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ROLE OF APOLIPOPHORIN-III IN THE IMMEDIATE ANTIBACTERIAL RESPONSES OF *Galleria mellonella* LARVAE (LEPIDOPTERA: PYRALIDAE)

by

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Not everything that can be counted counts,
Not everything that counts can be counted.
Albert Einstein

To Fouad

ABSTRACT

Apolipophorin-III is a hemolymph protein known for its role in lipid transport. Apolipophorin-III isolated from the hemolymph of last instar larvae of *Galleria mellonella* bound to the surface of the insect-pathogenic Gram-negative bacterium *Xenorhabdus nematophilus* and to the lipid A moiety of its lipopolysaccharide. This binding reduced the toxicity of the lipopolysaccharide to hemocytes and decreased the inhibitory effect of the lipopolysaccharide on phenoloxidase. Apolipophorin-III also bound to the Gram-positive bacterium *Micrococcus lysodeikticus* ; this enhanced the activity of hen egg lysozyme on the organism as well as the lytic activity of *G. mellonella* cell-free hemolymph.

The involvement of apolipophorin-III in the immune responses of *G. mellonella* larvae to lipoteichoic acids, surface components of Gram-positive bacteria, was examined. Lipoteichoic acids from *Bacillus subtilis, Enterococcus hirae* and *Streptococcus pyogenes* caused a dose- and time-dependent drop in the total counts of circulating hemocytes and a partial or complete depletion of plasmatocytes depending on the species of lipoteichoic acid. All lipoteichoic acids tested activated phenoloxidase *in vitro;* however, *in vivo,* only *B. subtilis* lipoteichoic acid elevated the phenoloxidase acivity while the other two suppressed it. Binding of apolipophorin-III to lipoteichoic acids was demonstrated. Apolipophorin-III prevented the complete depletion of plasmatocytes and depressed the activation of phenoloxidase by lipoteichoic acid from *B. subtilis*. The concentration of apolipophorin-III in hemolymph two hours post injections of lipopolysaccharides or lipoteichoic acids into

larvae of *G. mellonella* did not change with respect to control insects that received phosphate-buffered saline. The concentration of apolipophorin-III in hemolymph at the end of the feeding larval stage was 8-12 mg/mL of hemolymph. Apolipophorin-III was present in significant amounts in the prepupal, pupal and adult stages. The protein was detected immunologically in hemocyte lysates, plasma and fat body. Non-denaturing polyacrylamide gels and immunoblots of fresh hemolymph suggested that apolipophorin-III is associated with a 77 kDa protein.

RESUME

L'apolipoprotéine-III est une protéine de l'hémolymphe ayant un rôle reconnu dans le transport des lipides. L'apolipoprotéine-III, purifiée de l'hémolymphe du dernier stade larvaire de *Galleria mellonella*, s'associe à la surface de la bacterie Gram-negative entomopathogène *Xenorhabdus nematophilus* ainsi qu'au lipide A de son endotoxine. Cette association réduit la toxicité des lipopolysaccharides envers les hémocytes ainsi que l'effet inhibiteur de l'endotoxine sur la phenoloxydase. L'apolipoprotéine-III s'est aussi associée à *Micrococcus lysodeikticus*, une bacterie Gram-positive. Ceci a accru l'activité de la lysozyme de poule et l'action lytic d'une hémolymphe de *G. mellonella* dépourvue de cellules. Nous avons examiné la participation de l'apolipoprotéine-III chez la larve de *G. mellonella* dans les reactions immunitaires contre les acides lipoteiques, une composante de la surface bacterienne Gram-positive. Les acides lipoteiques de *Bacillus subtilis*, *Enterococcus hirae* et de *Streptococcus pyogenes* ont causé une diminution du nombre d'hémocytes proportionelle à la dose et au temps écoulé, ainsi qu'une réduction totale ou partielle des plasmocytes selon l'éspèce d'acide lipoteique étudiée. Toutes les éspèces d'acide lipoteique étudiées activèrent la phenoloxydase *in vitro*, cependant, *in vivo*, seul l'acide lipoteique du *B. subtilis* a élevé l'activité de phenoloxydase alors que les deux autres éspèces l'on diminuée. La liaison de l'apolipoprotéine-III à l'acide lipoteique est démontrée. L'apolipoprotéine-III a empêché la reduction totale des plasmocytes et a diminué l'activation de la phenoloxydase causée par l'acide lipoteique du *B. subtilis*. Deux heures après les injections de lipopolysaccharides et

d'acide lipoteique, la concentration de l'apolipoprotéine-III dans l'hémolymphe n'avait pas changé. La concentration de l'apolipoprotéine-III dans l'hémolymphe à la fin de l'étape active du dernier stade larvaire était de 8-12 mg/mL d'hémolymphe. L'apolipoprotéine-III était aussi présente dans la prépupe, la pupe et l'adulte en quantités significatives. La protéine fut detectée a l'aide d'anticorps dans le lysat d'hémocytes, dans le plasma ainsi que dans le corps adipeux. Des gels de polyacrylamide en milieu non-denaturant et des immunotransfers d'hémolymphe suggérèrent que l'apolipoprotéine-III est en faite associée a une autre protéine de 77 kDa.

SUGGESTED SHORT TITLE

Apolipophorin-III and the immune responses of *Galleria mellonella*.

CONTRIBUTIONS OF AUTHORS

The author of this thesis has held discussions and received guidance from Dr. G.B. Dunphy and Dr. D.F. Niven.

Dr. G.B. Dunphy purified LPS and assisted in the experiments involving phenoloxidase assays and hemocyte counts (chapter 2). Dr. G.B. Dunphy also provided technical support in obtaining antibodies used in the experiments described in chapter 5.

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

Dr. G.B. Dunphy and Dr. D.F. Niven have corrected and edited the manuscripts.

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LIST OF ABBREVIATIONS

AKH	Adipokinetic hormone
apoLp-III	Apolipophorin-III
DAG	Diacylglycerol
DMB	Dimethylmethylene blue
DOPA	D,L-dihydroxyphenylalanine
EDTA	Ethylenediamine tetraacetic acid
HDLp	High density lipophorin
KDO	2-keto-3-deoxyoctonate
LBP	LPS-binding protein
LDLp	Low density lipophorin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
UV	Ultraviolet

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

In addition to the intrinsic interest in understanding a biological phenomenon, the need to study the immunology of insects has stemmed from the use of microbes as control agents of insect pests in place of chemical insecticides.

A serious pest of honeybees, *Apis mellifera*, the greater wax moth, *Galleria mellonella*, is used in laboratories to study the immunology and physiology of Lepidoptera because it is easy to rear, has a relatively short life cycle (under laboratory conditions) and has a useful size. Despite the variations that exist between different insect species, *G. mellonella* shares with other lepidopterans basic features of the immune system (Chadwick and Aston, 1991). *G. mellonella* can elicit cellular and humoral responses against Gram-negative and Gram-positive bacteria invading the hemocoel. The intensities of these responses vary with the bacterial species. The insect can succeed or fail at overcoming the invading microbes depending on the virulence mechanisms of the bacteria and the ability of the insect to respond to them. A greater understanding of the antimicrobial system would be useful for increasing microbial control efficacy.

The Gram-negative insect pathogenic bacterium, *Xenorhabdus nematophilus*, is a symbiont of a nematode, *Steinernema carpocapsae*, commercially produced as a microbial insecticide (Kaya and Gaugler, 1993). Investigations of the interactions of *X. nematophilus* with the hemolymph of insects have been carried out with the ultimate aim of improving the virulence of the nematode/bacterium complex (Dunphy and Webster, 1991). *X. nematophilus* releases lipopolysaccharides (LPS) into the insect body cavity and impairs the immune system of the insect by damaging hemocytes and inhibiting prophenoloxidase (Dunphy and Webster, 1988; Dunphy and Webster, 1991). Dunphy and Hurlbert (1995) observed that a critical concentration of released LPS was necessary before the hemocyte damage occurred. This suggested that a mechanism that detoxifies LPS is present in the insect. In the course of their research (Dunphy and Hurlbert, 1995), the supernatant resulting from heating and centrifuging hemolymph of *G. mellonella* larvae was found to contain a 17 kDa protein that was suspected to bind to LPS.

The detection and identification of an LPS-binding protein and its interactions with components of the immediate cellular and humoral antimicrobial systems of *G. mellonella* larva were the central objectives of this thesis. In this thesis, immediate responses are defined as immune responses occurring within two hours of a bacterial challenge. This excludes the participation in this response of antibacterial proteins synthesized *de novo*, since induced antibacterial activity in *G. mellonella* hemolymph appears following a lag of eight hours (Croizier and Croizier, 1980; Chadwick and Aston, 1991).

In *G. mellonella*, the immediate immune reactions to bacteria are not specific and Gram-positive as well as Gram-negative bacteria trigger

cellular responses and activate prophenoloxidase (Dunn, 1986; Chadwick and Aston, 1991). One of the surface components of Gram-positive bacteria, lipoteichoic acid (LTA), resembles LPS in that they are both amphipathic molecules that are anchored in bacterial membranes by hydrophobic interactions, and they share some of the biological activities known to LPS in mammalian infections (Wicken and Knox, 1977). The role played by LTA in the response of insects to Gram-positive bacteria has received very little attention. This area is addressed in this study in terms of both the effects of LTA on hemocytes and prophenoloxidase activation and the contribution of the LPS-binding protein to the defence mechanisms of the insect against LTA.

In chapter 1 the rationale for the studies carried out is introduced, as well as the test insect and its immediate antibacterial responses and the protein, apolipophorin-III (apoLp-III). In chapter 2, the presence of two LPS-binding proteins, LBP-1 and LBP-2, in the hemolymph of *G. mellonella* larvae is reported and their detoxifying role is assessed. Chapter 3 reveals by means of N-terminal sequencing, that LBP-1 is apoLp-III. In this chapter, the interaction of apoLp-III with hen egg lysozyme and *G. mellonella* lysozyme is examined. Chapter 4 is a study on the effects of LTA on the immediate cellular and humoral responses of *G. mellonellla* and their interaction with apoLp-III. In chapter 5 are reported some aspects of apoLp-III, such as its concentration in different stages of the insect, its presence in selected tissues and its possible association in its native state with other hemolymph proteins. The effects of octopamine, LPS and LTA on the concentration of apoLp-III in hemolymph and the effect of octopamine on lipid-loading of high density lipophorin are also investigated. Chapter 6 is a general

discussion and summary of the results and conclusions of chapters 2-5. Elements of the thesis, considered to be "contributions to original knowledge", are listed in this chapter.

LITERATURE REVIEW

Galleria mellonella

The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae, subfamily: Galleriinae), is a worldwide pest of honeybee products that can cause major losses to beekeepers (Singh, 1962; Covell, 1984). The moth infests *Apis* colonies (Singh, 1962), nests of meliponids (stingless bees) and bumble bees, *Bombyx spp.* (Oertel, 1963). 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 hosts for mass propagation of dipteran and hymenopteran parasites (DeBach, 1964; Mohamed and Coppetl, 1983).

The life cycle of the moth lasts 4 weeks to 6 months (Marston *et al.*, 1975). The eggs are whitish, 0.4-0.5 mm, glued together in sheets of 50-150 eggs. Eggs are laid in crevices which prevent their removal by worker bees (Singh, 1962). Eggs hatch 3-5 days after oviposition (Dutky *et al.*, 1962). Newly-hatched creamy white larvae (3 mm) eat honey, nectar or pollen, if available, or beeswax. Developing larvae burrow tunnels into the comb where they feed protected from the worker bees; they feed on all products of the honeybee and can attack the bee brood (Nielson and Brister, 1979). Larvae can double in body weight daily for the first 10 days (Beck, 1960). Seven larval instars are reported for *G. mellonella*; a molting tube is spun before every larval ecdysis (Wani *et al.*, 1997). At the beginning of the 7th instar, the larvae weigh 50-80 mg and by the end of this stage weigh 200-300 mg. Following this feeding stage, larvae wander for a couple of days and spin cocoons in which they pupate. The

pupae are 14-16 mm long and dark brown when mature; the pupal stage lasts about 2 weeks (Beck, 1970). The adults, which are fairly small, the female being larger than the male (wingspan, 1.7-2.5 cm), do not feed or drink and live 3-30 days. The female lays 300-600 eggs although individual moths may lay up to 1,800 eggs.

G. mellonella is attacked by a number of microorganisms including a nuclear polyhedrosis virus (Vago, 1968), various bacteria including *Proteus mirabilis, Serratia marcescens* and *Bacillus thuringiensis* (Bucher, 1963; Barjac and Thompson, 1970), and two microsporidia (*Nosema* spp.) (Lipa, 1977).

Apolipophorin-III

In insects, lipid transport is mediated by a single lipoprotein called lipophorin (Chino and Kitazawa, 1981). Lipophorin is composed of two apolipophorins (apoLp), apoLp-I (230-250 kDa) and apoLp-II (70-80 kDa) in a 1:1 ratio; a third apolipophorin, apoLp-III (17-20 kDa) is sometimes associated with lipophorin in amounts that vary with the lipid content of the lipophorin particle (Shapiro *et al.*, 1984; Surholt *et al.*, 1992).

To date, apoLp-III has been detected in different developmental stages of 29 species of insects belonging to 4 orders (Table 1.1). ApoLp-III was not found in the adult of the cockroach *Blatella germanica* (Sevala *et al.*, 1999) or *Periplaneta americana* (Chino *et al.*, 1992) or in the larva and adult of *Musca domestica* (DeBianchi *et al.*, 1987). The concentration of apoLp-III in the hemolymph depends on the developmental stage of the insect. In *Bombyx mori*, the concentration of apoLp-III in the hemolymph is higher in the adults than in the larvae or pupae

Table 1.1: Distribution of apolipophorin-III among insects.

Species	Order	kDa	Insect stage	Insect tissues	Comments	Ref
Acheta domesticus	Orthoptera	18	adult	hemolymph	Complete sequence determined	1
Barytettix psolus	"	20	adult	hemolymph	Glycosylated; N-terminal sequence determined	2
Gastrimargus africanus	"	20	adult	hemolymph	Glycosylated	3
Gryllus integer	"	—	—	—	—	4
Locusta migratoria	"	19	adult	hemolymph	Glycosylated; complete sequence and crystal structure determined	5
Melanopus differentialis	"	20	adult	hemolymph	Glycosylated; N-terminal sequence determined	2
Abadus herberti	Hemiptera	—	—	—	—	6
Acantocephala granulosa	"	—	—	—	—	6
Dipetalogaster maximus	"	18	adult	—	—	7
Lethocerus medius	"	19	adult	hemolymph	N-terminal sequence determined; free or bound to biliverdin	8

Table 1.1 (cont'd)

Panstrongylus megitus	"	18	adult	hemolymph	—	7
Podisus maculiventris	"	20	adult	hemolymph	Bound to apoLp-I, -II and biliverdin	9
Ranatra quadredenata	"	—	—	—	—	6
Rhodnius prolixus	"	16	adult female	hemolymph	Glycosylated	10
Thasus acutangulus	"	20	adult	hemolymph	N-terminal sequence determined	11
Triatoma infestans	"	18	adult	hemolymph	—	7
Cotinus texana	Coleoptera	—	—	—	—	6
Derobrachus geminatus	"	18	adult	hemolymph	Glycosylated; complete sequence determined	12
Acherontia atropos	Lepidoptera	—	—	—	—	13
Agrius convolvuli	"	18	—	—	Complete sequence determined	14
Bombyx mandarina	"	—	—	—	—	15
Bombyx mori	"	17	adult; pupa; larva	hemolymph	Complete sequence determined	15

Table 1.1 (cont'd)

Diatrea grandiosella	"	17	adult; larva	hemolymph	N-terminal sequence determined	16
Galleria mellonella	"	18	adult; pupa; larva	hemolymph	Complete sequence determined	17
Hyalophora cecropia	"	18	adult; pupa	hemolymph eggs	N-terminal sequence determined	18
Hyles lineata	"	—	—	—	—	6
Hyphantria cunea	"	18	adult; pupa	hemolymph; ovary; testis	Absent from larval hemolymph	19
Manduca sexta	"	18	adult; larva	hemolymph	Complete sequence determined	20
Spodoptera litura	"	18	adult; larva	hemolymph; ovary; testis	Complete sequence determined	21

Key to references: Smith *et al.*, 1994; Strobel *et al.*, 1990 (1). Ryan *et al.*, 1990 (2). Haunerland *et al.*, 1986 (3). Hendrick, A. unpublished, as cited by Soulages and Wells, 1994 (4). Breiter *et al.*, 1991; Chino and Yazawa, 1986; Kanost *et al.*, 1988; Van der Horst *et al.*, 1991 (5). Smith, A.F. and Wells, M.A. unpublished, as cited by Soulages

and Wells 1994 (6). Canavaso and Rubiolo, 1995 (7). Kanost *et al.*, 1995 (8). Haunerland *et al.*, 1992 (9). Gondim *et al.*, 1989 (10). Fernando, G.J.P. and Wells, M.A. unpublished, as cited by Soulages and Wells, 1994; Wells *et al.*, 1985 (11). Smith *et al.*, 1994 (12). Surholt *et al.*, 1992 (13). Ragland *et al.*, 1997 as cited by Limura *et al.*, 1998 (14) Miura and Shimizu, 1988 as cited by Limura *et al.*, 1998; Miura and Shimizu, 1989; Yamauchi *et al.*, 1996 (15). Burks *et al.*, 1992 (16). Limura *et al.*, 1998; Mandato *et al.*, 1996; Weise *et al.*, 1998 (17). Telfer W.H. as cited in Telfer *et al.*, 1991 (18). Yun and Kim 1993, 1996 (19). Cole *et al.*, 1987; Cole and Wells, 1990; Kawooya *et al.*, 1984; Ziegler *et al.*, 1995 (20). Kim *et al.*, 1998 (21).

(Miura and Simizu, 1989). The expression of the gene encoding apoLp-III in *Manduca sexta* was found to be maximal in larval stages during the feeding periods of the fourth and fifth instars. The apoLp-III gene was not expressed in the pupae; however, in the adults, the level of expression was 10 fold higher than in the fifth larval instar (Cole and Wells, 1990). In the adult fat body of *Spodoptera litura*, the gene encoding apoLp-III was expressed at significantly higher levels than in the larval fat body (Kim *et al.*, 1998). *G. mellonella* seems to be an exception since the concentration of apoLp-III in the larvae and the adults did not differ greatly (Limura *et al.*, 1998).

While apoLp-III assists in the transport of a variety of lipids to various tissues (Chino and Kitazawa, 1981), most of the present understanding of the structure and function of apoLp-III is derived from studies related to fueling of muscles during sustained flight of adult *M. sexta* and *Locusta migratoria*. In response to flight, the peptide, adipokinetic hormone (AKH), is released from the corpora cardiaca (Beenakkers *et al.*, 1985). Under the action of a lipase sensitive to AKH, triacylglycerol stores in the fat body are converted to diacylglycerol (DAG) (Ziegler *et al.*, 1995; Arese *et al.*, 1996; Arrese and Wells, 1997). DAG molecules leave the fat body and associate with preexisting high density lipophorin particles (HDLp); as the diacylglycerol content of HDLp increases, apoLp-III molecules associate with the increasingly hydrophobic surface of HDLp to form a low density lipophorin (LDLp) (Wells *et al.*, 1987). LDLp transports diacylglycerol to its site of utilization where the lipid is discharged and the apoLp-III molecules dissociate from LDLp regenerating HDLp. HDLp and apoLp-III repeat this cycle acting as a shuttle for DAG in the hemolymph (Chino and Kitazawa,

1981). The binding of apoLp-III to the hydrophobic DAG on the lipophorin allows the uptake of additional DAG, stabilizes the particle and prevents it from aggregating (Soulages and Wells, 1994; Singh *et al.*, 1994). Recently, Ziegler *et al.* (1995) demonstrated that following injections of AKH, apoLp-III present in non-feeding molting larvae of *M. sexta* associates with HDLp and lipids to form LDLp, suggesting that apoLp-III assists in lipid metabolism in larvae as well as in adults. Exceptions to this mechanism have been reported: the flightless grasshopper *Barytettix psolus* (Ziegler *et al.*, 1988), the solitary-phase locust *L. migratoria* (Chino *et al.*, 1992) and the corn borer *Diatrea grandiosella* (as cited by Burks *et al.*, 1992) do not form LDLp in response to injections of AKH even though their hemolymph contains functional HDLp and apoLp-III.

Octopamine, a phenolic analog of noradrenaline released in response to stress, induced lipolysis in the fat body of *L. migratoria* (Orchard *et al.*, 1982) and *Acheta domesticus* (Fields an Woodring, 1991) and mediated the release of AKH (Orchard *et al.*, 1981; Panabecker and Orchard, 1986). The effect of octopamine on lipid loading of HDLp and its association with apoLp-III have been limited to *in vitro* studies on the locust fat body (Orchard *et al.*, 1982; Van Heusden *et al.*, 1984); octopamine-induced lipolysis was not accompanied by lipid loading of HDLp.

The complete amino acid sequences of apoLp-III from two orthopteran, one coleopteran and five lepidopteran species have been determined (Table 1.1). The sequences comprise 161-166 amino acid residues. *G. mellonella* apoLp-III contains 163 amino acid residues, lacks cysteine, tryptophan and methionine and shows no potential

glycosylation site (Weise *et al.*, 1998). N-terminal glycosylation of apoLp-III has been reported for some orthopterans and the coleopteran *Derobradus geminatus*. Comparing a recombinant non-glycosylated and a native apoLp-III from the locust, Soulages *et al.* (1998) demonstrated that glycosylation decreased the lipid-binding property of apoLp-III possibly by preventing the exposure of hydrophobic domains or decreasing the conformational flexibility of the protein. Sequence identity was high (64-90%) among lepidopteran species and relatively low among orthopteran species and between orders (Weise *et al.*, 1998). Although the percent identity between apoLp-III of certain insect species is low, the pattern of distribution of hydrophobic and hydrophilic amino acids is similar and a common structural motif of five up and down amphipathic α-helices connected by small loops to form a globular protein is shared by apoLp-III of all insects studied (Fig. 1.1) (Smith *et al.*, 1994). Hydrophobic residues are oriented toward the interior of the protein and the hydrophilic residues to the aqueous environment. This molecular architecture is similar to that of exchangeable human apolipoprotein E (Wilson *et al.*, 1991) and is believed to permit reversible binding activity (Wang *et al.*, 1998). In order for the amphipathic helices to bind to a lipid surface, a reorganization of the structure of the protein is required to expose its hydrophobic interior (Kawooya *et al.*, 1986; Breiter *et al.*, 1991). It was proposed that apoLp-III binds to the lipid surface via one end and opens up with the loops located between helices 2 and 3 and helices 4 and 5 acting as hinges (see Fig. 1) (Narayanaswami *et al.*, 1996); this results in helices 3 and 4 moving away from helices 1, 2 and 5 and exposing the hydrophobic sites of the protein. A partially folded intermediate of *M. sexta* apoLp-III

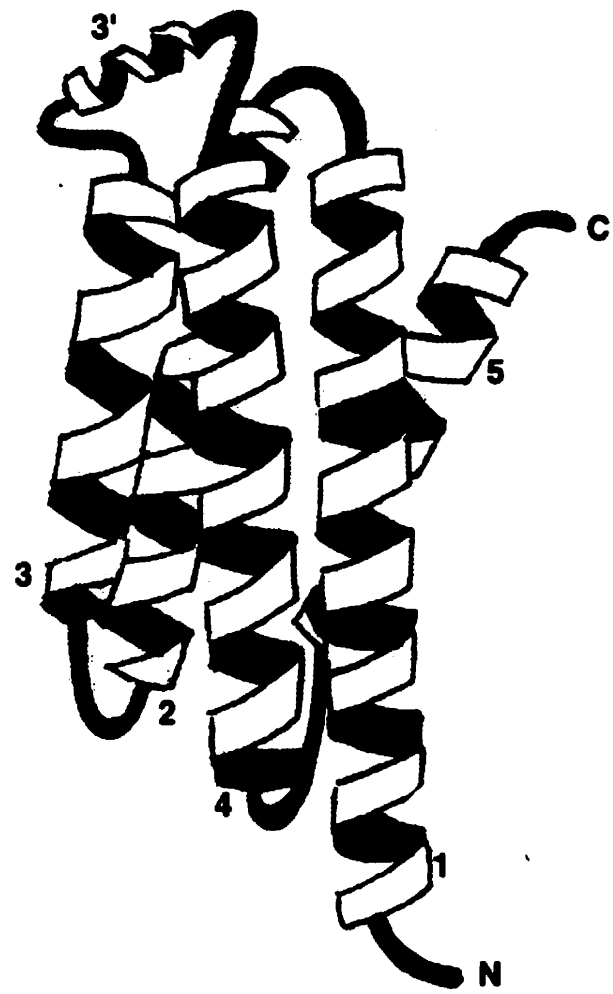

Figure 1.1 Schematic representation of apolipophorin-III of *M. sexta*.
Adapted from Narayanaswami *et al.*, 1999.

under physiological conditions, represents the most active lipid-binding state of the protein (Soulages and Bendavid, 1998). Recently, it was demonstrated that in the apoLp-III of *M. sexta*, a small helix composed of 6 residues connects helices 3 and 4 and serves as a site of recognition for hydrophobic surfaces and triggers the opening of the helix bundle; one of the six residues, a valine, appears critical for the recognition and initiation of binding (Narayanaswami *et al.*, 1999). The small helix named 3' and the valine residue are conserved in all the lepidopteran species. Considering the high sequence similarity between apoLp-III of *M. sexta* and *G. mellonella* (Weise *et al.*, 1998), it seems reasonable to expect the apoLp-III of *G. mellonella* to behave in a similar fashion.

