Grasshopper hemagglutinin : immunochemical localization in hemocytes and confirmation of non-opsonic properties

by Roger Steven Bradley

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

Abstract:
Hemagglutinins are lectins: proteins that recognize and bind to specific carbohydrate residues on cell surfaces. Soluble hemagglutinins have been demonstrated in the hemo-lymph of a wide variety of invertebrates. In light of their carbohydrate recognitory capabilities, agglutinins have been implicated in invertebrate defense responses against pathogens, and nonself or foreign tissues and cells. Consistent with this possiblity, agglutinins are known to be associated with the circulating hemocytes in many invertebrate species, including molluscs and insects. Opsonic capacity has been demonstrated for molluscan agglutinins, however, no solid evidence for opsonic activity has been found among the insect agglutinins studied.

The objectives of this study are to localize hemagglutinin to grasshopper hemocytes using immunochemical techniques, and to investigate the in vitro opsonic properties of grasshopper agglutinin. Results of immunocytochemical staining of monolayer hemocytes reveal the presence of agglutinin in the adult hemocytes of both Melanoplus differential is and sanguinipes. Granular cells only, stain for the presence of agglutinin, the phagocytic plas-matocytes do not stain.. The hemocytes bind asialo human erythrocytes, Nosema locustae spores, and Bacillus thuringiensis bacteria. Only the resetting, of asialo human erythrocytes can be inhibited by the addition of agglutinin-specific carbohydrates. No opsonic activity is found when asialo human erythrocytes or thuringiensis is incubated in either grasshopper serum or affinity purified hemagglutinin prior to over layering hemocytes. While a potential opsonic effect is seen for Nosema spores incubated in grasshopper serum, no opsonic activity is demonstrated for Nosema incubated in purified hemagglutinin prior to over layering hemocytes. Neither increased immunofluorescence nor increased phagocytosis was observed when hemocytes, in vitro, were incubated with purified agglutinin. Phagocytosis of Nosema spores is not inhibited by the addition of agglutinin-specific monoclonal antibody to hemocytes. These results indicate that although grasshopper hemagglutinin is demonstrated in a percentage of grasshopper hemocytes, it does not appear to have an in vitro role in the recognition of foreign particles by hemocytes. Potential physiological roles of agglutinins in grasshoppers have not previously been described. Results reported here are consistent with the non-opsonic character and hemocytic location of other described insectan agglutinins.

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

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ABSTRACT

Hemagglutinins are lectins: proteins that recognize and bind to specific carbohydrate residues on cell surfaces. Soluble hemagglutinins have been demonstrated in the hemolymph of a wide variety of invertebrates. In light of their carbohydrate recognitory capabilities, agglutinins have been implicated in invertebrate defense responses against pathogens, and nonself or foreign tissues and cells. Consistent with this possiblity, agglutinins are known to be associated with the circulating hemocytes in many invertebrate species, including molluscs and insects. Opsonic capacity has been demonstrated for molluscan agglutinins, however, no solid evidence for opsonic activity has been found among the insect agglutinins studied.

The objectives of this study are to localize hemagglutinin to grasshopper hemocytes using immunochemical techniques, and to investigate the *in vitro* opsonic properties of grasshopper agglutinin. Results of immunocytochemical staining of monolayer hemocytes reveal the presence of agglutinin in the adult hemocytes of both *Melanoplus differentialis* and *M. sanguinipes*. Granular cells only, stain for the presence of agglutinin, the phagocytic plasmatocytes do not stain. The hemocytes bind asialo human erythrocytes, *Nosema locustae* spores, and *Bacillus thuringiensis* bacteria. Only the rosetting of asialo human erythrocytes can be inhibited by the addition of agglutinin-specific carbohydrates. No opsonic activity is found when asialo human erythrocytes or *B. thuringiensis* is incubated in either grasshopper serum or affinity purified hemagglutinin prior to overlayering hemocytes. While a potential opsonic effect is seen for *Nosema* spores incubated in grasshopper serum, no opsonic activity is demonstrated for *Nosema* incubated in purified hemagglutinin prior to overlayering hemocytes. Neither increased immunofluorescence nor increased phagocytosis was observed when hemocytes, *in vitro*, were incubated with purified agglutinin. Phagocytosis of *Nosema* spores is not inhibited by the addition of agglutinin-specific monoclonal antibody to hemocytes. These results indicate that although grasshopper hemagglutinin is demonstrated in a percentage of grasshopper hemocytes, it does not appear to have an *in vitro* role in the recognition of foreign particles by hemocytes. Potential physiological roles of agglutinins in grasshoppers have not previously been described. Results reported here are consistent with the non-opsonic character and hemocytic location of other described insectan agglutinins.

INTRODUCTION

Insects exhibit a defense system which is remarkably effective at protecting them from pathogenic organisms. Because of their huge numbers and extreme species diversity, the importance of this defense system has been increasingly recognized. An understanding of the insect defense responses may provide workers with clues to the origin of vertebrate immunity, and may uncover novel defense reactions as yet undetected in higher organisms (1). In addition, studies on insect defense mechanisms may lead to new biological control agents to help man in his constant battle with insect pests.

Like the vertebrate immune system, insect defense responses involve both cellular and humoral components. Unlike the vertebrates, insects, along with other invertebrates, neither possess immunoglobulins nor the complement system (2). Thus, invertebrate defense reactions do not show the specificity or memory critical to the vertebrate immune system. Invertebrates have, however, developed a defense response that is rapid, efficient, and exhibits a broad degree of specificity. Invertebrates are able to distinguish self tissue from non-self, and a variety of mechanisms have evolved for dealing with the latter.

