Hemagglutinin from Acrididae (Grasshopper) : preparation and properties

by Mark Richard Stebbins

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

Abstract:
The proteinaceous hemagglutinin (lectin) present in the hemolymph of Melanoplus sanguinipes (F.), was isolated and biochemically characterized. The protein was purified to homogeneity by affinity chromatography on a column of Sepharose-galactose. The hemagglutinin showed broad specificity and agglutinated several erythrocyte types. Gel filtration and electrophoresis showed that grasshopper hemagglutinin was a high molecular weight (600-700 K dalton) non-covalent aggregate of 70 K dalton subunits. The 70 K dalton subunits contained two disulfide-linked polypeptide chains of molecular weight 40,000 and 28,000 respectively. The purified hemagglutinin contained a preponderance of acidic and polar amino acid residues and a small amount of glucosamine. Hemagglutination activity toward human asialo erythrocytes was destroyed by treatment of the hemagglutinin with trypsin, heat or EDTA. Hemagglutination inhibition studies showed that low concentrations (<5 mM) of both galactosidic and glucosidic carbohydrates are bound by the hemagglutinin and cause inhibition of erythrocyte agglutination. The strongest inhibitors of hemagglutination were the alpha anomers of D-galactose. Hemolymphatic hemagglutinin isolated from Melanoplus differentiaIis yielded identical physicochemical results as did hemagglutinin from Melanoplus sanguinipes. It was concluded that a single hemagglutinin protein was the substance responsible for all hemagglu-tinating activity present in the hemolymph of either species.

Research directed toward the elucidation of the possible roles that grasshopper hemagglutinin plays in grasshopper immune/defense mechanisms was initiated by producing antibodies to purified hemagglutinin in rabbits and mice. These antibodies were specific for grasshopper hemagglutinin as shown by gel double diffusion and immunoelectrophoresis. Individual subunits were recognized by rabbit antiserum as shown by an indirect immunochemical detection procedure where rabbit antiserum (bound to protein subunits immobilized on a nitrocellulose filter) is recognized by an enzyme-conjugated "second" antibody. Applications of this research toward future immunological localization studies of grasshopper hemagglutinin in insect hemocyte maintainance cultures are discussed.

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Mark Richard Stebbins

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

The proteinaceous hemagglutinin (lectin) present in the hemolymph of Melanoplus sanguinipes (F.), was isolated and biochemically characterized. The protein was purified to homogeneity by affinity chromatography on a column of Sepharose-galactose. The hemagglutinin showed broad specificity and agglutinated several erythrocyte types. Gel filtration and electrophoresis showed that grasshopper hemagglutinin was a high molecular weight (600-700 K dalton) non-covalent aggregate of 70 K dalton subunits. The 70 K dalton subunits contained two disulfide-linked polypeptide chains of molecular weight 40,000 and 28,000 respectively. The purified hemagglutinin contained a preponderance of acidic and polar amino acid residues and a small amount of glucosamine. Hemagglutination activity toward human asialo erythrocytes was destroyed by treatment of the hemagglutinin with trypsin, heat or EDTA. Hemagglutination inhibition studies showed that low concentrations (<5 mM) of both galactosidic and glucosidic carbohydrates are bound by the hemagglutinin and cause inhibition of erythrocyte agglutination. The strongest inhibitors of hemagglutination were the alpha anomers of D-galactose. Hemolymphatic hemagglutinin isolated from Melanoplus differentialis yielded identical physicochemical results as did hemagglutinin from Melanoplus sanguinipes. It was concluded that a single hemagglutinin protein was the substance responsible for all hemagglutinating activity present in the hemolymph of either species.

Research directed toward the elucidation of the possible roles that grasshopper hemagglutinin plays in grasshopper immune/defense mechanisms was initiated by producing antibodies to purified hemagglutinin in rabbits and mice. These antibodies were specific for grasshopper hemagglutinin as shown by gel double diffusion and immunoelectrophoresis. Individual subunits were recognized by rabbit antiserum as shown by an indirect immunochemical detection procedure where rabbit antiserum (bound to protein subunits immobilized on a nitrocellulose filter) is recognized by an enzyme-conjugated "second" antibody. Applications of this research toward future immunological localization studies of grasshopper hemagglutinin in insect hemocyte maintainance cultures are discussed.

INTRODUCTION

An organism's survival is often dependent in part upon endogenous "immune" protection systems that render the host exempt from the potentially harmful effects of pathogens and other foreign substances. Higher vertebrate organisms possess an integrated cellularly and humorally mediated antibody immune system, the hallmarks of which are the immunoglobulins, complement proteins, and lymphocytes [1].

