GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST SCORPION TOXIN AaIT AS GLUTATHIONE S-TRANSFERASE FUSION PROTEIN

By

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A thesis submitted to the Department of Microbiology and Immunology in conformity with the requirements for the degree of Master of Science

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ABSTRACT

Xiaoling Song: Generation and Characterization of Monoclonal Antibodies against Scorpion Toxin AalT as Glutathione S-transferase Fusion Protein

Spruce budworm (*Choristoneura fumiferana*) is a major Canadian forest pest, and causes damage to spruce and pine trees. To control this forest pest, biological insect control agents are an attractive alternative to chemical insecticides because they do not pose the environmental and health risks associated with chemical residues. Spruce budworm *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus (C*f*MNPV), a major pathogen of the spruce budworm, has considerable potential for insect control. However, naturally occurring C*f*MNPV is not virulent enough to be used as an effective control agent. Genetic engineering provides a means of improving C*f*MNPV for use as specific pest control agents. One approach to genetically modify C*f*MNPV was carried out by inserting a foreign gene which encodes for the insect-specific scorpion toxin AalT to produce a recombinant virus. The insects fed with recombinant C*f*MNPV carrying a scorpion toxin AalT (Spt-AalT) were found to stop feeding earlier than those fed with wild-type C*f*MNPV.

In order to study the expression of scorpion toxin AalT in spruce budworm infected with recombinant C*f*MNPV/Spt-AalT and to detect the persistence of the scorpion toxin AalT in the affected environmental samples, monoclonal antibodies

against the scorpion toxin AalT were developed in this investigation. A novel glutathione s-transferase (GST) protein expression system was used to express scorpion toxin AalT as a GST fusion protein with high level expression and easy purification. Using a modified hybridoma fusion protocol, we have produced specific monoclonal antibodies against the scorpion toxin AalT. These monoclonal antibodies have been characterized with their reactivities and specificities. The expression of scorpion toxin AalT in insects infected with recombinant *Cf*MNPV/Spt-AalT has been also analyzed with these specific monoclonal antibodies. The initial results demonstrated that the expression of scorpion toxin AalT in infected insects could be detected in dead insects after 9-10 days of viral infection, indicating these specific monoclonal antibodies could be a potential tool for further studies.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-[3-ethyl-benzthiazoline sulfonates
AcMNPV	autographa californica multicapsid nuclear polyhedrosis virus
AalT	scorpion androctonus australis toxin
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
Bt	bacillus thuringiensis
CfMNPV	spruce budworm *choristoneura fumiferana* multicapsid nuclear polyhedrosis virus
CPSR-3	controlled process serum replacement-type 3
DTT	dithiothreitol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMEM	dulbecco's modified eagle's medium
E. coli	escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoassays
GST	glutathione S-trasferase
HAT	hypoxanthine, aminopterin and thymidine
HT	hypoxanthine and thymidine
IPTG	isopropyl-1thio-β-D-galactoside
kb	kilobase
LB	Luria-Bertani broth
NOVs	non-occluded viruses
OB	occluded body
OD	optical density at the stated wavelength
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	polyhedrin-derived virus
PEG	polyethylene glycol
pGEX-2T	GST gene fusion plasmid vector
SDS	sodium dodecyl sulfate
SPT	scorpion toxin
TAE	Tris-acetate-EDTA
TE	Tris-EDTA buffer

INTRODUCTION

Spruce budworm, a major Canadian forest pest, destroys spruce and pine trees and costs million of dollars every year (Anon, 1993, 1994). The control of spruce budworm is very important not only economically but also by adversely affecting the aesthetic value of our forests. Traditionally, chemical insecticides have been used for pest control. Although efficient, these chemicals have undesirable side effects on the environment such as damage to wild life. Furthermore, some of their by-products are carcinogens. In order to overcome these problems, numerous attempts have been made to introduce pest parasites or pathogenic organisms into spruce budworm in an effort to control the pest without chemical intervention (Anon, 1976). These biological controls have become increasingly more environmentally acceptable. *Bacillus thuringiensis* (Bt) delta-endotoxin provided some effective control of agriculture food pest, but the results for spruce budworm were found not to be consistent and not very specific. Some insects developed Bt resistance (McGaughey and Whalen; Gould et al., 1992). Thus, agencies involved in forest pest control are committed to developing spruce budworm specific viral insecticide.

Baculoviruses, especially nuclear polyhedrosis viruses (NPVs), have potential for insect control (Granados and Federici, 1986) and recently have been proven to be efficient vectors for the expression of foreign genes (Luckow,1988; Maeda, 1989a).

Consequently nuclear polyhedrosis viruses are considered to be the best choice for developing specific viral pest control agents. The spruce budworm *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus (C*f*MNPV) is a major pathogen of spruce budworm. However, the wild type of C*f*MNPV is not virulent enough to be an effective control agent for the spruce budworm. In order to use this spruce budworm specific virus as forest pest control agent, it is necessary to improve the virulence of C*f*MNPV by genetic engineering. The preliminary results show that a recombinant C*f*MNPV carrying a insect-specific toxin gene from the scorpion *Androctonus australis* (SPT-AalT) could provide a safe and effective insecticide for the insects.

Since the new recombinant C*f*MNPV/Spt-AalT is a potential biological insecticide in controlling spruce budworm, the use of such a biological insecticide requires registration. To facilitate registration, it is necessary to study the expression of inserted scorpion toxin AalT as function of time in the spruce budworm and also to monitor the concentration of scorpion toxin AalT in any potentially affected environmental samples (e.g. soil and water). My research strategy focuses on the generation of scorpion toxin Aalt as glutathione S-transferase (GST) fusion protein, and production of specific monoclonal antibodies against scorpion toxin AalT. These monoclonal antibodies specific for scorpion toxin AalT would then be used to investigate the *in vivo* expression of the scorpion toxin AalT in the infected spruce budworm and also to monitor any environmental contamination.

REVIEW OF THE LITERATURE

I. Spruce Budworm *(Cristoneura fumiferana)*

Spruce budworm is the most widely distributed and destructive defoliator of spruce forests in North America. This native insect poses a threat to over 150 million acres of susceptible forests in the Eastern United States and Canada (Talerico, 1994).

I.1. Hosts of Spruce Budworm

The spruce budworm can be found in a large area ranging form Virginia to Newfoundland, and across Canada throughout the boreal forest region to the McKenzie River (Powell 1980). Larvae feed on a number of conifers, white spruce (*Picea glauca* Voss) and red spruce (*Picea rubens* Sarg.), which are the major forests type trees in the forest of eastern North America. Regional differences in the forest types of eastern North America vary from west to east. In the Great Lakes states region, white spruce and black spruce are the major sources of food for the spruce budworm. In Maine and the Canadian Maritimes Provinces, the patchy pattern gives way to extensive areas of soft woods. In this region, red spruce trees are a major component of the forest.

I.2. Life Cycle of Spruce Budworm

The spruce budworm has a 1-year life cycle (Figure 1). In Canada, the adult moths lay about 180 eggs in clusters or masses of about 20 eggs each in July. The hatched larvae progress through a series of developmental stages and molt between stages from first-instar to sixth-instar larvae. Hatching of first-instar larvae is usually complete by mid-August, and some may spin down on silken threads and be carried away by air currents which then spread the larvae over a wide area. Others remain on host foliage rarely feeding but spin cocoon-like shelters within which they soon molt to the second instar. The budworm overwinter in this stage. In April or May of the following year, they begin to feed on suitable host foliage forming feeding shelter until the populations reach outbreak levels and foliage is exhausted. Then, they move to the new foliage.

Pupation occurs within the feeding shelters in late June with apparent colour difference between the sexes. The male is often gray and the female often brown. Moths (adult) are present in the field from late June to mid-August, and live about 2 weeks during which time they do not eat. The male locates the female for mating when she releases a sex pheromone, or attractant scent, from small glands near the tip of the abdomen. Given suitable weather conditions, both male and female moths may be transported great distances by wind and storms (Talerico 1994).

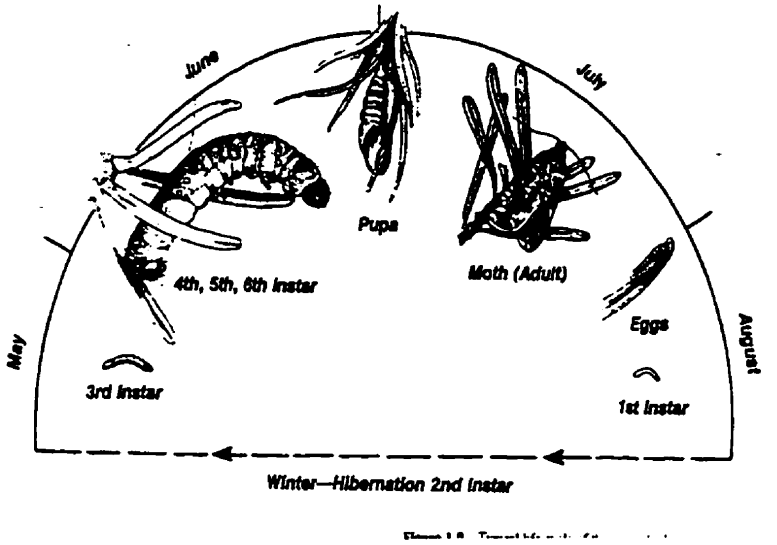


Figure 1 Typical life cycle of the spruce budworm. (From Ref. Talerico, 1994)

II. Baculoviruses-Major Insect Viruses

II.1 The Features of Baculoviruses

Four types of viruses (nuclear polyhedrosis, granulosis, cytoplasmic polyhedrosis, and an entomopoxvirus) have been isolated from budworm population in Canada, in which the baculoviruses are the largest group of viruses pathogenic to insects.

Baculoviruses are large, enveloped viruses (approximately 50 x 250nm) containing a circular double-stranded DNA genome ranging in size from 80 to 150 kb in length (Matthews, 1982). All baculoviruses produce virions which consist of

enveloped, rod-shaped nucleocapsids. Baculoviruses are divided into three subgenera based on their morphological properties. The nuclear polyhedrosis viruses (NPVs) belong to subgroup A in which several virions are occluded within a polyhedral occlusion body (OB). There are two morphological types of viruses in this group (Figure 2): single nucleocapsid NPV (SNPV; type species: *Bombyx mori*) with only one nucleocapsid per viral envelope and multiple nucleocapsid NPV (MNPV; type species: *Autographa californica* MNPV and *Choristoneura fumiferana* MNPV) with multiple nucleocapsids in a single viral envelope. The granulosis viruses (Gvs) belong to the subgroup B in which virions contain single nucleocapsid per occlusion body. Subgenus C consists of virions which are not packaged into occlusion bodies at any stage of their life cycle, these are the non-occluded viruses (NOVs) (Harrap 1979, Matthews 1982).

Baculoviruses have a very restricted host range, limited to specific insect species and do not infect vertebrates or plants (Groner 1986). Some baculoviruses, like the *Autographa californica* nuclear polyhedrosis virus (A*c*MNPV), have been well studied and have been found to infect over 30 species of insects. Some baculoviruses like B*m*SVPV or C*f*MNPV, on the other hand, have a very strict host range which is limited to the silkworm *bombyx mori* or spruce budworm, respectively.

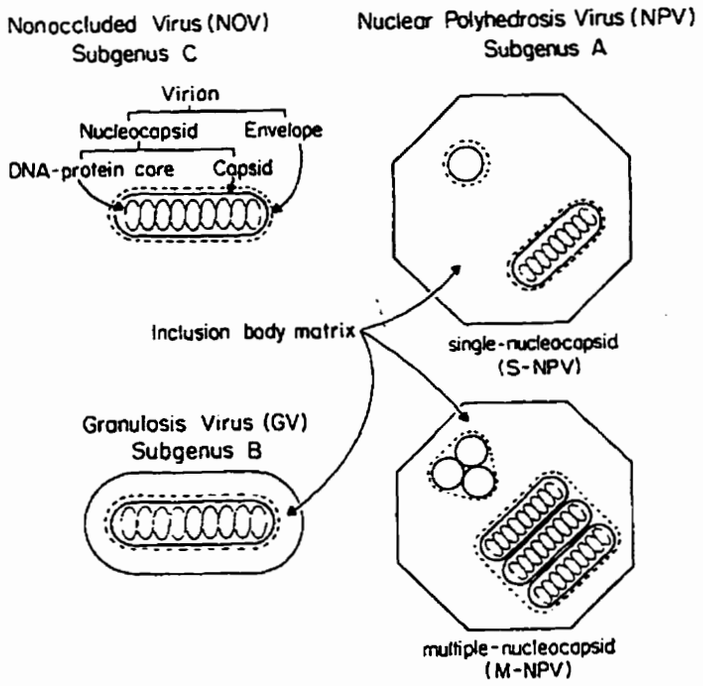


Figure 2 Schematic representation of the Baculovirus. The SNPVs and MNPVs contain numerous virions per inclusion body; MNPV virions contain as many as 17 nucleocapsides per envelope. Not drawn to scale. (From Bilimoria et al., 1986)

II.2 The Life Cycle of the NPVs

The replication cycle of NPVs and other baculoviruses is very complex (Fig.3). Virus particles exist in two forms in insects, a polyhedrin-derived (PDV) and budded or non-occluded (NOV) form. Insects feeding on infected plants ingest the occluded viral particles. The alkaline nature in the midgut region results in dissolution of the

crystal, releasing the PDV particles that enter the midgut epithelial cells by endocytosis (first infection). The particles migrate into the nucleus through the nuclear pore where they uncoat and replicate. Early in the infection cycle, the progeny nucleocapsids bud through the nuclear membrane into the cytoplasm. The membrane is removed in the cytoplasm, and another membrane surrounds the nucleocapsid as it buds through the plasma membrane. These enveloped nucleocapsids are referred to as budded viruses or NOVs. They are highly infectious to tissue culture cells, but are usually noninfectious if fed to insect larvae. These NOVs enter the hemolymph of the insect and spread the infection to other tissues (secondary infection). In the late replication cycle, progeny nucleocapsids become membrane bound within the nucleus. At this time, large amounts of polyhedrin protein are synthesized. The polyhedrin protein crystallizes around the membrane-bound particles forming polyhedra. PDV in polyhedra is not very infectious to tissue culture cells. The synthesis of polyhedrin is driven by a very strong late promoter. According to Smith's report, viruses containing deletions of the polyhedrin gene are viable and stably maintained in cultured cells, indicating that this gene is not essential for replication or production of the coded form of the virus (Smith et al., 1983). The polyhedrin gene loci make ideal cloning site for foreign genes for the construction of recombinant baculoviruses.

