Resistance against Endotoxin from *Bacillus thuringiensis* in Lepidopteran Insects

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A Thesis Submitted for the Degree of
Doctor of Philosophy
in the Faculty of Sciences at the University of Adelaide

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December 2002

To my Mother and Father

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Summary

Insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* (*Bt*) in sprays and transgenic crops are extremely useful for environmentally sound pest management. However, some important agricultural insect pests show increasing resistance to *Bt*-toxin. This resistance problem has prompted intensive investigations on the mechanisms of resistance to *B. thuringiensis,* including genetic and molecular aspects of the mode of toxin action. Although a great deal of information exists on the mode of action of *Bt*-toxin, the mechanisms of toxin insertion into the membrane during the process of pore formation is still not known.

The major aim of this study was to investigate the possible molecular mechanism of resistance against Bt-toxin based on observations that Bt-toxin binds to a soluble immune-defence component. Since the binding protein may also be involved in cellular adhesion and endocytosis processes, the proposed resistance mechanism may be discussed in the context of a speculative mechanisms of toxin insertion into the membrane during the process of cellular uptake of the toxin. The finding of an immune-related resistance mechanism can be useful to combat evolution of resistance in insect pests as it may provide novel *B.thuringiensis* resistant management strategies in the field. The specific aim of this study was to examine whether Bt-toxins act like a lectin and whether lectin functions can be used as a paradigm for *Bt*-toxicity. Observations that oligomeric lectins are involved in cell spreading, macropinocytosis and coagulation reactions, suggest a basic functional involvement of oligomeric adhesion molecules in a range of cellular functions.

Findings in this study suggest that Cry1Ac toxin has sugar-binding specificity similar to GalNAc-binding lectins, which confirms previous observations that Cry1Ac potentially interacts with GalNAc-specific glycoproteins like a lectin (Burton et al, 1999; Knowles et al, 1991). However, in contrast to studies involving BBMVs, our analysis of whole gut extracts revealed that one of the major Cry1Ac-binding proteins is a soluble GalNAc-containing glycoprotein of 85 kDa (p85), which is possibly involved in coagulation reactions. In the presence of oligomeric lectins, soluble hemolymph components, such as p85, coagulate to form insoluble coagulation products. Since lectins also interact with membrane-anchored receptors to perform endocytosis on the cell surface, Bt-toxins with lectin-properties may be involved endocytosis reaction in conjunction with GalNAc-containing membrane-receptors, such as aminopeptidase N, and hemomucin. During endocytosis reactions, a lectin-like toxin may be inserted into the membrane.

We tested this possible alternative mechanism by predicting specific molecular features of Bt-toxin. For example, in our model Cry1Ac is expected to be an oligomeric lectin before it interacts with membrane receptors. This implies that mature Cry1Ac can form multimers in the absence of membranes and lipids. This was confirmed by analysing proteolytic digestion products of protoxin, which form high molecular weight complexes, corresponding to tetrameric complexes of mature toxin. This suggests that Cry1Ac may interact with membrane receptors as a tetrameric molecule similar to lectins. The mechanism for toxin-insertion may be based on a novel endocytosis mechanism, which has the potential to generate the configurational energy required for the insertion of an oligomeric toxin complex into the membrane. This is different from present models, which assume that Bt-toxin is unable to form a tetrameric complex in

the absence of lipid, since the assembly into a tetrameric channel-forming complex occurs inside the membrane bilayer.

In addition, the properties of p85, an immune-related protein in the gut lumen was investigated in resistant and susceptible insects. Our findings suggest that the target sites of the toxin in the gut lumen are changed in caterpillars that are resistant to a low level of toxin. As a result of immune-induction in these insects, the toxin may interact with small globular structures in the gut lumen around the peritrophic membrane, which resemble coagulation products. This could indicate that the toxin may be inactivated in resistant insects by coagulation and melanization reactions. A tentative resistance mechanism suggests that the toxin is inactivated in the gut lumen by coagulation reactions, before it can reach the epithelial membrane. If *Bt*-resistance is based on immune induction these strains may also be resistant to other pathogens. Our observations suggest that Bt-resistant insects are not only resistant to the Bt-toxin but also to other non-related pathogens such as baculovirus.

Statement

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

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

Muhammad Sarjan
December 2002

Acknowledgements

I would like to express my greatest appreciation to my principle supervisor Professor Otto Schmidt who continuously gave me his ideas and criticisms, without his support, things wouldn't be working the way they are. His compassion, energy, enthusiasm, have been invaluable. I have enjoyed his academic philosophy throughout the course of study. Also to Dr. Ulrich Theopold and Assoc Prof Rick Roush for their guidance, support and encouragement throughout the course of this study. Many thanks also to Ms Nicki Featherstone for her help during this study as well as thanks are also due to all the members of the Insect Molecular Biology laboratory.

Furthermore, special thanks to Mrs. Sue Eaton for her consistent help and friendship, Ms Heather Fraser and Dr. Gary Taylor and all the others in the Department of Applied and Molecular Ecology who always gave contributions in this study. I acknowledge a scholarship from Australian Government under AusAID scholarship for the financial support.

This work is dedicated to my mother and father, also to my wife Nung and my daughter, Fiona and my son, Rama who constantly encourage and support me in this study.

Thank GOD for giving me spiritual power during the course of my study.

List of Abbreviations

Ab	Antibody
APN	Aminopeptidase-N
Apo	Apolipophorin
APS	Ammonium persulfate
p-APMSF	4-Amidinophenylmethanesulfonyl fluoride
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
b-Me	b-Mercaptoethanol
bp	Base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
Bs-1	*Bandeiraea simplicifolia*
Bt	*Bacillus thuringiensis*
Cry	Crystal protein of Bt-toxin
Con A	Concanavalin A
cDNA	Complementary deoxyribonucleic acid
DAB	3,3'-Diaminobenzidine
DABCO	1,4-Diazabicyclo-(2,2,2)-octane
DBM	Diamondback Moth
dCTP	Deoxycytosine triphosphate
DDW	Double distilled water

DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DL-DOPA	DL-3,4-Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GalNAc	N-Acetyl-D-galactosamine
GluNAc	N-Acetyl-D-Glucosamine
HPL	*Helix pomatia* lectin
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kb	Kilobase pair(s)
kDa	KiloDalton(s)
LB	Luria-Bertani
M	Molar
mA	Milliampere(s)
mbn-2	*melanotic blood neoplasm*, a *Drosophila* mutant from which a cell line was derived with hemocyte properties (Gateff *et al.*, 1980).
µg	Microgram(s)
µl	Microlitre(s)
min	Minute(s)
µM	Micromolar
mM	Millimolar

MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
NADPH	b-Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NAPCU	Nucleic Acid and Protein Chemistry Unit
NBT	Nitro blue tetrazolium chloride
ng	Nanogram(s)
nm	Nanometre(s)
NP-40	Nonylphenoxy polyethoxy ethanol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraform aldehyde
PHGPX	Phospholipid-hydroperoxide glutathione peroxidase
PMSF	Phenylmethanesulfonyl fluoride
PNA	Peanut Agglutinin
PPO	Prophenoloxidase
PTU	Phenylthiourea
PDVs	Polydnaviruses
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
r.p.m.	Rounds per minute
RT	Room temperature

SDS	Sodium dodecyl sulphate
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TBST	Tris-buffered saline + Tween-20
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetrametylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris-hydroxymethyl-aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Unit(s)
UV	Ultraviolet light
V	Volt(s)
v	Volume(s)
VLPs	Virus-like particles
VVL	*Vicia Villosa* Lectin
v/v	Volume per volume
w/v	Weight per volume
WBL	Winged Bean Lectin
WGA	Wheat Germ Agglutinin

Chapter 1: General Introduction

Bacillus thuringiensis is a gram-positive bacterium that produces insecticidal crystal proteins (ICPs) during sporulation, which are toxic to certain insect larvae. The target of *B. thuringiensis* crystal delta-endotoxins is the midgut (Hofte and Whiteley, 1989). This is one of the most successful pathogenic micro-organisms that has been used as a biological control agent against a large variety of insect pests. However, some lepidoptera of economic importance, including the diamondback moth (*Plutella xylostella,* DBM*)* and cotton bollworm (*Helicoverpa armigera*), show increasing resistance to chemical insecticides (Bryan, 1991). Currently DBM has the only field populations known with resistance to Bt-toxin (Tabashnik *et al*, 1997; Schnepf *et al*, 1998). This resistance problem initiated intensive investigations on the mechanisms of resistance to *B. thuringiensis*. Findings suggest that the mechanisms of resistance are complicated, involving multiple genes. Although a great deal of information exists on the mode of action of *B. thuringiensis* toxin, the unknown aspect is the molecular mechanism of toxin insertion into the membrane during the process of pore formation.

As part of the mode of action of *B. thuringiensis*, toxins are released as protoxins, which are solubilized in the midgut of insects and activated by gut proteases. It is assumed that the trigger for the insertion of the pore-forming domain of the toxins into the epithelial cell membrane is a conformational change in the toxin, which occurs when another domain of the toxin binds to a receptor present on the brush border membrane. The toxins bind to target cells through a two-stage process in pore formation. The first step involves reversible binding to a receptor followed by an

irreversible step leading to membrane insertion (Aronson *at al*, 1999; Gerber and Shai, 2000). Aronson and Shai (2001) suggested that the binding of a monomeric toxin molecule to receptors on larval midgut cells is followed by three processes including toxin insertion into membrane bilayer, aggregation and formation of functional channel. All these processes are probably dependent upon all three toxin domains. However, the process of toxin insertion into membranes and the mechanism of oligomerization to form ion channels as well as the factors involved in this toxin insertion step are still not fully understood, which is also true for pore-forming toxins in general.

Since the formation of pore-forming oligomeric complexes inside the membrane bilayer is a common feature of bacterial pore-forming toxins (Olson et al., 1999), the main issue for understanding bacterial toxicity is how soluble proteins convert into membrane-spanning proteins (Lacy and Stevens, 1998). Most models predict toxin interactions with membrane receptors, which lead to membrane insertion in the form of a monomer by an unknown process (Bhakdi *et al*, 1996). The assumption is that pore-forming toxins, such as Bt-toxins, are unable to form oligomeric complexes in the absence of a lipid, since assembly into oligomeric channel-forming complexes occurs inside the membrane bilayer (Aronson, 2001).

Presently, the umbrella model suggests that the toxin protein undergoes conformational changes upon membrane binding, in which structural rearrangements of domain I (Fig 1) occur (Gazit *et al*, 1998). Presumably, this is followed by insertion of a hydrophobic protein domain into the phospholipid bilayer, whereas the amphipathic helices are spread on the surface (Fig 2). After multiple insertions of

monomeric toxin molecules, oligomerization of toxin monomers occurs, and a channel or pore is formed (Knowles and Dow, 1993). The ion channels formed by δ-endotoxins consist of toxin-oligomers (Aronson and Shai, 2001) probably tetramers, as revealed by crystal-structure analysis (Ellar, 1990). Previously, Manoj Kumar and Aronson (1999) demonstrated that α5, a hydrophobic helical hairpin that initiates pore formation is involved in the oligomerization of the toxin molecules and that its ability to oligomerize directly affects protein toxicity.

Furthermore, mutations in α5 domains are directly correlated with the loss of oligomerization and loss of Cry1Ac toxicity, suggesting that α5 can play an important role both in membrane insertion and in the oligomerization of proteins to form a channel (Aronson *et al*, 1999; Gerber and Shai, 2000). Aronson *et al* (1999) also showed aggregation of *B. thuringiensis* Cry1A toxins upon binding to target molecules in insect larval midgut vesicles. Aggregation may occur at the surface of the membrane or after integration of toxin monomers within the membrane. They concluded that tight binding of virtually the entire toxin molecule to the membrane and the subsequent oligomerization are both important steps in toxicity. Binding may be important for mobilizing toxin at the membrane surface, either for oligomerization or for a concentration–dependent insertion step (Aronson and Shai, 2001). However, the nature of this process, whether it occurs at the membrane surface or within the membrane, the number of toxin monomers involved, and whether there are any interaction with soluble or membrane components is not known.

Insights regarding the insertion of the *B. thuringiensis* into membrane could significantly advance understanding of the mode of action of pore-forming toxins and advance the design of more potent toxins and help to combat *B.thuringiensis* resistance. In order to achieve these advantages, clarification of the mechanism of toxin insertion into the membrane is essential. This study demonstrates an alternative model of *B. thuringiensis* toxin insertion into the cell membrane. It also reveals that the immune status of the resistant strains of lepidopteran caterpillars is correlated with resistance against low levels of *B. thuringiensis* toxin.

Chapter 2: Literature Review

2.1 Introduction

In this review, several aspects of the molecular mechanism of Bt-resistance as a possible defense reaction to *B. thuringiensis* toxin in the midgut of Lepidoptera will be discussed: the mode of action of *B. thuringiensis* on the insect gut, the characteristics of receptor and recognition molecules, and the major defense reactions of insects. The first part will focus on the present knowledge of the mode of action, whereas in the second part the possible receptors of Bt- toxin will be discussed in the context of aspects of recognition molecules such as the lectins. In addition, the cellular defence reactions such as lectinophagocytosis and endocytosis as well as inducible bactericidal proteins and non-bactericidal proteins that are actively involved in the humoral defence reaction are discussed.

2.2 The structure of toxin

B. thuringiensis insecticidal proteins can be grouped into different classes based upon their insecticidal spectrum, and separated into families according to protein sequence relationships (Crickmore *et al*, 1998). Hofte and Whiteley (1989) proposed a toxin classification according to a taxonomic system (Table 1). Generally the toxins are divided into three size classes: the Cry1 (130-138 kDa) and Cry2 (71 kDa) proteins which are primarily toxic to Lepidoptera: the Cry3 (65-129 kDa) proteins which are toxic to Coleoptera and, the Cry4 (containing toxins of the 128-134 and 72-78 kDa size classes) proteins, which are toxic to Diptera, such as mosquitoes and blackly larvae (Pietrantonio et al, 1993).

Most of the research has been done with toxins that are active on Lepidoptera, designated Cry1 and Cry3 δ-endotoxins. The crystal structures of endotoxins have been determined, showing that toxins are composed of three distinct domains (I-III) (Ellar, 1994). These three domain structures (Fig 1) consist of seven α -helix bundles for domain I, the pore forming domain in the intestinal epithelium of the target organism. One of the proposed mechanisms of Cry toxin activity suggests six α-helices surrounding a central helix α5 (Gazit *et al*, 1998). Domain II and III of the δ-endotoxins have functions in specifically binding to receptors and for domain III, in modulating ion channel activity (Schnepf *et al*, 1998). These are comprised of β–sheets in different conformations, with domain III containing two antiparallel β–sheet sandwich structures (Li *et al*, 1991). The β–sandwich (domain III) is critical for the structural integrity of the toxin.

Table 1. Classification of the crystal protein genes of *B. thuringiensis*

Gene taxon	Spectrum of activity	Number of amino acids	Predicted mass (kDa)
Cry 1A(a)	Lepiodptera	1176	134
Cyy 1A(b)	Lepidoptera	1155	131
Cry 1A(c)	Lepidoptera	1178	133
Cry 1B	Lepidoptera	1207	138
Cry 1C(a)	Lepidoptera	1189	135
Cry 1C(b)	Lepidoptera	1177	134
Cry 1D	Lepidoptera	1165	133
Cry 1E	Lepidoptera	1171	132
Cry 1F	Lepidoptera	1174	134
Cry 1G	Lepidoptera	1156	130
Cry 2A	Lepidoptera/Diptera[a]	633	71
Cry 2B	Lepidoptera	633	73
Cry 3A	Coleoptera	644	71
Cry 3B	Coleoptera	659	74
Cry 3C	Coleoptera	1138	129
Cry 3D	Coleoptera	650	73
Cry 4A	Diptera [a]	1180	134
Cry 4B	Diptera [a]	1136	128
Cry 4C	Diptera [a]	675	78
Cry 4D	Diptera [a]	643	72
Cyt A	Diptera [a] Cytolytic [b]	248	27

Adapted from Hofte and Whitley (1989)

[a]Toxic to larva of suborder Nematocera , such as mosquitoes and blackly larvae, but not to other dipterous larvae

[b] Cytolytic to many different cell types, both vertebrate and invertebrate, *in vitro.*

2.3 The mode of action of *B. thuringiensis* on the insect gut

Discussion of the mode of action of *B. thuringiensis* is complicated by the number of subspecies of Bt or pathogens studied by different investigators, the number of susceptible hosts, the production of more than one toxin by the bacterium, the possible interaction of spores and crystals in toxicity to some larvae, and the fact that toxic materials may act at several sites in susceptible hosts (Beegle and Yamamoto, 1992). The mode of action of this biocontrol agent has been studied *in vitro* by using a variety of systems including insect cell lines, whole insect guts, and brush border membrane vesicles (BBMV's) purified from mid-guts (Knowles *et al*, 1991).

B. thuringiensis is specific to a narrow range of pests (mostly insects but also nematodes) in the orders Lepidoptera, Diptera and Coleoptera, depending on the subspecies of *B. thuringiensis* being used. Within these groups of insects, *B. thuringiensis* is only effective on the larval stage. No direct effect is seen on egg, pupal or adult stages, although indirect effects of reduced growth and fecundity may result from sublethal application (Bryan, 1991).

Many theories have been proposed to explain the mechanism by which the bacteria kill insects. Perhaps the most important characteristic of *B. thuringiensis* is the route of uptake and its implication for application (Aronson *et al,* 1986; Luthy and Ebershold, 1981). Recently the molecular mechanisms of *B. thuringiensis* endotoxin have been discussed intensively, with several hypotheses based on multistep activity of *B. thuringiensis* Cry toxins (Adang, 1998; Knowles and Dow, 1993).

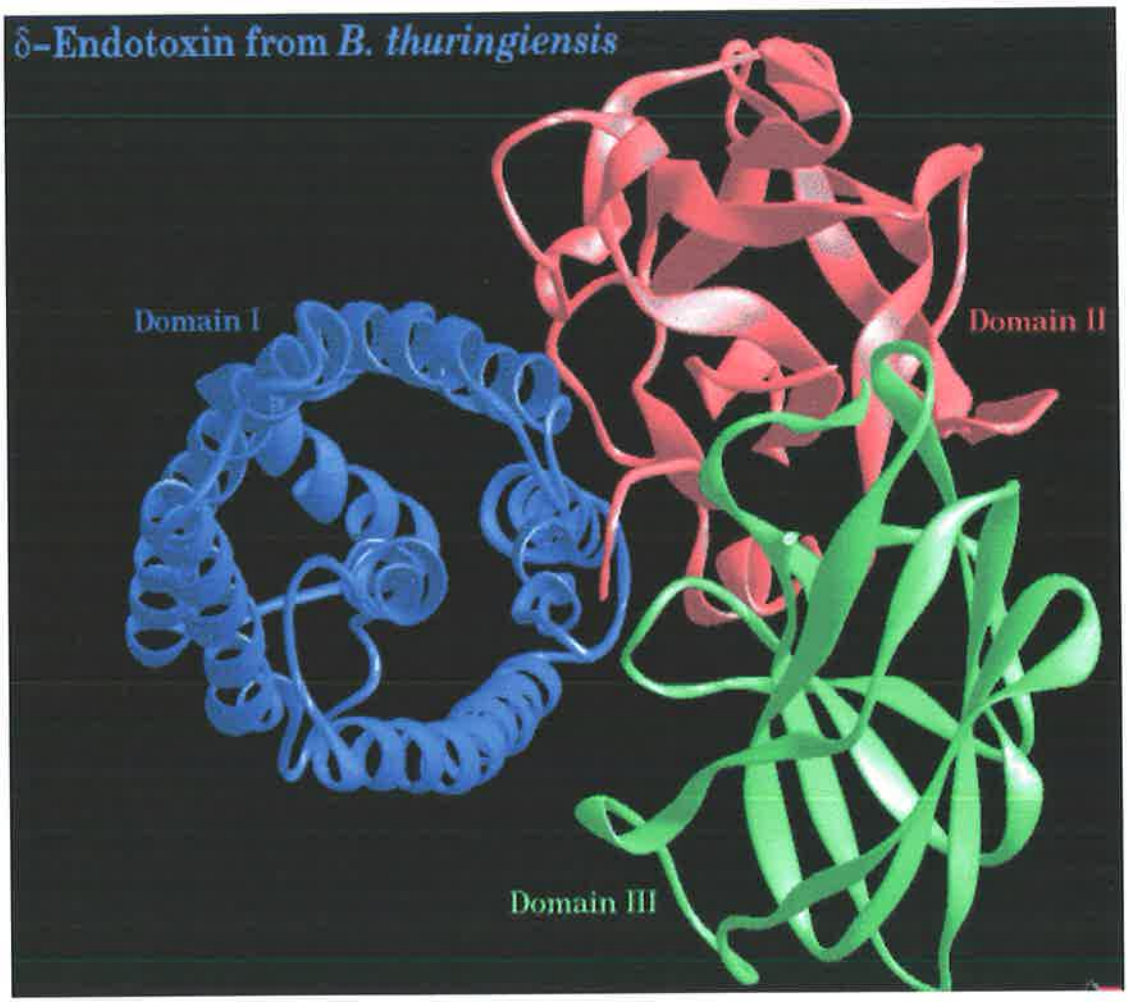


Fig 1. Structure of *Bacillus thuringiensis* Cry3A δ-endotoxin with schematic ribbon representations showing the domain organization. Domain I, the pore–forming domain; domain II and domain III, the receptor-binding domains; (Adapted from Schnepf *et al*, 1998).

The mechanism of action of *the B. thuringiensis* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptors and insertion of the toxin into the apical membrane to create ion channels or pores. These steps are discussed in the following sections.

2.3.1 Ingestion

Because the mode of action of *B. thuringiensis* is limited to the midgut of susceptible larvae, *B. thuringiensis* is only effective if ingested along with the insects' usual food substrate (Aronson *et al*, 1986; Luthy and Ebershold, 1981). Unlike many conventional chemical insecticides, where multiple routes of uptake offer flexibility of application, *B. thuringiensis* has no vapour activity or direct or residual contact activity. This means the target for deposition must be carefully identified as the food source for the pests, not necessarily the pest itself (Bryan, 1991).

2.3.2 Solubilization and proteolytic activation

The toxin proteins are synthesised as insoluble crystalline protoxins, which dissolve and become activated in the insect gut before exerting their effects. The Cry1 subclass consists of lepidopteran-active Bt-protoxins with apparent molecular masses of approximately 130 kDa, which are solubilized and processed (activated) by gut enzymes to approximately 65 kDa proteins (Hofte and Whiteley, 1989), primarily by removal of the carboxyl halves (Aronson and Shai, 2001). The gut of most target

insects has a very high pH and this is essential for dissolving most *B. thuringiensis* protoxins, which are usually soluble only above pH 9.5 (Knowles and Dow, 1993).

The major proteases of lepidopteran insect midgut are trypsin-like or chymotrypsin-like enzymes (Schnepf *et al*, 1998). For many of the Cry toxins the proteolytic activation of protoxin is intimately connected with solubilization and removes large segments of the molecule to leave a protease-resistant active core toxin (Ellar, 1990). In the case of the 130 kDa Cry toxins, the C-terminal half of the molecule is removed in this process, indicating that the insecticidal determinants are contained with the first 600 amino acid residues. This has been confirmed by genetic deletion studies. Structural comparison suggests that the Cry2 and Cry3 genes may be truncated versions of the 130 kDa Cry toxins. It is therefore interesting that proteolytic activation of these latter toxins occurs primarily at the N-terminus (Ellar, 1990).

2.3.3 Receptor binding

Binding of toxins to midgut receptors is a key step in the mode of action of *B. thuringiensis toxins* (Van Rie et al, 1989), which is based on the recognition of the toxin by highly specific binding proteins or receptors in the larval midgut. Receptor binding has been shown to be an important factor in insect specificity of ICPs, because *B. thuringiensis* toxins bind with high affinity to receptors on BBMVs of the midgut in susceptible insects. A critical step for insecticidal activity is the binding of activated toxins to receptors located on the apical microvilli membrane of epithelial midgut cells (Van Rie *et al*, 1989). It has become apparent from binding and competition studies (using Cry1 toxins and membrane from the gut of susceptible and

resistant insects) that there are many different toxin-binding proteins (Gill *et al*, 1992). Recent data suggest that toxicity correlates with irreversible binding (Ihara *et al*, 1993) that is probably associated with the insertion of the toxin into the larval membrane. This and subsequent aggregation and the formation of functional channels are dependent upon all three domains (Aronson and Shai, 2001), whereas reversible binding to larval membrane receptors mostly depends upon domains II and III. Ballester *et al.* (1999) also strongly suggested that domain II is involved in binding and/or receptor interaction with the midgut epithelial membrane as one major determinant of toxin specificity. In summary, domain I is mostly involved in membrane insertion to form the pore, and domains II and III are important determinants of toxin specificity through a role in high–affinity binding (Ballestee *et al*, 1999).

2.3.4 Formation of the toxic lesion

After binding to a specific receptor, the next step involves the formation of a pore or lesion in the plasma membrane. This leads to breakdown of the permeability barrier of the membrane, cell lysis, disruption of gut integrity and finally death of the insect from starvation or septicaemia. Pore-mediated cytolysis is a common strategy adopted by pathogenic bacteria (English and Slatin, 1992). The effect of pore formation in a plasma membrane will depend on the environment of the cell. It has been proposed that *B. thuringiensis* toxins kill cells by pH-mediated damage (Harvey *et al*, 1986) or osmotic lysis (Knowles and Ellar, 1986), since osmotic imbalance across the cell membrane leads to cell death (Pietrantonio *et al*, 1993).

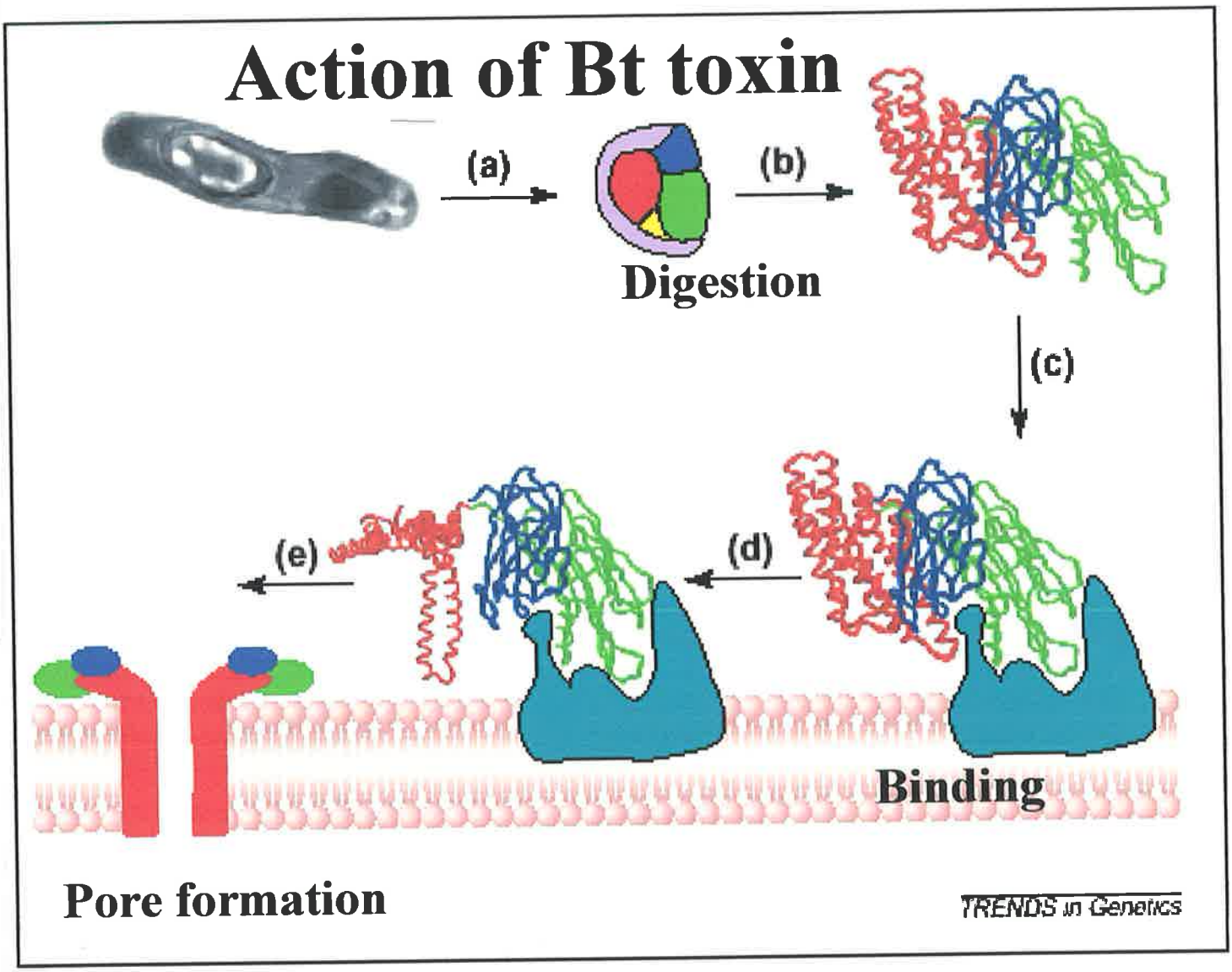


Fig. 2. Bt toxicity in the gut of lepidopteran caterpillars showing interaction of monomeric toxin with a receptor and insertion into the membrane by an unknown process (Adapted from de Maagd, *et al*, 2001).

2.4 Receptors and recognition molecules

2.4.1 Receptor characterization

Receptor characterisation is currently an active area of study as a result of the increasing cases of insect resistance to *B. thuringiensis*. For example Ferre *et al* (1991) suggested that receptor binding is a key factor in the specificity of toxins and different toxin types may bind to distinct receptors in one insect (Tabashnik *et al,* 1997). Toxin receptors have also directly been implicated in insect resistance to Bt toxins; for example in the case of *Plodia interpunctella*, Cry1Ab resistance is assumed to be due to a reduced binding affinity for this toxin (Ferre *et al*, 1991). The Cry1Ab resistance of *P. xylostella* is also due to a modified interaction of the toxin with its receptor. Furthermore, Valaitis *et al* (1997) pointed out that high affinity-binding of a *B. thuringiensis* toxin to specific receptors on the surface of midgut epithelial cells is a key factor in determining the insecticidal activity or resistance to different *B. thuringiensis* toxins. Because of the important role of receptors in the mode of action of *B. thuringiensis* toxin, much attention has recently been focused on the identification and characterisation of these binding proteins (receptors).

Receptor in this context refers to a molecule on the surface of an insect cell to which a *B. thuringiensis* toxin binds (Knowles and Dow, 1993). Many pore-forming toxins bind to specific sugar moieties on glycoproteins that have been described as receptors on the cell surface (Fig 3) (Cortajarena et al, 2001; Hatakeyama et al, 1996; Mercy and Ravindranath, 1994; Saha and Banerjee, 1997;

Sandros *et al*, 1994). Haider and Ellar (1986) found that the toxin of *B. thuringiensis* var. *aizawai* bound to membrane proteins of 120 kDa and 68 kDa in lepidoptera-derived cell lines, whereas dipteran cell lines possessed a toxin-binding membrane protein of 90 kDa. Additionally, a 146 kDa membrane glycoprotein from *Christoneura fumiferana* cells (CF1 cells) bound toxins of *B. thuringiensis* var. kurstaki HD 1 (Knowles and Ellar, 1986). Other findings indicate that CrylAc binding may involve sugar residues on an aminopeptidase, particularly N-Acetyl-galactosamine, because the presence of this sugar in excess reduces Cry1Ac binding and has been used to elute the aminopeptidase from toxin affinity columns (Knowles *et al*, 1991; Knight *et al*, 1994). Two major receptor types have been identified and are the subject of much published work, one consists of aminopeptidase N, the other of cadherin-like molecules.

2.4.1.1 Aminopeptidase N

The first *B. thuringiensis* toxin-binding protein to be identified was purified from *Manduca sexta*. Knight *et al* (1994) and Sangadala *et al* (1994) found that it is an aminopeptidase N (APN). Aminopeptidases catalyse the hydrolysis of amino acid residues from the amino terminus of peptide substrates. Taylor (1993) noted that APNs are distributed in many tissues and cells, and play an important role in several biological functions, for example, in the digestion and uptake of proteins in the insect midgut. They are more active than carboxypeptidases (Terra and Ferreira, 1994).

More recently, Valaitis *et al* (1997) reported that toxin binding to APN-1 (100kDa) is directly inhibited by N-Acetylgalactosamine, and it is suggested that this amino-sugar configuration forms an integral part of the binding site. Jenkins *et al* (2000) empirically determined two Cry1Ac surface regions that are involved *in vitro* in toxicity and APN-binding. Mutations within domain III affected binding rates to APN site 1, while mutations in domain II affected binding rates to APN site 2. Finally, domain III contact is completely inhibited by the presence of N-acetylgalactosamine, indicating that loss of domain III-binding eliminates all APN binding. From these data, emerged a model that implicates a functional cavity in lectin-like domain III, which initiates docking through recognition of an N-acetylgalactosamine moiety on *Lymantria dispar* APN. However, Valaitis *et al* (1997) found that not all APN molecules in larval midguts serve to function as toxin binding proteins. In addition, Knight *et al* (1995) and Sangadala *et al* (1994) suggested that the 120 kDa receptor has a normal function in insect nutrition as an aminopeptidase, because their studies suggest that proteins with other functions in insect physiology are utilized as toxin receptors and that the prevalence and properties of these receptors determine both host specificity and resistance.

2.4.1.2 Cadherin-like proteins

Cadherins are a group of integral membrane proteins with a molecular weight of ~ 130 kDa that mediate calcium dependent cell-cell adhesion (Takeichi, 1991). They are involved in various biological processes, such as morphogenesis and maintenance of tissue structure.

Recently, a receptor, BT-R1, for CrylAb has been identified and characterized in BBMVs of *M. sexta* midguts (Martinez-Ramirez *et al*, 1994). It is a glycoprotein, which migrates on sodium dodecyl-sulfate polyacrylamide gels (SDS-PAGE), with an apparent molecular mass of 210 kDa. A cDNA for this protein encodes a 172 kDa protein with an amino acid sequence that suggests that it is a member of the cadherin superfamily of proteins (Vadlamudi *et al*, 1995). In *M.sexta*, the CryIAb receptor is believed to be a cadherin-like 210 kDa membrane protein (Bulla, 1997; Keeton *et al*, 1997). Nagamatsu *et al* (1999) also showed that *B. thuringiensis* Cry IAa toxin binds to a cadherin-like protein (Bt R175) on the brush border membrane of the *Bombyx mori* midgut columnar cells, which are the target. They show that the Bt BTR 175 protein is an essential factor for the specificity, determination and cytotoxic action of the *B. thuringiensis* Cry1Aa toxin. However, little is known about the role of these molecules in binding. Therefore, further study of characterization and identification of these proteins is important to clarify their role in the binding processes. Gahan *et al* (2001) demonstrated a relationship between high levels of resistance to the Bt toxin Cry1Ac in *Heliothis virescens* and disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion.

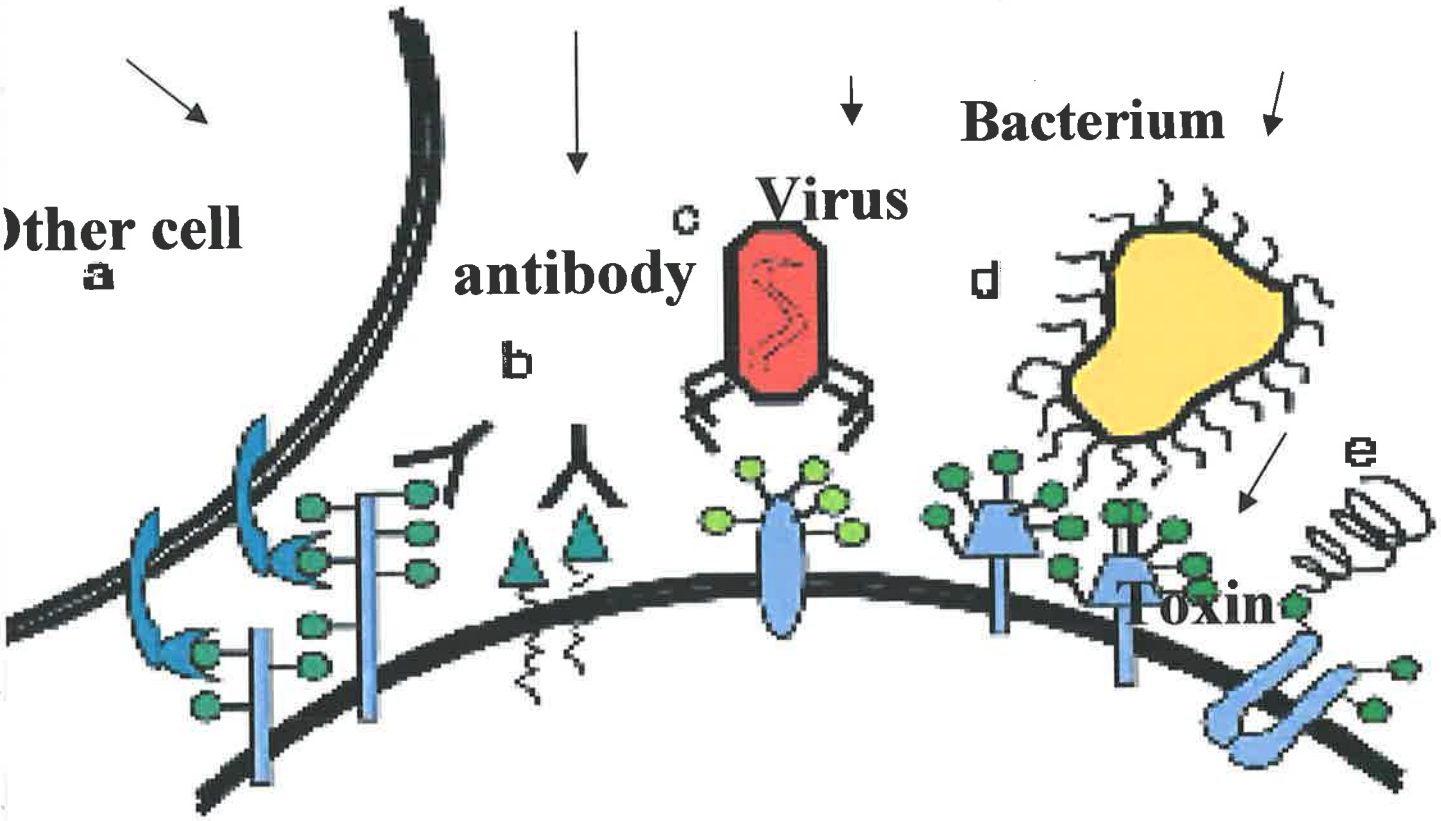


Fig 3.Cell surface receptors and their response to extra cellular signals (Adapted from Saxon and Bartozzi, 2001)

2.4.1.3 Sugar dependence of toxin binding

The sugars, GalNAc and GlcNAc are able to reduce Cry1A toxin binding. The sugar GalNAc was found to specifically decrease the cytolytic activity of a Cry1Ac toxin towards *Choristoneura fumiferana* CFI cells, to completely abolish toxin binding to *M. sexta* BBMVs, to partially inhibit binding to *Heliothis virescens* BBMVs and to have no apparent effect on binding to *Pieris brassicae* BBMVs (Knowles *et al*, 1991). The toxin binding proteins of *M. sexta* and *H. zea* also bound the GalNAc-specific lectin from soybean agglutinin. Furthermore, Burton *et al* (1999) showed that binding of the insecticidal *B. thuringiensis* Cry1Ac toxin to the putative receptor aminopeptidase N is specifically inhibited by GalNAc, suggesting that this toxin recognises GalNAc on the receptor. Taken together, these findings suggest that N-acetylgalactosamine might be a target-specific component of a Cry1Ac toxin receptor of CFI cells and of at least two of the insects tested (Knowles *et al*, 1991).