What do we know about the immune systems of lower vertebrates and invertebrates, particularly insects? It has been speculated that the mammalian immune system may have evolved from the hemocyte cells of invertebrates [2]. Certainly the success of the insects' strategy for survival is manifest in their numbers. Over 700,000 currently living species of insects have been identified, which amounts to over one-half of all living things found on the earth. Several lines of evidence are available to clearly indicate that the immunoglobulin-complement system is not present in the insect. Two basic events are known to occur upon the introduction of foreign matter into the hemocoel of an insect: phagocytosis of smaller particles (< 10 micrometers, um) and encapsulation of larger

particles [3]. Phagocytosis initially requires recognition of the foreign substance. This is followed by chemotactic attraction and subsequent attachment of the foreign substance to the phagocyte. The final phase is ingestion and neutralization of the foreign material. Particles too large to be phagocytosed are encapsulated and neutralized in a membranous capsule. Encapsulated bacteria have been shown to aggregate into melanized nodules [4]. What, then, is the nature of nonself recognition in insects? Is recognition accomplished by cells alone? Are humoral factors involved in recognition? One clue may lie in a group of proteins called agglutinins that occur ubiquitously in hemolymph, the blood of insects.

Agglutinins are polyvalent lectins that can recognize and bind to specific carbohydrate molecular structures on cell surfaces of bacteria and vertebrate erythrocytes. The cells are thus crosslinked and clumped or aggregated. In the case of red cells, the term hemagglutination is descriptive of the activity. The clumping activity can be visually observed, and this fact forms the basis for the convenient hemagglutination assay for detection of hemagglutinins.

Soluble hemagglutinin activity is generally present in the hemolymph of invertebrate organisms [5,6] and most current research on invertebrate hemagglutinins is

directed toward their putative (carbohydrate) recognitory capabilities as humoral and/or cellular immunosubstances [7]. Hemagglutinins have been implicated as opsonins in some invertebrates including crayfish [8], mollusks [9] and oysters [10,11]. Nonetheless, the in vivo function(s) of invertebrate hemagglutinins is unknown and experimental data supportive of their involvement in immune mechanisms is largely circumstantial and inconclusive.

The reasons for studying insect immune systems are two-fold. First, annual losses of food crops in the United States caused by insects have been estimated at 13% [12]. In the western United States grasshoppers alone destroy more than 30 million dollars worth of food crops each year [13]. Our rapidly increasing human population demands increased global food production capacity which is increasingly dependent on the effective control of insect pests. Insect control involving toxic pesticides is facing many drawbacks including technological limitations due to evolving genetic resistance, nonspecificity and sociological-legal restraints [14]. These facts along with recent advances in biotechnology have opened the door for research and development of biological control systems; that is, the strategic introduction of bacteria or viruses that are pathogenic to a specific (pest) organism [15]. The major obstacle limiting progress in this area is the highly successful immunodefense system of

insects, the biochemical basis of which is poorly understood.

Another reason for studying insect hemagglutinins is that lectins bind to cell surface polysaccharides, glycoproteins, and glycolipids in a specific fashion and provide a way to study the architecture of cell surfaces [16]. Some lectins differentially agglutinate certain mammalian cells in culture, depending on the structure of their surface polysaccharides. Since these cell surface carbohydrates are often antigenic in a nonself environment, lectins can provide a means by which to indicate donor-recipient tissue compatability in tissue transplants based on a similar agglutination pattern [17]. Also, some lectins preferentially agglutinate virally or chemically transformed mammalian cells in culture as well as cells from spontaneous tumors [18] and are therefore useful tools for the cytogeneticist and the cancer researcher [19].

The possible involvement of hemagglutinins in insect defense systems is described in reviews by Whitcomb, et al. [20] and more recently by Lackie [17] and Ratner and Vinson [21]. Insect agglutinins may be active in both recognitory and processing (phagocytosis, encapsulation) phases of immunosurveillance and protection. Elucidation of the <u>in vivo</u> function of insect agglutinins has been hampered by the unavailability of highly purified and

characterized agglutinins, and associated immunological detection procedures.

Hemagglutinating activity has been described in the hemolymph of several insect specimens including grasshoppers [22,23], crickets [24], flesh fly [25], cockroaches [26,27,28,29,30,31], beetles [32], locusts [28], milkweed bug [33], and butterflies and moths [34,35].

An injury induced hemagglutinin from Sarcophaga peregrina larvae that is also detected in high amounts in the early pupal stage was isolated and characterized by Komano, et al. [25]. It is a 190,000 molecular weight (MW) protein consisting of four 32,000 MW and two 30,000 MW noncovalently associated subunits. Only the 32,000 MW subunit is present in normal larvae. The authors suggest that the 30,000 MW subunit may be produced from 32,000 MW subunits by a protease that is activated commensurate with body wall injury [36]. This idea is based on indirect evidence that both 32,000 MW and 30,000 MW subunits show similar tryptic peptide maps. Alternatively, the 30,000 MW subunit may be synthesized de novo. In a further study using a radioimmunoassay Komano, et al. [37] showed that the lectin was synthesized in the fat body and secreted into the hemolymph both on injury and on pupation. Also, the amount of lectin on the outer hemocyte surface increased upon injury and on pupation. The authors suggest that this hemagglutinin may be involved in

nonspecific recognition in an immune system that culminates in phagocytosis of foreign substances or fragments of tissue undergoing metamorphosis.

