EFFECT OF THE AXENIC NEMATODE *Steinernema carpocapsae* ON THE IMMUNE RESPONSES OF TWO LEPIDOPTERAN LARVAE, *Galleria mellonella* (F. Pyralidae) AND *Malacosoma disstria* (F. Lasiocampidae)

NDONKEU TITA WALTER

Department of Natural Resource Sciences,

McGill University, Montreal

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ABSTRACT

Live adult and juvenile entomopathogenic as opposed to freeze-killed *Steinernema carpocapsae* DD136 (P. Nematoda) did not have adhering haemocytes of lepidopteran insect larvae of *Galleria mellonella* or *Malacosoma disstria in vitro* or *in vivo*. Haemocyte accumulation on dead nematodes was associated with cuticular activation of host phenoloxidase. Live nematodes and their exudates did not activate the enzyme. Live-nematode exudate but not dead-nematode exudate inhibited granular cell and some plasmatocyte adhesion to slides, increased granular cell but not plasmatocyte dissociation from preformed haemocyte monolayers and *in vivo* elevated total haemocyte counts while impairing bacterial removal from the haemolymph. Immunosuppressant activity by third stage juvenile nematodes may represent the release of non-cuticular inhibitors.

Initial characterization of the immunosuppressant properties revealed the suppressant(s) were weakly hydrophobic, were not affected by repeated freeze-thawing but most were destroyed at 65^{o}C. A residual heat-stable suppressant remained in the exudate, attributed to lipopolysaccharides. Exudate with a cocktail of protease inhibitors partially increased haemocyte adhesion with a small decline in total protein occurring indicating that non-enzymic proteins are also involved. A decrease in exudate total protein but not immunosuppression in exudate with trypsin and chymotrypsin-conjugated agarose beads indicated the presence of non-suppressant trypsin and chymotrypsin sensitive proteins. Correlation analysis between enzyme activities and level of adhering haemocytes implied several enzyme types including trypsin and chymotrypsin may participate in immunosuppression.

Herein, is reported for the first time that the serine proteases, trypsin and chymotrypsin released by live axenic *Steinernema carpocapsae* inhibits haemocyte adhesion to slides. Increasing concentrations of enzyme-specific inhibitors and polyclonal antibodies to trypsin and chymotrypsin in the exudate inhibited the enzymes and

increased haemocyte adhesion, compared to the controls. Incubating exudate with chymotrypsin specific inhibitor and trypsin substrate showed no reaction indicating that trypsin-like and chymotrypsin-like enzymes react only with their respective substrates. Antibodies targeting specific serine proteases in exudate elevated haemocyte adhesion but in PBS alone, neither enzyme antibody affected haemocyte adhesion, suggesting the haemocytes do not react with these foreign proteins. There was no observed cross-reactions of antibodies with the opposite enzyme targets. In conjunction with chemical inhibitors, the antibodies confirmed the presence of suppressive trypsin-like and chymotrypsin-like enzymes versus non-suppressive chemically inhibited enzymes. Both chemical inhibitors and antibodies in the exudate increased bacterial removal from the haemolymph *in vivo*. The release time of both enzymes from the nematode in artificial serum was 2 h with a peak release at 4 h for trypsin and 6 h for chymotrypsin.

Index descriptors: *Steinernema carpocapsae, Galleria mellonella, Malacosoma disstria,* insect, inhibition haemocytes, adhesion, phenoloxidase, nematode encapsulation, protease inhibitor, trypsin, chymotrypsin, Apizym, chemical inhibitor, polyclonal antibody, trypsin, chymotrypsin, serine protease.

RESUME

Les entomopathogénes vivants juvéniles et adultes comparément aux *Steinernema carpocapsae* DD136 (P. Nématodes) tués par congélation, ne contenaient pas d'hemocytes adhérent provenant de larves d'insectes lépidoptères de *Galleria mellonella* ou de *Malacosoma* disstria *in vitro* ou *in vivo*. L'accumulation des hemocytes sur les nématodes morts était associée à la stimulation de la cuticule de la phenoloxidase.

Les nématodes vivants et leurs exsudats n'avaient point stimulé l'enzyme. L'exsudat de nématodes vivants avait inhibé des cellules granuleuses et quelques plasmocytes adhérents aux larme, cependant l'exsudat de nématodes morts n'avait pas inhibé ces substances. L'exsudat de nématodes vivants avait aussi provoqué une quantité élevée de cellules granuleuses mais pas de division des plasmocytes provenant de couches mono moléculaires des hemocytes préformés, aussi *in vivo* il amplifiait le nombre total des hemocytes tout en ralentissant le retrait des bactéries de l'hémolymphe. L'activation des immunosuppresseurs par les nématodes juvéniles de la 3eme phase pourrait représenter le dégagement d'inhibiteurs non cuticulaire.

La première caractéristique des immunosuppresseurs indiquait que les suppressants avait une faible hydrophobicité et ils n'étaient pas touchés par l'alternance répétée de la température, mais plusieurs d'entres eux étaient détruit sous une température de 65˚ C. Un supprésant résiduel thermostable est resté dans l'exsudat ceci étant le résultat des *lipopolysaccharides*. Un exsudat accompagné d'un mélange d'inhibiteurs de protéase avait augmenté l'adhérence des hemocytes avec une chute négligeable du totale de protéines présent, ceci dévoilant la participation de protéines non-enzymatique. Une diminution total de protéines de l'exsudat mais pas d'immunosuppresseurs présent dans l'exsudat contenant la trypsine et la chymotrypsine combiné à l'agarose, indiquait la présence de trypsine non-suppresseurs et de protéines sensibles de chymotrypsine. Les analyses sur la relation entre les activités de l'enzyme et le niveau d'hemocytes adhérent affirment que les types d'enzymes à savoir la trypsine et la chymotrypsine, participent à l'immunosuppression.

Il est ainsi établit pour la première fois que les serines de protéase, la trypsine et la chymotrypsine, relâchées par *l'anexic steirnernema capocapsae* inhibe l'adhérence des hemocytes aux larme. Une haute concentration d'inhibiteurs spécifiques d'enzymes et d'anticorps polyclonal à la trypsine et la chymotrypsine dans l'exsudat avait inhibé les enzymes et avait accrus l'adhérence des enzymes, comparément aux examens.

L'incubation de l'exsudat avec des inhibiteurs AEBSF et des substrats de chymotrypsine ne démontrait aucune réaction ce qui indique que les enzymes de trypsine sont spécifiquement inhibés et réagissent seulement avec leur substrats respectifs. Isolé, aucun anticorps d'enzyme agissait sur l'adhérence des hemocytes. Ceci voulant dire que les hemocytes ne réagissent pas au contact de protéines étrangères. On observait aussi des réactions croisées d'anticorps avec l'enzyme opposé cible. Fusionné aux inhibiteurs chimiques, les anticorps confirmaient la présence d'enzymes de chymotrypsine et de trypsine suppresseurs contre des enzymes chimiques non-suppresseurs, inhibés. Les inhibiteurs chimiques aussi bien que les anticorps présent dans l'exsudat, avait accéléré le retrait des bactéries de l'hémolymphe *in vivo*. La durée de relâchement des deux enzymes provenant des nématodes de sérum artificiels, était de 2 heures avec un relâchement accentué de 4heures pour la trypsine et de 6heures pour la chymotrypsine.

SUGGESTED SHORT TITLE

Axenic nematode modulators and the immune responses of lepidopterans

CONTRIBUTIONS OF AUTHORS

The author of this thesis has held discussion and received guidance from Dr. G.B Dunphy and Dr. C.A Mandato.

Dr. G.B Dunphy assisted in the experiments involving the use of chemical inhibitors and antibodies with the nematode exudate (chapter four).

In all cases, the experimental designs and collection of data were carried out by the author.

Dr. G.B Dunphy and Dr. C.A Mandato have corrected and edited the manuscripts.

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LIST OF ABBREVIATIONS

apoLp-III: apolipophorin-III

AEBSF: 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride

BA*p*NA: N-α-benzoyl-*DL*-Arg-*p*-nitroanilide

βGRPs : β-1,3-glucans-binding protein

BNZTYRpNA: N-benzoyl-L-tyrosine-P-nitroanilide

DMSO: dimethyl sulfoxide

Dscam : Down syndrome cell adhesion molecule

***flh*DC**: flagella master operon

GNBP 1: Gram-negative binding protein 1

***Imd* :** immune deficiency system

LPS: lipopolysaccharides

LTA: lipotechoic acids

PAMPs: pathogen-associated molecular patterns

PAP : prophenoloxidase -activating proteinase

PBS: phosphate buffered saline

PGRPs: peptidoglycan recognition proteins

PGRP-LC: peptidoglycan recognition proteins-long transcript

PGRP-SA: peptidoglycan recognition proteins-short transcript

PLA_2: phospholipase A_2

proPO: prophenoloxidase

PRRs: pattern recognition receptors

SCP3a: surface coat protein 3a

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The entomopathogenic nematode, *Steinernema carpocapsae* is widely used as an alternative to chemicals for the biological control of pest insects (Dunphy and Thurston, 1990; Georgis and Gaugler, 1991; Samish and Glaser, 2001). These nematodes are ubiquitous, natural microscopic pathogens that infect and kill more than 200 different insect pests (Poinar, 1979; Klein, 1990). In the infective juvenile stage, *S. carpocapsae* vectors a mutualistic micro-organism, the Gram-negative γ-proteobacterium, *Xenorhabdus nematophila* (Vivas and Goodrich-Blair, 2001), within an intestinal vesicle (Martens and Goodrich-Blair, 2005). The mutualistic relationship is based on the fact that *X. nematophila* is necessary for rapidly killing the host and establishing conditions for nematode reproduction (Poinar and Thomas.,1967; Boemare *et al*., 1983), whereas the nematode is required to vector *X. nematophila* into an insect (Poinar and Thomas, 1967) and providing protection for *X. nematophila* outside the insect and inside against induced antimicrobial proteins (Poinar, 1979; Morgan *et al*., 1997). The nematode in the infective juvenile stage allows the bacteria to multiply in its vesicle outside the host (Martens *et al*., 2003).

The basis of the initial stages of infectivity of the nematode-bacterial complex against the host antimicrobial defenses is well understood for different species of *Steinernema* and several lepidopteran host species including the greater wax moth, *Galleria mellonella* (Akhurst and Dunphy, 1993; Dunphy and Webster, 1984; Ribeiro *et al*; 1999). Under ideal conditions, host death occurs within 24-48 hr after infection (Poinar, 1990).

Nematodes gain access to the haemocoel through the natural openings of the insect body and release their symbiotic bacteria into the blood (haemolymph) in a later

stage of infection. The time of release depends on bacterial strain and insect species, e.g. *X. nematophila* is released 4-8 h post-parasitism in the Japanese beetle, *Popillia japonica* (Wang *et al*., 1995), 4-5 h in *G. mellonella* (Dunphy and Webster, 1988a) and 2 h in *Manduca sexta* (Synder *et al*., 2007). Part of the success of the nematode-bacterium complex in biocontrol prior to bacterial release is the initial interaction of the nematode with the non-self innate cellular and/or humoral antimicrobial responses of the insect. During the initial interaction, entomopathogenic nematodes including *S. carpocapsae* may elicit a rapid cellular immune response in some insect species that results in encapsulation (encasement of nematode by host blood cells, haemocytes), harmful melanization, and killing of the nematode (Wang *et al*., 1994, 1995; Ehlers *et al*., 1997). However, many steinernematids escape encapsulation and overcome host immunity, e.g. *S. glaseri* in *P. japonica* (Wang *et al*., 1995), *S. feltiae* [(= *carpocapsae*) 1983-1986, Kaya and Gaugler, 1993)] in *G. mellonella* (Dunphy and Webster, 1986), and *S. carpocapsae* in the migratory locust, *Locusta migratoria* (Brehélin *et al*., 1990). Both *S. carpocapsae* and its symbiont are known to suppress the host antimicrobial immune responses of lepidopterans e.g. the Egyptian cotton leaf worm, *Spodoptera littoralis* (Abdel-Razek, 2006), the armyworm, *Mythimna unipuncta* (Ribeiro *et al*., 1999) and *G. mellonella* (Boemare *et al*., 1982; Dunphy and Webster, 1986). The symbiotic bacteria are known to produce proteases and toxins capable of inhibiting the immediate innate cellular immune response and the production of induced antimicrobial peptides (Dunphy and Thurston, 1990; Wang *et al*., 1994; Hatab *et al*., 1998; Jarosz, 1998; Caldas *et al*., 2002). Since the bacteria act at a later stage during infection, it is reasonable to attribute the short-term host-parasite interaction to the nematode.

The axenic steinernematid nematode cuticle is the interface between the nematode and the humoral and cellular immune responses of the host haemolymph (Dunphy and Webster, 1987). The physiochemical nature of the cuticle of the nematode influences haemocyte activity in several pest insects including, *G. mellonella* (Dunphy and Webster, 1987) and its cuticular extracts affect the humoral cellular defenses (Brivio *et al*., 2002). Partially purified lipopolysaccharide-like molecules from *S. feltiae* cuticle sequester (and interact with) lipopolysaccharide-binding proteins present in the host haemolymph, which inhibits normal melanotic encapsulation of the free-living nematode, *Panagrolaimus*

rigidus in *G. mellonella* (Brivio *et al*., 2004). Mastore and Brivio (2008), report that *S. feltiae* cuticular lipids are able to bind a variety of *G. mellonella* haemolymph molecules, by attracting the host's haemolymph proteins creating a coat around the nematode, to disguise itself against *G. mellonella* haemocyte recognition. Mastore and Brivio (2008), further investigated the role of the parasite lipids in the disguise process by simulating the nematode body surface with agarose micro-beads covered with purified cuticular components, preventing the host haemocytes from recognizing and encapsulating the beads. Other cuticular components may affect cellular responses depending on the nematode species. Surface coat protein 3A from axenic *S. glaseri* suppresses the host immune system of *P. japonica,* protecting unrelated and thus readily encapsulated nematode species from encapsulation and latex beads from phagocytosis (Wang and Gaugler 1999).

The interaction of non-cuticular metabolites from the axenic nematode with the internal environment of the insect host has rarely been considered. Axenic *S. carpocapsae* releases a protein toxin lethal to insects (Boermare *et al*., 1982; Burman, 1982). The present study will enhance the understanding of the mode of action of the immunosuppressive metabolites from axenic *S. carpocapsae* DD136, against the cellular immune responses of two economic insect pest of importance to agriculture (Covell, 1984) and forestry (Furniss and Carolyn, 1977), *G. mellonella* and *Malacosoma disstria,* respectively*,* with emphasis on the former. This study will also lead to an improved understanding of the physiological and biochemical virulence determinants of the entomopathogenic nematode towards their hosts.

The specific objectives of the thesis are as follows:

1. Determine if *S. carpocapsae* inhibits the cellular non-self responses of two insect species from different niches to selected antigens.
2. Determine if active *S. carpocapsae* releases metabolites inhibiting these responses as opposed to inhibiting responses by cuticular structural components.
3. Identify the cellular immunosuppressive metabolites.

LITERATURE REVIEW

ENTOMOPATHOGENIC NEMATODES

Among the obligate entomopathogenic nematodes two soil dwelling genera, *Steinernema* and *Heterorhabditis*, have attracted the most interest for the use in biological control against soil-borne pest insects (Ehlers, 1996), as well as pest insects in cryptic environment (Strauch and Ehlers, 1998). Heterorhabditids are similar to steinernematids in general life cycle and gross morphology (Wouts, 1984). The major difference between the two families is in the reproductive strategies. Heterorhabditid adults as opposed to steinernematid adults have infective juveniles that are hermaphrodites; therefore, only one juvenile is needed to enter the host for progeny production (Tanada and Kaya, 1993). Infective juveniles of *Steinernema* develop into amphimictic adults. Their offspring either develop to dauer (infective) juveniles or to an F1 adult generation (Ehlers, 1996). These entomopathogenic nematodes have been used in the control of insects in different orders such as in orthopterans (Nguyen and Smart, 1990), coleopterans (Poinar, 1992), isopterans (Nguyen and Smart, 1994), dipterans (Poinar, 1988), hymenopterans (Fuhrer and Ficher, 1990). For this study, emphasis will be placed on the lepidopterans, since much is known about their control using steinernematids especially *S. carpocapsae*.

LIFE CYCLE

The natural life cycle of *S. carpocapsae* includes reproductive and non-reproductive stages (Forst and Clark, 2002). The infective (dauer) juvenile stage is central to the *S. carpocapsae- X. nematophila* relationship (Poinar, 1966). The nematode contains a vesicle at the anterior section of the intestine that usually contains 50-200 colony-forming units of bacteria (Forst and Clark, 2002; Goetsch *et al*., 2006; Martens *et al*., 2005; Sicard *et al*., 2005), but less than five colony-forming units directly injected into the blood of most insects can kill the insect within 48-72 h (Goodrich and Clarke,

2007). However, the vesicle may form without bacteria (Bird and Akhurst, 1983; Martens *et al*., 2005). Within the lumen of the vesicle is an intravesicular structure to which *X. nematophila* adheres; this structure, which lacks internal organization, is surrounded by a mucus-like substance that reacts with wheat germ agglutinin (Martens and Goodrich-Blair, 2005), a lectin which binds to *N*-acetyl-glucosamine and *N*-acetyl neuraminic acid glycoconjugates (Burger and Goldeberg, 1967; Monsigny *et al*., 1980).

The non-reproductive, non-feeding, soil dwelling, infective juvenile is sheathed in an outer cuticle (Poinar and Leutenegger, 1968), enabling them to survive for long periods of time before finding an insect host (Kung *et al*., 1990). The infective juveniles locate their host through stimuli released by the host such as carbon dioxide (Gaugler *et al*., 1980). The behavioral responses of the infective juvenile to host cadaver changes as the state of the nematode-infected host changes, indicating that the repellent effect of infected host exudates may lead to an infective juveniles selecting between a suitable and an unsuitable host (Kunkel *et al*., 2006). Once inside the host gut, the nematode sheds its outer cuticle and migrates to the blood cavity (haemocoel) of the insect (Sicard *et al*., 2004). The nematode while in the insect cavity, ingests blood (haemolymph) triggering the release of *X. nematophila*, which colonizes the distal region of the anterior section of the receptacle (Synder, 2007). The haemolymph trigger produces a forward movement of the bacteria through the nematode's esophago-intestinal junction, causing the distal receptacle's narrow passage to widen, and allowing movement of the bacteria down the intestine and out the anus through pharyngeal pumping, bacterial motility not being required for release of the bacteria (Synder, 2007).

The release time varies with the insect and nematode-bacterial complex (Dunphy and Webster, 1988a; Yokoo *et al*., 1992; Synder *et al*., 2007). *X. nematophila* multiplies in the insect blood (Poinar, 1966; Sicard *et al*., 2004) releasing toxins some of which suppress the immune systems of the insect and others that cause the insect's septicemic death (Kim *et al*., 2005; Park *et al*., 2006; Sergeant *et al*., 2006). Depending on the infective juvenile infection intensity and bacterial load and the insect species, death occurs within 24-48 h (Tanda and Kaya, 1993; Kocan *et al*., 1998). The bacteria also produce antimicrobial substances e.g. xenorhabdicin (Boemare *et al*., 1992), a phage tail that protects the cadaver from secondary or opportunistic saprophytic infections by

bacteria and fungi (Jarosz, 1996; Ji and Kim, 2004; Sicard *et al*., 2005). In the stationary stage the bacteria produce broad spectrum antibiotics that suppress contamination of the cadaver by other microorganisms (Forst and Nealson, 1996). Maxwell *et al*., (1994) measured the *in vivo* production and stability of antibiotic activity from *G. mellonella* infected with *X. nematophila*.

The nematodes develop rapidly to the adult stage feeding on degenerated host tissues and molecules (caused by the bacterial proteases and lipases) and bacteria, mate and produce eggs (Poinar, 1990). During the infective juvenile developmental process, the nematodes become re-colonized by *X. nematophila* symbionts in a process initiated by 1-2 founder cells (Martens *et al*., 2003). After the infective juveniles develop, the founder cells grow to fill the vesicle (Martens *et al*., 2003; Synder *et al*., 2007), the bacteria rapidly multiplying using host haemolymph nutrients (Martens *et al*., 2005; Goetsch *et al*., 2006). Colonized infective juveniles emerged from the insect cadaver due to ammonia, a product of nematode defecation (San-Blas *et al*., 2008), thus completing the life cycle.

Initiation of the process of colonization of the nematode by the bacteria requires three bacterial genes, nil (nematode intestinal location) A, B and C. These genes are of particular interest because of the predicted localization of their products (Cowles and Goodrich-Blair, 2004). nil A is predicted to encode a 90-amino acid inner-membrane protein, nil B is predicted to encode a 460-amino acid outer membrane β-barrel protein (Heungens *et al*., 2002) while nil C encodes a 282-amino acid periplasmically-oriented lipoprotein (Cowles and Goodrich-Blair, 2004). Each of the nil genes is necessary for the colonization: nil A mutants colonize at reduced levels compared to wild type, while nil B and nil C mutants do not colonize above the detection level (Heungens *et al*., 2002; Cowles and Goodrich-Blair, 2004). The bacterial symbiont of *S. carpocapsae* encounters stress during the colonization process. The *X. nematophila* stress and stationary-phase sigma factor σ^s coded by the gene rpoS is required to counteract stress and is necessary for colonization (Vivas and Good-Rich, 2001). Although the rpoS mutants survive as well as the wild type in the colonization assay, it does not colonize the vesicle (Vivas and Goodrich-Blair, 2001; Heungens *et al*., 2002).

The natural life cycle of *S. carpocapsae* and *X. nematophila* benefits both participants: The infective juveniles of nematode contribute to the nutrition and growth of *X. nematophila* in the colonization site, while *X. nematophila* in turn contributes to *S. carpocapsae* reproductive fitness (Sicard *et al*., 2003; Mitani *et al*., 2004, Martens *et al*., 2005). The bacterial load in the vesicle however, declines as the vesicle becomes shorter and narrow as the colonized infective juvenile ages, thus reducing their efficiency in virulence or biological control (Flores-Lara *et al*., 2007).

Life cycle of *Galleria mellonella*

The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), is a world wide economic pest of honey bee products causing major financial losses to beekeepers (Singh, 1962; Covell, 1984). The larval stage of this pest is known to destroy bee colonies and beeswax (Nielson and Brister, 1979) by tunneling through honeycombs and consuming wax, honey and pollen (Eischen *et al*., 1986).

Outside the beekeeping industry, *G. mellonella* is a useful insect; its larvae are raised commercially to be used as fish bait, for use as test organisms in the laboratory and as artificial host for mass propagation of dipteran and hymenopteran parasitoids (Mohamed and Copetl, 1983).

The life cycle of this moth ranges from four weeks to sixth months (Marston *et al*., 1975), and comprises of about six to seven generations per year (Smith, 1938). Approximately 150 white eggs per female, cemented together in long sheets, are laid in small crevices in the beehive (Singh, 1962). Eggs hatch four to five days after oviposition.

Seven larval instars are reported for *G. mellonella*; a molting tube is spun before every larval ecdysis (Wani *et al*., 1997). The insect, weighing approximately 200-250 milligram by the seventh instar, spins a cocoon and pupates after which a beige-molted adult moth emerges (Beck, 1970). The female imagos are larger than the males (wingspan, 1.7-2.5 cm*),* do not feed or drink and live 3-30 days. The female lays a total of 300-600 eggs although individual moths may lay up to 1,800 eggs (Beck, 1970).

Life cycle of *Malacosoma disstria*

The forest tent caterpillar, *Malacosoma disstria* (Lepidoptera: Lasiocampidae), is the most widely distributed indigenous tent caterpillar in North America (Furniss and Carolyn, 1977), defoliating a variety of species of deciduous hardwood trees (Batzer and Morris, 1978). Traditionally, *M. disstria* does not cause mortality to host trees, though mortality can occur when concurrent with other disturbances e.g. drought (Dodds and Seybold, 2005). Although this insect is called tent caterpillar, it is unlike other *Malacosoma* species in that the larvae do not construct tents and are relatively harmless to people although a few people have an allergic reaction to the nettles contacted while handling the insects (James, 2001).

M. disstria has only one generation per year throughout its range. Over-wintering within egg masses, larvae begin emerging in early spring concurrent with the swelling and expanding of buds on host trees (James, 2001). The young larvae are gregarious and initially feed together on the expanding buds, foliage and flowers (James, 2001), the individual differences reflecting nutritional status culminating in the final aggregation of the social caterpillars (Dussutour *et al*., 2008).

As *M. disstria* develops through five larval instars, they devour entire leaves (James, 2001). The pale-yellow pupal cocoons are variously located amongst webbed leaves, bark crevices, shrubbery and other protective places such as the sides and under overhangs of buildings (James, 2001). Pupation takes 10-14 days, and involves the formation of yellowish silken cocoon, from which brown colored adults will emerge (Meeker 1997).

Pathogenicity of *Steinernema carpocapsae*-bacterial complex.

The pathogenic process induced by *S. carpocapsae* has been studied in few species of insects. *G. mellonella* larvae infected by axenic *S. carpocapsae* (Breton strain) show a progressive histolysis in the gut, dorsal blood vessel, Malphighian tubules, and haemocytes starting 3-6 h post-penetration of the infective juveniles into the insect

haemocoel and ending in a general histolysis at the time of death (34-38 h post-penetration) (Simões, 1992). Drees *et al*., (1992) reports that a given dose of *S. carpocapsae* All strain killed the red imported fire ant, *Solenopsis invicta*, in the larva, pupa and alate adult stages with a mortality decreasing with maturation. The All strain was pathogenic also to larvae of the cerambycid beetle, *Plectrodera scalator*, a cottonwood borer, killing 50% of the larvae (Fallon *et al*., 2006). *S. carpocapsae* is also reported to be pathogenic when applied curatively against larvae of the annual bluegrass weevil, *Listronotus maculicollis* (McGraw and Koppenhöfer, 2007, 2008). Sicard *et al*., (2008) demonstrated that both axenic and monoxenic *S. carpocapsae* are pathogenic to isopods, even though the hosts represent a reproductive dead-end for the nematodes The pathogenicity of *S. carpocapsae* has also been reported in the tick *Ixodes ricinus* (Hartelt *et al*., 2008) and the engorged adult blacklegged ticks, *Ixodes scapularis*, the nematode eventually killing the ticks (Zhioua *et al*., 1995). This nematode typically does not infect, complete its life cycle or produce infective juveniles in *I. scapularis* unless the tick body is slit open before *S. carpocapsae* infection (Zhioua *et al*., 1995). The normal lack of development, even as infection occurs, may reflect a physiological incompatibility elicited by the nematodes for some members of the Acarina. Female ticks of *Boophilus annulatus* exposed to different concentrations of *S. carpocapsae* exhibit in high mortality (greater than 90%) at nematode concentrations a low as 500 infective juveniles per dish within 8 days (Samish and Glazer, 1992).

The pathogenicity of *S. carpocapsae* is correlated with toxic factors (e.g. proteases) released by the nematodes and subsequently by *X. nematophila* in the insect haemocoel, resulting in a synergistic action of both participants of the complex (Boemare *et al*., 1982; Burman 1982). A few cells of *X. nematophila* released by *S. carpocapsae* were able to kill *G. mellonella* larvae (see Akhurst and Dunphy, 1993) and *Ma. sexta* (see Forst and Nelson, 1996). The lethality of these pathogens has been attributed to highly potent toxins, extracelluar enzymes, and lipopolysaccharides [(a major component of the outer cell membrane of Gram-negative bacteria, consisting of a lipid A moiety, core oligosaccharides and an *O*-chain; (Erridge *et al*., 2002)] produced during parasitism (Forst and Nealson, 1996). Monexenic *S. carpocapsae* produce in the host haemolymph and in growth medium, toxic proteins and proteases which cause insect death a few hours

post-injection (Simões and Rosa, 1986). The potential of axenic steinernematids infective juveniles to kill insects e.g. lepidopterans has been reported for *S. carpocapsae* (Boemare *et al*., 1982; Burman, 1982; Ehlers *et al*., 1990; Han and Ehlers, 2000) and *S. feltiae* (Ehlers *et al*., 1997). A single axenic *S. carpocapsae* is able to kill 80% of *G. mellonella* in 1day (Ehlers *et al*., 1997*).*

INFECTION

The Steinernematid nematodes are intra-haemocoelic insect pathogens. There are two stages of parasitism which consist of the nematodes (i) invading of the haemocoelic cavity and (ii) the evasion of the host innate immune reactions by the infective juveniles, allowing its establishment and further development (Simões and Rosa, 1976).

Host invasion

Ishibashi and Kondo (1990) emphasize the importance of penetration in the susceptibility of the insect to the nematode, considering it to be a key factor in the pathogenic process. The routes of entry through which the infective juveniles invade the cavity of their host insect, varies with the nematode and/or insect species. Infective juveniles of *S. carpocapsae* penetrate larval *G. mellonella* through the spiracles (Triggiani and Poinar, 1976) and the peritrophic membrane (Forschler and Gardner, 1991). *S. feltiae* infects through anus of the house fly maggots, *Musca domestica*, and leafminers [Diptera: Agromyzidae, (Renn, 1998)], and directly through the cuticle of the tipula fly, the leatherjacket, *Tipula paludosa*, (Peters and Ehlers, 1994) and the cuticle of the white grubs *Popillia japonica*, *Anomala orientalis*, *Cyclocephala borealis*, *Rhizotrogus majalis* (Koppenhöfer., 2007). *S. feltiae* and *S. carpocapsae* enter through the mouth and anus of *P. japonica* (Cui *et al*., 1993). In the adult mole crickets, *Scapteriscus* spp, *S. scapterisci* penetrates through the mouth and spiracles, rather than the anus (Nguyen and Smart, 1991). Direct penetration by steinernematids through the insect cuticles occurs also (see Dunphy and Akhurst, 1993), which is similar to *H. zealandica* and *H. bacteriophora* which penetrates through the cuticle of *P. japonica*, *A.*

orientalis, *C. borealis*, *R. majalis* (Koppenhöfer., 2007). However, some *Heterorhabditis* spp gain entry by abrading the intersegmental membranes of the insect using a dorsal tooth (Bedding and Molyneux, 1982). Variations in infection portal and host stages include infective juveniles entering the pre-pupae of the web-spinning larch sawfly, *Cephalcia lariciphila*, through the spiracles, with the anus and mouth being less favored entry portals (Georgis and Hague, 1981). *S. carpocapsae* are also able to penetrate through the pneumostatic lobes of the adult tsetse fly, *Glossina morsitans morsitans* (Poinar *et al*; 1977), and the pupae of the white-spotted sawyer, *Monochamus scutellatus* (Schmiege, 1963). Penetration through these barriers often requires mechanical processes commonly used by insect parasitic nematodes such as *S. carpocapsae* invading *G. mellonella,* (Poinar and Himsworth, 1967) and other rhabditoids such as *Parasitorhabditis ipsophila* in the bark beetle, *Ips sexdentatus* (Lieutier, 1984). Extraneous bacteria may influence host infection e.g. penetration through the midgut wall of *Cyclocephala hirta* (Coleoptera: Scarabaeidae) by *Heterorhabditis bacteriophora* is facilitated when the insects are infected by the stressor bacterium *Bacillus popilliae* which increases their susceptibility to infection (Thurston *et al*., 1992) and may apply to steinernematids. Synergistic interaction between *S. carpocapsae* or *S. feltiae* and *B. thuringiensis* subsp. i*sraelensis* against *T. paludosa* occurring in the laboratory but not in the field was observed by Oestergaard *et al*., (2006). *B. thuringiensis* subsp. *aizawai*, by damaging the gut wall of larval *Spodoptera exigua*, facilitates the entry of *X. nematophila* from *S. carpocapsae* into the haemocoel without mediation by the nematode (Jung and Kim, 2006). The occurrence of steinernematids and heterorhabditids within the same host is not unusual. *S. carpocapsae*, competing with *H. bacteriophora* simultaneously for *G. mellonella* significantly infected the insect hosts more than *H. bacteriophora* (Alatorre-Rosas and Kaya 1991) while for *Tenebrio molitor*, *S. feltiae* was dominant over *H. bacteriophora* when both species were applied together at different temperatures (Aydin and Susurlux, 2005). For intra-specific competition, *S. glaseri* develops more rapidly than *S. carpocapsae* and *S. feltiae*, within the same host (Kaya and Koppenhofer, 1996).

Studies on the midgut epithelial tissues of *G. mellonella* parasitized by *S. carpocapsae* demonstrated the occurrence of small holes at early stages of parasitism (Simöes, 1992). Small round holes have also been reported in the cuticles of *Tipula spp*

penetrated by *S. feltiae* (Peters and Ehlers, 1994). The holes maybe caused by enzymatic activity from the nematode (Simões and Rosa, 1996). The midgut epithelium cells of *G. mellonella* showed a marked histolysis in response to secretions of axenic *S. carpocapsae* (Simões, 1998). Penetration by *S. feltiae* through the cuticle of leatherjackets might be attributed to the absence of an epicuticular wax layer, the layer possibly blocking the activity of histolytic enzymes (Dowds and Peters, 2002). There is evidence that steinernematid protease secretions are involved in penetration. Protease inhibitors decreased penetration of *S. glaseri* through the gut wall of *G. mellonella* (Abuhatab *et al*., 1995). The secretions/excretions from *S. carpocapsae* (Breton) fractionated by ion exchange chromatography produce three proteolytic peaks, one of which caused disruption of the insect midgut, leading to the belief that it was a proteolytic penetrating factor (Roque *et al.*, 1994).

Evasion of insect host defenses by entomopathogenic nematodes

The nematodes, once in the gut, pass through the peritrophic membrane (Forschler and Gardner, 1991) and enter the haemolymph. In the haemocoel, the nematodes must contend with the host's innate immediate humoral and cellular defences (see section on Insect Immunity for detailed review). Evasion of host defenses by entomopathogenic nematodes and non-encapsulation of the nematodes might occur by the following possible mechanisms: (1) the nematodes avoid eliciting responses by being recognized as self, (2) nematodes are not recognized as non-self (Dunphy and Webster, 1986, 1988a) and (3) the pathogen suppress responses after being recognized as non-self (Dunphy and Webster, 1987; Brehélin *et al.,* 1990). Avoidance of activating host defences may occur by mimicry in which the nematodes are not recognized as proposed for *S. carpocapsae* in some lepidopteran species (Bréhelin *et al*., 1990). However, in situations were evasion is not avoided as with *S. carpocapsae* encapsulation in the Japanese beetle, *P. japonica,* and the house cricket, *Acheta domesticus,* insect death still occurs because bacterial release from the infective juveniles occurs before capsule completion (Wang *et al.,* 1994).

S. carpocapsae and *S. feltiae* is not encapsulated by *G. mellonella* possibly because the nematodes are not recognized as foreign due the non-adhesive lipid-like molecules of the cuticle (Dunphy and Webster, 1985, 1986; Mastore and Brivio, 2008). Another evasion mechanism includes the destruction by the nematode-bacteria complex of antimicrobial proteins such as cecropins which are induced in the larvae by the presence of a foreign object after the initial innate immune response. Proteinases of *S. carpocapsae* are able to destroy antibacterial factors produced by the lepidopteran *Hyalophora cecropia* (Götz *et al.*, 1981) and those induced in *G. mellonella, Bombyx mori* and the coleopteran *Tenebrio molitor* (Bettencourt *et al*., 1992). The nematode *S. feltiae* and its associated bacterium, *Xenorhabdus boivenii,* when parasitizing larval *G. mellonella* produces a toxic proteolytically active substance, which shows some similarity to the immune inhibitors released by certain bacterial pathogens of insects such as the virulent *Pseudomonas aeruginosa* (Dunn and Drake, 1983; Jarosz *et al*., 1991; Balcerzak, 1992a). Similarities include the heat sensitive proteinases (Jarosz *et al*., 1991) selectively destroying the antibacterial activity of immune haemolymph cecropins *in vitro* and *in vivo* (Balcerzak, 1992b). Jarosz *et al*., (1991), suggest that these toxic proteinases maybe produced also by *X. nematophila*. The enzyme(s) do not prevent lysozyme synthesis or activity (Balcerzak, 1992b), yet lysozyme and cecropin-like polypeptides disappeared in *G. mellonella* (Balcerzak, 1992b). Ji and Kim (2004) state that live but not dead *X. nematophila* inhibits expression of cecropin production in the beet armyworm, *Spodoptera exigua* but not by the eicosanoid pathway which is an important mediator in the immune signaling, resulting in an immunodepressive condition in the infected insect host (see Park and Kim, 2000).

Entomopathogenic nematode growth and reproduction in the haemocoel of a susceptible host in addition to depending on the *Xenorhabdus* spp and host tissue and molecule digestion products, initially depends significantly on their ability to either tolerate host defenses or evade host responses (Simões and Rosa, 1996). Resistance to steinernematid infection by insect species from numerous orders has been attributed to host blood cells (haemocytes) forming cellular capsules around steinernematids spp; *S. carpocapsae* in *G. mellonella* (Poinar and Himsworth, 1967), the corn root worm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (Jackson and Brooks, 1989)

and the pineweevil, *Hylobius abietis* (Coleoptera: Curculionidae) (Pye and Burman, 1978). Cellular encapsulation of *S. carpocapsae* has been reported also in the orthopteran *A. domesticus* (Wang *et al*; 1994) and in the coleopteran *P. japonica* (Wang *et al*, 1994), the armyworm moth, *My. unipuncta,* (Lepidoptera: Noctuidae) (Simões *et al*; 2001), thus leading to nematode death by encapsulation.

Humoral encapsulation [(immunity involving melanin layer(s) produced about foreign materials by means of the adhesion of the enzyme phenoloxidase (Gotz, 1986; Vey, 1993)] of *S. carpocapsae* nematodes is observed in mosquito larvae of *Aedes* spp (Welch and Bronskill, 1962), *Culex restuans* and *Culex pipiens* (Poinar and Leutenegger, 1971), and larval *Tipula olaraceae* (Peters, 1998), all dipterans with few haemocytes. *Steinernema anomaly* is restrained in melanin capsules of *C. pipiens* larvae (Poinar and Leutenegger, 1971), and *S. feltiae, S. glaseri, S. kraussei* and *S. intermedium* are encapsulated in *T. paludosa* larvae (Peters, 1998).

Toxins production by entomopathogenic nematodes

Although an immune inhibitor excreted by axenic *S. carpocapsae* destroys induced antibacterial activity in immune haemolymph of *H. cecropia* both *in vivo* and *in vitro* (Götz *et al*., 1981), most research deals with toxins produced *in vivo* with cytolytic activity. A correlation exists between the production of an unidentified toxin by axenic *S. carpocapsae* (Breton strain) in parasitized *G. mellonella* larvae and host death (see Simões and Rosa, 1996). Shortly after nematode penetration, toxins occur in the insect haemocoel, reaching a peak just before insect death and declining a few hours thereafter. Injection of sub-lethal doses of the toxin(s) causes an increase of the heartbeat followed by temporary tentanization and then paralysis of the insect (see Simões and Rosa, 1996). The toxin exhibits differential toxicity *per os* with higher mortality of the tobacco budworm, *Ma. sexta* than in *G. mellonella* and no activity against the beetle *Diabrotica undecimpunctacta* (see Simões and Rosa, 1996). *G. mellonella* haemocytes are more susceptible to the toxins than those of *My. unipuncta* under the same conditions (Ribeiro *et al*., 1994).

In addition to the toxins released by *S. carpocapsae*, secretions or excretions from the bacteria show cytotoxicity and proteolytic activity (see Simões and Rosa, 1996). After the release of *X. nematophila*, at undefined stages of infection, different extracellular enzymes are released by the bacteria (e.g. protease, lipase, lecithinases, DNAses and phosphatases) (Boemare and Akhurst, 1998; Schmidt *et al*., 1998). Caldas *et al* (2002) purified a heat-labile 60 kDa protease, protease II, secreted by *X. nematophila* that destroys cecropins present in the haemolymph of *G. mellonella* and *P. unipuncta*. However, the role of the metalloproteinase-like in initial nematode-insect defence interaction is not relevant since it is released long after nematode enters haemocoel.

Substantial evidence on the cytolytic and cytotoxic activity of the *X. nematophila* has been reported, with very few studies on the role of the toxic activity of the secretory/excretory products produced by the axenic nematode. Part of the focus on this study will be based on partially characterizing toxins produced by axenic *S. carpocapsae* DD136. The role of the extracellular metabolites produced by the nematode in the pathogenic process is not clear. Aspects of this is addressed in the thesis as it relates to haemocyte reactions with glass and two species of bacteria, *X. nematophila* and *Bacillus subtilis*, influenced by exudates of axenic *S. carpocapsae.*

Xenorhabdus nematophila

The bacterium *Xenorhabdus nematophila* (Enterobacteriaceae) is a Gram-negative insect pathogenic bacterium having a symbiotic relationship with the entomopathogenic nematode *S. carpocapsae* (Forst *et al.*, 1996, 1997). Two distinct physiological states at the end of a continuum in physiological attributes of *Xenorhabdus* spp occur *in vit*ro (Akhurst, 1980), the phase I and II variants (Boemare, 1988). Phase I variants absorb dyes on agar plates, produce several antibiotics, secrete a variety of proteins (e.g., lipases and proteases), and produce fimbriae and flagella, while these properties are either absent or greatly reduced in phase II variants (Boemare and Akhurst, 1988; Givaudan, 1995). The release of these compounds by the phase I variants is though to be facilitated by their more rigid membrane structure (Fodor *et al*., 1997). Both phases are pathogenic to insects, however, usually only the more virulent phase I variant

supports nematode growth and associates with infective nematodes parasitizing insects naturally (Ahurst, 1980). The set of mechanisms by which the *X. nematophila* bacteria are able to circumvent the host defense systems and cause insect death, as well as the benefits provided by the bacteria to their symbiotic nematodes, is frequently associated with the extracellular molecules produced by *Xenorhabdus* spp. (Dunphy and Webster, 1988a; Boemare *et al*., 1992; Forst *et al*., 1997). (See Nodulation section for details).

During the nematode-bacterium-insect complex, the bacteria, which is carried in the diverticulum of the gut of the infective juvenile (Akhurst, 1982), proliferates in the haemolymph (Forst, 1996) leading to insect death within 48 h of infection (see Akhurst and Dunphy, 1993). The bacteria produces a variety of antibiotics under both *in vitro and in vivo* conditions (Li *et al*., 1995; Hu *et al*., 1997), to help maintain an optimal environment for the developing nematode in the cadaver relatively free from competition from other bacteria, fungi, and nematode species (Akhurst and Boemare., 1990; Barbercheck and Kaya, 1990; Chen *et al*., 1994; Thaler *et al*., 1997; Walsh and Webster, 2003). Secondary contamination of the cadaver is also prevented by *Xenorhabdus* producing antimicrobial bacteriocins, xenoucoumacins and xenorhabdicins (McInerney, 1991; Boemare *et al.,* 1992, 1993; Thaler, 1997). Because the number of *X. nematophila* in the insect haemolymph is very low before insect death, Forst and Nealson (1996) hypothesized that the bacteria enter in an intraphagosomal phase during which it secrete some factors toxic to the insect. Sicard *et al* (2004) found an extracellular phase using green fluorescent protein-labelled *X. nematophila* that colonized an anterior gut region of *S. littoralis*. Since bacterial proliferation does not occur in the haemocoel before insect death it is suggested that the secretions of these pathogens are highly potent virulence factors. An organic extract from *Xenorhabdus* as a stationary culture contains uncharacterized substance(s) that inhibits the activity of phospholipase A_2 (Park and Stanley, 2005), an enzyme which participates in eicosanoid biosynthesis (Dennis, 1994) inhibiting nodulation and phenoloxidase activation (Park and Kim, 2003). Inhibition of eicosanoid biosynthesis in larvae of the tobacco hornworm *Ma. sexta* immediately prior to intrahaemocoelic infections with the bacterium *Serratia marcescens* strongly reduced the nodulation response (Miller, 1994). Schleusener (1996) shows that *Ma. sexta* haemocytes express PLA_2, which hydrolyzes arachidonic acid from phospholipids,

producing immune (cells) enhancing eicosanoids which mediates nodulation. Park and Stanley (2005) report that *X. nematophila* actively inhibits parameters of cellular immunity in adult male crickets, *G. firmus*, including haemocytic PLA_2 activity, haemocyte/bacterial microaggregation, and nodulation reactions. They also show that live bacterial injections result in reduced total haemocyte counts thus indicating the insect's capacity to respond to an infection is compromised. PLA_2 effects apply to several lepidopteran species. In *S. exigua*, PLA_2 was inhibited using *Photorhabdus temperata* and *Xenorhabdus* spp which caused highly virulent pathogenicity of the bacteria in a dose-dependent manner against the fifth instar larvae of the insect, as early as 24 h after the intra-haemocoelic injection (Kim *et al*, 2005). Both live and dead *X. nematophila* bind to haemocytes and are removed by limited nodulation (Dunphy and Webster, 1984; Dunphy *et al*., 2002) which may reflect binding of the bifunctional neuromodulator, octopamine and physiological status of the pathogen. The amine binds to live and dead *X. nematophila* surfaces accelerating removal of the bacteria from haemolymph (Dunphy and Downer, 1994). Only the live bacteria had an anti-nodulation effect, the heat-killed bacteria activated nodulation reaction 24 h post-injection in the armyworm, *S. exigua* (Kim *et al*., 2005). Eicosanoids are been implicated in phenoloxidase activity especially arachidonic acid-PLA_2 in *S. exigua* larvae (Shrestha and Kim, 2008).

Lipopolysaccharides (LPS) of *X. nematophila* prevent the conversion of prophenoloxidase into phenoloxidase in numerous insect species including the house cricket *A. domesticus* (da Silva, 2000 *et al*) and in *G. mellonella* (Dunphy and Webster, 1988a). Phenoloxidase is a key extracellular encapsulating enzyme responsible for the catalysis of melanin (Shelby and Popham, 2006; Zhao *et al*., 2007) resulting in the generation of toxic radicals (Nappi *et al*., 1995). Melanin is involved also in wound healing and humoral immune responses (Procházková *et al,* 2006). Activation of phenoloxidase is limited by *X. nematophila* by releasing LPS (Dunphy and Webster, 1988a) which chelates divalent cations; Fe^{2+} in *G. mellonella* (Dunphy *et al.*, 2002) and Ca^{2+} in *Agrotis segetum* (Yokoo *et al.*, 1995). Dunphy and Webster, (1988a, b) show that binding of lipid A to N-acetyl-D-glucosamine haemocyte receptors, damages *G. mellonella* haemocytes releasing entrapped bacteria and iron-binding proteins which, in

concert with iron-chelation by the endotoxin, limit the growth of bacteria that do not produce siderophores chelating iron molecules (Dunphy *et al.*, 2002).

X. nematophila displays swarming behavior on suitable media, but a spontaneous loss of motility is observed as part of the phenomenon of phase-variation (Givaudan and Lanois, 2000). The purified flagella master operon *flh*DC-dependent, 10.9kDa haemolytic cytotoxin peptide, called α-xenorhabdolysin triggers apoptosis and necrosis in insect granular cells and damages to the plasma membrane of *S. littoralis***,** leading to selective vacuolation of the endoplasmic reticulum, cell swelling, K^+ efflux due to possible pore formation and cell death by colloid-osmotic lysis (Ribeiro *et al*., 2003). Vigneux *et al*. (2007) describes a 70kDa binary toxin similar in biological activity to xenorhabdolysin in which the xenorhabdolysin A component is the apoptotic factor. A fimbrial subunit, MrxA, of *X. nematophila* form pores in cell membrane of haemocytes of larval *Helicoverpa armigera* leading to lysis of the haemocytes (Banerjee *et al*., 2006).

Herein, only dead *X. nematophila* were used to circumvent ongoing metabolic aspects of virulence, which might synergistically complicate the nematode effect on host haemocytes thus allowing direct observation of the interaction of the insect haemocytes with the surface of the bacteria.

Bacillus subtilis

Bacillus subtilis is a catalase-positive bacterium commonly found in soil (Madigan and Martinko, 2005; Morohashi *et al.*, 2007). The interaction of *Bacillus subtilis,* a non-pathogenic bacterium, with the antibacterial system of insects is known for few insect species. In the gypsy moth, *Lymantria dispar* and *G. mellonella* live and dead *B. subtilis* induce serum melanization and phenoloxidase activation *in vitro*, respectively (Brehélin *et al*. 1989, Dunphy and Bouchier 1992) and in the black-backed grasshopper, *Euprepocnemis shirakii*, the bacterium induces phagocytosis by both plasmatocytes and granular cells (Chang *et al*. 1992). *Bacillus subtilis* contains lipoteichoic acids [(a major membrane-associated amphiphilic molecule containing endotoxin found in Gram-positive bacteria, (Huff, 1982)] which elicit nodulation, deplete

the plasmatocyte concentration in the haemocoel and irreversibly damage granular cells while activating melanizing phenoloxidase of larval *G. mellonella* (Halwani *et al*., 2000) and *M. disstria* (Giannoulis *et al*., 2007). Phenoloxidase of *A. domesticus* and *L. dispar* binds to the surface of *B. subtilis* increasing the binding of the bacteria to haemocytes in *vitro* (da Silva *et al*. 2000; Giannoulis *et al*., 2007).

Nodulation of *B. cereus*, a bacterium closely related to *B. subtilis* (Mezes *et al*., 1985), in *G. mellonella*, maybe a product of octopamine elevation which binds to bacterial surfaces activating haemocytes (Dunphy and Downer, 1994). A multifunctional plasma protein apolipophorin-III, found in *G. mellonella* haemolymph, binds to lipoteichoic acids from *B. subtilis* impairing phenoloxidase activation and partially preventing the loss of plasmatocytes (Halwani *et al*., 2000).

B. subtilis without apolipophorin binds avidly to haemocytes of *G. mellonella* (Zakarian *et al*., 2002) and *M. disstria* which, in the latter insect, was associated with the bacterium being rapidly removed from the haemolymph by nodulation (Giannoulis *et al*., 2007). In *G. mellonella* dead *B. subtilis* does not alter iron levels (Dunphy *et al*., 2002).

INSECT IMMUNITY

Insect immunity is defined as the ability of an insect to defend itself from microbial invasion (Yamakawa and Tanka, 1999). Once past the structural-physio-chemical barriers of the cuticle and gut (Dunphy and Akhurst, 1993; Abuhatab *et al*., 1995) the antigen encounters the haemolymph. In the insect haemolymph, two forms of immunity are recognized: innate immunity and induced immunity. Yamakawa and Tanaka (1999) defines innate immunity as a nonspecific mechanism immediately responding to the invasion of foreign particles into the haemolymph. Induced immunity is an induced response to challenges from bacteria, viruses, fungi, parasites and foreign tissues resulting in the production of antimicrobial peptides (Hetru *et al*, 1998). Because *S. carpocapsae* first encounters innate immune activities of the insect, emphasis will be

placed on the humoral and cellular (haemocyte) non-self factors of the insect haemolymph of innate immunity.

Innate immunity

The innate immune response of insects involves both humoral and cellular components (Lavine and Strand, 2002). Humoral responses comprise the antimicrobial (Shelby and Popham, 2006) melanizing enzyme, phenoloxidase (Meister and Lagueux, 2003; Shelby and Popham, 2006; Zhao *et al*., 2007), the pattern recognition proteins lysozyme (Wilson and Ratcliffe, 2000), the immulectins and other C-type lectins, β -l,3-glucan binding proteins, hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002), apolipophorin-III (Halwani *et al,* 2000) and the lipopolysaccharide-inactivating lipophorin particle (Ma *et al,* 2006). The cellular defense reactions consist of phagocytosis, nodulation and encapsulation of invading particulate antigens predominantly by the haemocyte types, the plasmatocytes and granular cells in lepidopterans (Pech and Strand, 1995). These events eventually lead to the rapid and transient synthesis of antimicrobial polypeptides by the fat body (a functional equivalent to the mammalian liver and adipose tissues), haemocytes and the midgut epithelial cells (Lehane, 1997).

Plasma

Lepidopteran larvae have an advantage over most insects for biochemical analyses and studies of haemocyte functions because of their size and haemolymph volume which is composed predominantly of plasma (Kanost *et al*., 2004). The insect plasma is a biochemically rich solution with widely varying proportions of inorganic ions, high levels of free amino acids and trehalose, and sometimes substantial amounts of other solutes such as organic phosphates, organic acids (e.g. citrate), glycerol, peptides (Wyatt, 1961) and proteins as represented by apolipophorins-I, II and III (Dunphy and Halwani, 1997), hemolin (Lee *et al*., 2002), and scolexin (Kyrinkides *et al*., 1995). The haemolymph fills the body cavity or haemocoel and comprises about 10%-40% of the

body's volume depending on instar, species and environmental conditions (Wyatt and Pan, 1978).

In innate immunity, initial recognition of non-self is mediated by soluble and cellular pattern recognition receptors (PRRs) conserved from insects to mammals, encoded by germ line cells (Janeway and Medzhitov, 2002). The PRRs perform a surveillance role by binding to molecules known as pathogen-associated molecular patterns (PAMPs), molecules that are common to groups of micro-organisms but absent from animals (Hoffman *et al*., 1999) and include β-1,3 glucan of fungi (Gillespie and Kanost, 1997), lipoteichoic acids from Gram-positive bacteria and lipopolysaccharides from Gram-negative bacteria (Yu and Kanost, 2002).The salient PRR include lectins (Watanabe *et al*., 2006), immulectins (Yu and Kanost, 2004), hemolin (Bao *et al*., 2007) lysozymes (Wilson and Ratcliffe, 2000) and apolipophorin-III (Halwani *et al*., 2001) are briefly discussed herein.

The haemolymph proteins include lectins, a recognition molecule because they are able to bind to oligosaccharide structures present on cell surfaces (Gillespie and Kanost, 1997). Insect lectins have been purified from the haemolymph of the insects, the death head cockroach, *Blaberus discoidalis* (Chen *et al*., 1993), *B. mori* (Kotani *et al*., 1995), the blue bottle flies, *Calliphora vomitoria* (Mckenzie and Preston, 1992), the assassin bug, *Rhodnius prolixus* (Ratcliffe *et al*., 1996) and *Ma. sexta* (Yu and Kanost, 2005). Immulectins are C-type lectin humoral PRRs, present in the plasma (Yu and Kanost, 2003) and involved in innate immune responses including protection of *Ma. sexta* larvae by immulectin-2 from Gram-negative bacterial infection (Yu and Kanost, 2003), by stimulating prophenoloxidase (proPO) activation by hemolin in haemolymph (Yu and Kanost, 1999), promotion of *in vitro* cellular encapsulation by immulectin-3 (Yu *et al*., 2005) and *in vivo* melanization of agarose beads by immulectin-2 (Yu and Kanost, 2004). Immulectin-2 also binds to lipid A, lipotechoic acid and peptidoglycan triggering melanization (Yu and Ma, 2006).

Hemolin, a plasma protein has been identified in the haemolymph of lepidopteran species such as *H. cecropia* pupae (Rasmuson and Boman, 1979), and larvae of *Ma. sexta* (Ladendorff and Kanost, 1990), the fall webworm, *Hyphantria cunea*, (Shin *et al*., 1998)

and *L. dispar* (Lee *et al*., 2002). This PRR is an immune protein belonging to the immunoglobulin (Ig) superfamily, and synthesized mainly from the fat body (Lindstrom-Dinnetz *et al*., 1995). Hemolin, composed of four I-set immunoglobulin domains forming a horseshoe-shaped structure (Faye and Kanost, 1998; Su *et al*., 1998), is located also on the surfaces of *Ma. sexta* and *H. cecropia* haemocytes and may inhibit haemocyte aggregation (Lei and Kanost, 1996; Faye and Kanost, 1998), down-regulating excessive nodulation to prevent all the haemocytes from being used in nodule formation (Ladendorff and Kanost, 1991; Kanost *et al*., 1994). Hemolin also mediates cellular immune responses to bacteria in larval *Ma. sexta* by binding to molecular patterns present on the surface of bacteria and triggering a protective response involving humoral and cellular reactions (Ioannis *et al*., 2007). Hemolin may be a PRR triggering phenoloxidase reaction against bacterial infection as evident in *H. cecropia* after injecting silencing hemolin dsRNA in the insect causing a significant reduction in phenoloxidase activity suggesting that hemolin has the ability to bind to bacteria and that it acts through the prophenoloxidase activating pathway (Terenius *et al*., 2007). The plasma protein binding to the lipid A moiety of *E. coli* LPS (Sun *et al*., 1990), enhances phagocytosis of *E. coli* in haemocytes of *Ma. sexta* (Lei and Kanost, 1996) and the Drosophilia haemocyte cell line, mbn-2 (Lanz-Mendoza *et al*., 1996). Enhanced phagocytosis in *H. cecropia* elicited by the combined action of hemolin and LPS is controlled by the signal transduction enzyme, protein kinase C since the enzyme inhibitors staurosporine and H7 prevents phagocytosis (Lanz-Mendoza *et al*., 1996). Eleftherianos *et al.,* (2006a) described up-regulation of microbial PRP genes and antibacterial effector genes including hemolin in *Ma. sexta* against *P. luminescens* with prior vaccination by *E. coli*. However, suppression of immune responses mediated by hemolin by means of RNA interference causes increased susceptibility to the insect pathogen *P. luminescens* in the tobacco hornworm *Ma. sexta* (Eleftherianos *et al*., 2006b, 2007).

Fungal cell walls consist of β-1,3-glucans which serve as a pattern recognition molecules (Gillespie and Kanost, 1997). β- 1, 3-glucans-binding protein (βGRPs) has been isolated from haemolymph of *B. mori* (Ochiai *et al*., 1992) and *Ma. sexta* (Ma *et al*., 2000; Jiang *et al*., 2003). In *Ma. sexta*, βGRPs bind to β-1, 3-glucans and lipoteichoic

acid and agglutinate Gram-negative bacteria (which does not have either types of molecule) in addition to Gram-positive bacteria and yeast, stimulating activation of proPO in plasma (Ma *et al*., 2000; Jiang *et al*., 2003).

One important component of the innate immune system highly conserved from insects to mammals (Kang *et al*., 1998) but absent in nematodes and plants (Dziarski and Gupta, 2006), that recognizes unique bacterial cell wall components is the peptidoglycan recognition proteins (PGRPs) (Lee *et al*., 2004; Dziarski, 2004; Dziarski and Gupta, 2006). These 20-kDa proteins were first reported in *B. mori* with the ability to trigger the proPO cascade upon binding to peptidoglycan (Yoshida, 1996). Dziarski (2004) reports that there are four known effector functions of insect PGRPs that are unique for insect's activation of proPO cascade, activation of Toll receptors, activation of the *Imd* (immune deficiency system) pathway of Drosophila and induction of phagocytosis.

Lysozyme, a constitutive and inducible, heat and acid stable 14-16.5 kDa enzyme, is found both in the haemolymph plasma and insect haemocytes (Chung and Ourth, 2000; Yu *et al*., 2006). This peptidoglycan-lytic enzyme (Royet and Dziarski, 2007), can lyse bacteria by hydrolysing β-1,4 linkage between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan layer of bacterial cell walls (Nash *et al*., 2006), after the bacteria has been killed by cecropins and attacins (Boman *et al*., 1991). Lysozyme in *G. mellonella* haemolymph has binding affinity to cell-walls of Gram-negative, Gram-positive bacteria and the fungus *Candida albicans* (Lee, 2003). Dunphy *et al*., (2003) report that the avirulent myosin-I-defective yeast cells are rapidly removed from the haemolymph of *G. mellonella in vivo* because of lysozyme-mediated yeast agglutination and the possible binding of the yeast cells by lysozyme and apolipophorin-III. Lepidopteran C-type lysozyme retains antibacterial activity against Gram-negative and Gram-positive bacteria independently of enzyme activity (Ibrahim *et al.,* 2001; Yu *et al*., 2002) as does muramidase deficient lysozyme from mice that is bactericidal for both Gram positive and Gram-negative bacteria (Nash *et al*., 2006). Gandhe *et al* (2007) have similar conclusions in insect; these authors by cDNA cloning and protein sequencing, respectively, detected lysozyme-like proteins in *B.mori* and *Antheraea mylitta*, that are antibacterial and lack muramidase activity, however, some lysozyme isoforms are bacteriostatic but not bacteriocidal against *E. coli* and *M. luteus.* Adamo (2004) observed

an increase in lysozyme-like activity after an immune challenge and the greater the increase, the greater the chance that the cricket *Gryllus texensis* would survive infection by *Serratia marcescens* or *B. cereus*. Chadwick (1970) reports the presence of lysozymes in the larvae of *G. mellonella*, the concentration of lysozyme in the haemolymph of the larvae increasing after vaccination with *P. aeruginosa.* In *G. mellonella* and the cockroach *B. discoidolis*, lysozymes enhance bacterial attachment to haemocytes (Dunphy and Webster, 1991; Wilson and Ratcliffe, 2000). Dunn *et al*., (1985) observed that in non-immune insects, lysozyme may play a significant role in the digestion of bacteria cell walls and release of peptidoglycan fragments that act as signal molecules for the induction of the antibacterial proteins.