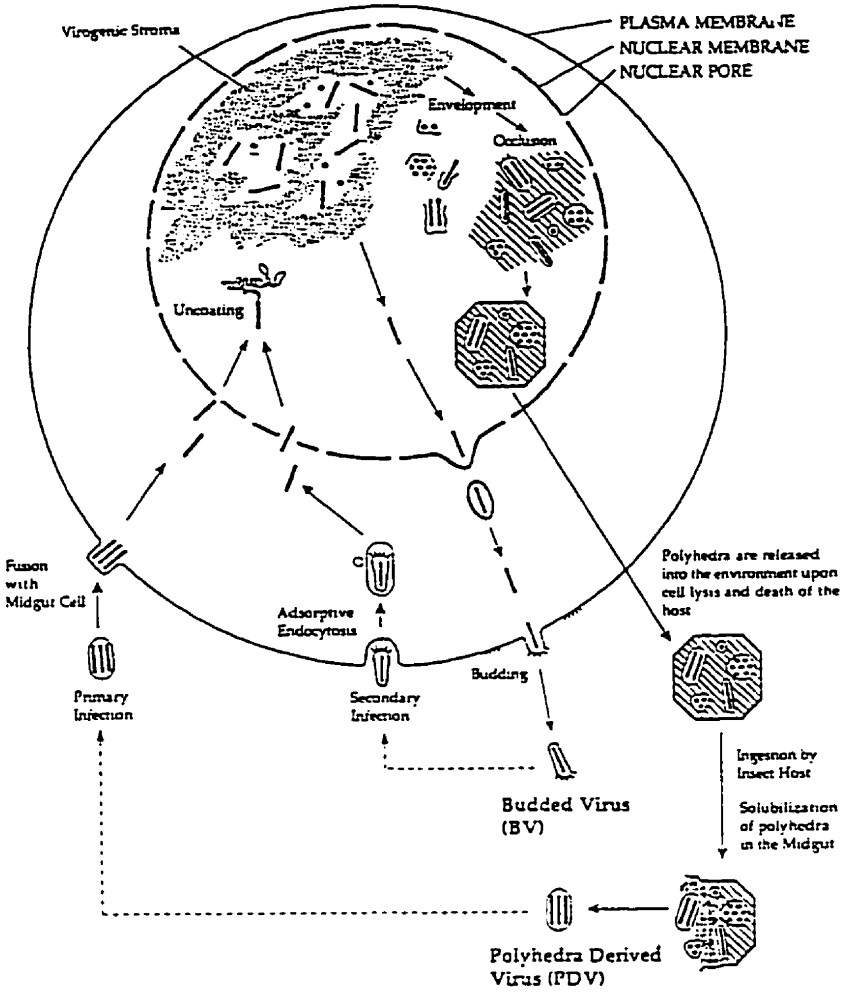


Figure 3 Cellular infection cycle of a nuclear polyhedrosis virus. Viruses are ingested by a insect and solubilized within the insect midgut. The polyhedra-derived virus (PDV) is released and enters midgut cells by fusion with microvilli. Uncoating of the DNA, followd by gene expression and viral DNA replication in the nucleus. Some progeny nucleocapsids (Ncs) leave the nucleus and bud through the cytoplamic membrane into the hemocoel. These budded virions (BV) can initiate the systemic infecton of tissues. Other NCs become enveloped within the nucleus and are occluded within polyhedra. Upon insect death and cell lysis, the polyhedra are released into the environment. (From Wood and Grandos, 1991)

III. Control of Canadian Forest Pest - Spruce Budworm

The spruce budworm, the major Canadian forest pest, destroys spruce and pine trees, and costs million of dollars every year. Protection of our forest resources against damaging insects is essential to sustain development and our economy (Anon, 1993).

III. 1. History of Insecticide

Traditionally, protection of forest resources from insect pests was by the use of chemical insecticides. Although they were very efficient and fast-acting, these chemicals had undesirable side effects on the environment. Most chemical insecticides used in forest insect pest control are known to be toxic to man, other animals, or plants at some level, and also are not species-specific. Some of these chemicals and their by-products are even carcinogens and tend to persist in the environment for long periods of time (Betz, 1986).

Biological control by the use of living organisms to control forest insect pests has increasingly replaced chemical insecticide control since 1980. One microbial product, the bioinsecticide *Bacillus thuringiensis* (Bt) delta-endotoxin, is the only alternative biological product registered in Canada for control of spruce budworm and other agricultural pests. There are a few drawbacks in the use of this biological insecticide in Canadian forests (Aronson et al., 1986). For example, there are

inconsistent control results, some insects develop Bt resistance, and there may be nonspecific infection of non-target insects. In order to overcome such problems, the forest pest control agencies of the government are committed to develop species specific control agents such as insect viruses.

III.2. Baculoviruses as Viral Insecticides:

Many of the baculoviruses have potential properties for insect control, especially the nuclear polyhedrosis viruses (NPVs) that are naturally occurring insect pathogens, and are considered to be host specific and environmentally safe (Ignoffo 1975). These viruses have many advantages for use as insecticides, including a unique biphasic infection cycle, a specificity for the spruce budworm, and a stability in nature, as well as being able to cause epizootic effects (virus spreads from one insect to the other).

1) History of Viral Insecticides

The first recorded attempt to use a baculovirus as an insecticide involved the nuclear polyhedrosis virus (NPV) in 1892 (Gehren 1892). After World War II, the development of the chemical pesticide industry was accompanied by an interest in developing baculovirus and other microbial control agents as pesticides. Consequently, extensive health and environmental safety testing was performed

(Groner 1986). The data indicated that naturally occurring baculoviruses posed no foreseeable hazards to the environment or to other arthropod hosts. The effective host ranges of these viral pesticides were generally limited to a small number of pests and did not include beneficial insects. Based on their potential as alternative pesticides and on their safety from 1950 to 1980, numerous insect viruses were tested in the field to evaluate their pesticidal properties to control forest pests or agricultural pest in (Cunningham 1982). But the major deterrent to the development of viral insecticides has been that it can take from 10 to 15 days postinfection to kill an insect pest. Based on this slow speed of action, baculoviruses were considered to have poor commercial efficacy.

Since 1980s, knowledge of the molecular biology and genetics of baculoviruses has accelerated greatly with the development of the baculovirus vector system (Smith et al., 1983; Pennock et al., 1984). The introduction of new genes into the baculovirus genome, which could deliver some deleterious gene product within the insect larva, could greatly increase the speed of kill or the host range (Maeda 1989a; Miller 1988). The identification of a foreign gene effective for insect control is a crucial for the construction of a baculovirus insecticide. Possible candidates include enzymes essential for metabolism, peptide hormones, and insect-specific toxins. Recently, an insect diuretic hormone (Maeda 1989b) and a juvenile hormone esterase (Hammock et al., 1990) have shown some insecticidal and physiological

effects on infected hosts when baculoviruses carrying these genes were applied to insects. However, these recombinant viruses did not show a strong increase in potency when compared to controls.

2). Development of a Specific Viral Insecticide for Spruce Budworm

The spruce budworm *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus (C*f*MNPV), is a major pathogen of spruce budworm and naturally found in this insect, and has been extensively studied by the Canadian Forest Service for over three decades (Arif et al., 1988). In nature, viral occlusion bodies (OBs) contaminating the foliage are eaten and dissolved by the larval midgut juices, releasing virus particles PDVs. These particles pass through the midgut epithelial cells and infect other body tissues of the host larva. Over a period of 15 days, the virus replicates in all susceptible tissue eventually causing insevt death. During the initial stages of infection the larvae still feed, and hence defoliation of forest trees can still occur. This feeding damage is inherent to the virus and is a result of the time lag between entry into the insect body and lethal infection. Therefore, like other natural insects viruses, wide-type C*f*MNPV is not very virulent in spruce budworm. Moreover, even when the spruce budworm is infected with C*f*MNPV, the larvae still ingest food (needles on the trees). Normally, it takes more than 10 days before the insects die, causing leave defoliation and forest damages. Therefore, naturally

occurring C*f*MNPV is not very efficient in protecting the Canadian forests. However, this virus is very specific to spruce budworm. In order to use this C*f*MNPV as a forest pest control agent, it is necessary to genetically modify the viral DNA of C*f*MNPV to enhance the effectiveness of this virus. Because of the developed resistance to the Bt delta-endotoxin in some insects, the introduction of an insect specific scorpion toxin gene into the DNA of C*f*MNPV has becomes a popular potential biological control strategy.

3). Scorpion *Androctonus australis* Toxin (Spt-AalT)

One of the toxin peptides found in scorpion *Androctonus australis* toxin is insect specific. This insect-specific toxin from venom of the scorpion *Androctonus australis* (Spt-AalT) consists of a single polypeptide chain of 70 amino acids (Darbon et al., 1982). Spt-AalT selectively affects the insect nervous system by targeting the insect sodium channel which is similar to the insecticidal mechanism of many widely used chemical control agents. Since the 1980s, Spt-AalT has been used to make a recombinant virus with A*c*MNPV or B*m*MNPV in the United States and in some European countries. Pest control results showed an increased speed of kill (McCutchen et al., 1991), and also a high insect specificity with no effect on isopods and mammals, even at high doses (Darbon et al., 1982; De Dianous, 1987). Spt-AalT is toxic only in the body cavity of susceptible insects. The genetically engineered

*Cf*MNPV carrying a insect-specific scorpion toxin AalT is thus an excellent candidate to improve the efficacy of *Cf*MNPV as insecticide. It is not only fast-acting but also very host specific.

IV. Study Model for Specific *Cf*MNPV/Spt-AalT Viral Control Agent

Preliminary results show that recombinant *Cf*MNPV/scorpion toxin AalT is a good insecticide (Dr. A. Pang, personal communication). After ingestion of the recombinant *Cf*MNPV/Spt-AalT, the insect stops feeding after 1 week and dies between 7-10 days. This is important in protecting foliage. With reference to the registration, it is necessary to answer such environmental assessment questions as: the fate of the virus, the persistence of the virus in the environment, and the detection of virus in environmental samples e.g. soil, water, or wild life etc.. As a result, my research strategies focus on expression of scorpion toxin AalT as a fusion protein with glutathione S-transferase (GST) in *Escherichia coli,* and the generation of the specific monoclonal antibody against scorpion toxin AalT as a GST fusion protein. In the future, the specific monoclonal antibodies to scorpion toxin AalT will be used to study the expression and the concentration of scorpion toxin as a function of time in the spruce budworm infected with recombinant *Cf*MNPV carrying the scorpion toxin AalT. It will also be used to monitor the persistence and concentration of scorpion toxin AalT in affected environment samples e.g. soil and water. In the latter

case, ELISA immunoassays will be developed, because they could be handled by non-technical foresters.

V. The Glutathione S-transferase (GST) Gene Fusion System

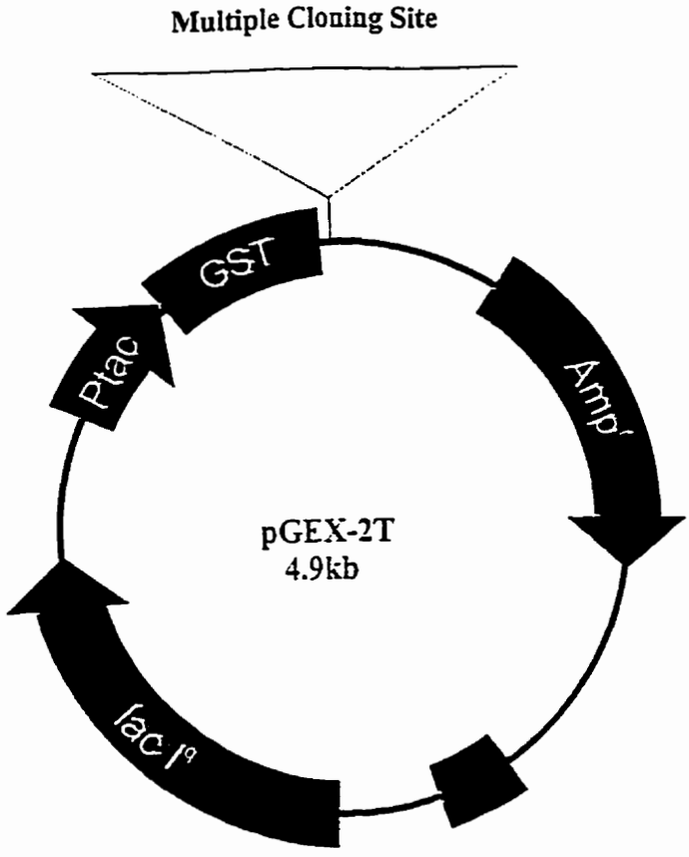
Several vectors have been constructed that simplify the purification of foreign peptides expressed in *E.coli*, for example, polypeptides expressed as a fusion with *E.coli* β-galactosidase (Gray et al., 1982) can be purified from crude cell lysates by substrate or immun affinity chromatography (Germino et al., 1983). Other vectors direct the synthesis of polypeptides as fusions with staphylococcal protein A that can be purified by affinity chromatography on IgG-Sepharose (Uhlen et al., 1983; Lowenadler et al., 1986). A disadvantage of these methods is that the denaturing reagents used during purification can be expected to alter the antigenicity and functional activity of the purified product, while an additional problem with the protein A system is that the binding of fusion proteins to IgG complicates immunological analysis. Alternative purification strategies involve the synthesis of polypeptides containing poly-arginine at their C terminus that can be purified by cation-exchange chromatography were not studed well (Sassenfeld and Brewer, 1984). In order to avoid several of the difficulties described above, we chose the novel glutathione S-transferase (GST) expression system.

