Lectin expression in grasshopper (Melanoplus differentialis) fat body, ovary and testis tissue
by Layla Gedik

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
Lectins play an important role in the humoral and cellular defense response of invertebrates. The carbohydrate-binding capacity of these proteins mediates recognition of specific carbohydrates on the surface of microorganisms, leading to their opsonization.

Agglutinin that binds specific carbohydrates has been isolated from hemolymph of grasshopper, M. differentialis. This protein is a C-type lectin, requiring calcium for sugar binding activity. Two related lectin cDNA clones have been isolated, namely clone 3 and clone 4. The full length cDNAs of clone 3 and clone 4 are 2.1 Kb and 2.0 Kb, respectively, and they show 80% sequence identity within the open reading frame.

The objective of this study is to determine lectin expression in grasshopper fat body, ovarian and testicular tissue using molecular biology techniques. Results would confirm or contradict previous published data that indicates synthesis of agglutinins in the three respective tissues (Stiles et al. 1988).

Total RNA and polyA+ RNA were isolated from predissected and frozen fat body, ovary and testis tissue, for use in Northern analysis and reverse transcription-polymerase chain reaction (RT-PCR). Northern analysis using a 32P-labeled lectin cDNA probe was successful with fat body polyA+ RNA, indicating lectin transcription in this tissue. RT-PCR with clone 3- and clone 4-specific primers gave expected bands with fat body, ovary and testis polyA+ RNA. Southern analysis of the RT-PCR products with a radioactively labeled lectin cDNA probe showed that the RT-PCR products were a result of amplification of lectin mRNA. Interpretation of RT-PCR and Southern analysis results indicate that genes encoding clone 3 and clone 4 are expressed in fat body, ovary and testis tissues. Additional lectin-specific bands' with the three tissues in Southern analysis data suggest expression of other lectin genes. Overall results confirm that grasshopper fat body, ovarian and testicular tissues are synthesizing lectin molecules.

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Layla Gedik

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Lectins play an important role in the humoral and cellular defense response of invertebrates. The carbohydrate-binding capacity of these proteins mediates recognition of specific carbohydrates on the surface of microorganisms, leading to their opsonization.

Agglutinin that binds specific carbohydrates has been isolated from hemolymph of grasshopper, *M. differentialis*. This protein is a C-type lectin, requiring calcium for sugar binding activity. Two related lectin cDNA clones have been isolated, namely clone 3 and clone 4. The full length cDNAs of clone 3 and clone 4 are 2.1 Kb and 2.0 Kb, respectively, and they show 80% sequence identity within the open reading frame.

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Total RNA and polyA$^+$ RNA were isolated from pre-dissected and frozen fat body, ovary and testis tissue, for use in Northern analysis and reverse transcription-polymerase chain reaction (RT-PCR). Northern analysis using a ^{32}P-labeled lectin cDNA probe was successful with fat body polyA$^+$ RNA, indicating lectin transcription in this tissue. RT-PCR with clone 3- and clone 4-specific primers gave expected bands with fat body, ovary and testis polyA$^+$ RNA. Southern analysis of the RT-PCR products with a radioactively labeled lectin cDNA probe showed that the RT-PCR products were a result of amplification of lectin mRNA. Interpretation of RT-PCR and Southern analysis results indicate that genes encoding clone 3 and clone 4 are expressed in fat body, ovary and testis tissues. Additional lectin-specific bands with the three tissues in Southern analysis data suggest expression of other lectin genes. Overall results confirm that grasshopper fat body, ovarian and testicular tissues are synthesizing lectin molecules.

INTRODUCTION

Insect (Invertebrate) Immunity

Insects have been very successful in evolution, populating all niches apart from the seas (Hoffman 1995). This achievement is largely due to the insects' ability to defend themselves against many different microorganisms such as bacteria and fungi, as well as viruses and parasites. The defense is both external, that includes avoiding discovery, resisting attack and physical barriers such as the insects' cuticle, or internal, involving the insects' innate immune system.