Phenoloxidase, a copper containing enzyme (Lu and Jang, 2007), is activated by modification of zymogenic prophenoloxidase as part of the innate immune response of insects and catalyses the formation of quinones [which are reactive intermediates for melanin synthesis (Marmaras, 1996)], with subsequent melanin formation around the invading pathogens (Christensen and Nappi, 2005; Xue *et al*., 2006) that participate in sequestrating and/or killing the pathogens (Nappi and Vass, 2001). Zhao *et al.,* (2007), showed that *Ma. sexta* phenoloxidase has a broad spectrum antimicrobial activity against bacteria and fungi depending on the enzyme substrate and microorganism, the activation of prophenoloxidase involving a multitude of cascading enzymes which immobilize and kill invading microorganism by the production of cytotoxic radicals and quinones through oxidation of phenolic compounds (Christensen and Nappi, 2005; Xue *et al.*, 2006). Although prophenoloxidase (proPO) can be activated in the plasma by proteolytic cleavage at a specific site near its amino-terminus by serine proteases, the zymogen is localized generally in the haemocytes oenocytoids, plasmatocytes, spherulocytes and granular cells (Ashida and Brey, 1998; Ling *et al*., 2005; Neuwirth, 2005). Arachidonic acid, thus eicosanoids, triggers phenoloxidase release from oenocytoids of *S. exigua* through cell rupture possibly mediating proPO activation at the post-transcriptional level in the haemocytes (Shrestha and Kim, 2008**)**. ProPO adheres to *Ma. sexta* haemocytes participating in haemocyte capsule melanization (Ling and Yu, 2005). Schmidt *et al* (1997) histochemically detected proPO in the granular cells of *G. mellonella* using DL-3, 4-dihydroxyphenylalanine as a substrate. Prophenoloxidase is usually activated by

conversion into phenoloxidase by interaction with bacterial surface components including most LPS types, β-1, 3 glucans and peptidoglycan (Charalambidis *et al*., 1996; Ashida and Yamazaki, 1990). The proteolytic activation of proPO is mediated by a proPO-activating proteinase (PAP) and its cofactor in the *Ma. sexta* (Jiang *et al*., 2003). The cofactor consists of two serine proteinase homologs which associate with immulectin-2, a calcium-dependent lectin that binds to lipopolysaccharides (Jiang *et al*., 2003). PAP-1 requires an auxiliary factor for generating active phenoloxidase (Yu *et al*., 2003) while PAP cofactor greatly enhanced proPO activation by PAP-3 (Wang and Jiang, 2004). Gupta *et al* (2005) reported that *Ma. sexta* proPO activation requires active PAP-1 and serine proteinase homologs simultaneously; one for limited proteolysis and the other as a cofactor. This proteolytic activation is the last step in a serine proteinase cascade that is triggered upon recognition of aberrant surfaces or microbial polysaccharides (Gillespie *et al*., 1997; see Ashida and Brey, 1998; Jiang and Kanost, 2000).

Apolipophorin-III (apoLp-III), a heat-stable, 18-24 kDa plasma lipid transport protein participates also in humoral and cellular antimicrobial responses in *M. disstria* and *G. mellonella* larvae including phagocytosis of the yeast *Saccharomyces cerevisiae* (Wiesner *et al*., 1997; Dettloff and Wiesner., 1998; Dettloff *et al*., 2001; Halwani *et al*., 2001). The protein can be found as a lipid-free haemolymph protein that associates with lipophorin during adipokinetic hormone-induced lipid mobilization (Weers and Ryan, 2006). Lipophorin is the major haemolymph protein responsible for lipid transport between tissues of insects, (Pennington and Well, 2002) and the adhesion of *P. americana* haemocytes to cover slips (Goodin and Caveney, 1992). Lipophorin particles from *G. mellonella* retain LPS and other hydrophobic toxins with assistance of the LPS-binding proteins immulectin-2 and β-1, 3 glucans binding proteins (Ma *et al*., 2006). Giannoulis *et al.* (2007) isolated an apolipophorin-III-like protein that once bound to bacteria slowed their removal from the haemolymph and also when incubated with haemocytes, limited their responses to glass slides and bacterial adhesion. Mandato *et al*., (1996) reported that apolipophorin-I but not recombinant apoLp-III inhibited *G. mellonella* haemocyte adhesion to slides in the presence of sodium dodecyl sulphate. The purified protein, apoLp-III enhances the phagocytic response of plasmatocytes to yeast cells *in vitro*, and induced antibacterial activity when injected into larvae of *G. mellonella*

(Wiesner *et al*., 1997). ApoLp-III caused agglutination of erythrocytes (Limura *et al*., 1998) and bound to the surface, and detoxified the LPS of the Gram-negative insect pathogenic bacterium *X. nematophila* (Dunphy and Halwani, 1997). It bound also to the Gram-positive bacterium, *Enterococcus hirae*, and reduced the haemocyte toxicity of the lipoteichoic acids (Halwani *et al*., 2000).

Other plasma haemocyte modifying components

The pupal haemocytes of *H. cecropia* exhibit increasing haemocyte adhesion and spreading activated by haemokinins, a 50-kDa injury-factor released from damaged epidermal cells into the plasma, (Cherbas, 1973). A plasmatocyte spreading peptide from the haemolymph of *P. includens*, upon injection, elicits a transient adhesion of the haemocytes to insect tissues (Clark *et al*., 1997). The protein may be similar to the plasmatocyte depletion factor in *G. mellonella*, which induced plasmatocyte adhesion to the tissues (Chain and Anderson, 1983). The peptide was subsequently detected in the plasma and fat body (Clark *et al*, 1998). A paralytic peptide in *Ma. sexta* stimulated spreading and aggregation activities of plasmatocytes *in vitro*; the peptide precursor was synthesized in the fat body, absent from haemocytes, and was not induced by injecting bacteria (Wang *et al*, 1999). Kanost *et al*., (1994) isolated a 50kDa haemocyte aggregation inhibitor protein, which was antigenically distinct from haemolin in *Ma. sexta* and was found in other lepidopteran including *G. mellonella*. An arylphorin-like protein in *Ps. separata* was induced by parasitization by the ichneumonid *Cotesia kariyai,* and suppressed haemocyte function in the host (Hayakawa, 1994).

Haemocytes

The insect haemocytes, which make up about 10% of the volume of the haemolymph, are nucleated blood cells that do not contain respiratory pigments and are classified according to morphology, antigenic properties and functions (Mullett *et al*., 1993). A typical lepidopteran haemogram is characterized as a combination of haemocyte types, the prohaemocytes, granular cells, plasmatocytes, spherulocytes and oenocytoids

(Gardiner and Strand, 1999). There are four haemocyte types in the late last larval instar of *G. mellonella*: (1) plasmatocytes [round to spindle shaped cells, 10-20µ long and 5-10µ wide with cytoplasm containing no distinguishing inclusions, and an abundance of Golgi complexes, rough endoplasmic reticulum and free ribosomes], (2) granular cells [oval shaped cells, 10-20 µ long and 5-10 µ wide, made up of acidic mucosubstances and contain lipid droplets at some stage of their development], (3) spherule cells [oval shaped, 15-20 µ long and 5-10 µ wide and made up of acidic mucosubstances] and (4) oenocytoids [the largest cells, 20 by 40 µ, with the cytoplasm containing an eccentric nucleus free ribosomes and microtubules] (Neuwirth, 1973).

Gardiner and Strand (2000) believe that haemocytes are generally produced in the haematopoietic tissues in the mesothorax and metathorax of lepidopteran larvae. All circulating haemocytes with the possible exception of oenocytoids proliferate in *S. frugiperda* and *P. includens* larvae (Gardiner and Strand, 2000). In Lepidoptera, maintenance of haemocyte populations during the larval stage also depends on both proliferation of cells already in circulation and the release of haemocytes from hematopoietic organs (Gardiner and Strand, 1999; 2000; Yamashita and Iwabuchi, 2001). Functions ascribed to a given haemocyte type vary due to variation in observation techniques and insect species (Mullet *et al*., 1993) and include hormone transport, wound repair and antimicrobial activity (Prasada *et al*., 1984).

Granular cells and plasmatocytes are recognized as the immunocompetent cells in most lepidopterans (Ribeiro *et al*., 1996; Brillard *et al*., 2001). Depending on the insect species and antigen type and concentration (Howard *et al*., 1998), they participate in phagocytosis with the plasmatocytes playing a dominant role (Tojo *et al*., 2000) while spherulocytes are believed to contain heparin-like molecules which probably prevents the coagulation of the haemolymph (Akhurst, 1982) and the oenocytoids, contain components of the prophenoloxidase system (Schmidt *et al*., 1977). However, functions vary with the insect species and/or stage as seen for the oenocytoids which phagocytose latex beads in larvae of the ground beetle, *Carabus lefebvrei* whereas plasmatocytes phagocytose latex beads in larvae and adult of this insect species (Giglio *et al*., 2008), and the granular cells and the oenocytoids of the scarabaeid beetle *Cetonischema aerugnninosa* are the haemocyte types involved in phagocytosis (Giulianini *et al*., 2003.).

Ling *et al*., (2005) observed that plasmatocytes and granular cells of *Ma. sexta* perform different phagocytotic roles, with the plasmatocytes being the major haemocyte involved in phagocytosis of non-self microsphere beads, while the granular cells were the only haemocytes that participate in the phagocytosis of self dead cells. A subpopulation of plasmatocytes in *Ma. sexta* known as hyperphagocytic cells, recognize and attach to large numbers of bacteria and act as nuclei for subsequent nodule formation (Dean *et al*., 2004). Table 1 summarizes the immunological functions attributed to the lepidopteran haemocytes.

Table 1.1 Summary of the haemocytes types found in Lepidoptera and their generalized functions.

Types	**Descriptions**	**Functions**	**References**
Prohaemocytes	7% of total number of haemocytes, small round cells, 3-7 μm in diameter, found in haemolymph of larva, in adults they are considered as stem cells. They show numerous small (less than 0.25 μm) membrane-bound cytoplasmic inclusions; large central nucleus.	Stem cells, divide to produce plasmatocytes and granular cells.	Beeman *et al.*, 1983; Butt and Shields, 1996; Gupta and Sutherland, 1966; Yamashita and Iwabuchi, 2001; Ling, 2005; Ribeiro and Brehélin, 2006.
Plasmatocytes	35% -50% of total number of haemocytes, spherical or oval cells with a regular shape, sometimes spindle-shaped, 10-20 μm long and 5-10 μm wide, round-spindle shaped in suspension but adhesive, flattened and often motile over substrate both *in vivo* and *in vitro*, agranular, contain lysosomal enzymes, most numerous class of circulating haemocytes.	Phagocytic, involved in encapsulation and nodule formation.	Bréhelin and Zachary, 1986; Pech and Strand, 1995; Butt and Shields, 1996; Zakarian *et al.*, 2002; Neuwirth, 2005; Ribeiro and Brehélin, 2006.

	Golgi complexes, rough endoplasmic reticulum and free ribosomes are abundant; large bilobed nucleus.		
Granular cells	30-50% of total number of haemocytes, round-oval in shape 4-13 μm in diameter, contains abundant cytoplasmic granules. 10-20 μm long and 5-10 μm wide. The granules, their most characteristic feature, have a diameter of 0.2 μm, a microtubular sub-structure, and are made up of acidic mucosubstances; central nucleus (3–6 mm diameter), containing several patches of heterochromatin	Aid in nodule extra cellular matrix formation, activation of plasmatocytes, granules are expelled from cells to aid the capturing of floating bacteria from haemolymph; limited phagocytosis.	Lackie, 1988; Geng and Dunn, 1989; Yokoo *et al*, 1995; Butt and Shields, 1996; Pendland and Boucias, 1996; Falleiros *et al*., 2003; Tojo *et al*., 2000; Neuwirth, 2005; Ribeiro and Brehélin, 2006.

Spherulocytes	5% of total number of haemocytes, 2-4 µm in diameter, oval or round cells with large cytoplasmic inclusions, spherules have a highly ordered substructure and are made up of acidic mucosubstances; small (3–4 mm diameter) centrally located nucleus. They are relatively stable in monolayers.	Process neutral mucopolyssacharides and mucoproteins, fine granular material is released into the haemolymph by the matured cells; possible role in encapsulation and phenoloxidase	Lackie, 1988; Geng and Dunn, 1989; Butt and Shields, 1996; Neuwirth, 2005; Ribeiro and Brehélin, 2006.
Oenocytoids	2% of total haemocytes, are the largest cells, spherical, 8-20 µm in diameter, the cytoplasm contains an eccentric nucleus mostly free ribosomes and microtubules.	Role uncertain but contains prophenoloxidase enzyme; possible coagulation.	Lackie, 1988; Butt and Shields, 1996; Neuwirth, 2005; Ribeiro and Brehélin, 2006.

Innate haemocyte non-self responses

The salient innate antimicrobial responses of the haemocytes consist of phagocytosis, nodulation and encapsulation (Ratcliffe and Rowley, 1987). The efficacy of these responses varies with the species of microorganism and insect species (Gardiner and Strand, 2000).

Phagocytosis

Phagocytosis is the internalization of microorganisms and/or cellular debris by haemocytes (Ling and Yu, 2006). It is considered to be the primary response of haemocytes to small foreign particles and bacteria (Gillespie and Kanost, 1997). The response is a multiple step process that involves recognition of the antigen which maybe generated by microbial metalloproteinases which mediate sensing of invading pathogens (Altincicek *et al.,* 2007), antigen attachment to humoral and cellular pattern recognition receptors, signal transduction, activation of pseudopodia formation, ingestion of the antigen and assembly of phagosomes within the haemocyte (Rohloff *et al*, 1994; Gillespie and Kanost, 1997). Granular cells and plasmatocytes have been reported to be the main phagocytic cells of Lepidoptera larvae *in vitro* (Wago, 1991; Tojo *et al*., 2000).

Phagocytosis can be triggered by haemolymph proteins such as lectins in the dictyopteran, *Blaberus discoidalis* (Wilson *et al*., 1999) and hemolin in *Ma. sexta* (Eleftherianos *et al*., 2007). In Diptera, humoral mediation is more complex, Kurtz and Armitage (2006) reporting that the Immunoglobulin-superfamily protein Down Syndrome Adhesion Molecules (Dscam) in soluble form and the soluble and membrane bound form of hypervariable PRR Down syndrome cell adhesion molecule in the mosquito, *Anopheles gambiae*, AgDscam (Dong *et al*., 2006), binds to and mediates bacterial phagocytosis by haemocytes. *D. melanogaster* immune competent cells has the ability to make more than 1800 isoforms of Dscam which, based on RNA interference-depletion of Dscam, are associated with bacterial phagocytosis, the protein causing bacterial binding (Watson *et al*., 2005). Research on the Down syndrome cell adhesion molecule (Dscam) is to date, limited to dipteran immunity. A transmembrane protein, Eater, has been implicated in phagocytosis in *Drosophilia* by significantly reducing binding and internalization of bacteria after transcriptional silencing of the eater genes in a macrophage cell line (Kocks *et al*., 2005).

The process of phagocytosis involves modulation of the cytoskeleton of both granular cells and plasmatocytes (Diehl-Jones *et al*., 1996). Calcium, depending on the concentration, enhances plasmatocyte attachment (a prelude to phagocytosis) and actual phagocytosis (Tojo *et al*., 2000). Attachment of foreign particles to insect haemocytes is energy-independent, and in *G.mellonella*, may be mediated by opsonic proteins of the prophenoloxidase cascade (Leonard *et al*., 1985; Ratcliffe, 1986; Brookman *et al*., 1988). Calreticulin a ubiquitous 47-60 kDa Ca^{2+} binding protein with multi-functional properties (Michalak *et al*., 1992) is present on the surface of two types of lepidopteran haemocytes and as an early stage encapsulation protein it binds to differently charged sepharose beads (Choi *et al*., 2002). Calreticulin is involved also in immune-related phagocytosis of yeast cells (Choi *et al*., 2002; Asgari and Schmidt, 2003). Asgari and Schmidt (2003) observed that in *G. mellonella*, anti-calreticulin antibodies did not block yeast phagocytosis but did block phagocytosis in *Pieris rapae* suggesting there is a difference in the dependence on Calreticulin in the phagocytosis of yeast cells by haemocytes from different insects. Kuraishi *et al*., (2007) identified calreticulin as the first molecular marker for phagocytosis of apoptotic cells by Drosophila phagocytes by observing that phagocytosis of apoptotic S2 cells of Drosophila haemocyte-derived l(2)mbn cells, was inhibited by the addition of anti-calreticulin antibody, the inhibition observed when the target cells, but not phagocytes, were pre-incubated with the antibody. Unexpectedly a calreticulin-like protein from the ichneumonid parasitoid, *Cotesia rubecula,* inhibits plasmatocyte spreading in *Pieris rapae* (Zhang *et al*., 2006). In *B. mori* calreticulin is not up-regulated by immunogens (Takahashi *et al*., 2006).

Haemocyte non-self responses in Lepidoptera are also mediated by the cytoskeletal microfilaments (Wago, 1982; Davies and Preston, 1987). Ratcliffe and Rowley (1974) observed that both pseudopodial production and cell membrane invagination around *E. coli* by plasmatocytes of *G. mellonella* produced phagosomes within which bacterial cell wall degradation occurred as the bacteria were attacked by β-glucuronidase and β -glucosaminidase contained with the phagosomes (Walters and Ratcliffe, 1983). The internalized or entrapped bacteria are secondarily killed by various killing mechanisms involving reactive oxygen intermediates produced by melanisation in *D. melanogaster* (Carton and Nappi, 1997; Nappi and Vass, 1998). Phagocytosis may occur concertedly with nodule formation (Walters and Ratcliffe, 1983). Adhesion of *E. coli* on *S. littoralis* granular

haemocytes is inhibited with polyinosinic acid, a specific anionic ligand of scavenger receptors, suggesting that these receptors are probably involved in phagocytosis in Lepidoptera (Costa *et al*., 2005).

Observations of the haemocytes of the Mediterranean fruitfly, *Ceratitis capitata,* show that the haemocytes respond to *E. coli* LPS by the activation of the three major mitogen-activated protein kinases subfamilies (1) extracellular signal-related kinase, (2) c-jun N-terminal kinase and (3) p38 in a Ras/Rho-dependent manner (Guha *et al*., 2001; Soldatos *et al*., 2003; Lamprou *et al*., 2005). The activation mitogen-activated protein kinases regulates *E. coli* LPS-dependent release, as well as phagocytosis, in insect haemocytes (Sweet and Hume, 996; Yamamori *et al*., 2000; Soldatos *et al*., 2003; Lamprou *et al*., 2005). Besides the MAPK pathway, the focal adhesion kinase (FAK)/Src pathway is also of central importance in the process of phagocytosis because their activation upon antigenic challenge regulates phagocytosis via haemocyte secretion and activation of the prophenoloxidase cascade (Lamprou, 2007). In *G. mellonella*, type 1 protein kinase A (PKA) inhibition enhanced bacterial phagocytosis both *in vitro* and *in vivo* (Brooks and Dunphy, 2005). Mandato *et al*., (1997) reported that active phospholipase A_2 and cyclooxygenase are required for spreading of and phagocytosis by *G. mellonella* haemocytes. Baines *et al*., (1992) has shown that the biogenic amines 5-hydroxytryptamine and octopamine enhances phagocytosis in *P. americana.* Octopamine contributes to bacterial–haemocyte adhesion in *G. mellonella,* increasing the efficiency of haemocytes in removing microorganisms from circulation (Dunphy and Downer, 1994).

Nodulation

Nodulation is mainly a cellular defense reaction against microbial infections in insects (Phelps *et al*., 2003; Miller, 2005; Buyukguzel *et al*., 2007; Gandhe *et al*., 2007). It is a biphasic response to large numbers of antigens [(LPS, LTA, β-1, 3 glucan, laminarin or selected glycoproteins (Leonard *et al*., 1985; Ratcliffe, 1986; Brookman *et al*., 1988; Lackie, 1988)] in which the antigens adhere to proteins discharged around the granular cells forming on the antigen-coagulum complex which is subsequently walled off by the plasmatocytes (Ratcliffe *et al*., 1985). Granular cells of *Ma. sexta* discharge an extracellular matrix

protein(s) (Nardi *et al*., 2005) which bind(s) to plasmatocytes enhancing adhesiveness while integrin receptors cluster on granular cells increasing their binding avidity to foreign surfaces (Nardi *et al*., 2005). Live or dead microorganisms are entrapped in the matrix protein, the complex binding to other granular cells in *G. mellonella* and the lepidopteran larvae of *Pieris brassicae* (Gagen and Ratcliffe, 1976). Multiple layers of plasmatocytes adhere to the accretion complex forming a nodule. Concomitant with this, in *G. mellonella* is the release of a plasmatocyte-depletion factor, which removes plasmatocytes from the haemolymph, and the activation of the prophenoloxidase cascade system (Dunn, 1986; Boman and Hultmark, 1987). A subpopulation of plasmatocytes in *Ma. sexta* known as hyperphagocytic cells, recognize and attach to large numbers of bacteria and act as nuclei for subsequent nodule formation (Dean *et al*., 2004). Gandhe *et al*., (2007) identified noduler, a protein up-regulated in the haemolymph of the saturniid silkmoth, *Antheraea mylitta*, involved in mediating the nodulation response against bacteria, yeast and haemocytes by specifically binding LPS, lipotechoic acid, and β-1, 3 glucan components of the microbial cell walls. The consequences of nodulation may vary with the microbe species. Giannoulis *et al*., (2007) report that *B. subtilis in vivo* elicit a nodulation-based decline in total haemocyte counts and do not affect haemocyte viability, while dead and live *X. nematophila* elevate haemocyte counts damaging the haemocytes as LPS levels increase and *X. nematophila* emerge into the haemolymph from the few nodules formed.

Live *X. nematophila* are known to impair insect innate cellular immune reactions including nodulation by inhibiting the biosynthesis of eicosanoids (Park and Kim, 2000; Park *et al*., 2003). Major groups of eicosanoids including prostaglandins, epoxyeicosatrienoic acids, and various products of lipoxygenase pathway, mediate cellular and some humoral immune reactions including nodule formation (Phelphs *et al*., 2003; Stanley, 2006) in numerous insect species and orders to bacterial infections (Miller *et al*., 1994, 1996, 1999; Jurenka *et al*., 1997; Morishima *et al*., 1997; Stanley, 2000; Park and Stanley, 2006). Treating infected larvae of the moth *S. exigua* with arachidonic acid reduces the mortality caused by *X. nematophila* whereas pharmaceutical inhibitors especially for PLA_2 exacerbate the lethality of *X. nematophila* infection (Park and Kim, 2000). The ability of haemocytes to spread and nodulate foreign particles decreases also when haemocytes are incubated with phospholipase A_2 and cycloxygenase inhibitors as observed in larval wax moths, *G.*

mellonella (Mandato *et al*., 1997). Cyclooxygenase products, prostaglandins, mediate nodulation response also to viral infection in *G. mellonella* (Buyukguzel *et al.,* 2007). Eicosanoids mediate nodulation reactions to challenge by protozoans in *Rhodnius prolixus* (Garcia *et al*., 2004) and in *Ma*. *sexta* (Stanley, 2005). Prostaglandins but not lipoxygenase products mediate haemocyte microaggregation reactions (another step in nodulation) to bacterial challenge (Miller and Stanley, 2001; Phelps *et al*., 2003; Miller and Stanley, 2004). Eicosanoids contribution to entomopathogenic fungal nodulation may vary with fungal stage and/or species, *Beauveria bassiana* blastospores being nodulated by *Ma. sexta* haemocytes using lipoxygenase pathway in *Ma. sexta* (Lord *et al*., 2002) whereas conidia of *Metarhizium anisopliae* in the same insect are nodulated by haemocytes using cycloxygenase products (Dean *et al*., 2002).

There are no publications of nematodes interacting directly with the eicosanoids metabolism of insect haemocytes. In the case of the Steinermatid-bacteria complex, the bacteria may assist the nematodes by inhibiting the eicosanoid pathways of the haemocytes, but this can only occur after the bacteria are released and not during initial nematode interaction with the defences.

Encapsulation

Encapsulation refers to the binding of usually melanizing granular cells to large targets like parasitoids, nematodes and chromatographic beads, materials larger than the haemocytes (Peters and Ehlers, 1997; Hernadez-Martinez *et al*., 2002; Pech and Strand, 2002), which are subsequently covered by an overlapping sheath of granular cells and plasmatocytes around a target (Lavine and Strand, 2002). In insects, two types of encapsulation are distinguished (1) cellular encapsulation which occurs mainly in insects with large numbers of haemocytes i.e. lepidopteran and (2) melanotic (humoral) encapsulation found in insects with low number of haemocytes, i.e. most of the *Diptera* (Nappi *et al*., 1995; Strand and Pech, 1995). In both cases, adhesion molecules are essential to the capsule formation. Ling and Yu (2006) report that plasma components like lectins are responsible for haemocyte aggregation around foreign entities.

Encapsulation in *G. mellonella* is a biphasic process similar to nodule formation where phase one is modified clot formation resulting from granular cell lysis, and phase two, consists of plasmatocyte attachment to the non-self material resulting in capsule formation (Walters and Ratcliffe, 1983). Lavine and Strand (2002) observed that in lepidopterans like *P. includens*, binding of granular cells or humoral PRRs to a foreign surface induces a rapid change in the adhesive state of plasmatocytes which are the main capsule-forming cells. The haemocytic encapsulation response of the moth *P. includens* was found to involve an RGD (Arg-Gly-Asp)-dependent cell adhesion mechanism (Pech and Strand, 1995). Multiple subunits of RGD-dependent α- and β-integrin receptors with differential expression on the granular cells and plasmatocytes of *P. includens* participate in haemocyte adhesion (Lavine and Strand, 2003). All plasmatocytes of *Ma. sexta* express a β-integrin detected by monoclonal antibody MS13 (Levin *et al*., 2005), but only a few have the cell adhesion molecule neuroglian which is essential for plasmatocyte encapsulation (Nardi *et al*., 2006). Integrin-β1 is unique to haemocytes since its mRNA, based by Northern analysis, occurs in haemocytes and not in other tissues of *Ma. sexta* (Levin *et al*., 2005). Of the three known α subunits associated with haemocyte-specific integrin, the α1 subunit is the predominant subunit of haemocytes (Levin *et al*., 2005). The α2 subunit is mainly expressed in epidermis and Malphigian tubules, whereas the α3 subunit is primarily expressed on haemocytes and fat body cells (Zhuang *et al*., 2008). Zhuang *et al*., (2008) further observed that of these three, only the α2 subunit is related to integrins containing RGD-binding motifs. However, all three α-integrin subunits participate in encapsulation because they are expressed on haemocytes during encapsulation, and exposure of haemocytes *in situ* to dsRNAs (disrupting expression of each of these three α subunits) disrupts also encapsulation. In lepidopteran larvae, granular cell contact of a foreign particle releases sticky and chemotactic components that attract plasmatocytes which subsequently form a multicellar sheet several layers thick (Götz, 1986; Pech and Strand, 2000).

Cellular encapsulation and melanization of agarose beads is enhanced in *Ma. sexta* when the beads are coated with immulectins (Yu and Kanost, 2004; Ling and Yu, 2006). However, immulectin-1 did not cause melanization but enhanced encapsulation (Ling and Yu, 2006). Yu and kanost (2004) also report that coating of the agarose beads with recombinant carboxyl-terminal carbohydrate-recognition domain (CRD2-II) of immunlectin-

2 enhanced encapsulation of the beads *in vitro* by haemocytes and melanization of the beads *in vivo* in *Ma. sexta*. Inhibitors of serine proteinases (e.g. para-aminophenylmethylsulfonyl fluoride) inhibit cellular encapsulation of immulectin-coated agarose beads (Ling and Yu, 2006), suggesting that humoral pattern recognition receptors may enhance cellular encapsulation and melanization by activating phenoloxidase.

Phenoloxidase associated with melanization may be directly released onto the alien from haemocytes or indirectly released from oenocytoids into plasma and then contact non-self matter (Yoshida and Ashida, 1986). Melanization is triggered by nematodes (Götz, 1986), β-1, 3-glucans (Leonard and Ratcliffe, 1985; Gunnarsson, 1988), bacterial capsules (Götz and Vey, 1986), peptidoglycan fragments, techoic acids and anionic polydextran (Götz, 1986; Yoshia and Ashida, 1986) and LPS (Bréhelin *et al*., 1989) In *B. mori* and *Chironomus spp*., melanization is initiated by the conversion of prophenoloxidase to phenoloxidase by means of calcium dependent serine protease(s) (Götz, 1986; Yoshida and Ashida, 1986).

In *P. includens*, capsule formation ended when a monolayer of granular cells attaches to the outermost plasmatocyte layer (Pech and Strand, 2000). The granular cells became apoptotic due to the outer plasmatocytes releasing unidentified factors ending capsule development (Pech and Strand, 2000).

Induced Immunity

Microbial antigens such as LPS, β-1, 3 glucan and intact bacteria and fungi, elicit *de novo* synthesis of antimicrobial proteins (Hoffman *et al.,*1996), representing a class composed of approximately 150 types of protein synthesized mainly in the fat body, and some in the haemocytes and epidermis and released into the haemolymph (Lamberty *et al*., 1999). The types of antimicrobial peptides produced vary with the antigens, insect tissue, insect species and life cycle of the insect (Lamberty *et al*., 1999). Table 2 shows the major antibacterial peptides within the haemolymph of lepidopterans and their putative/actual functions. The structural genes and their control have been elucidated (Cheng *et al*., 2006), including those in haemocytes (Lavine *et al*., 2005).

Table 1. 2. Antimicrobial peptides induced in Lepidoptera in response to antigenic stimulation

Peptide family	Examples	Characteristics	Activity spectrum	References
Cecropin	Cecropin A from *Hyalophora cecropia* Cecropin A,B,C,D from *B. mori*	Basic cationic peptides; approximately 4 kDa, heat-stable, synthesized in fat body, testis, haemocytes, gut epidermis; bactericidal by forming pores in bacteria; genes sequenced and cloned.	Mainly Gram-negative bacteria, some Gram-positive, some fungicidal activity except against entomopathogenic *B. bassiana.*	Ekengren and Hultmark, 1999; Yamakawa and Tanaka, 1999; Gillespie *et al.*, 1997; Hetru *et al*, 1998; Bulet and Stocklin, 2005; Cheng *et al*., 2006.
Attacin	Attacin E and F from *H. cecropia* Attacin B from *B. mori*	Acid, basic and neutral. Approximately 20-28 kDa, heat stable, synthesized in same tissues as cecropins; bacteriostatic, inhibiting protein synthesis by inhibiting gene transcription which impairs bacterial cell division.	Gram-negative bacteria.	Bulet, 1999; Hultmark *et al*, 1983; Cheng *et al*., 2006; Engstrom *et al.*, 1984

Defensin	Gallerimycin from *G. mellonella* and *S. frugiperda* Heliomycin from *Heliothis virescens* Spodoptericin from *S. frugiperda*	Cationic-cysteine-rich; approximately 5.4 kDa, similar to drosomycin	Gram-positive bacteria and filamentous fungi	Lamberty *et al*., 1999; Schuhmann *et al.*, 2003; Volkoff *et al.*, 2003.
Gloverins	Gloverin from *H. cecropia, H. armigera* and *B. mori*	Basic, heat-stable protein containing a large number of glycine residues but no cysteine; synthesized in the fat body, midgut, testis, silk gland and ovaries	Interacts with lipopolysaccharides and specifically inhibits the formation of the outer membrane of Gram-negative bacteria, leading to increased permeability of the outer membrane	Axen *et al*., 1997; Cheng *et al*., 2006

Moricin	Moricin from *S. litura, Ma. sexta, G. mellonella* and *B. mori.*	Highly alkaline peptide, synthesized in fat body, testis and haemocytes, contains a high level of glycine residues, heat stable.	Active against both Gram-negative and Gram-positive bacteria; affect structural integrity of bacterial plasma membrane. Four of this family from *G. mellonella* are fungicidal	Hara and Yamakawa, 1995, 1996; Furukawa *et al*., 1999; Oizumi *et al*., 2005. Dai *et al*., 2008.
Viresin	Viresin from *H. virescens*	12 kDa antibacterial protein, showed no amino acid sequence homology with any antibacterial protein	Shows antibacterial actions against Gram-negative bacteria including *E. cloacae* but not Gram-positive.	Chung and Ourth, 2000.
Lebocin	Lebocin from *B. mori and Trichoplusia ni*.	A proline-rich protein, synthesized in the haemocytes and fat body	Acts against Gram-negative bacteria and non pathogenic *E. coli;* When glycosylated or exists together with cecropin D, its antibacterial activity becomes much higher	Hara and Yamakawa, 1995; Furukawa *et al*., 1997; Liu *et al*., 2000

Recognition of Non-self in Innate Immunity in Lepidoptera

The recognition of any invading organism as foreign is the first critical step in any immune response. Once such recognition has taken place, it may trigger a protective response involving a combination of haemocytes and plasma proteins (Yu *et al*., 2002; Ma *et al*., 2006). Innate immune systems in both mammals and arthropods including insects utilize soluble proteins known as pattern recognition proteins or receptors, such as apolipophorin-III (Dettloff *et al*., 2001, Halwani *et al*., 2001); β-1, 3-glucan binding proteins in *G. mellonella* (Matha *et al.*, 1990), *B. mori* (Ochiai *et al*., 1992), *Ma. sexta* (Yu *et al*., 2002) (see section Insect Immunity: Plasma for additional examples). Pattern recognition proteins lack the binding specificity of antibodies, and instead function by binding to classes of polysaccharides, such as LPS from Gram-negative bacteria, LTA and peptidoglycan from Gram-positive bacteria, β-1, 3-glucan and mannan from yeast and double stranded RNA from viruses (Hoffmann *et al*., 1999; Aderem and Ulevitch, 2000; Yu *et al*., 2002). Binding of pattern recognition proteins to these molecular patterns trigger responses such as phagocytosis, nodule formation, encapsulation, activation of protein cascades, and synthesis of antimicrobial peptides (Gillespie *et al*., 1997; Ashida and Brey, 1998; Bulet *et al*., 1999; Yu *et al*., 2002).

Constitutive lectins present in the plasma and haemocytes have an opsonic role in several insect species and maybe part of the recognition system (Richards and Ratcliffe, 1990) although the evidence is not unequivocal for all insect species (Rowley *et al*., 1986). The lectins enhancing bacterial attachment to haemocytes may act as bridging molecules between antigen and haemocyte or promote the activation of the prophenoloxidase system. Morton *et al*., (1987) established the existence of carbohydrate receptors on the plasmatocytes and granulocytes of *G. mellonella*, thus suggesting that recognition of non-self material(s) by the haemocytes may occur independently of plasma factors. Also, pattern recognition receptors found on the surfaces of haemocytes may bind directly to microorganism and pathogen-associated molecular pattern such as LPS, LTA and β-1, 3-glucan in Lepidoptera (Ohta *et al*., 2006), Diptera (Ramet *et al*., 2001). PGRP-LC receptors found on S2 cell lines of Diptera bind to LPS and peptidoglycan (Werner *et al*., 2003; Kaneto

et al., 2004). *B. mori* C-type lectins are the first pattern recognition receptors on lepidopteran haemocytes which recognizes a variety of pathogen-associated molecular patterns leading to the induction of nodule formation (Ohta *et al*., 2006).

Some classes of humoral proteins are known to bind directly to Gram-negative bacteria in insects and serve as recognition molecules; two of such are the Gram-negative-binding protein (GNBP) and C-type lectins (Jomori and Natori, 1991; Lee *et al*., 1996; Yu and Ma, 2006). However, since Gram-positive bacteria lack LPS, other recognition molecules including immulectin-2 (Yu and Ma, 2006) must be used for their recognition. Another candidate is the peptidoglycan recognition protein, PGRP (Kang *et al*., 1998; Ochiai and Ashida, 1999). In many insects, they induce a strong antibacterial response, and trigger the activation of phenoloxidase, leading to melanin formation in infected wounds. Humoral PGRPs with a high affinity for peptidoglycan (Liu *et al*., 2000) have been isolated in the fat body and haemocytes (Kang *et al*., 1998; Ochiai and Ashida, 1999) of the moths *Trichoplusia ni* (Kang *et al*., 1998) and *B. mori* (Yoshida *et al*., 1996; Ochiai and Ashida, 1999). The *B. mori* PGRP is required for the activation of the phenoloxidase cascade (Yoshida *et al*., 1996). Insects have multiple PGRP genes that are classified into short (S) and long (L) transcripts and are often alternatively spliced into up to 19 different types proteins (Dziarski, 2003; Steiner, 2004). The short forms are present in the haemolymph, cuticle, and fat-body cells, and sometimes in epidermal cells in the gut and haemocytes, whereas the long forms are mainly expressed in haemocytes (Dziarski and Gupta, 2006). The long form encodes intracellular and membrane-spanning proteins (Werner *et al*., 2000). Silkworm (*B. mori*) and mealworm (*T. molitor*) PGRP-S, present in the haemolymph and cuticle, binds bacteria by lysine- and diaminopimelic acid-peptidoglycan components activating the prophenoloxidase cascade (Yoshida *et al*., 1996; Park *et al*., 2006), and expresses activity through the Toll-like pathway (see signal transduction) whereas PGRP-LC which recognize diaminopimelic acid-type PGN, produced by Gram-negative bacteria (Kaneko, 2005) express activity through *imd* pathway (see signal transduction) (Michel *et al*., 2001; Choe *et al*., 2002).

Signal transduction

Most research on signal transduction in insect is in Diptera especially pertaining to the induction of antimicrobial proteins in fruit fly, *D. melanosgaster*, herein focus will be on Diptera unless otherwise stated.

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. Innate immune recognition is mediated by a system of germline-encoded receptors that are coupled to signal transduction pathways that control expression of a variety of inducible immune-response genes (Kopp and Medzhitov, 1999). Rutschmann *et al.,* (2002) has shown that the Toll pathway is involved in the anti-Gram-positive bacterial and antifungal (Rutschmann *et al.,* 2002) response. A secreted factor pro-Spätzle is activated through cleavage by a so far unidentified serine protease in response to invasion by fungi and Gram-positive bacteria. The stimulation of Toll by Spätzle activates the cytoplasmic serine/threonine kinase Pelle via the adaptor protein DmMyD88 and Tube (Hoffmann and Reichhart, 2002). Activation of Pelle promotes degradation of the ankyrin-repeat protein, Cactus, which associates with the Rel-type transcription factor, Dorsal and Dorsal-related immune factor (DIF), in the cytoplasm. Once Cactus is degraded in response to the Toll-mediated signal, Dorsal and DIF translocate into the nucleus, where they induce the transcription of specific target genes (Hoffmann and Reichhart, 2002). A category of the petidoglycan recognition proteins, PGRP-SA, is essential for the activation of Toll during Gram-positive bacterial infections (Michel *et al*., 2001). Toll-dependent activation by Gram-positive bacteria requires the cooperation of at least two distinct pattern recognition proteins, Gram-negative binding protein 1 (GNBP1) and PGRP-SA (Gobert *et al.*, 2003). In fungal infections, a serine protease that is encoded by the *persephone* gene, is required for activation of Toll (Ligoxygakis *et al*., 2002). Ligoxygakis *et al*., (2002) show that ethylmethane sulfonate-induced mutations in the *persephone* gene, blocks induction of the Toll pathway by fungi and lessens resistance to this type of infection. Another category of the PGRP family, PGRP-LC, has been shown to be involved in the activation of the *Imd* pathway (Choe *et al*.,

2002; Gottar *et al*., 2002; Ramet *et al*., 2002), involved in the induction of apoptosis as well as the response against Gram-negative bacteria.

Biochemical and molecular evidence for the possible existence of an extracellular proteinase cascade, a Spätzle-like cytokine and its receptor, and an intracellular signaling pathway leading to upregulated transcription of immunity-related genes is known for *B. mori* and *Ma. sexta* (Wang *et al*., 2007). Their findings suggest that active Spätzle is required for the induced production of antimicrobial peptides in *B. mori* and *Ma. sexta.*

The Drosophila Toll pathway has been shown to be involved in cellular innate immunity by regulating the serine protease inhibitor Serpin 27A (Spn27A), which normally functions as a negative regulator of phenoloxidase (Nappi *et al*., 2005). Introduction of this serine protease inhibitor (Spn27A) into normally highly immune competent *D. melanogaster* larvae significantly reduces their ability to form melanotic capsules around eggs of the hymenopteran parasitoid, *Leptopilina boulardi*, confirming the role of Spn27A in the melanization cascade (Nappi *et al*., 2005). Cloning and characterization of a gene encoding *Ma. sexta* Toll receptor from the haemocyte suggests that its presence in haemocytes is in response to microbial infection, particularly to *E. coli* (Ao *et al.,* 2008).

Signal transduction mechanisms within insects are regulated by protein kinase C (PKC) through phosphorylation (Mellor and Paker, 1998), and involved in apoptosis in fat body cell lines of *L. dispar* (Malagoli *et al*., 2002), cell proliferation and tissue differentiation (Huttere *et al.*, 2004) and secretion (Tobe *et al*., 2005). PKC induces antibacterial proteins (Lanz-Mendoza *et al*., 1996) and influences haemocyte adhesion (Zakarian *et al*., 2003). Protein kinase C affects the phagocytosis of particulates by mbn-2, an established culture of malignant haemocytes of the fruitfly, *Drosophila melanogaster* (Lanz-Mendoza *et al*., 1996). Zakarian *et al*., (2003) showed that isoforms of active protein kinase C modulated by transiently elevating intracellular calcium levels limits the adhesion of larval *G. mellonella* plasmatocytes and granular cells.

Protein kinase A, which has been implicated in the regulation of antimicrobial peptide synthesis in insects, consist of a catalytic domain (Muller, 1999), which upon phosphorylation turns the PKA complex into an active form (Kim *et al*., 2006). This enzyme

is one of the necessary factors for the LPS-induced activation of cecropin B genes in isolated *B. mori* haemocytes (Taniai *et al.,* 1996). In addition, the expression of the gene induced by LPS or lipid A is inhibited by H89, a generalized PKA inhibitor (Shimabukuro *et al.,* 1996; Taniai *et al.,* 1996). The gene expression being triggered by dibutyryl cAMP, a cell-permeable cAMP analog (Shimabukuro *et al.,* 1996). Yoshihara *et al* (2000), observed that cyclic adenosine monophosphate (cAMP) with the insect cells act as an activator to PKA. PKA activity has been demonstrated in the fat body of *G. mellonella* larvae eliciting humoral responses of the insect to bacterial LPS (Cytryńska *et al.,* 2006). The PKA activity was increased about 2.5-fold and 1.5-fold, after *M. lysodeikticus* and *E. coli* injection, respectively in *G. mellonella* (Cytryńska *et al.,* 2007). Active PKA has been demonstrated to limit the haemocyte response of *G. mellonella* against *X. nematophila* and *B. subtilis in vitro* and *in vivo* (Brooks and Dunphy, 2005) with possible mediation by cAMP (Marin *et al*., 2005).

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CHAPTER 2

Metabolites of *Steinernema carpocapsae* DD136 limit the non-self adhesion responses of haemocytes of two lepidopteran larvae, *Galleria mellonella* (F. Pyralidae) and *Malacosoma disstria* (F. Lasiocampidae)

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Abstract

Live adult and juvenile entomopathogenic *Steinernema carpocapsae* DD136 (P. Nematoda) did not have haemocytes from lepidopteran insect larvae of *Galleria mellonella* or *Malacosoma disstria* adhering to their cuticular surfaces *in vitro* or *in vivo*. *In vitro* freeze-killed nematodes exhibited haemocyte attachment, the intensity increasing with time. Accumulation of haemocytes on the dead nematodes was associated with host phenoloxidase activity; live nematodes and their exudates did not activate the enzyme whereas dead nematodes but not their exudate did activate phenoloxidase. Live-nematode exudate inhibited granular cell and some plasmatocyte adhesion to slides, increased granular cell but not plasmatocyte dissociation from preformed haemocyte monolayers and *in vivo* elevated total haemocyte counts and changed the floating haemocyte types while impairing bacterial removal from the haemolymph. Dead-nematode exudate did not affect these parameters thus immunosuppressant activity by live nematodes may represent the release of inhibitors not associated with their cuticle. The third stage juveniles released the inhibitors.

Index descriptors: *Steinernema carpocapsae, Galleria mellonella, Malacosoma disstria,* insect, inhibition haemocytes, adhesion, phenoloxidase, nematode encapsulation

Introduction

The insect pathogenic nematode-bacterium complex, *Steinernema carpocapsae* (F. Steinernematidae)-*Xenorhabdus nematophila* (F. Enterobacteriaceae) controls economically important pest insects (Georgis and Gaugler, 1991). As part of the infection mechanism, once the infective juveniles pass through the host orifices (Nguyen and Smart, 1990) and the alimentary tract (Poinar and Himsworth, 1967), they encounter the haemolymph before voiding the phase one form of *X. nematophila* (located on the intravesicular element) from its intestinal vesicle (Martens and Goodrich-Blair, 2005). However, prior to bacterial release, which takes 4-5 h post-parasitism (Yokoo *et al*., 1995; Wang *et al*., 1995; Dunphy and Webster, 1988), the nematodes encounter the humoral and cellular non-self recognition factors of the haemolymph as they rapidly develop from infective juvenile into third stage nematodes (Poinar and Himsworth, 1967). Major humoral factors in lepidopteran larvae include the antimicrobial melanizing enzyme, phenoloxidase (Shelby and Popham, 2006), the pattern recognition proteins lysozyme (Wilson and Ratcliffe, 2000), immulectins and other C-type lectins, β-1,3-glucan binding proteins, hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002), apolipophorin-III (Halwani *et al*., 2000) and the lipopolysaccharide-inactivating lipophorin particle (Ma *et al*., 2006). The lipophorin particle composed of apolipophorin I and II, is affiliated with many of the aforementioned humoral factors and participates in cell-free immunity producing molecular cages around soluble toxic antigens limiting their effects (Rahman *et al*., 2006). Many of these humoral factors elicit binding of the haemocytes to foreign materials (Schmidt and Schreiber, 2006).

Cellular immune responses are the domain of predominantly the haemocyte types, the granular cells and plasmatocytes. Either haemocyte type, depending on the insect species and antigen species and concentration (Howard *et al*., 1998) participates in phagocytosis (Tojo *et al*., 2000) and nodule formation and encapsulation (Ratcliffe *et al*., 1985). Nodulation in lepidopteran larvae is a complex of activities initiated by the granular cells (Ratcliffe and Gagen, 1977; Schmidt and Ratcliffe, 1977) and possibly the haemocyte type the spherulocytes (Cook *et al.*, 1985), discharging extracellular matrix proteins. Granular cell matrix proteins entrap foreign

matter as the clustering adhesion receptors on the haemocytes increase in number (Nardi *et al*., 2005). Some of the matrix proteins contact receptors on the plasmatocytes inducing these haemocytes to become adhesive (Nardi *et al*., 2005). The latter haemocyte type walls off the granular cell-antigen aggregate forming a nodule within which toxic melanization products form. Encapsulation is much like nodulation except the antigen is too large for phagocytosis (Levine and Strand, 2003). RGD-binding integrin receptors participate in lepidopteran encapsulation (Lavine and Strand, 2003; Levin *et al.*, 2005; Zhuang *et al*., 2008) and RGDS-fibronectin receptors affect phagocytosis of the yeast, *Saccharomyces cerevisae* by *G. mellonella* plasmatocytes (Witter and Wiesner, 1996). In Lepidoptera neither granular cells nor plasmatocytes may elicit encapsulation when isolated as described for *Pseudelatia includens* (Pech and Strand, 1995) but, in concert with plasmatocytes with neuroglian, both haemocyte types may aggregate (Nardi *et al*., 2006).

Steinernematids in an insect species in which they do not develop are often encapsulated. Phenoloxidase, with eicosanoid mediation (Shrestha and Kim, 2008) released from the lepidopteran haemocyte type, the oenocytoids, (Yu and Kanost, 2004; Ling and Yu, 2005) produce toxic radicals and melanin by-products (Shelby and Popham, 2006) about the nematode cuticle (Wang *et al*., 1994). The fate of these steinernematids and numerous host insect species is known. Encapsulation of *S. carpocapsae* in the Japanese beetle, *Popillia japonica*, and the house cricket, *Acheta domestica*, does not preclude the demise of the insects, bacterial release from the infective juveniles occurring before capsule completion (Wang *et al*., 1994). Limited haemocytic encapsulation of *S. carpocapsae* in the Colorado potato beetle, *Leptinotarsa decemlineata*, occurs with increasing nematode burden circumventing encapsulation efficiency increasing insect mortality (Thurston *et al*., 1994). *Steinernema glaseri* and *Steinernema scapterisci* evade cellular immune responses in *P. japonica* and *A. domestica* producing rapid host death (Wang *et al*., 1994). The specificity of *S. glaseri*, and *S. scarabaei*, like the aforementioned complexes, is associated with either the propensity of the nematodes to avoid non-self recognition by the host immune systems or their ability to escape melanotic encapsulation in larvae of numerous insect orders; this ability varies with the nematode strain, host species and host interaction with environmental factors (Li *et al*., 2007).

In hosts where there is no nematode encapsulation, the nematode may be recognized as self, avoids non-self recognition or the host immune system is impaired (Dunphy and Webster, 1987; Brehelin *et al*., 1994; Mastore and Brivio, 2008). Short-term exposure of larval *G. mellonella* haemolymph to axenic *S. carpocapsae* DD136 *in vitro* does not elicit nematode encapsulation, or inhibit the binding of the bacterium, *Bacillus subtilis*, to haemocytes (Dunphy and Webster, 1985). *S. feltiae,* although impairing phenoloxidase (Brivio *et al.,* 2002, 2004), does not block cellular encapsulation of the free-living nematode, *Panagrolaimus rigidus* in *G. mellonella* (Mastore and Brivio, 2008). In the short term, *S. carpocapsae* does not impair phenoloxidase or lysozyme and *in vivo* the nematodes do not alter bacterial removal from the haemolymph (Dunphy and Webster, 1985). Both studies imply neither nematode species releases cytotoxic factors during the early stages of host infection. Prolonged incubation of *S. carpocapsae in vivo* alters the total and types of haemocytes (Dunphy and Webster, 1985) implying either nematode integumental components and/or on-going metabolism influences the haemocytes in the later stages of infection as the nematodes develop into the J_3 nematode stage.

Cuticular components of steinernematids affect non-self responses of insects. Sodium dodecyl sulphate extractions of cuticles from live and dead *S. feltia* limit haemolymph protease activity and the activation of prophenoloxidase in *G. mellonella* by lipopolysaccharides from *Escherichia coli* 055:B5 and *Salmonella minnesota* (Brivio *et al*., 2002). *S. feltiae* cuticle and lipid extracts with antigenic similarities to bacterial lipopolysaccharides, inhibit normal melanotic encapsulation of the free-living nematode, *Panagrolaimus rigidus* in *G. mellonella* by removing host-interacting proteins (Brivio *et al*., 2004). These lipids also impair bacterial removal from the haemolymph (Brivio *et al*., 2006). A surface coat protein, SCP3a, from axenic *S. glaseri* inhibits the encapsulation of *Heterorhabditis bacteriophora* and phagocytosis of latex beads by haemocytes of *P. japonica* (Wang and Gaugler, 1999). However, these proteins do not inhibit immunity in all insect species: surface coat proteins of *S. glaseri* NC do not impair nematocidal melanotic encapsulation of *H. bacteriophora* in the tobacco hornworm, *Manduca sexta*, but do in the oriental beetle, *Exomala orientalis* (Li *et al*., 2007).

Metabolism by axenic *S. carpocapsae* produces toxins that destroy induced bactericidal cecropins of diapausing *Hyalophora cecropia* pupae (Götz *et al*., 1981) and impair

G. mellonella antimicrobial defenses by unknown mechanisms (Boemare *et al*., 1982). The toxins, postulated to be proteases, kill the insects (Laumond *et al*., 1989). Differences in virulence between two strains of *S. carpocapsae* for *G. mellonella* in nematode excretion/secretions in haemolymph-supplemented Tyrode's solution are attributed to enhanced proteolytic activity and N-acetyl-D-glucosaminidase but their effect on immunity is uncertain with both strains not being encapsulated (Simões *et al*., 2000).

Haemocyte non-self response studies may involve analysis of haemocytes adhering to glass slides *in vitro* (Zakarian *et al*., 2002) and the removal of selected bacterial species, *B. subtilis* and *X. nematophila*, from the haemolymph *in vivo* (Giannoulis *et al*., 2007). Herein, the effects of live and freeze-killed axenic *S. carpocapsae* DD136 and their phosphate-buffered saline extracts on the immediate adhesion of haemocytes to slides and bacterial removal from the haemolymph *in vivo* by nodulation were determined using two pest insects, the greater wax moth, *G. mellonella* and the forest tent caterpillar, *Malacosoma disstria*. Emphasis was placed on immunosuppression of the haemocytes of *G. mellonella* unless otherwise stated. This is the first report of steinernematid metabolites, as opposed to cuticular components, inhibiting phenoloxidase activation and haemocyte adhesion responses. Such inhibition is considered as immunosuppression.

Materials and Methods

Nematodes

Monoxenic cultures of *S. carpocapsae* DD136 (Agriculture Canada, St. Jean sur le Richlieu, Canada) were established using dauer juveniles collected from parasitized *G. mellonella* larvae (Poinar and Himsworth, 1967) on White's water traps (White, 1927). Briefly, infective juveniles were gravity-washed three times in sterile distilled water (10 ml) in 15 ml sterile conical centrifuge tubes, disinfected in thimersol (10 ml, 0.4% w/v distilled water) for 20 min and aseptically gravity-washed in sterile, distilled water (Dunphy and Webster, 1987) before plating on Wouts' lipid agar (Wouts, 1981). Prior to inoculating with nematodes, Wouts' lipid agar had been inoculated with the phase one form of *X. nematophila* [(the form isolated from the infective juvenile (Akhurst, 1980)] and incubated in darkness at 27°C for 24 h producing a bacterial lawn upon which the pathogens feed. The nematodes were subcultured every two weeks to fresh bacterial lawns.

Axenic nematodes were obtained from eggs by dissolving gravity-washed gravid females in 10 ml axenizing solution [0.4 M NaOH, 0.2 M $NaHClO_4$ (Popiel *et al*., 1989)] for 10 min. The suspension was centrifuged (1000 x g, 1 min, 25°C) in 1.5 ml sterile microcentrifuge tubes and the pellet resuspended in fresh agenizing solution (1 ml) for 10 min, after which only nematode eggs remained. The eggs, rendered free of this solution by centrifugation (500 x g, 25°C, 10 min) and resuspension three times in 1 ml of filter-sterilized PBS [135 mM NaCl, 2.7 mM KCl, 8.0 mM H_2PO_4, 1.5 mM KH_2PO_4, adjusted with 1 M HCl to pH 6.5 (Giannoulis *et al*., 2007)], were allowed to hatch in PBS (5 ml) for 16 h at 25°C (Popiel *et al*., 1989). The resulting juveniles were aseptically washed by centrifugation (hereafter described as centrifuge-washing) in sterile distilled water, and then placed on sterile liver agar (Dunphy and Webster, 1986) supplemented with filter-sterilized antibiotics [100 µg/ml medium, gentamycin sulphate; 100 µg/ml medium, streptomycin sulphate and 20 µg/ml medium, kanamycin sulphate (Wang and Gaugler, 1999)]. Cultures were kept at 25°C and subcultured to fresh antibiotic-supplemented liver agar every three weeks.

The bacterial free status of nematodes was confirmed by incubating the pathogens in Luria broth (5 ml) in 25 ml scintillation vials on a horizontal gyratory shaker (250 rpm) for 72 h at 25°C and 30°C after which 10 µl aliquots were plated on Luria agar and incubated at 25°C and 30°C for 72 h. These temperatures were selected to increase the chances of detecting isolates of *X. nematophila* and other possible bacterial species with different temperature preferences surviving axenizing protocols. The absence of a change in broth turbidity (measured spectrophotometrically at 660 nm) and colony formation on the agar medium indicated axenic nematodes.

Axenic nematode excretion/secretion product (exudate)

Live and freeze-killed (-20°C, 24 h) mixed stage nematodes (J_{1+2} = 100, J_3 = 580, J_4 = 120, adults = 45 females and 65 males) previously rinsed from liver agar were used to obtain excretion/secretion metabolites (exudate). The axenic nematodes were centrifuge-washed twice in sterile distilled water (1 ml) with a final resuspension to 1000 nematodes per ml of PBS. After incubating 5 ml of suspension in 25 ml sterile beakers (capped with sterile paraffin) at 25°C on a horizontal gyratory shaker (100 rpm) for designated times (0.5-24 h), the nematodes were removed by centrifugation (1000 x g, 2 min, 25°C) and the resulting exudate frozen (-80°C) until used. Exudate sterility was based on the absence of change in turbidity of Luria broth (5 ml) in 20 ml scintillation vials that had been inoculated with exudate (20 µL) and incubated for 72 h at 25°C and 30°C as previously described.

Insects and bacteria

G. mellonella larvae were reared on a multigrain diet supplemented with glycerol and vitamins at 30°C (Dutky *et al*., 1962) under constant light. Fifth instar larvae weighing 200 ± 10 mg were used. Laboratory reared *M. disstria* (supplied by the Canadian Forestry Service, Sault Ste. Marie, Ontario) were maintained on a casein dextrose diet at 25°C (Addy, 1969). Seventh instar larvae, 3 days into the stadium and weighing 550 ± 30 mg (Etilé and Despland, 2008), were used.

Gram-negative, entomopathogenic, *Xenorhabdus nematophila* (F. Enterobacteriaceae, ATCC strain 19061), in the phase one form (Akhurst, 1980) were cultured on Luria agar supplemented with triphenyltetrazolium chloride (4 mg/ml) and bromthymol blue (2.5 mg/ml). Non-pathogenic *Bacillus subtilis* (F. Bacillaceae, Boreal Biological Co., St. Catherines, Ontario) were grown on Luria agar. Both bacterial species were subcultured every fortnight and kept in darkness at 25°C.

For experimental purposes both bacterial species were grown in Luria broth (10 ml) in 25 ml scintillation vials at 25°C on a horizontal gyratory shaker (250 rpm) until the cells reached the midlog growth stage (optical density of 0.6 at 660 nm). Bacteria were washed three times by centrifugation (12,000 x g, 2 min, 20°C) and resuspension of the pellet in PBS (1 ml). The bacteria were killed by ultraviolet irradiation for 2 h, stored at 5°C over night and centrifuge (12, 000 x g, 2 min, 20^{o}C) -washed in PBS (1 ml). The use of dead, washed bacteria removed haemocyte-activating formyl peptides (Alavo and Dunphy, 2004), detectable intracellular proteases [one of which from *X. nematophila* may be an immunosuppressant (Caldas *et al*., 2002)], eliminated from *X. nematophila* water-soluble metabolites that elicit apoptosis (Wang *et al*., 1994; Cho and Kim, 2004) and precluded inhibition of nodulation (Park *et al.,*2003). Bacterial death was confirmed based on the absence of both a change in optical density of Luria broth (10 ml) inoculated with bacteria (20 µl) and colony formation from aliquots (10 µl) plated on Luria agar and incubated for 72 h at 25°C and 30^{o}C.