The GST expression system has been used successfully in many applications

such as molecular immunology (Toye et al., 1990) and the production of vaccines (Fikrig et al., 1990; Johnson et al., 1989). GST expression system provides a series of plasmid vectors pGEX that express foreign polypeptides as fusions with GST, and simplify the purification of foreign polypeptides produced in *E.coli* under non-denaturing conditions by affinity chromatography on immobilized glutathione.

Each pGEX vector contains a strong *tac* promoter for inducible and high-level expression which is repressed with a *lac* repressor (product of the *lacI* gene) until induction with Isopropyl-1,thio-β-D-galactoside (IPTG), an internal $lacI^q$ gene for use in any *E. coli* host, an Amp^r gene (Ampicillin resistant), and an open reading frame encoding GST, followed by multiple cloning sites, and TAG stop codons in all three frames (Fig 4. a). Several pGEX vectors are available, in which each vector has different multiple cloning sites including different restriction sites. The pGEX-2T which contains a thrombin recognition site for cleaving the desired protein from the fusion product, *Bam*HI and *Eco*RI restriction sites (Fig. 4. b), has been chosen as expression vector for this project. The pGEX-2T vector has been widely applied to express and purify fusion proteins for use as immunogens and as biochemical reagents (Toye et al 1990).

(a)



(b)

Thrombin

	Pro	Lys	Ser	Asp	Leu	Val	Pro	Arg	Gly	Ser	Pro	Gly	Ile	Glu	Arg	Asp	***				
pGEX-2T	CCA	AAA	TCG	GAT	CTG	GTT	CCG	CGT	GGA	TCC	CCG	GGA	ATT	GAA	CGT	GAC	TGA	CTG	ACG	ATC	TG

BamHI SmaI EcoRI

Figure 4 Structure of the pGEX-2T vector. (a) Schematic presentation of pGEX-2T. The pGEX-2T expresses a cloned gene as a fusion protein to GST. The *lac* repressor (product of the *lacI* gene) binds to the *tac* promoter, repressing the expression of fusion protein. Upon induction with IPTG, derepression occurs and GST fusion protein is expressed. The scorpion toxin gene can be inserted into the multiple cloning site at the end of the GST gene. (b) Nucleotide sequences of the multiple cloning site, which encode the restriction endonuclease cloning sites for *Bam*HI, *Sma*I and *Eco*RI and protease cleavage site for thrombin (underlined by brackets), as well as TGA stop codons in all three frames (underlined).

MATERIALS and METHODS

I. Cell Culture and Medium

Mouse Sp2/0 myeloma cell line was obtained from Dr. S. P. Cole (Cancer Research Labs, Queen's University, Kingston, Ontario, Canada), and was maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Company, St. Louis. MO), supplemented with 10% controlled process serum replacement-type 3 (CPSR-3) (Sigma), 2 mM L-glutamine (Sigma), and 50 μg/ml gentamicin (Sigma) at 37^0C in a CO_2 incubator at 5% CO_2-in-air with 98% relative humidity. Hybridoma cells in the first two weeks after fusion were maintained in the HAT medium containing complete DMEM and 1 x HAT media supplement (5×10^{-3} M hypoxanthine, 2×10^{-5} M aminopterin, 8×10^{-4} M thymidine) (Hybri-Max, Sigma) in addition of 1 x OPI media supplement (0.15 g oxaloacetate, 0.05 g pyruvate, an 0.0082 g bovine insulin) (Hybri-Max, Sigma). After 2 weeks, the HAT medium was changed to 1 x HT ((5×10^{-3} M hypoxanthine, and 8×10^{-4} M thymidine). The hybridomas were grown in the HT medium until the completion of two cloning procedures.

II. Chemicals

The solutions and buffers used for production of GST fusion protein were as

follows: TE (10 mM Tris, 1 mM EDTA), TBE (1 x 89 mM Tris-borate, 89 mM borate, 0.2 mM EDTA), TAE (50 x: 2 M Tris, 50 mM EDTA, 5.7% glacial acetic acid, pH 7.2), PBS (0.14 M NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2), S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose, pH 7.0), LB medium (0.5% NaCl, 1% tryptone, 0.5% w/v Bacto-Yeast extract) (Difco, Detroit, MI), LB plates (1.2% bacto-agar in LB broth).

For enzyme-linked immunosorbent assay (ELISA) and western blot, buffers included: Bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), Washing buffer (0.05% Tween-20 and 0.03% bovine serum albumin in PBS), Blocking buffer (3% BSA in PBS).

III. Generation of Glutathione S-transferase (GST) / Scorpion toxin AalT Fusion Protein in *Escherichia coli*

III.1 Polymerase Chain Reaction (PCR)

1) Designation of primers: The sequences of the oligonucleotides used as primers in the PCR reaction were derived from the published gene sequences of scorpion toxin AalT (Spt-AalT) (Darbon et al., 1982), and designed using the OLIGO computer program. This program is used for selecting the optimal oligonucleotides for PCR amplification. The oligonucleotides were synthesized by Cortex Laboratory,

Department of Biochemistry, Queen's University. Synthetic oligonucleotides were purified by heating at 70^{0}C for 90 min, passing the solution over a Sephadex G-50 column, and eluting with 0.1 x TE buffer (10 mM Tris, 1 mM EDTA). Ten drop fractions were collected, and quantified by measuring absorbency at 260 nm in a DU-640 Beckman spectrophotomer. The concentration was calculated in pmol/µl as follows: [A260/(0.01 × N)] × dilution factor, where N is the # of bases of the oligonucleotides. For cloning purposes, the 5' (sense) primer contained a *Bam*HI restriction site (GC GGA TCC), and the 3' (antisense) primer contained an *Eco*RI site (CG GAA TTC /A/).

2) PCR reaction: Each PCR reaction mixture contained 15 mM of dNTP, 100 pmol of each primer, 1 µl of Pfu DNA polymerase (2.5 U/µl) (Stratagene, La Jolla, CA) and 10 µl of recombinant C*f*MNPV transfer plasmid with scorpion toxin AalT gene as template DNA (100 µg). The total volume is 100 µl. PCR was performed in an automated thermal cycler (Thermolyne) in Dr. S. P. Cole's laboratory (Cancer Research, Queen's University, Kingston, Ontario, Canada), as following: 96°C for 5 min for the initial denaturation step, then 35 cycles of denaturation at 96^{0}C for 40 s, annealing at 56°C (Optimal primer annealing temperature from OLIGO program) for 1 min, and extension at 72°C for 3 min.

3) Analysis of the PCR product: 9 µl of the reaction mixture were electrophoresed in a 1% agarose gel in TAE buffer containing 0.5 µg/ml ethidium

bromide at 80 voltage for 30-60 min. A 100 bp DNA molecular size ladder (100-1500 bp) (GIBCO BRL, Gaithersburg, MD) was run on the gel as markers. Following electrophoresis, the gel was visualized under UV light and photographed using a IBI Quickshooter Photosystem and Polaroid Type 667 positive/negative instant film.

4) Purification of the PCR product: The PCR product was purified by phenol extraction and ethanol precipitation. Briefly, the PCR product was mixed with phenol/chloroform at equal volume, and spun down at 10,000 x g for 10 min in microcentrifuge at room temperature. Upper aqueous layer was collected in a new tube carrying out phenol/chloroform extraction again, and then mixed with chloroform at equal volume, and centrifuged at the same speed. Following phenol extraction, ethanol precipitation was carried out. The upper aqueous layer was precipitated with 1/10 volume of 3 M Na Acetate and 3 volume of 95% ethanol at -70°C for 30 min. The precipitated DNA was collected by centrifugation at top speed in a microcentrifuge at 4°C for 15 min, and washed with 75% ethanol (pre-cooled) by centrifugation at 4°C for 15 min. After removal of the ethanol and air drying the pellet, the DNA pellet was resuspended in 30 μl of H_2O and stored at -20°C. The DNA concentration was estimated by absorbance measurements at 260, 280 and 320 nm using a Beckman DU 640 spectrophotometer equipped with a program to calculate nucleic acid concentration.

III.2 Cloning the PCR Product into pGEX Expression Vector

1) Restriction digestion and purificaton of PCR product: 2.5 μg of the purified PCR product was digested with *Bam*HI and *Eco*RI (GIBOCO BRL, Gaithersburg, MD) at 37°C for 3 hours, and then purified using GeneClean II kit (Dianova, Hamburg, Germany) as following: three volumes of sodium iodide (NaI) stock solution and 10 μl of silica matrix were added, the tube was placed at room temperature for 10-15 min to allow binding while mixing every 2-5 min. The silica was pelleted by centrifugation at 800 rpm for 10 min and washed with 200-600 μl of ice cold 'New Wash' solution three times, then resuspended with 10 μl of water at 55°C for 3 min. Finally, the DNA was eluted by centrifugation for 30 s at top speed in a microcentrifuge. The supernatant was carefully collected and stored at -20°C. The concentration of purified DNA was estimated as described in section III.1.4

2) Ligation: The purified DNA was ligated into the *Bam*HI and *Eco*RI sites of expression vector pGEX-2T (Pharmacia Biotech, Uppsala, Sweden) in the correct reading frame. The pGEX-2T vector was linearized by digestion with *Bam*HI and *Eco*RI and then dephosphorylated using calf intestinal phosphatase (Promega, Madison, WI). The 20 μl of ligation reaction was prepared containing purified DNA 40 ng, 100 ng of linearized vector DNA, 2 μl of T4 ligase (1U/μl, GIBCO BRL) and 4 μl of 5 x ligase buffer (0.25 M Tris-HCl pH 7.4, 50 mM MgCl, 0.2 M DTT, 5 mm ATP, 25 μg/ml BSA). A parallel reaction lacking the insertion DNA was included

as a control ligation. The ligation was incubated at room temperature for 2 hr. The resulting constructs encoded a fusion gene containing the glutathione S-transferase (GST) gene at their N terminus and the scorpion toxin AalT coding gene at their C terminus (GST/Spt-AalT). The fusion gene is under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter and contains stop codons in all three reading frames at the 3' end.

3) Transformation: The competent DH 5α *E.coli* cells (GIBCO BRL, Gaithersburg, MD) were transformed with the ligation reaction and control ligation by the heat shock method. Briefly, 50 µl of DH 5α *E.coli* cells were mixed with 2 µl of ligation reaction, kept in ice for 30 min, heated at in 37°C water bath for 20 seconds, and then put in ice for 2 min, finally mixed with 1 ml of S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose, pH 7.0) and incubated at 37°C for 1 hr. The transformed cells were plated onto LB plates containing 100 µg/ml ampicillin and incubated at 37°C overnight to allow colony development.

III.3 Analysis of Transformants with Expression of Recombinant Fusion Protein

After selection of transformants on LB/ampicillin plates, 10 sample colonies were picked using sterile wood sticks and grown in 2ml of LB/ampicillin liquid medium at 37^0 C to an A600 (600 nm) of over 0.5 O.D., and then induced to express

fusion protein by the addition of isopropyl-β-D-galactoside (IPTG) to a final concentration of 2 mM. Cells were grown in the presence of IPTG for 4 h at 37^{0}C, then 1 ml of cells were harvested by centrifugation at top speed for 20 seconds. Cell pellet was resuspended with 50 ul of 1 x SDS-PAGE buffer (50 mM Tris HCl pH 6.8, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromphenol blue), and aliquots of each sample were analysed by 12% sodium dodecyl sulfate-polyacrlamide gel electrophoresis (SDS-PAGE) as described below in section III.5.

III.4 Large-Scale Expression and Purification of Fusion Protein

The recombinant colonies expressing correct size of GST/Spt-AalT fusion protein, as determed by section III.3, were inoculated into 50 ml LB/ampicillin medium and grown overnight at 37^{0}C in a shaking incubator. This culture was diluted 1:20 into 1 litre of fresh LB/ampicillin medium, grown at 37^{0}C for 1 ½ hr to an A600 of over 0.5, and induced with IPTG (2mM). After 4 hours of growing in the presence of IPTG, the cell culture was collected and centrifuged at 5000 × g (~5500 rpm) for 10 min in a Beckman JA-10 rotor at 4^{0}C. The cell pellet was resuspended in 20 ml of ice cold PBS (in 2 tubes), and lysed with a 5-mm-diameter sonicator probe for a few seconds to minutes depending on the change of colour of whole cell lysates. 10% Triton X-100 was added into the whole cell lysates to a final concentration of 1%, and cellular debris were pelleted by centrifugation at 10,000 × g (~9500 rpm) for 5 min

in JA-20 rotor at 4^0C. The fusion protein was present in the supernatant and was purified with glutathione agarose beads as following: the supernatant (20 ml/each tube) was mixed at room temperature in a 50-ml polypropylene tube on a rotating platform with 1 ml of 50% slurry of glutathione-agarose beads (Sigma). After absorption up to 1 ½ hr, beads were collected by brief centrifugation at 500 × g for 30 seconds and washed three times with 10 ml ice-cold PBS. The beads were resuspended in 2ml ice-cold PBS and transferred to microcentrifuge tube (1 ml/each tube) and collected again by centrifugation at 500 × g for 10 seconds. Fusion protein was eluted by competition with free glutathione with 1 ml of 50 mM Tris HCl (pH 8.0) containing 5 mM reduced glutathione, and repeated for two more times. The eluted protein was aliquoted and stored at -70^0C. The concentration of fusion protein was determined by SDS-PAGE analysis with BSA as standards.

III.5 SDS-PAGE Analysis

1) Sample preparations: The transformed bacterial cell pellet was resuspended with 50 µl of 1 x SDS sample buffer, or the eluted protein (8 µl) was adjusted to 10 µl with 5 x SDS sample buffer, then boiled at 100°C for 10 min prior to separation. IPTG-noninduced transformed whole cell lysates and the cells only carrying a pGEX vector were used as controls. A broad range protein molecular weight marker (2-212 kDa) (Bio-Labs, Beverly, MA) was run on each gel.

