Heterogeneity of Lobster Agglutinins. I. Purification and Physiochemical Characterization†

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ABSTRACT: The agglutinins of the lobster, *Homarus americanus*, were characterized as part of a study of the role of agglutinins in the defense mechanisms of invertebrates. Multiple agglutinins were identified and two apparently independent agglutinins (LAg-1 and LAg-2) were purified from hemolymph by ammonium sulfate precipitation, pevikon block electrophoresis, and gel chromatography. Each agglutinin had a unique molecular size, electrophoretic mobility, and binding specificity. The molecular size of LAg-1 was in excess of 19 S and the size of LAg-2 was 11 S; LAg-1 reacted with human and mouse erythrocytes, whereas LAg-2 only reacted with mouse erythrocytes. The agglutinins were labile when heated at 56° for 15 min, required calcium ions for activity, and were inactive after treatment with trypsin or after reduction and alkylation. Both agglutinins dissociated into subunits of equal size (molecular weight 55,000) in 6 M urea indicating that the subunits were joined by noncovalent bonds. Although heterogeneity of invertebrate agglutinins has been suspected for many years, this study represents the first instance in which more than one agglutinin has been isolated from an invertebrate.

Phagocytosis is the primary mechanism of defense against foreign invaders in the invertebrates (Reade, 1968). Participation of humoral substances, particularly as recognition factors, in this process has been postulated (Cushing, 1967). Because of their functional similarity to vertebrate antibodies, invertebrate agglutinins have been considered as candidates for such recognition factors. *In vitro* studies have, in fact, shown that invertebrate agglutinins do facilitate phagocytosis (McKay and Jenkin, 1970; Pauley *et al.*, 1971b; Prowse and Tait, 1969; Tripp, 1966; Stuart, 1968) while structural studies have, however, demonstrated significant differences between these agglutinins and mammalian antibodies (Acton *et al.*, 1969; Hammarström *et al.*, 1972; Marchalonis and Edelman, 1968; Finstad *et al.*, 1972; Weinheimer, 1971; Jenkin and Rowley, 1970; Pauley *et al.*, 1971a; Gilboa-Garber, 1972). Despite lack of structural homology to antibodies, invertebrate agglutinins could nevertheless function to recognize and opsonize foreign substances. Such a function would be best served, as it is in vertebrates, by a heterogeneous group of recognition factors.

Heterogeneity of invertebrate agglutinins has been suspected for many years. Serologic studies have shown that agglutinating activity to one type of erythrocyte can be absorbed by that type of erythrocyte, leaving residual agglutinating activity to other types of erythrocytes (Noguchi, 1903b; McDade and Tripp, 1967; McKay *et al.*, 1969; Tyler and Metz, 1945). In every case thus far reported purified invertebrate agglutinins have failed to display physical–chemical evidence of heterogeneity (Acton *et al.*, 1969; Hammarström *et al.*, 1972; Marchalonis and Edelman, 1968; Finstad *et al.*, 1972; Weinheimer, 1971; Jenkin and Rowley, 1970; Pauley *et al.*, 1971a; Gilboa-Garber, 1972). The purpose of our experiments was to provide a chemical basis for the serologic heterogeneity observed in invertebrate agglutinins, by indentifying and purifying more than one agglutinin from a representative invertebrate, the lobster, *Homarus americanus*. An agglutinin in the hemolymph of this lobster has been previously described (Noguchi, 1903a), partially characterized (Cornick and Stewart, 1968, 1973), and partially purified (Hall *et al.*, 1972).

Materials and Methods

Hemolymph Collection. Live, fresh, male lobsters (*Homarus americanus*) were obtained by air freight from the Main Coast Seafood Corp., Spruce Head, Maine. Hemolymph serum (Morimoto and Kegeles, 1971) and citrated plasma (Fuller and Doolittle, 1971) were obtained, pooled, and stored at −10° until used. Extracts of lobster internal organs were prepared by excising the organ, washing with 0.9% saline, and homogenizing in a tissue grinder in 0.9% saline. Hemocytes were collected from plasma by sedimentation at 2000 rpm and washed three times in 0.01 M *N*-ethylmaleimide, and extracts were prepared using a tissue grinder as described above.

Erythrocytes (E)[1] from vertebrates were obtained as follows: hamster, mouse (Grand Island Biological Co.); horse, chicken (Miles Laboratories); human, group O negative (outdated bank blood; Hospital of the University of Pennsylvania).

Chemicals. The following chemicals were obtained from the sources indicated: acrylamide, *N'*,*N'*-methylenebisacrylamide, *N*,*N*,*N'*,*N'*-tetramethylethylenediamine, ammonium persulfate (Canalco); 2-mercaptoethanol (Eastman Organic Chemicals); trypsin soybean inhibitor, trypsin (bovine) Type III, iodoacetamide, bovine serum albumin, sperm whale myoglobin, egg albumin, blue dextran edestin (Sigma Chemical Co.); Freund's complete adjuvant, noble special agar (Difco); Sephadex G-200, DEAE-Sephadex A-25, Sepharose 4B and 6B (Pharmacia Fine Chemical Co.); Amberlite MBI (Mallinckrodt Chemical Co.); pevikon C-870 (Mercer Chemical Co.);

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[1] Abbreviations used are: TBD, Tris-buffered diluent; TBS, Tris-buffered normal saline; E, erythrocytes.