In vitro interaction of nematodes and exudate with haemocytes

Initial interaction of live and dead nematodes with larval haemocytes was described using an antigen-haemocyte adhesion system (Miller and Stanley, 2001). Wells (in 96 well tissue culture plates Gibco, USA) were inoculated with PBS (90 µl) containing 100 nematodes followed by 10 µl of haemolymph from individual larvae. Haemolymph was obtained by amputating a prothoracic leg of chilled larvae (5 min, 4°C) and collecting the blood in a micropipette tip. Control wells contained PBS and haemolymph. Suspensions were maintained by incubating the plates on a horizontal gyratory shaker (50 rpm) at 25°C. At designated times

the number of nematodes with at least two adhering haemocytes and the number of freely-floating haemocytes were determined using a stereo dissecting microscope (200 x magnification) and a haemocytometer, respectively, the latter with phase contrast microscopy (400 x magnification).

Dead as opposed to live nematodes in the present study elicited melanization. To ensure melanization was due to phenoloxidase and not haemocyte peroxidases, the phenoloxidase specific inhibitor, tropolone (Li *et al*., 1996) at selected concentrations, was added to diluted haemolymph (10% v/v PBS) of larval *G. mellonella* containing dead nematodes or the phenoloxidase activator, laminarin (1mg / ml PBS) and incubated over time. The supernatants of 10 µl samples from each replicate, clarified by centrifugation (12,000 x g, 2 min, 20°C), were added to PBS containing the phenoloxidase substrate, L-dihydroxyphenylalanine (1 mg/ml PBS). After incubation for 20 min dopachrome formation was determined spectrophotometrically by absorption at 490 nm (Giannoulis *et al*., 2007). Samples were blanked on fresh substrate solution to preclude autooxidation effects. Specific phenoloxidase activity was calculated (Leonard *et al*., 1985).

Herein dead nematodes enhanced phenoloxidase which may reflect the absence of an inhibitor in its exudate and/or the presence of an enzyme activator on the cuticle. To ensure the absence of an inhibitor, dead-nematode exudate was incubated with haemolymph laminarin (1 mg/ml) (Leonard *et al*., 1985) for 30 min after which clarified samples were assayed for dopachrome formation. Live-nematode exudate was similarly tested for suppression of phenoloxidase activation by laminarin.

The effect of phenoloxidase on the haemocyte adhesion frequency on dead nematode required incubating the nematodes using the described antigen-haemocyte adhesion system with selected tropolone levels known to inhibit phenoloxidase. Similarly the effect of 24 h exudate from live nematodes on the haemocyte adhesion frequency on dead nematode encapsulation was determined at 30 min incubation.

Haemocytes of both insect species avidly adhere to glass (Zakarian *et al*., 2002; Giannoulis *et al*., 2005). Two protocols consisting of haemocyte monolayers were used to determine if exudate type affected haemocytes binding to slides. Protocol one, designed to

detect inhibition of haemocyte adhesion to slides, involved adding 60 µl and 120 µl of haemolymph from six chilled (5°C, 10 min) larvae of *G. mellonella* and *M. disstria*, respectively, to PBS (1 ml, 4°C) per replicate. Ten and 20 µl of haemocyte suspension of the former and latter larvae were added to slides (previously rendered endotoxin-free by heating to 350°C for 24 h) containing 10 and 20 µl of live or dead nematode exudate, respectively, in a 95 mm^2 area. Different volumes of materials were used between insect species to ensure the inherent differences in total haemocyte counts resulted in similar numbers of haemocytes being used in the experiments. Haemolymph plasma (3% v/v final concentration) was not removed because humoral factors affect lepidopteran haemocyte reactions (Lanz-Mendoza *et al*., 1996). Haemocyte mixtures, as opposed to isolated haemocytes, were used to reflect the *in vivo* situation and to preclude isolation artifacts (Yokoo *et al*., 1995). Liver agar may have haemocyte modulating components carried by the nematodes and released into PBS. Such agar without nematodes was rinsed and treated as live-nematode extract, the liver agar serving as a negative control. Nematode exudates were initially produced by incubating live or dead nematodes in PBS for 30 min.

The haemocyte suspensions on the slides were incubated for 30 min [the time for maximum haemocyte adhesion for both insect species (Zakarian *et al*., 2002; Giannoulis *et al*., 2005)] at 25°C and 95% RH. Subsequently the slides were rinsed three times with PBS (2 ml) to remove non-adhering haemocytes. Attached haemocytes were fixed in glutaraldehyde-formaldehyde vapour and mounted in 20% (v/v) glycerol in PBS. Where possible, haemocyte types were identified according to Price and Ratcliffe (1974). In situations where morphological differentiation was limited, phase bright, spherical, cells with granules and oval, phase dark cells with few granules were regarded as granular cells and plasmatocytes, respectively. The total number and types of adhering haemocytes were determined by phase contrast microscopy.

In protocol two, detachment of adhering haemocytes by exudate type was addressed. Either exudate type was added to previously formed monolayers of *G. mellonella* (10 µl exudate) and *M. disstria* (20 µl exudate). Slides were incubated, rinsed, fixed and adhering haemocytes analyzed as in protocol one.

In vivo interaction of nematodes and exudate with haemocytes

Both the live nematodes and their exudate limited haemocyte adhesion *in vitro* (this study), which, if it occurs *in vivo,* could impair the host's cellular immunity. Axenic live and dead nematodes in PBS (100 nematodes /10 µl PBS), their exudates (10 µl) or PBS (10 µl) were injected into the larvae and changes in total haemocyte counts for all treatments determined after incubating the insects at 25°C during 24 h. Haemocyte types were identified according to Price and Ratcliffe (1979) and tallied from larvae injected with either exudate type or PBS. Haemocyte viability was determined for *G. mellonella* larvae injected with live and dead nematodes, PBS and exudate at 5 min, 8 h and 24 h post-injection based on exclusion of the vital stain, trypan blue, (0.4% w/v PBS) by suspended haemocytes (Lamprou *et al*., 2005).

The impact of the exudate from the live nematodes on haemocyte-antigen binding was based on injections of exudate (10 µl) containing *B. subtilis* or *X. nematophila* (1.7 bacteria x 10^{10}). Control larvae received comparable bacterial levels in 10 µl of PBS. Larvae, incubated for 0.5 h and 8 h [times during which haemocyte profiles were significantly altered (this study)] at 25°C were bled and the number of non-attached bacteria determined by phase contrast microscopy on a haemocytometer.

Source of immunosuppressants

To determine which nematode stage produced immunosuppressants the different stages from axenic cultures consisting predominantly of J_3 and adults were separated by density gradient centrifugation. The chilled (8^oC) sucrose-PBS gradient ranged from 10-25% sucrose in 5 ml volumes in 30 ml round bottom centrifuge tubes. PBS (1 ml) containing 1000 axenic nematodes were layered on the top of the column and the nematode stages separated by centrifugation (270 x g, 5 min, 8^oC) in a fixed angle rotor head. The fractions containing 52 females (0/10% interface), 43 males and 85 J_4 stage nematodes (10/15% interface) were pooled whereas the fractions with 665 J_3 and 30 J_4 stage nematodes (20/25% interface) were kept separate. Approximately 110 J_{1+2} nematodes isolated at the bottom of the column on the

centrifuge tube were similarly kept isolated. The fractions were diluted with PBS and, as previously alluded to, centrifuge-washed twice to remove sucrose. Sucrose affects *G. mellonella* haemocyte reactions with bacterial antigens by modifying phenoloxidase (Dunphy and Chadwick, 1989). Based on nematode mobility, the viability levels of all fractions exceeded 95%. Centrifugation controls consisted of all nematode stages pooled together to reflect the original non-centrifuged nematode mixture used to produce the original exudate. All fractions and pooled fractions were incubated and assayed for immunosuppression as previously described. Negative controls consisted of PBS without nematodes and the positive controls were exudates produced from nematodes rinsed into PBS without centrifugation. To further preclude early stage J_4s and establish J_3s as the suppressive stages, the enriched J_3 fraction was incubated on axenic liver agar until 50% and 80% of the J_3s had developed into J_4s, the nematodes were rinsed from the medium and incubated in PBS for immunosuppressant production. Suppression activity was assayed as previously detailed.

Statistics

All graphic data represents the mean ± standard error of the mean, of at least10 temporally separated replicates, from each of which contained 5-10 individual samples were taken for analysis. A significance α level of 0.05 was chosen. Haemocyte counts and bacterial levels were analyzed using 95% confidence limits overlap (Sokal and Rohlf, 1969). Differential counts and percentage data were analyzed using arc sin $\sqrt{}$ p-transformed data. Tabulated mean differential counts and mean percentage data in the text were presented as the decoded mean (with 95% confidence limits of the transformed data).

Results

In vitro interaction of nematodes and exudate with haemocytes.

Three stages (adult and two juvenile stages) of the live nematodes were not encapsulated by haemocytes of either insect species (Fig. 2.1 A, B). Actively motile nematodes were seen colliding with free-floating haemocytes, displacing the cells while other nematodes rolled over haemocytes on the bottom of the wells. Freeze-killed nematodes exhibited adhering haemocytes (Fig. 2.1 C, D), adhesion intensity increasing with incubation time (Fig.2.2). Haemocyte adhesion on the dead nematodes was correlated ($r = 0.97$, $P < 0.05$) with a decline in total haemocyte counts. No significant ($P > 0.05$) change in haemocyte counts occurred in the nematode-free control wells. A minor but not significant ($P > 0.05$) decline in haemocytes occurred in wells with live parasites establishing the absence of haemocyte adhesion to the parasites was not due to lysis. The experiment did not exclude inhibition of haemocyte adhesion by the nematodes.

Live nematodes did not activate phenoloxidase compared with the negative controls whereas dead forms enhanced enzyme activity above the spontaneously activated phenoloxidase control (haemolymph) to values comparable to the laminarin-activated samples ($P>0.05$) during the first 7 min incubation (Fig.2.3). By 12 min phenoloxidase activities in haemolymph with dead nematodes and with laminarin were similar ($P > 0.05$). Live-nematode exudate with or without dead nematodes or with the phenoloxidase activator, laminarin, did not activate phenoloxidase (Fig.2.3). Thus live-nematode exudate contained inhibitor(s) that affected either phenoloxidase discharge from the haemocytes and/or phenoloxidase activation. Dead-nematode exudate did not impair laminarin-activated enzyme or phenoloxidase activated by dead nematodes. Hence dead-nematode exudate may have lacked a phenoloxidase inhibitor. The cuticle structure of the dead nematodes was probably altered by freeze-killing favoring phenoloxidase activation.

Dopachrome formation was not attributed to haemocyte peroxidase because the phenoloxidase inhibitor, tropolone, inhibited pigment production. Ten μM tropolone totally inhibited dopachrome production in nematode-free haemolymph (Fig.2.3). In haemolymph with dead nematodes tropolone inhibition of dopachrome formation was concentration-dependent with 100μM tropolone being most effective. Tropolone inhibited also dead nematode encapsulation, the effect increasing with drug concentration (Table 2.1) with 100 μM tropolone inhibition being comparable to that of live nematode exudate ($P > 0.05$). Thus, phenoloxidase activated by dead nematodes was associated with haemocytes binding to dead nematodes.

Figure 2.1 Interaction of larval *Galleria mellonella* haemocytes with live (A, B) and dead (C, D.) *S. carpocapsae* nematodes *in vitro.* (A) Juvenile stage and (B) adult female stage after 30 min incubation. (C) freeze-killed nematode with adhering haemocyte (arrow) 15 min p.i. (D) freeze-killed nematode with extensive granular cell adhesion (arrow) 30 min p.i. (100 X magnification).

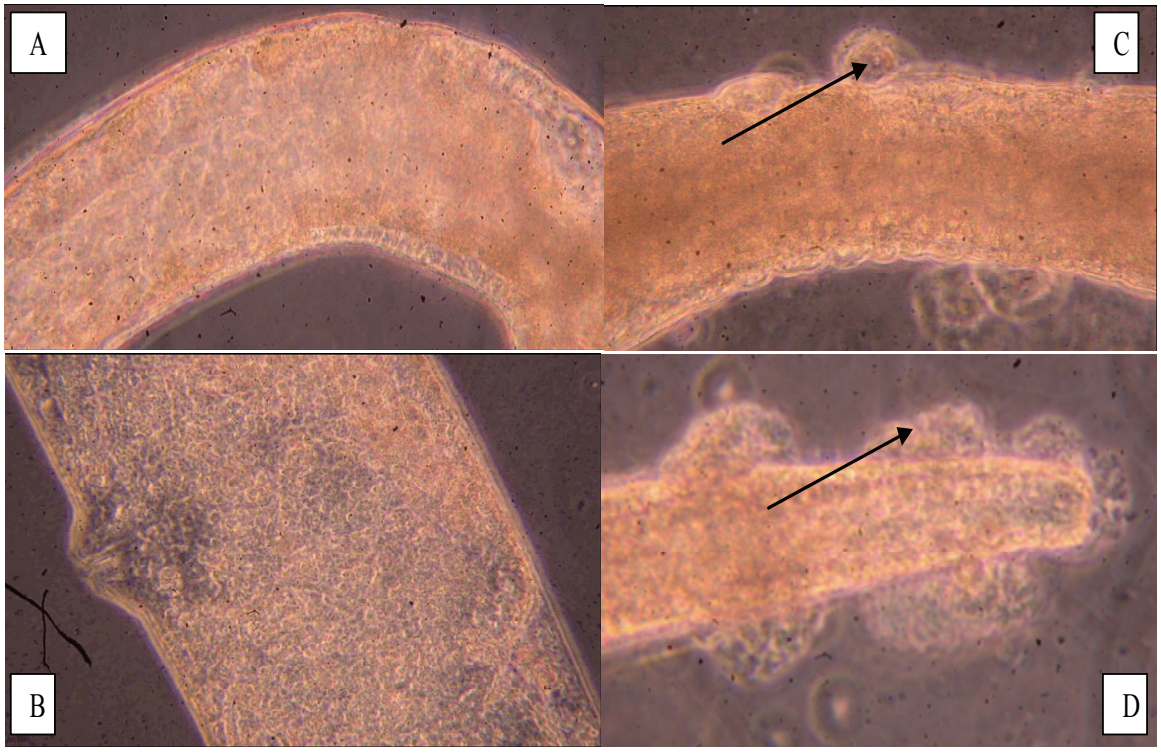

A
C
B
D

Figure 2.2 Changes in larval *Galleria mellonella* haemocyte counts and live and dead nematode encapsulation levels during 0-30 min incubation in diluted haemolymph. Dead nematode encapsulation increased over time as haemocyte counts declined whereas live nematodes did not alter haemocyte counts compared with control values. Values are expressed as the mean ± SE, $n \geq 10$.

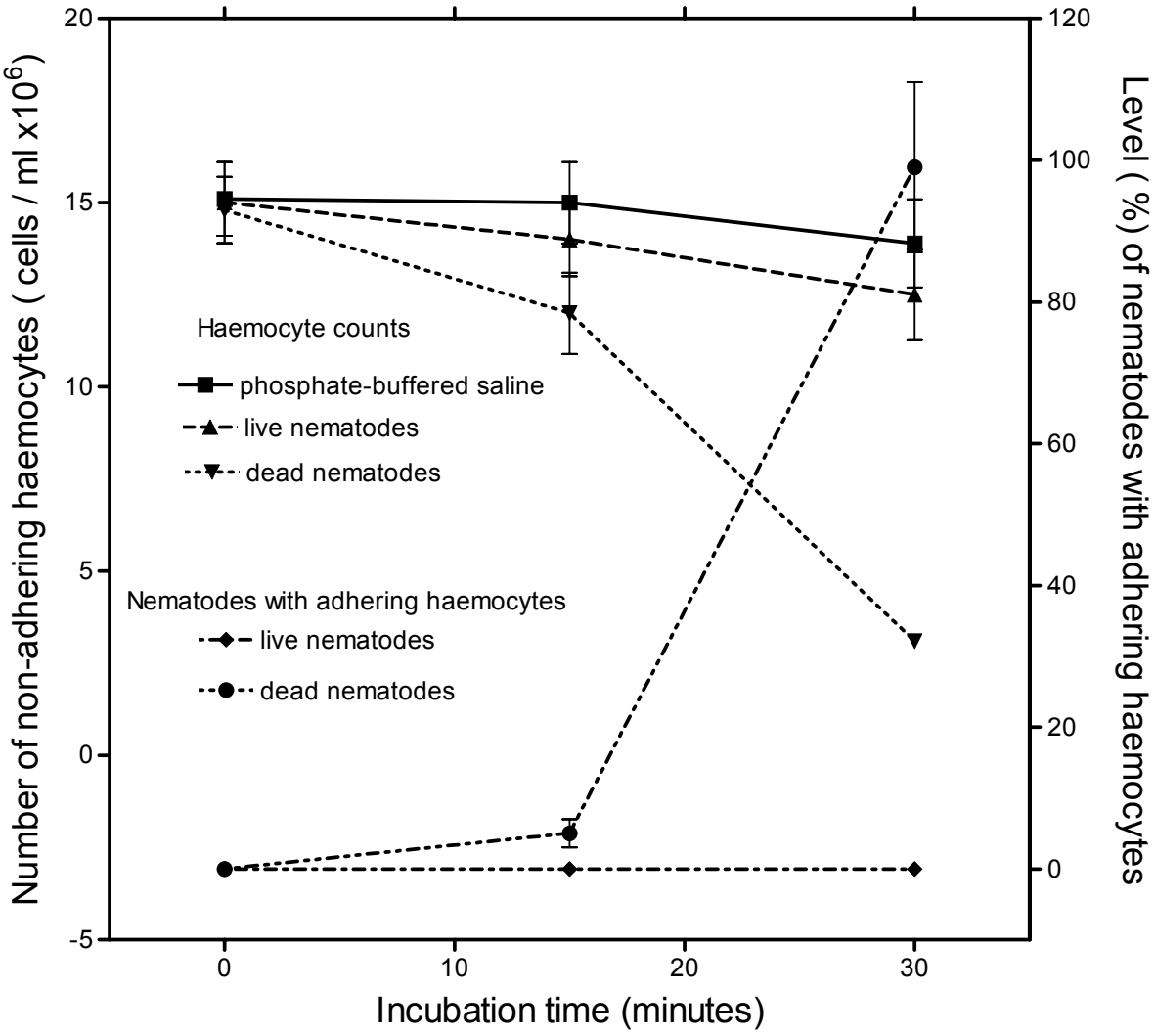
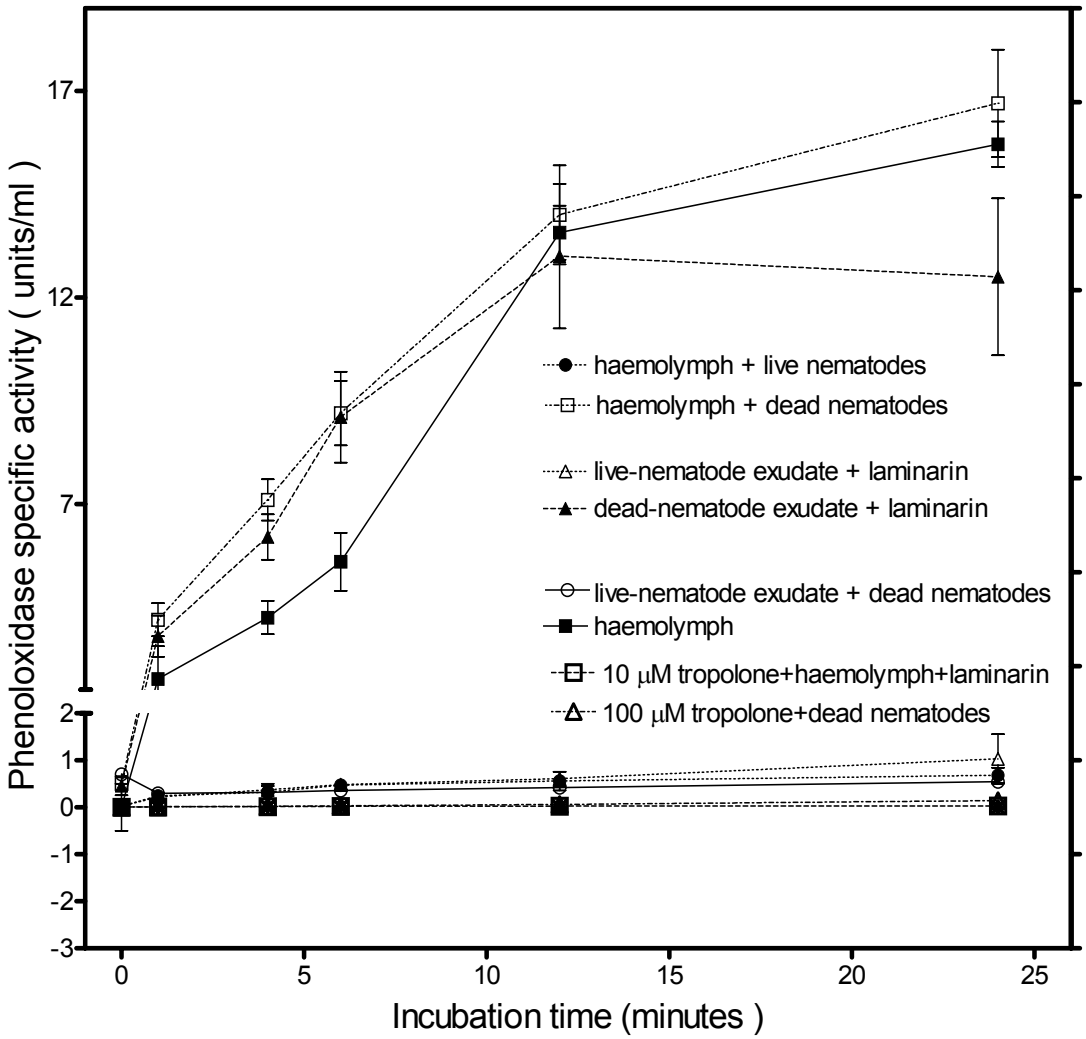

Number of non-adhering haemocytes (cells / ml x10^6)
Level (%) of nematodes with adhering haemocytes
Incubation time (minutes)
Haemocyte counts
phosphate-buffered saline
live nematodes
dead nematodes
Nematodes with adhering haemocytes
live nematodes
dead nematodes
20
15
10
5
0
-5
120
100
80
60
40
20
0
0
10
20
30

Figure 2.3 Live and dead *Steinernema carpocapsae* inhibited and enhanced phenoloxidase activity, respectively, in *Galleria mellonella* diluted haemolymph compared with spontaneous phenoloxidase in diluted haemolymph alone. Spontaneous phenoloxidase was readily inhibited by 10μM tropolone but with dead nematodes in haemolymph inhibition varied with the tropolone concentration, 100μM being the most effective. Non-diluted live-nematode exudates, but not dead-nematode exudate inhibited phenoloxidase activation. Values are expressed as the mean ± SE. n>10.


Phenoloxidase specific activity (units/ml)
Incubation time (minutes)
haemolymph + live nematodes
haemolymph + dead nematodes
live-nematode exudate + laminarin
dead-nematode exudate + laminarin
live-nematode exudate + dead nematodes
haemolymph
10 μM tropolone+haemolymph+laminarin
100 μM tropolone+dead nematodes

Live-nematode exudate, as opposed to liver agar extract, impaired the adhesion of the granular cells and plasmatocytes of *G. mellonella* to slides (Fig.2.4A). The extent of inhibition varied with haemocyte type, 80% (56.7-70.6) and 50% (40.8-51.3) for granular cells and plasmatocytes, respectively. Comparable results were seen with *M. disstria* haemocytes (Fig. 2.4B). Dead-nematode exudate did not impair or increase adhesion of the haemocyte types of either insect species which may indicate the exudate did not contain immune activating materials released from cold-damaged nematodes. Because the exudate did not activate detectable phenoloxidase (Fig.2.3), this confirms that no enzyme was released from cold-damaged nematodes that would subsequently alter haemocyte activity.

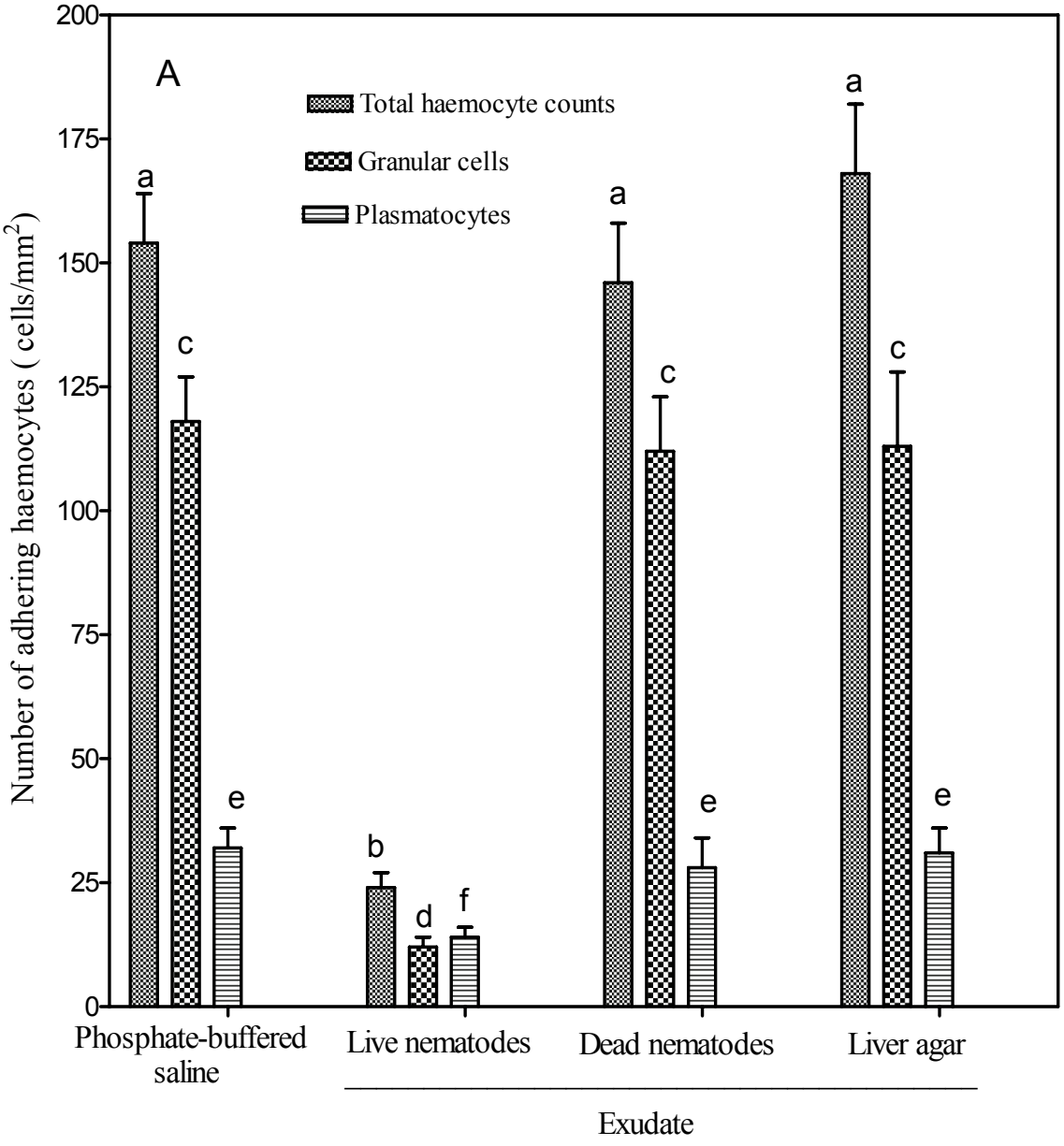
The release kinetics of immunosuppressant(s) from live nematodes varied with the haemocyte type of *G. mellonella* examined (Fig.2.5). Inhibition of adhesion of the total haemocytes and granular cells was detected within 0.5 h of incubating nematodes in PBS with maximum and constant inhibition levels by 12 h to 24 h incubation. Adhering plasmatocyte levels declined continuously to greatest inhibition by 12 to 24 h. All adhering granular cells and plasmatocytes appeared normal whereas nonattached haemocytes exhibited pycnotic nuclei, cytoplasmic blebbing and often fibrous thin filaments around the cell membrane. Because of the maximum effect on both haemocyte types occurred at 24 h, exudate 24 h old was used in subsequent experiments. The total exudate protein increased in concentration as the level of haemocyte attachment decreased implying that protein(s) may be an immunosuppressant. The anti-adhesion effect was dependent also on the exudate concentration and haemocyte type, granular cell adhesion increasing with increasing dilution of the 24 h old live–nematode exudate whereas the plasmatocytes were less affected (Fig. 2.6). To ensure maximum effect on both haemocyte types non-diluted exudate was used in subsequent experiments.

Table 2. 1 Effect of phenoloxidase inhibitors on the encapsulation of freeze-killed *Steinernema carpocapsae* DD136 by haemocytes of larval *Galleria mellonella*.

Treatment		Percentage of encapsulated nematodes[a]
Tropolone[b]	0 μM	87 (77.89 - 60.47) [c]
Tropolone	10 μM	52 (51.06 - 42.42) [d]
Tropolone	100 μM	10 (21.57 - 13.56) [e]
Live-nematode exudate		6 (17.98 - 9.10) [e]

[a] Mean (with 95% confidence limits of arcsin √p-transformed data), n ≥10 replicates which contained 5 samples. Values with the same superscript were not significantly different, P > 0.05. [b] Tropolone dissolved in PBS, pH 6.5.

Figure 2.4. Effect of 24 h exudate from live and dead *Steinernema carpocapsae* and the negative controls (exudate from liver agar and phosphate-buffered saline (PBS)) on the adhesion of haemocytes of (A) larval *Galleria mellonella* and (B) larval *Malacosoma disstria* to glass slides. Diluted haemolymph incubated with exudates and control solutions for 30 min to allow haemocyte adhesion was rinsed away with the PBS and the total number and types of adhering haemocytes were determined. Values represent the mean ± SE, $n \geq 10$.


A
Total haemocyte counts
Granular cells
Plasmatocytes
Number of adhering haemocytes (cells/mm2)
200
175
150
125
100
75
50
25
0
a
c
e
b
d
f
a
c
e
a
c
e
Phosphate-buffered saline
Live nematodes
Dead nematodes
Liver agar
Exudate

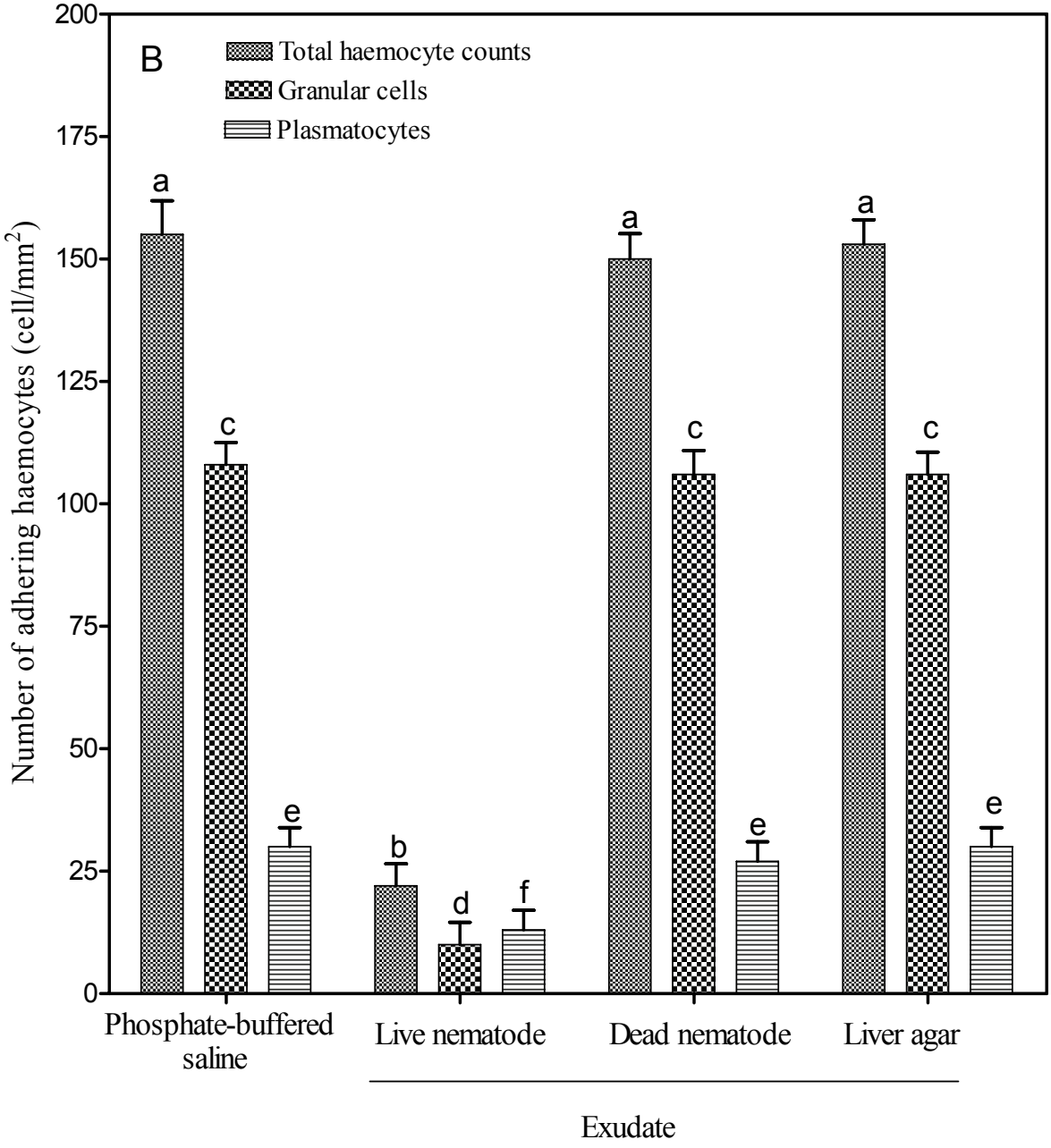

B
Total haemocyte counts
Granular cells
Plasmatocytes
Number of adhering haemocytes (cell/mm2)
200
175
150
125
100
75
50
25
0
a
c
e
b
d
f
a
c
e
a
c
e
Phosphate-buffered saline
Live nematode
Dead nematode
Liver agar
Exudate

Figure 2.5 Production of immunosuppressants based on the inhibition of adhesion of granular cells and plasmatocytes from larval *Galleria mellonella* to slides. Phosphate-buffered saline incubated with live nematodes for selected times and clarified by centrifugation was placed on slides prior to the addition of dilute haemolymph. After 30 min incubation slides were rinsed and adhering haemocytes analyzed with phase contrast microscopy. Values represent the mean ± SE, $n \geq 10$.

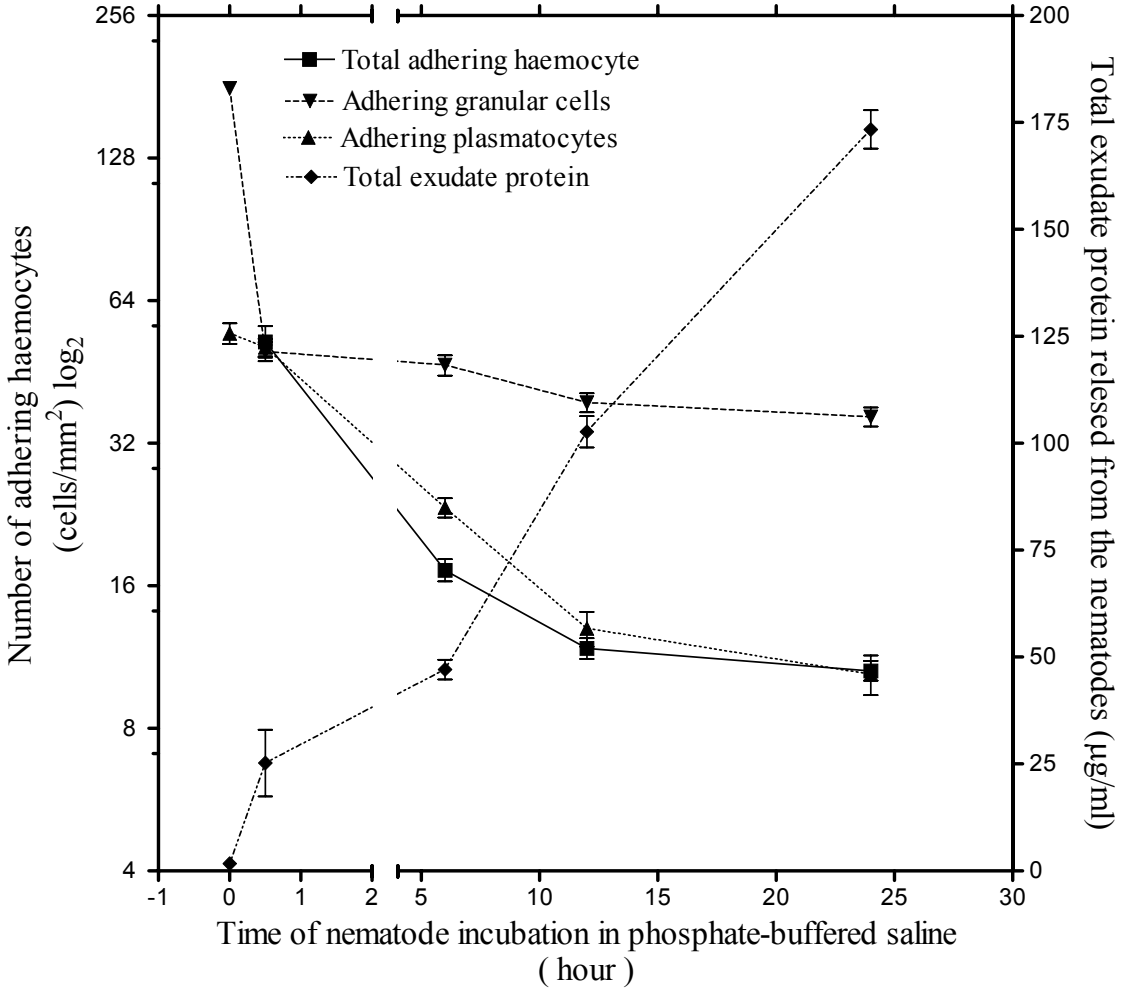
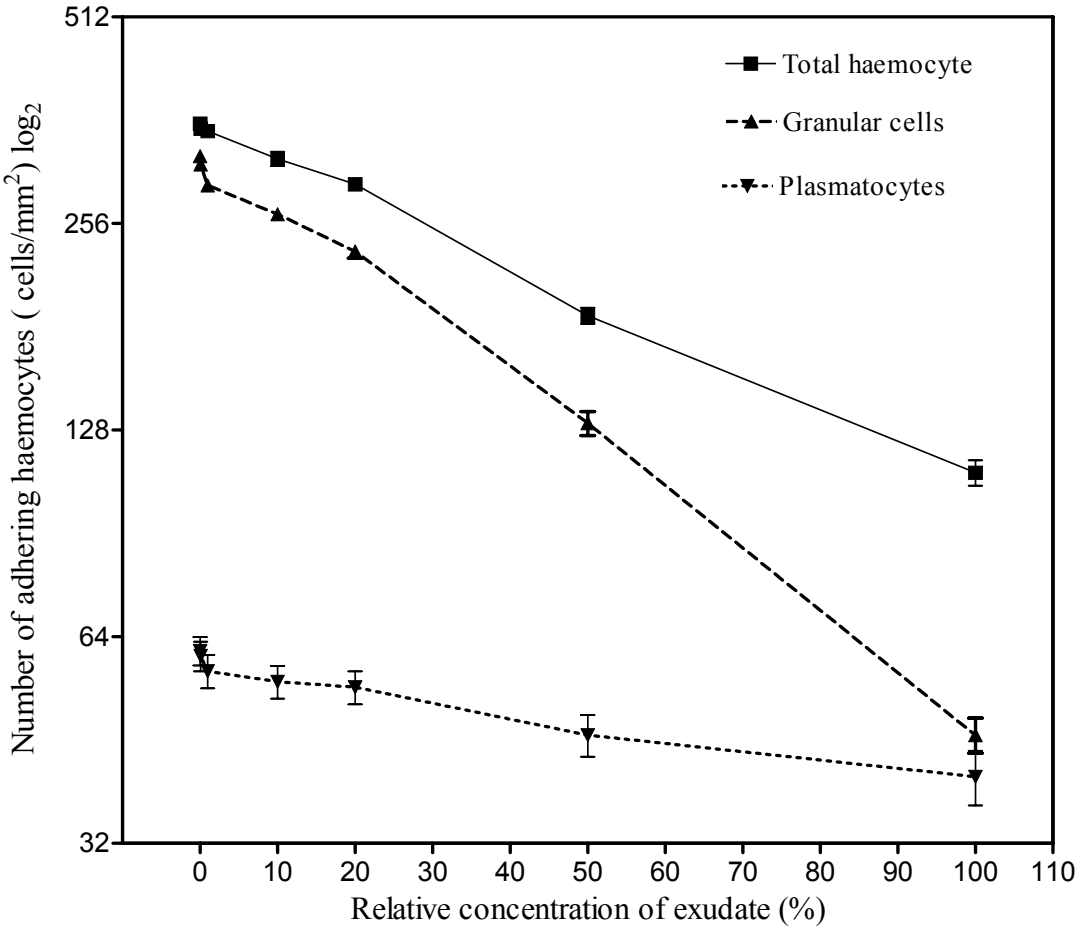

Total adhering haemocyte
Adhering granular cells
Adhering plasmatocytes
Total exudate protein
Number of adhering haemocytes (cells/mm^2) log$_2$
Total exudate protein relesed from the nematodes (μg/ml)
Time of nematode incubation in phosphate-buffered saline (hour)
256
128
64
32
16
8
4
200
175
150
125
100
75
50
25
0
-1
0
1
2
5
10
15
20
25
30

Figure 2.6. Effect of different concentrations of live-nematode exudate on the adhesion of larval *Galleria mellonella* haemocytes. Values represent the mean ± SE, n = 10.


512
256
128
64
32
Number of adhering haemocytes (cells/mm^2) log$_2$
Total haemocyte
Granular cells
Plasmatocytes
0
10
20
30
40
50
60
70
80
90
100
110
Relative concentration of exudate (%)

The aforementioned haemocyte adhesion assay may represent a combination of diminished adhesive capacity and increased detachment of bound haemocytes. Incubating live-nematode exudate with bound haemocyte monolayers triggered a rapid decline from 0-60 min in both total haemocyte counts and granular cells compared with the PBS control slides (Fig.2.7). Inhibition levels of granular cell adhesion at 30 min incubation (80%, 51.1-74.5) were equal to granular cell detachment (74%, 48.2-75.1, $n \geq 10$, $P > 0.05$). On the control slides granular cell levels were constant for 60 min and thereafter declined. Pre-adhering plasmatocyte levels did not change in the presence of exudate or PBS.

***In vivo* interaction of the nematodes and exudate with haemocytes**

PBS-injections into *G. mellonella* increased total haemocyte counts to a plateau value by 6 h post-injection (p.i.) (Fig. 2.8A). Haemocyte counts in larvae with dead nematodes declined over 8 h p.i.(Fig. 2.8A), the haemocytes adhering to the nematode epicuticle by this time. Live nematodes elevated haemocyte counts by approximately 2-fold within 24 h p.i.; the haemocytes did not adhere to the nematodes. Comparable changes occurred in *M. disstria* larvae (Fig. 2.8B). There was no significant difference in the viability of floating haemocytes of larval *G. mellonella* regardless of treatment [(viability range) %: PBS, 98 (79.7-89.5); dead nematodes, 96 (79.7-82.7); live nematodes, 84 (68.6-81.3), $n \geq 10$, $P > 0.05$]. There was no sign of haemocyte aggregation or morphological degeneration in the presence of live nematodes in either host species.

Live-nematode exudate injections in to *G. mellonella* increased total haemocyte counts at a linear rate until 4 h p.i., followed by a 4 h plateau and a decline by 24 h p.i. (Fig.2.9). Haemocyte counts after 0 h were always significantly greater than in the insects injected with PBS ($P < 0.05$). Although live-nematode exudate increased total haemocyte counts by 0.5 h p.i. in *M. disstria*, the pattern differed from *G. mellonella* with the plateau occurring over 2.0 h p.i. in *M. disstria* being followed by a linear increase from 2-8 h p.i. and thereafter a rapid decline to values less those in control larvae (Fig. 2.9). PBS control insects exhibited constant total haemocyte levels throughout 24 h.

Figure 2.7 Dissociation of haemocytes from previously formed *Galleria mellonella* haemocyte monolayers after the addition of live *Steinernema carpocapsae* exudate. Compared with the PBS negative controls, exudate detached granular cells but not plasmatocytes by 30 min incubation. Values represent the means ± SE, $n \geq 10$.

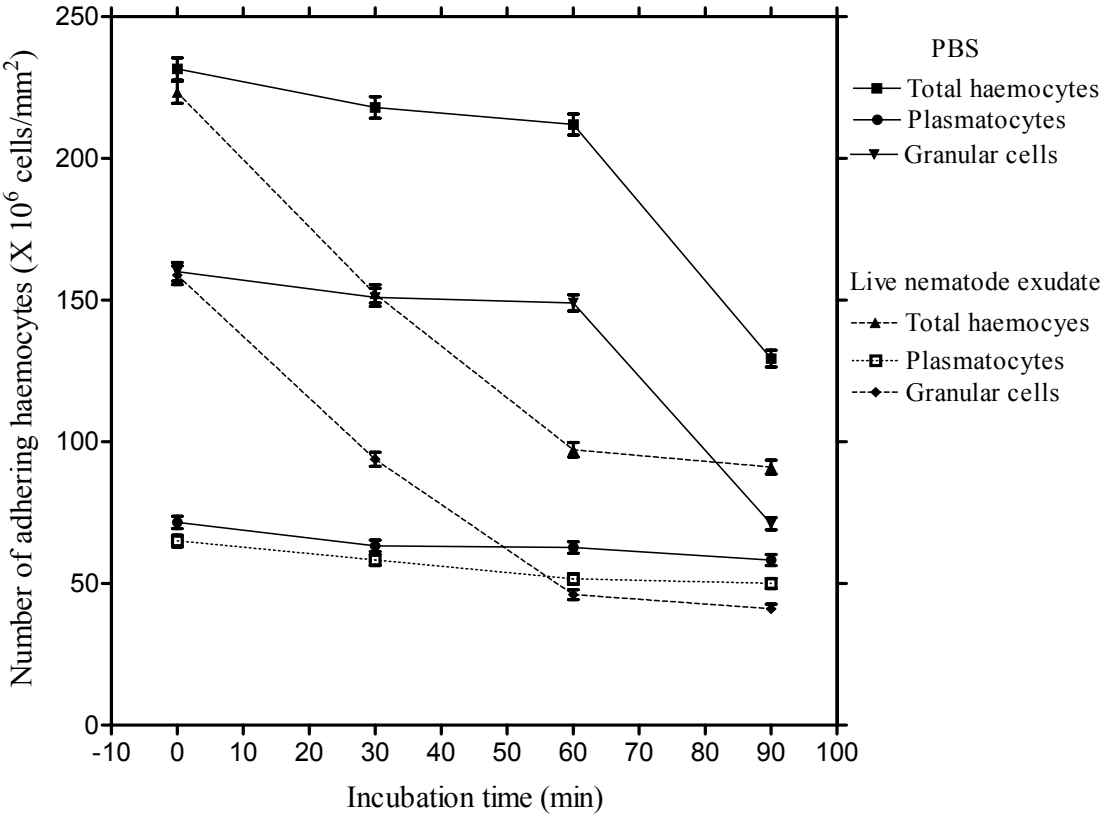
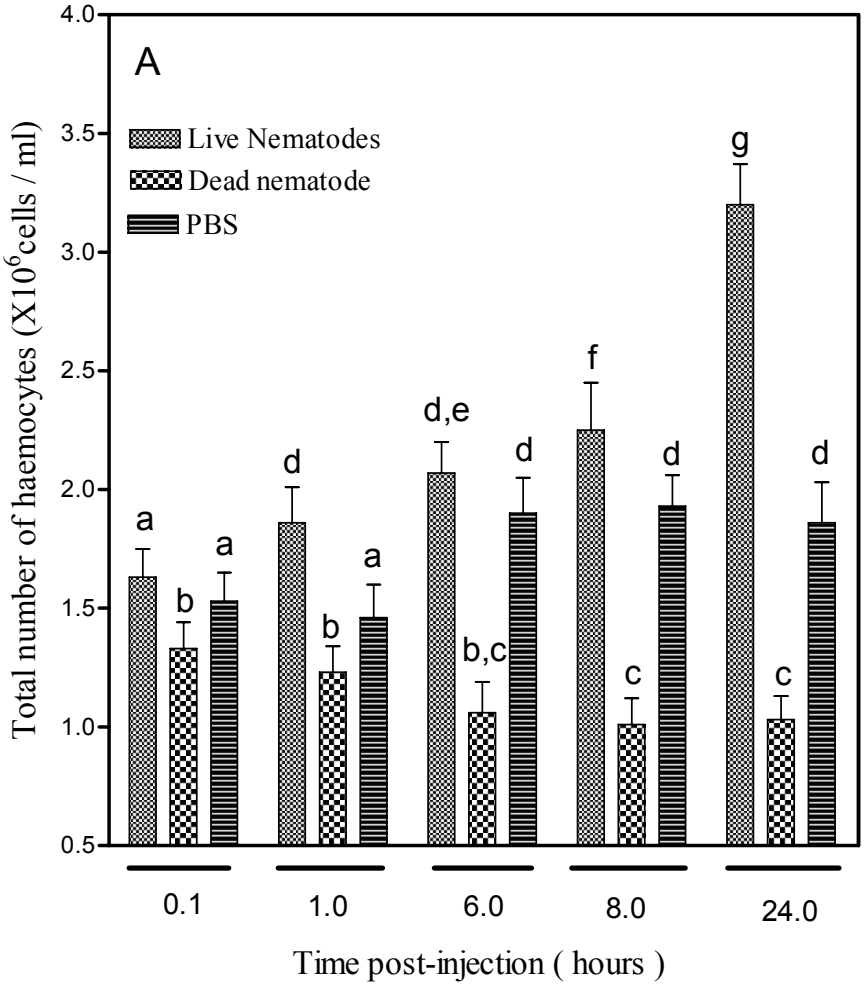

Number of adhering haemocytes (X 10^6 cells/mm^2)
250
200
150
100
50
0
-10
0
10
20
30
40
50
60
70
80
90
100
Incubation time (min)
PBS
Total haemocytes
Plasmatocytes
Granular cells
Live nematode exudate
Total haemocyes
Plasmatocytes
Granular cells

Figure 2.8 Effect of live and dead *Steinernema carpocapsae* injected into (A) larval *Galleria mellonella* (B) larval *Malacosoma disstria* on total haemocyte counts compared with the phosphate-buffered saline control. Values represent the mean ± SE, $n \geq 10$.


A
Live Nematodes
Dead nematode
PBS
Total number of haemocytes (X10^6cells / ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
a
b
a
d
b
a
d,e
b,c
d
f
c
d
g
c
d
0.1
1.0
6.0
8.0
24.0
Time post-injection (hours)

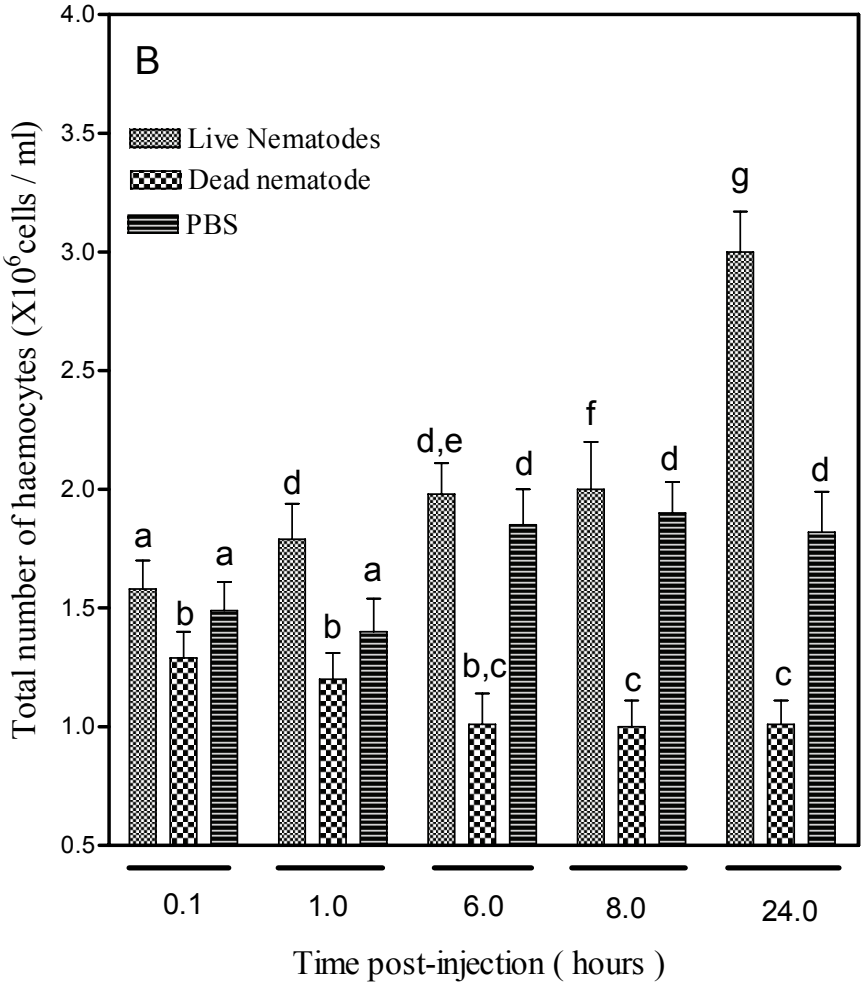
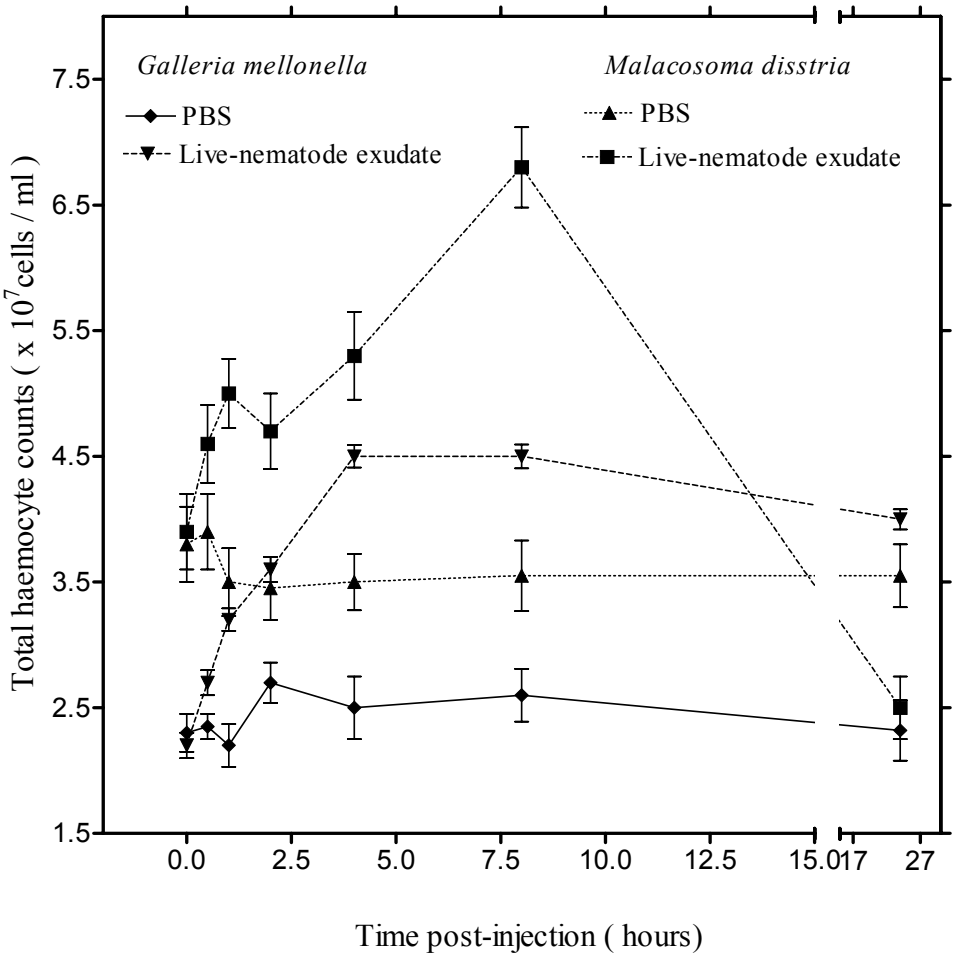

B
Live Nematodes
Dead nematode
PBS
Total number of haemocytes (X10^6cells / ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
a
b
a
d
b
a
d,e
b,c
d
f
c
d
g
c
d
0.1
1.0
6.0
8.0
24.0
Time post-injection (hours)

Figure 2.9 Effect of live *Steinernema carpocapsae* exudate injected into larval *Galleria mellonella* and *Malacosoma disstria* on total haemocyte counts. PBS is the negative control. Values represent the means ± SE, n ≥ 10.

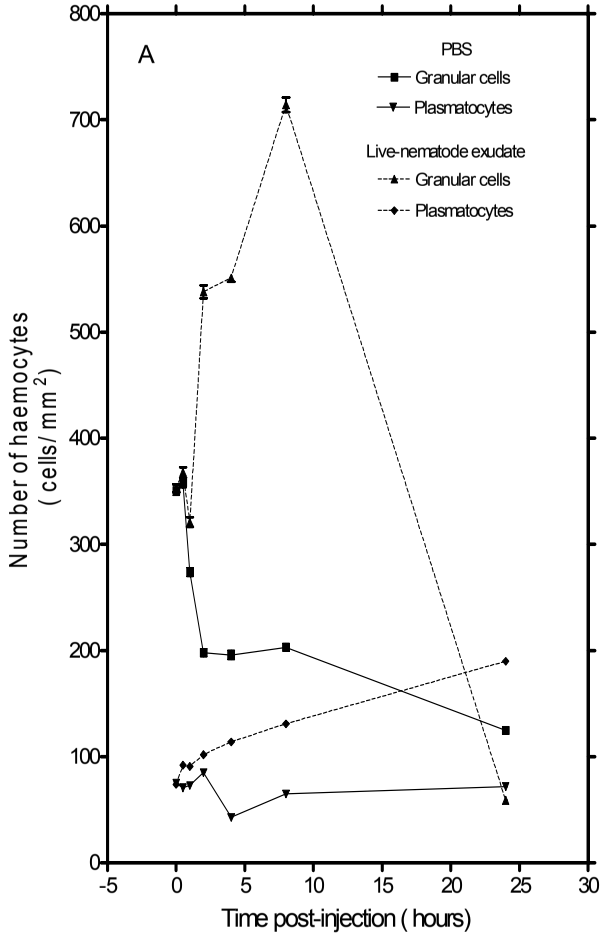

Galleria mellonella
PBS
Live-nematode exudate
Malacosoma disstria
PBS
Live-nematode exudate
Total haemocyte counts (x 10^7 cells / ml)
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17
27
Time post-injection (hours)

In *G. mellonella* granular cells increased to a maximum 8 h p.i. with live-nematode exudate whereas control larvae exhibited a rapid decline by 1 h p.i. followed thereafter by a gradual decline; both insect groups had comparable granular cell levels 24 h p.i. (Fig. 2.10A). Plasmatocyte levels remained essentially constant in the control larvae but increased continuously over 24 h in exudate-injected larvae. Increasing counts of spherulocytes and oenocytoids exhibited a bimodal pattern in insects with live-nematode exudate, peaking at 2 h and 8 h p.i., the levels at 8 h p.i. being greater than at 2 h p.i. In control insects oenocytoids did not change appreciably but spherulocytes peaked at 8 h p.i., the levels of both haemocyte types being less than in larvae with live-nematode exudate ($P < 0.05$). *Malacosoma disstria* plasmatocytes in both control and live-nematode exudate-treated larval groups did not change (Fig. 2.10B). Exudate increased oenocytoids and spherulocytes to a maximum by 8 h p.i. The lowest oenocytoid and spherulocyte levels occurred in the PBS control larvae by 4 h p.i. but by 8 h p.i. counts of both haemocyte types increased; the levels were always less than the larvae injected with live-nematode exudate ($P < 0.05$).

