Grasshopper agglutinin : preparation and characterization by MALDI/TOF-MS
by Donald Lee Wenzlick

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

Abstract:
A lectin from the grasshopper, Melanoplus differentialis has been previously isolated and partially characterized (Stebbins and Hapner 1985). The protein (GHA) is a presumed component of the insect's immune/defense system and is thought to have a role in self/non-self recognition. The protein is known to act in an opsonic fashion toward fungal blastospores having surface carbohydrate; aiding in their phagocytosis by hemocytes (Wheeler et al. 1993). Further research, reported here, into GHA is part of ongoing studies to increase knowledge of GHA's structure and functional roles. This research focuses on purification of the affinity chromatography isolated protein, its molecular mass, glycoprotein character, and sulfhydryl content. Techniques include classical mass determination using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and the more recently developed matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI/TOF-MS).

Results show that GHA consists of two closely-related, dimeric molecules, GHLAla and GHAlb. Masses of GHAla and GHAlb are measured to ~62.6 kDa using SDS-PAGE while MALDI/TOF-MS shows 72.0 and 72. 1kDa, respectively. Reduction of GHAla and GHAlb suggests that the dimers are composed of covalently-linked monomers with masses of ~36.9 kDa on SDS-PAGE and -36.1 kDa on MALDI/TOF-MS. The anomalous masses between the two techniques are thought to be related to the protein's carbohydrate content. Enzymatic removal of the carbohydrate moiety by peptide-N-glycosidase F causes a mass difference of 2.5- 2.8 kDa on SDS-PAGE. This value is consistent with the presence of 4 N-acetylglucosamine and 10-12 hexose residues per dimer, roughly equal to 4% carbohydrate by weight. Peptide-N-glycosidase F treatment of reduced protein shows that both monomers are glycosylated. Titration of GHAla and GHAlb with p-hydroxymercuribenzoate followed by MALDI/TOF-MS indicates each protein dimer to contain two free sulfhydryls, presumably one per monomer. All of the data obtained show GHAla and GHAlb to be essentially indistinguishable. The basis for their separation on reverse-phase HPLC is unknown, but possibly related to carbohydrate content.

GRASSHOPPER AGGLUTININ: PREPARATION AND CHARACTERIZATION BY MALDI/TOF-MS

by

Donald Lee Wenzlick

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY - BOZEMAN
Bozeman, Montana

August 1996

APPROVAL

of a thesis submitted by

Donald Lee Wenzlick

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.

Kenneth D. Hapner Kenneth D Hapner 9-6-96
(Signature) Date

Approved for the Department of Chemistry and Biochemistry

David M. Dooley David M Dooley 9/6/96
(Signature) Date

Approved for the College of Graduate Studies

Robert L. Brown R L Brown 9/23/96
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University - Bozeman, I agree that the Library shall make it available to borrowers under rules of the library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Ronald Unterseher

Date 06 Sept 96

ACKNOWLEDGMENTS

I wish to thank my major professor Dr. Kenneth D. Hapner, for his guidance and support throughout my studies at Montana State University- Bozeman. I thank the remainder of my Graduate Committee: Dr. Martin Teintze and Dr. Larry L. Jackson for their input and support. I thank Dr. Lyle Joseph "Joe" Sears for his outstanding help and support in using the Mass Spectrometry Facility at Montana State University. I thank the faculty and staff of Montana State University - Bozeman for their support during my period of study. Lastly, I thank my wife, Stephanie, and my family who have supported me since the beginning.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF FIGURES (continued)

Figure Page

ABSTRACT

A lectin from the grasshopper, *Melanoplus differentialis* has been previously isolated and partially characterized (Stebbins and Hapner 1985). The protein (GHA) is a presumed component of the insect's immune/defense system and is thought to have a role in self/non-self recognition. The protein is known to act in an opsonic fashion toward fungal blastospores having surface carbohydrate; aiding in their phagocytosis by hemocytes (Wheeler et al. 1993). Further research, reported here, into GHA is part of on-going studies to increase knowledge of GHA's structure and functional roles. This research focuses on purification of the affinity chromatography isolated protein, its molecular mass, glycoprotein character, and sulfhydryl content. Techniques include classical mass determination using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and the more recently developed matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI/TOF-MS).

Results show that GHA consists of two closely-related, dimeric molecules, GHA1a and GHA1b. Masses of GHA1a and GHA1b are measured to ~62.6 kDa using SDS-PAGE while MALDI/TOF-MS shows 72.0 and 72.1kDa, respectively. Reduction of GHA1a and GHA1b suggests that the dimers are composed of covalently-linked monomers with masses of ~36.9 kDa on SDS-PAGE and ~36.1 kDa on MALDI/TOF-MS. The anomalous masses between the two techniques are thought to be related to the protein's carbohydrate content. Enzymatic removal of the carbohydrate moiety by peptide-N-glycosidase F causes a mass difference of 2.5- 2.8 kDa on SDS-PAGE. This value is consistent with the presence of 4 N-acetylglucosamine and 10-12 hexose residues per dimer, roughly equal to 4% carbohydrate by weight. Peptide-N-glycosidase F treatment of reduced protein shows that both monomers are glycosylated. Titration of GHA1a and GHA1b with p-hydroxymercuribenzoate followed by MALDI/TOF-MS indicates each protein dimer to contain two free sulfhydryls, presumably one per monomer. All of the data obtained show GHA1a and GHA1b to be essentially indistinguishable. The basis for their separation on reverse-phase HPLC is unknown, but possibly related to carbohydrate content.