Ampholine Ampholytes (LKB Instrument Co); β-galactosidase (Worthington Biochemicals).

Agglutination Assay. Serial twofold dilutions of sample were made in Tris-buffered diluent (TBD) (0.05 M Tris–0.15 M sodium chloride–0.02 M calcium chloride (pH 8.0)) using microtiter "V" plates (Microbiological Associates) (Zmijewski, 1968). Washed E in 1.5% suspensions in TBD were added and the plates sealed and incubated at 20°. The agglutinin titer (units of agglutinin activity) was considered to be the reciprocal of the highest dilution of sample that gave positive agglutination.

Protein Assay. Protein concentrations of sample solutions and bovine serum albumin standards were determined by the Folin reaction (Lowry *et al.*, 1951) or by absorbance at 280 nm. Results of the Folin reaction on a series of fractions were often expressed as "OD 700 nm" which refers to the absorbance of the final product of the Folin reaction at 700 nm.

Polyacrylamide Gel Electrophoresis. The method used for polyacrylamide gel electrophoresis is that described in the instruction manual for the Buchler equipment (Buchler Instruments, 1970), except that a 6% gel concentration was used. Fixation and staining with Amido Black and periodic acid–Schiff reagents were carried out as described previously (Buchler Instruments, 1970). Eluates were prepared from a series of identical gels by sectioning the gels at 2.5-mm intervals and extracting the corresponding sections into TBD by mincing and homogenizing in a tissue grinder at 10°, incubating at 4° for 24 hr, and sedimenting at 10,000 rpm for 10 min.

Ultrafiltration. Samples of partially purified agglutinins were concentrated by ultrafiltration using collodion bags with a 70,000–100,000 molecular weight cutoff (Schleicher and Schuell).

Ultracentifugation. Preparative ultracentrifugation of samples of purified hemolymph proteins and standards was carried out at 4° in 10–37° sucrose gradients at 35,000 rpm for 16 hr in the Beckman L3-50 ultracentrifuge with a 50.1 swinging bucket rotor. A 0.2-ml quantity of sample (1–5 mg/ml) was layered on top of the 4.8-ml gradient, in a 2 × 0.5 in. cellulose nitrate ultracentrifuge tube. Fractions were collected from the bottom of the centrifuge tube (Martin and Ames, 1961; Olins and Edelman, 1964). Parallel standards in each experiment included serum from opossums immunized to bacteriophage F2. IgM and IgG antibody activity of fractions was determined by phage neutralization (Adams, 1959).

Samples of purified agglutinin were analyzed by sedimentation in a Beckman Spinco Model E3 ultracentrifuge equipped with phase-plate schlieren optics and ultraviolet absorption.

Ultraviolet Spectroscopy. The ultraviolet spectrum of the purified agglutinin was obtained using a Beckman DB-GT spectrophotometer.

Preparation of Antibody. Antisera were prepared in New Zealand White rabbits (Hillandale Farms, Franklinville, N. J.). Lobster hemolymph serum and plasma (30 mg/ml) and purified agglutinins (1 mg/ml) were emulsified in Freund's complete adjuvent in a 1 : 1 ratio and 1.0-ml quantities injected subcutaneously in divided doses into each of two rabbits per antigen. Several weeks later similar booster immunizations were performed and 10–14 days later the rabbits were bled and the antisera harvested. When purified agglutinins were used as the antigen, booster immunizations were performed using homogenized polyacrylamide gel sections.

Physical and Chemical Modifications of Hemolymph. Samples of hemolymph were heated to 50 and 56° for 15 and 30 min in a constant-temperature water bath and then immediately cooled and assayed. To study metal ion dependence, samples of hemolymph were dialyzed into TBS (0.05 M Tris–0.15 M sodium chloride (pH 8.0)) containing 0.005 M EDTA for 48 hr with three changes of buffer. The samples were then assayed for agglutinating and hemolytic activity using TBS diluent which was made 0.05 M in EDTA, 0.03 M in calcium chloride, 0.03 M in magnesium sulfate, or 0.03 M in both calcium chloride and magnesium sulfate. The pH dependence of agglutinating activity was studied using 0.9% saline diluents made 0.1 M in the following buffers: acetate (5.0), maleate (6.0 and 6.5), Tris-maleate (7.0), and Tris (7.5, 8.0, 8.5, and 9.0).

