



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

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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, GHLa and GHAb. Masses of GHLa and GHAb are measured to ~62.6 kDa using SDS-PAGE while MALDI/TOF-MS shows 72.0 and 72.1 kDa, respectively. Reduction of GHLa and GHAb 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 GHLa and GHAb 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 GHLa and GHAb to be essentially indistinguishable. The basis for their separation on reverse-phase HPLC is unknown, but possibly related to carbohydrate content.

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MONTANA STATE UNIVERSITY - BOZEMAN
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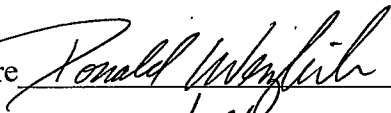
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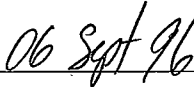
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ABSTRACT

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).

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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, dipterocins, defensins (sapeccins), ceratotoxins, 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 *Beauveria 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 -C 2_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 fimbriae 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 $[\text{NH}_4]_2\text{SO}_4$ 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 KH₂PO₄, 8mM Na₂PO₄, 0.9mM CaCl₂, 2.7mM KCl, 0.5mM MgCl₂, 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 μ l of GHA with 25 μ l of DPBS through 11 wells using the 12th well as a control (Stebbins and Hapner 1985). Suspended asialo erythrocytes (25 μ l) 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' VoyagerTM BiospectrometryTM Workstation. The instrument contains a N₂ 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/ μ l. 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 μ l water, 333 μ l acetonitrile, and 100 μ l 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% NaN₃) 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 μ l of 1 mg/ml), 1 μ l of 5X buffer, and 1 μ l 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 μ l, ~5 μ g) in HPLC solvent (pH ~3) were reduced with 4 μ l 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 μ l of 1389 pmol/ μ l 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 μ l of 277 pmol/ μ l in 2% NH_4OH) was added to 10 μ l, ~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 μ l (~6 pmol) of GHA1a or GHA1b on the sample plate with 1 μ l of 3490 pmol/ μ l TCEP (~60X molar excess over cysteine) for 10 minutes at room temperature

(~20°C). pHMB (1 μ l of 5490 pmol/ μ l) 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

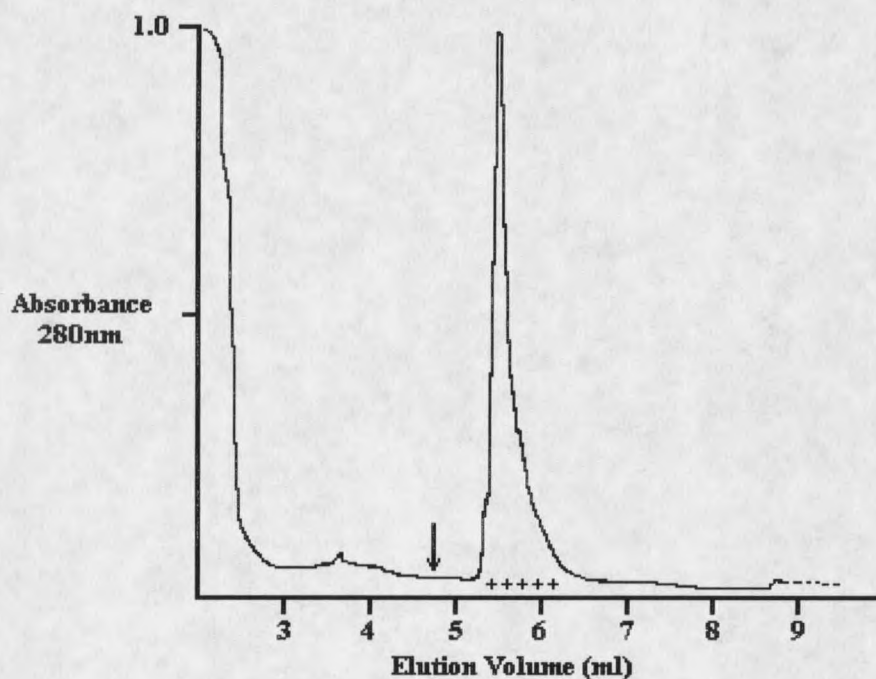


Figure 1. Affinity chromatography of GHA on Sepharose -D-galactose. Initially the addition of beads to the column caused the absorbance to rise but the absorbance returned to near zero before buffer containing EDTA was added (arrow) to elute the agglutinin. A single protein peak was eluted and approximately 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 $400\text{ }\mu\text{g}$ of total protein was isolated from the 25ml of diluted hemolymph used in this preparation.

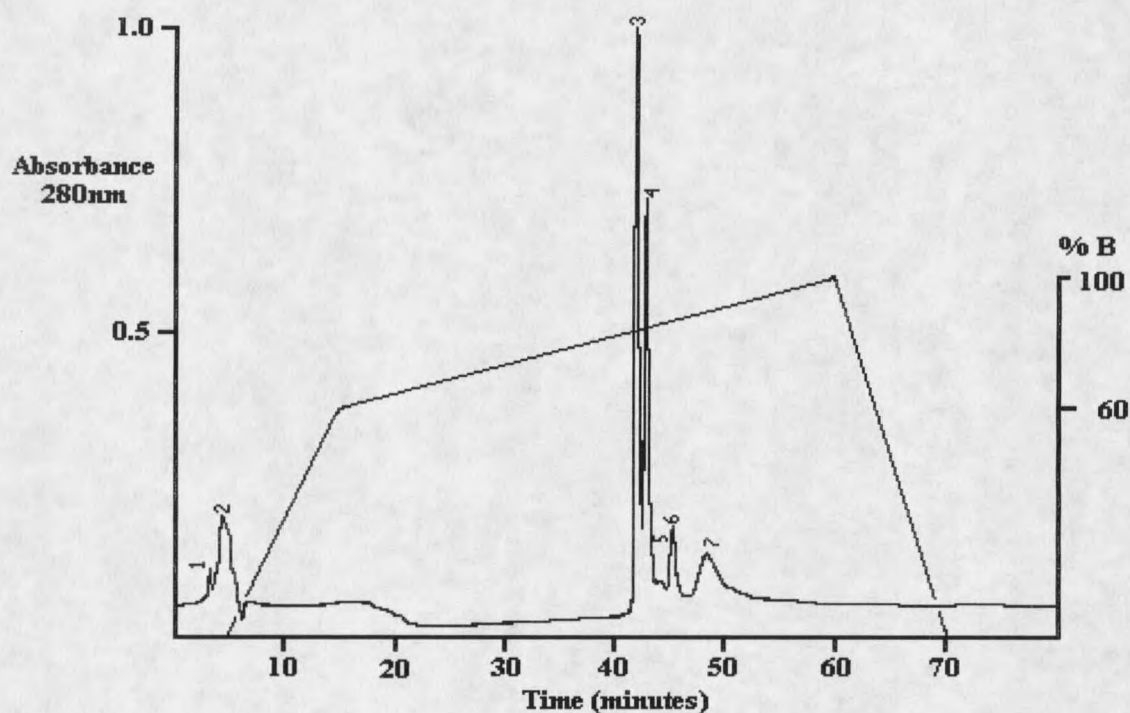


Figure 2. Separation of affinity chromatography-purified GHA by reverse phase-HPLC. The prominent initial split peak elutes at ~72% acetonitrile and accounts for ~75% of the total protein. This peak is collected in two fractions and labeled GHA1a (3) and GHA1b (4). The separation time between them is ~0.5 minutes. Sometimes the GHA1a peak was approximately equal in height of the GHA1b peak (see text), however they are always separated by ~0.5 minutes. Two additional small, broad peaks, designated GHA2 (6) and GHA3 (7), are eluted at higher acetonitrile concentration. Peaks 1 and 2 represent buffer salts and preservative at the column void. Peak 5 is tiny and was not collected. The solvent gradient used for these separations is shown by the wavy line. The collected fractions were stored at -20°C.

two smaller peaks were designated GHA2 and GHA3. These four fractions were manually collected for later analysis and stored at -20°C. The split peak constituting GHA1a and GHA1b contains approximately 75 percent of the total protein. GHA1a is the most abundant protein of the four fractions and is recovered in a ratio of ~1.3 to 1 over GHA1b, the next most abundant fraction. Occasionally GHA, that had been stored for long periods, was observed to have larger amounts of GHA1b relative to GHA1a, suggesting possible interconversion of 1a into 1b. No satisfactory explanation for this is available, however speculations are made later (see Discussion). Typical volumes of the collected fractions were ~40µl for GHA1a and ~70µl for GHA1b. Peaks corresponding to GHA2 and GHA3 are broad and were collected in volumes of ~100µl for GHA2 and ~200µl for GHA3.

Protein Mass Determination

SDS-PAGE

Molecular masses were determined using two different but complementary techniques: SDS-PAGE and MALDI/TOF-MS. Molecular weights have been determined for both the GHA1a and GHA1b fractions from HPLC on polyacrylamide gel electrophoresis. Figure 3 shows GHA1a and GHA1b to migrate as single pure bands of 60.1 kDa with an average of 62.6 kDa for multiple measurements. Figure 3 also shows

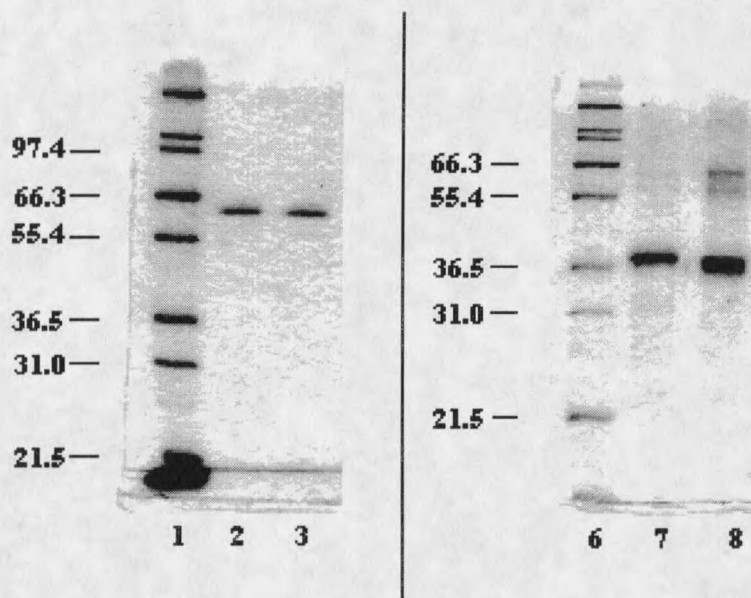


Figure 3. SDS-PAGE of GHA1a and GHA1b under reducing and non-reducing conditions. Two PAGE experiments are shown here with the non-reducing conditions on the left and reducing conditions on the right. Lanes 1 and 6 contain MW standards. Lanes 2 and 3 contain HPLC separated GHA1a and GHA1b respectively. Lanes 7 and 8 contain the same GHA1a and GHA1b material as lanes 2 and 3 but they have been reduced with BME. (A) Average masses of the dimers from multiple experiments are 62.6 kDa. No mass differences are observed between dimeric GHA1a and GHA1b on SDS-PAGE. (B) The average masses of the GHA1a-SH and GHA1b-SH samples are 36.9 kDa (from multiple experiments). Averages were compiled from measurements of multiple runs using the same type of gel.

that reducing SDS-PAGE also produces single bands, identical in migration, with a mass of 36.9 kilodaltons for GHA1a and GHA1b. These results suggest that each protein is composed of two monomers having equivalent masses.

MALDI/TOF-Mass Spectrometry

A mass spectrum of the affinity chromatography-purified lectin was obtained through MALDI/TOF-MS and shown in Figure 4. The large peak at 72.2 kDa is the singly-charged peak. This peak is sharp but some spreading on each side of its base indicating additional mass species. A small peak at ~144 kDa is consistent with a dimer peak of the singly-charged mass peak. The mass of 49.5 kDa is not a multiple of the 72.2 kDa peak and can only be another singly-charged protein species. The 36.1 kDa peak is likely a doubly-charged peak of the 72.2 kDa peak suggested by its appropriate mass and peak intensity. The peak with the smallest mass, 25.8 kDa, is in the correct mass range for being a triply-charged peak of the prominent 72.2 kDa peak but could also be a doubly-charged species resultant from the 49.5 kDa protein species. These results indicate the presence of two or more species in the affinity chromatography-purified GHA.

Figure 5 shows a comparison of affinity chromatography-purified GHA and the individual HPLC fractions subsequently collected. Each mass peak from the individual fractions of HPLC-separated GHA can be accounted for in the mass spectra of affinity-purified GHA. Protein from the GHA1a and GHA1b fractions presents itself in the mass

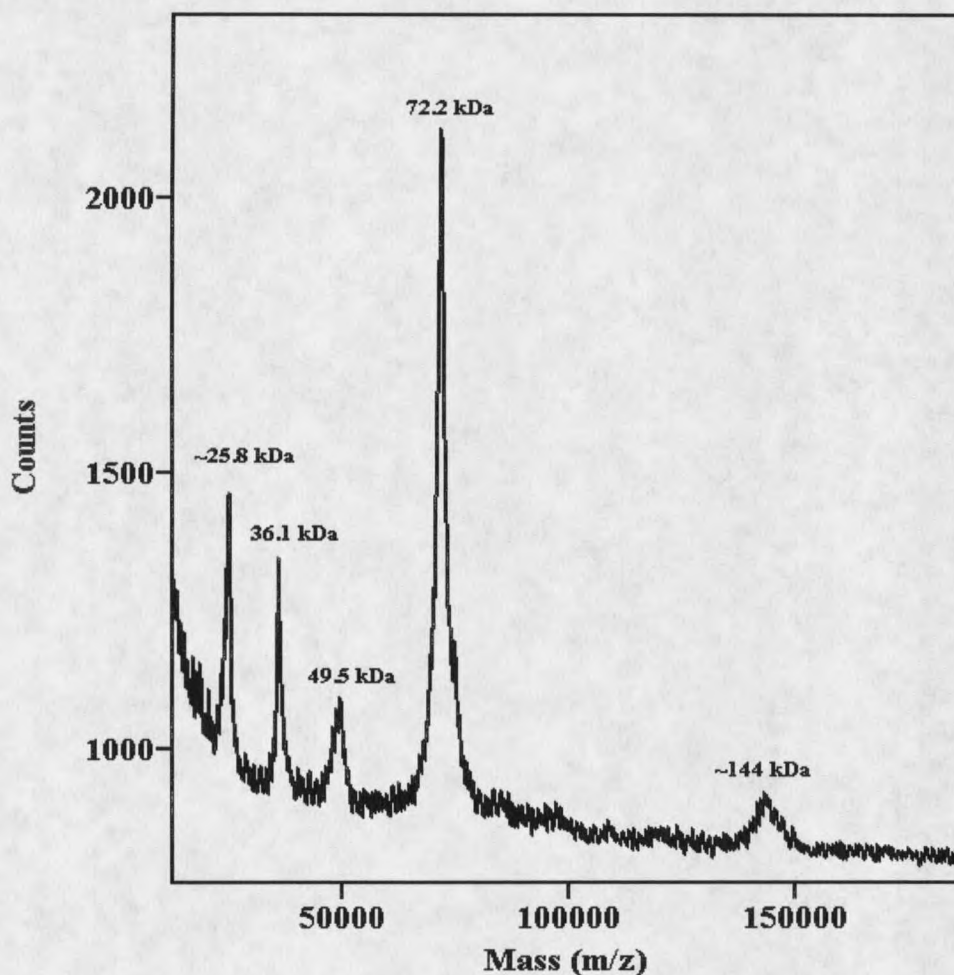


Figure 4. MALDI/TOF-mass spectrum of GHA prior to HPLC separation. Two protein species are evident in this spectrum. The most intense peak corresponds to a GHA protein having a mass of 72.2 kDa. This main protein species also creates a dimer peak at ~144 kDa, a doubly-charged mass specie at 36.1 kDa, and a triply-charged mass specie at ~25.8 kDa. The peak at 49.5 kDa is not a multiple of the 72.2 kDa peak and thus must be another protein. This mass peak can also generate a doubly-charged mass specie at 25.8 kDa, adding to the total intensity of that peak.