In invertebrates, and insects in particular, both hemocytes and serum proteins remove foreign objects from circulation. In general, insects exhibit three main mechanisms for dealing with invading microorganisms. These mechanisms include the cellular responses phagocytosis, encapsulation and nodule formation, and the humoral antimicrobial proteins (1,2,3).

Phagocytosis in insects is similar to that of vertebrate macrophages. Insect contain specialized phagocytic cells, generally termed plasmatocytes, which circulate in the hemolymph. In addition, insects often have sessile phagocytic cells found lining the body wall and internal organs. These cells are responsible for removing small foreign particles from circulation. The particles are engulfed by the cell and subsequently destroyed by lysosomal enzymes (for review of invertebrate phagocytosis see 4,5,6).

Encapsulation and nodule formation also are cellular responses involving the circulating hemocytes. Encapsulation generally occurs with foreign particles larger than 10 um (6). Nodule formation is similar to encapsulation and occurs when microorganisms exceed the number that can be cleared by phagocytosis (1). This process involves the formation of large aggregates of particles and hemocytes, usually 5 to 30 layers, packed tightly against the foreign particle. A capsule is formed against an organ or body wall, trapping the invader, thus removing it from

circulation, (1,2,6,7). The adhesion of the hemocytes to the foreign particle is achieved by a variety of sticky molecules formed during hemocyte degranulation. Encapsulation and nodule formation is usually accompanied by melanization of the aggregates. Melanization is the result of the prophenoloxidase enzyme cascade, believed to be triggered by hemocyte degranulation. This cascade produces toxic quinones and melanin, which may then cause microbial death. The prophenoloxidase cascade has been reviewed elsewhere (8,9).

Antimicrobial proteins are a third, humoral, component of invertebrate defense systems. Antibacterial factors have been found in a wide variety of insects and show activity towards both Gram-negative and Gram-positive bacteria. One of the most widespread antibacterial factors in invertebrate hemolymph is lysozyme, which causes the lysis of Gram-positive bacterial cell walls (1). The cecropins and attacins are non-lysozymal lepidopteran antimicrobial proteins that show broad spectrum antibacterial activity (1,10,11). Cecropins have been shown to cause bacterial lysis and may function in membrane disruption (1,11). Additional cecropin-like proteins have been identified in dipterans (12), and have been proposed to be widespread in insects (1). In addition, antiviral hemolymph factors have been identified in some insects (10).

The cellular responses of phagocytosis and encapsulation involve the interaction of foreign particles with hemocytes. A prerequisite for this interaction is the recognition of foreignness. In the vertebrate immune system immunoglobulins and the complement system are responsible for this initial recognition. These components act as opsonins, coating foreign particles and targeting them for removal by the macrophages. How do insects discriminate between self and non-self tissue? Specifically, do invertebrates have molecules which function in the recognition of foreign material?

Invertebrate hemolymph can cause the agglutination of bacteria and vertebrate erythrocytes *in vitro*. This agglutininating activity is due to the presence of soluble polyvalent lectins, called hemagglutinins. As lectins, agglutinins recognize and bind to specific carbohydrate residues present on the surfaces of cells. Lectins have been demonstrated in the hemolymph of a wide variety of invertebrates, including the arthropods (13,14,15), and they are considered to be ubiquitous among living organisms (16,17). In insects, hemagglutinins have been found in grasshoppers (18,19), crickets (20), flesh flies (21), cockroaches (22,23,24,), beetles (25), locusts (26), and moths (27,28,29).

Due to their carbohydrate binding capacity, insect agglutinins have been proposed to be the initial recognitory

step in pathogen clearance (30,31,32,33). Agglutinins, by binding to carbohydrate residues on foreign particles, may serve as the link between the invader and the hemocytes responsible for encapsulation or phagocytosis.

The following models have been proposed for the roles of agglutinin in insect defense responses (7,21,30,31,33).

1. Agglutinins may be membrane bound components of the circulating hemocytes. Agglutinin would then act directly, by binding the foreign particles via surface carbohydrates, causing the adherence of the foreign particle to the hemocyte.

2. Humoral hemagglutinins, circulating in the hemolymph, may serve as opsonins. The agglutinins would bind to and coat foreign particles and as a result of the agglutinin polyvalent nature, bind the invader to a phagocytic hemocyte via specific hemagglutinin carbohydrate receptors located on the hemocyte surface. Thus the foreign particle and hemocyte would be crosslinked by an agglutinin bridge.

3. Humoral hemagglutinins may help neutralize foreign particles by agglutinating the particles into larger aggregates. This would then allow encapsulation or nodulation to proceed more efficiently.

4. Hemagglutinins may be involved in wound healing and metamorphosis by binding disintegrating pieces of tissue thereby targetting them for removal. This mechanism would be especially relevant to holometabolous insects.

5. Hemagglutinins may not be directly involved in insect defense. Rather they may serve as carrier molecules for glycoprotein, glycolipid, and carbohydrate transport processes.

The proposed roles for agglutinin in invertebrate defense systems are mainly inferred from the capacity of invertebrate hemolymph to agglutinate bacteria and vertebrate erythrocytes *in vitro*. In addition, some similar recognitory lectin functions have been demonstrated in vertebrates (31). However attempts to define hemagglutinin function in invertebrates have been inconclusive, and the majority of these studies have centered on noninsect species. These experiments have generally concentrated on two possible roles for hemagglutinin, that of hemocytic membrane-bound agglutinin, and a humoral hemagglutinin-opsonin.

