provided in the brackets. Identical residues with Vn50 are black boxed. Conserved cysteine residues in the clip domain and serine proteinase domain are marked by asterisks. Residues in the catalytic triad of serine proteinases His, Asp and Ser are indicated by solid circles. The substitution of serine by glycine in the active site of SPHs is shown by an arrow head.

Figure 4:

(A)



(B)

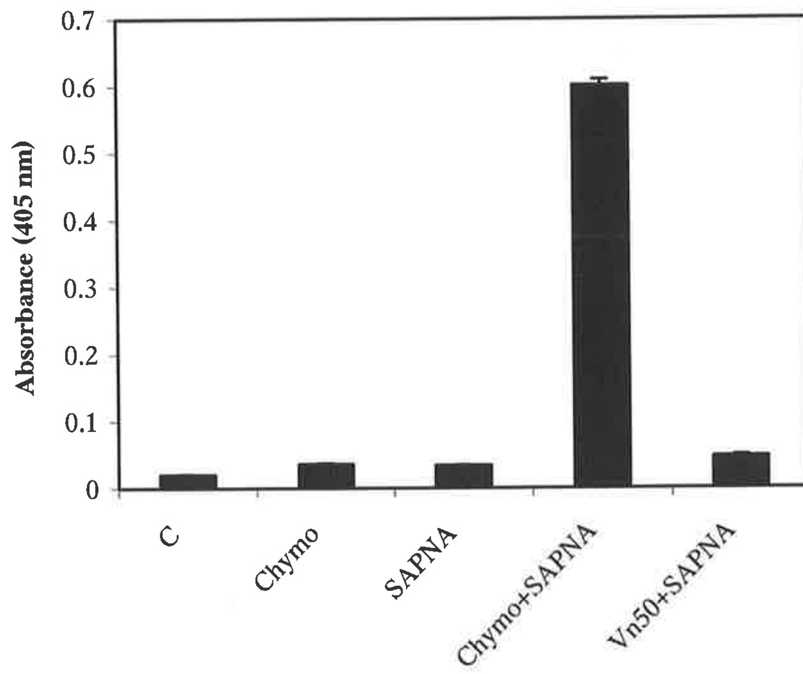


Fig. 4-4: Protease activity assay of Vn50 using synthetic substrates BAPNA (A) and SAPNA (B) for trypsin and α-chymotrypsin (chymo) enzymes, respectively. Hydrolyzed chromogenic substrates were detected spectrophotometrically at 405 nm after 2 h. C, 50mM Tris, pH 8.0 and 10mM $CaCl_2$ (buffer); Trypsin/Chymo, 1U/0.15U enzyme plus buffer; BAPNA/SAPNA, 4mM substrate in buffer; Trypsin+BAPNA/Chymo+SAPNA, enzymes plus substrates mixed (quantities as above); Vn50+BAPNA/Vn50+SAPNA, reactions including four wasps equivalent purified Vn50 (ca. 0.5μg) and 4mM of substrates in buffer. Vn50, a serine proteinase homolog did not show any enzyme activity with neither of the substrates whereas control enzymes digested the substrates. Bars indicate standard errors of the means.

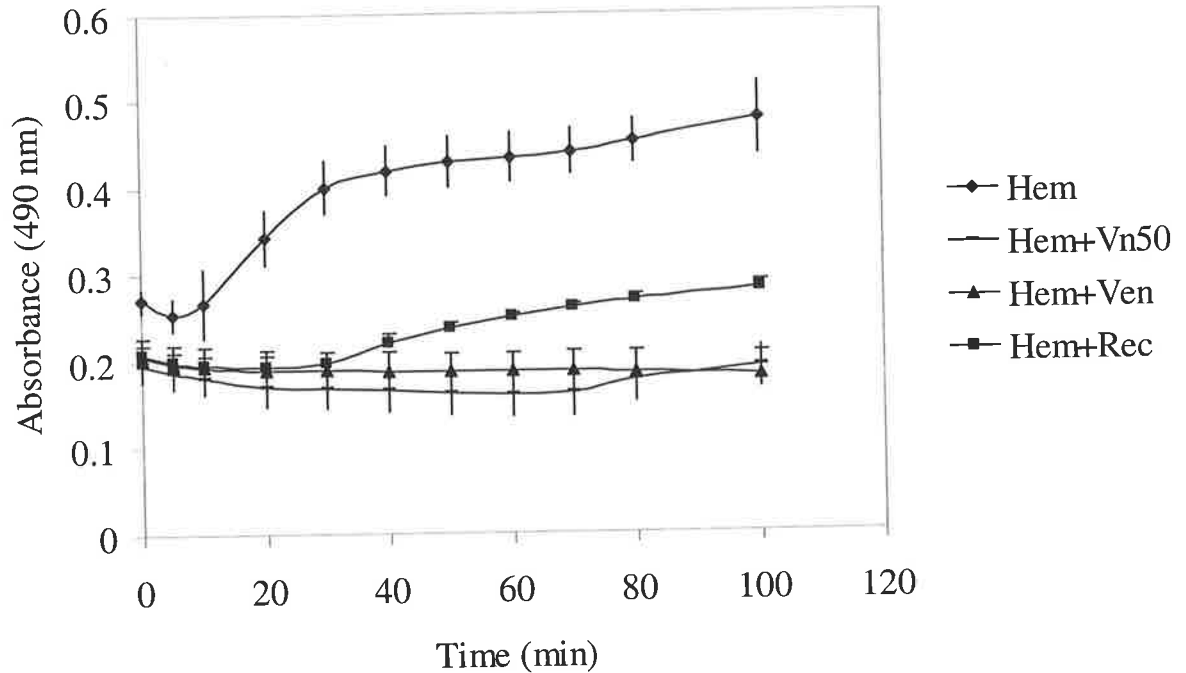


Fig. 4-5: Inhibition of prophenoloxidase activity by Vn50. Cell-free hemolymph from two 4th instar *P. rapae* larvae was mixed with 900 μl 20 mM DOPA substrate and absorbance was monitored at 490 nm for 100 min (Hem). In other treatments, total venom from four wasps (Hem+Ven), 0.5 μg purified Vn50 on rpHPLC (four wasps equivalent; Hem+Vn50) or 0.5 μg recombinant Vn50 (Hem+Rec) were added to the reaction. Total venom and purified native and recombinant Vn50 proteins similarly abolished proPO activity. Bars indicate standard errors of the means.

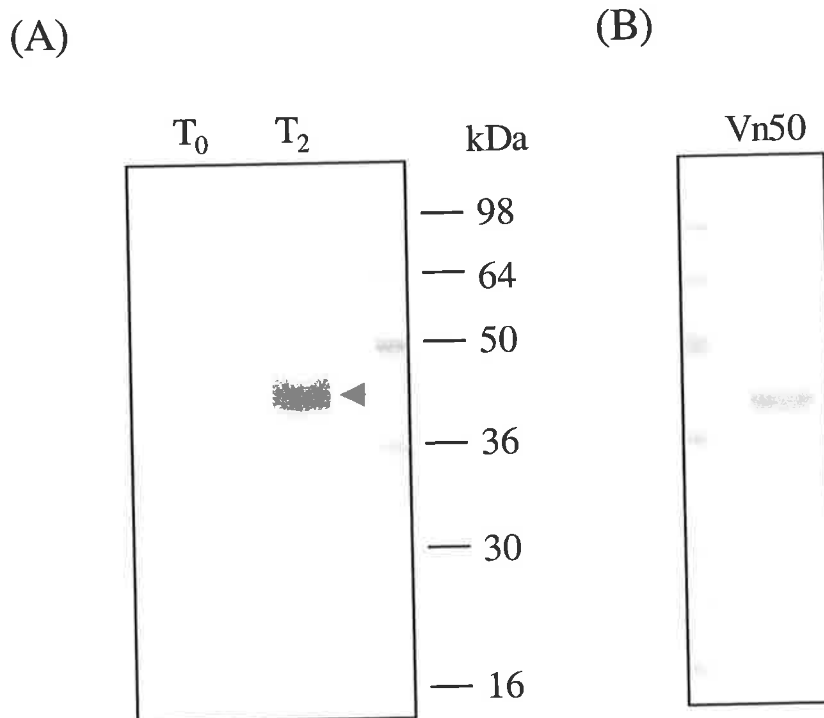


Fig. 4-6: Expression of Vn50 in *E. coli*. The coding region for the secreted Vn50 (see Fig 4-2) was expressed in *E. coli* under *lac*Z promoter. (A) A ca. 42 kDa fusion protein with 6×His residues, corresponding to the predicted size, was detected 2 h after induction with 1 mM IPTG (T_2) using monoclonal antibodies to poly-His (arrowhead) in a Western blot analysis. (B) The identity of the expressed protein was confirmed by anti-venom antibodies which cross-reacted with the protein.

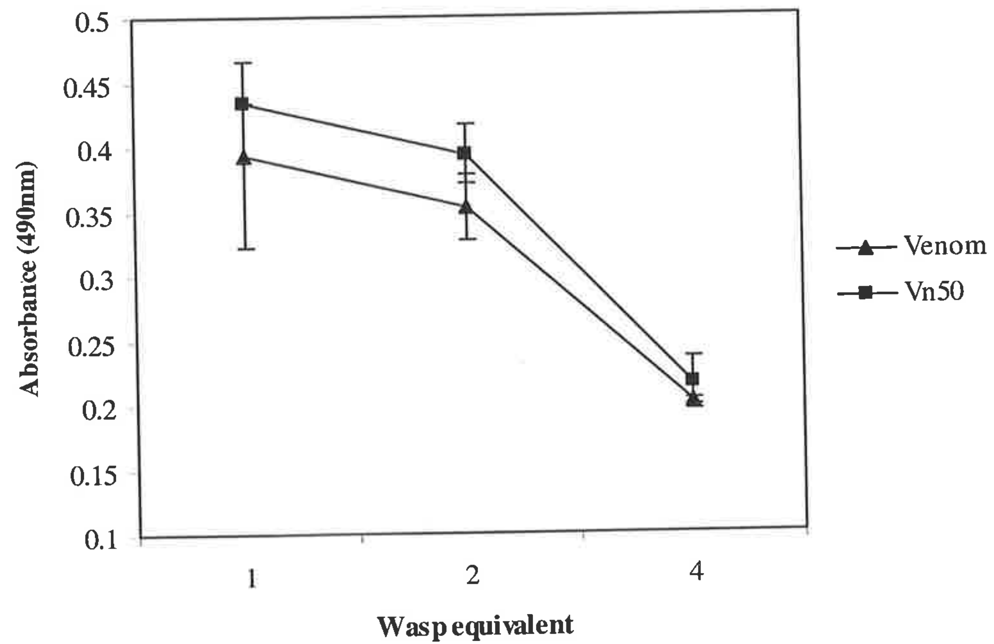


Fig. 4-7: Dose-dependent inhibition of hemolymph melanization by Vn50. Melanization assays were carried out as in Fig. 4-5 using total venom and purified Vn50 from 1, 2 and 4 wasp equivalents. Only four wasp equivalents of total venom or Vn50 completely inhibited hemolymph melanization. Absorbence at 490 nm after 70 min is shown. Bars indicated standard errors of the means.

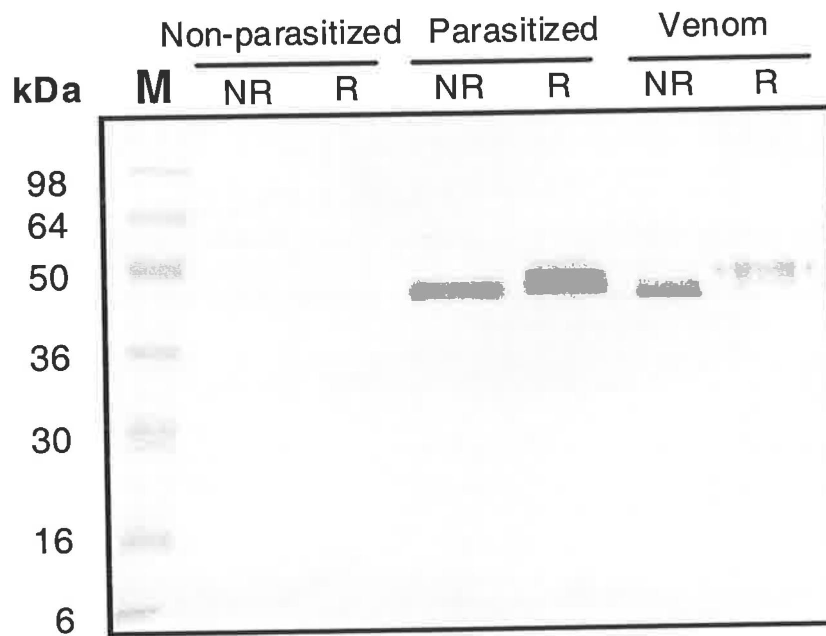


Fig. 4-8 Status of Vn50 in *P. rapae* hemolymph following parasitization. Cell-free hemolymph was collected from non-parasitised or parasitised (2 h) larvae and analysed on a Western blot under reducing (R) and non-reducing (NR) conditions. The blot was probed with Vn50 antibodies. Vn50 was not detected in the ~~naïve~~ non-parasitised caterpillars but in parasitised ones. The result also indicated that the protein was not cleaved into the clip and proteinase-like domains. Molecular masses of the protein standards (M) are marked on the left.

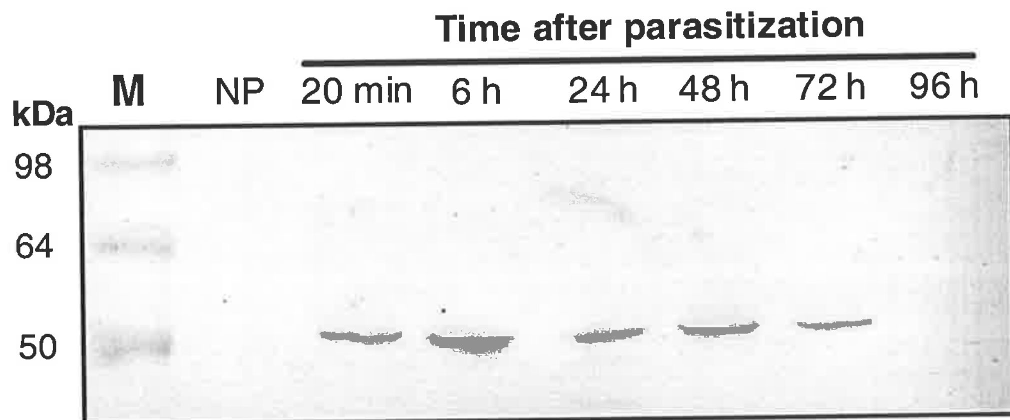


Fig. 4-9: Stability of Vn50 in the host hemolymph. Western blot analysis of cell-free hemolymph samples from third instar *P. rapae* larvae at various times after parasitization showed that the protein is not degraded by host proteinases and persists for at least 72 h. In each well, ca. 20 µl hemolymph from each parasitized larva was loaded. The blot was probed with anti-Vn50 antibodies. NP: hemolymph from non-parasitised larvae. Sizes of the molecular weight standards (M) are indicated on the left.

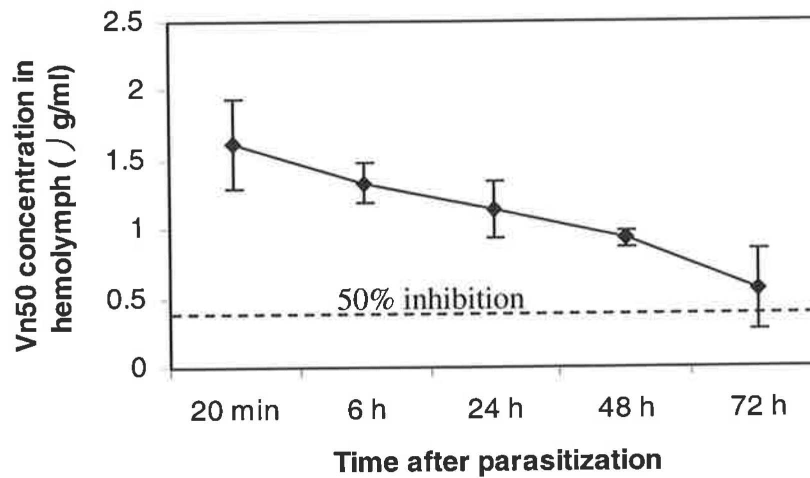


Fig. 4-10: Quantification of Vn50 in *P. rapae* hemolymph following paraistization. Concentration of Vn50 in *P. rapae* larval hemolymph was estimated at various times after parasitization by comparative western blotting. Three larvae were used for each time point. Dotted line represents the Vn50 concentration at which 50 % reduction of proPO activation occurred *in vitro* (Zhang *et al.*, 2004). Bars indicate standard errors of the means.

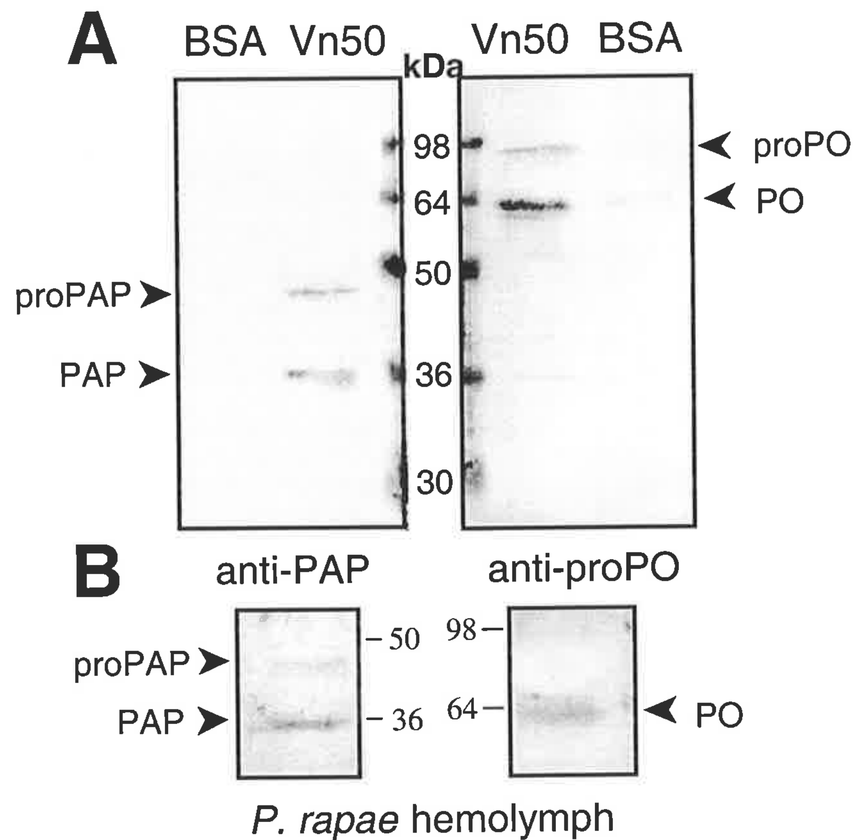


Fig. 4-11: Interaction of Vn50 with host hemolymph components. (**A**) Cell-free hemolymph from *P. rapae* larvae was incubated with beads coated with bovine serum albumin (BSA, control) or recombinant Vn50 and washed with buffers and salt as described in Materials and methods. Proteins bound to the beads were then analysed by SDS-PAGE and Western blotting, which were probed with PAP-1 (left panel) or proPO (right panel) antibodies. Both antibodies recognized the corresponding proteins only on the beads that were coated with Vn50. The enzymes were both found in their inactive (proPAP and proPO) and cleaved forms. This indicates that Vn50 may specifically interact with components of the proPO activation cascade. Sizes of the molecular weight markers are indicated. (**B**) The anti-proPO and anti-PAP-1 antibodies recognized the corresponding proteins in *P. rapae* hemolymph.