Although the 170-kDa Bt-binding protein is a GalNAc-containing glycoprotein, endoglycosidase treatment does not prevent the binding of Cry1Aa or Cry1Ab toxin. This indicates that the sugars are not the only structural requirement for the binding of these toxins. Other findings suggest that sugars appear to be important during the infection process of pathogens such as bacteria (Ishikawa *et al*, 1994), because bacteria might use sugar determinants rather than protein sites for binding to the lining of tissues. Because of this fact, it is important to understand the importance of sugars for toxin-binding to the receptors. In addition, because of the diverse results of investigations on the characteristics of receptors, clarification of the role of these proteins in the pore-forming process requires further molecular

characterisation and/or isolation of one or more toxin receptors, and determination of their function in insect midgut epithelial cells.

2.4.1.4 Hemomucin

Theopold *et al.* (1996) described an O-glycosylated protein in *D. melanogaster* that exhibited similar characteristics to mucins in the immune system. A GalNAc-specific lectin from *Helix pomatia* (HPL), which binds to mucins such as episialin and leukosialin on vertebrate leukocytes, attached to a membrane-bound mucin on hemocytes and hemocyte derived *Drosophila* cells (*mbn-2* cell line). This mucin, now named hemomucin (Hmu), was found to have a role in insect immunity, inducing an immune response measured by induction of cecropin mRNA when lectin was applied to *mbn-2* cells (Theopold *et al.* 1996).

In addition to hemocytes, the membrane bound form of Hmu was discovered on the peritrophic membrane of the gut and in ovaries. In the ovaries, hemomucin was deposited on the outer membrane of the eggshell - the chorion. Follicle cells, the cells that secrete the protein components for the chorion, showed a strong signal in *in situ* hybridisation experiments and are postulated to be the source of chorion Hmu (Theopold *et al.* 1996).

Two forms of Hmu were discovered in this study, distinguished by their size. The 100 and 220 kDa forms of hemomucin are products of the same gene as they have the same protein sequence, but it is unknown how the 220 kDa form is produced (Theopold *et al.* 1996). More recently, a 105 kDa form was reported (Theopold *et al*, 2001). The difference between the 100 and 105 kDa form is the presence of

one type of sugar. The 105 kDa form contains an 0-linked galactose attached to the GalNAc, which is recognized by the plant lectin peanut agglutinin (PNA). Interestingly, this form of the glycoprotein is only found in the ovary and the 100 kDa form is the predominant protein (Theopold *et al*, 2001).

2.4.2 Recognition molecules

Like all other organisms, recognition is an essential mechanisms for insects to distinguish between 'self' and 'non-self' by identifying foreign invaders (Sharon and Lis, 1989; Ratcliffe, 1993). The recognition molecules of the innate immune system are called pattern recognition receptors (PRRs, Medzhitov and Janeway, 1997). The most frequently recognised structures are lipopolysaccharides (LPS), peptidoglycans (PG) and ß-1, 3-glucans. Quite a number of binding proteins in arthropods have been reported (Cerenius *et al*., 1994; Duvic and Söderhäll, 1990) and several binding proteins in insects have been characterised at the molecular level (Xu, 1995; Matha *et al.*, 1990). Some other proteins that can bind foreign structures and thus induce or adjust insect immune reactions have been detected (Trenczek, 1998). In addition to LPS from gram-negative bacteria, the peptidoglycans from gram-positive bacteria and the mannans or beta-1,3-glucans from fungi have been described as evoking immune reactions [Sugumaran, 1993 #844; Söderhäll, 1994 #220]. Ashida and colleagues were able to purify two soluble proteins from the hemolymph of the silkworm *B mori*, one of which is specific for peptidoglycans from bacterial cell walls, and the other for β-1,3-glucans from fungal cell walls [Ashida, 1990 #681]. When bound to their respective ligands, these proteins activate a proteolytic cascade, named the

prophenoloxidase activating system, that leads to the activation of the enzyme phenoloxidase, which is generally considered to be the key enzyme for the synthesis of melanin [Sugumaran, 1993 #844]. Three putative classes of recognition molecules, such as lectins (hemagglutinins), hemolin and proteins involved in the activation of prophenoloxidase have been identified and those will be described and discussed in more detail.

2.4.2.1 The lectins (hemagglutinins)

Lectins are carbohydrate-binding proteins that interact specifically with sugar determinants through non-covalent linkages, which are mainly water-mediated hydrogen bonding or van der Waals forces. Animal lectins are recognized as molecules playing important roles in a variety of biological processes through binding to glycoconjugates and as lectin-like receptors (Hebert, 2000).

Lectins are possible recognition molecules because of their ability to bind to oligosaccharide structures present on cell and microbial surfaces. They are specific carbohydrate-binding proteins with multiple binding sites that play a key role as opsonins in the control of various pathological processes in living organism such as insects (Sharon, 1984; Komano and Natori, 1985). For example, Wheeler *et al* (1993) demonstrated that a lectin from the grasshopper *Melanoplus differentialis* has an opsonic activity against fungal blastospores. Generally, lectins and carbohydrates are partners in biological recognition. In addition, lectins mediate the adhesion of many pathogenic microorganisms to host cells, a precondition for infection and are involved in the destruction of cells as well as lymphocyte

migration (Sharon, 1984; Sharon and Liz, 1989). Those activities require the binding of lectins to complementary sugars on opposing cells. In insects, they are functionally described as hemagglutinins among soluble hemolymph proteins (Ratcliffe, 1993).

Induction of agglutinins by injury or by introduction of microorganisms into haemocoel of the flesh-fly, *Sarcophaga peregrina,* indicated a possible role in insect immunity, specifically in binding to hemocytes or phagocytic activity. Minnick *et al* (1986) claimed that inducible lectins are an essential component of cell-based immunity in insects. The carbohydrate binding capacity of insect agglutinins also signifies their proposed participation in early recognition steps in pathogen clearance (Sharon, 1984; Gupta, 1991). In addition, Knowles *et al* (1991) found that the lectins, soybean agglutinin and wheat germ agglutinin, which are GalNAc-specific, also inhibit toxicity of the *B. thuringiensis* toxin. Knowles *et al* (1991) also demonstrated that toxin binding to a number of proteins of lepidopteran BBMVs is specifically inhibited by GalNAc. Theopold *et al* (1996) found that a snail lectin HPL binds to *Drosophila* blood cells and induced an antibacterial response. In addition to induction experiments, Dimopoulos *et al* (1997) reported that a bacterial recognition protein, antibacterial peptides and lectins are induced in the gut of *Anopheles gambia* after infection with *Plasmodium berghei.*

The majority of invertebrate agglutinins are regulated during development and attempts to stimulate the production of new proteins or enhance existing levels have met with varying degree of success. The most detailed study on induction of

an agglutinin comes from work on the flesh fly, *S. peregrina*. This lectin is present in the embryo, disappears during larval stage and reappears at the early pupal stage when decomposition of larval tissues takes place.

Because of similarities between mammalian and insect immune responses, it was predicted that insects might rely on lectins to recognize pathogens (Franc and White, 2000). The authors also state that lectins are important in both the activation of the PPO activating cascade and in phagocytosis. In addition, the hemagglutination activity of most insect lectins suggests that they might play a role in micro-organism and hemocyte aggregation during nodule formation. A list of lectins used in the present study is given in Table 2.

Table 2. Properties of lectins conjugates that have been used in the following experiments

No	Common Name	Taxonomic Name	Mol.Wt X 10^3	Sub Unit	Sugar specificity
1	Winged Bean	*Psophocarpus tetragonolobus*	35	1	Gal
2	Hairy vetch	*Vicia villosa*	139	4	GalNac
3	Roman Snail	*Helix pomatia*	79	6	GalNac
4	Peanut	*Arachis hypogaea*	120	4	Gal, GalNac
5	Wheat germ	*Triticum vulgaris*	36	2	GlcNac,GalNac,NEuNac
6	Con A	*Concavalin A*	102	4	Man, Glc

2.4.2.2 Hemolin

Hemolin, a hemolymph protein with a molecular mass of 47 kDa, is a member of the immunoglobulin superfamily (Sun *et al*, 1990). It contains four immunoglobulin (Ig)-like domains, which are similar to neuroglian, a cell–adhesion molecule (Franc *et al.*, 1999). Earlier studies demonstrated that hemolin together with another protein in the hemolymph can bind to the bacterial surface (Schmidt *et al*, 1993). This protein is assumed to play a role in immune recognition and in modulation of defensive responses in *H. cecropia* and *M. sexta* (Sun *et al*, 1990).

Hemolin's structural similarity to cell adhesion molecules, and the fact that its synthesis is elicited by bacterial infection, point toward function in antibacterial defence or immune clearance. Hemolin inhibits the aggregation of hemocytes, elicited by plastic surfaces or signals such as LPS, which suggests that hemolin may regulate the adhesive properties of hemocytes during defensive responses (Faye and Hultmark, 1993; Schmidt *et al*, 1993).

2.4.2.3 Prophenoloxidase activating system

The prophenoloxidase (PPO) activation system is an important regulatory component of the defence system of animals including insects (Millar and Ratcliffe, 1994), and has been suggested to be involved in "self" and "non-self" discrimination (Ashida *et al*, 1988). PPO is a widely distributed enzyme, which generates melanin by oxidising phenolic substances. In addition, the cells, which

contain components of the PPO system, also produce lectins that are important putative recognition molecules (Millar and Racliffe, 1994).

An opsonic role of the activated PPO system has been demonstrated in haemocytes. For example, phagocytosis of non-self particles by crustacean and insect hemocytes can be enhanced by the presence of beta-1,3-glucan or LPS, known elicitors of PPO activation (Millar and Ratcliffe, 1994).

The activated phenoloxidase is involved in several reactions, including melanin production, production of reactive oxygen species (Nappi *et al*, 1995) and sclerotization as part of the formation of the cuticle, capsule formation and wound healing. The concept of pattern recognition has gained strong support through the identification of a number of proteins that recognize microbial elicitors and subsequently activate the PPO cascade (Lee *et al.*, 2000; Söderhäll *et al.*, 1994; Jomori *et al.*, 1990; Duvic and Söderhäll, 1990; Cerenius *et al.*, 1994; Ma and Kanost, 2000; Yu and Kanost, 2000).

2.5. Pre-coagulation complexes

Insect hemolymph coagulation depends on the interaction of cellular components and soluble serum factors (Grégoire, 1974). It is widely accepted that lipophorin is one of the clotting factors, which play an important role in insect clotting cascades. However, other hemolymph molecules engaged in coagulation and their functions in the coagulation cascade remain unknown.

Independent observations suggest that under certain conditions hemomucin binds to lipophorin (Theopold and Schmidt, 1997), a lipid carrier in insects (Pho *et al.*, 1996; Ryan and van der Horst, 2000). In addition to a function as a lipid carrier, lipophorin is involved in other functions, including coagulation (Li *et al.*, 2002), immune induction (Wiesner *et al.*, 1997), detoxification of microbial compounds (Kato *et al.*, 1994; Vilcinskas *et al.*, 1997) and endocytosis (Dantuma *et al.*, 1999; Kang *et al.*, 1995). Recently, a viral immune suppressor CrV1 (Asgari *et al.*, 1997) was found to form a complex with lipophorin via coil-coil structures enabling the suppressor to gain access to the cytoplasm of hemocytes (Asgari and Schmidt, 2002).

2.6 Insect defence reactions

Insects are continuously exposed to potentially pathogenic microorganisms, but only few encounters result in infection. A complex defensive response induced in insects after infection is stimulated by non-pathogenic and a few pathogenic bacteria (Kaaya, 1993). Several observations suggest that there are similarities between invertebrate and vertebrate' defence systems. For example, insects have an immunoglobulin-like protein in hemolymph (Sun *et al*, 1990), and common antibacterial proteins (Boman and Hultmark, 1987). Although insects lack immunoglobulins, they possess a complex and efficient system of biological defence against pathogens and parasites (Pathak, 1993). This system involves the integument and gut as physical barriers to infection, coordinated responses of several subpopulations of hemocytes when these barriers are breached, and the induced synthesis of antimicrobial peptides and proteins. The defence mechanisms of insects principally involve both cellular and humoral responses.

2.6.1 Cellular reactions

The cellular immune reactions involve mainly the insect blood cells or hemocytes and consist of adhesive reactions of hemocytes against microbes or parasites. Insect cellular defense reactions have been studied for about 100 years. Changes in morphology, behavior and the types of cell involved during an infection have been widely investigated using microscopic techniques, lectins and monoclonal antibody markers (McKenzie and Preston, 1992; Strand, 1994; Theopold *et al*, 1996). Hemocytes phagocytose bacteria, trap microbes in nodules and encapsulate large parasitoid eggs (Vinson and Hegazi, 1998). Hemocytes are also involved in other immune responses such as the phenoloxidase cascade and hemolymph coagulation (Brehelin, 1979; Gregoire, 1974).

Hemocytes from different insect species vary in their morphological and functional characteristics. Among hemocytes, the granulocytes and plasmatocytes are thought to be the most important hemocyte types involved in insect cellular defence reactions (Ratcliffe, 1993). According to the number and size of the foreign invaders in the insect hemocoel, three major cellular defence reactions can be classified: phagocytosis, encapsulation and nodule formation.

2.6.1.1 Phagocytosis

Phagocytosis is a major defense reaction in arthropods and is accomplished by two hemocyte morphotypes, the granulocytes (GRs) and plasmatocytes (PLs), collectively called phagocytes (Gupta, 1991). This process is common to all

eukaryotic organisms and is primarily performed in vertebrates by dedicated phagocytes like neutrophils, macrophages and dendritic cells. Phagocytosis is an actin-dependent clathrin-independent process that leads to the uptake of particles (Cardelli, 2001). It consists of multiple stages that include (i) particle binding to the cell surface via interaction with receptor, (ii) activation of a signaling pathway that leads to temporal and spatial regulation of F-actin formation, (iii) pseudopodia extension, dependent on actin polymerization, to engulf the particle, vesicle trafficking from endo-lysosomal compartments, and (iv) removal of the actin coat from the newly formed phagosome, followed by fission and fusion reactions to generate a mature phagolysosome (Gupta, 1991)

At least two cell surface recognition systems have been proposed to exist that account for internalization of particles. One 'receptor' is proposed to mediate binding of particles such as bacteria containing terminal glucose residues, while the other 'receptor' (termed the non-specific receptor) mediates binding of particles, such as latex beads by more hydrophobic interaction (Cardelli, 2001).

Since phagocytosis is a ubiquitous process within the animal kingdom, occurring in all invertebrate phyla and vertebrates, it may be regarded as the primary cellular response against invading microorganisms. The main steps for phagocytosis are recognition, endocytosis, ingestion and killing (Gupta, 1991; Götz and Boman, 1985). These processes have been studied in different insect species. Generally, granulocytes and plasmatocytes are the major hemocytes involved in phagocytosis. The surface of the hemocytes contains binding sites that recognize cell wall components of foreign objects. Thus, when the foreign object encounters the

hemocytes, the surface components of its cell wall will be recognised by the hemocytes. The bound objects are then surrounded by pseudopods and ingested by coated vesicles. Finally the engulfed particles are lysed by lysozyme and antibacterial peptides (Götz and Boman, 1985)

2.6.1.1.1 Lectinophagocytosis

Many of the initial interactions leading to coagulation and phagocytosis reactions in insects are sugar-lectin binding reactions, involving homeostasis- and immune-related receptors, such as scavenger receptors (Franc *et al*, 1999) and mucin-like immune receptors (Theopold *et al*, 1996). A number of sugar-lectin interactions have been documented to be involved in phagocytosis events (Wilson *et al*, 1999). Interactions involving mucin-like glycoproteins containing Gal and GalNAc are particularly interesting given the presence of these receptors in immune related tissues, such as epithelial (Pendland and Boucias, 1996) and blood cells (Chen *et al*, 1993; Theopold and Schmidt, 1997). The *Drosophila* immune receptor hemomucin is a major glycoprotein on larval hemocytes (Kramerov *et a*l, 1997; Theopold *et al*, 1996) and hemocyte-like *mbn-2* cells (Theopold *et al*, 1996), which are used as a model system for the study of lectinophagocytosis. Addition of HPL to *mbn-2* cells causes immune induction (Theopold *et al*, 1996) and large endocytotic vesicles called macro-pinocytosis. Although the process of lectin-mediated endocytosis, which is frequently described as lectinophagocytosis, is mainly restricted to the uptake of microbes (Ofek *et al*., 1995; Oka *et al*., 1998; Pendland and Boucias, 1996; (Rainho et al., 1999), Gal-specific glycoconjugants (Pendland and Boucias, 1996), the corresponding lectins (Abe *et al*, 1999) may play a central role in the initiation

and functional completion of endocytotic processes in general. We therefore use the term lectinophagocytosis in a broad context including endocytosic processes involving microbes, inert objects, and soluble substances, such as bacterial toxins.

2.6.1.1.2 Endocytosis and cell adhesion: Integral part of cell signalling

Endocytosis is the process that occurs in all eukaryotic cells by which cells internalise portions of the plasma membrane, including associated proteins and a portion of the extracellular space. This is the route by which cells absorb nutrients, recycle membrane proteins and lipids, receive some chemical and molecular signals, and down-regulate membrane receptors in response to ligands and/or environmental changes (Cardelli, 2001). At least two cell surface recognition systems have been proposed to exist that account for internalization of particles. One 'receptor' is proposed to mediate binding of particles such as bacteria containing terminal glucose residues, while the other 'receptor' (termed the non-specific receptor) mediates binding of particles including latex beads by more hydrophobic interaction (Cardelli, 2001).

2.6.1.2 Encapsulation

Foreign particles such as nematodes, insect parasitoids and copepods, too large to be phagocytosed are often encapsulated. In contrast to phagocytosis, encapsulation is a multicellular process. Similar to phagocytosis, both the granulocytes and plasmatocytes are involved. However, in *D. melanogaster,* which is lacking

granulocytes, the plasmatocytes and their modification, lamellocytes are the cells forming the capsule around the foreign intruders (Russo *et al,* 1996).

The major steps for encapsulation are the recognition of foreign invaders by granulocytes, the degranulation of the granulocytes on the surface of non-self objects, the attraction of plasmatocytes and the formation of a capsule (Rowley and Ratcliffe, 1981). However, Pech and Strand (1996) reported that granulocytes complete the capsule. Wiegand *et al* (1996) also showed that plasmatocytes initiated a cellular encapsulation or aggregates around large particles during *in vitro* culture.

2.6.1.3 Nodule formation

Some invertebrates that are unable to deal with large numbers of small foreign particles, such as bacteria, using phagocytosis, use nodule formation in addition (Ratcliffe, 1993). This defence response includes hemocyte degranulation followed by the formation of round spheres around microorganisms and microbial debris. Generally, nodules comprise a central core of entrapped foreign particles, surrounded by multicellular haemolytic aggregates. This formation can be induced by surface components of foreign invaders such as LPS, zymosan, laminarin and some glycoproteins (Lackie, 1988). During nodule formation, the granulocytes degranulate after having recognised the intruder. The degranulated and lysed granulocytes together with the entrapped and phagocytosed foreign particles form a coagulum. Around this coagulum, further hemocytes start to attach and flatten, finally forming large multicellular hemocytic aggregates or nodules. The nodule may adhere to tissue and eventually become melanised.

2.6.2 Humoral related reactions

Many studies have been conducted to examine the roles and factors of the humoral related system in insects. These reactions principally comprise a number of inducible antibacterial proteins and peptides in the hemolymph. In addition, some findings suggest that the immune response induced by bacterial infection involves the production of both anti-bacterial molecules and proteins with functions not yet fully understood (Shiotsuki and Kato, 1999). Recent studies using insect hemolymph have detected the presence of a variety of proteins formed in response to elicitors, which can be divided into inducible bactericidal proteins and inducible nonbactericidal proteins.

2.6.2.1. Inducible bactericidal proteins

Bacterial proteins are known to play an important role in insect immunity against bacterial infection. In insects, the synthesis of antibacterial peptides is induced by bacterial challenge and commonly requires several hours for their effect to be recognized (Koizumi *et al*, 1999). Today, more than 150 microbial peptides have been characterized from various insect species, and the peptides are often grouped into four families based on sequence similarity (Hoffmann and Reichhart, 1997).

The cecropins, the first inducible antibacterial peptides to be characterized, were found first in *H. cecropia* and later in a number of other insect species (Boman, 1991). These proteins are small and basic with a sequence of 37 amino acid residues (Qu *et al*, 1987), representing an important part of the inducible humoral related of lepidopteran and dipteran insects. Cecropins are frequently C-terminally amidated

and kill gram-positive and gram-negative bacteria (Lamberty *et al*, 1999). They have a molecular mass of 4 kDa. Although the mechanism of cecropin induction by bacterial infection is not completely understood in detail, some bacterial cell wall components such as LPS, peptidoglycan and beta-1,3-glucan (Boman, 1991) have been identified as inducers. This highlights the importance of elicitor clearance systems. Kato *et al* (1994) demonstrated that *B. mori* has an LPS clearance system in the hemolymph, which is sufficient to terminate LPS mediated antibacterial protein induction.

The attacins are larger than the cecropins, forming a family of closely related proteins with molecular weights around 23 kDa that have been identified in several lepidopteran and dipteran species (Hultmark *et al*, 1980). These proteins have narrow antibacterial spectra and are active only against gram-negative bacteria (Gillespie *et al*, 1997).

Insect defensins are active against gram-positive bacteria. They have been found in many insect orders and belong to a group of cysteine-rich peptides with molecular masses between 2 and 6 kDa (Lamberty *et al*, 1999). These proteins were originally identified in *Sarcophaga peregrina* and *in Phormia terranovae* (Gillespie *et al*, 1997). Two other antifungal activities in this group have been characterized. Drosomycin from the dipteran *D. melanogaster* (Fehlbaum et al., 1994) and a 5 kDa peptide with eight cysteine residues that are engaged in the formation of four intramolecular disulfide bridges (Lamberty *et al*, 1999). This peptide is inactive against bacteria but shows antifungal activity. Thanatin from the hemipteran *Podisus*

maculiventris (Fehlbaum *et al*, 1999) exhibits both antifungal and antibacterial activities (Lamberty *et al*, 1999).

Lysozymes occur constitutively in insects, but can also be induced by prior immunization (Boman and Hultmark, 1987). This enzyme hydrolyses beta-1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan of bacterial cell walls. Lysozyme is a naturally occurring enzyme found in the hemolymph of most Lepidoptera and tends to increase in abundance at the same rate as protective immunity develops, but persists well beyond the duration of bactericidal activity (Millar and Ratcliffe, 1994). The main role of lysozyme in insects is its specific hydrolysing activity (Boman and Hultmark, 1987), because it degrades the bacterial cell wall of food and potential invaders. Pathak (1993) reported that both heat-killed and live bacteria stimulate lysozyme production in adult tsetse flies.

Another groups of inducible antibacterial peptides are the proline-rich peptides that have been isolated from a number of insect species such as Hymenoptera, Diptera, Lepidoptera and Hemiptera (Gillespie *et al*., 1997). Some proteins in this group such as drosocin and metchnikowin from *D. melanogaster* and lebocin from *B. mori* are O-glycosylated (Gillespie *et al.*, 1997; Lamberty *et al*, 1999). Some members of this group are reported to be active against gram-negative and gram-positive bacteria as well as fungi (Lamberty *et al*, 1999), apparently interacting with the bacterial cell membrane.

2.6.2.2 Inducible nonbactericidal proteins

Since a number of proteins that have no direct bactericidal action have been identified, it is necessary to analyze these proteins for possible participation in the defence system and its involvement in novel insect defence reactions. An example of inducible proteins, which have no bactericidal activity, is an inducible glucose-binding lectin of *M. sexta* (M13), which appears to be involved in coagulation (Spence and Minnick, 1991)

Other findings by Minnick et al (1986) showed that protection against pathogenic microorganism correlates with the anatomical and biochemical changes of the midgut lumen. For example, using oral feeding approaches, Rupp and Spence (1985) demonstrated that a sub-lethal dose of the crystal endotoxin of *B. thuringiensis*, ingested into the midgut of several lepidopteran insects, induce a number of proteins in the midgut and hemolymph. This is assumed to be the result of toxin damaged midgut cells.

2.7 Aims of the Study

2.7.1 *B. thuringiensis* toxicity

2.7.1.1 The process of receptor-mediated endocytosis in insects

The cellular capacity to internalise objects, involving attachment, engulfment and uptake by endocytotic processes, exists in virtually all organisms. Many endocytosis reactions are based on cell-cell interactions. However, the mechanisms that cause the formation of endocytotic vesicles are not known. This is particularly true for macropinocytosis, which involves the formation of large vesicles containing soluble components.

Since only soluble molecules are internalized during macropinocytosis, this type of endocytosis is cited as a typical example for RME (Dantuma *et al.*, 1999; Hussain, 2001; (McPherson et al., 2001). The driving forces for the inverse membrane curvature are believed to be located in the cytoplasm and lectins are assumed to act as a trigger for the signaling process. In this context, the binding of the carbohydrate-recognising domain (CRD) to the glycodeterminant of the receptor is essential for signal transduction, which implies that monomeric lectins are expected to be functional ligands. In this context, therefore, arises the question whether macropinocytosis is induced by monomeric lectins. The major aims of this study are: (1) To test the induction of cell-spreading and macropinocytosis, (2) To investigate the role of lectins in coagulation reactions.

2.7.2 Possible alternative mechanisms of Bt-toxin insertion into the membrane during toxicity processes

Many pore-forming toxins bind to specific sugar moieties on glycoproteins that have been described as receptors on the cell surface (Cortajarena et al., 2001; (Frankel et al., 1996); Hatakeyama et al., 1996; Konska et al., 1994; Mercy and Ravindranath, 1994; Saha and Banerjee, 1997; Sandros et al., 1994; (Tonevitsky et al., 1996). We suggest that toxin-insertion into the membrane may be facilitated by an endocytosis mechanism, where the toxin interacts with receptors as an oligomeric lectin. We tested this assumption using the bacterial endotoxin of *B. thuringiensis.* Present models assume that Bt-toxin is unable to form a tetrameric complex in the absence of lipid, since assembly into a tetrameric channel-forming complex occurs inside the membrane bilayer. However this study will present a possible alternative model of the Bt toxin insertion into the membrane that differs from present models. The specific aims of this study are: (1) To examine, whether lectins have similar functions to the Bt-toxins, (2) To determine whether Bt binding proteins are involved in coagulation reactions.

2.7.3 Immune Induction in insects resistant against low levels of Bt toxin

We have recently observed that insect cells internalize lectins and insect toxins after forming a complex with lipophorin. This raises the question whether Bt-toxicity and Bt-resistance mechanisms are functionally correlated with a dual function of lipophorin-like molecules. In this context, toxin-insertion into the lipid membrane-bilayer may be mediated by an endocytosis reaction of a membrane-

bound lipoprotein-toxin complex, whereas toxin-inactivation may be caused by a coagulation reaction with soluble lipophorin molecules. To test this assumption, we examined the immune-status of Bt-resistant and susceptible insects *of H. armigera* using melanisation assays. We wanted to test the assumption whether immune reactions in the Bt-resistant strain are constitutively induced. If Bt resistance is based on immune induction this strain may also be resistant to other pathogens. Both toxin and pathogen resistance may be based on changes in immune components in the gut lumen. The study therefore includes the localisation of immune proteins using confocal microscopy. The aim of this project is to explore a large area of different experimental approaches and to bring together concepts of immunity, physiology, toxicology and molecular biology, the time limitation prevented investigations beyond exploration stages. Therefore, the experiment will be put into experimental context by summarising it as "Results and Discussion" in a single chapter. Conclusions and working hypotheses will be discussed in a "General Discussion"chapter.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Chemicals

Acrylamide	Sigma Chemical Co., St. Louis, MI, USA
Agarose	Sigma Chemical Co., St. Louis, MI, USA
Ammonium persulphate	ICN Biomedicals Inc., Aurora, OH, USA
Benzamidine hydrochloride	Sigma Chemical Co., St. Louis, MI, USA
Boric acid	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)	Sigma Chemical Co., St. Louis, MI, USA
Bromophenol blue	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Coomassie brilliant blue R250	Sigma Chemical Co., St. Louis, MI, USA
EDTA	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Formaldehyde	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Freund's adjuvants (complete and incomplete)	Sigma Chemical Co., St. Louis, MI, USA
Glycine	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Hydrochloric acid	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Hydrogen peroxide	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Imidazole	Sigma Chemical Co., St. Louis, MI, USA
IPTG	Sigma Chemical Co., St. Louis, MI, USA
Isopropanol	BDH, Merck Pty. Ltd., Kilsyth, Vic.,
Kanamycin	Sigma Chemical Co., St. Louis, MI, USA
KBr	Sigma Chemical Co., St. Louis, MI, USA

KH_2PO_4	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Low-melting agarose	Bio-Rad Laboratories, Hercules, CA, USA
Magnesium chloride	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Magnesium sulphate	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Maltose	Sigma Chemical Co., St. Louis, MI, USA
ß-Mercaptoethanol	Riedel-deHaën AG, Seelze, Germany
Methanol	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Mineral oil	Sigma Chemical Co., St. Louis, MI, USA
MOPS	Sigma Chemical Co., St. Louis, MI, USA
NADPH	Sigma Chemical Co., St. Louis, MI, USA
$NaHCO_3$	Sigma Chemical Co., St. Louis, MI, USA
Na_2HPO_4	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
NITRO BLUE TETRAZOLIUM (NBT)	Sigma Chemical Co., St. Louis, MI, USA
NH_4HCO_3	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
NP-40	Sigma Chemical Co., St. Louis, MI, USA
PFA	Sigma Chemical Co., St. Louis, MI, USA
Phenol	Sigma Chemical Co., St. Louis, MI, USA
Polyvinylpyrrolidone	Sigma Chemical Co., St. Louis, MI, USA
Ponceau S	Sigma Chemical Co., St. Louis, MI, USA
Potassium acetate	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Potassium chloride	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
PTU	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
RbCl	Sigma Chemical Co., St. Louis, MI, USA
SDS	BDH, Merck Pty. Ltd., Kilsyth, Vic.,

	Australia
Sodium azide	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Sodium chloride	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Sodium citrate	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
Sodium hydroxide	APS Ajax Finechem, Auburn, NSW, Australia
Sucrose	BDH, Merck Pty. Ltd., Kilsyth, Vic., Australia
TEMED	Progen Industries Ltd., Darra, Qld., Australia
TFA	Sigma Chemical Co., St. Louis, MI, USA
Triethanolamine	ICN Biomedicals Inc., Aurora, OH, USA
Trizma Base	Sigma Chemical Co., St. Louis, MI, USA
Tryptone	Oxoid, Basingstoke, Hampshire, England
Tween-20	Sigma Chemical Co., St. Louis, MI, USA
X-gal	Promega Corp., Madison, WI, USA
Yeast extract	Oxoid, Basingstoke, Hampshire, England

3.1.2 Enzymes and Proteins

Protein kaleidoscope standard, prestained	Bio-Rad Laboratories, Hercules, CA, USA
Protein markers, SeeBlue® & Mark12®	Novex, San Diego, CA, USA
Proteinase K	Sigma Chemical Co., St. Louis, MI, USA

3.1.3 Antisera and Lectins

Goat anti-rabbit IgG (Fc), alkaline phosphatase-conjugated	Pierce, Rockford, IL, USA

Goat anti-rabbit IgG, FITC-conjugated	Sigma Chemical Co., St. Louis, MI, USA
Lectins peroxidase labelled	Sigma Chemical Co., St. Louis, MI, USA
Unlabelled Lectins	Sigma Chemical Co., St. Louis, MI, USA
FITC and TRITC labelled lectins	Sigma Chemical Co., St. Louis, MI, USA

Lectins used are also listed in table 2.

3.1.4 Commonly Used Solutions

Antifade solution	2% (v/v) 1,4-diazabicyclo-(2,2,2)-octane in 9 parts glycerol : 1 part 1M Tris-HCl pH 7.5
PBS	1.47 mM KH_2PO_4, 7.3 mM NaH_2PO_4, 138 mM NaCl, 2.7 mM KCl; pH 7.5
Phenol	Saturated with 10 mM Tris-HCl pH 8, 1 mM EDTA; pH 6.7
SDS gel-loading buffer (3x)	100 mM Tris-HCl pH 6.8, 20% glycerol, 10% (v/v) Me, 4% SDS, 0.2% BPB
SM buffer	Per liter: 5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml 2% gelatin Solution; autoclave for 20 min at 15 lb/sq. in. on liquid cycle
Sonication buffers	50 mM NaH_2PO_4, 300 mM NaCl; Adjust to pH 8 with NaOH
TAE	40 mM Tris-acetate, 1 mM EDTA
TBST	10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20
Wash buffer	50 mM NaH_2PO_4, 300mM NaCl, 10% glycerol; pH adjusted to 6.0 with NaOH

3.2 Methods

3.2.1 Insect culture

Galleria mellonella larvae were reared on an artificial diet in the dark.

Drosophila melanogaster Canton S flies were kept on cornmeal/yeast food at a photoperiod of 14 h light: 10 h dark at 25^{o}C.

Fourth instar of *P. xylostella* larvae, used in the described experiments, were reared on fresh cabbage leaves at 23^{o}C with a photoperiod of 16 hrs light and 8 hrs darkness.

H. armigera strains were reared on artificial diet. The Cry1Ac resistant lab-selected population and the susceptible lab population were obtained as eggs from Dr Ray Akhurst (CSIRO Entomology Division, Canberra). Both of these populations were reared at 25^{o}C with 50-60% RH. Multi-well (Falcon) trays and small plastic cups (28 ml) were used for rearing small and large larvae respectively. The recipe for preparing the artificial diet was the same as used by CSIRO (Entomology) at the Australian Cotton Research Institute (ACRI) as follows:

1 liter	hot water (+ approx.400 ml to top up)
60 g	wheat germ
53 g	Brewers yeast
130 g	soybean flour
3.3 g	nipagin (methyl 4-hydroxybenzoate)
1.7 g	sorbic acid
13.5 ml	10% formaldehyde
2 teaspoons	cold pressed sunflower oil (if flour is processed)
24 g	agar
300 ml	water
add 5.3 g	ascorbic acid

3.2.2 Cell Culture

mbn-2 cells were kept in Schneider medium at 25°C (LanzMendoza et al., 1996) and *Plutella* cells were kept in lepidopteran medium. Schneider cells are derived from embryonic tissues of *Drosophila melanogaster*. *mbn-2* cells are derived from a blood tumor mutant (Gateff et al., 1980). These cells were grown in Schneider's Insect Medium supplemented with 10% FCS and grown in the dark at 25°C. Schneider and *mbn*-2 cells were supplied by the Biochemistry Department at the University of Adelaide.

3.2.3 Bt-toxin and resistant insect strains

The Cry1Ac protoxin was purified from *B. thuringiensis* subsp. *kurstaki* HD73 (Aronson *et al*, 1999). Protoxins were solubilized in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6 and digested with trypsin or gut juice extracts. Digested toxin was sterilised by passing through a FP point 2 filter (0.2 µm) and used after dialysis against PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6,8) buffer.

According to (Akhurst et al., 2002) a laboratory strain of *H. armigera* was selected for Bt-resistance after being exposed to low levels of Cry1Ac. Resistant insects were repeatedly crossed with susceptible insects before subsequent exposure to the toxin. Crosses were repeated four times resulting in nearly isogenic resistant (ISOC4) and susceptible (ANGR) insect strains R. Akhurst, pers. comm.).

3.2.4. Isolation of hemolymph, hemocytes, fat body and gut

Final instar *G. mellonella* larvae were surface sterilised with 70% ethanol and a pair of prolegs was cut off with microscissors. The larvae were bled into a drop of insect ringer solution on a piece of Parafilm 'M' (American National Can, Chicago, IL, USA) on ice. To avoid melanogenesis, 10 larvae were bled into 200 ml of insect ringer and immediately centrifuged at 760g to precipitate the hemocytes. The recovered supernatant is the cell-free hemolymph.

To isolate fat body and gut, the sterilised caterpillar was dissected in PBS with fine forceps under a stereo-microscope. Fat body and gut were collected in PBS and kept at $-80^{O}C$ or used directly to extract soluble proteins. Fat body tissue was mixed and incubated with 2X volume of DDW and homogenized. Cell debris was pelleted by centrifugation at 13,000 rpm (full speed) for 10 minutes, and supernatant, containing most of the hemolymph-specific proteins, was used immediately for experiments to avoid spontaneous aggregation.

3.2.5 SDS polyacrylamide gel electrophoresis

SDS-PAGE was used to separate proteins according to their size (Laemmli, 1970). SDS, an ionic detergent, denatures the proteins as they migrate through the gel due to electric current. SDS-PAGE consists of a stacking and a separating gel. The stacking gel on the top of the separating gel, concentrates the proteins whilst the separating gel separates the proteins based on size. 30% prepared solution of 19.2:0.8

(acrylamide:Bis-acrylamide) was used to prepare 7.5% vertical SDS PAGE gels. Proteins were dissolved in loading buffer and heated for 10 minutes at 65°C. Gels were run at 25 mA for approximately 1 1/2 hr on Mini Protean II Dual Slab Cell until dye was 0.5 cm from the bottom of the gel. SeeBlue^{TM} Pre-stained molecular weight standards were used to determine the size of the protein samples.

3.2.6 Western blots

Proteins separated by SDS- PAGE were transferred to nitrocellulose filter at 200 mA for 1 hr using the Mini Trans - Blot Electrophoretic. The transfer time of proteins extracts to the nitrocellulose filter was kept low so that the glycoproteins would not blot through the filter. The gel was soaked in transfer buffer for 5 min. The Whatman paper, nitrocellulose and sponges were also soaked in transfer buffer and added to the holder in this order: sponge, 1 x Whatman paper, nitrocellulose, gel, 4 x Whatman paper and sponge. After transfer, the nitrocellulose filter paper was washed 3 times in 1 x TBST for 5 min each and then once for 10 minutes.

3.2.7 Lectin staining

Lectins conjugated with peroxidase (PNA lectin was added to the nitrocellulose filter at a 1/10,000 concentration, diluted in 1 x TBST. The nitrocellulose filter was incubated at room temperature overnight with gentle agitation. The filter box was covered with aluminium foil to prevent evaporation. The filter was washed with 1 x TBST three times for 5 min and then once for 10 min. 30 µl of H_2O_2 and a small amount (1mg) of DAB were added to 30 ml of peroxidase developing solution and left at room temperature with gentle agitation until bands of brown precipitate were

detected. The detection reaction was stopped with H_2O and the nitrocellulose filter air-dried.

3.2.8 Antibody staining

Polyclonal serum from rabbits challenged with various insect proteins was used to detect protein on nitrocellulose filters. Polyclonal serum was diluted to a 1/10,000 concentration in 1 x TBST and added to the nitrocellulose filter. The filter was incubated at room temperature overnight with gentle agitation and covered with aluminium foil to prevent evaporation. The filter was washed with 1 x TBST 3 times for 5 min and then once for 10 min. Alkaline Phosphatase labelled secondary antibody, usually goat anti-rabbit, was added at a 1/10,000 dilution in 1 x TBST and incubated at room temperature for 2 hr, covered with aluminium foil to prevent evaporation. The filter was washed in 1 x TBST 3 times for 5 min and once for 10 min. 100 µl of NBT and 50 µl of BCIP was added to 50 ml of Alkaline Phosphatase developing solution composition. The filter was left at room temperature with gentle agitation until purple bands appeared. The colour reaction was stopped with H_2O and the nitrocellulose filter air-dried.

3.2.9 Coomassie staining

10 % SDS-PAGE was performed to separate proteins due to size. The gel was stained with Coomassie stain solution to detect the protein concentration in each sample. A 10 % SDS-PAGE was loaded and run as described in 3.2.7.1. The gel was removed from the holder and washed in DDW. The gel was covered in Coomasie Stain and incubated for 30 min with gentle agitation. The Coomassie stain was returned to the bottle and the destain solution added to the gel. The gel was incubated for 30 min with gentle agitation. The destain solution was returned to the bottle and new destain solution added to the gel and left overnight with tissue paper to absorb the colour. The gel was removed and placed on wet Whatman paper and wrapped in plastic wrap to preserve.

3.2.10 Preparation of antiserum

For the preparation of a specific antiserum, proteins were purified by differential centrifugation and further purified on a preparative polyacrylamide gel and used for immunization. The proteins band was cut from the gel and mixed with an equal volume of complete Freund's adjuvant for first injection and incomplete Freund's adjuvant for further boosters. Immunisations were performed according to (Harlow and Lane, 1988).