Samples of partially purified agglutinin were digested with trypsin by incubating equal volumes of sample and trypsin solution (2.0 mg/ml in TBS) at 37° for 2 hr. During the incubation, aliquots were removed, immediately cooled, combined with a slight excess of soybean trypsin inhibitor, and assayed. Reduction and alkylation of samples of hemolymph and partially purified agglutinin were accomplished as follows: a 1.0-ml quantity of sample was mixed with 6.0 ml of a 0.1 M solution of 2-mercaptoethanol in 0.5 M Tris buffer (pH 8.0) and incubation for 1 hr at 20° in the dark with stirring. Alkylation was accomplished by adding 0.3 g of iodoacetamide (final concentration 0.2 M) and stirring for 30 min at 4°. The reduced and alkylated samples were then dialyzed for 24 hr in three changes of TBS.

In order to test the ability of antisera of hemolymph proteins to inactivate the agglutinin samples of partially purified agglutinins were combined with the purified antibody at ratios near equivalence (as determined by a capillary tube precipitin test), incubated at 37° for 1 hr and 4° for 2 hr, and centrifuged, and the supernatant was assayed for activity.

The effect of urea on the purified agglutinin was studied as follows: samples of agglutinin were made 3 and 6 M in urea. Portions of each urea-treated sample were dialyzed against TBD for 24 hr with three changes of buffer. Dialyzed and undialyzed samples were then assayed, the latter in both TBD and TBD containing 3 M urea. In addition, samples of purified agglutinins were made 6 M in urea and placed on a Sephadex G-200 column (1.5 × 30 cm) previously equilibrated with Tris-buffered urea. This buffer was prepared by dissolving fresh urea in deionized water to give a 6 M solution, filtering the solution through an Amberlite MB1 column (2.5 × 45 cm), and making the eluate 0.1 M in Tris (pH 7.5). Void and total volumes were measured with blue dextran and phenol red; fractions (2.0 ml) were collected and assayed for protein and agglutinin activity.

Results

Distribution of Agglutinins among Lobster Tissues. Agglutinating activity to human and mouse E was present in the extracts of the lymphatic gland, pericardium, testis, ovary, hepatopancreas (liver), and green gland (kidney) but the specific activity was low (1–3 U/mg). Greater concentrations of agglutinins were found in the hemolymph (3–6 U/mg) and hemocyte extracts (10–20 U/mg).

Preparation of Crude Agglutinins. Crude agglutinins were prepared from native hemolymph serum by precipitation with 35% ammonium sulfate at 4° in the presence of 0.02 M calcium chloride. The precipitate, containing the agglutinins, was washed with 35% ammonium sulfate and dissolved in 0.1 M barbital buffer (pH 8.6). This procedure resulted in a 30–60-fold increase in specific agglutinating activity and proceeded in 85% yield.

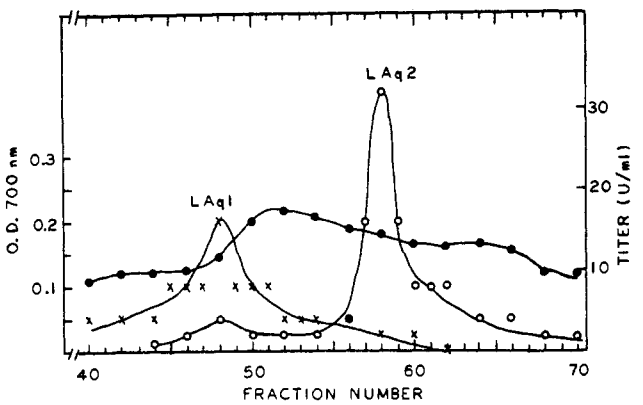

FIGURE 1: Chromatography of crude agglutinins on Sepharose 4B. The column, 1.5 × 90 cm, containing Sepharose 4B was equilibrated with 0.05 M Tris-buffered normal saline, pH 8.0. The redissolved ammonium sulfate precipitate (30 mg) was applied and elution commenced with the same buffer, and 3.0-ml fractions were collected and tested for activity: (●) optical density of the Folin reactions at 700 nm; (×) activity to human E; (○) activity to mouse E; (LAg-1, 2) lobster agglutinins 1 and 2.

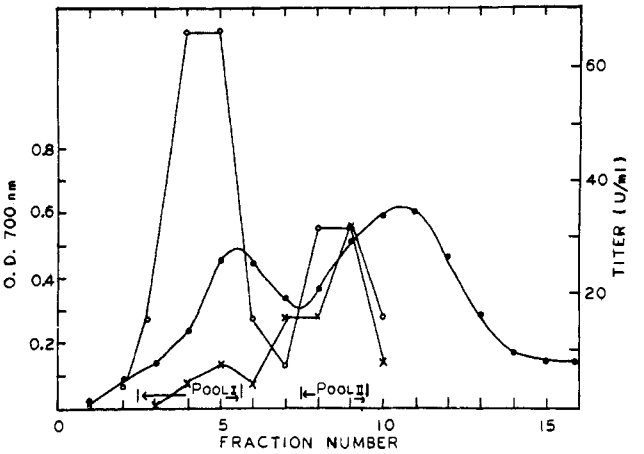

FIGURE 2: Pevikon block electrophoresis of crude agglutinins. Pevikon in 0.1 M barbital buffer (pH 8.6) was formed into a 22 × 45 cm block, and the redissolved ammonium sulfate precipitate (70 mg) applied 10 cm from the cathodal end of the block. Following electrophoresis for 20 hr at 150 V, parallel 1-cm sections were made from the origin toward the anode and the protein eluted into 0.1 M Tris buffer (pH 7.5): (●) optical density of the Folin reaction at 700 nm; (×) agglutinin activity to human E; (○) agglutinin activity to mouse E.