The stability of apoLp-III of *M. sexta* was assessed by examining the effect of pH and temperature on the secondary stucture of the protein. A marked loss of helical content at pH values greater than 10 and less than 4 and a temperature-induced denaturation mid-point of 52°C were observed. Heat-denaturation was fully reversible upon cooling as indicated by the totally restored α-helix content (Ryan *et al.*, 1993). Like apoLp-III from *M. sexta*, apoLp-III from *L. migratoria, Acheta domesticus, Lethocerus medius* and *G. mellonella,* appears to be very stable to heat (Kanost *et al.*, 1988; Strobel *et al.*, 1990; Kanost *et al.*, 1995; Weisner *et al.*, 1997). This makes possible the separation of the apoLp-III from most hemolymph proteins by heat-precipitation. It was postulated that the lack of disulfide bonds is in part responsible for the heat stability of apoLp-III (Weise *et al.*, 1998).

Though apoLp-III assists in loading of lipids from the midgut or fat body and delivers them to tissues without internalization (Van Antwerpen *et al.*, 1988), it was reported that apoLp-III accumulated in

oocytes during oogenesis in *M. sexta* (Kawooya and Law,1988) and *Hyalophora cecropia* (Telfer *et al.*, 1991). In eggs of *M. sexta* , upon entering the follicle, HDLp is stripped of 80% of its lipids and two molecules of apoLp-III dissociate from HDLp resulting in a very high density lipophorin (VHDLp) (Kawooya and Law, 1988); none of the proteins is recycled. ApoLp-III has also been reported to accumulate in the ovaries and testis of *Hyphantria cunea* (Yun and Kim, 1996) and *Spodoptera litura* (Kim *et al.*, 1998). In the latter, the presence of apoLp-III mRNA, although at low levels compared to fat body, suggests that the protein is also synthesized in the gonads. In *M. sexta,* only 5% of the egg lipid is accounted for by HDLp and the role of apoLp-III in the gonads remains an open question. Sun *et al.* (1995) demonstrated that apoLp-III expression is up-regulated during apoptosis of neurons and intersegmental skeletal muscles of *M. sexta;* the authors reject the hypothesis that apoLp-III is involved in lipid transport as apoLp-I and apoLp-II were absent from these tissues. Another example of involvement of apoLp-III in a function other than lipid transport is provided by the giant water bug *Lethocerus medium,* where a portion of the apoLp-III molecules in the hemolymph is bound to the blue chromophore, biliverdin, and does not associate with apoLp-I and apoLp-II (Kanost *et al.*, 1995). ApoLp-III in the hemolymph of *G. mellonella* appears to participate in the cellular and humoral immune responses of the insect (Wiesner *et al.*, 1997; Limura *et al.*, 1998) and is discussed further in the next section.

Immediate Immunity in Lepidoptera

The immediate (as defined in the introduction) defense systems of insects against bacteria invading the hemocoel comprises two major interactive mechanisms, one cellular and one humoral. Cellular responses include phagocytosis, nodulation and encapsulation while humoral responses include the action of antibacterial proteins constitutively present in the hemolymph (mainly lysozyme) and activation of the phenoloxidase cascade leading to melanization.

Cellular mechanisms

Insect hemocytes are divided into classes according to morphology, antigenic properties and function (Mullett *et al.*, 1993; Gardiner and Stand, 1995). Typically, a lepidopteran hemogram is characterized by a combination of prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids (Trenczek, 1996). While granulocytes and plasmatocytes are recognized as the immunocompetent cells (Lackie, 1988), spherulocytes are believed to contain heparin-like molecules (Ashhurst, 1982) and oenocytoids, components of the prophenoloxidase system (Schmit *et al.*, 1977).

Phagocytosis is the internalization by hemocytes of microorganisms and/or cellular debris. This cellular response is a multiple step process that involves recognition, attachment, signal transduction, activation of pseudopodium formation, ingestion and assembly of phagosomes (reviewed by Bayne, 1990 and Gupta, 1991). The types of hemocytes involved in phagocytosis remains uncertain, due perhaps to variations among insect species, differences in experimental designs and problems in identifying hemocyte types (Ratcliffe, 1993;

Trenczek, 1996). In *G. mellonella, Bacillus subtilis* was observed to be internalized by granulocytes as well as plasmatocytes (Ratcliffe and Rowley, 1974). Monolayer assays indicated that plasmatocytes are the main phagocytic cells in *G. mellonella* (Ratcliffe *et al.*, 1984; Wiesner and Gotz, 1993; Rohloff *et al.*, 1994) while *in vivo*, foreign particles were engulfed preferentially by granulocytes (Ehlers *et al.*, 1992). Up to 10^3 bacteria per microliter of hemolymph can be removed by phagocytosis (Walters and Ratcliffe, 1983) while higher bacterial numbers trigger the formation of hemocyte aggregates or nodules in which the bacteria are trapped (Ratcliffe and Gagen, 1976; Horohov and Dunn, 1983). The formation of nodule-like aggregates of hemocytes is also triggered by wounding and by molecules of microbial origin such as LPS from Gram-negative bacteria, zymogen and β–1,3-glucans from yeast and laminarin from algae (Lackie, 1988). The number of nodules per insect is a function of the numbers, the pathogenicity and the species of the bacteria (Ratcliffe and Walters, 1983) but is unrelated to insect dimensions (Howard *et al.*, 1998). Organisms too large to be phagocytosed or trapped in nodules are encapsulated. Events leading to the formation of nodules or capsules are basically the same. Following recognition of non-self, granulocytes discharge components of the prophenoloxidase system onto the foreign surface. Plasmatocytes are then recruited by endogenous factors (wound factors or hemokines) and they form multi-layered sheets around the granulocytes. The inner layer of the cellular capsule may melanize (Pech and Strand, 1995), and the resulting capsule usually adheres to tissues within the hemocoel (Ratcliffe and Gagen, 1976, 1977).

Hemocytopenia, a drop in hemocyte numbers, follows wounding or injection of phosphate-buffered saline, inert particles or bacteria. In *G.*

mellonella, this event is immediate and affects mainly plasmatocytes. Cell numbers return to normal levels an hour after wounding or injections of phosphate-buffered saline but stay low for several hours following injections of bacteria (Gagen and Ratcliffe, 1976; Chain and Anderson, 1982; Ratcliffe and Walters, 1983; Dunphy *et al.*, 1986). The reduction in cell counts is believed to occur in response to a plasmatocyte depletion factor that is released by stimulated hemocytes and that affects hemocyte adhesiveness (Chain and Anderson, 1982a, 1982b, 1983). Hemocytes utilised in a cellular response may be replaced by cells released from the hemopoietic organ and/or internal organs to which they attach. There is evidence that the hemogram of insects is under endocrine control. Endocrine glands influence the total and differential hemocyte population by increasing the mitotic index and releasing hemocytes from the hemopoietic organ and sessile hemocytes from tissues (Pathak, 1986).

In insects, at least three distinct groups of compounds affect the activities of hemocytes, namely, peptides and protein factors, biogenic amines and eicosanoids.

Evidence for factors with cytokine-like functions and referred to as hemokines (Chadwick and Aston, 1991) has been reported for *G. mellonella*. A proteinaceous phagocytosis-promoting factor is released following injections of latex beads and may be responsible for the engulfment of *B. thuringiensis* which usually escapes phagocytosis (Mohrig *et al.*, 1979). In *M. sexta*, as well as in *G. mellonella*, a plasmatocyte depletion factor was secreted by hemocytes following injection of bacteria (Chain and Anderson, 1983a; Geng and Dunn, 1989). A factor derived from granulocytes enhances the phagocytic activity of

G. mellonella plasmatocytes towards *Bacillus cereus*, indicating cell-cell cooperation during hemocytic responses (Anggreani and Ratcliffe, 1991). This was confirmed by the work of Pech and Strand (1996) on encapsulation in *Pseudoplusia includens*; in this study, spreading granulocytes and plasmatocytes released proteins into the medium. A peptide purified from the hemolymph of *P. includens* triggered spreading of plasmatocytes (Clark *et al.*, 1997). Phagocytic activity of plasmatocytes in *G. mellonella* towards yeast cells was enhanced by apoLp-III when apoLp-III was added directly to the plasmatocyte monolayers or when apoLp-III was preincubated with yeast cells or with isolated plasmatocytes (Wiesner *et al.*, 1997). Peptides and protein factors also modulate hemocyte adhesion to foreign surfaces. Pech and Strand (1995) found that peptides with RGD sequences interfered with the adhesion of plasmatocytes, but not granulocytes, of *P. includens* to plastic surfaces. In *G. mellonella*, the enhancement of the phagocytic activity of plasmatocytes by fibronectin is inhibited by RGD (Arg-Gly-Asp) peptides implying that the hemocytes have an RGD receptor (Wittwer and Wiesner, 1996). The inhibition of cell adhesion *in vitro* appears to be mediated by lipophorin which is present in nodules (Mandato *et al.*, 1996). Lipophorin was also shown to disrupt aggregates that formed during harvesting and washing of *Hyalophora cecropia* hemocytes (Lanz-Mendoza *et al.*, 1996). Hemolin, an invertebrate member of the immunoglobulin superfamily, may modulate adhesiveness; hemolin prevented aggregation of hemocytes of *M. sexta* and *H. cecropia* and stimulated the phagocytic activity of hemocytes through intracellular signalling events involving protein kinase and protein tyrosine phosphorylation (Landendorff and Kanost, 1991; Lanz-

Mendoza *et al.*, 1996). A hemocyte aggregation inhibitor (HAIP), a glycoprotein, from *M. sexta* inhibited aggregation of hemocytes but not their adhesion to polystyrene vessels (Kanost *et al.*, 1994).

The biogenic amine, octopamine, accelerated the removal of bacteria from the hemolymph (Dunphy and Downer, 1994) and affected the locomotory behavior, polarity and cytoskeleton of plasmatocytes of *G. mellonella* (Diehl-Jones *et al.*, 1996). Octopamine also enhanced the phagocytic activity of hemocytes in the cockroach *Periplaneta americana* (Baines *et al.*, 1992).

The role of eicosanoids in mediation of cellular immune responses was demonstrated by the decreased ability of *M. sexta* to clear bacteria from hemolymph and from nodules when injected with dexamethasone, an inhibitor of eicosanoid synthesis (Stanley-Samuelson *et al.*, 1991; Miller *et al.*, 1996). Similarly, in *G. mellonella*, inhibition of eicosanoid synthesis prevented phagocytosis and hemocyte spreading *in vitro* and nodulation and prophenoloxidase activation *in vivo*; this effect was abolished by the addition of arachidonic acid (Mandato *et al.*, 1997).

Humoral mechanisms

Lysozyme is constitutively present at low levels in lepidopterans (Hultmark *et al.*, 1980; Lockey, 1996; Powning and Davidson, 1973; Russel and Dunn, 1990). This enzyme can lyse bacteria by hydrolysing β-1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan layer of bacterial cell walls (Jollès and Jollès, 1984). Lysozyme is a basic protein of 14-16.5 kDa. The lysozymes in lepidopterans are similar to hen egg-white lysozyme with respect to

amino acid composition, molecular weight, net charge, stability to heat, optimum pH and specificity of action (Powning and Davidson, 1973; 1976; Jollès *et al.*, 1979). The fat body and hemocytes of insects contain lysozyme and increase their lysozyme contents in response to antigen injection (Anderson and Cook, 1979; Chain and Anderson, 1983; De Verno *et al.*, 1984; Dunn *et al.*, 1985). High levels of lysozyme persist even after induced immunity and bactericidal activity have subsided (Chadwick, 1970; Jarosz, 1979). Despite the fact that lysozyme kills Gram-positive bacteria and generates, by lysing bacterial cells, signal molecules necessary for the induction of antibacterial protein synthesis (Dunn and Dai, 1990), it has been suggested that its main function lies in the removal of the murein sacculus that results from the activities of antibacterial peptides (Boman, 1991). On the other hand, lysozyme, when in association with *Gal*lysin (an inducible antibacterial protein in *G. mellonella*), had bactericidal activity against Gram-negative bacteria (Chadwick and Aston, 1991). It appears that the precise role of lysozyme in antibacterial immunity is still not clear.

Phenoloxidase (monophenol, L-dopa: O_2 oxidoreductase) is a copper-containing enzyme present in insect hemolymph that catalyzes the oxidation of mono-phenols to *o*-diphenols and *o*-diphenols to *o*-quinones leading to the formation of melanin (for recent reviews see Sugumaran and Kanost, 1993; Ashida and Brey, 1997). Melanization is involved in sclerotization and wound healing of the cuticle (Lai-Fook, 1966; Sugumaran, 1988) and in nodulation and encapsulation (Gupta, 1991). Moreover, quinones have cytotoxic properties and may contribute to the killing of pathogens (Nappi *et al.*, 1995). The precursor of phenoloxidase, prophenoloxidase, is present in hemolymph, plasma

and/or hemocytes, and in the cuticle (Pye, 1974; Iwama and Ashida, 1986; Ashida and Brey, 1995). Prophenoloxidase was detected histochemically in hemocytes, mainly granulocytes and oenocytoids, of *G. mellonella* (Schmit *et al.*, 1977) but not in immunized *G. mellonella* (Pye, 1978). Prophenoloxidase is activated during an immune response through a cascade of enzyme activities triggered by microbes or microbial components including peptidoglycan, β-1,3- glucans, and LPS (Seyhold *et al.*, 1975; Ashida and Yoshida, 1988). This activation is achieved by proteolytic cleavage of the proenzyme (Ashida and Dohke, 1980). Prophenoloxidase from hemolymph of *G. mellonella* has been purified and characterized; the enzyme has the same amino acid composition as that of *B. mori* and is antigenically similar to that of *M. sexta*. Its activation, using chymotrypsin, produced two proteins of 67 and 50 kDa (Kopacek *et al.*, 1995). Prophenoloxidase activation depends on at least one serine protease, itself present as a proenzyme and activated through a cascade (Yoshida and Ashida, 1986; Saul and Sugumaran, 1987). The early events that trigger the activation of the first protease are not known; a calcium-dependent enzyme appears to take part in these events (Yoshida and Ashida, 1986; Ashida and Brey, 1995). The existence of proenzymes of the phenoloxidase system and their activation by cascade reactions allows for regulation of the system. A number of serine protease inhibitors have been isolated from lepidopteran insects. However, their functions as regulators of phenoloxidase activation are still hypothetical (Sugumaran and Kanost, 1993).

Interactions of LPS and LTA with the immune system

LPS, a component of Gram-negative bacterial cell walls, also referred to as endotoxin, is an amphipathic polyanionic molecule. LPS is generally composed of three parts, lipid A, a glucosamine disaccharide esterified to fatty acids and present in all LPS species, a core oligosaccharide attached to lipid A and the O-specific chain. The invasion of the insect hemocoel by Gram-negative bacteria is followed by release of LPS. LPS of the insect pathogenic bacterium *Xenorhabdus nematophilus* is released in hemolymph of *G. mellonella* probably under the action of lysozyme, carbohydrases or proteases of the insect (Dunphy and Webster, 1984, 1991) and LPS of *Escherichia coli* is liberated in hemolymph of *Bombyx mori* following phagocytosis and enzymatic digestion of bacteria (Taniai *et al.*, 1997). Released LPS triggers a range of events that can vary with the LPS and the insect. Although *X. nematophilus* LPS is not toxic to hemocytes *in vitro* (Ribeiro *et al.*, 1999), *X. nematophilus* and *Photorhabdus luminescens* LPS injections caused an increase in hemocyte numbers due to damaged cells (Dunphy and Webster, 1988, 1991). Other species of LPS provoked a typical plasmatocyte depletion (Trenczek *et al.*, 1994), hemocytopaenia and nodulation (Dunphy and Webster, 1988) and aggregation of hemocytes (Lanz-Mendoza *et al.*, 1996).

The binding of LPS to hemocytes has been documented. Hemocytes of *G. mellonella* bound *X. nematophilus* LPS (Dunphy and Webster 1988) and *B. mori* hemocytes bound *E. coli* LPS (serotype 0111:B4) *in vitro* (Xu *et al.*, 1995). In both of these cases, inhibition studies indicated that binding occurred via the lipid A portion of LPS. In *Hyalophora cecropia* and *G. mellonella*, LPS was bound and internalized

within minutes by granulocytes but not by plasmatocytes (Trenczek and Peik-Steinhof, 1992; Trenczek *et al.*, 1994).

The presence of LPS receptors in mammals prompted investigations on LPS receptors and LPS-binding proteins in insects. In *B. mori*, an 11 kDa protein on the surface of granulocytes bound LPS specifically and induction of cecropin gene expression correlated with this binding (Xu *et al.*, 1995). LPS activation of the signal transduction pathway in hemocytes has been studied mainly in the dipteran *Ceratitis capitata* (Charalambidis *et al.*, 1995, 1996a, 1996b). In these studies, *E. coli* LPS (serotype 026:B6) bound to a 47 kDa protein on the hemocytes before it was internalized; internalization was associated with phosphorylation of intracellular proteins and the secretion of prophenoloxidase activators. Activation of a hemocyte cell line of the lepidopteran *Estigmene acraea* by *E.coli* LPS (serotype 0111:B4) enhanced phagocytosis and increased lysozyme release (Wittwer *et al.*, 1997). These events were dependent on the LPS-triggered release of proteases by the *E. acrea* hemocyte (Wittwer and Wiesner, 1998). Few LPS-binding proteins have been identified in insects. Two lectins (carbohydrate-binding proteins) isolated from hemolymph of *Periplaneta americana* (an orthopteran) bound to the inner core region of LPS and participated in the clearance of *E. coli* cells (Jomori and Natori, 1992; Kawasaki *et al.*, 1993). A putative LPS-binding protein with a molecular mass of 24 kDa was isolated by affinity chromatography from *G. mellonella* hemolymph (Kluner *et al.*, 1994). The binding of hemolin exclusively to the lipid A portion of *E. coli* LPS has recently been demonstrated (Daffre and Faye, 1997). In *B. mori*, LPS was detoxified and cleared from hemolymph through complex formation with lipophorin (Kato et al., 1994a, 1994b). It was

proposed that this is a hydrophobic association via lipid A but over eight hours were required for the formation of this complex. In *G. mellonella,* agglutination of erythrocytes by apoLp-III was inhibited by LPS and the LPS component, KDO (2-keto-3-deoxyoctonate), suggesting possible interactions of apoLp-III with the endotoxin (LPS). However, lipid A from *Salmonella minnesota* (strain: RE 595) and a few of the intact LPS species tested had no effect (Limura *et al.*, 1998).

Activation of prophenoloxidase by LPS is variable and sometimes inconsistent. LPS from *E. coli* activated rapidly prophenoloxidase in plasma of *M. sexta* (Saul and Sugumaran, 1987) and *G. mellonella* (Ehlers and Beetz, 1996) while LPS from several *E. coli* strains and *Salmonella enteritidis* LPS failed to activate prophenoloxidase in *B. mori* (Yoshida and Ashida, 1986). *X. nematophilus* LPS inhibited the activation of prophenoloxidase in *G. mellonella* (Dunphy and Webster, 1991) and in the moth *Agrostis segetrum* (Yokoo *et al.*, 1992).

In addition to triggering immediate responses, LPS also has long term effects such as the induction of resistance to bacterial pathogens (Chadwick and Vilk, 1969; Chadwick, 1971) and of synthesis of antibacterial proteins such as lysozyme, *Gal*lysin, hemolin and cecropins (Chadwick and Aston, 1991; Faye and Kanost, 1997; Beresford, *et al.*, 1997).

Lipoteichoic acids (LTAs) are amphipathic molecules anchored in the cytoplasmic membranes of a wide variety of Gram-positive bacteria and are characterized as glycolipid-linked polymers of glycerol phosphate units joined by phosphodiester bonds (Fischer, 1988). Many of the biological properties of LPS are shared by LTAs (Wicken and Knox, 1977). The cytotoxicity of LTAs in mammals is well established and is

presently a dynamic area of research. On the other hand, studies on the interactions of LTA with insects have only been conducted by Chadwick's group. Injections of LTAs from *Bacillus subtilis* into *G. mellonella* larvae induced synthesis of low molecular weight proteins, increased lysozyme and bactericidal activities in the hemolymph, caused a 40-50% reduction of hemocyte numbers and conferred a protective immunity to the insect against bacteria comparable to that induced by LPS injections (Alexander *et al.*, 1992).

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CHAPTER 2

HEMOLYMPH PROTEINS OF LARVAE OF *Galleria mellonella* DETOXIFY ENDOTOXINS OF THE INSECT PATHOGENIC BACTERIUM *Xenorhabdus nematophilus* (ENTEROBACTERIACEAE).

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ABSTRACT

Hemolymph of *Galleria mellonella* larvae contains two proteins, LBP-1 (17.2 kDa) and LBP-2 (26 kDa) that (1) bound to the surfaces of the insect pathogenic bacterium, *Xenorhabdus nematophilus*, (2) prevented lipid A-binding dye attaching to the lipid A of *X. nematophilus* endotoxin and (3) reduced endotoxin activity on the hemocytes. Protein LBP-1 also blocked the inhibition of prophenoloxidase activation by the endotoxin. It is proposed that proteins LBP-1 and LBP-2 are part of the containment responses of the insects to bacteria.

Keywords: *Galleria*, hemolymph, proteins, *Xenorhabdus* , endotoxins.

INTRODUCTION

The short-term responses of Lepidopteran larvae to bacteria consist of a combination of interactive humoral and hemocytic actions. The efficiency of the reactions to contain the bacteria varies with the insect species and the bacterial species (Dunphy and Bourchier, 1992). The main hemocytic response is nodule formation which consists of bacteria adhering to granular cells which in turn aggregate, and eventually the complex is walled off by the plasmatocytes (Ratcliffe and Gotz, 1990). During these responses, the hemocytes release hemokines which, depending on the insect species, may include components of the phenoloxidase system (Chadwick and Aston, 1991). The generation of phenoloxidase from its zymogenic precursor by serine proteases is triggered by bacterial compounds and has been implicated in increased attachment of foreign materials to the hemocytes (Brookman *et al.*, 1989). However, bacterial attachment to hemocytes occurs also in the absence of phenoloxidase activity (Rizki and Rizki, 1990). Lectins, humoral enzymes and octopamine also facilitate nodule formation (Dunphy and Webster, 1991; Dunphy and Downer, 1994).

Lipopolysaccharides (LPS), endotoxins of Gram-negative bacteria, are complex molecular species composed of three parts (i) O-side chains (polymers of carbohydrates) which are linked to (ii) a polysaccharide core which, in turn, connects to (iii) lipid A, (a glucosamine disaccharide esterified to fatty acids) (Hammond *et al.*, 1984). LPS may initiate nodule formation and enhance phagocytosis (Gunnarsson and Lackie, 1985). Depending on the endotoxin species and species of insect, LPS may

enhance or prevent phenoloxidase activation in insects (Dularay and Lackie, 1985; Ratcliffe *et al.*, 1985, Dunphy and Webster, 1991). The insect pathogenic bacterium, *Xenorhabdus nematophilus*, is carried into insect hemolymph within the insect parasitic nematode, *Steinernema carpocapsae*, and releases LPS into the hemolymph that eventually damages hemocytes. Hemocyte damage occurs after a lag period suggesting that insects have a means of detoxifying LPS until a critical level of LPS is reached (Dunphy and Webster, 1988; Dunphy and Hurlbert, 1995).

Hemolymph and hemocyte proteins capable of binding to endotoxins are known (Sun *et al.*, 1990; De Lucca *et al.*, 1995; Xu *et al.*, 1995). With the exception of an opsonic lectin binding to LPS in *Periplaneta americana* (Jamori and Natori, 1990), the role of LPS-binding proteins in the initial antibacterial defences of insects is unknown. Herein is reported that two LPS-binding hemolymph proteins, LBP-1 and LBP-2, detoxify LPS from virulent *X. nematophilus*. Emphasis is placed on LBP-1 with comparison with LBP-2.

MATERIALS AND METHODS

Insects, larval serum, microorganisms and LPS extraction.

Galleria mellonella larvae were reared on an artificial diet (Dutky *et al.*, 1962) supplemented with 2% (w/w) brood comb at 30°C under constant light until the insects weighed around 180 mg.

Partially heat-inactivated serum (henceforth referred to as serum) was prepared from hemolymph obtained by amputation of a prothoracic leg. The hemolymph was diluted 1:1 (v/v) with phosphate-buffered saline (NaCl, 8 g/L, KCl, 0.2 g/L, Na_2HPO_4, 1.15 g/L, KH_2PO_4, 0.2 g/L: pH 6.5) and the hemocytes removed by centrifugation (12000 x g, 4 min, 25°C). The supernatant was heated (90°C, 30 min) to precipitate heat-labile proteins and totally inactivate hemolymph enzymes (Dunphy and Hurlbert, 1995). Serum was expressed by centrifugation (12000 x g, 25°C, 4 min) and frozen at -20°C.

The ATCC 19061 and DD136 strains of the insect pathogenic bacterium *Xenorhabdus nematophilus* (Enterobacteriaceae) in the phase one form, the phase infecting insects (Akhurst, 1982), were cultured on tergitol-7-agar supplemented with 1% (w/v) triphenyl tetrazolium chloride (Dunphy and Webster, 1988). The ATCC 19061 strain possesses LPS deficient in O-side chain and most of the core sugars and the DD136 strain possesses complete LPS (Dunphy and Hurlbert, 1995). Stock cultures were maintained at 25°C in darkness and were subcultured monthly.

For experimental purposes, the bacteria were grown for 24 hours in 10 mL tryptic soy broth in 50 mL Erlenmeyer flasks at 24°C on a

horizontal gyratory shaker (200 rpm). Bacteria were washed by centrifugation (8 500 x g, 5°C, 5 min) and resuspension in phosphate-buffered saline (Dunphy, 1994).