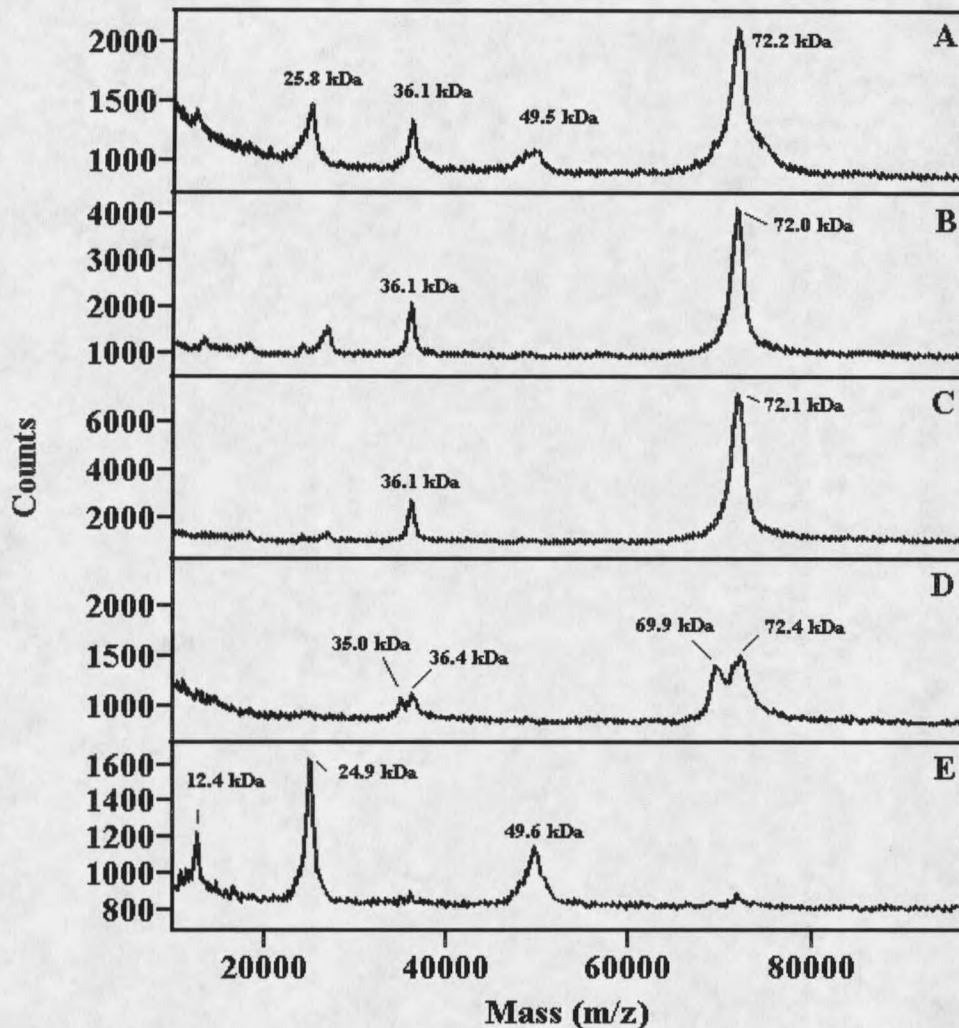


Figure 5. Comparison of mass spectra from GHA, GHA1a, GHA1b, GHA2, and GHA3. (A) GHA prior to HPLC. (B) GHA1a. (C) GHA1b. (D) GHA2. (E) GHA3. All mass peaks found in the fractions can be accounted for in the mass spectra of affinity purified GHA prior to HPLC shown in (A). The 72.2 and 36.1 kDa peaks in (A) are a composite of signals from (B), (C), and (D). The slight peak broadening and presence of shoulders in (A) is due to multiple species of slightly differing masses present in (B), (C), and (D). GHA3 is the only HPLC fraction that contributes the signal for the 49.5 kDa mass peak in (A). The doubly-charged mass specie for GHA3 is seen in the 25.8 kDa mass peak of (A). The peak also includes small amounts of the triply-charged mass species from GHA1a and GHA1b. The 12.4 kDa specie seen in (E) is a doubly-charged specie of the mass peak at 24.8 kDa in GHA3.

spectra of the affinity-purified GHA at masses of ~72.2, 36.1, and 25.9 kDa. The species seen at 49.5 kDa in the mass spectrum of the pre-HPLC GHA can only be attributed to the GHA3 fraction. The GHA3 fraction also gives rise to signal at the 25.9 and 12.5 kDa mass species. Broadening of peaks at 72.2 and 36.1 kDa in the GHA mass spectrum can be attributed to ionization of multiple protein species including GHA1a, GHA1b, and 2 species from GHA2.

The mass of GHA1a is found to be 72.0 kDa as shown in Figure 6. This value comprises an average of 36 values from separate measurements with a standard deviation of 0.2 kDa. The doubly-charged peak's mass was determined to be 36.1 kDa (standard deviation of 0.2 kDa). Triply-charged ($[M+3H]^{3+}$) and quadruply-charged ($[M+4H]^{4+}$) peaks are also seen and have masses of 26.4 and 17.3 kilodaltons respectively. Also present in the spectrum is the singly-charged dimer ($[2M+H]^+$) ion, which is formed through the ionization of noncovalent dimers of the dimer. It has a mass approximating twice that of the dimer, ~144 kDa. All of the mass peaks present are derived from a single, pure protein.

The mass of GHA1b is determined to be 72.1 kDa as seen in Figure 7. The mass values are an average of 35 separate measurements with a standard deviation of 0.2 kDa. The doubly-charged peak had a mass of 36.1 kDa (standard deviation of 0.1 kDa). Masses of the triply-charged and quadruply-charged peaks have also been determined but are not shown in Figure 7. They have masses of 25.8 and 17.0 kilodaltons, respectively. Also

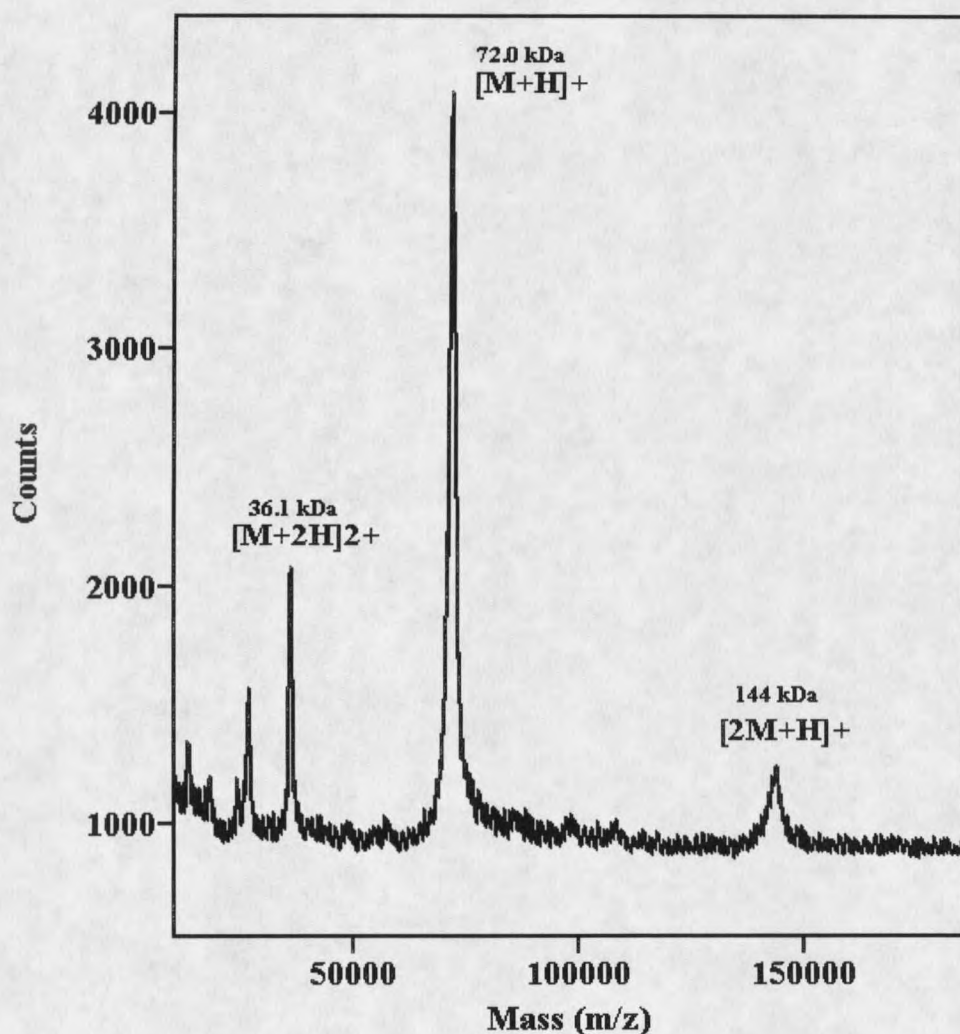


Figure 6. MALDI/TOF-mass spectrum of the GHA1a HPLC fraction. The prominent peak at 72.0 kDa represents singly-charged protein; all other peaks on this spectrum are derived from this single protein. The peak at ~144 kDa represents a singly-charged dimer of the 72.0 kDa protein peak sometimes seen at high laser power settings. The smaller peaks at 36.1 kDa and below are multiply-charged protein species of the 72.0 kDa protein mass peak.

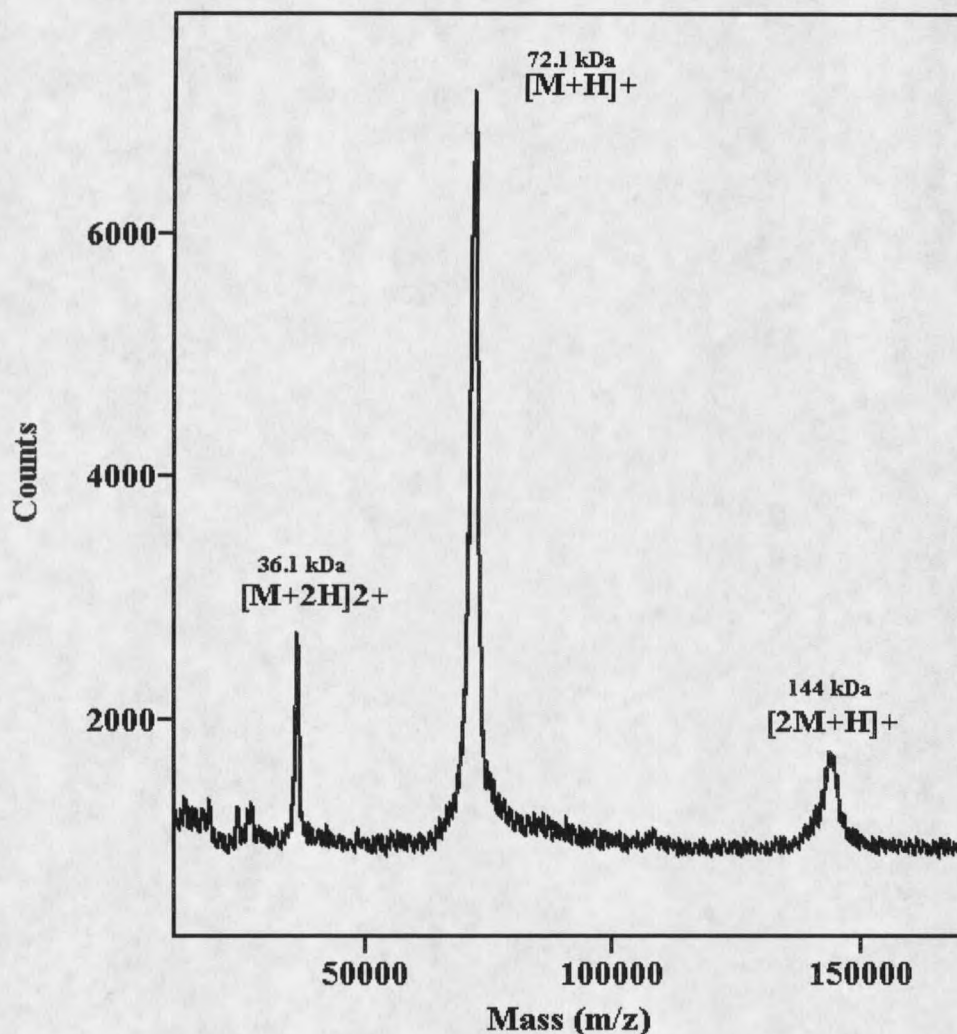


Figure 7. MALDI/TOF-mass spectrum of the GHA1b HPLC fraction. The protein mass peak at 72.1 kDa represents a singly-charged protein specie. All of the other mass peaks observed are multiply-charged or dimer species of this protein. The sharp, symmetrical peaks indicate a single, pure protein sample. The peak at ~144 kDa is a dimer ion and the peak at 36.1 kDa is a doubly-charged specie of the main protein mass peak. This spectrum is almost identical to the spectrum of GHA1a. Within the limits of our resolution, GHA1a and GHA1b have the same mass.

present in the spectrum is the singly-charged dimer ($[2M+H]^+$) ion at ~144 kDa. This mass spectrum shows the GHA1b HPLC fraction to contain a single, pure protein.

Although not the primary focus of this research, the masses GHA2 and GHA3 have been measured. Figure 5 shows the masses of both proteins present in the GHA2 fraction; 72.4 and 69.9 kilodaltons. Doubly-charged peaks for both proteins were measured and have masses of 36.4 and 35.0 kDa, respectively. The GHA3 fraction also contains multiple proteins as seen in Figure 5. The largest specie is 49.6 kDa and corresponds to a singly-charged protein. The second is 24.9 kDa and likely represents both a singly-charged and a doubly-charged specie. The third is 12.4 kDa and corresponds to a doubly-charged specie of the 24.9 kDa peak. GHA2 contains proteins that are similar in mass and may be related to GHA1a and GHA1b. GHA3 protein masses are distinctly different from the GHA1a and GHA1b protein masses, possibly suggesting that they are non-related proteins that are isolated on galactose affinity matrix or that they bind to the proteins of interest, GHA1a and GHA1b.

Mass spectra of reduced GHA1a (GHA1a-SH) and GHA1b (GHA1b-SH) are shown in Figure 8. Both proteins were reduced using excess TCEP in an overnight reaction to a mass of 36.1 ± 0.1 kDa. Complete reduction to the monomer is evident by the absence of any dimer mass peak at ~72.2 kDa. Also apparent from this side-by-side comparison of GHA1a-SH and GHA1b-SH is their indistinguishable mass value indicating that GHA1a and GHA1b are very similar in size. Both spectra in Figure 8 suggest that GHA1a and GHA1b are homodimers by the presence of only one singly-

charged specie. To determine the length of time needed for complete reduction of the GHA1a and GHA1b dimers, the progress of the reduction was followed. Aliquots of the reaction were taken at various times and measured by MALDI/TOF-MS. Figure 9 shows the progress of reduction on GHA1a by TCEP. Reduction of the dimer is nearly complete by 90 minutes of incubation and is complete after 16 hours. Figure 10 shows an almost identical result for GHA1b, indicating that they act similarly with TCEP.

Evidence supporting reduction of either protein is seen by the growth of the peaks at 36.1 and ~18 kDa and the disappearance of peaks at 72.2 and 26.3 kDa in Figures 9 and 10. MALDI/TOF-MS of proteins produces ions of each protein that are singly- and doubly-charged. Singly- and doubly-charged protein peaks are seen in almost every mass spectrum with the +2 peak being smaller in intensity. The disappearance of the +1 peak in GHA1a or GHA1b at 72.1 kDa must also result in the disappearance of the +2 peak at 36.1 kDa and any other multiply-charged species derived from the 72.2 kDa mass, unless a new protein specie is formed in the process. In this case, the new specie formed is the +1 peak of the monomer of GHA1a or GHA1b at 36.1 kDa. The presence of the 18.1 kDa peak corroborates this result because it is a +2 peak, that is commonly seen in MALDI/TOF- mass spectra.