Heterogeneity of the Agglutinins. During purification agglutinin activity was assayed using E obtained from several different species. In this way, agglutinins could be defined according to their relative reactivity with various types of E. When the crude agglutinin preparation was subjected to gel chromatography on Sepharose 4B, activity maxima to mouse E and human E appeared in different fractions (Figure 1). The same elution activity pattern was obtained when crude hemolymph from a single lobster was used instead of the crude agglutinin preparation from pooled lobster hemolymph. These two areas of agglutinin activity, representing heterogeneity based on molecular size, were designated LAg-1 and LAg-2 for heavier and lighter lobster agglutinins, respectively. LAg-1 reacted to variable degree with human, horse, hamster, chicken, sheep, and mouse E, while LAg-2 reacted only with mouse, horse, and hamster E. No other activity maxima were found when these other E were used to assay the fractions obtained in the Sepharose 4B purification procedure.

Heterogeneity of lobster agglutinins could also be demonstrated on pevikon block electrophoresis of crude agglutinin preparations. Analysis of the fractional eluates from the pevikon block (Figure 2) showed two separate areas of agglutinin activity (pool I and pool II); pool I contained an agglutinin that reacted with mouse E only (later shown to be LAg-2), while pool II contained an agglutinin that reacted with both mouse and human E (later shown to be LAg-1). Therefore, the two agglutinins which separated on gel chromatography on the basis of their difference in molecular size were also different in electrophoretic mobility.

The heterogeneity of lobster agglutinins was confirmed by two additional experiments. Sucrose density gradient ultracentrifugation of the crude agglutinins resolved the mixture of agglutinins into several components. Activity maxima with human, horse, and mouse E appeared in different parts of the gradient (Figure 3). The activity maximum to human E was at the very bottom of the gradient (usually with a higher titer than that in the experiment shown in Figure 3), while the major activity to mouse and horse E was present at a position in the gradient corresponding to 11 S. A minor peak of activity to horse E only was also present at a position corresponding to 20 S. Finally, preparative polyacrylamide gel electrophoresis resolved the mixture of crude agglutinins into two major and one minor components (Figure 4). The component near the top of the gel, reacting with mouse, human, and horse E, was identified as LAg-1 by comparison with gels of LAg-1 purified by gel chromatography. The second major component which reacted with only mouse and horse E was similarly identified as LAg-2. The minor component, migrating slightly further into the gel, reacted with all three E tested.

Purification of the Agglutinins. LAg-1 was purified by a combination of pevikon block electrophoresis and chromatography on Sepharose 6B. The active fractions from the pevikon block electrophoresis (Figure 2, pool II) were concentrated by ultrafiltration and applied to a Sepharose 6B column. The elution pattern (Figure 5) shows that the protein peak con-

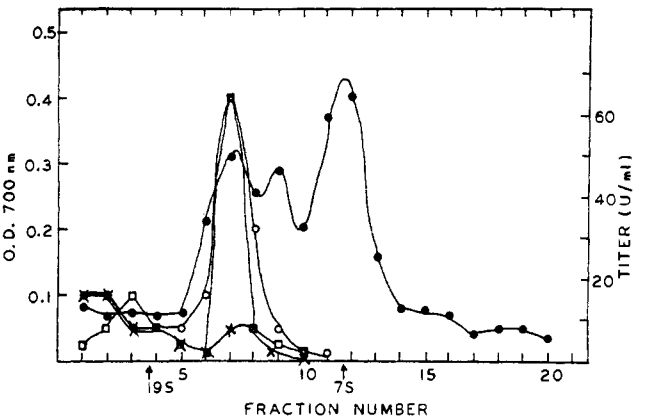

FIGURE 3: Sucrose density gradient ultracentrifugation of crude agglutinins. A 0.2-ml (1.2 mg) quantity of active pevikon block eluates (pool I plus pool II) was placed at the top of 4.8 ml of a 10–37% sucrose gradient in TBS in an ultracentrifuge tube. The tube was placed in a 50.1 swinging bucket rotor and sedimented for 16 hr at 39,000 rpm. Fractions of equal volume (0.24 ml) were collected and assayed for protein and agglutinin activity. A parallel gradient was run with immune oppossum serum to provide 7S and 19S markers: (●) optical density of the Folin reaction at 700 nm; (×) activity to human E; (○) activity to mouse E; (□) activity to horse E.