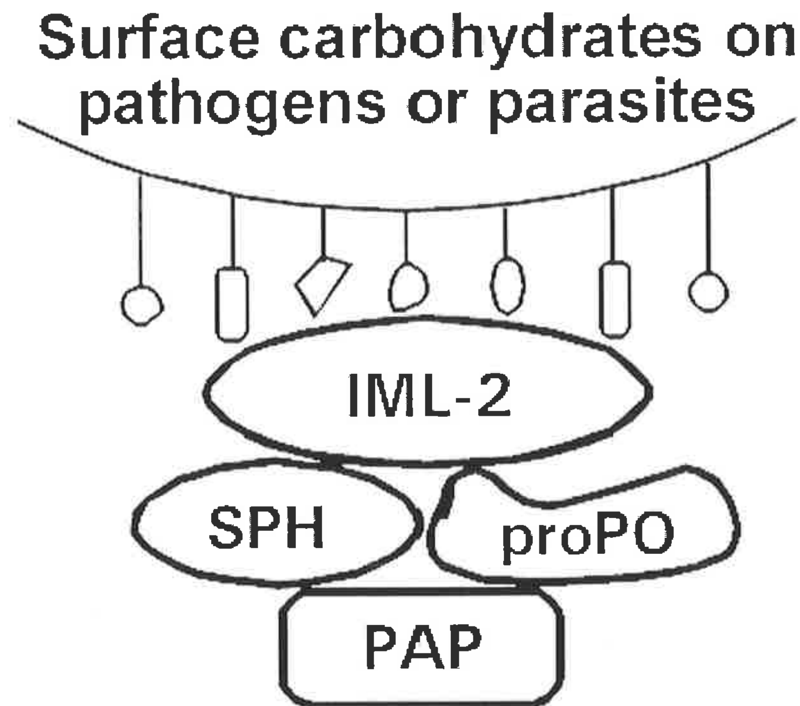


Fig. 4-12: Proposed model for IML-2 initiated protein-protein interactions (Adopted from Yu *et al.*, 2003). IML-2 recognizes and binds to surface carbohydrates on pathogens or parasites, initiates protein-protein interactions with serine proteinase homolog (SPH) and prophenoloxidase (proPO), while SPH functions as a mediator to recruit other plasma proteins such as prophenoloxidase-activating proteinase (PAP) to the surface of pathogens or parasites to activate proPO.

Chapter 5: Isolation and characterization of a novel venom protein, calreticulin

5.1 Introduction

Insects utilize humoral and cellular defense responses against invading parasites and pathogens. Humoral immune responses involve mainly the synthesis of antimicrobial proteins (Boman, 1991), melanization and blood clotting regulated by the prophenoloxidase activating system (Söderhäll & Cerenius, 1998). The cellular immune responses include phagocytosis, nodule formation and encapsulation (Strand & Pech, 1995; Lavine & Strand, 2002). Encapsulation is a major defense response against eggs and larvae of parasitoid wasps. For successful parasitism and development, parasitoids have developed various mechanisms to evade or suppress host encapsulation responses by interfering with every possible step during the cellular reaction: recognition of the parasitoid as a foreign object, adhesion, spreading and melanization (Strand & Pech, 1995; Gillespie *et al.*, 1997). The most uncertain and controversial aspect of encapsulation is how hemocytes recognize foreign objects and what molecules are involved in this process?

Since insects lack specific receptors generated by somatic mechanisms during ontogeny, diagnostic molecular patterns are used to recognize pathogens and parasitoids as non-self (Medzhidov & Janeway, 1997; Janeway & Medzhidov, 1997). A few pattern recognition proteins have been identified including C-type lectins (Mckenzie & Preston, 1992; Chen *et al.*, 1995), hemolin (Sun *et al.*, 1990; Zhao & Kanost, 1996) and LPS-binding proteins (Xu *et al.*, 1995; Charalambidis *et al.*, 1996). Cho *et al.* (1999a, b) described three early-stage encapsulation-relating proteins (ERPs) from the colepteran insect, *T. molitor* larvae. Recently, Choi *et al.* (2002) isolated and characterized an ERP, calreticulin (CRT), from *G. mellonella* larvae, which may be involved in the non-self recognition process in cellular defense reactions.

CRT is a ubiquitous calcium (Ca^{2+})-binding protein with multifunctional properties (Michalak *et al.*, 1992). It was originally found in the endoplasmic reticulum (ER) (Michalak *et al.*, 1992) and plays a variety of important roles in the regulation of key cellular function (Michalak *et al.*, 1999; Fraser *et al.*, 2000). However, CRT has also been isolated in a variety of non-ER locations, which include the cytoplasm, nucleus, cytotoxic granules in T cells, tick saliva, blood serum, sperm acrosomes and cell surface (Spiro *et al.*, 1996; review in Ferreira *et al.*, 2004). CRTs contain three domains: N-terminal, proline-rich and C-terminal domains (Nakhasi *et al.*, 1998). The P-rich domain has a Ca^{2+} binding site, lectin-binding and chaperone functions (Nakhasi *et al.*, 1998). CRT was also involved in many host-parasite interactions (Nakhasi *et al.*, 1998; Prichard *et al.*, 1999; Jaubert *et al.*, 2002; Ferreira *et al.*, 2004). In addition, CRT was found to mediate cell spreading and adhesion (Yao *et al.*, 2002; Ferreira *et al.*, 2004). There is growing evidence suggesting that calreticulin may mediate a broad array of cellular functions.

The wasp *C. rubecula* has evolved effective mechanisms against host immune responses, including passive evasion of the host defense (Asgari & Schmidt, 1994; Asgari *et al.*, 1998) and active suppression of host cellular and humoral immune responses (Asgari *et al.*, 1996, 1997, 2003a, c; Glatz *et al.*, 2003; Zhang *et al.*, 2004a). Both mechanisms are essential for the completion of wasp development inside the host caterpillar. The immunoprotective protein Crp32 is produced in the calyx cells of the female wasp ovaries and attached to the surface of egg. This protein was found to be a major protective component and effectively protects the egg against the immediate attack by host components before the expression of PDV genes (Asgari *et al.*, 1998).

This chapter describes the isolation and characterization of a calreticulin (CrCRT) from *C. rubecula* venom glands. The protein was also found associated with CrPDV particles (Asgari *et al.*, 2003b). To understand the CrCRT function from the venom in host regulation, we decided to produce the protein in a bacterial expression system to examine the functional properties of the recombinant protein. The results show that this protein can inhibit hemocyte aggregation and spreading. It also protects abiotic objects

against the host encapsulation response. Since host-specific calreticulin was identified from the host *P. rapae*, this suggests that the soluble parasite-specific protein might function as an antagonist molecule, competing for the binding sites with the host calreticulin.

5.2 Materials and Methods

5.2.1 Insects

Both the parasitoid *C. rubecula* and its host were maintained as described in Chapter 2.

5.2.2 Screening a cDNA library using anti-venom antibodies

A *C. rubecula* ovary/venom gland expression cDNA library was constructed using SMART cDNA library construction kit according to the manufacturer's instructions (Clontech, and see Chapter 2). Anti-venom antibodies were used to screen the cDNA library as described in Chapter 2. Positive plaques were detected by a secondary antibody and isolated for re-screening. Confirmed single positives were re-circularized as plasmids according to the manufacturer's instructions (Clontech). Inserts were sequenced in both directions.

5.2.3 Recombinant protein expression and purification

The calreticulin was expressed in bacteria containing the mature protein from amino acid 28 to 385. The coding region was amplified by PCR. The forwards primer (CRT-BamHI-F, 5'-GCGCGGATCCGAAGTTTTCTTTGAAGAG-3', restriction sites are underlined) and reverse primer (CRT-HindIII-R, 5'-CGCGAAGCTTTTACAATTCGTCGTGCTC-3') were designed to contain *Bam*HI and *Hind*III restriction sites, respectively. The PCR amplified product was digested with *Bam*HI and *Hind*III enzymes and cloned into the corresponding sites in pQE30 expression vector (Qiagen). Transformed bacteria were induced with 1mM IPTG for 2 h

and analyzed on a 12% SDS-PAGE gel as described (Laemmli, 1970). The identity of the protein was confirmed by Western blotting using a polyclonal antiserum raised against *G. mellonella* calreticulin (1:5000). Alkaline phosphatase-conjugated anti-rabbit IgG antibodies were used as secondary antibodies (1:5000, Sigma).

Since the expressed protein was found in the insoluble fraction, it was purified under denaturing conditions according to the manufacturer's instructions (Qiagen). The fusion protein was purified by affinity chromatography using Ni-NTA technology (Qiagen) and eluted with 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris at pH 4.5. To refold the protein, it was dialyzed against 150 mM NaCl, 10 mM Tris (pH 7.5) for 16 h at 4°C.

5.2.4 Production of a specific antibody

For the preparation of a specific antiserum, purified calreticulin expressed in bacteria was run on preparative polyacrylamide gels, stained in 4% Coomassie blue in water and used for immunisation. The corresponding bands were excised from the gel, smashed and mixed with an equal volume of complete Freund's adjuvant for first injection and incomplete Freund's adjuvant for further boosters. Immunisations were performed as described in Chapter 2.

5.2.5 SDS-PAGE and Western blotting analyses

Protein samples were separated on 12 % SDS-PAGE gels (Laemmli, 1970), transferred onto nitrocellulose membranes as described in Chapter 2. Anti-CRT antibodies (1:5000) were used as a probe followed by detection with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (1:5000).

5.2.6 Isolation of RNA and slot blotting

Total RNA was extracted from venom glands and wasp carcasses devoid of venom glands and ovaries as described in Chapter 2. RNA samples (3 μg for each) were blotted

onto a nylon membrane under denaturing conditions according to the manufacturer's instructions (BioRAD). The membrane was pre-hybridized in pre-hybridisation buffer at 65 °C for 2 h and then hybridized with a [^{32}P]-labelled PCR product coding for the CrCRT fragment overnight at 65 °C. After washing twice in 2 × SSC containing 0.1 % SDS at 65 °C for 10 min, twice in 0.2 × SSC containing 0.1 % SDS at 65 °C for 10 min, the membrane was exposed to XAR film (Kodak).

5.2.7 Inhibition of hemocyte aggregation and spreading

Fourth instar *P. rapae* larvae were surface sterilized in 70 % ethanol and directly bled into wells with 100 µl of PBS saturated with phenylthiourea by removing one of the legs. After 10 min incubation to allow the cells to attach, 20 µl of 4-fold, 2-fold and non-diluted purified recombinant CrCRT (60 µg/ml) were added, respectively. Hemocytes were incubated for additional 1 h at 25 °C and observed under a phase contrast microscope.

5.2.8 *In vitro* encapsulation assay

The assay was performed as described previously with minor modifications (Asgari *et al.*, 1998). Briefly, one day prior to the assay, several wells in a microtiter plate were seeded with Sf21 cells (*Spodoptera frugiperda* cell culture). Cells were removed from the wells and replaced by fresh Grace's insect culture medium (GIBCO/BRL) saturated with phenylthiouria. This leaves a layer of secreted proteins with an apparent effect on adhesion and degranulation of hemocytes that are in contact with the plastic surface. Two 4th instar *P. rapae* caterpillars were bled directly in each well to which resin beads were also added. Ni-NTB beads (Qiagen) were covered with recombinant CrCRT or with bacterial proteins by incubating the beads with the soluble fractions of the bacteria containing the plasmid (pQE30-CrCRT) and the bacteria containing the vector pQE30 for one hour at RT with mild shaking. After that, the beads were washed three times with PBS.

5.2.9 Early-stage encapsulation-relating protein assays

Triggering of an early-stage encapsulation response and recovery of the early-stage ERPs from *P. rapae* larvae were carried out as previously described (Choi *et al.*, 2002). Briefly, beads (DEAE Sepharose CL-6B) were resuspended with insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 6.0). 10 µl of bead suspension was injected into each 4th instar *P. rapae* larva that had been anesthetized on ice. Hemolymph was harvested after 10 min, by injecting the larvae with 20 µl of modified anti-coagulation buffer (30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.5). The proleg of each larva was cut off using fine scissors and the hemolymph harvested on ice in a test tube. The beads were collected from the hemolymph by centrifugation (750 × *g*, 10 sec), and washed twice with anti-coagulation buffer. The materials coating the beads were recovered by using 6 M guanidine-HCl in 50 mM Tris-HCl containing 1 mM EDTA solution (pH 7.0) and were analyzed on 12 % SDS-PAGE under denaturing conditions. Western blot with anti-CrCRT antibodies was performed as described above or Chapter 2.

5.3 Results

5.3.1 Isolation and characterization of calreticulin

Using anti-venom antibodies to screen a *C. rubecula* venom/ovary expression cDNA library, several positives were obtained. Sequencing analysis of the positives showed that one of the cDNAs contained an open reading frame coding for a putative protein that showed similarity to calreticulins (CrCRT). The cDNA obtained coding for CrCRT is 1367 bps in length coding for 403 amino acids. A methionine at position 1 was identified as the putative initiation site (Cavener & Ray, 1991) and a stop codon at position 1210 (Fig. 5-1). Using PSORT II (http://psort.nibb.ac.jp/form2.html) a putative cleavage site was found between amino acids 18 and 19 consistent with other calreticulins (Fig. 5-1). At the end of the C-domain, a conserved HDEL motif was identified (Fig. 5-2). In mammalians a KDEL sequence is conserved and believed to be

an endoplasmic reticulum (ER) retention signal. However, calreticulins isolated so far from insects all have a HDEL sequence instead. As mentioned above, sequence alignment in GenBank showed a high similarity between the gene product of the isolated cDNA and calreticulins. The highest similarities were found with *G. mellonella* (73.8%) and *Anopheles gambiae* (69.2%) calreticulins.

In a preliminary experiment, we used anti-calreticulin antibodies from *G. mellonella* in a Western blot containing the venom fluid from the venom storage sac. The antibody cross-reacted with a 58 kDa protein (Fig. 5-3). The predicted size for CrCRT is 46.5 kDa with a pI of 4.40, although calreticulins usually runs atypically at 60 kDa on SDS-PAGE (Opas *et al.*, 1996, Spiro *et al.*, 1996). The fact that the protein is found in the venom fluid suggests that the protein is secreted by venom gland cells and stored in the venom sacs.

5.3.2 Production of recombinant CrCRT in *E. coli*

In an attempt to produce recombinant CrCRT for functional analyses, we constructed a plasmid which encodes the mature CrCRT attached to a hexahistidine tag at its amino terminus. SDS-PAGE analysis showed the presence of a 55 kDa protein that was highly up-regulated in induced cells (Fig. 5-5 A) and produced mainly in an insoluble form at a low concentration (data not shown). Ni-NTA resin beads were used to purify the recombinant protein from the denatured soluble fraction. After dialysis, a ca. 55 kDa protein was only visible in recombinant bacteria (Fig. 5-5 B). The identity of the purified protein was confirmed by its cross-reactivity with anti-calreticulin antibodies raised against *G. mellonella* calreticulin (Fig. 5-5 C). Purified protein from the insoluble fraction was used for injection into rabbits and production of anti-CrCRT antibodies.

5.3.3 Tissue-specific localization of CrCRT

To examine the localization of CrCRT in adult *C. rubecula* female wasps, we collected the proteins from ovaries, venom glands, venom reservoirs and gut. As shown in Fig. 5-

4 A, CrCRT was detected in all the organs examined. A significant amount of protein can also be detected in venom reservoir, indicating that CrCRT is an extracellular component of the maternal secretions in this endoparasitoid. In addition, RNA slot blot analysis showed that CrCRT is more expressed in the venom glands compared to the rest of the body (Fig. 5-4 B).

5.3.4 Inhibition of hemocyte aggregation and spreading by CrCRT

Several components from this wasp venom have been identified to suppress host immune responses by inactivating the phenoloxidase activating system (Asgari *et al.*, 2003a, c; Zhang *et al.*, 2004a) and facilitating the expression of CrBV genes (Zhang *et al.*, 2004b). To elucidate the function of the venom CrCRT in host regulation, we investigated a possible role in host immune suppression. Phenoloxidase enzyme activity assay showed that CrCRT did not interfere with melanization of host hemolymph (data not shown).

We therefore examined a possible interference with cellular defence reactions. In the absence of recombinant CrCRT hemocytes migrated to form well-defined aggregates and spread more readily after 1 h incubation (Fig. 5-6 A). In the presence of recombinant CrCRT hemocytes were prevented from aggregation and spreading. CrCRT displayed concentration-dependent inhibition of aggregation shown in Fig. 5-6 B-D. When 4-fold diluted recombinant CrCRT was applied, the aggregation and spreading of hemocytes was partially inhibited after 1 h incubation (Fig. 5-6 B). When 2-fold diluted CrCRT was added, few hemocyte aggregations were observed (Fig. 5-6 C). Addition of non-diluted recombinant CrCRT completely inhibited hemocyte spreading and aggregation after 1 h incubation (Fig. 5-6 D).

5.3.5 Effect of CrCRT on encapsulation *in vitro*

To investigate whether CrCRT is involved in conferring protection against foreign objects intruding the insect body, an *in vitro* encapsulation assay with resin beads covered with

recombinant CrCRT was carried out. When the CrCRT-coated beads were incubated with isolated *P. rapae* hemocytes for 4 h, no evidence of encapsulation was observed (Fig. 5-7 C), whereas both control beads devoid of CrCRT were partially surrounded by hemocytes (Fig. 5-7 A, B). The encapsulation rate observed in the control beads is more than half, and is somewhat influenced by the ratio of hemocyte and bead numbers added. Under these conditions, no encapsulation was observed on CrCRT-coated beads.

5.3.6 Identification of an early-stage encapsulation-relating protein

Calreticulin was identified as an early-stage ERP in *G. mellonella* larvae and suggested to be involved in non-self recognition in encapsulation response (Choi *et al.*, 2002). To elucidate the molecular mechanism of CrCRT interference with the encapsulation response, putative early-stage ERPs were examined. DEAE-Sepharose beads were injected into naïve *P. rapae* larvae and recovered after 10 min incubation. The bound proteins were separated on 12 % SDS-PAGE gel, and blotted with anti-CrCRT antibodies. A 52 kDa *P. rapae* CRT was found to be enriched (Fig. 5-8), which suggests that CrCRT might function as an early-stage ERP.