2) Electrophoresis: Electrophoresis was carried out utilizing a 12% running gel with a 4% stacking gel, using a Bio-Rad mini-gel system. After loading 10 µl of each samples onto the gel, electrophoresis was done in running buffer (0.025 M Tris HCl, 0.192 M glycine, and 0.1% SDS)) at 80 V for 1 hr, and 120 V for 30 min. Gels were removed and stained with Commassie blue solution (50% methanol, 10% acetic acid, and 0.05% Commassie brilliant blue) for 30 min and destained in 20% methanol and 7% acetic acid for couple of hours to overnight to visualize the overexpressed GST and the GST/Spt-AalT fusion protein.

III.6 Isolation of Plasmid DNA Containing GST/Scorpion Toxin AalT Gene

Plasmid DNA was isolated using Wizard Miniprep Kit (Promega), following the manufacture's instruction. Briefly, 1-3 ml of transformed *E.coli* cells cultured overnight were centrifuged for 1-2 min at top speed in microcentrifuge. Two tubes of cell pellet were resuspended in 200 µl of cell resuspension solution, and then transferred to one microcentrifuge tube. Two hundred µl of cell lysis solution was added and mixed by inverting the tube several times. The cell suspension was clear almost immediately and mixed with 200 µl of neutralization solution by inverting several times, and centrifuged at top speed for 5 min to collect the cleared supernatant to a new tube. The supernatant was mixed with 1ml of the Wizard Minipreps DNA purification Resin by inverting. The mixture of DNA/Resin was pushed into mini-

column with syringe plunger and washed with 2 ml of column wash solution. After centrifugation at top speed for 20 seconds to dry the Resin, and 50 µl of water was applied to the Mini-column and kept for 1 min. The plasmid DNA was eluted by centrifugation at top speed for 20 seconds, and stored in - 20°C. The concentration of plasmid DNA was determined as described in section III.1.

III.7 DNA Sequencing

1) Sample preparation: 4.7 µg of double-stranded GST/Spt-AalT plasmid DNA templates per 50 µl were denatured by incubation with 5.5 µl of fresh 2 M NaOH containing 20 mM EDTA at 37°C for 30 min, and then precipitated by adding 1/10 volume of 3 M sodium acetate and 3 volume of 100% ethanol at -70°C for 15 min. The pellet was collected by centrifugation at top speed for 15 min in cold room, washed with 500 µl of cold 75% ethanol by centrifugation, and air dried at room temperature for at least 20 min. The dried DNA pellet was resuspended in 7 µl of water immediately before the sequencing reactions were performed.

2) Sequencing reaction: The reactions were performed by the dideoxy chain termination method (Sanger 1977) with DNA Sequenase ™ (version 2.0) T7 DNA polymerase kit (United States Biochemica, Cleveland, OH). The DNA fragments were labelled with 0.5 µl of [α-^{35}S] dATP (1000 Ci/mmol; 10 µCi) (Amersham. Arlington Heights, IL) and resolved on 6% polyacrylamide sequencing gel using a

Model S2 sequencing Gel Electrophoresis apparatus (GIBCO BRL Life Technologies, Inc.) for 3 h at 80V. After fixation in 10% ethanol and 10% acetic acid in water, the gel was removed from the glass plates onto Whatman 3 MM paper and dried using a Mode 583 Gel Drier (Bio Rad, Melville, NY). The dried gel was exposed to X-Omat AR X-ray film overnight at room temperature.

IV. Generation of Monoclonal Antibodies Against Scorpion Toxin AalT

IV.1 Animals

Female BALB/c mice (Jackson Labs, Bar Harbor, Maine) were used for immunization at an age of 6 to 8 weeks.

IV.2 Immunization of Mice

The GST/Spt-AalT fusion protein was suspended in PBS at a concentration of 500 μg/2 ml, injected into the vial of adjuvant (RIBI-700 MPL+TDM emulsion) (Cedarlane Laboratories, Hornby, Ontario, Canada) and then mixed by vigorous vertex for 2-3 min at room temperature. Two hundred μl of this mixture containing 50 μg of fusion protein was injected into a mouse intraperitoneally. Injections were repeated 2 times at tri-weekly intervals. To determine the antibody titer in the serum, the blood was collected by retroorbital bleeding 7 days after second immunization, and tested by ELISA. The immunized mice were rested for at least 4 weeks before the

final boost. For the final boost, mice were given with 50 μg of GST/Spt-AalT fusion protein in a 0.2 ml of PBS without adjuvant by intravenous injection into a tail vein. Before injection, fusion protein was sterilized by filtering through a 0.22 μm filter. The cell fusions took place 72-96 hours following the final boost.

IV.3 Preparation of Mouse Feeder Cells

To prepare peritoneal exudate feeder cells, unimmunized BALB/c mice were sacrificed by cervical dislocation, and immersed in a beaker containing 70% ethanol. The lower part of the rib cage and abdomen were exposed by snipping skin at diaphragm level and pulling back. 10 ml of serum-free Dulbecco's Modified Eagle's Medium (DMEM) were injected into the mouse peritoneal cavity, and withdrawn again after gently squeezing the abdomen. The peritoneal feeder cells were transferred to a 50-ml centrifuge tube and washed twice with DMEM. Cell counts were performed using haemocytometer.

IV.4 Production of Hybridomas

1) Preparation of the Sp2/0 myeloma cells: Sp2/0 cells were thawed at least 10 days in advance from liquid nitrogen, and cultured in DMEM containing 10% CPSR-3 (Hybri-Max, Sigma). Cell were challenged with 0.132 mM of 8-azaguanine (Hybri-Max, Sigma) for one passage at least 1 week before fusion. The Sp2/0 cells

were then diluted ½ in fresh medium every 24 hours for 3 days before fusion. On the day of fusion, the Sp2/0 cells were collected, and washed twice in DMEM without serum. Cell viability was determined by trypan blue exclusion using a haemocytometer, and only cells of >90% viability were used. The final Sp2/0 cells were resuspended in 10 ml of DMEM.

2) Isolation of the mouse spleen cells: Each immunized mouse was killed by cervical dislocation 3 days after the final boost, and immersed in a beaker containing 70% ethanol and laid out on a dissecting board. The spleen was removed aseptically from the mouse, and placed in a 100-mm petri dish containing 5 ml of serum-free DMEM. After carefully dissecting away surface fat and other adhering tissue, the spleen was transferred to sterilized stainless-steel strainer in 100 mm petri dish containing 10 ml DMEM, pressed against the screen of the strainer with a syringe plunger of the 3-cc syringe until only fibrous tissue remained in top of the screen, and the screen of strainer was rinsed with 3 ml of DMEM. The suspension of spleen cells was collected in a 50-ml tube, and left for 3 min at room temperature. The top 95% of the cell suspension was transferred to a new 50-ml centrifuge tube, washed in serum-free DMEM, and then resuspended in 10ml DMEM. A viable cell count was performed as described in section V.4.1. Approximately 1.8×10^8 cells were recovered from one mouse spleen.

3) Fusion pretreatment with polyethylene glycol (PEG): Cells used for the fusion were pre-treated with PEG as previously described (Orlik and Altaner, 1988). Spleen cells (18×10^7) were split into 2 equal aliquots and each mixed with Sp2/0 cells (3.4×10^7) at a ratio of 2.3 :1 in 20 ml of serum-free DMEM. The mixture was then centrifuged at 100 × g for 5 min, and pellet was then resuspended in 20 ml of DMEM containing 10% CPR-3, 50 μg/ml gentamicin, 2mM L-glutamine and 100 μl of 50% (w/v) PEG 1500 (Boehringer-Mannheim, GmbH). The cell suspension was incubated at 37°C, 5% CO_2 for 90 min, and then centrifuged at 100 × g for 5 min. The cell pellet was warmed by placing the tube in a 37°C water bath in a beaker for 2 min and resuspended.

4) Cell fusion: The spleen and Sp2/0 cells were fused by the addition of 1ml of 50% PEG(pre-warmed) at 37°C for 1 min. The cell suspension was stirred with the tip of a pipette for another minute, then diluted drop wise with 2 ml of serum-free DMEM for 2 min and with 10 ml of DMEM over 3 min. The cells were collected and washed by centrifugation at 100 × g for 5 min at room temperature, and then resuspened in 150 ml of prewarmed DMEM containing 10% CPR-3, 50 μg gentamicin per ml, 2mM L-glutamine and OPI (Hybri-Max, Sigma). The cell suspension was incubated at 37°C, 5%CO_2 for 30 min, and then seeded into 96-well plates containing feeder cells at concentration of 1.6×10^5/100 μl/well. 24 hours later, 100 μl of medium were removed and replaced with 2 x HAT medium. The cells were

fed with 1 x HAT medium every 3-4 days for 2 weeks, and then with 1 x HT.

5) Screening and subcloning of hybridomas: The supernatant of cell culture in 96-well plates was firstly screened by ELISA for antibody reaction with GST/Spt-AalT fusion protein. The positive colonies were transferred to 24-well plates (Flow Laboratories. Inc., Molean, Virginia). The supernatant of cell culture from 24-well plates was secondly screened with both of GST/Spt-AalT fusion protein and GST protein in ELISA. The colonies with specific antibody reaction with only GST/Spt-AalT fusion protein but not with GST protein were subcloned by limiting dilution (Zola, 1987) for 3 more rounds.

6) Production of ascites: Positive hybridomas derived from single population were grown in tissue culture flask (25-cm^2) in complete DMEM under log-phase growth, and collected by centrifugation at 100 × g for 5 min. 5 × 10^6 cells resuspended in PBS or serum-free DMEM were intraperitoneally injected into each mouse which was pretreated with 0.5 ml Pristane (Sigma, Chemical Company, St. Louis, MO) 7-10 days before. Ascites fluid was collected by needle puncture into the abdominal cavity using 18-G needle over the next 1-2 weeks (Yokoyama, 1992). Ascites fluid was used to characterize the antibody by ELISA, western blotting, and isotyping kit.

IV.5 Determination of Isotype of Antibody

This assay was performed with IsoStrip™ mouse monoclonal antibody isotyping kit (Biehringer Mannheim, Laval, Quebec, Canada), following manufacturer's instructions. Briefly, ascites or culture supernatant was diluted in PBS (pH 7.2) at 1:20,000 or 1:100 respectively, and then mixed with lyophilized latex beads bearing anti-mouse light chains kappa and lambda in each tube. Isotyping strip bearing goat anti-mouse antibody isotypes was inserted into each reaction tube, and incubated at room temperature for 5 min. Results were visualized by a blue band on strip.

IV.6 Enzyme-Linked Immunosorbent Assays (ELISA)

1) Optimizing concentration of coating protein: This assay was performed by the criss-cross serial dilution titration analyses as described by Cooper and Paterson (1992). Briefly each row of the ELISA plate was serially diluted with GST/Spt-AalT fusion protein at 10, 5, 2.5, 1.25, and so on, to a final concentration of 0.15625 µg/ml in coating buffer. Each column of the ELISA plate was serially diluted by ½ with mouse antiserum to GST/Spt-AalT fusion protein. Enzyme conjugated goat anti-mouse antibody was added at an optimal concentration (1:5000) to all wells. Experimental detail was followed as standard protocol described in section V.5.2.

2) ELISA for screening specific hybridomas: GST/Spt-AalT fusion protein or

GST protein was dissolved in ELISA coating buffer at an optimized concentration of 2.5 μg/ml. Each well of a 96-well plate was coated with 50 μl of this protein, and incubated overnight at 4°C or 37 °C for 2 hr. After removing the coating protein solution, the plates were washed three times in washing buffer and blocked by incubating with 100-200 μl of blocking buffer (3% BSA in PBS) per well for 1 hr at room temperature. Peroxidase-conjugated goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch Labs. Inc., West Grove, PA) antibody was used as secondary antibody at concentration of 1:5,000 or 1:10,000, and 50 μl of them were added to each well. After incubation at 37°C for 1 hr, plates were washed 3 times in washing buffer and incubated with ABTS [(2,2'-azino-di-[3-ethyl-benzthiazoline sulfonates)] substrate (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD) at room temperature for 20 min to develop a blue-green colour. Its colour absorbency was measured on the microplate reader (Flow Titertek Multiskan Plus Mark II) (ICN Biomedicals, Inc., Costa Mesa, CA) at a wavelength 410 nm. For each assay, mouse antiserum from immunized mice with GST/scorpion toxin AalT fusion protein was used as positive control, and mouse normal serum as negative control.

3) ELISA for determination of specific antibody titre: 10-fold serially diluted ascites were incubated in microtiter wells previously coated with the GST/Scorpion toxin AalT fusion protein (2.5 μg/ml). The bound antibody was detected by employing peroxidase-conjugated goat anti-mouse antibody as described as above

(V.5.2). BSA and GST were used as control antigens. An irrelevant antibody was used as control antibody. The amount of specific antibody in the ascites was determined from a standard curve at 410 nm OD.

4) Reactivity of specific antibody with purified scorpion toxin AalT as GST fusion protein in ELISA: A series of two fold diluted concentrations of purified scorpion toxin AalT as fusion protein (at 1.25 μg/ml, 0.625 μg/ml, 0.313 μg/ml, 0.156 μg/ml, 0.078 μg/ml, and 0.039 μg/ml) were incubated with monoclonal antibody G10 at three optimal dilutions (10^{-5}, 10^{-6}, and 10^{-7}) in microtitre plates. Goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs. Inc., West Grove, PA) was used to detect the bound antibody. After addition of ABTS substrate, the colour reaction was measured at 410 nm. BSA was used as a antigen of negative control.

IV.7 Western Blotting

GST/scorpion toxin AalT fusion proteins and GST protein were electrophoresed on 12% SDS-PAGE as described as above in section III.5., and electroblotted in 50 mM 3-(cyclohexylamino)-1-propanesulfonate (pH 11.0: Sigma Chemical Co., St. Louis, MO) onto Immobilon-P membrane (Millipore, Mississauga, Ontario, Canada) using a Bio-Rad mini transfer apparatus for 2 hrs. Membranes were washed with PBS washing buffer (pH 7.2, 1% Tween 20, and 0.03% BSA) for 2-3

times, and then blocked with blocking buffer (3% BSA and 5% calf serum in PBS) for 3 hrs at room temperature. After rinsing in washing buffer for 3 times, membranes were incubated with specific monoclonal antibody in ascites (1:1,000 or 1:5,000 dilution in blocking buffer) for 2 hr at room temperature. Mouse polyclonal antiserum to GST/Scorpion toxin AalT was used as control. After washing, membranes were incubated with goat anti-mouse IgG conjugated with peroxidase (1:10,000 dilution) in blocking buffer at room temperature for 1 hr, washed extensively, and then incubated with chemiluminescence blot (ECL) substrate (Amersham, Oakville, Ontario, Canada) for 1 min. The membranes were drained, wrapped in Saran Wrap, and overlaid with Kodak X-Omat film and exposed for 30 seconds to 1hr.