In both insect species approximately 60% and 90% of the granular cells and plasmatocytes, respectively, from larvae with live-nematode exudate failed to adhere to slides at 0.5 and 8 h p.i., compared with haemocytes from the control larvae. Larvae with dead-nematode exudate produced results comparable to the PBS control insects ($P>0.05$). Many of the haemocytes from insects with live-nematode exudate, unlike those in larvae with live nematodes, exhibited blebbing and nuclear degeneration. The data imply the granular cells and plasmatocytes were non-functional due to damage produced by the live-nematode exudate which may reflect the amount of inhibitor(s) injected in the exudate exceeding that produced by live nematodes during this time *in vivo*. Spherulocytes and oenocytoids from live-nematode exudate-injected insects did not exhibit degeneration at 0.5 and 8 h p.i..

That haemocytes of both insect species containing live-nematode exudate were non-functional was confirmed additionally by the higher levels of *X. nematophila* and *B. subtilis* in both larval species compared with insects injected with bacteria in PBS and

Figure. 2.10A Changes in haemocyte types of larval *Galleria mellonella* injected with live-nematode exudate or PBS (negative control). Haemolymph, removed from the larvae over 24 h post-injection was placed in PBS on glass slides to allow haemocyte differentiation. Values were expressed as the mean ± SE, $n \geq 10$.


A
PBS
Granular cells
Plasmatocytes
Live-nematode exudate
Granular cells
Plasmatocytes
Number of haemocytes
(cells/ mm 2)
Time post-injection (hours)
800
700
600
500
400
300
200
100
0
-5
0
5
10
15
20
25
30

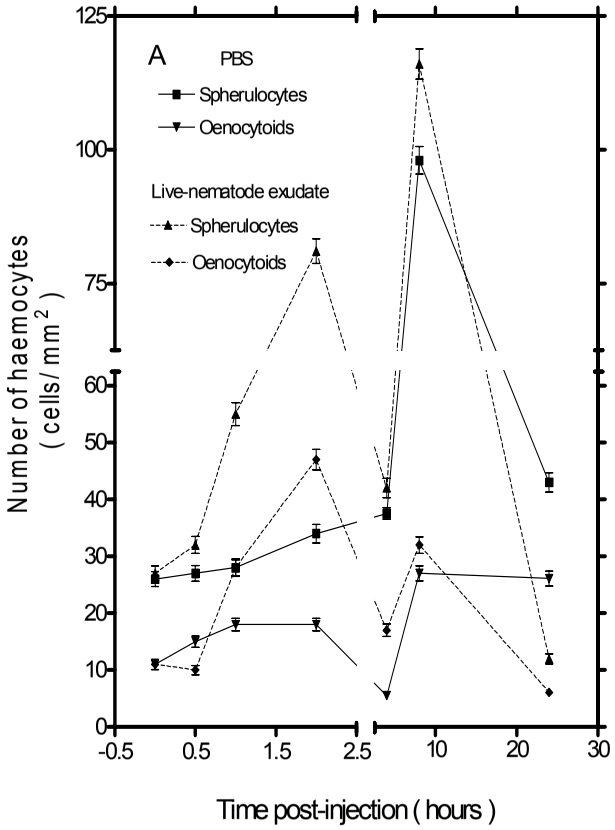
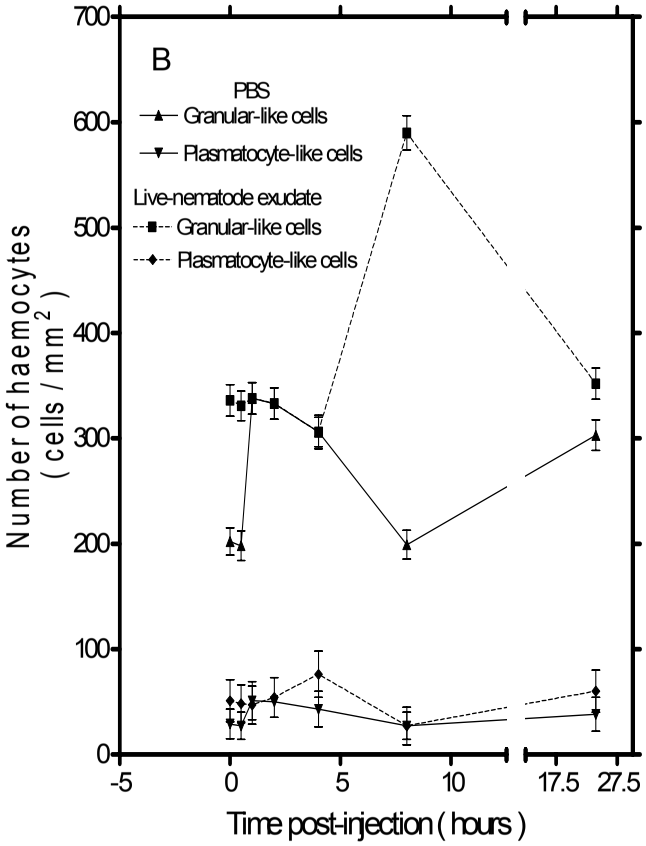
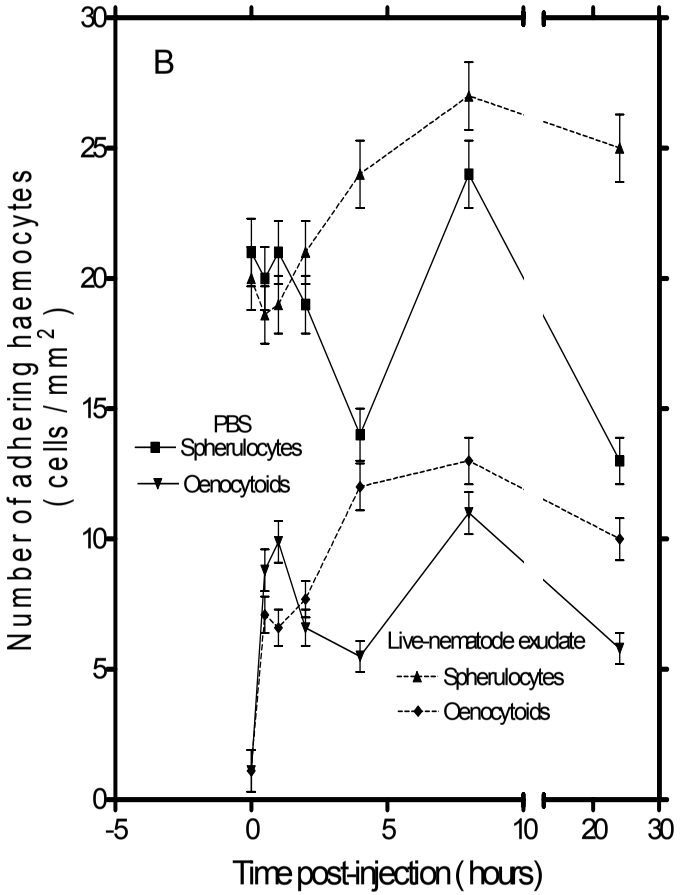

A
PBS
Spherulocytes
Oenocytoids
Live-nematode exudate
Spherulocytes
Oenocytoids
Number of haemocytes
(cells/ mm 2)
Time post-injection (hours)
125
100
75
60
50
40
30
20
10
0
-0.5
0.5
1.5
2.5
10
20
30

Figure 2.10B Changes in haemocyte types of larval *Malacosoma disstria* injected with live-nematode exudate or PBS (negative control). Haemolymph, removed from the larvae over 24 h post-injection was placed in PBS on glass slides to allow haemocyte differentiation. Values were expressed as the mean ± SE, $n \geq 10$.


B
PBS
Granular-like cells
Plasmatocyte-like cells
Live-nematode exudate
Granular-like cells
Plasmatocyte-like cells
Number of haemocytes (cells / mm2)
Time post-injection (hours)
700
600
500
400
300
200
100
0
-5
0
5
10
17.5
27.5


B
PBS
Spherulocytes
Oenocytoids
Live-nematode exudate
Spherulocytes
Oenocytoids
Number of adhering haemocytes (cells / mm2)
Time post-injection (hours)
30
25
20
15
10
5
0
-5
0
5
10
20
30

dead-nematode exudate (Table 2.2). Dead-nematode exudate produced results similar to the PBS control groups. Prolonged incubation of the insects equally lowered *B. subtilis* levels in larvae injected with PBS and dead-nematode exudate (P>0.05) whereas live-nematode exudate lowered bacterial levels to a lesser degree. Results with *X. nematophila* were complex; hosts with live-nematode exudate exhibited higher bacterial counts at 8 h than 0.5 h p.i. as did the PBS control group and insects with dead-nematode exudate, the counts in the latter two groups being similar to each other (P>0.05). These values were lower (P<0.05) than those in insects injected with live-nematode exudate. However with all three groups, levels of *X. nematophila* increased with incubation time.

By 8 h p.i. the *B. subtilis* control larvae and insects with the dead-and live-nematode exudate exhibited comparable elevation of morphologically damaged total haemocytes (90%, 56.9-75.8; P>.0.05) which were greater than haemocyte damage levels in bacteria-free larvae (12%, 2.2-4.4; n = 10, P < 0.05); the haemocytes exhibiting blebbing and chromatin degeneration. Dead *X. nematophila* in PBS and in dead-nematode exudate produced degenerative symptoms by 8 h p.i. in approximately 85% (63.4-71.7) of the haemocytes but in the presence of live-nematode exudate haemocyte damage was reduced (37%, 30.6-40.5).

Source of immunosuppressants

Centrifugation did not affect the immunosuppression production by the mix of nematodes produced by pooling the different fraction from the density gradient when compared with the positive control exudate and the negative control PBS groups (Table 3, P>0.05). Samples containing male and female adults and J_4 nematodes and the J_{1+2} fraction only did not detectably suppress or enhance haemocyte adhesion compared with the negative controls implying that these stages may not produce immune modulators. However, the enriched J_3 fraction which contained few J_4 nematodes substantially limited the number of haemocytes on slides, suppression representing 93% of the positive control level. Allowing the J_3s to develop into J_4s resulted in a dramatic increase in haemocyte adhesion as the J_3 levels decreased suggesting that the J_4s did not release suppressants whereas the J_3s did produce suppressants. Collectively the results establish that the adults and J_{1+2} and J_4s as opposed to the J_3s did not produce exudates with discernible haemocyte

modulation ability. It validates also the use of nematode mixes of known ratios and culture age as source of immunosuppressant(s).

Table 2.2. Effect of *Steinernema carpocapsae* DD136 exudate on bacterial removal from the haemolymph of two insect species *in vivo*.

Insect species	Treatment	Bacterial concentration (x 10^6/ml haemolymph)[a]			
		30 min p.i		8h p.i	
		X. nematophila[b]	*B. subtilis*[b]	*X. nematophila*	*B. subtilis*
Galleria mellonella	PBS[b]	$4.1 \pm 0.6^{c,c}$	$0.9 \pm 0.5^{c,c}$	$18.2 \pm 0.3^{c,d}$	$0.1 \pm 0.1^{c,d}$
	Live-nematode exudate	$6.2 \pm 0.7^{d,c}$	$2.1 \pm 0.2^{d,c}$	$10.1 \pm 0.7^{d,d}$	$2.9 \pm 0.4^{d,c}$
	Dead-nematode exudate	$3.7 \pm 0.2^{c,d}$	$0.4 \pm 0.6^{c,d}$	$16.1 \pm 1.1^{c,d}$	$0.0 \pm 0.5^{c,d}$
Malacosoma disstria	PBS	$1.9 \pm 0.5^{e,c}$	$0.4 \pm 0.3^{c,c}$	$14.5 \pm 0.8^{c,d}$	$0.0 \pm 0.5^{c,d}$
	Live-nematode exudate	$3.1 \pm 0.9^{f,c}$	$1.7 \pm 0.5^{d,c}$	$8.7 \pm 0.6^{f,d}$	$2.1 \pm 0.4^{d,c}$
	Dead-nematode exudate	$1.1 \pm 0.3^{e,e}$	$0.0 \pm 0.2^{e,f}$	$4.3 \pm 0.2^{h,g}$	$0.0 \pm 0.0^{c,c}$

[a] Mean ± standard error of the mean, n = 5 replicates each containing 10 individual larvae. Values within a column with the same left superscript are not significantly different ($P > 0.05$). Values between rows for a given bacterial species with the same right superscript are not significantly different ($P > 0.05$). [b]p.i. post-injection; *X. nematophila, Xenorhabdus nematophila; B. subtilis, Bacillus subtilis*; exudate, live-nematode exudate; dead exudate, dead-nematode exudate.

Table 2.3. Effect of exudates from different stages of *Steinernema carpocapsae* DD136 on the adhesion of *Galleria mellonella* larval haemocytes to glass *in vitro.*

Exudate source	Total adhering haemocytes (cells / mm^2) [a]	Immunosuppressive activity (%) [b]
PBS	176.1 ± 3.4 [e]	0 (0.0-1.8) [h]
Regular exudate with mixed nematode stages [c]	80.4 ± 2.7 [f]	53 (45.0-50.3) [i]
Centrifuged mixed nematode exudate [d]	96.6 ± 2.5 [f]	45 (39.2-48.9) [i]
Pooled J_4 stage and both sexes of adults	173.1 ± 3.1 [e]	2 (4.1-32.6) [h]
50% J_3 grown into J_4 stage nematodes	103.1 ± 2.7 [g]	42 (35.8-49.8) [i]
80% J_3 grown into J_4 stages nematodes	129.3 ± 2.8 [e]	26 (26.5-33.2) [j]
J_{1+2} stage nematodes	167.6 ± 4.2 [e]	3 (7.9-15.2) [i]

[a] Mean ± standard error of the mean, n=4. Values with the same superscript are not statistically different (P>0.05). [b] Percentage (with arcsin√P 95% confidence limits) of the degree of change in adhering haemocytes compared to those in the negative phosphate-buffered saline (PBS) control. Values with the same superscript are not statistically different (P>0.05). [c] Mixed stage nematodes rinsed from axenic liver agar media, centrifuged-washed and incubated in PBS for 24 h to produce regular exudate. [d] Pooled density-gradient fractions containing $J_{1,2,3+4}$ stage juveniles and both males and female nematodes.

Discussion

Nematodes in the insect host haemolymph often limit the innate non-self cellular responses by parasite-induced haemocyte lysis, suppression of haemocyte activity, the parasite possessing a non-adhesive surface or by the nematode surface not experiencing loss-of-self antigens and/or exhibiting molecular mimicry (Blaxter *et al*., 1992; Politz and Phillips, 1992). The latter event would not activate or impair the adhesive activities of the haemocytes to slides and particulates and thus was not part of detected immunosuppression by live nematodes in the present study. Herein, live axenic stages of *S. carpocapsae,* unlike dead nematodes, did not have haemocytes adhering *in vitro* or *in vivo.* This contrasts with dead monoxenic *S. feltiae* which were not encapsulated (Mastore and Brivio, 2008) in *G. mellonella.* In the present study live *S. carpocapsae* did not lyse haemocytes during short- or long-term exposure. The short-term results collectively imply that the nematode stages possess a non-adhesive lipoidal surface as proposed by Dunphy and Webster (1987) which would allow the host to respond to wounding and the innate immune system to contain adventitiously introduced micro-organisms. In the case of *S. feltiae* epicuticular lipids may have masked non-self recognition by acquiring host plasma proteins (Mastore and Brivio, 2008). Haemocyte adhesion to dead *S. carpocapsae* was not due to haemocyte activation factors released from freeze-damaged tissues through body orifices or damaged cuticle because the dead-nematode exudate did not enhance haemocyte binding to slides or accelerate bacterial removal from the haemolymph. Haemocytes adhering to the dead nematodes may reflect perturbations in the epicuticle caused by freezing including the release of possible surface immunosuppressive lipids similar to the surface limiting factors known for *S. feltiae* (Brivio *et al*., 2002, 2004, 2006, 2008) and *S. glaseri* (Wang and Gaugler, 1999). However, immunosuppressant release from the epicuticle seems unlikely since haemocytic suppressants were not detected in the exudate of the dead nematodes. Possibly killing the nematodes by freeze-thawing caused cuticular lipid and protein re-organization generating an adhesive surface by altering molecular mimicry or unmasking non-self antigens. Rapid freeze thawing is known to alter membrane lipid structure of the outer membrane of *Escherichia coli* (Souzu, 1989; Souzu *et al.*, 1989) and cell membranes of Dunning AT-1 prostate cancer cells (Bischof *et al.,*2002).

In the present study initiation of dead nematode encapsulation was associated with activation of phenoloxidase. Phenoloxidase and/or its byproducts, including extracellular matrix protein reactive and nematocidal radicals (Kamarov *et al*., 2005), in lepidopteran haemolymph (Shelby and Popham, 2006) facilitate melanotic encapsulation of immulectin-2-coated agarose beads by haemocytes (Ling and Yu, 2005). Also, phenoloxidase once discharged from stimulated haemocytes enhances antigen binding to lepidopteran granular cells and plasmatocytes (Giannoulis *et al*., 2007). Lipids from the cuticle of *S. feltiae* prevented phenoloxidase activation by depleting host interaction proteins precluding humoral encapsulation of *P. rigidus* (Brivro *et al*., 2002). Although in the present study, it is possible that live-nematode exudate may contain cuticular extracts limiting phenoloxidase activation, this seems unlikely since exudates from dead nematodes, which should have the same surface components as live nematodes, did not inhibit laminarin-induced phenoloxidase activation. Alternatively phenoloxidase release from the haemocytes, which is required for enzyme activation, may be inhibited by products released only from the metabolically active epicuticle of live nematodes. The inhibition of phenoloxidase activity in haemolymph with live-nematode exudate may explain part of the absence of haemocytes adhering to live *S. carpocapsae* and the diminished bacterial removal from the haemolymph *in vivo* by melanotic nodulation.

Because live-nematode exudate limited granular cell and plasmatocyte adhesion and increased granular cell detachment *in vitro*, an increase in total haemocyte counts and granular cells *in vivo* was anticipated and observed relative to PBS. For unknown reasons the changes in absolute magnitude of the haemocyte types varied with insect species. However, the percentage of change was similar for both species implying the same proportion of haemocytes exhibited differential sensitivity to the exudate, the granular cells being more sensitive than the plasmatocytes. The inability of the salient immunocytes, the granular cells and plasmatocytes, to adhere to slides once removed from insects injected with immunosuppressive exudate may indicate intracellular haemocyte modification limiting haemocyte function.

Haemocyte-antigen adhesion represents a complex of interactive humoral and cellular factors and activities (Schmidt and Schreiber, 2006). In *G. mellonella*, after antigen-haemocyte contact, the granular cells discharge extracellular sticky proteins (Ratcliffe and Gagen, 1977; Ashida and Brey, 1998), possibly collagen (Adachi *et al.,* 2005; Altincicek and Vilcinskas,

2008). Nardi *et al.,* (2001, 2005) and Zhuang *et al.*(2007) describe *Ma. sexta* granular cell activation, part of which involves the discharge of the extracellular cell adhesion matrix proteins lacunin and neuroglian and their binding to granular cells and subsequently plasmatocytes as both haemocyte types re-arrange their matrix protein binding receptors. RGD-dependent integrin and fibronectin receptors mediate lepidopteran haemocyte adhesion (Pech and Strand, 1995; Levin *et al.,* 2005, Nardi *et al.,* 2006; Zhuang *et al.,* 2007, 2008) including haemocytes of *G. mellonella,* the receptors participating in the phagocytosis of yeast cells (Wittner and Wiesner, 1996). Haemocyte integrins trigger apoptotic killing of *Onchocerca onchengi* microfilaria in the blackfly, *Simulium damnosum,* (Hagen and Kläger, 2001) but herein did not discernibly affect live *S. carpocapsae* in *G. mellonella* and *M. disstria.*

In the present study the live-nematode suppressive exudate, the product of nematode stage J_3, may have affected the adhesion events at two levels *in vitro.* At one level the differential inhibition of granular cells and plasmatocytes may indicate imperfect inhibition of the discharge of matrix proteins and zymogenic phenoloxidase. The latter is implied by the absence of phenoloxidase activity in haemocyte suspensions with live nematodes and their exudate. Phenoloxidase is released from the haemocyte types, the oenocytoids and spherulocytes (Schmidt and Ratcliffe, 1977, Cook *et al.*, 1985; Kurihara *et al.,* 1992; Ochiai *et al.*, *1992;* Lee *et al.,* 2005) and from young granular cells and plasmatocytes (Liing *et al.,* 2005), the latter two cell types participating in the adhesion based responses of phagocytosis (Tojo *et al.,* 2000) and nodulation and encapsulation (Ling and Yu, 2005). At the second level either alternatively or in concert with haemocyte discharge limitations, exudate from the live nematodes may have damaged the matrix proteins limiting the adhesion of granular cells and plasmatocytes and caused granular cell detachment from preformed monolayers. Why attached plasmatocytes did not detach is unknown. Boemare *et al.,* (1982) describe unidentified metabolites of axenic *S. carpocapsae* impairing non-defined *G. mellonella* antimicrobial defenses, proteases being the lethal agents (Laumond *et al.,* 1989). A plasmin-cathepsin-D-like enzyme that is released from steinernematids into *G. mellonella* haemolymph and is marginally affected by host protease inhibitors (Kučera and Mracek, 1989), may attack the matrix proteins of the granular cells more effectively than those of the plasmatocytes.

Immunosuppression of insect haemocytes in this study was established also *in vivo* by the higher concentrations of both bacterial species in larvae with live-nematode exudate compared to dead-nematode exudate and PBS at a given time p.i.. Higher bacterial numbers means less bacterial removal whether by haemocyte-based nodulation and/or humoral induced bacterial agglutination. In the present study bacterial agglutination was not seen in either the *in vitro* or *in vivo* control groups precluding it as a factor in bacterial removal and thus haemocyte-mediated nodulation may be the mechanism. The level of *B. subtilis* in PBS- and dead-nematode exudate-injected larvae equally decreased in both *M. disstria* and *G. mellonella* with incubation time which confirms the absence of either haemocyte activating or inhibiting factors in the dead-nematode exudate. Live-nematode exudate prevented haemocytic reactions limiting bacterial adhesion to haemocytes by either directly influencing haemocyte integrity, stimulating bacterial release from the haemocytes and/or by limiting phenoloxidase effects. Dead *X. nematophila* in the PBS-control insects and those with live- or dead-nematode exudate increased over time as reported for bacterial septicemia in *G. mellonella* (Dunphy and Webster, 1988) and *M. disstria* (Giannoulis *et al*., 2005). In both insect species dead *X. nematophila* induces apoptotic symptoms in the haemocytes due to the release of lipopolysaccharides from the bacterial outer membrane exceeding the endotoxin-neutralizing capacity of the humoral immune system of the larvae; haemolymph apolipophorin-III protein limits lipopolysaccharide toxicity (Dunphy and Halwani, 1997; Ma *et al*., 2006; Giannoulis *et al*., 2008). The lipopolysaccharides in *G. mellonella* bind to N-acetyl-D-glucosamine receptors on the haemocytes by means of the lipid A moiety of lipopolysaccharide (Dunphy and Webster, 1984, 1988) increasing the levels of damaged haemocytes and freeing bacteria associated with haemocytes (Dunphy and Webster, 1988). Both *X. nematophila* lipopolysaccharide and its lipid A suppress phenoloxidase activation (Dunphy and Webster, 1988) but in view of the lateness with which the bacteria emerge from the nematodes their contribution to protecting the monoxenic nematodes from haemocyte attack during early infection is unlikely. The nematode cuticle does not initially react with the insect haemocytic factors allowing time for the release of the exudate effectively shutting down the haemocytes and preventing phenoloxidase activity. The exudate may protect the nematode from encapsulation [should the cuticle eventually be modified by the host plasma enzymes (Dunphy and Webster, 1987)] ensuring the release of the *X. nematophila* into a noncompetitive environment. Live-nematode exudate limited haemocyte damage by *X. nematophila* by either

damaging the endotoxin-binding receptors, preventing endotoxin release from the bacteria or neutralizing lipopolysaccharide toxicity as it impairs bacterial removal from the haemolymph.

The present study determined that the J_3 stage of *S. carpocapsae* produced immune inhibitors. This is biologically sound because the other stages would be present later in development after or near the point of host demise (Poinar and Himsworth, 1969) long after the haemocytes and phenoloxidase are inhibited (Dunphy and Webster, 1984, 1985, 1986). A tentative infection model for *S. carpocapsae* would involve shedding the J_2 stage cuticle during gut invasion after which the epicuticle of the rapidly developing J_3 stage would passively avoid humoral and cellular responses without producing cytotoxic metabolites during the early stages of infection. Later development of J_3 would release metabolites that inhibit phenoloxidase and haemocyte activity allowing the nematodes to complete development into non-reproductive insecticidal axenic adults (Ehlers *et al.,* 1997) and for monoxenic nematodes, the release of entomocidal *X. nematophila*. This differs from monoxenically cultured *S. feltiae* in which epicuticular lipopolysaccharides binding to host plasma proteins blocks humoral activity (Brivio *et al.,*2004) and lipids bind host proteins masking the nematode from the haemocytes (Mastore and Brivio, 2008). Such differences establish that host-pathogen association and consequences are related to steinernematid species and/or culture protocols since the same insect species was used.

In summary, exudate released from the live J_3 stage of *S. carpocapsae* inhibited granular cell and plasmatocyte adhesion to foreign matter *in vivo* and *in vitro* and the detachment of granular cells from preformed haemocyte monolayers. *In vitro* the exudate prevented either the discharge of haemocytes containing phenoloxidase and/or phenoloxidase activation, both mechanisms explaining the absence of phenoloxidase activity in media with live nematodes. A decrease in haemocyte-induced removal of *B. subtilis* and *X. nematophila* was observed in larvae with live-nematode exudate but not those with dead-nematode exudate. Collectively these data explain the absence of haemocytic encapsulation of live as opposed to dead nematodes, haemocyte activation is blocked.

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CONNECTING STATEMENT 1

In chapter two, it was observed that live axenic *Steinernema carpocapsae* release exudate with immunosuppressant activity which inhibits haemocyte adhesion *in vitro* and limits bacterial removal from the haemolymph *in vivo*. The nature of possible exudate immunosuppressants was further investigated in terms of haemocyte suppression in chapter three.

CHAPTER 3

Components of *Steinernema carpocapsae* exudate and their impact on insect immunosuppression

A version of this chapter will be submitted to the Journal of Experimental Parasitology as follows,

Walter, T. N., Dunphy, G.B. and Mandato, C.A. 2008. Components of *Steinernema carpocapsae* and their impact on immunosuppression of *Galleria mellonella.*

Abstract

Initial characterization of the properties of the immunosuppressant(s) released by axenic *Steinernema carpocapsae* revealed weakly hydrophobic immunosuppressant(s) based on batch hydrophobic interaction chromatography. The immunosuppressant(s), although not affected by repeated freeze-thawing, were essentially destroyed at 65^{o}C. However, a 10% level of residual suppression remained in heat-treated exudate which was attributed to heat-stable LPS-like molecules. The possible presence of LPS-like molecules was confirmed by the reduction in exudate suppression using polymyxin B-conjugated agarose beads. Exudate treated with a cocktail of protease inhibitors immediately increased haemocyte adhesion to slides. With prolonged incubation of the exudate with the cocktail of protease inhibitors, adhesion increased without a change in total protein. However, there was a decline in the total number of protein bands and band intensity suggesting that non-enzymic proteins maybe involved. A decrease in total protein in the exudate incubated with trypsin and chymotrypsin-conjugated agarose beads but without a change in immunosuppressive activity indicated the presence of trypsin-like and chymotrypsin-like sensitive proteins in the exudate which did not affect haemocyte adhesiveness. Correlation analysis between exudate enzyme activities and level of adhering haemocytes implied other enzymes in addition to trypsin and chymotrypsin may participate in immunosuppression.

Index descriptors: *Steinernema carpocapsae, Galleria mellonella,* protease inhibitor, trypsin, chymotrypsin, Apizym, haemocyte inhibition.

Introduction

The Steinernematid insect pathogenic nematodes are effective and environmentally safe agents for controlling a variety of economically important pest insects (Dunphy and Thurston, 1990; Georgis and Gaugler, 1991; Samish and Glazer, 2001). The nematodes are symbiotically associated with the entomopathogenic bacterium of the genus *Xenorhabdus* (Poinar and Thomas., 1967; Boemare *et al*., 1993). The infectivity process of nemato-bacterial complex against the host antimicrobial defenses is understood for different species of *Steinernema* and several lepidopteran host species including the greater wax moth, *Galleria mellonella* (Akhurst and Dunphy, 1993; Dunphy and Webster, 1984; Ribeiro *et al*., 1999), and *Manduca sexta* (Li *et al*., 2007).

Steinernematid infective juvenile nematodes passing through the host orifices (Nguyen and Smart, 1990) by means of protease digestion of epithelial and muscle cells (Abuhatab *et al*., 1995), reach the host blood (haemolymph) after which it releases the phase one form of the Gram-negative γ-proteobacterium *X. nematophila* (Vivas and Goodrich-Blair, 2001) from the distal region of its receptacle (Synder *et al*., 2007). The nematode, as it rapidly transforms into the third stage (dauer) juvenile (Poinar and Himsworth, 1967; Poinar and Leutenegger, 1968; Grewal *et al*., 1997), encounters the humoral and cellular non-self factors of the haemolymph before it releases the bacteria, which occurs 4-5 h after parasitization (Dunphy and Webster, 1988; Yokoo *et al*., 1995, Wang *et al*, 1995). The symbiotic bacteria and/or toxic proteases produced by the axenic nematode in the insect blood contribute to insect death (Burman, 1982; Sicard *et al*., 2004; Kim *et al*., 2005; Park and Stanley, 2006; Sergeant *et al*., 2006). Although *Xenorhabdus* spp produce toxic enzymes and proteases (Bucher, 1960; Caldas *et al*., 2002), in view of its time of release from the nematode by haemolymph generated esophagealintestinal activity via the anus (Synder *et al*., 2007), the bacterium contributes to the pathology in the later infection stages. During earlier parasitization an immune

inhibitor excreted by axenic *S. carpocapsae* destroys induced antimicrobial proteins in immune haemolymph of *H. cecropia* both *in vivo* and *in vitro* (Götz *et al*., 1981), however, most research deals with cytolytic toxins produced *in vivo* by the monoxenic nematode in the more commonly encountered non-induced larvae (see Simões, 1992). A correlation exists between the production of toxic activity by axenic *S. carpocapsae* (Breton strain) in parasitized *G. mellonella* larvae and host death (see Simões, 1992). Ribeiro *et al*., (1999) describe proteases released from the nematode-bacterial complex, *S. carpocapsae-X. nematophila*, which lowers or suppresses the ability of haemocytes to stick to each other limiting the formation of capsules around nematodes and nodules about *X. nematophila*.

Insects usually respond in the initial stages of general infections with immediate innate humoral and cellular immune reactions (Götz and Boman, 1985; Hoffman *et al*., 1996; Bulet *et al*., 1999). In Lepidoptera, the main humoral factors include phenoloxidase (Shelby and Popam, 2006) [(which in many lepidopteran species is often released from the haemocyte type, the oenocytoids (Kurihara, 1997)], and the pattern recognition molecules lysosome (Wilson and Ratcliffe, 2000), apolipophorin III (Halwani *et al*., 2000), immulectins, (Ohta *et al*., 2006), hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002). Many of these humoral factors participate in cell-free immune responses but also elicit binding of the foreign materials to haemocytes (Schmidt and Schreiber, 2006). The major haemocyte types, the granular cells and the plasmatocytes carry out the salient innate cellular immune responses of lepidopterans to pathogens and parasites. These responses include phagocytosis (Tojo *et al*., 2000), and nodulation and encapsulation (Ratcliffe *et al*., 1985). The type and intensity of the reaction depends on the size, number and characteristics of the foreign bodies (Dunphy and Thurston, 1990; Rohloff *et al*., 1994) and is initiated by antigens reacting with both humoral and cellular pattern recognition receptors (PRR). PRR on haemocytes includes the peptidoglycan recognition protein (PGRP-LC) which binds to peptidoglycan and lipopolysaccharides [(an outer surface membrane component on most Gram-negative bacteria that strongly stimulates innate immune activity diversity of eukaryotic species ranging from

arthropods, including insects, to humans (Alexander and Rietschel, 2001)] (Werner *et al*., 2003; Kaneto *et al*., 2004). Other haemocytic PRR include Gram-negative bacteria-binding protein (DGNBP-1) which binds to LPS and β-1,3-glucan (Kim *et al*., 2000; Werner *et al*., 2000) scavenger receptor which binds to lipotechoic acid (Ramet *et al*., 2001) and C-type lectins which binds to LPS (Koizumi *et al*., 1999) and LTA (Yu *et al*., 2005).

Nodule initiation may vary with lepidopteran species. The granular cells, discharging extracellular matrix proteins, are responsible for initiating the nodulation process in *G. mellonella* (Ratcliffe and Gagen, 1977; Schmidt and Ratcliffe, 1977) whereas hyperphagocytic cells in *Ma. sexta* may act as the nodular nucleation center (Dean *et al*., 2004). In *Ma. sexta* the granular cell matrix proteins entrap alien materials as the clustering adhesion receptors on the haemocytes increase in number (Nardi *et al*., 2005). In Lepidoptera, haemocytes may exhibit interaction in that neither the granular cells nor plasmatocytes elicit encapsulation as occur with isolated haemocytes from the soybean looper, *Pseudoplusia includens* (Pech and Strand, 1995) but, in concert with plasmatocytes with neuroglian, both haemocyte types aggregate (Nardi *et al*., 2006). Noduler, a protein up-regulated in the haemolymph of *Antheraea mylitta* has been shown to bind to LPS, LTA and β-1, 3-glucan during nodulation and possibly part of the extracellular matrix (Gandhe *et al*., 2007). Calreticulin, thought to be an early stage encapsulation protein (Choi *et al*., 2002), binds to LPS (Takahashi *et al*., 2006) and may be part of nodulation since encapsulation is similar to nodulation and refers to haemocyte aggregation around larger pathogens like parasitoids and nematodes (Gandhe *et al*., 2007). Cellular encapsulation, as opposed to nodulation, entraps foreign antigens too large to be phagocytosed (Lavine and Strand, 2003) as observed for pathogenic nematodes (Jackson and Brooks, 1989; Wang *et al*., 1994; Cruz *et al*., 2001). The haemocyte type, the spherulocytes, also releases antigen-trapping proteins (Cook *et al*., 1985) that have defensive abilities (Horohov and Dunn, 1982). The plasmatocytes wall-off the granular cell-antigen aggregates forming nodules within which form antibacterial

melanotic by-products such as 5, 6-dihydroxyindole (Shelby and Popham, 2006; Zhao *et al*., 2007) and toxic radicals (Nappi and Vass, 1998).

Encapsulation types vary with the insect species even when the same macroantigen is used. Humoral encapsulation [(innate, non-cellular immunity involving melanin layer(s) produced about foreign materials by means of the adhesion of the enzyme phenoloxidase (Götz, 1986)] of *S. carpocapsae* nematode occurs in larvae of the cranefly, *Tipula olaraceae* (Peters, 1988), while cellular encapsulation of *S. carpocapsae* results in the house cricket, *Acheta domestica* (Wang *et al*., 1994), the armyworm, *Pseudaletia unipuncta* (Cruz *et al*., 2001), and the Japanese beetle, *Popillia japonica* (Wang *et al*., 1994). Despite encapsulation in these cases, insect death occurs because bacterial release from the nematode is ongoing before encapsulation is completed (Wang *et al*., 2004). The occurrence and extent of nematode cellular encapsulation varies with the insect species e.g. in *A. domestica* and *P. japonica*, infecting *S. glaseri* and *S. scapterisci* are not encapsulated because they either evade cellular immune responses or avoid non-self recognition, each mechanism allowing bacterial release leading to rapid host death (Wang *et al*., 1994). Although host recognition underlies some of the differences between resistant and susceptible host species, nematode species that are usually encapsulated in an insect species may avoid haemocyte attack following recognition if another non-recognized nematode species or its cuticular structural components co-exist in the host, e.g. co-injected surface-coat proteins from *S. glaseri* protects normally encapsulated *H. bacteriophora* in *Exomala orientalis*, the oriental beetle larva (Li *et al*., 2007). However, *H. bacteriophora* is not protected by these proteins in *Ma. sexta*. Surface coat proteins do not universally convey host susceptibility (Li *et al*., 2007). In insect hosts where no nematode encapsulation occurs, the nematode may be recognized as self, passively avoid non-self recognition or the host immune system maybe actively impaired (Dunphy and Webster, 1987; Brehélin *et al*., 1994; Mastore and Brivio, 2008).

The pathogenicity of the *X. nematophila*-*S. carpocapsae* complex is correlated with toxic factors (e.g. proteases) released by the nematode and subsequently by *X.*

nematophila into the insect haemocoel, resulting in a synergistic action of both participants of the complex in *G. mellonella* (Boemare *et al*., 1982; Burman, 1982). Following invasion of an insect haemocoel by the nematode, the released bacteria produce a myriad of extracellular substances during multiplication (e.g. proteases, lipases, lecithinases, antibiotics and lipopolysaccharides (Dunphy and Webster, 1988 a, b; Schmidt *et al*., 1988, Boemare and Akhurst, 1998; Clarke and Dowds, 1995), some of which inhibits antimicrobial peptides synthesis (Caldas *et al*., 2002; Brivio *et al*., 2006).

The physiochemical lipid nature of the epicuticle of the nematode, which is the interface between the nematode and the humoral and cellular immune responses of the host haemolymph, influences haemocyte activity in several pest insects including, *G. mellonella* to *S. feltiae* [(= *S. carpocapsae*; Kaya and Gaugler, 1993) (Dunphy and Webster, 1987)]. Similarly, that live or dead monoxenic *S. carpocapsae* are not encapsulated in the larval stage of the turnip moth, *Agrotis segetum* (Yokoo *et al*., 1992) nor the live or dead monoxenic *S. feltiae* in *G. mellonella* (Mastore and Brivio, 2008) suggesting cuticular factors limit recognition. Cuticular lipids of *S. feltiae*, by binding a variety of the insect haemolymph molecules, produce a coat around the nematode, disguising the pathogen against *G. mellonella* haemocytes recognition (Mastore and Brivio, 2008). Epicuticular extracts of *S. feltiae* affect also the humoral (phenoloxidase) and cellular defenses (Brivio *et al*., 2002). Partially purified lipopolysaccharide-like molecules from *S. feltiae* cuticle sequester lipopolysaccharide-binding proteins present in the host haemolymph limiting prophenoloxidase activation, a process which is thus responsible for a molecular disguise strategy against cellular encapsulation (Brivio *et al*., 2004). Other molecular species such as SCP3A, a surface coat protein from axenic *S. glaseri*, suppresses the host haemocytic immune system of *P. japonica,* protecting unrelated and thus usually readily encapsulated nematode species from haemocyte attack and latex beads from phagocytosis (Wang and Gaugler, 1999).

Axenic *S. carpocapsae* are known to produce toxin(s) that destroy induced antibacterial cecropins of diapausing *Hyalophora cecropia* pupae (Götz *et al*., 1981). The axenic nematodes, in both the haemolymph and in growth medium, produce proteins and

protease which cause insect death after a few hours post-injection (Laumond *et al*., 1989; Simões and Rosa, 1996) while in nutrient rich medium, monoxenic nematodes barely release their bacterium (Snyder *et al*., 2007) indicating that toxin release by the axenic nematode is independent of bacterial release. Kucera and Mracek (1989a) partially purified a 90 kDa-plasmin-cathepsin D-like enzyme from axenic *S. kraussei* with an optimum pH of 5.0, which is pathogenic for larval *G. mellonella* (Kucera and Mracek, 1989b). The role of these cidal toxins and enzymes in immunity is not known.

In chapter two, I observed that live-nematodes as opposed to dead nematodes are not encapsulated and that metabolites from live J_3 stage *S. carpocapsae* but not dead axenic *S. carpocapsae* inhibited the adhesion of granular cells and some plasmatocytes to slides, increased granular cell but not plasmatocyte dissociation from preformed haemocyte monolayers and *in vivo* elevated total haemocyte counts changing the haemocyte types while impairing bacterial removal from the haemolymph of both larval *G. mellonella* and the forest tent caterpillar, *M. disstria*. The results imply that immunosuppressive activity by live nematodes represents the release of inhibitor(s) that may not be physically associated with the nematode epicuticle. As suppression of haemocyte adhesion increased, total protein increased indicating that the immunosuppressant maybe proteineous in nature (chapter 2). Herein, the partial identity of possible suppressants in the nematode exudate is addressed. Other potential immunosuppressant(s) candidates include lipopolysaccharide-like molecules and lipids. It is also considered that the immunosuppressant(s) in live-nematode exudate may predominantly be protein(s) either without or with enzyme activity. Putative suppressants will be tested for their anti-adhesion effects on the haemocytes of the pest insects, *G. mellonella* and *M. disstria* with emphasis on *G. mellonella,* unless otherwise stated.

Materials and Methods

Nematode culture

Monoxenic cultures of *S. carpocapsae* DD136 (Agriculture Canada, St. Jean sur le Richelieu, Canada) were established using dauer juveniles collected from infected *G. mellonella* larvae (Poinar and Himsworth, 1967) on White's water traps (White, 1927). Briefly, infective juveniles were gravity-washed three times in sterile distilled water (10 ml) in 15 ml sterile conical centrifuge tubes, disinfected in thimersol (10 ml, 0.4% w/v distilled water) for 20 min and aseptically gravity-washed in sterile, distilled water (Dunphy and Webster, 1987) before plating on Wouts' lipid agar (Wouts, 1981). Prior to inoculating with nematodes, Wouts' lipid agar had been inoculated with the phase one form of *X. nematophila* [(the form isolated from the infective juvenile (Akhurst, 1980)] and incubated in darkness at 27°C for 24 h producing a bacterial lawn upon which the pathogens feed. The nematodes were subcultured every two weeks to fresh bacterial lawns.

Axenic nematodes were obtained from eggs by dissolving gravity-washed gravid females in 10 ml axenizing solution [0.4 M NaOH, 0.2 M $NaHClO_4$ (Popiel *et al*., 1989)] for 10 min. The suspension was centrifuged (1000 x g, 1 min, 25°C) in 1.5 ml sterile microcentrifuge tubes and the pellet resuspended in fresh sterilizing solution (1 ml) for 10 min, after which only nematode eggs remained. The eggs, rendered free of this solution by centrifugation (500 x g, 25°C, 10 min) and resuspension three times in 1 ml of filter-sterilized PBS [135 mM NaCl, 2.7 mM KCl, 8.0 mM H_2PO_4, 1.5 mM KH_2PO_4, adjusted with 1 M HCl to pH 6.5 (Giannoulis *et al*., 2007)], were allowed to hatch in PBS (5 ml) for 16 h at 25°C (Popiel *et al*., 1989). The resulting juveniles were aseptically washed by centrifugation (hereafter described as centrifuge-washing) and resuspension in sterile distilled water, and then placed on sterile liver agar (Dunphy and Webster, 1986) supplemented with filter-sterilized antibiotics [100 µg/ml medium, gentamycin sulphate; 100 µg/ml medium, streptomycin sulphate and 20 µg/ml medium, kanamycin sulphate

(Wang and Gaugler, 1999)]. Cultures were kept at 25°C and subcultured to fresh antibiotic-supplemented sterile liver agar every three weeks.

The bacterial free status of nematodes was confirmed by incubating the pathogens in Luria broth (5 ml) in 25 ml scintillation vials on a horizontal gyratory shaker (250 rpm) for 72 h at 25°C and 30°C after which 10 µl aliquots were plated on Luria agar and incubated at 25°C and 30°C for 72 h. These temperatures were selected to increase the chances of detecting isolates of *X. nematophila* and other possible bacterial species with different temperature preferences surviving axenizing protocols. The absence of a change in broth turbidity (measured spectrophotometrically at 660 nm) and colony formation on the agar medium indicated axenic nematodes.

Axenic nematode excretion/secretion product (exudate)

Live and freeze-killed (-20°C, 24 h) mixed stage nematodes (J_{1+2} = 100, J_3 = 580, J_4 = 120, adults = 45 females and 65 males) previously rinsed from liver agar were used to obtain excretion/secretion metabolites (exudate). The axenic nematodes were centrifuge-washed twice in sterile distilled water (1 ml) with a final resuspension to 1000 nematodes per ml of PBS. After incubating 5 ml of suspension in 25 ml sterile beakers (capped with sterile paraffin) at 25°C on a horizontal gyratory shaker (100 rpm) for designated times (0.5-24 h), the nematodes were removed by centrifugation (1000 x g, 2 min, 25°C) and the resulting exudate frozen (-80°C) until used. Exudate sterility was based on the absence of change in turbidity of Luria broth (5 ml) in 20 ml scintillation vials that had been inoculated with exudate (20 µL) and incubated for 72 h at 25°C and 30°C as previously described.

Insects

G. mellonella larvae were reared on a multigrain diet supplemented with glycerol and vitamins at 30°C (Dutky *et al*., 1962) under constant light. Fifth instar larvae weighing 200 ± 10 mg were used. Laboratory reared *M. disstria* (supplied by the

Canadian Forestry Service, Sault Ste. Marie, Ontario) were maintained on a casein dextrose diet at 25°C (Addy, 1969). Seventh instar larvae, 3 days into the stadium and weighing 550 ± 30 mg (Etilé and Despland, 2008), were used unless otherwise state.

Characterization of immunosuppressive properties of exudate proteins

Hydrophobic interaction chromatography

Cuticular lipids, lipopolysaccharides (LPS) and proteins are alluded to have immune inhibiting activities in lepidopteran larvae (Boemare *et al*., 1982; Dunphy and Webster, 1987; Brivio *et al*., 2002, Mastore and Brivio, 2008) many of which are hydrophobic. Initial characterization of the immunosuppressant(s) in the exudates was thus based on batch hydrophobic interaction chromatography. The exudate was treated with hydrophobic beads that differed in their hydrophobicity, herein listed in descending order of grouped hydrophobicity; highly hydrophobic HiTrap phenyl sepharose HP, HiTrap phenyl FF (high substitution) sepharose and HiTrap phenyl FF (low substitution) sepharose, moderately hydrophobic HiTrap octyl FF sepharose, and HiTrap butyl FF sepharose with low hydrophobicity (Amersham Bioscience, Canada; 1 ml). The beads had been previously centrifuge-washed three times with PBS (1000 xg, 25^{o}C, 10 sec) to remove fines. Aliquots (25 µl) of the hydrophobic beads were incubated with 50 µl of the nematode exudate in 1.5 ml microcentrifuge tubes for 30 min on a horizontal gyratory shaker (100 rpm, 25^{o}C). The control consisted of aliquots (25 µl) of the hydrophobic beads incubated with of PBS (50 µl) in 1.5 ml microcentrifuge tubes. After centrifugation (1000 xg, 25^{o}C, 10 sec) the supernatant was divided into two parts; one part was used to test for the suppression of haemocyte monolayer formation. Monolayers were formed using haemocyte suspensions of 60 µl and 120 µl of haemolymph from six chilled (5°C, 10 min) larvae of *G. mellonella* and *M. disstria,* respectively, in PBS (1 ml, 4°C). Ten and 20 µl of aliquots of the former and latter suspensions were added to slides (previously rendered endotoxin-free by heating to 350°C for 24 h) containing 10 and 20 µl of exudate,

respectively, in a 95 mm^2 area. Different volumes of materials were used between insect species to ensure the inherent differences in total haemocyte counts resulted in similar numbers of haemocytes being used in the experiments to preclude possible results being due to different haemocyte concentrations (Walter *et al*., 2008). The haemocyte suspensions on the slides were incubated for 30 min [the time for maximum haemocyte adhesion for both insect species (Zakarian *et al.,* 2002; Giannoulis *et al.,* 2005)] at 27 °C and 95% RH. Subsequently the slides were rinsed three times with PBS (2 ml) to remove non-adhering haemocytes. Attached haemocytes were fixed in glutaraldehyde-formaldehyde vapour and mounted in 20% (v/v) glycerol in PBS. The total numbers of adhering haemocytes were determined by phase contrast microscopy. The second protocol consisted of subjecting the supernatant and the protein on the resin beads to sodium dodecyl sulphate polycrylamide gel electrophoresis using 12% polycrylamide separating gel (Laemmle, 1970). Changes in the intensity of the protein bands were determined using Coomasie blue stain. Protein was extracted from the beads using the electrophoretic solubilizing buffer.

Hydrophobic interaction chromatography may also react with lipids (Fischer, 1991; 1996) and heat-stable, LPS-like molecules (Fischer, 1990) in *S. carpocapsae* exudate. The possible involvement of immunosuppressive lipids was tested by extraction of exudate (1ml) with 2 ml of chloroform in 20 ml volume scintillation vials. Magnetic flea stir bars were used to ensure mixing of exudate and chloroform for 1 h at 25^{o}C after which solvent separation was encouraged by centrifugation (14000 xg, 4 min, 25^{o}C). The chloroform phase was removed and evaporated in a 15 ml Pyrex test tube in a 50^{o}C water bath using N_2 gas. The lipids were suspended in PBS (1 ml) using ultrasonication for 60 sec. Thereafter the suspension was frozen (-80^{o}C) until used. Prior to use, samples were ultrasonicated for 1-5 min while chilling in an ice bath.

The collected chloroform-deproteinized aqueous layer was bubbled with N_2 to remove residual chloroform and stored at -80^{o}C. Chloroform treated PBS was used as the negative control. Deproteinized-lipid extract (which would indicate the possible involvement of proteins), lipid extracts and negative controls were assayed for

immunosuppression as previously described and compared with the positive control, whole nematode exudate.

Thermal sensitivity of immunosuppressant in exudate

Hydrophobic interaction chromatography established the suppressants to be hydrophobic, and chloroform extraction indicated that lipids were not involved and that proteins were largely contributing to immunosuppression. I sought to determine if proteins were the immunosuppressants by determining the thermal sensitivity of the immunosuppressant(s). Cold tolerance of the immunosuppressant activity was based on repeatedly freeze (-80^{o}C)-thawing (25^{o}C) exudate (50 µl) several times. Heat stability was determined by incubating 50 µl of exudate in 1.5ml microcentrifuge tubes for 1 h in water baths set at 25^{o}C, 35^{o}C, 50^{o}C, 65^{o}C and 90^{o}C. The negative controls contained PBS only at these temperatures. The resulting freeze-thawed and heated exudates were then tested for immunosuppression of haemocyte adhesion to slides using haemocyte monolayers as previously described.

Cold tolerance and heating implied the suppressant(s) could be either non-enzyme proteins or enzymes. The involvement of proteins was further explored by incubating exudate at room temperature and -80 ^{o}C, for 48 h and measuring the suppressant activity remaining based on the total number of adhering haemocytes on slides. Non-digested exudate total protein concentration was determined using the Comassie Bio-Rad (Bio-Rad Labs. Ltd) assay. The protein assay consisted of nematode exudate (8 µl) being diluted in 152 µl of PBS to which Bio-Rad reagent (40 µl) was added, the resulting samples were read on a plate reader at 595nm. Bovine serum albumin (Sigma Co.) was used as the protein standard.

The exudate might contain heat-stable, phenoloxidase-inhibiting, LPS-like molecules (Brivio *et al*., 2004) which would explain the residual suppressive activity in heated exudate detected in this study. The heat stable molecules in the exudate were extracted using polymyxin B-conjugated agarose heads (Sigma Co.). The nona-peptide

antibiotic, polymyxin B, which binds to hydrophobic molecules (Wise *et al*., Zhong *et al*., 2008) binds to the lipid A moiety of LPS (Vaara and Vaara, 1983; Moore *et al*., 1986), effectively linking LPS to the beads. Prior to use, the beads were exhaustively centrifuge (250 xg, 30 sec, 25^{o}C)-washed in PBS. The beads (5 µl) were added to 250 µl aliquot of exudate in 1 ml volume (24 wells) tissue culture plates producing a bead concentration ranging from 0 to 1 x 10^4 beads/ml. Control PBS was inoculated with 1 x 10^5 beads/ml. This control had the most beads to detect possible dissociation of polymyxin B from the agarose beads; the drug is known to activate sheep B-cells (Smith and Hammar-Ström, 1978) and murine cytolytic natural killer cells (Zhong *et al*., 2008) and damage lepidopteran haemocytes (Giannoulis *et al*., 2008). After incubation at 25^{o}C on a horizontal gyratory shaker (250 rpm) for 1 h, the material was centrifuged (12,000 xg, 30 sec, 25^{o}C) and the supernatant, separated from beads with potential polymyxin B-reactive molecules, was frozen at -80^{o}C until samples and controls were assayed for immunosuppression of haemocyte activity as previously described. The positive control consisted of whole exudate.

Effect of trypsin, chymotrypsin and protease inhibitors on nematode exudate

To determine if exudate proteases may be directly reacting with and inhibiting haemocytes adhesion or indirectly by digesting non-enzymic proteins affecting immunosuppressant activity, fresh exudate was divided into two portions; the first contained exudate (50 µl) incubated with 40 µl of a dimethyl sulfoxide (DMSO) solution containing a cocktail of protease inhibitors [4-(2-aminoethyl) benzenesulphonyl fluoride, 104mM; aprotinin, 0.08mM; leupeptin, 2mM; bestatin, 4mM; pepstatin A, 1.5mM and E. 64; 1.4mM (Sigma)]. The cocktail is a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine-, and aspartic-proteases and aminopeptidases (Dye *et al*., 2005). These enzyme groups have been found in bacteriophagous *Caenorhabditis elegans* and vertebrate parasitic nematodes, *Strongyloides stercoralis* (Blaxter *et al*., 2000; Tcherepanova *et al*., 2000), both genera

showing evolutionary affinity with Steinernematids (Tadeusz and Stefanski, 1999), thus the use of this cocktail inhibitor was justified. The second portion was non-inhibited exudate (50 µl) with DMSO (40 µl) as control. The samples were incubated at room temperature for 0, 24 and 48 h. The following parameters measured for both experiments included: haemocyte attachment, total protein and changes in protein bands, the latter determined by sodium dodecyl sulphate polycrylamide gel electrophoresis using 12% polycrylamide as a separating gel (Laemmle; 1970, see Hydrophobic Interaction Chromatography).

Herein the observed decline in total protein was linked to a decline in haemocyte adhesion and inhibition of the aforementioned protease types, leading to the possibility of non-enzymatic protein immunosuppressants that are sensitive to serine protease digestion. This was determined by incubating the exudate with trypsin or chymotrypsin-conjugated to agarose beads (Sigma, Co.). Ten µl of beads previously centrifuge-washed (1000 xg, 25^{o}C, 10 sec) three times in Ca^{2+} (10mM)-supplemented PBS [(pH 6.5 (Giannoulis *et al*., 2007)] and resuspended in 25 µl of the same buffer were added to exudate (50 µl) producing a final enzyme activity of 100 units which was at least 20 fold in excess of the maximum activities of these proteolytic enzymes in the exudate (determined by Apizym analysis). Results were compared to exudate diluted with PBS. Negative control samples contained beads in the buffer to determine if trypsin and/or chymotrypsin dissociated from the beads inhibiting haemocyte attachment when compared to haemocyte adhesion in PBS only. After incubation on a horizontal gyratory shaker (100 rpm, 30^{o}C) samples were removed, centrifuged (1000 xg, 25^{o}C, 10 sec) to remove the beads and the supernatant analyzed for both immunosuppression using the haemocyte monolayers assay and total protein.

Apizym analysis

Because protein degeneration was associated with a decline in exudate suppression (thus study) and proteases and N-acetyl-D-glucosaminidase have been

implicated in undefined immunosuppression of *G. mellonella* haemocytes by *S. carpocapsae* (Simões *et al*., 2000), an APIZYM (Biomerieux), semi-quantitative micro assay (described in the following paragraph) was used to determine the enzymatic activity in the exudate produced by incubating the axenic nematodes in PBS for various times. The Apizym test has substrates designed to detect the activity of the following 19 hydrolases: alkaline phosphatase, acid phosphatase, esterase, esterase lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β glucosaminidase, α-mannosidase. The APIZYM strip is composed of 20 microtubes, the bottom of which contains the enzymatic substrates and buffer salts. The humidifying incubation tray with 5 ml of distilled water contained the test strips. Aliquots (65 µl) of exudate obtained from nematodes incubated for 6 h incubation (time suppression was first observed), 12 h and 24 h (times maximum suppression was observed) were placed in each well. The first well served as a control since it has no substrate. A lid was placed on the tray and the assembly was incubated for 4 h at 25^{o}C. After the incubation period, one drop of developer ZYM A was added into the well containing the exudate, immediately followed by one drop of developer ZYM B. The strip was further incubated for 5 min to allow the colours to develop. A value ranging from 0-5 was assigned corresponding to the developed colours as per the API ZYM colour chart recommended by the manufacturer. Enzyme activity was recorded as nmoles of substrate hydrolysed/4 h/ 580 J_3 nematodes. Adhesion effects of the exudates on the total number of adhering haemocytes were determined and used in product-moment correlation analyses (Sokal and Rohlf, 1969) with enzyme activities to determine possible relationships between enzymes in exudate and suppression of haemocyte adhesion.

Effect of commercial trypsin and chymotrypsin on *G. mellonella* haemocytes *in vitro*

Based on the findings of the correlation analysis of the APIZYM data and the use of a cocktail of protease inhibitors establishing the presence of trypsin and chymotrypsin

in the nematode exudate, and chymotrypsin gene sequenced from *S. carpocapsae* (Lucerna *et al*., 2007), it is possible that these enzymes have an effect on the haemocytes of *G. mellonella*. This was determined by dissolving the 1 mg of commercial trypsin (Sigma Co.) and chymotrypsin (Sigma Co.) in $CaCl_2$ (50mM)-Tris (100 mM) buffer (pH 6.5). The enzymes were adjusted by dilution with the buffer to10 nmoles/ml PBS to reflect the amount of both enzymes from the Apizym analysis. The treatments consisted of the following: 10 µl of either enzyme incubated with 10 µl of *G. mellonella* diluted haemolymph; 10 µl of trypsin plus 10 µl of chymotrypsin co-incubated with 20 µl of *G. mellonella* haemolymph; the negative control consisted of 10 µl of PBS with 10 µl of haemolymph, while the positive control consisted of 10 µl nematode exudate with 10 µl of haemocytes for 30 min. The treatments and controls were assayed for immunosuppression of haemocyte activity as previously described.

Statistics

All graphic data represent the mean ± standard error of the mean, of at least10 temporally separated replicates, from each of which 5-10 individual samples were taken for analysis. A significance α level of 0.05 was chosen. Haemocyte counts were analyzed using the 95% confidence limits overlap protocol (Sokal and Rohlf, 1969). Percentage data were analyzed using arc sin √ p-transformed data. Tabulated mean differential counts and other mean percentage data in the text were presented as the decoded mean (with 95% confidence limits of the transformed data).