In an attempt to establish the validity of the agglutinin as a membrane-bound component of hemocytes, researchers have tried to localize hemagglutinin on hemocytes. This has been done successfully for three mollusc species (34,35,36). Using immunochemical techniques, hemocytes from the snail *Lymnaea stagnalis*, (34) and the oyster *Crassostrea virginica* (36) have been demonstrated to contain a cell surface hemagglutinin. In both cases antibodies directed toward the hemagglutinin were prepared by injecting rabbits with serum-agglutinated and washed rabbit erythrocytes. It

was assumed that the hemagglutinin responsible for the erythrocyte clumping was the major antigenic protein that elicited antibody production.

In a similar experiment, Renwrantz and Stahmer (35) demonstrated the presence of a cell membrane hemagglutinin on the hemocytes of the mussel Mytilus edulis. For this experiment, the anti-agglutinin antiserum was prepared by injecting rabbits with Mytilus hemolymph, followed by affinity purification of the antibodies on a Sepharose-agglutinin column.

To date, hemocyte-associated agglutinins have been demonstrated in four insect species (27,37,38,39). Amirante and Mazzalai (37) localized hemagglutinin in the cytoplasm and on the plasma membrane of the granular and spherule hemocytes of the cockroach Leucophaea maderae L., and Yeaton (27) demonstrated an agglutinin on the surface of plasmatocytes and in the cytoplasm of the granular hemocytes of the silkmoth, Hyalophora cecropia. Both of these studies utilized anti-agglutinin antisera prepared in rabbits injected with hemolymph-agglutinated rabbit erythrocytes. Similarly, Lackie (38), using an antibody directed against purified lectin, showed that the hemagglutinin from the cockroach Periplaneta americana is associated with all hemocyte types in that insect.

Using an alternative technique, Komano and associates (39) demonstrated hemocyte-associated hemagglutinin in the

flesh fly Sarcophaga perigrina. Purified flesh fly agglutinin was radioiodinated with ^{125}I. This labelled lectin was then shown to bind to hemocytes in vitro. This association with hemocytes appears to be mediated via lectin-carbohydrate interactions, as binding was completely inhibited by galactose.

The standard method for demonstrating hemagglutinin opsonic activity consists of the addition of serum-treated or serum-untreated test particles to hemocytes in vitro. If opsonic activity is present, serum pretreatment will increase the binding or phagocytosis of the particles. These experiments most often use mammalian erythrocytes as the test particle, thus the role of hemagglutinin as the serum opsonin is inferred from the in vitro agglutination of the test particles. In this manner, a serum opsonin has been demonstrated in the hemolymph of the mussel Mytilus (35) and the snail Lymnaea (40). Similarly, a serum opsonin from the snail Helix pomatia has been studied in vivo. Renwrantz and colleagues (41,42) have demonstrated that the elimination of mammalian erythrocytes and yeast cells from the circulation of the snail is greatly increased by pretreatment of the particles in snail serum. Clearance of these particles can be inhibited by N-acetylated hexosamines. As before, the humoral factors responsible for recognition of the foreign particles were believed to be hemagglutinins.

A more precise determination of the opsonic activity of molluscan hemagglutinin has been attempted by Renwrantz and Stahmer (35). The agglutinating activity in Mytilus hemolymph was isolated by affinity chromatography on Sepharose-mucin. This purified material, which still exhibited erythrocyte agglutination, was then used to pretreat yeast cells prior to phagocytosis assays. Mytilus hemocytes phagocytosed agglutinin-incubated yeast cells much more readily than saline-incubated yeast cells. The phagocytosis stimulating activity of the isolated hemagglutinin was comparable to the activity of Mytilus hemolymph. In Mytilus, hemagglutinin appears to have opsonic properties in vitro.

Attempts to demonstrate the presence of hemolymph opsonizing factors in insects have been unsuccessful (43,44,45). Insects studied for the presence of serum opsonins have been the cockroaches Blaberus craniifer (43), and Periplaneta americana (44,45), and the stick insect Clitumnus extradentatus (45). In each case hemocytes, incubated in vitro, with hemolymph-pretreated particles failed to show increased adherence or phagocytosis. In fact, Scott (44) and Rowley and Ratcliffe (45) found that sheep erythrocytes incubated in hemolymph adhered less avidly to hemocytes.

In a related study, Pendland and Boucias (29) have shown that a purified lectin from the beet armyworm binds to fungal cell wall surfaces. Binding of the agglutinin to the

fungus was detected by incubating spores with fluorescence-labeled agglutinin. Although binding to the spore would be a prerequisite for hemagglutinin opsonic activity, phagocytosis experiments were not done, leaving the question of the function of the hemagglutinin unanswered.

To date, experiments on insect hemagglutinins have not supported an opsonic role for the lectins. However, no purified hemagglutinins have been tested for opsonic activity. It may be that in vitro opsonic activity of agglutinins in insects is masked when using molecularly complex whole hemolymph preparations. Therefore, use of purified insect serum in phagocytosis studies should prove valuable. In addition, due to the large number of insect species and their morphogenic diversity, opsonization studies in only three species cannot give generally conclusive results. Additional studies on insect hemagglutinins are necessary before generalizations on their role in defense reactions can be made.

Grasshoppers (Melanoplus differentialis and Melanoplus sanguinipes) contain a hemolymph hemagglutinin (GHA) which agglutinates humanasialo erythrocytes as well as weakly agglutinating several other animal erythrocytes (18,19). Biochemical characterization has shown that a single hemagglutinin accounts for all observed hemagglutination activity (46). The hemagglutinin has been purified by affinity chromatography and exists as a 600-700 Kd molecular

weight noncovalent aggregate of 70,000 molecular weight subunits (46). Hemagglutination of asialo human erythrocytes can be inhibited by a broad range of carbohydrates including D-galactosidic and D-glucosidic residues. Grasshopper agglutination can also be inhibited by EDTA and shows a requirement for calcium (19,46). Mono-specific rabbit polyclonal antibody and a mouse monoclonal antibody directed towards purified M. differentialis agglutinin have been developed. Both antibodies show specificity towards the 70,000 molecular weight hemagglutinin subunit on Western blots of M. differentialis and M. sanguinipes whole hemolymph (47).