3.2.11 Endocytosis assays

Cell lines from *mbn*-2 *and Plutella xylostella* were placed into tissue Culture Plates (24 well) with 1 ml of medium until the cells attached onto the surface. After attachment, the cells in each well were treated with different lectins in different concentrations (0.5, 1.0 and 2 ul/ ml medium) from 1 mg/ml lectin stock solution. The treated and non-treated cells were kept at room temperature and observed after 3 hr, 6 hr, 9 hr, 24 hr, 48 hr and 72 hr after treatment. The percentage of endocytic and foam-like cells were recorded.

3.2.12 Low-density gradient centrifugation

Lipophorins were isolated from whole larval hemolymph by single spin density gradient ultracentrifugation in a Beckman SW28 (Swing) rotor, using a procedure modified from (Chung and Ourth, 2002). Fifth instar larvae were bled through prolegs into phosphate buffered saline (PBS, 0.15 M NaCl)/0.10M sodium phosphate /0.1 % of EDTA pH 7.0) containing phenylthiourea (PTU) to inhibit melanization. Approximately 0.5 ml hemolymph was collected per gradient (usually about 10 *G. mellonella* larvae). Hemocytes were sedimented by centrifugation for 15 minutes at 5000 rpm, the supernatant collected, and 44.3 g KBr/100 ml of PBS solution were added. The KBr/hemolymph mixture was placed into Beckman 39 ml tube, 19.5 ml/tube, and overlaid with another 19.5 ml of fresh 0.9 % NaCl. Tubes were placed into a Beckman SW28 rotor and centrifuged at 24,000 rpm overnight at 10°C and 20 fractions (ca 1,5 ml each) were collected from the bottom of the tube using a glass needle. Fractions were measured for their density by weighing equal volumes and desalted in dialysis tubes or in MicroSpin G-25 columns using PBS. Aliquots were

analyzed by SDS–PAGE electrophoresis. Gradients were conducted with additional components mixed with hemolymph such as lectins and Bt-toxins.

3.2.13 Insect gut staining

Gut tissues were dissected from caterpillars and midgut separated with microscissors leaving the peritrophic membranes intact and fixed in paraformaldehyde (4%), containing 0.5% Tween 20 in the presence or absence of PTU for several hours. After extensive washing overnight, gut tissues were treated with gut juice-activated Cry1Ac (ca 10ug/ml), antibodies against Cry1Ac (1:1000 dilution), and (FITC or TRITC)-conjugated secondary antibodies (1:5000 dilution) by incubating for at least four hours, followed by four washing steps each. Lectins (conjugated with FITC or TRITC) were applied (0.1ug/ml) together with the secondary antibodies in double-staining experiments, using different combinations of fluorescent lectins and antibodies. To keep unspecific staining to a minimum, incubations were performed in the presence of PTU to prevent coagulation or melanisation reaction during the process.

3.2.14 Coagulation and melanization assays

Hemolymph from a single 5^{th} instar *H. armigera* caterpillar was collected in 1.5 ml ice-cold PBS containing PTU and hemocytes were removed by centrifugation (5,000g, 5 min at 6^{o}C). Supernatant was divided into aliquots, and lectins and Cry1Ac (0.1 ug each) were added and mixed gently. After 5 min at room temperature the mixture was centrifuged for 5 min at 13 000g and loading buffer added to both pellet and supernatant and analysed by SDS-PAGE. For melanisation assays, cell-free

hemolymph from a single caterpillar was collected in the absence of PTU and light absorbance measured at a wavelength of 490 nm. Gut extracts were obtained by homogenizing gut tissue in 1 ml PBS and removing debris by centrifugation (5,000g, 5 min) and measuring light absorbance of the supernatant in the presence of 10 mM DOPA.

3.2.15 Baculovirus assay

Similar sized third instar larvae from resistant (ISOC4) and susceptible (ANGR) *H. armigera* strains were fed with suspensions of 10^7/ml of *Autographa californica* multiple nuclear polyhedrosis virus (*Ac*MNPV). Since *H. armigera* is semipermissive to *Ac*MNPV, lethality was determined by counting the dead larvae over several days. Each treatment was performed with at least 20 caterpillars.

3.2.16 Immune-induction assay

Fourth instar larvae from diamondback moth (*P. xylostella*) were fed with leaf dip assays bioassay method. Leaf discs of 10 cm diameter were cut with a metal punch hole from the fully expanded leaves of Chinese cabbage (Pak Choi) plants. Leaf disc were dipped for five seconds in freshly prepared distilled water suspensions of various inducers such as Bt-toxin (Delfin WG) (1 ppm), heat-killed Bt (1 ppm), *E.coli* (1 ppm), Bt-toxin (Delfin WG) (0.1 ppm), heat-killed Bt (0.1 ppm), *E.coli* (0.1 ppm), combination of all inducers and control (distilled water) in 100 ml beaker. Each leaf disc was placed on a corrugated sheet of aluminium foil for drying at room temperature. The leaf discs were placed into petri dishes of 14 cm diameter

containing a single 70 mm filter paper (*Whatman No.1) moistered with distilled water. 10 larvae of fourth instar were placed in each petri dish. After 48 hrs the survivors from each treatment were dissected for induction of p85.

Chapter 4 : Results and discussion

4.1 Resistance to *B. thuringiensis* endotoxin

4.1.1 A soluble Bt-binding protein in the gut lumen

It is widely accepted that there is more than one *Bt*-binding protein in the insect midgut. The proteins that bind *Bt*-toxin in lepidopteran insects described so far are aminopeptidase N proteins of 120 to 220 kDa in size, which are integral parts of the brush border membrane vesicle (BBMV) fraction (Martinez-Ramirez *et al.*, 1994; Knight *et al.*, 1994; Sangadala *et al.*, 1994). In addition, cadherin-like receptors have been associated with high levels of resistance (Gahan *et al.*, 2001).

However, when proteins were extracted under denaturing conditions directly from the gut, additional *Bt*-binding proteins were isolated. For example, in the diamondback moth (*P. xylostella*), an 85 kDa protein (p85) was found as the major glycoprotein band labelled with both the Bt-toxin and HPL (Fig 4). This suggests that under denaturing conditions (SDS-PAGE and Western blots), Bt-toxin and HPL bind predominantly to p85. The fact that p85 is not found in BBMVs suggests that the glycoprotein is probably lost during the BBMV fractionation process. Therefore, it is probably not attached to the brush border membrane micro-environment, but is prevalent in the gut lumen of dissected larvae and in the whole insect. This glycoprotein shows weak Coomassie staining in several tissues compared to the strongly stained triple bands at 78, 80, 82 kDa, which according to size comparisons, correspond to arylphorin, phenoloxidase (PO) and apolipophorin II (Fig 5A). With HPL-staining the p85 protein is the predominantly labelled glycoprotein (Fig 5B). p85 glycoprotein is detected in several tissues of DBM, such as gut, fat body,

hemolymph and gut content (Fig 6). In hemolymph and fat body p85 frequently co-exists with glycoproteins of 150 and 230 kDa in size, which have the same glycodeterminants (Fig 7). The presence or absence of p85 is correlated with a 150 kDa protein (Fig 7), which could suggest that p85 is derived from the 230 kDa protein by proteolytic cleavage (see also Fig 10C). This and other observations suggest that p85 is probably produced in the fat body and released into the gut lumen (Fig 5A). The observation that isolated peritrophic membrane contains relatively low amounts of p85 (Fig 5B), suggests that the protein is associated with, but not a structural part of the peritrophic membrane.

To analyse the possible function of p85 as a toxin-binding protein further, resistant and susceptible DBM larvae were examined (kindly provided as ethanol-fixed caterpillars by Dr. Tony Shelton, Cornell University, Ithaca, USA). In the resistant DBM strain, HPL binding to p85 is stronger than in the susceptible strain (Fig 8). A possible explanation for this is that the amount of p85 in the gut lumen is increased in the resistant strain. Since resistant DBM larvae are available in Australia only in ethanol fixation due to quarantine restrictions, detailed comparison with susceptible larvae was not possible. We therefore used nearly isogenic resistant and susceptible strains of *Helicoverpa armigera* (kindly donated by Dr. Ray Akhurst, CSIRO Canberra) to confirm the DBM observations and to study the possible role of p85 further. In contrast to DBM, HPL staining of p85 is variable, but on average stronger in susceptible larvae *of H. armigera* compared to those of the resistant strain (Fig 9). This suggests that p85 differs in resistant and susceptible strains in both species. The observation that the relative amounts of p85 in resistant and susceptible strains is opposite in the two species could indicate that p85 may be highly unstable and that

extraction of Bt-binding proteins under different conditions may yield variable amounts.

Recently, the identification of glycoproteins involved in coagulation revealed a similar variation (Li *et al*, 2002). For example, proteins of immune-induced larvae formed non-soluble complexes, whereas proteins from non-induced larvae remained soluble (D.Li, unpubl. data). Given its presence in hemolymph, one possible role of p85 would be an involvement in coagulation reactions.

4.1.2 Coagulation of a GalNAc-containg protein (p85) in the resistance strain

The coagulation of insect hemolymph requires the interaction between soluble hemolymph components and cellular components derived from hemocytes (Bohn, 1986). This process is important in insect defence reaction to inactivate microbes and to seal off wounds upon injury to the body wall (Gregoire, 1974).

To confirm specific binding of the toxin to native (non-denatured) p85, cell-free hemolymph was mixed with various lectins and toxin. After short incubation times protein-aggregates were subsequently removed by centrifugation. Analysis of the pellets revealed two bands at 85 and 80 in all fractions, but no differential amounts of re-dissolved aggregates due to the lectin specific precipitation (Fig 10). Analysis of the remaining hemolymph supernatant revealed a selective removal of the 85 kDa protein in mixtures containing Cry1Ac and oligomeric GalNAc-specific lectins, such as HPL and *Vicia villosa* lectin (VVL) (Fig.10B). In the presence of monomeric GalNAc-specific lectins, such as WBL and Gal-specific tetrameric lectins, such as PNA, the 85 kDa protein remained in solution (Fig 10A). This suggests that

oligomeric GalNAc-specific lectins and Cry1Ac interact with hemolymph proteins to form large protein aggregates. The observation that the lectin-binding proteins were not recovered from the pellet, suggests that these aggregates were insoluble, whereas spontaneous aggregates were found in all hemolymph fractions, which were (at least partly) dissolved in loading buffer. Lectin-mediated aggregation also occurred in protein extracts from fat body, where apolipophorin I (230 kDa) was precipitated by GalNAc-specific lectins similar to those that precipitated p85 in the hemolymph (Fig 10C). Taken together these observations suggest that lectin-binding to p85 occurs under native conditions, causing aggregation of p80 (apolipophorin II) and p85 in the hemolymph. When hemolymph from resistant *H. armigera* caterpillars was analyzed on SDS-PAGE, most lipophorin-specific hemolymph proteins detected in the susceptible strain are absent (Fig 9). This could indicate that hemolymph from resistant caterpillars is more reactive, causing spontaneous aggregation into insoluble protein complexes.

Since p85 is probably produced in fat bodies, extracts of fat body cells were examined on Western blots. Binding studies using different lectins, indicate that p85 probably consists of two glycoforms in the fat body, namely a GalNAc-specific glycoform (HPL form) of ca 85 kDa and a Gal-specific glycoform (PNA form) of ca 88 kDa (Fig 11). In addition to p88, PNA-staining revealed a strong staining at the top of the gel, which could suggest that PNA-stained glycoproteins form high molecular-weight aggregates. These findings raise the question whether the two p85 glycoforms are involved in coagulation. The observed difference between p85 and p88 may be due to the absence or presence of specific lectins in the protein extract. For example, a Gal-specific lectin involved in immunity, was found to be expressed

in the fat body (Natori, 2002), and PNA-like lectins are present in large amounts in hemolymph (Castro et al, 1987).

In contrast, GalNAc-specific lectins are either not present or are not very active in hemolymph. If the two glycoforms are able to react with lectins to form coagulation products, the glycoproteins may be prevented from reacting with hemolymph-specific lectin by separation of the glycoproteins in hemocyte secretory granules. For example Gal-containing glycoproteins are stored in granules and only form coagulation product if released by immune-induction into hemolymph, which contains Gal-specific lectin. Conversely, GalNAc-containing glycoproteins are abundant in hemolymph but are not exposed to GalNAc-specific lectins, unless these are released from hemocytes or activated from inactive forms in the hemolymph.

To explore this question, hemocytes from the two strains of *H. armigera* were analysed by lectin-staining of hemocytes after fixation in the presence of mild detergents (Triton X100 or Tween20), which allows cytoplasmic granule contents to be stained. When hemocytes from resistant and non-resistant strains were examined with a GalNAc-binding lectin (HPL) and a Gal-binding lectin (PNA), the two lectins produced inverse staining patterns. Hemocytes from the non-resistant strain stained more strongly with HPL and less with PNA, whereas hemocytes from the resistant strain bound more PNA and less HPL (Fig 12). Under these conditions the major staining was located in large granules, located in the cytoplasm of hemocytes. This was confirmed in granulocytes that were extensively spread on the glass surface (Fig 13). In preparations where the cells were allowed to spread for extended time periods in the presence of hemolymph deposits, hemocytes expanded over a large area

displaying individual granules, which can be detected in phase contrast microscopy (Fig 13). Since most granules became black during fixation reactions due to melanisation reactions, these granules are likely to be secretory vesicles containing immune components, such as PO.

To confirm Bt-toxin binding to GalNAc-specific glycoproteins, we incubated hemocytes with HPL and PNA after fixation in the absence of detergents, where only the cell surface was stained. Under these conditions HPL stained granulocytes and plasmatocytes (Fig 14A), whereas PNA only stained granulocytes (Fig 14 B). When Bt-toxin was bound to hemocytes both granulocytes and plasmatocytes were stained (Fig 14 C), which suggests that Bt-toxin correlates with HPL-staining. Since PNA-staining is also found in granules inside the granulocytes (Fig 15), this indicates that PNA-binding may be restricted to an immune-reactive component stored inside granulocytes.

This suggests that hemocytes may accumulate Gal-containing glycoforms inside granules. Since PNA-glycoforms differ from HPL-glycoforms by the addition of galactose, a galactosyltransferase may add a galactose molecule to existing GalNAc-specific determinants of glycoproteins (see also Fig 11). However, other more complicated sugar modifications are also possible. Taken together, these observations suggest that the PNA-glycoforms of hemolymph proteins are enriched in hemocyte granules of Bt-resistant insects.

4.2 Function of lectins in coagulation and adhesion

If lectin-binding coagulation proteins are present in high levels in the gut lumen, this could lead to a coagulation of proteins, such as Bt-toxin molecules, which may thus be unable to reach the gut membranes, as also discussed in nematodes (Griffits *et al.*, 2001).

Lectins mediate a number of diverse processes, such as cell-agglutination, cell-attachment and spreading, and lectinophagocytosis. Our observations suggest that two seemingly unrelated processes can be added to this list, namely coagulation and macropinocytosis. Since hemocytes in *Bt* -resistant and susceptible insects differ with regard to the relative distribution of protein glycoforms, we examined the behaviour of hemocytes in the presence of lectins. Since some lectins interact with hemolymph components to form insoluble complexes (Fig 10B), and are also required by hemocytes to perform cell attachment, we asked whether some lectins are involved in both coagulation and adhesion functions. Addition of lectins to hemocyte-like *Drosophila* cells showed that adhesion and spreading is increased with the addition of HPL (unpubl, observations). When hemocytes are allowed to settle on a glass slide in the presence of hemolymph, the addition of lectin affected adhesion depending on the time before the lectin was added (Fig 16 B). When hemolymph is bled directly into a lectin buffer solution, the complex formation may occur before the proteins have an opportunity to interact with the hemocyte surface. When GalNAc-specific lectins are present in hemolymph dilution buffer, the hemocytes stay round and inactive, instead of attaching to the glass surface (Fig 16 B).

Since modified lipophorin is the major GalNAc-containing glycoprotein in the hemolymph, and is removed by GalNAc-specific lectins, this suggests that modified lipophorin may interact with the hemocyte surface at the time of transition between the inactive state inside the hemocoel and the active state. When hemolymph is removed from the hemocoel, diluted in physiological buffer solutions and exposed to oxygen, lipophorin is altered by an unknown mechanism. For example, lipophorin is glycosylated, proteolytically digested and acquires different densities when hemolymph is oxidised and lipoproteins are separated by low-density gradient centrifugation (data not shown) or HPLC chromatography (G.Ma, unpubl.results). The type of attachment of lipophorin to hemocytes is not known, but probably involves protein or lipid interactions to membrane-anchored proteins (Theopold and Schmidt, 1997). This suggests that hemocytes may require a soluble hemolymph component, such as lipophorin, to interact with the cell surface as a precondition for cell adhesion and macropinocytotic functions.

To test this assumption, we added cell-free hemolymph (plasma) to hemocytes, which were previously collected in lectin-buffer. Under these conditions, hemocytes resumed cell spreading on glass surfaces (Fig 17). This suggests that hemolymph components are essential for cellular functions of hemocytes. Since these functional requirements are correlated with lectins and their interaction with modified lipophorin, we concluded that hemocytes require the attachment of lipophorin to perform cellular functions in conjunction with lectin-like molecules.

To examine whether glycoproteins on the hemocyte surface interact with Bt-toxin Cry1Ac, hemocytes from resistant and susceptible insects were fixed without

detergent, incubated with the toxin and stained with antibodies. Whereas PNA stains only granulocytes, Cry1Ac toxin stains both granulocytes and plasmatocytes (Fig 14). This staining pattern of the toxin coincides with HPL (Fig 15) and VVL (not shown). No differences between the two strains were observed. The mechanisms of *Bt* binding to hemocytes are not clear. Since glycosylated lipophorin binds to both cell-types, *Bt*-binding to hemocytes may be mediated by lipophorin attached to a receptor, but sugar-mediated binding to membrane receptors is also possible.

Since lectins and modified lipophorin appear to play an important role in cellular hemocyte functions and since lectins interact with modified lipophorin to form coagulation products, we wanted to examine whether lectins play a role in coagulation reactions. When external lectins are added to hemolymph, GalNAc-specific lipophorin is aggregated into insoluble complexes (Fig 10). If lipophorin is recovered with hemolymph, modified lipophorin may interact with hemocytes first and if lectins are added subsequently, the result is a range of cellular activities, such as adhesion and macropinocytosis. Obviously coagulation and cellular functions both depend on lectins, but in the process the timing of lectin-interaction is apparently crucial.

Since coagulation reactions produce both fibrous and small globular structures (SGS), the question is which structures are formed in the presence of oligomeric lectins. To examine how lectins interact with the content of hemocyte granules, live hemocytes were incubated with lectins. In the presence of HPL, some hemocytes are covered with hemolymph glycoproteins (Fig 16 AC). In the presence of PNA, new structures emerged as intensively stained round sphere-like globules. These

structures were frequently attached to live granulocytes, which indicated that hemocytes discharge granules, which contain components that form round spheres on the hemocyte surface (Fig 16 B, D). Since PNA-binding proteins are absent in cell-free hemolymph (Fig 10), and SGS only formed in the presence of hemocytes, the components interacting with PNA to form globules are probably stored in granules and released into the hemolymph. In contrast to cellular function, such as adhesion and endocytosis, the discharge of granules is independent of external lectins or lipophorin (unpubl. observation).

To test whether globule formation depends on modified lipophorin and lectins, we added PNA and HPL to fat body extracts, which contained PNA-glycoforms of lipophorin and compared the structures formed. Under these conditions PNA formed globules (Fig 16, F), whereas irregular fibers are formed in the absence of lectins (Fig 16 E). This indicates that glycosylated lipophorin can interact with PNA to form round spheres, which resemble globules in insect coagulation reactions. Globules are formed around microbes and microbial debris and become attached to basement membranes. It is possible that globule-formation is more prevalent in immune-induced caterpillars or hemolymph from prepupae, but this remains to be examined. Globules may become part of nodules, which are detected after infection with bacteria (Ratcliffe and Walters, 1983) and viruses (Washburn *et al.*, 1996). Since lipophorin is known to be involved in the detoxification of LPS (Kato *et al.*, 1994) and cyclosporin (Vilcinskas *et al.*, 1997), a pattern recognition complex may comprise lectins and modified lipophorin to form globules.

Hemocytes inside the hemocoel of a healthy insect are round and in general circulate without adhering to surfaces or without aggregation to each other (Arnold, 1974). However, when hemolymph is exposed to immune-elicitors or oxygen, hemocytes quickly assume cellular functions, such as adhesion, spreading, aggregation and endocytosis. The transition between inactive and active hemocyte states can be observed in blood smears and takes usually several minutes. It is well known that the time of transition varies in different species and in individual insects of the same species. For some lepidopteran species that are slow in acquiring cellular activities, such as *G. mellonella*, the immediate separation of hemocytes from serum sometimes appears to slow down the initiation of cellular activities (O. Schmidt, pers. comm.)

Moreover, when live hemocytes were examined in blood smears some lectins inhibited cellular processes, instead of increasing adhesion or endocytosis. This could indicate that soluble plasma components may be inactivated in the presence of some lectins and thus not available to attach to hemocytes. To test whether hemocytes perform cellular activities by acquiring soluble components from the hemolymph, we bled hemolymph into a lectin-containing buffer and compared hemocyte attachment and spreading with hemocytes recovered in the absence of lectins.

When hemocytes were inspected under these conditions differences were observed (Fig.18). More than fifteen percent of hemocytes were spread in the absence of lectins, whereas less than five percent cells were spread in the presence of tetrameric GalNAc-specific VVL (Fig. 18A). When hemolymph was recovered in lectin-free buffer and mixed with lectin at different time periods, the percentage of spreading

hemocytes was increased after 15 minutes (Fig. 18B). This suggests that inactive hemocytes may require soluble hemolymph components to perform cellular functions.

To test this assumption, we isolated hemolymph in a lectin-containing buffer, washed lectin-containing plasma from hemocytes several times and mixed hemocytes lectin-free plasma. Under these conditions lectin-treated and washed hemocytes were less spread than treated hemocytes (Fig 19B), an aliquot of which were subsequently mixed with untreated hemolymph fractions (Fig.19 C-E). When spreading was examined using extracellular staining and phalloidin, it was apparent that hemocyte spreading differed in untreated hemocytes and in lectin-treated hemocytes that were restored with hemolymph fractions. When plasma fractions, containing lipophorin were added to lectin-treated hemocytes spreading resumed in filopodia-like extensions (Fig 19 D,E), whereas high-density fractions did not show significant changes (Fig. 19 C).

In summary, in the presence of lectins, hemocytes can either form coagulation products, such as globules, or perform cellular functions, such as adhesion and macropinocytosis. Since coagulation and cell activities are quite disparate processes, the balance between the two types of reactions is quite relevant for the overall outcome. In cell-free hemolymph (plasma) only coagulation reactions occur in the presence of externally added GalNAc-specific lectins. This suggests that GalNAc-lectin-mediated coagulation reactions may not normally occur in plasma, but are confined to Gal-containing glycoproteins stored inside granules and Gal-specific lectins found in the plasma (Castro *et al*, 1987). In the presence of hemocytes

GalNAc-containing glycoproteins may attach to the cell surface to mediate cellular functions. Thus the balance between the two processes is dependent on the presence of the two types of lectins in the process. If GalNAc–specific lectins are present before lipophorin can bind to hemocytes, coagulation dominates. If lectins are added after lipophorin has bound to hemocytes, cell adhesion and endocytosis processes dominate. This suggests that the two processes are interrelated in a dynamic process, which depends on the presence of active lectins and the modification of lipophorin. We therefore examined the role of lectins and lipophorin in these processes.

4.3 Induction of macropinocytosis in the presence of lectins

Endocytosis is a vital process that occurs in all eukaryotic cells, by which cells internalize portions of the plasma membrane, including associated proteins and a portion of the extracellular space (Fig. 20). This is the route by which cells absorb nutrients, recycle membrane proteins and lipids, receive chemical and molecular signals, and down-regulate membrane receptors in response to ligands and/or environmental changes (Cardelli, 2001; Baggett and Wendland, 2001). In particular, macropinocytosis is an endocytic process associated with the formation of large vesicles. In the gut lumen the process of food uptake is intrinsically linked with a process that potentially allows the insect to recognize toxic components in food stuff. Thus food uptake and related reaction are probably closely related processes. Given that the glycosylation is important for endocytosis and coagulation, we analysed glycosylation patterns in hemolymph.

4.3.1 Glycodeterminants in insects

To determine lectin patterns of hemolymph proteins from various insect species, we tested four different lectins on Western blots containing protein extracts from lepidopteran and one dipteran larvae (*Drosophila*). Using GalNAc-specific (HPL) and GlcNAc-specific (ConA) lectins we observed dramatic differences in the complexity of stained glycoproteins (Fig 21). Whereas most lepidopteran species contained several hemolymph glycoproteins with GalNAc and GlcNAc-specific determinants, *Drosophila* showed only one major band at 100 kDa, which represents hemomucin (Theopold *et al.,* 1996) and a minor band at 200 kDa (Fig. 21) according to hemomucin-specific antibody staining (not shown).

When protein extracts from lepidopteran hemolymph and tissues were stained with a Gal-specific lectin (PNA), no significant staining was observed (not shown). Similar results were obtained in *Drosophila*, except that in protein extracts from ovaries a 105 kDa hemomucin band was identified, which was stained with PNA (Theopold *et al.*, 2001). The PNA-stained band was also identified in hemocyte-like *Drosophila* cell lines (Theopold *et al.*, 2001) to be a PNA-glycoform of hemomucin. In addition to hemomucin, a 150 kDa protein with protein sequence similarities to glue proteins is stained with PNA (Fabbri et al, 2000). This suggests that GalNAc-specific lectins bind to a limited number of glycoproteins from *Drosophila* hemolymph and tissues and that there is little or no staining with GlcNAc-specific lectins. This is in contrast to lepidopteran glycoproteins, which bind to GalNAc-specific lectins over a wide range of different sized glycoproteins and are heavily stained with GlcNAc-specific lectins. To investigate GalNAc-specific lectins for the induction of macropinocytosis,

we used a hemocyte-like cell line *mbn-2* from *Drosophila* (Gateff *et al.*, 1980). To compare GalNAc- with GlcNAc-specific lectins we used a lepidopteran cell line from *Plutella xylostella*.

4.3.2 Lectin-mediated spreading and endocytosis

A number of lectins (Table 2) were tested in the two cell lines for induction of cell-spreading and macropinocytosis. The GalNAc- and Gal-specific tetrameric lectins from *Vicia villosa* (VVL) and *Arachis hypogaea* (peanut agglutinin, PNA) were found to induce spreading and macropinocytosis in *mbn-2* cells and *Plutella* cells (Fig. 22, 23). In the *mbn*-2 cell line, the presence of HPL and ConA had little effect on the induction of macropinocytosis (Fig. 24).

In contrast, HPL induced macropinocytosis in *P. xylostella* cells together with VVL and PNA, although the induction process was delayed compared to *mbn2*-cells (Fig. 25). Despite ConA-binding glycoproteins present in lepidopterans (Fig. 21), ConA was not an effective inducer of macropinocytosis (Fig. 26A,B). Since nothing is known about the properties of GlcNAc-containing proteins in lepidopteran cells, further experiments are required to examine the difference in binding and endocytosis induction. The fact that HPL binds to *mbn2*-cells, causing hemomucin clustering (Theopold and Schmidt, 1997) and immune signaling (Theopold *et al.*, 1996), but is unable to induce macropinocytosis, suggests that additional factors may be required to induce endocytosis. Alternatively, hexameric HPL may not interact with relevant components as do tetrameric VVL. To examine possible structural features of lectins, we used monomeric and oligomeric lectins.

4.3.3 Induction of cellular functions by oliogomeric lectins

In both cell lines, the Gal/GalNAc-specific monomeric lectin from *Psophocarpus tetragonolobus* (winged bean, WBL) had no significant effect on spreading and macropinocytosis in *mbn-2* and *Plutella* cells (Fig. 22, 23). To investigate whether VVL, PNA and WBL bind to the same sites on glycoproteins, we performed competition experiments, where we pre-treated the cells with WBL before adding VVL and PNA. In the presence of WBL the induction of macropinocytosis by VVL or PNA was reduced (Fig. 27). This suggests that WBL binds to glycodeterminants that are related to those used by VVL and PNA, but instead of inducing cell spreading and macropinocytosis, WBL is inactive under the conditions used in the experiment. The reasons for the lack of adhesive cross-linking activities are not known. Recent reports on the lectin conformation (Manoj et al., 2001) and stability of the tetrameric complex (Srinivas et al., 1998) suggest that under certain conditions the lectin may be unstable and act as a monomer. Since lectins are known to retain sugar-binding specificity as a monomer (Reddy et al., 1999), it is possible that monomeric WBL can compete with tetrameric lectins for binding sites.

4.3.4 Lipophorin-mediated endocytosis

When tetrameric lectins were added to cells after incubation with purified lipophorin, the induction of macropinocytosis was higher than with each of the two components alone (not shown). To examine whether the two components interact with each other we mixed cell-free hemolymph with peroxidase-conjugated lectins and separated lipophorin by low-density gradient-centrifugation. When fractions were analysed for

the presence of lectins on a dot blot, a significant amount of the lectin was found in low-density fractions (not shown). When these fractions were added to cells, induction of macropinocytosis was observed. This suggests that some plant lectins interact with lipophorin to form a complex.

In summary, in the presence of lipophorin, which is attached to membrane-anchored receptors, tetrameric lectins induce macropinocytosis. Since lipophorin is required for the processes as well as GalNAc-containing determinants, it is not known, whether lectin interacts with bound lipophorin or with glycoprotein receptors on the cell membrane or with both.

4.4 *B. thuringiensis* - toxins are tetrameric complexes

Given that Bt-toxins react with lipophorin like oligomeric lectins (Fig 10), we examined the structure of Bt-molecules. Cry1Ac forms high molecular weight complexes when processed in the presence of gut juice extract, which are stable in SDS at 65°C and revert to low molecular weight proteins at 100°C (Fig. 28A). To test whether the toxin can form oligomeric complexes in the absence of lipids, the 130 kDa toxin precursor was incubated with trypsin in a lipid-free buffer. Under these conditions a ca 60 kDa band was detected, which is smaller than the size of the mature toxin (69 kDa) and in addition, a ca 250 kDa band, which corresponds in size to an oligomeric toxin-complex (Fig. 28A). Trypsin-incubation of the protoxin for more than two hours produced smaller diffuse bands, which suggests a possible proteolytic degradation of the mature toxin, similar to those occasionally observed in

gut juice activated protoxin (Fig. 28*B*). If the 60 kDa protein corresponds to the mature toxin molecule, a 230 kDa complex may be close to a tetrameric complex.

To study the emergence of large complexes protoxin was processed over several time periods, which produced different ratios of 60 and 69 kDa proteins. Since both proteins form oligomeric structures, mixtures of hetero-oligomeric complexes were produced (Fig 28C). Further analysis on Western blots confirmed that the hetero-oligomeric were found in a size range between 250 and 270 kDa which fits with a tetrameric complex of 60 and 69 kDa proteins (Fig 28 D)

4.5 *Bt*-toxin binding protein is a low density lipophorin complex

To examine possible toxin interactions with lipoproteins, we mixed gut juice activated protoxin with cell-free hemolymph and separated the mixture by low-density gradient centrifugation. Oligomeric toxin complexes were detected at low-density regions of the gradient, which remained intact during SDS-extraction at 65^{o}C (Fig. 29, fractions 11-15). Tryptic peptide sequences from the oligomeric CryIAc-containing fraction revealed similarities to apolipophorin (G.Ma unpubl.results), which suggests that Bt-toxins potentially interact with lipophorin-like molecules to form a soluble toxin-lipoprotein complex. In addition to soluble complexes, CryIAc formed large lipoprotein aggregates (Fig. 29, fractions 1-19), which were distributed over the length of the gradient, resembling a fibrous coagulum (Theopold et al., 2002) or long threats of vitellin-like molecules (Hall et al., 1999).

In summary, the oligomeric but not the monomeric toxin, interacts with lipophorin to form a stable complex that can be separated from other proteins by low–density gradient centrifugation.

4.6 Glycosylation status of glycoproteins in the gut lumen

Since *Bt*-toxin binds to a GalNAc-containing glycoprotein in the hemolymph, which exists in two glycoforms in the fat body (Fig 11), we examined the binding of the two corresponding lectins to structures in the gut and compared the patterns with toxin-staining. Using confocal microscopy the putative location of p85 in lepidopteran gut lumen was examined with two different lectins, such as HPL and PNA and compared with Bt-toxin. In gut preparations of DBM, HPL binds to glycoproteins on the basement membrane (Fig 30 AB), inside the gut lumen (Fig 30 B) and inside of the cell (Fig 30 D). In combination with Cry1Ac toxin, HPL partly coincides with toxin-staining to the space between cells (Fig 31). On the surface of mid-gut tissues basement membranes and trachea also bound Cry1Ac strongly (Fig 32B).

While PNA staining is very weak on the basement membrane and most gut cells, a structure inside gut cells, which resembles Golgi vesicle is stained (Fig 33). When the gut of DBM was incubated with anti-PPO antibodies, staining is visible at sphere-like structures at the peritrophic membrane (Fig 34).

Several observations mentioned above and in the literature, suggest that the binding mechanisms of Cry1Ac proteins may be analogous to that of lectins at least in the

initial step of the insecticidal action. However, Bt-toxin also binds to GalNAc-containing proteins in the hemolymph (Fig 10) and co-localize with a Gal-containing proteins in the gut (Fig 38). The significance of this observation is not clear. It could suggest that Bt-toxin can bind to both sugar-determinants, or that lectin-binding is only an initial step of a complex protein-protein interaction, or that Gal-containing (PNA-binding) proteins form globules together with GalNAc-containing (Bt-binding) proteins. Since some proteins that are recognized by lectins participate in immune responses Gupta (1991), it was necessary to explore the location of the immune-related proteins in the gut lumen.

4.6.1 Location of the immune-related proteins in the gut of caterpillar

There is an indication that in gut tissues of resistant caterpillars hemolymph molecules, containing a toxin-binding glycoprotein, accumulate in the gut lumen, particularly in the space between gut and peritrophic membrane. Quantitative and/or qualitative changes in hemolymph molecules may alter toxin processing and sequestration in the gut lumen of resistant caterpillars.

In the context of our previous findings that lipophorin is a pro-coagulant, it is possible that soluble Bt-binding molecules prevent mature toxin from reaching the gut cells by a coagulation reaction in the gut lumen. To test this assumption the amount of lipophorin and phenoloxidase (PPO) in resistant and susceptible gut tissues were examined and the location of immune-related proteins determined using confocal microscopy.

Gut tissues flatten between glass slides and epithelial cells forming two layers with peritrophic membranes in between. In confocal microscopy the focal plane can be adjusted to cut through the space between the undulating peritrophic membranes and epithelium. In whole gut preparations of susceptible larvae lipophorin staining was localized to diffuse background staining and in some individual caterpillars to dot-like structures at the peritrophic membrane and in the space between the gut epithelia (Fig 35). Since these dot-like structures resemble globules found in hemolymph and fat body extracts, we refer to these dot-like structures as globules. Only few of the lipophorin–stained structures were co-stained with PNA in the gut lumen of susceptible larvae (Fig 35, A,C). In the gut of resistant larvae, lipophorin-staining was only marginally increased (Fig 35, A,D), whereas the PNA-staining was significantly higher and was co-localised to most of the lipophorin–stained globules (Fig. 35, B,E). The localisation of lipophorin–stained globules to the peritrophic membrane was confirmed in gut preparations, where the gut content was separated from the gut epithel and surrounded only by peritrophic membrane (Fig 37). When PPO-staining was compared in whole gut preparations of susceptible and resistant larvae, almost no staining was observed in susceptible larvae, whereas PPO-staining was significantly increased in resistant larvae (Fig. 36, A.D). Interestingly, staining of PPO together with PNA showed co-localisation, which increased in resistant larvae (Fig 36, B,E). This suggested that in the gut lumen of resistant larvae a proportion of phenoloxidase is found together with glycosylated lipophorin in globules.

When optical sections were adjusted at the level of peritrophic membranes, TRITC-conjugated HPL stained the peritrophic membrane, showing a smooth, folded

structure (Fig. 37B). While only few globules were observed in susceptible gut preparations, the number of globules significantly increased in resistant gut preparations (Fig. 37E). These globules were also detected with the TRITC-laser in the absence of TRITC-conjugated substances (Fig 37H), suggestive of autofluorescing dopamine-like substances, which may also have cross-linking properties (Nappi *et al*, 1995). This was confirmed in preparations incubated with various unrelated antibodies and FITC-conjugated secondary antibodies (Fig. 37G-I), which showed low but significant binding to globules in preparations from resistant but rarely in susceptible caterpillars.

When activated Cry1Ac was incubated with resistant and susceptible gut preparations, toxin-binding was detected to SGSs, which contain lipophorin-like proteins (Fig 38). Toxin-binding increased in resistant tissue (Fig. 38, D) together with PNA-stained globules (Fig 38E). This suggests that resistant caterpillars accumulate more modified lipophorin molecules in the guts, which are co-localized with other immune components, such as PPO. Initial hybridization experiments, using a PPO-encoding cDNA fragment as a probe, were performed to test total RNA from resistant and susceptible caterpillars on a dot-blot filter. These blots revealed no significant difference in PPO gene expression. This suggests that the increase in PPO in the gut lumen may be due to increased transport of the protein from the hemocoel across the gut epithel into the gut lumen. This could suggest that other immune-related proteins are also accumulated in the gut lumen, resulting in a higher level of immune-reactivity.

Activated Bt-toxin, which is structurally and functionally similar to oligomeric lectins, interacts with modified lipophorin to form globules. These globules are attached to the peritrophic membrane and removed from the gut in the process of food processing into fecal pellets. If this assumption is correct, a number of predictions can be experimentally tested. For example, the gut lumen of resistant caterpillars is expected to melanise more readily than that of susceptible. Moreover, resistant caterpillars are expected to be resistant to other gut-associated pathogens that interact with GalNAc-containing glycoproteins.

4.7 Immune reactions in *Bt*-resistant insects

4.7.1 Immune elicitors in the gut

Since p85 is found in hemolymph and produced by fat body cells, the question is whether the protein is part of the related system. Although p85 is found in insects in variable amounts, attempts to stimulate the production of new proteins or enhance existing levels have met with varying degree of success. The question is: Are immune elicitors capable of stimulating the insect to defend itself through the accumulation of related molecules?

In this study an analysis of soluble midgut proteins for their potential to react to microbial infection is of interest. Therefore, attempts to correlate Bt-binding with the immune status were made involving the induction of related reactions in the diamondback moth by feeding of insect pathogenic bacteria and other elicitors. To analyse a possible immune induction by gut derived pathogens or immune elicitors,

lethal and sub-lethal concentrations of Bt-toxin were fed to fourth instar larvae of DBM and the amount of p85 monitored after 48 hrs of treatment with different inducers. Lethal concentration of Bt caused damage in the gut, which appeared to prevent the induction or accumulation of p85 (Fig 39). In sub-lethal concentrations, Bt-toxin is able to induce p85 similar to other inducers, such as LPS, *E.coli* (Fig 39; 1, 3, 7). However, when inducers are mixed with a lethal concentration of toxin, their ability to induce p85 is reduced (Fig 39; lane 2,4,6). This suggests that an intact gut epithel is required to accumulate immune-related components in the gut lumen.

4.7.2 Melanization reactions

The enzyme phenoloxidase (PO) is responsible for the melanisation-reactions in caterpillars and plays a role in defence reactions. Since spontaneous melanisation of hemolymph, which is exposed to air, is generally used as a reliable indicator for the immune-status of an insect, we performed melanisation assays with individual resistant and susceptible caterpillars. Cell-free hemolymph from Bt-resistant caterpillars differed from susceptible caterpillars in that the melanization reaction started from a higher level and increased to a higher level compared to hemolymph from susceptible insects, where the start of the melanization reaction was delayed for several minutes and produced less melanized reaction products (Fig. 40*A*).