Hapner and Jermyn [24] isolated a hemagglutinin from the cricket Teleogryllus commodus (Walker) on an affinity matrix of Sepharose-fetuin. Cricket hemagglutinin was completely desorbed from the affinity column with buffer that contained 0.1 M N-acetyl neuraminic acid, and incompletely with buffer containing 0.1 M 2-acetamido-2-deoxy-D-glucose. Purified cricket hemagglutinin activity was inhibited by the two desorbants as well as by N-acetyl-D-glucosamine (10 mM), N-acetyl-D-galactosamine (50 mM), and EDTA (10 mM). The unpurified cricket hemagglutinin was shown to be a high molecular weight glycoprotein complex of disulfide-linked 31,000 MW and 53,000 MW polypeptide chains.

Amirante, et al. [38] described the presence of two hemagglutinins in the hemolymph of the cockroach Leucophaea maderae L. Amirante and Mazzalai [39] used fluorescein-labeled antiserum, to show that both hemagglutinins were synthesized in granular hemocytes and spherule cells. The authors propose that the two hemagglutinins are probably released into the hemolymph where they may be responsible for "cellular immunological reactions".

Grasshopper hemolymph nonspecifically agglutinates all human ABO and many animal erythrocytes. The hemagglutinin activity exhibits a broad pattern of carbohydrate inhibition of hemagglutination and shows highest sensitivity to inhibition by both galactosidic and glucosidic structures [22]. This broad range of hemagglutination and carbohydrate inhibition resides in individual insects [23] and is not the result of the pooling of hemolymph from many insects. Individual grasshoppers are therefore viewed as either containing complex mixtures of agglutinins of various specificities (heteroagglutinins) or a single hemagglutinin of broad red cell and carbohydrate binding capability.

RESEARCH OBJECTIVES

The specific objectives of this study are:

a. Purify and characterize the hemagglutinin from the hemolymph of Melanoplus sanguinipes and Melanoplus differentialis.

b. Immunize rabbits and mice using purified hemagglutinin as the immunogen.

c. Develop methodology for indirect immunochemical localization procedures for grasshopper hemagglutinin.

MATERIALS AND METHODS

Isolation of Grasshopper Hemagglutinin

Collection of hemolymph. Adult M. sanguinipes and M. differentialis grasshoppers were provided from permanent colonies at the USDA Rangeland Insect Laboratory, Bozeman, MT. Hemolymph was collected with a capillary pipette from ether-anaesthetized insects as previously described [22]. The hemolymph was pipetted into an equal volume of ice 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. Hemocytes and coagulum were removed by centrifugation at 3000 g and the clear yellow supernatant was stored at -20°C.

Hemagglutination assay. Human ABO erythrocytes were a gift from Physicians Laboratory Service, Inc. (Bozeman, MT) and animal erythrocytes were purchased from Colorado Serum Company (Denver, CO). Erythrocytes were washed four times by centrifugation in ice cold DPBS prior to use. Hemagglutination activity was detected at 22°C by serial two-fold dilution of 25 microliter (ul) hemagglutinin sample with 25 ul DPBS using plastic V-bottom microtiter dishes. After dilution of the sample, 25 ul of a 2.5%

suspension of erythrocytes in DPBS was added and agglutination was visually determined after 30 min. The reciprocal of the highest dilution causing agglutination of erythrocytes was the hemagglutination titer. Controls not containing hemagglutinin were always performed.

Preparation of asialo erythrocytes. Asialo human erythrocytes were prepared by incubating for one hour at 37°C 0.5 ml human O^+ erythrocytes with 3 mg neuraminidase (type 5, Sigma Chemical Co., St. Louis, MO) in 10 ml DPBS at pH 5.7. The asialo red cells were washed four times with ice cold DPBS prior to use.

Affinity chromatography. D-Galactose was covalently attached to Sepharose 4B (Pharmacia, Piscataway, NJ) by the divinylsulfone method [40]. A 0.5 x 3 cm column was prepared and washed with 100 volumes of ice cold DPBS. About 50 ml of grasshopper hemolymph (previously diluted with an equal volume of DPBS, 1 mM PTU) was passed through the column (10 ml/hr) at 4°C. The column was then washed with ice cold DPBS until the absorbance of the effluent at 280 nm returned to zero. Adsorbed hemagglutinin activity was released from the column upon elution with DPBS that contained 0.2 M D-galactose. After the first one ml fraction was collected, the column flow was stopped and the column was allowed to incubate for several hours at 22°C before the second one ml fraction was collected. Subsequent fractions were collected after similar

incubation periods. Hemagglutination titer was immediately determined by hemagglutination assay using asialo human O^+ erythrocytes.

Protein assay. Protein concentrations of eluted fractions were determined relative to a bovine serum albumin standard by the method of Bradford [41] with the Bio Rad protein assay kit (Bio Rad Laboratories, Richmond, CA). Collected fractions were stored at -20°C.