5.4 Discussion

Venom proteins introduced by endoparasitoids into host insects at oviposition have diverse functions, but mainly in manipulations of host physiology. Depending on whether the endoparasitoid produces virus-like particles such as polydnaviruses or not, venom may complement or replace PDV functions in host regulation and immune suppression. In endoparasitoids with PDVs, a combination of maternal factors is used to suppress host immune responses, whcre venom proteins were considered to play a synergistic role with PDVs (Kitano, 1986; Tanaka, 1987; Stoltz *et al.*, 1988; Wago & Tanaka, 1989). In addition, venom proteins were found to mediate short-term immune suppression before the PDV gene expression (Webb & Luckhart, 1994).

In the *C. rubecula/P. rapae* system, maternal factors introduced into the body of the host by the wasp at oviposition are essential for successful parasitism. An immunosuppressive gene (*CrV1*) from *C. rubecula* bracoviruses is involved in the destabilization of endo-cytoskeleton in host hemocytes (Asgari *et al.*, 1996, 1997). In addition, a calyx protein (Crp32) on the surface of CrBV particles and eggs, provides short-term protection of the parasitoid eggs against host encapsulation reactions before CrBV genes are expressed (Asgari & Schmidt, 1994; Asgari *et al.*, 1998). When crude venom preparations, isolated from female *C. rubecula*, were separated by rpHPLC, over a dozen proteins were found in the venom extract, some of which are involved in inhibition of melanization reactions by blocking the activation of prophenoloxidase (Asgari *et al*, 2003a, c; Zhang *et al.*, 2004a). Some components interact with CrBVs and are required for the expression of CrBV genes (Zhang *et al.*, 2004b).

In the present study, we explored the functional role of a novel venom protein CrCRT from *C. rubecula* in cellular defence reactions. Experimental evidence presented here indicates that CrCRT is able to inhibit hemocyte aggregation and spreading (Fig. 5-6), where the inhibition is dose-dependent (Fig 5-6). The recombinant protein attached to Ni-resin beads precludes hemocytes *in vitro* from attaching to the surface, thus protecting the beads from being encapsulated (Fig. 5-7 C). Hemocyte de-adhesion might have synergistic effects in conjunction with the immunoprotective action of another calyx protein Crp32, which protects the parasitoid eggs before the CrBV genes are expressed to suppress hemocyte adhesive functions. However, the molecular mechanism of CrCRT-mediated protection is not clear.

CRT has been reported to be a multifunctional Ca^{+}-binding protein in many intra- and extra-cellular processes (Michalak *et al.*, 1992; Gray *et al.*, 1995; White *et al.*, 1995). CRT can be released from the cells by either active secretory processes or cell death (Zhu *et al.*, 1997; Ferreira *et al.*, 2004). Interestingly, the tick *Amblyomma americanum*, while feeding on its host, secretes CRT (Kovacs *et al.*, 2001), presumably as a mechanism to divert host defensive responses. In host vertebrates, the protein is a target for both cell-mediated and innate immune responses by generating antibodies that are

cross-reactive with host CRT (Kovacs *et al.*, 2001; Ferreira *et al.*, 2004). In our study, a CRT from *P. rapae* was identified as a putative early-stage encapsulation response protein (Fig. 5-8), which might be involved in non-self recognition. It is therefore possible that CrCRT might function as an antagonist molecule competing for binding sites with the host calreticulin.

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GTACGTAAAATGCGAGCTCTCGTTGCTGTACTGGCAATTGCCGCAATTGCCACCGCGTCCGCTGAA   57
         M  R  A  L  V  A  V  L  A  I  A  A  I  A  T  A  S  A  E     19
GTTTTCTTTGAAGAGAGATTTTCAGACGATTCATGGGAAAAGAACTGGGTATACTCAGAGCATCCC  123
V  F  F  E  E  R  F  S  D  D  S  W  E  K  N  W  V  Y  S  E  H  P     41
GGCAAAGAATTCGGAAAGTTCAAGCGGACTGCTGGAAAATTCTACCACGAAGAATTACAAGACACA  189
G  K  E  F  G  K  F  K  R  T  A  G  K  F  Y  H  E  E  L  Q  D  T     63
GGTATCCAAACAACTGAAGATGCCAGGTTCTACGCTCTCAGCAATAAGTTCAAGCCATTCACGAAC  255
G  I  Q  T  T  E  D  A  R  F  Y  A  L  S  N  K  F  K  P  F  T  N     85
AAGGACAAGCCATTGGTTGTCCAATTCACCGTCAAGCATGAACAGATGATCGATTGCGGTGGTGGC  321
K  D  K  P  L  V  V  Q  F  T  V  K  H  E  Q  M  I  D  C  G  G  G    107
TACGTGAAAGTCTTCGACTGTTCATTGGACCAGAAGGACATGCACGGAGAGACACCATACCTTCTT  387
Y  V  K  V  F  D  C  S  L  D  Q  K  D  M  H  G  E  T  P  Y  L  L    129
ATGTTTGGACCTGACATTTGTGGACCTGGAACCAAGAAAGTTCACGTAGTCTTCAACTACAAGGGA  453
M  F  G  P  D  I  C  G  P  G  T  K  K  V  H  V  V  F  N  Y  K  G    151
AAGAATCTGTTGATCAAGAAAGACATTCGTTGCAAGGACGACGTATACACTCACTTGTACACTTTG  519
K  N  L  L  I  K  K  D  I  R  C  K  D  D  V  Y  T  H  L  Y  T  L    173
ATTGTCAAACCAGACAACACTTACGAGGTTCTTATCGACAATGAAAAGGTAGAGTCTGGAGAACTT  585
I  V  K  P  D  N  T  Y  E  V  L  I  D  N  E  K  V  E  S  G  E  L    195
GAAGCTGACTGGGACTTCTTGCCACCCAAGAAAATCAAGGACCCGAATGAGAAGAAACCCGAAGAC  651
E  A  D  W  D  F  L  P  P  K  K  I  K  D  P  N  E  K  K  P  E  D    217
CGGGATGACCGTGCCACCATTCCTGATCCAGAAGACACCAAACCCGAAGATTGGGACAAGCCTGAG  717
R  D  D  R  A  T  I  P  D  P  E  D  T  K  P  E  D  W  D  K  P  E    239
ACCATCCCCGACCCTGAAGCCACCAAACCAGAAGACTGGGACGATGAAATGGACGGAGAATGGGAA  783
T  I  P  D  P  E  A  T  K  P  E  D  W  D  D  E  M  D  G  E  W  E    261
CCACCAATGATCGACAATCCAGACTTCAAAGGTGAATGGAAGCCAAAGCAAATTGACAACCCAGCT  849
P  P  M  I  D  N  P  D  F  K  G  E  W  K  P  K  Q  I  D  N  P  A    283
TACAAAGGACCATGGCTTCACCCTGAAATCGACAACCCAGAATACGTTAAGGACGAAGAACTCTAC  915
Y  K  G  P  W  L  H  P  E  I  D  N  P  E  Y  V  K  D  E  E  L  Y    305
AAGAAAGACGAAGTCTGCGCCATTGGTTTCGATCTTTGGCAAGTAAAGTCTGGAACCATCTTCGAT  981
K  K  D  E  V  C  A  I  G  F  D  L  W  Q  V  K  S  G  T  I  F  D    327
AACGTCCTCATCACTGATGAACCAGAGGCCGCCAGCAAATTCGCCGAAGATGTCTGGAAACCAAAC 1047
N  V  L  I  T  D  E  P  E  A  A  S  K  F  A  E  D  V  W  K  P  N    349
TTTGAAGGTGAAAAGAAAATGAAAGAAGCTCAAGATGAGGCTGAAAGAAAATCAATGGAGGCGGAG 1113
F  E  G  E  K  K  M  K  E  A  Q  D  E  A  E  R  K  S  M  E  A  E    371
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K  P  E  A  P  E  E  D  D  D  E  D  D  D  D  A  D  D  E  D  N  T    393
GTACCAGAAGTTGAGGAGCACGACGAATTGTAAATAAAAAATATGTCGCGCGTAGCATGAGAAGCA 1245
V  P  E  V  E  E [H  D  E  L] *                                     403
GACTGTATTCCAGGAGCTGTTGGGCAAGAAAAAGACACACCAGCTATTTCCAACACTACACAATCC 1311
ATTTATAAAATAACTTTAAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAACATGTC           1367
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Fig. 5-1: Nucleotide sequence and deduced amino acid sequence of cDNAs encoding for a calreticulin from *C. rubecula*. The numbers of nucleotides and amino acid residues are shown at the end of each line. In each sequence a putative initiation codon (ATG) is shown in bold letters and the stop codon is underlined. The conserved ER retention signal found in calreticulins is boxed. Putative cleavage site are indicated by arrow.

```
1   M R A L V - A V L A I A A I A T A S A E V F F E E R F S D D S W E K N W V Y S E H P G K E F G K F K  CR-CRT.PRO
1   M K S L V L I I V S L L T I S S I N C E V Y F E E K F P D D S W E S N W V Y S E H P G K E F G K F K  GM-CRT.PRO
1   M R T L A V L F A A F L A V - - - N A K V Y F E E G F K D D S W Q K T W V Q S E H K G V E Y G K F V  AG-CRT.PRO

50  R T A G K F Y H E E L Q D T G I Q T T E D A R F Y A L S N K F K P F T N K D K P L V V Q F T V K H E  CR-CRT.PRO
51  L T A G K F Y N D P E E D K G L K T S E D A R F Y A L S R K F K P F S N R D K P L V I Q F S V K H E  GM-CRT.PRO
48  H T A G K F Y N D A E A D K G L Q T S Q D A R F Y A L S N K F T P F S N K D D T L V I Q F S V K H E  AG-CRT.PRO

100 Q N I D C G G G Y V K V F D C S L D Q K D M H G E T P Y L L M F G P D I C G P G T K K V H V V F N Y  CR-CRT.PRO
101 Q E I D C G G G Y L K V F D C K L D Q K D M H G E S P Y E I M F G P D I C G P G T K K V H V I F S Y  GM-CRT.PRO
98  Q N I D C G G G Y L K V F D C S V D Q K D L H G E T P Y L V M F G P D I C G P G T K K V H V I F S Y  AG-CRT.PRO

150 K G K N L L I K K D I R C K D D V Y T H L Y T L I V K P D N T Y E V L I D N E K V E S G E L E A D W  CR-CRT.PRO
151 K G K N H L I K K D I R C K D D V Y T H L Y T L V V K P D N T Y E V L I D N E K V E S G E L E A D W  GM-CRT.PRO
148 K G K N H L I N K D I R C K D D V F T H F Y T L V V R A D N T Y E V L I D N E K V E S G S L E D D W  AG-CRT.PRO

200 D F L P P K K I K D P N E K K P E D R D D R A T I P D P E D T K P E D W D K P E T I P D P E A T K P  CR-CRT.PRO
201 D F L P P K K I K D P E A K K P E D W D D R A T I P D P D D K K P E D W D K P E H I P D P D A N K P  GM-CRT.PRO
198 D F L P P K K I K D P E A K K P E D W D D R A T I A D P D D T K P E D W D K P E H I P D P D A T K P  AG-CRT.PRO

250 E D W D D E M D G E W E P P M I D N P D F K G E W K P K Q I D N P A Y K G P W L H P E I D N P E Y V  CR-CRT.PRO
251 E D W D D E M D G E W E P P M I D N P E Y K G V W A P K Q I D N P A Y K G A W I H P E I D N P E Y T  GM-CRT.PRO
248 D D W D D E M D G E W E P P M I D N P E Y K G E W K P K Q I D N P A Y K G V W V H P E I D N P E Y E  AG-CRT.PRO

300 K D E E L Y K K D E V C A I G F D L W Q V K S G T I F D N V L I T D E P E A A S K F A E D V W K P N  CR-CRT.PRO
301 P D A N L Y K R D E L C A V G L D L W Q V K S G T I F D N F L F T D D I E V A K E R G E Q I K K T Q  GM-CRT.PRO
298 E D K S L Y L R E E V C A V G I D V W Q V K S G T I F D N F M I T N D L E E A K K V A A S V K E T Q  AG-CRT.PRO

350 F E G E K K M K E A Q D E A E R K S M E A E K P E A P E E D D D E D D D D A D D E D N T V P - - - -  CR-CRT.PRO
351 - E G E K K M K N Q Q D E A E R E K E K A E K P - - - - - - D D E D D E D L D D E A G E A S - - - -  GM-CRT.PRO
348 - E G E K K V K D A Q E A E E R K K A E G E A A A E E A A K D D E D E D D E D D A D N A L F G E A T  AG-CRT.PRO

396 - - E V E E H D E L  CR-CRT.PRO
390 - - P I E D H D E L  GM-CRT.PRO
397 E L D D E G H D E L  AG-CRT.PRO
            * * * *
```

Fig. 5-2: Amino acid sequence alignment of CrCRT with calreticulins from *Galleria mellonella* (BAB79277) and *Anopheles gambiae* (AAL68781). The GenBank accession numbers are in brackets. The conserved ER retention signal (HDEL) is shown by asterisks.

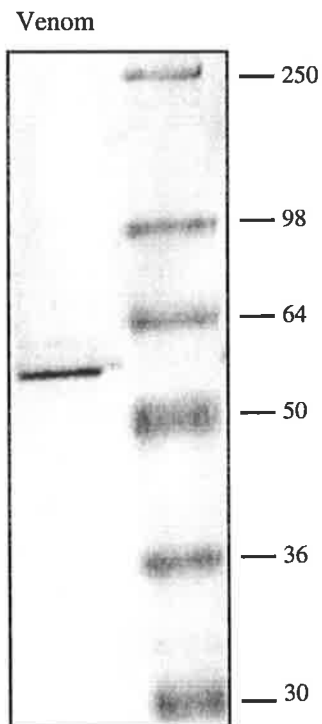


Fig. 5-3: Western blot analysis of venom proteins. 0.5 wasp equivalent of venom proteins were applied and analysed on a Western blot (12 % SDS-PAGE) using anti-calreticulin antibodies raised from *G. mellonella.* A 58 kDa protein was recognized by the antibodies.

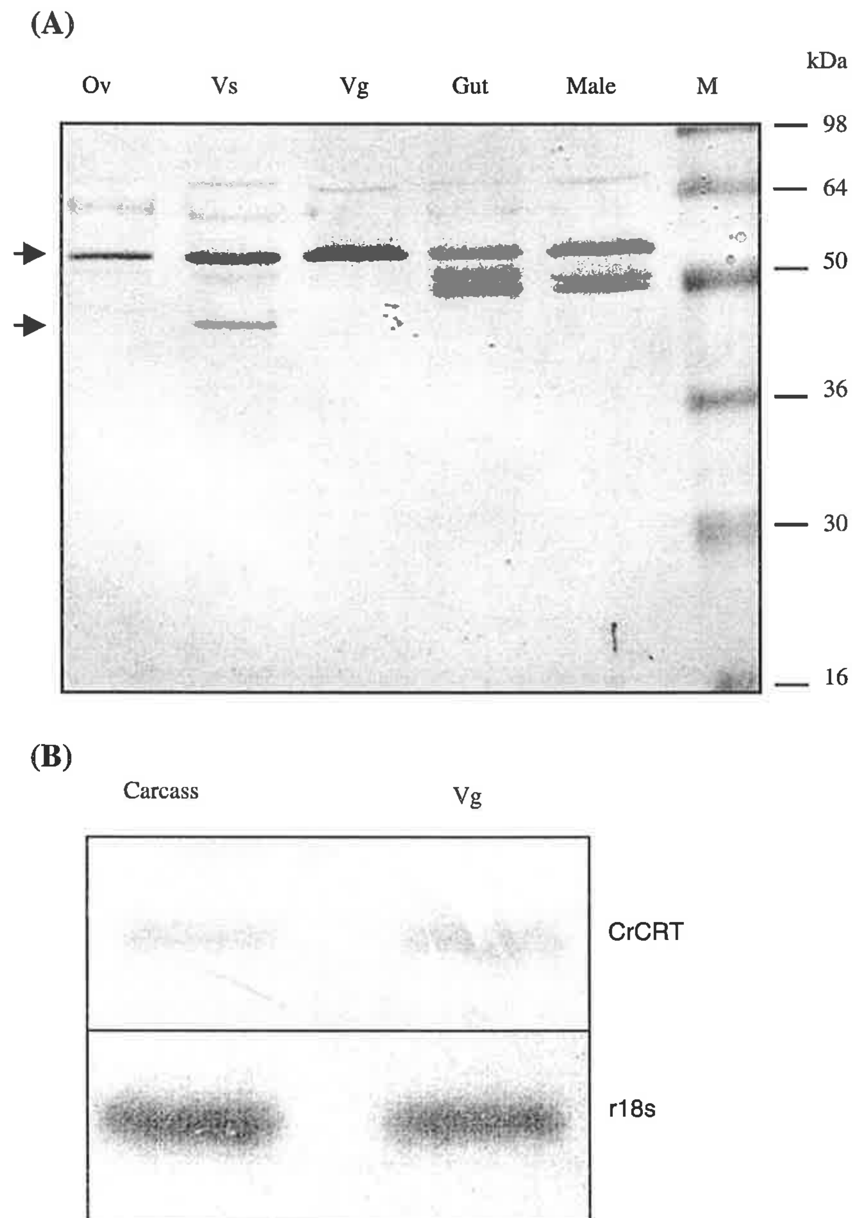


Fig. 5-4: (A) Determination of localization of CrCRT by Western blot analysis. Proteins were collected from ovaries (Ov), venom glands (Vg), venom reservoirs (Vs), gut and whole male wasps and analysed on a Western blot (12 % SDS-PAGE) using anti-CrCRT antibodies. A ca. 58 kDa and a ca. 45 kDa proteins were detected (indicated by arrows). (M) Molecular mass marker. (B) Slot blot analysis of RNA isolated from the venom glands and the remaining carcasses of *C. rubecula* wasps. 3 μg RNA was loaded onto each slot and probed with [^{32}P]-labelled cDNA fragment coding for *C. rubecula* calreticulin. CrCRT was more expressed in the venom glands compared to the remaining carcasses.