V. Analysis of Expression of Scorpion Toxin AalT in spruce budworm Infected with Recombinant C*f*MNPV/Spt-AalT

Insect samples were prepared in Sault Ste Marie. Briefly, spruce budworm were infected with recombinant viruses (C*f*MNPV/Spt-AalT) by feeding in which a drop of virus (10,000 viral particles) in sucrose was hanged inside a microtube. Insects (5th-instar) would be placed in a diet cup after consuming the droplet. Insects who did not consume the droplet would be discarded. After viral infection, insects were collected at different time to determine the expression of scorpion toxin Aalt.

To detect the expression of scorpion toxin in insect samples, the insects were homogenized by homogenizer, and then centrifuged at top speed in microcentrifuge for 15 min. The supernatant was collected and stored at 4°C.

The 96-well ELISA plates were coated with 100 µl of supernatant per well at 4°C overnight, and blocked by blocking buffer (3% BSA and 0.02% Tween-20 in PBS) at room temperature for 2 hrs, and then reacted with specific monoclonal antibody Spt-E1 (1:1000) at 37°C for 2 hrs. After washing the plates, goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs. Inc., West Grove, PA) was added to detect the bound antibody. After addition of ABTS substrate, the colour reaction was assessed by eye assessment.

RESULTS

In order to study the expression of scorpion toxin AalT (Spt-AalT) in insects infected with recombinant baculovirus C*f*MNPV carrying Spt-AalT, the plasmid vector pGEX-2T was used for the expression of scorpion toxin AalT as fusion protein with glutathione S-transferase (GST). The GST/Spt-AalT fusion protein was expressed at high level in *Escherichia coli,* and easily purified from whole bacterial lysates under non-denatured condition as soluble protein. Furthermore, monoclonal antibodies were developed against scorpion toxin AalT as a fusion protein, using a modified cell fusion protocol. Preliminary application of specific monoclonal antibodies against scorpion toxin AalT as fusion protein was also made to detect the expression of scorpion toxin AalT in spruce budworm infected with recombinant C*f*MNPV/Spt-AalT. These results are described below in different sections.

I. PCR Amplification and Cloning of Scorpion Toxin AalT Gene

The scorpion toxin AalT gene was amplified from the C*f*MNPV transfer vector carrying the scorpion toxin AalT gene by the PCR method using primers depicted in Figure 5. (a). The nucleotide sequences of the primers were derived from the published gene sequence of scorpion toxin AalT (Darbon et al., 1982). The gene encoding scorpion toxin AalT is located behind a secretion signal sequence which was

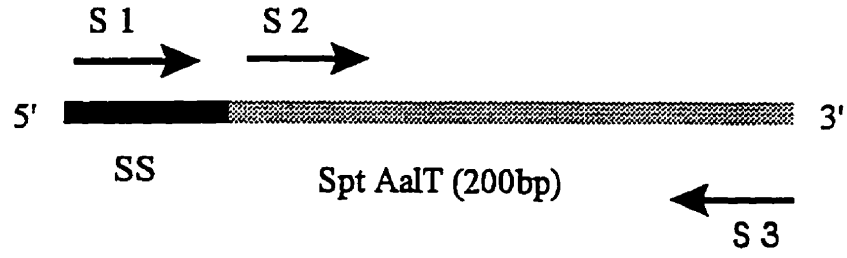
introduced into this viral transfer vector to induce the secretion of products of virus in the infected insects. The first PCR assay used the nucleotide sequences of the 5' primer (S1 primer) which was derived within this signal sequences. The fusion protein failed to be isolated from whole cell lysates of transformed *E.coli* because the signal sequence caused the secretion of fusion protein into the culture medium. Figure 5 (b) shows the PCR amplification products of scorpion toxin AalT (lane 1,2 and 3) without containing the secretional signal sequences. The product gives a expected size of 210 bp. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into *Bam*HI- and *Eco*RI-digested pGEX-2T (Fig. 6). The resulting constructs encoded a fusion protein containing the glutathione S-transferase (GST) gene at their N terminus and the scorpion toxin AalT (Spt-AalT) coding region at their C terminus. The recombinant gene under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter contained stop codons in all three reading frames at the 3' end. The transformation was carried out with DH 5α *E.coli* using heat shock method and generated 3-fold more colonies than control reaction.

II. Expression of Fusion Protein

In order to analysis the recombinant transformants, DH5α *E. coli* cells transformed with the recombinant plasmid or with pGEX-2T were grown to an A_{600} of 0.5 and subsequently induced with IPTG at 37°C to express either the recombinant

Figure 5 PCR amplification of scorpion toxin AalT gene. (a) Scheme of the primers used to amplify scorpion toxin AalT genes. Primer S1 and S3 were used in the first PCR assay to amplify the whole sequence (signal sequence + scorpion toxin AalT gene). Amplification of scorpion toxin AalT gene was carried out with primers S2 and S3. The sequence of the PCR primers are shown at the bottom. For cloning purposes, the 5' primer contains a *Bam*HI restriction site, and 3' primer contains an *Eco*RI site. (b) 1% agarose gel electrophoresis of PCR products obtained using the primers S2 and S3. In lanes 1, 2 and 3, PCR products of scorpion toxin AalT appearing at 200 bp indicated by an arrow, respectively. Lane M contains a 100 bp DNA ladder (GIBCO BRL). SS means signal sequences.

a



S2 primer: 5' GCGGATCC AAAAACGGCTACGCTGTTG3'
BamHI

S3 primer: 5' CGGAATTCAGTTGATGATAGTAGTGTCGCA 3'
EcoRI

b

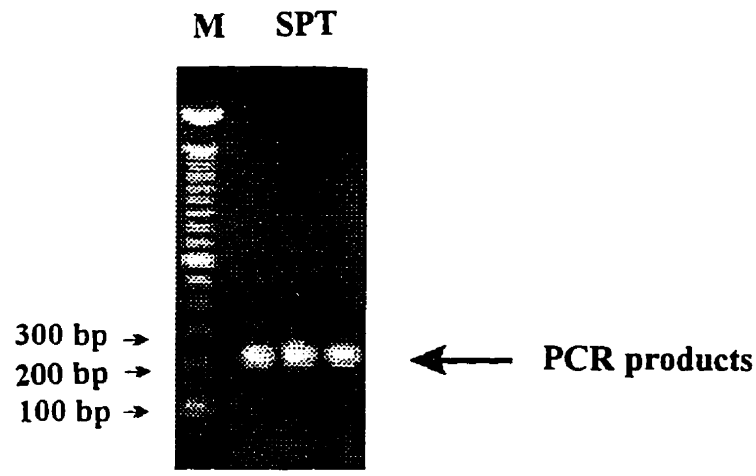
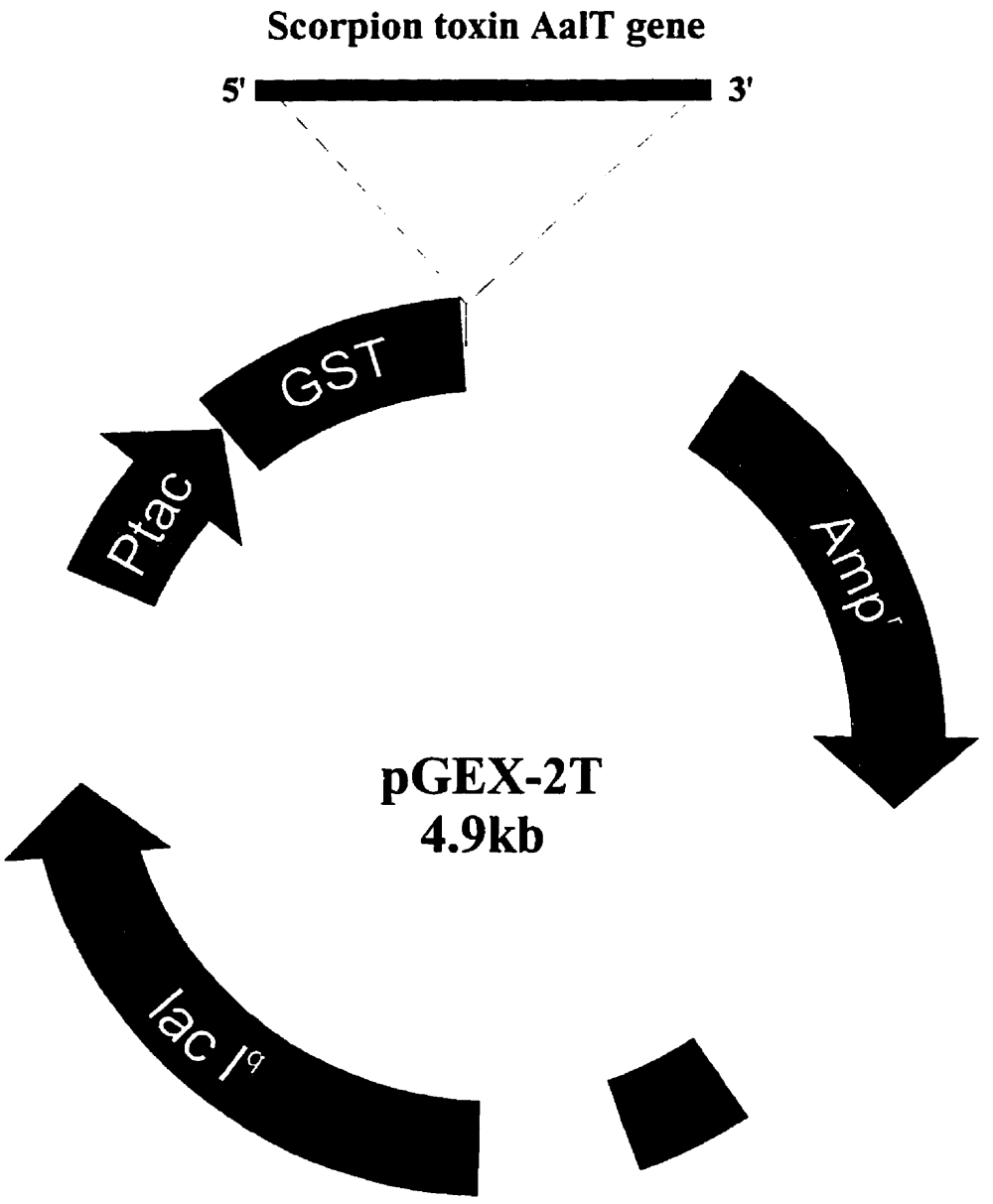


Figure 6 Subcloning of PCR products into the GST fusion vector pGEX-2T. pGEX-2T vector offers a *tac* promoter for IPTG inducible, high-level expression, and an open reading frame encoding GST, followed by a cloning site for *Bam*HI and *Eco*RI restriction endonuclease enzymes. The PCR product of scorpion toxin AalT was cloned into the cloning site at the N-terminal of GST and expressed in-frame with GST as fusion protein in *E.coli*.


Scorpion toxin AalT gene
5'
3'
GST
Ptac
Amp^r
pGEX-2T
4.9kb
lac I^q

fusion proteins or the GST protein. The whole-cell lysates of IPTG-induced and non-induced *E. coli* DH5α cells were analysed by SDS-PAGE gel, and the results of protein expression are shown in Fig. 7. 5 out of 8 colonies analysed have induced expression of GST/Spt-AalT fusion protein with correct molecular weight of 34 kDa while 3 colonies have GST expression with molecular weight of 27 kDa. In contrast, the noninduced 2 samples have non visible expression of protein band.

As noted in Fig. 7, the expression level of GST/Spt-AalT fusion protein is relatively less than GST. In order to enhance the amount of expression of GST/Spt-AalT fusion protein, different culture conditions were attempted to see if temperature had an effect on the expression of fusion protein. One *E. coli* cell colony bearing GST/Spt-AalT fusion plasmid and one bearing only GST plasmid were grown in the different temperatures (30°C or 37°C). Figure 8 shows the IPTG-induced or non-induced GST/Spt-AalT or GST protein expression under 30°C and 37 °C on SDS-PAGE gel. As indicated in Fig. 8, there are no difference in the level of protein expression at 30°C or 37°C.

III. Large-Scale Expression and Purification of GST/Spt-AalT Fusion Protein

The GST/Spt-AalT fusion protein was purified by affinity chromatography of disrupted cells on immobilized glutathione. The first time of purification in Fig. 9 showed eluted fusion protein in lanes 3-6 appearing at correct molecular weight of

Figure 7 Analysis of GST/scorpion toxin AalT fusion protein. Coomassie blue-stained SDS-PAGE gel showing whole cell lysates of IPTG induced and non-induced *E. coli* DH 5α harboring recombinant plasmid (Lanes 1-10) or only pGEX-2T (GST control). IPTG non-induced samples (Lanes 7-8) and IPTG non-induced GST control show no expression of foreign proteins. Lanes 1-4 and 9 are IPTG induced samples, respectively, showing the GST/Spt-AalT fusion protein at the expected molecular weight of 34 kDa (arrow). Lanes 5, 6,10 and GST control lane IPTG (+) show the GST expression at the exact molecular weight of 27 kDa (arrow). Lane M contains protein molecular weight markers (Bio-Labs, broad range 2-212 kDa at 20, 27, 36 and 47 kDa).