Dissection of gut tissues from resistant caterpillars frequently revealed a blackening of the peritrophic membrane and gut content due to melanisation reactions (Fig. 41). This could indicate the presence of hemolymph-derived immune molecules in the gut lumen confirming the observation from confocal microscopy. To examine

melanisation at a quantitative level we performed melanisation assays in gut extracts, which showed an increased melanisation in the gut of Bt-resistant caterpillars (Fig. 40*B*).

4.7.3 The *B. thuringiensis*–resistant strain may be resistant to other pathogens

If the Bt-resistant strain is constitutively immune-induced producing immune-reactive components in the gut lumen, Bt-resistance may protect against other pathogens as well. We tested this assumption by feeding caterpillars with baculoviruses. Although it is not known, whether baculoviruses use GalNAc-containing glycoproteins to gain access to gut cells, the prevalence of these glyco-determinants in the gut (Fig 30) could indicate a virus-glycoprotein interaction. When *Autographa californica* M Nucleopolyhedrovirus (AcMNPV) was applied to caterpillars from both strains, Bt-resistant caterpillars were less vulnerable to the virus than susceptible caterpillars (Fig. 42. Similar results were obtained when virions were injected into the hemocoel (not shown).

Chapter 5 : General Discussion

5.1 Coagulation versus endocytosis in insect hemolymph

Insect hemolymph generally contains a single major lipoprotein particle, lipophorin, which is found in relatively large quantities. Lipophorin consists of three protein components, apolipophorin I (apo-I, ~ 240 kDa), apolipophorin II (Apo-II, ~ 80 kDa) and apolipophorin III (Apo-III, ~ 18 –20 kDa), a component attached to lipophorin thereby increasing the lipid content in flying insects (Ryan and van der Horst, 2000). Apo-I and apo-II, the two major lipophorin components are produced as a single precursor in the fat body and released into the hemolymph as a functional lipid carrier. In addition to a major role as a lipid carrier, lipophorin is involved in other functions, including hemolymph coagulation (Li *et al.,* 2002), immune induction (Wiesner *et al.*, 1997), detoxification of microbial compounds (Kato et al., 1994; Vilcinskas *et al.*, 1997) and endocytosis (Dantuma *et al.*, 1999; Kang *et al.*, 1995). Recently, lipophorin has been identified as a major pro-coagulant in insects (Theopold *et al*, 2002; Li *et al*, 2002). Our observations suggest that lectins interact with lipophorin to form large complexes.

Given that oligomeric lectins are involved in endocytosis (section 4.3.2) and at the same time form insoluble aggregates (section 4.1.2), the possibility exists that lectin-lipophorin complexes form the structural basis for both processes. For example, lectin-lipophorin complexes could form asymmetric conical structures, which may constitute the building stones for small globular-like structures (Fig. 16F). If lectin-lipophorin interactions occur in cell-free medium, the result may be coagulation in

the form of globules (Fig. 43). If lectins interact with lipophorin on the cell surface, the result may be adhesion and endocytosis (Fig. 43).

Since lipophorin is the major pro-coagulant in lepidopteran insects (Li *et al*, 2002) and involved in endocytosis (Fig. 18), this study may provide a functional relationship of coagulation versus endocytosis (Fig. 43). The model presented in figure 43 is highly speculative but consistent with available data and generates a number of testable hypotheses. The model proposes that in insect systems, there are two types of lipophorin molecules. One is a functional lipid carrier, the other is a defense-related molecule. It is not known what exactly differentiates the two types of lipophorin molecules. Our observations suggest two modifications potentially involved in the transition of lipophorin fronm a lipid-carrier into immune-related molecules: glycosylation modification and proteolytic cleavage of the apolipophorin I into a 85 kDa (p85) molecule. Some of these changes occur in fat body cells, where lipophorin is produced.

Since lipophorin can interact with lectins as a plasma component or being attached to receptors on the cell surface, such as hemomucin (Theopold and Schmidt, 1997), the outcome is either formation of small globular structures (Fig. 16F), or macropinocytosis. In insect hemolymph the two processes appear to be separated and performed by two distinct glycoforms of lipophorin: the GalNAc-containing glycoform is probably involved in cell functions, such as macropinocytosis, whereas the Gal-containing glycoform is involved in coagulation reactions (Fig. 43). The difference could be due to structural features of glycosylated lipophorin or availability of each of the two relevant lectins. For example, the only lectins found in

large amounts in the plasma of lepidopteran insects are PNA-like lectins (Castro *et al*, 1987). If these lectins cause coagulation of Gal-containing glycoproteins, this could explain, why in cell-free hemolymph PNA-glycoforms are virtually absent. Instead these glycoproteins may be stored in granules of hemocytes to be released after immune activation.

Hemocytes (insect blood cells) play an important role in the internal related system of insects against parasites and pathogens. Elimination of potentially damaging organisms that enter the insect hemocoel requires hemocytes to recognize and respond to the invaders (Ratcliffe et al., 1996). For example, pathogens are either phagocytized or enveloped in nodules by the aggregations of hemocytes and plasma components, leading to the formation of capsules. Hemocytes also participate in cellular aggregations at wound sites after the integument is damaged.

Many lectins interact with glycodeterminants on the surface of animal blood cells, causing adhesion and agglutination reaction. Millar and Ratcliffe, (1994) illustrated that there are three ways in which lectins are able to mediate attachment of foreign particles to blood cells: 1) the lectins may be associated with the hemocyte membrane, either as integral membrane proteins, or as cytophilic hemolymph factors. In this case, the membrane–bound lectins bind directly to carbohydrate moieties on the non-self particle. 2) the lectin may be humoral and act as a bridge between complementary carbohydrates on both the foreign particle and the immunocompetent cell. 3) Lectins present on microorganism such as bacteria recognize carbohydrate residues on the phagocyte surface.

Our observations suggest a fourth type of interaction, in which the lectins can either be pattern recognition molecules or abundant pro-coagulant components to interact with modified lipophorin to assemble a cage-like envelope around microbes and toxic compounds to form small globular structures. Likewise, the lectins could interact with modified lipophorin or glycosylated receptors to induce adhesion or endocytosis. The outcomes of both reactions are the inactivation of microbes or microbial debris under cell-free conditions or by the cell. The initiation for globule formation and endocytosis reactions may be regulated at the lectin level. For example, the formation of oligomeric lectins may only occur after binding to the sugar determinant. Another level of regulation is the modification of lipophorin. For example, oxidation of lipids may trigger glycosyl-transferases or tryptic enzymes to modify lipophorin which is then able to react with lectins.

In some arthropods, lectins interact with their respective glycoproteins like a two-component glue system, which are compartmentalized under normal conditions but combined during immune-induction (Theopold *et al*, 2002). Present observations indicate that oligomeric GalNAc-specific lectins, such as VVL and HPL, when added to cell-free hemolymph interact with soluble glycoproteins to form insoluble aggregates. Gal-specific PNA only interacts with components that are released from granules stored inside hemocytes. This indicates that PNA-like lectins, which are present in the hemolymph (Castro *et al.*, 1987), interact with discharged Gal-containing glycoproteins to form globules, whereas GalNAc-binding lectins may interact with membrane-attached glycoproteins to mediate macropinocytosis. If globules formation and endocytosis reactions are indeed based on similar components, these glycoproteins may exist in two glycoforms and interact with two lectin types

performing alternative functions (Fig. 43). For example, in *Drosophila* hemocyte-like cells, where hemomucin, a membrane-anchored glycoprotein, exists in both glycoforms (Theopold, 2001), both lectin-types can induce macropinocytosis. Although the lectin-induced formation of globules in conjunction with cell-free glycoproteins, and of macropinocytosis with membrane-anchored glycoproteins is very different in appearance, the structural features and molecular mechanisms underlying these reactions may be similar.

In summary, in the presence of oligomeric lectins lipophorin molecules can either form coagulation products (globules) or perform cellular functions, such as adhesion and macropinocytosis. Since coagulation and cell activities are quite disparate processes, the regulatory balance between the two pathways may be crucial and can potencially open up new lines of investigation. For example, if Bt-toxins act like oligomeric lectins, interaction with lipophorin can either lead to inactivation through globule formation or uptake by cells through endocytosis processes. Since Bt-toxin may be adapted to use the endocytosis process for membrane insertion, the two processes may explain toxicity and resistance (Fig. 43). Much remains to be tested in order for this model to be valid: For example, are lectin-lipophorin interactions the only functional complexes or are there any other potential factors involved in this process? Is hemomucin also involved in the binding process of lepidopteran lipophorin, or are other receptor molecules involved, such as aminopeptidases and cadherin-like molecules? Are protein-protein or protein-lipid interactions involved in addition to carbohydrate-lectin interactions?

5.2 Lectin-mediated macropinocytosis

Given a possible lectin function of Bt-toxins the role of lectins in immune reactions requires further discussion. Lectins that bind to microbes mediate lectinophagocytosis based on their ability to interact with glycoprotein receptors on phagocytotic cells, thus enwrapping the cellular membrane around the microbe or triggering cell-surface sculpturing (Ofek *et al*, 1995). Interactions involving mucin-like glycoproteins containing Gal- and GalNAc-specific glycoconjugants are observed in immune-related tissues, such as epithelial tissues (Pendland and Boucias, 1996) and blood cells (Chen *et al*., 1993; Theopold and Schmidt, 1997).

Since tetrameric Gal-specific lectins exist in the hemolymph of insects (Castro *et al*., 1987; Wilson *et al*., 1999), cross-linking of cellular receptors with Gal-containing glycodeterminants provide a possible functional basis for immune-related processes, such as phagocytosis, cell aggregation and clearance of immune-derived components. For example, lectinophagocytosis is mediated by lectins that bind to microbial surface carbohydrates and to cell surface glycoproteins, causing internalization of microbes by a zipper-mechanism (Ofek *et al*., 1995). The driving force for the inverse membrane curvature during lectinophagocytosis is derived from attachment of the cellular membrane to an already curved particle like a zipper (Huttner and Zimmerberg, 2001). Similar driving forces are implied for the budding of viral nucleocapsids and formation of secretory granules from the trans-Golgi network, with the particle being the condensed cargo (Huttner and Zimmerberg, 2001).

In contrast to zipper-like mechanisms, the sculpturing of membranes during endocytosis of soluble substrates is assumed to be based exclusively on intracellular driving forces (Huttner and Zimmerberg, 2001). One possible mechanism is mediated by the polymerization and binding of cytosolic coat proteins, such as clathrin, to integral membrane constituents, where the bending of the membrane is presumably driven by the curvature of the assembling coat proteins (Kirchhausen, 2000). A second driving force is derived from membrane-attachments to cytoskeleton components capable of driving the membrane curvature by 'pushing' the budding membrane into extracellular space or by 'pulling' the membrane into the cytoplasmic interior (Mellman, 1996; (Russell-Jones, 2001). These intracellular driving forces are triggered by cytoplasmic signals, which are the result of ligand interactions with membrane-anchored receptor molecules. In general, the process of receptor-mediated endocytosis (RME) is based on two steps, one involving signalling by ligand-receptor interactions, which in turn activates the assembly of cytoplasmic motor proteins to produce the membrane curvature (Schwartz, 1995).

Since only soluble molecules are internalized during macropinocytosis this type of endocytosis is generally cited as a typical example for RME (Dantuma *et al.*, 1999; Hussain, 2001; McPherson et al., 2001). Since soluble material is unable to provide structural support to bent membranes around it, the driving forces for the inverse membrane curvature are believed to be located in the cytoplasm and lectins are assumed to act as a trigger for the signalling process. In this context, the binding of the carbohydrate-recognising domain (CRD) to the glycodeterminant of the receptor is essential for signal transduction, which implies that monomeric lectins are functional ligands.

We found that some lectins mediate cell attachment and endocytosis, which allowed us to test some of the assumptions of RME by using different types of lectins in different cell lines. For example, the *Drosophila* immune receptor, hemomucin, is the predominant GalNAc-containing membrane receptor on hemocytes and hemocyte-like cells and interaction with the GalNAc-specific HPL triggers antibacterial protein synthesis in *mbn2*-cells (Theopold *et al.*, 1996) and Schneider cells (Theopold *et al.*, 2001). When monomeric, tetrameric and hexameric GalNAc-specific lectins were applied to *mbn2*-cells, only the tetrameric lectin caused significant macropinocytosis, whereas monomeric and hexameric lectins were less effective.

Since hemomucin exists in two glycoforms that can interact with Gal and GalNAc-binding lectins (Theopold *et al.*, 2001), we also applied the tetrameric Gal-specific peanut agglutinin (PNA), which also caused significant macropinocytosis. In contrast, the monomeric Gal and GalNAc-specific lectin from winged bean (Manoj *et al*, 2001) was ineffective in causing macropinocytosis. This suggests that the interaction is not only based on sugar-determinants, but on the tertiary structure of the lectin as well.

To confirm that the monomeric lectin (WBL) competes for the same binding-sites in macropinocytosis, it was applied in conjunction with other tetrameric lectins and shown to reduce macropinocytosis (Fig. 27). Although this experiment was only performed twice, the observation that WBL may compete with GalNAc-specific VVL and Gal-specific PNA could be due to dual specificities of WBL (Manoj *et al*, 2001) or spatial hindrances of closely located lectin binding-sites on the mucin

domain. Taken together, these observations suggest that binding of the carbohydrate-recognising domain (CRD) to a glycoprotein receptor is a precondition but not sufficient for macropinocytosis induction. Induction of macropinocytosis may require specific structural arrangements and cross-linking properties, which are only possible in oligomeric lectins. The observation that HPL is able to aggregate hemomucin on the cell surface (Theopold and Schmidt, 1997) and induce an immune responses (Theopold et al., 1996), but is unable to induce macropinocytosis in *mbn2*-cells (Fig. 24), suggests that clustering of receptors is probably required but not sufficient for endocytosis. Interestingly, HPL was able to induce macropinocytosis in *P. xylostella* cells, after a time delay, which suggests that additional components may be required for macropinocytosis and may be induced in lepidopteran, but not in *Drosophila* cells.

Recent reports of lipophilic domains in some lectin isoforms (Ortiz et al., 2000) suggested to us a possible interaction of some lectins with lipoproteins. To test whether lectins interact with lipoproteins, we added lectins to cell-free hemolymph from *G. mellonella* and separated the mixture by low-density gradient centrifugation. In this assay, the two tetrameric lectins (VVL and PNA) are found in low-density fractions. In this context, it is also worth noting that some low-density fractions induced macropinocytosis in the absence of lectins. Protein analysis revealed that in addition to lipophorin additional proteins are present in low-density gradient fractions, which may contain endogenous lectins. For example, a lipophorin-complex containing an endogenous lectin, which has similarities to imaginal disc growth factors, was isolated from these low-density fractions (M. Fabbri, unpubl. results). Imaginal disc growth factors are dimeric chitinase-like

proteins (Homma et al., 1996; Kawamura et al., 1999), which bind sugar, but lack catalytic activity (van der Holst *et al.*, 2001). If lipoproteins induce macropinocytosis in conjunction with endogenous lectins, the addition of VVL and PNA to hemolymph fractions may mimic the formation of an internalization-complex, involving cross-linking of adhesion molecules and lipoproteins. Internalization-complexes may interact with the cell surface by lipoprotein-receptor interactions or by lectin-receptor interactions, involving lectin-CRDs and receptor-glycodeterminants (Fig. 44).

Previous observations indicated that under certain conditions hemomucin binds to lipophorin (Kinuthia *et al.*, 1999; Theopold and Schmidt, 1997), a lipid carrier in insects (Pho *et al.*, 1996; Ryan and van der Horst, 2000). This is supported by sequence similarities of a hemomucin domain to strictosidine synthases (Theopold *et al*, 1996), paraoxogenases (Fabbri *et al.*, 2000) and arylesterases (NCBI database). The latter two gene families are known to bind to lipoproteins in human.

Recently a viral immune suppressor protein, CrV1 was found to form a complex with lipophorin via coil-coil structures enabling the suppressor to prevent hemocyte function by depolymerisation of actin cytoskeleton (Asgari *et al.*, 1997)(Asgari and Schmidt, 2002). In conjunction with our observations, the possible function of CrV1 could be explained in two ways: One is that Cr VI interacts with a cell-surface receptor and after internalisation interacts with the cytoskeleton. The other is that CrV1 interacts with lipophorin in the plasma and thus preventing it from interacting with hemocytes. The later possibility does not require internalisation of CrVI by hemocytes. Finally, indirect evidence for a dual role of lipophorin in coagulation and cellular

functions emerged from protein sequence analysis of apolipophorin I (Gang Ma, unpubl results).

Insect lipophorins contain two conserved protein domains with similarities to adhesin and the van Willebrand factor (VWF) domain, present in coagulation proteins. The adhesin similarity starts at around position 2300 of the lipophorin precursor protein. Adhesin has the ability to form tetrameric adhesion complexes (Henrich *et al*, 1993). The VWF domain is located at position 2900. If these two domains are part of the p85 (Gang Ma, unpubl.results), the protein may be able to form tetrameric adhesion molecules and multiple coagulation aggregates depending on the domain activated in the process. This suggests that lipophorin is potentially able to interact with immune components to facilitate clearance of detrimental substances or coagulation of hemolymph (Li *et al*, 2002). Since hemomucin can potentially interact with lipophorins, mediated by the strictosidine synthase domain (Fabbri *et al*., 2000) and with lectins, mediated by the mucin-like domain (Theopold and Schmidt, 1997), a putative endocytosis complex may consist of lipophorin, lectin and mucin-like glycoproteins, such as hemomucin or aminopeptidase N.

Given the structural features of the putative endocytosis complex, it is tempting to speculate that the tetrameric lectin, in assembling four hemomucin molecules around bulky lipoproteins such as a tetrameric p85, generates the leverage necessary for bending the membrane-anchored hemomucin molecules (Fig. 44). If multiple endocytosis complexes form by lateral aggregation, the extracellular structure would be sufficient to produce the conformational energy to induce an inverse membrane curvature. The prediction is that only combinations of oligomeric

adhesion molecules and receptors that can produce the necessary leverage will induce endocytosis.

5.3 Alternative model of *Bacillus thuringiensis* toxin-insertion into the membrane

Increasing limitations on the use of chemical pesticides stimulate interest in alternative strategies in pest control. Obviously, *B. thuringiensis* is now the most widely used biologically produced pest control agent. However, intensive Bt-usage in the field, has led to Bt-resistance in DBM and possibly other pest species. To minimize this negative effect of Bt-resistance, development of resistance management strategies require a molecular understanding of the mode of action of Bt-toxin. This includes a molecular mechanism of a possible toxin-insertion into the membrane bilayer. Our results suggest that lipophorin components may be involved in Bt-toxicity by a lectin-mediated endocytosis process and in resistance mechanisms by coagulation reactions in the gut lumen.

Since membrane insertion of Cry1Ac may depend on its lectin function, a systemic modification of glycodeterminants in gut and hemolymph proteins would explain increased toxicity if the Bt-binding protein is attached to receptors on the gut cell surface. However, if the Bt-binding protein is present predominantly in the gut lumen or more reactive as a pro-coagulant, the overall result is Bt resistance. Hemocytes from resistant caterpillars show increased PNA-staining (Gal) and relatively less HPL-staining (GalNAc) compared to hemocytes from susceptible caterpillars. Likewise, PNA and PPO-staining is increased in the gut lumen of resistant insects.

This suggests that in resistant insects coagulation may be predominant over endocytosis, where PNA shows strong binding to modified lipophorin in globules. If PNA and PPO-binding to globules are indicators of immune-reactivity, Bt-toxin molecules may be bound to globules in an irreversible fashion, thereby removing mature toxin molecules from the gut lumen. The observation that GalNAc-containing proteins are relatively low in the gut lumen of resistant compared to susceptible insects is counter-intuitive, since Bt-toxin may require GalNAc-sugar to bind to coagulation molecules. However, further experiments are necessary to determine the functional binding properties to Gal-containing coagulation proteins.

This model allows a number of predictions that can be experimentally tested. A major difference between the model described in the literature and the model described here is the mechanism of toxin insertion into the membrane bilayer (Fig. 45). Bacterial toxins inactivate eukaryotic cells by creating pores, but the mechanisms by which water-soluble toxins are inserted into the cellular membrane are not known (Lacy and Stevens, 1998). Current models assume that bacterial toxins assemble into a membrane–associated complex after the insertion of a pore-forming domain into the membrane (Olson *et al*, 1999). The general view is that monomeric toxins interact with a receptor and are inserted into the membrane forming an oligomeric pore-forming complex inside the membrane bilayer (Bhakdi *et al.*, 1996). Thus the *B. thuringiensis* endotoxin is assumed to form a pore-forming oligomer complex within the lipid bilayer after binding to a cell surface receptor as a monomer. This study shows that tetrameric Cry1Ac complexes are formed in the absence of lipid and are able to bind to lipophorin forming a complex. Since

lipophorin can be attached to receptors on the cell surface, interactions of Cry1Ac with cell surface receptors could resemble a lectin-induced endocytosis process.

5.4 Are oligomeric lectins of Bt –toxins involved in endocytosis?

Thus an alternative mechanism for toxin-insertion can be based on a lectin-mediated endocytosis mechanism, which has the potential to generate the configurational energy required for the insertion of an oligomeric toxin complex into the membrane. It is suggested that toxin-insertion into the membrane may be facilitated by an endocytosis mechanism, where the toxin interacts with receptors or with glycosylated lipophorin as an oligomeric lectin. We tested this assumption using the *in vitro* processing of the bacterial endotoxin. Present models assume that Bt-toxin is unable to form a tetrameric complex in the absence of lipid, since assembly into a tetrameric channel-forming complex occurs inside the membrane bilayer. However, when Cry1Ac protoxin was incubated in the presence of high salt, a heat- and SDS-stable tetrameric toxin was formed (Fig. 29). These experiments suggest that the toxin may potentially interact with gut-receptors in the form of a multimeric cross-linking ligand. An interaction of multimeric toxins with membrane-anchored glycoproteins such as aminopeptidase N, cadherin-like molecules or hemomucin, may lead to a process similar to the initial phase of lectin-mediated endocytosis. Toxicity may be the result of osmofragility due to insertion of the lipophilic domains of the toxin complex into the underlying membrane, thereby forming an ion-channel (Schmidt et al., in prep.).

Recent observations suggest that brush border membrane vesicles (BBMs) interact with large *B. thuringiensis* (Aronson, 1999) and *Clostridium perfringens* enterotoxin

complexes (Wieckowski et al., 1998). Many non-bacterial pore-forming toxins also have lectin activities. For example, CEL-III is one of four Ca-dependent, Gal-specific lectins purified from the sea-cucumber (*Cucumaria echinata*) that cause hemolysis of rabbit and human erythrocytes by specific binding to membrane-specific carbohydrates and formation of pores (Sallay et al., 2000); Fujisawa et al., 1997; Hatakeyama et al., 1996; Nakano et al., 1999; (Oda et al., 1997). Hemolysis is a highly cooperative event that requires a subsequent oligomerization of individual units of the toxin (Gray, 1998).

Taken together, our observations in insects point towards an endocytosis process that is driven by multimeric ligands that are able to cross-link receptors around lipoproteins or bulky protein complexes. In hindsight, it is possible to derive this lectin-mediated endocytosis mechanism conceptually from a 'zipper' mechanism that mediates microbial phagocytosis and by extension, to a clustering of receptors around protein complexes (Fig. 46). This may be called a leverage-mediated endocytosis mechanism, since it involves adhesion-based linkages of membrane-anchored receptors around protein or lipoprotein complexes, causing bending of receptors and formation of an inverse curvature of the cell surface, leading to the internalization of the complex. This means that endocytosis processes comprise two functional elements, one involving assembly of a complex, the other the generation of an inverse curvature of the cell membrane, which drives the reaction towards internalization.

Leverage-mediated endocytosis or receptor-internalization also facilitates cytoplasmic signaling through rearrangement of receptor-complexes connecting the

receptor to cytoplasmic components, including the cytoskeleton. For example, the formation of an endocytosis complex changes the orientation of membrane-anchored receptor molecules relative to the cytoskeleton, creating structural tensions at the cytoplasmic attachment sites of the cell surface receptors.

Receptor-mediated endocytosis (RME) is described as a two-step mechanism (Schwartz, 1995), where extracellular signals are assumed to initiate cytoplasmic-driven endocytosis reactions (Hussain, 2001; Cardelli, 2001). Although a two-step RME mechanism is not ruled out, our observations are also compatible with a one-step endocytosis mechanism driven by extracellular driving forces. In this model, soluble adhesion molecules (SAMs), such as tetrameric lectins, cross-link membrane-anchored molecules (MAMs) around lipoproteins or bulky endocytosis complex molecules (ECMs) tilting MAMs to cause a local inversion of the membrane curvature (Fig. 46 *a,b*). This 'leverage-mediated endocytosis' mechanism (LME) involves lateral clustering of MAMs by SAMs, generating the configurational energy, which drives the reaction towards internalization of the complex.

Since bending of MAMs is part of the process, LME may be a precondition for cell signaling, which is fundamentally different from RME, where an external signal is required for a cytoplasmic response to initiate endocytosis. LME-mediated slant of MAMs may produce an intracellular signal if the leverage is strong enough to dislodge cytoplasmic proteins (Fig. 46*b*). In this model the signaling capacity of SAMs, such as dimeric and oligomeric binding proteins is based on their ability to form a complex that can affect anchorage of MAMs to cytoplasmic components.

For example, receptor-specific antibodies may be able to cluster MAMs on the cell surface but may lack the configurational energy to initiate internalisation and signaling. Conversely, GPI-anchored molecules may be involved in endocytosis without creating a cytoplasmic signal (Shin and Abraham, 2001). This suggests that signal transduction may not constitute a precondition of endocytosis, but is intrinsically linked with the bending of MAMs during LME (Grakoui et al., 1999); Cochran *et al*, 2001). In LME, an outside signal to the cytoplasm is transmitted when the combined leverage of an extracellular endocytosis complex supersedes intracellular MAM-associated attachments to the cytoskeleton.

In summary, the endocytosis model provides an alternative toxicity mechanism, where an oligomeric toxin may interact with cell surface membrane-attached lipophorin and receptors to form an endocytosis complex, inserting the toxin into the membrane during receptor-internalization. This model is different from previous models, because the mature toxin molecules form an oligomeric complex before being inserted into the membrane.

5.5 Resistance against Bt-toxin

Genetically, laboratory development of resistance may involve polygenes (multiple-genes, each having a small impact on the selected trait). In contrast, development of resistance in the field is more likely to involve a single major gene (Ferre *et al*, 1991). There are several biochemical modes of resistance such as altering the proteolytic processing of protoxin and binding site modifications (Schnepf *et al*, 1998). A subsequent study demonstrated a genetic linkage between decreased susceptibility to CryIAc and the absence of a major gut protease (Oppert, 1999).

Cry1Ac resistance in *P. xylostella* was demonstrated to be due to dramatically reduced binding (Tabashnik et al., 1994).

This study demonstrates a possible resistance mechanism based on an increased level of immune defence reactions. Based on the assumption that Bt-toxin is a tetrameric lectin, a model of a *Drosophila* immune receptor complex has been used to explore *Bt*- resistance as a result of glycosylation modification. Genetic resistance to low levels of Bt-endotoxins has been selected in several species in the laboratory, but is common in field populations of diamondback moth (Ferre and Van Rie, 2001). Whereas different mechanisms have been observed in resistant population selected under laboratory conditions, only one major mechanism has been reported so far for resistance developed under field conditions. We used a *Helicoverpa armigera* strain, which has been selected in the laboratory for resistance to moderate levels of *Bt*-toxin. Resistant insect populations were subsequently back-crossed four times with a susceptible population to generate nearly isogenic lines of resistant (ISOC4) and susceptible (ANGR) insects (Akhurst et al., 2002).

Our observation that oligomeric lectins interact with modified lipophorin to form coagulation products may provide a basis for a novel *Bt*-resistance mechanism in insects (Fig 47). In this model the amount of modified lipophorin in the gut lumen is increased which increases the chance of *Bt*-toxin to interact with soluble lipophorin before interacting with lipophorin attached to brush border membranes (Fig. 47).
Several observations support this model:

1) The amount of p85 is increased in resistant DBM larvae or gut extracts. In *H. armigera* p85 is co-localised with increased amounts of PPO. The increase

may lead to increased reactivity, which may cause aggregation of lipophorin into insoluble coagulation complexes. This may remove and inactivate Bt-toxin in the gut lumen. It also can explain why p85 may be reduced in protein extracts of resistant *H. armigera* insects.

2) When *Bt*-binding proteins are examined in the gut lumen *in situ*, the toxin binds to small globular structures and the number of globules is increased in resistant insects.

3) The increase of soluble *Bt*-binding molecules in the gut lumen of resistant insects correlates with increased resistance against other pathogens. This is compatible with the assumption that the Bt-binding protein in resistant insects is an immune complex that potentially inactivates toxins and other pathogens before they can reach the gut epithel.

In summary, the binding of *Bt*-toxin to glycosylated lipophorin in the gut lumen is consistent with a model where the toxin is inserted into the membrane by an endocytosis mechanism (Fig. 44). In resistant insects, the primary *Bt*-target is an immune-complex in the gut lumen and at the peritrophic membrane, which inactivates the toxin by a coagulation process.

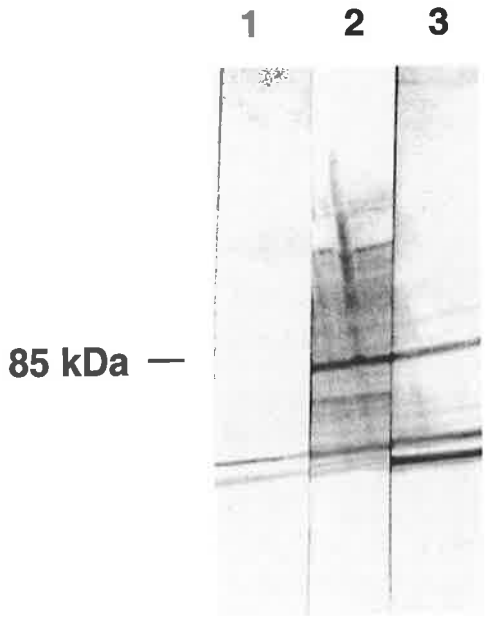


Fig 4. *Bt*-toxin Cry1Ac binds to lepidopteran GalNAc-containing glycoproteins. Western blot containing total protein extracts from fourth instar diamondback moth (*P. xylostella*) caterpillars were probed with 1) pre-immune serum of anti-Cry1Ac-antibodies and peroxidase-conjugated secondary antibodies, 2) Peroxidase-conjugated HPL, 3) Cry1Ac incubation followed by antibodies against Cry1Ac and peroxidase-conjugated secondary antibodies.

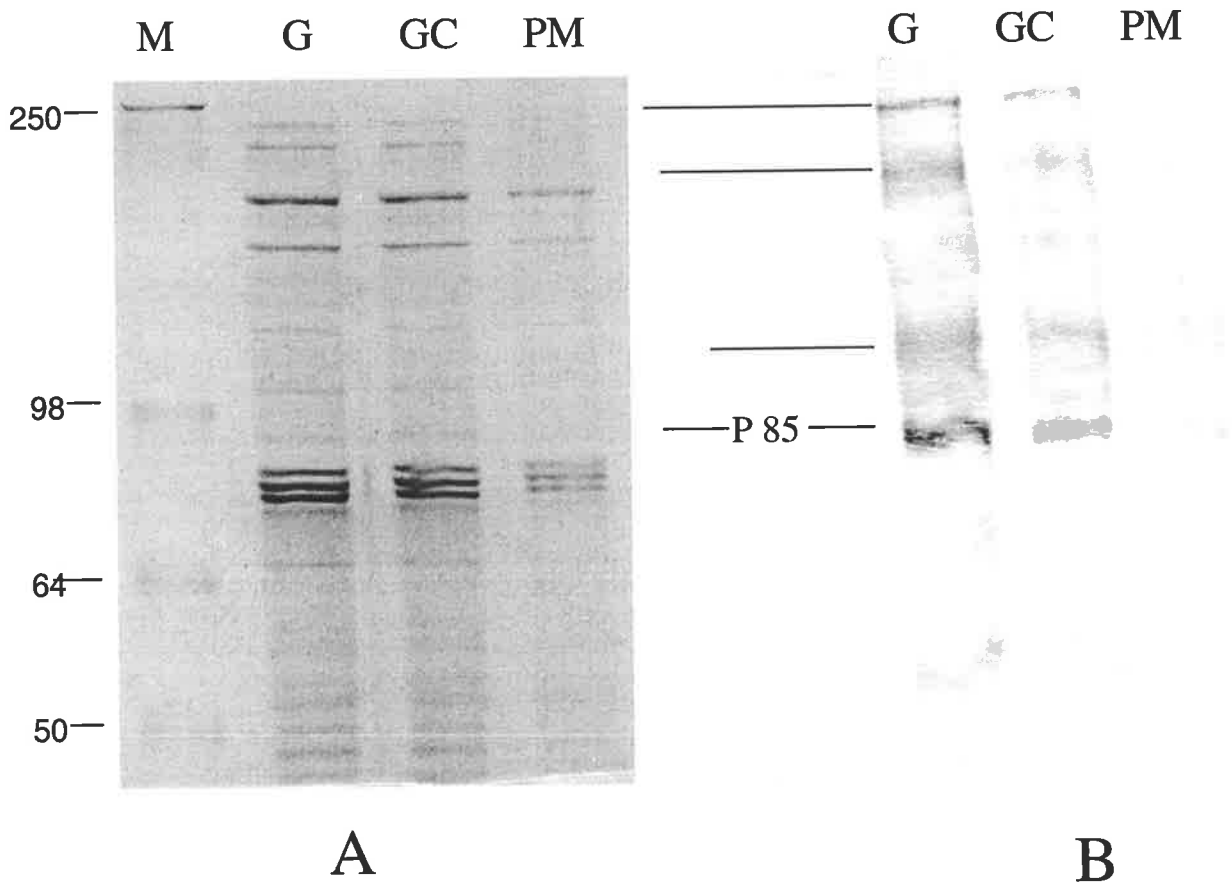


Fig. 5 Protein extracts from various tissues such as gut (G), gut content including peritrophic membrane (GC) and peritrophic membrane from fourth instar DBM caterpillars.

A) Coomassie blue staining of protein extracts showing the relative intensity of p85 staining compared to a characteristic triplet of bands at similar size (78, 80 and 82 kDa), which were identified as arylphorin, PPO and apoII using specific antibodies (Li *et al*, 2002).

B) Western blots of aliquots of the same protein extracts stained with peroxidase-conjugated HPL showing the sugar-staining of p85 in gut and gut content, and less in peritrophic membrane (PM).

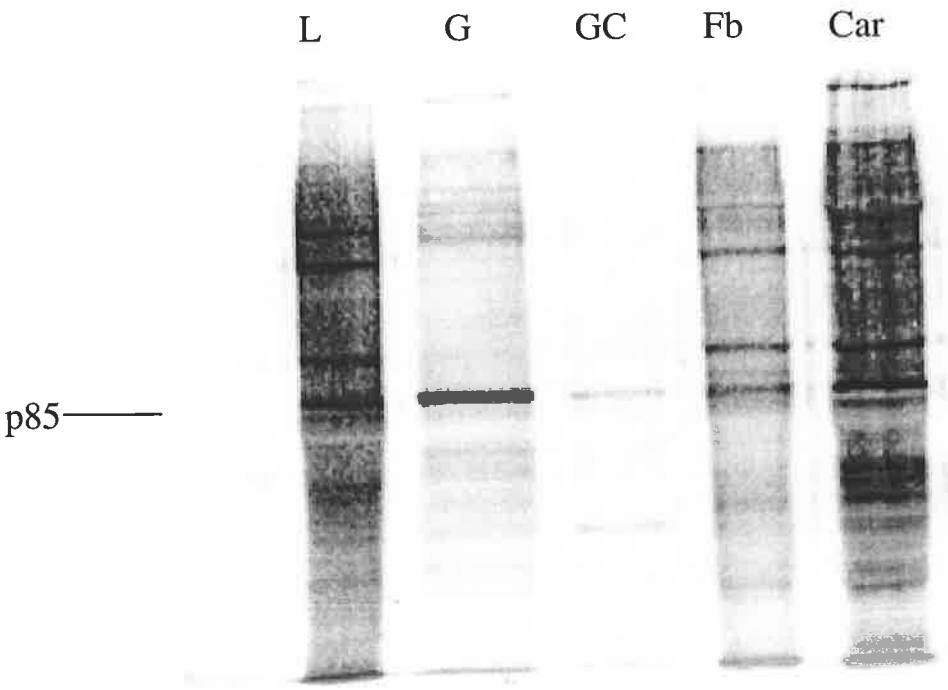


Fig 6 . Western blot of protein extracts from various tissues of DBM caterpillars stained with peroxidase-conjugated HPL. The p85 glycoprotein is found in several tissues of DBM such as whole larvae (L), gut (G), gut content (GC), fat body (FB), carcass (Car).

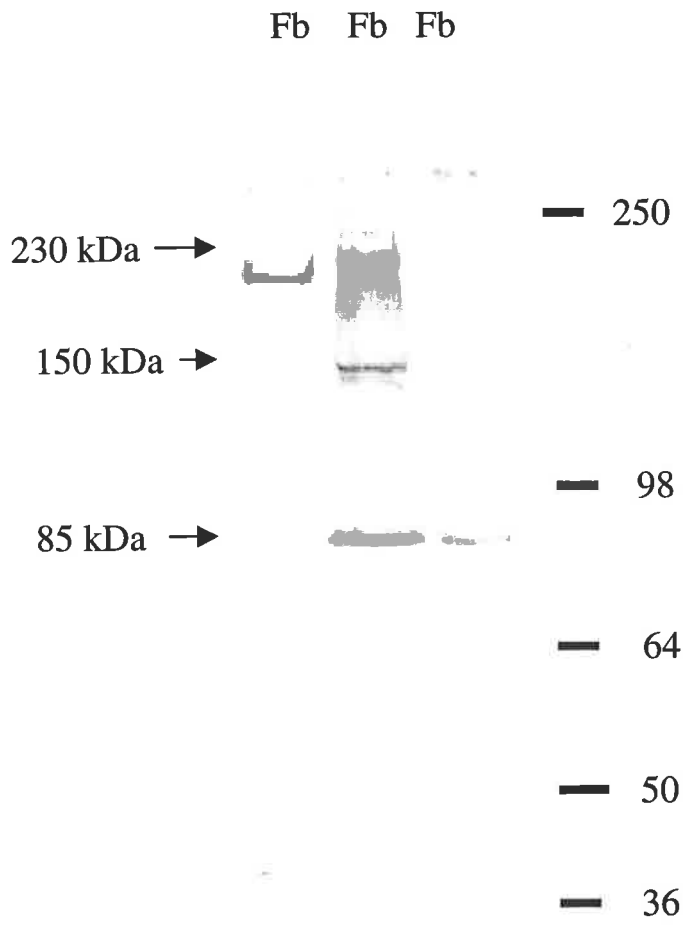


Fig. 7 Western Blot of protein extracts from DBM fat body stained with peroxidase-conjugated HPL, showing co-existing glycoprotein band of p85 with glycoproteins of 150 and 230 kDa in three different protein extracts of fat body (arrows). The HPL-stained bands above 150 kDa could be due to proteolytic degradation of a precursor (glycosylated apolipophorin I), or due to complex formation with another protein (arylphorin).