LPS was extracted from the DD136 strain of *X. nematophilus*. Bacteria were grown in 250 mL of tryptic soy broth in 1L Erlenmeyer flasks (25°C, 200 rpm) until achieving stationary phase. The culture was washed three times by centrifugation (10 000 x g, 5°C, 5 min) and resuspension in phosphate-buffered saline (Dunphy, 1994). Bacteria were lyophilized and LPS extracted using phenol-water (Westphal and Jann, 1965) followed by purification by ultracentrifugation (100 000 x g, 3.5 h, 4°C) and resuspension in LPS-free distilled water (Dunphy and Webster, 1988).

Larval injections, hemocyte counts and phenoloxidase assays.

To detect LPS-binding proteins by their detoxifying properties, insects were injected with 10 µL of serum or phosphate-buffered saline containing selected serum proteins at various concentrations with and without 2 µg of LPS. The samples were incubated *in vitro* at 30°C for 30 min and were then injected into larvae. The larvae were kept at 30°C for 30 min before bleeding. Other insects were injected twice to examine the effects of increasing concentrations of LPS-binding proteins *in vivo* by injecting first with the protein, incubating the larvae at 30°C for 10 min to allow protein to disperse in the hemolymph and subsequently injecting 10 µL of phosphate-buffered saline containing selected amounts (10^{-2} to 10^{5} ng) of LPS. Insects were incubated for 30 min at 30°C before bleeding.

Larvae were bled by amputating the left prothoracic leg and the total hemocyte counts determined on a Petroff-Hausser counter using phase contrast microscopy. Hemocytes are rapidly damaged by the lipid A moiety of *X. nematophilus* LPS; they become vacuolated, fail to exclude trypan blue and those on tissues float into the hemolymph raising hemocyte counts several fold within 5 min of injection. Hence an increase in such hemocytes is a valid indication of LPS-induced damage (Dunphy and Webster, 1988; Dunphy and Hurlbert, 1995).

LPS of *X. nematophilus* inhibits the conversion of prophenoloxidase to phenoloxidase (Dunphy and Webster, 1991) . To determine if LPS-binding protein could abrogate such inhibition, insects were injected (10 µL) with phosphate-buffered saline containing LPS and one test protein. Insects were bled 30 min post-injection, by which time LPS alone inhibits prophenoloxidase activation (Dunphy and Webster, 1991), and analyzed for phenoloxidase activity . This approach, as opposed to incubation of the solutions with hemolymph *in vitro*, was necessary to preclude high phenoloxidase levels immediately generated by the trauma of bleeding. Ten µL of hemolymph were added to 100 µL of laminarin (1 mg/mL) in 10% (v/v) phosphate-buffered saline and incubated at 25°C for 20 min. Laminarin activates any non-inhibited prophenoloxidase to phenoloxidase. One mL of the prophenoloxidase substrate DL-dihydroxyphenylalanine (2 mg/mL in 10% phosphate-buffered saline) was added and the level of dopachrome determined spectrophotometrically at 490 nm. Total protein was determined (Bradford, 1976) and phenoloxidase titers expressed as units/mg protein.

Electrophoresis purification and identification of LPS-binding proteins.

LPS-binding proteins in serum were tentatively identified by adhesion to both strains of *X. nematophilus* as determined by a decrease in the intensity of a protein band in a sodium dodecyl sulphate (SDS) polyacrylamide gel. Bacteria were killed by UV-irradiation for one hour and washed by centrifugation (8500 x g, 5 min, 5°C) and resuspension in phosphate-buffered saline to preclude bacterial proteolytic enzymes influencing results. Broad spectrum exoproteases are known for *X. nematophilus* (Boemare and Akhurst, 1988). Bacteria were pelleted by centrifugation (16000 x g, 4 min, 25°C) and resuspended in 200 µL serum to a bacterial concentration of $7x10^9$/mL.The suspensions were incubated at 30°C for 30 min and the bacteria removed by centrifugation. Proteins in the supernatants were separated by SDS-PAGE using a 12% polyacrylamide separating gel (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue.

To ensure bacterial proteases did not influence results, 50 µL of bacterial-adsorbed serum were incubated with non-adsorbed serum at 30°C for 0, 30 and 60 min. Samples were analysed for total protein by the method of Bradford, (1976) and for changes in protein banding by SDS-PAGE.

Putative LPS-binding proteins in the heat-inactivated serum were partially purified by precipitating the other protein species with 85% (w/v) ammonium sulfate at 0°C for 15 min. Precipitates were removed by centrifugation (10 000 x g, 10 min, 0°C) and the soluble proteins in the supernatant were separated by SDS-PAGE (Laemmli, 1970). Possible LPS-binding proteins were isolated by cutting the bands from electrophoresis

gels that had been stained with copper chloride (Lee *et al.*, 1987). The bands were destained in 0.25 M ethylenediamine tetraacetic acid in 0.25 M Tris (pH 9.0), dialysed overnight against 2 L of Tris-glycine buffer (pH 8.3) and electroeluted for 2 hours at 100 V (Maniatis *et al*, 1982). Proteins were exhaustively dialysed against 1% (v/v) phosphate-buffered saline and lyophilized. Protein purity was established for both proteins by SDS-PAGE (Laemmli, 1990) and silver staining of the bands (Harlow and Lane, 1988) and for the 17.2 kDa protein, by acid gel electrophoresis using a 15% acrylamide separating gel (pH 4.3) (Gabriel, 1971) and staining with Coomassie Brilliant Blue.

Chemical identification of the proteins as LPS-binding proteins was based on a metachromatic shift that occurs when the dye 1,9-dimethylene blue binds to the lipid A moiety of LPS (Keller and Nowotny, 1986). The inhibition of dye binding to LPS by the lipid A-binding protein, cecropin A, lowers the absorbance at 535nm (De Lucca *et al.*, 1995). Solutions (25 µL final volume) containing 25 µg of *X. nematophilus* LPS and various concentrations of two possible LPS-binding proteins were incubated at 30°C for 30 min. The dye solution (160 µg/mL) was added and the absorbance recorded at 535 nm (A535). Controls consisted of LPS only and proteins only.

Statistics

Hemolymph data were analysed by the 95% confidence interval overlap procedure (Sokal and Rholf, 1969). Unless stated otherwise, "n" refers to 3 group means representing data from 5 larvae per group. Data are presented as the mean ± the standard error.

RESULTS

LPS-binding proteins in larval serum

Larvae injected with 2 μg of LPS had a two fold increase in hemocyte counts compared with the phosphate-buffered saline control group (Fig. 2.1). Injections of LPS that had been incubated *in vitro* with different concentrations of serum (and thus different concentrations of total serum proteins) caused a decline in hemocyte levels proportional to the serum protein concentrations. Serum proteins alone did not influence hemocyte counts.

Serum adsorbed with bacteria with complete and incomplete LPS revealed a decline in the intensities of three bands including two major protein bands corresponding to proteins with apparent molecular weights of 17.2 kDa and 26 kDa (Fig. 2.2). Bacteria with incomplete LPS adsorbed the 17.2 kDa protein more extensively than bacteria with complete LPS. The prolonged incubation of adsorbed serum with non-adsorbed serum did not statistically change the total serum protein (non-adsorbed serum only: 18.2 ± 0.1 μg/mL; adsorbed serum with non-adsorbed serum for 0 min : 18.4 ± 0.3 μg/mL; 30 min : 19.6 ±0.8 μg/mL ; 60 min, 18.3 ± 1.8 μg/mL) nor the electrophoretic profiles (data not shown).

Ammonium sulphate precipitation lowered the complexity of the remaining soluble proteins and diminished the concentration of the 17.2 kDa and 26 kDa proteins (Fig. 2.3A). The latter two proteins electroeluted from the gels were electrophoretically pure up to 16 μg protein per well, the 17.2 kDa

Figure 2.1 Changes in the hemocyte counts of *G. mellonella* larvae following injection with various amounts of heat-inactivated serum with *X. nematophilus* LPS (2μg) or without.

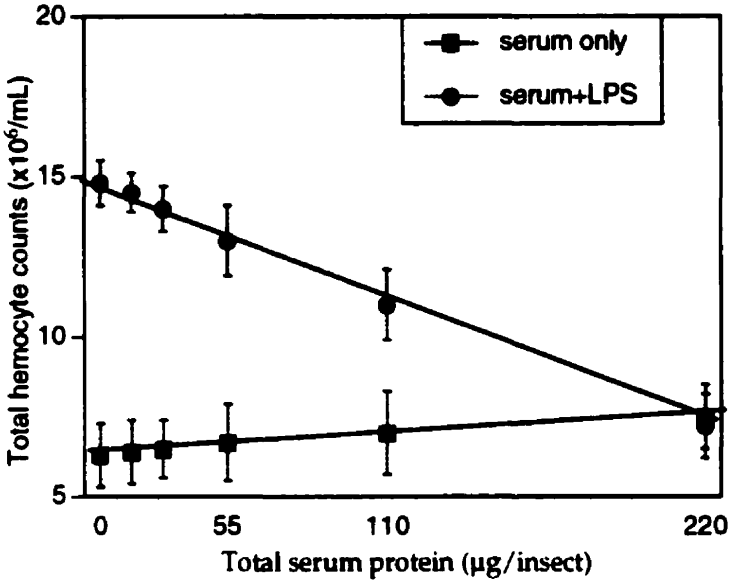

20
15
10
5
Total hemocyte counts (x10⁶/mL)
serum only
serum+LPS
0
55
110
220
Total serum protein (µg/insect)

Figure 2.2 Effect of *X. nematophilus* on the removal of the 17. 2 and 26 kDa proteins (arrows) from the heat-inactivated larval serum of *G. mellonella*. Lane 1, the DD136 strain of bacteria with complete LPS, lane 2, the ATCC 19061 strain of bacteria with incomplete LPS and lane 3, control serum.

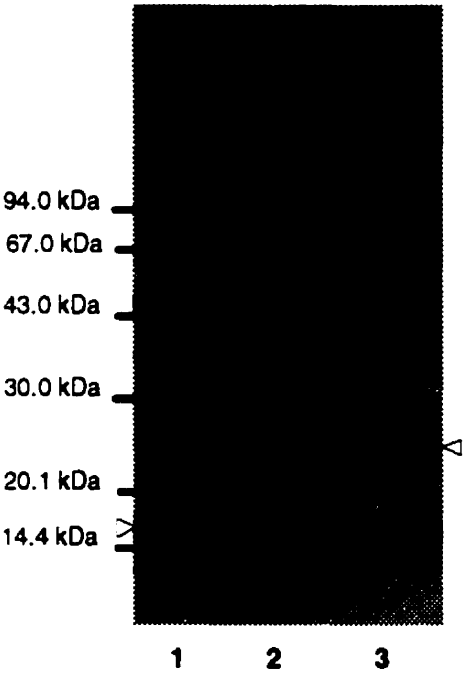
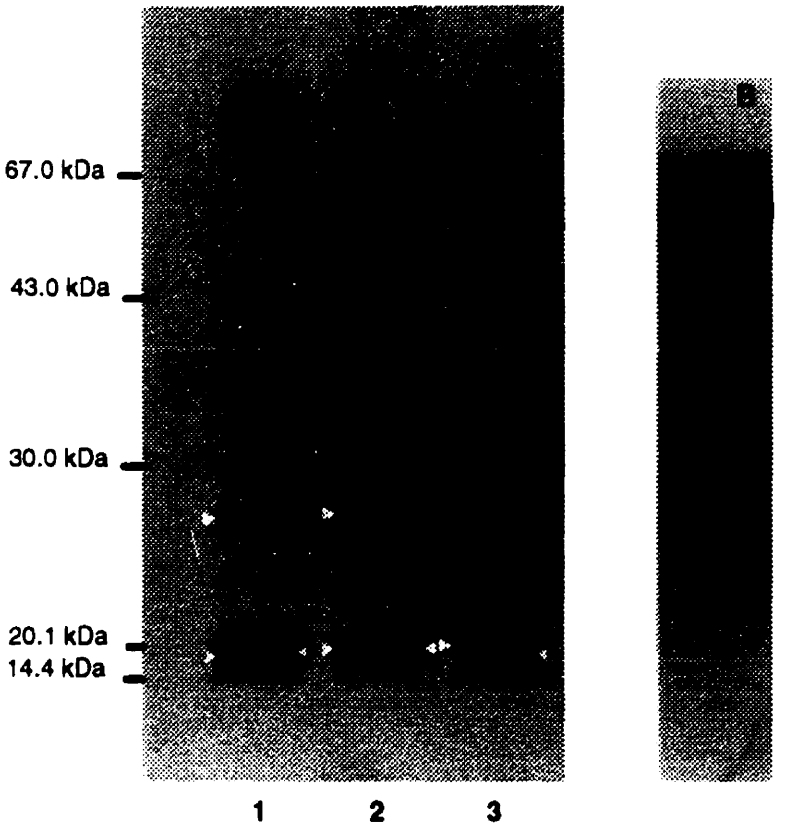

94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
1
2
3

Figure 2.3 Purification of the 17.2 kDa protein (arrows) from heat-inactivated larval serum of *G. mellonella*. **A**. SDS-PAGE (silver stained); Lane 1, heat-inactivated serum showing the 26 kDa (arrow) and 17.2 kDa protein bands, lane 2, soluble serum proteins remaining after ammonium sulfate precipitation (the 26 kDa band is not readily visible) and lane 3, purified 17.2 kDa protein. **B**. The 17.2 kDa protein in a 15% acrylamide gel, pH 4.3 (Coomassie Blue stained).


67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
1
2
3
B

protein was pure in both SDS (Fig. 2.3A) and acidic polyacrylamide gels (Fig. 2.3B) and the 26 kDa band was pure in the SDS-PAGE (data not shown).

In the dye-binding assay, both the 17.2 kDa and 26 kDa proteins decreased the absorbance at 535 nm, the effect increasing with increasing protein concentration (Fig. 2.4). Thus, the proteins were regarded as LPS-binding proteins (LBP) and designated as LBP-1 and LBP-2 for the 17.2 kDa and 26 kDa proteins, respectively.

LPS-binding proteins detoxify LPS.

Both LBP-1 and LBP-2 incubated *in vitro* with LPS (2 μg) reduced LPS toxicity for hemocytes, the levels of damaged hemocytes decreasing with increasing protein concentration (Fig. 2.5A). LBP-1 was more effective than LBP-2. Neither protein alone altered hemocyte counts compared with the control larvae injected with phosphate-buffered saline.

Supplementing indigenous LBP-1 with injections of LBP-1 (0, 1 and 2.5 μg/larva) increased the amounts of LPS required to elevate hemocyte counts (Fig. 2.5B). The amounts of LPS required to increase the numbers of hemocytes by 20 % for larvae injected with 0.0, 0.1 and 2.5 μg LBP-1 were 0.25, 6.3 and 100 μg/larva respectively.

LPS injections lowered phenoloxidase activity by 75% compared with the specific activity in larvae injected with phosphate-buffered saline (Fig. 2.6). Injections of LPS previously incubated *in vitro* with concentrations of LBP-1 known to detoxify LPS (from Fig. 2.5B) reduced the inhibition of prophenoloxidase activation (Fig. 2.6). Phenoloxidase activity increased to 90% of the buffer control titer in larvae injected

Figure 2.4 Absorbance of the dye 1,9-dimethylene blue bound to complete LPS of *X. nematophilus* in the presence of phosphate-buffered saline and selected amounts of LBP-1 and LBP-2.

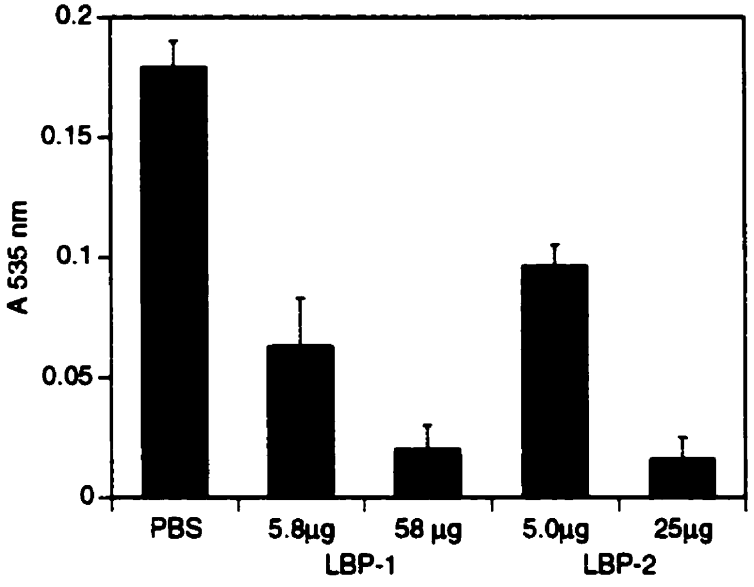

0.2
0.15
0.1
0.05
0
A 535 nm
PBS
5.8µg
58 µg
5.0µg
25µg
LBP-1
LBP-2

Figure 2.5 Influence of LPS-binding proteins on the level of hemocytes damaged by LPS. **A**. Hemocyte levels in larvae responding to injections of LBP-1 or LBP-2 and LBP-1 or LBP-2 with LPS (incubated *in vitro*). **B**. Hemocyte counts in larvae after initial injection with 0, 1 and 2.5 μg of LBP-1 followed by selected levels of LPS.

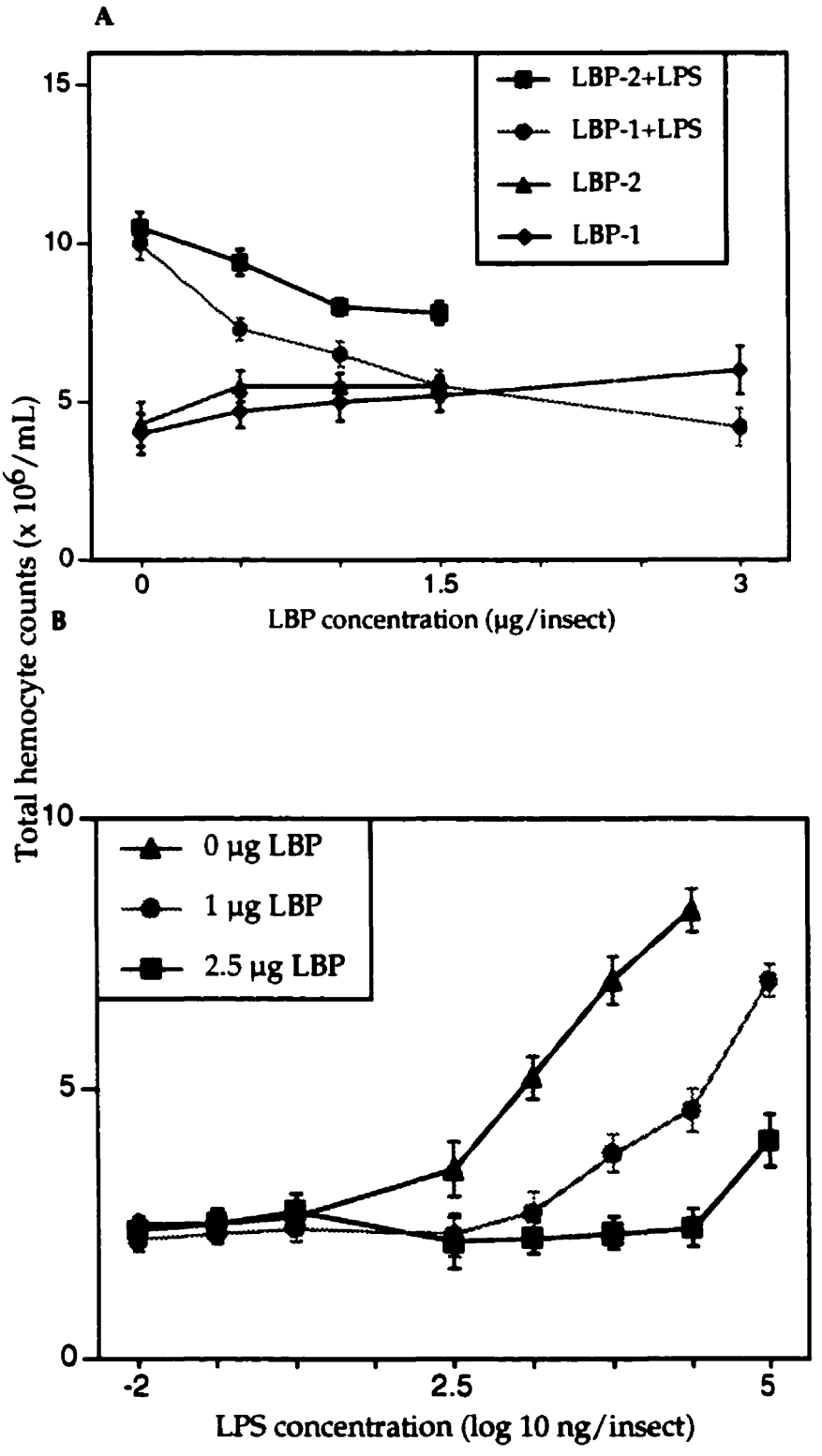
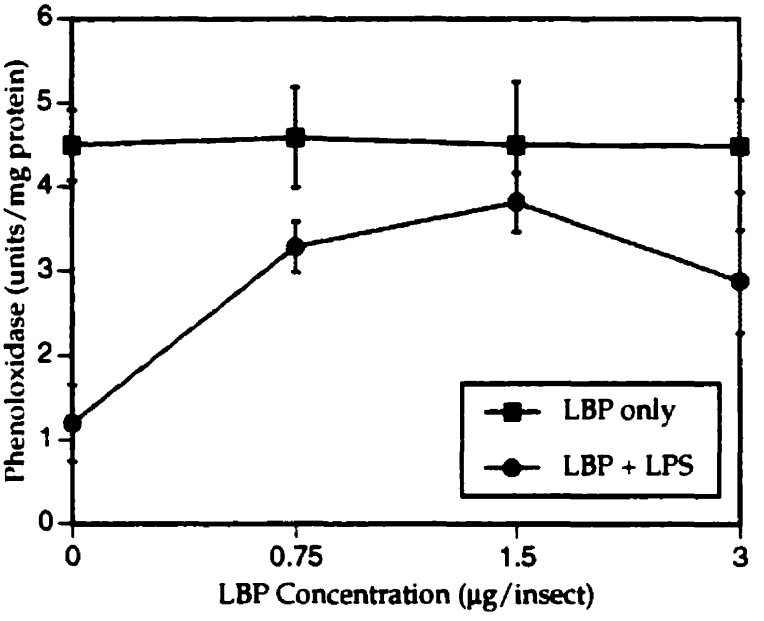

A
LBP-2+LPS
LBP-1+LPS
LBP-2
LBP-1
15
10
5
0
0
1.5
3
LBP concentration (µg/insect)
B
Total hemocyte counts (x 10^6/mL)
0 µg LBP
1 µg LBP
2.5 µg LBP
10
5
0
-2
2.5
5
LPS concentration (log 10 ng/insect)

Figure 2.6 Effect of LBP-1 in the presence and absence of LPS on phenoloxidase titers in *G. mellonella* larvae.


Phenoloxidase (units/mg protein)
6
5
4
3
2
1
0
0
0.75
1.5
3
LBP Concentration (µg/insect)
LBP only
LBP + LPS

with LPS and 1.5 μg of LBP-1 per insect. Inexplicably, LBP-1 at 3.0 μg/insect inhibited either prophenoloxidase activation and /or phenoloxidase activity. LBP-1 alone did not alter phenoloxidase titres.

DISCUSSION

LPS of *X. nematophilus* damages the hemocytes and impairs the activation of the prophenoloxidase system of many insect species (Dunphy and Webster, 1988, 1991;Yokoo *et al.*, 1992) thus facilitating the survival of the nematode *S. carpocapsae* within the insect (Akhurst and Dunphy, 1993). However, in the presence of whole bacteria, the impairment of prophenoloxidase activation requires a critical titre of bacterial LPS in the hemolymph (Dunphy and Hurlbert, 1995) which may indicate that the insects have an LPS-detoxifying system. In the present study, the inhibition of hemocyte damage induced by *X. nematophilus* by increasing concentrations of larval serum support this hypothesis .

Bacterial adsorption of larval serum decreased the levels of two major proteins. Decreases in the levels of these proteins were not due to residual protease activity because serum incubated with adsorbed serum did not show a decline in serum total protein or changes in serum protein electrophoretic profiles. The decreases in the 17.2 kDa and 26 kDa proteins were greater for bacteria with incomplete LPS than for bacteria with complete LPS; the former bacteria would expose more lipid A to the environment (Hammond *et al.*, 1984; Dunphy and Hurlbert, 1995).

Both the 17.2 kDa and 26 kDa proteins competed for the lipid A portion of LPS with the lipid A-specific dye 1,9-dimethymethylene blue confirming their identity as LPS-binding proteins. Also, this experiment revealed that the heat-stable proteins may be hydrophobic or have hydrophobic domains by virtue of their binding to lipid A.

Lipid A of *X. nematophilus* LPS is toxic to hemocytes of *G. mellonella* due to the fatty acids of lipid A intercalating into the hemocyte membrane after the glucosaminylglucosamine disaccharide becomes attached to the hemocyte receptor (Dunphy and Webster, 1988). Both LBP-1 and LBP-2, incubated *in vitro* with LPS, detoxified the endotoxin and thus prevented hemocyte damage. To preclude the incubation system giving artifactual results, other larvae were injected with LBP-1 to elevate the protein level in the hemolymph and then challenged with increasing concentrations of LPS. Enhancing indigenous levels of LBP-1 significantly increased the amount of LPS required for a given level of damaged hemocytes. Hence, the LBPs are LPS-detoxifying proteins.