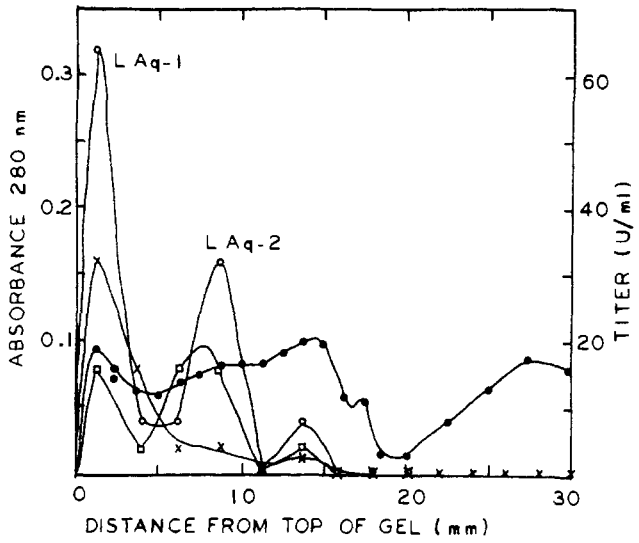

FIGURE 4: Preparative polyacrylamide gel electrophoresis of crude agglutinins. After application of 1.2 mg of active pevikon block eluate (pool I plus pool II) to each of a series of nine 6% gels, they were subjected to 100 V for 5 hr. One gel was stained with amido black and the remainder were sectioned at 2.5-mm intervals and the corresponding sections from each gel pooled, minced, and eluted into TBS. Eluates were assayed for protein concentration and activity: (●) absorbance at 280 nm; (×) activity to human E; (○) activity to mouse E; (□) activity to horse E; (LAg-1, 2) lobster agglutinins 1 and 2.

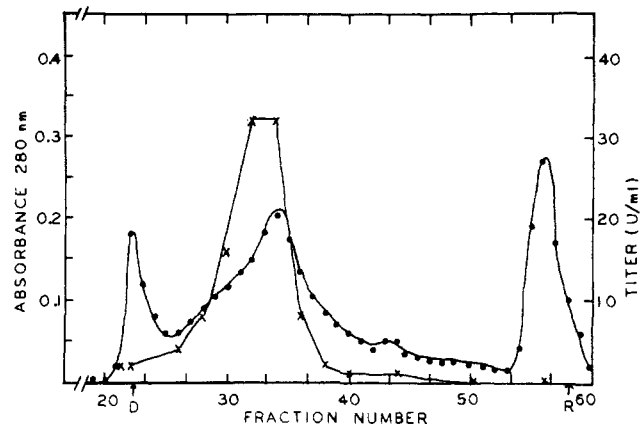

FIGURE 5: Chromatography of purified LAg-1 on Sepharose 6B. The column, 1.5 × 90 cm, containing Sepharose 6B was equilibrated with 0.1 M Tris buffer (pH 7.5) containing 0.005 M calcium chloride. Pevikon block eluate (pool II) (6.0 mg) was applied and elution commenced with the same buffer, and 3.0-ml fractions were collected: (●) absorbance at 280 nm; (×) agglutinin activity to human E: (D) elution maximum of blue dextran; (R) elution maximum of phenol red.

taining the activity is slightly asymmetric. Analysis of the purity of the intermediate and final products by polyacrylamide gel electrophoresis (Figure 6B–D) shows that the final product consists of several species represented by three to five closely spaced bands near the top of the gel. The overall purification of LAg-1 resulted in a 400-fold increase in specific activity and a 17% yield based on the starting material (Table I). LAg-1 was also purified from the crude agglutinin preparation by chromatography on DEAE-Sephadex A-25 and elution by a linear 0.1–0.5 M ionic strength gradient at pH 7.8. Recycling of the product over the same column under the same conditions resulted in a final product that was of equal purity to that obtained by the original method described above, but the yield was quite low (overall 5%).

LAg-2 was purified from the crude agglutinin preparation by a similar procedure to that of LAg-1, in which the pool I eluate from the pevikon block electrophoresis (Figure 2) was concentrated and applied to a Sepharose 4B column. The protein peak in the elution curve containing the activity was symmetric (Figure 7) and polyacrylamide gel electrophoresis of the intermediate and final products verified the degree of purification achieved (Figure 6B, F, and G). The overall yield and increase in specific activity were similar to those reported for LAg-1 (Table I). Attempts to purify the agglutinins by isoelectric focusing resulted in irreversible precipitation of the agglutinins, while efforts to elute the agglutinins from E or E stroma following specific absorption were unsuccessful.

Both LAg-1 and LAg-2 could be purified from the agglutinin preparation by preparative polyacrylamide gel electrophoresis. The resulting products gave single major bands on subsequent polyacrylamide gel electrophoresis (Figure 6E and H) but were obtained in low yield (5%).