Reverse phase HPLC separates GHA1a and GHA1b by about 0.5 minutes indicating some chemical difference between the two molecules. An experiment to determine a difference in mass between the two was devised using samples containing different ratios of GHA1a to GHA1b. Changing the ratio of proteins in the mixed

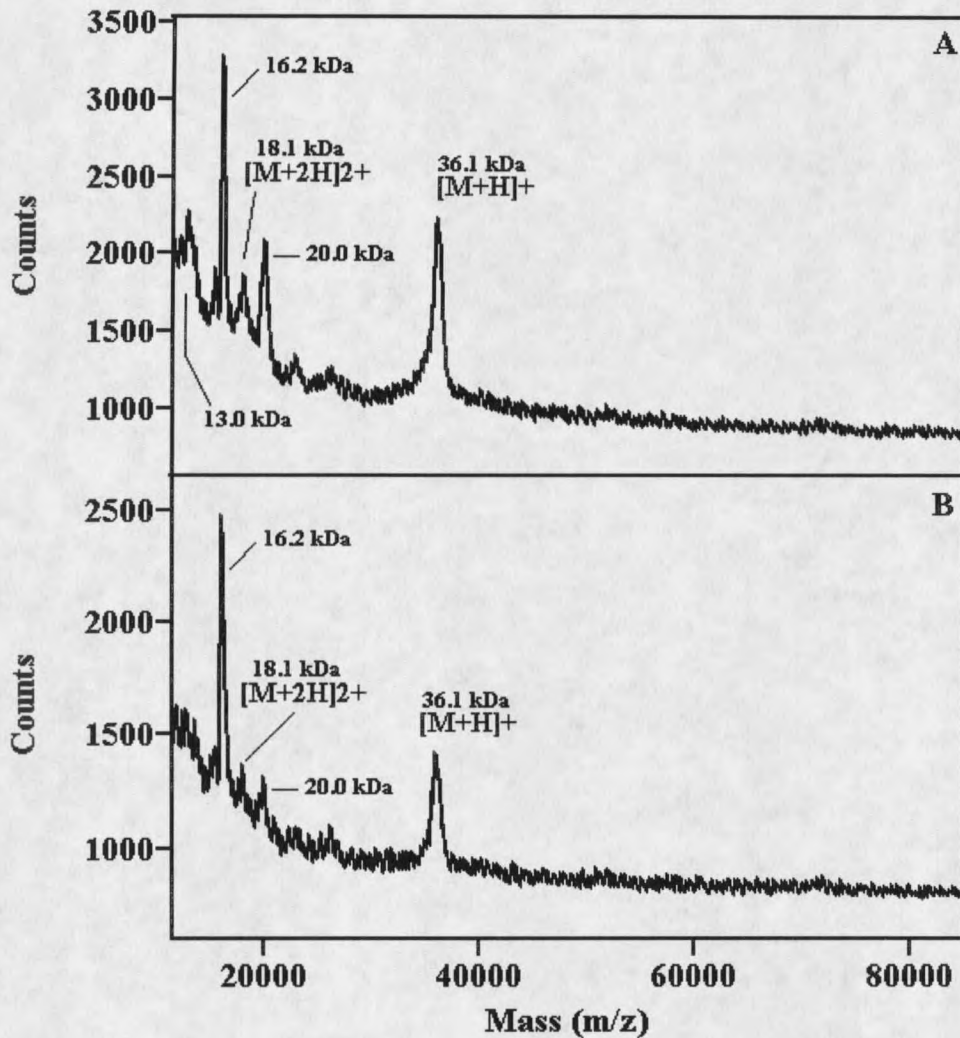


Figure 8. Comparison of the mass spectra of reduced GHA1a and GHA1b. (A) Reduced GHA1a (GHA1a-SH) is confirmed through absence of the dimer mass (72.0 kDa). (B). Reduced GHA1b (GHA1b-SH) is confirmed by the absence of the GHA1b peak at 72.1 kDa. Both proteins were reduced with TCEP. The singly-charged peaks indicate almost identical masses of the reduced proteins. The presence of a single prominent specie in each spectrum implies that GHA1a and GHA1b are homodimers. The peaks at 18.1 and 13.0 (GHA1a-SH only) are multiply-charged specie of the parent peak at 36.1 kDa. The spectra show two unusual masses; 16.2 and 20.2 kDa. The source of these peak is unknown and they do not appear in other spectra of reduction products.

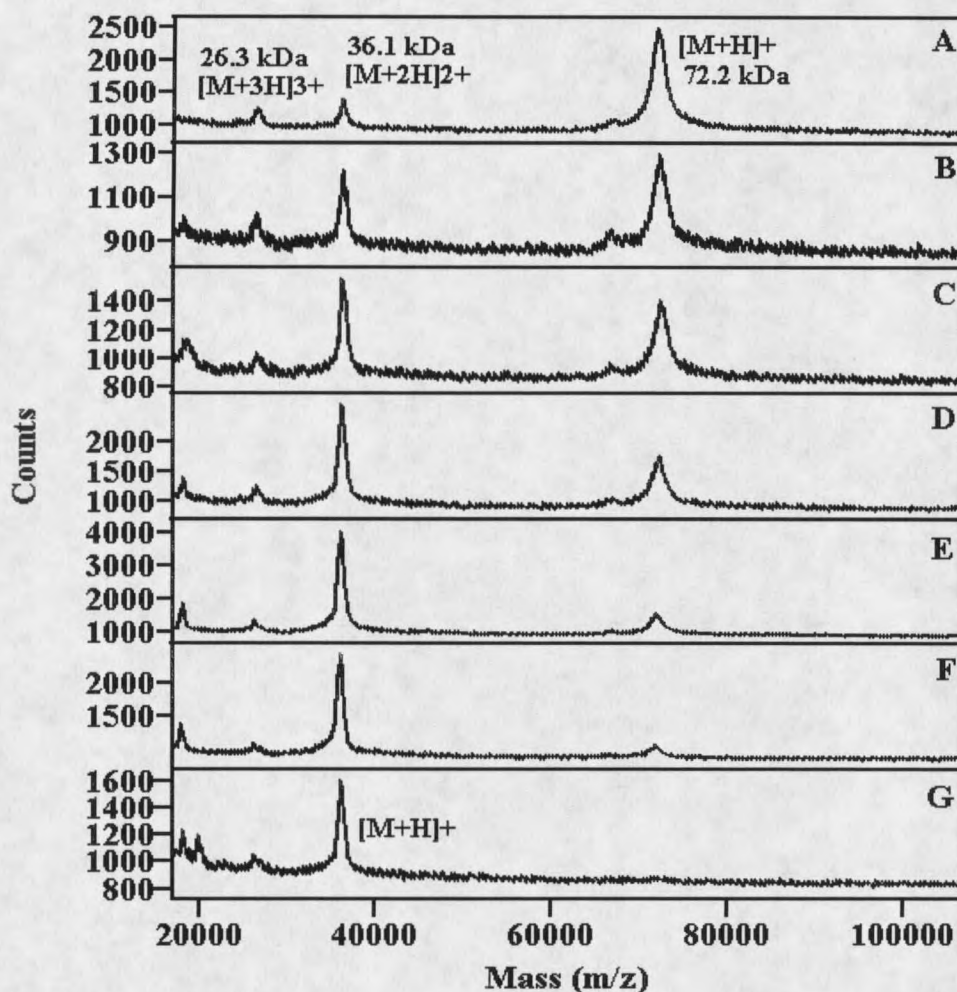


Figure 9. Mass spectra showing the progress of TCEP reduction of GHA1a. (A) Mass spectra of GHA1a prior to reduction, (B) after 10 minutes of incubation with TCEP at 30°C, (C) 20 minutes, (D) 30 minutes, (E) 60 minutes, (F) 90 minutes, and (G) after almost 16 hours of incubation with TCEP. A decrease in the 72.2 and 26.3 kDa masses with a continued presence of the peak at 36.1 kDa and formation of a peak at 18.0 kDa (in G, the last peak on the left) indicates that the reduction has occurred. The 20.0 kDa peak seen in the last spectrum (G) is unknown in origin and does not form in the reduction of GHA1b (Figure 10).

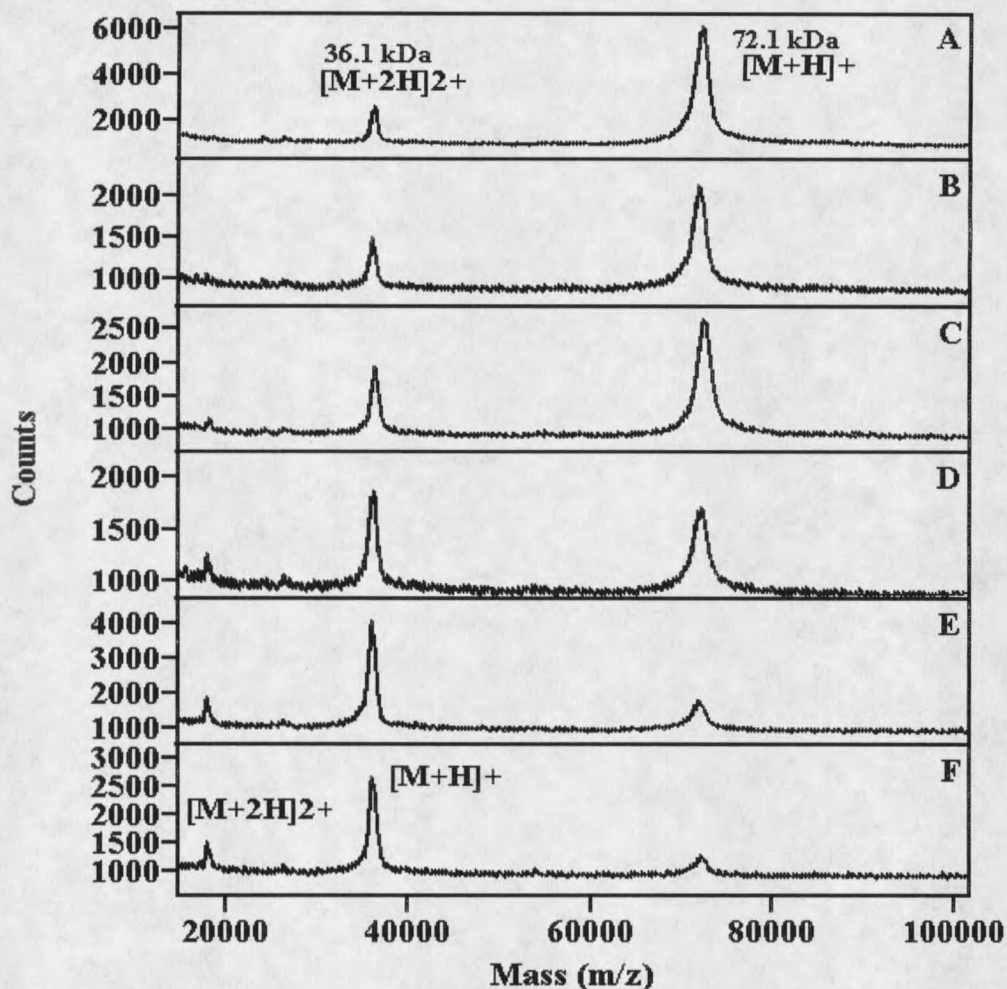


Figure 10. Mass spectra following the progress of reduction of GHA1b by TCEP. (A) Mass spectra of GHA1a prior to reduction, (B) after 10 minutes of incubation with TCEP at 30°C, (C) 20 minutes, (D) 30 minutes, (E) 60 minutes, and (F) after 90 minutes. This reduction to the monomer mass has not been completely fulfilled at 90 minutes but the reduction can be thought to behave similarly to GHA1a's reduction (Figure 9). A decrease in 72.2 and 26.3 kDa peaks with increased presence of 36.1 and 18.0 kDa peaks suggests that reduction of the dimer is occurring. Although not shown in this comparison, the overnight reaction with TCEP has been shown to reduce GHA1b to its monomer form (Figure 8).

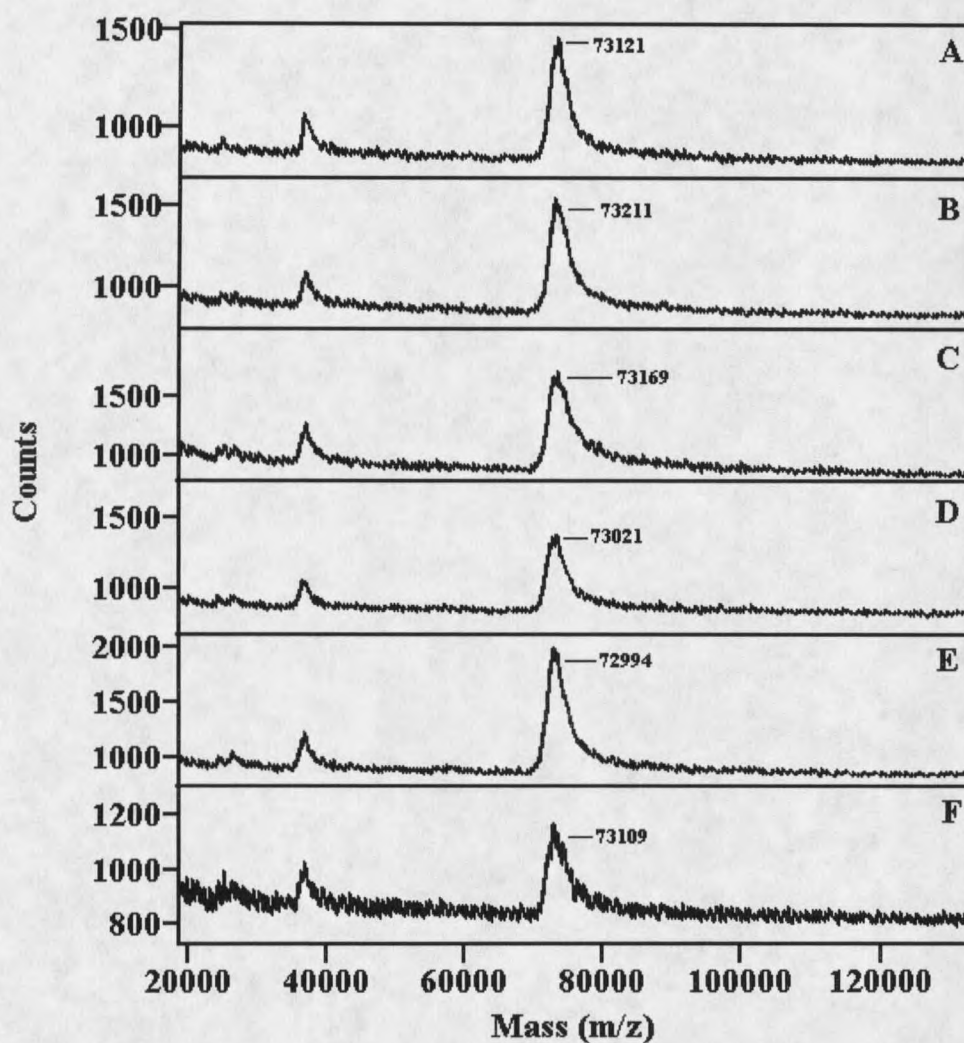


Figure 11. Mass spectra of samples containing known ratios of GHA1a and GHA1b. (A) A sample containing 10 parts GHA1a to 0 parts GHA1b; (B) 8:2; (C) 6:4; (D) 4:6; (E) 2:8; and (F) 0:10. A mass difference between the singly-charged peaks cannot be detected at the current resolution of our protein samples (~ 200 Da). The average of these masses is 73.1 kDa and the range is 217 daltons.

samples did not show a change in mass as a function of sample ratio. All spectra of the mixed samples in Figure 11, show single peaks that have increased peak width when compared to the mass spectra of the individual proteins. Increased width of the peak could suggest the presence of two species of slightly different mass. This suggests that a mass difference, if it exists, between GHA1a and GHA1b is less than 200 daltons, the present limit of our resolution for the GHA1a and GHA1b proteins. It is possible that a difference in carbohydrate content or composition could account for the HPLC separation of the two peaks.

Comparison of Masses Values from SDS-PAGE and MALDI/TOF-MS

Masses of GHA1a or GHA1b determined through SDS-PAGE and MALDI/TOF-MS are substantially different. The differences shown in Table 1 are thought to be related to the anomalous migration of glycoproteins on SDS-PAGE. Values for the masses of the GHA1a and GHA1b dimers varied over a range of ~14 kilodaltons on SDS-PAGE but only 900 daltons on MALDI/TOF-MS for all measurements taken during this project. These ranges account for ~10% and 0.6% of the total mass of the protein, indicating that MALDI/TOF-MS had greater reproducibility.

Table 1. Comparison of GHA1a and GHA1b Masses From Different Techniques (masses in kilodaltons).

	MALDI/TOF-MS*	SDS-PAGE [#]	Percent Difference
GHA1a	72.0 ± 0.2	62.6 ± 2	13.1
GHA1a-SH	36.1 ± 0.1 [†]	36.9 ± 2 [§]	2.2
GHA1b	72.1 ± 0.2	62.6 ± 2	13.3
GHA1b-SH	36.1 ± 0.1 [†]	36.9 ± 2 [§]	2.0

Notes: [†] Protein reduced with TCEP. [§] Protein reduced with β -mercaptoethanol. * These masses are averages of multiple experiments. [#] The values presented are the average of masses determined over multiple experiments on 10% BioRad ReadyGels.