Results

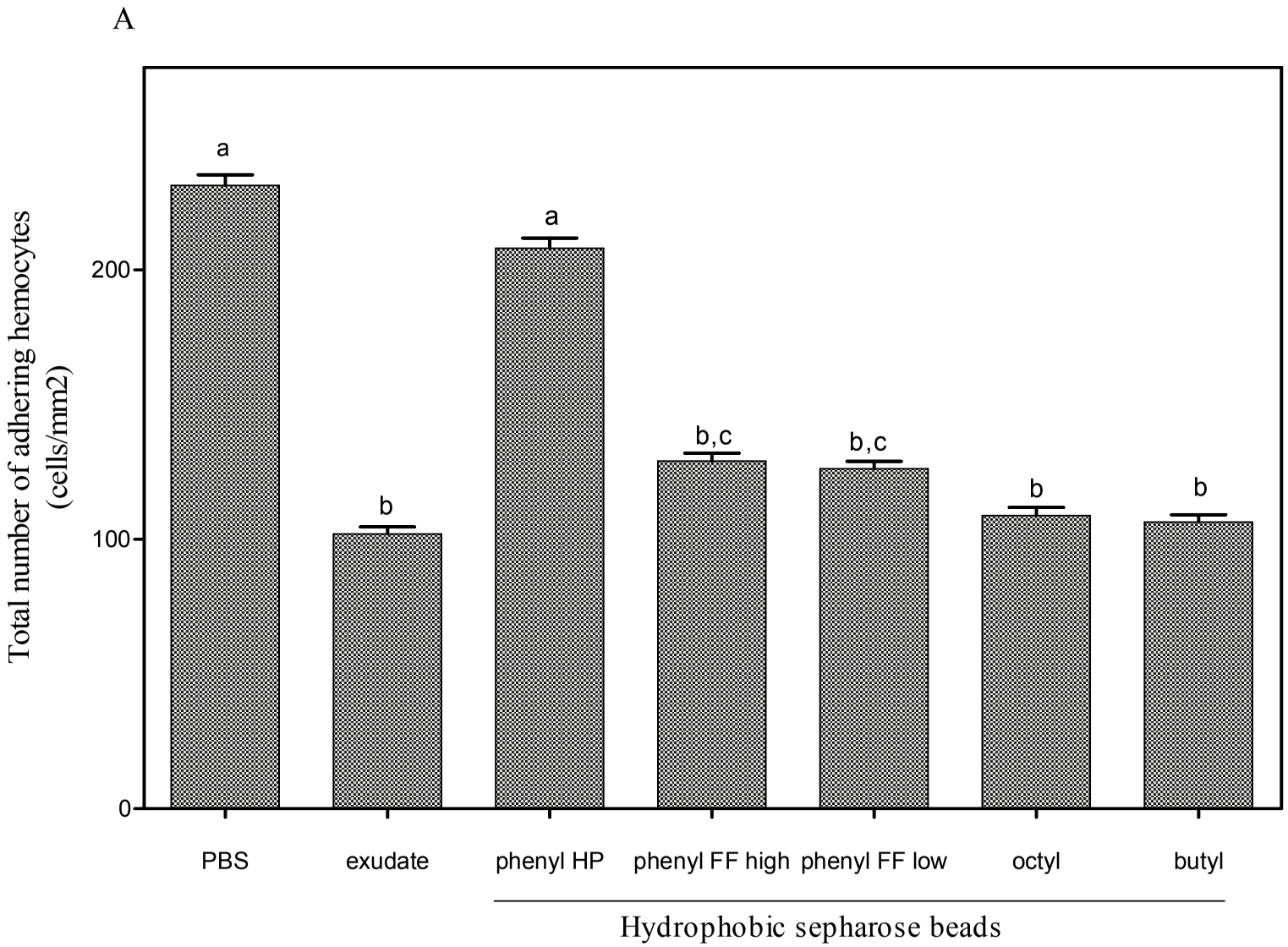
Characterization of immunosuppressive properties of exudate

Hydrophobic interaction chromatography

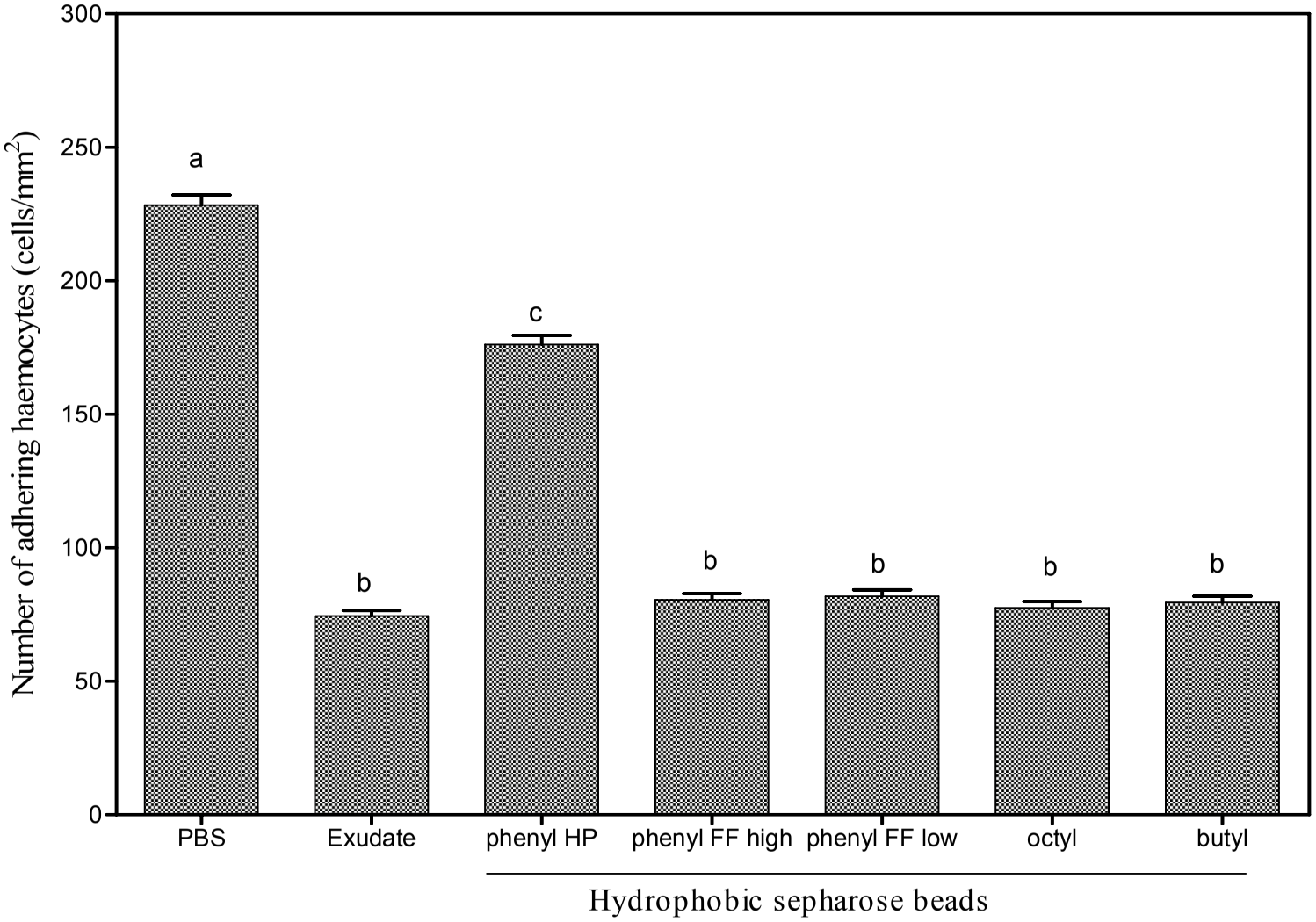
The strongly hydrophobic resin with the larger total surface area, phenyl high performance (HP) sepharose bead, produced supernatants with low immunosuppressive activity based on a comparison of the numbers of adhering haemocytes with treated exudate and the non-inhibiting PBS control ($p>0.05$) which were elevated compared with suppressive exudate (Fig. 3.1A). Exudates incubated with equally hydrophobic but smaller interactive surfaces of phenyl fast flow high substitution, phenyl fast flow low substitution, and with moderate hydrophobic octyl agarose beads and least hydrophobic butyl resin beads, resulted in supernatants containing significantly more haemocyte-adhesion inhibitory activity compared with phenyl HP agarose ($p<0.05$) and the PBS negative control ($p<0.05$). Inhibition of haemocyte adhesion with exudate modified by the least hydrophobic beads was comparable to those with the positive non-treated exudate control ($p>0.05$). Approximately 10% suppression of *G. mellonella* haemocyte adhesion to slides remained in the exudate treated with the phenyl HP sepharose beads compared to the PBS control. Comparable results were seen with *M. disstria* haemocytes (Fig 3.1B), the percentage change between the levels of adhering haemocytes being marginally more affected by the immunosuppressant(s) with approximately 15% suppression of haemocyte adhesion remaining in exudate incubated with phenyl HP beads when compared to non-suppressive PBS control groups.

Changes in the intensity of the proteins bound to different beads showed that the phenyl high performance (HP) beads, which dramatically lowered the inhibition of haemocyte adhesion, had the most bound exudate proteins (Fig. 3.1C), compared to the other hydrophobic resin beads (Fig. 3.1C). The major proteins adhering to phenyl HP beads included those with molecular weights 58.4 kDa, 49.7 kDa

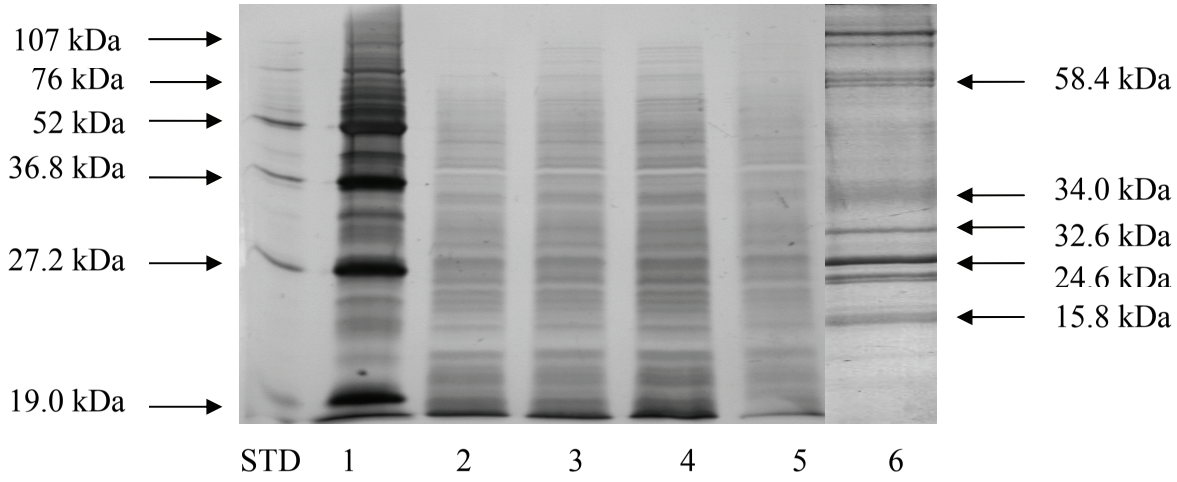
Figure 3.1 Effect of batch hydrophobic interaction chromatography on exudate of *Steinernema carpocapsae* in terms of the total number of haemocytes adhering to glass slides using haemolymph from (A) *Galleria mellonella* and (B) *Malacosoma disstria*. Values represent the mean ± SE, $n \geq 10$. Values with same letters are not significantly different ($p<0.05$). (C) Binding of the proteins to beads of different hydrophobicity and substitution values [molecular weights of bound proteins on phenyl HP (right side) represent the mean of three replicates]. (D) Proteins remaining in the exudate after incubation with the hydrophobic beads.


A
Total number of adhering hemocytes (cells/mm2)
0
100
200
a
b
a
b,c
b,c
b
b
PBS
exudate
phenyl HP
phenyl FF high
phenyl FF low
octyl
butyl
Hydrophobic sepharose beads

B


Number of adhering haemocytes (cells/mm2)
300
250
200
150
100
50
0
a
b
c
b
b
b
b
PBS
Exudate
phenyl HP
phenyl FF high
phenyl FF low
octyl
butyl
Hydrophobic sepharose beads

C



STD = standards

1 = phenyl HP sepharose,

2 = phenyl FF high substitution sepharose,

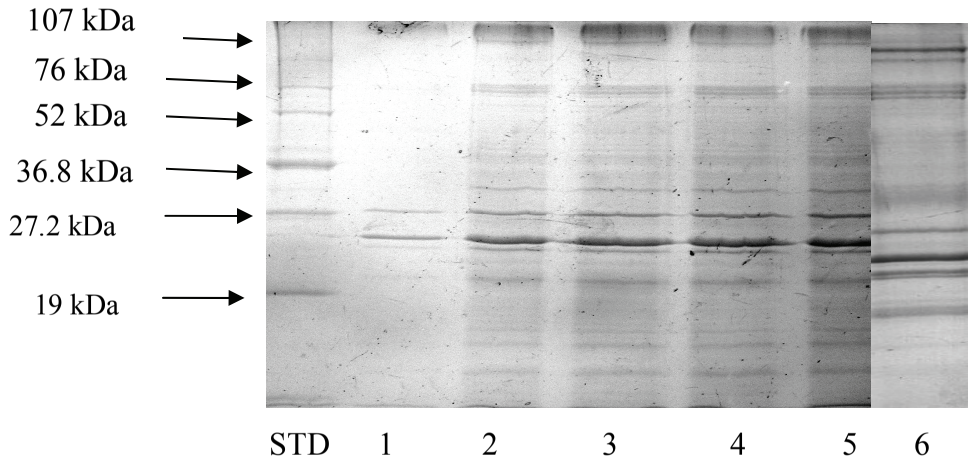
3 = phenyl FF low substitution sepharose,

4 = octyl sepharose,

5 = butyl sepharose

6 = diluted exudate

D



STD = standards

1 = phenyl HP sepharose,

2 = phenyl FF high substitution sepharose,

3 = phenyl FF low substitution sepharose,

4 = octyl sepharose,

5 = butyl sepharose,

6 = diluted exudate

and the medium sized proteins had molecular weights of 34.0 kDa, 32.6 kDa and 24.6 kDa [the latter being approximately equal to the molecular weight of bovine chymotrypsin, (25 kDa) or bovine trypsin, (24 kDa)]. The lowest molecular weight of a major band was 15.8 kDa. The phenyl high performance beads left a low level of haemocyte inhibition in the exudate (Fig. 3.1 A and 3.1B), and decreased the number and intensity of proteins bands in the exudate supernatant (Fig. 3.1D). The hydrophobic interaction chromatography results did not preclude lipids and related molecules as possible binding suppressants.

Effect of chloroform on nematode exudate

To determine if fatty acids and other chloroform-soluble lipid classes and/or peptides had suppressive activity, exudate was extracted with chloroform. Treatment of the exudate with chloroform separated the exudate into two fractions: the lipid fraction and the aqueous fraction. Chloroform essentially removed exudate protein in the aqueous phase to barely detectable levels (0.07 ± 0.03 μg/ml). The lipid fraction had no significant effect on haemocyte adhesion of *G. mellonella*, adhesion being similar to the PBS control ($p>0.05$) (Fig. 3.2) which favored haemocyte attachment in excess of haemocyte adhesion in exudate ($p<0.05$) (Fig. 3.2). Thus lipids did not affect haemocyte adhesion. The delipided and deprotinized aqueous exudate exhibited low inhibition implying proteins maybe major immunosuppressants. In lipid and protein-free exudate, a small level of inhibition remained, approximately10% of exudate inhibitory activity.

Thermal sensitivity of immunosuppressant in exudate

Exudate freeze-thawed several times compared with the fresh exudate (positive controls) did not alter ($p>0.05$) the number of total adhering haemocytes of either insect species, the counts always being equally less than haemocyte counts in the PBS controls (Fig 3.3 A, B) establishing the suppressant(s) as freeze-thaw robust. Both the total haemocyte counts and adhering granular cell levels were approximately 50% less than the

Figure 3.2 Effect of chloroform extraction on the exudate of *Steinernema carpocapsae* in terms of the total number of adhering haemocytes of *Galleria mellonella*. The lipid extract and aqueous fractions of the de-lipided and protein-denatured exudate were compared to PBS (negative control) and exudate (positive control). Values represent the mean ± SE, $n \geq 10$.

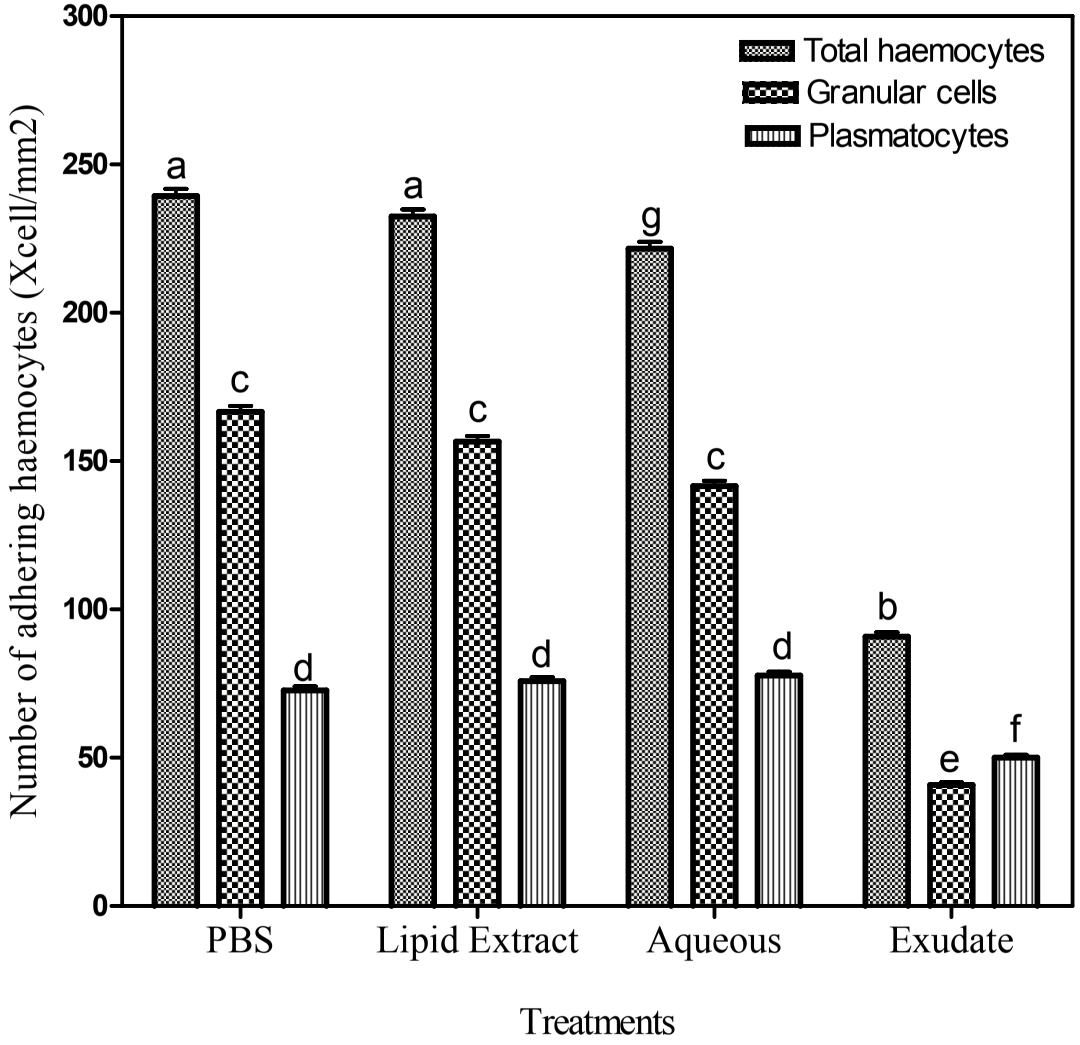
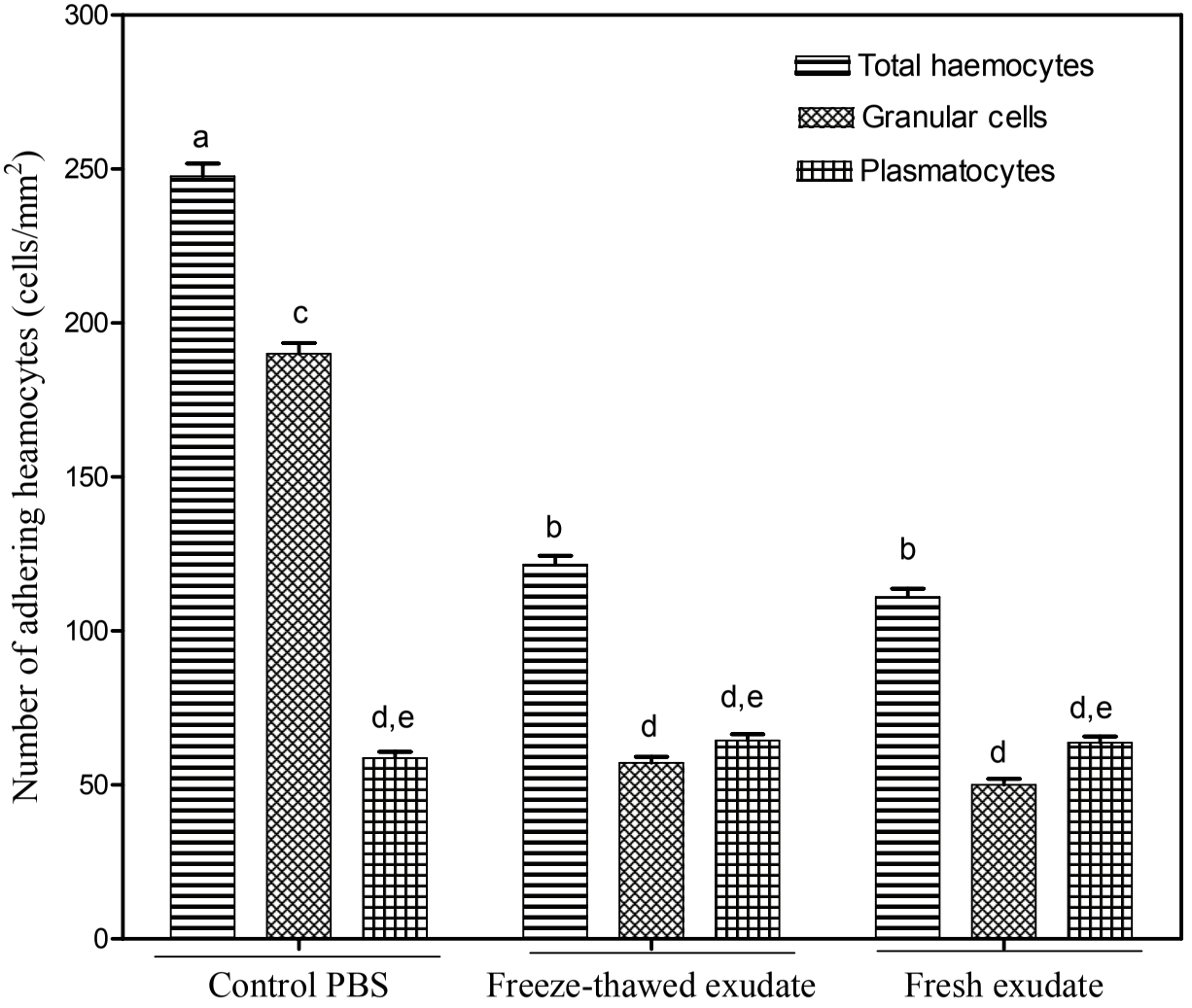
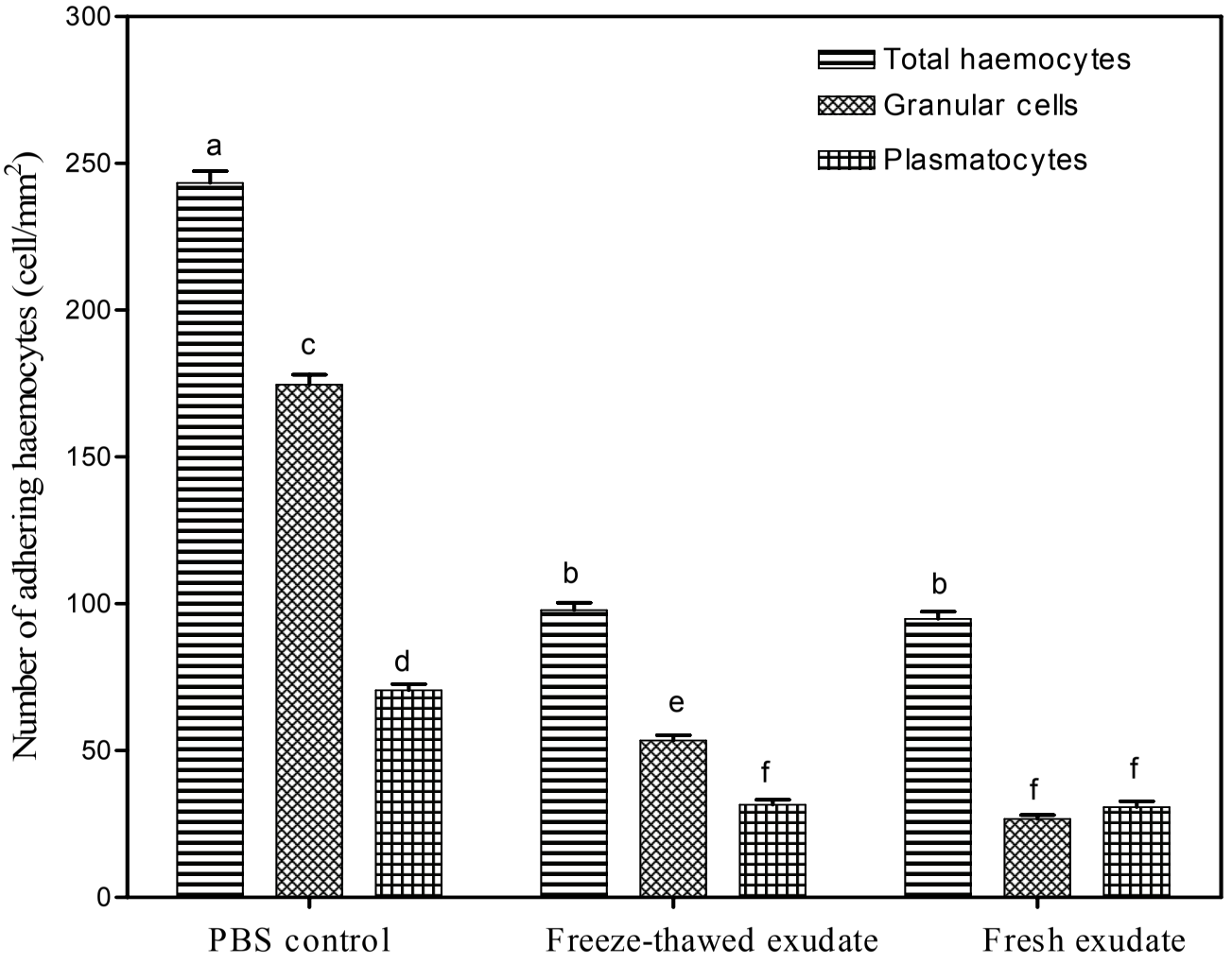

Total haemocytes
Granular cells
Plasmatocytes
Number of adhering haemocytes (Xcell/mm2)
300
250
200
150
100
50
0
a
c
d
a
c
d
g
c
d
b
e
f
PBS
Lipid Extract
Aqueous
Exudate
Treatments

Figure 3.3 The total number of haemocytes of (A) *Galleria mellonella* (B) *Malacosoma disstria* adhering to slides after incubating with *Steinernema carpocapsae* exudate previously subjected to repeated freeze-thawing (25^{o}C to -80^{o}C), compared with PBS (negative control) and fresh exudate (positive control) *in vitro*. Values represent the mean ± SE, n ≥ 10. Values with same letters are not significantly different ($p<0.05$).

A


Total haemocytes
Granular cells
Plasmatocytes
Number of adhering heamocytes (cells/mm2)
300
250
200
150
100
50
0
a
c
d,e
b
d
d,e
b
d
d,e
Control PBS
Freeze-thawed exudate
Fresh exudate

B


Total haemocytes
Granular cells
Plasmatocytes
Number of adhering haemocytes (cell/mm2)
300
250
200
150
100
50
0
a
c
d
b
e
f
b
f
f
PBS control
Freeze-thawed exudate
Fresh exudate

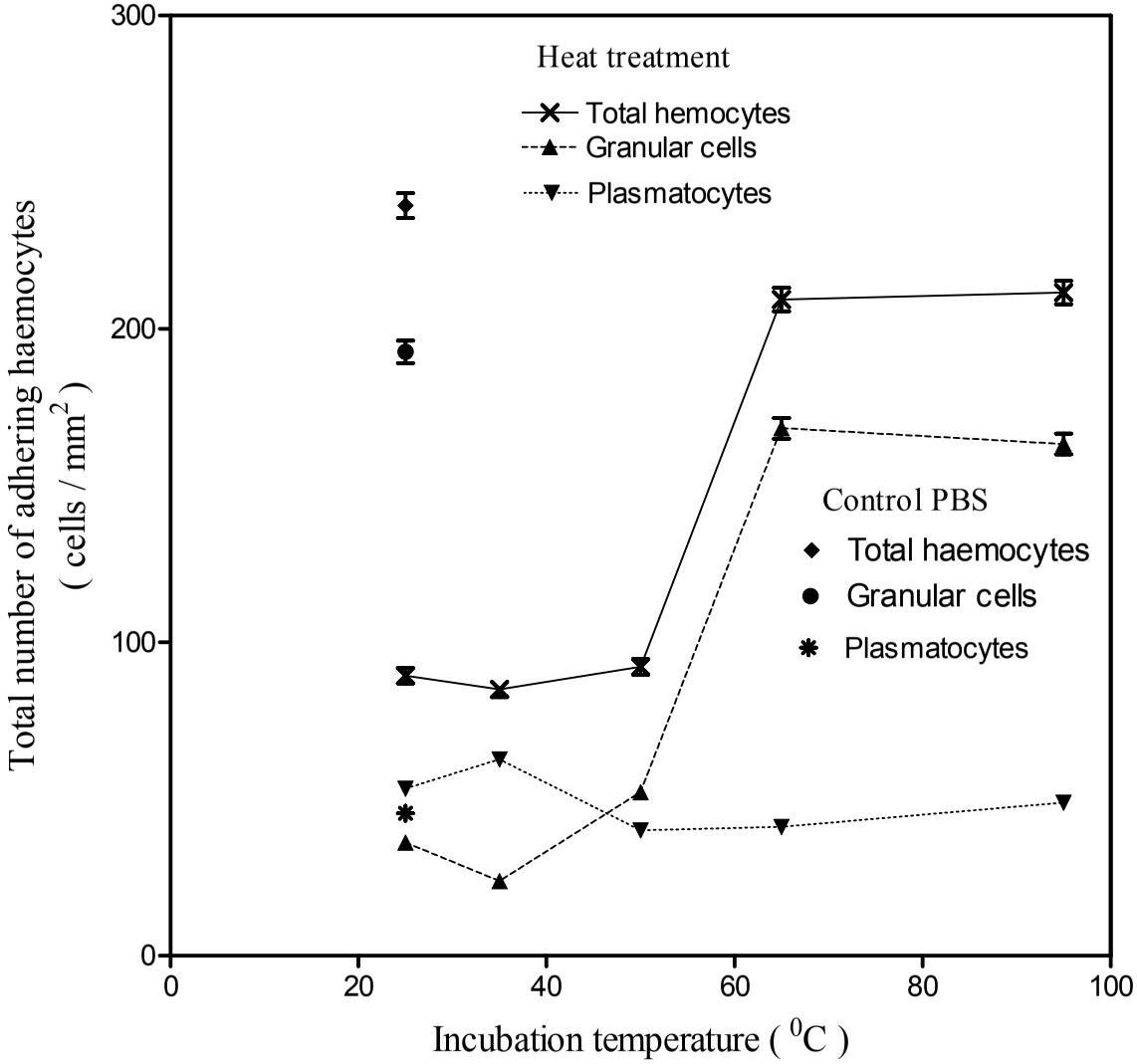
non-inhibiting PBS control in *G. mellonella* and 70% less in *M. disstria* compared to PBS. For both insect species, plasmatocyte adhesion declined by approximately 25% for *G. mellonella* and 45% for *M. disstria* compared with the PBS controls.

Subjecting the exudate to increasing temperatures increased significantly ($p<0.05$) the total number of adhering haemocytes and granular cells of *G. mellonella* (Fig. 3.4A) and *M. disstria* on slides (Fig. 3.4B) indicating heat lability of a proteinaceous suppressant(s). The maximum haemocyte adhesion values for both insect species occurred when exudate was incubated at temperatures greater than and equal to 65^{o}C. Despite the increase in granular cells and total haemocytes adhesion 65^{o}C, these values were still less (approximately 10%) for *G. mellonella* than for the non-suppressive PBS negative controls ($p<0.05$) implying both heat-sensitive and heat-stable immunosuppressants exist in the exudate. Heat denatured exudate had no effect on the plasmatocytes as observed by the consistently low counts that were equal to the positive control values regardless of the incubation temperature. A similar trend in terms of the number of adhering haemocytes was observed with *M. disstria* (Fig 3.4B) but with approximately 36% suppression left at 65^{o}C. This was significantly ($p>0.05$) greater than *G. mellonella* results again indicating possible species differences in suppression susceptibility.

Heat-stable, hydrophobic molecules with immunosuppressive activity have been identified as LPS-like molecules associated with the epicuticle of *S. carpocapsae* (Brivio *et al*., 2002; 2004). In the present study, lipid-like molecules were removed by treating the exudate with high amounts of polymyxin B beads ($1x10^4$ beads/ml). This revealed a small, but significant ($p<0.05$) amounts of polymyxin B-reactive molecules in the exudate suppressing adhesion by approximately 15% for *G. mellonella* (Table 3.1). This may explain the similar level of inhibition seen in the chloroform-extracted aqueous exudate (Fig. 3.2) and heat-treated exudate (Table 3.1). No significant difference ($p>0.05$) was observed in haemocyte adhesion or the appearance of cells in PBS only and PBS with the beads, indicating that the antibiotics beads did not come off the agarose beads in PBS.

Figure 3.4 The total number of haemocytes of (A) *Galleria mellonella* (B) *Malacosoma disstria* adhering to slides after incubating with *Steinernema carpocapsae* exudate previously subjected to heating (25^{o}C-95^{o}C) compared with PBS *in vitro*. Values represent the mean ± SE, n ≥ 10.

A


Heat treatment
Total hemocytes
Granular cells
Plasmatocytes
Control PBS
Total haemocytes
Granular cells
Plasmatocytes
Total number of adhering haemocytes
(cells / mm2)
Incubation temperature (0C)
0
100
200
300
0
20
40
60
80
100

B

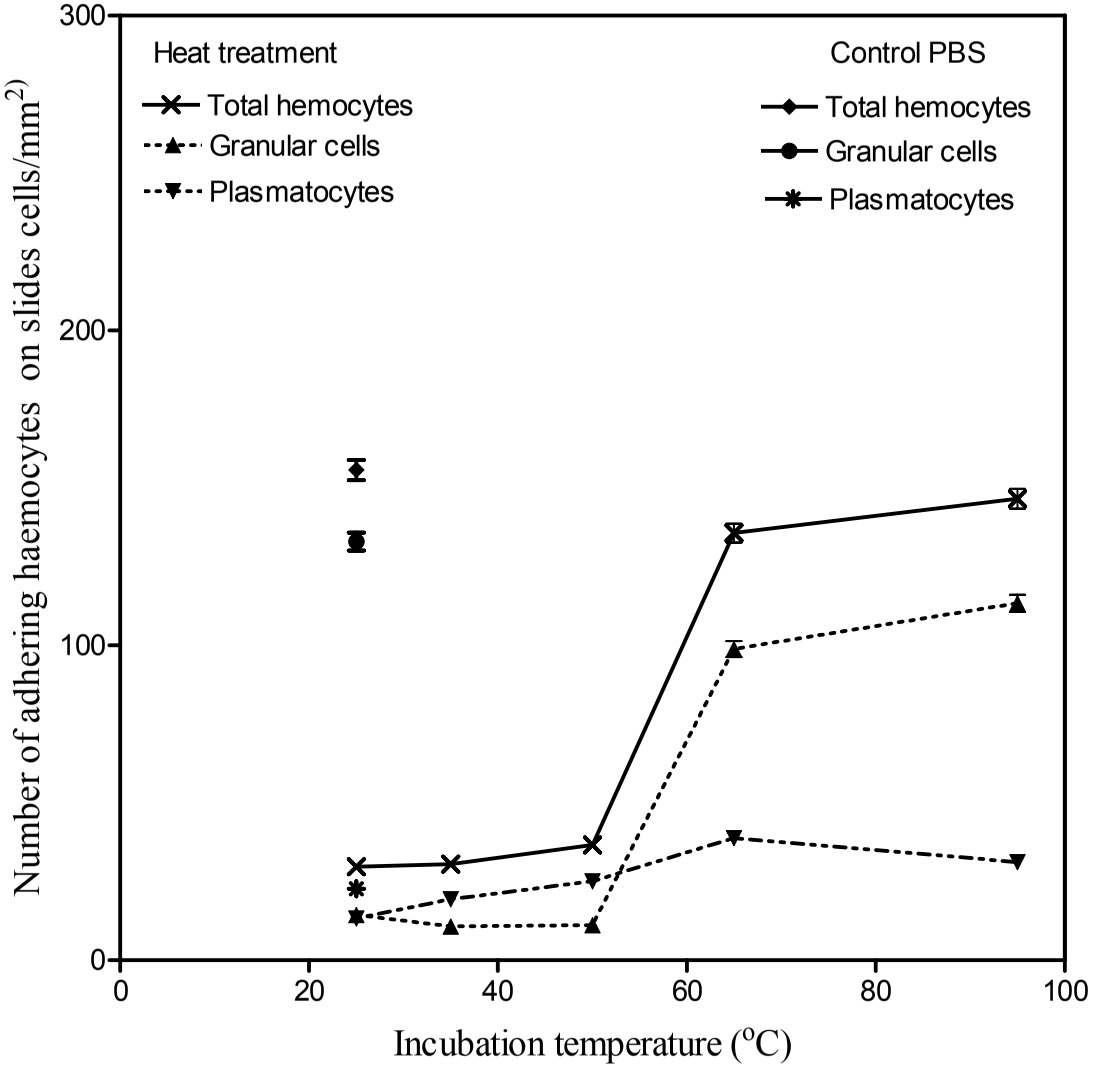

Heat treatment
Total hemocytes
Granular cells
Plasmatocytes
Control PBS
Total hemocytes
Granular cells
Plasmatocytes
Number of adhering haemocytes on slides cells/mm2)
Incubation temperature (°C)
0
100
200
300
0
20
40
60
80
100

Table 3.1 Effect of polymyxin B-conjugated agarose beads in axenic *Steinernema carpocapsae* exudate on the adhesion of larval haemocytes of *Galleria mellonella* to slides

Treatments	Level of adhering haemocyte (mm^2)		
	*THC	$^{+}$GR	^{1}PL
PBS only	239.4 ± 3.9^{a}	166.6 ± 3.3^{e}	72.7 ± 2.2^{j}
PBS + beads ($1x10^5$/ml)	225.0 ± 3.8^{a}	156.6 ± 3.2^{e}	67.1 ± 2.1^{j}
Exudate + beads ($1x10^4$/ml)	106.6 ± 2.6^{c}	60.0 ± 2^{g}	46.6 ± 1.7^{i}
Exudate + beads ($5x10^3$/ml)	96.6 ± 2.5^{d}	47.7 ± 1.7^{h}	49.1 ± 1.8^{i}
Exudate + beads ($1x10^3$/ml)	95.0 ± 2.5^{d}	54.1 ± 1.8^{h}	42.0 ± 1.6^{i}
Exudate only	90.8 ± 2.4^{d}	40.8 ± 1.6^{i}	50.0 ± 1.8^{i}

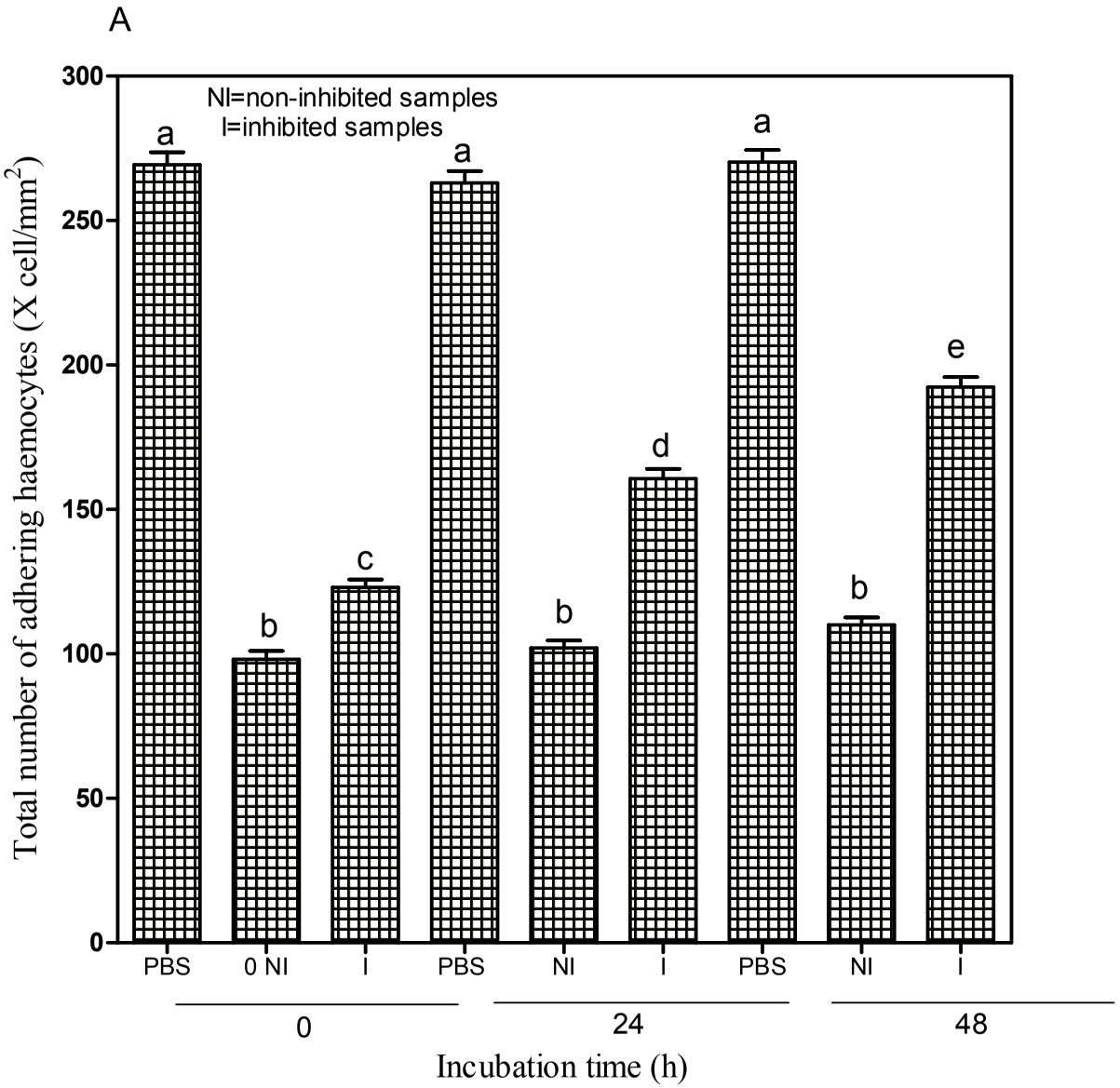
*THC; total haemocyte counts, GR^{+}, granular cells; ^{1}PL, plasmatocytes, values expressed mean ± standard error of the mean, n = 15
Values with the same superscript are not significantly different ($p>0.05$).

Effect of trypsin, chymotrypsin and protease inhibitors on nematode exudate

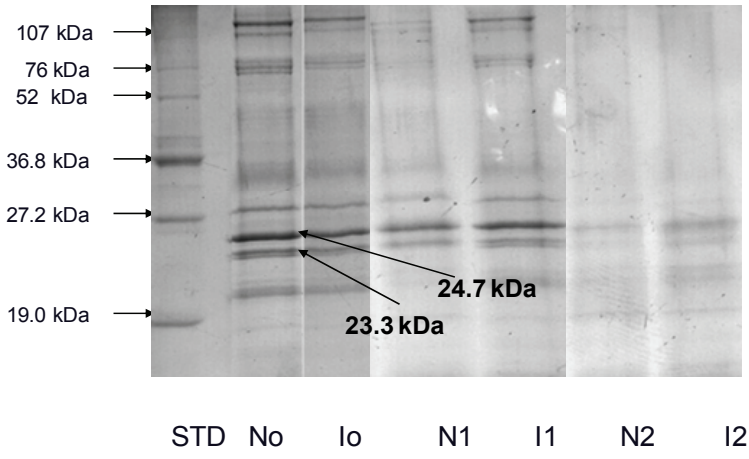
To identify immunosuppressant(s) as possible protease(s), the exudate was incubated with a cocktail of protease inhibitors. Immediately inhibited exudate favored significantly more haemocytes on slides than did non-inhibited exudate (Fig. 3.5A) implying proteases affect haemocyte adhesion. Throughout the 48 h incubation period both non-inhibited exudate limited haemocyte adhesion to a constant level that was below the negative control values (Fig. 3.5A). Unexpectedly, haemocyte adhesion linearly increased in the inhibited exudate treatments over time (Fig. 3.5A). The increase in haemocyte adhesion in enzyme-inhibited exudate did not reflect proteolytic activity by some enzyme species that were not inhibited by the protease cocktail inhibitors because the total protein did not significantly decline (0 h: 161 ± 14 µg/ml exudate; 24 h: 154 ± 11µg/ml exudate; 48 h: 147 ± 12µg/ml exudate, $p>0.05$) and there was no discernible correlation with haemocyte adhesion and total protein levels ($r = -0.072$, $p>>0.05$). Immunosuppressive non-enzymic proteins maybe denaturing with prolonged room temperature incubation increasing adhesion. Non-inhibited exudate exhibited a decline in total protein (0 h: 175 ± 14 µg/ml exudate; 24 h: 148 ± 12 µg/ml exudate; 48 h: 47 ± 8µg/ml exudate, $p<0.05$) that was not correlated with adhesion ($r = -0.83$, $p<0.05$, $n= 5$) reflecting the direct suppression of haemocytes by enzymes interacting with the cell surface rather than possible spontaneous denaturation of non-enzyme proteins with suppressant activities and/or their degradation by the enzymes.

The decrease in band number and intensity (Fig 3.5A) in inhibited samples at 24 h and 48 h reflect denaturation of the proteins which precipitated and were removed by centrifugation. Two of the bands in the inhibited samples which showed a decrease in intensity had molecular weights of 24.7 kDa and 23. 5 kDa, which correspond approximately to the molecular weights of bovine chymotrypsin [25 kDa (Totowa, 1993) and bovine trypsin (24 kDa (Walsh, 1970)] respectively. In non-inhibited exudate there was no detectable precipitation, the decline in band number and intensity reflecting digestion by the enzymes. Thus, the decline in non-enzymic proteins may not be associated with the increase in haemocyte adhesion.

Figure 3.5 Effect of protease inhibitor on exudate of *Steinernema carpocapsae* in terms of (A) changes in the total number of adhering haemocytes of *Galleria mellonella* compared with PBS (negative control) and the non-inhibited exudate samples (positive control) (B) Sodium dodecyl sulphate polycrylamide gel electrophoresis in terms of proteins remaining in the treated exudate. Molecular mass values in the non-inhibited exudate represent the mean ± SEM, $n \geq 10$.


A
NI=non-inhibited samples
I=inhibited samples
Total number of adhering haemocytes (X cell/mm2)
300
250
200
150
100
50
0
a
b
c
a
b
d
a
b
e
PBS
0 NI
I
PBS
NI
I
PBS
NI
I
0
24
48
Incubation time (h)

B



STD= standards

No= non-inhibited exudate 0 h; Io = inhibited exudate 0 h

N1= non-inhibited exudate 24 h; I1 = inhibited exudate 24 h

N2= non-inhibited exudate 48 h; I2 = inhibited exudate 48 h

Concomitantly with these experiments, the enzyme profile of the exudate (see Apizym analysis) revealed serine proteases with trypsin-like and chymotrypsin-like activity. To determine if these serine proteases by digesting sensitive proteins would lead to a decrease in haemocyte attachment, the 24 h old exudate was incubated with trypsin- and chymotrypsin-conjugate agarose beads resulting in a decline in exudate total protein [trypsin: 0 h: 181 ± 14 µg/ml exudate; 1 h: 133 ±15 µg/ml exudate, 2 h: 71 ± 20 µg/ml exudate, 4 h: 65 ± 3 µg/ml exudate ($p<0.05$). chymotrypsin: 0 h: 181 ± 16 µg/ml exudate, 1 h: 125 ± 12 µg/ml exudate, 2 h: 84 ± 7 µg/ml exudate, 4 h: 51 ± 9 µg/ml exudate ($p<0.05$)]. However, neither enzyme treatment produced a decline in immunosuppression, the number of adhering haemocytes being the same in monolayers with or without enzyme treated exudate (Fig. 3.5A). Thus, although serine-protease sensitive proteins occur in the exudate, they were not discernibly involved in suppression of haemocyte adhesion which supports the contention derived from the electrophoretic and total protein results. There was no difference ($p>0.05$) in haemocytes on slides in PBS without and with enzyme conjugated beads (Fig. 6), establishing that the enzymes did not dissociate from the agarose beads and remain in the exudate to affect haemocyte adhesion.

Apizym analysis

Apizym analysis of the semi-quantitative changes in enzyme activity in the exudates over time revealed rapid increases in lysosomal acid and alkaline phosphatases to maximum values by 6 and 12 h, respectively (Table 3.2). A maximum value was also observed for naphthol-AS-BI-phosphohrolase by 24 h. Moderate increases were observed for esterase-lipase and lipase (maximum occurring at 12 h for the latter and 24 h for the former) (Table 3.2). The hydrophobic peptidase, leucine aminopeptidase was constant at 6, 12 and 24 h, while valine aminopeptidase and cystine aminopeptidase were constant from 12 h to 24 h and the serine protease, trypsin but not chymotrypsin, showed a gradual increase throughout the study. The carbohydrases were essentially and consistently low including lysosomal β-glucuronidase, the exception being α- and β- glucosidase and N-acetyl-β glucosaminidase with a maximum value of 20 nmoles/4 h/ 580 J_3 for the former and 10 nmoles/4 h/ 580 J_3 for the latter two enzymes, respectively, by 24 h.

Figure 3.6 Effect of trypsin-and chymotrypsin-conjugate agarose beads incubated with nematode exudate on haemocyte adhesion of *Galleria mellonella in vitro* compared with PBS control.

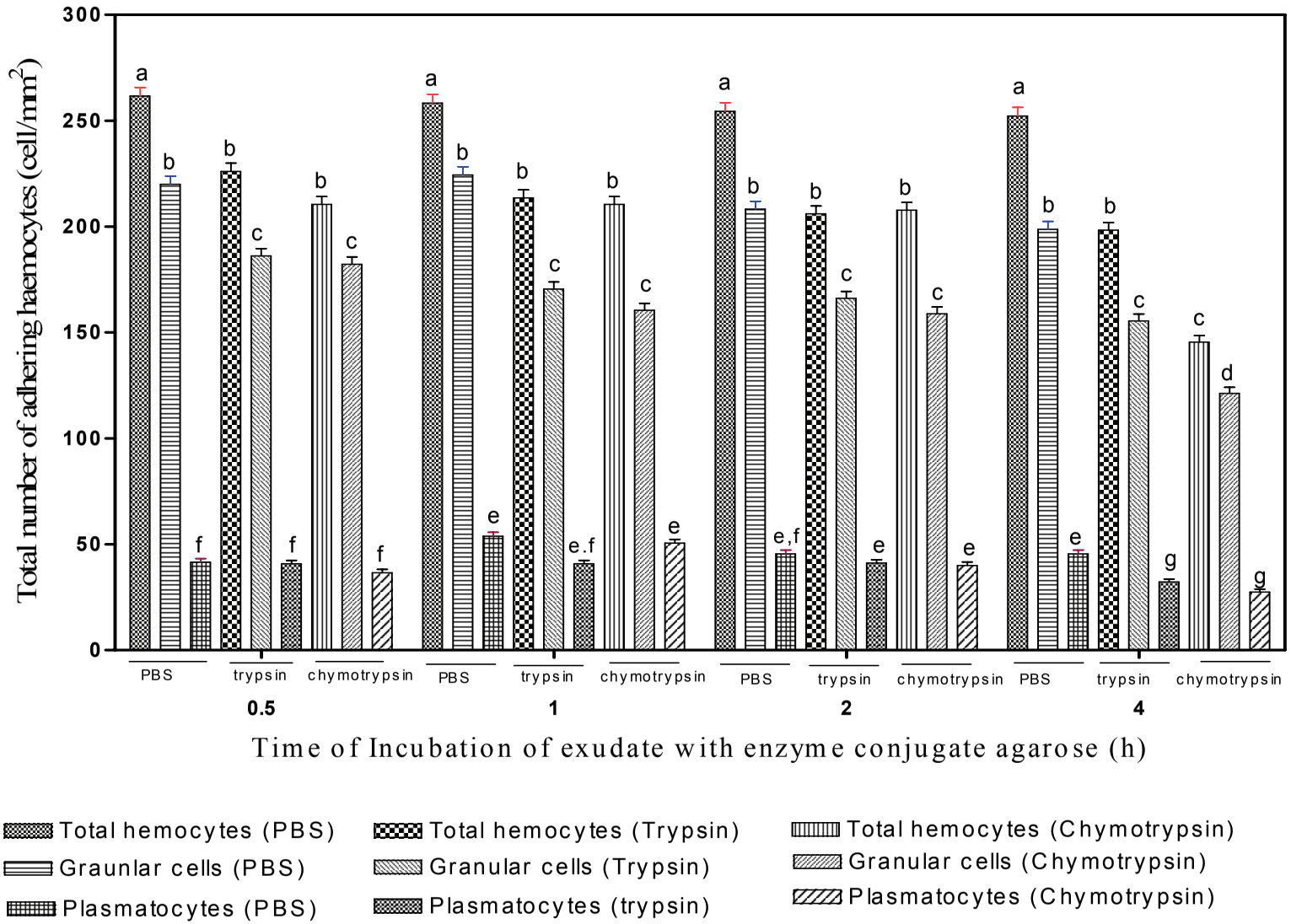

Total number of adhering haemocytes (cell/mm2)
300
250
200
150
100
50
0
a
b
f
b
c
f
b
c
f
a
b
e
b
c
e.f
b
c
e
a
b
e,f
b
c
e
b
c
e
a
b
e
b
c
g
c
d
g
PBS
trypsin
chymotrypsin
PBS
trypsin
chymotrypsin
PBS
trypsin
chymotrypsin
PBS
trypsin
chymotrypsin
0.5
1
2
4
Time of Incubation of exudate with enzyme conjugate agarose (h)
Total hemocytes (PBS)
Graunlar cells (PBS)
Plasmatocytes (PBS)
Total hemocytes (Trypsin)
Granular cells (Trypsin)
Plasmatocytes (trypsin)
Total hemocytes (Chymotrypsin)
Granular cells (Chymotrypsin)
Plasmatocytes (Chymotrypsin)

Table 3.2 Enzyme activity (nmoles hydrolysed substrate/4 h/ 580 J_3 nematodes) of live *Steinernema carpocapsae* nematode exudate during prolonged incubation of the nematode in PBS

Enzymes[a]	Time of incubation (h)			
	0	6	12	24
Alkaline phosphatase	0[b]	30.0 ± 3.1[c]	>40.0 ± 3.6[d]	>40.0 ± 3.6[d]
Acid phosphatase	0	>40.0 ± 3.6[d]	>40.0 ± 3.6[d]	>40.0 ± 3.6[d]
Esterase	0	5.0 ± 1.2[g]	10.0 ± 1.8[f]	10.0 ± 1.8[f]
Esterase lipase	0	10.0 ± 1.8[f]	10.0 ± 1.8[f]	20.0 ± 2.5[e]
Lipase	0	0	0	0
Leucine aminopeptidase	0	10.0 ± 1.8[f]	10.0 ± 1.8[f]	10.0 ± 1.8[f]
Valine aminopeptidase	0	0	5.0 ± 1.2[g]	5.0 ± 1.2[g]
Cystine aminopeptidase	0	0	5.0 ± 1.2[g]	5.0 ± 1.2[g]
Trypsin	0	2.8 ± 0.9[g]	5.2 ± 1.2[g]	9.5 ± 1.7[g]
Chymotrypsin	0	2.8 ± 1.2[g]	5.2 ± 1.2[g]	9.8 ± 1.2[g]
Naphthol-AS-BI-phosphohydrolase	0	20.0 ± 2.5[e]	20.0 ± 2.5[e]	>40.0 ± 3.6[d]

α- galactosidase	0	2.5 ± 0.9^{h}	5.0 ± 1.2^{g}	5.0 ± 1.2^{g}
β- galactosidase	0	2.5 ± 0.9^{h}	2.5 ± 0.9^{g}	2.5 ± 0.9^{h}
β- glucosidase	0	5.0 ± 1.2^{g}	5.0 ± 1.2^{g}	10.0 ± 1.8^{f}
N-acetyl-β glucosaminidase	0	5.0 ± 1.2^{g}	5.0 ± 1.2^{g}	10.0 ± 1.8^{f}
α- mannosidase	0	2.5 ± 0.9^{g}	2.5 ± 0.9^{g}	2.5 ± 0.9^{h}

[a]Values for a given enzyme in a row with the same superscript are not significantly different, $p>0.05$, $n = 3$.

A significant negative correlation was observed between haemocyte adhesion and the activities of alkaline phosphatase, acid phosphatase and esterase lipase and esterase (Table 3.3). Amongst the serine proteases present in the exudate, trypsin (Fig. 3.7A), chymotrypsin (Fig. 3.7B) and the carbohydrase N-acetyl-D-glucosaminidase (Fig. 3.7C) [all of which are resistant to freeze-thawing (Saborowski *et al*., 2004; Ozdemir *et al*., 2005) and were heat sensitive (Dance *et al*., 1969; Saborowski *et al*., 2004)], based on correlation analysis herein showed strong suppressant roles. The following enzymes also show significant negative correlation between activity and haemocyte adhesion: Naphthol-AS-BI-phosphohrolase, β- galactosidase, β- glucuronidase, α- glucosidase (Table 3.3). (see Appendix for the other enzyme-haemocyte correlation figures).