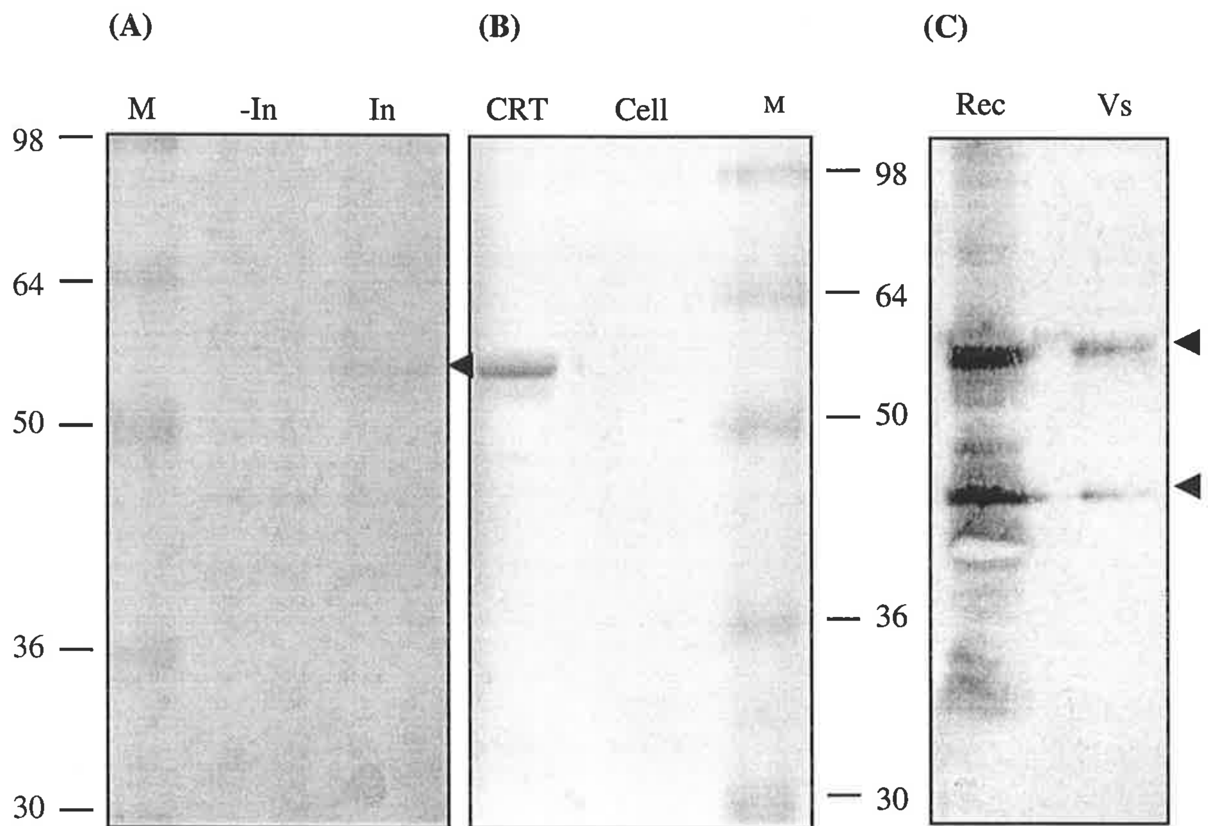


Fig. 5-5: Expression of CrCRT in *E. coli.* (A) SDS-PAGE (12 %) analysis of proteins from 2 h IPTG-induced (in) and –uninduced (-in) recombinant bacteria containing pQE30-CrCRT plasmid. A ca. 58 kDa protein is only visible in induced sample (arrowhead). (B) SDS-PAGE analysis of proteins from purified recombinant CrCRT and *E. coli* cells containing the expression vector. A ca. 58 kDa protein is only visible in recombinant CrCRT sample. (C). Western blot analysis of recombinant proteins (CrCRT) and venom reservoirs using anti-CRT antibodies from *G. mellonella* (Vs). A ca. 58 kDa and a ca. 45 kDa proteins were detected (indicated by arrows). (M) Molecular mass marker.

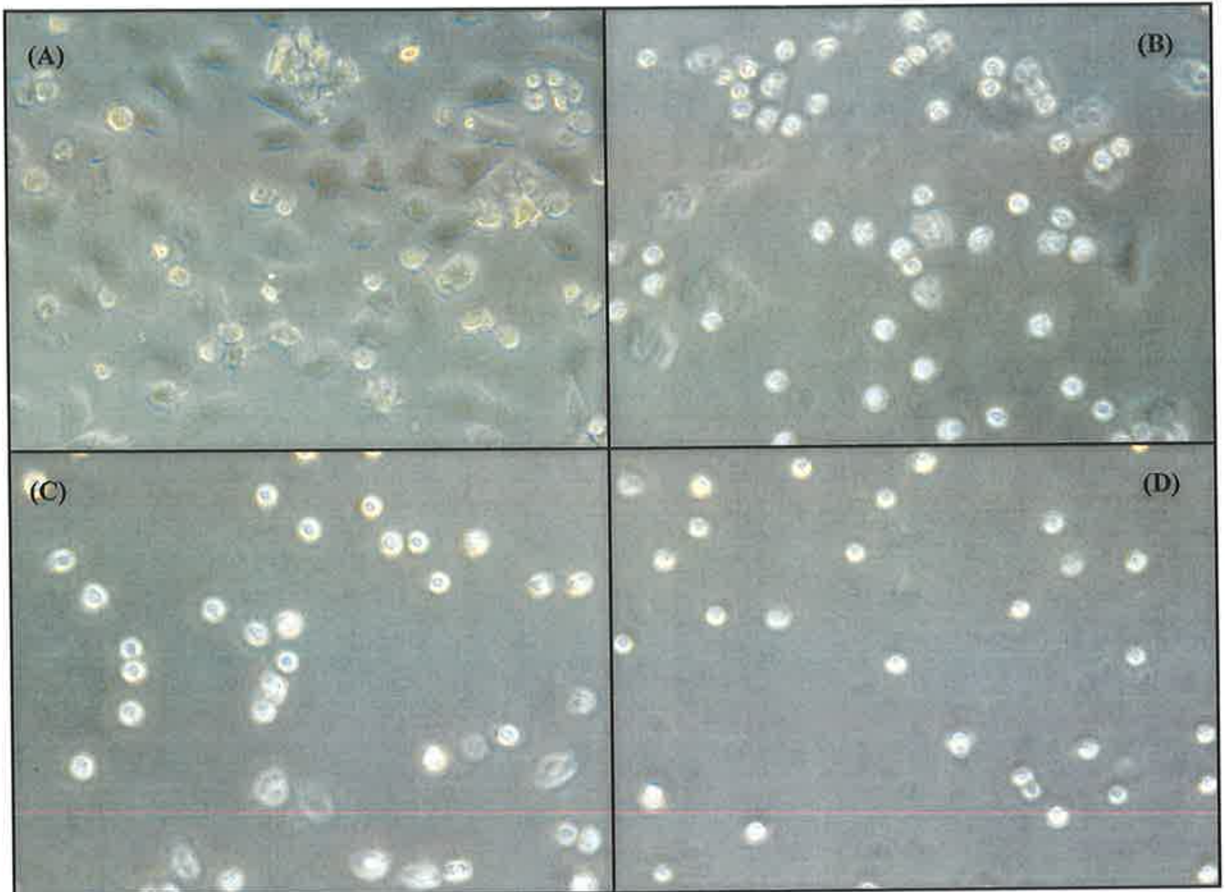


Fig. 5-6: Inhibition of hemocyte aggregation by recombinant CrCRT. To prepare hemocytes, 4th instar larvae of *P. rapae* were bled into wells with 100 µl of PBS saturated with phenylthiourea. After 10 min incubation to allow the cells to attach, 20 µl of 4-fold (B), 2-fold (C) and original (D) recombinant CrCRT (60 µg/ml) were added, respectively. (A) is the control. Mixtures were incubated for additional 1 h. Aggregation and spreading of hemocytes was observed under phase contrast microscope. The adhesion of hemocytes was inhibited by recombinant CrCRT and the inhibition is dose-dependent.

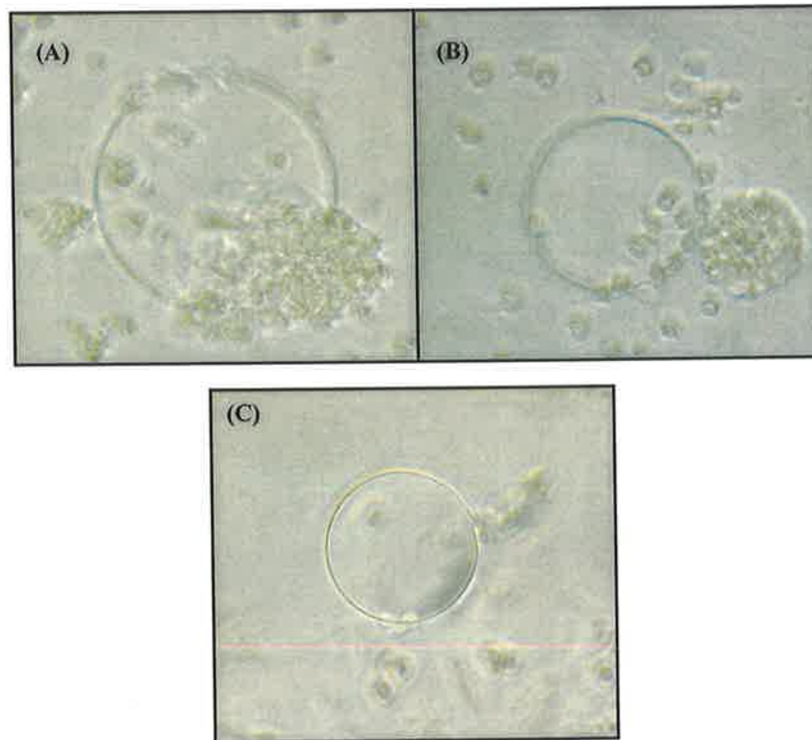


Fig. 5-7: Effect of recombinant CrCRT on encapsulation response *in vitro*. Control beads (A), covered with proteins from bacteria containing the expression vector (B) and beads coated with recombinant CrCRT (C) were incubated with *P. rapae* hemocytes for 4 h. In CrCRT-covered beads no signs of hemocyte attachment were detected.

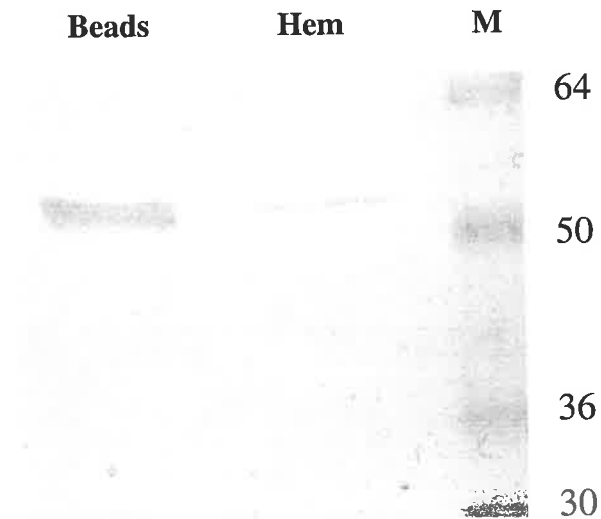


Fig. 5-8: Western blot analysis of the early-stage encapsulation-relating proteins using anti-CrCRT antibodies. Triggering of an early-stage encapsulation response and recovery of the early-stage encapsulation-relating proteins from naïve *P. rapae* larvae were performed as described in materials and methods. The proteins were separated on 10 % SDS-PAGE gel under reducing condition and transferred onto a membrane, which was probed with CrCRT antibodies.

Chapter 6: A novel venom peptide is required for expression of polydnavirus genes in host hemocytes

6.1 Introduction

Polydnaviruses are injected at oviposition together with other maternal components into the host hemocoel, where virus genes are expressed in the absence of viral DNA replication (Fleming *et al.*, 1983; Theilmann & Summers, 1986). Virions are able to enter most host cell types (Strand *et al.*, 1992; Harwood & Beckage, 1994). Viral transcripts are detected within a few hours following parasitization and viral genes are expressed transiently or persistently (Strand *et al.*, 1992; Asgari *et al.* 1996). The presence of PDVs has been shown to be essential for successful parasitism and development of the parasitoid inside the hosts (Edson *et al.*, 1981; Stoltz & Guzo, 1986; Fleming & Summers, 1991).

In certain host-parasitoid systems, PDVs are only effective when they are complemented by venom proteins. For example, in *C. glomeratus* (Kitano, 1986) and *Apanteles kariyai* (Tanaka, 1987), venom is an essential requirement for successful parasitism. In *C. melanoscela*, it was reported that venom proteins promoted the release of virions into the cytoplasm after uptake by host hemocytes and facilitated the uncoating of PDVs at nuclear pores in host hemocytes. Also the venom proteins enhanced virus persistence in the host (Stoltz *et al.*, 1988).

In the present study, we found that venom proteins are essential for the expression of CrBV genes in host cells although not required for CrBV entry. A novel venom peptide consisting of 14 amino acids with a molecular mass of 1598 Da (Vn1.5) was found to be sufficient for CrBV gene expression in host cells. In the absence of Vn1.5, CrBV transcription was not detected in the host hemocytes and hemocyte behaviour was not changed by CrBVs, despite the entrance of viruses into host cells. In the presence of Vn1.5, hemocyte behaviour was changed by CrBV, which indicates that both CrBV particles and Vn1.5 are required for the expression of CrBV genes in host hemocytes.

6.2 Materials and methods

6.2.1 Insect culture

The endoparasitoid wasps, *C. rubecula* (Hymenoptera: Braconidae) and its host insect *P. rapae* were maintained as described in Chapter 2.

6.2.2 Bracovirus isolation

CrBVs were purified from newly-emerged female wasps as described previously in Chapter 2 (Beckage *et al.*, 1994).

6.2.3 Reverse phase HPLC and mass spectrometry

Venom reservoirs from 50 female *C. rubecula* wasps were dissected and disrupted in PBS by micro-scissors to release venom proteins into the buffer solution. Reverse phase high pressure liquid chromatography (rpHPLC) of samples was carried out in a Hewlett Packard 1090 Liquid Chromatograph as described in Chapter 2. Protein fractions were collected manually. For *in vitro* hemocyte infection assays (see below), individual fractions were vacuum-dried and dissolved in sterile water.

To determine the molecular masses of these peptides, electrospray ionisation mass spectrometry was carried out by using a PE Sciex API-100. Generally, an amount equivalent to ca. 7 picomoles of purified protein was directly infused and then the results were analyzed using the BioMultiView 3.1.1 software.

6.2.4 Peptide sequencing

Approximately 100 picomol of the purified peptide from rpHPLC was vacuum-dried, reconstituted in 8 M urea containing 0.1 M NH_4HCO_3 and 4 mM DTT (DL-Dithiothreitol) and finally alkylated by addition of sodium iodoacetate to the final

concentration of 10 mM. The sample was acidified with TFA to stop the reaction. Peptide sequencing was carried out using a Hewlet Packard G1000A Protein Sequencer.

6.2.5 Peptide Vn1.5 synthesis

Vn1.5 was synthesized by Auspep Pty. Ltd. and analysed by mass spectral analysis for the molecular weight and purity. The lyophilized peptide was reconstituted in sterile water. Various concentrations of the peptide were used in CrBV infection of host cells (see below).

6.2.6 CrBV infection of hemocytes *in vitro*

Fourth instar *P. rapae* larvae were surface sterilized in 70 % ethanol and bled into cold PBS (saturated with phenyl-thiuorea to prevent melanization) by removing one of the prolegs. Hemocytes were collected by centrifuging the hemolymph at 750 × *g* for 5 min and removing serum. Hemocytes were resuspended in 100 µl HyQ-SFX insect cell culture medium (HyClone) per insect bled, and transferred into wells of a 24-well tissue culture plate. 200 µl of cell suspension were allowed to attach for about half an hour at room temperature. Then the purified CrBVs, two wasp equivalents, were added to each well. In different treatments, total venom proteins, individual or mixture of purified peptides by rpHPLC were also added to wells and gently mixed by pipetting up and down. The mixtures were incubated for additional 6 h at 25 °C, after which hemocytes were collected by resuspending the hemocytes in the medium and centrifuging at 750 × *g* for 5 min. Total RNA was then isolated from hemocytes for RT-PCR or slot blot assays.

6.2.7 DNA and RNA isolation

Total DNA and RNA were isolated from *P. rapae* hemocytes as previously described in Chapter 2.

6.2.8 Reverse transcription-PCR (PT-PCR)

Two pairs of primers specific to two CrBV genes (*CrV1* and *CrV2*) CrV1-*Bam*HI-F and CrV1-*Kpn*I-R; and CrV2-*Sph*I-F and CrV2-*Hind*III-R (Asgari *et al.*, 1996; Glatz *et al.*, 2003) were used in reverse transcription reactions using AMV (Avian Myeloblastosis virus) reverse transcriptase as described in Chapter 2.

Primers used:

CrV1-*Bam*HI-F: 5'-CGCGGGATCCCAAGCTTATCCATCCGAATAC-3'

CrV1-*Kpn*I-R: 5'-CGCGGGTACCTCAAAAAAAAGTTTGCGATGGG-3'

CrV2-*Sph*I-F: 5'-CGCGGCATGCCCGTTGCAAGACAGAAG-3'

CrV2-*Hind*III-R: 5'-GCGCAAGCTTTTAGGGATGATCTCGAGC-3'

Resulting cDNA fragments were then used in the PCR amplification by adding 5 µl of 10 × reaction buffer, 1 µl of forward primer (0.1 µg/µl), 1 µl of forward primer (0.1 µg/µl), 0.5 µl deoxynucleotide triphosphates (15 mM), 0.5 µl of Taq DNA polymerase (Promega) and 27.5 µl of H_2O. After 5 min at 94 °C, 35 amplification cycles were run including denaturing at 94 °C for 50 sec, annealing at 56 °C for 50 sec, extension at 72 °C for 1.5 min. Final extension at 72 °C was carried out for 10 min. All reaction products were electrophoresed on 1 % agarose gels and visualized by using ethidium bromide.

6.2.9 DNA and RNA Slot blot

DNA or RNA samples were blotted onto a nylon membrane (Amersham Biosciences) under denaturing conditions according to the manufacturer's instruction (BioRad). The blot was hybridized with ^{32}P-labelled PCR products encoding CrV1 (about 900 bp) at 65 °C overnight. For RNA slot blot, a *P. rapae* 18S ribosomal DNA fragment probe was used as a control.

6.2.10 Hemocyte behaviour Changes after CrBV infection with or without venom proteins and Vn1.5

Fourth instar *P. rapae* larvae were surface sterilized in 70 % ethanol and bled into cold PBS (saturated with phenyl-thiuorea to prevent melanization) by removing one of the prelegs. Hemocytes were collected by centrifuging the hemolymph at 750 × *g* for 5 min and removing serum. Then hemocytes were resuspended in 120 μl HyQ-SFX insect cell culture medium (HyClone) per insect equivalent. 30 μl of the cell suspension was transferred into each well of a multi-well slide and incubated for 10 min to allow hemocytes to attach. Four treatments were set up by adding the following ingredients to the cell monolayers: 1) purified viruses and venom proteins or synthesized Vn1.5, 2) CrBVs alone, 3) synthesized Vn1.5 alone or venom proteins alone, and 4) PBS. Hemocytes were incubated for an additional 6 h at 25 °C and fixed with 4 % paraformaldehyde (PFD). Hemocyte behaviours were observed under a phase contrast microscope.