Glycoprotein Content

Confirmation of the glycoprotein character of GHA1a and GHA1b was attempted through enzymatic cleavage of N-linked carbohydrate and measurement of the resultant mass changes. Peptide-N-glycosidase F (PNGase F) was used to cleave the N-linked carbohydrate from GHA1a and GHA1b in overnight reactions. Mass measurements on native and deglycosylated GHA1a and GHA1b were done with SDS-PAGE. Figure 12 shows the SDS-PAGE of native and PNGase F-treated protein. Mass differences between 2.5 and 2.8 kDa are measured for the GHA1a and GHA1b dimers in repeated reactions. The presence of single bands before and after treatment with PNGase F and a mass shift of the bands after treatment suggests that the removal of carbohydrate was complete. The masses determined for the carbohydrate on the dimer are consistent with the presence of 4 N-acetylglucosamine (GlcNAc) and 10-12 hexose units. This

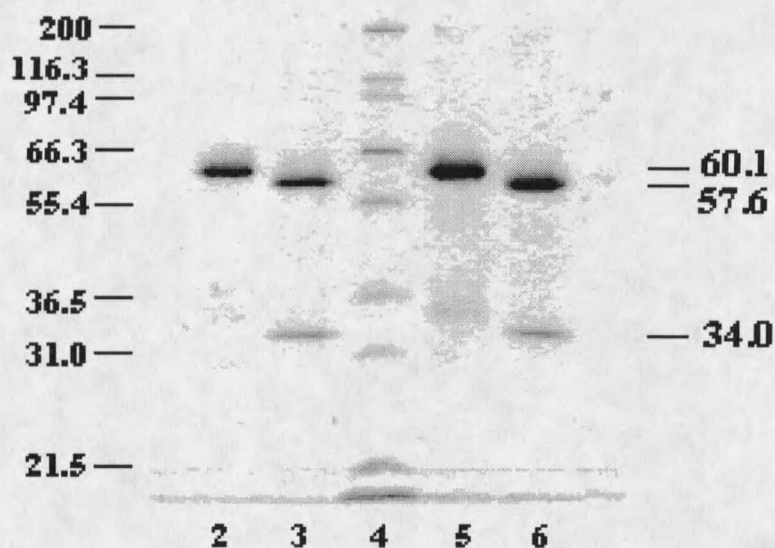


Figure 12. Non-reducing SDS-PAGE of native and deglycosylated GHA1a and GHA1b. Lanes 2 and 3 contain GHA1a. Lanes 5 and 6 contain GHA1b. Lane 4 contains MW standards. Lanes 3 and 6 contain protein deglycosylated with PNGase F. The change in mass assumed to be carbohydrate is approximately 2580 daltons. Other deglycosylation reactions with PNGase F on GHA1a and GHA1b dimers show carbohydrate masses from ~2.5 to 2.8 kDa as measured with SDS-PAGE. All protein samples show single bands. This indicates both sample homogeneity and complete removal of the carbohydrate by PNGase F.

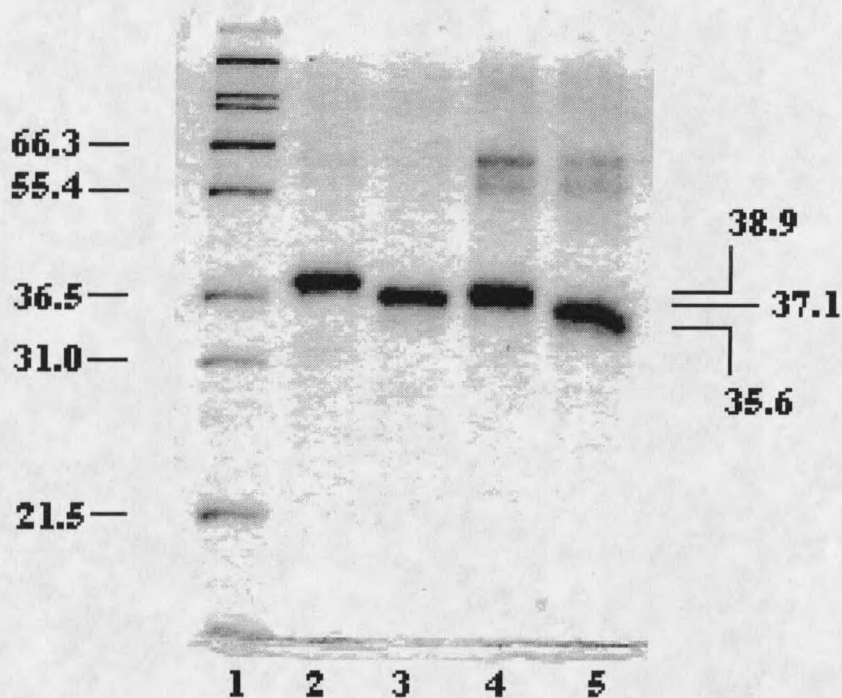


Figure 13. Reducing SDS-PAGE of GHA1a and GHA1b before and after deglycosylation. Lane 1 contains molecular weight standards. Lanes 2 and 3 contain GHA1a. Lanes 4 and 5 contain GHA1b. Deglycosylated proteins are in lanes 3 and 5. Light bands in lanes 4 and 5 at the dimer mass range indicate possible incomplete reduction of the dimer. The reason for unequal migration of samples is unknown, but other gels indicated no difference between the proteins. The abnormal migration may be related to local gel effects. Mass of the carbohydrate is ~1750 daltons for GHA1a-SH and ~1500 daltons for GHA1b-SH. The masses of carbohydrate measured on the monomers of GHA1a and GHA1b is consistent with ~half of the mass of carbohydrate on the dimers. The deglycosylation reactions were carried out in BME to reduce GHA1a and GHA1b.

conclusion is made assuming that the monomers are equivalent in carbohydrate content and that the N-linked carbohydrate conforms to the common oligosaccharide structure of eukaryotes, hexose residues attached to 2 GlcNAc residues on the asparagine of the protein. Figure 13 shows a SDS-PAGE of reduced GHA1a and GHA1b before and after deglycosylation. The monomers of GHA1a and GHA1b show mass changes of between 1.5 and 1.8 kDa on SDS-PAGE after PNGase F treatment. Single bands on the SDS-PAGE gel indicate that both monomers contain carbohydrate. The mass of the carbohydrate removed from the monomers is consistent with a presence of 2 GlcNAc and 7-8 hexose units. These results are consistent with both monomers having carbohydrate that is approximately half the mass of the total carbohydrate on the dimer.

Table 2: Comparison of Glycosylated and Deglycosylated Protein Molecular Weights as Determined from SDS-PAGE (masses in daltons). [†]

	Glycosylated	Deglycosylated	Carbohydrate Mass
GHA1a	60125	57571	2581
GHA1a-SH	38904	37153	1751
GHA1b	60125	57571	2581
GHA1b-SH	37153	35645	1508

[†] Data derived from Figures 12 and 13.

MALDI/TOF-MS mass measurement of reaction products from PNGase F treatment of the dimer or monomer has not yet been successful. MALDI/TOF-MS is not compatible with the combination of buffers and detergents used for the enzymatic reaction because they disrupt the crystallization of the matrix and analyte. When the

deglycosylation reaction products were added to matrix for subsequent mass measurement, the analyte/matrix mixture formed a viscous "syrup" on the sample plate preventing any ionization of analyte or matrix by the MALDI. Deglycosylation experiments in the absence of detergents with reduced and alkylated protein are anticipated.

Free Sulphydryl Content of GHA1a and GHA1b

Complete characterization of GHA1a and GHA1b requires that cysteines be documented as free sulphydryl groups or those involved in disulfide bonds. Free sulphydryl content of GHA1a and GHA1b has been determined. Alkylation with pHMB on the "native" protein resulted in a mass shift consistent with 2 reactive sulphydryls per dimer. Every indication so far is that the monomers comprising the dimers are equivalent. This suggests that each monomer should have one reactive sulphydryl group. Figures 14 and 15 show the progress of alkylation of GHA1a and GHA1b, respectively, over 20 minutes. After 20 minutes of incubation, the protein appears to revert to its original non-alkylated mass -- indicating the loss of the alkylating groups. A conclusive explanation to, or mechanism for, this reversal is unknown.

Alkylation of the single free sulphydryl on β -lactoglobulin was reproducible and occurred within 10 minutes or less. Figure 16 shows the mass change of about 321

daltons observed for HPLC β -lactoglobulin (non-reduced) when incubated with pHMB. Figure 17 confirms the attachment of only one p-mercuribenzoate to β -lactoglobulin over 20 minutes of incubation similar to the reactions of GHA1a and GHA1b with pHMB. Reversion of the single alkylation is not witnessed with β -lactoglobulin as measured by MALDI/TOF-MS.

Complete reduction of all the disulfides in GHA1a and GHA1b by TCEP has not yet been accomplished and therefore the total cysteine content has not been determined conclusively through pHMB alkylation. Upon reaction of GHA1a with TCEP and pHMB on the sample plate, peaks corresponding to various alkylated dimer species were present indicating incomplete reduction. Figure 18 shows mass shifts consistent with 4 to 21 alkylations, with the majority of the protein receiving 4 p-mercuribenzoate groups. Four alkylations caused a mass shift of the centroid of about 1.3 kDa. Significant shoulders on the peaks on the high mass side show that further alkylations have occurred. The higher mass values presented in Figure 18 are not reliable enough to corroborate nor refute the ~10 cysteines for GHA1a shown by amino acid analysis in Table 7 in the appendix.

A and B chain insulin were used as control systems for this reaction. The TCEP-reduced insulin was separated with HPLC and the individual chains were incubated with pHMB. MALDI/TOF-MS data from the A and B chain insulin alkylation with pHMB is consistent with the data presented by Zaluzec et al. (1995), indicating alkylation of all cysteine residues in the molecule. Additional data is presented in the appendix.

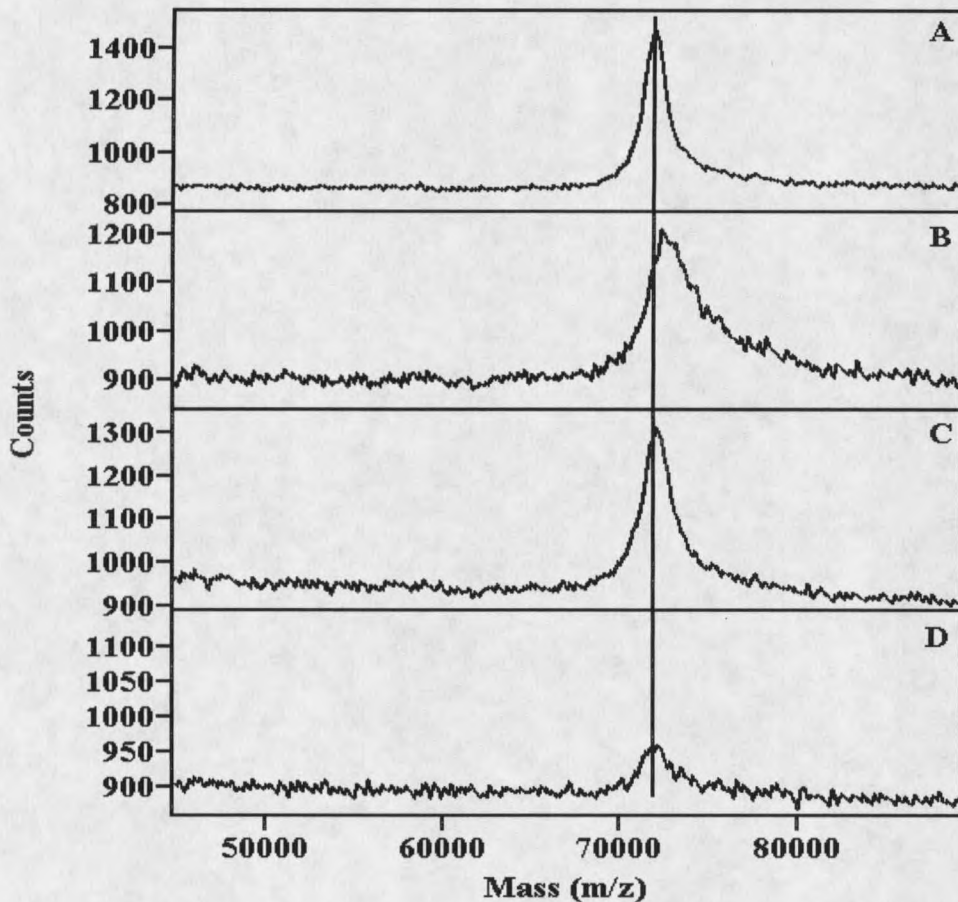


Figure 14. Mass spectra of the reaction of GHA1a with pHMB. (A) GHA1a prior to reaction with pHMB. The long vertical line indicates the centroid of the native mass peak and extends through the other spectra as a reference. (B) After 5 minutes incubation, a shift of ~650 daltons is produced. This addition is consistent with the presence of 2 free sulfhydryls on GHA1a. (C) After 10 minutes incubation, a loss of ~330 daltons is observed. (D) After 20 minutes, the mass of the peak has returned to the value of the protein prior to pHMB alkylation. Reasons for this are unknown.

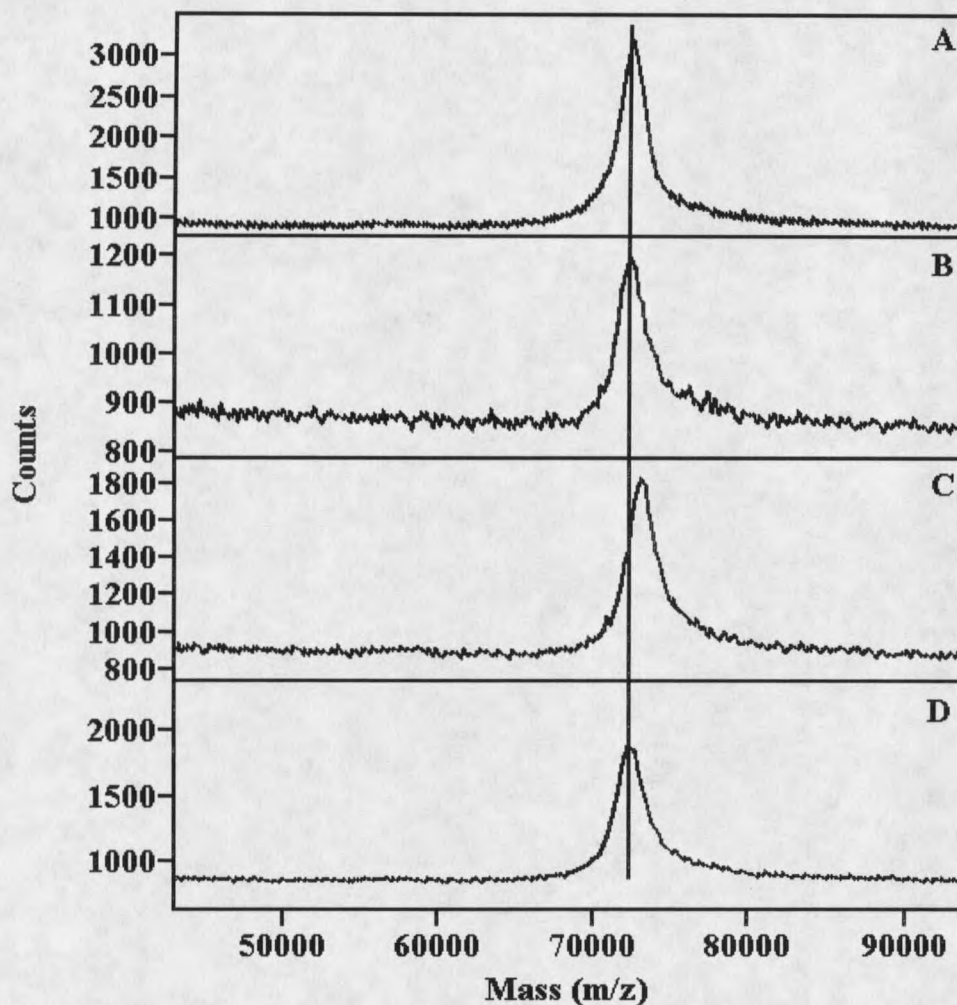


Figure 15. Mass spectra of the pHMB alkylation of GHA1b sulfhydryl groups. (A) GHA1b prior to reaction with pHMB. The long vertical line indicates the centroid of the native mass peak and extends through the other spectra as a reference. (B) After 5 minutes incubation, no shift in mass is evident. (C) After 10 minutes of incubation, a mass shift of ~660 daltons is observed. This mass shift indicates the presence of 2 free sulfhydryl groups on GHA1b. (D) After 20 minutes, the mass of the peak reverts to its normal position with a loss of ~660 daltons. An explanation for this result has not been found.