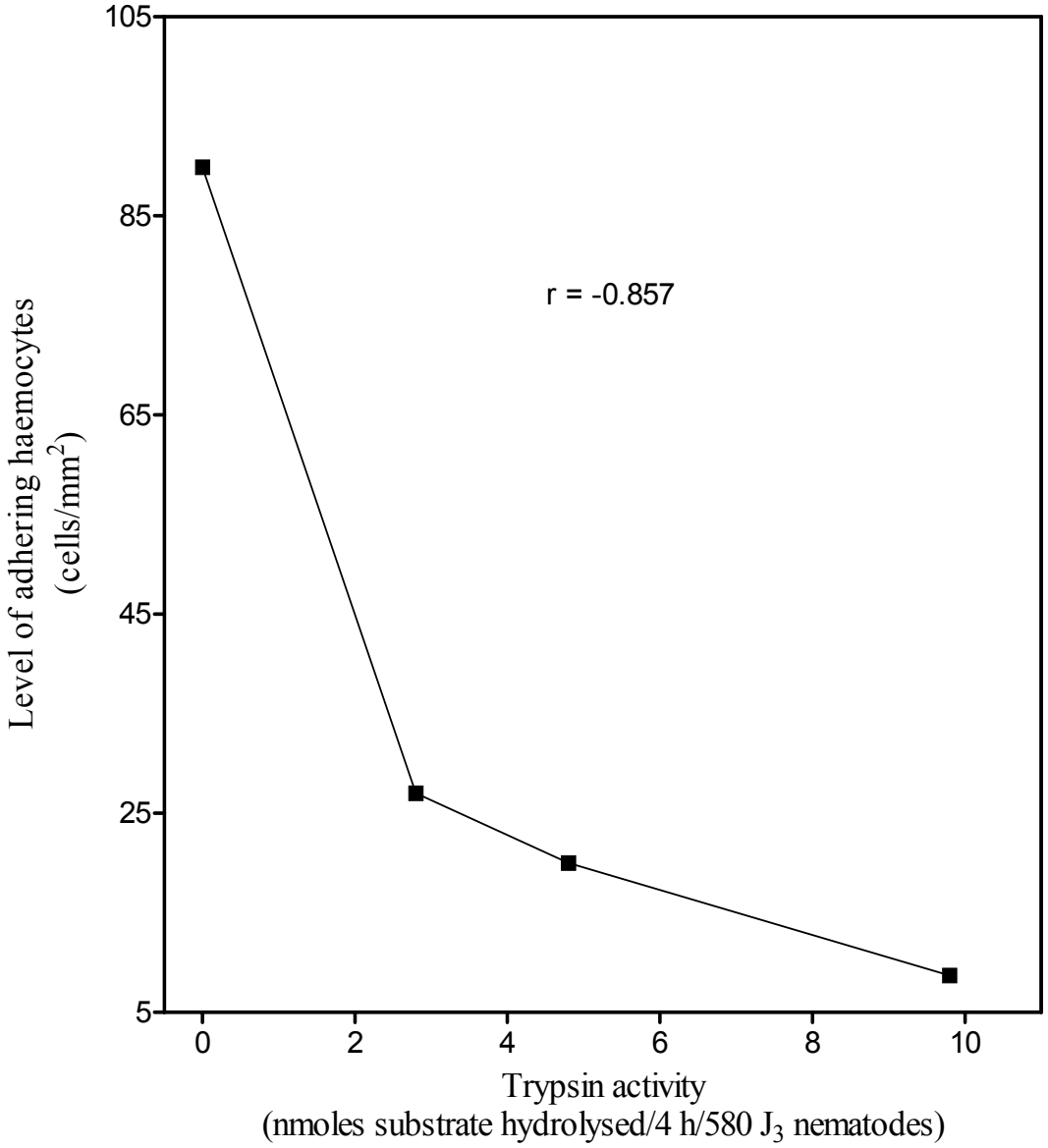
Table 3.3 Product-moment correlation of enzyme activity in the exudate of *Steinernema carpocapsae* and the adhesion of haemocytes of *Galleria mellonella.*

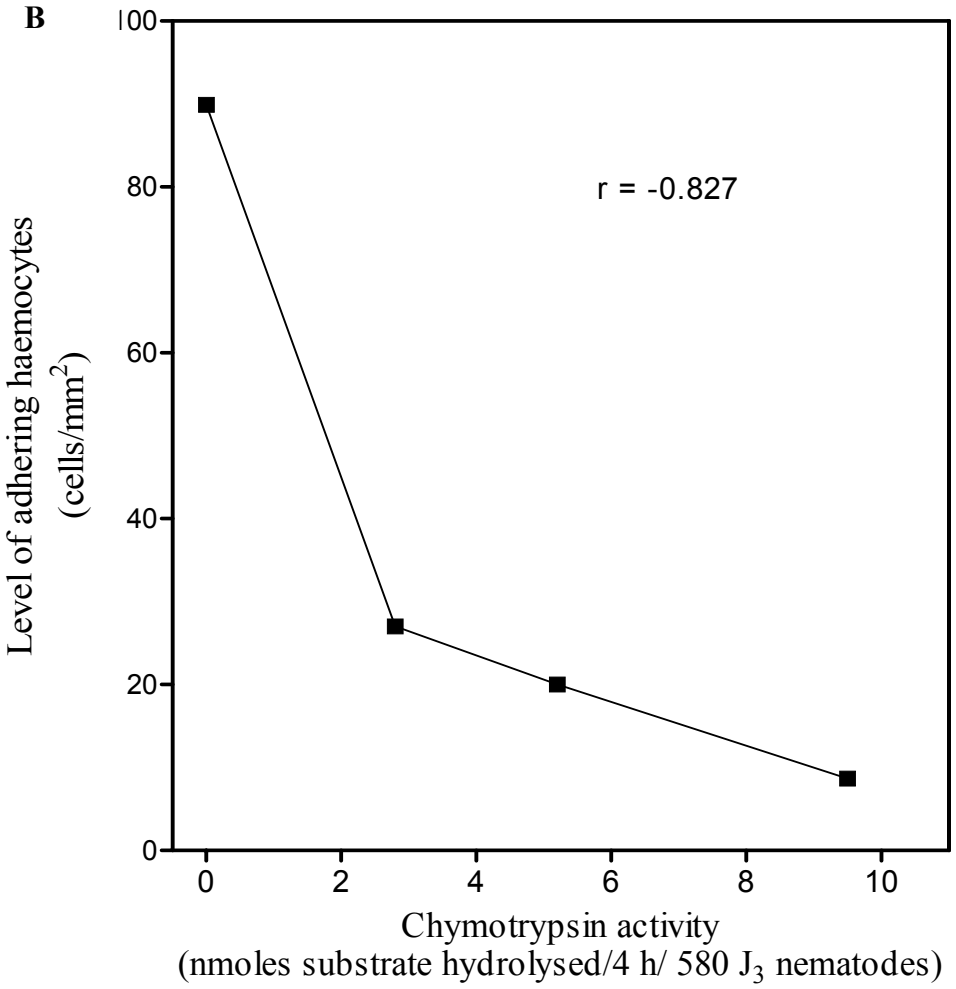
Enzyme[a]	r values
Alkaline phosphatase	-0.980[b]
Acid phosphatase	-0.966[b]
Esterase	-0.932[b]
Esterase lipase	-0.899[b]
Lipase	0.00
Leucine aminopeptidase	-0.453
Valine aminopeptidase	-0.802[b]
Cystine aminopeptidase	-0.802[b]
Trypsin	-0.857[b]
Chymotrypsin	-0.827[b]
Naphthol-AS-BI-phosphohydrolase	-0.909[b]
α -galactosidase	-0.613
β- galactosidase	-0.978[b]
β- glucuronidase	-0.909[b]
α- glucosidase	-0.899[b]
β-glucosidase	-0.525
N-acetyl-β glucosaminidase	-0.525
α- mannosidase	-0.710

[a]Graph of relationship in Appendix I, [b] values with the same superscript are not significantly different from a slope of zero, $p>0.05$

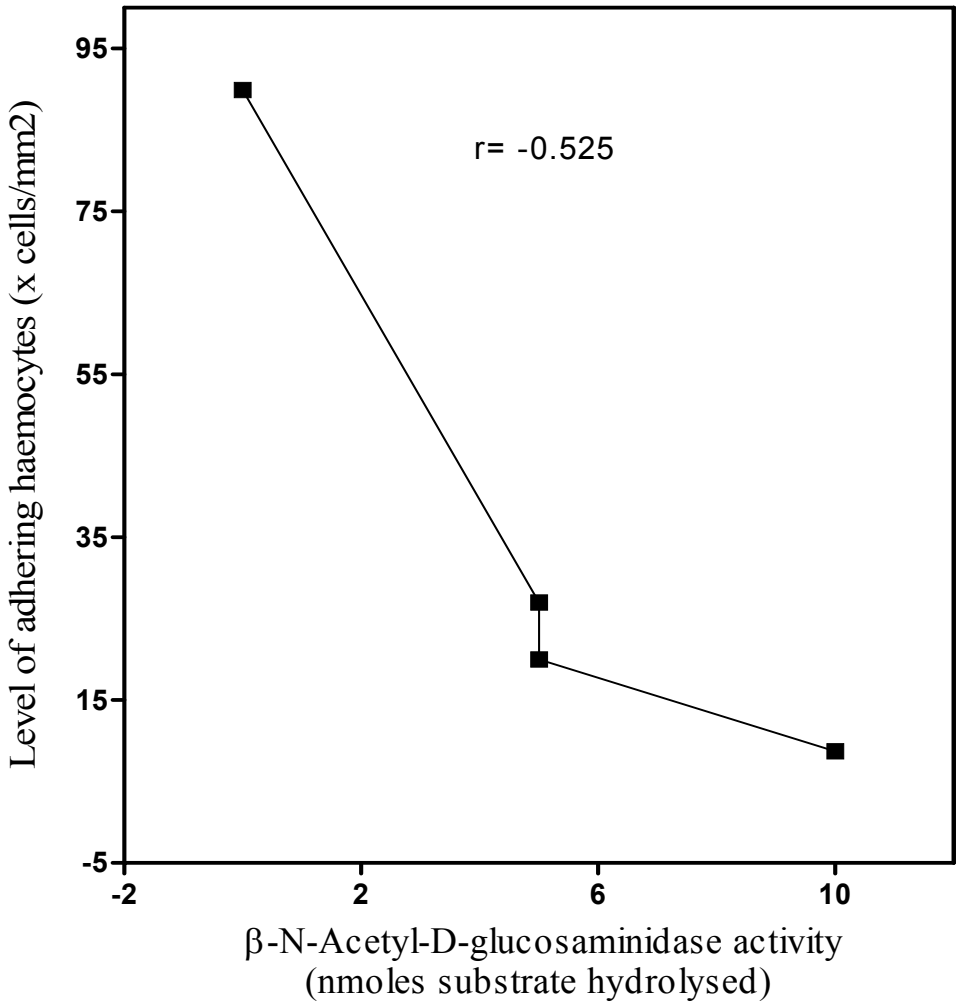
Figure 3.7 Representative samples of the correlation between (A) trypsin, (B) chymotrypsin and (C) N-acetyl-β glucosaminidase enzyme activities in *Steinernema carpocapsae* exudate and levels of adhering haemocytes of *Galleria mellonella*..

A


105
85
65
45
25
5
r = -0.857
Level of adhering haemocytes
(cells/mm2)
0
2
4
6
8
10
Trypsin activity
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)


B
100
80
60
40
20
0
r = -0.827
Level of adhering haemocytes
(cells/mm2)
0
2
4
6
8
10
Chymotrypsin activity
(nmoles substrate hydrolysed/4 h/ 580 J3 nematodes)

C


95
75
55
35
15
-5
-2
2
6
10
r= -0.525
Level of adhering haemocytes (x cells/mm2)
β-N-Acetyl-D-glucosaminidase activity
(nmoles substrate hydrolysed)

Effect of commercial trypsin and chymotrypsin on *Galleria mellonella* haemocytes *in vitro*

Haemocyte adhesion was lowered by treatment of the haemocyte suspension with commercial trypsin, chymotrypsin and both enzymes together compared with PBS (Table 3.4), the granular cells being more sensitive than the plasmatocytes. Treatment of the haemocytes with both commercial enzymes together significantly lowered haemocyte adhesion ($p<0.05$) compared to the enzymes separately, but the combined effect was still less than the level of inhibition for the exudate only (Table 3.4), indicating that other suppressant(s) might be present in the exudate.

Table 3.4 Extent of adhesion of larval *Galleria mellonella* haemocytes to slides in the presence of commercial trypsin and chymotrypsin

Treatments	*THC	$^{+}$GR (cells/mm^2)	^{1}PL
Haemocytes + commercial trypsin	217.4± 3.8^{a}	150.8 ± 3.1^{e}	66.6 ± 2.1^{i}
Haemocytes + commercial chymotrypsin	224 ± 3.8^{a}	152.2 ± 3.1^{e}	71.8 ± 2.1^{i}
Haemocytes + commercial trypsin + chymotrypsin	161.6 ± 3.2^{b}	97.0 ± 2.5^{f}	64.6 ± 2.0^{i}
Haemocytes + exudate	133.6 ± 2.9^{c}	80.5 ± 2.3^{g}	53.1 ± 1.8^{j}
Haemocytes + PBS (no enzyme)	292.8 ± 4.4^{d}	218.3 ± 3.8^{h}	74.5 ± 2.2^{i}

*THC: total haemocyte counts, $^{+}$GR: granular cells, ^{1}PL: plasmatocytes, Values with the same superscript in a row are not significantly different $p>0.05$, n = 15

Discussion

Treatment of the live-nematode exudate by batch hydrophobic interaction chromatography showed that the immunosuppressant(s) bound to the highly hydrophobic beads resulting in higher numbers of adhering haemocytes of both insect species incubated with bead-modified exudate compared with adhering haemocyte numbers of the exudate control groups. The weakly hydrophobic immunosuppressant(s), could be proteins, lipids or other molecules because hydrophobic interaction chromatography binds with proteins [based on their biological and physio-chemical properties including molecular size, net charge, biospecific characteristics and hydrophobicity (Garcia and Pires, 1993)], lipids (Fischer, 1991) and lipoproteins (Hashimoto *et al*., 2004). Chloroform extracts of the exudate established that exudate lipids did not have an anti-adhesion effect on the haemocytes thus the decline in exudate immunosuppressant activity by hydrophobic beads was not due to lipids binding to the beads. That does not mean other lipid sources might affect haemocyte adhesion. Dunphy and Webster (1987) found cuticular lipid of *S. carpocapsae* to be anti-adhesive for haemocytes. This differs from *S. feltiae* in which cuticular lipids from monoxenic *S. feltiae* participate in masking the live and dead nematodes from cellular encapsulation in *G. mellonella* by acquiring host plasma proteins (Mastore and Brivio, 2008). Epicuticular lipopolysaccharides of the monoxenic *S. feltiae* prevent phenoloxidase activation by binding to host plasma proteins (Brivio *et al*., 2004), the bacterium, *X. bovienni*, possibly indirectly protecting the nematodes by coating the hydrophobic cuticle with LPS during nematode growth and developmemt *in vitro*. This is plausible since Gram-negative bacteria routinely shed LPS into culture media (Mackowiak, 1984) as does *X. nematophila* in *G. mellonella* haemolymph (Dunphy and Webster, 1988b). Herein, chloroform effectively denatured protein and substantially lowered immunosuppression establishing exudate proteins as major suppressants but it did not remove heat stable molecules from the aqueous extract, possibly explaining the 10% residual suppression in heated exudate. Using polymyxin B-conjugated agarose beads, a decline in suppressant activity was similar to suppressant activity remaining in both heat-denatured and chloroform-extracted exudate. The polymyxin B-reactive

molecules from the axenic nematode exudate needs additional characterization because there were no discernible bacteria associated with the axenic nematode or in their exudate that could provide LPS-like molecules. LPS is a major virulence factor of *X. nematophila* in insects preventing phenoloxidase activation, limiting Ca^{2+} (Yokoo *et al*., 1995) and Fe^{2+} availability and thus limiting haemocyte adhesion (Dunphy *et al*., 2002) and ultimately lysing the blood cells (Dunphy and Webster, 1988a, b; Forst *et al*., 1997). However, live-nematode exudate did not cause *G. mellonella* haemocytes to lyse (Walter *et al*., 2008). Unless the LPS-like molecules were below a critical lysis level, this and the absence of bacteria question the actual identity of the heat-resistant molecules. It clearly was an amphiphilic molecule which, in low salt level in PBS, would not be expcted to bind to the hydrophobic beads (Fischer, 1991) even though polymyxin B establishes its hydrophobic nature (Moore et al., 1996; Wang et al., 2000).

Heat-inactivation of most of the anti-haemocyte activity in the exudate implied the existence of heat-labile factors in addition to heat-stable LPS-like molecules**.** Numerous heat sensitive substances from *X. nematophila* have immunosuppressive activity including an organic factor from *X. nematophila* culture medium that disables eicosanoid-mediated cellular immune reactions in *Ma. sexta* (Park *et al*., 2003) but do not contribute to suppression herein with axenic nematodes. Ribeiro *et al*. (1999) characterized two heat-sensitive immunosuppressants from *Steinernema carpocapsae*, one with susceptible protease cytotoxic activity from *X. nematophila* and the other, a weak protease from both the axenic nematode and the nemato-bacterial complex, with unsticking effect on the plasmatocytes of the armyworm, *Pseudaletia unipunctata.* Much is not known about heat-stable toxins released by the nematode-bacterial complex other than LPS.

Immediate incubation of the exudate with cocktail of broad spectrum protease inhibitors decreased haemocyte adhesion indicating that enzymes maybe involved. However, with prolonged incubation adhesion continued to increase in inhibited samples even when total protein did not decline implying non-enzymic proteins may also be suppressing haemocyte adhesion and in the protease inhibited samples they are denatured at room temperature during prolong incubation loosing activity. The decline in the total protein concentration after the trypsin and chymotrypsin-conjugate agarose beads were incubated with the exudate indicates the presence of trypsin-like and chymotrypsin-like sensitive proteins in the exudate. Since the

number of adhering haemocytes for both the enzyme digested exudate and non-enzyme treated exudate was the same, the serine protease sensitive proteins were not regarded as suppressants. It is possible that other non-enzymic proteins susceptible to non-serine protease digestion may affect haemocyte adhesion. However, this seems unlikely because non-inhibited exudate, which lowered protein content, band types and intensity, had the same suppressant activity throughout the study. A possible non-enzymic immunosuppressive protein from *S. carpocapsae* could be a surface coat protein as reported for axenic *S. glaseri* which inhibits the encapsulation of *H. bacteriophora,* and the phagocytosis of latex beads, lyses haemocytes and suppresses melanization in *P. japonica* (Wang and Gaugler, 1999). In *S. feltiae*, a surface coat protein immunosuppresses *G. mellonella* by down-regulating the prophenoloxidase pathway (Brivio *et al*., 2002). For some insect-pathogenic nematodes, surface coat proteins may suppress host immune systems as they do for insect-vectored mammalian parasitic nematodes; *B. malayi* releases a 15 kDa cuticular protein, cystatin (cysteine protease inhibitor) and serpins from L3 infective nematodes blocking antigen processing pathways of B cells and inhibiting neutrophil proteases, respectively (Maizels *et al*., 2004).

Serine proteases in *S. carpocapsae* exudate limited haemocyte adhesion. Proteases are released by *S. feltiae* and *S. carpocapsae* during gut invasion (Rogue *et al*., 1999; Abuhatab *et al*., 1995) and implicated in the virulence of *S. carpocapsae* (Götz *et al*., 1981; Laumond *et al*., 1989) but there is little available information on their involvement in immunosuppression in insects. Proteases released by entomopathogenic fungi participate in the suppression of lepidopteran host immune responses. Isolated *G. mellonella* plasmatocytes incubated with sub-lethal amounts of proteases from *Metarhizium anisopliae* or *Beauveria bassiana* show impaired phagocytotic activity, attachment, spreading and cytoskeleton formation (Griesch and Vlicinskas, 1998). The above processes can be explained by proteolytic digestion of the cell membrane-associated receptor molecules that mediate the contact between the plasmatocytes and the foreign surfaces (Griesch and Vilcinskas, 1998). It is not known if S. carpocapsae exudate proteases directly modified the haemocyte surfaces or indeirectly affected adhesion by digesting plasma adhesion factors.

Based on the highly conserved presence of serine proteases in *St. stercoralis* and *C. elegnans* (Blaxter *et al*., 2000; Tcherepanova *et al*., 2000) which are designed for the acquisition of nutrients (Tort *et al*., 1999) and in the former species for processing vertebrate host immunoglobulins during infection (Tcherepanova *et al*., 2000), the proposed functions of trypsin and chymotrypsin, and possibly other feeding-related enzymes (Blaxter *et al*., 2000) seem to be common to the members of the dendrogramic clade containing these and the steinernematid nematodes (Blaxter *et al*., 2000). That many of these enzymes are not found in all infective stages of parasitic nematodes is evidenced by their absence from *Ancylostoma tuberforme* and *Necator americanus* (Dresden *et al*., 1985), nematodes from a different clade (Dorris *et al*., 1999).

Commercial chymotrypsin and trypsin suppressed haemocyte adhesion in the present work which is similar to results of Chen and Bayne, (1994) for bivalve haemocytes in which trypsin reduced haemocyte adhesion of the California mussel, *Mytilus californianus,* to slides. The level of suppression observed with each enzyme type and with both enzymes together represents 60%, 70% and 80%, respectively, of the inhibitory capacity of *S. carpocapsae* exudate. Insect haemolymph, including *G. mellonella* contain serine protease inhibitors, serpins (Frobius *et al*., 2000). Possibly, in the early stage of infection, host serpins limit trypsin and chymotrypsin activity until serine proteases released from J_3 exceeds this ability. Alternatively other factors including other types of enzymes in the exudate maybe involved but either way the aforementioned serine proteases are the major immediately acting factors.

Although correlation analysis between enzyme activities and levels of adhering haemocytes may represent a myriad of complex interactions of membrane damaging enzymes, the Apizym analysis and enzyme cocktail results together implied enzymes including trypsin and chymotrypsin may participate in immunosuppression validating correlation analysis using Apizym data. Based on ‘r’ values, the activities of both acid and alkaline phosphatase were very high in the nematode exudate. In the cestode, *Ligula intestinalis*, alkaline phosphatase is known to take part in active transport of metabolites through cellular membranes, while the lysosomal acid phosphatase plays an important role in regulating metabolic processes by indirectly providing information concerning the intra-cellular digestion processes (Arme, 1966). High

amounts of acid and alkaline phosphatase have also been reported in excretory-secretory products and extracts in juvenile and mature specimens of the nematode *Cystidicola farionis*, a parasite which occurs in the swim bladders of salmonid fish (Dziekonska-Rynko *et al*., 2003). The high amount of these enzymes is an indication that the nematodes can easily penetrate and digest host tissues. Collectively, these phosphatases are active in cellular degradation and thus in *S. carpocapsae* exudate they maybe active on phospholipids (Guo *et al*., 2005), their targets being cell membranes which would thus limit haemocyte adhesion.

Esterase lipase and esterase were active in the *S. carpocapsae* exudate, both showing suppressant roles based on their strong correlation values. Esterases have been found active in the hatching fluid of *Ascaris suum* (Rogers, 1963).

Alpha-mannosidase and N-acetyl-β glucosaminidase were also active in the exudate, but did not show strong suppressant roles based on their correlation with the haemocytes. N-acetyl-D-glucosaminidase has been implicated in undefined immunosuppression of *G. mellonella* haemocytes by *S. carpocapsae* (Simões *et al*., 2000). The carbohydrase has been found also on the cuticle of the zooparasitic [(which includes insects, (Albuquerque and Ham, 1996)] nematode, *Brugia pahangi* (Devaney, 1985) and the free-living bacteriophagous nematodes *C. briggsae* and *C. elegance* (Zuckerman *et al*., 1979). The three nematode genera occupy the same clade as the steinernematids based on rRNA and rDNA analysis thus these enzymes may have a conserved digestive function as observed for some animal parasitic nematodes. Alpha- and β-galactosidase were also active in the exudate, with β-galactosidase showing a suppressant role based on its strong negative correlation with the haemocytes. These carbohydrases have been reported in the haemolymph and fat body of the fifth instar larvae of *B. mori*, and thought to be involved in the inactivation of lectin activity after humoral lectin protein is rapidly activated by neuraminidase on the spinning day (Kato and Takeuchi, 2006). The presence of these carbohydrases in nematode exudate may decrease host lectin binding to the pathogen's cuticle, decreasing or preventing recognition and preventing host response such as phagocytosis which happens with the haemocytes of mollusks to parasites (Boswell and Bayne, 1985; Loker *et al.,* 1989; Ford and Ashton-Alcox, 1998). This, however, is unlikely since β- N-acetly-D-glucosamine and mannose occur on the epicuticle of *S. carpocapsae* and their digestion did not

enhance haemocyte adhesion to the nematode (Dunphy and Webster, 1987). Galactosidases similarly did not enhance haemocyte adhesion, possibly because the sugars existed as N-acetyl-sugars (Dunphy and Webster, 1987) conferring steric hindrance upon enzyme-substrate contact. Hence α-and β-glucosidase may facilitate tissue digestion of the host by *S. carpocapsae*. This would apply to α-galactosidase which is correlated also with suppression of haemocyte activity. These three carbohydrase species would be expected to react with insect haemocytes altering their membrane structure and possible haemocyte adhesion because lectin studies have established N-acetyl-D-glucosamine, D-galactose and D-mannose moieties on granular cell surfaces (Richards *et al*., 1989).

Aminopeptidases, which are important activators of hormone-and enzyme precursors during the hatching and molting of juveniles of *A. suum* (Rhoads *et al*., 1997), were detected in *S. carpocapsae* exudate with low activity, and their corresponding high r-value except for leucine aminopeptidase, indicates that they may contribute to the development of the *S. carpocapsae* molting from one juvenile stage to the next. This was observed with *Schistosoma mansoni*, molting from L3 to L4 (Auriault *et al*., 1981; Xu and Dresden, 1986), and *A. suum* juveniles molting from L3 to L4, while also triggering disintegration of host's immunoglobulin on the parasite's surface (Rhoads *et al*., 1997). The enzymes might also contribute in dietary protein digestion in insects (Wang *et al*., 2005) and for pathogenic nematodes in general or steinernematids in particular this might extend to the host tissue including haemocyte proteins responsible for antigen recognition.

The immunosuppressant activity against granular cells and plasmatocytes of both insect species in the exudate was not affected by repeated freeze-thawing. In chapter two, it was observed that changes in absolute magnitude of the haemocyte types in terms of level of haemocyte adhesion varied with insect species and that the same proportion of haemocytes exhibited differential sensitivity to the exudate. The differences in sensitivity of the granular cells and plasmatocytes of both insect species, the plasmatocytes of *M. disstria* being more sensitive than those of *G. mellonella*, maybe attributed to the fact that *M. disstria* is more closely related to the field larvae with only about 20 generations of rearing (Mick Gail, 2008, pers. comm.), and *G. mellonella* less so with more than 360 generations of rearing (personal observation) leading to

much inbreeding. Another possibility could be the different diets these two pests are fed leading to their differential development (see Literature Review: Lifecycles) or affect haemocyte activity as described for *Lymantria dispar* (Dunphy and Bouchier, 1992). Differential sensitivity may occur with subpopulations of plasmatocytes within a given species e.g. the dessert locust, *Schistocerca gregaria,* their granular cell lysate supernatant, a source of components of the prophenoloxidase enzyme sequence, induces positive chemokinesis in a heavily granulated, phenoloxidase-containing, subpopulation of plasmatocytes but has no effect on a less granular subpopulation of plasmatocytes (Huxham and Lackie, 1986). A hyperphagocytic subpopulation of plasmatocytes in *Ma. sexta* cells recognizes and attaches to large numbers of bacteria and act as nuclei for subsequent nodule formation whereas the other plasmatocyte type do not (Dean *et al*., 2004), while Nardi *et al*., (2006) observed that neuroglian-positive plasmatocytes from *Ma. sexta* that segregate with most granular cells adhere firmly to a substratum and that if the neuroglian-positive plasmatocytes are separated from most granular cells, attachment of these plasmatocytes to foreign surfaces is suppressed.

In summary, exudate released from the live axenic J_3 stage of *S. carpocapsae* suppressed haemocyte adhesion to foreign particles. Lipids did not affect haemocyte adhesion whereas proteins and heat-stable, polymyxin B-reactive molecules limited adhesion. In addition to immunosuppressive proteases, it is possible that non-enzymic proteins, other than serine-protease sensitive proteins, may inhibit haemocyte adhesion. Partial characterization of the immunosuppressant in the exudate revealed the main suppressants to be weakly hydrophobic proteins, which might be proteases, possibly trypsin and chymotrypsin. The enzymes in the exudate may have two roles, the primary being in food and tissue digestion as previously discussed, since many of these enzymes in free-living nematodes and parasitic helminthes including nematodes, play a role in feeding. As these enzymes can digest molecules around the haemocytes, it is assumed they play a secondary role in suppression of the insect immune system.

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CONNECTING STATEMENT II

In chapter three, numerous candidates with possible immunosuppressive activity in the *S. carpocapsae* exudate were detected including LPS-like, non-enzymic proteins and a myriad of proteinases, peptidases, carbohydrases and lipases. However, it was observed that the major immunosuppressants in the axenic nematode exudate were trypsin-like and chymotrypsin-like enzymes. These enzymes were further investigated in chapter four.

CHAPTER 4

Chymotrypsin and trypsin as modulators of innate haemocyte responses

A version of this chapter will be submitted to the International Journal of Parasitology as follows,

Walter, T.N., Dunphy, G.B. and Mandato, C.A. 2008. Trypsin and Chymotrypsin as modulators of the innate haemocyte responses of the pest insect, *Galleria mellonella*.

Abstract

It is reported here for the first time that serine proteases with chymotrypsin-like and possibly trypsin-like activity released by live axenic *Steinernema carpocapsae*, inhibits haemocyte adhesion to slides. Subjecting the exudate to increasing concentrations of enzymic chemical inhibitors and specific polyclonal antibodies limited trypsin-like and chymotrypsin-like activities in the exudate as evident by a decline in spectrophotometric-based biochemical assay and the increasing adhesion level of larval *G. mellonella* haemocyte, compared with the non-inhibited exudate control groups. Based on haemocyte adhesion the blood cells did not react with the antibodies or the chemical inhibitors. Further, treatment of the exudate with chymotrypsin specific inhibitor I and trypsin-specific substrate showed no change in the trypsin-like reaction with the substrate indicating that trypsin-like enzyme reacts only with its substrate. Comparing the effect of the antibodies and the chemical inhibitors on the enzymes in terms of changes in optical density readings of the treated samples and haemocyte suppression, indicates that these enzymes are trypsin-like and chymotrypsin-like but a subset of these enzymes, which are sensitive to the antibodies are the major haemocyte suppressants *in vitro*. Blocking the action of the enzymes in the exudate with chemical inhibitors and antibodies increased bacterial removal from the haemolymph *in vivo*. Initial detection of the time of release of the enzymes from the nematode in artificial serum, based on enzyme hydrolysis of their specific substrates, was 2 h with a peak release at 4 h for trypsin and 6 h for chymotrypsin.

Index descriptors: *Steinernema carpocapsae, Galleria mellonella,* chemical inhibitor, polyclonal antibody, trypsin, chymotrypsin, serine protease.

Introduction

Entomopathogenic nematodes are widely used as alternatives to chemicals for the biological control of both cryptic and non-cryptic insect pests (Poinar, 1979; Dunphy and Thurston, 1990; Georgis and Gaugler, 1991; Samish and Glazer, 2001). These insect pathogens are symbiotically associated with a bacterium *Xenorhabdus nematophila* (F. Enterobacteriaceae) that are lethal to the host. The infection mechanism is best understood using the *Steinernema carpocapsae* (F. Steinernematidae)-*X. nematophila* complex in a typical host, the greater wax moth, *Galleria mellonella*, (Insecta, Lepidoptera) (Akhurst and Dunphy, 1993; Ribeiro *et al*., 1999). The infective nematodes, after gaining entry into the host haemolymph through natural openings (Nguyen and Smart, 1990) facilitated by protease digestion of epithelial cells, release the bacterium (Vivas and Goodrich-Blair, 2001), from within the nematode's intestinal vesicle (Martens and Goodrich-Blair, 2005) 4-5 h post-parasitism (Dunphy and Webster, 1988a; Wang *et al*., 1995; Yokoo *et al*., 1995). The nematodes, before it releases its bacterium, encounters the humoral and cellular non-self recognition factors of the haemolymph as they rapidly develop from infective juvenile into third stage nematodes (Poinar and Himsworth, 1967).

Insect larvae usually respond to bacterial or parasite infections with humoral and cellular immune reactions (Götz and Boman, 1985; Loker, 1994; Hoffmann *et al*., 1996). In lepidopteran larvae, the principal humoral factors include the antimicrobial melanizing enzyme, phenoloxidase (Shelby and Popham, 2006), the pattern recognition proteins lysozyme (Wilson and Ratcliffe, 2000), immulectins and other C-type lectins, β-1,3-glucan binding proteins, hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002), apolipophorin-III (Halwani *et al*., 2000) and the lipopolysaccharide-inactivating lipophorin particle (Ma *et al*., 2006). Various types of circulating haemocytes are responsible for the cellular immune responses to these parasites, the reactions includes phagocytosis, encapsulation and nodule formation (Götz and Boman, 1985; Ratcliffe *et al*., 1985; Götz, 1986; Tojo *et al*., 2000). The reacting haemocyte types and intensity of the reactions depend on the size, number, and characteristics of the foreign

bodies (Dunphy and Thurston, 1990; Rohloff *et al*., 1994). Details of nodulation and encapsulation are presented in the section on Insect immunity.

After invasion of the of the host haemocoel the nematode-bacterium complex releases the phase one form of the bacterium which produces extracellular compounds [(e.g. proteases, lipases, lecithinases, antibiotics (Dunphy and Webster, 1988 a, b; Schmidt *et al*., 1988; Clarke and Dowds, 1995; Boemare and Akhurst, 1998)], some of which are toxic to the insects, and others inhibit the immediate innate cellular immune responses (Dunphy and Thurston, 1990; Sicard *et al*., 2004; Kim *et al*., 2005; Sergeant *et al*., 2006). In the case of protein II, a 60 kDa metalloproteinase, secreted by *X. nematophila*, the enzyme destroys the antibacterial cecropin (Caldas *et al*., 2002) much like that of the phase one form of *P. luminescens* released from the monoxenic entomopathogenic *Heterorhabditis bacteriophora* (Jarosz, 1998). The *S. carpocapsae-X. nematophila* complex releases proteases suppressing haemocyte-haemocyte adhesion, capsule formation and nodulation (Ribeiro *et al*., 1999). However, because *X. nematophila* proteases and other metabolites contribute to pathology in the later stages of infection (Bucher, 1960; Caldas *et al*., 2002), the nematodes mechanism(s) of dealing with host haemocytes during early infection is herein considered.

The cuticle from axenically cultured *S. carpocapsae* (Dunphy and Webster, 1987) and cuticular extracts from monoxenically grown *S. feltiae* (Brivio *et al*., 2002) influence haemocyte activity in *G. mellonella*. Lipid components of *S. feltiae* binds a variety of the insect haemolymph molecules creating a coat around the nematode to disguise it against *G. mellonella* haemocyte-recognition (Mastore and Brivio, 2008). LPS-like molecules affect the humoral and cellular defenses of the insect by sequestering lipopolysaccharide-binding proteins present in the host haemolymph limiting prophenoloxidase activation (Brivio *et al*., 2004; Mastore and Brivio, 2008), a process which is responsible for impairing cellular encapsulation (Brivio *et al*., 2002; 2004). During early infection, an immune inhibitor excreted by axenic *S. carpocapsae* destroys induced antimicrobial proteins in haemolymph of vaccinated *H. cecropia* both *in vivo* and *in vitro* (Götz *et al*., 1981). Axenic *S. carpocapsae*, releases excretory/secretory products with unidentified proteolytic activity against *G. mellonella* with impact on unidentified immune factors (Boemare *et al*., 1982; Burman, 1982; Simões *et al*., 1999). Proteases are involved also in

mammalian host infections by other helminthes in terms of penetrating host tissues, digestion of host proteins and evasion of host immunity (McKerrow, 1989; Monroy *et al*., 1989; Fishelson *et al*., 1992).

In chapter two, I observed that immunosuppressive metabolites from live axenic *S. carpocapsae* in the J_3 stage inhibited haemocyte adhesion to slides, and *in vivo* elevated total haemocyte counts, while impairing bacterial removal from the haemolymph of both larval *G. mellonella* and the forest tent caterpillar, *Malacosoma disstria*. In chapter three, I observed that of the potentially numerous immunosuppressants, the main suppressants were proteins, especially serine proteases. Trypsin-like and chymotrypsin-like enzymes were likely immunosuppressants based on (i) correlation analysis of activities of these enzymes in Apizym assays with haemocyte adhesion and (ii) the effect of commercial trypsin and chymotrypsin on adhesion of *G. mellonella* and *M. disstria* haemocytes to slides. Herein, the biochemical properties of trypsin-like and chymotrypsin-like enzymes will be elucidated in terms of immunosuppression of *G. mellonella* haemocytes. For this study, *M. disstria* was not used because of unavailability of the insect at the time.

Materials and Methods

Nematode cultures

Monoxenic cultures of *S. carpocapsae* DD136 were established using dauer juveniles collected from infected *G. mellonella* larvae (Poinar and Himsworth, 1967) on White's water traps (White, 1927). Briefly, infective juveniles were gravity-washed three times in sterile distilled water (10 ml) in 15 ml sterile conical centrifuge tubes, disinfected in thimersol (10 ml, 0.4% w/v distilled water) for 20 min and aseptically gravity-washed in sterile, distilled water (Dunphy and Webster, 1987) before plating on Wout's lipid agar (Wouts, 1981). Prior to inoculating with nematodes, Wout's lipid agar had been inoculated with the phase one form of *X. nematophila* [(the form isolated from the infective juvenile (Akhurst, 1980)] and incubated in darkness at 27°C for 24 h producing a bacterial lawn upon which the pathogens feed. The nematodes were subcultured every two weeks to fresh bacterial lawns.

Axenic nematodes were obtained by dissolving gravity-washed gravid females in 10 ml axenizing solution [0.4 M NaOH, 0.2 M $NaHClO_4$ (Popiel *et al*., 1989)] for 10 min, the suspension was centrifuged (1000 x g, 1 min, 25°C) in 1.5 ml sterile microcentrifuge tubes and the pellet resuspended in fresh axenizing solution (1 ml) for 10 min, after which only nematode eggs remained. The eggs, rendered free of this solution by centrifugation (500 x g, 20°C, 10 min) and resuspension three times in 1 ml of filter-sterilized phosphate-buffered saline [PBS,135 mM NaCl, 2.7 mM KCl, 8.0 mM H_2PO_4, 1.5 mM KH_2PO_4, 10 mM $CaCl_2$, adjusted with 1 M HCl to pH 6.5 (Giannoulis *et al*., 2007), the pH of *G. mellonella* haemolymph 6.0-6.5 (Dunphy and Chadwick, 1989)], were allowed to hatch in PBS (5 ml) for 16 h at 27°C (Popiel *et al*., 1989). The resulting juveniles, aseptically washed by centrifugation (hereafter described as centrifuge-washing) in sterile distilled water, were placed on sterile liver agar (Dunphy and Webster, 1986) supplemented with filter-sterilized antibiotics [100 µg/ml medium ,gentamycin sulphate; 100 µg/ml medium, streptomycin sulphate and 20 µg/ml medium, kanamycin sulphate (Wang and Gaugler, 1999)]. Cultures were kept at 27°C and subcultured to fresh antibiotic-supplemented liver agar every three weeks.

The bacterial-free status of nematodes was confirmed by incubating the nematodes in Luria broth (5 ml) in 25 ml scintillation vials on a horizontal gyratory shaker (250 rpm) for 72 h at 25°C and 30°C after which 10 µl aliquots were plated on Luria agar and incubated at 25°C and 30°C for 72 h. These temperatures were selected to increase the chances of detecting isolates of *X. nematophila* and other possible bacterial species with different temperature preferences surviving axenizing protocols. The absence of a change in broth turbidity (measured spectrophotometrically at 660 nm) and colony formation on the agar medium indicated axenic nematodes.

Axenic nematode excretion/secretion product (exudate)

Live and freeze-killed (-20°C, 24 h) mixed stage nematodes (J_{1+2} = 100, J_3 = 580, J_4 = 120, adults = 45 females and 65 males) previously rinsed from liver agar were used to obtain excretion/secretion metabolites (exudate). The axenic nematodes were centrifuge-washed twice in sterile distilled water (1 ml) with a final resuspension to 1000 nematodes per ml of PBS. After incubating 5 ml of suspension in 25 ml sterile beakers (capped with sterile paraffin) at 25°C on a horizontal gyratory shaker (100 rpm) for designated times (0.5-24 h), the nematodes were removed by centrifugation (1000 x g, 2 min, 25°C) and the resulting exudate frozen (-80°C) until used. Exudate sterility was based on the absence of change in turbidity of Luria broth (5 ml) in 20 ml scintillation vials that had been inoculated with exudate (20 µL) and incubated for 72 h at 25°C and 30°C as previously described.

Insects and bacteria

G. mellonella larvae were reared on a multigrain diet supplemented with glycerol and vitamins at 30°C (Dutky *et al*., 1962) under constant light. Fifth instar larvae weighing 200 ± 10 mg were used. Gram-negative, entomopathogenic, *Xenorhabdus nematophila* (F. Enterobacteriaceae, ATCC strain 19061), in the phase one form (Akhurst, 1980) were cultured on Luria agar supplemented with triphenyltetrazolium chloride (4 mg/ml) and bromthymol blue

(2.5 mg/ml). Non-pathogenic *Bacillus subtilis* (F. Bacillaceae, Boreal Biological Co., St. Catherines, Ontario) were grown on Luria agar. Both bacterial species were subcultured every fortnight and kept in darkness at 25°C. For experimental purposes both bacterial species were grown in Luria broth (10 ml) in 25 ml scintillation vials at 25°C on a horizontal gyratory shaker (250 rpm) until the cells reached the midlog growth stage (optical density of 0.6 at 660 nm). Bacteria were washed three times by centrifugation (12,000 x g, 2 min, 20°C) and resuspension of the pellet in PBS (1 ml). The bacteria were killed by ultraviolet irradiation for 2 h, stored at 5°C over night and centrifuge (12, 000 x g, 2 min, 20^{o}C) -washed in PBS (1 ml). The use of dead, washed bacteria removed haemocyte-activating formyl peptides (Alavo and Dunphy, 2004), detectable intracellular proteases [one of which from *X. nematophila* may be an immunosuppressant (Caldas *et al*., 2002)], eliminated from *X. nematophila* water-soluble metabolites that elicit apoptosis (Wang *et al*., 1994; Cho and Kim, 2004) and precluded inhibition of nodulation (Park *et al.,*2003). Bacterial death was confirmed based on the absence of both a change in optical density of Luria broth (10 ml) inoculated with bacteria (20 µl) and colony formation from aliquots (10 µl) plated on Luria agar and incubated for 72 h at 25°C and 30^{o}C.

Optimization of trypsin and chymotrypsin detection in nematode exudate in terms of optimum exudate volume and incubation time

Recognizing the limitations of Apizym assay for enzyme detection, the assay not being able to adequately identify enzyme, as it could not reliably distinguish between isolates of *Haemophilus somnus*, a Gram-negative fermentative coccobacillus from different anatomical sites or types of secretions (Groom *et al*., 1986), a more vigorous proof for the presence of trypsin and chymotrypsin in the exudate was required. It was deemed necessary to detect these enzymes using assays with maximized sensitivity. Optimization of the assays required determining the optimum exudate volume and incubation time to maximize chromogen production, the chromogen being released from substrate enzyme. Trypsin-specific substrate [(N-α-benzoyl-*DL*-Arg-*p*-nitroanilide (BA*p*NA), Ramalho-Ortigão *et al*., 2003, Oppert *et al*., 2006)] 1 mg; Sigma

Co.) and the chymotrypsin-specific substrate, N-benzoyl-L-tyrosine-P-nitroanilide [(BNZTYRpNA, 1 mg; Sigma Co.) (Lam *et al*., 1999; Oppert *et al*., 2000)], were dissolved in dimethyl sulfoxide (DMSO, 1 ml) to a concentration of 84 mM which was subsequently diluted in PBS giving a working substrate concentration of 0.084 mM which was used in all assays. Both assays consisted of increasing aliquots of exudate (0-100 µl) in PBS (the volumes of which varied to produce a total of 100 µl) in 96 well tissue culture plates. Enzyme-specific substrate was added in 15 µl volumes. Negative controls consisted of varying exudate and PBS volumes (100 µl final volume) with 15 µl PBS added subsequently *in lieu* of substrate. A positive control consisted of 25 µl of commercial enzyme, bovine pancreatic trypsin or chymotrypsin (Sigma Co.) stock solution (1mg/ml of PBS) added to PBS (65 µl) and 15 µl of corresponding substrate. Samples were incubated at 25^{o}C on a horizontal gyratory shaker (50 rpm) for selected times (0-16 h) and the absorbance read on a plate reader at 405nm (Ramalho-Ortigão *et al*., 2003).

To characterize the trypsin-like and chymotrypsin-like enzymes further their reaction with serine protease inhibitors were determined. Exudate (25 µl) was inoculated with selected concentrations (0-100 µM) of the general serine protease inhibitor (15 µl) 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride [(AEBSF; Sigma Co.) (Awata *et al*., 2001)], or the chymotrypsin-specific inhibitor, chymotrypsin inhibitor I (Liu *et al*., 2001) and 60 µl of PBS in tissue culture plates (96 wells, Gibco). The plates were incubated at room temperature for 15 min prior to using to test for enzyme activity which was measured after adding trypsin substrate or chymotrypsin substrate (15 µl) and reading the absorbance of the samples on a plate reader 15 min later at 405nm (Ramalho-Ortigão *et al*., 2003). Aliquots (25 µl) of commercial trypsin and chymotrypsin (1mg/ml PBS) were in the positive control samples containing PBS (75 µl) and 15 µl of the corresponding enzyme substrate. Commercial enzyme negative controls consisted of enzymes in 100 µl PBS without substrate.

AEBSF was chosen as the serine protease inhibitor because it is an irreversible inhibitor of trypsin and chymotrypsin isoforms (Citron *et al*, 1996), it is both water soluble and membrane permeable and non toxic for human blood monocytes (Megyeri *et al*., 1995) and the non-self activities of haemocytes of the oyster, *Crassostrea virginica* (La Pere *et al*., 1999). Additionally the inhibitor blocks metalloproteinases (Fosang *et al*., 1998), which are associated with

activating the innate haemocytic responses of *G. mellonella* (Altincizek and Vilvinskas, 2008). There was no trypsin specific inhibitor available that could be used to circumvent the generalized inhibitory activity of AEBSF including the reversible soybean trypsin inhibitor (Ozawa and Kaskowski, 1966) which reacts with chymotrypsin (Ryan, 1966; De Vonis Bidlingmeyer *et al*., 1972) and, although permeable to nuclear membranes (Reynold and Tedeschi, 1984), strongly inhibits human endothelial cell adhesion activities (Shakiba *et al*., 2007). The properties of AEBSF were required to assess the contribution of serine proteases in the nematode exudate to haemocyte adhesion alluded to herein. Chymotrypsin I inhibitor was chosen because it is water and membrane soluble and a very weak to negligible inhibitor of trypsin (Melville and Ryan, 1972).

To determine if serine proteases in general and chymotrypsin-like enzymes in particular have anti-haemocyte adhesion activity, haemocytes monolayers were formed using haemocyte suspensions of 60 µl haemolymph from six chilled (5°C, 10 min) larvae of *G. mellonella*, in chilled PBS (1 ml, 4°C). Ten µl of the haemocyte suspension placed on a 95 mm^2 area on the slides were incubated with 10 µl of the exudate solutions with and without AEBSF or chymotrypsin I inhibitor for 30 min [the time for maximum haemocyte adhesion for the insect species (Zakarian *et al.,* 2002) at 27 °C and 95% RH. The negative control consisted of 10 µl of haemocytes incubated with 10 µl of PBS with the different inhibitor concentrations *in lieu* of the exudate. Subsequently the slides were rinsed three times with PBS (2 ml) to remove non-adhering haemocytes. Attached haemocytes were fixed in glutaraldehyde-formaldehyde vapour and mounted in 20% (v/v) glycerol in PBS. The total number and types of adhering haemocytes were determined by phase contrast microscopy.

To confirm the specificity of chymotrypsin inhibitor I for chymotrypsin, the effect of exudate generated hydrolysis of the trypsin-specific substrate was assayed as follows: selected amounts of chymotrypsin inhibitor I in15 µl solution were added to 25 µl of nematode exudate to which was subsequently added corresponding volumes of PBS and 15µl of trypsin substrate. This shows specificity for chymotrypsin by not affecting trypsin. The negative control consisted of 15 µl inhibitor added to 85 µl of PBS and 15 µl of substrate with no exudate. The samples

were incubated in a tissue culture plate (96 well, Gibco) on a horizontal gyratory shaker (50 rpm) at room temperature and the absorbance read on a plate reader at 405 nm at selected times.

Effect of chymotrypsin and trypsin antibody on nematode exudate and on *Galleria mellonella* haemocytes *in vitro*

It is possible the exudate may contain enzymes that are not closely related to trypsin or chymotrypsin but are sensitive to the AEBSF and chymotrypsin inhibitor, react with trypsin or chymotrypsin substrates and affect haemocyte adhesion. To increase the validity that chymotrypsin-like or trypsin-like enzymes act as an immunosuppressant, the exudate (10 µl) was treated with selected amounts (0,5,10 and 50 µg/µl) of anti- chymotrypsin or anti-trypsin rabbit polyclonal antibody (Abcam, Co.) in PBS (10 µl). The negative controls consisted of 20 µl of PBS with the same amount of antibodies of either type, while the positive controls consisted of 10 µl of exudate and 10 µl of PBS. Samples were incubated in tissue culture plates (96 wells, Gibco) on a horizontal gyratory shaker (50 rpm) for 30 min and centrifuged (14000 xg, 25^{o}C, 2 min) in micro-centrifuge tubes (1.5 ml) to remove precipitates. Aliquots (10 µl) of the supernatant were transferred to tissue culture plates containing the chymotrypsin or trypsin substrate (15 µl), respectively. The samples were further incubated for 15 min and absorbance read at 405 nm. A second aliquot of the supernatants were used to test for antibody-induced immunosuppression using *G. mellonella* larval haemocyte monolayers as previously described for the chemical inhibitor study.

To determine the potential of antibody cross-reaction with opposite enzyme type, the following biochemical assay design was used. A fixed volume of exudate (10 µl) was mixed with selected antibody concentrations in PBS (15 µl) and 75 µl of PBS to which was added 15 µl of substrate that was opposite to the antibody enzyme target (e.g. chymotrypsin antibody reaction contained the trypsin-specific substrate to determine if chymotrypsin antibody neutralized trypsin-like enzymes). Samples were incubated in 96 well polystyrene plates (Gibco) at 25^{o}C, for 15 min on a horizontal gyratory shaker and the absorbance read on a plate reader at 405 nm.

Effect of nematode exudate, chymotrypsin and chymotrypsin antibody and serine protease chemical inhibitors on the removal of *X. nematophila* and *B. subtilis* from *G. mellonella* haemolymph *in vivo*

The ability of the nematode exudate treated with selected amounts of chymotrypsin or trypsin antibody, AEBSF or chymotrypsin inhibitor I to modify the removal of *B. subtilis* and *X. nematophila* from the haemolymph of *G. mellonella* was tested by co-injecting the larvae with either bacterial species in the test solutions. The concentration of the test bacteria *B. subtilis* or *X. nematophila* injected was 1.7 x 10^{10} bacteria in 10 µl of test solution. The bacterial suspensions consisted of the following: exudate (positive control), exudate with 50 µg of chymotrypsin or trypsin antibody (the concentration that most effectively inhibited enzyme activity), exudate with 100µM AEBSF or chymotrypsin inhibitor I (concentrations that maximally inhibited the enzymes). The exudate-free bacterial suspension controls consisted of PBS, PBS with chymotrypsin or trypsin antibody, PBS with AEBSF or chymotrypsin inhibitor I. Thirty min post-injection, the insects were bled and the number of non-attached bacteria determined by phase contrast microscopy on a haemocytometer.

Detection of trypsin-like and chymotrypsin-like enzyme release from axenic *S. carpocapsae* *in vitro*

Axenic *S. carpocapsae* has been reported to release toxic proteins and proteases lethal to insect within few hours post-injection (Simões and Rosa, 1996) which would be before bacterial release from monoxenic *S. carpocapsae* at 4-5 h post-injection (Wang *et al*., 1995). In the present study chymotrypsin-like and possibly trypsin-like enzymes contributed to suppression of haemocyte adhesion to slides and bacterial removal from the insect haemocoel. To assign their contribution during early infection of the host, I sought to determine their time of release from the axenic nematodes. To achieve this, 500 µl of artificial *G. mellonella* serum (Dunphy and Webster, 1986) or 500 µl of PBS were placed in tissue culture plates (96 wells, Gibco), and 100 nematodes [(alive or dead (freeze-killed -20°C, 24 h)] in 100 µl of PBS were added in each well. The samples were incubated for 0, 0.5, 1, 2, 4, 6 and 8 h on a horizontal gyratory shaker (50

rpm). At each time, 25 µl of each of the samples were placed in another tissue culture well (96 wells, Gibco) to which were added 60 µl of PBS and 15 µl of either trypsin substrate or chymotrypsin substrate. The samples were incubated for 15 min on a horizontal gyratory shaker and chromogen read on a plate reader at 405 nm.

Statistics

All graphic data represents the mean ± standard error of the mean, of at least10 temporally separated replicates, from each of which 5-10 individual samples were taken for analysis. A significance α level of 0.05 was chosen. Haemocyte counts were analyzed using 95% confidence limits overlap (Sokal and Rohlf, 1969). Percentage data were analyzed using arc sin √ p-transformed data. Tabulated mean differential counts were presented as the decoded mean (with 95% confidence limits of the transformed data).

RESULTS

Optimization of trypsin and chymotrypsin detection in nematode exudate in terms of optimum exudate volume and incubation time

Both 10 µl and 30 µl exudate with trypsin substrate produced a low absorption profile for chromogen production by 15 min incubation that was essentially similar to subsequent incubation times until 16 h after which an inexplicable increase in optical density occurred (Fig. 4.1). Although 50 µl and 100 µl of exudate produced maximum sensitivity at 15 min incubation, 50 µl was more sensitive. Detectable chromogen precipitation occurred at subsequent incubation times. In the case of chymotrypsin, exudate with the chymotrypsin substrate showed a substantially different absorption profile than did the trypsin assay. The 100 µl exudate showed maximum sensitivity at 30 min incubation (Fig. 4.2). Both 10 µl and 30 µl revealed activity by 5 and 60 min respectively, the optical density values declining thereafter as precipitation occurred. Thus for all subsequent trypsin-like and chymotrypsin-like assays 50 µl and 100 µl exudate volumes (or scaled down versions) were incubated for 15 min for both enzymes since the optical density value from 0-15 min for chymotrypsin was significantly greater ($p>0.05$) than the increase from 15 min to 30 min.

Effect of enzyme inhibitors on nematode exudate enzyme activity and haemocyte adhesion

Subjecting the exudate to various amounts of the serine protease inhibitor AEBSF or chymotrypsin inhibitor I decreased the optical density value with increasing inhibitor concentration compared with the positive control values (Table 4.1, 4.2) suggesting the presence of serine proteases reacting with trypsin-specific and chymotrypsin-specific enzymes. Chymotrypsin I inhibitor also decreased optical density to a plateaued maximum extent at 50 µM (Table 4.2) establishing that some of the decline in values in Table 4.1 may represent trypsin-like enzymes. It is unlikely that chymotrypsin inhibitor I affected trypsin-like activity because increasing the chymotrypsin inhibitor concentration did not alter hydrolysis of the trypsin substrate (Fig 4.3). There was no evidence of the inhibitors or substrates in PBS influencing optical density in the three assays (Table 4.1, 4.2, Fig 4.3).

Figure 4.1 Trypsin-like enzyme detection in live-nematode exudate based on hydrolysis of a trypsin-specific substrate *in vitro*. Values represent the mean ± SE, n ≥ 10.

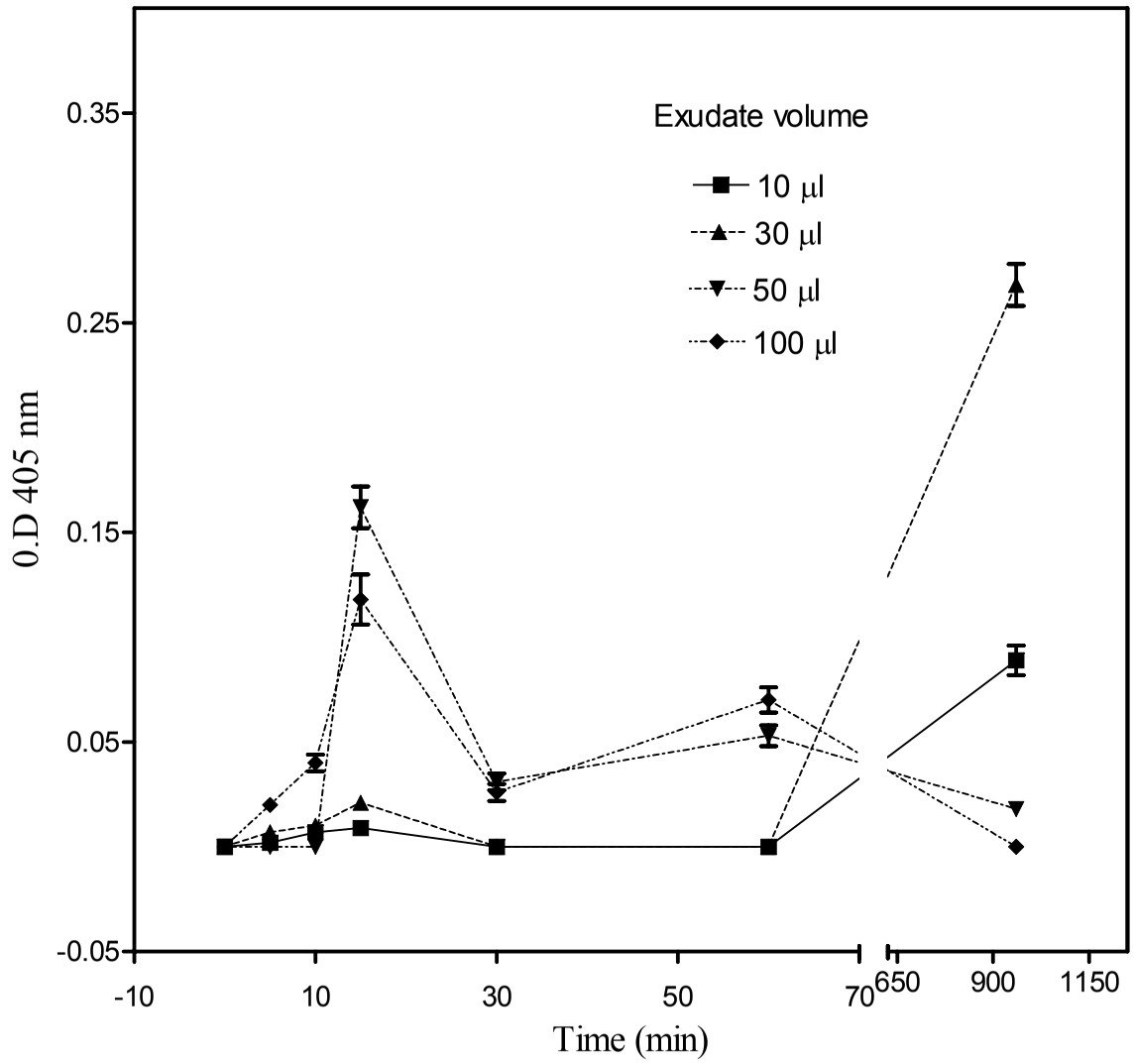

Exudate volume
10 µl
30 µl
50 µl
100 µl
0.35
0.25
0.15
0.05
-0.05
0.D 405 nm
-10
10
30
50
70
650
900
1150
Time (min)

Figure 4.2 Chymotrypsin-like enzyme detection in live-nematode exudate based on hydrolysis of a chymotrypsin-specific substrate *in vitro.* Values represent the mean ± SE, $n \geq 10$.