Physicochemical Properties of the Agglutinins. The agglutinins were labile when heated to 56° for 15 min, but not at lower temperatures. They were inactive in the presence of EDTA, but activity returned following dialysis and addition of calcium ions. Agglutinating activity using human E was highest at 4° (1024) and lower as the temperature increased to 37° (32). The optimum pH for activity was pH 7.5–8.0, and activity was irreversibly lost at pH 5.0 and below. Both treatment with trypsin and reduction (2-mercaptoethanol) and alkylation

TABLE I: Purification of Lobster Agglutinin-1 (LAg-1)[a]

Fraction	Protein (mg/ml)	Anti-human Titer (U/ml)	Specific Activity (U/mg)	Total Activity (1000 U)	Yield[b] (%)	Purity (fold)
Native hemolymph	23.0	128	5	140	100	1
Ammonium sulfate precipitate	14.0	4096	300	120	85	60
Pevikon block eluate (pool II)	2.0	2048	1000	6	25	200
Sepharose 4B eluate	0.7	2048	3000	4	17	400

[a] Native hemolymph (1100 ml) was 35% saturated with ammonium sulfate and the precipitate harvested, washed, and redissolved. About 15% of the redissolved precipitate (4.5 ml) at a time was subjected to pevikon block electrophoresis and the active eluates were pooled (pool II). The pooled eluate was placed on a Sepharose 4B column and the active fractions were pooled to give the final purified agglutinin. [b] Cumulative yield based on the starting material and adjusted for processing of smaller batches in the later steps (footnote *a*).

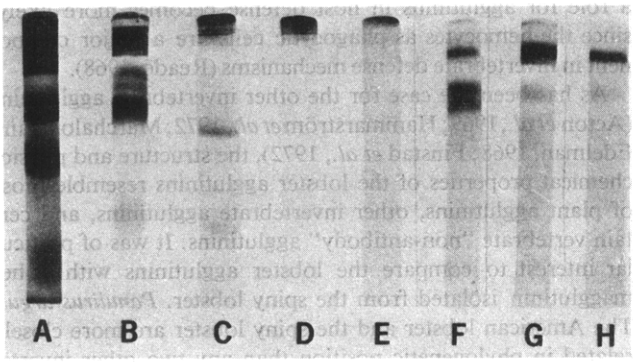

FIGURE 6: Polyacrylamide gel electrophoresis of crude and purified agglutinins. After application of 50–100 μg of sample the 6% gels were subjected to 100 V for 3 hr and stained with amido black: (A) native hemolymph; (B) ammonium sulfate precipitated agglutinins; (C) pevikon block eluate, pool II; (D) LAg-1 from Sepharose 6B chromatography; (E) LAg-1 from preparative polyacrylamide gel electrophoresis; (F) pevikon block eluate, pool I; (G) LAg-2 from Sepharose 4B chromatography; (H) LAg-2 from preparative polyacrylamide gel electrophoresis.

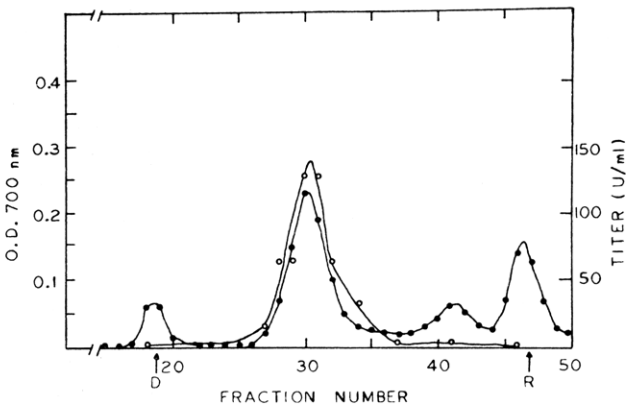

FIGURE 7: Chromatography of purified LAg-2 on Sepharose 6B. The column, 1.5 × 90 cm, containing Sepharose 6B was equilibrated with 0.1 M Tris buffer (pH 7.5) containing 0.005 M calcium chloride. Pevikon block eluate (pool I) (5.0 mg) was applied and elution commenced with the same buffer, and 4.0-ml fractions were collected: (●) optical density of the Folin reaction at 700 nm; (○) agglutinin activity to mouse E; (D) elution maximum of blue dextran; (R) elution maximum of phenol red.

(iodoacetamide) abolished agglutinin activity. The agglutinins were inactive in the presence of 3 M urea, but could be treated with 6 M urea for 3 days and dialyzed with partial retention of activity. The ultraviolet spectrum of LAg-1 showed a maximum at 275 nm ($\epsilon^{1\,cm}_{1\%}$ 6.7) and a minimum at 255 nm. Both LAg-1 and LAg-2 were stained with the periodic acid–Schiff stain in polyacrylamide gels, but not with the Schiff reagent alone, indicating the presence of carbohydrate.

The molecular size of LAg-2 as determined by sucrose density gradient ultracentrifugation using opossum IgM and IgG, β-galactosidase, and edestin as markers was 11.5 S. Analytical ultracentrifugation gave values of 10.8 S and 11.3 S, calculated using the ultraviolet absorption pattern. The molecular size of LAg-1 could not be accurately determined. On sucrose density gradient ultracentrifugation it was found at the bottom of the tube, well below the 19S marker, and on analytical ultracentrifugation gave a rapidly sedimenting broad peak from which an accurate *s* value could not be calculated. This was not unexpected, as purified LAg-1 was known to be heterogeneous since it showed multiple, closely spaced bands on polyacrylamide gel electrophoresis.