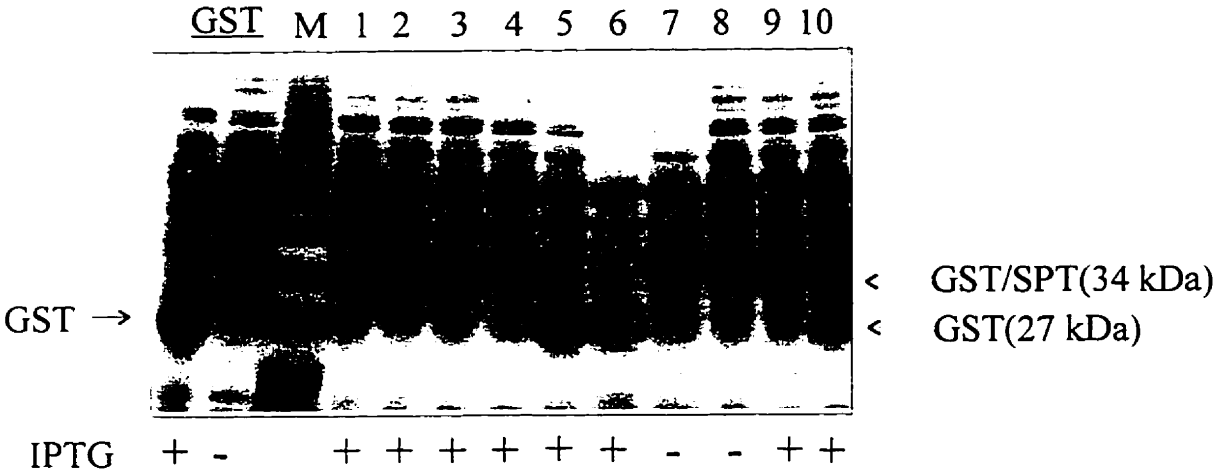
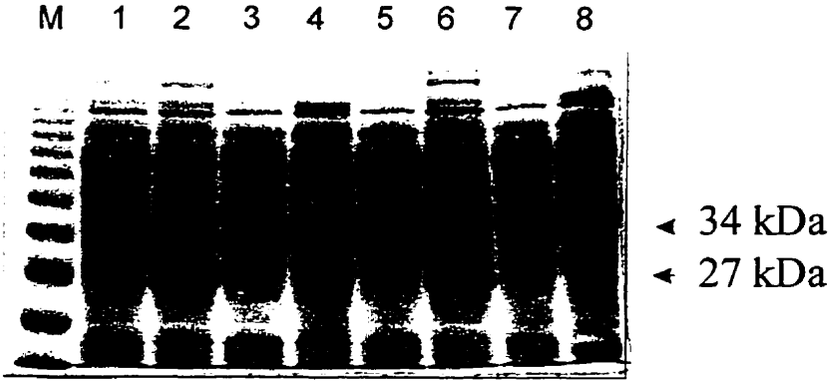

GST M 1 2 3 4 5 6 7 8 9 10
GST →
< GST/SPT(34 kDa)
< GST(27 kDa)
IPTG + - + + + + + + - - + +

Figure 8 Exp[illegible] under different temperatures. [illegible] of expression of [illegible] under different temperatures, [illegible] cell lysates of IPTG-induced [illegible] 4, [illegible] 0°C (lanes 8) show [illegible] molecular weight of 34 [illegible] fusion proteins (Lane [illegible] cells containing only plasmid; [illegible] 2) and 30°C (Lan[illegible] uninduced cells show no exp[illegible] protein molecular weight marke[illegible]

Figure 8 Expression of GST/scorpion toxin AalT fusion protein under different temperatures. Coomassie blue-stained SDS-PAGE gel shows the kinetics of expression of the GST/Spt-AalT fusion protein and GST protein under different temperatures, at 37°C (Lanes 1-4) and at 30°C (Lanes 5-8). Whole cell lysates of IPTG-induced *E.coli* cells bearing fusion plasmid grown at 37°C (lane 4) or at 30°C (lanes 8) show the expression of GST/ Spt-AalT fusion protein at expected molecular weight of 34 kDa, while the IPTG-noninduced cells show no expression of fusion proteins (Lane 3 and 7). Whole cell lysates of IPTG induced *E.coli* cells harboring only plasmid pGEX-2T vector show the expression of GST protein at 37°C (Lane 2) and 30°C (Lane 6) at right molecular weight of 27 kDa, and IPTG-noninduced cells show no expression of GST (Lane 1 and 5). Lane M contains protein molecular weight markers (Bio-Labs, broad range 2-212 kDa).


M 1 2 3 4 5 6 7 8
34 kDa
27 kDa

34 kDa, but the yield of elution protein was very low at 0.5 mg from 1 litre of cell culture. The concentration of purified fusion protein was estimated by comparing with the standard BSA protein. Using modified protocols, such as prolonging the sonication time, extending the period of absorption of cell lysates with glutathione agarose beads, and increasing the quantity of glutathione-agarose beads, about 8.75 mg of fusion protein was purified from 1 litre of bacterial culture as determined with the standard BSA protein (Fig. 10). After purification, a lower molecular weight of protein was also coeluted with GST/Spt-AalT. This could be result of protein degradation during purification.

IV. Sequence Analysis of GST/Scorpion Toxin AalT Recombinant Construct

In order to confirm that the inserting scorpion toxin AalT is in correct reading frame with GST, the GST/scorpion toxin AalT fusion plasmid was isolated and sequenced by the dideoxy chain termination method. The sequence result indicated that nucleotide sequences of scorpion toxin AalT were in accordance with the published sequences (Darbon et al., 1982) and also in the correct reading frame.

V. Determination of Optimal Concentration of Antigen for ELISA

To determine the optimal concentration of GST/Spt-AalT fusion protein as a coating antigen for screening hybridomas in an ELISA assay, microtitre plates were

Figure 9 Large-scale of expression and purification of GST/scorpion toxin AalT fusion protein with low yield of protein. Coomassie blue-stained SDS-PAGE gel showing: whole cell lysates of *E.coli* cells harboring fusion plasmid with the expression of GST/Spt-AalT fusion protein at the expected molecular weight of 34 kDa (Lane 2); fusion protein after purification with glutathion agarose beads (elution 1- 3) with correct molecular weight of 34 kDa (Lanes 3-5); whole cell lysates of *E.coli* cells bearing only plasmid pGEX-2T vector with the expression of GST at 27 kDa (Lane 1). Standard BSA shows different protein concentration in each band (20, 10, 5 to 0.625 μg). Lane M contains protein molecular weight marker (Bio-Labs, broad range 2-212 kDa at 20, 27, 36 and 47 kDa)

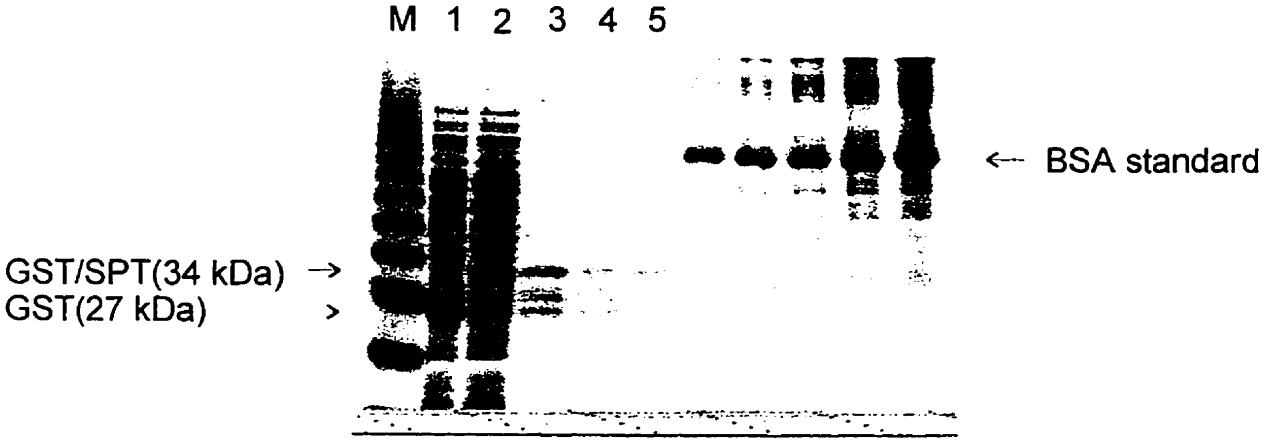
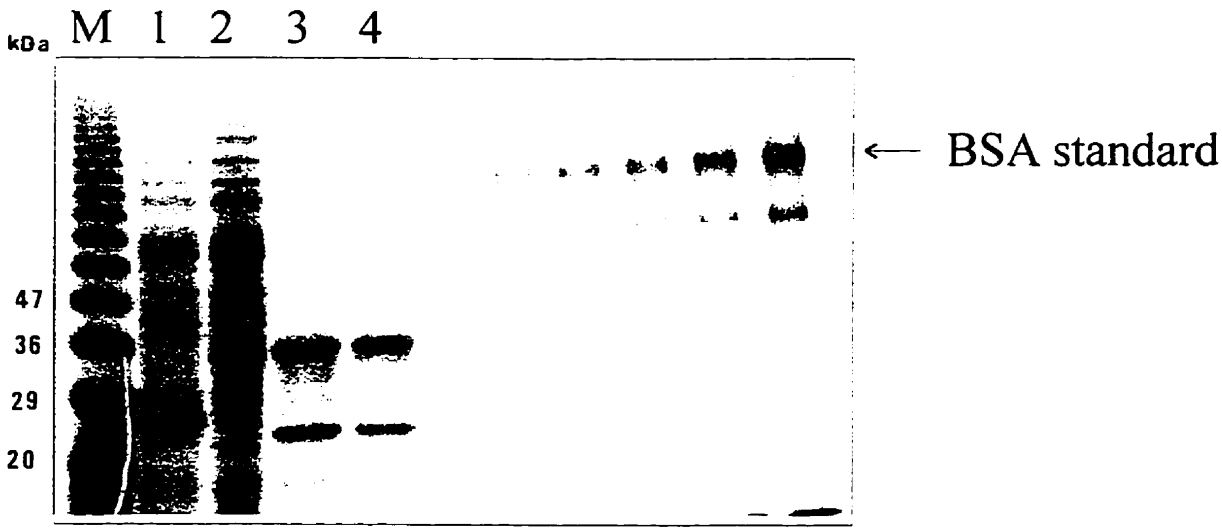

M 1 2 3 4 5
GST/SPT(34 kDa) →
GST(27 kDa) >
← BSA standard

Figure 10 Purification of GST/scorpion toxin AalT fusion protein under modification conditions. Coomassie blue-stained SDS-PAGE gel showing the second purification of the GST/Spt- AalT fusion protein after changing some conditions of purification. Whole cell lysates, lane 2 with fusion protein at correct molecular weight of 34 kDa, and lane 1 with GST at molecular weight of 27 kDa. Lanes 3 and 4, purified protein elution 1 and elution 2, with correct molecular weight of 34 kDa and with high yield of fusion protein compared with standard BSA. Standard BSA shows different protein concentration in each band (20, 10, 5 to 0.625 μg). Lane M contains the protein molecular weight markers (Bio-Labs, broad range 2-212 kDa).

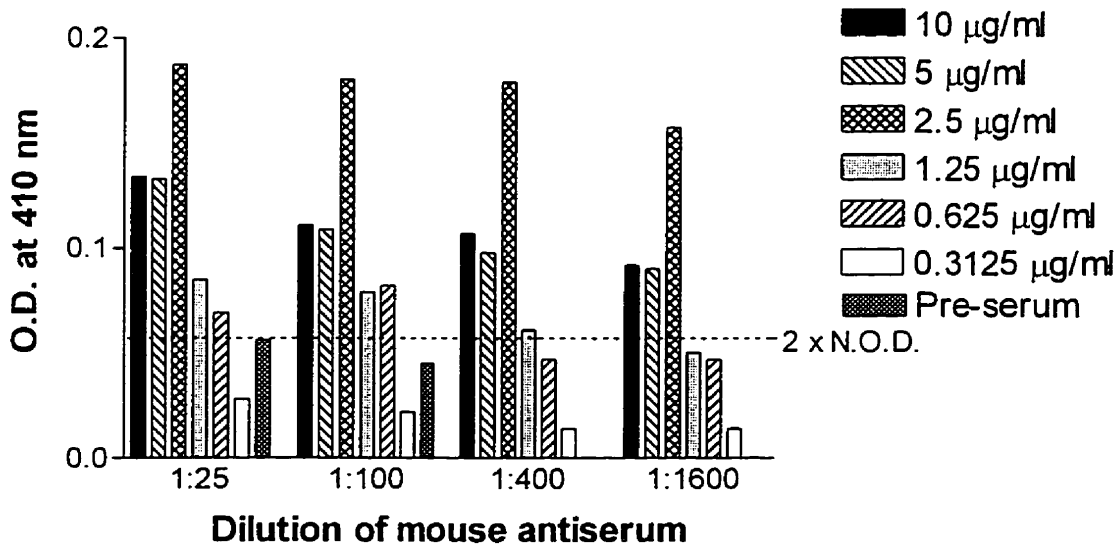

kDa
M
1
2
3
4
47
36
29
20
← BSA standard

coated with two-fold serial dilution of purified GST/Spt-AalT fusion protein, and were incubated with mouse polyclonal antiserum used as the primary antibody at different dilutions. The bound primary antibody was detected with goat anti-mouse IgG conjugated with horseradish peroxidase and substrate for colour development (Fig. 11). The concentration of 2.5 μg of GST/Spt-AalT fusion protein per ml was found to be the optimum to detect the primary antibody. The 1:1600 dilution of mouse polyclonal antiserum was used as positive control in hybridoma screening ELISA.

VI. Production and Screening of Hybridomas

Initial experiments using a standard fusion procedure (Oi and Herzenberg, 1980) failed to produce hybridomas secreting antibodies against the scorpion toxin AalT as GST fusion protein. A modified fusion protocol was developed that involved preincubating the mouse spleen cells with Sp2/0 myeloma cells in presence of 0.25% PEG at 37°C, 5% CO_2 for 90 min (Orlik and Altaner, 1988), and the fused cells were post-incubated in complete DMEM at 37°C, 5% CO_2 for 30 min (Fuller et al., 1988). One to two weeks after fusion, the hybridoma culture supernatant was screened for the presence of specific antibodies by a two-step ELISA. The first step was to screen the hybridomas secreting antibodies against GST/Spt-AalT fusion protein. The second step (Double Screening) was used to screen the antibodies only against GST/Spt-

Figure 11 Optimization of concentration of GST/scorpion toxin AalT fusion protein as coating antigen in ELISA. Titration bars represent the reactivities of mouse polyclonal antibody at 25-fold dilution for different concentrations of GST/scorpion toxin AalT fusion protein as antigen at 10 μg/ml, 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.625 μg/ml and 0.3125 μg/ml. A sample was considered positive if the calculated O.D. value was greater than the 2 x N.O.D. which represents the double average O.D. value of negative control (pre-serum).