Insects lack the immunoglobulin-like recognition molecules and immune memory characteristic of the vertebrate immune system (Lowenberger 1996). However, there are similarities between vertebrate and invertebrate immune responses. Insects use cellular and humoral components of their hemolymph to fight harmful microorganisms (Strand and Pech 1995, Glinski and Jarosz 1995). With the cellular facet, it is understood that most insects have several types

of cells in the open circulatory system that remove foreign material by phagocytosis or encapsulation (Lowenberger 1996, Hoffman 1995). However, little is known of the molecular mechanism of the cellular response involved in the recognition of the foreign particles. The humoral response mechanism is better grasped. This involves the induction of two proteolytic cascades - the hemolymph coagulation cascade and prophenoloxidase cascade (Hoffman et al. 1996). The cascades result in clotting and melanization of encapsulated pathogens. Another humoral response to a septic injury is the synthesis, predominately by the insect fat body, of antimicrobial proteins and peptides that are released directly into the circulating hemolymph (Lowenberger 1996, Hoffman et al. 1996). The response has similarities to the vertebrate acute-phase response.

Lectins are recognition molecules that have suspected roles in insect defense against pathogens. They will be described below.

Lectins

Lectins are ubiquitous and diverse proteins or

glycoproteins that bind to carbohydrates and glycoconjugates with considerable specificity (Rini 1995, Kennedy et al. 1995). Many lectins have been isolated from plants, animals and microorganisms (Sharon 1993). They serve to mediate biological recognition events through the binding of ligands to their one or more carbohydrate recognition domains (Rini 1995, Kennedy et al. 1995). Agglutinins are lectins in which binding of ligands at the carbohydrate binding sites leads to precipitation or agglutination of the cell or glycoconjugate (Lis and Sharon 1986).

There are four classes of animal lectins: C-type, S-type (galactins), I-type and P-type (Rini 1995, Drickamer 1993, Powell and Varki 1995). The C-type lectins require one calcium ion at each carbohydrate-recognition domain (CRD) to directly coordinate with the carbohydrate (Kolatkar and Weis 1996, Weis et al. 1992). Additional domains, if any, of the protein dictate the role and location of the lectin molecules (Drickamer 1993). Most C-type lectins bind to galactose and its derivatives (Gal-type ligands) or to D-mannose, D-glucose and derivatives (Man-type ligands) (Kolatkar and Weis 1996). Selectins, collectins, endocytic glycoprotein receptors and the macrophage mannose receptor

are all members of the C-type family (Rini 1995, Weis et al. 1992).

C-type vertebrate lectins are found in serum, membranes and extracellular matrix (Drickamer 1993). For example, type II endocytic receptors that mediate endocytosis of glycoproteins have been described and include the mammalian hepatocyte asialoglycoprotein receptor, which is the most understood of the Gal-binding C-type lectins (Kolatkar and Weis 1996). The rat serum mannose-binding protein (MBP) is the best described Man-binding C-type lectin and its crystal structure is known (Weis et al.1991). By recognizing self from nonself, MBP functions independently of immunoglobulins in host defenses against mannosidic-containing pathogens.

Agglutinins have been discovered in many species of invertebrates (Mori et al. 1992). It has been proposed that they play an important role in the humoral and cellular immunity in insects (Ratcliffe et al. 1985; Wheeler et al. 1993). Lectins in the invertebrates *Sarcophaga peregrina* (flesh fly; Komano et al. 1983), *Periplaneta americana* (cockroach; Kawasaki et al. 1996), *Drosophila melanogaster* (fruit fly; Natori et al. 1996), *Bombyx mori* (silkworm; Mori

et al. 1992), *Manduca sexta* (tobacco hornworm; Minnick et al. 1986) and *Spodoptera exigua* (beet armyworm; Boucias and Pendland 1993) have been described and they all possess hemagglutinin activity. The expression of the *Sarcophaga* lectin is induced by injury to the body wall of the larvae (Sugiyama and Natori 1991). Two *Periplaneta* hemolymph lectins show enhanced expression after injection of *E.Coli* into the body cavity of the adult (Kawasaki et al. 1996). These lectins act as opsonins and have been implied to have functions in insect defense systems.