X. nematophilus LPS also inhibits the activation of prophenoloxidase (Dunphy and Webster, 1991). LBP-1 prevented the inhibition of the melanization system by LPS and since LBP-1 binds to lipid A, this report states that lipid A inhibits prophenoloxidase activation.

In summary, hemolymph of *G. mellonella* larvae contain two LPS-binding proteins. The proteins detoxify LPS preventing hemocyte damage and LBP-1 blocks inhibition of the melanization system.These previously undescribed proteins protect the antimicrobial systems of the insects by binding to lipid A.

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CONNECTING STATEMENT 1

In chapter 2, LBP-1, a hemolymph protein of *G. mellonella*, is reported to bind to the LPS of *X. nematophilus* as well as to the surface of this bacterium. During the experimentation, an increase in lysozyme activity against *M. lysodeikticus* in the presence of LBP-1 was observed. This was investigated further as described in chapter 3.

CHAPTER 3

APOLIPOPHORIN-III IN *Galleria mellonella* POTENTIATES HEMOLYMPH LYTIC ACTIVITY.

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ABSTRACT

Heat-inactivated serum of *Galleria mellonella* larvae enhanced the lytic activity of larval cell-free hemolymph against *Micrococcus lysodeikticus*. The increase in bacterial lysis was due to a 17.2 kDa protein known previously to bind to bacterial lipopolysaccharides. The protein enhanced the lytic activity of insect cell-free hemolymph and hen egg lysozyme *in vitro* and insect hemolymph *in vivo*. The hydrophobic protein, which adhered to *M. lysodeikticus*, was identified by its amino acid sequence homology as apolipophorin-III. Apolipophorin-III did not bind to lysozyme. A possible mode of action of apolipophorin-III with lysozyme in the insect is proposed.

Keywords: Apolipophorin-III, *Enterococcus faecalis*, *Galleria mellonella*, hemolymph, lysozyme, *Micrococcus lysodeikticus*.

INTRODUCTION

The first response of insects to bacteria in the hemocoel is a combination of interactive cellular and humoral reactions. Hemocytic responses, phagocytosis and nodulation, may contain the invading bacteria (Whitcomb *et al.*, 1974) and are usually accompanied by the proteolytic activation of the phenoloxidase cascade (Sugumaran and Kanost, 1993). Recognition of the bacteria as non-self is a prerequisite for these events and is mediated by bacterial components such as lipopolysaccharides (LPS) and peptidoglycan (Gillispie and Kanost, 1997).

Lysozyme, an enzyme found in the hemolymph of insects, has anti-microbial properties partially due to its ability to hydrolyze ß-1,4-linkages in the peptidoglycan layer of bacterial cell walls (Jollés and Jollés, 1984). The low constitutive level of lysozyme present in the hemolymph of insects (Powning and Davidson,1973; Hultmark *et al.*, 1980; Russell and Dunn, 1990; Lockey and Ourth, 1996) plays a significant role in the digestion of bacterial cell walls and the release of peptidoglycan fragments that act as signal molecules for the activation of the antibacterial cecropin, attacin and lysozyme genes (Dunn *et al.*, 1985). The amino acid composition, molecular size, net charge, stability to heat, optimum pH and specificity of action of *Galleria mellonella* lysozyme are similar to those of hen egg lysozyme (Powning and Davidson, 1973; 1976).

Recently, we reported a 17.2 kDa hemolymph protein in *G.mellonella* larvae that bound and detoxified LPS (Dunphy and Halwani, 1997). The similarities between the apparent molecular weight (17.2 kDa), heat stability and initial purification steps of this LPS-binding

protein and apolipophorin-III (apoLp-III) (Wiesner *et al.*, 1997) from *G. mellonella* suggested that the two molecules were identical. During our previous study, when the LPS-binding protein was reacted with hen egg lysozyme, an increase in lytic activity towards *M. lysodeikticus* was observed. Induction of lysozyme activity 24 hours post-injection of apoLp-III in *G. mellonella* was reported by Weisner *et al* (1997). The present work examined the immediate effect of apoLp-III on the lytic activity of hen egg lysozyme and *G. mellonella* cell-free hemolymph.

This communication confirms the LPS-binding protein as apolipophorin-III, reports synergy between this protein and both *G. mellonella* lysozyme and hen egg lysozyme and suggests an additional role for the protein in the antibacterial response of the insect.

MATERIALS AND METHODS

Insects and Materials

Larvae of the greater wax moth, *G. mellonella,* were reared at 30°C in constant light on an artificial diet composed of cereal grains (Dutky *et al,* 1962) supplemented with approximately 2% (w/w) brood comb. Last instar larvae weighing 200-250 mg were used in all experiments.

Freeze-dried *M. lysodeikticus,* hen egg lysozyme and lactalbumin were from Sigma. Octyl-Sepharose was purchased from Pharmacia Biotech.

Preparation of heat-inactivated insect serum, protein purification and amino acid sequence determination.

Hemolymph, collected from larvae by amputation of a prothoracic leg, was mixed (1:1, v/v) with phosphate-buffered saline (NaCl, 8 g /L, KCl, 0.2 g/L, Na_2HPO_4, 1.15 g/L, KH_2PO_4, 0.2 g/L; pH 6.5) and centrifuged (14 000 x g, 4 min, 20°C) to pellet hemocyte debris. Heat-stable peptides were partially purified by precipitating heat labile proteins at 100°C for 30 minutes. After a second centrifugation (14 000 x g, 4 min, 20°C), the supernatant (henceforth referred to as heat-inactivated serum) was collected and frozen at -20°C. The heat-inactivated serum did not have lysozyme activity.

The 17.2 kDa serum protein previously shown to be LPS-binding protein (hereafter referred to as apoLp-III as explained later in the text) was purified as described earlier (Dunphy and Halwani, 1997). Briefly, heat inactivated serum proteins were further purified by partial

precipitation with 85% (w/v) ammonium sulfate (Harris and Angal, 1992) and centrifugation (10 000 x g, 10 min, 0°C). The supernatant was dialyzed against 10% (v/v) phosphate-buffered saline and the proteins separated by electrophoresis on a sodium dodecyl sulphate (SDS) 12% polyacrylamide gel (Laemmli, 1970). After staining with copper chloride (Lee *et al.*, 1987), the 17.2 kDa band was excised, destained in 0.25 M ethylenediamine tetraacetic acid in 0.25 M Tris (pH 9.0), dialyzed overnight against 2 L of Tris-glycine buffer (pH 8.3) and electroeluted for 2 hours at 100 V (Maniatis *et al.*, 1982). The electroeluted protein was dialyzed against 10% (v/v) phosphate-buffered saline and lyophilized. Protein was determined using the Bradford (1976) method with bovine serum albumin as standard.

N-terminal sequencing of 20 residues was performed by automated Edman degradation using a Procise sequencer (Applied Biosystems).

Lysozyme activity assays

Lysozyme activity was based on the lysis of *M. lysodeikticus* using the agar plate method (Mohrig and Messner, 1968). Plates were prepared with 10 mL of 1.5% agar in phosphate-buffered saline (pH 6.5) mixed with freeze-dried *M. lysodeikticus* cells (1 mg/mL). Heat-inactivated serum or apoLp-III was incubated (30°C for 30 min) with hen egg lysozyme or cell-free hemolymph. The latter was prepared by pelleting the hemocytes by centrifugation (14000 x g, 6 min, 20°C). Aliquots (10 µL) of test solutions were pipeted into 0.5 cm diameter wells cut in the agar. Plates were incubated for 24 h at 30°C and the diameters of the clearance zones measured. Hen egg lysozyme was used as the standard.

Preliminary studies were done to select for a dilution series of cell-free hemolymph that would not show an increase in lysozyme content within the limitations of the assay to facilitate detecting a treatment effect.

Injections and bleeding

To study the effect of apoLp-III on *G. mellonella* lysozyme *in vivo*, insects were injected at the base of the third left proleg with 10 µL of phosphate-buffered saline containing 0, 5, 10 or 20 µg of apoLp-III and kept at 30°C for 30 min. Hemolymph was collected by puncturing the cuticle of the larvae with a sterile needle and drawing the blood into a chilled pasteur pipette. Cell-free hemolymph was made by centrifuging the samples (14 000 x g, 6 min, 20°C). Samples from individual larvae were kept separate and used fresh.

Adherence of heat-inactivated proteins to *M. lysodeikticus* and Octyl-Sepharose resin

To detect binding of serum proteins to *M. lysodeikticus*, freeze-dried bacterial cells (1 g) were hydrated in 5mL of phosphate-buffered saline and 50 µL volumes of diluted suspensions (25, 50 or 100 µg/µL) or phosphate-buffered saline were added to 20 µL volumes of heat-inactivated serum. Following incubation (30 min, 30°C), the bacterial cells were removed by centrifugation and the proteins in the supernatant were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) (Laemmli, 1970). Protein bands were stained with Coomassie Brilliant Blue.

Hydrophobic interaction chromatography was used to determine the nature of proteins in heat-inactivated serum that bound to the bacteria. Twenty-five µL of heat-inactivated serum were incubated with 25 µL of phosphate-buffered saline containing different amounts of Octyl-Sepharose beads (1, 0.5 and 0.2 mg/mL) for 20 min at 30°C. The resin beads were pelleted by centrifugation (16 000 x g, 5 min, 25°C) and the supernatant proteins analyzed by SDS-PAGE. To ensure that the effect with apoLp-III was not an artefact of heat-inactivated serum, cell-free hemolymph was similarly treated.

Acid polyacrylamide gels

To detect possible hydrophobic or ionic binding of apoLp-III to hen lysozyme, 40 µg of apoLp-III were reacted with 25 µg of hen egg lysozyme (30°C, 30 min). Controls consisted of apoLp-III or hen lysozyme in phosphate-buffered saline.The samples were subjected to polyacrylamide gel electrophoresis using a ß-alanine-acetic acid buffer (pH 4.3) (15% acrylamide separating gel) or diethyl barbituric acid buffer (pH 7.0) (12% acrylamide separating gel) (Gabriel, 1971) and the separated proteins stained with Coomassie Brilliant Blue.

Statistical Analysis

The effect of heat-inactivated serum and apoLp-III on the lysozyme in cell-free hemolymph (*in vitro* and *in vivo*) and on hen egg lysozyme were analyzed by ANOVA followed by least significant differences. The correlation between concentrations and effects

were tested using the product moment correlation coefficient (Sokal and Rohlf, 1969). Probability values of $P \leq 0.05$ were taken to indicate statistical significance.

RESULTS AND DISCUSSION

The sequence of the N-terminal twenty amino acids of the 17.2 kDa protein (DASTPLQDLEKHAAEFQKTF) establishes the molecule as apoLp-III as described by Wiesner *et al.* (1997).

The addition of heat-inactivated serum to cell-free hemolymph increased significantly the level of lysozyme activity regardless of the percent cell-free hemolymph present in the reaction (Fig. 3.1). Ions or small molecules (< 14 kDa) in the serum did not contribute to this increase in lysis since the same results were obtained when heat-inactivated serum was previousely dialyzed against phosphate-buffered saline. Because apoLp-III is a major protein in heat-inactivated serum (Dunphy and Halwani, 1997), purified apoLp-III was reacted with cell-free hemolymph and with hen egg lysozyme (Figs. 3.2 and 3.3, respectively). The lytic activity increased with the concentration of the test protein ($r= 0.97$ and 0.92 for insect hemolymph and hen egg lysozyme, respectively; $P\leq 0.05$) achieving a two fold increase at the highest concentrations. Compared to the amount of lysozyme in cell-free hemolymph (0.7-0.8 mg/mL; Chadwick, 1970) and the total protein content (178.75 ± 50.53 mg/mL), the amount of apoLp-III added was negligible. To confirm that the increase in lysis was not due to a non-specific stabilization of lysozyme by proteins, apoLp-III was replaced by an equal concentration of lactalbumin (14 kDa protein) and the lactalbumin had no effect (data not shown).

The synergy between apoLp-III and lysozyme, whether from hen egg or in *G. mellonella* cell-free hemolymph, is further confirmation of the closeness of the two enzymes and thus hen egg lysozyme may

Figure 3.1 Effect of five µL of heat-inactivated serum (total protein: 0.965±0.142 mg/mL) on the lytic activity of *G. mellonella* cell-free hemolymph. (Means ± SE, n=5).

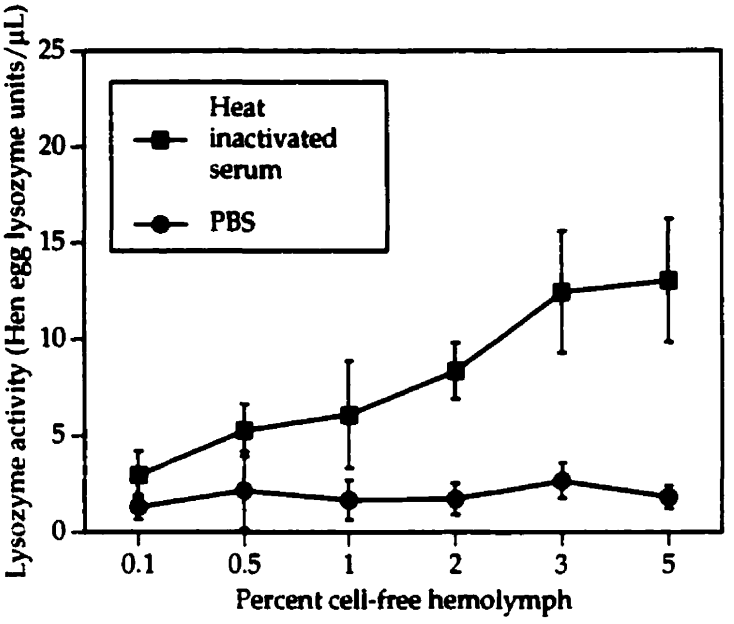

Lysozyme activity (Hen egg lysozyme units/µL)
25
20
15
10
5
0
Heat inactivated serum
PBS
0.1
0.5
1
2
3
5
Percent cell-free hemolymph

Figure 3.2 Effect of apoLp-III on the lytic activity of *G. mellonella* cell-free hemolymph. *in vitro*. (Means ± SE, n= 5). Columns labeled with identical letters are not significantly different ($p>0.05$) from each other.

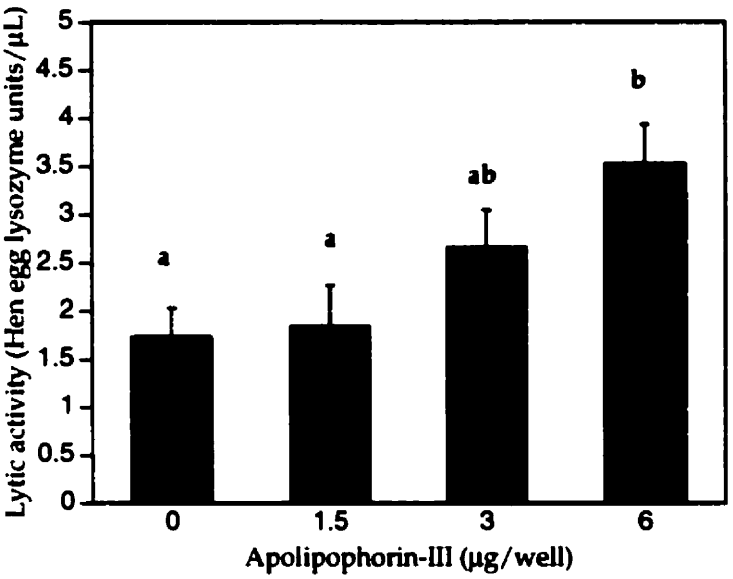
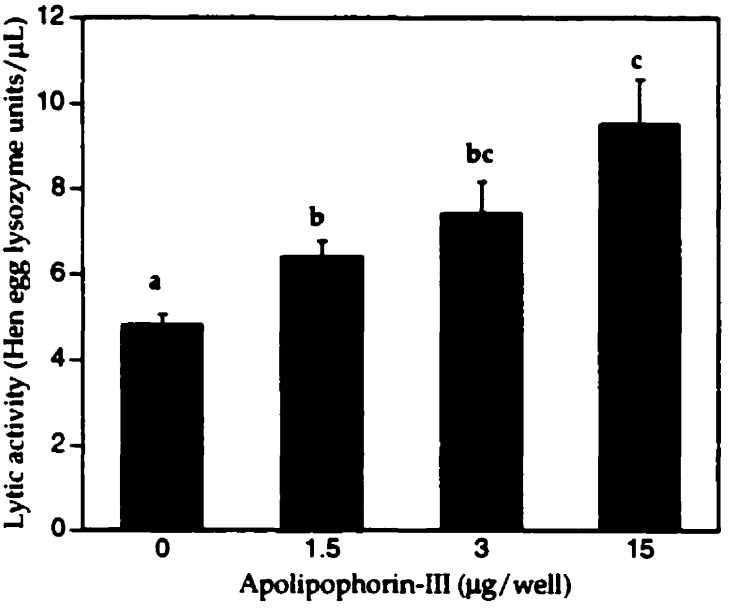
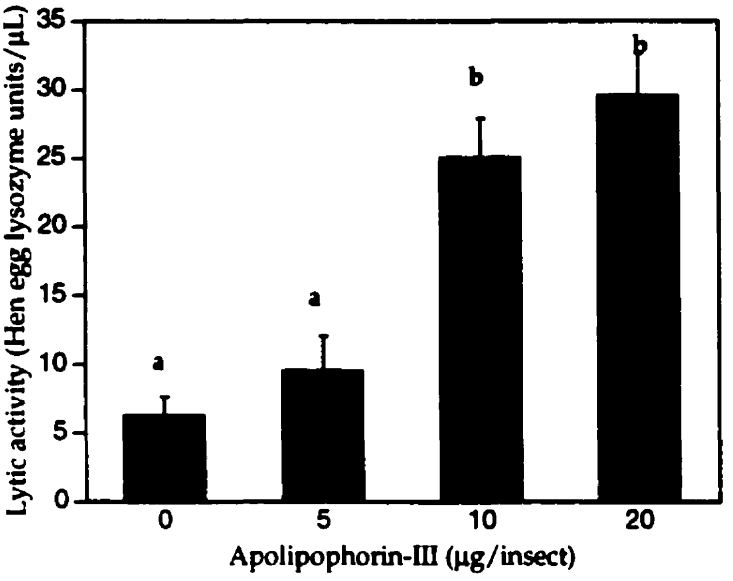

Lytic activity (Hen egg lysozyme units/μL)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
ab
b
0
1.5
3
6
Apolipophorin-III (μg/well)

Figure 3.3 Effect of apoLp-III on the lytic activity of hen egg lysozyme. (Means $\pm$ SE, n= 5). Columns with identical letters are not significantly different ($p>0.05$) from each other.


Lytic activity (Hen egg lysozyme units/μL)
12
10
8
6
4
2
0
a
b
bc
c
0
1.5
3
15
Apolipophorin-III (μg/well)

replace insect lysozyme in preliminary tests. Synergy of induced antibacterial proteins of insects with lysozyme has been reported previously (Boman and Hultmark, 1987; Bang *et al*, 1997). In the present context, the synergy could be a direct effect of apoLp-III on lysozyme (which is discounted elsewhere in the text) or an indirect effect due to interactions of apoLp-III with the bacteria. Because purified apoLp-III increased hen egg and *G. mellonella* lysozyme activity *in vitro* and since apoLp-III was rendered lipid-free by SDS, it is unlikely lipids participated in the observed effect. Injections of 10 or 20 µg of apoLp-III *G. mellonella* larvae elevated significantly the level of lysozyme activity by 3 to 4 fold compared with the control, the effect being proportional to the concentration of apolipophorin-III ($r= 0.9330$, $P \leq 0.05$, Fig. 3.4). The increase in lysozyme activity 30 min post-injection was not the result of *de novo* synthesis of the enzyme since the induction of such antibacterial proteins is known to be preceded by a lag of several hours (Dunn, 1987) nor was it due to an increase in the numbers of lysozyme-containing hemocytes because apoLp-III did not alter the hemogram (Dunphy and Halwani, 1997). The increase in lytic activity following injections was greater than that observed *in vitro* suggesting the possible contribution of lysozyme released from the hemocytes; hemocyte activation by apoLp-III has been reported (Wiesner *et al*, 1997). Injections of 20 µg of apoLp-III into a 200 mg insect represents less than 4% of the total apoLp-III in the system (based on an estimated concentration of apoLp-III of 8-12 mg/mL of hemolymph, see chapter 5); the results are thus physiological as opposed to pharmacological. However, it is not yet known whether apoLp-III in *G. mellonella* is found free, or associated with other proteins or lipids. An innate low

Figure 3.4 Levels of lytic activity in *G. mellonella* larval serum 30 minutes after injecting the insects with apoLp-III. (Means ± SE, n=5). Means with identical letters are not significantly different ($p>0.05$) from each other.


Lytic activity (Hen egg lysozyme units/μL)
35
30
25
20
15
10
5
0
a
a
b
b
0
5
10
20
Apolipophorin-III (μg/insect)

level of free apoLp-III in the hemolymph of the larvae would explain why addition of a small amount of apoLp-III would have an effect.

The effects reported herein were not attributed to denatured apoLp-III because apoLp-III from insect species in three orders, including *Manduca sexta*, readily renatures after heating and is regarded as heat stable (Strobel *et al.*, 1990; Ryan *et al.*, 1993; Kanost *et al.*, 1995). In addition, apoLp-III in *G. mellonella*, which shows homology with that of *M.sexta*, does not loose physiological properties following heating and cooling (Wiesner, 1997; Dettloff and Wiesner, 1998; Weise *et al.*, 1998).

The band intensity of apoLp-III in the supernatant previously exposed to *M. lysodeikticus*, decreased with increasing bacterial concentrations indicating that apoLp-III binds to the bacterial surface (Fig 3.5A). This occurred with other proteins including the 26 kDa protein. The fact that both proteins declined in band intensity with increasing Octyl-Sepharose resin concentration in cell-free hemolymph and in heat-inactivated serum (Fig 3.5B, C) establishes that the binding to bacteria and resin in heat-inactivated serum was not an artefact of the heating procedure. Both apoLp-III and the 26 kDa protein bound to hydrophobic LPS isolated from the outer membrane of the bacterium *Xenorhabdus nematophilus* (Enterobacteriaceae) as well as to the bacteria (Dunphy and Halwani, 1997) implying that they were hydrophobic proteins. Other proteins that herein bound to *M. lysodeikticus* were similarly hydrophobic.

The non-denaturing acid gel (pH 4.5) shows that hen lysozyme and apoLp-III migrate separately (Fig. 3.6) and at pH 6.8 apoLp-III but not lysozyme entered the gel (data not shown) indicating that the two molecules do not bind to each other. This suggests that the elevated

Figure 3.5 Interaction of heat-inactivated serum proteins with (A) *M. lysodeickticus* and (B) Octyl-Sepharose resin beads in SDS 12 % polyacrylamide gels; (C) interaction of cell-free hemolymph with Octyl-Sepharose resin beads in SDS 12% polyacrylamide gels. Solid arrows for apoLp-III; white arrows for 26 kDa protein. A: Lanes 1-4: heat-inactivated serum proteins following incubation with 0, 25, 50 and 100 mg/mL of *M. lysodeikticus* cells; lane 5: control, *M .lysodiekticus* only (100 mg/mL). B: Lanes 1-3: heat-inactivated serum proteins following incubation with 0.2, 0.5 and 1 mg/mL of Octyl-Sepharose resin beads; lane 4: no resin beads. C: Lanes 1-4: hemolymph proteins following incubation with 0, 0.2, 0.5 and 1 mg/mL of Octyl-Sepharose resin beads.