6.2.11 Transmission electronic microscopy

Hemocytes were collected from 4th *P. rapae* larvae as described above and were resuspended in HyQ-SFX insect cell culture medium (HyClone). Hemocyte monolayers were inoculated with purified CrBVs plus synthesized Vn1.5 or with CrBVs alone as above for 2 h at 25 °C. Hemocytes were then collected by centrifuging at 750 × g for 5 min and washed three times. The cells were fixed overnight in 4 % glutaraldehyde (EM grade) in PBS, with 4 % sucrose at pH 7.2. Cells were washed in PBS plus 4 % sucrose-2 changes of solution 10 min each. Samples were post-fixed in 2 % osmium tetroxide (OsO4) for 1 h on a rotator and dehydrated in ethanol and propylene oxide. Samples were embedded in resin and micrographs were taken by using a Jeol 1010 transmission electron microscope.

6.3 Results

6.3.1 Venom is required for CrBV gene expression

In order to show whether venom components are essential for CrBV gene expression in host cells, *in vitro* assays were carried out by using *P. rapae* hemocytes. Specific primers from the *CrV1* gene coding region, which is highly expressed in host hemocytes at 6 h following parasitization (Asgari *et al.*, 1996), were used as markers in RT-PCR reactions. When hemocytes were infected with only purified CrBV particles isolated from female wasps, no *CrV1* transcripts were detected in the cells after 6 h (Fig. 6-1 A). However, when CrBV particles were added to cells together with venom fluid, *CrV1* was found to be expressed strongly in these infected cells (Fig. 6-1 A). The results were confirmed by three independent repeats of the experiment. In addition, a slot blot analysis using *CrV1* cDNA as a probe showed that the gene is not expressed in inoculated hemocytes in the absence of venom (Fig. 6-1 B). This indicated that venom components are required for CrBV gene expression in host hemocytes, although they are not essential for CrBV entry into host cells (see below).

6.3.2 rpHPLC separation of venom proteins and bioassay

When crude venom preparations from female *C. rubecula* endoparasitoid wasps were fractionated on a rpHPLC column more than a dozen peptides and proteins were identified (Fig. 6-2 A). In order to examine which venom component(s) are involved in CrBV gene expression, we collected 12 main fractions and arbitrarily divided the fractions into three groups (A: 1-4, B: 5-8 and C: 9-12) each containing four major fractions (Fig. 6-2 A). The eluted proteins corresponding to each fraction were vacuum-dried and resuspended in sterile water. Proteins or peptides from each group were mixed together (equivalent to two wasps) and applied together with purified CrBV particles to hemocyte monolayers. After 6 h incubation, total RNA was isolated from the infected cells and subjected to RT-PCR using CrV1-specific primers as markers.

CrV1 transcripts were detected in the inoculated cells when a mixture of 12 fractions was added together with purified CrBVs (Fig 6-3 A). When a mixture of individual groups was added together with purified CrBVs, *CrV1* transcripts were only found in group A fractions, but not in the other two groups (Fig. 6-3 B). Since component(s) required for CrBV gene expression are in group A, individual fractions from the group were used in a subsequent assay. *CrV1* transcripts were found in cells inoculated with CrBVs in the presence of fractions 1, 3 or 4 (Fig. 6-3 C, D). All subsequent experiments were performed with fraction 1.

6.3.3 Mass spectrometry and peptide sequence analyses

The size of the peptide in fraction 1 isolated on rpHPLC (Fig. 6-2 A) was determined by mass spectrometry as 1598 Da and designated as Vn1.5. The peptide was also N-terminally sequenced providing 14 amino acids (Fig. 6-2 B) with the predicted molecular weight matching the mass of the peptide determined by mass spectrometry. The estimated isoelectric point of Vn1.5 is 10.01. The sequence of Vn1.5 was compared with other proteins and peptides in protein databases, but no significant similarity was found with existing sequences.

6.3.4 Effect of synthetic peptide Vn1.5 on the CrBV gene expression

To further confirm the function of Vn1.5 in CrBV gene expression the peptide was synthesized and used for *in vitro* bioassays. Hemocytes were inoculated with purified CrBV particles isolated from female wasps in the presence or absence of synthetic Vn1.5. To examine possible concentration dependence, three different magnitudes of peptide concentrations 1 μg/ml, 100 μg/ml and 10 mg/ml were applied. *CrV1* transcripts were detected in infected cells at all three concentrations (Fig. 6-4 A). Although the intensity of signals increased slightly with the increase in concentration (Fig. 6-4 A, B), the difference in gene expression did not reflect three magnitudes in peptide concentrations. These results further confirm that peptide Vn1.5 alone was sufficient to promote the expression of CrBV genes at low concentrations.

To examine whether other CrBV genes were also dependent on the venom peptide for their expression, we analyzed expression of *CrV2*. When *CrV2*-specific primers were used for RT-PCR, *CrV2* transcripts were also detected in the infected cells in the presence of 1.5 μg/ml Vn1.5 and one wasp equivalent total venom proteins (Fig. 6-5). Conversely, no transcripts were detected in the cells inoculated with CrBVs alone (Fig. 6-5).

6.3.5 Hemocyte changes after CrBV incubation with or without venom

To further confirm the effect of venom proteins and Vn1.5 on the expression of CrBV genes, hemocyte changes, such as spreading behaviour after CrBV infection with or without the synthesized peptide, were investigated. When hemocytes of *P. rapae* were only incubated with purified CrBV particles or Vn1.5 (1.5 μg/ml) for 6 h, no changes were found in hemocytes compared to the control (PBS). However, when hemocytes were incubated with a mixture of purified CrBV particles and venom proteins (one wasp equivalent) or Vn1.5 together for 6 h, hemocytes were found to aggregate and spread less frequently on the glass surface compared to the control (Fig. 6-6, Fig. 6-7), which is reminiscent to the hemocyte behaviour after natural parasitization (Asgari *et al.*, 1996, 1997). This further confirms the role of venom proteins and Vn1.5 in facilitating the expression of CrBV genes at the functional level.

6.3.6 Observation by electron microscopy

To examine whether CrBV particles are able to enter host hemocytes in the absence of venom components, hemocytes were inoculated with purified particles and the cytoplasm analysed for the presence of electron-dense particles, using transmission electron microscopy (Fig. 6-8). Under these conditions, electron-dense particles are detected in endosomes, free of membranes in the cytoplasm and associated with nuclear membranes (Fig. 6-8 B), similar to naturally infected hemocytes (Stoltz *et al.*, 1988). This implies that the peptide might be involved in chromatin restructuring or uncoating

of the virus genome at the nuclear membrane. Alternatively, the peptide may be required for viral gene expression at the transcriptional level (eg. as a transcription factor). Further investigations are required to elucidate the exact mechanism by which Vn1.5 facilitates CrBV gene expression in host hemocytes.

6.4 Discussion

In contrast to insect ectoparasite-host interactions, in which venom mainly has a paralysing function, venom proteins have different functions in endoparasitoids, where they are mainly involved in alterations of host physiology. In addition, depending on whether the endoparasitoid produces virus-like particles such as PDVs or not, venom may complement or replace PDV functions in host regulation and immune suppression. For example, in *Pimpla hypochondriaca* (Braconidae), which seems to lack any virus-like particles, venom adversely affects the morphology, viability, and the immune function of hemocytes of the tomato moth, *Lacanobia oleracea* (Richards & Parkinson, 2000). In endoparasitoids with PDVs, a combination of maternal factors is used to suppress host immune responses, where venom proteins play a synergistic role with PDVs (Kitano, 1986; Tanaka, 1987; Stoltz *et al.*, 1988; Wago & Tanaka, 1989). In *C. melanoscela*, total venom proteins were found to promote release of virions into the cytoplasm of infected host hemocytes and enhance persistence of the viruses in the host (Stoltz *et al.*, 1988).

In *C. rubecula*, maternal factors also introduced into the body of the host by endoparasitoid wasps at oviposition are essential for successful completion of parasitism. An immunosuppressive gene (*CrV1*) from *C. rubecula* bracoviruses (CrBVs) is involved in the destabilization of cytoskeleton in host hemocytes (Asgari *et al.*, 1996, Asgari *et al.*, 1997). In addition, a calyx protein (Crp32) on the surface of CrBV particles and eggs, provides short-term protection of the parasitoid eggs against host encapsulation reactions before CrBV genes are expressed (Asgari & Schmidt, 1994; Asgari *et al.*, 1998). When crude venom preparations, isolated from female *C. rubecula* endoparasitoid wasps, were separated by rpHPLC, over a dozen proteins were

found (Fig. 2A), some of which are involved in inhibition of melanization by blocking the activation of prophenoloxidase (Asgari *et al.*, 2003a, c; Zhang *et al.* 2004a and See chapter 5).

In present study, we explored the interaction between venom proteins and CrBVs with regards to virus entry and gene expression. When *P. rapae* hemocytes were incubated with CrBVs *in vitro* in the presence or absence of venom proteins, viral transcripts were only detected in the infected cells in conjunction with venom proteins. It is clear that venom is required for CrBV gene expression in host hemocytes, although electron microscopy observations revealed that venom components were not essential for CrBV entry into host hemocytes. Using various mixtures of venom proteins and peptides separated by rpHPLC, we found that a 1598 Da peptide (Vn1.5) was sufficient for the expression of CrBV genes when used together with purified CrBV particles in *in vitro* assays. In addition, two other fractions were also found to promote the expression of CrBV genes. Synthetic Vn1.5 peptide and total venom proteins in conjunction with purified virus were shown to facilitate expression of CrBV genes, while synthetic peptide or CrBV particles alone were not able to cause behavioural changes in host hemocytes (Fig. 6-7). This suggests that calyx-derived virus particles and venom-derived Vn1.5 peptide are both required to express CrBV genes in host hemocytes similar to natural parasitism (Asgari *et al.*, 1996, 1997).

Transmission electron microscopy observation showed that venom proteins, including Vn1.5, are not required for the virus entry, release into the cytoplasm from endosomes or attachment to the nuclear membrane. This suggests that the peptide Vn1.5 might facilitate virus chromatin restructuring, uncoating of genomic DNA at the nuclear pore, or expression of CrBV genes at transcriptional level. Further investigations are required to elucidate the exact involvement of Vn1.5 and perhaps other venom proteins in facilitating expression of polydnaviral genes. However, this is the first report about the characterization of a venom peptide from an endoparasitoid wasp involved in promoting expression of polydnaviral genes at the molecular and functional level.

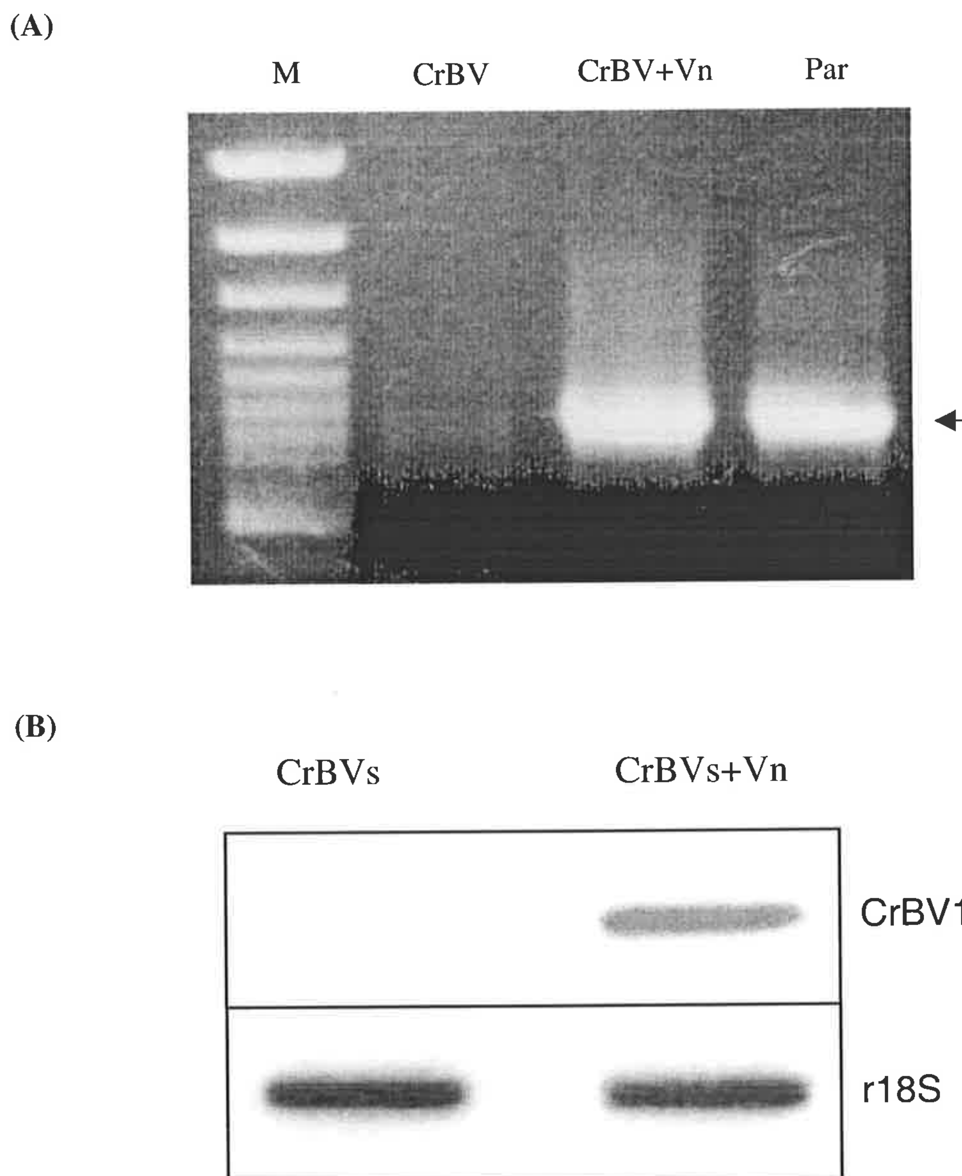
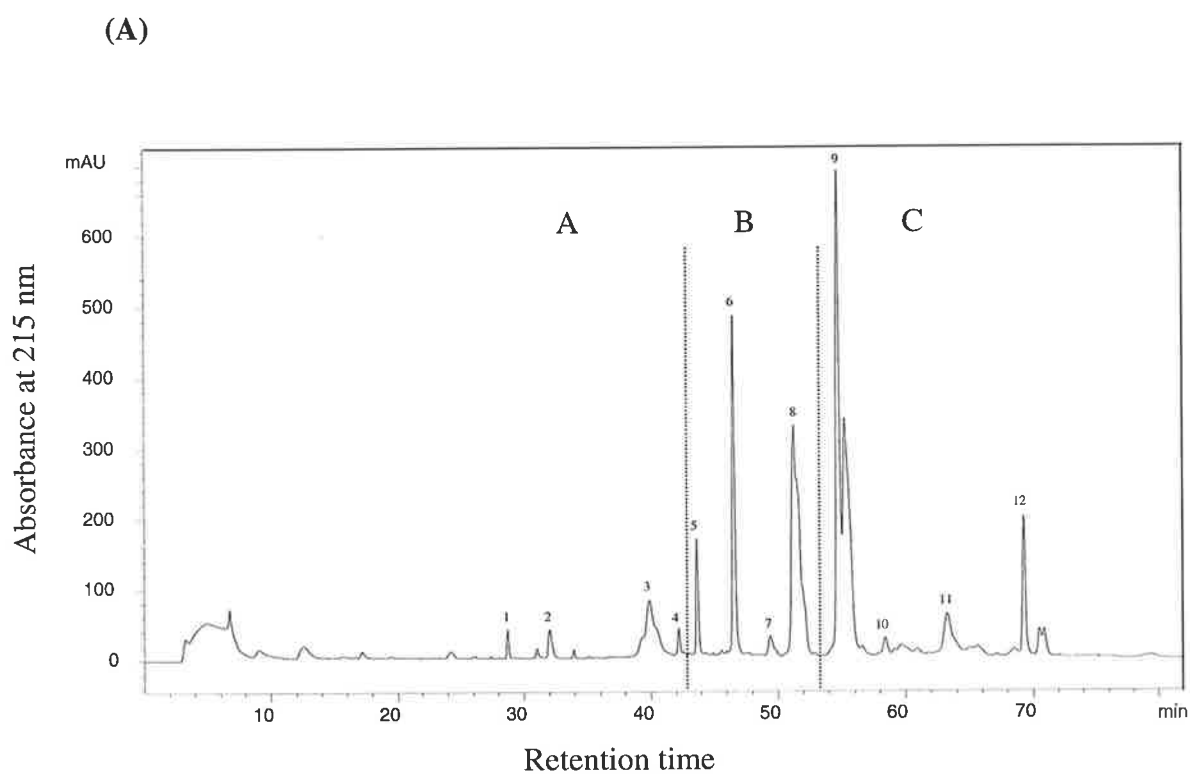


Fig. 6-1: Effects of venom proteins on expression of *CrV1*. *P. rapae* hemocytes were allowed to attach for 10 min. CrBVs purified from 2 wasp equivalents were added to the medium and incubated at 25 °C for an additional 6 h in the absence and presence of total *C. rubecula* venom proteins (1 wasp equivalent). Cells were collected by centrifugation as described in methods. Total RNA was extracted, and 2 μg RNA for each sample was used for (A) RT-PCR analysis with primers specific to *CrV1*, and (B) slot blot hybridisation using *CrV1* cDNA fragment and *P. rapae* 18S rDNA as probes. No *CrV1* expression was detected in the absence of venom components. DNA molecular weight marker (M); cells incubated with purified particles without (CrBV) and with total venom proteins (CrBV+Vn); RNA from 6 h parasitised hemocytes (Par).

(A)

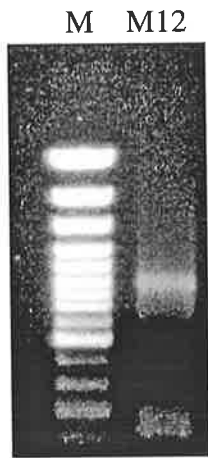


(B)

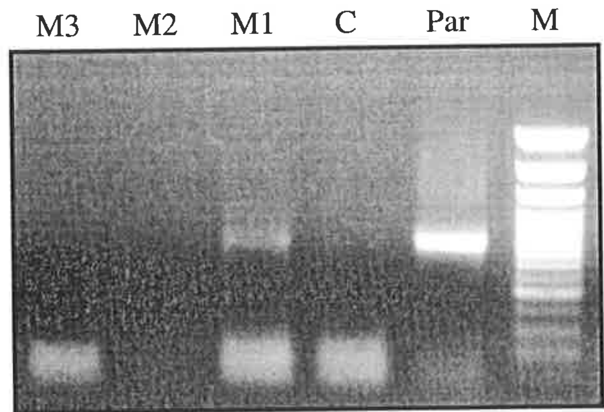
H_2N-**KLTSKHTNIMLNAK**-COOH

Fig. 6-2: Venom proteins separation and Vn1.5 sequence. (A) Separation of crude *C. rubecula* venom proteins by reverse phase high pressure liquid chromatography (rpHPLC). Twelve main proteins or peptides were divided into three groups (A, B and C). Group A included fractions 1-4, group B included fractions 5-8; Group C included fractions 9-12. (B) Amino acid sequence of fraction 1 (Vn1.5 peptide) obtained from N-terminal microsequencing.