Agglutinin Subunits. A purified preparation of LAg-1 was made 6 M in urea and applied to a Sephadex G-200 column equilibrated with 6 M urea; the effluent was monitored for absorbance at 280 nm and fractions were collected, dialyzed into TBD, concentrated four times, and assayed for agglutinin activity. The elution position of LAg-1 in 6 M urea fell between that of ovalbumin and bovine serum albumin standards, corresponding to a molecular weight of 55,000. Purified LAg-2 in 6 M urea eluted at the same position, suggesting that it also was composed of subunits of a molecular weight of 55,000. Reduction and alkylation of one of the agglutinins (LAg-2) with 2-mercaptoethanol and iodoacetamide in 6 M urea followed by passage through the 6 M urea–Sephadex G-200 column gave a protein concentration peak also corresponding to a molecular weight of 55,000.

Antigenic Comparison of the Agglutinins. The most highly purified preparations of LAg-1 and LAg-2 were compared on Ouchterlony plates using anti-agglutinin serum. The precipitin lines which formed cross, indicating nonidentity of the two agglutinins. Immunoelectrophoresis of the purified agglutinins using anti-agglutinin serum gave only one major precipitin arc with each agglutinin, although a minor arc joined as a line of identity to the major arc was present on immunoelectrophoresis of LAg-2. When anti-agglutinin serum was incubated with LAg-2 at 37° for 1 hr the agglutinin activity was completely neutralized, whereas control rabbit serum had no effect on agglutinin activity.

Discussion

At least two agglutinins have been identified and purified from a representative invertebrate, the American lobster. These agglutinins (LAg-1 and LAg-2) differ in molecular size, in electrophoretic mobility, and in binding specificity. One agglutinin (LAg-1) has a sedimentation rate in excess of 19 S, a moderately low electrophoretic mobility at pH 8.6, and agglutinates both human and mouse E. The second agglutinin (LAg-2) has a sedimentation rate of 11 S, a very low electrophoretic mobility at pH 8.6, and agglutinates mouse E but not human E. The two agglutinins may be independent, unique proteins, or they could represent different polymeric forms of an identical subunit or subunits. Because comparison of the two agglutinins using anti-agglutinin serum showed that each agglutinin had unique antigenic determinants, we favor the interpretation that the agglutinins are independent proteins. These two agglutinins were found in hemolymph from individual lobsters as well as pooled hemolymph, so that the heterogeneity exists within a single lobster rather than between lobsters.

Although only two agglutinins from the lobster were purified and characterized, there is evidence that additional agglutinins are present. On preparative polyacrylamide gel electrophoresis a third minor peak of activity is present, but was not investigated further. Likewise in sucrose density gradient ultracentrifugation a third minor peak, reacting only with horse erythrocytes, was present having an *s* value of 20.

Our identification of multiple lobster agglutinins represents the first substantial chemical evidence of agglutinin heterogeneity among the invertebrates. This is an important piece of evidence in support of theories that involve the agglutinins as recognition factors in defense processes of the invertebrates. In fact, it would be difficult to explain how one agglutinin alone could effectively discriminate between "self" and the multiplicity of "not self" materials. The finding that inverte-

brate agglutinins are heterogeneous also establishes one interesting point of similarity with vertebrate antibodies in the face of substantial evidence of structural dissimilarity. This comparison leads us to raise a question that is very basic to antibody heterogeneity, yet not completely resolved, namely, how is the heterogeneity (or diversity) generated? Is it possible that the same mechanism of generating the extensive diversity of vertebrate antibodies might be operative in invertebrates as well?

Lobster agglutinins, which comprise less than 1% of the total hemolymph protein, were purified from hemolymph by a series of procedures. Crude preparations of LAg-1 and LAg-2 were obtained by precipitation from hemolymph in 35% ammonium sulfate followed by electrophoresis on pevikon block. LAg-1 was further purified on a Sepharose 6B column giving a product that had a 400-fold greater specific acitvity than the starting material but which gave three to five closely spaced bands on polyacrylamide gel electrophoresis. Only preparative polyacrylamide gel electrophoresis gave a product that was homogeneous on gel electrophoresis. LAg-2 was further purified from the crude preparation by chromatography on Sepharose 4B giving a product that also had a 400-fold greater specific activity than the starting material. This product had only one major band and two minor bands on polyacrylamide gel electrophoresis and gave one peak on analytical ultracentrifugation. One of the minor bands present 1 cm further down the polyacrylamide gel probably represents a breakdown product of the complete agglutinin molecule, since it was also present on analysis of LAg-2 purified by preparative polyacrylamide gel electrophoresis. This conclusion is supported by immunoelectrophoresis of purified LAg-2 using anti-agglutinin serum which showed a minor arc that was joined to the major arc in a continuous line of identity. Although most of the other invertebrate agglutinins which have been purified have not been analyzed by high resolution electrophoretic techniques, the two studies which have employed such analyses have revealed multiple species of the agglutinin. Immunospecific purification of snail agglutinin gave a product that had three major closely spaced bands on polyacrylamide gel electrophoresis (Hammarström *et al.*, 1972). Isoelectric focusing of purified crab and starfish agglutinins also showed multiple closely related species (Finstad *et al.*, 1972).