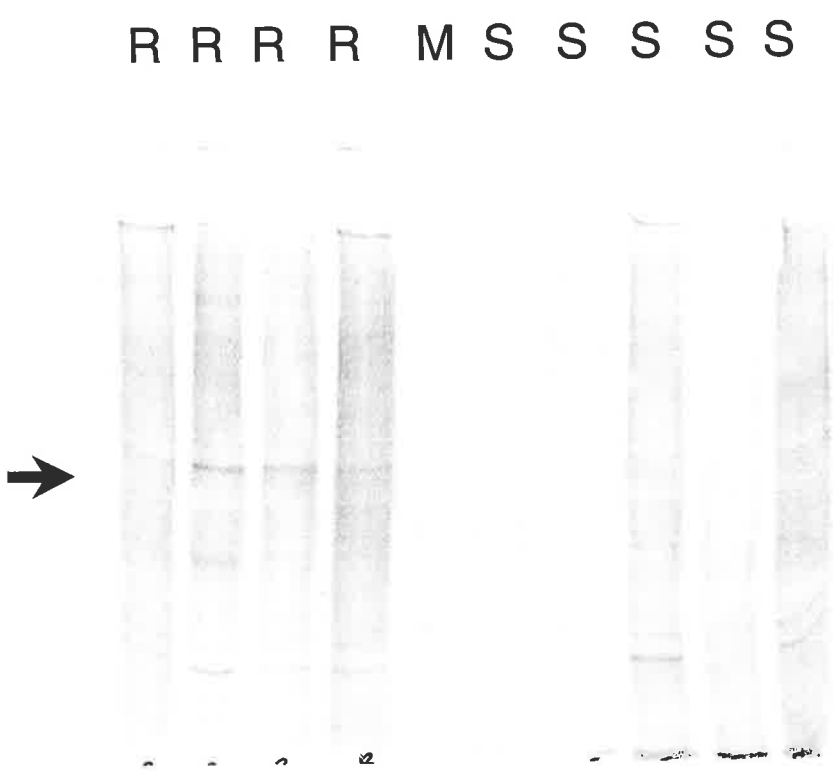


Fig 8. Western blot of total protein extracts from individual resistant (R) and susceptible (S) DBM caterpillars stained with peroxidase-conjugated HPL, showing a somewhat stronger staining of p85 in the resistant strain compared to p85-staining in the susceptible strain. Protein amounts loaded onto the gel where adjusted to be similar in resistant and susceptible using several Coomassie blue-stained proteins below 60 kDa.

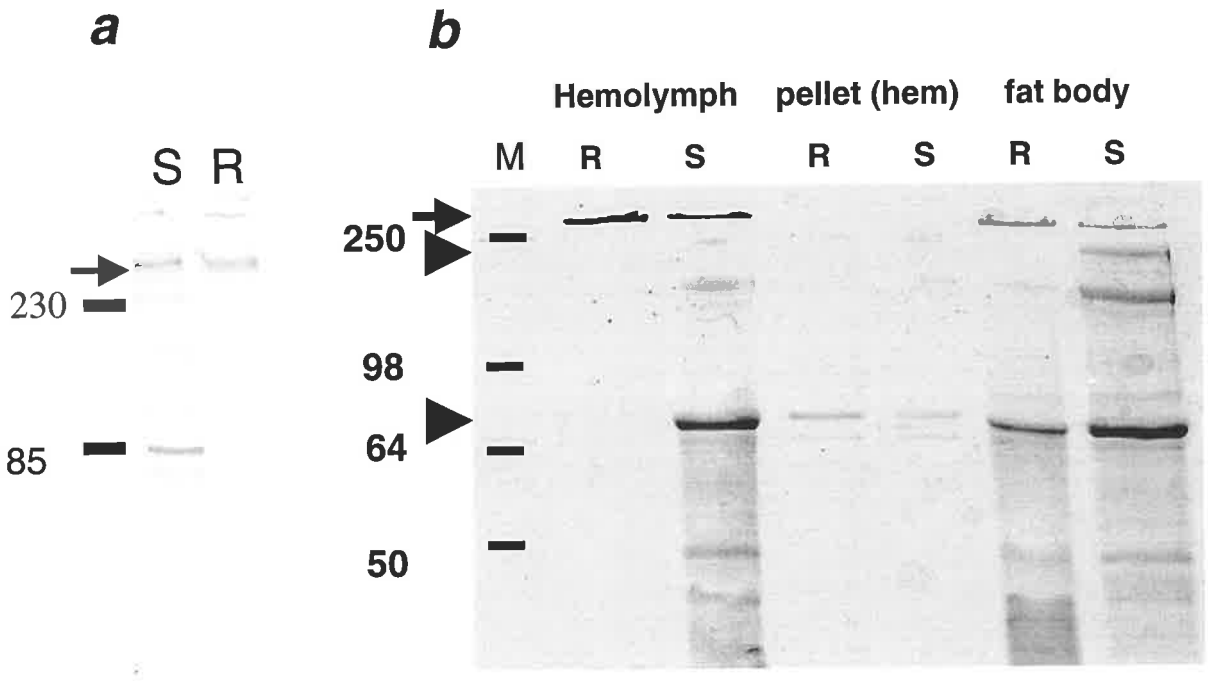


Fig 9. HPL binding to gut glycoproteins and lectin-induced coagulation in *H. armigera*. *a*) Western blot of gut extract from *H. armigera* resistant (ISOC4) and susceptible (ANGR) 3rd instar caterpillars incubated with peroxidase-conjugated HPL. The Bt-binding glycoprotein is present in the gut lumen of resistant caterpillars, as shown by *in situ* staining (see below Fig. 38), but is co-localised with PPO (see Fig. 37) and may form insoluble aggregates, which are not dissolved during extraction. *b*) Coagulation of hemolymph and fat body extracts in the presence of *Vicia villosa* lectin, a GalNAc-specific tetrameric lectin. Cell-free hemolymph and soluble proteins from osmotically shocked fat body cells from 4th instar caterpillars (ISOC4 and ANGR) were incubated with the lectin for five minutes and large protein aggregates removed by centrifugation. Pellets were dissolved in loading buffer. The position of intact apolipophorin I and apolipophorin II is indicated (arrowheads).Note that proteins from resistant caterpillars are more reactive, forming large complexes (arrows), whereas proteins from susceptible caterpillars remain soluble for longer periods. The large aggregates (arrow) contain arylphorin (Gang Ma, unpubl results) and GalNAc-containing glycoproteins (compare gut preparations *a*, arrow).

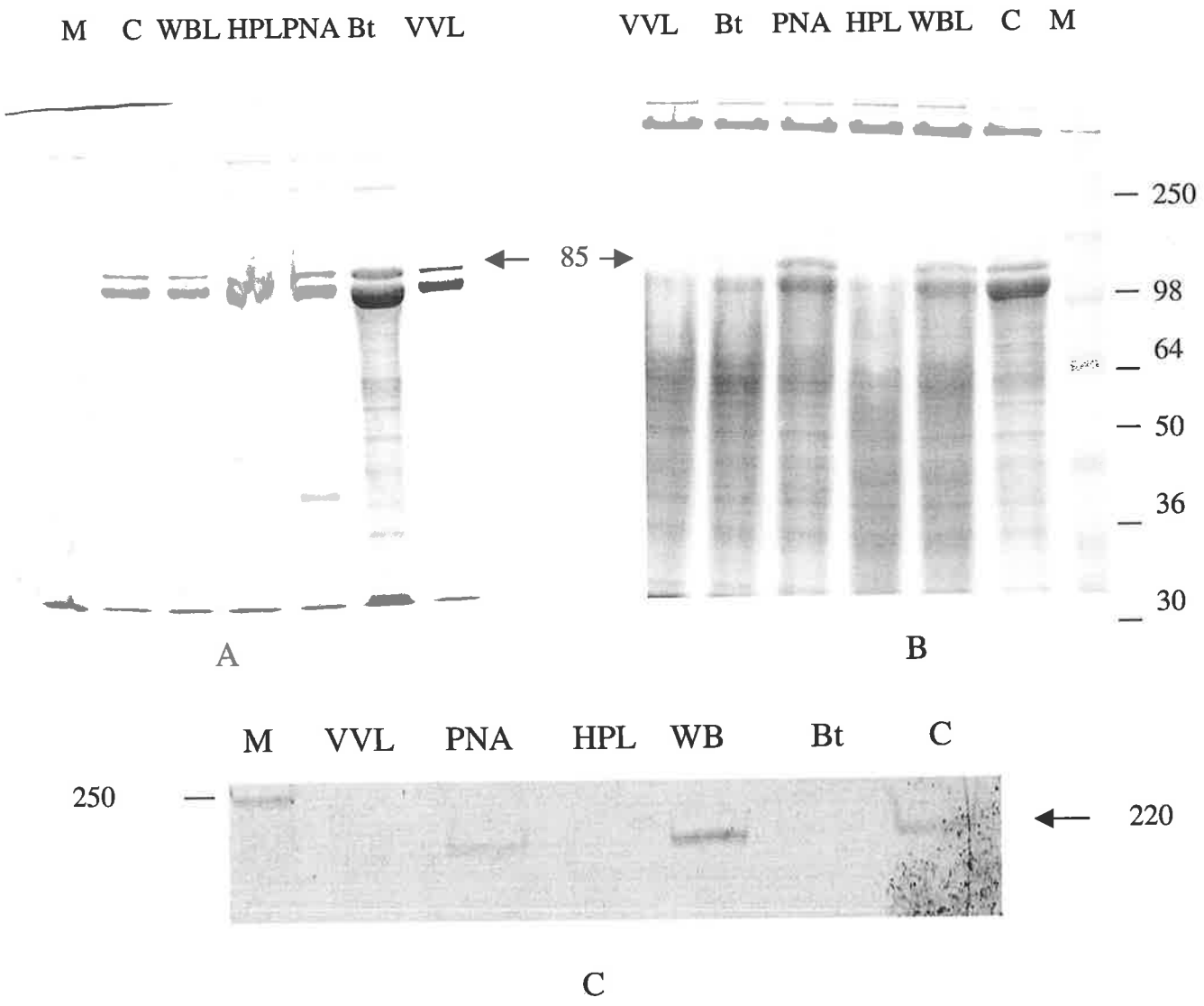


Fig. 10 Aggregation of hemolymph and fat body glycoproteins in the presence of oligomeric lectins and toxin. Addition of oligomeric GalNAc-specific lectins and Cry1Ac to cell-free hemolymph (plasma) causes aggregation of the 85 kDa protein together with other proteins. *V. villosa* lectin (**VVL**, a tetrameric GalNAc-specific lectin); **Bt** (gut juice-activated Cry1Ac); peanut agglutinin (**PNA**, a tetrameric Gal/GalNAc-specific lectin); *H. pomatia* lectin (**HPL**, a hexameric GalNAc-specific lectin); winged bean lectin (**WBL**, a monomeric GalNAc-specific lectin). Addition of buffer **C**) or gut juice did not have an effect. A). Amount of protein recovered after centrifugation is small (less than 0.1% of total protein). It is not known whether this is due to the absence of aggregates in the pellet or whether aggregates did not dissolve even in the presence of SDS, urea or guanidyl-chloride. *B*) Aliquots from each of the supernatants containing ca 5 ug of the plasma mix (total amount ~ 500 ug for each treatment). *C).* Addition of oligomeric GalNAc-specific lectins (VVL,PNA, HPL, WB) and Cry1Ac to fat body extracts causes aggregation and selective precipitation of the large lipophorin subunit apo I.

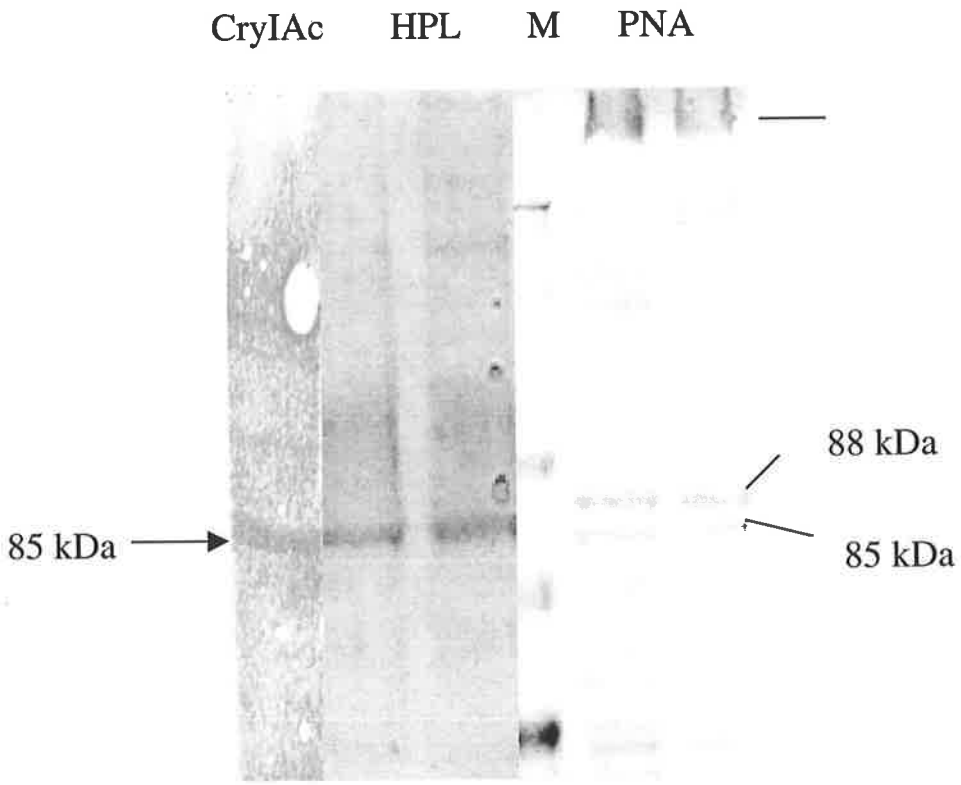


Fig 11 Staining of fat body extract from *H. armigera* with Cry1Ac, peroxidase-conjugated HPL and peroxidase-conjugated PNA. Cry1Ac-binding was visualised with anti-toxin antibodies. HPL and Cry1Ac bind to the same protein namely p85, whereas PNA binds to two glycoprotein such as 85 kDa and 88 kDa as well as high molecular weight aggregates.

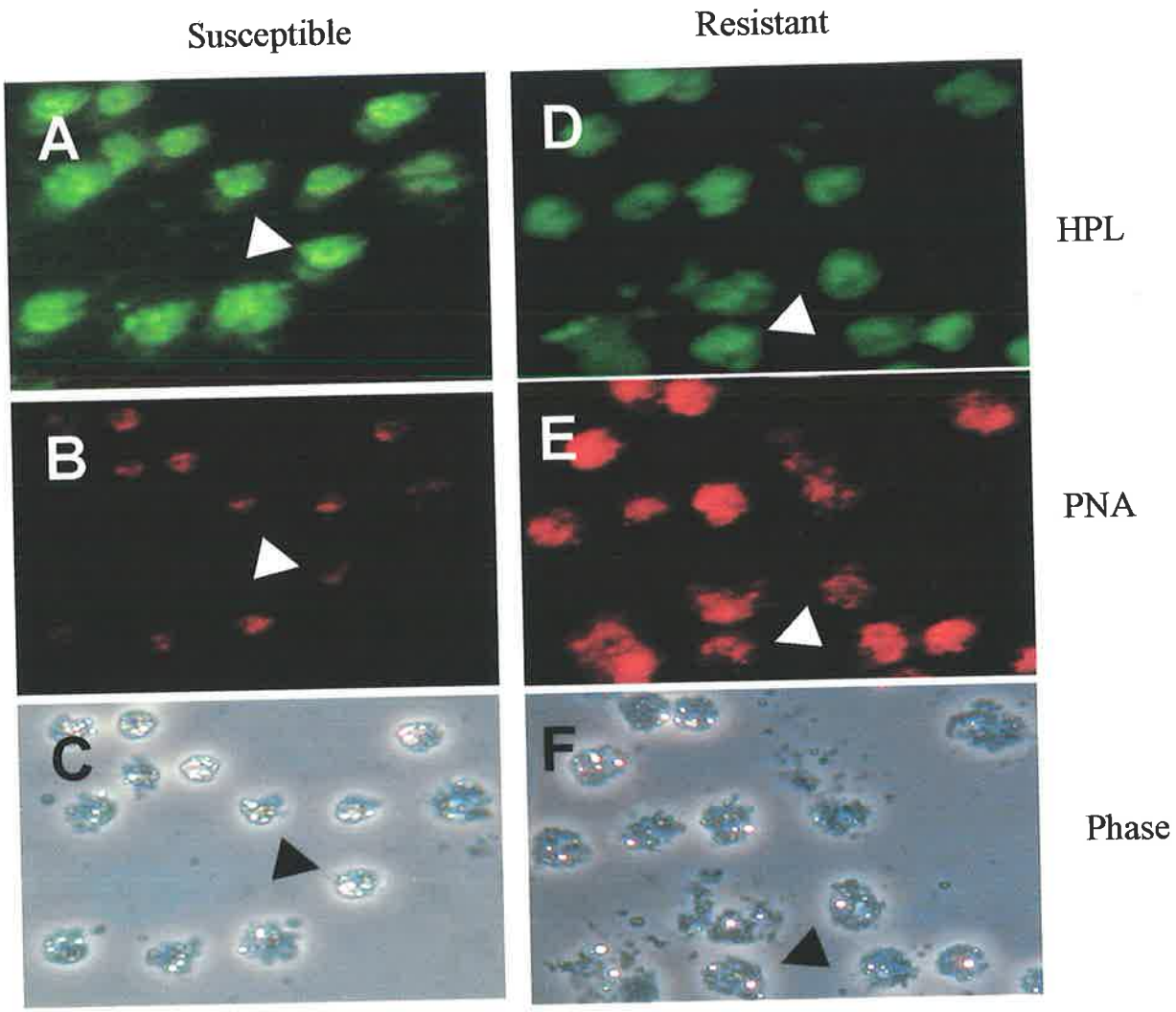


Fig. 12 Lectin–staining of hemocytes from susceptible and Bt-resistant strains of *H. armigera,* using a mixture of FITC-conjugated HPL and TRITC-conjugated PNA. Hemocytes from fourth instar caterpillar were attached to a glass slide and fixed with paraformaldehyde (4%) containing non-ionic detergent (Tween20). Under these conditions lectin stains cell surface and cytoplasmic components (granules). A) FITC-conjugated HPL on hemocytes from susceptible insects. B) TRITC-conjugated PNA on the same hemocytes from susceptible insects. C) Corresponding phase contrast picture. D) FITC-conjugated HPL on hemocytes from resistant insects. E) TRITC-conjugated PNA on the same hemocytes from resistant insects. F) Corresponding phase contrast picture. Granulocytes, but not plasmatocytes are stained with the two lectins. HPL-staining was higher in susceptible insects, whereas PNA-staining was increased in resistant insects. Similar results were obtained with TRITC-conjugated HPL and FITC–conjugated PNA.

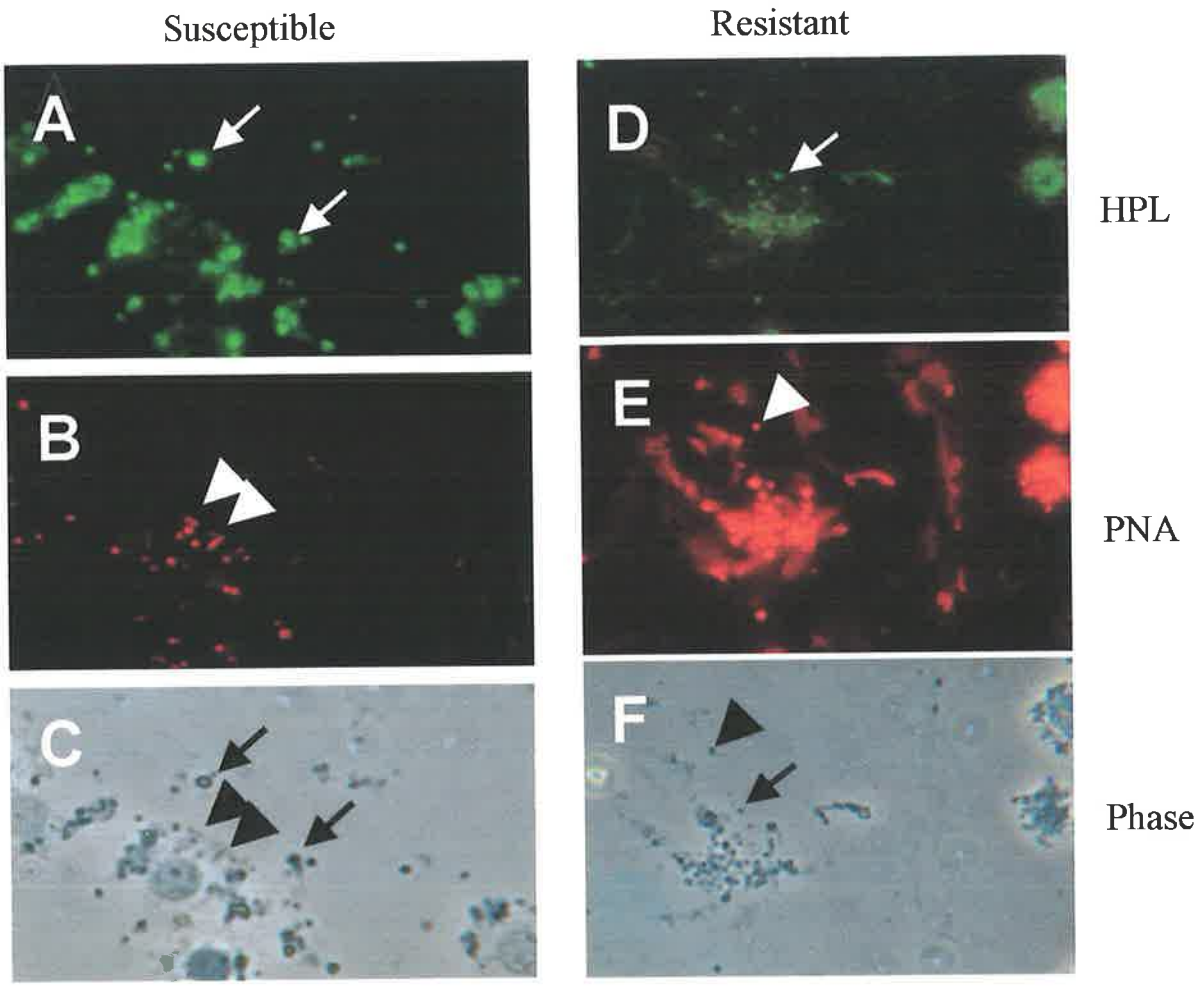


Fig. 13 Glycosylation status of hemocyte glycodeterminants from resistant and susceptible caterpillars of *H.armigera*. Hemocytes were spread for 1-2 hours in the presence of calcium, and after fixation in the presence of mild detergents, stained with FITC-conjugated HPL and TRITC-conjugated PNA. Granule–staining patterns from susceptible hemocytes using A) FITC-conjugated HPL, B) TRITC-conjugated PNA, C), Phase contrast picture. Granule–staining patterns from resistant hemocytes using D) FITC-conjugated HPL, E) TRITC-conjugated PNA, F) Phase contrast picture. Only melanized granules and the nucleus are visible in phase contrast. Note the difference in melanization between HPL-stained (arrow) and PNA stained (arrowheads) granules from susceptible and resistant granulocytes.

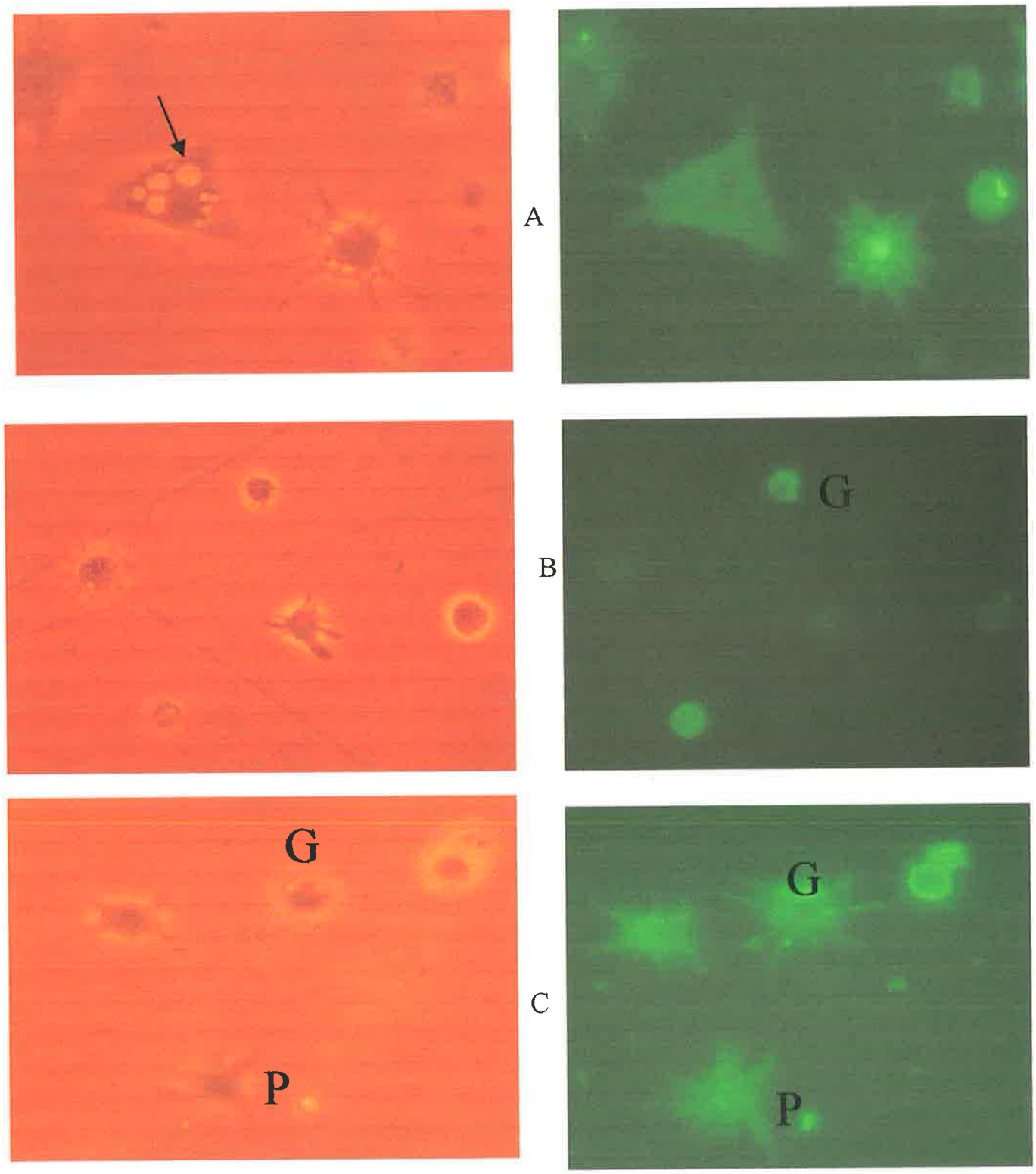
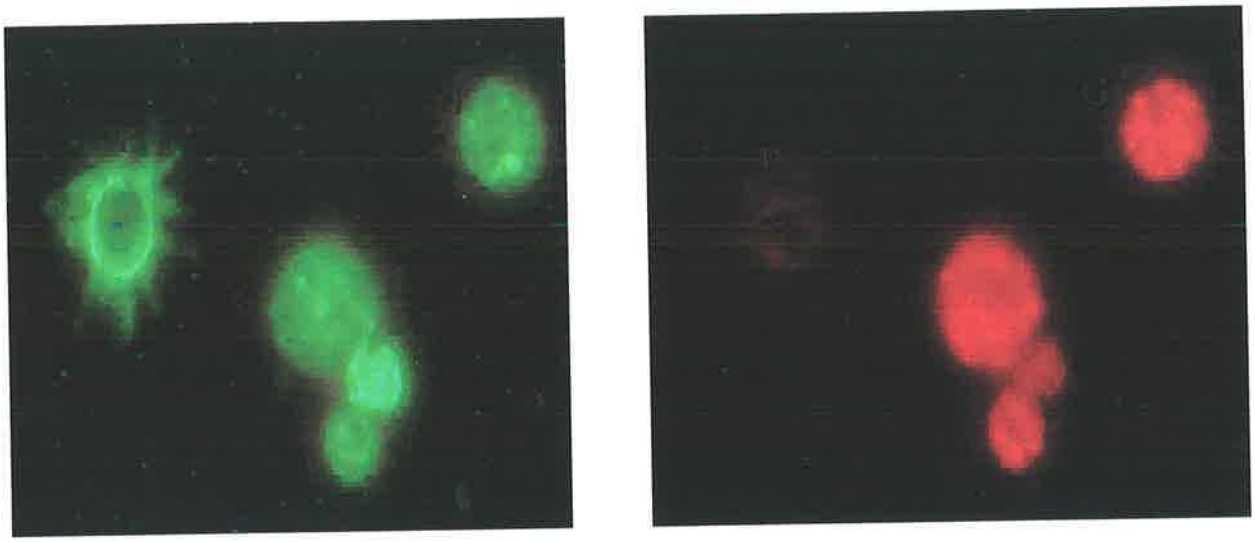
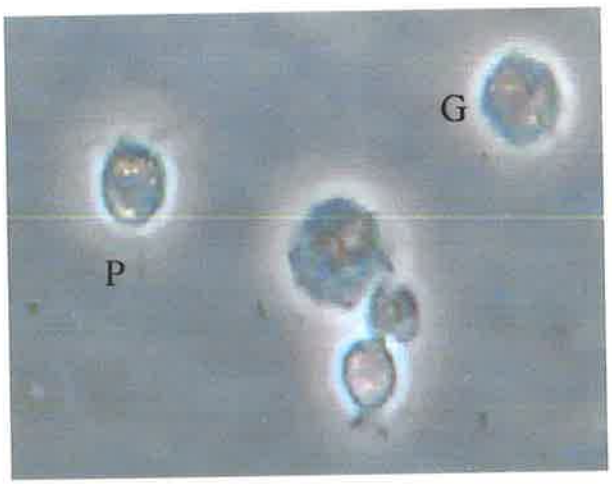


Fig .14 Staining of hemocytes from *H. armigera (susceptible strain)* after fixation in the absence of mild detergent. (A) FITC-conjugated HPL stained granulocytes (G) and plasmatocytes (P), large vesicles of macropinocytosis events that precede staining are not labelled (arrow), whereas small newly induced vesicles are stained. (B) FITC-conjugated PNA stained small round hemocyte only (Granulocytes?). (C) Cry1Ac toxin was visualised with anti-toxin antibodies and stained both granulocytes and plasmatocytes. No staining was observed with antibodies alone.



A B



C

Fig. 15 Hemocytes from *H. armigera* (susceptible strain) fixed with paraformaldehyde and mild detergent was treated with A) FITC-conjugated HPL B) TRITC-conjugated PNA C) Phase contrast picture. Granulocytes (G) and plasmatocytes (P) are stained strongly with HPL, while PNA only stains granulocytes (G) and granules.

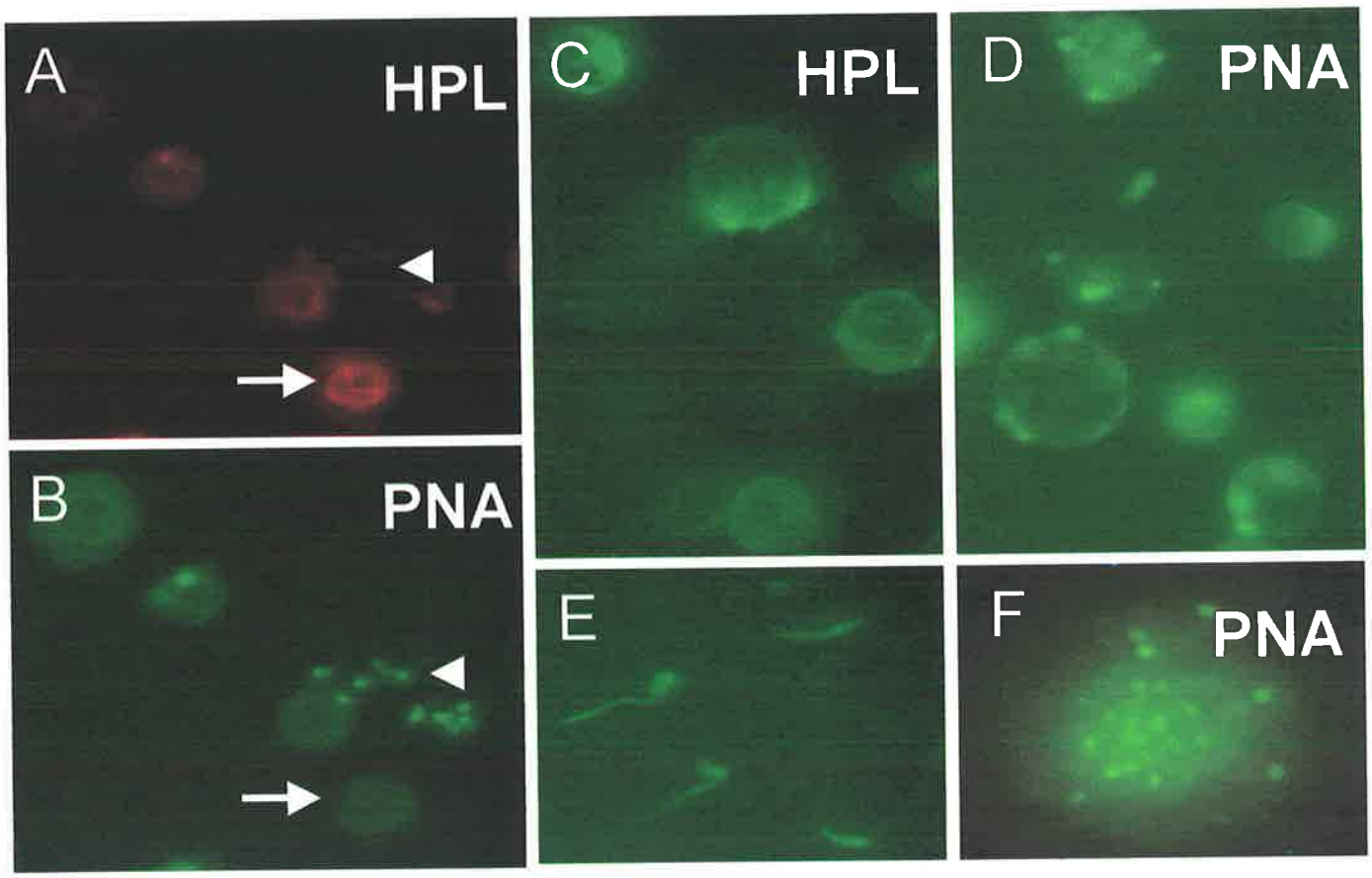


Fig 16 Formation of small globular structures on the surface of hemocytes from *H. armigera* . A-B) Hemocytes were collected in lectin-free buffer and within minutes transferred to a buffer, containing TRITC-conjugated HPL and FITC-conjugated PNA. A) No staining of globular structures was observed in the red light (HPL), but some cells are strongly stained on the surface. B) With green light staining emerged on the cell surface in the form of small globular structures and faint staining on the cell surface. No spreading was observed in stained cells or in phase contrast (not shown). Note that staining of cells with the two lectins is almost mutually exclusive. C) Incubation with hemolymph (lectin-free) increased staining on cell surface after incubation with FITC-conjugated HPL or D) FITC-conjugated PNA. Extracts from fat body. E) Incubation in the absence of lectin or in the presence of FITC-conjugated HPL stained fibrous structures. F) Incubation in the presence of FITC-conjugated PNA stained small globular structures.

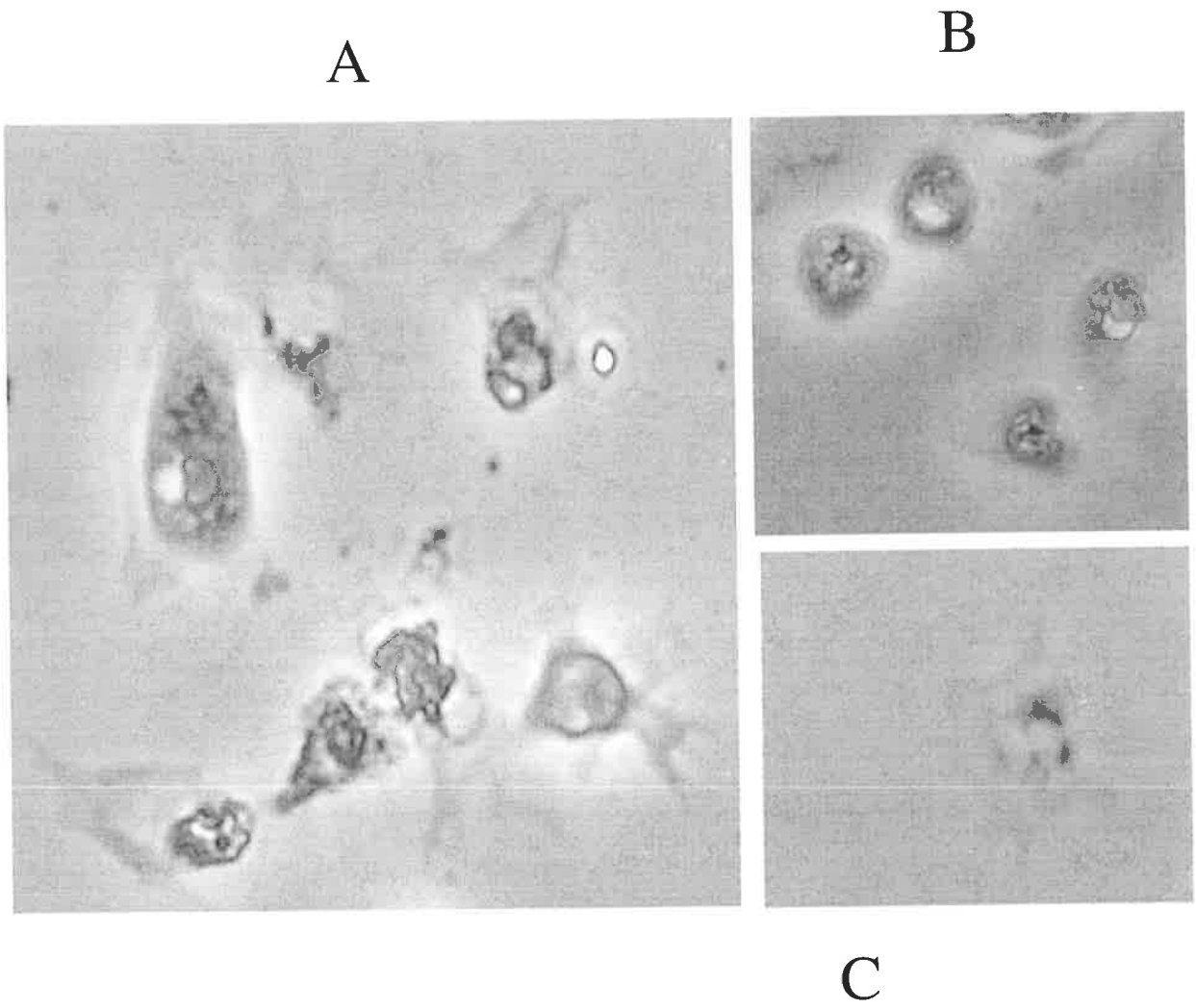
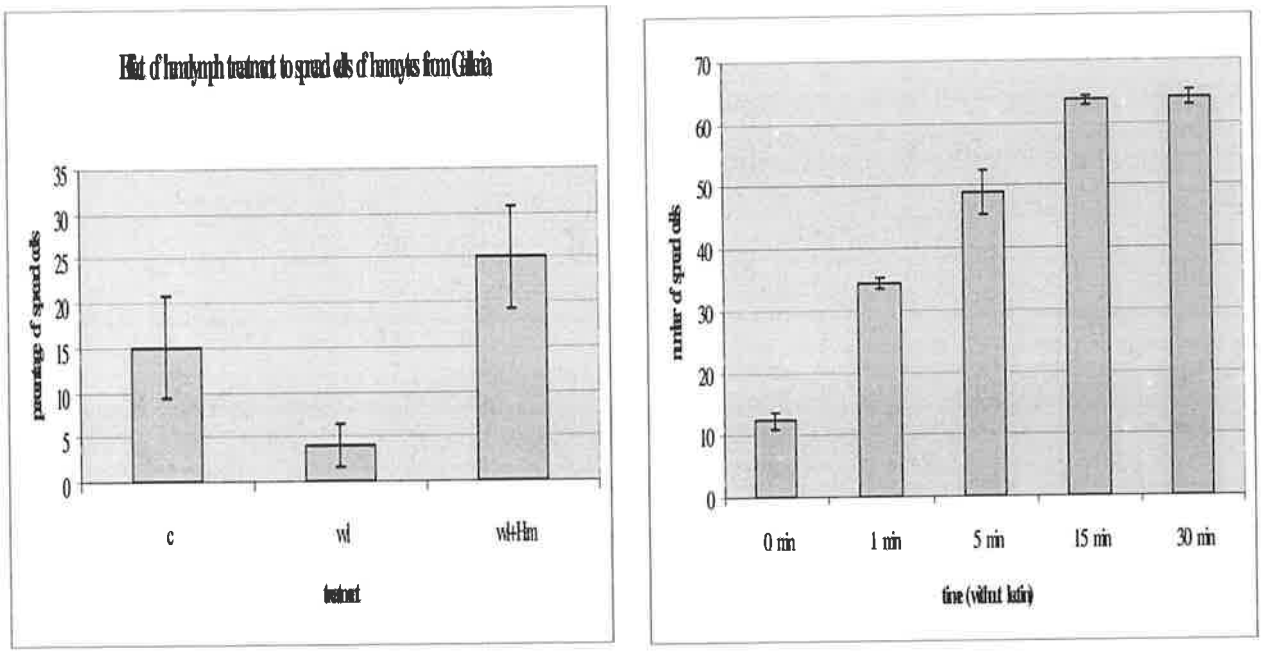


Fig 17 Hemolymph containing hemocytes from *G. mellonella* larvae after different lectin treatments show different amounts of hemocyte spreading and attachment A). Control showing attachment and spreading of hemocytes in blood smears (no lectin treatment). B) Hemolymph mixed with lectin (VVL) immediately after bleeding. Under these conditions hemocytes stay round (without spreading). C) Hemocytes from lectin-treated hemolymph as in (B) were subsequently washed with PBS three times. Cell-free hemolymph (plasma) was added to washed hemocytes and hemocytes plated onto glass slide. Hemocytes show spreading.