INTRODUCTION

Insect (Invertebrate) Immunity

Invertebrates, like other animals, are constantly bombarded by challenges to their immune systems. Wounds, parasites, and infectious agents are continually dealt with by the immune system so that the insect does not become incapacitated. Invertebrates and vertebrates are inherently different in their approach to self-defense. Invertebrates contain neither antibodies nor specialized cells for specific interaction with foreign particles (Pathak 1993). Absence of antibodies or specialized cells results in lack of specificity and memory normally associated with immune responses in vertebrates. Even in the absence of antibodies or specialized cells, invertebrates are able to non-specifically destroy foreign invaders with great efficiency.

Immunity in invertebrates is less well known than that in vertebrates. Cellular immunity aspects are sketchy at best with most of the knowledge in this area being related to cell morphology with little knowledge of interactions between cell types (Hoffmann 1995). Humoral immunity is better understood and is the focus of most current research. Ratcliffe et al. (1985) provide a substantial review of invertebrate immunity.

Immune response in invertebrates is a complex soup of reactive molecules, peptides, proteins, and cells. Many act in concert to achieve the common goal of removal or inactivation of foreign invaders. Initiation of the response is accomplished after recognition of foreign cells or debris. Several different molecules are known to be recognized as foreign by invertebrates, including: lipopolysaccharide, peptidoglycan, and β-1,3-glucan (Hoffmann 1995). Upon detection of non-self matter, several different responses may occur. These include induced production of bactericidal peptides and agglutinating proteins (leading to localized blood coagulation), initiation of the prophenoloxidase cascade, formation of nodules and capsules, and phagocytosis of foreign material (Ratcliffe et al. 1985, Hoffmann 1995). Humoral and cellular immune responses are briefly described below.

Cellular Immunity.

Most insects have several types of common cells including granular hemocytes (3 subtypes), prohemocytes, plasmatocytes, oenocytoids, and spherule cells. Certain species lack one or more of these cell types and others may have additional cell types including thrombocytoids, "peculiar granular hemocytes", coagulocytes, adipohemocytes, and "hemocytes with numerous small granules" (Ratcliffe 1985, Pathak 1993). Overall, identity of some cell types is still in question due to lack of convenient biochemical markers or structural diversity that could be used to tell them apart. Because different

researchers have applied many different names to cells that may be related in structure and function, analysis of related immune functions is hard to determine and compare with other cell types.

Cellular aspects of invertebrate immunity are largely related to phagocytosis and encapsulation of foreign materials (Boman and Hultmark 1987). Other reactions include cell aggregation and nodule formation (Hoffmann 1995). The main cellular participants in phagocytosis are plasmatocytes and granular cells. These two types also play major roles in encapsulation reactions, with granular cell components forming the core of the capsule. Capsule formation results from layering of cells around a foreign object or pathogen. This process is a technique that isolates the foreign substance from invertebrate tissues. The capsule may emit toxic phenolic compounds and thereby contribute to the host's resistance (Pathak 1993).

Humoral Immunity.

Humoral immunity addresses the aspects of immunity that do not directly involve cells. Immune reactions of this type usually involve proteins and other small molecules found in the tissues and hemolymph of the invertebrate. The chief humoral immune reactions have been studied in detail. These types of interactions include inducible antibacterial peptides, lectins, prophenoloxidase and hemolymph coagulation cascades,

and humoral encapsulation. Many of these immune reactions are triggered together and cooperate to rid the invertebrate organism of foreign material.

Inducible Antibacterial Peptides. The response involving antibacterial peptides in invertebrates is similar to the acute phase response in vertebrates (Hoffmann 1995). Most invertebrates contain several different classes of peptides including lysozymes, cecropins, diptericins, defensins (sapecins), ceratoxins, magainins, proline-rich peptides, and the glycine-rich family of peptides (attacins, sarcotoxins II) (Boman and Hultmark 1987, Hoffmann 1995, Lowenberger 1996). By 1995, at least 50 different antibacterial peptides had been discovered from bacterially-challenged insects (Hoffmann 1995). Each insect can be responsible for the production of 10 to 30 different immune peptides (Boman and Hultmark 1987).

These peptides are known to be produced in the fat body, gut cells, salivary gland cells, ovaries/testes, and some blood cell types (Hoffmann 1995, Hoffmann et al. 1996). The genes encoding these peptides are inducible through challenge by bacteria and sometimes by fungal or viral sources. Once triggered, their products serve to rid the insect of foreign invaders. Exact functions of most of these antibacterial peptides are not known but a few of these peptides are responsible for pore formation in the surface of bacteria (Lockey and Ourth 1996).