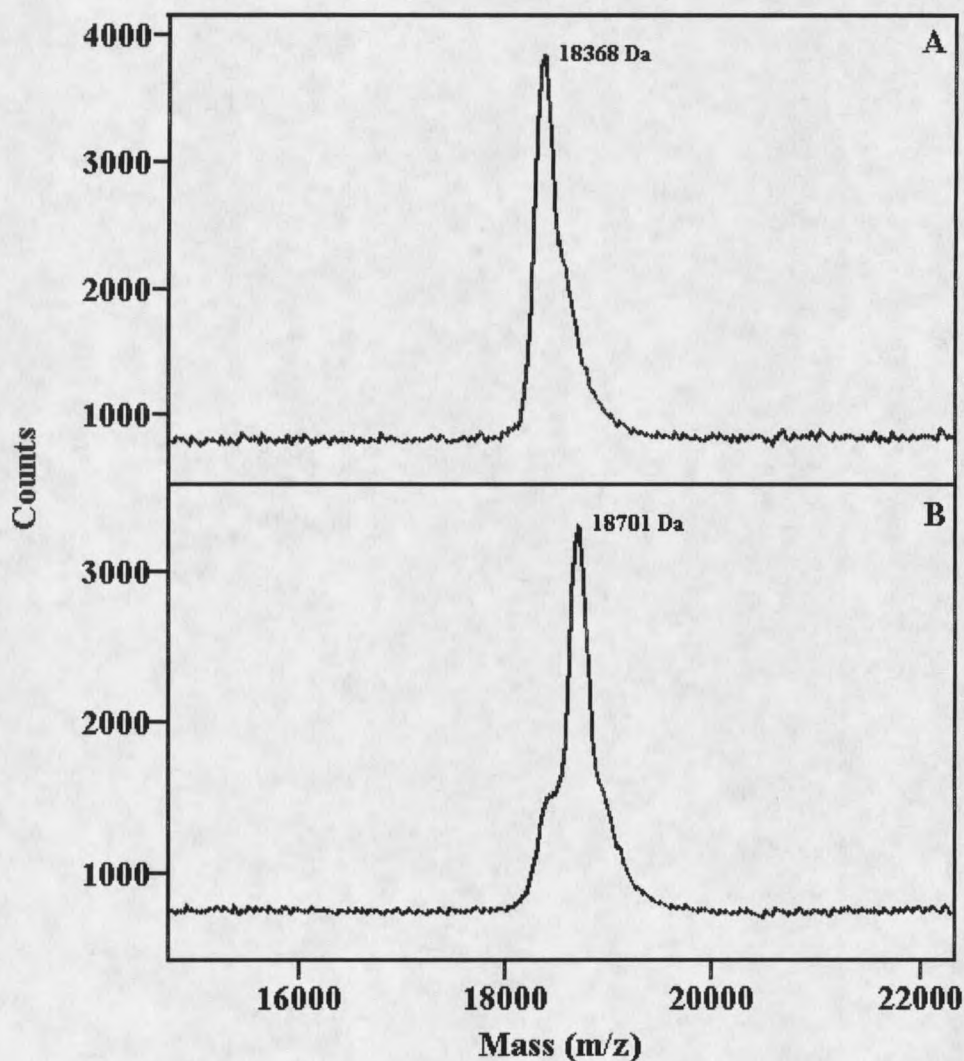


Figure 16. MALDI/TOF-mass spectra of β -lactoglobulin before (A) and after alkylation of its single sulfhydryl group with pHMB (B). Alkylation took place in 10 minutes and was consistent with addition of one pHMB group to the single free sulfhydryl on the protein. The shoulder on the low mass side of the alkylated protein peak in (B) is consistent with residual non-alkylated protein.

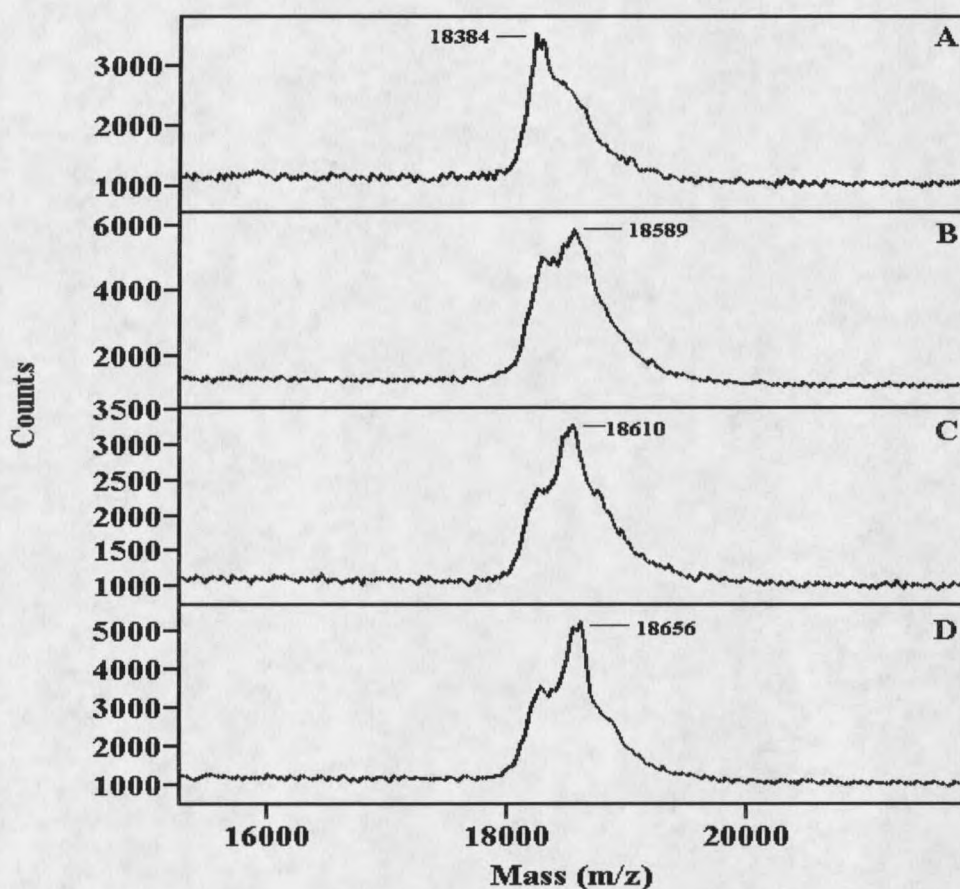


Figure 17. Progress of the alkylation of the single sulfhydryl on β -lactoglobulin. (A) β -lactoglobulin prior to alkylation with pHMB. (B) After 5 minutes incubation with pHMB, a large split in the peak can be observed. The mass difference between the forks of the split is consistent with 1 alkylation of ~ 321 daltons. (C) After 10 minutes of incubation, the mass shift is still observed and the intensity of the peak consistent with 1 alkylation continues to grow. (D) After 20 minutes, the native peak has declined more in intensity and the alkylated peak continues to grow. The alkylation of the single free sulfhydryl group is conclusive over all 20 minutes.

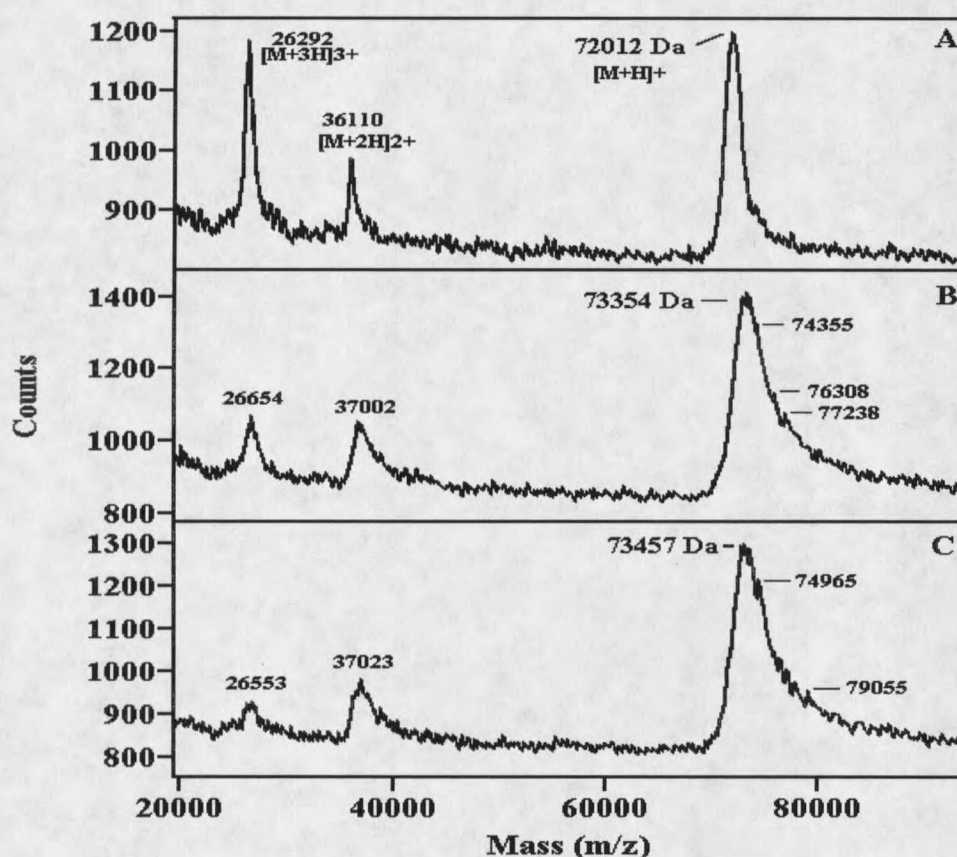


Figure 18. MALDI/TOF-mass spectra of GHA1a dimer before and after TCEP reduction and pHMB alkylation. (A) HPLC prepared GHA1a dimer prior to any other reactions. (B) Incomplete reduction by TCEP is observed with this reaction. The mass shift of the centroid indicates an alkylation of ~4 cysteines. The significant shoulder on the peak implies further alkylation of 15 and 16 cysteines at 76,308 and 77,238 daltons. (C) Reduction of GHA1a is also incomplete in this reaction. The mass shift of the peak's centroid, ~1500 daltons, indicates alkylation of over 4 cysteines. The significant shoulder indicates further alkylations occurred. A shoulder peak of 74,965 daltons indicates alkylation consistent with ~9 cysteines. A peak at 79,055 daltons suggest alkylation of a possible 21 cysteines. The doubly-charged peaks in the last two spectra (B and C), confirm the average alkylation of 4 cysteines in each spectrum. Table 7 in the appendix contains data from amino acid analysis of reduced and alkylated GHA1a showing a cysteine content of ~9.7.

DISCUSSION

Preparation of Grasshopper Agglutinin

Isolation of the hemolymph lectin through affinity chromatography occurred much the same as previously published (Wheeler et al. 1993). One particular change involved the amount and use of the Sepharose-D-galactose matrix used for isolation of the lectin. Previously, lectin was purified by passing hemolymph through a chromatography column containing Sepharose-D-galactose . Affinity chromatography using this "flow-through" method is not as convenient as the currently used "batch" method. The batch method involves incubation of matrix with clarified hemolymph prior to addition of the matrix to the chromatography column and subsequent elution. The "batch" method provides a complete removal of lectin from the hemolymph with fewer steps than the "flow-through" method. Its advantage is that the titre can be tested during the absorption step. Flow-through affinity chromatography is also not conducive to testing of the completeness of the complexation of the lectins to the matrix. If, after several hours absorption, the effluent still contains lectin as indicated by significant hemagglutination titre, the hemolymph must be readded to the column for additional

complexation. Use of the batch method caused little change in the elution pattern of the protein peak from that seen previously.

EDTA causes rapid elution of GHA that has bound to the affinity matrix (Figure 1). This suggests that removal of Ca^{2+} from the proteins results in their subsequent loss of binding activity and their release from the matrix. Immediate re-introduction of calcium restores the agglutinating/binding activity, as evidenced from recovery of hemagglutination activity. This same approach, re-introduction of required metal ions following EDTA elution of an affinity matrix, is used by Kubo and Natori (1987) and Saito et al. (1995) to isolate invertebrate lectins from the American cockroach and Japanese horseshoe crab, respectively.

Affinity chromatography-purified GHA shows multiple peaks corresponding to several different protein species on mass spectrometry (Figure 4) that can be traced to each separate protein fraction from the HPLC (Figure 5). Six proteins, as observed in the mass spectra of the fractions obtained through HPLC (Figure 5), are eluted with EDTA in the single peak on affinity chromatography. This implies that all the proteins eluted have a metal ion requirement for binding the Sepharose-D-galactose matrix.

The 0.5 minute separation of GHA1a and GHA1b on reverse-phase HPLC, observed as a split peak, indicates a chemical difference between them (Figure 2). Contrary to this result, amino acid analysis, seen in Table 7, and amino terminal sequence, seen in Figure 23, suggest that GHA1a and GHA1b are indistinguishable. The factor or factors causing the peak separation are not discernible with mass measurement

on either MALDI/TOF-MS (Figures 6 and 7) or SDS-PAGE (Figure 3), indicating that it is smaller than our current resolution or that the difference on HPLC is not related to mass. If the separation is related to a mass difference, the resolution of MALDI suggests that this difference is smaller than 200 Da. Differences of this size between similarly isolated glycoproteins are normally related to carbohydrate content. Glycoforms with a mass difference of one hexose unit between them could not be reliably visualized on MALDI/TOF-MS with the current resolution of GHA1a and GHA1b. MALDI/TOF-MS measurement of the carbohydrate may support this idea but has not been accomplished to date because of the incompatibility of the technique with required detergents and buffers.

We also propose that the separation of GHA1a and GHA1b may be related to possible glutathione attachment to the protein. Glutathione (GSH) is used in the bleeding buffer to retard oxidation and enables the hemolymph to be kept for long periods of time without oxidative degradation. The -SH form of glutathione could undergo disulfide interchange with a lectin intramolecular disulfide bond during collection and storage of hemolymph. Both GHA1a and GHA1b contain reactive sulfhydryl groups in addition to several disulfide bonds and interaction with glutathione may be possible. A mixed disulfide bond between GHA and glutathione could account for separation on the HPLC. If about 60% of the protein had been complexed to form this new mixed disulfide, the HPLC chromatogram would look similar to the one for GHA separation.

An interesting observation occurs that may lend itself to this hypothesis. On several HPLC runs of older preparations of GHA protein, the GHA1a peak intensity

seems to diminish as GHA1b becomes larger. The decline of the GHA1a peak could be related to a reversal of some form of glutathione exchange intermediate. The small mass of glutathione (MW 307) likely could not be seen on SDS-PAGE and probably could not have been detected on MALDI with our current resolution limits. Investigation into the question of glutathione attachment and its possible relation to the GHA1a and GHA1b peaks is underway.

The GHA1a and GHA1b fractions appear to contain a single protein with little contamination from other molecules. The purified proteins electrophorese on SDS-PAGE as single, sharp bands indicating a single protein specie in each fraction (Figure 3). When reduced, SDS-PAGE gives single bands with a mass roughly half the mass of the dimeric form of the protein (Figure 3). Mass spectra of the HPLC fractions confirm that they contain single, pure proteins because mass peaks for molecular species other than for those attributable to GHA1a or GHA1b were not detected (Figures 6 and 7).

Mass spectra of GHA2 and GHA3 (Figure 5) indicate that each fraction is composed of multiple members. No further characterization has been done on these proteins but speculation links GHA2 to Clones 3 and 4. Clone 3 and Clone 4 are C-type grasshopper lectins being investigated in our laboratory currently with various molecular biology methodologies. The protein sequences of the proteins, derived from cDNA sequence, indicate that they are of a size consistent with the species found in GHA2. Clone 3 and clone 4 proteins have been shown to be different from, but related to, GHA1a and GHA1b HPLC fractions (unpublished data). Figure 23 shows the known

sequences of Clones 3 and 4 with some preliminary CNBr fragment sequences from GHA1b. The sequences are similar but not identical. In addition, the amino acid compositions of GHA1a and GHA1b, shown in Table 8, do not closely match that of clones 3 and 4. While not identical, several characteristics of GHA1a, GHA1b, Clone 3 and Clone 4, such as galactose-binding, similar sequences, molecular weight, and amino acid compositions indicate that they are likely homologous members of a family of lectin proteins in the grasshopper. It appears that while GHA1a and GHA1b represent the major lectin present in hemolymph, clones 3 and 4 from fat body represent minor components.