A

B

Fig. 18 Hemocyte spreading in lectin-treated hemolymph. A) Hemolymph, containing hemocytes, was treated with lectin (VVL) and hemocytes were separated from serum by repeated washes. Non-treated serum was added and hemocytes were allowed to attach and spread on a glass surface. Percentage of hemocytes spread in non-treated hemolymph (C), hemocyte spreading in lectin-treated hemolymph (VVL) and hemocyte spreading after lectin-treated hemolymph was replaced with non-treated serum. Average of two independent experiments is shown. Differences between control and VVL + hem are not significant. B) Hemolymph, containing hemocytes was collected in PBS buffer and lectin was added after different time periods as indicated.The number of total cells were similar in each experiments but number of spread cells increased with time.

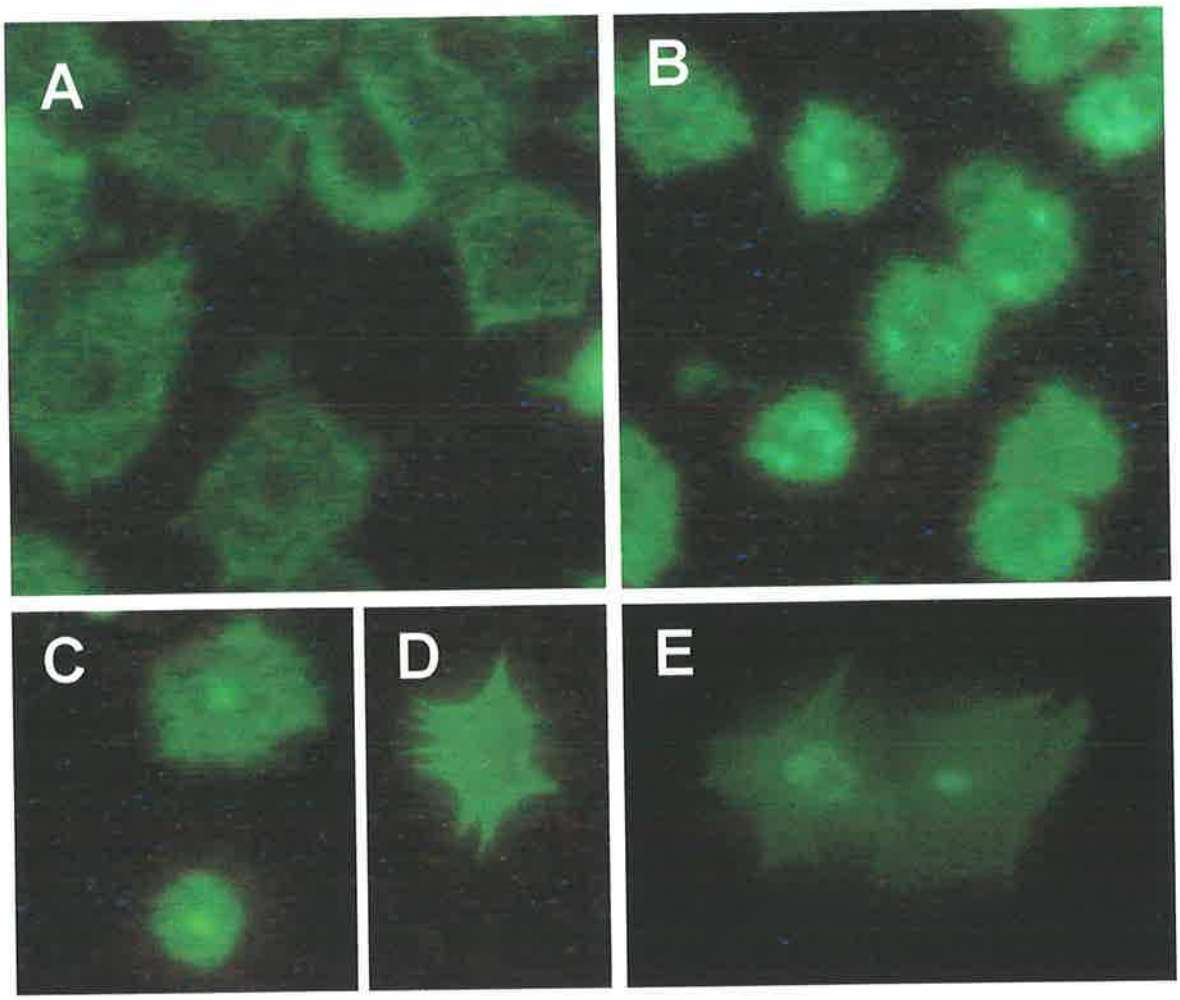


Fig. 19 Hemocyte spreading after different treatments. Hemolymph containing hemocytes was treated with lectin (HPL) and hemocytes were separated from plasma by repeated washes. Dialysed fractions from low-density gradient centrifugation of non-treated serum were added and hemocytes were allowed to attach and spread on a glass surface. Hemocytes were fixed and actin-cytoskeleton visualized with FITC-conjugated phalloidin. **A**) Hemocytes from non-treated hemolymph. Note the evenly spread actin-cytoskeleton with a gap of staining over the nucleus. **B**) Hemocytes from lectin-treated hemolymph. Note the reduced spreading and the dotted phalloidin-staining over the cytoplasm and around the nucleus. **C**) Hemocytes from lectin-treated cells complemented with fraction 6 (high-density fraction) from a low-density gradient centrifugation of serum. **D**) Hemocytes from lectin-treated cells complemented with fraction 17 (low-density fraction, containing mainly lipophorin) from a low-density gradient centrifugation of serum. Note the star-like spreading, which is different from non-treated hemocytes involving filopodia-formation. Note also the dotted phalloidin-staining around the nucleus, which resembles lectin-treated hemocytes.

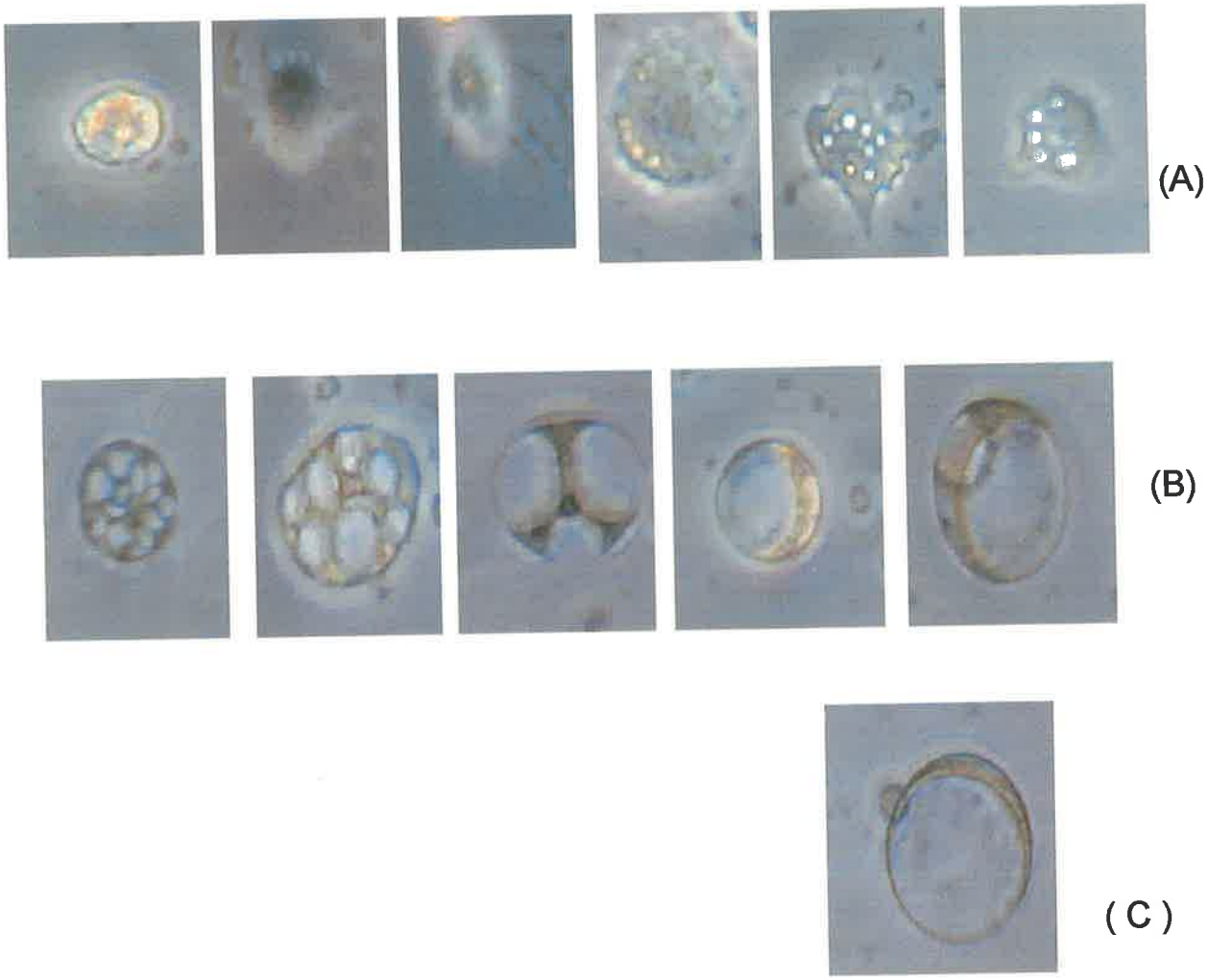


Fig 20. The process of macropinocytosis and osmofragility in a cell line of *P. xylostella*. Single cells were observed from non-attached to attached and spread forms to initiation of multiple macropinocytosis events. A) In some cells endosomes (macropinocytosis vesicles) increased in size B), leading to foam cells C).

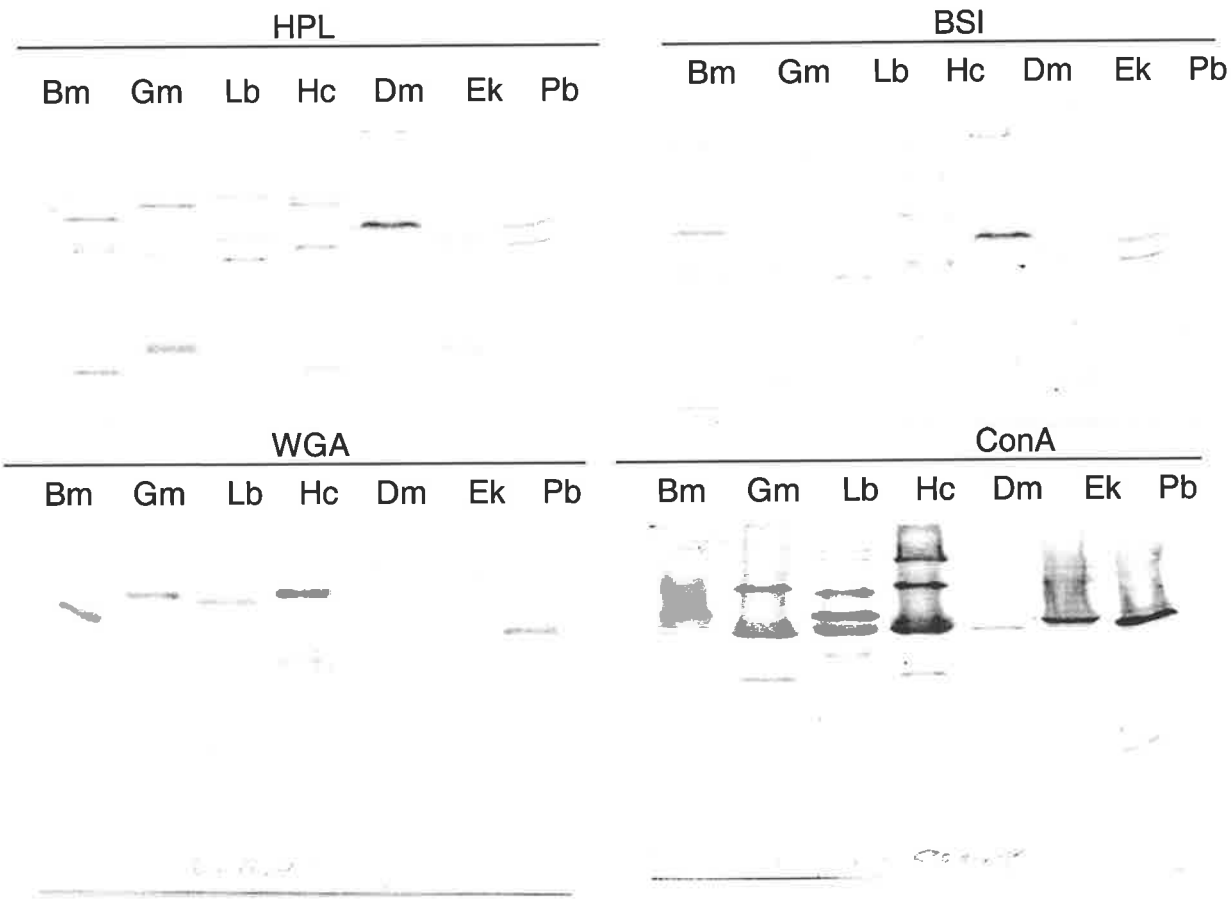


Fig. 21 Relative distribution of glycodeterminants in hemolymph protein extracts from *Drosophila* and lepidopteran insects. Hemolymph from late larval instars was extracted with SDS-containing buffer separated on SDS-PAGE and exposed to various lectins on Western blots.

A) Peroxidase-conjugated *H. pomatia* lectin (HPL). B) Peroxidase-conjugated *Bandeiraea simplicifolia* (BSI). C) Peroxidase-conjugated wheat germ agglutinin (WGA). D) Peroxidase-conjugated concanavalin A (ConA). Note the predominant staining of *Drosophila* protein extracts with GalNAc-specific lectins mainly at a 100 kDa protein (hemomucin). In contrast hemolymph from most lepidopteran species contain multiple glycoproteins stained strongly with GlcNAc-specific lectins.

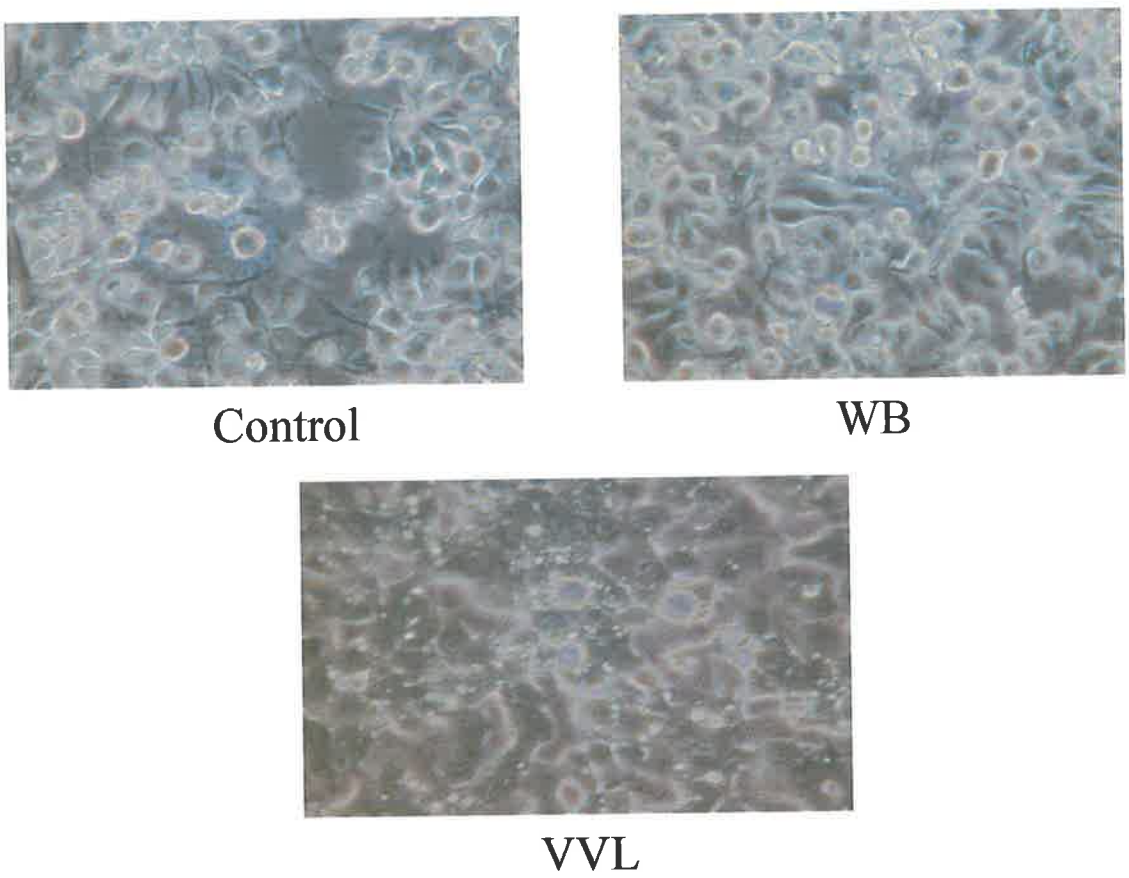


Fig. 22. Attachment and spreading of *mbn-2* cells in the presence and absence of lectins. Cells exposed to lectins from *P. tetragonolobus* (winged bean, WB) were similar to control and did not show any significant attachment and macropinocytosis. In contrast, tetrameric lectins with GalNAc- and Gal/GalNAc-specific binding properties such as VVL and PNA (not shown), induced attachment and macropinocytosis.

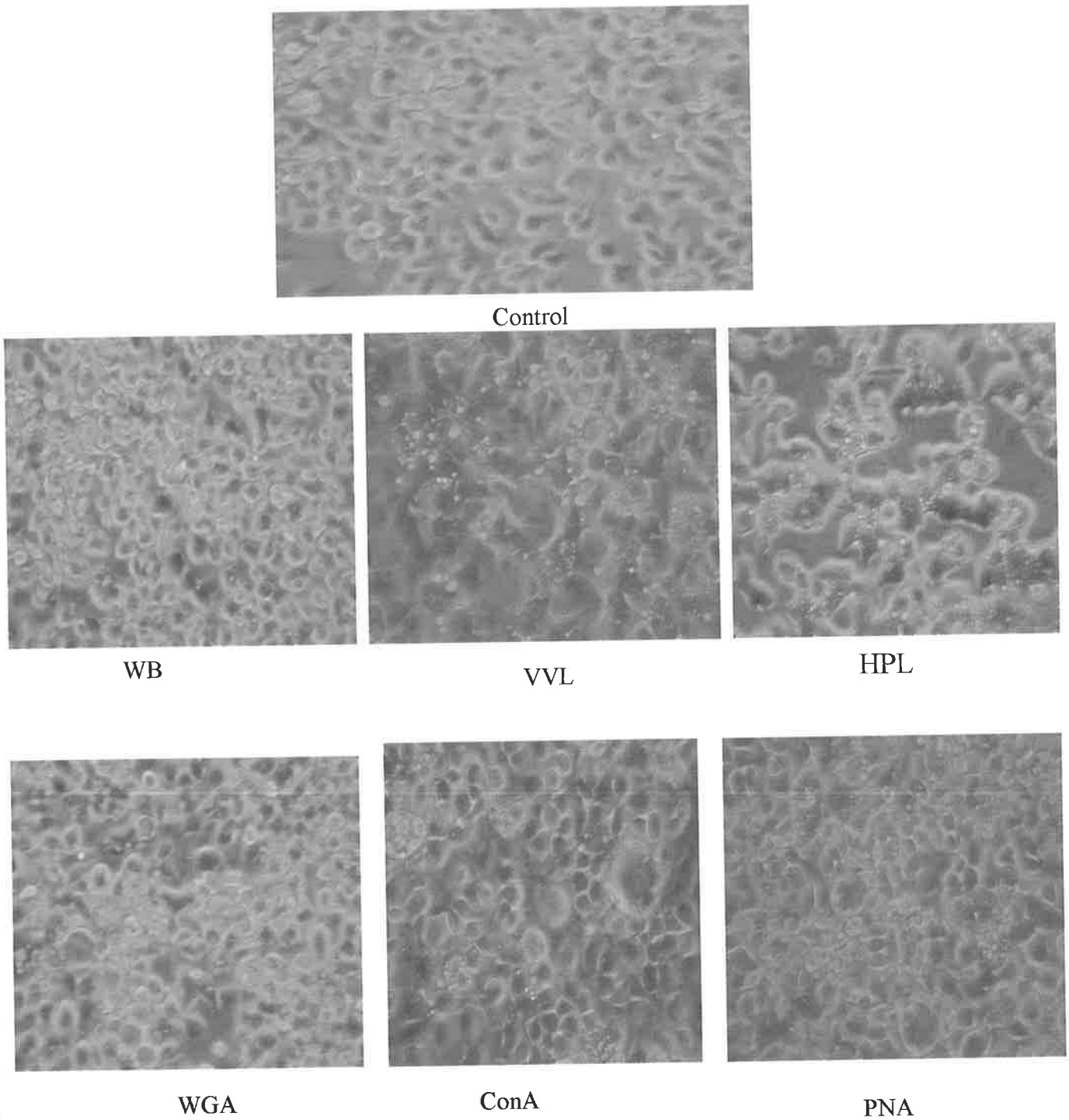


Fig. 23 Attachment and spreading of *Plutella* cells in the presence and absence of lectins. Lectins from *P. tetragonolobus* (winged bean, WB) were similar to control and did not show any significant attachment and macropinocytosis. In contrast, tetrameric lectins with GalNAc- and Gal-specific binding properties such as VVL and PNA, induced attachment and macropinocytosis.

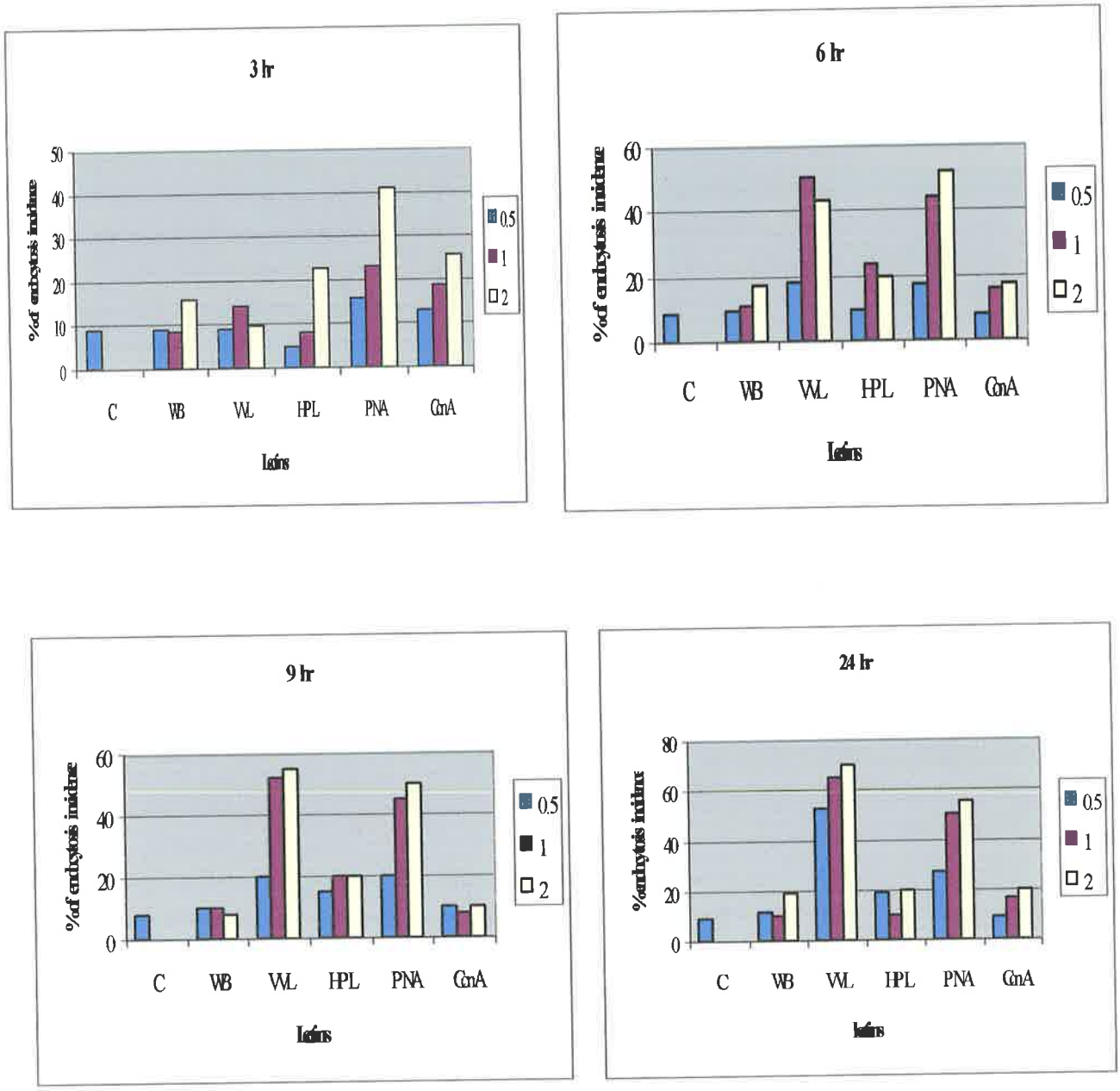


Fig. 24 Macropinocytosis induced by oligomeric lectins in *mbn2*-cells. After addition of lectins at various concentrations, the relative number of cells with macropinocytosis events was monitored over different time points. Tetrameric lectins, such as VVL and PNA showed significant higher macropinocytosis events, whereas WB and hexameric HPL were less effective.

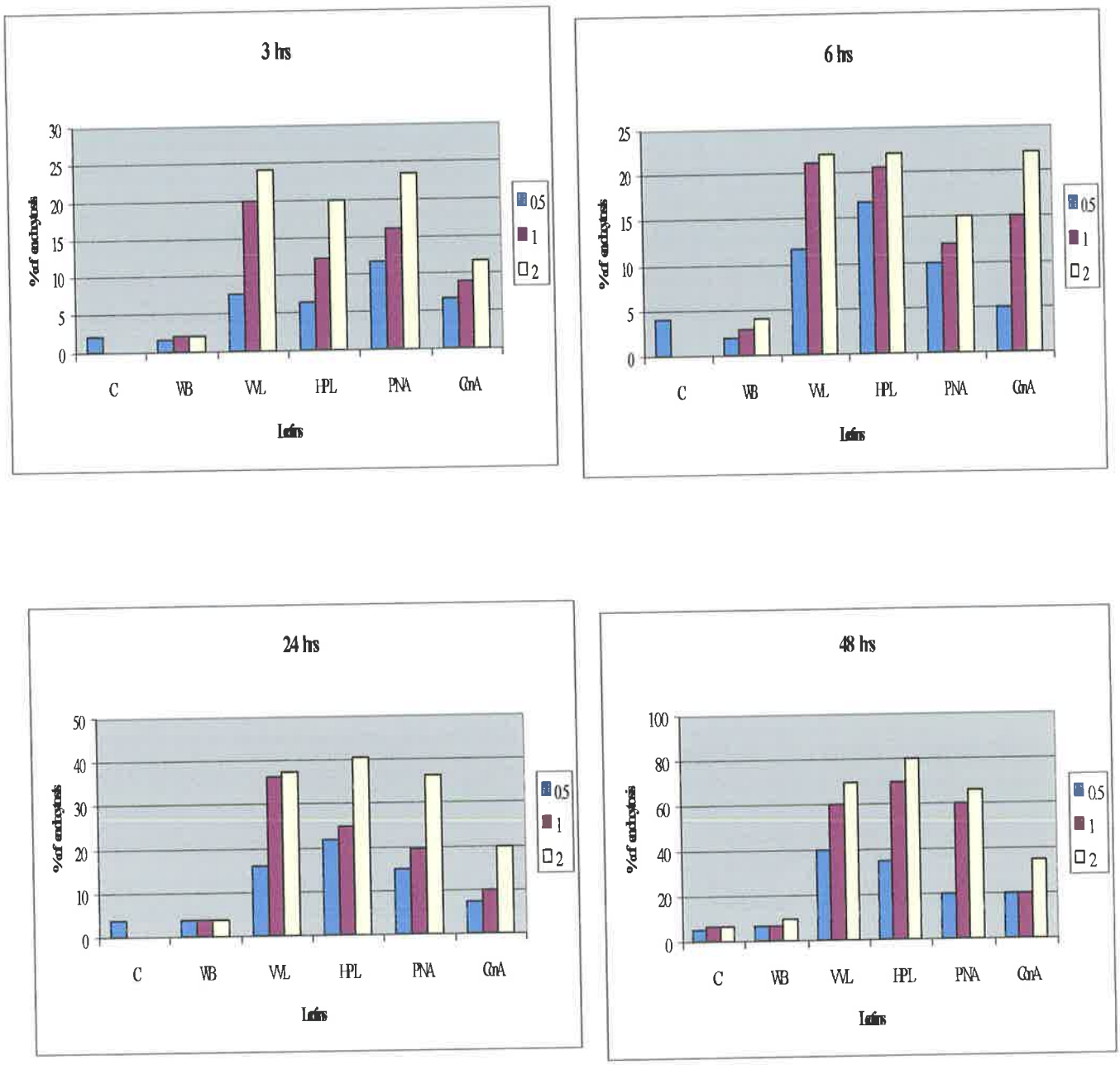
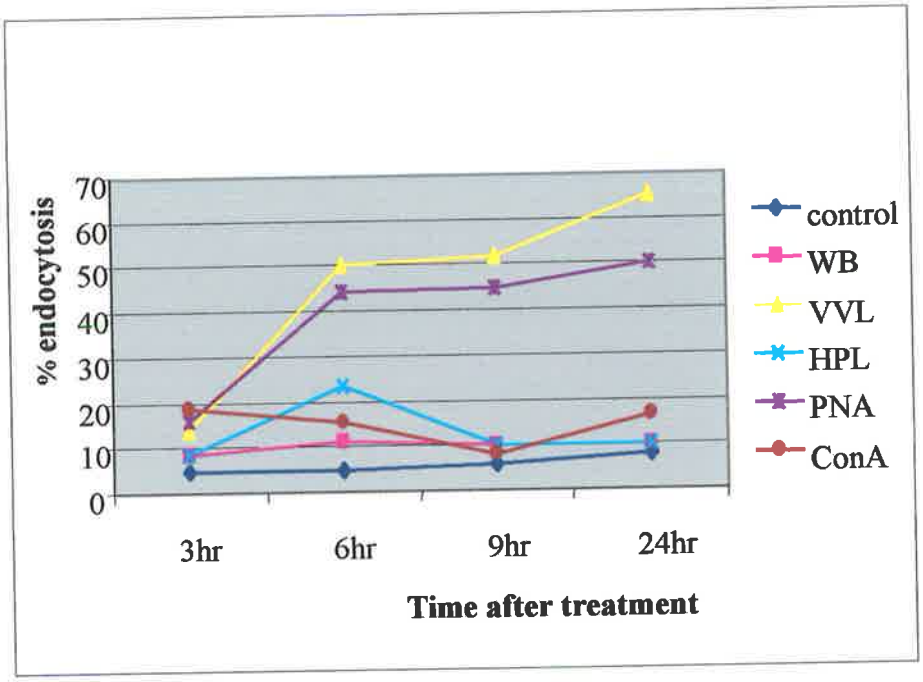
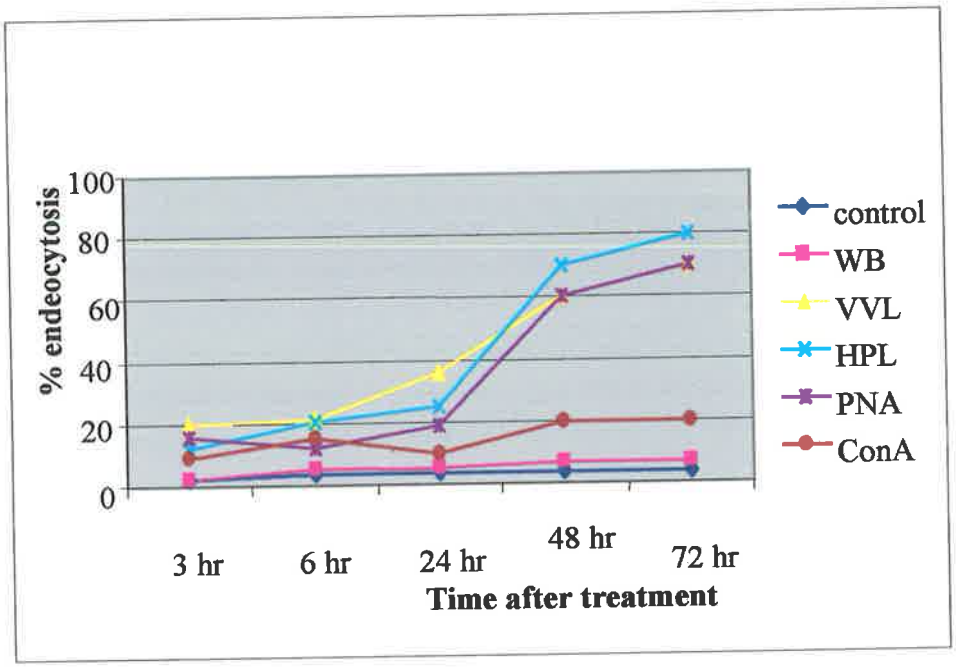


Fig. 25 Lectin-mediated macropinocytosis in *P. xylostella* cells. After addition of lectins at various concentrations, the relative number of cells with macropinocytosis events was monitored over different time points. Compared to *mbn2*-cells (see Fig. 24), induction in *P. xylostella* cells is delayed. In these cells HPL is an effective inducer of macropinocytosis, whereas ConA is less effective.



A



B

Fig . 26 Number of macropinocytosis events after treatment with various lectins. A) Diagram of macropinocytosis induction in *mbn2*-cells. B) Diagram of macropinocytosis induction in *P. xylostella* cells.

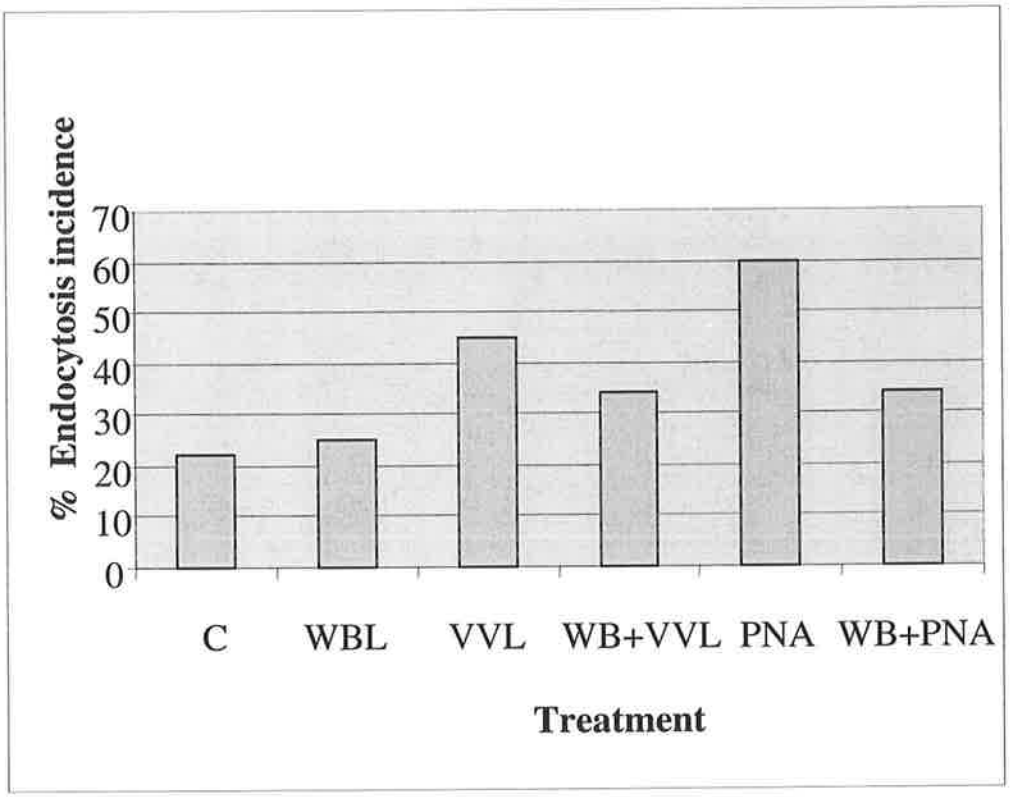
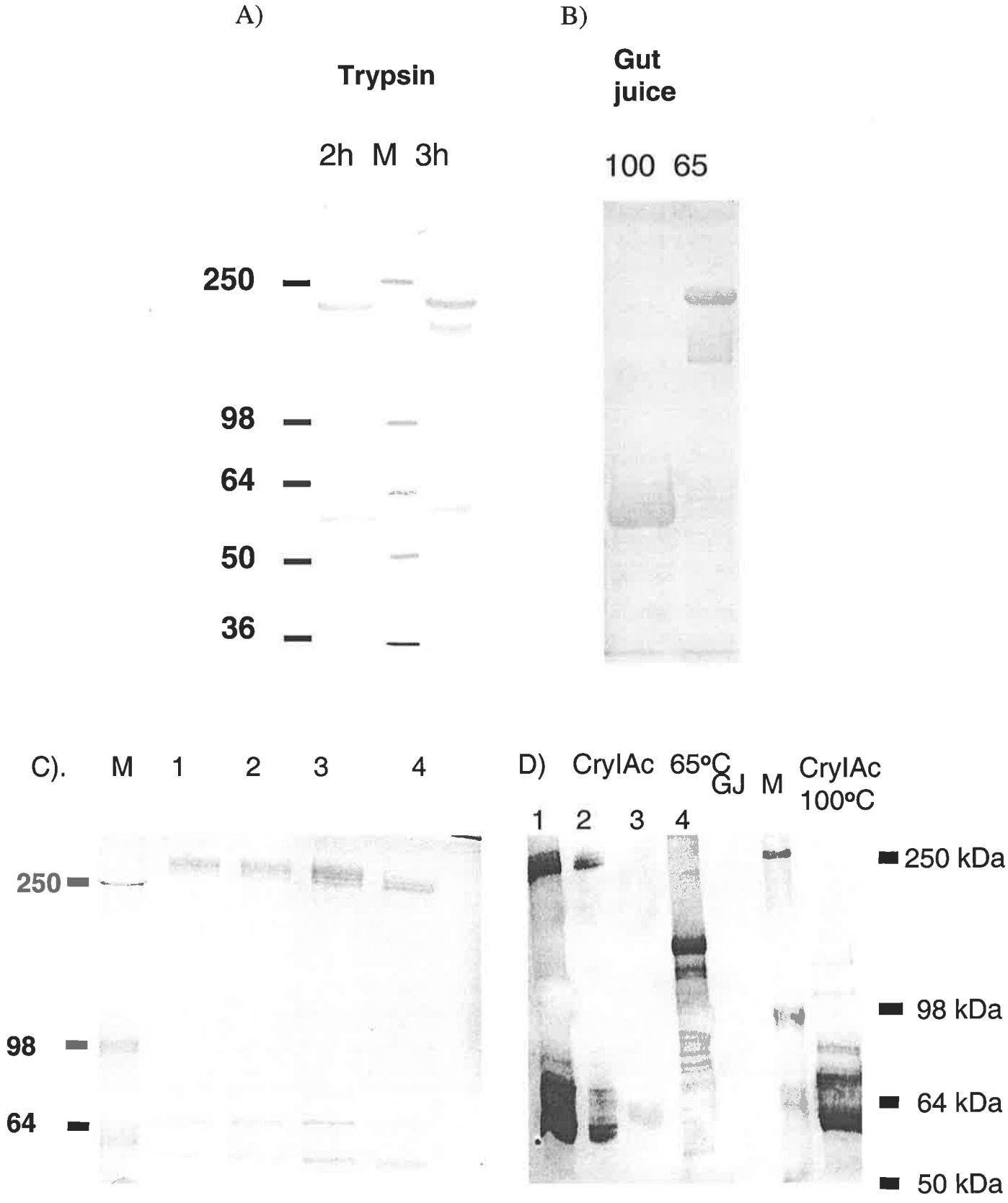


Fig. 27 Macropinocytosis induction in *mbn2*-cells by oligomeric lectins is reduced in the presence of WBL. To test, whether WBL was able to compete for binding sites of tetrameric lectins, the induction of macropinocytosis was monitored in the presence of both lectins. Each bar represents an average of two independent experiments.