Lectins. Lectins are important in the immune responses of invertebrates. They are likely produced in all invertebrate organisms. They are directly involved in many carbohydrate recognition interactions that deal with removal or inactivation of foreign invaders. Self/non-self recognition may involve binding sugar residues on the surface of bacteria and other parasites allowing for identification of "foreignness" (Ratcliffe et al. 1985). Recognition of foreign cells by means of carbohydrate binding is their major role in invertebrate defense and they are thought to aid in phagocytosis through action as an opsonin (Wheeler et al. 1993). The galactose-binding lectin from grasshopper, GHA, acts as an opsonin against *Beauvaria bassiana* blastospores but not against those from *Nomurea rileyi*, whose surface lacks galactose (Wheeler et al. 1993). Previous to Wheeler et al., it was shown that the grasshopper agglutinin did not act in an opsonic fashion against erythrocytes, protozoan spores, or bacterial cells, but did bind to them (Bradley et al. 1989). These results suggest that lectins have selective opsonic activity through recognition of specific carbohydrate moieties.

Prophenoloxidase Cascade and Humoral Encapsulation. Encapsulation is the process which involves the deposition of multiple layers of hemocytes around an object. Normally encapsulation proceeds through a combination of cellular and humoral events (Richman and Kafatos 1995). However, some invertebrates have been shown to

accomplish 'encapsulation' without direct involvement of hemocytes (Vey 1993), hence the name "humoral encapsulation".

Activation of the prophenoloxidase (proPO) cascade starts a chain of events leading to both cellular and humoral immune reactions. The method of initiation of the prophenoloxidase cascade is not completely known. It is triggered through recognition of lipopolysaccharides, peptidoglycans, and β-1,3-glucan on the surface of bacteria (Soderhall and Aspan 1993, Hoffmann 1995, Marmaras et al. 1996). Lowered Ca^{2+} concentration can also trigger the cascade and may be involved in response to wounding (Soderhall and Aspan 1993). Prophenoloxidase, a stable precursor form of phenoloxidase, is cleaved through a series of reactions comprising many different catalytic steps. Several reactive quinone intermediates are formed during this cascade, and are thought to have bactericidal and fungicidal activity (Boman and Hultmark 1987, Soderhall and Aspan 1993). This cascade is somewhat analogous to the reactions of complement in vertebrates (Soderhall and Aspan 1993, Cerenius and Soderhall 1995, Marmaras et al. 1996). The end of the cascade is production of melanotic compounds, melanin and protein polyquinone complexes similar to melanin, some of which are toxic.

The melanotic compounds are deposited around foreign bodies during encapsulation. Initially the capsule covering is soft, but hardens and thickens within minutes. The capsule has been shown to aid in growth prevention and entrapment of fungi, bacteria (Boman and Hultmark 1987), and nematodes (Vey 1993).

Hemolymph Coagulation Cascade. Coagulation of hemolymph in a localized area after encounter with a foreign entity could slow or trap that entity, enabling other processes to deal with the problem (Muta and Iwanaga 1996). This process is triggered through contact of serine protease zymogens with LPS on bacteria and (1→3)-β-D-glucans on fungi (Hoffmann 1996, Muta and Iwanaga 1996). Lectins are thought to aid in the initial recognition of the foreign molecules. The zymogens are 'autocatalytically activated' and result in the triggering of other cascade events. Molecules having bactericidal or fungicidal activity are also released during events in the cascade. Ultimately coagulin, an insoluble protein, is formed and traps the foreign invaders. Together, these events lead to isolation and killing of the foreign cells. (Muta and Iwanaga 1996).

Lectins

Lectins are a class of carbohydrate-binding proteins of non-immunoglobulin origin that agglutinate cells or precipitate glycoconjugates (Barondes 1988, Sharon and Lis 1987). It is now known that some lectins have protein binding sites in addition to those for carbohydrate (Barondes 1988), however their function is unknown.

Source and Functions.

Lectins are ubiquitously present in plants, animals, and microorganisms. Since the first discovery of a 'lectin' by Stillmark (1888), lectins have been determined to have many functions (Sharon and Lis 1987). Roles of lectins have been extensively studied in the past two decades. Lectins assist in glycoprotein transport and absorption, allow for cell to cell attachment and cellular homing, and act in an opsonic fashion to promote phagocytosis.

Additionally, researchers have determined that lectins are useful for biochemical analysis of other carbohydrate molecules. Lectins have been used to type blood, purify other lectins or glycoproteins, characterize cell surfaces and tissues, and determine the presence of certain micro-organisms in culture (Kennedy, et al. 1995).

Nomenclature and Classification:

Lectins are usually named according to the carbohydrate they bind most strongly (mannose-binding protein, MBP) or the tissue of origination (β-D-galactoside-binding lectin from the electric eel organ of *Electrophorus electricus*). Some lectins are named by common name (grasshopper agglutinin, GHA) or from the scientific name of the species from which they originate (Concanavalin A, Con A from *Canavalia ensiformis*).

Although lectins can be classified/grouped by their ligand specificity, they are more likely to be grouped according to subcellular location, dependence on cations, biological function, physical/structural attributes, or source (Drickamer and Taylor 1993, Kennedy et al. 1995). Plant lectins are further split into two groups: the cereal and the legume lectins. Microbial lectins are grouped according to their source (Sharon and Lis 1995). Four types of animal lectins have been identified thus far and grouped as I-type, C-type, P-type, and galectins (S-type). C-, P-, and S-type lectins are the most prominent members (Drickamer 1993, Arason 1996).