Mass Determination

Identical mass values are observed for GHA1a and GHA1b in SDS-PAGE with both reducing (Figure 3) and non-reducing conditions (Figure 3). Identical masses are also determined for GHA1a and GHA1b by MALDI/TOF-MS, at least within the limits of resolution for the instrument. Previous data by Stebbins and Hapner (1985) determined the mass of affinity chromatography-purified GHA to be ~70 kDa by SDS-PAGE. Currently, the two major components, GHA1a and GHA1b, are determined to be $\sim 62.6 \pm 2$ kDa on SDS-PAGE. The masses determined for GHA1a and GHA1b on SDS-PAGE are significantly different than the mass spectrometry values. MALDI/TOF-MS

gives 72.0 ± 0.2 and 72.1 ± 0.2 kDa for GHA1a and GHA1b, respectively. Table 1 shows a comparison between the two techniques, which differ by as much as 13%. The anomalous migration on SDS-PAGE, relative to the mass observed with MALDI/TOF-MS, could be related to the carbohydrate portion of the glycoproteins. Glycoproteins are known to have abnormal migration patterns on SDS-PAGE (See and Jackowski 1989). Another possible explanation for the abnormal migration is a compact structure of GHA1a and GHA1b caused by extensive disulfide bonding or hydrophobic character. Proteins with high hydrophobic character travel at faster rates than those that are more flexible in their structure owing to binding of less SDS and smaller Stokes radius (See and Jackowski 1989).

GHA1a (Figure 6) and GHA1b (Figure 7) have similar mass spectra suggesting that they have similar masses. Current resolution of the mass spectra is near 200 daltons and any mass differences smaller than this cannot be differentiated with certainty. An experiment designed to detect a small difference between GHA1a and GHA1b measured the masses of mixed samples of the two proteins. As the mixture changes from 100% GHA1a to 100% GHA1b, it was thought that a trend might be seen in the peak mass. Figure 11 shows that no definitive difference or trend can be seen in the peak masses of these samples. This supports the closely related or identical masses of the two proteins. Range of determined masses is 217 daltons which approaches the limits of our resolution for GHA1a and GHA1b. The average mass of the centroid is ~ 73 kDa, showing the

increased mass of the mixed samples as compared to each individual protein. Reasons for the apparent increased masses of the mixed protein samples is unknown.

The observation of a mass change after BME reduction of the protein on SDS-PAGE confirms previous data showing some degree of disulfide bonding between the monomers. The extent of this intermolecular disulfide bonding is unknown from these experiments. A single band after reduction suggests that the monomers are of equivalent or nearly equivalent mass at 36.9 kDa (Figure 3) further contributing to the idea that GHA1a and GHA1b are similar in mass and gross structure.

Reduction of both GHA1a and GHA1b for MALDI/TOF-MS was accomplished with TCEP. Figure 8 shows the mass spectra of these reduction products. The absence of a 72 kDa mass peak indicates that all of the dimer was converted to monomer. The mass spectra in Figure 8 show that monomeric GHA1a and GHA1b are identical in mass within the resolution of MALDI/TOF-MS. A single, prominent monomer mass peak is seen in Figure 8(A) at 36.1 ± 0.1 kDa, indicating monomers of equal mass in the GHA1a dimer. Figure 8(B) indicates monomers of equal mass for the GHA1b dimer at 36.1 ± 0.1 kDa. This confirms the SDS-PAGE data showing monomers of equivalent masses comprising the native dimer.

Progress of the reduction reactions was monitored by taking aliquots of the reaction at various times and analyzing them on MALDI-MS. Figure 9 shows the progress of GHA1a reduction and Figure 10 shows the equivalent reaction for GHA1b. These spectra indicate that more than 90 minutes are needed for TCEP to reduce the

disulfide bonds between the monomers. The length of time needed to reduce the disulfide bond(s) is rather long and suggests that the intermolecular bonds are inaccessible and/or resistant to the reagent. The reduction of GHA1a and GHA1b was substantially different from that of insulin in that the amount of TCEP used to accomplish the reduction of GHA1a and GHA1b was enormous compared to the amount used on insulin. This may suggest that these proteins are tightly folded or that the reagent was not able to reduce the disulfide bonds in stoichiometric fashion as indicated by Gray (1993). Perhaps the difference in susceptibility to TCEP simply relates to the much larger protein structure (72.2 kDa) relative to that of insulin (5.7 kDa).

Glycoprotein Character of GHA1a and GHA1b

Previous amino acid analysis showed affinity chromatography-purified GHA to contain 1-2% glucosamine by weight (Stebbins and Hapner 1985). Deglycosylation experiments on that same material showed loss of mass when the sample was treated with PNGase F, indicating some N-linked carbohydrate present. Furthermore, GHA showed no change in mass when treated with O-Glycosidase, suggesting the absence of o-linked carbohydrate (Hapner unpublished results).

Current work with PNGase F on the HPLC separated proteins confirms GHA1a and GHA1b as glycoproteins with N-linked carbohydrate. Masses determined through

SDS-PAGE are consistent with a carbohydrate moiety of 4 GlcNAc residues and 10-12 hexose residues per dimer (Figure 12). This is calculated to be about 4% carbohydrate content for the GHA1a and GHA1b dimers. Similarly, removal of carbohydrate from monomeric GHA1a and GHA1b is consistent with this finding. Data in Figure 13 show the mass of the carbohydrate on the monomer to be approximately half the carbohydrate mass of the dimer, 1508 and 1751 daltons. Enzymatic removal of carbohydrate from the monomers also indicates that each monomer has attached carbohydrate. Deglycosylated monomers of GHA1a and GHA1b also have equivalent masses as determined by SDS-PAGE, Figure 13. The number of carbohydrate sites on the protein can not be discerned from these data, but they suggest one site per monomer chain. Previous experiments where deglycosylation of the dimer produced three electrophoretic bands also suggests that one site is present on each monomer (Hapner unpublished data). It may be of interest that two potential glycosylation sites are present in the amino acid sequences of clones 3 and 4 (Radke and Hapner unpublished data).

MALDI/TOF-MS was not successful on the deglycosylated proteins. The protocol used for enzymatic deglycosylation involves detergents for denaturation of the protein. SDS and high amounts of n-OG are incompatible with mass spectrometry sample preparation. Beavis and Bridson (1993) have shown that the detergent interferes with the ionization of the protein while in the matrix crystal. Successful deglycosylation of GHA1a and GHA1b without detergent has not been successful. It is assumed that this is possible because the control glycoprotein, RNase B, was deglycosylated without

inclusion of detergent. Both GHA1a and GHA1b have been previously reduced and pyridylethylated for other analytical procedures. Use of these (denatured) samples for deglycosylation would presumably remove the need for detergents in the PNGase F reaction and thus would be compatible with MALDI/TOF-MS. An experiment to test this possibility has been designed.

Complete deglycosylation of RNase B was carried out as a control for similar studies on carbohydrate removal from GHA1a and GHA1b by peptide-N-glycosidase F. Figure 19 shows the mass spectra of RNase B before and after enzymatic deglycosylation. Detergent was not required for removal of the carbohydrate.

Reduction of Disulfide Bonds with TCEP

TCEP reduction of GHA1a and GHA1b followed by MALDI/TOF-MS provided an accurate mass of the monomer and determined the composition of the dimer to be two monomers of equivalent mass.

A particular problem with reduction of GHA1a and GHA1b involved the acidic pH of the samples and the applicability of the reducing agent to MALDI/TOF-mass spectrometry. MALDI/TOF-MS uses matrices that are inherently acidic. Most reducing agents, such as β -mercaptoethanol, dithiothreitol, and sodium borohydride, are most

effective at basic pH ranges. Applicability of these reagents to matrix crystallization in MALDI/TOF-MS is questionable. Tris(2-carboxyethyl)phosphine (TCEP) has advantages over other reducing agents in this area. TCEP is an active reductant at acidic pH's (Han and Han 1994), making it an ideal reagent for use on RP-HPLC separated proteins and peptides that are acidic due to use of trifluoroacetic acid (TFA) as a counter ion in HPLC solvents. One of the biggest advantages of using TCEP is applicability to mass spectrometry. TCEP does not hinder crystal formation or suppress ionization of the analyte (Fischer et al. 1993).

Reduction reactions of small proteins and peptides with TCEP are nearly stoichiometric and are driven by formation of an irreversible phosphorus-oxygen bond (Burns et al. 1991, Fischer et al. 1993, Gray 1993, Han and Han 1994). The reduction of larger proteins or "unusually stable" proteins may require elevated temperatures, higher concentrations of TCEP, or longer reaction times (Gray 1993). Gray found that large excesses of TCEP were beneficial in reducing peptides. This idea could be extended to large proteins which may require even larger concentrations of TCEP to get complete reduction. Gray also noted a particular resistance to TCEP by some peptides that are also resistant to proteases and found that elevated temperature and longer time may be necessary to complete reduction. TCEP reductions are more dependent on accessibility of the disulfide to TCEP rather than the conformation of the disulfide bond (Gray 1993). TCEP reduction is not known to modify any other residues and is compatible with many alkylating agents (Ruegg and Rudinger 1977). These conditions allowed for wide

latitude in choosing reagents and reaction conditions for use with GHA and MALDI/TOF-MS.

Disappearance of the dimer peak at 72.2 kDa and a large increase in intensity of the 36.1 kDa peak over time provided quantitative evidence for the complete reduction of the GHA dimer to the monomer. Figure 9 shows the reaction for GHA1a and Figure 10 shows the reaction for GHA1b. This reduction does not prove, however, that the cysteine residues internal to the monomer are completely reduced. The experiment shows that one or more intramolecular disulfide bonds have been reduced and that the dimer is made of two monomers with equivalent masses. The reactions studied here confirm that while TCEP works almost stoichiometrically on small proteins and peptides, it does require a substantial excess for large proteins such as GHA1a and GHA1b.

MALDI/TOF-MS vs. SDS-PAGE.

MALDI/TOF-MS shows the GHA1a and GHA1b dimers to have masses of 72.0 ± 0.2 and 72.1 ± 0.2 kDa, respectively. The monomer masses of GHA1a and GHA1b have been measured at 36.1 ± 0.1 kDa via MALDI/TOF-MS. This measurement is in contrast to the results from SDS-PAGE. Recent SDS-PAGE shows the mass of the monomer to be between 34.6 and 38.9 kDa and the mass of the dimer to be between 60.1 and 64.6

kDa. This discrepancy presumably follows from the anomalous mobility of the protein in polyacrylamide gels due to carbohydrate content as discussed earlier.

Another interesting aspect stems from the source of the gels and standards used for analysis. Gels that were purchased gave different results than gels that were made in the laboratory. Different masses were also determined from different concentrations of separating gels from the same source. Molecular weight standards from two companies also gave varying masses of the same samples on the same gels. In order to combat this variability, all recent samples used to calculate masses for this thesis were run using the same gel type and only one type of molecular weight standards.

SDS-PAGE has been used as protein separation technique for many years and will likely continue in usage. SDS-PAGE allows for widely varying conditions that other techniques for mass determination can not. Buffers, detergents, and other high concentration reagents normally do not hinder the measurement of mass by SDS-PAGE but may harm other techniques, such as mass spectrometry. Accuracy of PAGE measurements is probably the biggest disadvantage of the technique. Overall accuracy is only about ± 1000 daltons on average, with anomalous results from glycoproteins, glycopeptides, and proteins with extensive disulfide bridging or high hydrophobic character (See and Jackowski 1989). MALDI/TOF-mass spectrometry, on the other hand, is able to provide accurate ($\sim 0.3\%$ at 72.2kDa, ± 0.2 kDa), fast mass measurements with little consideration to size of protein or types of modifications, an aspect unparalleled by classical protein techniques used to measure masses. MALDI/TOF-MS

will not replace those techniques because it currently has limitations. MALDI/TOF-MS is incompatible with certain buffers and reagents normally used in protein biochemistry. Further developments may yield better sample preparation methods and matrices to combat this problem. Both techniques are useful and have their advantages and disadvantages.

Free Sulfhydryl Content of GHA1a and GHA1b

Knowledge of cystine and sulfhydryl content are important to protein characterization. Undoubtedly the cysteines involved in disulfide bonds perform an important role in the overall structure of the molecule by providing links between subunits and within individual polypeptide chains. Sulfhydryls are one of the most reactive groups in a protein and can account for a large part of the chemical behavior of a protein or enzyme.

Measurement of protein cysteine content is also one way to approximate the similarity between proteins, such as GHA1a and GHA1b. Cysteines are usually highly conserved between related proteins because a majority of them are involved in structural roles in the protein, with only tryptophan having a smaller replacement rate within homologous proteins (Creighton 1993; Table 3.1). In our case, the cysteine data could enhance our knowledge about the relationships between GHA1a, GHA1b, Clone 3, and

Clone4. Clones 3 and 4 are C-type lectins under study in this lab and their primary sequences have been derived from cDNA data. Attempted sequencing of CNBr fragments of GHA1a and GHA1b has previously shown some sequence relationship between their partial sequences and sequences derived from the cDNAs for Clones 3 and 4 (Figure 23, Appendix). Comparison of the known amino acid composition of Clones 3 and 4 and the amino acid analysis data on GHA1a and GHA1b has also suggested that they are similar proteins (Table 7).

A particularly useful technique has been developed to aid in the determination of the numbers of Cys residues within a protein via alkylation with p-hydroxymercuribenzoate and mass measurement with MALDI/TOF-mass spectrometry (Zaluzec et al. 1994). This technique was originally applied to peptides and smaller proteins such as bovine insulin (MW 5734.5), β -lactoglobulin (MW 18,367), and somatostatin (MW 1637.9). Both free sulfhydryls and total numbers of cysteines were analyzed by this technique using these control proteins. We decided to apply this procedure to GHA1a and GHA1b.

Our choice of organomercurial reagent was pHMB (p-hydroxymercuribenzoate). This reagent is compatible with MALDI/TOF-MS and produces a mass shift of 321 Da for each Cys residue alkylated. The 321 dalton mass shift associated with addition of one p-mercuribenzoate group is needed at the high mass values we investigate because our current resolution is not good enough to see smaller shifts by other alkylating agents such as vinylpyridine

Evidence of two sulfhydryls per dimer of GHA1a and GHA1b has been shown using pHMB alkylation and mass measurement by MALDI/TOF-MS. The alkylation that occurs on the two proteins is mysterious. The protein appears to be alkylated in the first 10 minutes but eventually returns to its native mass within 20 minutes (Figures 14 and 15). Researchers using pHMB with mass spectrometry have not reported any findings of this type (Zaluzec et al. 1995) but it is possible that reaction with another thiol could result in removal of an organomercurial reagent from its complex with a cysteine residue (Means and Feeney 1971). Possibly, rearrangement of disulfide bonds close to the alkylation site may provide for removal of the p-mercuribenzoate group through a reaction between the alkylated cysteine and another cysteine. Perhaps the glutathione used in the preparation of GHA plays a role in the stability of the p-mercuribenzoate adduct.

Alkylation of β -lactoglobulin with pHMB showed no such reversal. β -lactoglobulin was chosen as a control because it contains one sulfhydryl group in its native structure. The control reaction of pHMB and β -lactoglobulin confirmed the ability to determine the presence of free sulfhydryls via pHMB alkylation coupled with MALDI-MS. The single free sulfhydryl of β -lactoglobulin alkylates within 10 minutes resulting in a mass shift of ~323 daltons as seen in Figure 16. This increase in mass is consistent with only one addition of p-mercuribenzoate. Figure 17, observation of the pHMB/ β -lactoglobulin reaction over 20 minutes, suggests that the mass shift, consistent with one alkylation, does not reverse itself.

The total cystine content of GHA was previously determined through performic acid oxidation followed by amino acid analysis and reported to be 14 per dimer (Stebbins and Hapner 1985). This result is now thought to be incorrect because we know that GHA is a group/family of proteins that can be separated on reverse-phase HPLC. Current amino acid analysis has shown that the GHA1a and GHA1b monomers have ~9.7 and ~10 cysteines, respectively, as measured through presence of reduced and pyridylethylated cysteine residues (Table 7, Appendix). Average efficiency of amino acid analysis is 80-90%, allowing for the possibility of 11 or 12 cysteines in GHA1a and GHA1b. It is interesting that clone 3 and 4 contain 12 and 11 cysteine residues respectively.