Carbohydrate Inhibition of Hemagglutination

Minimal inhibitory concentrations of carbohydrates were determined by performing the hemagglutination assay in the presence of carbohydrates ranging downward in concentration from 100 mM to 0.3 mM. The initial sample of hemagglutinin was adjusted by dilution to a titer of 64-128. The serially diluted hemagglutinin and the added carbohydrate (25 ul) were incubated 5 min prior to addition of 25 ul human O^+ asialo erythrocytes. All determinations were done in duplicate and titer was visually estimated after one hour. Controls in which DPBS was substituted for the carbohydrate solution were done concurrently. Direct comparison of the hemagglutination titer for the inhibited and noninhibited assays allowed determination of the inhibitor concentration which decreased the titer by 50% (one agglutination well).

Biochemical Characterization of Grasshopper Hemagglutinin

Gel filtration. Whole hemolymph and purified hemagglutinin were chromatographed separately on a 1.5 x 120 cm column of Sepharose 6B. The column was developed at 22°C at a flow rate of 12 ml/hr with a pH 7.2 buffer consisting of 10 mM tris, 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM D-galactose. Calibration standards were thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and aldolase (151,000) (Pharmacia). Column effluent was monitored at 280 nm and 2 ml fractions were collected. Aliquots were assayed for hemagglutinin activity using asialo erythrocytes.

Polyacrylamide gel electrophoresis (PAGE). Protein samples were electrophoresed, under various conditions, in 140 x 160 x 1.5 mm polyacrylamide slab gels using the apparatus and procedures from Hoefer Scientific (San Francisco, CA). Acrylamide and sodium dodecyl sulfate were from Sigma. N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine were from Aldrich Chemical Company (Milwaukee, WI). Enzyme grade urea was from Bethesda Research Laboratories (Gaithersburg, MD) and protein molecular weight markers were from Pharmacia. All other chemicals were of reagent grade.

Nondenaturing discontinuous PAGE. Nondenaturing discontinuous PAGE was carried out using a 3.1%, pH 7.5 stacking gel and a 5%, pH 8.3 separating gel, both

containing 0.2 M D-galactose. Samples contained approximately 10 ug protein mixed with 1/10 volume 50% sucrose. Electrophoresis was carried out at 20 mA/gel for 6 hr at 13°C. Gels were fixed for 1 hr in 12.5% (w/v) trichloroacetic acid in water, stained 2 hr in 0.25% (w/v) Coomassie Blue G-250 in water and destained 24 hr in methanol/acetic acid/water (5:7:88 by vol). The position of non-denatured haemagglutinin was related to the standard protein markers: thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and lactate dehydrogenase (140,000).

Sodium dodecyl sulfate (SDS) PAGE. Electrophoresis in SDS polyacrylamide gel slabs was done using 4%, pH 6.8 stacking and 12%, pH 8.8 separating gels according to Laemmli [42]. Samples that contained 10-30 ug protein were denatured by heating 2 min at 95 °C in an equal volume of pH 6.8 buffer (0.1 M tris·HCl, 4% SDS and 20% glycerol) that did or did not contain 2-mercaptoethanol. Standard molecular weight markers were treated similarly. Electrophoresis was continued for 3 hr at 22°C and 30 mA/gel. Gels were stained 4 hr in 0.12% (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (5:1:4 by vol), destained in the same solvent for 1 hr and then destained 24 hr in methanol/acetic acid/water (5:7:88 by vol). Standard curves were calculated from the migration positions of bovine serum albumin (68,000), ovalbumin

(43,000), chymotrypsinogen (25,700) and lysozyme (14,300) relative to that of the phenol red marker dye. Apparent molecular weights for reduced and non-reduced conditions were extrapolated from plots relating migration and log molecular weight for the reduced and non-reduced standard protein markers, respectively.

Urea PAGE. Electrophoresis using urea as the denaturant was performed as in the nondenaturing system except that both stacking and separating gels contained 6 M urea and 0.02 M EDTA. Before application to the gel, solid urea was added to all protein samples to give a final concentration of 6 M. Reduced protein samples were prepared by incubation in 5% 2-mercaptoethanol for 2 min at 95°C prior to addition of urea. Samples contained 10-20 ug protein. Electrophoresis was performed at 200 volts for 18 hr at 22°C.

Isoelectric focusing. Isoelectric focusing of purified hemagglutinin was done in 5 x 90 mm 6% polyacrylamide gel rods with a Hoefer DE 102 Tube Gel Unit according to Wrigley [43]. Carrier ampholyte in the pH range 3-10 was from LKB Products (Bromma, Sweden). All nondenaturing gels contained 0.2 M D-galactose. Isoelectric focusing under denaturing conditions was performed in gels containing 6 M urea. Isoelectric focusing was continued for 4 hr at 1 mA/tube and at 13°C. Focused gels were fixed 2 hr in methanol/water (3:7 v/v)

that contained 3.45% (w/v) sulfosalicylic acid and 11.5% (w/v) trichloroacetic acid. They were then soaked in destain solution (ethanol/acetic acid/water, 25:8:67 by vol) for 2 hr and stained 20 min at 50°C with 0.12% (w/v) Coomassie Blue R-250 in destain solution. Destaining was continued 12-24 hr or until bands were visible.