The *Sarcophaga*, *Periplaneta* and *Spodoptera* lectins are synthesized in the fat body of the insect (Mori et al. 1992, Komano et al. 1983). The fat body is an amorphous tissue that lines the hemocoel of the insect (Lowenberger 1996). One of its roles is to synthesize immune compounds that are then released into the hemolymph. The hemocyte is the site of synthesis of the lectins in *B. mori* (Mori et al. 1992).

Although it appears that certain lectins are involved in insect immunity, this is not their only role. Lectins are involved in cell-cell interactions. For instance, the *Sarcophaga* lectin and *Drosophila* lectin are suspected to function in development as well as host defense, since their

expression is induced during embryonic and pupal stages of the fly (Sugiyama and Natori 1991, Natori et al. 1996). In most hemimetabolous invertebrates, agglutinins occur constitutively and are apparently not induced by foreign material. These agglutinins probably have roles in various tissue interactions and developmental processes (Lis and Sharon 1989). A well-studied lectin from sea urchin sperm binds carbohydrates on the egg surface in a species specific manner, prerequisite to fertilization (O'Rand 1988).

Previous Work

Previous work has given indication of agglutinin synthesis in grasshopper fat body, ovary and testes (Stiles et al. 1988). Agglutinin release from hemocytes was not detected, although hemagglutinin was located to hemocytes via potential hemocytic membrane receptors (Bradley et al. 1989). The work involved two techniques on primary cultures of the respective tissues, (1) the use of ELISA assays to determine whether primary cultures of the three tissues were releasing agglutinin into the medium, and (2) metabolic labeling of newly synthesized proteins using L-[^{35}S]

methionine followed by immunoprecipitation with agglutinin-specific antibody to determine if the cultured cells were synthesizing agglutinin in addition to releasing it from cellular storage.

Agglutinin release from fat body was not stimulated by the addition of microbial cell wall components to fat body cultures or injection of these constituents into the grasshopper hemocoel. It appears that the agglutinin is constitutively present in the hemolymph and is an opsonin toward *Beauvaria bassiana* blastospores, since it stimulates adhesion to hemocytes leading to removal of the fungal blastospores from the circulation (Wheeler et al. 1993). The agglutinin is not opsonic toward certain bacteria, protozoa or asialo erthyrocytes, indicating strict selectivity (Bradley et al. 1989).

More recently, two positive lectin clones were isolated and sequenced from a *Melanoplus differentialis* grasshopper cDNA λgt11 library, namely clone 3 and clone 4 (Rognlie, unpublished results). The clones were subsequently inserted into Promega pGem®-7zf(+) plasmid vector (see Figure 1). It was found that the two clones were only partial length

representing incomplete copies of the two clones. Clone 4 was missing the extreme 5' end and clone 3 was absent approximately 30 percent of the 3' end. It has been recently shown that both truncated clones were the result of cleavage at internal EcoRI sites during library construction. Currently, using RACE (Rapid Amplification of cDNA Ends) methods, the extreme 5' end of clone 4 and the 3' portion of clone 3 were isolated and sequenced in this laboratory (Radke, unpublished results). This work resulted in full length cDNAs of approximately 2.1 Kb and 2.0 Kb for clone 3 and clone 4, respectively. Clone 3 and clone 4 code a protein of 324 and 326 amino acid residues, respectively. The open reading frames of clone 3 and clone 4 show 80% sequence identity. See Figure 2 for alignment of clone 3 and clone 4 nucleotide sequence, indicating homology between the two clones. Each clone contains two CRDs that are 30% identical to each other as well as other invertebrate C-type lectins, indicating considerable time since divergence of the ancestral form (Doolittle 1995). There are two potential N-glycosylation sites in each clone.