Preliminary data has been retrieved on total cysteine content of GHA1a and GHA1b through use of pHMB and mass spectrometry. Reduction of GHA1a with TCEP followed by alkylation with pHMB on the MALDI sample plate resulted in a mass shift of about 1300 daltons for the centroid of the peak (Figure18). The 1300 dalton mass shift is equivalent to about 4 p-mercuribenzoate molecules. A significant shoulder on the high mass side of the shifted peak was observed, most likely due to smaller amounts of highly alkylated species. Small peaks on the shoulder indicate alkylations of up to 16-21 cysteines. The fully reduced protein with all cysteines alkylated should be in the mass range of 36-40 kDa indicating that the number of alkylations can not be the total number of cysteines. Further work on reduction and alkylation is needed to confirm the total numbers of cysteines in each protein.

Insulin was used to check the procedures and reagents in the alkylation reaction needed to quantify total cysteine content because it has 6 cysteines involved in disulfide bonds and no free -SH groups. Insulin was reduced to its separate chains (A and B) to expose the cysteines present in the molecule. Alkylation over 10 minutes with pHMB followed by MALDI/TOF-MS produced a series of mass peaks corresponding to alkylation of all the cysteines present in each chain. Figure 20 and 21, in the Appendix, show the results of the alkylation of A chain and B chain insulin respectively.

Optimization of MALDI/TOF-MS

Sample preparation is ultimately the most important aspect of MALDI/TOF-mass spectrometry. Proper ratios and concentrations are necessary to insure proper crystallization of the protein with the matrix. High buffer concentrations and the smallest amounts of detergents do not allow for ionization of the protein sample, proper crystallization of the matrix, or inclusion of the protein within the matrix crystal (Beavis and Bridson 1993, Xiang and Beavis 1993, Vorm et al. 1994) thereby interfering with analyses. Other reagents, e.g. β -mercaptoethanol and NaN_3 , are not conducive to matrix crystallization and should be avoided (Xiang and Beavis 1993).

The method of crystallization also plays a role in whether the analyte ionizes easily or not. Various methods have been developed and may be used for differing

proteins or intended outcomes. The most popular crystallization method is "dried-drop". This method requires the analyte and matrix be mixed and placed on the sample plate where they dry at a rate controlled by atmospheric conditions. Crystal sizes can range from small to large depending on the time the crystal formation requires. The crystals form at any angle or orientation and may be grouped into large masses of crystals while other areas of the sample well may have no crystals. This method has inherent complications because the atmospheric conditions in the laboratory are rarely the same from one day to the next. This may allow for slightly varying values of masses from day to day. This method is useful in that it does not require extra equipment nor long periods to get crystal formation.

"Rapid crystallization" uses a vacuum source to form crystals. Theoretically, quick crystallization forms crystal of uniform shape and coverage in the sample well. This allows for equivalent spectra from all parts of the sample well. This has been tried with success in our laboratory using a vacuum dessicator. A variation on this method, "fast evaporation", relies on the use of volatile organic solvents for matrix and analyte solvation (Vorm et al. 1994). This method presumably approaches the speed of "rapid crystallization" without the extra equipment. Another technique involves slowly growing matrix crystals in a solution containing analyte, allowing for large crystals doped with protein. The applicability of this technique to our protein was not pursued.

Several papers have provided insight into matrix conditions/ concentrations on ionization and "mass preference" as they apply to 4-hydroxy- α -cyanocinnamic acid

(α CHCA) (Arkady et al. 1995, Billeci and Stults 1993, Cohen and Chait 1996). α CHCA is the most used MALDI/TOF-MS matrix for peptides and small proteins. Interestingly, the conditions by which the matrix crystallizes controls the formation of ions in the analysis. The outcome of the laser pulse can be controlled by changing various aspects of the matrix solution. Solvents, concentration, co-matrices, and pH can all be optimized to achieve increased resolution and intensity of the mass peaks.

Laser settings, vacuum level, number of scans, voltages, and use of the low mass gate all have great degrees of importance on the spectra that are obtained from the MALDI/TOF instrument. The low mass gate, which blocks low mass molecules from reaching the detector, is very useful in obtaining reasonable spectra from large proteins with small concentrations. The low mass gate is able to cut off most of the matrix ion signal and still leave the signals for the larger ionized species. This effectively increases the numbers of ions of interest that reach the detector because the total number of ions has decreased.

Ionization of the analyte and the resulting intensity of peak are dependent on instrument settings and on a protein's chemical characteristics. Generally, these settings were determined through empirical work with the protein(s) in question. In the case of large proteins, high laser power is used to get increased ionization of analyte. This allows for increased intensity on the mass spectra but hinders good resolution. This loss of resolution is of small consequence because large proteins have low resolution, regardless

of laser power. In addition, some large proteins require more laser power to be ionized than other proteins of the same size.

BSA was chosen as a calibrant for this work because it had a mass close to GHA1a and GHA1b. BSA was used both as an external and internal calibrant for MALDI/TOF-MS analysis of GHA samples. Internal standards are generally held to be more accurate by most researchers in the field (Beavis and Chait 1994). In the case of GHA, it could not be determined if the internal standard "runs" were indeed better values than the external standard runs.

Possible Future Work

Further characterization of the proteins isolated from the hemolymph of *Melanoplus differentialis* is necessary to get a clear understanding of their structure and function. Areas that need attention in the near future:

1. The nature of the difference between GHA1a and GHA1b. Isolation of GHA through affinity chromatography and HPLC without glutathione may provide answers to this question. Repeating pHMB alkylation of sulfhydryl groups on GHA1a and GHA1b using "non-glutathione" isolated protein may yield answers to reversal of alkylation by pHMB.

2. MALDI/TOF-MS mass measurements from GHA1a and GHA1b deglycosylation reactions without use of detergents. The use of reduced and pyridylethylated protein may circumvent the need for detergent in the deglycosylation reaction.
3. Total cysteine content of GHA1a and GHA1b as determined through complete reduction, alkylation with pHMB, and mass measurement with MALDI/TOF-MS. This can be compared with traditional oxidative and exhaustive alkylation procedures.

CONCLUSIONS

The objectives for this research project have been met, giving further insight into the structure of GHA1a and GHA1b. The major accomplishments of this project are:

1. The lectin from grasshopper hemolymph, GHA, has been successfully purified into single electrophoretic and mass spectroscopic components through Sepharose-D-galactose affinity chromatography and reverse phase-HPLC. The major fractions GHA1a and GHA1b are disulfide stabilized dimeric molecules consisting of two indistinguishable glycosylated monomers.
2. Masses have been measured for GHA1a and GHA1b dimers and monomers with SDS-PAGE and MALDI/TOF-MS. The dimeric GHA1a and GHA1b molecules have been measured to 72.0 ± 0.2 and 72.1 ± 0.2 kDa, respectively, by MALDI/TOF-MS. Both proteins are measured at 62.6 ± 2 kDa by SDS-PAGE. The dimers have been determined to be comprised of disulfide-linked monomers, with the equivalent mass of 36.1 ± 0.1 kDa by MALDI/TOF-MS. SDS-PAGE gives 36.9 ± 2 kDa for the mass of the monomers. Anomalous behavior of the proteins on SDS-PAGE is presumed to be due to glycoprotein character.
3. MALDI/TOF-MS gives a more accurate mass measurement than SDS-PAGE and is independent of protein structural characteristics. Mass measurements on MALDI/TOF-

MS are also faster and easier to obtain than SDS-PAGE mass measurements.

MALDI/TOF-MS does not, however, have a toleration of certain reagents commonly used in protein chemistry, such as buffers and detergents.

4. GHA1a and GHA1b were confirmed to be glycoproteins through enzymatic removal of carbohydrate by PNGase F and mass measurement by SDS-PAGE. The mass of the N-linked carbohydrate has been estimated by SDS-PAGE to be within 2.5 and 2.8 kilodaltons. This mass is consistent with a carbohydrate content of 4 GlcNAc and 10-12 hexose residues per dimer. Additional deglycosylation on the monomers of GHA1a and GHA1b indicate that both monomers contain carbohydrate. The mass of the carbohydrate removed from the monomers of GHA1a and GHA1b is consistent with half the mass of carbohydrate on the dimer and is consistent with a composition of 2 GlcNAc and 6-7 hexose residues.
5. The free sulfhydryl content of GHA1a and GHA1b has been determined through pHMB alkylation and MALDI/TOF-mass spectrometry. The dimers of GHA1a and GHA1b were determined to have 2 reactive sulfhydryl groups each, presumably one per monomer. Reversal of the mass increase associated with 2 alkylations results after brief incubation, a phenomenon not presently understood.

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APPENDIX

Deglycosylation of RNase B

The various glycoforms grouped under the title RNase B were reduced to the mass of the native unglycosylated protein (RNase A). RNase A has a mass of 13683 daltons and the major glycoform has a mass 1217 daltons greater. This carbohydrate mass is known to be composed of 5 mannose units at 162 daltons each and 2 N-acetylglucosaminyl residues (GlcNAc) at 203 daltons each (Finnigan MAT 1996). Up to 5 glycoforms (5 to 9 mannose units attached), starting at a mass of 14,875 daltons, could be recognized from the mass spectra of RNase B in Figure 19(A).

Table 3. Comparison of various RNase B glycoforms and the unglycosylated form (RNase A).

Glycoform	Mass (calculated)	Mass (observed)
RNase B (GlcNAc)2(Mann)9	15548	15516
RNase B (GlcNAc)2(Mann)8	15386	15363
RNase B (GlcNAc)2(Mann)7	15224	15219
RNase B (GlcNAc)2 (Mann)6	15062	15038
RNase B (GlcNAc)2 (Mann)5	14900	14875
RNase A	13683	13659

Total Cysteine Content of β -Lactoglobulin

Upon reduction of the protein, the reaction with pHMB was allowed to alkylate all the cysteine residues. Although the resolution is diminished, the total number of cysteines can be deciphered. Figure 20 shows the fully alkylated β -lactoglobulin in comparison to native and singly-alkylated β -lactoglobulin.

Reduction and alkylation produced 6 peaks on the mass spectra. Figure 20 shows the comparison of "native" β -lactoglobulin (no alkylation), the protein with one alkylated cysteine, and the fully reduced and alkylated form. The peaks shown in Figure 20(C) are consistent with a series of alkylation products, one for each cysteine in the molecule.

Table 4. Reduced β -lactoglobulin alkylated with pHMB: masses from MALDI/TOF-MS.

Number of Alkylations (Specie)	Expected mass (Da)	Observed mass (Da)
0 (native)	18393	18392
1 (native + 1 pHMB)	18715	18715
2 (native + 2 pHMB)	19000	18999
3 (native + 3 pHMB)	19303	19303
4 (native + 4 pHMB)	19665	19664
5 (native + 5 pHMB)	19986	19985

Alkylation of Reduced A and B Insulin with pHMB

Alkylating the six Cys residues in insulin, coupled with the possible side reactions, can lead to a large number of peaks on the MALDI/TOF spectrum. While it is possible to determine the number of cysteines in the reduced insulin, peak assignment is easier if the two insulin chains are separated by HPLC prior to alkylation. The two chains were separated on the HPLC and then reacted with about a 5-fold molar excess of pHMB. After alkylation with pHMB, the alkylated products were analyzed by mass spectrometry.

By taking the original mass of the chain (A chain: 2339.7 Da and B chain: 3999.9 Da) and subsequently adding 321 mass units for each cysteine, it is possible to calculate the masses that should be found in the spectrum. In addition to the theoretical mass increases, other mass shifts were observed. First, mercury metal can catalyze the oxidation of cysteine to cysteic acid which results in a mass shift of 48 Da for every Cys affected. Secondly, mercury ions (from contaminating mercury salts) have been shown to attach themselves to the chain by inserting itself into a disulfide bond arrangement (-S-Hg-S-) or by attaching itself to a sulfhydryl group (-S-Hg). This results in a mass shift of 201 Da. It is recommended to first rinse the pHMB salt with cold dilute trifluoroacetic acid prior to use, to remove the troublesome mercury salts (Zaluzec et al. 1994):

Alkylation of A chain resulted in eight major peaks on the mass spectrum (Table X and Figure 21B). Each mass determined is consistent with masses that can be calculated for the spectrum of alkylation products. Note that B chain masses are present in the A chain spectrum. Due to poor separation on the HPLC, a small portion of B chain peak was collected with the A chain peak. The B chain mass spectra shows five peaks, four of which can be identified/assigned (Table X and Figure 22B). The identity of the peak at 3502.14Da is a mystery. All cysteines in each reduced chain were alkylated. One of the side reactions possible, alkylation with mercury ions, was also present.

Table 5. Insulin A chain after pHMB alkylation: masses from MALDI/TOF-MS.

Specie	Calculated Mass (Da)	Experimental Mass (Da)
A chain	2340.7	2346.13
A chain +1pHMB	2661.7	2667.18
A chain +2pHMB	2982.7	2985.07
A chain +2pHMB + Hg	3183.3	3189.67
A chain +3pHMB	3303.7	3306.52
A chain +3pHMB + Hg	3504.3	3518.70
A chain +4pHMB	3624.7	3627.52
B chain + 2pHMB [†]	4042.9	4048.48

[†] Note: A and B chain insulin are not fully separated on the HPLC procedure used and a small amount of B chain was collected when A chain was collected.

Table 6. Insulin B chain after pHMB alkylation: masses from MALDI/TOF-MS.

Specie	Calculated Mass (Da)	Experimental Mass (Da)
B chain	3400.9	3379.90
B chain + ?	---	3502.14 [†]
B chain +1pHMB	3721.9	3699.61
B chain +2pHMB	4042.9	4018.47
B chain +2pHMB + adduct [‡]	---	4225.43 [‡]

[†] Specie of unknown origin. [‡] Matrix adducts are common occurrences in MALDI/TOF-MS. Adducts of sinapinic acid are normally 207 or 225 daltons.

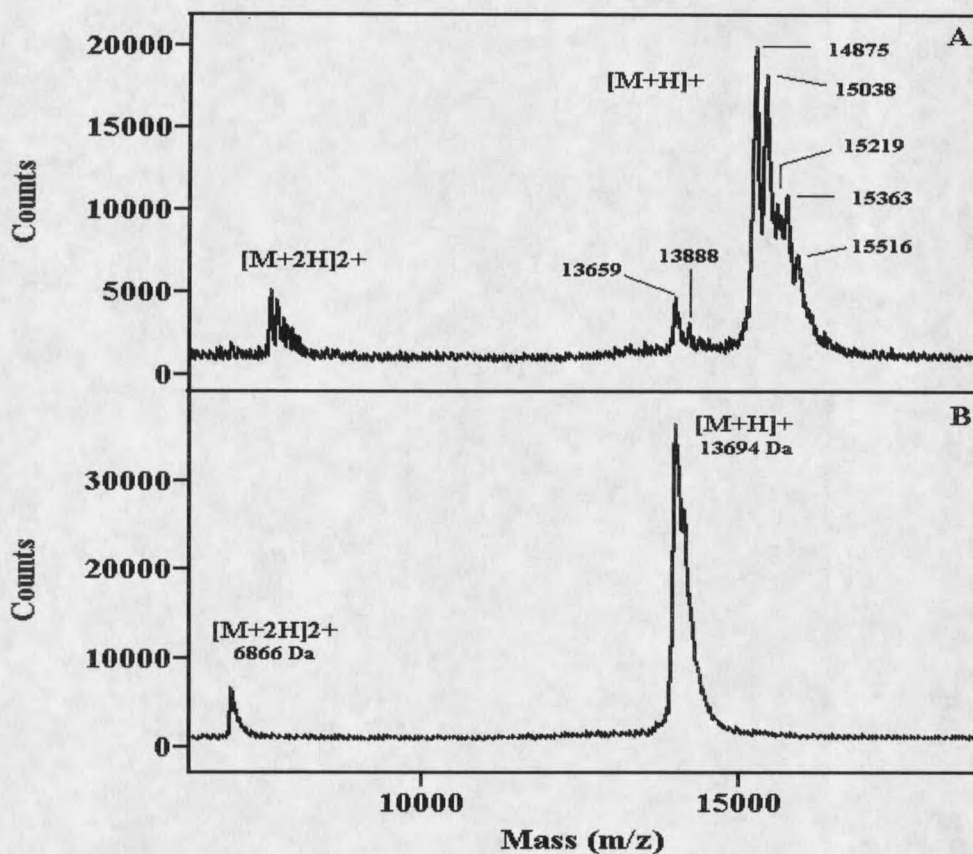


Figure 19. RNase B prior to (A) and after treatment with PNGase F (B). (A) Mass spectrum of RNase B showing unglycosylated protein (RNase A) at 13,659 and a series of glycoforms at 14,875 to 15,516 daltons. The peak at 13,888 is a matrix adduct of the unglycosylated protein. (B) After PNGase F treatment, two peaks consistent with singly- and doubly-charged unglycosylated protein species are seen.