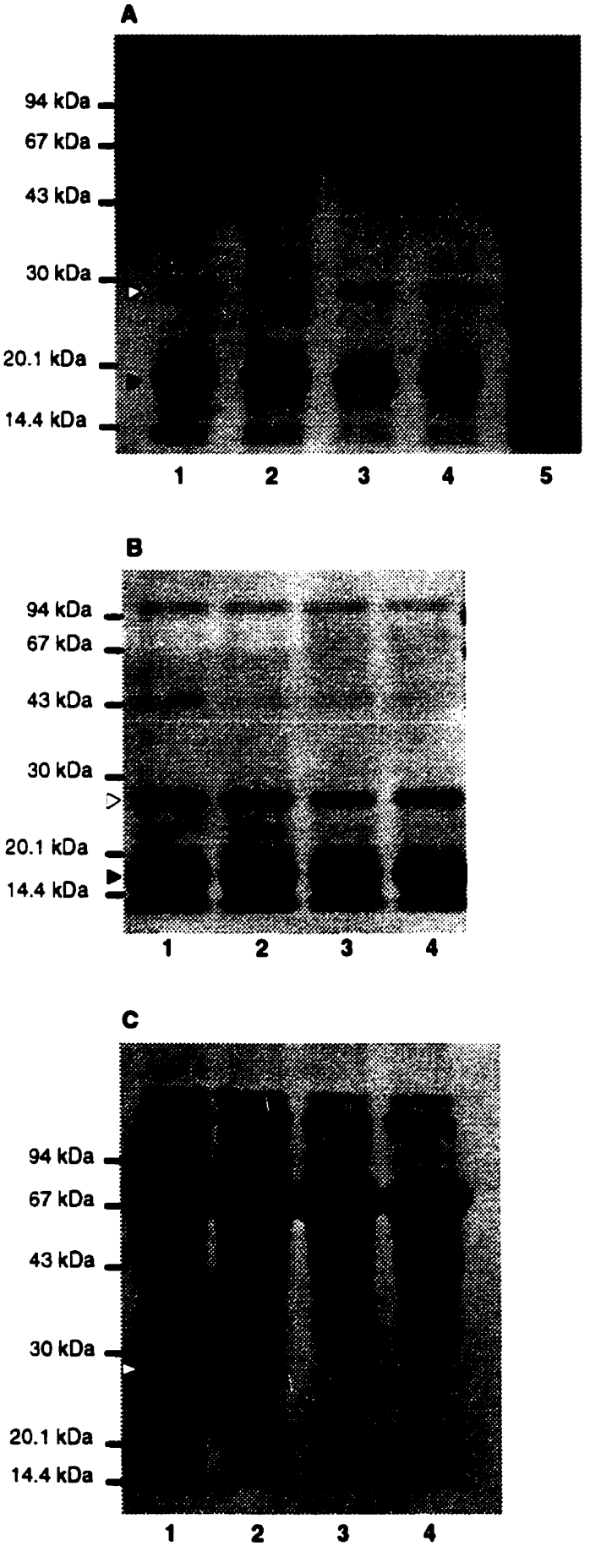
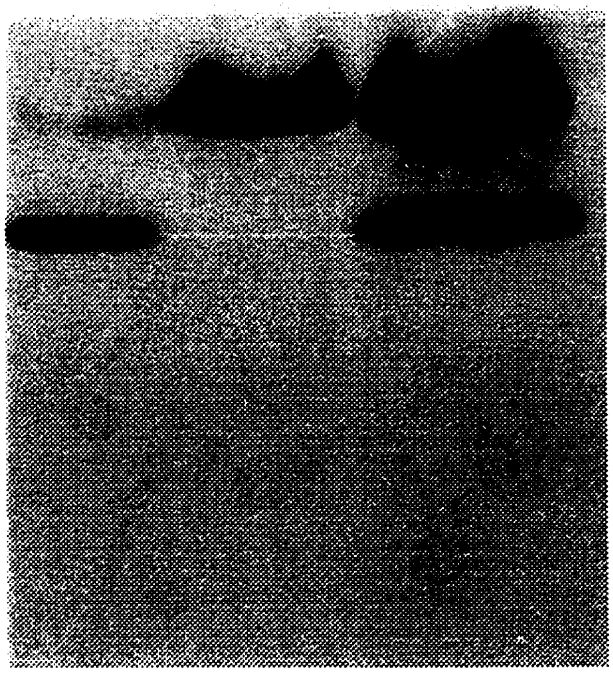

A
94 kDa
67 kDa
43 kDa
30 kDa
20.1 kDa
14.4 kDa
1
2
3
4
5
B
94 kDa
67 kDa
43 kDa
30 kDa
20.1 kDa
14.4 kDa
1
2
3
4
C
94 kDa
67 kDa
43 kDa
30 kDa
20.1 kDa
14.4 kDa
1
2
3
4

Figure 3.6 Interaction of apoLp-III with hen egg lysozyme in an acidic polyacrylamide gel (pH 4.3). Lane 1: hen egg lysozyme, lane: 2 apoLp-III, lane 3 : hen lysozyme and apoLp-III reacted for 30 min at 30°C.


1
2
3

activity of lysozyme observed in the presence of apoLp-III could be due to increased susceptibility of the bacterial cell to lysozyme as a result of alteration of its surface by apoLp-III. The digestion of susceptible microorganisms by lysozyme results in bacterial fragments that elicit non-self responses (Gillespie and Kanost, 1997). ApoLp-III, constitutively present in hemolymph, could immediately maximize the effect of lysozyme against bacteria that gain access to the hemocoel such as do gut bacteria during molting (Dunn *et al.*, 1994). Cell-free hemolymph and hen lysozyme were both capable of lysing *Enterococcus faecalis* isolated from the gut of *G. mellonella* last instar larvae (data not shown). Lysozymes have been known to destroy the symbionts that escape from mycetocytes (Gupta, 1989).

The results reported here support the suggestion of Wiesner *et al* (1997) that in *G. mellonella*, apoLp-III plays a role in antibacterial defense mechanisms in addition to its role in lipid transport.

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CONNECTING STATEMENT 2

In Chapter 3, LBP-1 is identified as apoLp-III, a protein that binds hydrophobically to lipids. ApoLp-III neutralized LPS toxicity by binding to the lipid A moiety (Chapter 2). The ability of apoLp-III to bind to and neutralize LTA, a surface component of Gram-positive bacteria that resembles LPS, was considered. The interaction of apoLp-III and LTA is examined in Chapter 4.

CHAPTER 4

APOLIPOPHORIN III AND THE INTERACTIONS OF LIPOTEICHOIC ACIDS WITH THE IMMEDIATE IMMUNE RESPONSES OF *Galleria mellonella*

A version of this chapter has been submitted to the Journal of Invertebrate Pathology as follows, Halwani, A.E., Niven, D.F., and Dunphy, G.B. Apolipophorin-III and the interactions of lipoteichoic acids with the immediate immune responses of *Galleria mellonella*.

ABSTRACT

In this study, we investigated the effects of lipoteichoic acids, surface components of Gram-positive bacteria, on the hemocytes and phenoloxidase activity in last instar *Galleria mellonella* larvae, as well as the binding of apolipophorin-III, an insect lipid-binding protein, to lipoteichoic acids. Binding of apolipophorin-III to lipoteichoic acid was studied using an assay based on 1,9-dimethylmethylene blue and an electrophoretic mobility assay using non-dissociating polyacrylamide gels. Apolipophorin-III bound the lipoteichoic acids from *Bacillus subtilis*, *Enterococcus hirae* and *Streptococcus pyogenes*, and to intact cells of *E. hirae*. *E. hirae* lipoteichoic acid promoted the binding of apolipophorin-III to the cells of this species. All lipoteichoic acids tested caused a dose- and time-dependent drop in the total counts of hemocytes and, depending on the species of lipoteichoic acid, partial or complete depletion of plasmatocytes. Granulocyte counts were not affected. Apolipophorin-III prevented partially the loss of plasmatocytes due to *B. subtilis* lipoteichoic acid. All three lipoteichoic acids studied activated phenoloxidase *in vitro*; injections of *B. subtilis* lipoteichoic acid into the larvae elevated the phenoloxidase activity while injections of *E. hirae* or *S. pyogenes* lipoteichoic acid, or apolipophorin-III alone, suppressed it. Apolipophorin-III decreased the activation of phenoloxidase by *B. subtilis* lipoteichoic acid.

Key words: *Galleria mellonella*, *Enterococcus hirae*, phenoloxidase, hemocytes, plasmatocytes, granulocytes, apolipophorin-III, lipoteichoic acid.

INTRODUCTION

Apolipophorin-III (apoLp-III) is a hemolymph protein that associates hydrophobically with lipoprotein surfaces to facilitate lipid transport in an aqueous medium (Blacklock and Ryan, 1994). The amphipathic helices of this molecule are such that their hydrophobic residues are oriented toward the center of the bundle and the hydrophilic residues are in contact with the solvent; it is thought that this molecular architecture is responsible for the reversible lipoprotein-binding property of apoLp-III (Wang *et al.*, 1998). In addition to lipid transport, apoLp-III has been linked to apoptosis of insect skeletal muscle (Sun *et al.*, 1995)

ApoLp-III in *Galleria mellonella* larvae has been recently implicated in immune responses; Wiesner *et al.* (1997) reported a significant increase in antibacterial activity 24 hours post-injection of larvae with apoLp-III and an enhanced phagocytic response *in vitro*. We have shown that apoLp-III binds to lipid A and detoxifies *Xenorhabdus nematophilus* lipopolysaccharide (LPS) (Dunphy and Halwani, 1997) and potentiates lysozyme activity (Halwani and Dunphy, 1999). Limura *et al.* (1998) observed that LPS prevented apoLp-III-mediated erythrocyte hemagglutination.

Lipoteichoic acids (LTAs) are amphipathic molecules that are anchored in the cytoplasmic membrane of Gram-positive bacteria by means of hydrophobic interactions; they are thought to be counterparts of LPS of Gram-negative bacteria (Wicken and Knox, 1977) and are generally characterized as glycolipid-linked polymers of glycerol phosphate units joined by phosphodiester bonds (Fischer, 1988). The effects of LTAs on the immune responses of mammals and their contribution to the virulence of Gram-

positive bacteria have recently received considerable attention. LTA has been shown to partially mediate bacterial adhesion to membranes of several types of mammalian cells (Op den Camp *et al.*, 1985; Chugh *et al.*, 1990; Courtney *et al.*, 1997). LTAs are also reported to stimulate macrophages and dermal and vascular muscle cells to release cytokines and nitric oxide (Longchamp *et al.*, 1992; DeKimpe *et al.*, 1995; English *et al.*, 1996; Baroni *et al.*, 1998; Hattori *et al.*, 1998)

The ability of LTA to induce immunity in *G. mellonella* larvae has been reported (Alexander *et al.*, 1992); however, studies on immediate non-immune responses of insects to Gram-positive bacterial antigens have been limited to the effects of peptidoglycan (reviewed by Dunn, 1986 and Gillespie *et al.*, 1997) and wall teichoic acids (Brookman *et al.*, 1989a, 1989b). The immediate responses of non-immune Lepidopterans to bacterial antigens include stimulation of hemocytes (Lackie, 1988) and activation of the prophenoloxidase cascade (Sugumaran and Kanost, 1993; Ashida and Brey, 1997).

Because apoLp-III is hydrophobic and was shown to bind LPS, we investigated the short-term immune response of *G. mellonella* larvae to LTA and the possible involvement of apoLp-III in this response.

MATERIALS AND METHODS

Insects and chemicals

G. mellonella larvae were reared on an artificial diet of infant mixed cereal wetted with a 1:1:1 (v/v/v) solution of sucrose, glycerol and distilled water that was supplemented with a complete vitamin mixture (Dutky *et al.*, 1962) and approximately 2% (w/w) brood comb. Insects were kept at 30°C in constant light. Last instar larvae weighing 200-250 mg were used in all experiments.

The dye 1,9-dimethylmethylene blue (DMB) was from Aldrich Chemical Co. D,L-Dihydroxyphenylalanine (DOPA), bovine serum albumin and LTAs from *Bacillus subtilis, Enterococcus hirae* (previously known as *E. faecalis*) and *Streptococcus pyogenes* were from Sigma. Tris and glycine were from Fisher Scientific. Chemicals for polyacrylamide gel electrophoresis were from Bio-Rad. Low molecular weight protein markers (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin) were from Pharmacia.

Preparation of heat-inactivated serum and purification of apoLp-III

Hemolymph was collected from *G. mellonella* larvae by amputation of a prothoracic leg and was diluted 1:1 (v/v) with phosphate- buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6.5 with HCl). Hemocytes were removed by centrifugation (12 000 x g, 4 min, 20°C). The supernatant was heated at 100°C for 30 min to precipitate heat labile proteins and inactivate hemolymph enzymes. Following centrifugation (12 000 x g, 4 min, 20°C), the heat-inactivated serum was frozen at -20°C. Heat-inactivated serum

proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). After staining with copper chloride (Lee *et al.*, 1987), the apoLp-III band (17.2 kDa) was excised, destained in 0.25 M ethylenediaminetetraacetic acid (tetrasodium salt) in 0.25 M Tris (pH 9.0 with HCl), dialyzed overnight against 2 L of 25 mM Tris, 192 mM glycine (pH 8.3) and electroeluted in the same buffer for 2 h at 100 V (Maniatis *et al.*, 1982). Purity was confirmed as reported previously (Dunphy and Halwani, 1997; Halwani and Dunphy, 1999). The electroeluted protein was dialyzed against 10 % (v/v) PBS and lyophilized.

Isolation of gut bacteria

E. hirae was isolated from the gut of *G. mellonella* last instar larvae. Whole guts were removed aseptically, homogenized in sterile PBS in a tissue grinder and plated on *Enterococcus faecalis* selective medium (Difco).

Bacteria were grown overnight in 1 L of tryptose broth (Difco), washed (10 000 x g, 4°C, 10 min) in sterile PBS, killed by UV irradiation for 90 min (cell viability was checked on tryptose agar), lyophilized and stored at -20°C.

DMB and LTA-binding assays

DMB reagent was prepared as described by Keler and Nowotny (1986) and contained 16 mg DMB, 4.28 g glycine, 3.33 g NaCl, 47 mL 1 N NaOH and 5 mL 80% ethanol in 1 L of distilled water. The mixture was stirred for 5 h at room temperature and stored in a brown bottle. Solutions of LTAs from *B. subtilis*, *E. hirae* or *S. pyogenes* (0, 1, 5, 10, 20,

30 and 40 µg in 50 µL of PBS) were mixed with 1 mL of DMB reagent and the absorbance at 535 nm was recorded.

To detect the possible binding of apoLp-III to LTA, 10, 20 and 40 µg of apoLp-III were reacted with 10 µg of LTA at 30°C for 30 min and the mixture was assayed using DMB as described above. The control, which was used as blank in the assay, consisted of apoLp-III (10, 20, or 40 µg) and DMB.

Mobility shift assay

The formation of LTA-apoLp-III complexes was also detected as a shift in electrophoretic mobility of apoLp-III. Mixtures of apoLp-III (10 µg) and lipoteichoic acid (0, 0.1, 0.5, 1, 5 and 10 µg), in a total volume of 3 µL of PBS, were incubated for 15 min at 30°C, diluted 1:2 (v/v) in electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3) and loaded onto a 0.75 mm thick, 6% polyacrylamide non-denaturing continuous gel. Electrophoresis was performed at a constant voltage of 200 V until the tracking dye (bromophenol blue) was 1 cm from the lower edge of the gel. Gels were stained with Coomassie Brilliant Blue (0.1% in methanol/acetic acid) and destained with 40% methanol/10% acetic acid.

Competition between LTA and *E. hirae* for binding to apoLp-III

Adherence of apoLp-III to *E. hirae* was determined as previously described for *Micrococcus lysodeikticus* (Halwani and Dunphy, 1999). Fifty µL of bacterial suspension (50 or 500 µg/µL in PBS) or 50 µL PBS was reacted with 50 µL of heat-inactivated serum (30 min at 30°C) and centrifuged (16 000 x g, 5 min, 20°C). Fifty µL of bacterial suspension (500 µg/µL) was centrifuged and used as control. Supernatant fractions were

subjected to SDS-PAGE (Laemmli, 1970) using 12% acrylamide. Polypeptides were visualized using Coomassie Brilliant Blue.

To determine if the binding of apoLp-III was mediated by LTA, competition between LTA and *E. hirae* for binding to the protein was examined by reacting 50 µL of heat-inactivated serum with 25 µL of PBS containing 0, 25 or 100 µg of LTA from *E. hirae* for 30 min at 30°C before incubating the serum mixture with 25 µL of bacterial suspension (50 µg/µL) for another 30 min at 30°C. Suspensions were centrifuged (16 000 x g, 5 min, 20°C) and proteins separated by SDS-PAGE as described above.

Because apoLp-III binds lipids hydrophobically (Narayanaswani and Ryan, 1997), its mode of binding to the bacterial cell surface was also suspected to be of a hydrophobic nature; consequently, in a separate experiment, the bacterial cells were replaced by Octyl Sepharose resin (Pharmacia). Heat-inactivated serum (50 µL) was reacted with 0 and 100 µg of LTA in 50 µL of PBS for 30 min at 30°C and 25 µL of 0, 0.125, 0.25 and 0.5 g/mL of resin in PBS added to the mixture, incubated (30 min, 30°C) centrifuged (16 000 x g, 5 min) and the supernatant electrophoresed as before.

Total and differential hemocyte counts

To examine the dose effect of LTA on total hemocyte counts, *G. mellonella* larvae were injected with 10 µL of PBS containing 0, 0.6, 1.25, 2.5 or 5 µg of *E. hirae* LTA and incubated at 30 °C. At 5 and 30 min post-injection, the insects were pricked at the base of a proleg with a needle and the hemocytes were counted on a hemocytometer using phase contrast microscopy. To monitor the cellular response to LTA over time, insects were injected with 10 µL of PBS containing 2.5 µg of one of 3

different species of LTA and incubated at 30°C. At 5, 10, 30 and 60 min post-injection, hemocytes were counted as described above.

To examine the effect of LTA on differential hemocyte counts, larvae were injected with PBS (10 µL) containing 2.5 µg of LTA from *B. subtilis*, *E. hirae* or *S. pyogenes* or with 5 µg of apoLp-III in PBS; controls received PBS alone. To test possible detoxification of LTA by apoLp-III, *B. subtilis* LTA (2.5 µg) was reacted (30°C, 30 min) with 5 or 25 µg of the protein in 10 µL of PBS, and the resulting solution administered to the insects. Ten min post-injection, 10 µL of hemolymph were collected from individual insects (n=10); samples were not pooled. Each sample was added to 50 µL of PBS on a slide between 2 cover slips; each diluted sample was dispersed by gentle addition of another 50 µL of buffer and a 22 x 40 mm cover slip gently lowered over the preparation. Blood cells were allowed to attach to the slide for 10 min and were then identified and counted. At least 100 hemocytes were counted per replicate, each replicate representing hemolymph sample from one insect (1 slide=1 replicate).

Phenoloxidase assays

Hemolymph was collected from an incision made at the base of a proleg and added to 100 µL of LTA solution (0, 0.6, 1.25, 2.5, 5.0, 10 µg/100 µL of sterile PBS). The resulting solution was mixed well and incubated at room temperature for 30 min. One mL DOPA solution (2mg/mL) was added to the hemolymph mixture. The production of dopachrome was allowed to proceed for 5 min and was detected spectrophotometrically at 490 nm.

To test the effect of LTA and apoLp-III on phenoloxidase *in vivo*, insects were injected with PBS (10 µL), PBS (10µL) containing 2.5 µg of LTA from *B. subtilis*, *E. hirae* or *S. pyogenes* , or PBS (10µL) containing 5 µg apoLp-III and 2.5 µg of *B. subtilis* or *E. hirae* LTA; the insects were incubated for 10 min at 30°C and the phenoloxidase activity assayed as described above. One unit of phenoloxidase activity was defined as a 0.001 change in absorbance at 490 nm per min.

Protein determination

Protein was determined using the BioRad protein assay (Bradford, 1976) with bovine serum albumin as the standard.

Statistical analyses

Data from DMB assays, data relating to the effect of lipoteichoic acid on phenoloxidase activity, and total hemocyte counts are presented as means ± SEM and were analyzed using 95% confidence limits. Relationships between DMB and LTA, LTA and phenoloxidase activity and total hemocyte counts were established using regression analysis. Differential hemocyte counts were analyzed using 95% confidence limits and are presented as means and 95% confidence intervals (Sokal and Rohlf, 1969).

RESULTS

Binding of apoLp-III to LTA

LTA from *B. subtilis*, *E. hirae* and *S. pyogenes* caused a colorimetric shift of DMB resulting in a dose-dependent increase in its absorbance at 535 nm (Fig. 4.1) (respectively, r = 0.9225, 0.8916, 0.9663; n=6). Overall, this increase did not differ significantly among the three LTA species.

Reacting different amounts of apoLp-III with a fixed amount of LTA from *E. hirae* or *S. pyogenes* caused a significant decrease in the absorbance of DMB at 535nm (Fig. 4.2). The absorbance decreased as the amount of apoLp-III in the reaction increased. The extent of decrease varied with the LTA species.

As revealed by the mobility shift assay (Fig. 4.3), increasing concentrations of all three species of LTA increased the mobility of the apoLp-III band under non-dissociating conditions. This was evidenced by a decrease in band intensity and a broader more diffuse band as the protein moved towards the anode.

Adherence of apoLp-III to *E. hirae* cells

Adherence of apoLp-III to Gram-positive bacteria was tested using *E. hirae*, a bacterium present in the gut of *G. mellonella*. The intensity of the apoLp-III band (arrow) decreased with increasing amounts of bacterial cells suggesting the protein bound to the surface of the bacteria (Fig. 4.4A). The decrease in the intensity of the apoLp-III band was greater when the heat-inactivated serum was first reacted with LTA (Fig. 4.4B). Band intensity also decreased when heat-inactivated serum was

Figure 4.1 Metachromatic activity of *B. subtilis*, *E. hirae* and *S. pyogenes* LTAs with DMB. Data points are means ± SEM (n=6).

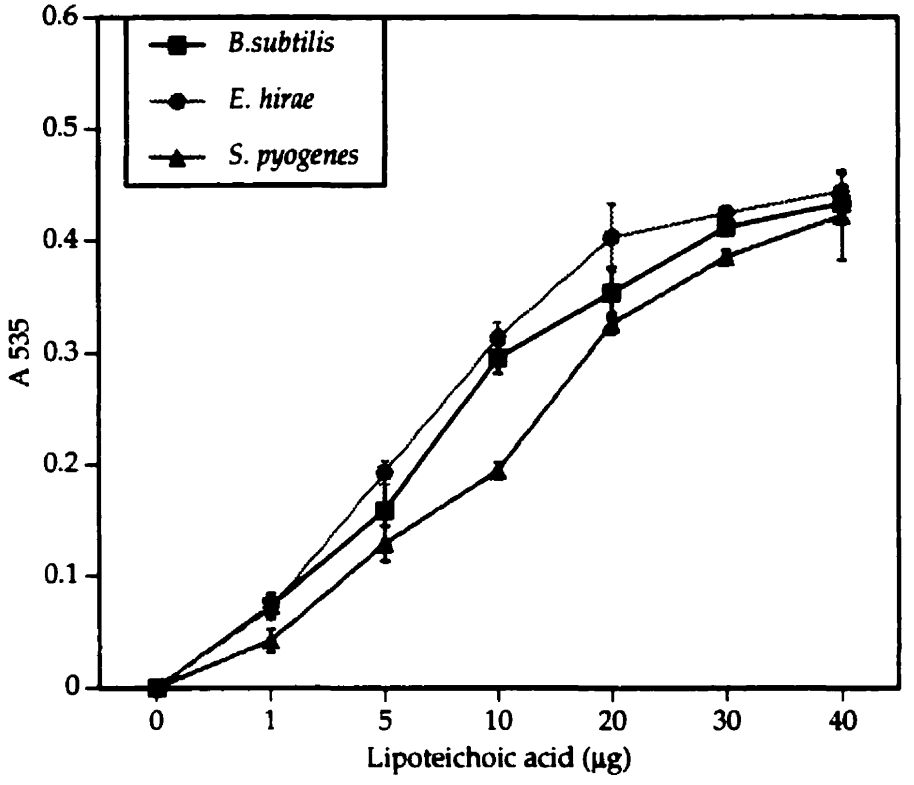

B.subtilis
E. hirae
S. pyogenes
A 535
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
5
10
20
30
40
Lipoteichoic acid (µg)

Figure 4.2 Dose effect of apoLp-III on the metachromatic activity of *E. hirae* and *S. pyogenes* LTA. Data are means ± SEM (n=4). Columns of same treatments labelled with same letters are not statistically different ($p<0.05$).

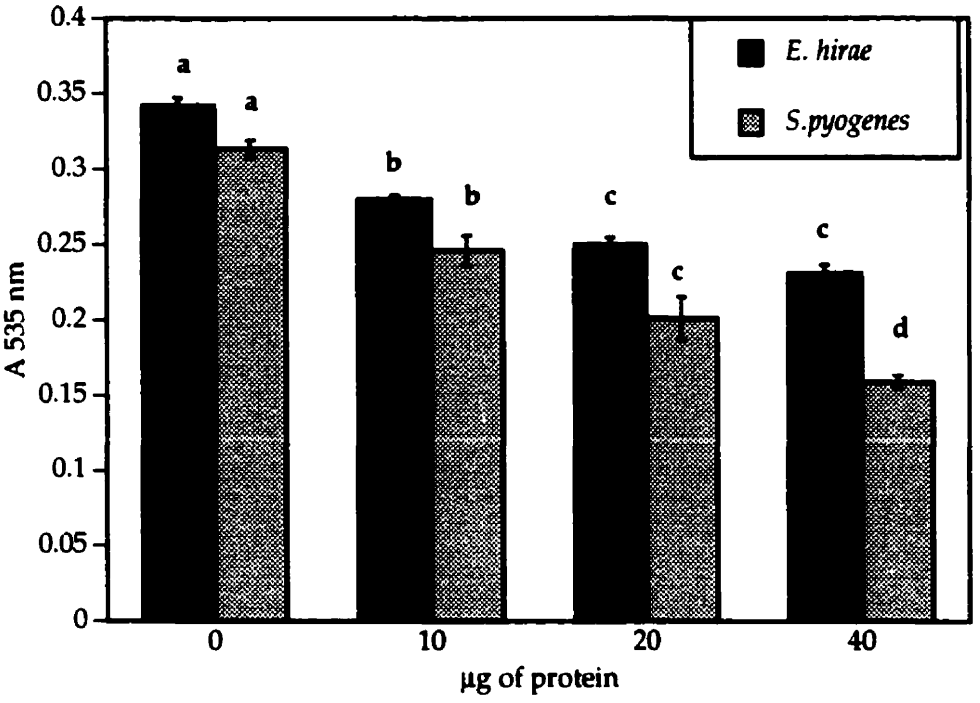

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
A 535 nm
a
a
b
b
c
c
c
d
E. hirae
S.pyogenes
0
10
20
40
µg of protein

Figure 4.3 Effect of LTA from (A) *B. subtilis*, (B) *E. hirae* and (C) *S. pyogenes* on the electrophoretic mobility of apoLp-III (arrow). Lanes 1-7, increasing amounts of LTA.