Fig . 28 Mature Bt-toxins form oligomeric complexes. *A*) Protoxin activation with gut juice extracts and trypsin. Protoxin purified from *B. thuringiensis* subsp. *kurstaki* HD73 was solubilized in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6 and digested with trypsin or gut juice extracts. Trypsin-digest for 2 hours (2h) and three hours (3h). Digest was extracted in the presence of SDS at 65°C and analyzed on SDS-PAGE. Extraction at 100°C showed a single band at 60 kDa (not shown). *B*) Gut juice-extract from the lepidopteran species *Pieris rape* incubated for five hours and extracted at 65°C and 100°C.

C) Protoxin activation with gut juice extracts. Protoxin purified from *B. thuringiensis* subsp. *kurstaki* HD73 was solubilized in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6 (Aronson et al., 1999) and digested with gut juice extracts. Protoxin and gut juice-extract from the lepidopteran species *Pieris rapae* incubated for 30 min (1), 1 h (2), 2 h (3) and five hours (4) and extracted at 65°C in SDS-containing buffer. The mature toxin (69 kDa) is predominant initially, but is replaced by a 60 kDa protein. Both proteins appear to form hetero-oligomeric complexes, which form a cluster of narrow bands above the 250 kDa marker band. The relative amounts and distribution of these narrow bands are correlated with the relative composition of the 60 and 69 kDa bands. Extraction at 100°C in SDS-containing buffers take off the bands above 250 kDa.

D) Western blot of gut juice-activated protoxin after incubation of one hour (1) and five hours (2) . Non-toxic recombinant protein (3), protoxin (4), marker (M), gut juice (GJ), gut juice-activated protoxin (as in 1) extracted at 100°C.


A)
Trypsin
2h M 3h
250
98
64
50
36
B)
Gut juice
100 65
C).
M 1 2 3 4
250
98
64
D)
CrylAc 65°C
GJ M CrylAc 100°C
1 2 3 4
250 kDa
98 kDa
64 kDa
50 kDa

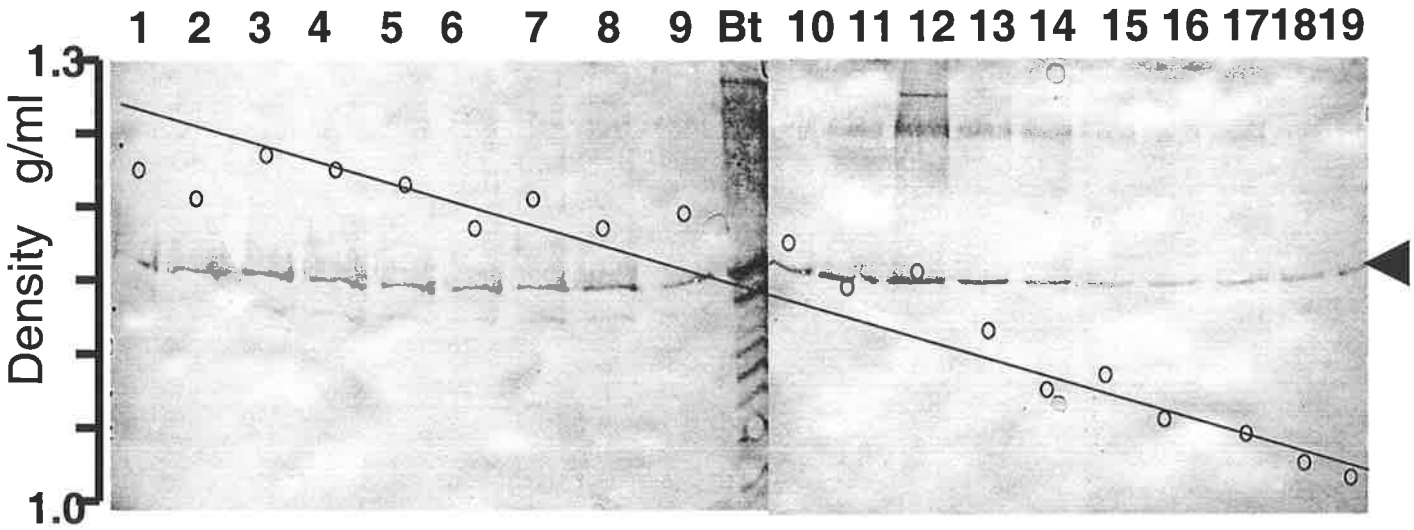


Fig. 29 Western blot of cell-free hemolymph mixed with activated Cry1Ac and separated into 20 fractions by low-density gradient centrifugation. Aliquots from each fraction were dissolved in SDS and separated on a SDS-PAGE and transferred on nitrocellulose filter and incubated with anti-Cry1Ac antibodies.

Densities were measured in a control gradient by weighing 1ml volumes from each fraction. Note the enrichment of high molecular weight Cry1Ac complex in fraction 11-15 in contrast to the monomeric toxin, which is homogeneously spread over the gradient. The fact that monomeric toxin is spread over the length of the gradient, including the low-density section (arrowhead), suggest that this form of the toxin may also form a complex with other proteins (fibers?), which are not stable at 65°C.

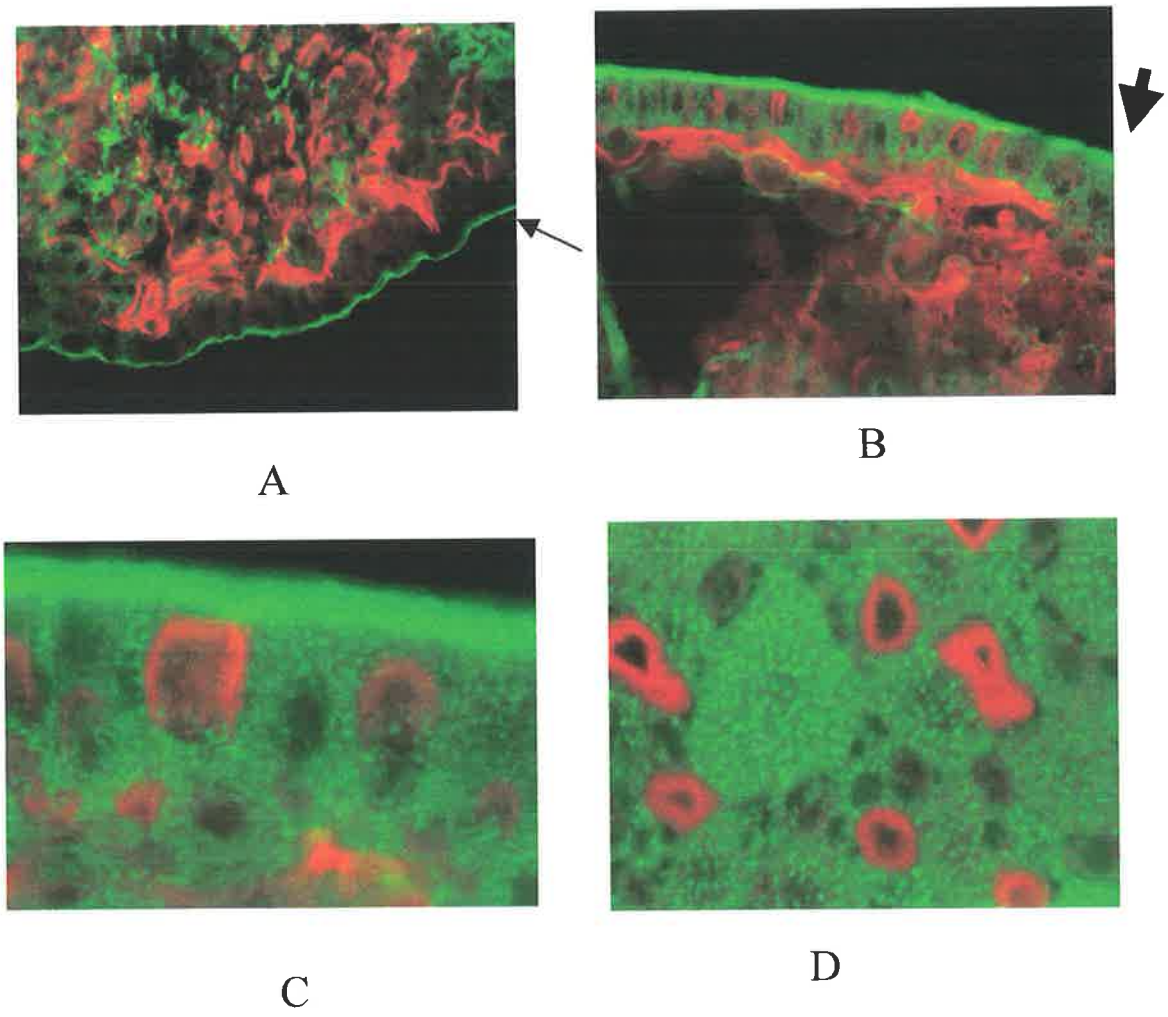


Fig 30 Staining of midgut epithelium from fourth instar DBM larvae with FITC-conjugated HPL with increasing magnification of optical sections cutting alongside epithelial cells (A-C) and across epithelial cells (D). C) and D) are the same magnification. A) HPL stains dot structures inside the gut lumen mainly at the peritrophic membrane. B) HPL stains the basal membrane C). and D) HPL stains dotted structures inside and at the interphase of gut cells. Note the strong staining to the basement membrane representing the lining of the hemocoel (arrows). The staining is also increased around the peritrophic membrane, and within the epithelial cells in a basal location towards the peritrophic membrane probably representing Golgi or secretory vesicles (see Fig 33). The red colour is due to autofluorescence visible in the red channel.

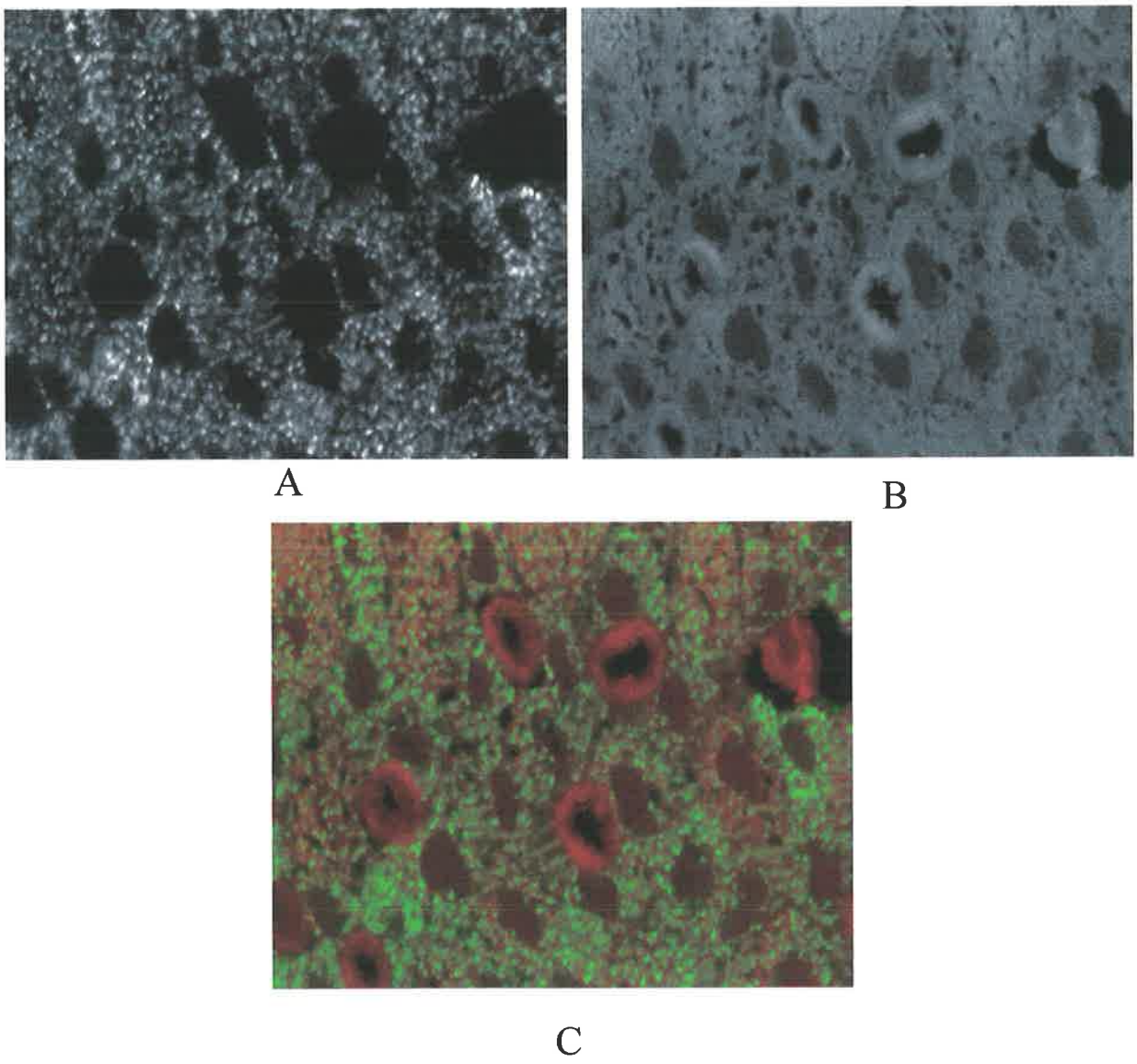
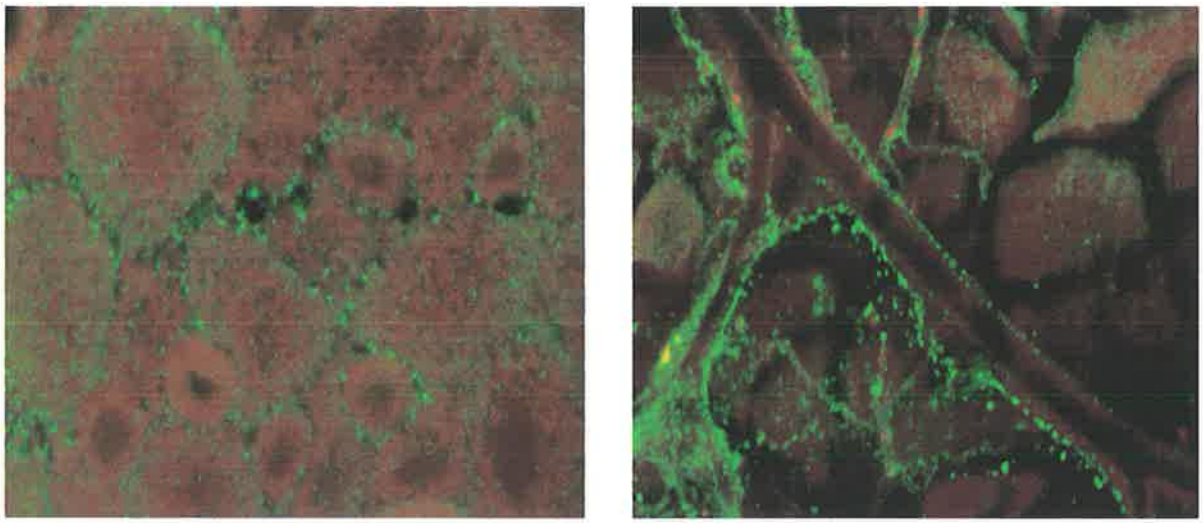


Fig. 31 Combination of HPL and toxin staining in the gut of DBM. A) FITC-conjugated HPL. B) Activated toxin (CryIAc) visualised with anti-Cry1Ac–antibodies and TRITC-conjugated secondary antibodies and C) Combination of HPL and Cry1A toxin staining; Green: HPL ; Red: Cry1Ac; Orange: Co-location of toxin and HPL. Note that most of the toxin-staining is to locations that appear to be part of the intercellular space. Compared to the toxin-staining to dot-like structures in the gut lumen, the staining in the epithelium is relatively low level (see Fig 35).



A B

Fig . 32 Localisation of Cry1Ac toxin with antibodies and FITC-conjugated secondary antibodies in the midgut of DBM. (A) In the outer layer of midgut epithelium, toxin binds to dot-like structures mostly in the space between the cells (intercellular space); (B) On the surface of midgut tissues, dot-like structures are stained by the toxin, which are attached to trachea and basement membrane.

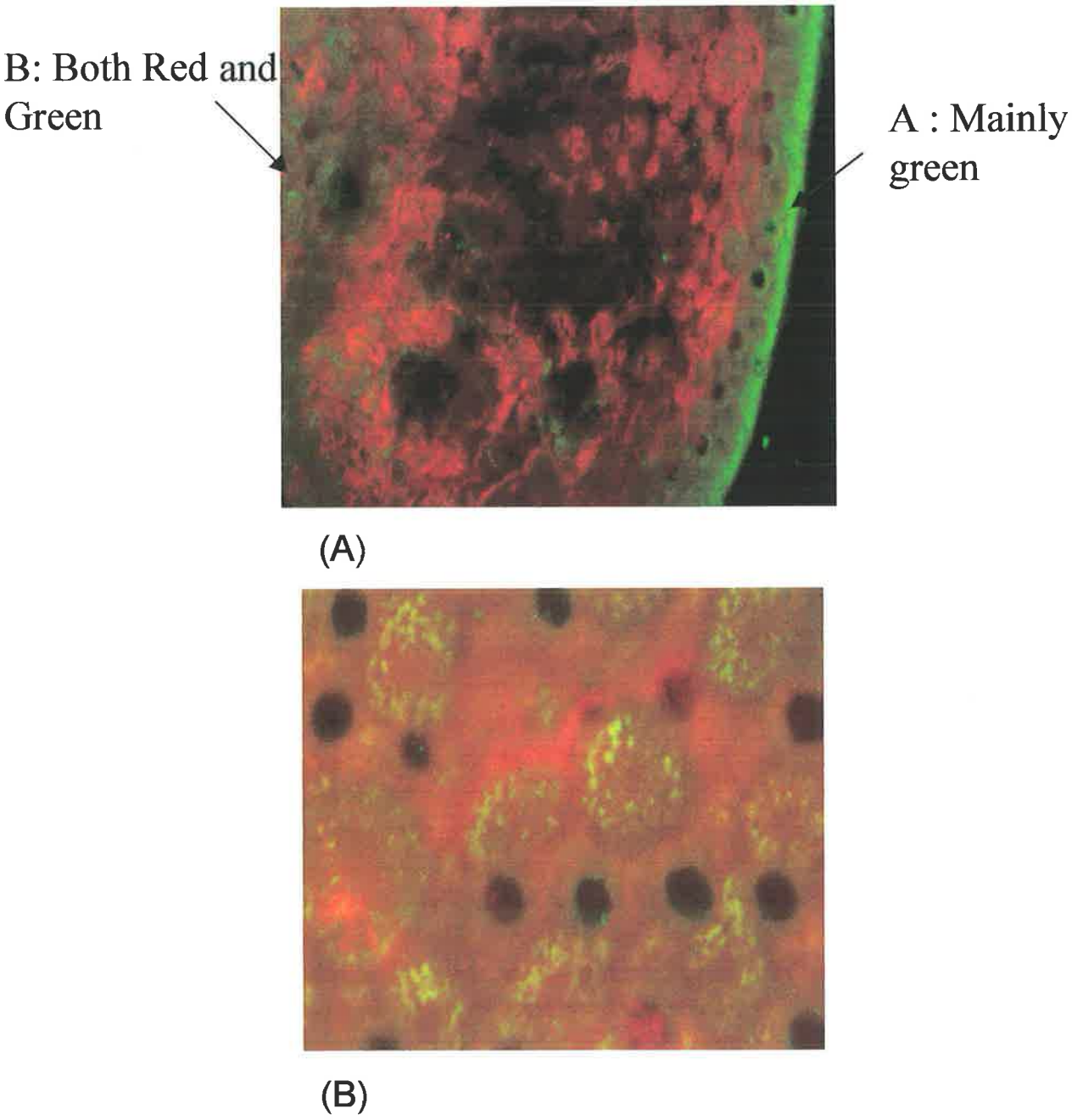
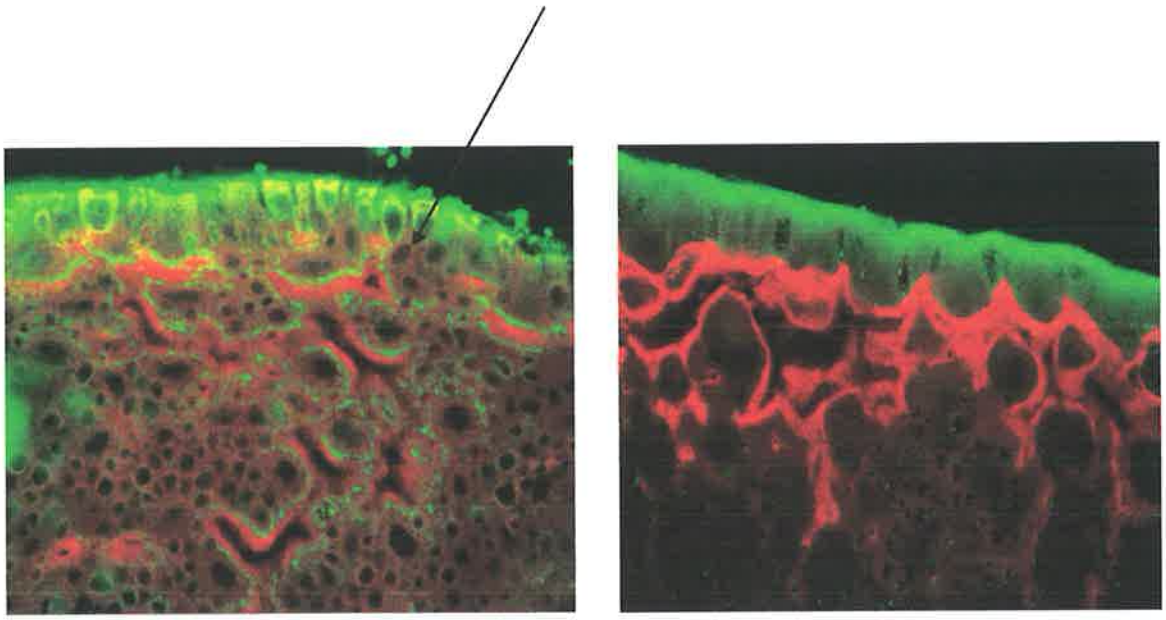


Fig . 33 Co-staining between PNA-conjugated TRITC and activated Bt-toxin Cry1Ac. Localisation of the toxin was visualised after incubation with anti-Cry1Ac antibodies and FITC-conjugated secondary antibody on the midgut of DBM. Overlay of red and green channels show outer layer of the gut corresponding to gut epithelium (mainly green) stained with toxin. Gut lumen stains with both PNA and Bt-toxin.

B) Enlargement of gut cells in overlay. The PNA staining inside gut cells could be due to staining of Golgi vesicles, according to the basal location within epithelial cells.



A B

Fig 34 Staining of midgut from DBM larvae with antibodies against prophenoloxidase and FITC-conjugated secondary antibodies, showing intensive staining to dot-like structures around cell borders (arrow in A) and peritrophic membrane.

A) and B) show the variation of staining visible among different caterpillars, which may be due to differences in immune-induction. In contrast to HPL-staining (Fig 30 B), PPO-staining is more intensive in intracellular space.

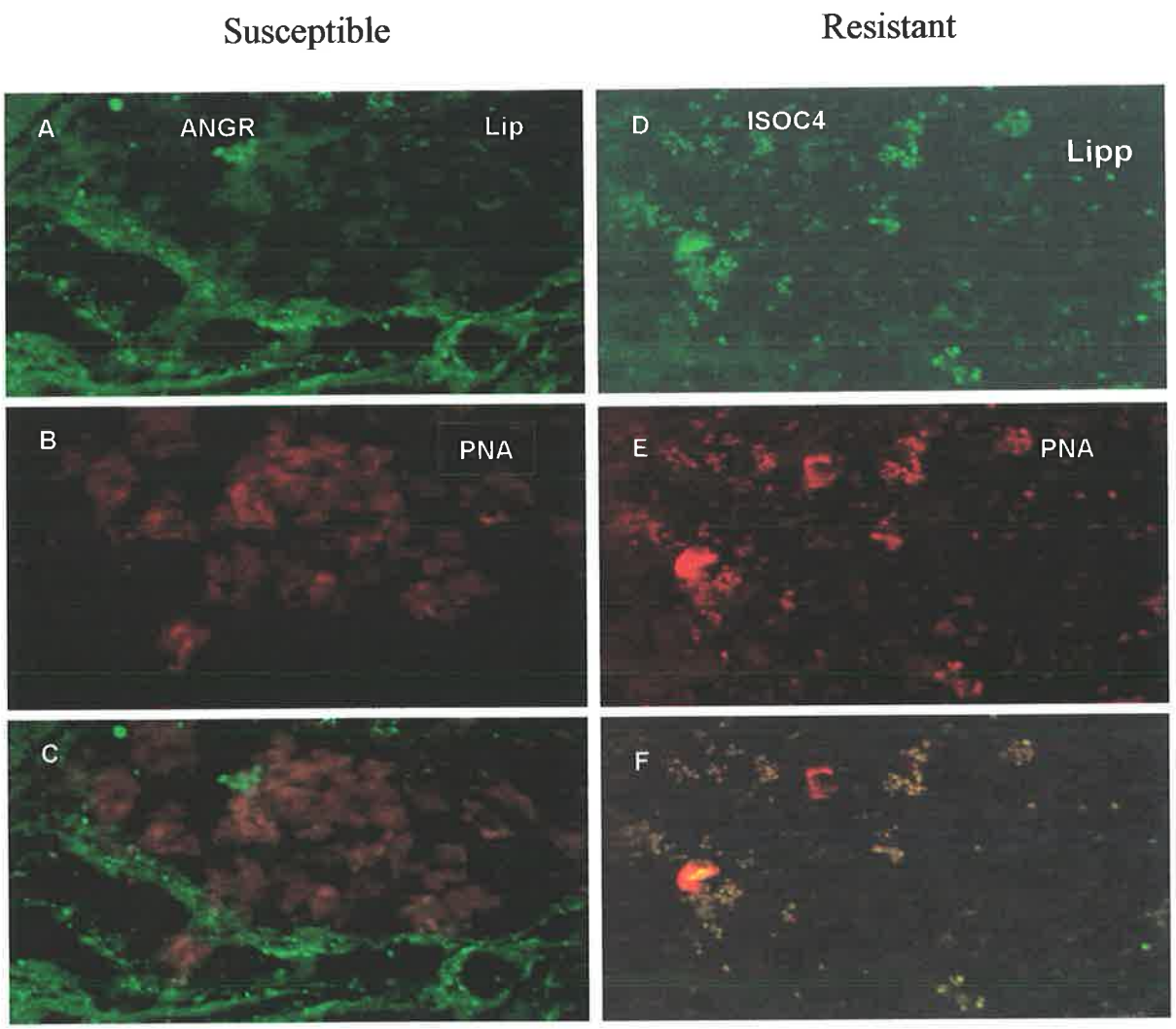


Fig. 35 Presence of lipophorin in the gut lumen. Gut sections of resistant (ISOC4) and susceptible (ANGR) *H. armigera* 3rd instar caterpillars were incubated with antibodies against lipophorin, stained with FITC-conjugated secondary antibodiesand inspected under indirect UV-light using confocal microscopy. TRITC-conjugated peanut agglutinin (PNA) was added together with the secondary antibodies. Weak autofluorescent staining of the undulating peritrophic membrane was visible in non-treated gut tissues, whereas dot-like structures were only detected in treated gut preparations (not shown). Little or no dot-like staining was detected in tissues treated with preserum or FITC-conjugated secondary antibodies alone (Fig 37). **A)** Lipophorin-staining in gut lumen of susceptible (ANGR) caterpillar **B)** PNA-staining in gut lumen of susceptible (ANGR) caterpillar **C)** Overlay of lipophorin and PNA-staining in gut lumen of susceptible (ANGR) caterpillar. **D)** Lipophorin-staining in gut lumen of resistant (ISOC4) caterpillar **E)** PNA-staining in gut lumen of resistant (ISOC4) caterpillar **F)** Overlay of lipophorin and PNA-staining in gut lumen of resistant (ISOC4) caterpillar.

Peritrophic

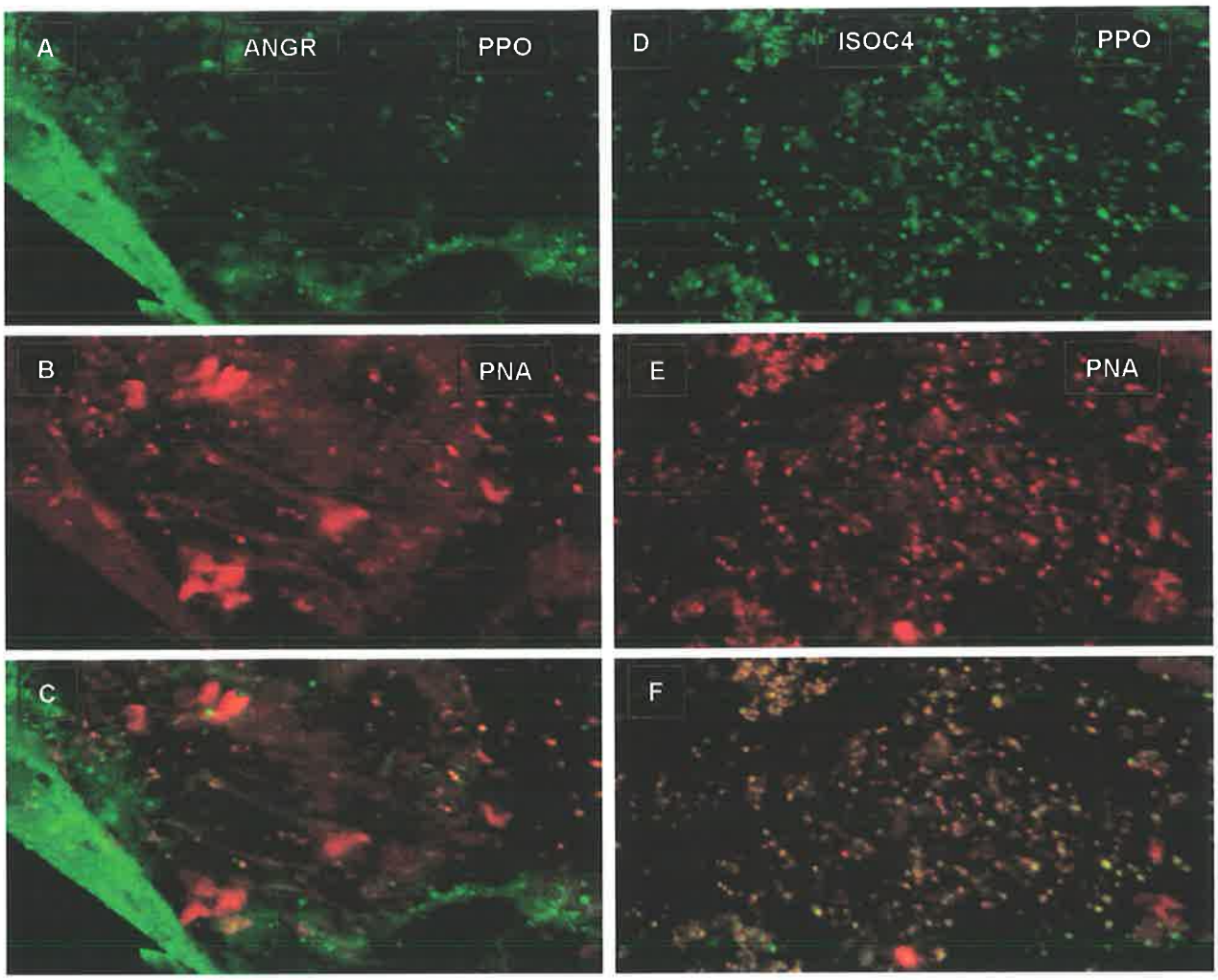


Fig. 36 Presence of phenoloxidase in the gut lumen.. Gut sections of resistant (ISOC4) and susceptible (ANGR) *H. armigera* 3rd instar caterpillars were incubated with antibodies against prophenoloxidase (Jiang, 1997), stained with FITC-conjugated secondary antibodiesand inspected under indirect UV-light using confocal microscopy. TRITC-conjugated peanut agglutinin (PNA) was added together with the secondary antibodies. Dot-like structure were absent in tissues treated with preserum or FITC-conjugated secondary antibodies alone (Fig 37). **A**) Phenoloxidase-staining in gut lumen of susceptible (ANGR) caterpillart **B**) PNA-staining in gut lumen of susceptible (ANGR) caterpillar **C**) Overlay of phenoloxidase and PNA-staining in gut lumen of susceptible (ANGR) caterpillar. **D**) Phenoloxidase-staining in gut lumen of resistant (ISOC4) caterpillar **E**) PNA-staining in gut lumen of a resistant (ISOC4) caterpillar **F**) Overlay of phenoloxidase and PNA-staining in gut lumen of a resistant (ISOC4) caterpillar.

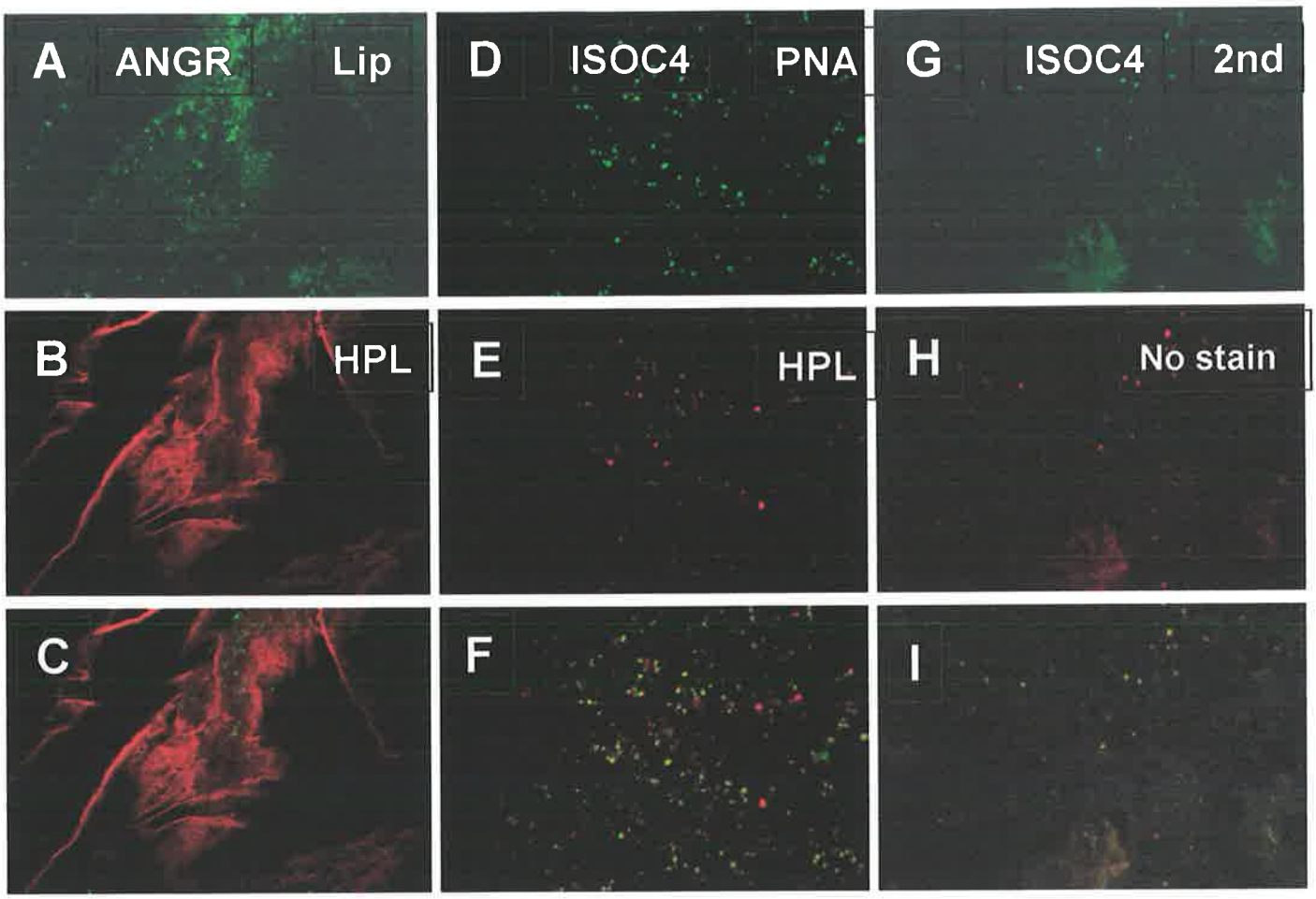


Fig. 37 Peritrophic membrane and dot-like structures. Gut sections of susceptible (ANGR) and resistant (ISOC4) *H. armigera* 3rd instar caterpillars were incubated with antibodies against lipophorin, stained with FITC-conjugated secondary and inspected under indirect UV-light using confocal microscopy. TRITC- or FITC-conjugated lectins were added together with the secondary antibodies. **A**) Lipophorin-staining in the gut lumen of susceptible (ANGR) caterpillar **B**) HPL-staining in gut lumen of a susceptible (ANGR) caterpillar. Note the absence of dot-like structures around the peritrophic membranes. **C**) Overlay of lipophorin and HPL-staining in gut lumen of a susceptible (ANGR) caterpillar. **D**) PNA-staining in gut lumen of a resistant (ISOC4) caterpillar. **E**) HPL-staining in gut lumen of a resistant (ISOC4) caterpillar. Note the increase of (autofluorescent) dot-staining relative to peritrophic membrane-staining **F**) Overlay of PNA-and HPL-staining in gut lumen of resistant (ISOC4) caterpillar. **G**) Unspecific binding of FITC-conjugated secondary antibodies to gut lumen of resistant (ISOC4) caterpillar. No significant binding is observed in susceptible (ANGR) caterpillars (not shown). **H**) Autofluorescence detected in the TRITC-channel in the absence of fluorescent dye. **I**) Overlay of the two channels.