Animal Lectins. C-type lectins are very diverse in carbohydrate-binding specificity and function (Drickamer 1996). C-type lectins require Ca^{2+} ions for function. They have a characteristic 15 kDa carbohydrate recognition domain (CRD) of about 120 amino acids that may be associated with other functional domains or CRDs in the same polypeptide chain. Numbers of carbohydrate-binding sites vary. C-type lectins, numbering more than 100, show between 20 and 60% amino acid sequence identity (Drickamer 1993). C-type lectins aid in (1) recognition and removal of glycoconjugates, (2) cellular attachment, and (3) self/non-self recognition. All C-type lectins are extracellular or membrane-bound (Drickamer and Taylor 1993) and are further categorized by structure and function to form the selectins, collectins, and endocytic glycoprotein receptors (endocytic lectins) (Sharon and Lis 1995). Drickamer 1993 (Table

1 and Figure 3 that reference) places the C-type lectins into 6 groups with group I comprised of proteoglycans, endocytic lectins in groups II, V, and VI, collectins as group III, and selectins as group IV. Grasshopper agglutinin would likely be put in Group III: molecules involved in humoral defense.

Galectins (previously S-type lectins) were once thought to have invariant sulfhydryl residues that were responsible for function but this has been disproven (Drickamer and Taylor 1993, Kasai and Hirabayashi 1996). However, all the cysteines that are present seem to be in the sulfhydryl form. The sulfhydryl groups appear to stabilize the protein but their removal, mutation, or alkylation does not result in protein inactivation (Drickamer and Taylor 1993, Kasai and Hirabayashi 1996). Galectins have characteristic 15 kDa carbohydrate recognition domains. This CRD type has been shown to contain a set of highly conserved amino acid residues of which 19 are invariant and 36 conserved (Sharon and Lis 1995). All known examples are soluble proteins. They preferentially bind carbohydrate moieties that contain galactose. Intracellular and extracellular forms exist with the majority forming receptors for β-galactosides (Drickamer and Taylor 1993, Rini 1995, Sharon and Lis 1995). Although precise roles for galectins have not been determined, evidence has shown connections between these lectins and human cancer metastases (Sharon and Lis 1995). Three classes of S-type lectins have been formed based on structural characteristics; L-36, L-14, and L-30. L-14 galectins are the simplest of the three with only one CRD that comprises most of the protein. They are usually found as homodimers and are proposed to act in cell-matrix

interactions and glycoconjugate attachment (Drickamer and Taylor 1993). L-30 galectins are 30 kDa monomers that consist of one CRD and one domain rich in proline and glycine. These lectins have been found inside and outside the cell and have proposed functions related to cell-surface interactions and immune activation. L-36 galectins are the newest subgroup. These lectins are comprised of two CRDs, both of which have carbohydrate-binding activities.

I-type lectins contain a characteristic V_1-$C2_n$ domain structure and thus belong to the immunoglobulin superfamily. All are integral membrane proteins with some having large cytosolic domains with large numbers of serine, tyrosine, and threonine residues (Powell and Varki 1995). They are thought to control certain biological events or processes including cellular adhesion and possible secondary activation events.

The mannose-6-phosphate receptors are the only known members of P-type lectins. These receptors are sub-grouped according to their dependence or independence on cations. The cation-dependent receptor is comprised of one CRD while the cation-independent has 15 CRDs. This P-type CRD is unique to this group of proteins and does not resemble the C- and S-type CRDs.

Plant Lectins. Although plant lectins were the first to be studied, only little is known about their functions in nature (Sharon and Lis 1995). Several proposals have been put forth. One contends that the lectins aid in attachment of symbiotic bacteria to

the roots of plants. Another contends that the lectins aid in host defense against insects and pathogens, particularly on seeds.

The largest group of plant lectins occurs in the legumes. These lectins, from the seeds of legumes, number about 70 (Sharon 1993). Legume lectins require Mn^{2+} or Ca^{2+} for binding activity . Some, such as Con A, use both. Each subunit of about 30 kDa has one carbohydrate binding site. They show no structural homology to the C-type domain.

Cereal lectins can best be exemplified by wheat germ agglutinin (Sharon 1993). Wheat germ agglutinin (WGA) has four carbohydrate-binding sites on a dimer of identical subunits (about 18kDa). The molecule is extremely stable, owing to its 16 disulfide bridges. Each subunit in the dimer contributes 2 carbohydrate-binding sites and may account for binding of 2 different carbohydrate moieties. For example, WGA binds both sialic acid and N-acetylglucosamine. Cereal lectins are unique in that they show multiple ligand specificity.

Microbial lectins. Lectins from micro-organisms are best characterized by their attachment properties. Many disease causing microbes have the ability to attach themselves to the cell/tissue that they infect. Lectins provide for the attachment by binding to surface glycoproteins and carbohydrate (Sharon and Lis 1995). Viral bodies have lectins on their surfaces allowing for host cell attachment. Bacteria have lectin

domains at the ends of the pili or fimbrae that subsequently allow for attachment and infection of the host cell.

Isolation and Purification.

Many techniques are available for isolation and purification of lectins (Stebbins and Hapner 1986). While many methods such as HPLC, size exclusion chromatography, ion exchange chromatography, dialysis, and $[NH_4]_2SO_4$ precipitation can be used for general protein extraction, one technique is specifically useful because it employs the binding specificities of the lectins. Affinity chromatography can be used to "single out" proteins with specific binding characteristics. In this case, the affinity matrix is coated with the appropriate carbohydrate ligand. The lectin binds to the matrix-sugar substrate while other proteins and extraction materials are washed away. The lectins can be eluted by addition of a specific ligand, change in pH or ionic strength, or by removal of required metal ions by EDTA (Wheeler et al. 1993, Kennedy et al. 1995).