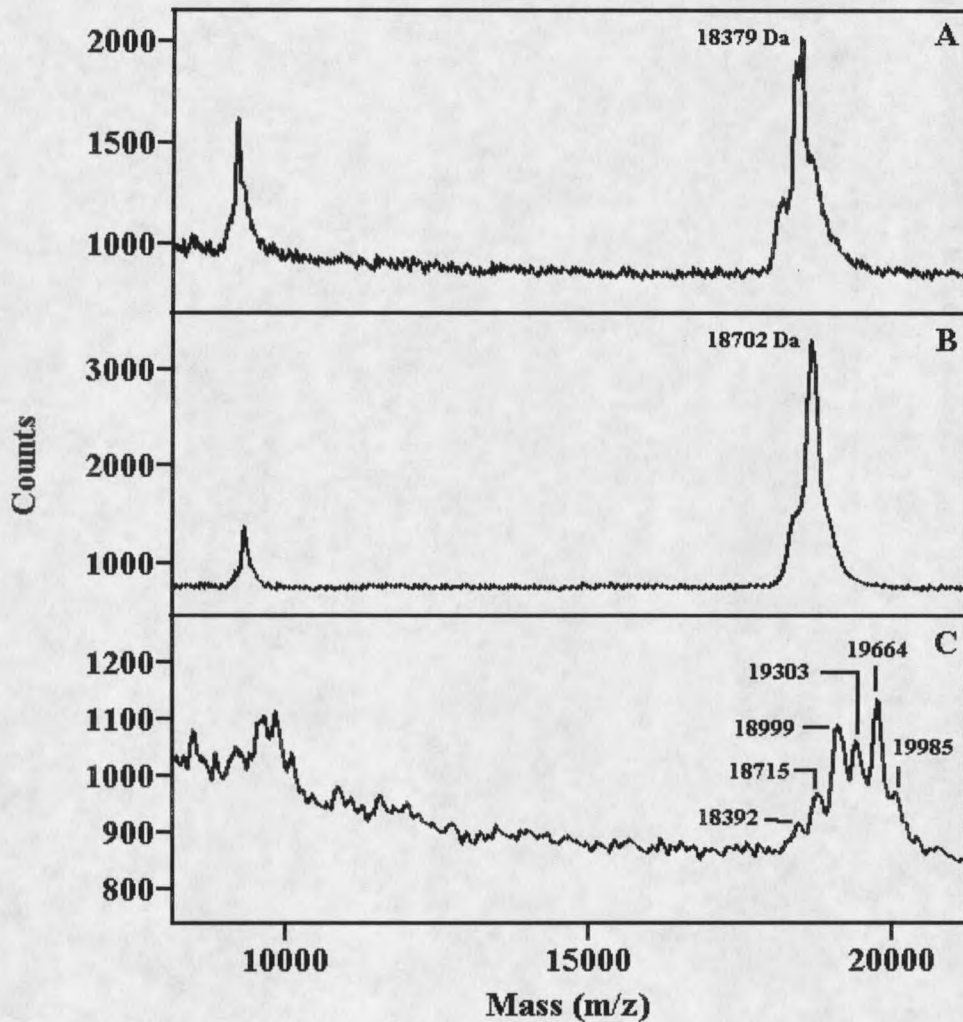


Figure 20. Mass spectra of β -lactoglobulin at varying degrees of alkylation with pHMB. (A) Prior to alkylation. (B) The peak mass has shifted 323 daltons and is consistent with pHMB alkylation of the single free sulfhydryl in the protein. (C) Reduced protein was alkylated with pHMB resulting in a series of alkylations. Each peak represents a different number of cysteines alkylated. The peak at 19,985 daltons represents the fully alkylated form of β -lactoglobulin with 5 cysteines.

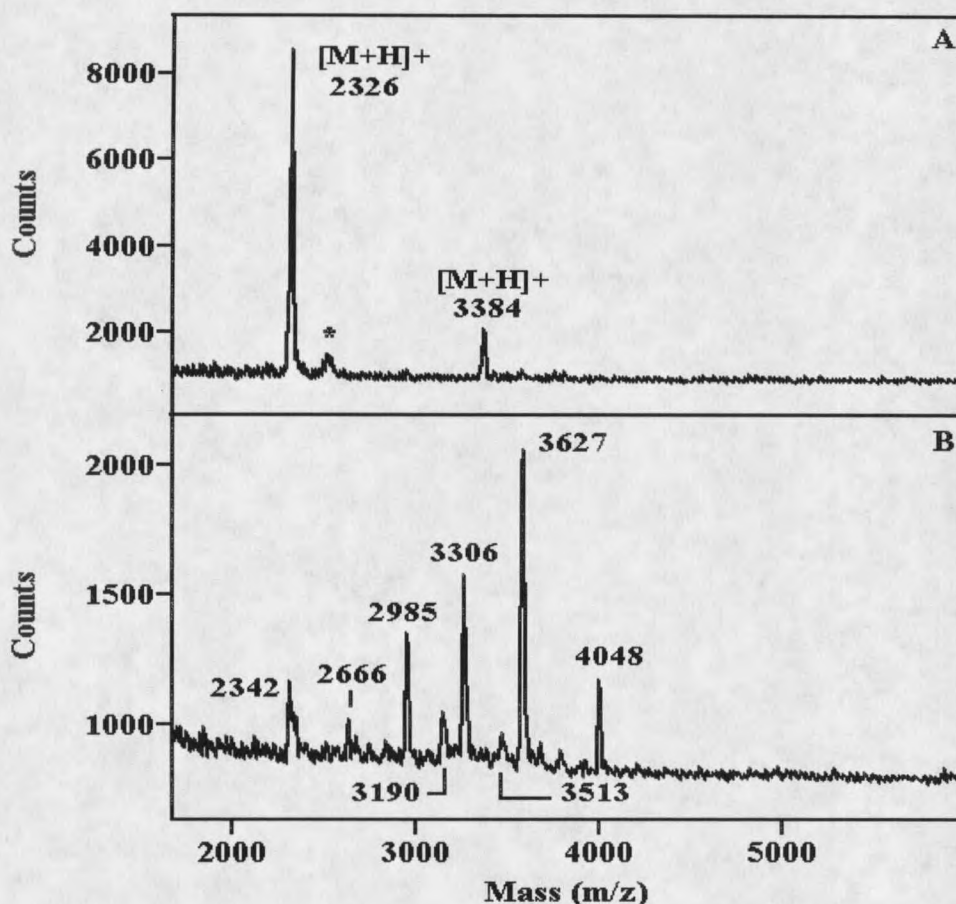


Figure 21. Mass spectra of A chain insulin before (A) and after (B) a 10 minute incubation with pHMB. (A) Collected HPLC fraction of A chain insulin containing a small amount of B chain insulin. The asterisk indicates a matrix ion adduct of A chain insulin. (B) The alkylation of all 4 cysteines in A chain is accomplished and can be observed at 2666, 2985, 3306, and 3627 daltons. The peak located at 2342 daltons is remaining unalkylated A chain insulin. Peaks at 3190 and 3513 daltons are consistent with alkylations of a single mercury ion in addition to the previous 2 and 3 alkylations by pHMB respectively. The peak at 4048 daltons is consistent with B chain insulin that has both cysteines alkylated by pHMB.

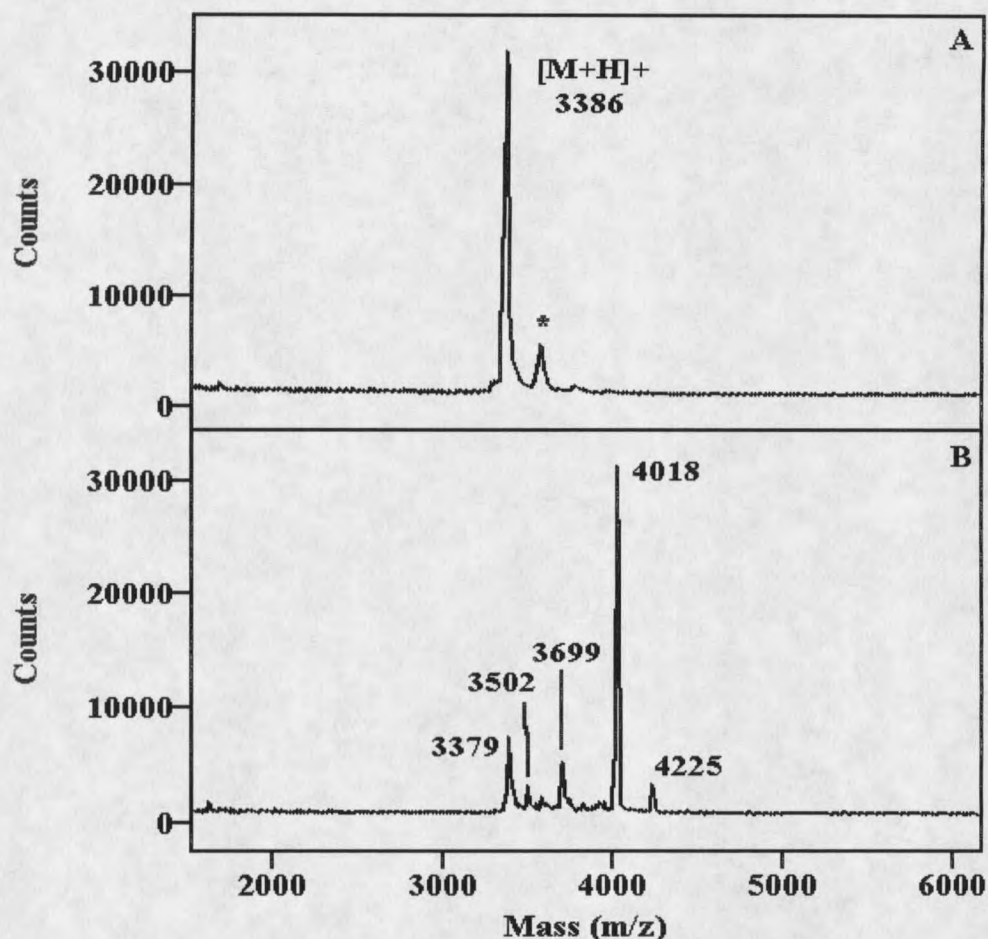


Figure 22. MALDI/TOF-mass spectra of B chain insulin before and after a 10 minute incubation with pHMB. (A) Mass spectra of B chain insulin with expected matrix ion adduct indicated by an asterisk. (B) Mass spectra of reaction product of pHMB and B chain insulin. Peaks at 3379, 3699, and 4018 daltons are consistent with 0, 1, and 2 alkylations of pHMB respectively. The peak at 4225 daltons is a matrix adduct of completely alkylated B chain insulin. The peak at 3502 daltons is of unknown origin.

Clone 3	M Q L V T V C A A L V A A T V P C T L A A V D L F	25
Clone 4	? ? ? N S <u>A V D L F</u>	
Clone 3	C S C Q V R H H R D S T T A V G C S G E Q S G N K	50
Clone 4	G G C K L R H H K E S T M A L H C S R D Q S G N K <u>C D C H V R H H R D X</u>	
Clone 3	T I S C Q K A Q V P D I P R D Y H Y V P G Y A L V	75
Clone 4	T : : C Q K A Q V P D I P R G Y H Y V P G Y A L V	
Clone 3	K L Y R I M M T W E E A K K A C E A E G A K L A V	100
Clone 4	K M Y R I M M T N E E A R K S C E S E G A I L A L	
Clone 3	P R D N H A Y D G L K Q I : : : F K L G F G V Y W	122
Clone 4	P K D S H A Y D G L K Q I I A E H K E E : G V Y W	
Clone 3	A N I G I T D H E S E G I F S G V D G H P V S F L	147
Clone 4	A N I G I T D Q Y S E G I F V G V D G L P V S Y L <u>M D G A P V S F I</u>	
Clone 3	P W N P N E P N N N G G N E N C V N V N D K G Q L	172
Clone 4	P W R P N E P N N F G G N E N C V Y V D D K G Q L <u>P D C P V</u>	
Clone 3	N D W H C G N T A P F F C E R R P S V G I P P S Y	197
Clone 4	N D W G C A N A E P F F C E R R L S V G I P P S Y <u>M C L N T A D L F C E R X L</u>	
Clone 3	V W L K D A S R F Y K V H A E K H V Y A E A A R V	222
Clone 4	V W L K D A S R F Y K V H A E K H V H A E A A R V	
Clone 3	C R S E N A T L A V P D T W D R V E T L L R L L E	247
Clone 4	C R S E N A T L A V P D T W D R V E A L L R L L E	
Clone 3	P K E E F Y L T G F T D E A V E G D F V T E T G R	272
Clone 4	P K E E F Y L T G F T D E A A E G D F V A E T G R	
Clone 3	H L K G M E F Q V W S P G E P N N D V D G K P E N	297
Clone 4	H L R G M E F Q V W K P G E P N N N F L G K P E N	
Clone 3	C L A F S G R G Y Y G D R S C E V E L P F I C E I	322
Clone 4	C L G F G G K G Y Y D D K S C D L E L P F I C E I	
Clone 3	A P	324
Clone 4	A P	

Figure 23. Amino Acid Sequence Alignment of Clone3, Clone 4, and CNBr of GHA1b. Numbering at the right indicates clone 3 residue positions only. The amino acid sequence of clone 3 includes the leader sequence (initial 20 amino acids). The mature protein amino terminus is Ala21 on Clone 3. Clone 4 is missing the leader sequence and 2 or 3 residues on the amino terminal end (denoted by question marks). Sequence of clones 3 and 4 courtesy of Jay Radke (unpublished work). Cyanogen bromide (CNBr) fragment sequences of GHA1b (underlined) courtesy of Brandon Walters and Kenneth Hapner (unpublished work). These data support the similarity but non-identity of GHA1b with Clones 3 and 4. Partial amino acid sequence of GHA1a is indistinguishable from GHA1b.

Table 7. Comparison of GHA1a and GHA1b amino acid composition with that of Clones 3 and 4.

	GHA1a [†]	GHA1b [†]	Clone 3 [‡]	Clone 4 ^{‡§}
D (aspartate)	39.2 ^{††}	39.1 ^{††}	18	18
E (glutamate)	35.6 ^{††}	36.1 ^{††}	26	28
S (serine) ^{††}	10.3	11.1	15	12
G (glycine)	28.9	28.3	25	26
H (histidine)	9.2	7.6	11	10
R (arginine) ^{††}	19.5	17.8	15	15
T (threonine) ^{††}	15.3	14.0	13	9
A (alanine)	27.8	27.0	23	25
P (proline)	22.8	24.8	19	18
Y (tyrosine)	15.9	15.9	12	14
V (valine)	21.1	22.7	24	19
M (methionine) ^{††}	2.5	2.7	3	5
SS (cystine)	0.2	0.2	---	---
I (isoleucine) ^{††}	8.4	7.8	10	11
L (leucine) ^{††}	22.0	20.2	18	22
F (phenylalanine)	8.9	8.9	14	12
K (lysine)	5.3	8.4	15	18
C (cysteine) [#]	9.7 [#]	10.0 [#]	12	11
N (asparagine)	--- ^{††}	--- ^{††}	17	15
Q (glutamine)	--- ^{††}	--- ^{††}	7	7
W (tryptophan)	--- ^{§§}	--- ^{§§}	7	7

[†] GHA1a and GHA1b compositions were obtained through amino acid analysis of reduced and pyridylethylated protein (Hapner and Walters unpublished work) [‡]

Composition of mature clones 3 and 4 were obtained from cDNA sequence by Jay Radke (unpublished work). [§] Clone 4 is missing approximately 3 or 4 residues on its amino

terminus. [#] Cysteine residues were alkylated with 4-vinylpyridine prior to amino acid analysis. ^{††} Amino acid analysis requires acid hydrolysis of the peptide bonds, which also results in conversion of asparagine and glutamine to aspartate and glutamate respectively. ^{††} Residues that are slowly hydrolyzed and could result in smaller values.

^{§§} Tryptophan is normally completely destroyed by acid hydrolysis in amino acid analysis.