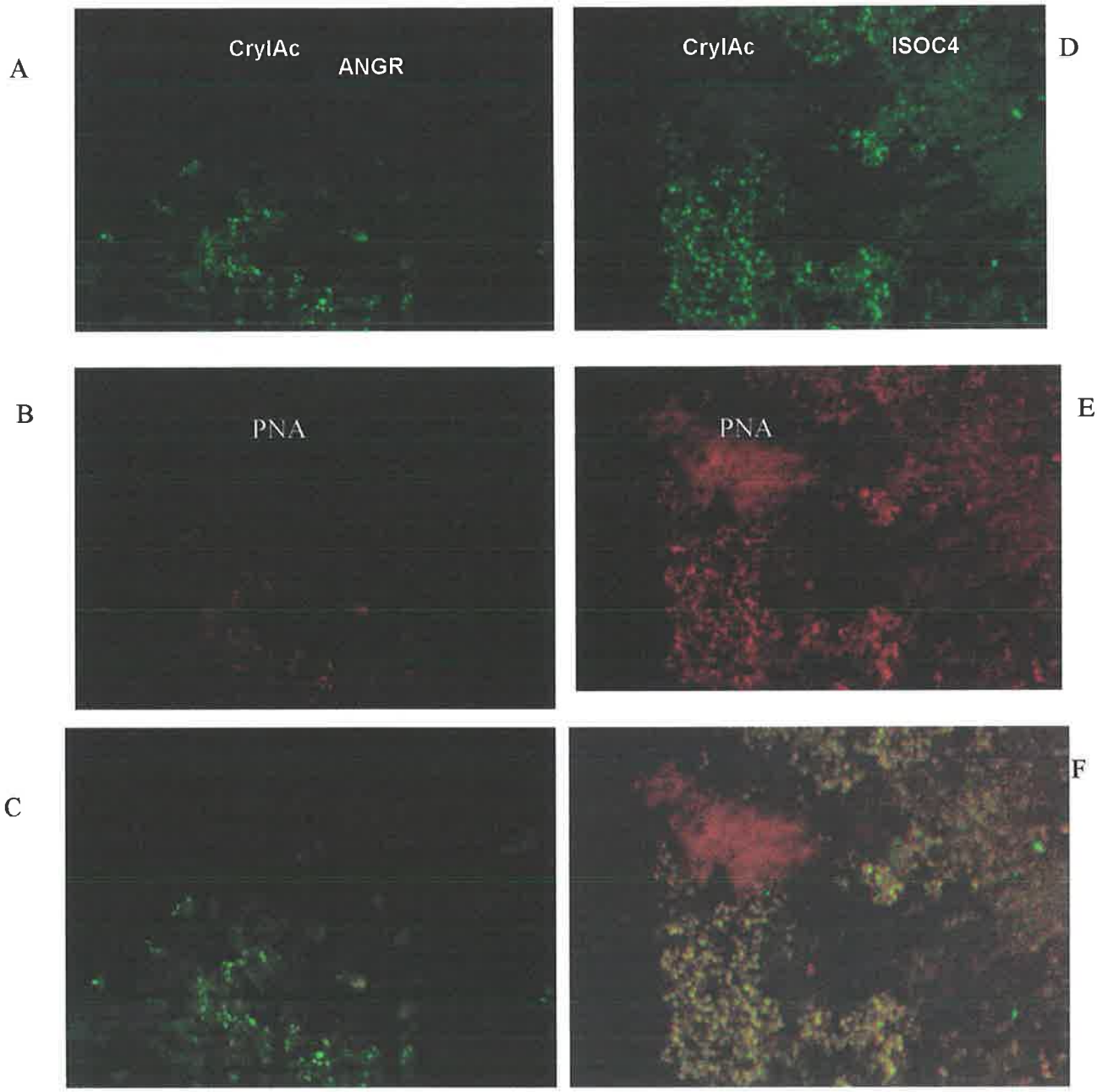


Fig. 38 Binding of activated Cry1Ac in the gut lumen. Gut sections of resistant (ISOC4) and susceptible (ANGR) 3rd instar *H. armigera* caterpillars were incubated with gut juice-activated Cry1Ac, bound toxin was identified with antibodies against Cry1Ac. TRITC-conjugated peanut agglutinin (PNA) was added together with the FITC-conjugated secondary antibody and inspected under indirect UV-light using confocal microscopy. Weak autofluorescent staining of the undulating peritrophic membrane was visible in non-treated gut tissues, whereas dot-like structures were only detected in treated gut preparations (not shown). Little or no dot-like staining was detected in tissues treated with preserum or FITC-conjugated secondary antibodies alone (not shown). **A**) Cry1Ac-staining in gut lumen of susceptible (ANGR) caterpillar. **B**) PNA-staining in gut lumen of susceptible (ANGR) caterpillar. **C**) Overlay of Cry1Ac- and PNA-staining in gut lumen of susceptible (ANGR) caterpillar. The Cry1Ac-stained dots outnumber the PNA-stained dots. **D**) Cry1Ac-staining in gut lumen of resistant (ISOC4) caterpillar. **E**) PNA-staining in gut lumen of resistant (ISOC4) caterpillar. **F**) Overlay of Cry1Ac- and PNA-staining in gut lumen of resistant (ISOC4) caterpillar.

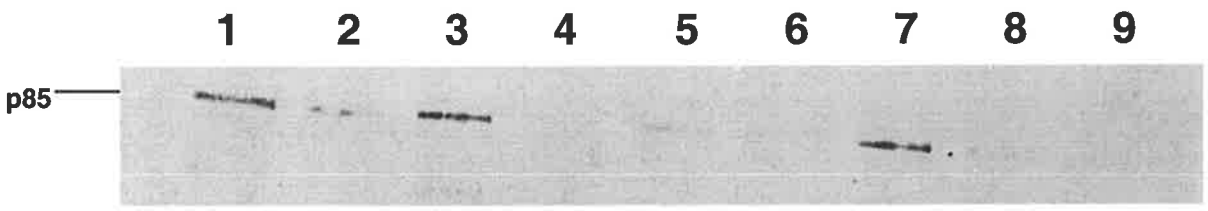
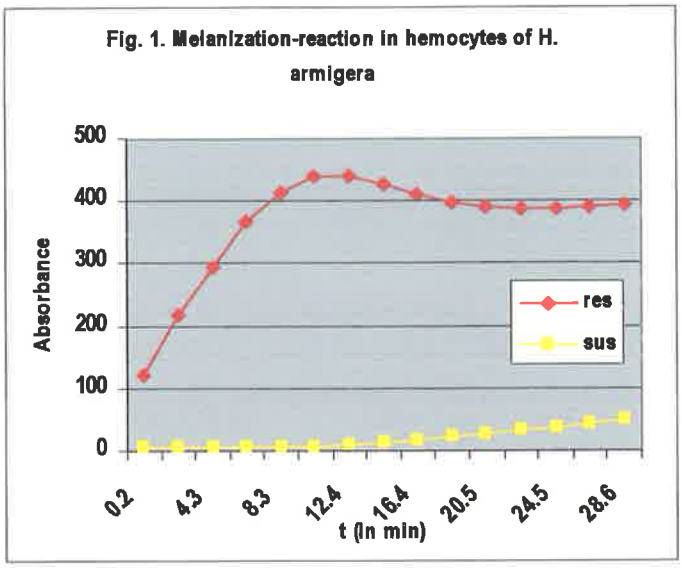


Fig. 39 Induction of the p85 in midgut of DBM 48 hrs after feeding caterpillar with several immune-elicitors. 1) LPS, 2) LPS + toxin (0.1 ppm), 3) *E. coli,* 4) *E. coli* + toxin (0.1 ppm), 5) Heat-killed *Bt*, 6) Haet-killed *Bt* + toxin (0.1 ppm), 7) toxin)0.01 ppm), 8) toxin (0.1 ppm), 9) Control (sterile water). Note that epithelial tissue structures were intact in all cases, except when toxin was used in lethal dosis (0.1 ppm). Under these conditions total protein patterns were altered and p85 disappeared.



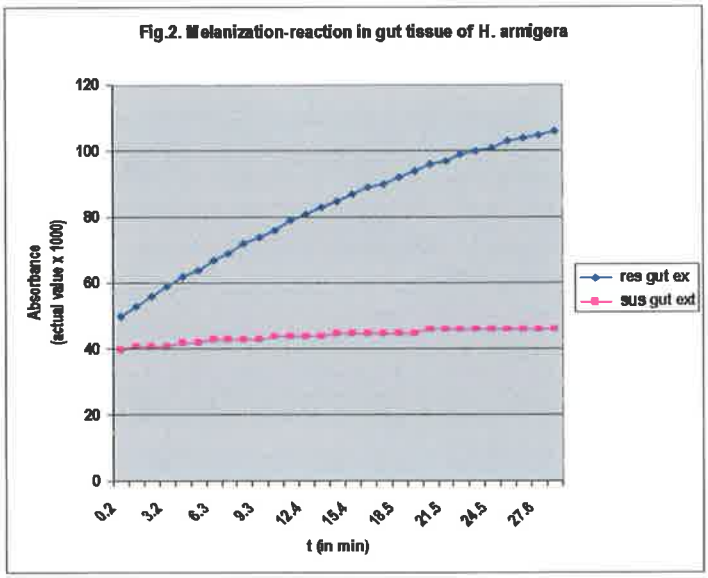


Fig. 40 Melanisation reactions in *H. armigera* strains that are resistant and susceptible to Bt-toxin.

A) Cell-free hemolymph from 3rd instar caterpillars was diluted in PBS-solution and relative absorbance measured over 30 min.Note a slight reduction of relative absorbance in hemolymph from the resistant strain due to coagulation reactions.

B) Gut extracts without gut contents were measured in the presence of 100mM 3,4-dihydroxyphenylalanine (DOPA).

Although the shape of absorbance curves varies slightly between hemolymph from individual caterpillars, the difference in absorbance between the two strains always exceeded 200 units, with hemolymph from susceptible caterpillars showing variability due to possible immune-induction by wounding or food derived elicitors.

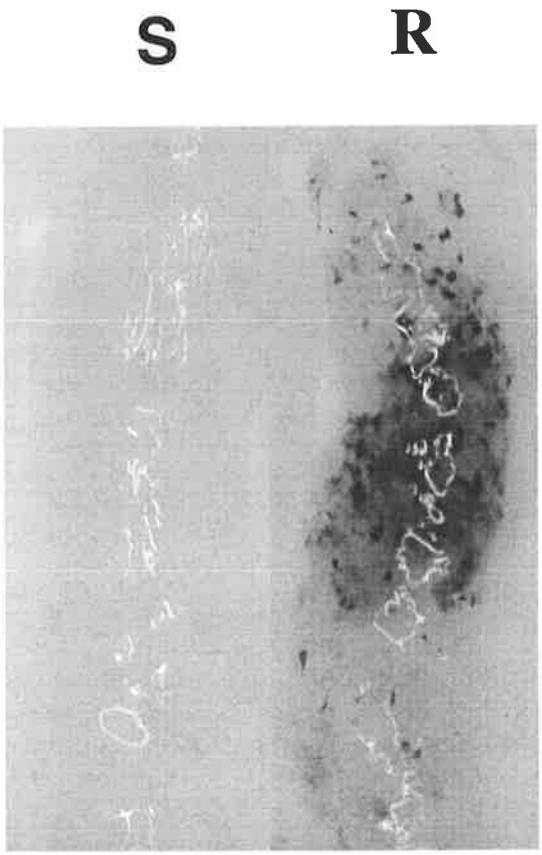


Fig 41 Dissected gut tissues from a susceptible caterpillar of *H. armigera* (A) and a resistant caterpillar (B) shows blackening of the peritrophic membrane and pieces of gut content in resistant caterpillars.

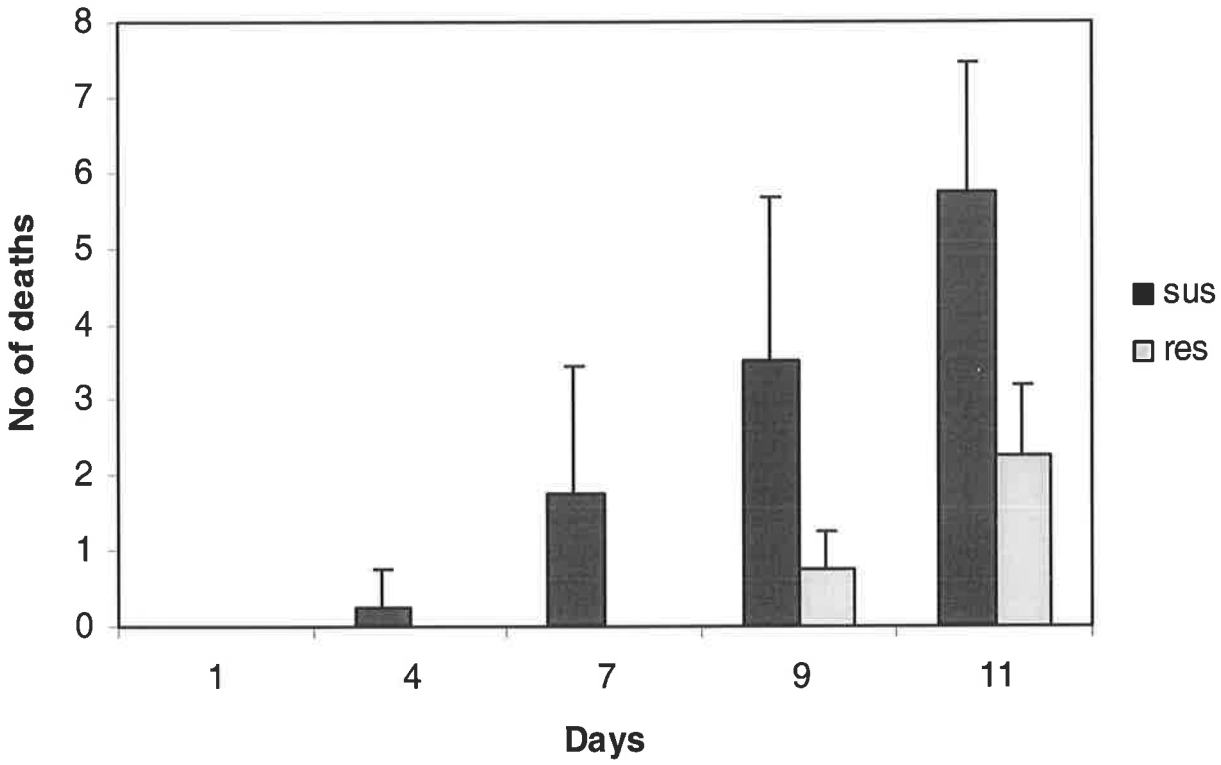


Fig 42. Mortality rate in baculovirus- treated caterpillars from Bt-resistant and susceptible strains. *H. armigera* 3rd instar larvae were fed on artificial food mixed with a suspension of 10^7/ml of *Autographa californica* multiple nuclear polyhedrosis virus (*Ac*MNPV). *H. armigera* is semipermissive to *Ac*MNPV. The virus titre, resulting in low mortality rates, was chosen to mimic low Bt-toxin levels. Each treatment was repeated three times with at least 20 caterpillars each. The difference in mortality rates was highly significant for each time point. No mortality was observed in non-treated insects.

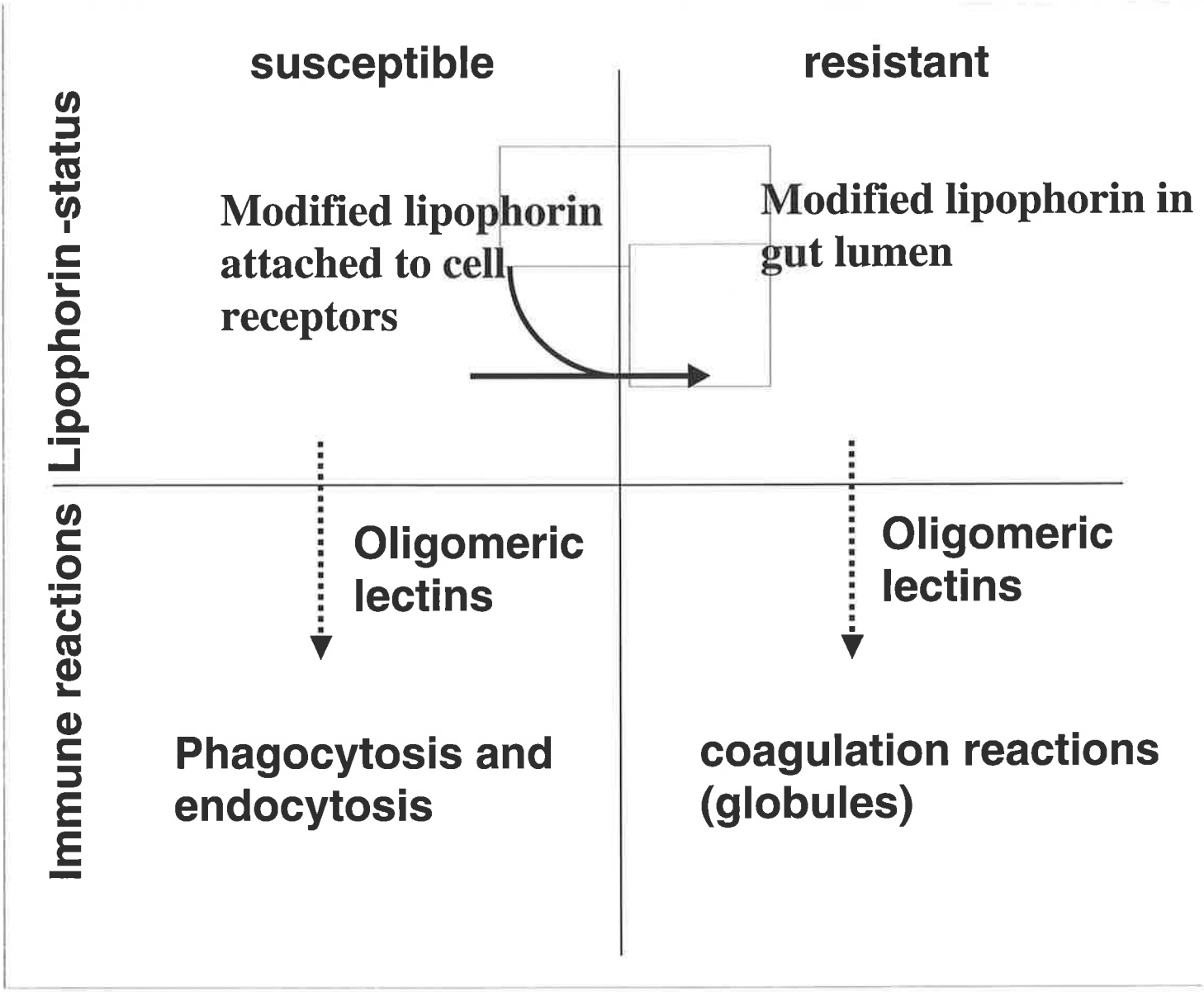


Fig. 43 Schematic diagram of putative glycosylation and immune status in susceptible and resistant strains of *H. armigera*

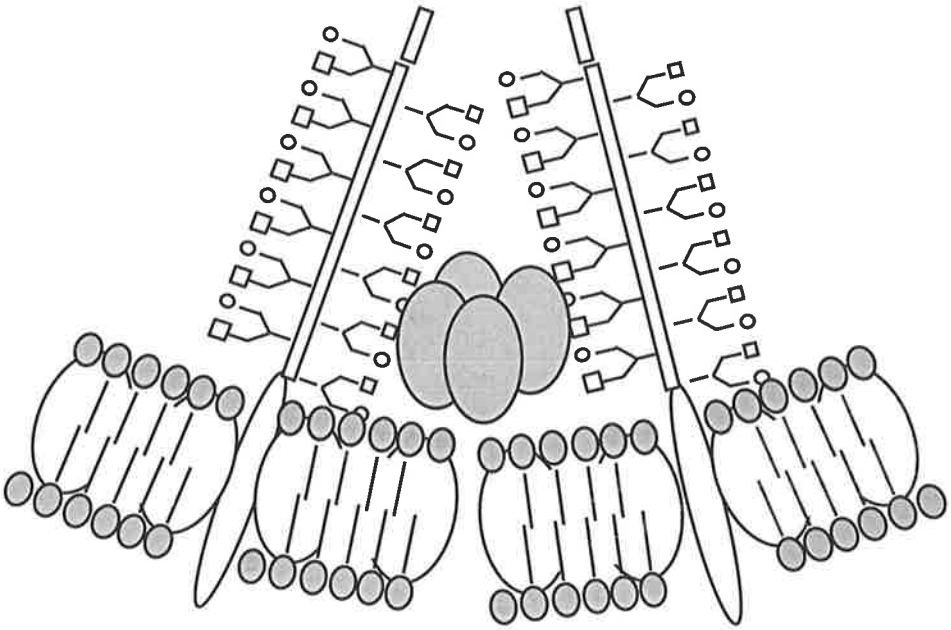


Fig 44. lipophorin-mucin complex (permission O. Schmidt)

Fig. 45 Essential features of an endocytosis complex according to the LM-mechanism and cellular processes affected by the process. The schematic illustrations depict a typical complex, consisting of membrane-anchored receptors and multimeric molecules connecting the receptors around a bulky protein, such as lipoprotein. The leverage created by the endocytosis complex creates an inverse curvature of the cell membrane, an essential requirement for the initiation of endocytosis. Membrane-anchored receptor molecules are dislodged from their cytoplasmic connections in the process thereby initiating cytoplasmic signaling as an integral part of endocytosis or receptor internalization.

Pathogen and parasite invasion : LM-mediated endocytosis processes are exploited by pathogens and parasites, which can induce and utilize endocytosis complexes to enter the cell and insert pore-forming toxins. *Cell surface sculpturing*: Release of cross-linking components onto the cell surface by microbes or other cells induces surface sculpturing and membrane ruffling. Multimeric complexes induce macropinocytosis by localized cross-linking of cell surface molecules, which can be used for *nutrient uptake, phagocytosis of biotic and abiotic objects. Phagosome maturation*: Receptor rearrangements inside endosomes can initiate cluster-mediated subdivision of vesicles relevant to receptor recycling and phagosome maturation. *Drug and DNA delivery*: The design of specific drug and DNA delivery vehicles can be based on the choice of cross-linking complexes, which define the target cell and the mode of uptake. *Cell signaling*: Since the intracellular signal is dependent on the bending of membrane-anchored molecules, cell signaling can be seen as a variation of a basic endocytosis or receptor internalization process, where the nature of the signal is based on the recruitment process of participating receptors. *Cell-cell interaction*: Cross-linking between two cells connects membrane receptors, while the cluster-mediated cross-linking of lateral membrane receptors creates an inverse curvature. If two cells have similar capacities to drive an inverse curvature of the membrane, the tug between the two cells is balanced forming a straight line. *Phagocytosis of apoptotic cells*: Cell interactions with an imbalance of intercellular and cluster-mediated linkages will not form straight lines of contact but will instead produce a curvature. This situation will lead ultimately to phagocytosis of one of the two cells (permission O. Schmidt).

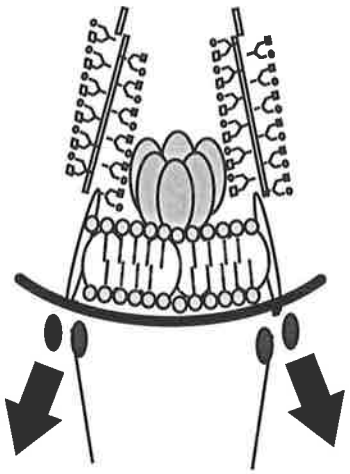
Toxin insertion

Pathogen and parasite invasion

Cell-cell interaction

Cell surface sculpturing

Phagocytosis of apoptotic cells



Removal of immune-reactive components

Cell signalling

Outside-in/Inside-inside

Receptor recycling

Innate immune recognition

Nutrient uptake

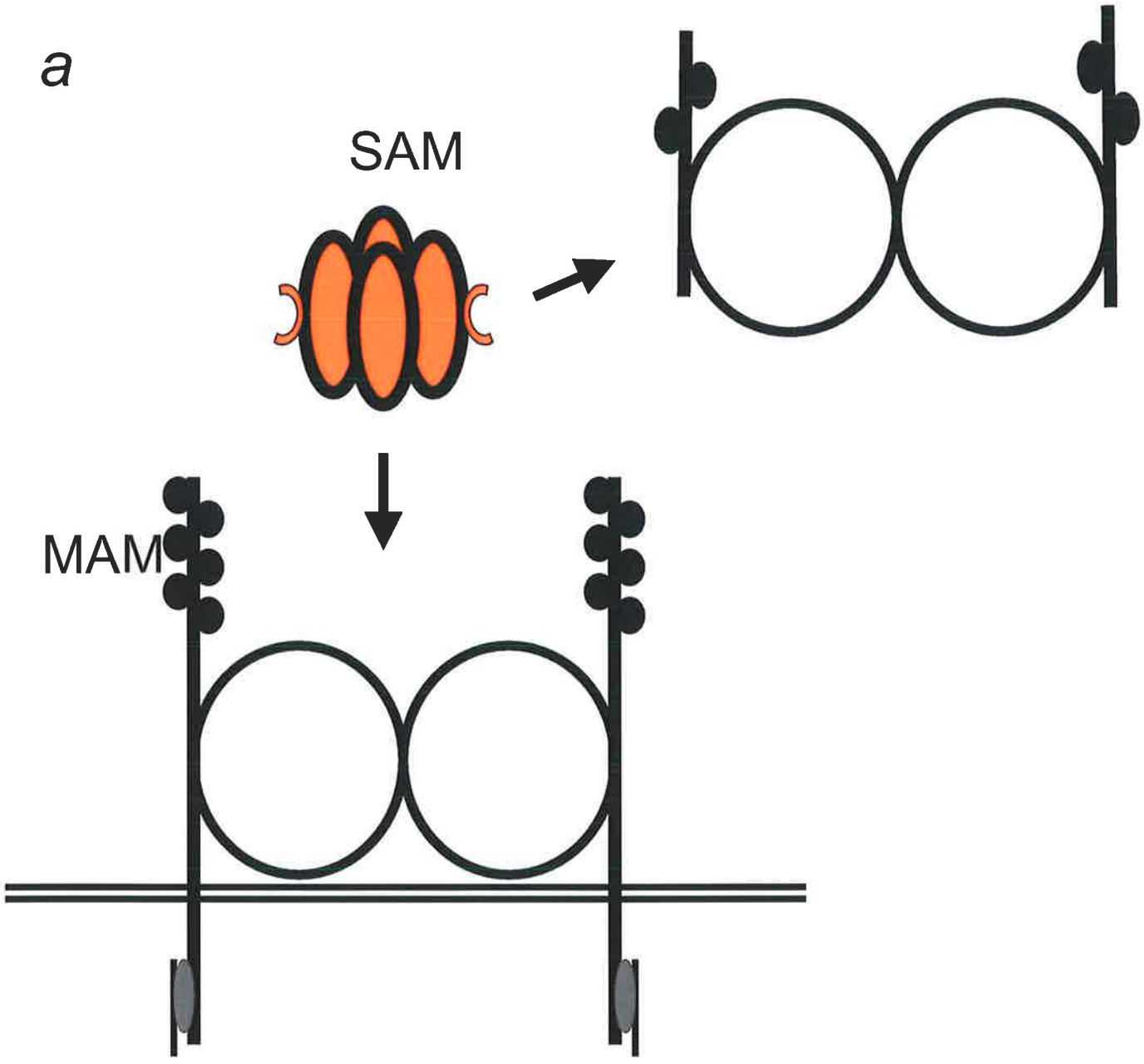
Drug and DNA delivery

Phagocytosis

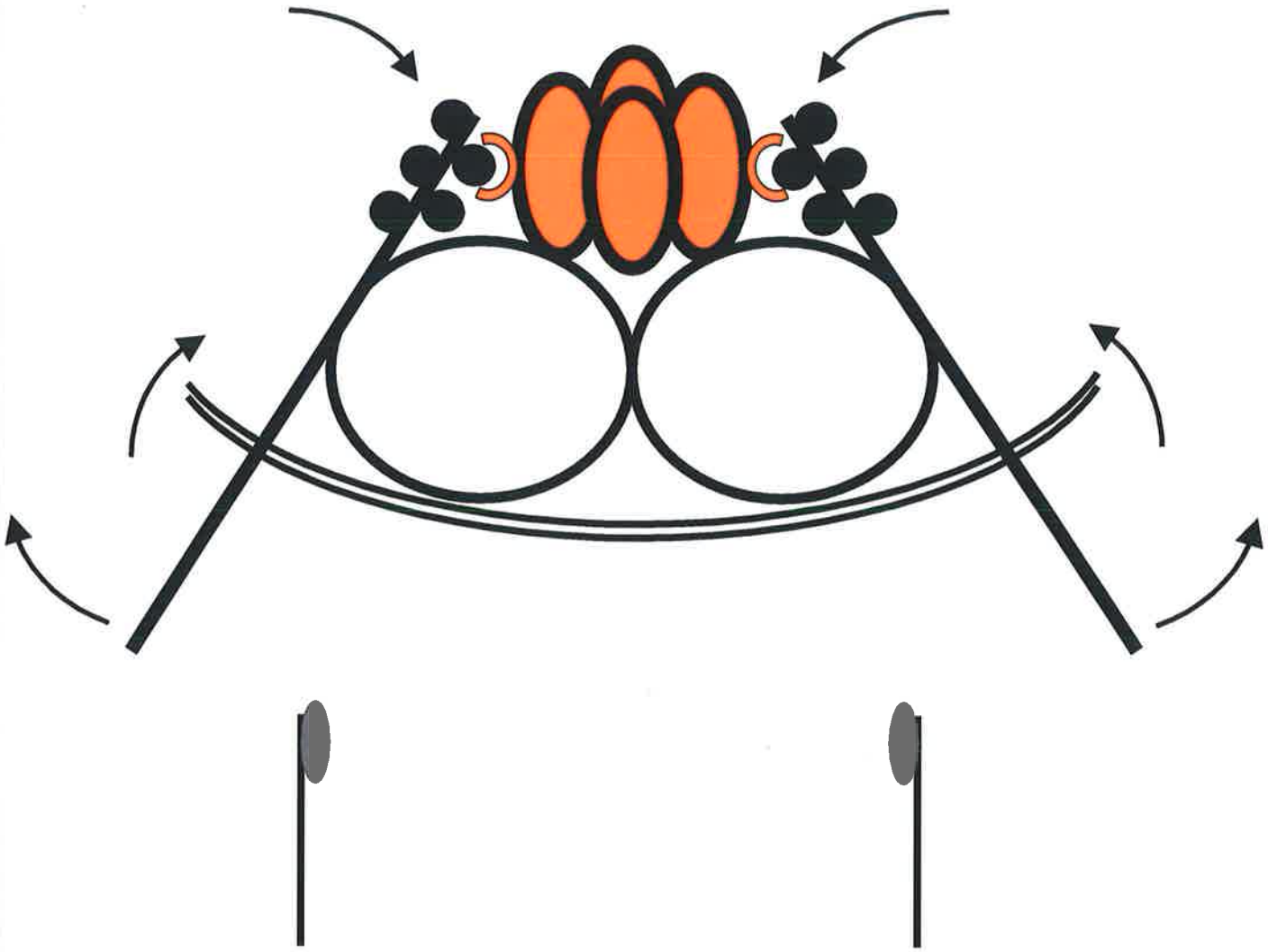
Biotic/abiotic

Phagosome maturation

Fig 46. Schematic illustration of an LME mechanism and coagulation reaction involving globules. a) LME is driven by an extracellular endocytosis complex, which is initiated by soluble adhesion molecules (SAMs), consisting of lipoproteins,and multimeric lectins, interacting with membrane-anchored molecules (MAMs), such as hemomucin molecules. An example is hemomucin, lipophorin and tetrameric lectins, which form hemomicin-lipophorin, and hemomucin-lectin interaction. Note that the three-dimentional structure of the lectin-lipophorin complex is not known. The schematic drawing, which is not to scale, is used as an example for the recruitment of receptors to endocytosis complexes. b) Once SAMs have made contact with MAMs, the complex can act as a template around which MAMs are bent, creating an inverse curvature of membrane. Bending of cell surface receptor may destabilise cytoplasmic linkages between intracellular MAM-domains and the actin cytoskeleton. c) Coagulation reaction producing round spheres as a result of oligomeric lectin (SAMs) interacting with soluble glycoproteins (permission O. Schmidt).


a
SAM
MAM

b **Leverage Mediated Endocytosis**



Coagulation

C

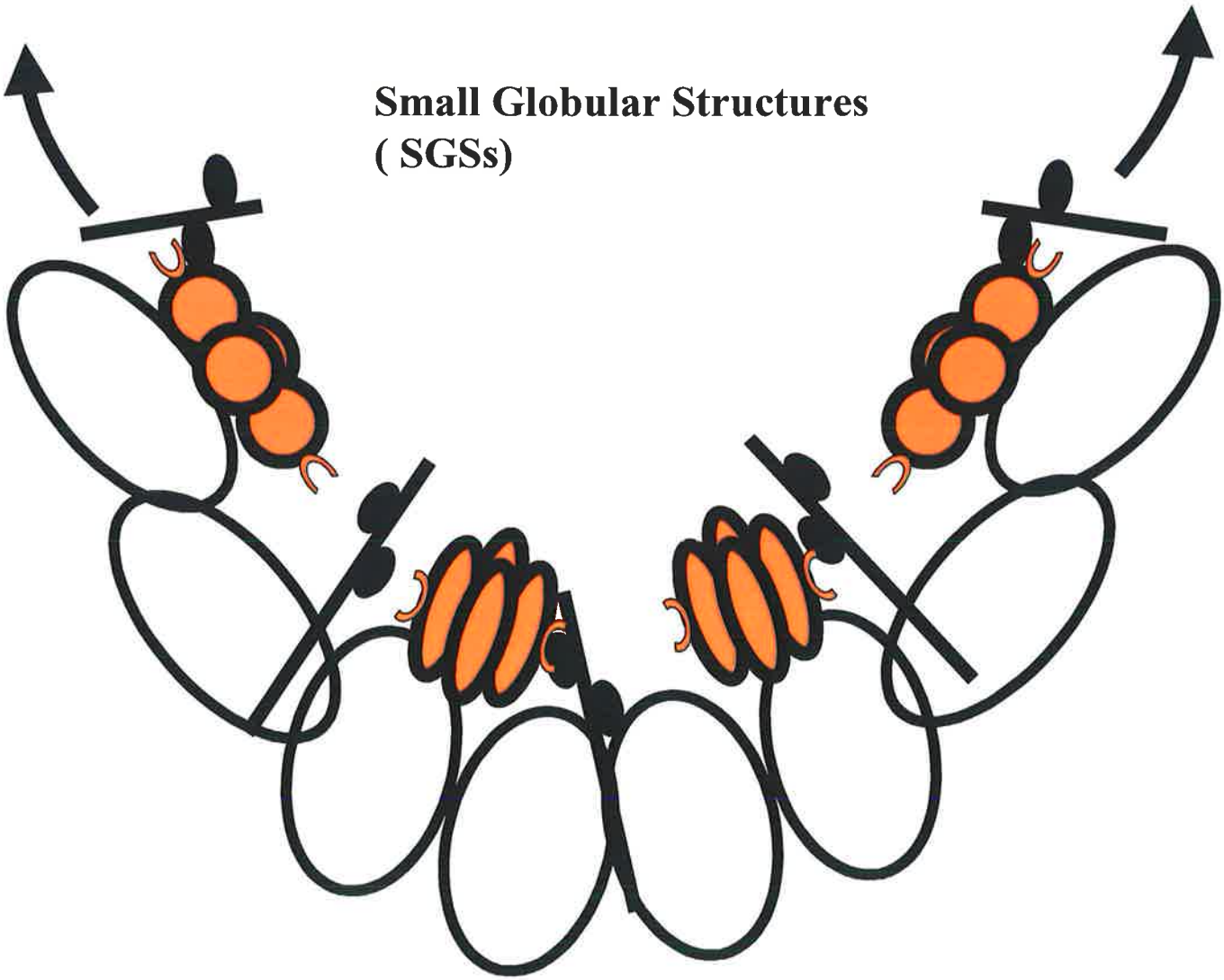
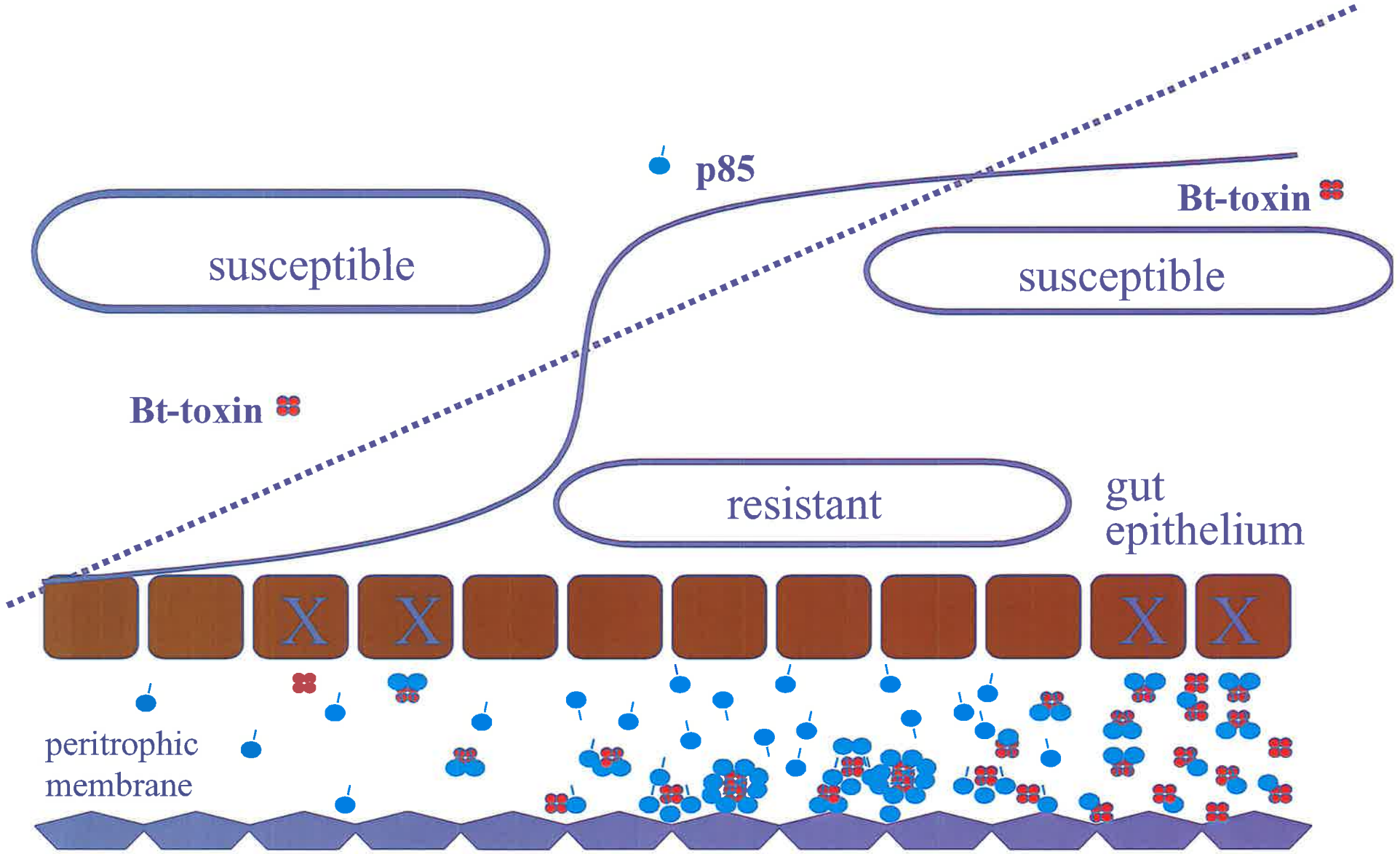


Fig 47. Model of Bt resistance: p85 is shown at two levels, which represent the induced and non-induced status. Increasing levels of Bt-toxin in the gut lumen cause toxicity at low p85 levels, but trap the toxin at high p85 levels. It the toxin is increased further, p85-mediated coagulation is exhausted and toxin molecules can reach the gut epithelium.


p85
Bt-toxin
susceptible
susceptible
Bt-toxin
resistant
gut
epithelium
X
X
X
X
peritrophic
membrane

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