Descriptions of the possible role of grasshopper hemagglutinin in the insect's defense system have not been reported in the literature. I propose to study this role using an approach similar to that used for the molluscan and insectan species described above. The availability of agglutinin-specific antibodies and purified GHA should provide this study with more definitive probes in the examination of possible roles for grasshopper hemagglutinin in insect defense mechanisms.

RESEARCH OBJECTIVES

The specific objectives of this study are:

1. To localize grasshopper hemagglutinin in hemocytes from *Melanoplus differentialis* and *Melanoplus sanguinipes*. This will be achieved by the development and application of appropriate immunochemical labeling procedures to detect the hemagglutinin.

2. To examine the possible opsonic character of insect hemagglutinin using the hemolymphatic hemagglutinin isolated from *M. differentialis*. This will be achieved by studying hemocytic adherence or phagocytic response to particles pretreated with hemolymph or purified hemagglutinin.

MATERIALS AND METHODS

Purification and Detection

Collection of Hemolymph

Adult *M. differentialis* and *M. sanguinipes* were provided from permanent colonies at the USDA Rangeland Insect Laboratory, Bozeman, MT. Hemolymph was collected by a capillary pipette from individual cold-anaesthetized insects as previously described (18). The hemolymph was pipetted into an equal volume of cold Dulbecco's phosphate buffered saline (DPBS) (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.135 M NaCl, pH 7.2) which contained 0.001 M phenylthiourea (PTU) to inhibit melanin formation and 0.001 M phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolytic destruction. Hemocytes and coagulated material were removed by centrifugation at 3000 x g and the hemolymph supernatant solution was stored at -20°C.

Hemagglutination Assay

Preparation of Asialo Human Erythrocytes. Human O^+ erythrocytes were obtained from Bozeman Deaconess Hospital (Bozeman, MT). Erythrocytes were washed four times by centrifugation at 4°C in DPBS. Asialo human erythrocytes (AHRBC) were prepared by incubating erythrocytes for 1 hr at

37°C with 2 mg neuraminidase (type V, Sigma Chemical Co., St. Louis, MO) in 10 ml DPBS, pH 5.7. The asialo erythrocytes were washed four times by centrifugation at 4°C in DPBS prior to use.

Hemagglutination Assay. Hemagglutination activity was assayed by serial two-fold dilution of 25 ul hemagglutination sample (whole hemolymph or purified agglutinin) with 25 ul DPBS using plastic V-bottom microtiter dishes (Dynatech Labs, Inc., Alexandria, VI). and addition of 25 ul of a 2.5% suspension of asialo human erythrocytes. Agglutination was visually determined after 1 hr. The reciprocal of the highest dilution causing agglutination of erythrocytes was the hemagglutination titer.

Purification of Grasshopper Hemagglutinin

Affinity Chromatography. The purification method outlined below represents a slight modification of a procedure reported elsewhere (46). Grasshopper hemagglutinin was purified by affinity chromatography on a column of Sepharose-galactose. A 0.5 x 2.5 cm column (0.5 ml) of Sepharose-galactose was prepared and washed with 50 volumes of DPBS. Approximately 35 ml M. differentialis hemolymph was passed through the column (6 ml/hr) at 25°C. The column was then washed with HEPES buffer (0.01 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.15 M NaCl, 0.9 mM

$CaCl_2$, pH 7.2) until the 280 nm absorbancy of the effluent returned to near zero. Bound hemagglutinin was released from the column upon elution with HEPES buffer containing 0.1 M alpha-methyl-D-galactoside. The elution buffer was added in 1.5 ml aliquots, with the column stopped between additions and allowed to incubate for 2 hours at 25°C. Hemagglutinin activity of the aliquots was determined by hemagglutination assay using asialo human O^+ erythrocytes as described above.

In addition to purifying hemagglutinin by this modified technique, hemagglutinin was isolated by the previously reported procedure. In the original procedure, all column washes were in DPBS, and elution of agglutinin was performed in DPBS containing 0.2 M galactose.

Concentration. Purified hemagglutinin samples obtained from the above procedure were concentrated to remove the eluting carbohydrate. The agglutinin was concentrated approximately 20 fold in DPBS using a 30,000 molecular weight cutoff microconcentrator (Amicon, Corp., Danvers, MA). The concentrate was then returned to the original volume with DPBS and titered to determine hemagglutination activity.

Production of Agglutinin-specific Antibodies

Monclonal Antibodies. Agglutinin specific monoclonal antibodies (MAB) were prepared previous to this study (47). Purified *M. differentialis* hemagglutinin was prepared by

affinity chromatography and used to immunize mice. Mouse spleen cells were then harvested and fused with mouse X-63 myeloma cells. Hybrids were selected and cloned according to the methods of Kohler and Milstein (48). Hybridomas producing hemagglutinin specific antibody were identified by detection of hemagglutinin on Western blots. Hybridoma supernates from these clones were collected and concentrated 5-fold in an ultrafiltration cell (Amicon Corp., Danvers, MA). Concentrated supernatant solutions were stored at -50°C. Frozen supernates were thawed and dilutedin an appropriate buffer to return them to their original concentration prior to use.