Amino acid analysis. Samples of hemagglutinin (100 ug) were refluxed in vacuo in 6 M HCl for 18 hr at 110°C. The hydrolyzates were dried in a vacuum desiccator, dissolved in pH 2.2 citrate sample buffer and analyzed on a Beckman 120C amino acid analyzer according to Spackman, et al. [44]. Performic acid oxidation of protein samples prior to hydrolysis was performed by the method of Hirs [45]. Cysteic acid was assumed to have a ninhydrin color value equal to that of aspartic acid. No corrections were made for incomplete hydrolysis or partial hydrolytic destruction of amino acid residues.

Stability. Heat stability of the hemagglutinin was examined by periodic hemagglutination assay of 250 ul samples incubated at 37°C and 55°C in DPBS. Susceptibility to trypsin was similarly determined by incubating hemagglutinin (250 ul) with 25 ug active trypsin (Sigma) at 37°C. The hemagglutination titer (human O^+ asialo erythrocytes) was adjusted to 1024 by dilution with DPBS for both whole hemolymph and purified hemagglutinin prior to each experiment.

Production of Antiserum in Rabbits

Female New Zealand white rabbits were each immunized with 100 ug of purified hemagglutinin according to the multiple intradermal injection method of Vaitukaitus [46]. The immunogen was prepared by emulsifying one ml of hemagglutinin solution (DPBS, 0.2 M D-galactose) with one ml of complete Freund's adjuvant that contained 5 mg/ml T. bacillus. For each animal, control serum was obtained before immunization and antiserum was collected weekly beginning 6 weeks post immunization.

Production of Antibodies in Murine Ascitic Fluids

Two BALB/c-BYJ mice were immunized with purified hemagglutinin according to the method of Tung [47]. Each mouse was injected intraperitoneally on days 0, 14, 21, 28, and 35 with an emulsion of complete Freund's adjuvant and purified hemagglutinin solution (9:1 v/v). Each 0.2 ml immunization contained approximately 40 ug purified hemagglutinin. Mice were tapped when ascitic fluid build-up became appreciable, usually every 3 days after the 5th injection. An 18 gauge needle (without syringe) was inserted into the peritoneal cavity and the fluid was collected directly into a centrifuge tube. Typically about 5 ml of hyperimmune ascitic fluid was obtained from each tap. Sodium azide was added to the ascitic fluid to a final concentration of 0.025% (w/v), and the mixture was

allowed to incubate overnight at 22°C. Cellular debris was removed by centrifugation at 8,000 rpm (SS-34 rotor, 5 min) and at 4°C. The supernatant was centrifuged again at 4 °C (15,000 rpm, 20 min) and the fatty layer formed in this step was removed by aspiration. The remaining solution was then filtered through glass wool to remove any residual lipid. Hyperimmune ascitic fluid was stored in 1 ml aliquots at -50°C.

Gel Double Diffusion

Antibody production from rabbit antiserum and murine ascitic fluid was monitored by double diffusion of antiserum and hemagglutinin in 0.5% agarose gels [48]. Agarose was dissolved in DPBS and in DPBS that contained 0.1 M each D-galactose and D-glucose. Titer values of antisera and ascites were the reciprocal of the highest dilution that produced a precipitin line after 48 hr incubation in a moist environment at 22°C.

Immunochemical Detection of Grasshopper Hemagglutinin

Immunoelectrophoresis. Purified grasshopper hemagglutinin were electrophoresed in 1.5 mm thick 0.5% agarose gels on plain 25 x 75 mm microscope slides according to the method of Grabar and Williams [49]. The gels were prepared using reservoir buffer (0.025 M barbital, 0.005 M sodium barbital, pH 8.6) that contained 0.2 M galactose and 0.025% (w/v) NaN_3. Electrophoresis was carried out at

1 mA/gel for 2-3 hr in a cooled (13°C) horizontal electrophoresis unit. Following electrophoresis the center trough was filled with 0.1 ml of rabbit antiserum and the gel was incubated at 37°C for 24 hr. Precipitin lines were evaluated visually and stained with amido black to obtain a permanent record. Gels to be stained were first washed in 300 ml of 10 mM PBS (pH 7.2) for 24 hr (1 change) to remove non-precipitated protein. Gels were then rinsed in deionized water (5 min), covered with wet filter paper, and air-dried at 37°C overnight. The slides were stained for 5 minutes at 22°C in 1% amido black (w/v) in destain solution, acetic acid/water (7:93 v/v), and destained with 3 successive 100 ml washes. The gels were air-dried and the blue banding patterns were evaluated visually.