The distribution of agglutinin activity within an individual invertebrate has not been investigated previously, except for one report of agglutinin activity in lobster hemocytes (Cornick and Stewart, 1973), although it is an important step toward understanding the biologic significance of agglutinins. The lobster agglutinins were present primarily in the hemolymph and hemocytes. The high specific activity of the agglutinins in the hemocytes, in contrast to the low activity in other organs, suggests that a special relationship exists between the agglutinins and the hemocytes. We considered the possibility that the hemocytes may either synthesize or sequester the agglutinins but we could not discriminate between these two alternatives on the basis of the present experiments. Hemocyte agglutinin synthesis would seem to be the more likely possibility however, and is supported by morphologic studies which have shown that the organelles required for protein synthesis (rough and smooth endoplasmic reticulum, Golgi apparatus, ribosomes) are present in lobster hemocytes[2] (Hearing and Vernick, 1967). If more definitive studies do establish that the hemocytes synthesize the agglutinins, then a role for agglutinins in host defense becomes more likely, since the hemocytes as phagocytic cells are a major component in invertebrate defense mechanisms (Reade, 1968).

As has been the case for the other invertebrate agglutinins (Acton *et al.*, 1969; Hammarström *et al.*, 1972; Marchalonis and Edelman, 1968; Finstad *et al.*, 1972), the structure and physiochemical properties of the lobster agglutinins resemble those of plant agglutinins, other invertebrate agglutinins, and certain vertebrate "non-antibody" agglutinins. It was of particular interest to compare the lobster agglutinins with a hemagglutinin isolated from the spiny lobster, *Panulirus argus*. The American lobster and the spiny lobster are more closely related in phylogenetic position than any two other invertebrates whose agglutinins have been purified. The *Panulirus* hemagglutinin was found to be heat labile at 50°, calcium dependent, contained carbohydrate, present in low (<1%) concentration in the hemolymph, had a low electrophoretic mobility, a sedimentation value of 10.2 S, and subunits of 68,000 molecular weight which were joined by noncovalent bonds (Weinheimer, 1971). In these respects the *Panulirus* hemagglutinin is very similar to LAg-2, although *Panulirus* hemagglutinin reacts with human E and LAg-2 does not. In his experiments on the spiny lobster, Weinheimer used only human E to monitor purification, which could be an explanation for his having found only one agglutinin.

In looking for proteins in vertebrates which are structurally similar to the lobster agglutinins, recent reports of antibody-like proteins in three lower vertebrates are of interest. An α-globulin that agglutinates human E by binding to "H" surface antigen has been described in an agnathan, the sea lamprey (Pollara *et al.*, 1970). A fructosan precipitin is present in the serum of a chondrostean, the nurse shark (Harisdangkul *et al.*, 1972), and an α-globulin that reacts with terminal L-fucose residues in agglutinating human group "O" E has been purified from a teleost eel (Bezkorovainy *et al.*, 1971). Each of these proteins shares with the lobster agglutinins a molecular structure in which the major subunits are held together by noncovalent bonds. The molecular size of the lamprey and shark proteins (320,000 and 280,000 or 10.6 S, respectively) are similar to LAg-2 (11 S) as is the subunit size (75,000 and 71,000 *vs.* 55,000 for LAg-2). In both the lamprey and shark proteins, reduction and alkylation fail to further reduce the size of the subunits, except for the possible dissociation of small fragments, which is also the case with the lobster agglutinins. In addition, the nurse shark contains a group of fructosan-specific proteins which show multiple closely spaced bands on polyacrylamide gel electrophoresis, similar to that of LAg-1 preparations. Certainly the lobster agglutinins are much more similar to these proteins than they are to immunoglobulins. Although no non-immunoglobulin agglutinins of this type have been described in mammals, it is entirely possible that they are present since they seem to have persisted in evolution through the transition from invertebrates to vertebrates. The question of what role such molecules might play in the natural defense mechanisms of mammals is intriguing, but so far unanswered.

Although invertebrate agglutinins show marked structural differences from vertebrate antibodies, investigation of their role in invertebrate defense processes should not be abandoned. These agglutinins, like antibodies, facilitate phagocytosis, are heterogeneous, and may be synthesized by a principal cellular component (the hemocyte) of the invertebrate defense system.

In addition, structurally similar molecules have been found in vertebrates, which suggests that the importance of non-

[2] Personal observation.

antibody agglutinins, as recognition factors, may extend throughout the animal kingdom. Ultimately, it is hoped that a full understanding of defense mechanisms in these relatively primitive animals will provide new insights into the operation of defense mechanisms in human diseases.

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