Rabbit Polyclonal Antibodies. During the course of this study, monospecific rabbit polyclonal antibodies were prepared in our lab by a coworker (47). Two female New Zealand white rabbits were immunized with 100 ug purified M. differentialis hemagglutinin according to the multiple intradermal injection method of Vaitukaitis (49). The affinity purified antigen (agglutinin) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electro-transfered to nitrocellulose paper. The agglutinin-bearing nitrocellulose was dissolved in dimethyl sulfoxide (DMSO) according to the method of Knudsen (50). The solubilized antigen was then emulsified in an equal volume of complete Freund's adjuvant and injected into the animals at weekly intervals. For each rabbit, control

serum was obtained before injection, and antiserum was harvested 8 weeks after immunization. Specificity of the antiserum was confirmed by Western blots of hemolymph and purifed agglutinin.

Detection of Grasshopper Hemagglutinin

Western Blot. Grasshopper hemolymph and purified hemagglutinin were subjected to electrophoresis in SDS-polyacrylamide gel slabs using the apparatus and procedures from Hoefer Scientific (San Francisco, CA). The stacking gel was 4%, pH 6.8, and the separating gel was 7.5%, pH 8.8, according to Laemmli (51). Electrophoresis was at 15 mA/gel until the proteins entered the separating gel (approximately 1 hr), then amperage was increased to 30 mA/gel for 3 hr.

Proteins separated by SDS-electrophoresis were transferred from the polyacrylamide gel onto nitrocellulose filter paper (Schleicher and Schuell, Keene, NH) using a Hoefer TE 42-Transphor Electrophoresis Cell. For review, see Gershoni and Palade (52). Proteins were blotted in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 90 minutes at a current of 0.8 A. Following transfer, the nitrocellulose was stained either with Amido Black (0.1% Amido Black 10B in 25% 2-propanol, 10% acetic acid, destained with 25% 2-propanol, 10% acetic acid), or by immunochemical means with the agglutinin-specific MAB, followed by a horseradish peroxidase (HRP) labelled secondary antibody (Appendix A).

Con A Probing. Purified grasshopper hemagglutinin subjected to electrophoresis and blotted to nitrocellulose was probed with biotinylated-Concanavalin A (EY Labs, San Mateo, CA) to detect possible contaminating glycoproteins. The nitrocellulose was incubated in a 5 ug/ml dilution of Con A in Tris-tween buffer (0.05 M Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.2) using the Hoeffer PR-200 decaprobe. The nitrocellulose was then given three 5 minute washes in Tris-tween, followed by incubation in HRP-conjugated Avidin (EY Labs) at a concentration of 1 ug/ml in Tris-tween. Simultaneous with this incubation, additional nitrocellulose lanes were incubated in the agglutinin-specific immunochemicals, and all lanes were then developed as described (Appendix A).

Silver Stain. Hemagglutinin purified by the modified affinity chromatography procedure was subjected to SDS-polyacrylamide electrophoresis, with subsequent silver staining of the gel. The silver stain method of Morrissey (53) was employed.

Localization

Hemolymph Probing

To determine possible *in vivo* receptors for GHA, hemolymph from *M. sanguinipes* was subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Individual sample lanes in the Hoefer decaprobe were then

incubated for 2 hr in either M. sanguinipes whole hemolymph (diluted 1:1 in Tris-tween), or left untreated. The nitrocellulose was given three 10 minute washes in Tris-tween, then all lanes were immunostained with horseradish peroxidase labeled conjugate as described in Appendix A.

Formation of Hemocyte Monolayers

Hemocyte monolayers were prepared in either 24 well plates (Nunclon, Denmark), or on 20 mm square glass coverslips placed inside 35 mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ). Adult grasshoppers were bled by capillary pipette as previously described. The hemolymph was pipetted into the culture dishes containing 1 ml cold DPBS to which had been added 4 ul/ml glycerol (to increase osmolarity to 400 mOsm), and 0.65 mg/ml glutathione (to prevent melanization). After 20 minutes at room temperature the hemocytes had spread and adhered to the bottom of the wells or glass coverslips. The hemocyte monolayer was then washed, by Pasteur pipette, with DPBS (adjusted for osmolarity) to remove hemolymph and non-attached cells.

Lysing Hemocyte Monolayers

Hemocyte monolayers formed in 24 well plates were lysed for subsequent Western blot analysis. Lysis was carried out by incubating monolayers for 1 hr in 100 ul 2% Nonidet P40 (NP-40; Bethesda Research Laboratories, Gaithersburg, MD) in

DPBS. Lysates were stored at -20°C until subjected to electrophoresis.

Indirect Immunofluorescent Staining (IFA)

Fixed Monolayer Hemocytes. Generally, the method of Willingham and Pastan (54) was followed, and the staining procedure is outlined in Appendix B. Monolayer hemocytes were fixed in 3.7% formalin in DPBS for 1 hr, 4°C. Monolayers were then incubated in the concentrated monoclonal antibody supernates, diluted to their original volume with DPBS. The monolayers were then washed, then incubated in a fluorescein conjugated-goat-anti mouse IgG (Hyclone Labs, Logan UT). The cells were again washed and mounted on glass slides for observation.

Fixed Hemocyte Suspensions. Hemocyte suspensions were prepared by bleeding cold-anaesthetized grasshoppers directly into polypropylene tubes containing 1 ml ice cold 3.7% formalin in DPBS. Fixation continued for 1 hr at 4°C. The hemocytes were then washed three times by gentle centrifugation (950 x g) and resuspended in DPBS. Suspended hemocytes were stained under identical conditions as that outlined for monolayers, with all incubations occurring in polypropylene tubes on a rocking table.