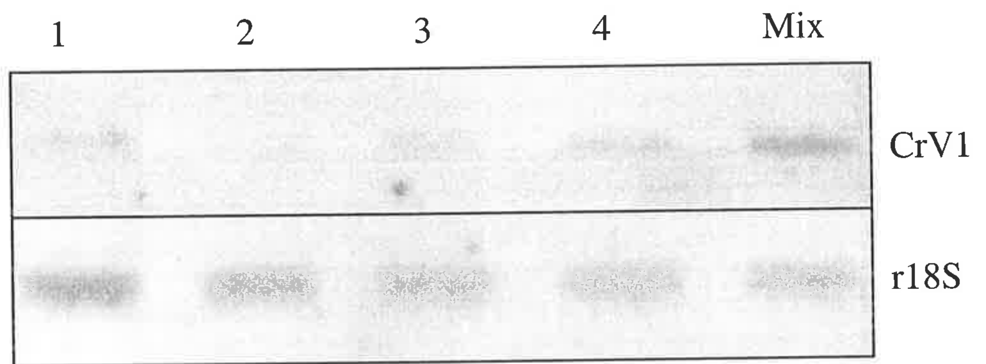
(A)



(B)



(C)



(D)

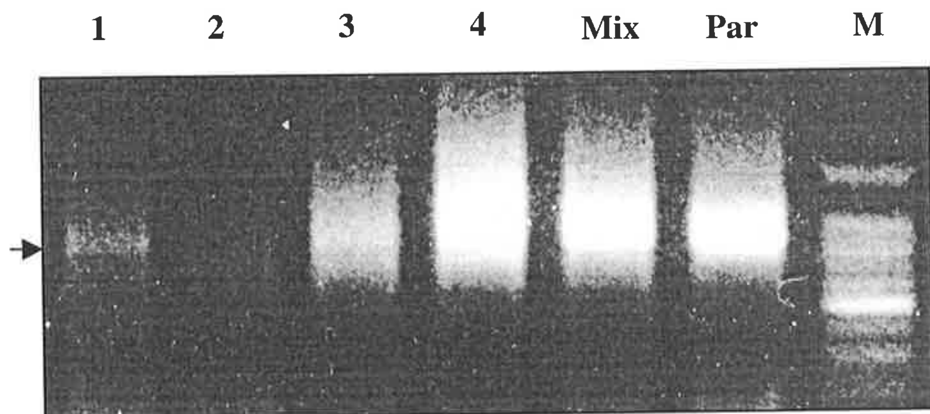


Fig. 6-3: Venom proteins and PDV expression. Effect of rpHPLC-isolated venom proteins or peptides on the expression of *CrV1* in *P. rapae* hemocytes by using RT-PCR and RNA slot blot hybridisation. *P. rapae* hemocytes were allowed to attach for 10 min and then incubated with purified CrBVs in presence of purified protein fractions (see Fig. 6-2) for an additional 6 h at 25 °C. Cells were collected and total RNA was extracted as described in methods. (A) Mixture of 12 fractions (M12) representing proteins from the total venom proteins was used for the assay. 2 μg RNA for each sample was used for RT-PCR analyses using *CrV1*-specific primers. (B) Mixtures of group A (M1), B (M2) and C (M3) were used for infection assay. 2 μg RNA for each sample was used for RT-PCR using *CrV1*-specific primers. (C) Venom fractions 1-4 from group A purified by rpHPLC were used individually for the assays and RNA from infected cells were analysed by slot blot hybridisation. Blots containing 3 μg RNA were hybridized with a *CrV1* cDNA fragment as a probe. A control blot was probed with a *P. rapae* 18S ribosomal DNA fragment (r18S), to monitor that similar amounts of RNA had been loaded for each sample. Mix, mixture of the four fractions 1-4. (D) Venom fractions 1-4 from group A purified by rpHPLC were used individually for the assays by RT-PCR using *CrV1*-specific primers.

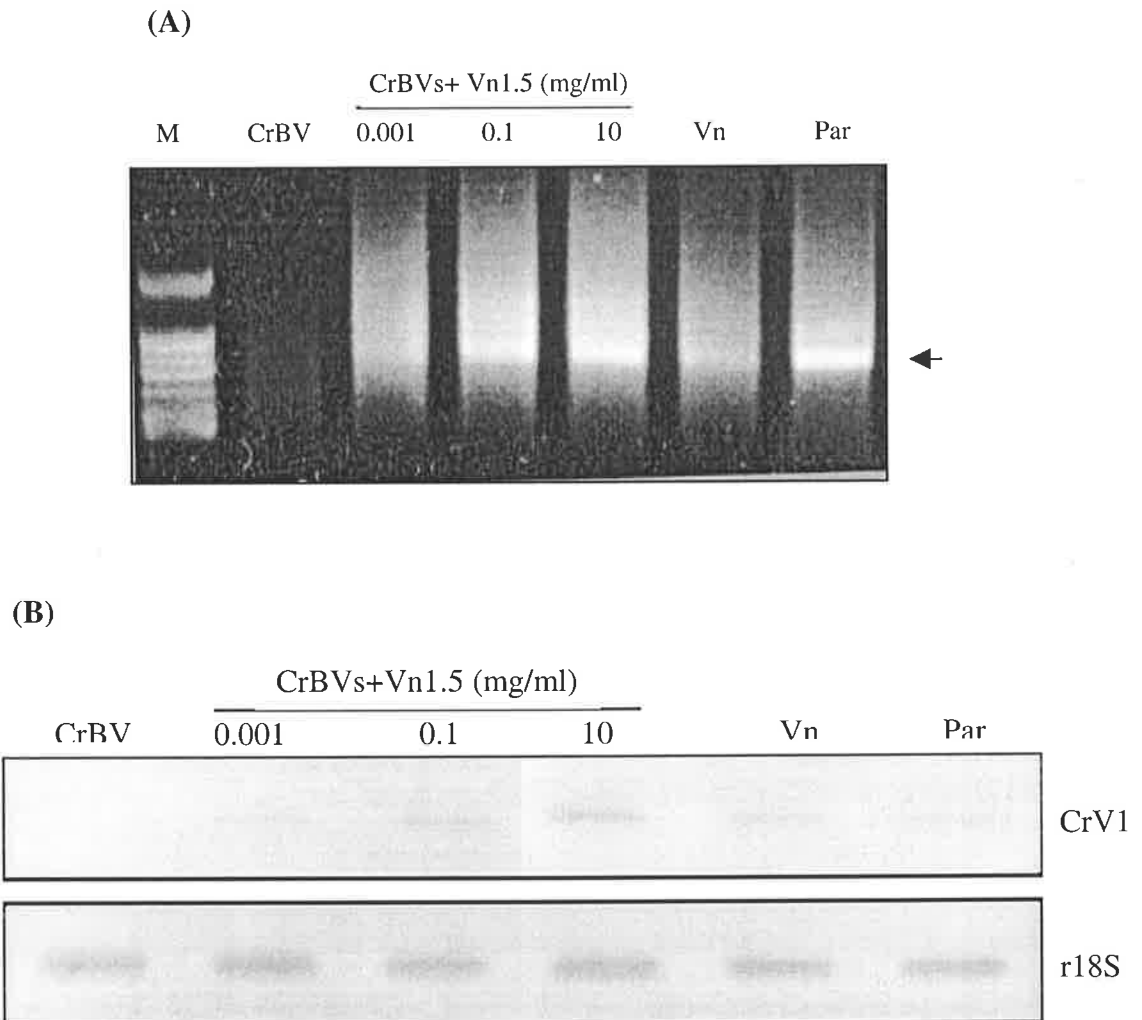


Fig. 6-4: Effect of synthesized peptide Vn1.5 on the expression of *CrV1*. Virus gene expression was monitored by RT-PCR and RNA slot blot hybridisation. (A) 2 μg of RNA for each sample was used for RT-PCR. (B) About 3.2 μg of RNA was loaded onto each slot and probed with a ^{32}P-labelled cDNA fragment encoding *CrV1*. A control blot was probed with a *P. rapae* 18S ribosomal DNA fragment (r18S), to monitor that similar amounts of RNA had been loaded for each sample. 1 μg of Vn1.5 (P1), 100 μg of Vn1.5 (P2), 10 mg of Vn1.5 (P3), with total venom proteins (Vn), RNA from 6 h parasitised hemocytes (Par).

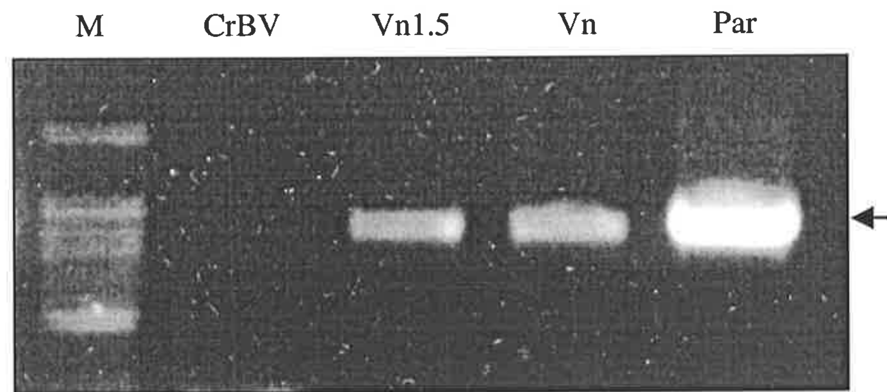


Fig. 6-5: CrV2 gene expression. Effect of synthesized Vn1.5 on the expression of *CrV2* in host hemocytes by RT-PCR analyses. About 1.2 μg of RNA was used for each reaction. CrV2 transcripts were not detected in hemocytes infected with CrBVs alone.

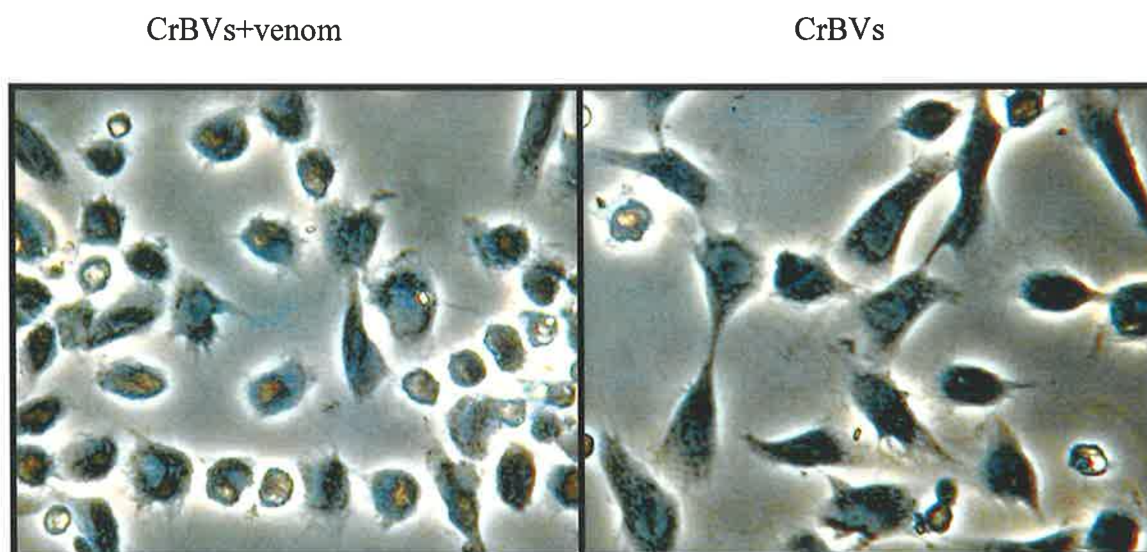


Fig. 6-6: Effect of CrBVs on hemocyte behaviour in the presence or absence of venom proteins. To collect hemocytes, 4th instar *P. rapae* larvae were bled into PBS saturated with phenyl-thiourea, followed by centrifugation 5 min at 750 × *g*. Hemocytes were resuspended in cell culture medium. 30 µl of cell suspension was added per well of a multi-well slide and incubated for 10 min to allow for attachment. Purified CrBVs and total venom proteins (0.2 wasp equivalent) were added and mixed gently. Mixtures were incubated for an additional 6 h. Then, hemocytes were fixed and observed under a phase contrast microscope.

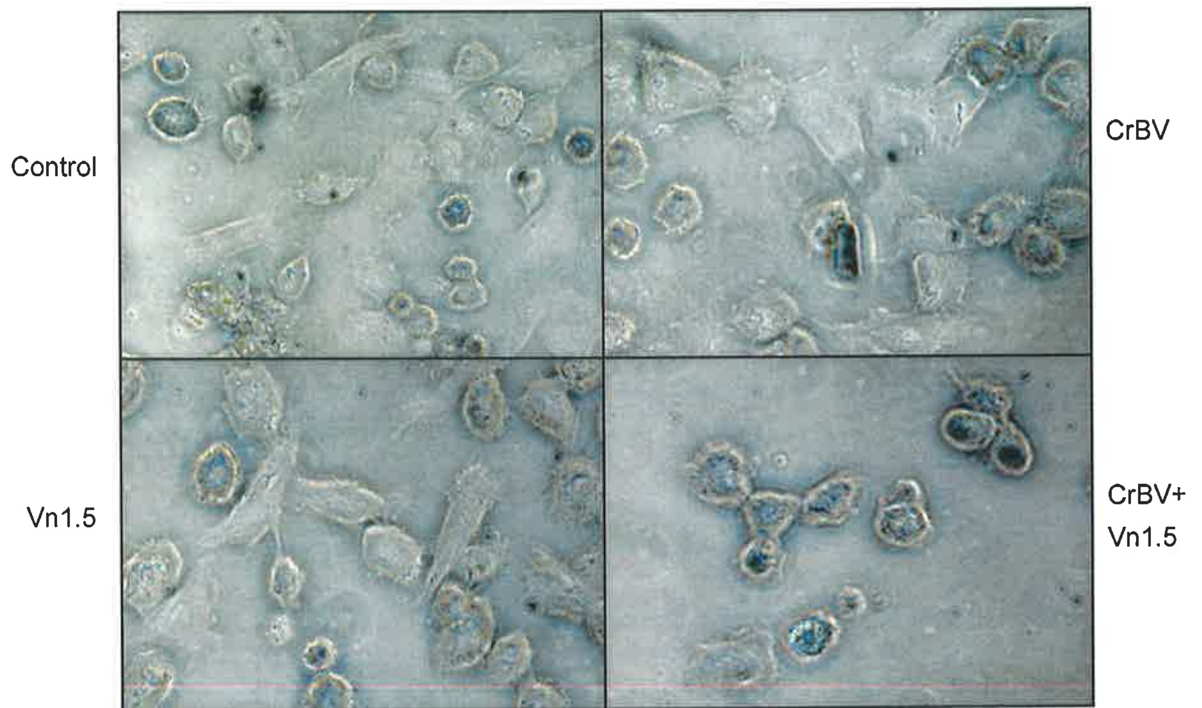


Fig. 6-7: Effect of CrBVs on hemocyte behaviour in the presence or absence of Vn1.5. Hemocytes were prepared as described above. Purified CrBVs and synthesized Vn1.5 (1.5 μg/ml) were added and mixed gently. Mixtures were incubated for an additional 6 h. Then, hemocytes were fixed and observed under a phase contrast microscope. No difference was found between cells inoculated with either CrBVs or Vn1.5 and the control (PBS). However, when the particles were added to cells together with Vn1.5, cells failed to spread.

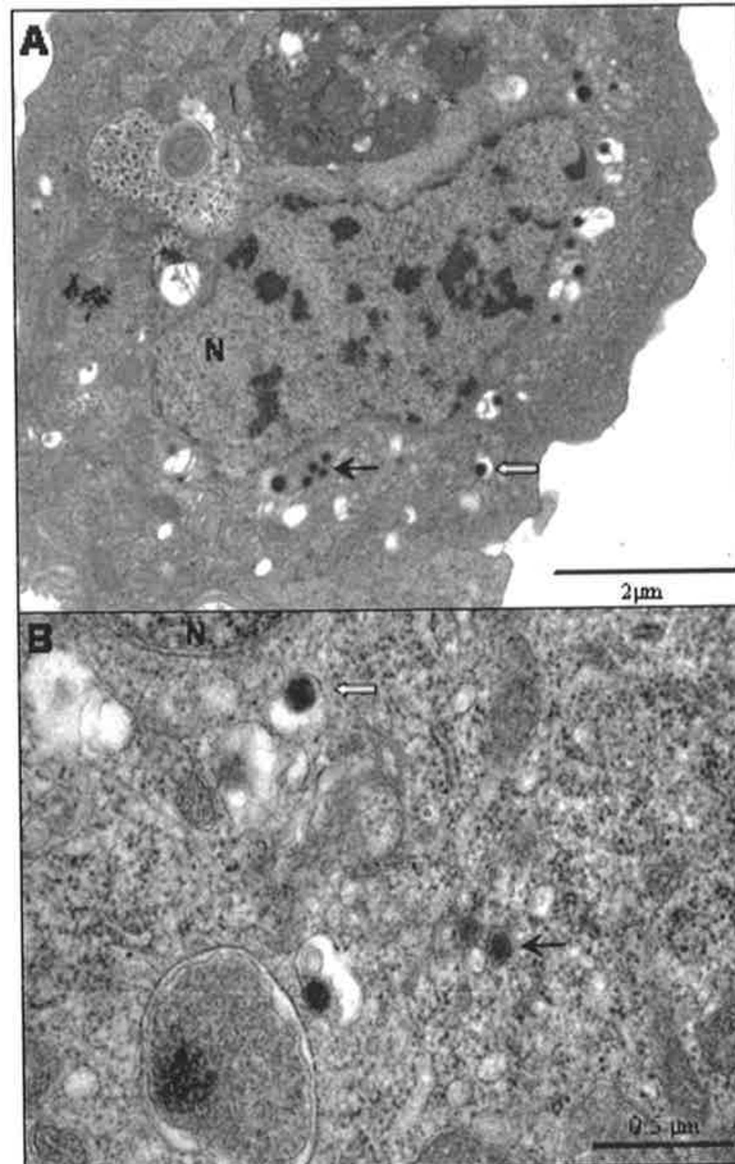


Fig. 6-8 Transmission electron microscopy of *P. rapae* hemocytes incubated with purified CrBVs. Hemocytes were incubated with the viruses in the absence of venom proteins for 2 h. Cells are shown at (A) lower and (B) higher magnifications. Large numbers of electron-dense virus particles were found in the cytoplasm either within endosomes (empty arrows) or released into the cytoplasm (solid arrows) and some associated with the nuclear membrane (N, nucleus). Similar observations were made when CrBV particles were accompanied with total venom or Vn1.5 (not shown). No apparent differences are observed with naturally infected hemocytes.