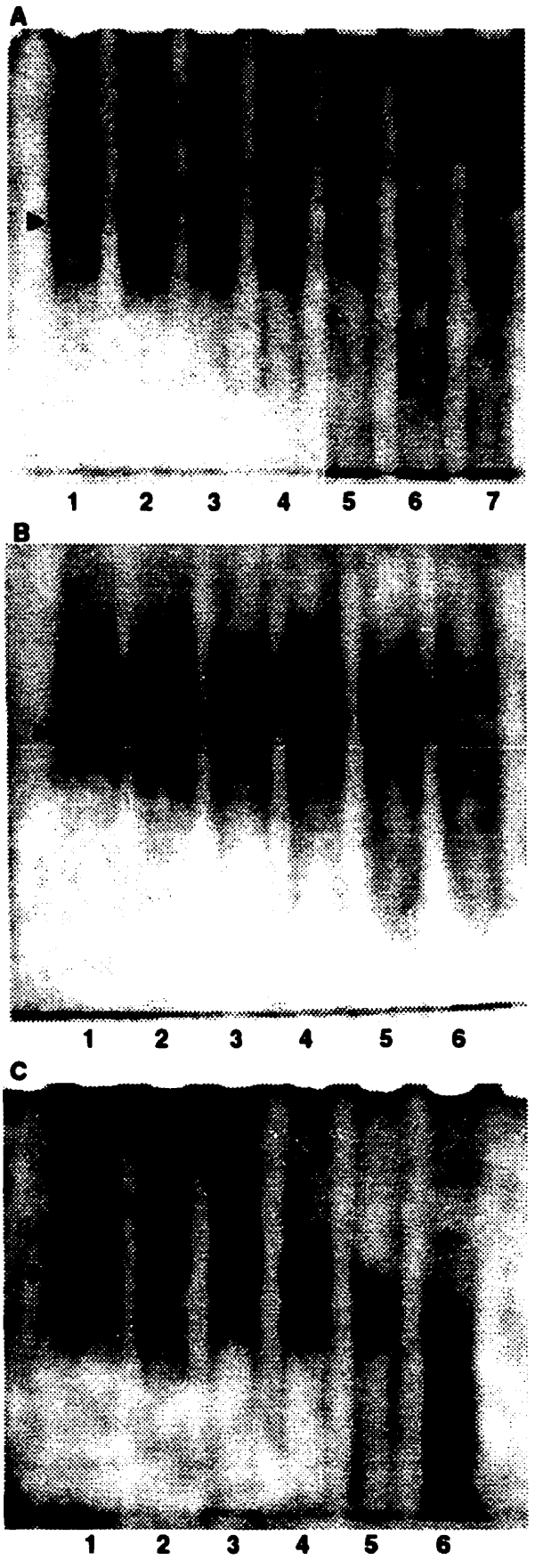

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6
C
1 2 3 4 5 6

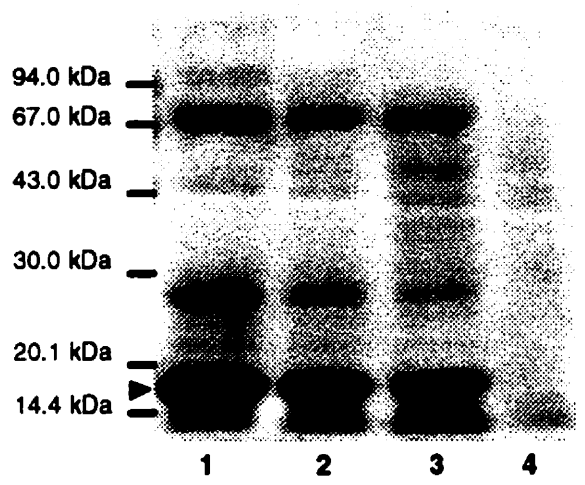
Figure 4.4 Effect of LTA on the binding of heat-inactivated serum proteins to *E. hirae* or Octyl Sepharose (arrow indicates apoLp-III).

A-Protein profile of heat-inactivated serum (50 µL) following incubation (30°C, 30 min) with 50 µL of 0 (lane 1), 50 (lane 2) and 500 (lane 3) µg /µL of *E. hirae* bacterial suspension. Lane 4: supernatant of a 500 µg/µL suspension of bacteria only.

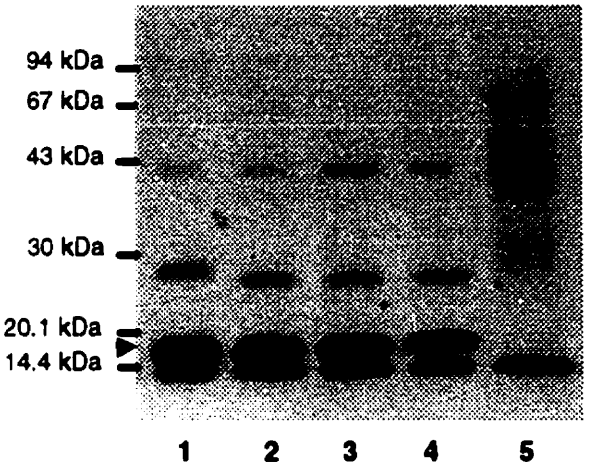
B-Protein profile of heat-inactivated serum reacted (30°C, 30 min) with *E. hirae* LTA before incubation with *E. hirae* cells. Lane 1: heat-inactivated serum (25 µL + 50 µL PBS); lane 2: heat-inactivated serum (25µL + 25 µL PBS) incubated with 25 µL of bacterial cell suspension (50 µg/µL); lanes 3 and 4: heat-inactivated serum (25 µL) reacted with 25 µL of PBS containing 25µg or 100 µg of LTA respectively, before incubation with 25 µL of bacterial cell suspension. Lane 5: supernatant of a 500 µg/µL suspension of bacteria only.

C-Protein profile of heat-inactivated serum proteins reacted with *E. hirae* LTA followed by Octyl Sepharose resin.
Lanes 1, 2, 4 and 6: heat-inactivated serum (25 µL+25 µL PBS)) incubated (30°C, 30 min) with 25µL of PBS containing 0, 0.125, 0.25 or 0.5 g/mL of resin. Lanes 3, 5 and 7: heat-inactivated serum (25 µL) reacted with 25 µL of PBS containing 100µg of LTA followed by 25 µL of 0.125, 0.25 and 0.5g /mL of Octyl Sepharose resin.

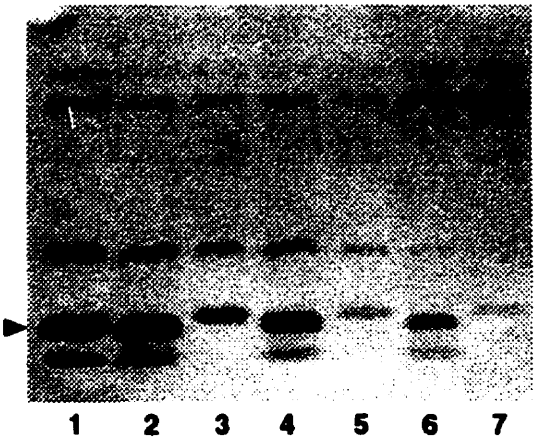
A


94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
1
2
3
4

B


94 kDa
67 kDa
43 kDa
30 kDa
20.1 kDa
14.4 kDa
1
2
3
4
5

C


1
2
3
4
5
6
7

incubated with increasing amounts of Octyl Sepharose resin and reacting heat-inactivated serum proteins with LTA caused greater binding of apoLp-III to the resin (Fig. 4.4C)

Effect of LTA on hemocyte counts

E. hirae caused a significant dose-dependent drop in the total hemocyte counts between 5 and 30 min post-injection (respectively, r= -0.8757 and -0.9395; n=6) (Fig. 4.5). Injections of all three species of LTA and PBS caused a significant drop in total hemocyte counts 5 min post-injection (Fig. 4.6). Pricking the insects with a needle also provoked a drop in the number of blood cells although less pronounced. While hemocyte counts of pricked insects had returned to normal levels within 30 to 60 min post-injection, and close to normal levels in PBS-injected insects, the level of hemocytes stayed significantly low in insects that received LTA. The effect observed 5, 10 and 30 min post-injection did not differ significantly among LTA species; however, hemocyte counts, 60 min post-injection of *B subtilis* LTA, were significantly lower than with the other two LTAs (Fig. 4.6).

Differential hemocyte counts per microscope field were carried out in an attempt to explain the results of the phenoloxidase assays. These counts revealed a decline in the number of plasmatocytes as a result of injections with *E. hirae* and *S. pyogenes* LTA, and complete depletion of plasmatocytes following treatment with *B. subtilis* LTA (Table 4.1). Of the LTA species tested, only *B subtilis* LTA caused shrivelling of granular cells (data not shown). Reacting *B. subtilis* LTA with apoLp-III (5 μg) prior to injections resulted in an increase in the number of plasmatocytes with respect to that observed with LTA alone;

Figure 4.5 Dose effect of *E. hirae* LTA injections on total hemocyte counts. Each point represents the mean $\pm$ SEM of the data obtained using 10 insects (5 min post-injection (PI), $r_{[0.05, 8]} = -0.8757$, 30 minutes PI, $r_{[0.05, 8]} = -0.9399$).

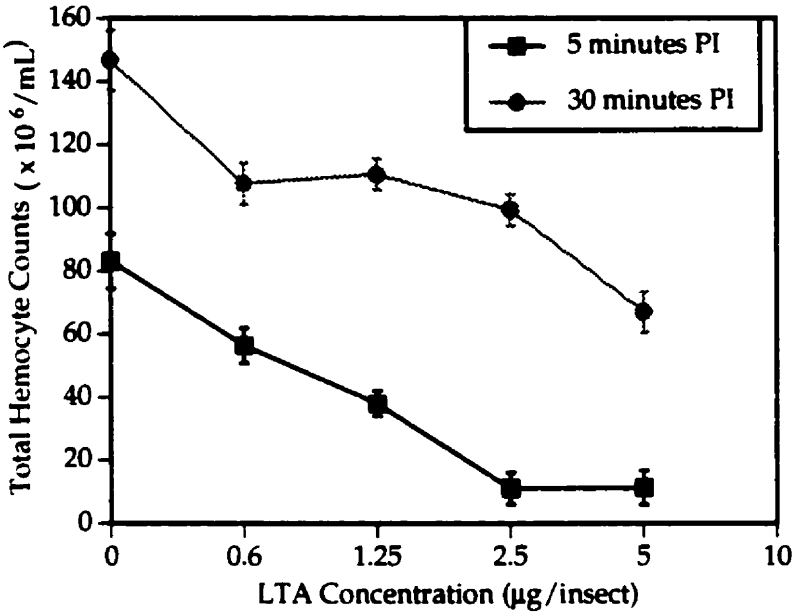

160
140
120
100
80
60
40
20
0
Total Hemocyte Counts (x 10 6/mL)
0
0.6
1.25
2.5
5
10
LTA Concentration (µg/insect)
5 minutes PI
30 minutes PI

Figure 4.6 Total hemocyte counts following injections of LTAs from *B. subtilis*, *E. hirae* or *S. pyogenes*. Each point represents the mean ± SEM of the data obtained using 6 insects.

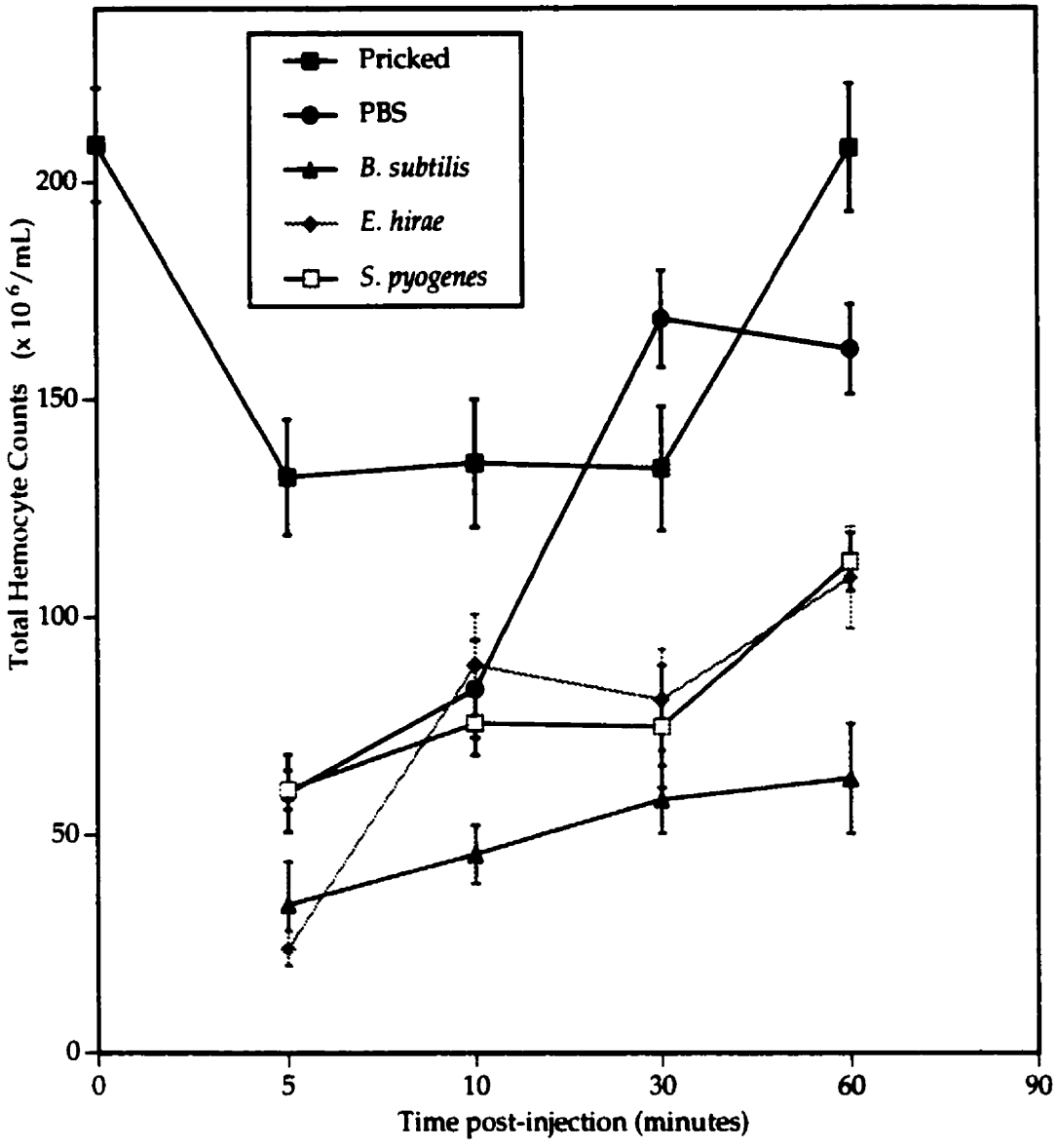

Pricked
PBS
B. subtilis
E. hirae
S. pyogenes
Total Hemocyte Counts (x 10 6/mL)
200
150
100
50
0
0
5
10
30
60
90
Time post-injection (minutes)

Table 4.1. Effects of LTA and apoLp-III on the hemogram of *G. mellonella* larvae at 10 min post-injection.

Treatments[2]	Absolute Hemocyte Counts per Microscope Field[1]		
	Total counts	Plasmatocytes	Granulocytes
PBS[3]	29.93 (26.02-33.84)a	2.43 (1.32-3.55)a	27.06 (23.34-30.78)a
LTA[4]			
B. subtilis	31.18 (27.13-35.22)a	0.00 (0.00-0.00)b	31.08 (27.09-35.07)a
E. hirae	26.70 (23.00-30.39)a	0.70 (0.10-1.30)c	25.60 (21.98-29.22)a
S. pyogenes	31.77 (27.74-35.80)a	0.68 (0.09-1.27)c	30.18 (26.25-34.11)a
ApoLp-III[5]	27.11 (23.38-30.83)a	4.81 (3.24-6,38)a	22.02 (18.66-25.38)a
B. subtilis LTA+ apoLp-III(5 µg)	27.20 (23.47-30.93)a	0.77 (0.14-1.40)ac	25.68 (22.06-29.30)a
B. subtilis LTA+ apoLp-III (25 µg)	25.49 (21.88-29.10)a	0.54 (0.01-1.07)c	24.44 (20.90-27.98)a

[1] Data are expressed as means (with 95% confidence limits), n=10; means with the same letter are not statistically different (P >0.05)
[2] 10 µL injections using PBS as solvent.
[3] PBS alone.
[4] LTA from the indicated organisms.
[5] ApoLp-III alone.

however, the number of plasmatocytes was still lower than that observed following the PBS injections and increasing the amount of apoLp-III (25 µg) had no further effect. ApoLp-III alone had no effect on the hemogram and granulocyte counts were not affected by any of the treatments (Table 5.1). Injection of *B. subtilis* LTA also caused shrivelling of granulocytes (data not shown).

Effect of LTA on phenoloxidase activity

LTA caused an increase in phenoloxidase activity *in vitro* (Fig. 4.7) . The increase was dose dependent for all three species of LTA although statistically significant only in the case of LTA of *E. hirae* and *S. pyogenes* (respectively, r= 0.9151 and 0.9973; n=6). Phenoloxidase activity measured 10 min following injection of *E. hirae* or *S. pyogenes* LTA, apoLp-III or *E. hirae* LTA reacted with apoLp-III, was significantly lower than in PBS- and *B. subtilis* LTA-treated insects. LTA of *B. subtilis* caused a three-fold increase in phenoloxidase activity with respect to PBS. This activation was weaker in the presence of apoLp-III although not statistically significant (Fig. 4.8).

Figure 4.7 Effect of LTAs from *B. subtilis*, *E. hirae* and *S. pyogenes* on phenoloxidase activity *in vitro*. Values are means ± SEM (n=6).

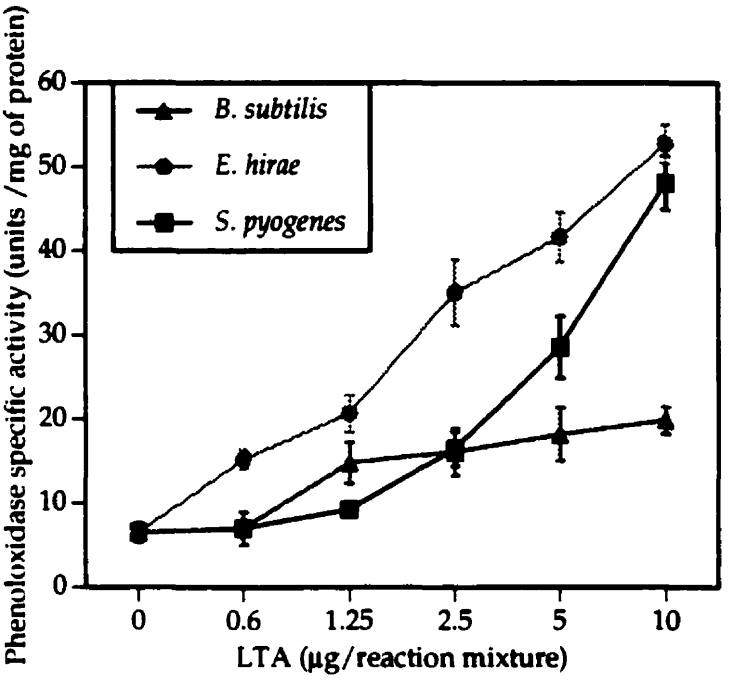
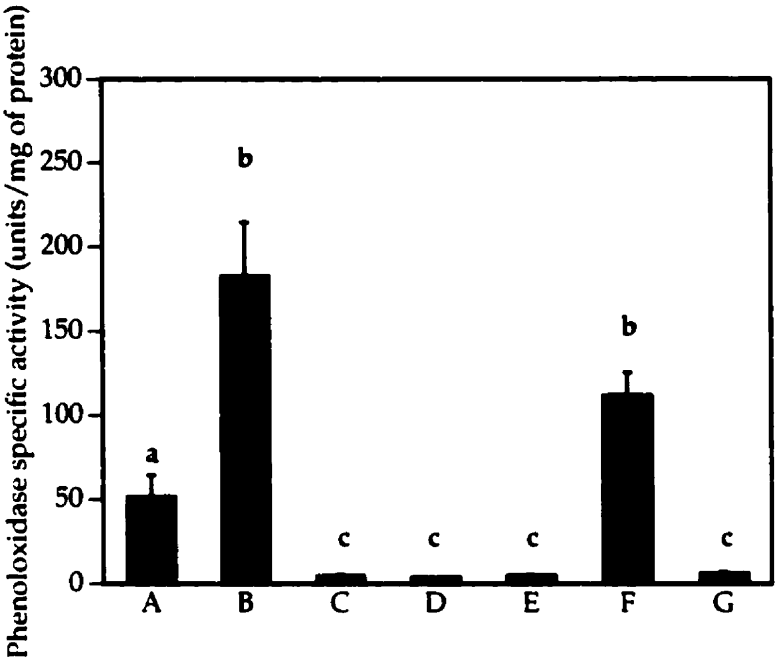

Phenoloxidase specific activity (units /mg of protein)
60
50
40
30
20
10
0
B. subtilis
E. hirae
S. pyogenes
0
0.6
1.25
2.5
5
10
LTA (µg/reaction mixture)

Figure 4.8 Effects of LTAs and apoLp-III on phenoloxidase activity *in vivo* . Phenoloxidase activity was assayed as described in Materials and Methods, 10 min post-injection of larvae with 10 µL of the following solutions: A, PBS; B, *B. subtilis* LTA; C, *S. pyogenes* LTA; D, *E. hirae* LTA; E, *E. hirae* LTA + apoLp-III; F, *B. subtilis* LTA + apoLp-III; G, apoLp-III. Values are means ± SEM (n=10).


Phenoloxidase specific activity (units/mg of protein)
300
250
200
150
100
50
0
a
b
c
c
c
b
c
A
B
C
D
E
F
G

DISCUSSION

This study addresses the role of LTA in triggering an immediate immune response in *G. mellonella* during a Gram-positive bacterial infection and the participation of apoLp-III in this response. It also is the first report on the use of the dye DMB to measure LTA.

DMB has been used to detect and measure LPS (Keler and Nowotny, 1986) and to investigate the binding of protein to LPS (DeLucca *et al.*, 1995; Dunphy and Halwani, 1997). It is believed that DMB reacts with the phosphorylated glucosamine of the lipid A moiety of LPS which causes a shift in the absorption maximum to shorter wavelength. The metachromatic shift caused by LTA did not differ among the three species of LTA tested suggesting that the dye reacts with the glycerol phosphate backbone common to all LTAs (Knox and Wicken, 1977).

The decrease in absorbance of DMB at 535 nm with increasing amounts of apoLp-III reacted with LTA suggests that the protein is complexing with LTA and thus preventing metachromasia. The increased electrophoretic mobility of apoLp-III as a result of the binding of polyanionic LTA confirmed that the two molecules interact. This interaction is possibly hydrophobic in nature since apoLp-III interacts hydrophobically with lipids (Narayanasami and Ryan, 1997).

E. hirae was chosen as the test bacterium since it is the main organism colonizing the gut of *G. mellonella* larvae (Bucher and Williams, 1967). Dunn *et al.* (1994) proposed that during the replacement of larval to pupal midgut epithelium, bacteria might escape from the lumen to the body cavity (Dunn *et al.*, 1994); hence, *E. hirae* would be the

Gram-positive bacterial species most likely controlled by the defense mechanisms of the insect.

ApoLp-III in heat-inactivated serum bound to the surface of the bacteria as evidenced by the decrease in band intensity on SDS-polyacrylamide gels. Whether LTA mediates this binding cannot be concluded from this study since LTA, rather than preventing the binding of apoLp-III to *E. hirae*, appeared to promote it. LTA also increased the binding of apoLp-III to Octyl Sepharose, a hydrophobic resin. It is possible that pre-incubation of LTA with apoLp-III causes the protein molecule to open up exposing more hydrophobic sites and hence amplifying its hydrophobic binding ability to the bacterial surface. This hypothesis is in keeping with proposed lipid binding mechanisms of apoLp-III (Kawooya *et al.*, 1986; Wang *et al.*, 1998). Increased binding of apoLp-III to the bacterial surface in the presence of LTA would be beneficial to the insect since apoLp-III renders bacterial cells more sensitive to lysozyme activity (Halwani and Dunphy, 1999). It is not known if this association increases the hemocytic responses against bacteria; Wiesner *et al.* (1997) reported that incubation of *Saccharomyces cerevisiae* with apoLp-III enhanced the phagocytic activity of hemocytes against this microorganism.

Whether Gram-positive bacteria shed LTAs into their insect host hemolymph is not known. However, LTA has been detected in bacterial culture media (Wicken and Knox, 1977) and in infected human skin or soft tissue (Baroni *et al.*, 1998). In addition, the hydrolysis of the peptidoglycan wall by lysozyme, constitutively present in the hemolymph (Powning and Davidson, 1973), could release LTA into the insect hemocoel.

To investigate the possible detoxification of LTA by apoLp-III, the effects of LTA on hemocyte counts and phenoloxidase activity were determined. Hemocytopaenia has been observed previously in *G. mellonella* as a result of wounding or injection of LPS or bacteria (Chain and Anderson, 1982; Ratcliff and Walters, 1983; Trencsek and Preik-Stenhof, 1992). Herein, injections of LTA into *G. mellonella* larvae were followed by rapid and drastic dose-dependent decreases in counts of circulating hemocytes. The low level of hemocytes recovered one hour post-injection of PBS; this low level persisted, however, in LTA-treated insects possibly as a result of nodulation (Gagen and Ratcliffe, 1976) (hemocyte clumping was detected in LTA-injected insect larvae; data not shown). Irreversible hemocyte damage evidenced by granulocyte crenation might also explain these results.