Live Monolayer Hemocytes. Live hemocytes were subjected to the indirect immunofluorescence protocol under similar conditions as for fixed monolayer hemocytes, with

the following exceptions. All solutions were adjusted to 400 mOsm by the addition of glycerol, and all incubations occurred at 4°C. In addition, the monoclonal antibody solution was heat treated (56°C for 30 minutes) to inactivate complement.

Horseradish Peroxidase Enzyme Immunoassay (EIA) Staining

Indirect Enzyme Immunoassay. Staining procedure for the indirect EIA technique was similar to that for immunofluorescence (Appendix B). Fixed monolayer hemocytes were incubated in monoclonal antibody, washed, then incubated in a horseradish peroxidase-conjugated secondary antibody. The HRP-conjugated goat anti-mouse IgG (BioRad, Richmond, CA) was diluted 1:100 in complete Iscove's Modified Dulbecco's Medium (IMDM) (Gibco Laboratories, Grand Island, New York). The monolayers were then washed, and incubated in developer solution (0.5 mg/ml diaminobenzidine, DAB, 0.01% hydrogen peroxide, in 0.05 M Tris, pH 7.2) for 30 minutes. The hemocytes were washed for 10 minutes in0.05 M Tris, and mounted under glycerol diluted 1:1 with DPBS.

Avidin-Biotin Complex. Monolayer hemocytes were stained for agglutinin using the reagents and protocol of an avidin-biotin complex (ABC) kit (Vectastain Laboratories, Burlingame, CA). Fixed hemocytes were first preincubated in whole goat serum, followed by two five minute washes in DPBS. The monolayers were then incubated in monoclonal

antibody for 1 hr, followed by three five minute DPBS washes. Biotinylated-goat anti-mouse IgG (1:100 in complete IMDM, 0.1 M galactose) was used as the secondary conjugate. The monolayers were washed in DPBS for 15 minutes and incubated in the Vectastain biotin/avidin complex for 30 minutes, according to kit directions. The cells were again washed for 15 minutes and developed in DAB substrate for 30 minutes. After an additional 10 minute wash in DPBS the cells were mounted under glycerol-DPBS.

Immunocytochemical Controls

Controls for nonspecific antibody binding were included for each immunocytochemical labeling experiment. In place of the primary antibody, control antibodies were either normal mouse serum (Cappel Laboratories, Cochranville, PA) at 1 ug/ml in complete IMDM, or an irrelevent mouse monoclonal antibody supernate prepared by Dr. Mickey McGuire (USDA Rangeland Insect Laboratory, Bozeman, MT).

Photomicroscopy

All hemocytes were observed under either phase contrast or 490 nm blue light (for FITC fluorescence) using an Olympus BH-2 microscope with a reflected light fluorescence attachment. Black and white photographs were taken with Kodak 400 ASA Tri-X pan film. Color photographs were taken with Kodak 400 ASA Ektachrome. Exposure times for fluorescence varied from 30 to 60 seconds, depending upon

the intensity of the stain.

Opsonic Characterization

Phagocytosis by Monolayer Hemocytes

Test Particles. Live monolayer hemocytes were incubated with the following particles: Asialo human erythrocytes (HRBC) at a concentration of 1000 HRBC: 1 hemocyte; *Nosema locustae* spores, 20 spores: 1 hemocyte; and *Bacillus thuringiensis* bacteria, 100 bacteria: 1 hemocyte. All incubations were performed in moist chambers at room temperature for 90 minutes. The hemocytes were then washed to remove nonadhering particles, and were observed for particle adherence or phagocytosis by microscopic examination.

Immunostain of Phagocytic Hemocytes. Hemocytes incubated with *Nosema* spores were also subjected to indirect immunofluorescence staining. The hemocytes were incubated in *Nosema* for 90 minutes, washed, and fixed in 3.7% formalin in DPBS. The hemocytes were then probed for the presence of agglutinin as described (Appendix B).

Carbohydrate Inhibition. Dependence of particle adherence or phagocytosis on carbohydrates was determined by incubating the particles on the monolayer in the presence of various agglutinin-specific carbohydrates (as determined by Stebbins and Hapner, 46). Hemocytes were incubated in the

carbohydrates (0.1 M in DPBS) for 15 minutes, then the particles were added to the monolayers in the presence of carbohydrate. Controls consisted of monolayers in which a non-inhibitory carbohydrate was used for incubation, and monolayers in which DPBS was substituted for the carbohydrate solution.

Opsonization. Opsonic properties of hemagglutinin were studied by overlayering live monolayer hemocytes with erythrocytes, Nosema spores, or B. thuringiensis bacteria that had or had not been previously incubated in varying concentrations (1:8, 1:32, and 1:256 in DPBS) of whole hemolymph or affinity purified agglutinin. Pretreated particles were incubated in whole hemolymph or purified agglutinin for 30 minutes, then overlaid on the monolayer while still in the incubating solution. In a second experiment, particles were pretreated as before, only subjected to a wash by centrifugation and resupended in DPBS prior to overlayering the monolayer. Due to the tendency of erythrocytes to clump during some of the hemolymph and agglutinin incubations, erythrocytes were ejected through a 23 gauge needle prior to overlaying the monolayers. Control particles were incubated in DPBS, otherwise treated identically to the experimental particles.

Hemocytic Agglutinin Receptors. Monolayers of live hemocytes were incubated for 30 minutes in purified

hemagglutinin (approximately 5 ug/ml in DPBS) prior to overlayering with erythrocytes and Nosema spores.

Live hemocytes incubated with agglutinin were also subjected to immunofluorescence assay to detect possible hemocytic agglutinin binding sites. After incubation in agglutinin the hemocytes were fixed in 3.7% formalin-DPBS and subjected to the normal immunofluorescence protocol (Appendix B).