Chapter 7: Factors influencing the entry of *Cotesia rubecula* bracoviruses into host cells

7.1 Introduction

Enveloped animal viruses enter their host cells by the fusion of the viral envelope with cellular membrane (Lenard & Miller, 1982; White, 1990). Virus fusion reactions can be divided into two general classes, low pH-dependent and pH-independent. For low pH-dependent viruses such as influenza, Semliki Forest and vesicular stomatitis (VSV) viruses, fusion requires exposure of the virus to mildly acid pH. These viruses enter into cells by a receptor-mediated endocytic pathway prior to fusion to endosomal membranes, which releases virus DNA into the cytoplasm (White *et al.*, 1981; Lenard & Miller, 1982). In contrast, for pH-independent viruses such as Sendai virus (Hsu *et al.*, 1982; Nagai *et al.*, 1983) and HIV-1 (Stein *et al.*, 1987), fusion does not require exposure to acid pH and might occur as a direct interaction between the virus envelope and the cellular membrane at neutral or slightly basic pH (Hsu *et al.*, 1982).

Braconid and ichneumonoid PDVs are two major groups of obligate symbiotic viruses, specifically associated with certain braconid and ichneumonoid endoparasitoids (Stoltz & Vinson, 1979; Stoltz *et al.*, 1988). Braconid polydnaviruses (bracoviruses) have cylindrical nucleocapsids surrounded by single unit membrane, but ichneumonid particles (ichnoviruses) have quasi-cylindrical nucleocapsids surrounded by two envelopes (Stoltz & Vinson, 1979). At parasitization, PDVs are injected into host larvae together with the parasitoid eggs and enter into host cells, where the packaged genomes are expressed. The virus-associated gene products act to suppress the host immune reactions resulting in the failure of hemocytes to encapsulate the parasitoid eggs (Edson *et al.*, 1981; Stoltz, 1986; Asgari *et al.*, 1996, 1998; Backage, 1998). In addition, they may lead to host physiological disorders by interfering with the host endocrine system (Webb & Dahlman, 1986; Balgopal *et al.*, 1996; Beakage & Gelman, 2004).

In some host-endoparasitoid systems, PDVs are only effective when accompanied by

venom proteins. In *C. melanoscela*, only in the presence of venom, nucleocapsids are released into the cytoplasm and subsequently uncoated *in vitro* (Stoltz *et al.*, 1988). Venom proteins also facilitate virus persistence *in vivo* (Stoltz *et al.*, 1988). In *C. glomeratus* (Kitano, 1986) and *A. kariyai* (Tanaka, 1987), venom is an essential requirement for successful parasitism. However, it is unknown whether venom proteins in *C. rubecula/P. rapae* parasitoid-host system are required for PDV entry into host cells or not.

C. rubecula bracoviruses express only 4 major genes in host hemocytes and fat body cells within a short period between 6 to 12 h after parasitization (Asgari *et al.*, 1996; Glatz *et al.*, 2003; 2004a). As with other PDVs, CrBV replication only occurs in calyx cells located in wasp ovaries. Because no particles are produced in lepidopteran hosts, relative levels of viral gene expression may depend largely on the gene copies that enter cells (Webb & Cui, 1998). Although some of the viral genes have been isolated and characterized, little is yet known about the mechanism of viral nucleocapsid entry into host hemocytes.

In this chapter, development of an *in vitro* infection system using a *P. rapae* cell line is discussed, which allowed monitoring virus DNA uptake into the cells by DNA slot blotting. Using this bioassay we investigated the uptake of virus particles and effects of temperature, pH value, ammonium chloride and venom proteins on this process. Together these experiments suggest that CrBVs most likely initiate uptake at low pH in a pathway analogous to that of pH-dependent viruses. Venom proteins are not essential for CrBV entry into host cells, although they are required for CrBV expression (Zhang *et al.*, 2004 b; also see Chapter 6, Vn1.5).

7.2 Materials and Methods

7.2.1 Insect cultures and virus purification

The endoparasitoid wasps, *C. rubecula* and its host insect *P. rapae* were maintained as

described in Chapter 2.

CrBVs were purified from newly-emerged female wasps as described previously in Chapter 2 (Beckage *et al.*, 1994).

7.2.2 Cell line culture

A *P. rapae* cell line, originally established from embryo cells (kindly donated by Dr. Peter Christian), was used throughout this study, because in preliminary studies, it was found that these cells supported CrBV transcription. Cells were maintained at 25 °C as monolayers in modified insect cell culture medium TC 199-MK (Sigma).

7.2.3 Virus uptake assays

To investigate CrBV uptake, 200 µl of *P. rapae* cell suspension was plated to each well in 24-well tissue culture plates to allow cell attachment for 0.5 h. Then the purified CrBVs from 2 wasp equivalents were applied in each treatment. After incubation at 25 °C for an additional 1 h, 1.5 h and 5 h, respectively, cells were collected by and resuspending in the medium and centrifugation at 750 × *g* for 5 min, and washed three times with PBS to remove the viruses attached on the cell surface. Then total cell DNA was extracted and used for DNA slot blots. All incubations were carried out in an incubator at 25 °C without CO_2 supplementation, under which conditions the pH remained stable.

7.2.4 Effect of temperature on CrBV entry into cells

From the uptake assays above, CrBVs were first detected after incubation for 1.5 h. Thus, incubation of CrBVs was performed for 1.5 h at 4 °C, 25 °C and 37 °C, respectively. Then uptake assays were carried out as described above. Total cell DNA was extracted and used for DNA slot blots.

7.2.5 Effect of pH on CrBV entry into cells

To examine pH effects on the uptake of CrBVs, a series of media and PBS solutions at pH 5.3, 6.3, and 7.3 were used for all incubations in virus uptake assays. The pH of medium and PBS solutions was adjusted to the indicated values with 1 M NaOH and 1 M HCl. Uptake of CrBVs into cells was performed at 25 °C for 1.5 h. The uptake assays were carried out as described above. Total cell DNA was extracted and used for DNA slot blots.

7.2.6 Treatment of cells with lysosomotropic agent

A 0.5 M stock solution of lysosomotropic agent ammonium chloride was freshly prepared in PBS prior to use. The stock solution was diluted to the indicated concentrations in medium just before use. Uptake of CrBVs into cells was performed at 25 °C for 1.5 h. The uptake assays were carried out as described above. Total cell DNA was extracted and used for DNA slot blots.

7.2.7 Effect of venom proteins on CrBV entry into cells

To examine the effect of venom proteins on the uptake of CrBVs into host cells, 200 µl of cell suspension were plated in 24-well plates and incubated at room temperature for 0.5 h to allow attachment. CrBVs isolated from 2 wasp equivalents were added in the presence or absence of total venom proteins and mixed with cells. After incubation for an additional 1.5 h at 25°C, cells were washed three times with PBS to remove the viruses attached to the cell surface. Then total DNA was extracted and used for analysis on DNA slot blots.

7.2.8 Total DNA isolation from infected cells and slot blot assays

Total DNA was isolated from cells as described in Chapter 2.

DNA samples were blotted onto a nylon membrane (Amersham Biosciences) under denaturing conditions according to the manufacturer's instruction and as described in Chapter 2 (BioRad). After 2 h prehybridization, the blot was hybridized with a ^{32}P-labelled *CrV1* PCR product (see Chapter 6). Following overnight hybridization at 65 °C, the membrane was washed twice in 2 × SSC, 0.1 % SDS at 65 °C, each for 15 min. Washing was then continued at 65 °C with 0.2 × SSC 0.1 % SDS twice, each for 15 min. The membrane was sealed with plastic bag and exposed onto film for 24 h or indicated time.

7.3 Results

7.3.1 Assay for CrBV uptake into cells

Since CrBVs are precluded from DNA replication in infected cells, plaque titration of progeny virus is not available as a tool to measure virus infection. We therefore used two critical steps involving the uptake of viral DNA into cell and its expression inside the cell, to monitor CrBV infectivity. For the first step, we measured virus attachment and uptake into the cell using cellular DNA containing slot blots and a fragment from the *CrV1* gene as a hybridization probe.

The time course of appearance of CrBV specific DNA in infected cells (Fig. 7-1) suggested that CrBVs are not attached or taken up by cells at 1 h after inoculation, although few CrBVs might enter into host cells, but remain below the detection power of DNA slot blot hybridization. The fact that no virus DNA was detected in virus-incubated cells before one hour shows that the washing procedure used in our experiments is sufficient to remove suspicious attachment of virus to cell surfaces. After 1.5 h incubation, large amounts of virus DNA are observed. After 5 h, the amount of CrBVs is still high, but less than that after 1.5 h. These results suggest that under conditions used in these experiments, the uptake of CrBVs occurs almost simultaneously and that after 5 h more DNA is degraded inside host cells than taken up (Fig 7-1). Since CrBV DNA thoroughly accumulates inside the host cells between 1 and

1.5 hours after incubation, we used 1.5 h of incubation in the following experiments.

7.3.2 Effect of temperature on CrBV uptake

To examine the effect of temperature on the virus uptake, CrBVs were incubated with cells at 4 °C, 25 °C and 37 °C, respectively, for 1.5 h, after which viruses remaining on cell surface were washed away as described above. As can be seen in Fig. 7-2, the rate of uptake into cells is the highest at temperature of 4 °C and lowest at 37 °C. These results suggest that CrBV uptake is temperature-dependent. Although at 25 °C the entry of CrBVs into cells is not the highest, in all subsequent experiments, infection of CrBVs was done at 25 °C, to avoid cellular effects at low temperature.

7.3.3 CrBV uptake at different pH values

The entry of many animal enveloped viruses requires mildly acidic environments. To determine whether the uptake of CrBVs is inactivated by low pH, CrBVs were incubated with cells at pH 5.3, 6.3 and 7.3, respectively, for 1.5 h. Viruses remaining on cell surface were washed away. Figure 7-3 shows CrBV uptake in host cells at different pH conditions. Optimal uptake was observed at pH 6.3. Under these pH conditions, the amount of viral DNA is higher than that detected at pH 5.3 and 7.3. Entry of CrBVs at pH 5.3 was higher than that at pH 7.3. These results suggest that CrBV might enter into host cells in a low pH-dependent fashion.

7.3.4 Lysosomotropic agent NH_4Cl inhibits CrBV uptake

We examined whether the presence of a lysosomotropic weak base affected the penetration of CrBVs into cells. Cells were pre-incubated in pH 7.0 cell culture medium with 5 mM, 20 mM and 40 mM of NH_4Cl, respectively for 0.5 h. CrBVs were then allowed to incubate with cells for 1.5 h in the presence of the corresponding concentrations of NH_4Cl. Fig. 7-4 shows that the amount of CrBVs detected in all three treatments with NH_4Cl are similar but markedly lower than that of control. These results

suggest that CrBV entry into host cells was inhibited by NH_4Cl, a further indication that CrBV uptake is low pH-dependent.

7.3.5 Venom proteins are not essential for CrBV entry into cells

In *C. melanoscela*, it was reported that venom proteins promoted uptake of the virions by the host hemocytes and facilitated the uncoating of PDVs in host hemocytes (Stoltz *et al.*, 1988). In addition, the venom proteins enhanced virus persistence in the host (Stoltz *et al.*, 1988). To examine whether venom components from *C. rubecula* are required for CrBV entry into the host cells, *in vitro* assays were carried out by using the *P. rapae* cell line and DNA slot blot hybridization to detect CrBVs. Cells were incubated with venom proteins for 1 h or 1.5 h in the presence or absence of venom proteins, respectively. Total DNA was exacted from cells and used for slot blot. DNA slot blot hybridization showed that CrBVs were not detected in cells in the presence or absence of venom when incubated for 1 h, but were detected in both infected cells after 1.5 h (Fig. 7-5). The amount of CrBVs was the same in both treatments with venom proteins (Fig. 7-5). These results indicate that venom components are not ~~relevant~~ required for CrBV entry into host cells, although venom proteins are required for CrBV gene expression in host hemocytes (Zhang *et al.*, 2004 and see Chapter 6).

7.4 Discussion

Polydnaviruses are a unique group of double DNA insect virus (Stoltz & Vinson, 1979; Stoltz & Guzo, 1986). Particles are only produced in specialized ovarian calyx cells, stored in the oviduct and introduced into host hemocoel together with eggs and various maternal protein secretions at oviposition (see Chapter 1). Although much is known regarding their replication strategy, chromosomal transmission and transforming capabilities (reviewed by Beckage, 1998; Glatz *et al.*, 2004b), little is known about how PDVs are taken up by host cells. To our best knowledge, this is the first study to address the possible mechanism of PDV uptake and factors influencing this process. We investigated the roles of temperature, pH values and venom proteins in the cellular

uptake process of CrBVs.

First, we developed an *in vitro* cell line from *P. rapae* for a slot blot hybridization assay to monitor CrBV uptake into the cells. As expected these cells supported CrBV transcription, but not replication. By using this assay, we successfully investigated the time course of entry of CrBV particles into host cells and the factors influencing this process. In our study, CrBVs were absent in cells after 1 h incubation but strongly accumulated in cells 1.5 h post inoculation (Fig. 7-1). This result is similar with previous observations in *C. congregata* parasitizing *M. sexta* (Stoltz & Vinson, 1977). In that system, nucleocapsids were firstly observed to penetrate cells in 0.75 h post inoculation by electron microscopy and then viral DNA was shown to accumulate in cells (Stoltz & Vinson, 1977).

In previous studies, it was found that PDVs from *C. congregata* might gain entry into host cells by means of membrane fusion, rather than phagocytosis (Stoltz & Vinson, 1977). In this context, it is relevant that CrBV fusion is low temperature-dependent. Classically, viruses that enter by membrane fusion are incubated with cells at 0 to 4 °C to limit penetration (Rigg & Schaller, 1992). Our observations show that at 4 °C CrBVs enter into cells in a very high rate. At 37 °C, very few CrBVs were detected in infected cells (Fig. 7-2). These results suggest that phagocytosis might be involved in the initiation of CrBV infection. This is corroborated by the observed pH-dependent uptake reactions, which is optimal at pH 6.3, but reduced above or below. Because low pH-dependent viruses enter cells efficiently at low temperature, but viruses fusing at neutral pH appear not to undergo fusion at or below 20 °C (White, 1990), we conclude that CrBVs might enter into cells in a low pH-dependent pathway.

In the main part of this study, we investigated whether low pH was involved in initiation of CrBV uptake. We found that the optimal pH for CrBV entry into cells is 6.3. At pH higher or lower than optimal pH 6.3, CrBVs were able to initiate infection, but were inhibited largely, especially, at pH 7.3 which is close to neutral pH (Fig. 7-3). Cells were also inoculated with CrBVs in the presence of weak base NH_4Cl, which raises the pH of

intracellular vesicles and inhibits infection by low pH-dependent viruses, but not pH-independent viruses (Helenius *et al.*, 1982; White, 1990). The results show that in the presence of NH_4Cl, penetration of CrBVs into cells was inhibited, but not completely (Fig. 7-4). This is consistent with previous analysis that CrBVs enter host cells in a low pH-dependent pathway.

In certain braconid endoparasitoid/host systems, venom played an important role for successful parasitism (Kitano, 1982; Guzo & Stoltz, 1985, 1987; Stoltz *et al.*, 1988). It is found that venom proteins from *C. melanoscela* are required for *in vivo* persistence of viral DNA in host larvae and promote the uncoating at nuclear pores. Observation at ultrastructural level suggested that venom proteins might enhance the initial uptake of polydnavirus particles (Stoltz *et al.*, 1988). Our study shows that CrBVs enter cells in the same rate in the presence or absence of venom proteins, which indicates that venom proteins are not essential for CrBV entry into host cells (Fig. 7-5). In further studies, venom proteins are found to be required for the CrBV successful expression in host cells (Zhang *et al*, 2004b).

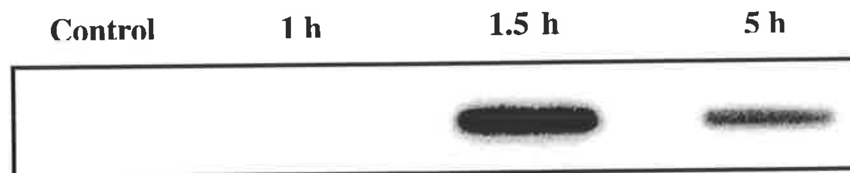


Fig. 7-1: Effects of incubation time on CrBV entry into host cells by DNA slot blot hybridization. 200 μl of cell suspension were plated in 24-well tissue culture plates to allow attachment for 0.5 h. Then CrBVs from 2 wasp equivalents were added into each well and incubated at 25°C for an additional 1 h, 1.5 h and 5 h. Control was incubated for 5 h without CrBVs. The cells were then collected by centrifugation at 800 × *g* for 5 min and washed 3 times with PBS as described in methods. Total DNA was extracted. 8 μg of DNA for each sample was used for slot blot hybridization by probing with *CrV1* cDNA fragment labelled by incorporation of ^{32}P-dCTP.

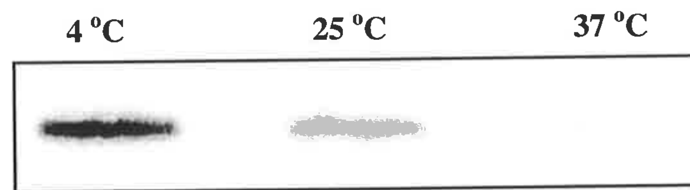


Fig. 7-2: Effect of temperature on CrBV uptake into host cells by DNA slot blot hybridization. 200 µl of cell suspension were plated in 24-well tissue culture plates to allow attachment for 0.5 h. Then CrBVs from 2 wasp equivalents were added into each well and incubated at 4 °C, 25 °C, 37 °C for an additional 1.5 h, respectively. The cells were collected by centrifugation at 800 × *g* for 5 min and washed 3 times with PBS as described in methods. Total DNA was extracted. 4 µg of DNA for each sample was used for slot blot hybridization by probing with *CrV1* cDNA fragment labelled by incorporation of ^{32}P-dCTP.

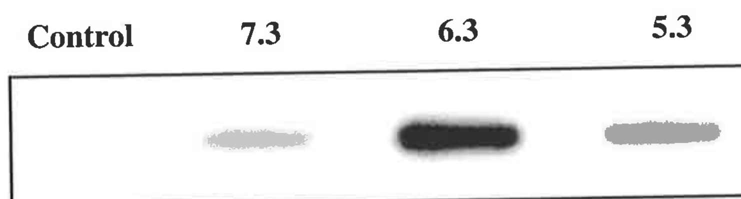


Fig. 7-3: Effect of pH values on CrBV uptake into host cells. 200 μl of cell suspension were plated in 24-well tissue culture plates to allow attachment for 0.5 h. Then CrBVs from 2 wasp equivalents were added into each treatment and incubated at 25 °C at pH 7.3, 6.3, 5.3, respectively, for an additional 1.5 h. Control was incubated without CrBVs for 1.5 h at pH 7.3. The cells were collected by centrifugation at 800 *g* for 5 min and washed 3 times with PBS as described in methods. Total DNA was extracted. 8 μg of DNA for each sample was used for slot blot hybridization by probing with *CrV1* cDNA fragment labelled by incorporation of ^{32}P-dCTP.