0.2
0.1
0.0
O.D. at 410 nm
1:25
1:100
1:400
1:1600
Dilution of mouse antiserum
10 µg/ml
5 µg/ml
2.5 µg/ml
1.25 µg/ml
0.625 µg/ml
0.3125 µg/ml
Pre-serum
2 x N.O.D.

AalT fusion protein but not against GST protien. Table 1 shows that from the one fusion using one mouse spleen, 221 out of 629 hybridoma clones tested were found to be producing antibodies for GST/Spt-AalT fusion protein, but only 49 clones were secreting specific antibodies for the scopion toxin AalT portion of the fusion protein. The percentage of specific antibody producing hybridoma clones were 7 % which was higher than that reported by Orlik and Altaner (1988). Some hybridomas producing specific antibodies were frozen in liquid N_2 and some were subcloned by limiting dilution and rescreened by the double screening ELISA test. After three times subcloning, four stable monoclonal cell lines showing the strongest reactivities with scorpion toxin AalT as fusion protein were designated Spt-E1, Spt-G10, Spt-H9, and Spt-C1, and were chosen for production of ascites from mice and for further characterization.

VII. Characterization of Monoclonal Antibody (mAb)

VII.1 Determination of monoclonal antibody isotype

IsoStrip kit (Boehringer Mannheim Co.) provides a wide range of class- and subclass-specific antibodies including κ and λ light chains and positive control. All immunoglobulin subclasses of four monoclonal antibodies were determinated to be IgG1 and κ light chain using this kit (Table 2).

Table 1 Results of generation and screening of specific antibody secreting hybridomas with modified cell fusion procedure. From one fusion on one mouse spleen, 221 out of over 629 hybridoma clones show producing antibodies for GST/scorpion toxin AalT fusion protein; 49 clones, after Double Screening, show producing specific antibodies only for the scorpion toxin AalT fusion protein but no for GST protein. The percentages of growing hybridomas and specific antibody producing hybridomas are 65.5% and 7% respectively.

Number of Fusion				Percentage of Fusion	
Wells	Wells/Clones	GST/SPT Ab-Screening	Double Ab-Screenig GST (-) GST/SPT (+)	Hybridomas	Specific Ab-Producing Hybridomas
960	629	221	49	65.5	7

Table 2 Determination of monoclonal antibody isotype. IsoStrip kit (Boehringer Mannheim Co.), providing a wide range of class- and subclass-specific antibodies including κ and λ light chains and positive control, shows the isotype IgG1 and κ light chain for all 5 monoclonal antibodies.

MAbs / Isotype	Spt-C1	Spt-D2	Spt-E1	Spt-G10	Spt-H9
Subclass	IgG1	IgG1	IgG1	IgG1	IgG1
Light-Chain	κ	κ	κ	κ	κ

VII.2 Determination of titer of monoclonal antibodies

Ten-fold serial dilution of two monoclonal antibodies from pooled ascites fluid were reacted with the optimal concentration (2.5 μg/ml) of scorpion toxin AalT fusion protein as shown above. GST was used as a antigen of negative control in the ELISA test. In Fig. 12, mAbs Spt-E1 and Spt-G10 have strong reactivities with scorpion toxin AalT as GST fusion protein, with half-maximal binding for Spt-E1 at 10^{-6} dilution and Spt-G10 at 10^{-7} dilution and with minimal detectable response above two times negative control for Spt-E1 at 10^{-7} dilution and for Spt-G10 at 10^{-8} dilution. Monoclonal antibodies did not react with GST, and an irrelevant ascites had no reaction with GST/Spt-AalT fusion protein. Two times negative control (2 x N. O.D.) represents the double average O.D. values of negative controls. The sample is considered positive if the calculated O.D. value was two times greater than the negative control O.D.

VII. 3 Determination of reactivity of mAb with scorpion toxin AalT

To detect the reactivity of specific monoclonal antibody with scorpion toxin AalT as GST fusion protein, different concentrations of purified scorpion toxin AalT fusion protein were incubated with three optimal dilutions of monoclonal antibody SPT-G10(10^{-5}, 10^{-6}, 10^{-7} dilutions), as shown in Fig. 12. The data indicated the lowest detectable concentration of scorpion toxin AalT as GST fusion protein at 0.039 μg/ml (Fig. 13).

Figure 12 Determination of titre of monoclonal antibody (mAb) in ELISA. Two monoclonal antibodies Spt-E1 and Spt-G10 from ascites fluid at ten-fold serial dilutions have different reactivities with a constant GST/scorpion toxin Aalt fusion protein (2.5 μg/ml): either half-maximal binding at 10^{-6} dilution for mAb Spt-E1 and 10^{-7} dilution for Spt-G10, or minimal detectable response above 2 x N.O.D. at 10^{-7} dilution for Spt-E1 and 10^{-8} dilution for Spt-G10. Both monoclonal antibodies show no reaction with GST protein; irrelevant ascites shows no reaction with GST/scorpion toxin AalT fusion protein. A sample was considered positive if the calculated O.D. value was greater than the 2 x N.O.D. which represents the double average O.D. value of two negative controls.

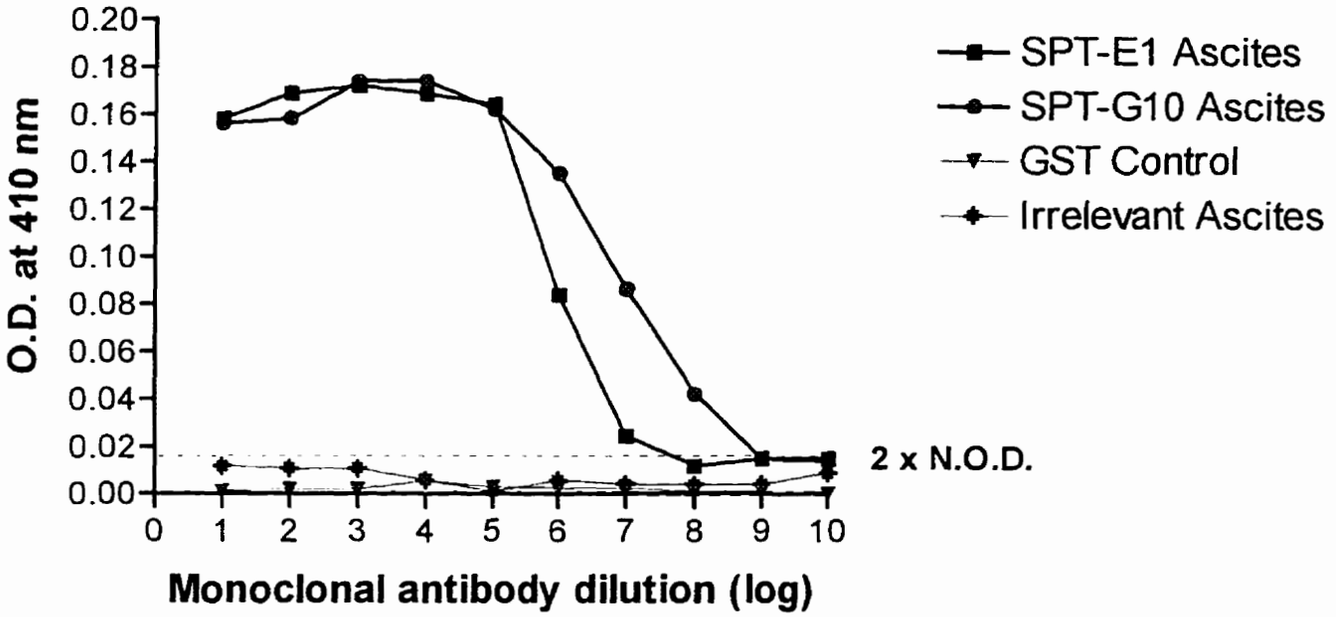
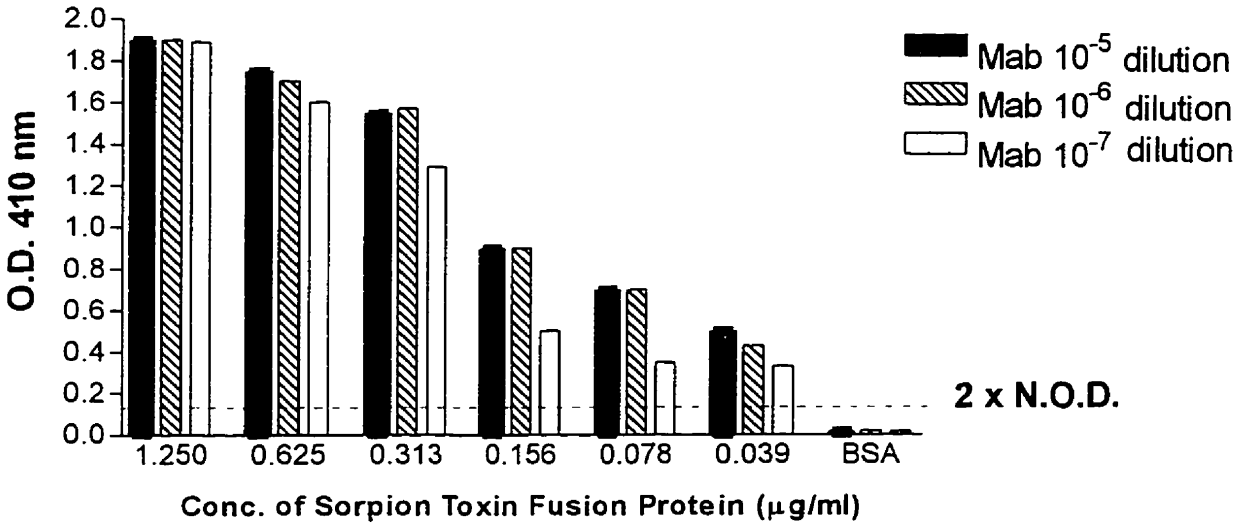

0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
O.D. at 410 nm
0 1 2 3 4 5 6 7 8 9 10
Monoclonal antibody dilution (log)
SPT-E1 Ascites
SPT-G10 Ascites
GST Control
Irrelevant Ascites
2 x N.O.D.

Figure 13 Reactivity of monoclonal antibody with purified GST/scorpion toxin AalT fusion protein in ELISA. Different concentrations of purified scorpion toxin AalT fusion protein were incubated with three optimal dilution of monoclonal antibody Spt-E1 (10^{-5}, 10^{-6}, 10^{-7} dilutions), as taken from Fig. 12. The data were plotted graphically showing the lowest detectable concentration of scorpion toxin AalT fusion protein above 2 x N.O.D. at 0.039 μg/ml. 2 x N.O.D. represents the double average O.D. value of BSA negative control.


O.D. 410 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.250
0.625
0.313
0.156
0.078
0.039
BSA
Conc. of Sorpion Toxin Fusion Protein (μg/ml)
Mab 10-5 dilution
Mab 10-6 dilution
Mab 10-7 dilution
2 x N.O.D.

VII. 4 Determination of specificity of monoclonal antibody in Western blotting

The specificity and reactivity of monoclonal antibody against scorpion toxin AalT as fusion protein were further examined in Western blots analysis. Whole cell lysates from IPTG induced *E. coli* containing recombinant plasmid or only pGEX-2T vector were run on SDS-PAGE, transferred to nylon membranes and incubated with one of the monoclonal antibodies Spt-E1, Spt-G10, Spt-H9 and Spt-C1 respectively. A mouse antiserum was used as positive control. The results are shown in Fig. 14. While mouse polycolonal antiserum reacted with both GST and GST/Spt-AalT fusion protein, the four monoclonal antibodies showed reactivities only with GST/Spt-AalT fusion protein, but not with GST protein.

To test the cross-reaction of monoclonal antibody with *E.coli* proteins (Fig.15), whole cell lysates from *E. coli* cells containing the recombinant pGEX-2T fusion plasmid or no plasmid grown in the presence or the absence of the IPTG were subjected to SDS-PAGE gel. Separated proteins were transfered to nylon membrane and reacted with Spt-G10 monoclonal antibody (Fig. 15. A), in which the scorpion toxin AalT as a GST fusion protein shows a band of reactivity with the monoclonal antibody Spt-G10 in IPTG induced whole cell lysates (Lane 1) and no reactions with Spt-G10 in IPTG non-induced whole cell lysates (Lane 2). *E. coli* proteins in IPTG induced cell lysates in lane 3 or non-induced in lane 4, show no cross-reaction with monoclonal antibody. The mouse polyclonal antiserum was used as a positive control

Figure 14 Specificity of monoclonal antibody in Western blot. The whole cell lysates of *E.coli* over-expressing GST and GST/Spt-AalT fusion protein were subjected to 12%SDS-PAGE, transferred to nylon membranes, and reacted with four monoclonal antibodies (Spt-G10, -E1, -H9, -C1) from ascites (1: 5,000) or mouse polyclonal antiserum (1:1,000).