Protein transfer from PAGE gels to nitrocellulose filters. The methods of Towbin [50] were used to electroelute proteins from various types of polyacrylamide gel slabs onto nitrocellulose filter paper of 0.2 um porosity (Schleicher & Schuell, Keene, NH). For review, see Gershoni and Palade [51]. The transfer apparatus was a Hoefer TE series transphor unit, and all transfers were performed at 13°. SDS-PAGE and nondenaturing-PAGE gels were transferred in buffer (25mM tris, 192 mM glycine, 20% methanol v/v, pH 8.3) with the nitrocellulose on the anodic side of the gel for 30-60 minutes (depending on the

percentage of acrylamide in the gel) at a current of 0.7-0.8 amps. Urea gels were transferred in 0.7% acetic acid with the nitrocellulose on the cathodic side of the gel for 45 minutes. Following the transfer, the gels were stained with Coomassie Blue in the usual fashion. Nitrocellulose strips were stained either with amido black or immunochemically with glucose oxidase (Appendix I).

Glucose oxidase conjugated goat anti-(rabbit IgG) IgG. This glucose oxidase immunoenzyme was purchased from Cappel Laboratories (Malvern, PA) and was used to identify native grasshopper hemagglutinin or subunits thereof that had been immobilized on nitrocellulose filters. Generally the method of Rathev, et al. [52] was followed, and the staining procedure is outlined in the Appendix. The glucose oxidase method was also used to stain strips of nitrocellulose onto which had been spotted 2 ul of various hemagglutinin and control protein solutions. Following disclosure, nitrocellulose strips were dried overnight between weighted blotter paper.

RESULTS

These results describe the isolation, biochemical characterization, and immunological characterization of the hemagglutinin from M. sanguinipes. During this work, parallel studies with hemolymphatic hemagglutinin from M. differentialis were performed which yielded virtually identical results.

Purification of Grasshopper Hemagglutinin

Affinity chromatography. The hemagglutinin present in grasshopper hemolymph was purified on a column of Sepharose-galactose as shown in Figure 1. In a typical affinity purification experiment, about 350 ug of hemagglutinin was isolated from a 50 ml hemolymph sample. This represented hemolymph collected from approximately 300-400 insects. The minimal concentration of purified hemagglutinin capable of agglutinating human O^+ asialo erythrocytes was 20 ng/ml. Hemolymph, prior to application to the column, typically had a hemagglutination titer in the range 512-1024. The titer value of hemolymph emerging from the column was in the range 8-16 showing that approximately 98% of the hemagglutinin activity in the original sample was adsorbed by the

affinity matrix. When desorbing buffer (DPBS, 0.2 M D-galactose) was applied to the affinity column (arrow in Fig. 1), a small peak of 280 nm absorbancy and a coincident peak of hemagglutinin activity emerged from the column. The hemagglutinin activity trailed somewhat behind the absorbancy peak indicating that release of activity from within the Sepharose-galactose matrix was not instantaneous.

Stepwise elution. In later experiments the adsorbed affinity matrix was incubated in one column volume of desorbing buffer for several hours prior to elution. This procedure resulted in a slightly higher yield and a more highly concentrated preparation of hemagglutinin.

Elution with other desorbants. Adsorbed hemagglutinin was also released from the affinity column by elution with either DPBS that contained 0.2 M sucrose or by 5 mM sodium phosphate buffer (pH 7.2) that contained 1% sodium dodecyl sulfate and 50 mM EDTA. In both cases, molecular characteristics of the desorbed protein were indistinguishable from those associated with hemagglutinin released from the affinity column by elution with DPBS that contained 0.2 M D-galactose.