Homology modeling of one clone 3 CRD has been performed using the three-dimensional x-ray crystal structures of rat

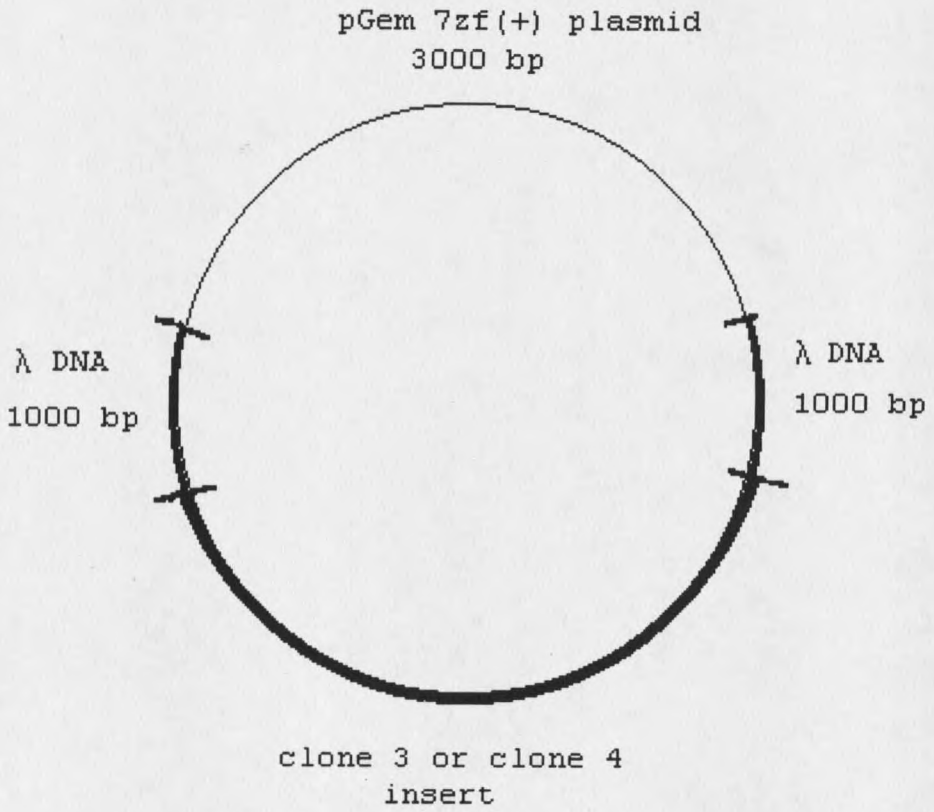

Figure 1: Plasmid vector pGem 7zf(+) with clone 3 or clone 4 inserts (bracketed with approximately 1000 bp of λ DNA from previous vector).

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GGGAGAGCACGGCTGCAGCTCGCGCCAGGAGGAGGAGTTGATAAGCAGGATGCAGCTGGT      60
                                          ATGGCCTGCCCCCTTTATGACGGTGT      26
GCGCGGCGCTGGTGGCAG--CGACAGTACCCTGCACCCTGGCCGCCGTG------GACCT      120
TATTATTTTTGAGACCAGACCAACTGTG---TTAATGGTAGGGACCGGGGCTCAGCAGAA      86
GTTCTGCAGCTGCCAGGTGCGCCACCACAGGGACTCGACGACGGCCGTGCACTGCTCAGG      180
TTCCGGCGGCTGCAAGCTGCGCCACCACAAGGAATCGACGATGGCCCTGCACTGCTCAAG      146
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GGATCAGAGTGGGAACAAAACG------TGTCAAAAAGCCCAAGTGCCGGACATTCCACG      206
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TGGCTACCACTACGTGCCAGGCTACGCTCTCGTCAAGATGTACCGCATAATGATGACATG      266
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