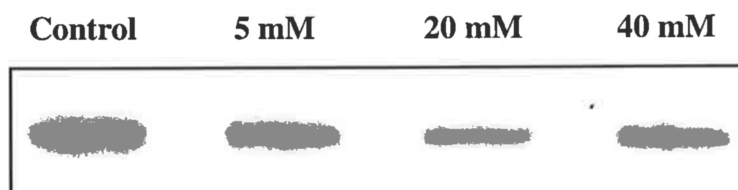


Fig. 7-4: Effect of a lysosomotropic agent, ammonium chloride, on the CrBV uptake into host cells. 200 µl of cell suspension were pre-incubated at 25 °C for 0.5 h in the absence or presence of 5 mM, 20 mM, 40 mM of NH_4Cl, respectively. Cells were then incubated with CrBVs from two wasp equivalents at the same conditions for an additional 1.5 h. The cells were collected by centrifugation at 800 × *g* for 5 min and washed 3 times with PBS as described in methods. Total DNA was extracted. 5 μg of DNA for each sample was used for slot blot hybridization by probing with a CrV1 cDNA fragment labelled by incorporation of ^{32}P-dCTP.

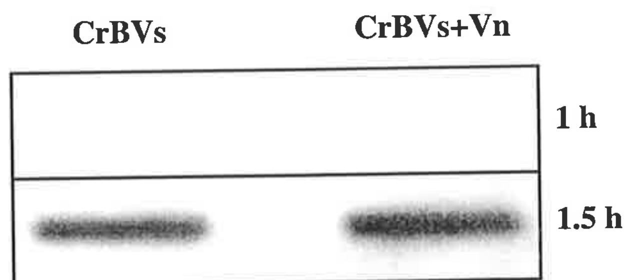


Fig. 7-5: Venom proteins from *C. rubecula* are not required for CrBV entry into host cells. 200 μl of cell suspension were plated in 24-well tissue culture plates to allow attachment for 0.5 h. Then CrBVs from 2 wasp equivalents were added into each treatment and incubated at 25 °C for an additional 1 h or 5 h in the absence or presence of venom proteins from two wasp equivalents, respectively. The cells were collected by centrifugation at 800 × *g* for 5 min and washed 3 times with PBS as described in methods. Total DNA was extracted and 5 μg of DNA for each sample was used for slot blot hybridization by probing with CrV1 cDNA fragment labelled by incorporation of ^{32}P-dCTP.

Chapter 8: General discussion

A hallmark of the parasite life cycle is its ability to adapt swiftly to the unique physiology of either invertebrate or vertebrate host. This involves adapting abiotic and biotic changes including temperature, pH and host defence strategies. This adaptation capacity is particularly true for hymenopterous endoparasitoids, which oviposit and develop in their host hemocoel where they are exposed to the humoral and cellular elements of host immune systems. Although insects apparently lack an acquired immune response, insects possess a complex and efficient immune system to defend against microbial pathogens and parasites. Encapsulation is a major defence response against parasitoid wasps in view of the sizes of parasitoid eggs and larvae (Strand & Pech, 1995; Gillespie *et al.*, 1997).

To complete development inside their hosts, endoparasitoids have necessarily evolved an amazing array of effective mechanisms to manipulate host physiology and biochemistry to create an environment that is favorable for parasitoids but detrimental to host insects (Beckage, 1985; Strand & Pech, 1995; Beckage & Gelman, 2004). Maternal factors introduced into their hosts during oviposition are believed to be evolutionary adaptations involved in the manipulation of host physiology and suppression of host immune responses. These factors include ovarian proteins, PDVs, VLPs and venom proteins (Vinson, 1990; Lavine & Backage, 1995; Strand & Pech, 1995; Webb & Luckhart, 1994, 1996; Beckage, 1998; Beckage & Gelman, 2004). The most interesting of all is thought to be PDVs.

PDVs are only produced in the calyx region, released into the oviduct lumen and injected into host together with eggs. Once inside the host, PDV particles infect various tissues, virus genes are expressed and the PDV-expressed products are assumed to be responsible for a range of physiological alterations and immunosuppression. PDVs are thought to play a major role in mediation of host immune responses (Edson *et al.*, 1981; Lavine & Beckage, 1995; Summer & Dib-Haji, 1995; Beckage, 1998; Glatz *et al.*, 2004). Many PDV genes have been isolated and

characterized from various endoparasitoids (review in Beckage, 1998; Glatz *et al.*, 2004). However, PDV-encoded proteins are not detected before a few hours after parasitization (Webb & Luckhart, 1996; Asgari *et al.*, 1996).

It is well known that insects react quickly to invading parasites and pathogens by utilizing humoral and cellular responses (Ratcliffe, 1993). Therefore, a delay in protection caused by the PDV gene expression might endanger the survival of the developing embryo of endoparasitoids. In this regard, other maternal factors such as ovarian proteins (Webb & Luckhard, 1994; Luckhard & Webb, 1996), venom proteins (Webb & Luckhard, 1994), and ovarian materials covering the egg surface (Davies & Vinson, 1986; Asgari & Schmidt, 1994) are required for successful parasitism and development. The rapid short-term immunosuppressive activity complements the slower, long-term immune suppression provided by PDV expression (Luckhard & Webb, 1996; Asgari *et al.*, 1998).

In insect endoparasitoids without PDVs, venom proteins may play major roles in host regulation and immune suppression. Venom proteins adversely affect the morphology, viability, behaviour and the immune function of hemocytes (Richards & Parkinson, 2000; Parkinson *et al.*, 2001; 2002a, b). In endoparasitoids with PDVs, a combination of maternal factors is used to regulate host physiology and suppress host immune responses, where venom proteins are thought to synergize the effect of PDVs (Tanaka, 1987; Stoltz *et al.*, 1988; Wago & Tanaka, 1989), although venom alone was shown to prevent encapsulation of eggs in the *C. glomeratus*/*H. virescens* system (Kitano, 1986). In *C. melanoscela*, venom proteins were found to promote release of virions into the cytoplasm of infected host cells and enhance persistence of the viruses *in vivo* (Stoltz *et al.*, 1988). Thus, venom proteins facilitated the action of the PDVs. However, very few specific components from venom have been identified in most endoparasitoids.

In the braconid endoparasitoid *C. rubecula*, maternal factors also introduced into the host body by endoparasitoid wasps at oviposition are essential for successful completion

of parasitism. Only a limited number of CrBV genes are transcribed in the host hemocytes and expressed in a highly transient fashion from 4 to 8 hours following parasitization (Asgari *et al.*, 1996). This is in contrast to other known parasitoid systems where a variety of viral multigenes are transcribed (Summers & Dib-haji, 1995) and transcripts persist for a large portion of parasitoid development (Theilmann & Summers, 1986; Strand *et al.*, 1992). An immunosuppressive gene (*CrV1*) from *C. rubecula* bracoviruses is identified to be involved in the destabilization of cytoskeleton in host hemocytes (Asgari *et al.*, 1996, Asgari *et al.*, 1997). In addition, a calyx protein (Crp32) on the surface of CrBV particles and eggs, provides short-term protection of the parasitoid eggs against host encapsulation reactions before CrBV genes are expressed (Asgari & Schmidt, 1994; Asgari *et al.*, 1998).

When crude venom preparations, isolated from female *C. rubecula* endoparasitoid wasps, were separated by rpHPLC, over a dozen proteins were found, some (Vn4.6 and Vn50) of which are involved in inhibition of melanization by blocking the activation of prophenoloxidase (Asgari *et al*, 2003a, b; Zhang *et al.*, 2004), some (CrCRT) of which are involved in suppression of host cellular immune responses. In addition, several peptides (such as Vn1.5) were found to be required for CrBV gene expression.

Vn4.6 cDNA consists of 277 bps containing an open reading frame of 129 nucleotides coding for 42 amino acids, which has 25.5 % identity with ω-atracotoxin-HV1A from the Australian funnel web spider *H. versuta* (Fig. 3-5). The ω-atracotoxins are small peptide neurotoxins that specifically block insect voltage-gated calcium channels with insecticidal activities (Wang *et al.*, 1999). Since Vn4.6 was not found to have the same function of ω-atracotoxins and phylogenetic studies suggest the evolution of endoparasitoids from ectoparasitoids, Vn4.6 might be reminiscent to paralyzing proteins present in ancestral ectoparasites. In addition, sequence analysis showed similarities of Vn4.6 to small Kunitz-type protease inhibitors, although not very significant. The family includes snake venom basic proteases, mammalian inter-alpha-trypsin inhibitors, trypstatin, etc. proPO inhibition assay suggests that Vn4.6 protein was able to significantly inhibit melanin formation but not completely. Although the protein may

lack Kunitz-type protease inhibitors properties, the proPO inhibition assays suggest that Vn4.6 may interfere with specific components of the prophenoloxidase-activating system, by competitive binding to the zymogen and thereby interrupting the immune-activation.

Vn50 is a glycoprotein containing 388 amino acids with similarity to SPHs, which completely inhibits melanization of the host hemolymph *P. rapae*. Similar to other SPHs, Vn50 consists of two domains, a carboxyl-terminal serine proteinase domain and an amino-terminal "clip" domain. However, in both SPHs and Vn50, the serine proteinase domain is not functional since they lack a serine at the conserved site, precluding enzymatic activity. Although Vn50 resembles SPHs at the structural level, it appears that Vn50 differs from SPHs at the functional level. Whereas SPHs interact with proPO to activate the enzyme complex (Yu *et al.*, 2003), Vn50 inhibits proPO activation. One explanation for this discrepancy is the unique functional context of parasitoid-host interactions, where evolutionary adaptations of regulatory proteins are exploited for host manipulations. A possible assumption for the mechanism by which Vn50 inhibits melanization is the protein might function as an antagonist molecule competing with host SPHs for binding sites of immunolectins and proPO, instead of activating the complex, because Vn50 has structural and sequence similarities to SPHs.

To explain how Vn50 exerts its function, Vn50-binding proteins in the host hemolymph were investigated. In our investigation, it was found that Vn50 might interact with proPO and PAP in *P. rapae* hemolymph (Fig. 4-11). The interaction among the proteins might confer a conformational change in proPO to facilitate its activation by the PAP (Yu *et al.*, 2003). The antibodies recognized the respective proteins in *P. rapae* hemolymph, confirming that proteins bound to the beads came from the larval hemolymph. Similar observations were made in *M. sexta* SPH-1, whose proteinase-like domain binds to proPO, PAP-1, and immulectin-2 (Yu *et al.*, 2003). These results suggest that Vn50 may interact with one or more of proteins involved in melanization. Complementary studies carried out by our collaborators using purified *M. sexta* proPO, PAP-1, and SPHs, as an *in vitro* system to test the possible role of Vn50 in regulating

the proPO activation reaction, indicated that Vn50 efficiently down-regulated proPO activation mediated by the proteins. Vn50 did not inhibit active PO or PAP-1, but it significantly reduced the proteolysis of proPO (Zhang *et al.*, 2004). While molecular details are still lacking, our results strongly suggest that interactions among the substrate, proteinase, and cofactor were impaired by Vn50.

In the present study, a CrCRT was isolated from venom fluid. CrCRT was found to inhibit hemocyte aggregation and spreading, where the inhibition is dose-dependent (Fig 5-6). The recombinant protein attached to Ni-resin beads precludes hemocytes *in vitro* from attaching to the surface, thus protecting the beads from being encapsulated. Hemocyte de-adhesion by CrCRT might have synergistic effects in conjunction with the immunoprotective action of another calyx protein Crp32, which protects the parasitoid eggs before the CrBV genes are expressed to suppress hemocyte adhesive functions. However, the molecular mechanism of CrCRT-mediated protection is not clear.

CRT is a multifunctional Ca^{+}-binding protein in many intra- and extra-cellular processes (Michalak *et al.*, 1992; Gray *et al.*, 1995; White *et al.*, 1995). CRT was identified from many parasites and might be involved in host/parasite interaction (review by Ferreira *et al.*, 2004). In tick *Amblyomma americanum*, CRT was considered to divert host defensive responses (Kovacs *et al.*, 2001). Choi *et al.* (2002) isolated and characterized an encapsulation response protein, calreticulin, from *G. mellonella* larvae, which may be involved in the non-self recognition process in cellular defence reactions. Asgari & Schmidt (2003) reported that *P. rapae* CRT was involved in the phagocytosis reaction. In our study, a CRT from *P. rapae* was identified as a putative early-stage encapsulation response protein (Fig. 5-8), which might be involved in non-self recognition. It is therefore possible that CrCRT might function as an antagonist molecule competing for binding sites with the host calreticulin.

Finally, we explored the interaction between venom proteins and CrBVs with regards to virus entry and gene expression. It is clear that venom is required for CrBV gene expression in host hemocytes, although slot blot assays and electron microscopy

observations revealed that venom components were not essential for CrBV entry into host hemocytes and release into the cytoplasm. A 1598 Da peptide (Vn1.5) was found to be sufficient for the expression of CrBV genes in *in vitro* assays. In addition, two other peptides were also found to promote the expression of CrBV genes. Synthetic Vn1.5 peptide and total venom proteins in conjunction with purified virus were shown to facilitate expression of CrBV genes, while synthetic peptide or CrBV particles alone were not able to cause behavioural changes in host hemocytes (Fig. 6-7). These results suggest that calyx-derived virus particles and venom-derived Vn1.5 peptide are both required to express CrBV genes in host hemocytes similar to natural parasitism (Asgari *et al.*, 1996, 1997).

A system of supposed recognition molecules and a series of reactions leading to activations of prophenoloxidase (in turn leading to melanization) have been proposed as possible pathways to immune recognition and defence responses (Söderhall, 1982). For successful parasitism, parasitoids have developed various mechanisms to evade or suppress host encapsulation responses by interfering with every possible step during the cellular reaction: recognition of the parasitoid as non-self, adhesion, spreading and melanization (Strand & Pech, 1995; Gillespie *et al.*, 1997). In the present study, venom proteins are found to interfere with both cellular and humoral immune responses, which suggests that venom proteins might play a more important role than that thought in endoparasitoid/host system with PDVs. Based on characterisation of CrBV genes, Crp32 and venom proteins, it is apparent that *C. rubecula* employs integrated pathways to actively and passively suppress host immune responses. Fig. 8-1A summarises the mechanisms employed by *C. rubecula* and compares them with those employed by *C. sonorensis* against *H. viriscens* (Fig. 8-1B).

From our study, not only do *C. rubecula* venom proteins co-manipulate host immune responses, but also venom peptides were required for CrBV gene expression and functional roles. In addition, venom, calyx fluid and PDVs share some similar genes and proteins such as Vn4.6 and Crp32 (Asgari *et al.*, 1998; 2003a), and CrCRT (Asgari *et al.*, 2003c, see Chapter 5). The coding region for Vn4.6 is located upstream in

opposite direction of a gene coding for a *C. rubecula* Crp32, although they are expressed in different areas. CrCRT was also found to be a PDV-associated particle protein (Asgari *et al.*, 2003c). These may reflect an evolutionary relationship between venom gland and polydnavirus in this wasp, which was previously reported in *C. sonorensis/H. viriscens* system (Webb & Summers, 1990). The synergistic effects of venom proteins in conjunction with the immunosuppressive action of CrBVs and particle- associated proteins might enhance parasitoid survival.

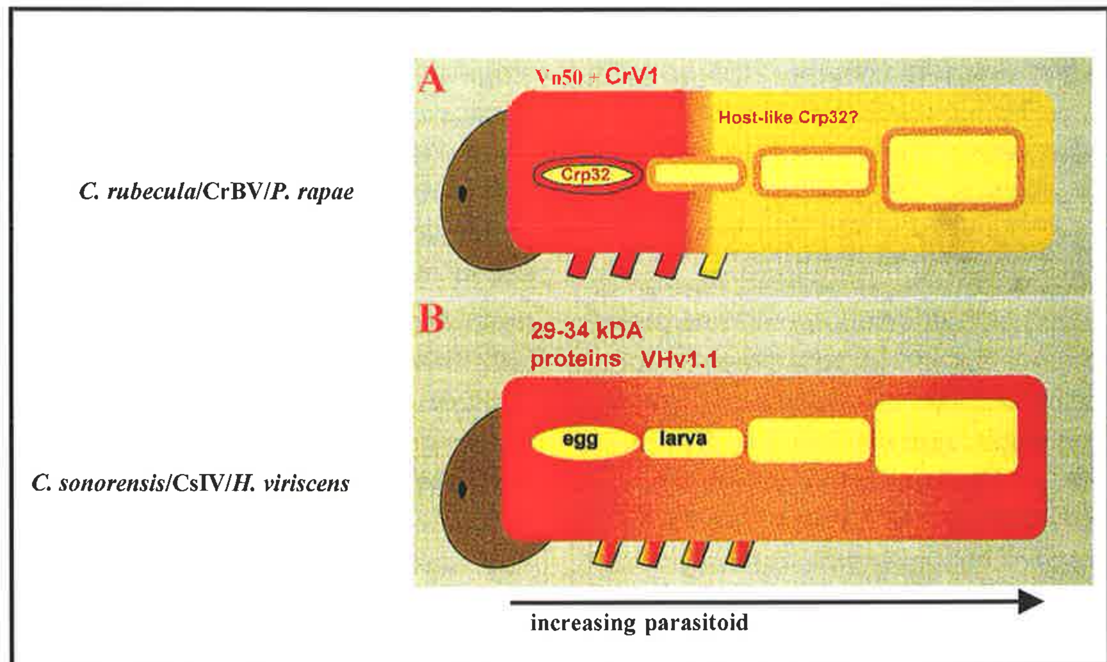


Fig. 8-1: Putative suppression of lepidopteran host immune response by bracovirus- and ichnovirus-associated hemenopteran endoparasitoids (adapted from Schmidt *et al.*, 2001). Parasitoid development (i.e. time) increases moving left to right, where red areas indicate the temporal and spatial distribution of active hemocytes (e.g. inside the egg and larva). A, *C. rubecula* applies active and passive means of immune-suppression against *P. rapae*. Venom proteins and Crp32 synergistically passively protect the developing eggs prior to active suppression by CrBV gene products such as CrV1. CrV1 is expressed by *P. rapae* hemocytes over a period of 4-10 hpp and provides systemic active suppression of host immune reactions by causing destabilization of their cytoskeleton. Hemocytes recover after2-3 days. The mechanism of immune-suppression after this time is not clear. B, *C. sonorensis* actively and systemically suppresses the *H. viriscens* immune responses over the total period of parasitoid larval development. A series of related 29-34 kDa calyx glycoproteins provide initial hemocyte inactivation. CsIV gene products, e.g. VHv1.1, are subsequently expressed and cause long-term inactivation. The calyx proteins and VHv1.1 both inactivate hemocytes by damaging their cytoskeleton.

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