All three species of LTA caused a significant drop in the number of plasmatocytes with *B. subtilis* LTA causing a complete depletion of plasmatocytes. These results agree with previous work where injection with *B. cereus* was found to deplete the numbers of plasmatocytes in *G. mellonella* larvae (Chain and Anderson, 1982). ApoLp-III prevented partially the loss of plasmatocytes caused by LTA from *B. subtilis* and improved the aspect of the granulocytes. However, use of a higher amount of apoLp-III (25 μg) did not prevent totally the drop in plasmatocyte numbers; it is possible that at this concentration, the apoLp-III molecules aggregate and are therefore less reactive with LTA.

In vitro phenoloxidase activity increased significantly with increasing concentration of LTA of *E. hirae* or *S. pyogenes*. LTA could be stimulating hemocytes to release the enzyme or activating the enzyme already present in the plasma. The LTA from *B. subtilis*, at the two

highest concentrations, was not as strong an activator of the enzyme. The effect of LTA on phenoloxidase *in vivo* did not support the results obtained *in vitro* in that *B. subtilis* LTA activated dramatically phenoloxidase *in vivo* while the other two LTA species inhibited it. LTAs have been reported to share some of the toxic properties of LPS (Fischer, 1990); LPS of *X. nematophilus* inhibited phenoloxidase in *G. mellonella* (Dunphy and Webster, 1991). Counts of phenoloxidase-positive granulocytes did not correlate with the low activity of the enzyme since their numbers relative to the total hemocyte counts did not change.

The inhibition of phenoloxidase by *E. hirae* and *S. pyogenes* LTA *in vivo* but not *in vitro* indicates that these LTAs interfere with the activation of the phenoloxidase cascade at its earliest steps, possibly discharge from hemocytes of components of the phenoloxidase system. *In vitro*, these steps are already triggered by the bleeding trauma. Based on the damaged appearance of the granulocytes caused by *B. subtilis* LTA, the observed high activity of phenoloxidase following injection of this LTA could be the result of increased discharge of the prophenoloxidase system from the cells. Differences in the effects of *B. subtilis* LTA compared to the other two LTAs studied could be accounted for by the reported differences in the length of the polyglycerophosphate chain and the nature and degree of substitution (Fischer, 1990).

ApoLp-III injected into the insect inhibited phenoloxidase. Reacting apoLp-III with *B. subtilis* LTA caused a drop in phenoloxidase; although not significant statistically, this result suggests that the apoLp-III plays a role either in neutralizing LTA or regulating the phenoloxidase cascade.

In summary, LTAs affect the immediate cellular and humoral responses of non-immune *G. mellonella* larvae. ApoLp-III binds to LTA and can protect the insect against the toxic effects of this molecule. ApoLp-III binds to the surface of Gram-positive bacteria.

Further research could be directed at the identification of the part of the LTA molecule responsible for its biological activity in the insect. It would also be of interest to investigate if LTA influences hemocytes directly by binding to their surface and if apoLp-III mediates this binding.

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CONNECTING STATEMENT 3

Chapters 2, 3 and 4 report the participation of apoLp-III in the immediate immune responses of *G. mellonella* to Gram-negative and Gram-positive bacteria. In view of the function of apoLp-III in the antimicrobial defense system of the insect, its concentration in hemolymph and its responses to antigen stimulation were investigated.

CHAPTER 5

FURTHER OBSERVATIONS ON APOLIPOPHORIN-III IN *Galleria mellonella* LARVAE.

A version of this chapter will be submitted to the
Journal of Insect Physiology as follows,
Halwani, A.E., Niven, D.F., and Dunphy, G.B. Further observations on apolipophorin-III in *Galleria mellonella* larvae.

ABSTRACT

Apolipophorin-III in hemolymph of *G. mellonella* participates in the antibacterial responses of this insect. Immunoblots using antibodies raised to apolipophorin-III and sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that the level of apolipophorin-III reached 8-12 mg/mL of hemolymph at the end of the feeding phase of the seventh larval instar. Apolipophorin-III was also present in significant amounts in prepupal, pupal and adult stages. Apolipophorin-III was detected immunologically in fat body tissue, hemocyte lysates and plasma. In its native state, apolipophorin-III may be associated with another protein with an apparent molecular mass of 77 kDa, possibly apolipophorin-II. Injections of octopamine, lipopolysaccharide or lipoteichoic acid into larvae of *G. mellonella* did not change the concentration of apolipophorin-III in hemolymph. Also, octopamine did not cause lipid loading of high density lipophorin.

Keywords: *Galleria mellonella*, apolipophorin-III, fat body, hemocytes, octopamine, lipopolysaccharides, lipoteichoic acids.

INTRODUCTION

Apolipophorin-III (apoLp-III) is one of three hemolymph apoproteins that constitute the lipid transport vehicle of insects (for recent reviews, see Van der Horst *et al.*, 1993; Blacklock and Ryan, 1994; Narayanaswami and Ryan, 1997). Based, mainly, on studies of the adult stages of the tobacco hawkmoth, *Manduca sexta*, and the migratory locust, *Locusta migratoria*, it has been proposed that, following the flight-induced signal of adipokinetic hormone and under the action of a hormone-sensitive lipase, diacylglycerol molecules leave the fat body and associate with high density lipophorin particles (HDLp) present in the hemolymph (Van der Horst *et al.*, 1979; Ziegler and Schultz, 1986). HDLp is composed of apolipophorin-I (≈250 kDa) and apolipophorin-II (≈80 kDa) as well as phospholipids, sterols, hydrocarbons and diacylglycerol (Soulages and Wells, 1994). The loading of HDLp with additional diacylglycerol is accompanied by the reversible binding of apoLp-III molecules to the increasingly hydrophobic surface of the HDLp particle to form low density lipophorin (LDLp). Kawooya *et al.* (1986) proposed that the role of apoLp-III is to stabilize LDLp. When the lipid component is discharged at its site of utilization, apoLp-III dissociates from LDLp regenerating HDLp.

ApoLp-III has been purified from the hemolymph of a number of Lepidopteran species and characterized. It is water-soluble, heat-stable and has a molecular mass of 17-20 kDa depending on the insect species (reviewed by Soulages and Wells, 1994). In addition to hemolymph, apoLp-III has been detected in ovaries and testis (Kawooya *et al.*, 1988; Telfer *et al.*, 1991; Yun and Kim, 1996) where its function has not yet

been defined. ApoLp-III is synthesized in the fat body (Prasad *et al.*, 1986), intersegmental muscles and neurons (Sun *et al.*, 1995) and gonads (Kim *et al.*, 1998).

ApoLp-III has been isolated from the hemolymph (Weisner *et al.*, 1997) and molting fluid (Limura *et al.*, 1998) of last instar *Galleria mellonella* larvae, characterized, and its complete amino acid sequence determined (Weise *et al.*, 1998). The protein has a molecular mass of 18.1 kDa and an isoelectric point of 6.5. The amino acid sequence has 64-90 % homology with apoLp-III molecules of other Lepidopteran species and implies an almost exclusively α-helical structure.

Studies of the function of apoLp-III in *G. mellonella* have been restricted to its immunological role. In its purified form, the protein was found to enhance the phagocytic response of plasmatocytes to yeast cells *in vitro*, to induce antibacterial activity when injected into larvae (Wiesner *et al.*, 1997) and to cause agglutination of erythrocytes (Limura *et al.*, 1998). In addition, apoLp-III was found to bind to the surface, and detoxify the lipopolysaccharide (LPS), of the Gram-negative insect pathogenic bacterium *Xenorhabdus nematophilus* (Dunphy and Halwani, 1997), and to bind to the Gram-positive bacterium, *Enterococcus hirae*, and reduce the toxicity of the lipoteichoic acids (LTAs) (Halwani *et al.*, 1999). ApoLp-III was also shown to increase the susceptibility of *Micrococcus lysodeikticus* to lysozyme through hydrophobic association with the bacterial surface (Halwani and Dunphy, 1999).

The present work was prompted by the newly discovered involvement of apoLp-III in the immune responses of *G. mellonella* larvae, and the lack of information pertaining to some basic aspects of

this protein. The concentration and native state of apoLp-III in the hemolymph and its presence at different developmental stages and in different tissues are reported for the first time. The effects of LPS, LTA and octopamine injections on the concentration of this protein in the hemolymph were also investigated.

MATERIALS AND METHODS

Insects and materials

Larvae of *G. mellonella*, the greater wax moth, were reared on an artificial diet at 30°C in constant light (Dutky *et al.*, 1962). Unless stated otherwise, last instar larvae (200-250 mg) were used.

Alkaline phosphatase-conjugated anti-rabbit monoclonal antibodies (γ-chain specific), and D,L-octopamine were from Sigma. Tris and glycine were from Fisher Scientific. Low molecular weight protein standards (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin) were from Pharmacia. Chemicals for polyacrylamide gel electrophoresis and immunodetection were from Bio-Rad. Other chemicals were certified ACS grade or better.

Purification of apoLp-III and preparation of antibodies

ApoLp-III was purified as follows. Hemolymph, obtained by amputation of a prothoracic leg, was diluted 1:1 (v/v) with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6.5 with HCl) and hemocytes removed by centrifugation (12 000 x g, 4 min, 20°C). The supernatant was heated (100°C, 30 min), centrifuged (12 000 x g, 20°C, 4 min) and the heat-inactivated serum (supernatant) was collected and frozen at -20°C. Heat-inactivated serum proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with copper chloride (Lee *et al.*, 1987). The apoLp-III band (17.2 kDa) was excised, destained in 0.25 M ethylenediamine tetraacetic acid (EDTA) (tetrasodium salt) in 0.25 M Tris (pH 9.0 with HCl), dialyzed overnight

against 2 L of 25 mM Tris, 192 mM glycine (pH 8.3) and electroeluted in the same buffer for 2 hours at 100 V (Maniatis *et al.*, 1982). The electroeluted protein was then dialyzed against PBS and lyophilized. Protein was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

Antibodies against apoLp-III were raised in male New Zealand white rabbits (1.5 kg). Following 3 injections of 0.25 mg of purified apoLp-III in 1 mL of Ribi adjuvant (RIBI ImmunoChem Research Inc.), at 4 week intervals, blood was collected from the ear vein and the sera purified using an Econo-Pac serum IgG purification kit (BioRad).

Polyacrylamide gel electrophoresis and immunological procedures

SDS-PAGE was performed according to Laemmli (1970) with separating gels containing 12 % or 15% acrylamide. A mini Protean II slab cell electrophoresis system was used (Bio-Rad); samples were run at a constant voltage of 200 V for 45 min. Separated polypeptides were stained with Coomassie Brilliant Blue. The apparent molecular masses were calculated by comparing the relative mobilities of the polypeptides to those of standards. For protein purification, a 16 cm x 18 cm vertical slab gel (Hoefer Scientific Instruments) was used. Non-denaturing gradient gels (4-20 % polyacrylamide) were prepared with a gradient maker and a mini Protean II multicasting chamber (BioRad). Electrode buffer was 25 mM Tris, 192 mM glycine (pH 8.3). Sample buffer consisted of electrode buffer containing 5% (w/v) bromophenol blue and 10% (v/v) glycerol.

For immunoblot analysis, samples were either spotted onto nitrocellulose membranes (Immobilon-NC, 0.2 μm pore size, Millipore)

in a Bio-Dot microfiltration apparatus (BioRad) or, following separation by polyacrylamide gel electrophoresis, transferred to the membrane electrophoretically (100 V, 120 min) using a mini-trans blot apparatus (BioRad). Non-specific binding sites were blocked overnight by incubation (20°C with gentle shaking) in a 5% (w/v) solution of skimmed milk. After 2 x 25 min washes in Tris-buffered saline (TBS) (20 mM Tris, 430 mM NaCl, pH 7.5) containing 0.05 % (v/v) Tween-20 (TTBS), the membranes were incubated (as above) for 5 h with anti-apoLp-III antibody diluted 1:1000 in TTBS. Following 2 x 15 min washes in TTBS, membranes were transferred to a 1:10 000 (v/v) solution of alkaline phophatase-conjugated monoclonal anti-rabbit antibody in TTBS, incubated for 2 h at 20°C and then washed (4 x 15 min) with TTBS. Immunoreactivity was detected by incubating the membrane in a reagent composed of a 1:1 (v/v) solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, both in aqueous dimethylformamide (AP Conjugate Substrate Kit, Biorad), diluted 50 x in 0.1 M Tris pH 9.5. The reaction was stopped by rinsing with distilled water.

Determination of apoLp-III concentration

The concentration of apoLp-III in the hemolymph was estimated using SDS-PAGE and a dot immunobinding assay. Purified apoLp-III (1, 3, 6, 9 and 12 µg) and 10 µL of sample buffer containing 1 µL of heat-inactivated serum, or 0.125 or 0.25 µL of hemolymph pooled from 10 insects, were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. For the dot immunobinding assay, purified apoLp-III (0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µg) and 10 µL of TBS containing 0.05

μL of hemolymph pooled from 3-4 insects were spotted on a nitrocellulose membrane and probed with anti-apoLp-III antibody as described above.

Change in apoLp-III content during development from last instar to adult

Classification of larval developmental stages were based on head capsule width, weight and time (Beck, 1970). Larvae at the end of the 6th instar (head capsule width < 1.8 mm; weight= 50-80 mg) were selected and placed in rearing jars with food. Larvae with head capsule width >1.8 mm and weighing over 80 mg were considered to be in the 7th or last instar. Larvae were weighed, head capsule width measured and hemolymph collected daily from groups of at least 8 insects. These insects were not used again. Larvae within a completed cocoon were classified as prepupal. Hemolymph from pupae and adults was collected with a capillary tube drawn to a fine tip. A few crystals of phenylthiourea were added to the samples to prevent melanization. Proteins in hemolymph samples from each stage were separated by SDS-PAGE; each sample consisted of 0.3 μL of hemolymph in 10 μL of sample buffer.

Detection of apoLp-III in hemocyte lysates, plasma and fat body tissue

The presence of apoLp-III in hemocyte lysates was investigated using one of three different anticoagulants. Anticoagulant 1 contained 93 mM NaCl, 0.1 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6, and had an osmolality of 440 mOsm (Leonard *et al.*, 1985). Anticoagulant 2 contained 146 mM NaCl, 98 mM NaOH, 41 mM citric acid, 17 mM EDTA, pH 4.5, and had an osmolality of

370 mOsm (Mead *et al.*, 1986). Anticoagulant 3 contained 186 mM NaCl, 13 mM KCl, 17 mM EDTA, 10 mM HEPES, 1 mM $NaHCO_3$, pH 6.8 (Mandato *et al.*, 1996). Insects were kept on ice for 10 min, injected with 60 µL of one of the previously chilled anticoagulants, a proleg severed and the hemolymph from 5-6 insects allowed to drip into 1 mL of cold anticoagulant in a microcentrifuge tube. Hemocytes were dispersed in the anticoagulant by gently inverting the tube, centrifuged (1000 x g, 10 min, 4°C), and the supernatant constituting the plasma preserved. The hemocyte pellet was washed 3 times in 1 mL of anticoagulant (1000 x g, 10 min, 4°C) and the hemocytes lysed in 10 % PBS by sonication (30 x 1 sec pulses, 50 W; Vibra Cell, Sonics and Materials Inc, Danbury, CT, USA).

To collect fat body tissues, larvae were dissected ventrally and flooded with PBS. Fat body fragments were collected, washed 4 times in 3 mL of PBS and homogenized in 1 mL of PBS using a hand-driven tissue homogenizer.

Samples of purified apoLp-III, hemocyte lysates, plasma and fat body homogenates were subjected to SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with anti-apoLp-III antibody (as described above).

Analysis of native apoLp-III

To identify the apoLp-III band on the native 4-20% gradient gel, hemolymph was collected from 8-10 7th instar larvae, pooled in the presence of a few crystals of phenylthiourea and used immediately. Purified apoLp-III (2 µg in 10 µL of sample buffer) and hemolymph (0.25 µL in 10 µL of sample buffer) were loaded in duplicate on a non-

denaturing 4-20% acrylamide gel. Following electrophoresis, as described above, the gel was sectioned; one half was stained with Coomassie Brilliant Blue and polypeptides in the other were electroblotted onto nitrocellulose membrane and probed with anti-apoLp-III antibody. To investigate possible association of apoLp-III with another protein, the band containing apoLp-III was excised from a non-denaturing 4-20% acrylamide gel and subjected to SDS-PAGE (12% acrylamide). Following electrophoresis, polypeptides were visualized using Coomassie Brilliant Blue.

Density gradient ultracentrifugation and the effect of octopamine on lipophorin densities

To investigate the native state of apoLp-III (free or bound to lipophorins) in the hemolymph of larvae and the effect of octopamine on lipid loading of lipophorins, hemolymph proteins were separated according to their density using a modification of the procedure described by Shapiro *et al.* (1984). One mL of hemolymph was collected as described above, centrifuged (5000 x g, 5 min, 4°C) to sediment hemocytes and the supernatant diluted with 4 mL of PBS. KBr was added to the hemolymph solution to a concentration of 44.3 g/100 mL (density : 1.3 g/mL); 2.5 mL of this solution were dispensed into 5 mL Quick-Seal centifuge tubes (Beckman) and overlayed with 0.9% NaCl. Ultracentrifugation (330 000 x g_{av}) in a Vti 65 rotor was for 2 hours at 10°C using the slow acceleration and deceleration feature. To determine the densities of the fractions, tubes containing only KBr and NaCl solutions were centrifuged simultaneously. Fractions (200 µL) were collected from the top of the tube using a syringe and needle and

desalted on a MicroSpin G-25 column (Amersham Pharmacia Biotech). Refractive indices of control fractions were measured using a Mark II digital refractometer (AO Scientific Instruments) and the refractive index converted to density using standard tables (CRC Handbook of Chemistry and Physics). Proteins in the fractions were separated by SDS-PAGE (12% acrylamide) and stained with Coomassie Brilliant Blue. To determine the effect of octopamine on lipid loading of lipophorins, insects were injected with 10 µL of PBS containing 0.0133 µg of octopamine; this amount increased the concentration of octopamine in the insect hemolymph to 1 µM based on 70 µL of hemolymph per insect (Dunphy and Downer (1994) reported the octopamine level to reach 1.25 µM 20 min post-injection of *Bacillus cereus*). Larvae were incubated one hour at 30°C before collection of the hemolymph. Control hemolymph was collected one hour post-injection of PBS.

Effects of LPS, LTA, and octopamine on the concentration of apoLp-III

The effects of LPS, LTA and octopamine on the concentration of apoLp-III in hemolymph were investigated since LPS and LTA were previously found to trigger an immune response and to interact with apoLp-III in *G. mellonella* (Dunphy and Halwani, 1997; Halwani *et al.*, 1999), and octopamine levels are known to increase as a result of infection or stress (Dunphy and Downer, 1994; Orchard *et al.*, 1981). Insects were injected with 10 µL of PBS containing *X. nematophilus* LPS (100 µg/mL), *Bacillus subtilis* LTA (100 µg/mL) or octopamine (1.33 µg/mL). Following 5, 30, 60 and 120 minutes of incubation at 30°C, hemolymph was collected and the samples collected at each time interval were pooled. Ten µL samples of hemolymph solution (2%, in

TBS) were applied to nitrocellulose membrane and probed with anti-apoLp-III antibody.

RESULTS

Concentration of apoLp-III in hemolymph

The concentration of apoLp-III in hemolymph and in heat -inactivated serum were estimated to be 8-12 μg/μL and 3 μg/μL, respectively (Fig. 5.1A). The immunoblot (Fig. 5.1B) indicated the concentration of apoLp-III in the hemolymph to be 8 ± 0.3 μg/μL.

Change in apoLp-III concentration during development

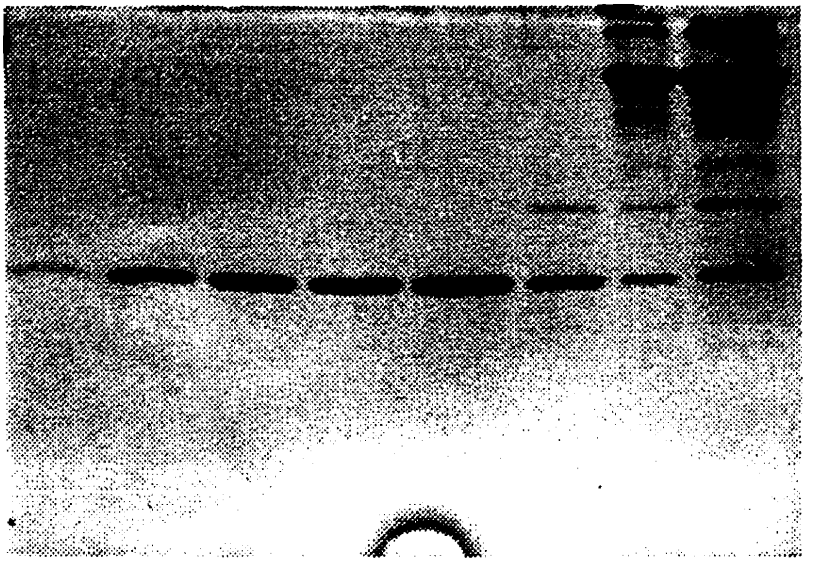
Electrophoretic separation of the proteins in hemolymph from insects at different developmental stages indicated that apoLp-III was present in insects of all stages (Fig. 5.2). The concentration of apoLp-III was lowest at the end of the 6th and at the beginning of the 7th instar and increased through the 7th instar. The apoLp-III concentration was at its peak in larvae that weighed 181-250 mg and decreased before pupation. The amount of apoLp-III present in the pupae and the adults was lower than in larvae at the peak of the 7th instar. The concentrations of the other hemolymph proteins appeared to follow the same pattern as apoLp-III.

Presence of apoLp-III in tissues

As revealed by the immunoblot (Fig. 5.3), apoLp-III was detected in the hemocyte lysate regardless of the anticoagulant used, as well as in the plasma and the fat body.

Figure 5.1 Concentration of apoLp-III in hemolymph. The concentration of apoLp-III in hemolymph was estimated visually using SDS-PAGE and a dot immunobinding assay. **A:** SDS-PAGE; lanes 1-5, 1, 3, 6, 9, and 12 μg of apoLp-III, respectively; lane 6, heat-inactivated serum, 1μL; lanes 7 and 8: 0.125 and 0.25 μL of hemolymph, repectively. **B:** immunoblot; row 1: increasing amounts of apoLp-III (0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 μg/dot); row 2: hemolymph samples (0.05 μL/dot).

A



1 2 3 4 5 6 7 8

B

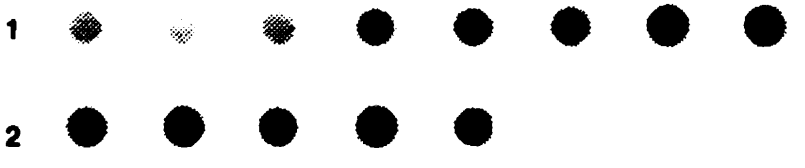


Figure 5.2 Hemolymph proteins from different developmental stages of *G. mellonella* separated on SDS-PAGE. (Closed arrow: apoLp-III). Lanes 1-10: 0.3 µL of hemolymph from larvae weighing 50-80, 81-90, 91-100, 101-120, 121-140, 141-160, 161-180, 181-200, 201-250 and >250 mg, respectively; lane 11: 0.3 µL of hemolymph from prepupae; lane 12: 0.3 µL of hemolymph from pupae; lane 13: 0.3 µL of hemolymph from adults.

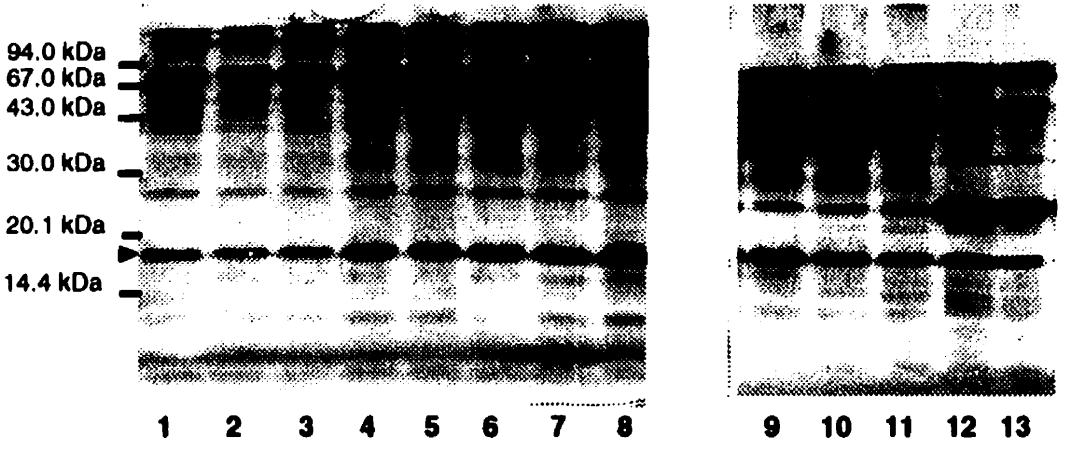
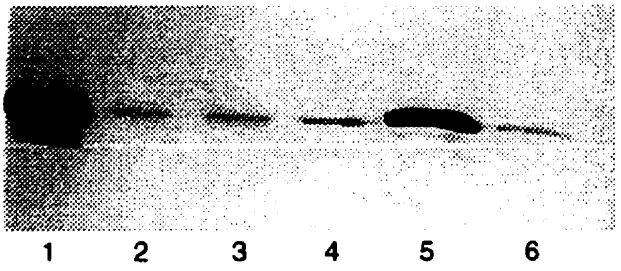

94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
1 2 3 4 5 6 7 8
9 10 11 12 13

Figure 5.3 Immunoblot of hemocyte lysates, plasma and fat body tisssues of *G. mellonella* larvae. Lane 1: purified apoLp-III; lanes 2, 3, and 4: hemocyte lysates using anticoagulants 1, 2, and 3, respectively; lane 5: plasma; lane 6: fat body tissues.