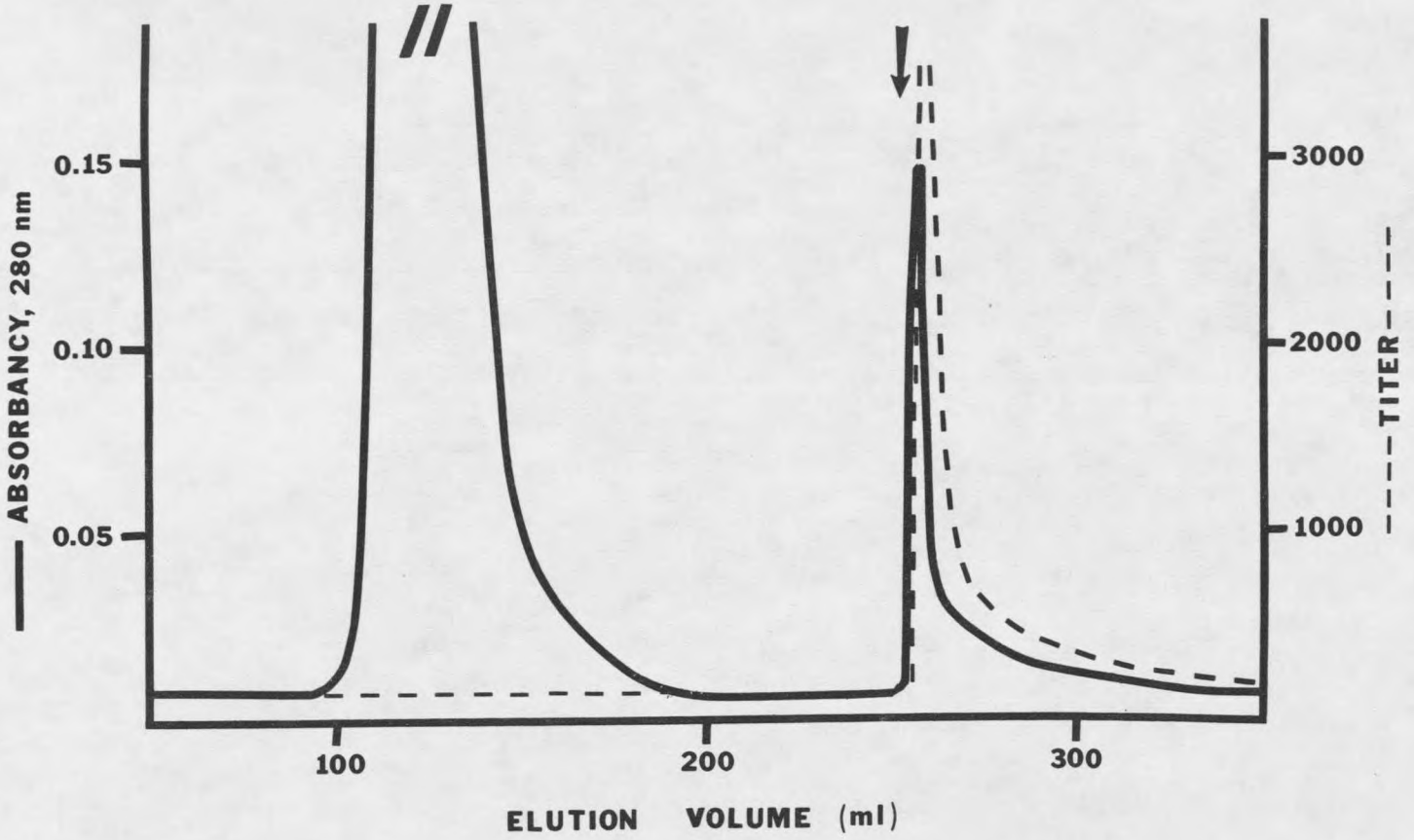

Figure 1. Affinity chromatography of grasshopper hemolymph. Elution of pure grasshopper hemagglutinin is with 0.2 M galactose in DPBS (arrow).

Erythrocyte agglutination

Hemolymph that had passed through the Sepharose-galactose column was examined for possible residual hemagglutinating activity toward other human and animal erythrocyte types. The ability of purified hemagglutinin and of whole (non-adsorbed) hemolymph to agglutinate these cells was also examined. These data are summarized in Table 1 and indicate that essentially all hemagglutinin activity was removed from the original hemolymph sample by one pass over the affinity matrix, and was regained upon elution with desorbing buffer (DPBS, 0.2 M D-galactose). This particular sample had generally low activity and, in contrast with previously shown data (Hapner, [23], did not agglutinate normal human red cells. Rabbit erythrocytes behaved anomalously since not only did adsorbed hemolymph show a high titer, but heat-denatured purified hemagglutinin showed a high amount of agglutinating capability as well. The control experiment, however, showed that rabbit cells are not agglutinated and settle normally in DPBS alone. Agglutination of rabbit erythrocytes was therefore viewed as being caused by nonspecific factors, perhaps hydrophobic protein interactions.

Table 1. Hemagglutination of Various Erythrocytes by Whole Hemolymph, Absorbed Hemolymph and Purified Hemagglutinin.

Cell type	Hemagglutination titer*		
	Whole Hemolymph	Adsorbed Hemolymph	Pure Hemagglutinin
A^+	NA	NA	NA
B^+	NA	NA	NA
AB^+	NA	NA	NA
O^+	NA	NA	NA
Asialo O^+	128	T	4096
Pig	128	T	4096
Cat	32	NA	1024
Calf	16	T	64
Chick	NA	NA	NA
Sheep	NA	NA	NA
Rabbit	2048	1024	4096

*T, Trace; NA, No Activity

Native Molecular Structure of Grasshopper Hemagglutinin

Gel filtration. The native molecular weight of grasshopper hemagglutinin was examined by gel filtration. As shown in Figure 2, the hemagglutinin activity from a sample of whole hemolymph emerged as a single peak of high molecular weight near 700,000. This peak emerged separately from major regions of protein absorbancy indicating that it was not associated with principal hemolymph proteins. When a sample of purified hemagglutinin was placed on the column, under identical flow conditions, the elution position of hemagglutinin activity was unchanged from that shown in Figure 2. The size of the molecular aggregate was therefore independent of other