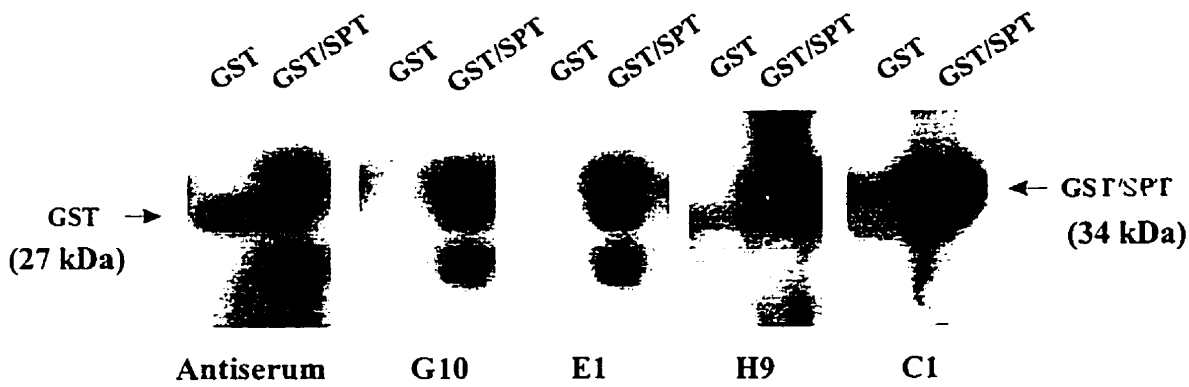
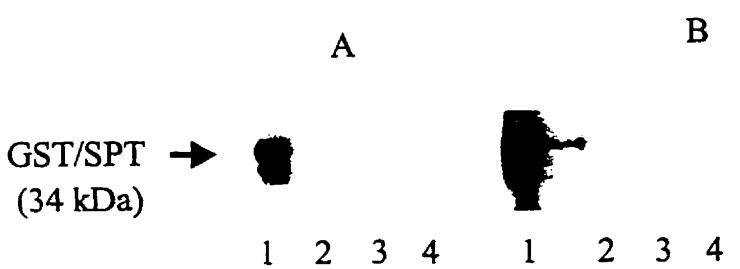

GST
GST/SPT
GST
GST/SPT
GST
GST/SPT
GST
GST/SPT
GST
GST/SPT
GST
(27 kDa)
GST/SPT
(34 kDa)
Antiserum
G10
E1
H9
C1

Figure 15 Cross-reaction of monoclonal antibody with *E.coli* protein in Western blot. *E.coli* whole cell lysates from IPTG induced cells containing the recombinant plasmid (lanes 1 in A and B) and no plasmid (lanes 3 in A and B), or from non-induced cells containing the recombinant plasmid (lanes 2 in A and B) and no plasmid (lanes 4 in A and B), were separated by SDS-PAGE and transferred to nylon membrane. Transferred membrane were incubated with monoclonal antibody Spt-G10 in A, or mouse polyclonal antiserum used as control in B.


A
B
GST/SPT
(34 kDa)
1 2 3 4
1 2 3 4

(Fig. 15. B), in which lane 1 shows reactivity of mouse polyclonal antiserum with GST/scorpion toxin AalT fusion protein, and lanes 2, 3 and 4 show no reactivities with non-induce GST/Spt-AalT fusion protein and *E. coli* proteins.

VIII. Analysis of Expression of Scorpion Toxin AalT in Spruce Budworm Infected with Recombinant C*f*MNPV/Spt-Aalt

To detect the expression of scorpion toxin AalT in spruce budworm infected with recombinant C*f*MNPV/Spt-AalT, the insect samples were collected at different days after viral infection. The insects which died at 9-10 days after viral infection were named D-09 or D-10 (Dead). Some insects still survived after 9-10 day of viral infection, in which samples were named D-09 or D-10 (Live). Supernatant isolated from insect samples were detected with specific monoclonal antibody Spt-G10 (1:1,000) in ELISA. The results are shown in Table 3, in which samples D-01, D-03, D-06, D-07, D-08 and D-09 (Live) show negative reaction with monoclonal antibody, sample D-10 (Live) shows weak positive reaction, and samples D-09 and D-10 (Dead) show positive reaction. These results demonstrated that the scorpion toxin AalT could be detected in dead insects after 9-10 days of viral infection.

Table 3 Analysis of expression of scorpion toxin AalT in insects infected with recombinant C*f*MNPV/Spt-AalT using specific monoclonal antibody. In insect samples, D stands for the day after viral infection. Samples D-09 and D-10 (Dead) refer to those insects that died after 9-10 days of viral infection, and samples D-09 and D-10 (Live) to those insects that still survived after 9-10 days of viral infection. Controls D-01 and D-10 were not infected with recombinant C*f*MNPV/Spt-AalT. ELISA results (color development) were assessed by eye assessment, in which - = no detectable color, +- = weak color, + = definite color, and ++ = strong color.

Samples	Results
D-01	-
D-03	-
D-06	-
D-07	-
D-08	-
D-09 (Dead)	+
D-09 (Live)	-
D-10 (Dead)	++
D-10 (Live)	+-
D-01 (Control)	-
D-10 (Control)	-

DISCUSSION

Spruce budworm *(Choristoneura fumiferana)* is the major Canadian forest pest. Traditional chemical insecticides used for pest control created numerous unacceptable agricultural, environmental, and human health problems (Betz, 1985). Biological insecticides such as spruce budworm, *Choristoneura fumiferana*, multicapsid nuclear polyhedrosis viruses (C*f*MNPVs) are an attractive alternative to chemical pesticides because they do not pose the environmental and health risks associated with chemical residues. They are also specific for insects , but the majority of these viruses possess an inherent property of low speed of kill that does not afford growers an economic level of pest control. One approach to enhance the insecticidal properties of naturally occurring viruses might be to produce new recombinant viruses by inserting a scorpion toxin gene that encodes the insect-specific toxin AalT. A synthetic gene encoding AalT was constructed and transferred to the C*f*MNPV. The insects fed with recombinant C*f*MNPV carrying scorpion toxin AalT (Spt-AalT) stop feeding earlier than those fed with wild-type virus (Dr. Pang, A., personnel communication). To study the expression of scorpion toxin AalT in spruce budworm infected with recombinant C*f*MNPV/Spt-AalT and to detect the persistence of scorpion toxin AalT in the affected environmental samples, development of monoclonal antibodies against scorpion toxin AalT was carried out in this study.

Monoclonal antibodies have proven to be very useful tools in different areas of biological science (Kohler and Milstein, 1975). A monoclonal antibody against any different molecular structure such as drugs, hormones, receptors for hormones, or any of the biologically derived or biologically active materials which interest the biological or medical scientist, can be used to identify it, locate it, purify it, and where appropriate, destroy it. Obviously, the potential uses for monoclonal antibodies are very widespread. In order to raise monoclonal antibodies, sufficient quantities of the immunogen are required to elicit a strong immune response as well as to screen specific antibodies. However, it is not always possible to prepare large amounts of protein in common expression system. Recently a new GST expression system has increased this possibility in which expression vectors based on plasmid with strong promoters and antibiotics secretion system can be used to express foreign polypeptide as fusion protein with glutathione S-transferase (GST) in *Escherichia coli*. The fusion protein typically remains soluble within the bacteria and can be purified from lysed cells because of the affinity of the GST moiety for glutathione immobilized on agarose beads (Smith and Jonson, 1988). Polymerase chain reaction (PCR) and the expression vector pGEX-2T were used to generate a fusion gene composed of an N-terminal glutathione S-transferase (GST) sequence and a C-terminal scorpion toxin AalT gene. Scorpion toxin AalT gene was amplified using PCR from a recombinant *Cf*MNPV transfer vector carrying the scorpion toxin AalT gene, in which the

complete scorpion toxin AalT gene includes a signal sequence. The results have shown that the expression and isolation of scorpion toxin AalT fusion protein in *E.coli* was possible only after the signal sequence was deleted. The reason for this phenomenon is that the signal sequence in recombinant C*f*MNPV/Spt-AalT was designed to lead the secretion of expressed scorpion toxin protein into the hemolymph of insects during the infection of C*f*MNPV/Spt-AalT. Morever, during the purification of fusion protein from crude bacterial lysates, the quantities of purified fusion protein was low in the first assay. However, the yield of the purified fusion protein was increased dramatically after changes of the serial condition of purification, including increasing the quantity of glutathione-agarose beads, longer time of sonication, and extending the period of incubation of whole cell lysates with glutathione agarose beads up to 1 hr. The yield of fusion protein was 8.75 mg from 1 litre of cell culture, a high level as expected in Smith and Corcoran's report (Smith and Corcoran, 1994). The temperature for expression of protein in bacteria is usually 37°C, but sometimes lower temperatures of 30°C (Smith and Corcoran, 1994) or 28°C (Gunzer and Karch, 1993) are used to increase the quantity and solubility of expressed protein. In the experiments presented in this thesis, cell growth at 37°C or 30°C had no significant effects on the yield of scorpion toxin AalT fusion protein. Purified scorpion toxin AalT fusion proteins analyzed on SDS-PAGE gel have shown some smaller bands below the predominant fusion protein bands. Since these bands were not observed in

normal or non-induced lysates, but were seen in the purified fusion protein preparation, they probably represent degradation products of the fusion protein.

The main advantage of the GST fusion system for expressing and recovering scorpion toxin AalT proteins from *E. coli* is that the fusion proteins remain soluble, and foreign polypeptides remain antigenic. Furthermore, this system offers additional features such as high level of inducible expression, efficient, rapid and easy purification under non-denaturing conditions (Smith, 1993). In this study, the strong immune response in mice had been stimulated using recombinant GST/scorpion toxin AalT fusion protein as immunogen, confirming that the GST carrier does not compromise the antigenicity of the foreign polypeptide.

Standard immunization and fusion protocols have been used for long time in the production of hybridomas (Oi and Herzenberg, 1980). Initial experiments, using the standard protocol, failed to produce high yields of hybridomas and specific antibody secreting clones to scorpion toxin AalT. In order to increase the yield of hybridoma clones, two modified fusion protocols were used together. These involved pre-incubating the mouse spleen cells/ myeloma Sp2/0 cells mixture with 0.25% PEG for 90 min at 37°C (Orlid and Altaner, 1988), and postincubating the PEG fused spleen and Sp2/0 cells for 30 min at 37°C (Fuller et al., 1988). This modification increased the cell fusion rate remarkably, giving the percentage of hybridoma clones up to 65.5%, compared to 50.2% as reported by Orlid and Altaner (1988). From more

than 629 hybridoma clones from one fusion on one mouse spleen, 49 clones were found to be producing antibodies specific for the scorpion toxin AalT. The percentage of specific antibody producing hybridoma clones was 7%, also higher than that 3.8% reported by Orlid and Altaner (1988).

The hybridomas were selected against GST and GST/Spt-AalT fusion protein. Only hybridomas reacted with GST/Spt-AalT fusion protein but not GST were subcloned further by limiting dilution. The reactivity of monoclonal antibodies with purified scorpion toxin AalT fusion protein was examined by ELISA analysis. As low as 0.039 μg/ml of scorpion toxin AalT fusion protein can be detected using specific monoclonal antibody. These data will be helpful in developing immunoassay systems for further applications. The specificity of monoclonal antibodies with scorpion toxin AalT was further confirmed by Western blotting. The results clearly demonstrated the specificity of the monoclonal antibodies for the scorpion toxin AalT. No reactivity was observed against GST protein and other proteins normally present in *E.coli*. In addition, some weak bands were also detected and most likely represented degradation products of the fusion protein. This is in accordance with other report (Campbell et al., 1995).

Scorpion toxin AalT is very specific for insects. It has been widely used as a foreign gene for insertion into various baculoviruses to make recombinant viruses for pest control. Polyclonal rabbit anti-scorpion toxin AalT antibody has been used to

confirm the scorpion toxin AalT expression in infected insects (McCutchen, 1991). Polyclonal antibody comprises a mixture of antibody isotypes with the capacity to engage in the different effector elements of the immune system depending upon the target and its antigenic characteristics. The polyclonal antibody preparations obtained from immunized animals contain a relatively small proportion of antigen-specific antibodies and a larger and variable proportion of antibodies that are potentially cross-reactive with nontarget antigens. This may give rise to undesirable incorrect interpretation of results. In contrast, monoclonal antibodies show much greater specificity, less likely to cross react with nontarget antigen, and lower background staining. Above all, monoclonal antibodies provide the opportunity for standardization. Once a monoclonal clone is established, it is possible to produce an identical antibody in unlimited amounts and without variation in specificity (Zola, 1987).

The monoclonal antibodies specific for scorpion toxin AalT were used to determine the expression of scorpion toxin AalT as function of time in the insects infected with recombinant C*f*MNPV/Spt-AalT, which was carried out in collaboration with CFS-Sault Ste. Marie. After infection with C*f*MNPV/Spt-AalT, insects collected at different time were used for analysis of the expression of scorpion toxin AalT in enzyme-linked immunoassay (ELISA). The preliminary results demonstrated that the expression of scorpion toxin AalT in infected insects could not be detected until 9-10

days after infection. Since the scorpion toxin AalT gene is inserted into the viral polyhedrin gene, a late gene, scorpion toxin AalT would be expressed in the late stage of viral infection (Dr. Anthony Pang, personal communication), which is consistent with the observation that the expression of scorpion toxin AalT was only detected in the dead insects after 9-10 days of viral infection, but not detected in those insects which still survived after 9 days of infection or were collected in the early days of infection. These initial results indicated that monoclonal antibodies specific for scorpion toxin AalT will be a useful tool to evaluate the efficacy of the recombinant CfMNPV/Spt-Aalt as biological insecticide.

In further studies, these specific monoclonal antibodies against scorpion toxin AalT will be used to examine the genetic system for the virus-directed intracellular expression of the scorpion toxin AalT gene, and to study the expression concentration of scorpion toxin AalT in the spruce budworm infected with C*f*MNPV-AalT. Also, the development of appropriate immunoassay system for routine use by non-experts will be standardized in the field to monitor the persistence of scorpion toxin AalT in affected samples such as water, soil, and wild life.

In summary, GST gene fusion system, an novel protein expression system with high level expression and ease purification in *E. coli,* has been used for the production of scorpion toxin AalT as GST fusion protein which was used as antigen to immunize mice to develop monoclonal antibodies against scorpion toxin AalT.

Several specific antibody producing hybridoma cell lines have been established. The specificity and reactivity have been characterized by ELISA and Western blotting. The expression of scorpion toxin AalT in insects infected with recombinant C*f*MNPV/Spt-AalT was analysed with specific monoclonal antibody against scorpion toxin AalT. The preliminary results showed that the scorpion toxin AalT which is inserted into the late polyhedrin gene of C*f*MNPV could be detected in dead insects after 9-10 days of viral infection, and indicated that these monoclonal antibodies would be a useful tool for further evaluation of recombinant C*f*MNPV/Spt-AalT as biologic insect control agent and for determination of the persistence of scorpion toxin AalT in environmental samples.

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