1
2
3
4
5
6

Native state of apoLp-III

Based on the native gradient gel and the immunoblot, purified apoLp-III had a faster electrophoretic mobility than apoLp-III in fresh hemolymph implying that apoLp-III in hemolymph might be associated with other proteins (Figs. 5.4A and 5.4B). The hemolymph apoLp-III band was excised from a native 4-20% polyacrylamide gel and subsequent electrophoresis (SDS-PAGE; 12% acrylamide) revealed 2 bands with apparent molecular masses of 17 and 77 kDa.

Density gradient ultracentrifugation

SDS-PAGE of the hemolymph fractions collected following KBr density gradient centrifugation revealed that apoLp-III was present mainly at high densities (1.19-1.22 g/mL) (Fig. 5.5). Fractions 6-10, found at densities of 1.09-1.10 g/mL, contained a yellow pigment indicative of the presence of carotenoids usually associated with lipophorins I and II. Traces of apoLp-III were also present in these fractions.

Injection of insects with octopamine or PBS had no effect on the density profile of the hemolymph proteins (Figs. 5.6A and 5.6B).

Effects of LPS, LTA and octopamine on the concentration of apoLp-III

The concentration of apoLp-III in the hemolymph was affected by injections of neither bacterial antigens nor octopamine (data not shown).

Figure 5.4 Analysis of native apoLp-III. **A:** non-denaturing gradient gel; lane 1 (closed arrow): purified apoLp-III; lanes 2 and 3: 0.3 μL of hemolymph (open arrow: band containing apoLp-III); **B:** immunoblot of proteins seen in A; lanes 1 and 2 (open arrow): apoLp-III in the hemolymph sample; lane 3 (closed arrow): purified apoLp-III. **C:** Preparative SDS-PAGE of apoLp-III excised from a non-denaturing 4-20% polyacrylamide gradient gel (closed arrow: apoLp-III, open arrow 77kDa protein).

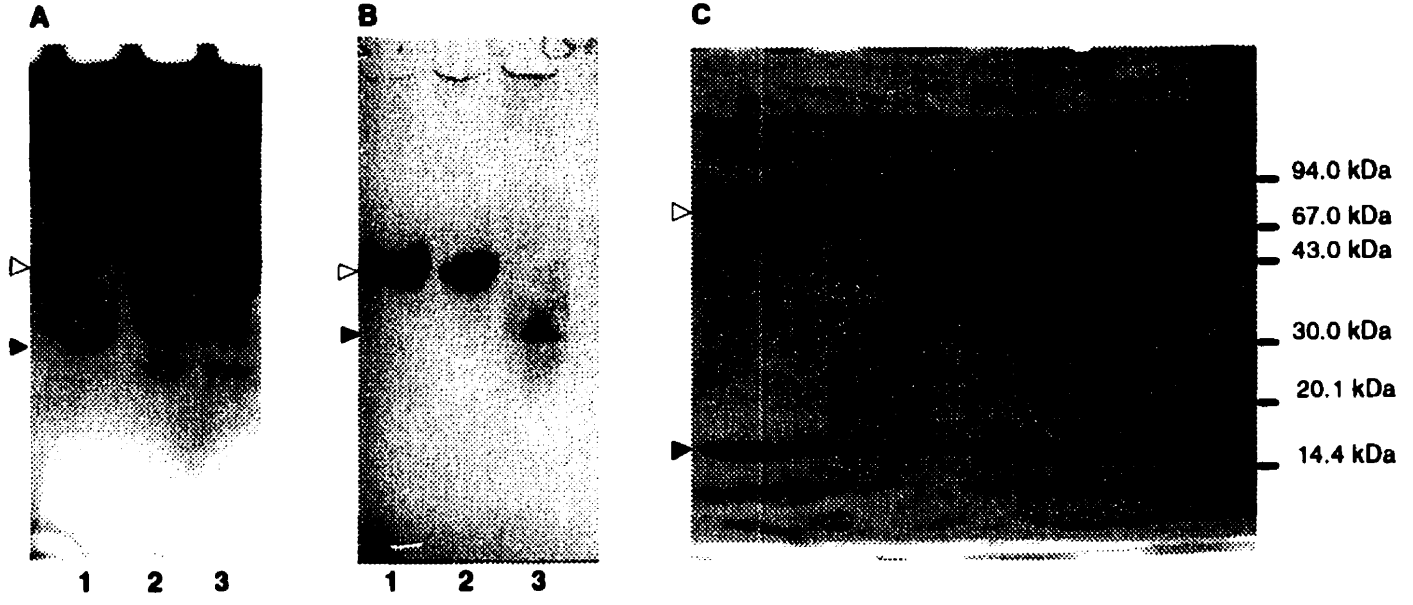

A
B
C
1
2
3
1
2
3
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

Figure 5.5 SDS-PAGE of hemolymph proteins in aliquots (5μL) from fractions (200μL) collected following density gradient centrifugation.

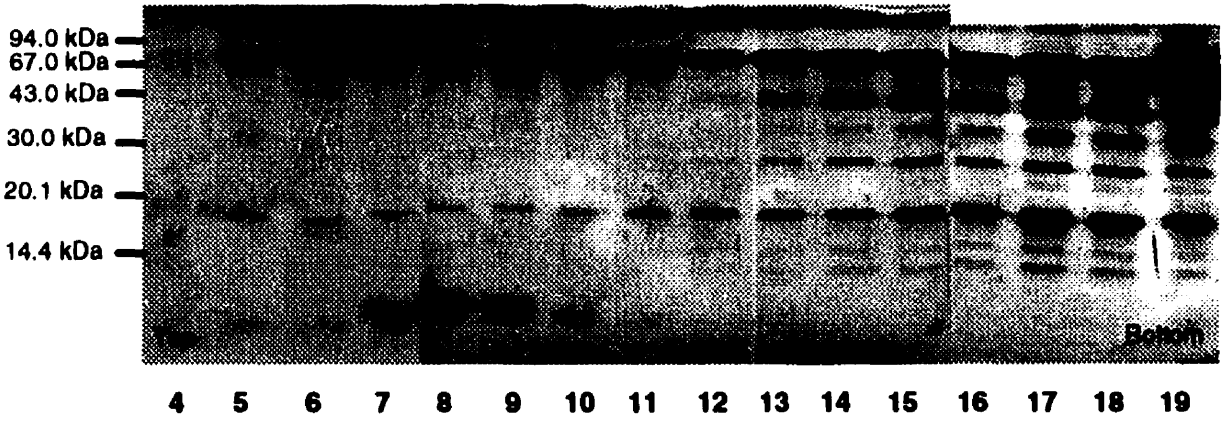
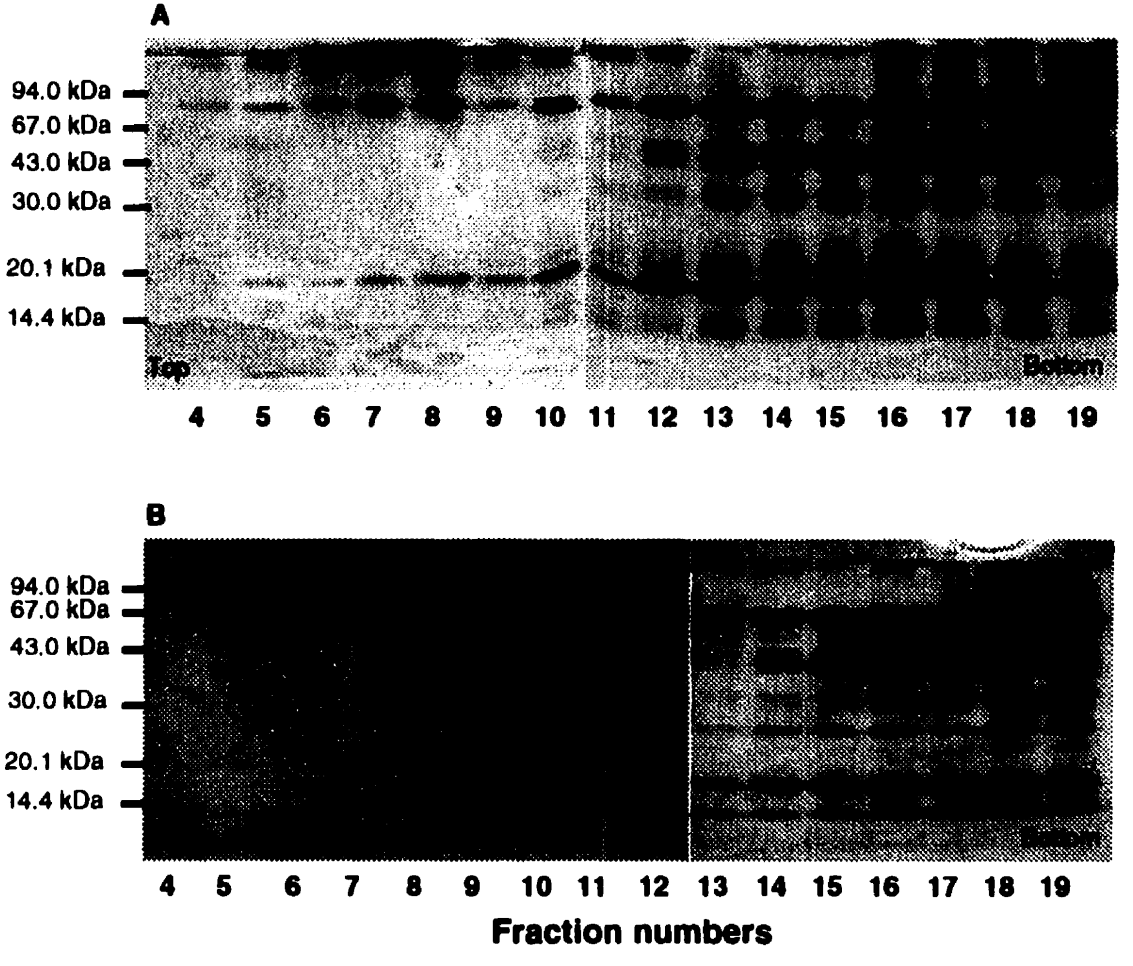

94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
Bohdin
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Fraction numbers

Figure 5.6 SDS-PAGE of hemolymph proteins in (5µL) aliquots collected from fractions (200µL) following density gradient centrifugation. **A**: hemolymph protein profiles of PBS-injected *G. mellonella* larvae. **B:** hemolymph protein profiles of octopamine-injected *G. mellonella* larvae.


A
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
Top
Bottom
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
B
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Fraction numbers

DISCUSSION

In hemolymph, the level of lipophorins as well as that of lipids is known to change with the developmental stage of the insect (Beenakkers *et al.*, 1985). Cole and Wells (1990) have shown that, in *M. sexta*, the apoLp-III gene is expressed in a cyclic manner during the fourth and fifth larval stadium; maximum expression was reported during the feeding periods. The apoLp-III gene was not expressed in the pupae, and in the adults, the level of expression of the gene was ten fold higher than the highest level of expression of the last larval stages. The concentration of apoLp-III was reported to be 0.46 mg/mL of hemolymph in larvae and 17 mg/mL of hemolymph in adults (Kawooya *et al.*, 1984). Here also, *G. mellonella* apoLp-III appears to increase during the feeding period; contrary to the situation with *M. sexta*, the concentration of apoLp-III, at the end of the feeding larval stage, is higher than in the adults. At its peak, the apoLp-III concentration in larval hemolymph was 8-12 mg/mL. Based on hemolymph volumes of 60-75 µL for 200-250 mg larvae (Gagen and Ratcliffe, 1976), the amount of apoLp-III per larva is at least 480 µg. The presence of apoLp-III in high amounts in larval, pupal and adult stages implies it may have multiple functions. Wiesner *et al.* (1997) reported 70 µg of apoLp-III per last instar *G. mellonella* larva; this low estimate could be due either to the size/stage of the larvae (300 mg), since in this study apoLp-III concentration decreased in larvae over 250 mg, or to a difference in the sensitivity of the technique used.

The observed fluctuations in apoLp-III concentration in the hemolymph with developmental stages do not correlate with the

concentrations of morphogenic hormones reported for the 7th larval instar and pupal and adult stages of *G. mellonella* (Hsiao and Hsiao, 1977). The decrease in the concentration of apoLp-III at the end of the last larval instar could be the result of an increase in hemolymph volume; such an increase is known to occur prior to moulting due to water absorption from the tissues and diminished excretion (Lee, 1961). Yun and Lee (1997) reported that in *G. mellonella*, apoLp-I and -II are synthesized *in vitro* by larval but not by pupal fat body; their presence in pupae and adults is thought to reflect carry over from the larval stage. A similar situation could be the case for apoLp-III.

The occurrence of apoLp-III in the fat body of *G. mellonella* was expected since the fat body is the major site of synthesis of apoLp-III in other insects (Kanost *et al.*, 1988; Smith *et al.*, 1994; Kim *et al.*, 1998). Contrary to the report of Mandato *et al.* (1996), apoLp-III was found to be present in the hemocytes of *G. mellonella* larvae. It is unlikely that the apoLp-III in the hemocyte pellet is a contaminant from hemolymph since similar results were obtained with three different anticoagulants and with the washing procedure used, a contaminant would have been diluted at least $1x\ 10^6$ fold to an undetectable concentration; the negative result of Mandato *et al.* (1996) might reflect a low number of hemocytes in the sample tested. It would be of interest to investigate whether apoLp-III is internalized or bound to the surface of the hemocytes and if hemocytes constitute another site of synthesis for this protein.

Because apoLp-III in hemolymph, identified by its reactivity with anti-apoLp-III antibody, had reduced electrophoretic mobility in a native polyacrylamide gel with respect to the purified protein, it was suspected that apoLp-III was bound to other proteins or to lipid molecules. SDS-

PAGE revealed that the material associated with apoLp-III was probably a protein with an apparent molecular mass of 77 kDa. This material was tentatively identified as apoLp-II based on its molecular mass (Yun and Lee, 1997) and its presence in the low density yellow band obtained after KBr gradient centrifugation. SDS-PAGE of the fractions collected following density gradient centrifugation revealed that apoLp-III as well as apoLp-II was found mainly in the fractions of the highest densities. Relatively low amounts of apoLp-III were associated with apoLp-I and -II at the lower densities. Since on the immunoblot, only one band was visible, it would appear that in the hemolymph of *G. mellonella* larvae, apoLp-III is bound to another protein, possibly apoLp-II; association of apoLp-III and lipophorin in the resting situation was observed in the Hemipteran, *Thasus acutangulus* (Wells *et al.* 1985). However, the identity of the 77 kDa protein has yet to be confirmed and the data presented do not rule out the possibility that this protein could be a subunit of arylphorin, a storage protein composed of subunits of 74-81 kDa (Ray *et al.*, 1987; Memmel *et al.*, 1992; Beresford *et al.*, 1997; Wiesner *et al.*, 1997) and usually associated with small amounts of lipids (Shapiro *et al.*, 1988).

Since apoLp-III was recently implicated in immune functions, a possible increase in its concentration following injections of LTA or LPS was investigated; these two bacterial toxins did not cause any detectable change in the concentration of apoLp-III in the hemolymph. The synthesis or release of apoLp-III to the hemolymph during an immune response might not be necessary since apoLp-III is already present in significant amounts and the molecule is known to be recycled (Van Heusden *et al.*, 1987).

Octopamine is a phenolic analog of noradrenaline and is secreted in response to stress. Dunphy and Downer (1994) observed that the octopamine levels in the hemolymph of *G. mellonella* larvae increased significantly following injections of the bacterium, *B. cereus*. Furthermore, octopamine produces either hyperlipaemia or hyperglycemia and mediates the release of adipokinetic hormone, a neurohormone responsible for lipid loading of lipophorins (Orchard, 1987). The effects of octopamine on the concentration of apoLp-III in hemolymph and on lipid-loading of lipophorins were investigated. An increase in the lipid content of the hemolymph caused by higher levels of octopamine during an infection, would mean less apoLp-III available to participate in the response to that infection. Octopamine did not change the concentration of apoLp-III or the density of lipophorin indicating that no lipid loading had occurred; *in vitro* studies on the locust fat body indicated that octopamine induced lipolysis but not loading of HDLp (Orchard *et al.*, 1982; Van Heusden *et al.*, 1984). However, that octopamine did not cause lipid loading, could also be related to the incubation time and/or the concentration of octopamine used, or to a hyperglycemic role for octopamine in *G. mellonella*.

In summary, the concentration of apoLp-III at the end of the last larval instar is higher than in the pupae or the adult. ApoLp-III is present in the fat body, the plasma and the hemocytes. In its native form, apoLp-III is bound to another protein, possibly apoLp-II. Injections of LPS, LTA, or octopamine had no effect on the apoLp-III concentration in hemolymph.

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CHAPTER 6

GENERAL DISCUSSION AND CONTRIBUTION TO ORIGINAL KNOWLEDGE

GENERAL DISCUSSION

The central objective of this work was to identify proteins in the hemolymph of *G. mellonella* last instar larvae that detoxify LPS. The study was also directed at the immediate effects of LTA on hemocytes and prophenoloxidase activation and the role played by LPS-binding protein in the insect immune response to LTA. In **Chapter 2,** it is reported that two hemolymph proteins with apparent molecular masses of 17.2 kDa and 26 kDa, adhered to two strains of the insect pathogenic bacterium *X. nematophilus* and were therefore suspected to be LPS-binding proteins. After several unsuccessful attempts at purification of these proteins by means of gel filtration and hydophobic interaction chromatography, purified preparations were finally obtained by means of SDS-PAGE followed by electroelution. These two proteins were found to bind to *X. nematophilus* LPS in that they decreased the availability of LPS for interactions with the dye dimethylmethylene blue. When the polypeptides in heat-inactivated serum were separated by means of non-reducing SDS-PAGE, the 26 kDa protein was no longer visible indicating that this polypeptide is bound to another via one or more disulfide bonds. This protein was not investigated further and the study focused on the 17.2 kDa protein. Future studies could include identification of

the hemolymph protein that associates with the 26 kDa protein and investigations of its role(s) in the immune responses of *G. mellonella*. LPS of *X. nematophilus* damages hemocytes of *G. mellonella* and inhibits the activation of prophenoloxidase (Dunphy and Webster, 1988; Dunphy and Webster, 1991). However, *G. mellonella* is able to partially detoxify *X. nematophilus* LPS. Studies pertaining to the participation of the 17.2 kDa protein in the neutralization of *X. nematophilus* LPS in hemolymph are also presented in **Chapter 2.** This protein reduced the detrimental effects of *X. nematophilus* LPS on hemocytes and phenoloxidase activity. At this stage in the research, the identity of the 17.2 kDa protein (LBP-1) was still unknown. In **Chapter 3,** LBP-1 is reported to be apoLp-III. Wiesner *et al.* (1997) demonstrated that apoLp-III of *G. mellonella* larvae opsonizes yeast cells and stimulates the phagocytic activity of isolated plasmatocytes. It remains to be determined if apoLp-III opsonizes bacteria and accelerates their removal from the hemolymph.

Does apoLp-III weaken the cell wall and render bacteria more susceptible to attack by other antibacterial proteins? In **Chapter 3,** the lysis of *M. lysodeikticus* by hen lysozyme or *G. mellonella* lysozyme is shown to be enhanced by the presence of apoLp-III. ApoLp-III did not associate with hen lysozyme but bound to *M. lysodeikticus,* without lysing the cells. It would appear that apoLp-III probably weakened the bacterial cell wall and increased the susceptibility of the bacteria to lysozyme. The mode of antibacterial action of apoLp-III is an open question for future investigations.

Since apoLp-III detoxified LPS (**Chapter 2**), it seemed appropriate to determine if it had a similar effect on the LTAs of Gram-positive

bacteria. The immune responses of insects to LTAs have been sudied mainly in terms of the capacity of LTAs to induce the synthesis of antibacterial proteins (Alexander, 1992). In **Chapter 4,** the short term effects of LTA from three different bacterial species on the hemocytes and phenoloxidase are described. The decrease in the hemocyte counts and the partial or complete depletion of plasmatocytes that were observed following injections of LTA into *G. mellonella* larvae indicated that LTA activated blood cells. Whether this activation is direct, in that it results from binding of LTA to hemocytes, or indirect, in that it is mediated by factors present in hemolymph, should be addressed in further research. LTA and LPS could interact with hemocytes via a common receptor. Receptors with broad specificities (common receptors for LPS, LTA and other molecules) have been reported in mammals and insects (Dziarski and Gupta, 1994; Pearson, 1995). All LTA species tested activated prophenoloxidase *in vitro.* However, of the three LTA species injected into the insect, only *B. subtilis* LTA activated prophenoloxidase; the other two species had the opposite effect. While a complete depletion of phenoloxidase-positive granulocytes by *E. hirae* LTA and *S. pyogenes* LTA, but not by *B. subtilis* LTA, was a possible explanation for this result, differential hemocyte counts did not support this hypothesis. Nevertheless, the damaged appearance of granulocytes in insects injected with *B. subtilis* LTA suggested that this species of LTA might be causing an increased discharge of the prophenoloxidase system. What step of the phenoloxidase cascade is inhibited by the LTAs from *E. hirae* and *S. pyogenes* is a question raised by these results. Since the effect of *B. subtilis* LTA was so pronounced, this LTA species was used to test the detoxifying property of apoLp-III. Based on the levels of phenoloxidase

activity observed following injections of LTAs and/or apoLp-III, it cannot be concluded whether apoLp-III partially neutralised LTA and/or decreased the activation of the phenoloxidase cascade.

In **Chapters 2, 3 and 4,** the participation of apoLp-III in the short term immune response of *G. mellonella* to bacteria and their toxic surface components is demonstrated. Does the concentration of apoLp-III in hemolymph change in response to an infection and is apoLp-III present in all stages of the insect? These questions are answered in **Chapter 5.** The concentration of apoLp-III in hemolymph was highest at the end of the last larval instar; this concentration was 8-12 mg/mL or 522-652 μg/larva weighing 200-250 mg. ApoLp-III was also present in pupae and adults but at lower levels. The concentration of apoLp-III in larval hemolymph did not change following injections of LPS or LTA; such a response is possibly not necessary as sufficient amounts of apoLp-III are already available. Also, apoLp-III was detected in hemocytes and fat body; if needed, the protein could have been quickly released into the hemolymph. The regulation of the release of lipophorins from the fat body has been documented only for apoLp-I and apoLp-II (Blacklock and Ryan, 1994). Modulation by antigen of fat body discharge of apoLp-III is unknown and should be investigated. In addition, octopamine, a stress hormone, did not induce a change in apoLp-III concentration nor did it trigger lipid loading of HDLp.

In **Chapters 2, 3 and 4,** it is reported that injections of relatively small amounts of apoLp-III had marked effects although apoLp-III was already present in large quantities in hemolymph. Is apoLp-III, in its native state, bound to HDLp or other proteins? In **Chapter 1** (Table 1.1), apoLp-III in some insect species is reported to be bound to apoLp-I and

apoLp-II or biliverdin. The apoLp-III band that was obtained when hemolymph proteins were separated by means of non-denaturing PAGE was identified by its reactivity with anti-apoLp-III antibodies. Analysis of this band by means of SDS-PAGE revealed that apoLp-III in hemolymph is associated with a 77 kDa protein, possibly apoLp-II or arylphorin. Future research should address the identity of this protein. That apoLp-III in hemolymph is bound to another protein and is therefore not free, may explain the potency of small amounts of added apoLp-III.

In conclusion, this research has explored aspects of the participation of apoLp-III in the immediate immune responses of *G. mellonella* last instar larvae to Gram-negative and Gram-positive bacteria and their toxic surface components. ApoLp-III neutralizes LPS and LTA and may mediate antibacterial responses by binding to bacterial surfaces.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. The binding of two hemolymph proteins of *G. mellonella* larvae to *X. nematophilus* LPS was demonstrated; this binding was mediated by the lipid A portion of the LPS molecule.

2. The detoxifying capacity of apoLp-III was demonstrated. ApoLp-III prevented the damage to hemocytes and the inhibition of the melanization system caused by *X. nematophilus* LPS.

3. Adherence of apoLp-III to the surface of *X. nematophilus* and *M. lysodeikticus* was observed. Although apoLp-III and lysozyme did not associate, the activities of hen egg lysozyme and *G. mellonella* lysozyme were enhanced by apoLp-III.

4. The dye, dimethylmethylene blue, was shown to be useful for the determination of lipoteichoic acid concentrations.

5. LTAs of *B. subtilis* , *E. hirae* and *S. pyogenes* were shown to cause a decrease in the numbers of circulating hemocytes and a decrease in the numbers of plasmatocytes.

6. The activation of prophenoloxidase by *B. subtilis* LTA *in vitro* and *in vivo* is reported.

7. Activation of prophenoloxidase by *E. hirae* LTA and *S. pyogenes* LTA *in vitro* and its inhibition *in vivo* are reported.

8. The binding of apoLp-III to lipoteichoic acids and its ability to detoxify LTA are demonstrated.

9. The concentration of apoLp-III in hemolymph of last larval instar was shown to be 8-12 mg/mL of hemolymph. The concentration of apoLp-III in larvae was higher than in pupae or adults.

10. The presence of apoLp-III in hemocytes and fat body of *G. mellonella* is reported.

11. Injections of LPS or LTA into the larva of *G. mellonella* were shown to have no effect on the concentration of apoLp-III in hemolymph.

12. Injection of octopamine into last instar larvae caused no change in the concentration of apoLp-III nor did it induce lipid loading of HDLp.

13. ApoLp-III in larval hemolymph was shown to be associated with a 77 kDa protein.

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