Rationale for this Research

This research project focuses on the lectin present in the hemolymph of grasshoppers native to Montana and the western United States. Lectins are regarded as

significant components of invertebrate immune response reactions. Montana and other states have major losses from the action of insects on crops/agriculture. In order to combat the insect-related crop losses, the United States Department of Agriculture has made available research monies to develop insect control methods. To determine new methods for controlling insects in their natural environment, it is necessary to be knowledgeable about their natural defense mechanisms. Researching aspects of the immune system in the grasshopper may lead to insights into their control. Once more is known about the immune response to foreign molecules and pathogens, this knowledge can be used to devise programs that circumvent the pest's natural defenses and perhaps encourage greater control by natural predators. This knowledge could also increase our ability to protect beneficial insects from disease or predation.

Previous Work

This research extends previous and ongoing work in Dr. Hapner's laboratory. Grasshopper lectin from *Melanoplus differentialis* has been isolated and partially purified from hemolymph through affinity absorption to Sepharose-D-galactose followed by EDTA elution (Wheeler et al. 1993). The lectin agglutinates human asialo erythrocytes providing a basis for an assay of its presence. Hemagglutination is inhibited most strongly by α-glucosides and α-galactosides (Stebbins and Hapner 1985). The native

lectin molecule is an aggregate of ~700 kDa consisting of 70 kDa subunits. The subunits were reported to consist of 40 and 28 kDa disulfide linked monomers (Stebbins and Hapner 1985). Amino acid analysis shows relatively high amounts of aspartic and glutamic acid. A significant quantity of glucosamine in the amino acid analysis indicated the presence of carbohydrate associated with the protein. Grasshopper lectin is unstable toward heat, trypsin, and EDTA but , in the case of EDTA, can be reactivated by addition of calcium ion.

The lectin is synthesized in fat body, ovaries, and testes as shown by metabolic incorporation of ^{35}S-methionine into the respective organ cultures (Stiles et al. 1988). This biosynthetic work is being extended with molecular biology methods in this laboratory. Attempts to induce the synthesis of the lectin in cultures were unsuccessful (Stiles et al. 1988). Selective opsonic activity of grasshopper agglutinin toward fungal blastospores, but not toward bacteria, protozoa, or erythrocytes has been observed (Bradley et al. 1989, Wheeler et al. 1993).

Recent unpublished work concerning the grasshopper lectin includes preliminary HPLC, amino acid sequence, additional amino acid analysis, cDNA sequence, Northern analyses, and Southern analyses of nucleic acid. Preliminary HPLC has shown GHA to separate into multiple peaks upon elution. The amino acid sequence of several cyanogen bromide fragments has been determined. An amino acid analysis on reduced and alkylated protein has been completed and compared to amino acid compositions from the cDNAs of two other grasshopper lectins. Southern analysis has provided insight into the

number and structure of lectin genes. Northern analysis and reverse-transcription PCR have yielded confirmatory evidence of lectin synthesis in fat body, ovary, and testes tissues.

Goals

The overall goal of this research is to increase knowledge of the biochemical properties of the grasshopper lectin molecule, with emphasis on its molecular mass and potential glycoprotein character. Specific goals are listed below.

(1). Isolation and purification of GHA: Complete the purification of grasshopper lectin by reverse-phase HPLC, confirm purity by SDS-PAGE and mass spectrometry.

(2). Mass Determination: Measure molecular mass of the purified grasshopper lectin by means of SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS). Compare the two methods.

(3). Glycoprotein character: Demonstrate, through enzymatic treatment and mass analysis, that grasshopper lectin is a glycoprotein and estimate the amount of the carbohydrate moiety

(4). Sulfhydryl content: Estimate the sulfhydryl content of grasshopper lectin through MALDI/TOF-MS analysis of p-hydroxymercuribenzoate (pHMB) alkylation products.

MATERIALS AND METHODS

Isolation and Purification of Grasshopper Lectin (GHA)

Collection of Hemolymph.

Grasshoppers (*Melanoplus differentialis*) were raised from a colony at the USDA/ARS Rangeland Insect Lab at Montana State University (Bozeman) and provided by Elaine Oma. Adult males and females were cold anesthetized for ~15 minutes at 4°C and kept under ice prior to collection of hemolymph. The left fore- and middle legs of the grasshopper were severed and 100µl capillary pipettes were used to draw the emerging hemolymph. The collected hemolymph was added to an equal volume of cold (4°C) Dulbecco's phosphate buffered saline (DPBS; 1.5mM KH_2PO_4, 8mM Na_2PO_4, 0.9mM $CaCl_2$, 2.7mM KCl, 0.5mM $MgCl_2$, 135mM NaCl, pH 7.2) with 1 mg/ml glutathione and 1mM phenylthiourea. The diluted hemolymph was centrifuged at ~3,000 rpm for 5 minutes. Yellow supernatant was transferred to glass vials and stored at -20°C until use Hemocytes and coagulum in the bottom of the microfuge tubes were discarded. This procedure closely follows that used by Wheeler et al. (1993).

Affinity Chromatography.