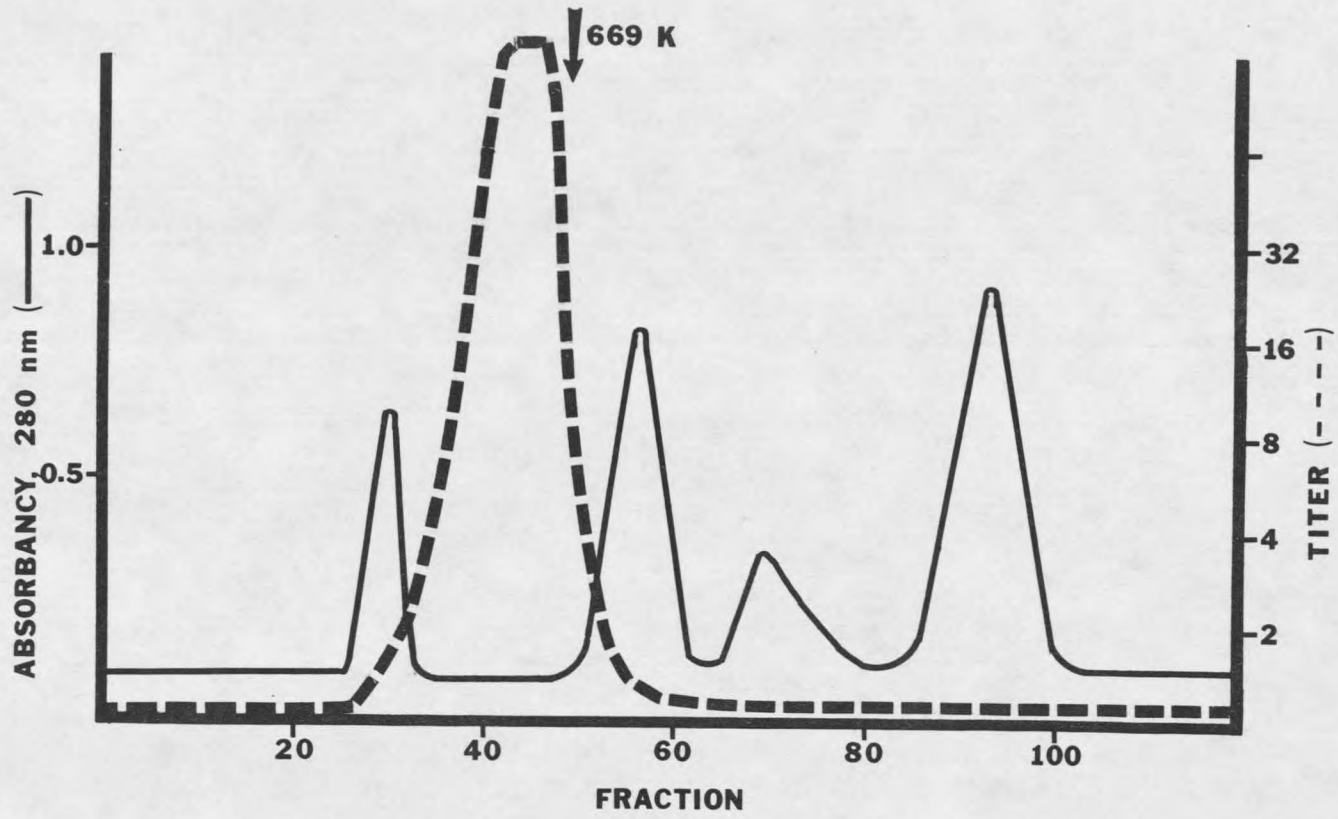

Figure 2. Gel filtration of grasshopper hemolymph. The arrow indicates the emerging position of a 669,000 MW standard protein.

hemolymph factors. If D-galactose was omitted from the elution buffer, no detectable hemagglutinin activity was recovered from the gel filtration column, suggesting a structural dependence of the hemagglutinin on the presence of D-galactose.

Nondenaturing electrophoresis. Electrophoresis of hemagglutinin under nondenaturing conditions produced a single protein band of molecular weight near 590,000 (Figure 3). Some diffuse lightly stained areas were detectable in the lower molecular weight regions of the gel slab indicating the possible presence of contaminants or protein fragments dissociated from the high molecular weight aggregate. Omission of D-galactose from the polyacrylamide slab, or prior incubation of the hemagglutinin sample in 5 mM EDTA resulted in the disappearance of the 590,000 molecular weight protein band, a result analogous to the above observation concerning gel filtration in the absence of D-galactose.

Isoelectric focusing. A single broad band in the slightly acidic pH range resulted upon isoelectric focusing of purified grasshopper hemagglutinin under nondenaturing conditions (Figure 6, gel 1). Purified, native hemagglutinin was therefore viewed as being a homogeneous population of protein of nearly identical ionic character.