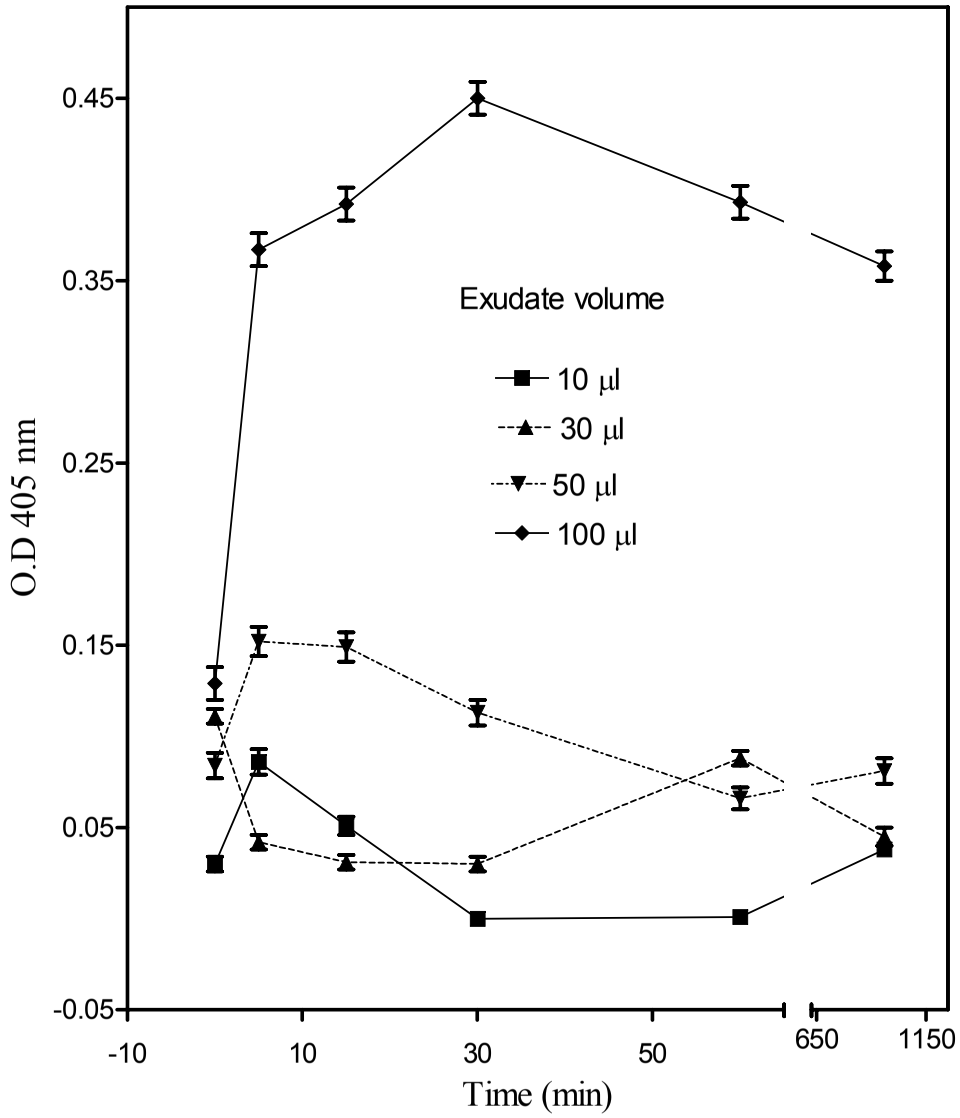

0.45
0.35
0.25
0.15
0.05
-0.05
O.D 405 nm
Exudate volume
10 µl
30 µl
50 µl
100 µl
-10
10
30
50
650
1150
Time (min)

Table 4.1 Effect of AEBSF[a] inhibitor on the hydrolysis of trypsin-specific substrate, BApNA[a], by enzymes in live-nematode exudate and commercial trypsin *in vitro*.

Treatment	Inhibitor concentration (μM)	OD 405nm[b]
Exudate + inhibitor + substrate	0	0.112 ± 0.004[c]
	10	0.074 ± 0.003[d]
	50	0.058 ± 0.003[e]
	100	0.025 ± 0.001[f]
Commercial trypsin + inhibitor + substrate	0	0.113 ± 0.004[c]
	10	0.074 ± 0.004[d]
	50	0.060 ± 0.003[e]
	100	0.027 ± 0.002[f]
Pooled controls[c]	0	0.002 ± 0.000[g]

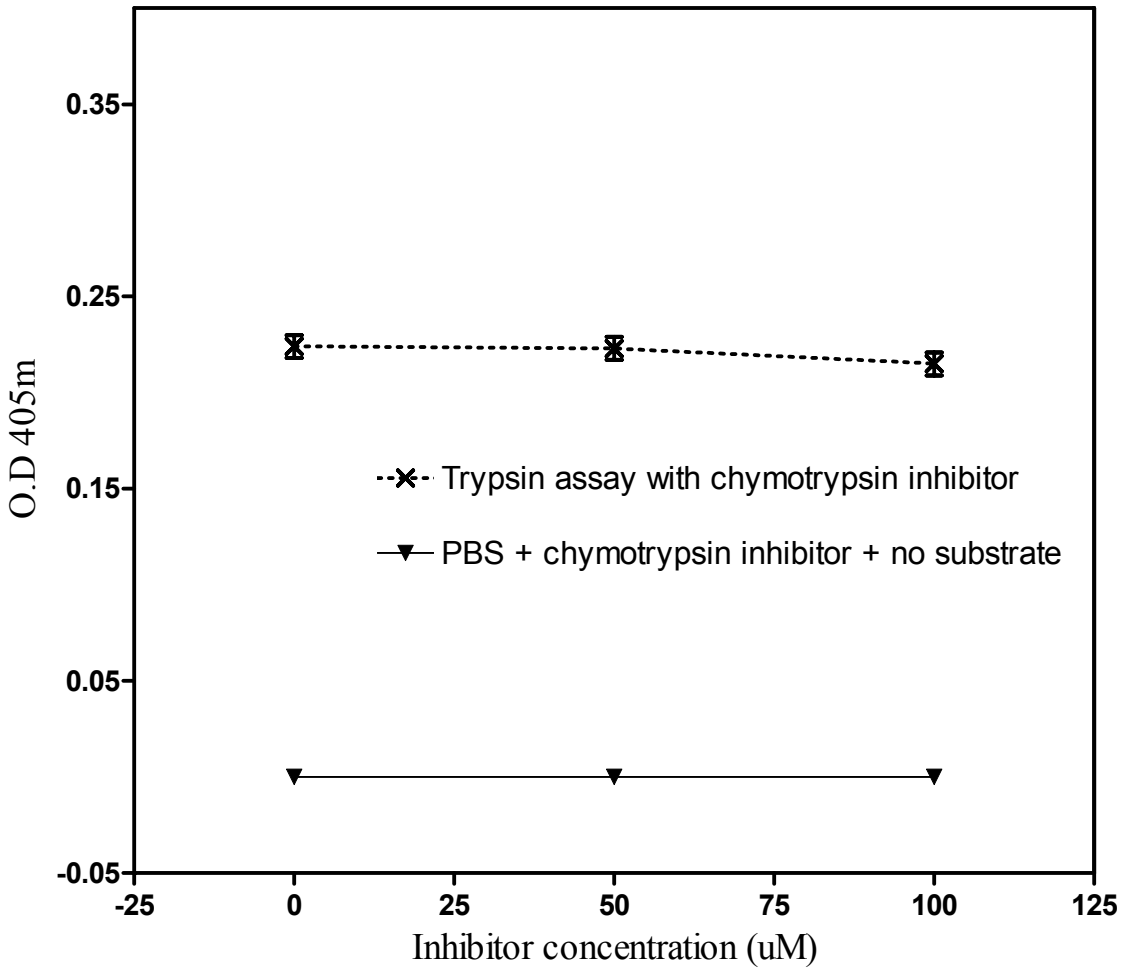
[a]AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], [a]BApNA [N-α-benzoyl-*DL*-Arg-*p*-nitroanilide], [b]Mean ± SEM, n= 10 replicates, values with the same superscript are not significantly different ($p>0.05$). [c]Pooled controls consisted of PBS + inhibitor, PBS + substrate, PBS + exudate and PBS + commercial trypsin. Because values were not significantly different ($p>0.05$) and data fit the assumption for the analysis of variance (Sokal and Roholf, 1968), they were pooled.

Table 4.2 Effect of chymotrypsin inhibitor I on the hydrolysis of chymotrypsin-specific substrate, BNZTYRpNA[a] by enzymes in live-nematode exudate and commercial chymotrypsin *in vitro*.

Treatment	Inhibitor concentration (μM)	OD 405nm[b]
Exudate + inhibitor + substrate	0	0.111± 0.004[c]
	10	0.082± 0.004[d]
	50	0.058± 0.004[e]
	100	0.053± 0.002[f]
Commercial chymotrypsin + inhibitor + substrate	0	0.112 ± 0.004[c]
	10	0.069 ± 0.004[d]
	50	0.060 ± 0.003[e]
	100	0.028 ± 0.002[f]
Pooled controls[c]	0	0.002 ± 0.000[g]

[a]N-benzoyl-L-tyrosine-P-nitroanilide [(BNZTYRpNA, [b]Mean ± SEM, n= 10 replicates. Values with the same superscript are not significantly different ($p>0.05$), n = 10, [c]Pooled controls consisted of PBS + inhibitor, PBS + substrate, PBS + exudate and PBS + commercial chymotrypsin. Because values were not significantly different ($p>0.05$) and data fit the assumption for the analysis of variance (Sokal and Roholf, 1968), they were pooled.

Figure 4.3 Trypsin assay with various concentrations of chymotrypsin inhibitor I in nematode exudate *in vitro* to determine specificity of the inhibitor on the trypsin-like enzyme. Values represent the mean ± SE, $n \geq 10$.


0.35
0.25
0.15
0.05
-0.05
O.D 405m
-25
0
25
50
75
100
125
Inhibitor concentration (uM)
Trypsin assay with chymotrypsin inhibitor
PBS + chymotrypsin inhibitor + no substrate

Increasing the amount of AEBSF inhibitor and chymotrypsin inhibitor I increased haemocyte adhesion to glass slides in terms of the total number of haemocytes, granular cells and plasmatocytes compared with exudate ($p<0.05$); the magnitude varied also with inhibitor type and haemocyte types (Table 4.3). AEBSF (100 μM) increased total haemocyte counts, granular cells and plasmatocytes by 75%, 30% and 50%, respectively, above the exudate values. For 100 μM chymotrypsin inhibitor, the increase was 40%, 75% and 0% for the total haemocyte counts, granular cells and plasmatocytes, respectively, above the exudate values. Thus serine proteases, chymotrypsin-like enzymes, affect haemocyte adhesion, however, because exudate contains a myriad of haemocyte-reactive factors, it cannot be unequivocally stated that active chymotrypsin-like enzymes directly produce lower haemocyte adhesion nor can it be stated if the effect is due directly to enzyme reaction with haemocte membranes or indirectly due to alterations in plasma recognition proteins. The inhibitor data revealed differential adhesion responses by the haemocyte types especially higher levels of granular cells with AEBSF than with chymotrypsin inhibitor I suggesting that the inhibitors may not have totally overlapping activity spectra. This was confirmed since co-incubating both inhibitor types increased the total number of adhering haemocytes, granular cells and plasmatocytes to values comparable to non-inhibited haemocyte in PBS (Table 4.4). Incubating exudate with chymotrypsin inhibitor and trypsin substrates showed that the inhibitor did not affect the trypsin-like enzyme (Fig.4.3) confirming inhibitor specificity for chymotrypsin-like enzymes. The broad inhibitory nature of AEBSF precluded further trypsin-like analysis.

No significant difference ($p>0.05$) was observed between PBS without and with the various inhibitors at the selected concentrations (Table 4.3) indicating that plasma serine proteases present outside the haemocytes did not affect adhesion. Since both inhibitor types are membrane permeable, the PBS data imply any insect serine proteases in the cytoplasm sensitive to the very broad inhibitor, AEBSF, and the lesser active chymotrypsin inhibitor I are not factors in haemocyte adhesion.

Table 4.3 Extent of adhesion of larval *Galleria mellonella* haemocytes to slides in the presence of *Steinernema carpocapsae* exudate with selected types of inhibitors.

Treatments	Inhibitor types					
	[#]AEBSF			Chymotrypsin Inhibitor I		
	*THC	[+]GR	[1]PL	*THC	[+]GR	[1]PL
Exudate + 100 µM inhibitor	208.8±3.7^{a,a}	119.8±2.8^{a,a}	89.0±2.4^{a,a}	200.1±3.6^{a,a}	146.6±3.1^{a,b}	53.5±1.8^{a,b}
Exudate + 50 µM inhibitor	163.3±3.2^{b,a}	109.4±2.7^{b,a}	54.4±1.9^{b,a}	189.6±3.5^{a,a}	138.1±3.0^{a,b}	51.5±1.8^{a,a}
Exudate + 10 µM inhibitor	125±2.8^{c,a}	94.4±2.5^{c,a}	30.6±1.4^{c,a}	155.2±3.2^{b,b}	112.5±2.7^{b,a}	42.7±1.6^{a,b}
Exudate + 0 µM inhibitor	119.0±2.8^{c,a}	85.1±2.3^{c,a}	33.9±1.5^{c,a}	118.0±2.8^{c,a}	81.6±2.3^{c,a}	36.4±1.5^{c,a}
PBS + 100 µM inhibitor	292.7±4.4^{d,a}	227.2±3.8^{d,a}	65.5±2.0^{d,a}	293.3±4.4^{c,a}	226.2±3.8^{d,a}	67.1±2.1^{c,a}
PBS + 50 µM inhibitor	295.0±4.4^{d,a}	212.0±3.7^{d,a}	72.7±2.2^{a,a}	296.6±4.4^{c,a}	229.1±3.9^{d,b}	67.5±2.1^{c,a}
PBS + 10 µM inhibitor	290.1±4.3^{d,a}	209.8±3.7^{d,a}	80.3±2.3^{a,a}	288.3±4.3^{c,a}	215.8±3.7^{d,a}	71.6±2.1^{c,a}
PBS + 0 µM inhibitor	291.1±4.4^{d,a}	230.0±3.9^{d,a}	61.1±2.0^{d,a}	301.6±4.4^{c,a}	230.4±3.9^{d,a}	71.2±2.1^{c,b}

[#]AEBSF: 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, *THC: total haemocyte counts/mm^2, [+]GR: granular cells/mm^2, [1]PL: plasmatocytes/mm^2, Mean±SEM, n=15 replicates. Values with same left superscript with a column are not significantly different ($p>0.05$). Values with same right superscript between rows for a given column type (e.g. THC) are not significantly different ($p>0.05$).

Table 4.4 Extent of adhesion of larval *Galleria mellonella* haemocytes to slides in the presence of *Steinernema carpocapsae* exudates with chymotrypsin inhibitor I co-incubated with 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) inhibitor.

Treatments	*THC	[1]GR	[+]PL
Exudate + 100 µM AEBSF inhibitor	211.4± 3.7[a]	128.9 ± 3.1[a]	82.5 ± 2.0[a]
Exudate + 100 µM chymotrypsin inhibitor I	206.6 ± 3.7[a]	139.9± 3.0[a]	66.6 ± 2.1[c]
Exudate + 100 µM AEBSF + chymotrypsin inhibitor I	271.4 ± 4.2[b]	198.7 ± 3.6[b]	72.7 ± 2.2[c]
Exudate + 0 µM inhibitor	136.3 ± 3.0[c]	82.8 ± 2.3[c]	53.5 ± 1.8[d]
PBS + 100 µM AEBSF + chymotrypsin inhibitor I	298.3 ± 4.4[d]	228.3 ± 3.9[d]	70.0 ± 2.1[b]
PBS + 0 µM inhibitor	303.0 ± 4.4[d]	228.7 ± 3.9[d]	74.3 ± 2.2[b]

*THC: total haemocyte counts/ mm^2, [+]GR: granular cells / mm^2, [1]PL: plasmatocytes / mm^2,

Mean ± SEM, n= 15 replicates, values with same superscript on a column are not significantly different ($p>0.05$).

Effect of chymotrypsin and trypsin antibodies on nematode exudate, and on *Galleria mellonella* haemocytes *in vitro*

Increasing amounts of chymotrypsin (Fig. 4. 4) and trypsin (Fig. 4.5) antibodies in the exudate lowered equally the optical density value by 25% for trypsin-like and chymotrypsin-like enzymes, followed thereafter by a plateau. That the absolute and relative decline in optical densities were less than those caused by AEBSF and chymotrypsin inhibitor I suggest not all the enzymes sensitive to the chemical inhibitors were chymotrypsin and trypsin-like. The similar antibody-based neutralization kinetics implied possible cross reactivity between opposite antibodies and antigens. This is not the case however, because increasing chymotrypsin antibodies did not affect trypsin substrate hydrolysis nor did trypsin antibodies influenced chymotrypsin activity (Fig. 4.6).

The enzymes neutralized by the antibodies had a significant impact on the haemocyte adhesion. Increasing the concentration of chymotrypsin antibodies (Table 4.5) and trypsin antibodies (Table 4.6) increased haemocyte adhesion to glass slides compared with exudate. The highest concentration of the trypsin antibody in the exudate, increased the total number of adhering haemocytes (52%), granular cells (47%) and plasmatocytes (29%) above the exudate values. These values were similar for chymotrypsin antibody inhibition, the total number of adhering haemocytes increasing by (53%), granular cells (51%) and plasmatocytes (17%), which were all significantly higher ($p<0.05$) than the non-treated exudate control, again indicating that the two antibodies maybe cross reacting with the enzymes. Alternatively, it is possible that the haemocytic surface factors (receptors) controlling adhesion were equally susceptible to both enzymes directly or these enzymes rendered the factors equally susceptible to other immunosuppressive agents. Maximum amounts of antibodies inhibiting the enzymes did not return adhesion levels to PBS control values suggesting that although each enzyme significantly affected adhesion, other suppressive factors exist. No significant difference ($p>0.05$) was observed in the level of adhering haemocytes between PBS without and with the different antibody concentrations (Table 4.5 and 4.6), indicating that haemocytes did not detectably react with foreign antibodies.

Figure 4.4 Effect of chymotrypsin antibody on the hydrolysis of chymotrypsin substrate by nematode exudate compared to PBS control. Values represent the mean ± SEM, n ≥ 10.

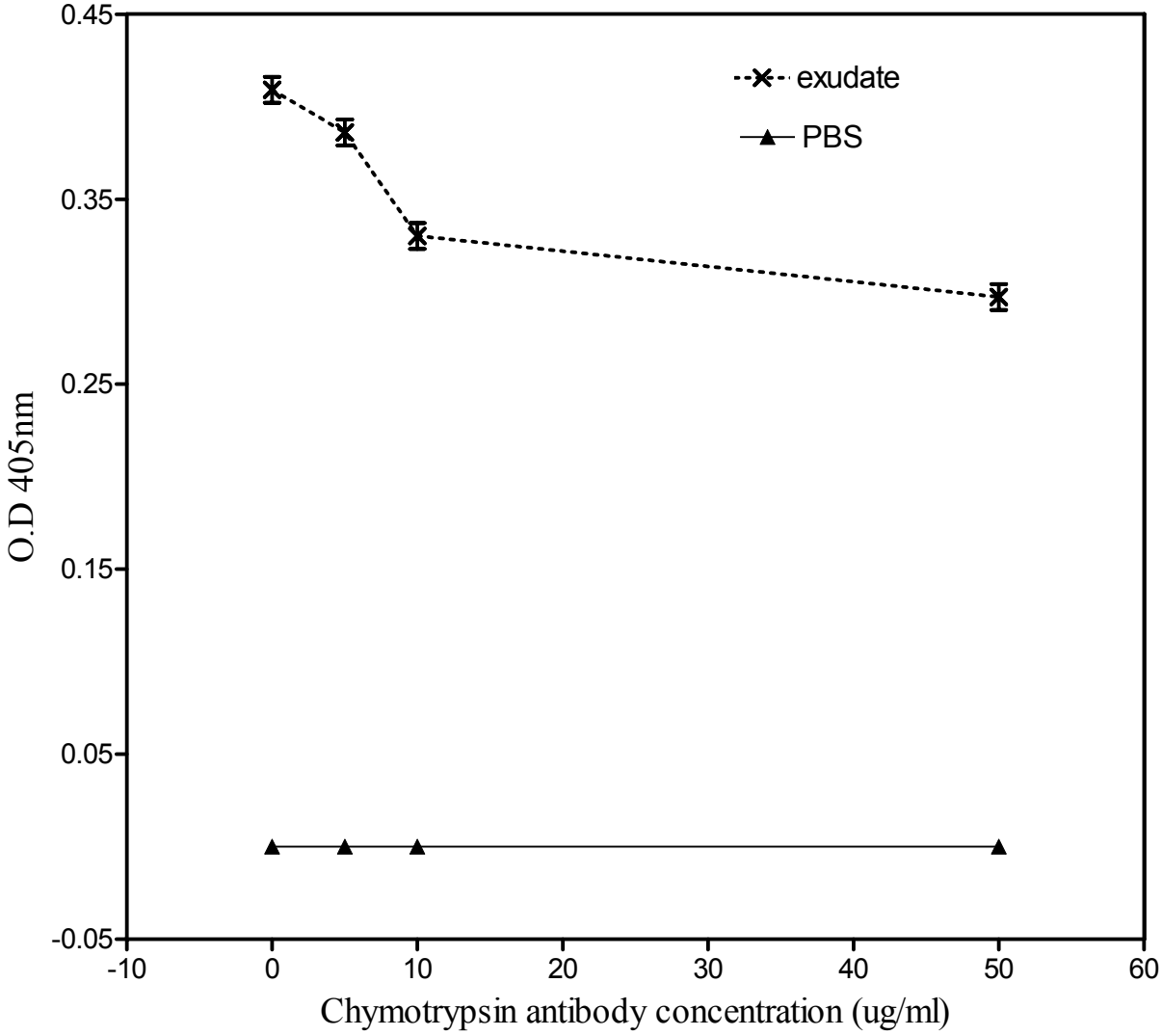

0.45
0.35
0.25
0.15
0.05
-0.05
O.D 405nm
exudate
PBS
-10
0
10
20
30
40
50
60
Chymotrypsin antibody concentration (ug/ml)

Figure 4.5 Effect of trypsin antibody on the hydrolysis of trypsin substrate by nematode exudate compared to PBS control. Values represent the mean ± SEM, n ≥ 10.

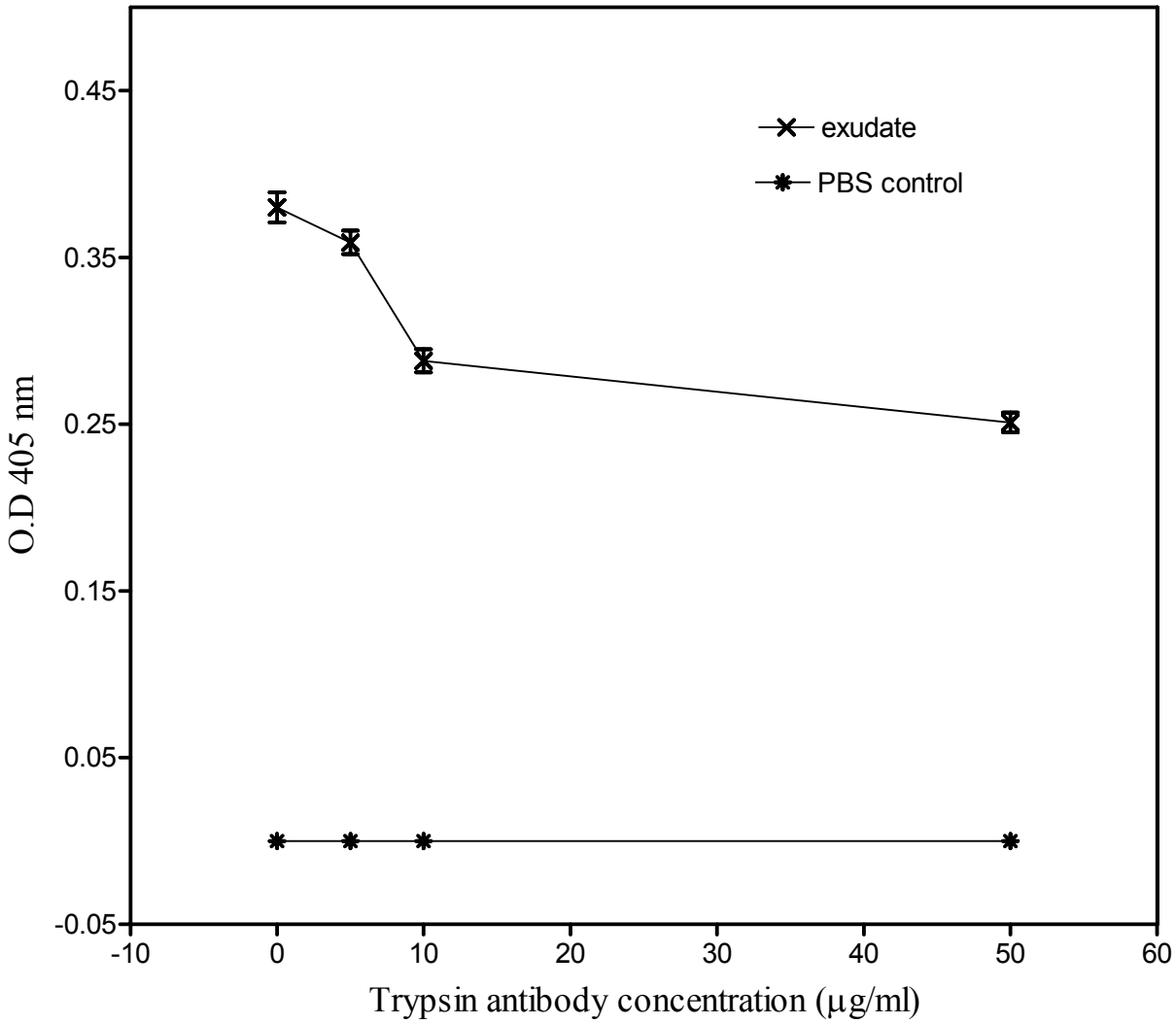

exudate
PBS control
O.D 405 nm
0.45
0.35
0.25
0.15
0.05
-0.05
-10
0
10
20
30
40
50
60
Trypsin antibody concentration (µg/ml)

Figure 4.6 Trypsin and chymotrypsin assays with various concentrations of their respective polyclonal antibodies on live-nematode exudate in *vitro* to determine the specificity of the antibodies on the enzymes. Values represent the mean ± SE, $n \geq 10$.

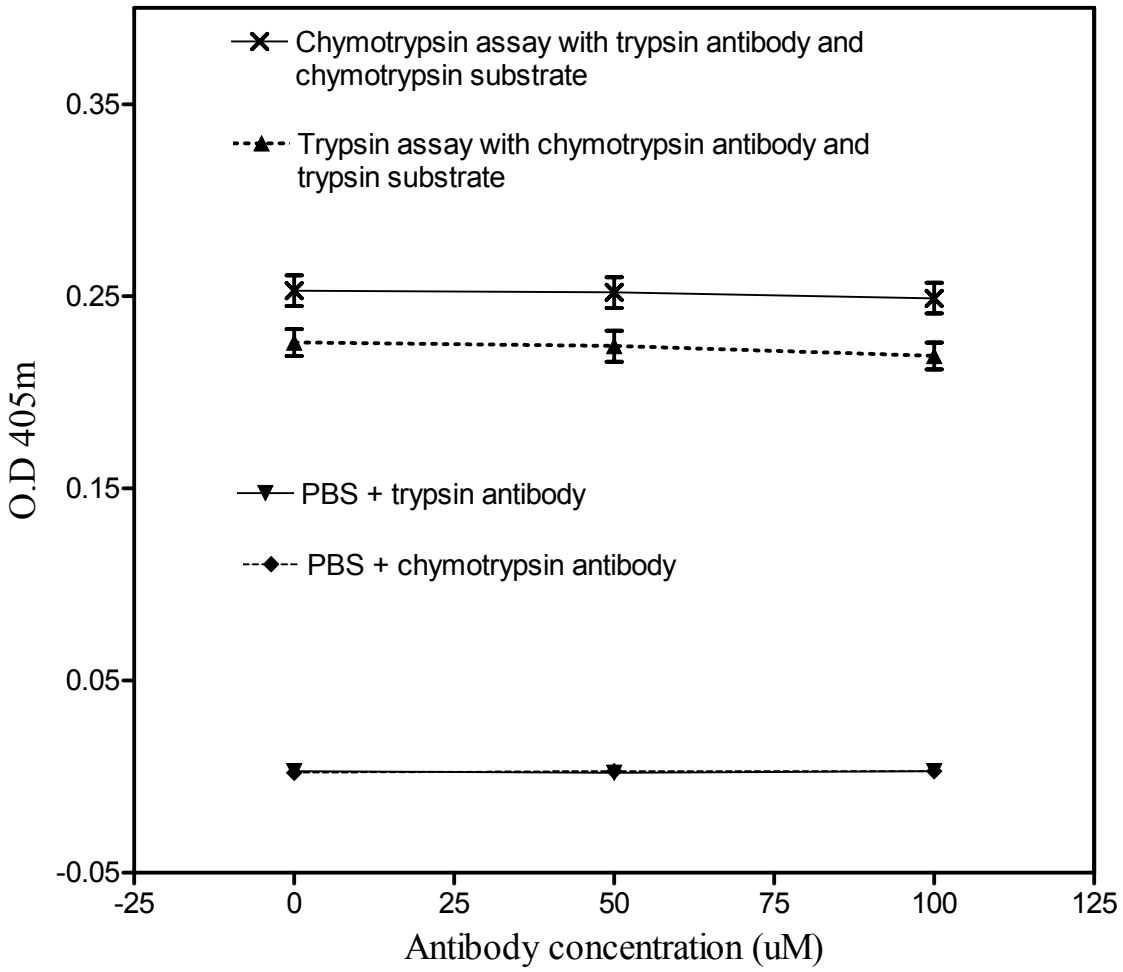

Chymotrypsin assay with trypsin antibody and chymotrypsin substrate
Trypsin assay with chymotrypsin antibody and trypsin substrate
PBS + trypsin antibody
PBS + chymotrypsin antibody
O.D 405m
0.35
0.25
0.15
0.05
-0.05
-25
0
25
50
75
100
125
Antibody concentration (uM)

Table 4.5 Effect of chymotrypsin polyclonal antibody in *Steinernema carpocapsae* exudate on *Galleria mellonella* haemocytes *in vitro*

Treatments	*THC	$^{+}$GR	^{1}PL
Exudate + 50µg antibody	218.6 ± 3.8^{a}	161.0 ± 3.2^{a}	57.6 ± 1.9^{a}
Exudate + 10µg antibody	189.1 ± 4.3^{b}	129.1 ± 3.5^{b}	60.0 ± 2.4^{a}
Exudate + 5µg antibody	158.8 ± 3.2^{c}	113.3 ± 2.7^{c}	45.5 ± 1.7^{b}
Exudate + 0 µg antibody	117.5 ± 2.7^{d}	83.3 ± 2.3^{d}	34.2 ± 1.8^{b}
PBS + 50µg antibody	287.2 ± 4.2^{e}	213.8 ± 3.7^{e}	73.4 ± 2.2^{c}
PBS + 10µg antibody	284.1 ± 4.3^{e}	209.1 ± 3.7^{e}	75.0 ± 2.7^{c}
PBS + 5µg antibody	292.7 ± 4.4^{e}	215.9 ± 3.7^{e}	76.8 ± 2.2^{c}
PBS + 0 µg antibody	288.3 ± 4.3^{e}	224.2 ± 3.8^{e}	64.1 ± 2.0^{c}

*THC: total haemocyte counts/ mm^{2}, $^{+}$GR: granular cells / mm^{2}, ^{1}PL: plasmatocytes / mm^{2},

Mean ± SEM, n= 15 replicates, values with same superscript on a column are not significantly different ($p>0.05$).

Table 4.6 Effect of trypsin polyclonal antibody in *Steinernema carpocapsae* exudate on the adhesion of larval *Galleria mellonella* haemocytes to slides *in vitro*

Treatments	*THC	$^{+}$GR	^{1}PL
Exudate + 50 µg antibody	228.8 ± 3.9^{a}	181.1 ± 3.4^{a}	47.7 ± 1.7^{a}
Exudate + 10 µg antibody	163.8 ± 3.2^{b}	109.4 ± 2.7^{b}	54.4 ± 1.9^{a}
Exudate + 5µg antibody	125.0 ± 2.8^{c}	94.4 ± 2.5^{c}	30.6 ± 1.4^{b}
Exudate + 0 µg antibody	119.4 ± 2.8^{c}	85.0 ± 2.3^{c}	34.4 ± 1.5^{b}
PBS + 50 µg antibody	292.7 ± 4.4^{d}	227.2 ± 3.8^{d}	65.5 ± 2.0^{c}
PBS + 10 µg antibody	295.0 ± 4.4^{d}	222.3 ± 3.8^{d}	72.7 ± 2.2^{c}
PBS + 5 µg antibody	285.0 ± 4.3^{d}	208.0 ± 3.7^{d}	77.0 ± 2.2^{c}
PBS + 0 µg antibody	291.1 ± 4.4^{d}	230.0 ± 3.9^{d}	61.1 ± 2.0^{c}

*THC: total haemocyte counts/ mm^{2}, $^{+}$GR: granular cells / mm^{2}, ^{1}PL: plasmatocytes / mm^{2},

Mean ± SEM, n= 15 replicates, values with same superscript on a column are not significantly different ($p>0.05$).

Comparing the adhesion results of a given enzyme inhibitor (see section: Effect of enzyme inhibitors on nematode exudate enzyme activity and haemocyte adhesion) and antibody type (present section) showed no significant difference in the total haemocyte adhesion or granular cell and plasmatocyte adhesion. The inexplicable exception was for plasmatocytes with the AEBSF inhibitor which favoured more adhesion than did the antibody.

Effect of nematode exudate, trypsin and chymotrypsin antibody and trypsin and chymotrypsin inhibitor on *X.nematophila* and *B. subtilis* removal from *G. mellonella* haemocytes *in vivo*

Both the chemical and antibody inhibitors of both enzymes limited enzyme activity and enhanced haemocyte binding to slides, thus these inhibitors should also enhance bacterial removal from the haemolymph *in vivo*. Co-injection of bacteria in exudate with maximum amounts of inhibitors accelerated removal of both *X. nematophila* and *B. subtilis* (lowered bacterial levels) from *G. mellonella* haemolymph *in vivo* compared with insects with bacteria in the non-inhibited exudate (which elevated bacterial counts). Inhibition of trypsin-like and chymotrypsin-like exudate enzymes with either chemical inhibitors or either type of antibodies equally accelerated *X. nematophila* removal by 50% (Fig. 4.7, 4.8). Similarly, removal of *B. subtilis* was equally accelerated by 40% with chymotrypsin-like inhibited enzymes (Fig. 4.9) and 45% with AEBSF inhibited enzymes (Fig. 4.10) compared with non-inhibited exudate. The removal of *B. subtilis* even with non-inhibited exudate and all other treatments under similar conditions was greater than the removal of *X. nematophila*. The types of inhibitors did not influence *X. nematophila* or *B. subtilis* removal when co-injected in PBS implying proteases participating in haemocyte-mediated removal of the bacteria were resistant to the inhibitors or that non-serine protease mechanisms in the plasma or on the haemocytes bonded the bacteria to the blood cells which is in agreement with the absence of serine protease inhibitors influencing haemocyte adhesion *in vitro.*

Figure 4.7 Effect of *Steinernema carpocapsae* exudate and chymotrypsin inhibitor and antibody on the removal of *Xenorhabdus nematophila* from *Galleria mellonella* haemolymph *in vivo* compared with non-inhibited exudate and PBS with the inhibitor and/or antibody containing the bacteria. Values represent the mean ± SE, n ≥ 10. Values with same letters are not significantly different ($p<0.05$).

Total number of floating bacteria (x 10^6 cells/ml)
0
1
2
3
4
5
6
7
a
a
a
b
a
a
PBS+Xn
PBS + clymotrypsin + antibody + Xn
PBS + clymotrypsin + inhibitor I + Xn
exudate + Xn
exudate + clymotrypsin antibody + Xn
exudate + clymotrypsin inhibitor I+ Xn

Figure 4.8 Effect of *Steinernema carpocapsae* exudate without and with and AEBSF and/or antibodies on the removal of *Xenorhabdus nematophila* from *Galleria mellonella* haemolymph *in vivo* compared with non-inhibited exudate and PBS with the inhibitor and/or antibody containing the bacteria. Values represent the mean ± SE, $n \geq 10$. Values with same letters are not significantly different ($p<0.05$).

Total number of floating bacteria (X10^6 cells/ml)
0
1
2
3
4
5
6
7
a
a
a
b
a
a
PBS+Xn
PBS + trypsin antibody + Xn
PBS + AEBSF inhibitor + Xn
exudate + Xn
exudate + trypsin antibody + Xn
exudate + AEBSF inhibitor + Xn

Figure 4.9 Effect of *Steinernema carpocapsae* exudate and chymotrypsin inhibitors and antibodies on the removal of *Bacillus subtilis* from *Galleria mellonella* haemolymph *in vivo* compared with non-inhibited exudate and PBS with the inhibitor and/or antibody containing the bacteria. Values represent the mean ± SE, n ≥ 10. Values with same letters are not significantly different ($p<0.05$).

Total number of floating bacteria (X 10^6 cells/ml)
0
1
2
3
4
5
6
7
a
a
a
b
a
a
PBS + Bs
PBS + chymotrypsin
antibody + Bs
PBS + chymotrypsin
inhibitor I+ BS
exudate + Bs
exudate + chymotrypsin
antibody + Bs
exudate + chymotrypsin
inhibitor I+ Bs

Figure 4.10 Effect of *Steinernema carpocapsae* exudate and AEBSF inhibitors and antibodies on the removal of *Bacillus subtilis* from *Galleria mellonella* haemolymph *in vivo* compared with non-inhibited exudate and PBS with the inhibitor and/or antibody containing the bacteria. Values represent the mean ± SE, n ≥ 10. Values with same letters are not significantly different ($p<0.05$).

Total number of floating bacteria (X 10^{6}cell/ml)
0
1
2
3
4
5
6
7
a
a
a
b
a
a
PBS + Bs
PBS + trypsin antibody + Bs
PBS +AEBSF inhibitor + Bs
exudate + Bs
exudate + trypsin antibody + Bs
exudate + AEBSF inhibitor + Bs

Detection of time of release of chymotrypsin and trypsin released by axenic *S. carpocapsae* based on enzyme-specific hydrolysis

Release of chymotrypsin-like enzyme by the live nematodes was detected by 2 h p.i with peak at 6 h p.i (Fig. 4.11) in both solutions. The level of chymotrypsin-like activity started declining by 8 h. p. i. with the value at 8 h in the case of PBS. Active trypsin-like enzyme released by live axenic *S. carpocapsae* both in PBS and artificial serum was first detected by 2 h post-incubation with peak activity at 4 h p.i. (Fig. 4.12). In both solutions, the level of trypsin-like activity started declining by 6 h p. i, but the value was still significantly ($p<0.05$) higher than at 2 h p. i. The value at 2 h p. i were similar to that of 8 h p.i. Neither trypsin-like or chymotrypsin-like enzymes were released from the dead nematodes in either solution type.

Figure 4.11 The time of release of chymotrypsin-like from axenic *Steinernema carpocapsae in vitro* compared to the live nematode in PBS control. Values represent the mean ± SE, n ≥ 10.

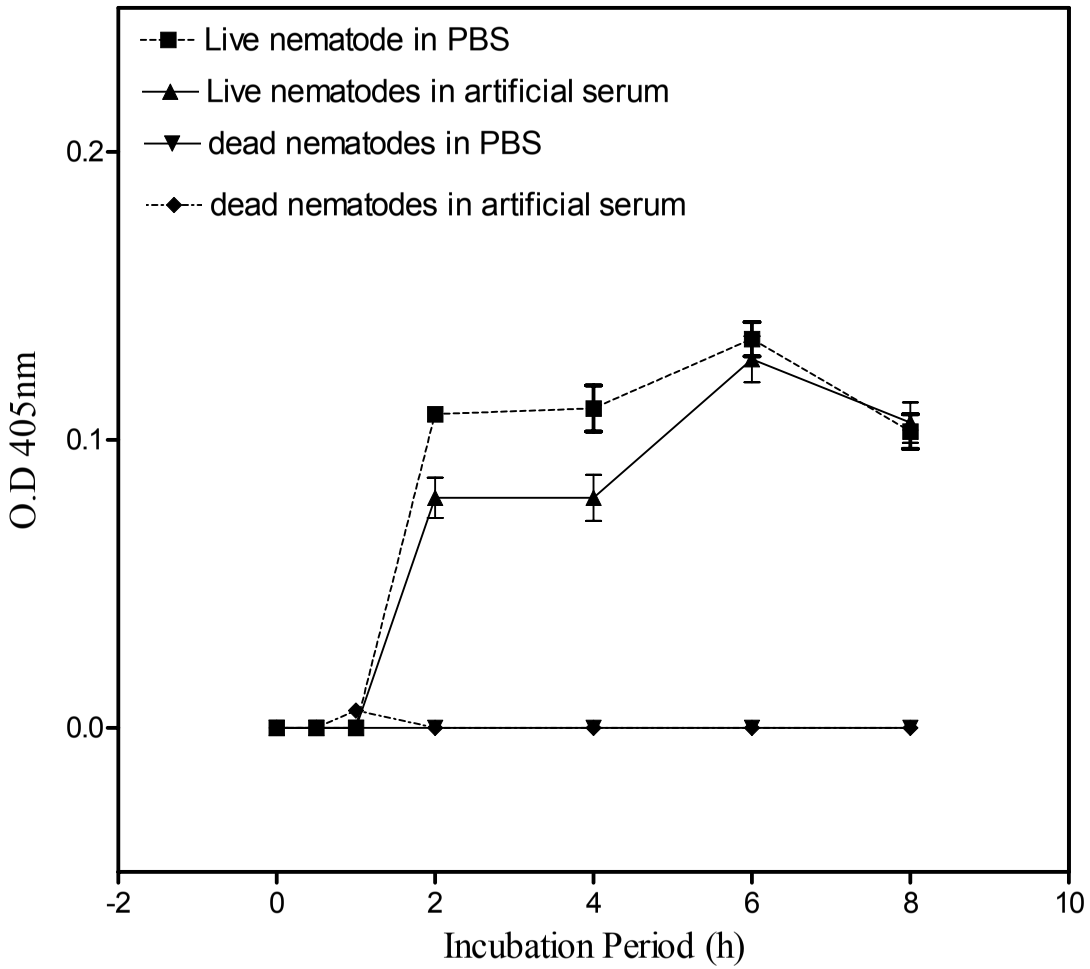
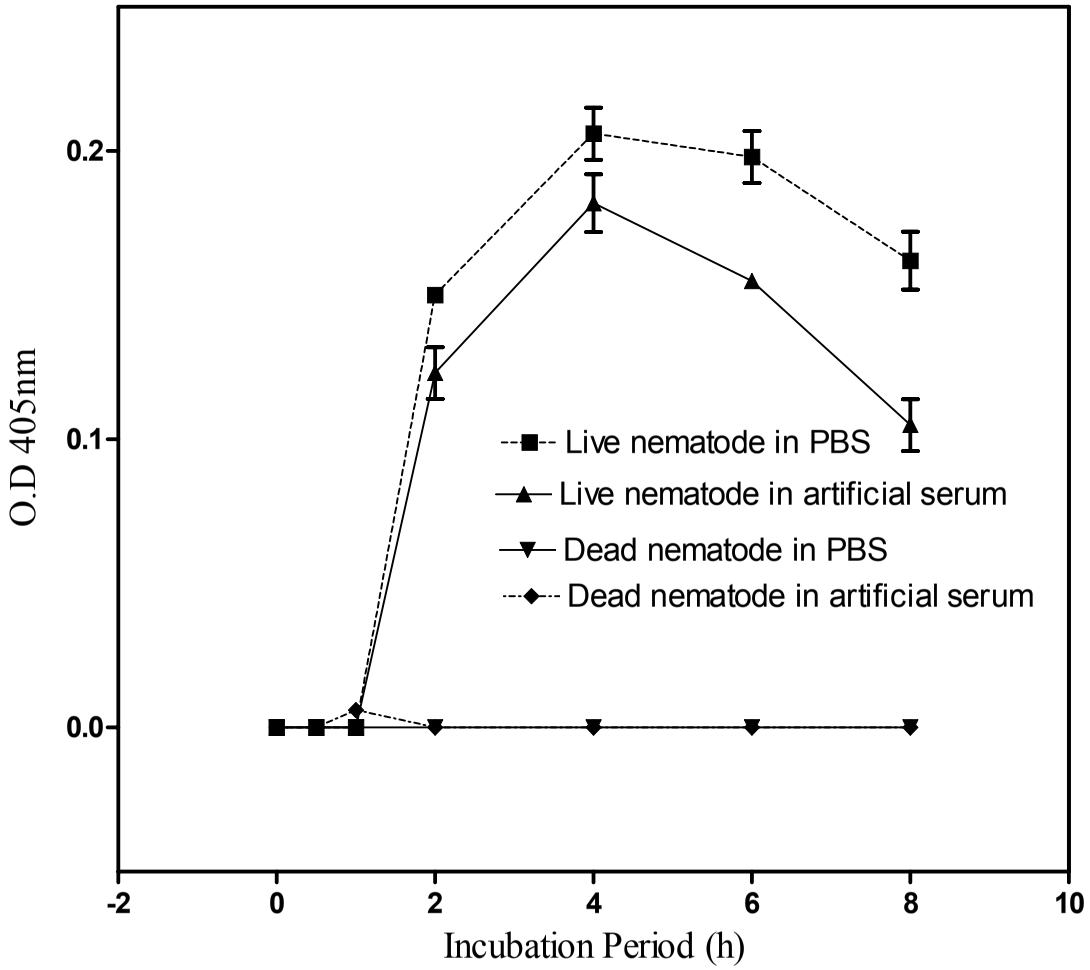

Live nematode in PBS
Live nematodes in artificial serum
dead nematodes in PBS
dead nematodes in artificial serum
O.D 405nm
0.2
0.1
0.0
-2
0
2
4
6
8
10
Incubation Period (h)

Figure 4.12 The time of release of trypsin-like from axenic *Steinernema carpocapsae in vitro* compared to the live nematode in PBS control. Values represent the mean ± SE, n ≥ 10.


0.2
0.1
0.0
O.D 405nm
-2
0
2
4
6
8
10
Incubation Period (h)
Live nematode in PBS
Live nematode in artificial serum
Dead nematode in PBS
Dead nematode in artificial serum

Discussion

Here is reported for the first time that serine proteases with chymotrypsin-like and trypsin-like activity are released from axenic *S. carpocapsae* DD136 based on (i) specific enzyme substrate hydrolysis reactions and (ii) inhibition of substrate hydrolysis by specific polyclonal antibodies and chymotrypsin inhibitor I. These enzymes, emerging (based on enzyme activity) from the nematode 2 h before *X. nematophila* would be released from monoxenic infective juveniles *in vivo* may be involved in host tissue destruction and metabolism associated with nutrition during the early stage of infection. Proteases of *S. feltiae* are involved in host gut invasion (Abuhatab *et al*., 1995) and are part of the undefined virulence mechanisms (Götz *et al*., 1981; Laumond *et al*., 1989; Simões *et al*., 2000). Numerous parasitic nematodes of vertebrates release serine proteases for nutritional purposes including *Necator americanus* (Kumar and Pritchard, 1992), *Trichinella spiralis* (Todorova and Stoyanov, 2000), *Dirofilaria imimtis* (Tamashiro *et al*., 1987), *Onchocerca volvulus* (Lackey *et al*., 1989), *Ascaris suum* (Knox and Kennedy, 1988) and *Anisakis simplex* (Mathews, 1984). These nematodes, which are not related to those in the clade containing steinernematidae (Blater *et al*., 2000) are similar in serine protease release as the bacteriophagous *Caenorhabditis elegans* and vertebrate parasitic *Strongyloides* spp (Blaxter *et al*., 2000; Tcherepanova *et al*., 2000), which are in the same grouping as *Steinernema* spp. Thus these enzymes from *S. carpocapsae* and other invasive nematodes may play a role in degrading connective tissue for nutrition as proposed by Sakanari and Mckerrow (1990) for parasitic nematodes in general. Since *S. carpocapsae* is both invasive and bacteriophagous release of serine proteases was expected. Herein, chymotrypsin-like and trypsin-like enzymes may contribute to host digestion. Chymotrypsin from the root-knot nematode, *Meloidogyne incognita*, digest plant tissues (Fragoso *et al*., 2005) and chymotrypsin-like gene, Scsp-1, isolated from *S. carpocapsae* (Lucerna *et al*., 2007) may have a digestive role.

For many parasitic helminthes serine proteases suppress host's innate and induced humoral immunity (Chen and Bayne, 1994; Strand and Clark, 1999). Weak protease activity associated with a larval *My. unipuncta* plasmatocyte detachment factor from axenic *S. carpocapsae* Breton strain is known (Ribeiro *et al*., 1999). Commercial trypsin also

elicited an unsticking effect against the haemocytes of this insect. Trypsin affects haemocyte activities in terms of haemocyte-substratum adhesion in other invertebrate species including those of the California mussle, *Mythilus californianus* (Chen and Bayne, 1994) and adherent larval plasmatocytes of the soybean looper, *Pseudoplusia includens* (Strand and Clark, 1999). In chapter two of this thesis, trypsin and chymotrypsin were more effective against *G. mellonella* granular cells than against plasmatocytes. It is possible live *S. carpocapsae* uses these enzymes to limit encapsulation, even as the nematodes degrades host tissues and plasma proteins. Haemocyte adhesion readily occurs against dead *S. carpocapsae* which does not release the enzymes.

Live-nematode exudate inhibits activation of *G. mellonella* phenoloxidase (Walter *et al*., 2008) by limiting discharge of prophenoloxidase from larval oenocytoids. Inhibition of haemocyte discharge is necessary since both chymotrypsin and trypsin activates the zymogen in other insect species (Yokoo *et al*., 1992; Kopácek *et al*., 1995) and could generate a systemic response activating phenoloxidase killing the nematodes. It is not known how these serine proteases limit haemocyte discharge although cleavage of cell surface receptors associated with signaling is likely as was proposed for the inhibition of *G. mellonella* haemocytes by entomopathogenic fungi (Griesch and Vlicinskas, 1998). Dead *S. carpocapsae*, which do not inhibit phenoloxidase production, lack these serine proteases, but still activate prophenoloxidase by a possible modification on its cuticle resulting in haemocyte adhesion to the cuticle (chapter 2). That the haemolymph of larvae with dead nematodes does not melanize suggests the non-self response is localized about the dead nematode and possibly controlled by serpins (see Kanost, 1999). Serpins may also limit the adhesion of haemocytes during the initial contact with *S. carpocapsae* before the release of the serine proteases explaining the failure of adhesion for haemocytes of *G. mellonella* (Dunphy and Webster, 1987), and *M. disstria* (Giannoulis *et al*., 2007). Subsequently, the serpin supply may be exhausted by the release of nematode serine proteases, effectively shutting down haemocyte adhesion. It is also possible that cuticular lipids limit haemocyte attachment at the beginning (Dunphy and Webster, 1987; Yokoo *et al*., 1992; Mastore and Brivio, 2008).

AEBSF, chymotrypsin I and anti-chymotrypsin and anti-trypsin antibodies limited enzyme activity and increased haemocyte binding to slides confirming the relationship

between serine protease activity and haemocyte function. That the antibodies limited serine protease activities to a lesser extent than did the chemical inhibitors implies the existence of subpopulations of chymotrypsin-like and trypsin-like enzymes which, although reacting with specific-enzyme substrates, differ in their reaction with the antibodies. Yet the antibody-sensitive enzymes produced enhanced removal of *X. nematophila* and *B. subtilis* in exudate to the same level as chemically inhibited exudate. Thus enzymes with greater similarity to chymotrypsin and trypsin were more effective than serine proteases that react with specific substrates. Additionally, co-incubating haemocytes with AEBSF and chymotrypsin inhibitor I did not increase adhesion to levels found for haemocytes in PBS implying other suppressive factors are involved e.g. possible LPS (Chapter 3) or possibly nematode produced immunosuppressive serpins. Serpins produced by *Brugia malayi* (in same clad as steinernematids) are known block protease released from human neutrophils (Maizels *et al*., 2001). The latter hypothesis is not unrealistic considering the similar functions and protein homologies in NADHoxidase between human neutrophils and insect haemocytes described by Bergin *et al*., (2005).

Other possible factors which could have contributed to suppression since the serine proteases did not produce complete inhibition of haemocyte adhesion maybe PRRs on the insect haemocytes that could recognize pathogens or PAMPs e.g. LPS directly (Dunphy and Halwani, 1997; Ohta et al., 2006). Watanabe *et al*., (2006) identified two types of C-type lectin (BmLBP and BmMBP) from *B. mori* haemocytes that recognize a variety of PAMPs leading to the induction of nodule formation and also enhanced haemocyte binding to microorganisms.

B. subtilis was removed more effectively than *X. nematophila* in both active and serine protease-inhibited exudate. These findings were similar to those of Dunphy and Bourchier (1992) in the gypsymoth, *Lymantria dispar*, *G. mellonella* (Dunphy and Webster, 1988a, b) and *M. disstria* (Giannoulis *et al*., 2007). *Pseudomonas aeruginosa* isolates of moderate virulent are removed more rapidly than the highly virulent isolates from the haemolymph of *G. mellonella* (Dunphy and Webster, 1986) which suggests that the bacterial rate of removal is correlated with virulence. However, Horohov and Dunn (1983) report that different strains of *P. aeruginosa* were removed at the same rate from circulation by nodulation even though the strains differed in their virulence for *Ma. sexta*.

The differences in bacterial removal between the two dead species maybe indicative of antigenic surface differences. Surface antigen LPS released from *X. nematophila* limits phenoloxidase activation (Dunphy and Webster, 1988a) and chelates divalent cations; Fe^{2+} in *G. mellonella* (Dunphy *et al.*, 2002) and Ca^{2+} in *Agrotis segetum* (Yokoo *et al.*, 1995) limiting haemocyte activities and phenoloxidase activation while the lipid A moiety of this LPS, binding to N-acetyl-D-glucosamine haemocyte receptors, damages *G. mellonella* haemocytes releasing entrapped bacteria (Dunphy and Webster, 1988a, b). The combining effects of inhibiting humoral and cellular factors and releasing antigens may have accounted for the higher number of bacteria in the haemolymph. In contrast, the antigen, LTA, from *B. subtilis* activates phenoloxidase in haemolymph of *G. mellonella,* depletes plasmatocyte levels and damages granular cells (Halwani *et al*., 2000)*,* while in *M. disstria, B. subtilis* binds avidly to haemocytes leading to the bacterium being rapidly removed from the haemolymph by nodulation (Giannoulis *et al*., 2007). These reactions may be generated by different signaling pathways than those inhibited by exudate, the difference representing the summation effects of signaling.

The time of release of both enzymes in artificial *G. mellonella* serum from the third stage juvenile nematode (Walter *et al*., 2008) was 2 h with a peak at 4 h for trypsin-like and 6 h for chymotrypsin-like enzymes. The reason why the enzymes peaked at different times may reflect as yet differential undefined interactions of the nematode with the enzymes. The decline in both enzyme activities was unexpected but may represent enzyme reabsorption by the nematode or the release of selective serpins. The fact that these enzymes were not detected in media having dead nematodes is further proof that these enzymes are released only by the live nematodes and are not associated with the cuticle. The enzyme release experiment was done in artificial serum to avoid detection problem as would occur with the presence of serine protease inhibitors (serpins) in the insect. Serine protease inhibitors are present in the haemolymph of many insects including *G. mellonella* (Frobius *et al*., 2000), *Ma. sexta* (Gan *et al*., 2001; Kanost *et al*., 1989; Kanost, 1990), *B. mori* (Eguchi and Shomoto, 1985; Narumi *et al*., 1993; Iwanaga *et al*., 1998) and other arthropods e.g. horseshoe crab, *Tachypleus tridentatus* (Kanost, 1999) and the crayfish, *Pacifastacus leniusculus* (Kanost, 1999) and are synthesized in the haemocytes of some arthropods (Miura *et al*., 1994, 1995; Argarwala *et al*., 1996) where they down-regulate

key proteinase activity in the haemolymph (Kanost, 1999; De Gregorio *et al*., 2001; Ligoxygakis *et al*., 2002b; Tong *et al*., 2005; Zou and Jiang, 2005).

Synder *et al*., (2007) report that the release of *X. nematophila* from *S. carpocapsae* is not triggered by complex or nutrient rich media, but by unknown factors in the haemolymph. That the enzymes were detected in PBS and artificial *Galleria* serum implies enzyme release maybe by a different triggering mechanism, the two solutions being less complex than the haemolymph. The source of the enzyme is unknown. However, in rhabditoid nematodes the phasmids are possible locations where the exudate could be released as observed for *C. elegans* (Politz and Philipp, 1992) and in other rhabditoids including *St. popillosus*, they are produced in and released from the anterior suboesophageal glands (Tadeusz, 1999).

In summary, immunosuppressive exudate released by the live axenic J_3 stage of *S. carpocapsae* suppressed haemocyte adhesion to foreign particles and limited bacterial removal from the haemolymph. Biochemical characterization of the immunosuppressants in the exudate revealed that trypsin-like and chymotrypsin-like enzymes are the major suppressants based on the enhanced haemocyte adhesion responses induced by use of serine protease chemical inhibitors and polyclonal enzyme-specific antibodies.

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CHAPTER 5

GENERAL DISCUSSION AND CONTRIBUTION TO ORIGINAL KNOWLEDGE

GENERAL DISCUSSION

The general objective of this study was to explain the absence of haemocyte adhesion to live axenic *Steinernema carpocapsae*, DD 136 and identify haemocyte immunosuppressive agents including possible cuticular factors and released metabolites. In **Chapter 2**, it is reported that live stages of *S. carpocapsae*, unlike dead nematodes, did not have haemocytes adhering *in vitro* and *in vivo*, which contrasts with dead monoxenic *S. feltiae* which were not encapsulated in *G. mellonella* due to their cuticular composition (Mastore and Brivio, 2008). There was no haemocyte lyses with short-or long-term exposure of live *S. carpocapsae* to the blood cells, the short-term exposure observation implying the nematode possesses a non-adhesive surface as proposed by Dunphy and Webster (1987), which would allow the host to respond to wounding and its innate immune system to contain adventitiously introduced microorganism. Adhesion of haemocytes to dead nematodes was not caused by haemocyte activation factors released from freeze-thawed tissues or damage cuticle since the dead nematode exudate did not enhance haemocyte binding to slides or accelerated bacterial removal from the haemolymph. The initiation of dead nematode encapsulation was associated with activation of phenoloxidase, which once discharged from stimulated haemocytes, enhanced antigen binding to the granular cells and plasmatocytes as reported by Giannoulis *et al*., (2007). The inhibition of phenoloxidase activity in haemolymph with live nematode exudate may partially explain the absence of haemocytes adhering to live *S. carpocapsae* and diminished bacterial removal from the haemolymph *in vivo* by melanotic encapsulation.

The exudate released by the third stage infective juveniles (Walter *et al*., 2008), caused granular cell and not plasmatocyte detachment from pre-formed monolayers. A toxin (Boemare *et al*., 1982), from the axenic nematode exudate, possibly a protease (Laumond *et al*., 1989; Ribeiro *et al*., 1999) is thought to be responsible for granular cell

detachment. Why the plasmatocytes did not detach in this study is unknown and needs to be investigated further. Studies pertaining to the role of immunosuppression of haemocytes and bacterial removal by the exudate are also presented in **Chapter 2**. The higher bacterial numbers observed in the haemocoel of bacteria infected larvae with exudate meant less removal by haemocyte-based nodulation. The live-nematode exudate prevented haemocyte antibacterial reaction by limiting bacterial adhesion to the haemocytes by influencing haemocyte integrity, stimulating bacterial release from the haemocytes and/or limiting phenoloxidase effects. It is therefore established in this chapter that the J_3 stage of *S. carpocapsae* produce immune inhibitors which will be investigated further in the preceding chapter.