Affinity chromatography-purification of GHA followed the procedure set forth by Wheeler et al. (1993) with several deviations. Thawed hemolymph was filtered through glass wool and centrifuged at 12,000 rpm for 20 minutes to remove small particulates and suspended lipid. Hemagglutination activity was assayed to confirm initial activity and as a reference for further purification steps. Sepharose beads complexed with D-galactose were added to the supernatant at ~0.6 ml for each 10 ml of supernatant. The mixture was gently agitated for several hours, centrifuged, and the supernatant tested for agglutination activity. Minimal activity (2^1 titre) indicated that most of the agglutinin had bound to the beads and thus indicated the end point of the incubation.

The beads were transferred to a 0.5 cm i.d. by 10 cm chromatography column and effluent absorbancy was monitored at 280 nm. The column was washed with application buffer (0.01M HEPES, 0.2M NaCl, 0.001M $CaCl_2$, pH 7.2) to remove non-absorbed material. Upon achieving a low baseline, elution buffer (0.01M HEPES, 0.2M NaCl, 0.001M EDTA, pH 7.2) was applied to elute the calcium-dependent, galactose-binding proteins at a rate of 0.5 ml/minute. Fractions were collected in 10 drop aliquots of ~200 µl total volume. Effluent was collected in tubes containing 5 µl 1M $CaCl_2$ yielding ~20mM final calcium concentration to restore hemagglutination activity. Tubes containing activity were combined and refrigerated for later HPLC separation. Freezing

of the affinity chromatography-purified GHA was avoided because sometimes the lectin partially precipitated.

Reverse Phase-HPLC.

Affinity chromatography-purified GHA was chromatographed on a LC-18 Vydac© narrow bore column (2.1mm X 25cm) with a High Performance Guard Column (Protein and Peptide C18, 5 micron narrow-bore cartridge). Solvents (A: 10% acetonitrile, 0.06% trifluoroacetic acid; B: 85% acetonitrile, 0.055% trifluoroacetic acid) were eluted at 100 µl/minute via two Shimadzu LC-6A Liquid Chromatograph pumps controlled by a Shimadzu SCL-6A System Controller. Monitoring was done at 280nm by a Spectroflow 757 Absorbance Detector (ABT Analytical, Kratos Division) and the Shimadzu C-R6A Chromatopac integrator/chart recorder. Samples were injected via a 100 µl injection loop connected to a #7125 Rheodyne manual injector port.

Manually collected peak volumes ranged from 40 to 200 µl. The elution profile consisted of 5 minutes 100% solvent A, changing to 60% B through a period of 15 minutes, changing to 100% B within another 45 minutes, and then back to 100%A in the next ten minutes. Collected peaks were stored at -20°C until use.

Hemagglutination Assay

Human Asialo Erythrocyte Preparation.

Preparation of asialo erythrocytes follows the procedure set forth by Stebbins and Hapner (1985) with minor changes. Approximately 2ml of human O positive blood (from the local hospital) were washed into a 15ml centrifuge tube with ~5ml of ice cold DPBS. The volume was adjusted to 12ml and the erythrocytes were suspended by gentle inversion. The cells were centrifuged for 4 minutes at 4000 rpm. The supernatant was discarded and the cells were resuspended in 12 ml of DPBS. Washing was repeated four times. After the last supernatant was discarded, the cells were resuspended in 10 ml DPBS adjusted to pH 5.5-6.0. One mg of neuraminidase (EC 3.2.1.18, Type V from Clostridium perfringens, Sigma) dissolved in 1 ml of DBPS, pH 5.5-6.0, was added and gently mixed. The tube was incubated for 10 minutes at 37°C and inverted every 2 minutes. The tube was cooled on ice for 10 minutes and then the cells were washed 3 times as before. The cells were resuspended in DPBS at a concentration of 2.5% (v/v) for use in the hemagglutination assay.

Assay Procedure.

The hemagglutination titre was conducted by serial two-fold dilution, in plastic V-bottom titer dishes, of 25 µl of GHA with 25 µl of DPBS through 11 wells using the 12th well as a control (Stebbins and Hapner 1985). Suspended asialo erythrocytes (25 µl) were immediately added to each well. After 30 minutes, the wells were observed for possible red cell agglutination. Agglutinated wells showed a button of cells in the center of the well. Non-agglutinated cells did not group together and remained evenly spread (carpeted) over the entire sloping bottom of the well. The titre was designated as 2 to the power of the last agglutinated well.

Mass Determination

SDS-PAGE.

SDS-PAGE was performed with BioRad 10% 8X10 cm X 0.75 mm Readygels using a BioRad Mini-Protean II gel electrophoresis apparatus. Conditions were according to Laemmli (1970). Samples were denatured by boiling 3 minutes at 95°C in a volume ratio of 1:1 with BioRad sample buffer that did or did not contain 5% β-mercaptoethanol. Electrophoresis was conducted at 75 volts (constant) and 25 mAmps (initial) for 1.5 hours. Standards consisted of Novex Mark12 MW Standards [myosin (200 kDa), β-

galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.3kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), B chain bovine insulin (3.5 kDa), A chain bovine insulin (2.5kDa)]. Gels were stained with 0.125% Commassie Blue R-250 (w/v) in 50% methanol/ 10% acetic acid for 30 minutes, and destained with 5% methanol/ 7% acetic acid. After 15 minutes incubation in 5% glycerol, gels were placed on filter paper and dried in a BioRad gel dryer.