Blocking of Phagocytosis with Immunochemicals. Live monolayer hemocytes were preincubated in monoclonal antibody and Nosema spores were then added to the monolayer while still in antibody solution. The monolayers were incubated in a moist chamber for 1 hr. As a negative control, additional monolayers were incubated in mouse whole serum (1 ug/ml in complete IMDM) instead of MAB.

Data Analysis

Six to ten female M. differentialis adults were used to form the monolayers for each of the above opsonization experiments. The percentage of hemocytes with adhering or phagocytosed particles and the average number of particles per hemocyte with one or more particle was determined for each monolayer by observing 500 hemocytes in randomly selected microscopic fields. Levels of significance for difference of means were determined using the Student-t test. The level of significance for each experiment was $p<0.05$.

RESULTS

Purification and Detection

Purification of Grasshopper Hemagglutinin

Grasshopper hemagglutinin is routinely purified in our laboratory by affinity chromatography on Sepharose-galactose. Previous purifications have used 0.2 M D-galactose in DPBS as the elution buffer, and on average isolates approximately 350 ug protein. Typical results have been published elsewhere (46). For the purposes of this study the technique was modified by incorporating 0.1 M alpha-methyl-D-galactoside in 0.1 M HEPES as the elution buffer. Alpha-methyl-D-galactoside was added to the column in aliquots, and incubated for several hours prior to elution, as opposed to a continuous elution with galactose. Hemolymph prior to application to the column had a hemagglutination titer of 4096. The titer value of hemolymph emerging from the column was 32, indicating that approximately 99% of the original activity was bound to the affinity matrix. Typical hemagglutination activity of the purified agglutinin aliquots was in the range of 4096-8192. This technique resulted in the isolation of about 700 ug of hemagglutinin from 35 ml *M. differentialis* hemolymph sample, as determined by a modified Lowry protein assay

(55). This is double the yield of the previous purification method, and is due to the incorporation of alpha-methyl-D-galactoside, a more effective competitor for the lectin, in the elution buffer, and the increased time of elution used.

After concentrating and washing the purified agglutinin to remove the carbohydrates used for elution, the titer dropped to 512-1024, representing an 8-fold loss in hemagglutination activity. This loss in activity is presumably due to agglutinin precipitating on the microconcentrator membrane.

Immunochemical Detection of Grasshopper Hemagglutinin by Western Blot Analysis

Both the agglutinin-specific monoclonal antibody, and the rabbit polyclonal antibody detect hemagglutinin in Western blots of whole hemolymph and purified agglutinin preparations. Staining of hemagglutinin is specific, as no other hemolymph proteins are immunostained. Western blotting and immunochemical staining of hemagglutinin can provide an accurate and sensitive technique for the detection of agglutinin in complex mixtures of proteins.

M. differentialis hemagglutinin is often detected as a doublet band of approximate molecular weights 59,000 and 53,000 (Fig. 1). *M. sanguinipes* hemolymph contains an antigenically similar hemagglutinin that also immunostains as a double band of molecular weights 59,000 and 53,000. These two bands indicate a slight agglutinin heterogeneity