In **Chapter 3**, the immune inhibitor(s) is/are characterized as weakly hydrophobic proteins based on batch hydrophobic interaction chromatography. When the exudate was incubated at 65^{o}C, the total number of adhering haemocytes and granular cells increased indicating heat-labile suppressant(s). However, elimination of all suppressive activity was not observed, the extent remaining varying with the insect species being tested. In *G. mellonella* the remaining 10% residual suppression was attributed to a heat-stable, hydrophobic, polymyxin B-reactive immunosuppressant. It was unlikely to be LPS because axenic nematode culturing should dilute out any LPS attached to the nematode cuticle and there were no bacterial source in the exudate precluding LPS contamination.

Does repeated freeze-thawing affects the suppressive nature of the immunosuppressant(s) against haemocyte adhesion? In **Chapter 3**, the immunosuppressant activity against granular cells and plasmatocytes of both *G. mellonella* and *Malacosoma disstria* in the exudate was not affected by repeated freeze-thawing, the plasmatocytes of *M. disstria* being more sensitive than those of *G. mellonella* for both freeze-thawed and fresh exudate. In **Chapter 2**, and **3** it was observed that changes in absolute magnitude of the haemocyte types varied with insect species and that the same proportion of haemocytes exhibited differential sensitivity to the exudate.

In **Chapter 3**, it was observed that the immediate reaction between the cocktail of protease inhibitors and the exudate limited a decline in exudate protein and increased haemocyte adhesion implicating immunosuppressive proteases in the exudate. Prolonged incubation of the protease-inhibited exudate led to an increase in haemocyte adhesion, an

indication that non-enzymic proteins, which are denatured at room temperature with prolong incubation, may be involved. The observed decline in total protein concentration after the exudate was incubated with trypsin and chymotrypsin-conjugated agarose beads without a decline in immunosuppression indicates the presence of trypsin and chymotrypsin sensitive proteins in the exudate that do not affect the haemocytes. In **Chapter 3**, is also observed that based on correlation analysis between the enzyme activity and the level of adhering haemocytes other enzymes in addition to trypsin and chymotrypsin present in the exudate maybe suppressing adhesion. The level of suppression observed when both commercial trypsin and chymotrypsin were incubated with the haemocytes was not equal to the exudate implying the involvement of other suppressive factors.

In **Chapter 4**, chymotrypsin-like and possibly trypsin-like serine proteases were established as the major suppressants released by axenic J_3 of *S. carpocapsae* based on enzyme substrate assays and chemical inhibitors and polyclonal antibodies inhibiting the suppressant(s) increasing haemocyte adhesion to slides and limiting of bacterial removal from the haemolymph *in vivo*. Proteases have been implicated in the virulence of *S. carpocapsae* (Götz *et al*., 1981; Laumond *et al*., 1989; Simões *et al*., 2000). The low optical density value observed when the exudate was treated with increasing amount of polyclonal antibodies of these enzymes further justifies the immunosuppressants as trypsin- and chymotrypsin-like. However, comparing the chemical inhibitor and antibody effects based on changes in optical density and immunosuppression of haemocytes suggest that both these enzymes are present but only those sensitive to the antibody are major suppressants. Blocking the action of trypsin and chymotrypsin in the exudate with the chemical inhibitor and antibody increased bacterial clearance from the haemolymph for both bacterial species further supporting the hypothesis that trypsin and chymotrypsin are the major immunosuppressants in the exudate. The rate of removal for avirulent *Bacillus subtilis* was not as effective as for virulent *Xenorhabdus nematophila* as also observed by Dunphy and Bourchier (1992).

The initial time of release of both enzymes was the same with trypsin-like enzymes peaking before chymotrypsin-like enzymes. The presence of serpins in the haemolymph (Kanost *et al*., 1989; Kanost, 1990; Frobius *et al*., 2000; Gan *et al*., 2001), justified the use

of artificial *Galleria* serum for the assay. The possible location in the J_3 were the exudate could be released are the phasmids as observed Politz and Philipp (1992). These enzymes maybe involved in host tissue digestion and metabolism of host proteins. It is thus proposed that these enzymes in the exudate may have two roles, the primary being in food and tissue digestion, and as these enzymes digest molecules around the haemocytes, it is assumed they play a secondary role in suppression of the insect immune system.

In conclusion, this research has explored possible suppressants released by live axenic J_3 *S. carpocapsae* and their mode of action in terms of immunosuppression and bacterial removal from the haemolymph. Trypsin-like and chymotrypsin-like enzymes were identified as the major suppressant.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. The first report on non-cuticular immunosuppressive metabolites in steinernematids.

2. The J_3 stage only released the immunosuppressant(s), however the use of mix stage nematodes to obtain immunosuppressive exudate was reported.

3. The pathogenicity of an entomopathogenic nematode and its metabolites against *Malacosoma disstria* was demonstrated for the first time.

4. Inhibition of phenoloxidase activation and haemocyte adhesion responses by steinernematid metabolites, as opposed to cuticular components was demonstrated.

5. Based on Apizym analysis, chemical inhibitors and polyclonal antibodies, chymotrypsin-like and trypsin-like enzymes were detected as the likely major immunosuppressants in the nematode exudate

6. Both of the enzymes inhibited bacterial removal from the haemolymph by suppressing the haemocyte activity.

7. First report of a heat-stable, LPS-like molecule from axenic *S. carpocapsae* nematode involved in haemocyte suppression.

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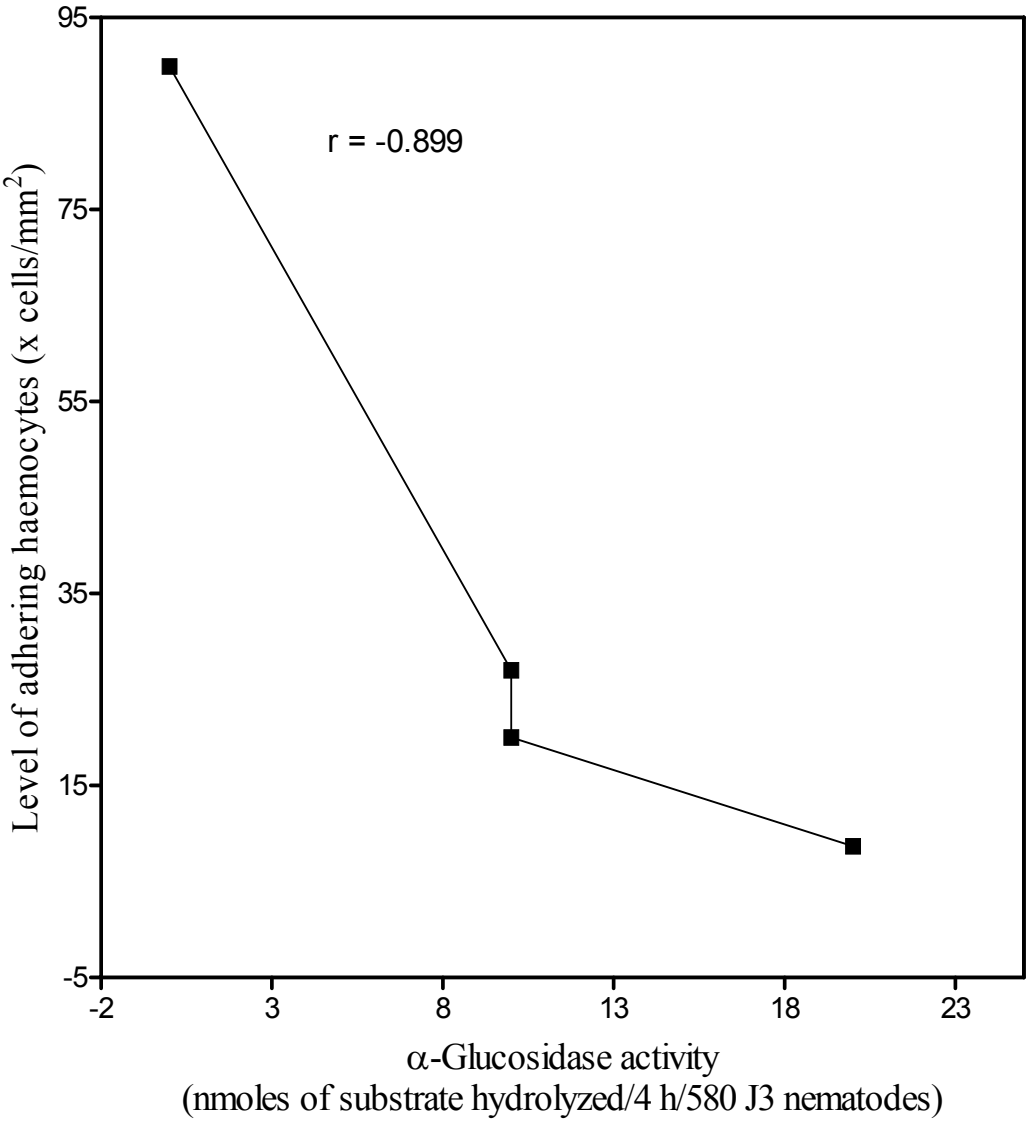
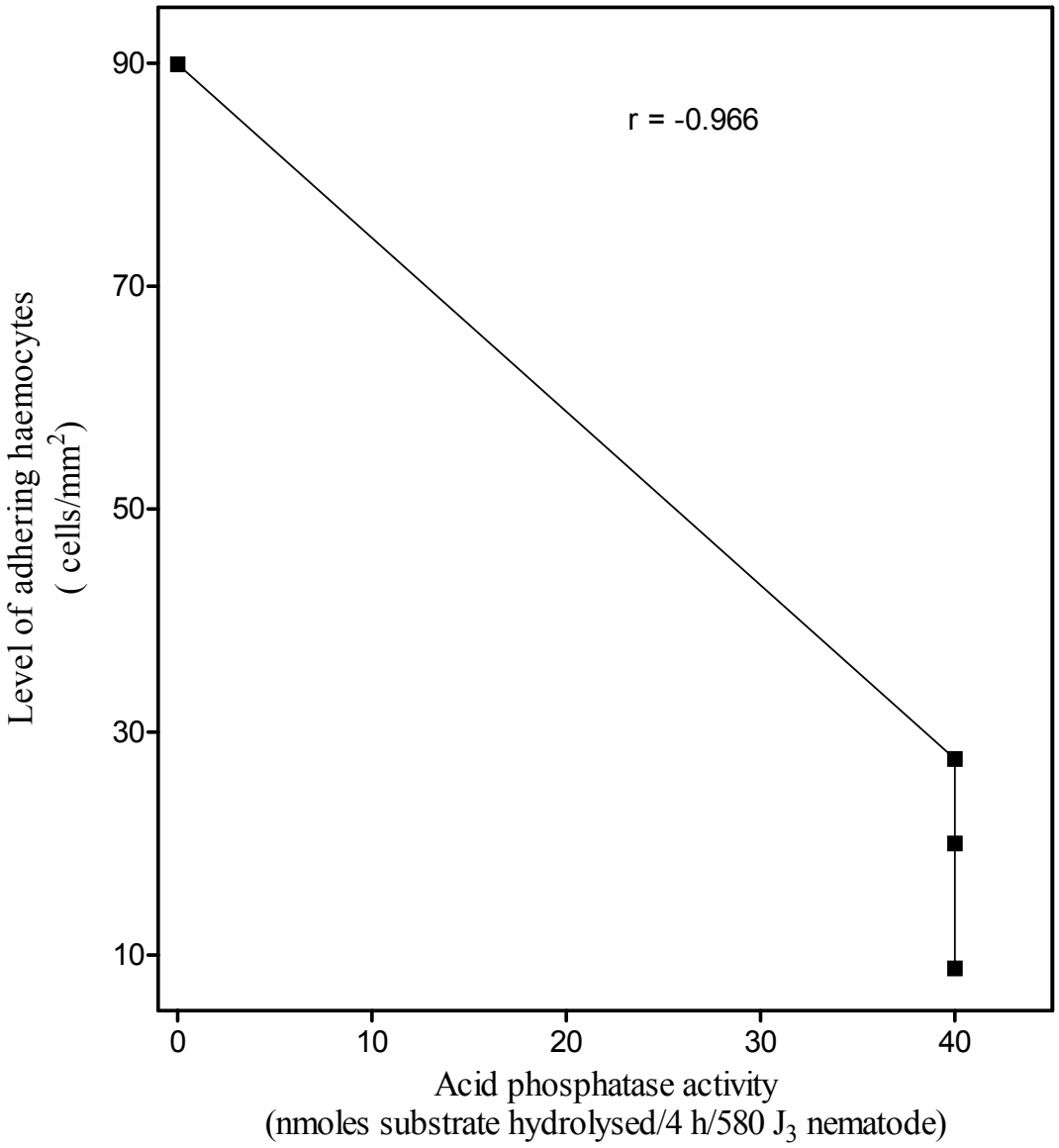
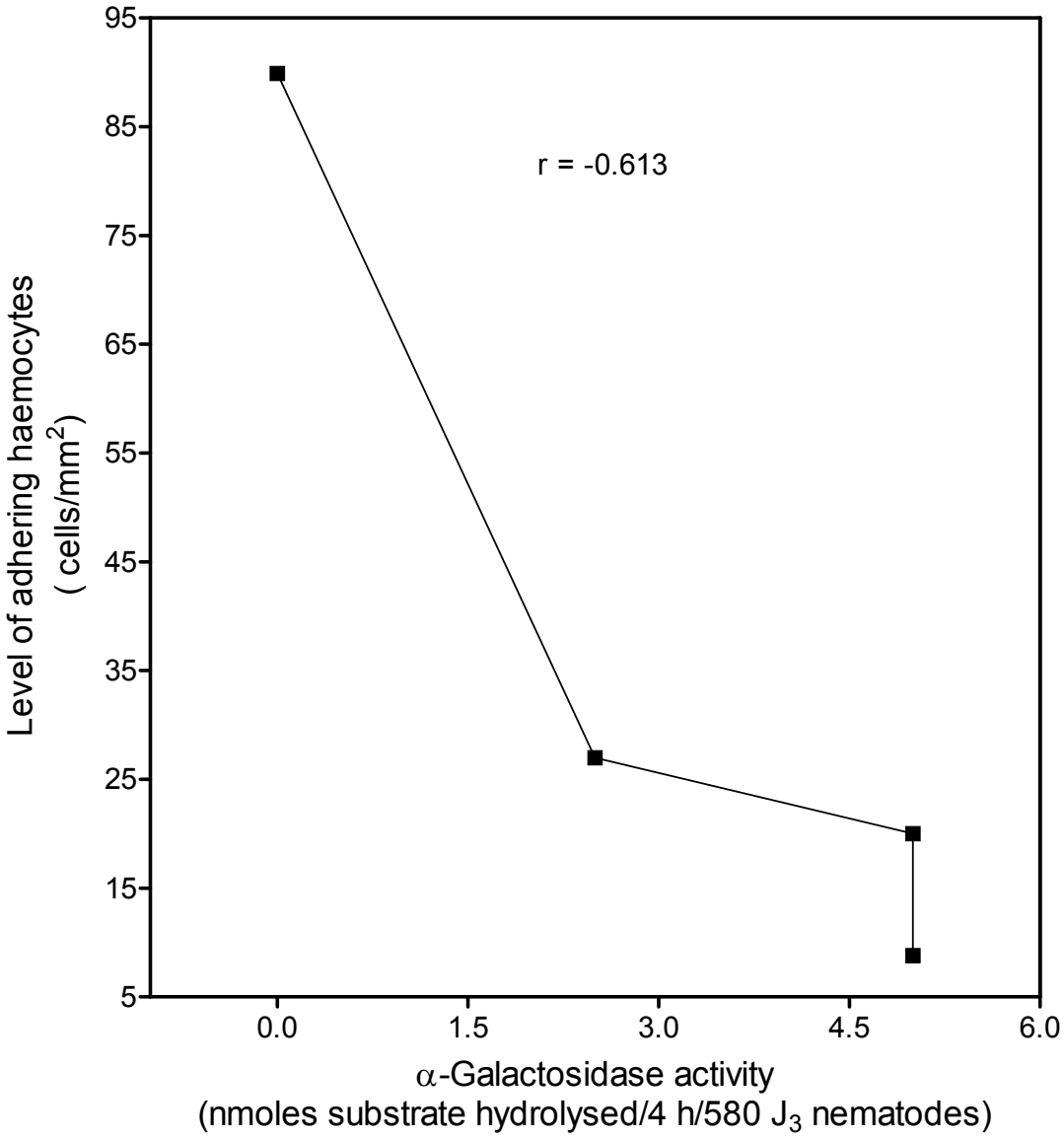
Appendix

Relationships between enzyme activities and level of haemocyte adhesion.


95
75
55
35
15
-5
r = -0.899
Level of adhering haemocytes (x cells/mm2)
-2
3
8
13
18
23
α-Glucosidase activity
(nmoles of substrate hydrolyzed/4 h/580 J3 nematodes)


r = -0.966
90
70
50
30
10
0
10
20
30
40
Level of adhering haemocytes
(cells/mm2)
Acid phosphatase activity
(nmoles substrate hydrolysed/4 h/580 J3 nematode)


95
85
75
65
55
45
35
25
15
5
r = -0.613
Level of adhering haemocytes
(cells/mm2)
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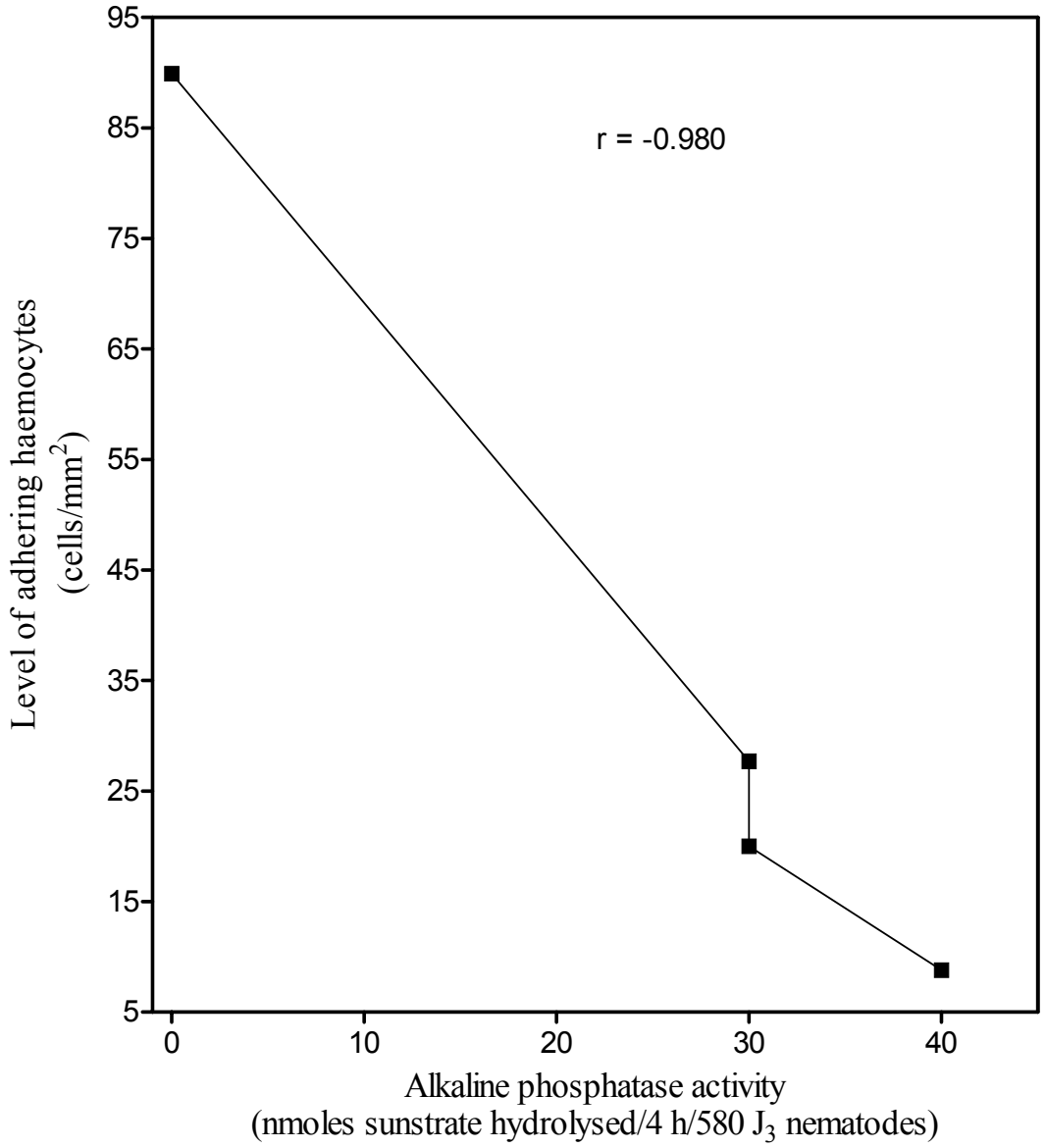
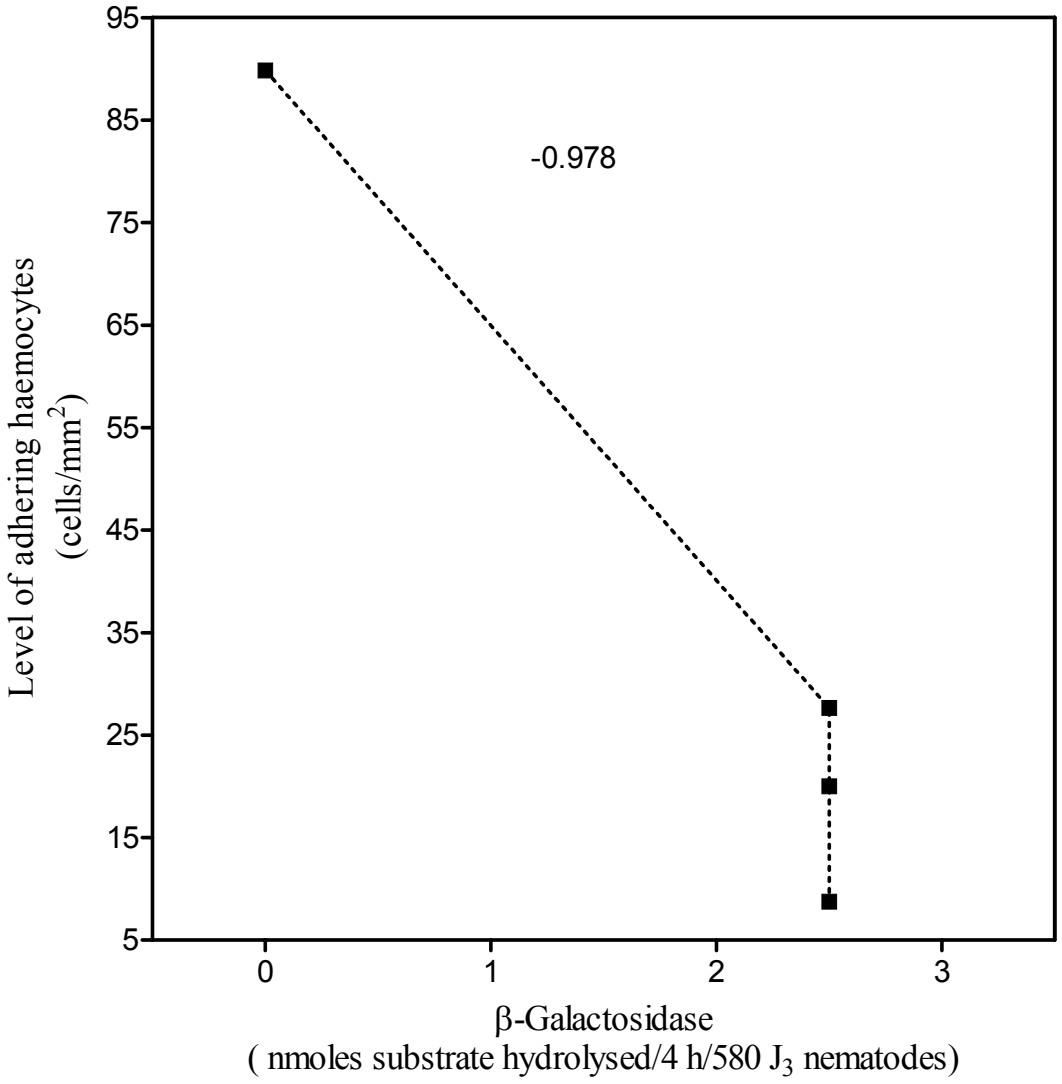
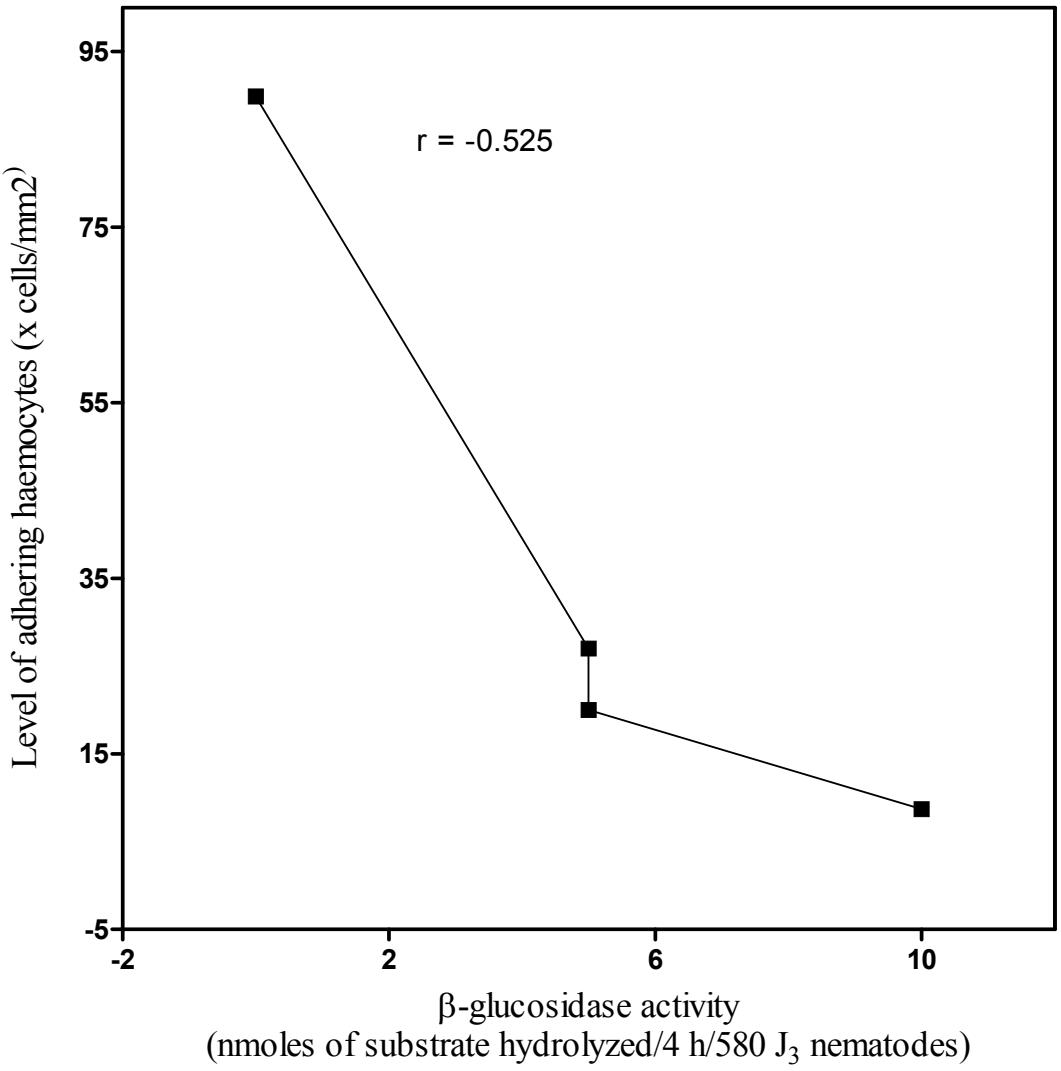
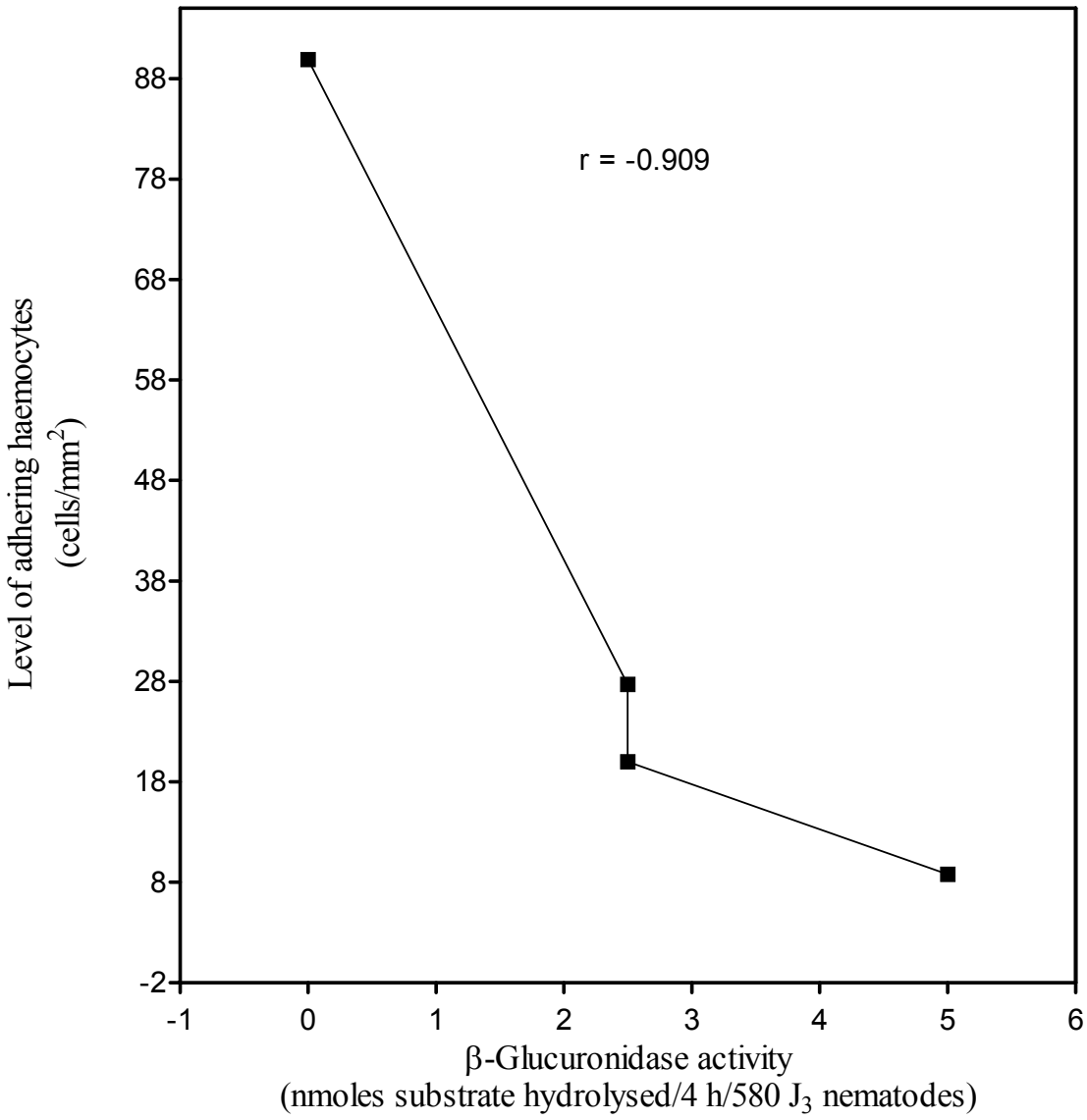
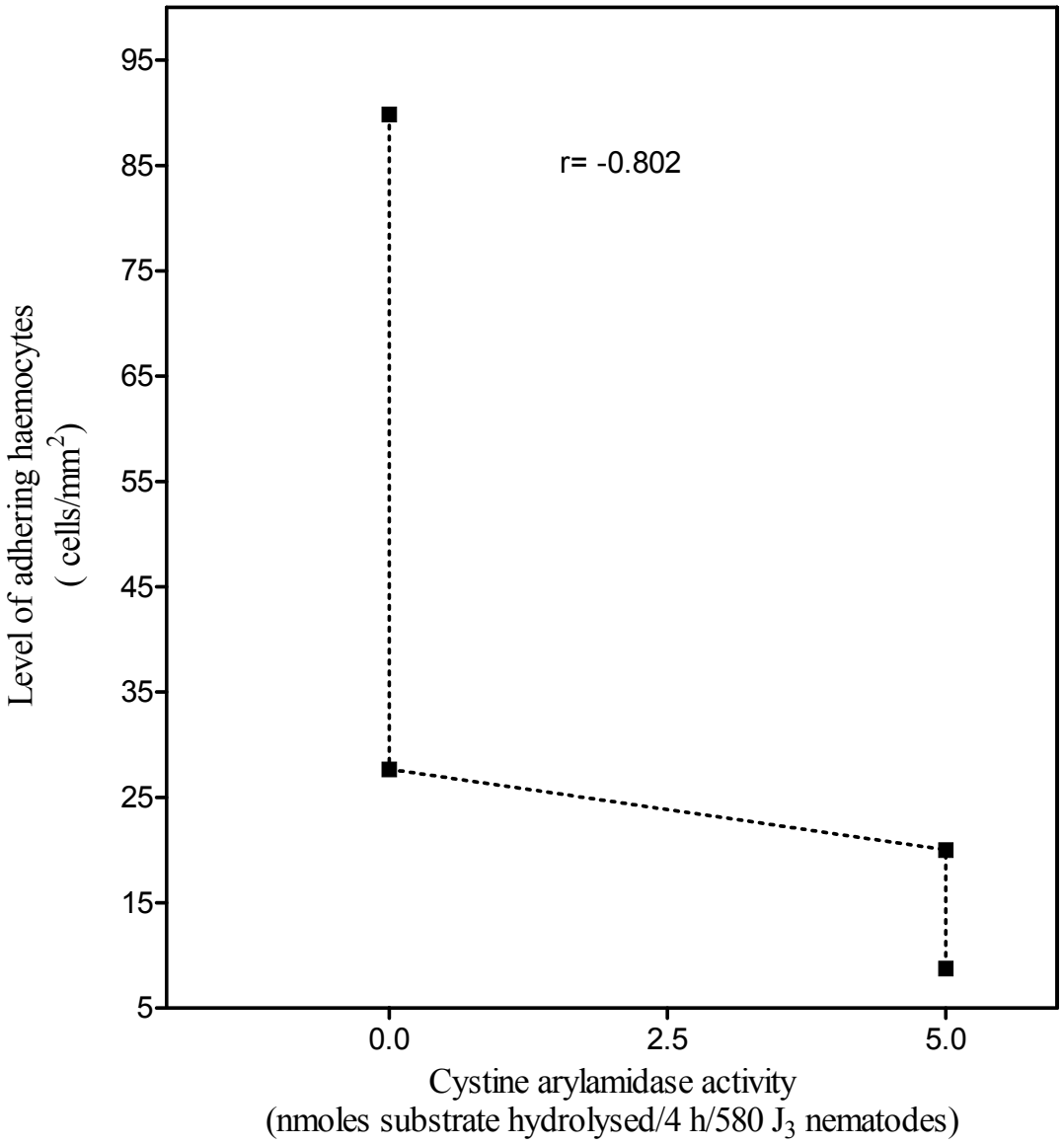
1.5
3.0
4.5
6.0
α-Galactosidase activity
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)

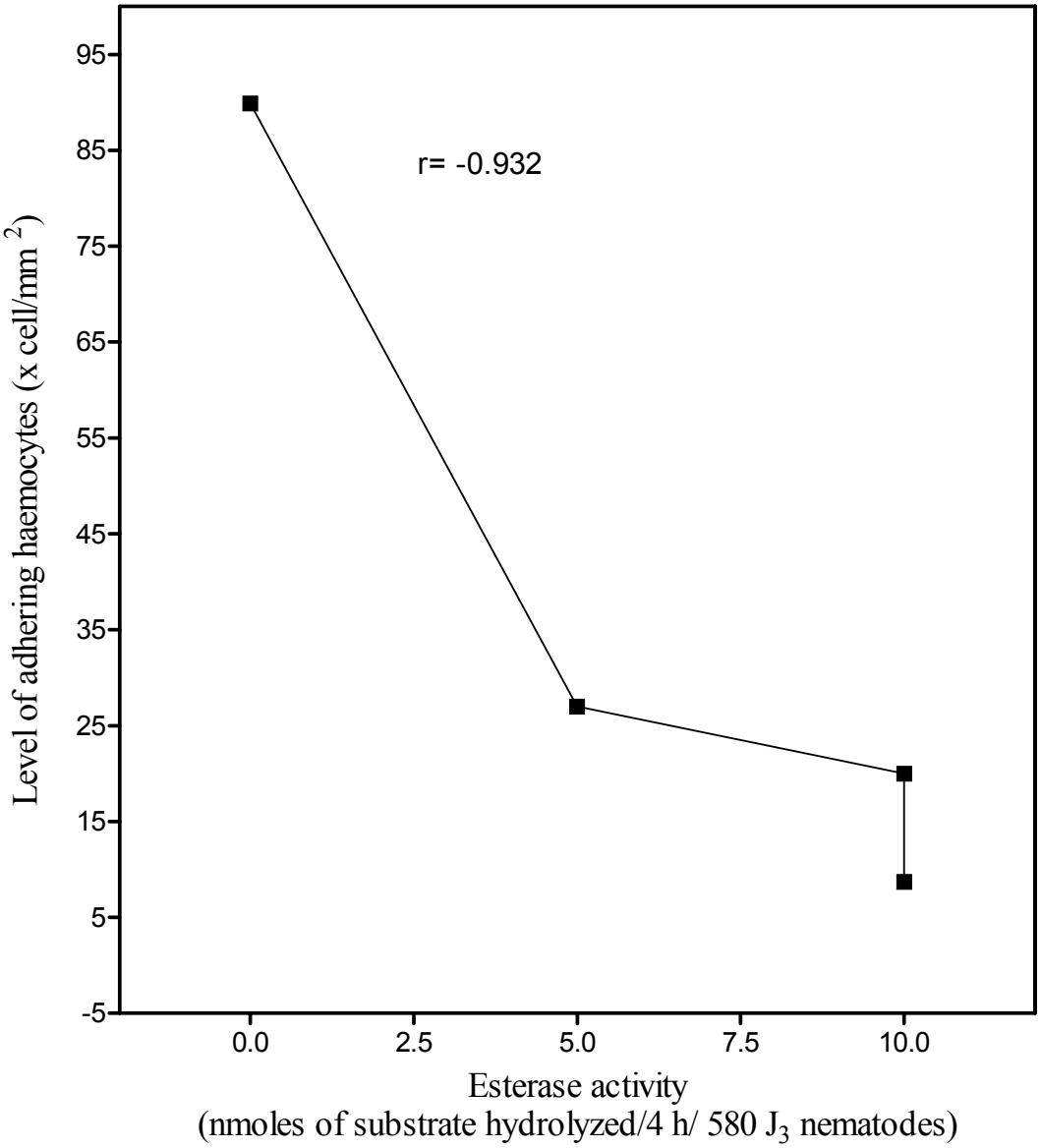

r = -0.980
Level of adhering haemocytes
(cells/mm2)
95
85
75
65
55
45
35
25
15
5
0
10
20
30
40
Alkaline phosphatase activity
(nmoles sunstrate hydrolysed/4 h/580 J3 nematodes)

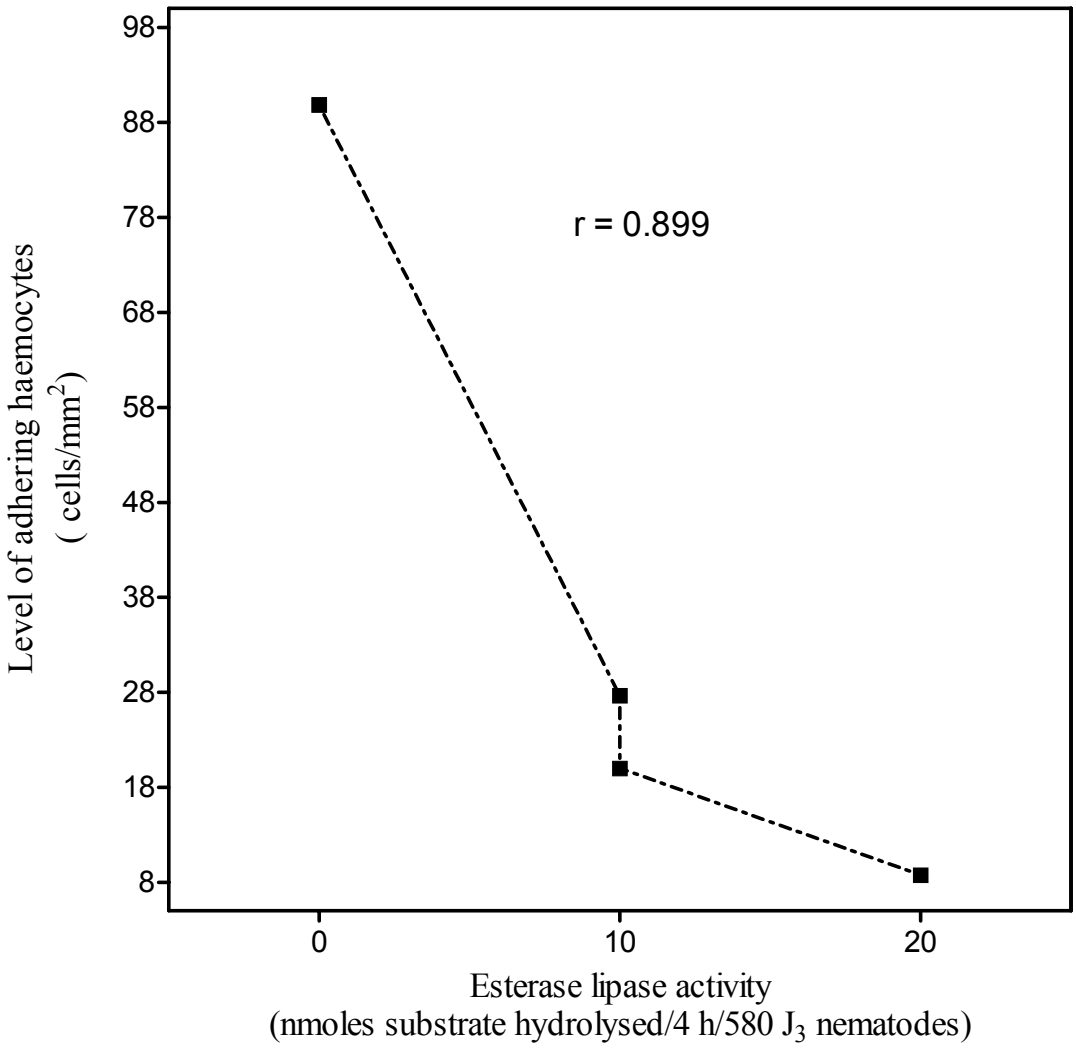

95
85
75
65
55
45
35
25
15
5
-0.978
Level of adhering haemocytes
(cells/mm2)
0
1
2
3
β-Galactosidase
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)

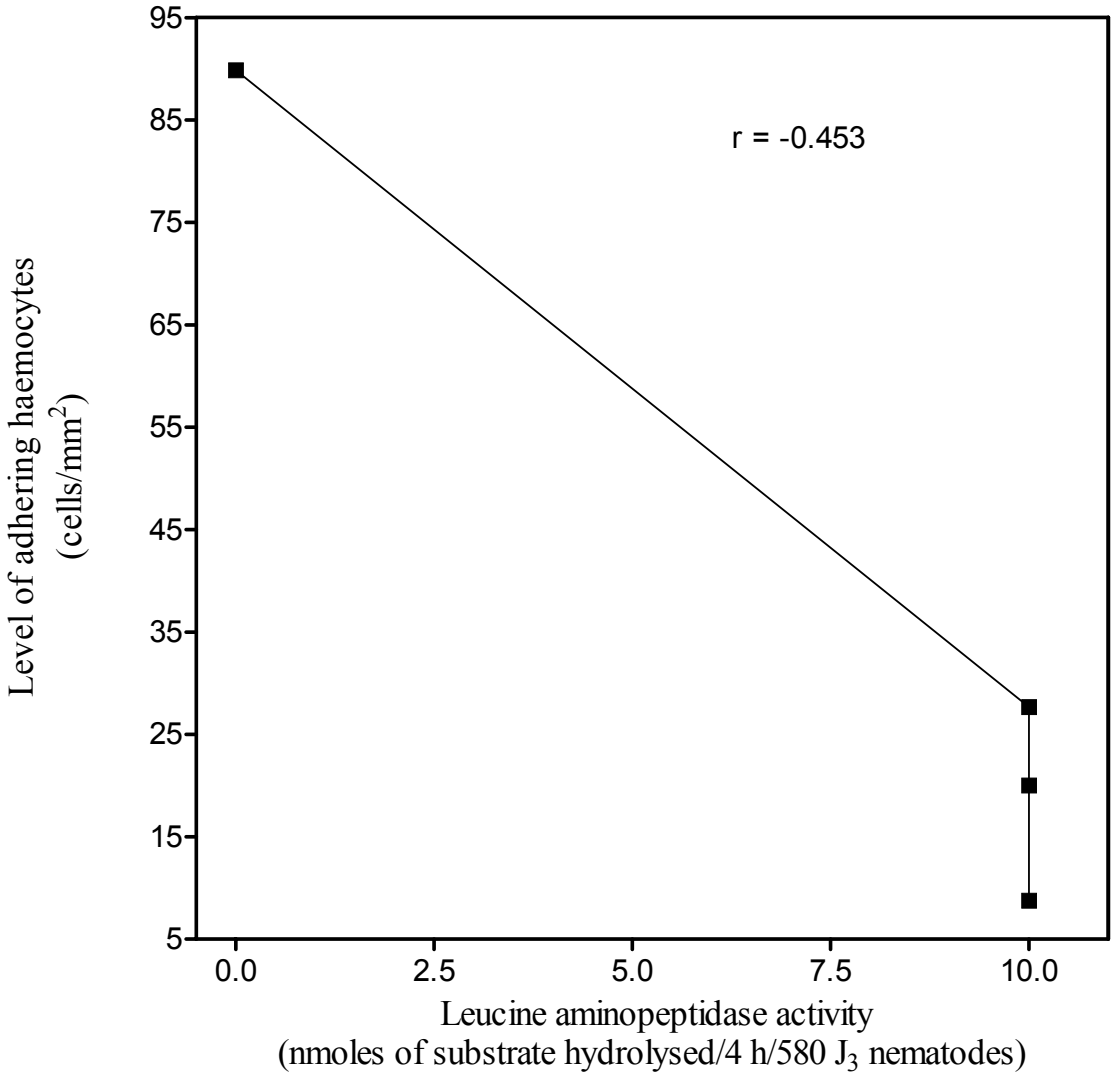

r = -0.525
95
75
55
35
15
-5
-2
2
6
10
Level of adhering haemocytes (x cells/mm2)
β-glucosidase activity
(nmoles of substrate hydrolyzed/4 h/580 J3 nematodes)

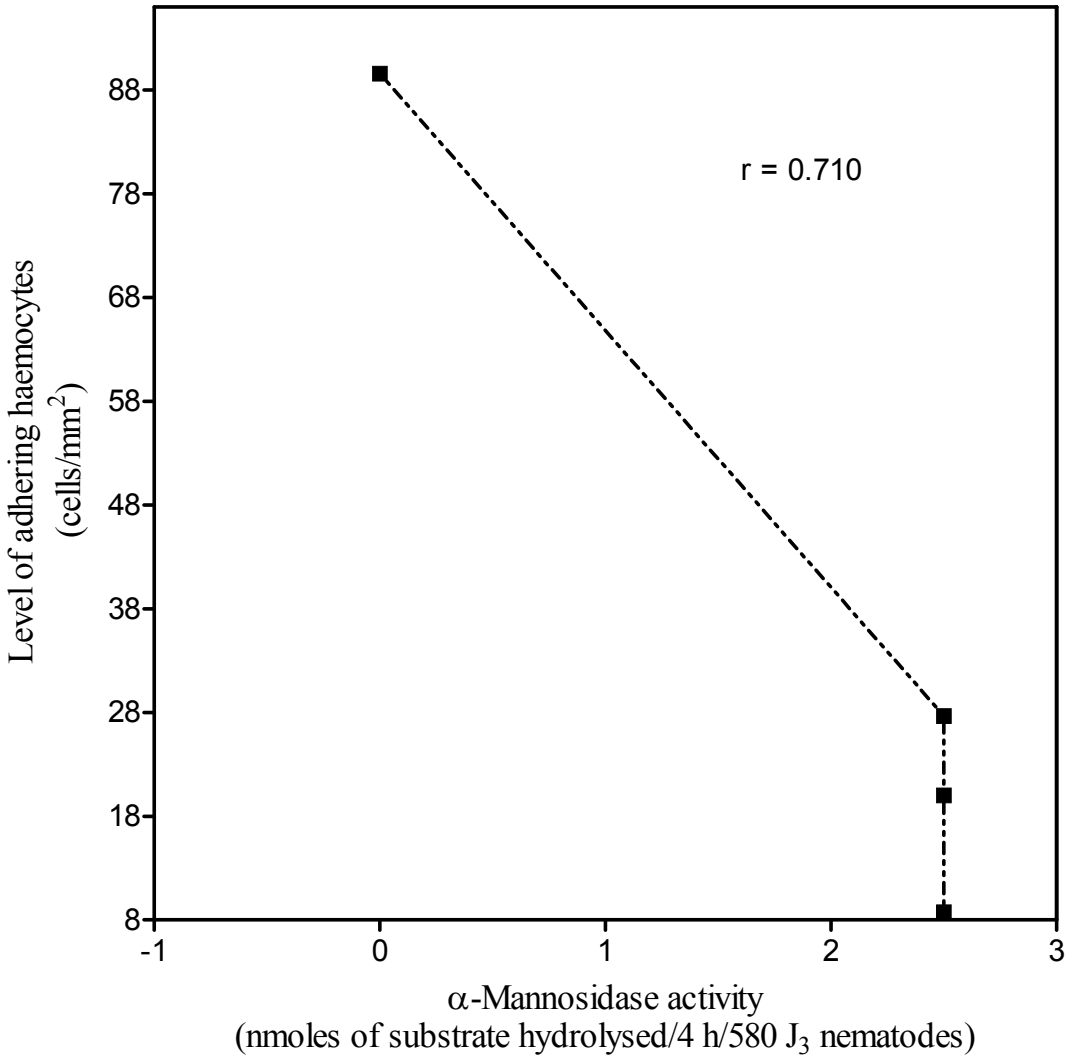

r = -0.909
Level of adhering haemocytes
(cells/mm2)
88
78
68
58
48
38
28
18
8
-2
-1
0
1
2
3
4
5
6
β-Glucuronidase activity
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)

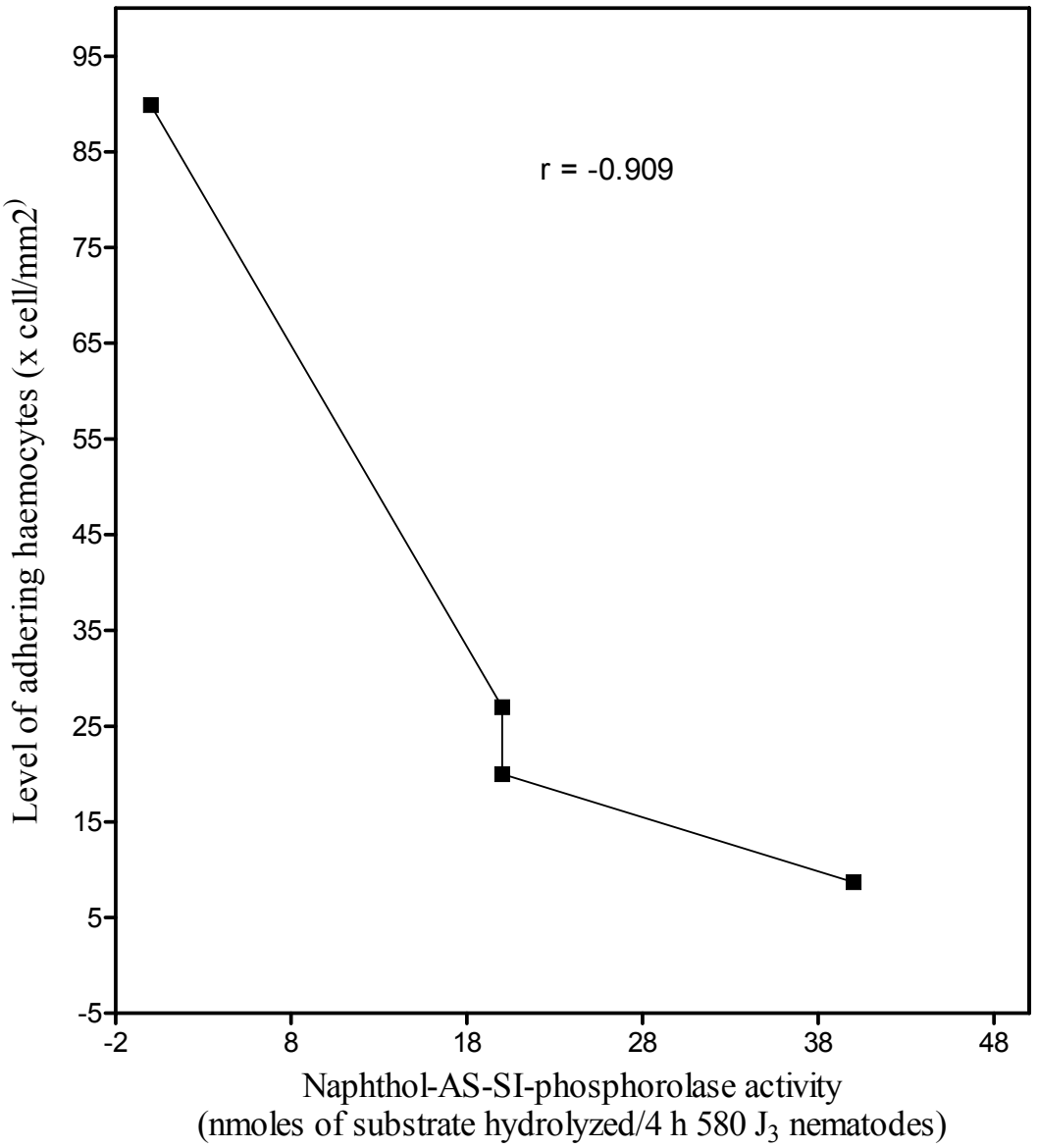

r= -0.802
Level of adhering haemocytes
(cells/mm2)
95
85
75
65
55
45
35
25
15
5
0.0
2.5
5.0
Cystine arylamidase activity
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)

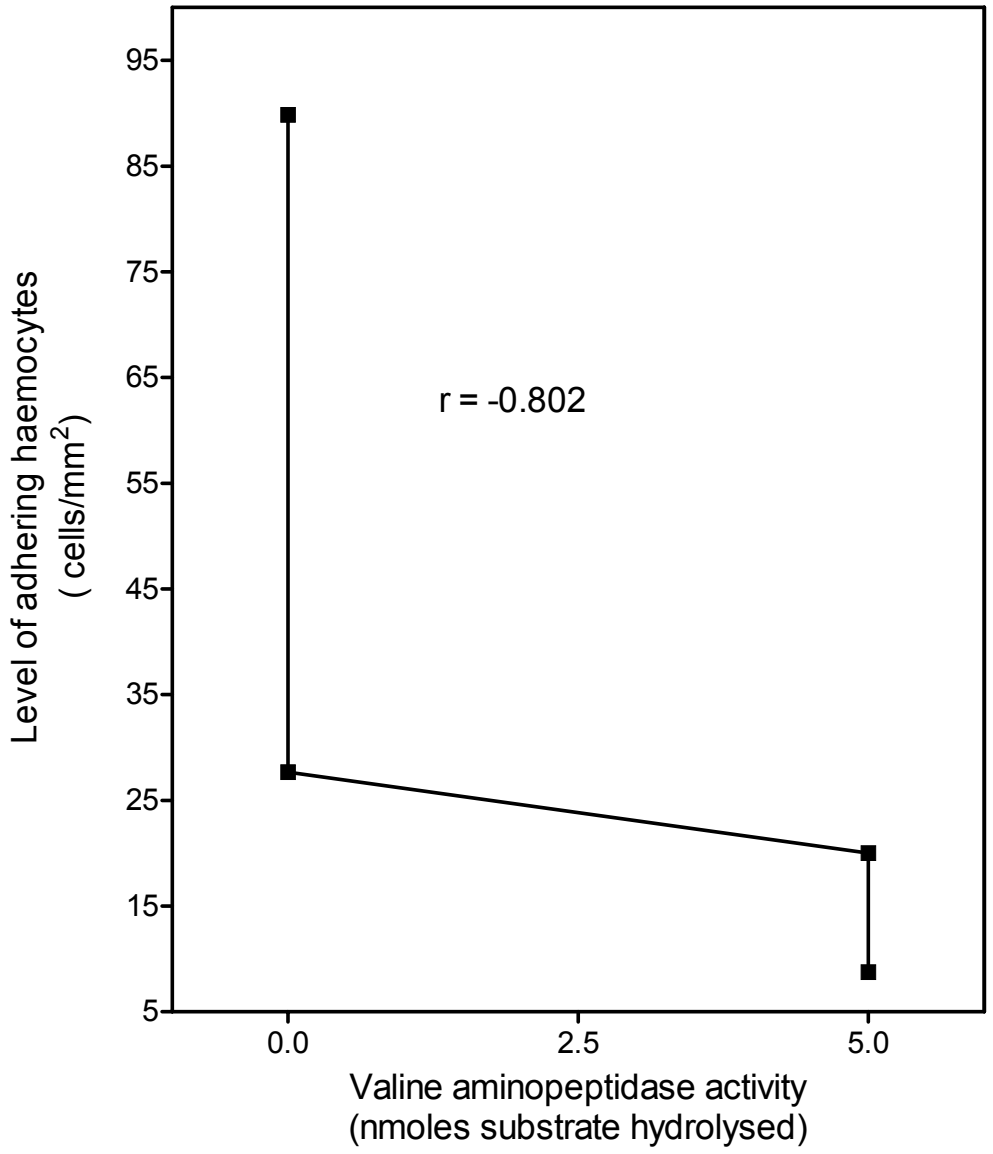

r= -0.932
Level of adhering haemocytes (x cell/mm 2)
95
85
75
65
55
45
35
25
15
5
-5
0.0
2.5
5.0
7.5
10.0
Esterase activity
(nmoles of substrate hydrolyzed/4 h/ 580 J3 nematodes)


98
88
78
68
58
48
38
28
18
8
r = 0.899
Level of adhering haemocytes
(cells/mm2)
0
10
20
Esterase lipase activity
(nmoles substrate hydrolysed/4 h/580 J3 nematodes)


95
85
75
65
55
45
35
25
15
5
r = -0.453
Level of adhering haemocytes
(cells/mm2)
0.0
2.5
5.0
7.5
10.0
Leucine aminopeptidase activity
(nmoles of substrate hydrolysed/4 h/580 J3 nematodes)


r = 0.710
Level of adhering haemocytes
(cells/mm2)
88
78
68
58
48
38
28
18
8
-1
0
1
2
3
α-Mannosidase activity
(nmoles of substrate hydrolysed/4 h/580 J3 nematodes)


r = -0.909
Level of adhering haemocytes (x cell/mm2)
95
85
75
65
55
45
35
25
15
5
-5
-2
8
18
28
38
48
Naphthol-AS-SI-phosphorolase activity
(nmoles of substrate hydrolyzed/4 h 580 J3 nematodes)


95
85
75
65
55
45
35
25
15
5
Level of adhering haemocytes
(cells/mm2)
r = -0.802
0.0
2.5
5.0
Valine aminopeptidase activity
(nmoles substrate hydrolysed)