MALDI/TOF-Mass Spectrometry.

Instrumentation. All work was accomplished on the PerSeptive Biosystems' Voyager™ Biospectrometry™ Workstation. The instrument contains a N_2 laser at 337nm (3 nanosecond pulses with up to 20 pulses per second) and an ion source capable of 30,000 volts, which allows for repulsion of sample ions from the sample plate. The impacts of ions were detected by a time-of-flight detector and stored in a Tektronix TDS-520A, two channel digitizing oscilloscope (500mhz, 500 MS/s). Stored data was transferred to a Gateway2000, 4DX-33V personal computer for processing via Grams/386 (version 3.0) software. Instrument settings for analysis were optimized from the manufacturer's recommendations for BSA. The accelerating voltage was set at 30,000 volts, the guide wire voltage at 0.30% of the accelerating voltage, and the grid voltage at

90.0% of the accelerating voltage. Linear mode was used for all spectra. The low mass gate and laser step settings were adjusted for each individual sample.

Sample Preparation. Analytes were in the range of 1 to 4 pmol/μl. Matrix was added to the analyte in a ratio ranging from 1:1 to 6:1. Sinapinic acid was used as the matrix for all experiments. Both analyte and matrix were added to a microfuge tube and vortexed for several seconds. Variations in the amount of analyte used per spot were accomplished by successive additions of analyte-matrix mixture to the same position on the sample plate, with intermittent drying. By using this method it is possible to increase the amount of analyte available without requisite increases in sample concentration. Samples were spotted on the sample plate using both "dried-drop" (air drying) and rapid crystallization (vacuum drying) methods . Protein standards, bovine serum albumin (Sigma, Fraction V), myoglobin (Sigma), and β-lactoglobulin (Sigma), were used in the 0.1 pmol to 10 pmol range. Data collected from the mass spectra of these standards was used to calibrate the instrument with the Grams/386 software. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Aldrich), 10 mg, was dissolved in 567 μl water, 333 μl acetonitrile, and 100 μl 0.1% TFA for preparation of the matrix solution.

Glycoprotein Deglycosylation

The enzyme used was PNGase F (peptide- N-glycosidase F) cloned from *Flavobacterium meningosepticum*. The procedure follows that recommended by the manufacturer of the enzyme, Oxford Glycosystems. Six microliters (8 ug) of protein was mixed with 1 µl of 5X incubation buffer (100 mM sodium phosphate, pH 7.5, with 250 mM EDTA and 0.02% NaN_3) and 4 µl of a solution of aqueous 0.6% SDS and 6 % BME (β-mercaptoethanol). This solution was vortexed for several seconds and centrifuged. The reaction mixture was boiled 3 minutes, cooled on ice, and centrifuged. Two microliters of 10% n-OG (n-octylglucoside, n-octyl-β-D-glucopyranoside, Sigma) were added to neutralize the SDS and the solution was boiled 3 minutes, cooled, and centrifuged. Four microliters, (2 units), of PNGase F was added and the tube centrifuged. The reaction was placed in a 37°C water bath for 18-24 hours. Reaction products were subjected to SDS-PAGE.

For mass determination of deglycosylation products by MALDI/TOF-MS, sodium dodecylsulfate (SDS) was omitted from the reaction and TCEP (tris-(2-carboxyethyl) phosphine hydrochloride, Pierce) was substituted for the reducing agent, β-mercaptoethanol. Four µl of 100 mM TCEP and 2 µl of 10% n-OG were added to the

protein sample prior to boiling for 3 minutes. After cooling and centrifugation, 2 units of PNGase F were then added. The reaction was incubated for 18-24 hours.

Ribonuclease B (Sigma), used as a control protein, was also subjected to PNGase F treatment. The protein (10 µl of 1 mg/ml), 1 µl of 5X buffer, and 1 µl of TCEP (0.4 mM in water) were heated for 5 minutes at 95°C. The reaction was cooled on ice and centrifuged before adding 0.5 units of PNGase F. Reactions were incubated 18-24 hours at 37°C, before subjecting to MALDI/TOF-mass spectrometry.

Reduction of Disulfide Bonds

GHA1a and GHA1b were reduced for molecular mass analysis of their respective monomers by MALDI/TOF-mass spectrometry. Aliquots of GHA1a and GHA1b (20 µl, ~5 µg) in HPLC solvent (pH ~3) were reduced with 4 µl of aqueous 100 mM TCEP overnight at 30°C. Reaction products were subjected to MALDI/TOF-MS. Reduction of insulin was used as a control for the reduction of GHA under similar conditions. Procedures followed work by William R. Gray (1993).

Alkylation of Cysteine Residues

Free sulfhydryl content of GHA1a and GHA1b was investigated through alkylation of cysteines by p-hydroxymercuribenzoate (pHMB). Our procedure followed the protocol set forth by Zaluzec, Gage, and Watson (1995) with minor changes. Para-hydroxymercuribenzoate (pHMB, Sigma) was washed with cold trifluoroacetic acid (TFA) three times to remove contaminating mercury salts prior to use for alkylation reactions. Six microliters (~18 pmol) of GHA1a or GHA1b were incubated with 1 µl of 1389 pmol/µl pHMB in 2% NH_4OH (~77X molar excess over protein) at 30°C and pH 8.5 Aliquots were taken at 5, 10, and 20 minutes and mixed with an equal volume of matrix. The analyte/matrix mixtures were subjected to MALDI/TOF-MS.