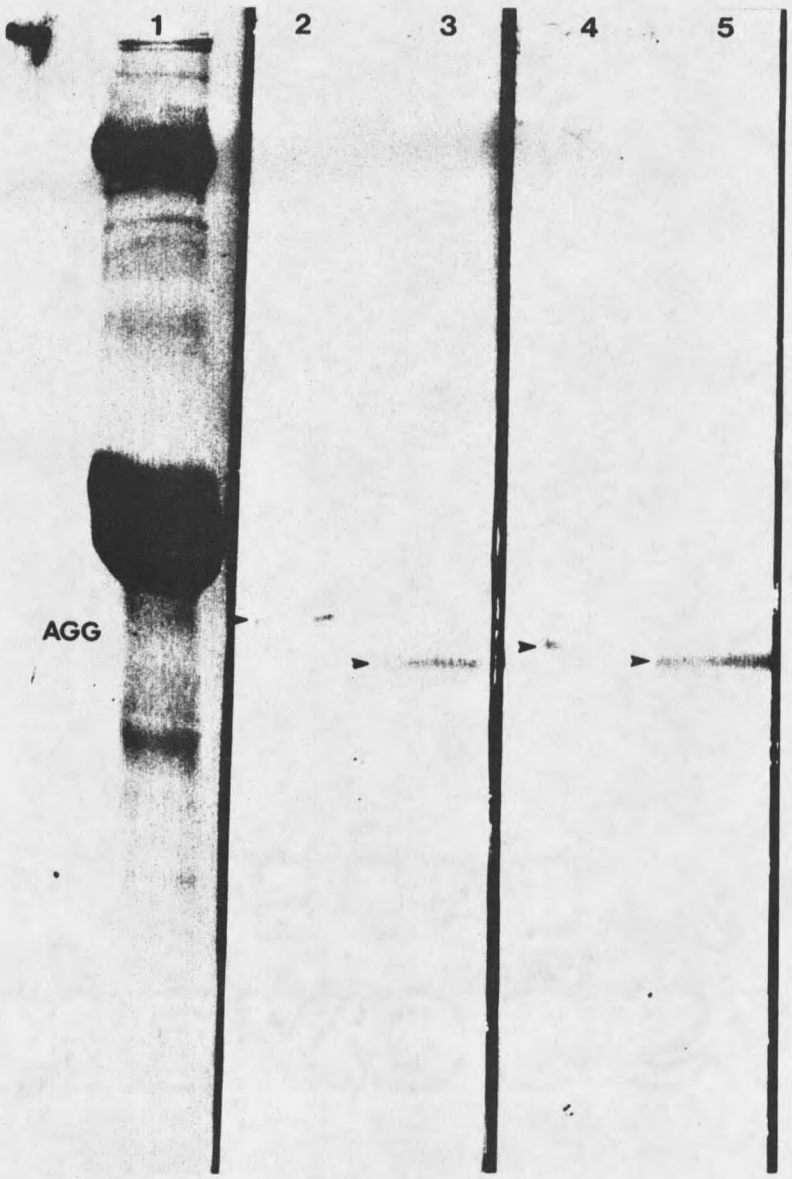

Figure 1. Western blot of whole grasshopper hemolymph and affinity purified agglutinin showing agglutinin-specific staining by both mouse monoclonal and rabbit polyclonal antibodies. Lanes 1,2,4 contain hemolymph, lanes 3,5 purified hemagglutinin. Lane 1 was stained with Amido Black, lanes 2,3 with monoclonal antibody, lanes 4,5 with rabbit polyclonal antibody (AGG-agglutinin).

in grasshoppers. These results are slightly different from previously published determinations of the grasshopper hemagglutinin molecular structure. Earlier, Stebbins and Hapner (46) had reported a single band of molecular weight 70,000 when purified hemagglutinin was examined by electrophoresis in SDS polyacrylamide gels and stained with Coomassie Blue G-250. In their procedure, electrophoresis was carried out at 30 mA/gel for 3 hr, while in the presently reported procedure electrophoresis was at 15 mA/gel for 1 hr, then 30 mA/gel for 3 hr. This initial low amperage allows for better stacking of the proteins at the stacking-separating gel interface. This would then allow for greater protein separation and may cause the increased disassociation of hemagglutinin from the other hemolymph components. Thus the molecular weight of SDS-denatured hemagglutinin may be more accurately represented as 53,000 to 59,000.

Concanavalin A probing

Seven horseradish peroxidase stained bands were observed when hemagglutinin purified by the techniques previously reported in the literature (46) was subjected to a modified Western blot-Con A probe (Fig. 2). One of these bands may correspond to the immunochemically detected agglutinin band. These bands represent humoral molecules bound by Con A. Con A is a plant lectin specific for alpha-D-mannose and glucose residues. The Con A bands presumably

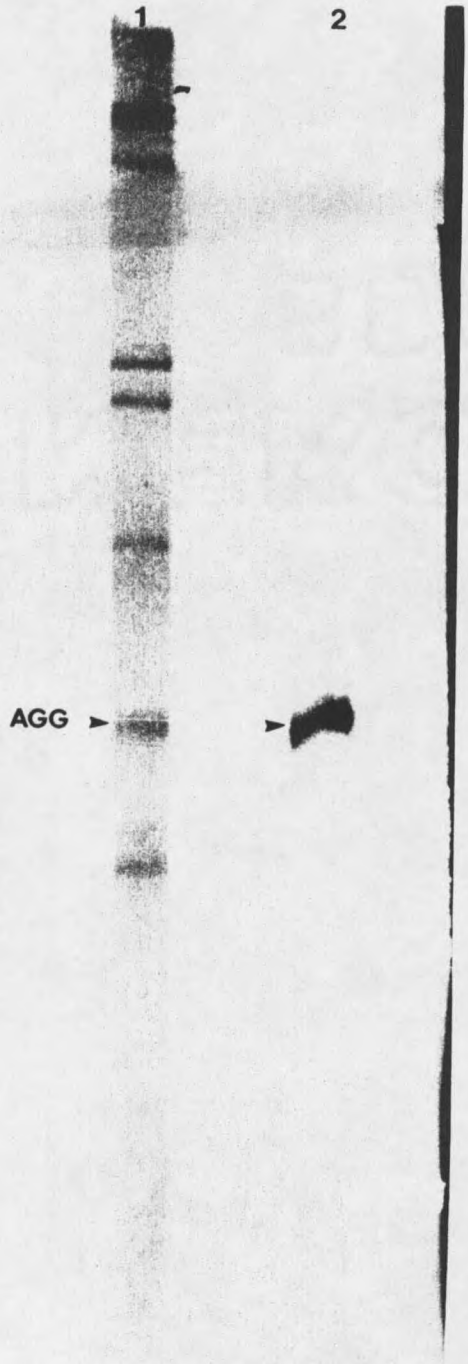

Figure 2. Modified Western blot-Con A probe of purified grasshopper hemagglutinin. Lane 1 contains purified agglutinin incubated in Concanavalin A, lane 2 contains purified hemagglutinin immunostained with monoclonal antibody (AGG-agglutinin).

represent glycoproteins that were copurified with hemagglutinin. A similar probe of hemagglutinin isolated by the modified technique reported in this study identified no Con A binding sites, indicating no detectable glycoprotein contaminants. Thus, the modified purification technique may represent a significant improvement over the previously reported purification method.

Silver Stain

A silver stain of SDS polyacrylamide gels containing the *M. differentialis* hemagglutinin purified by the modified technique reported in this study produced one major protein band of molecular weight 59,000, and a minor band at 56,000 M.W. (Fig. 3), both of which have been shown to be antigenic on Western blots of GHA (47). At loads of 5 ug protein per lane, only one slight contaminant was detected, at a molecular weight of 70,000. The modified purification technique thus provides a convenient method to obtain pure grasshopper hemagglutinin for subsequent opsonization studies.

Localization

Hemolymph Probing

Potential *in vivo* binding sites for grasshopper hemagglutinin were assayed by a modified Western blot-hemolymph probing experiment (Fig. 4). Electrophoresed *M. sanguinipes* hemolymph was blotted to nitrocellulose, then incubated in additional *M. sanguinipes* hemolymph. Upon