β-Lactoglobulin was used as a control protein for alkylation of its single sulfhydryl group. Ten-fold excess pHMB (1 µl of 277 pmol/µl in 2% NH_4OH) was added to 10 µl, ~25 pmols, HPLC purified β-lactoglobulin (Sigma) and allowed to react for ten minutes. Reaction products were subjected to MALDI/TOF-MS.

Attempts were made at determining total cysteine content of GHA1a and GHA1b by reacting 2 µl (~6 pmol) of GHA1a or GHA1b on the sample plate with 1 µl of 3490 pmol/µl TCEP (~60X molar excess over cysteine) for 10 minutes at room temperature

(~20°C). pHMB (1 μl of 5490 pmol/μl) was then added to the mixture and allowed to react for 10 minutes. Mass determination was carried out with MALDI/TOF-MS.

The ability to alkylate all cysteines in a protein was investigated using reduced β-lactoglobulin and reduced and separated insulin chains. Procedures for alkylation of each control protein with pHMB followed work by Zaluzec et al. (1995) and was similar to the above procedures.

RESULTS

Isolation and Purification of Grasshopper Agglutinin

Elution of the Sepharose-D-galactose affinity matrix with 1mM EDTA resulted in a single broad peak that was off scale at 1 OD and 280nm as seen in Figure 1. About 25ml of diluted hemolymph (approximately 200-300 grasshoppers) was used in a single preparation. A total yield of ~400 μg protein normally resulted. Elution occurs because EDTA binds the Ca^{2+} ion necessary for carbohydrate-binding affinity. Loss of calcium results in the loss of carbohydrate-binding affinity and consequent desorption of the lectin proteins. A hemagglutination titre of 2^{11} from the main peak fraction showed that activity was restored successfully by eluting the fractions into 20mM Ca^{2+}. The high concentration of Ca^{2+} saturated the EDTA present and bound to the protein ligands restoring activity. The affinity chromatography-purified GHA was stored at 4°C to avoid precipitation of the protein prior to further purification by HPLC.

The affinity chromatography-purified GHA was further fractionated by reverse-phase HPLC. Figure 2 shows the separation of affinity chromatography-purified GHA into 3 separate peaks within 50 minutes via C18 reverse phase-HPLC. Elution of the lectin began at approximately 72% acetonitrile with an initial large split peak followed by two smaller peaks. The split peak was designated GHA1a and GHA1b. The following

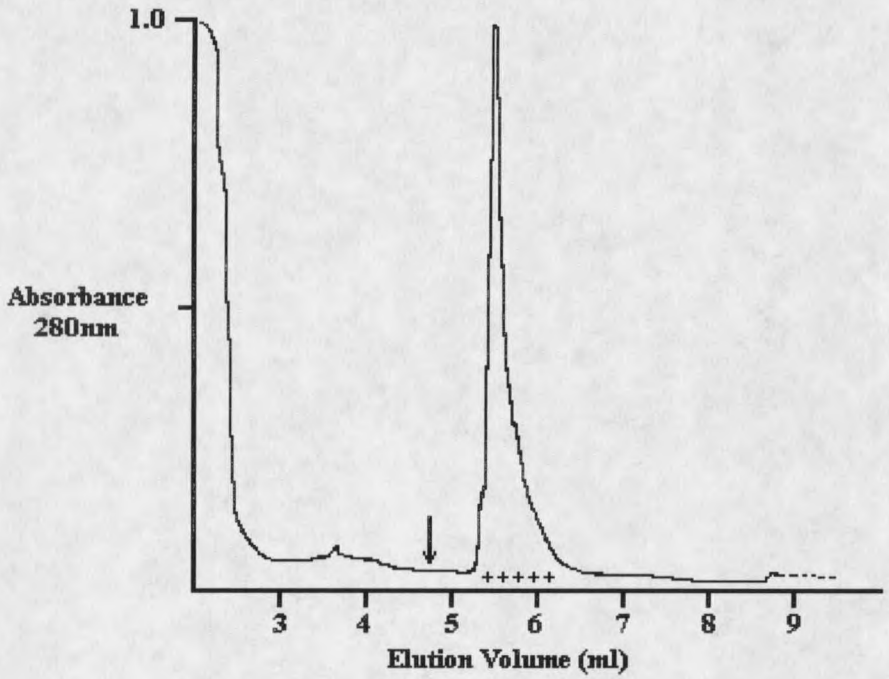

Figure 1. Affinity chromatography of GHA on Sepharose -D-galactose. Initially the addition of beads to the column caused the absorbance to rise but the absorbance returned to near zero before buffer containing EDTA was added (arrow) to elute the agglutinin. A single protein peak was eluted and approximately 2ml total volume was collected in Ca^{2+} to restore hemagglutination activity. The pluses (+ + + +) indicate effluent with significant hemagglutination activity. The collected fraction was stored at 4°C prior to separation on reverse-phase HPLC. Sodium azide was added to the samples to prevent microbial contamination while in storage. Approximately 400 μg of total protein was isolated from the 25ml of diluted hemolymph used in this preparation.