A New Multimeric Hemagglutinin from the Coelomic Fluid of the Sea Urchin *Anthocidaris crassispina*†

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ABSTRACT: A hemagglutinin was purified from the coelomic fluid of the sea urchin *Anthocidaris crassispina* by ion-exchange chromatography on DEAE-cellulose and affinity adsorption to glutaraldehyde-fixed ghosts of human erythrocytes, followed by elution with 10 mM EDTA. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, it showed a single protein band with a molecular weight of 13 000 and 26 000 in the presence and absence of 2-mercaptoethanol, respectively. The molecular weight of the native protein with a hemagglutinating activity was determined to be 300 000 by sedimentation equilibrium analysis. Its sedimentation coefficient, $s^0_{20,w}$, and Stokes radius were 13.7 S and 5.5 nm, respectively. The hemagglutinating activity of this protein required calcium ions. When calcium ions were depleted, no activity was observed and its sedimentation coefficient, $s^0_{20,w}$, decreased to 11.4 S while its Stokes radius increased to 6.7 nm without a change in its molecular weight. The purified hemagglutinin agglutinated human erythrocytes regardless of their ABO and MN blood types. The hemagglutination reaction was not affected appreciably by various simple sugars but was inhibited by tryptic fragments released from human erythrocyte membranes. The results of alkaline borohydride treatment of the inhibitory tryptic fragments showed that the receptor sites for this hemagglutinin were mainly composed of alkali-labile carbohydrate chains with the structure AcNeuα2→3Galβ1→3(AcNeuα2→6)GalNAc→serine (or threonine). Since removal of sialic acid residues from the fragments did not affect their inhibitory activity while destruction of the terminal galactose of the desialyzed tryptic fragments brought about the loss of their inhibitory effect, it was assumed that the inner disaccharide structure linked to serine (or threonine) was important for the binding of this hemagglutinin.

Since the hemagglutinating activity in the hemolymph of the horseshoe crab *Limulus polyphemus* was reported [Noguchi (1903) as cited by Yeaton (1981a)], a number of proteins with such activity have been isolated from the coelomic fluid and tissue extracts of various invertebrate species (Yeaton, 1981a), as well as from plants. The ability of these proteins to recognize foreign cells and, in some cases, to enhance phagocytosis (Tripp, 1966; Komano et al., 1980; Yeaton, 1981b) led to the idea that such hemagglutinin played some roles in the defense mechanism of invertebrates, while the structure of hemagglutinins differed in many respects from that of immunoglobulins (Marchalonis & Edelman, 1968a; Finstad et al., 1972; Kaplan et al., 1977).

Sea urchins belong to the phylum Echinodermata and are currently considered to be phylogenetically rather close to the vertebrates. Some of them have a large quantity of coelomic fluid by which the internal organs seem to be protected, though the physiological role of the fluid remains obscure. The fluid, in some cases, has a total protein concentration of up to 1 mg/mL and has been reported by Ryoyama (1974) to have a hemagglutinating activity. We initiated characterization of proteins in the coelomic fluid with an aim to understand its function and, as the first step, purified a protein responsible for its hemaglutinating activity. As stated above, Ryoyama (1974) reported the hemagglutinating activity of the coelomic fluid of three species of sea urchins, *Anthocidaris crassispina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*, but the molecular reality and the specificity for carbohydrate binding of such activity have not been studied so far.

In this study we obtained the substance with hemagglutinating activity from *A. crassispina* for the first time as a pure protein by using glutaraldehyde-fixed ghosts of human erythrocytes as an affinity adsorbent. We describe the purification procedure, the physical and chemical properties, and the carbohydrate-binding specificity of this hemagglutinin. It is a multisubunit protein that requires calcium ions (Ca^{2+}) and the O-glycosidically linked oligosaccharide chains on the human erythrocyte membranes for its activity.

Materials and Methods

Materials. Lactosamine, thiodigalactoside, methyl α-D-glucoside, methyl α-D-mannoside, sialidase (*Clostridium perfringens*), and *Phaseolus vulgaris* hemagglutinin (type V) were all purchased from Sigma Chemical Co. (St. Louis, MO). D-Glucose, D-galactose, D-glucosamine, L-fucose, D-mannose, *N*-acetyl-D-galactosamine, lactose, sucrose, *N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid were all purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). *Agaricus bisporus* hemagglutinin was purchased from E. Y. Laboratories (San Mateo, CA). Trypsin (TPCK[1] treated) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Glycophorin isolated from human erythrocytes (type O^{MN}) by the method of Marchesi & Andrews (1971) was generously supplied by Dr. Akira Hamada (Showa University, Tokyo, Japan). Phosphocholine–Sepharose affinity resin was

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[1] Abbreviations: TPCK, N^{α}-tosylphenylalanine chloromethyl ketone; Tris-HCl, 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; DEAE, (diethylamino)ethyl; AcNeu, *N*-acetylneuraminic acid; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; $CaCl_2$–NaCl buffer, 20 mM imidazole buffer (pH 7.0) with 1 mM $CaCl_2$ and 0.5 M NaCl; EGTA–NaCl buffer, 20 mM imidazole buffer (pH 7.0) with 0.5 mM EGTA and 0.5 M NaCl.

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prepared as described (Chesebro & Metzger, 1972). Human erythrocytes were donated by Tokyo University Hospital (Tokyo, Japan). Erythrocytes of other species were donated by Japan Immunological Research Laboratories Co. Ltd. (Takasaki, Japan).

Coelomic Fluid. Anthocidaris crassispina sea urchins were obtained from the Misaki Marine Biological Station of the University of Tokyo. The coelomic fluid was collected by pipetting, after the peristomial membrane was cut around the mouth and Aristotle's lantern was removed. The coelomic fluid was allowed to clot for about 1 h at 0 °C and centrifuged at 2500*g* for 30 min. The precipitated coelomocytes and clotted proteins were discarded. The resulting supernatant was dialyzed against 50 mM Tris-HCl (pH 7.5) and stored at 0 °C.

Preparation of Glutaraldehyde-Fixed Ghosts as an Affinity Adsorbent. Human erythrocytes were separated from 200 mL of blood by centrifugation at 1000*g* for 20 min and washed 3 times with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. Hemolysis was allowed to occur by pouring the erythrocytes into 20 volumes of 10 mM Tris-HCl (pH 7.5), and the mixture was gently stirred for 15 min at 4 °C. Hemoglobin-free ghosts were centrifuged at 20000*g* for 40 min, washed 5 times with the same hypotonic buffer, and then fixed with 1% glutaraldehyde for 5 h at room temperature. The reaction was terminated by addition of 0.1 volume of 1 M lysine (pH 7.3). Glutaraldehyde-fixed ghosts were washed 5 times with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and suspended in 10 mL of 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 20 mM $CaCl_2$.

Affinity Adsorption to Glutaraldehyde-Fixed Ghosts. The protein solution containing hemagglutinin(s) was mixed with an equal volume of a suspension of glutaraldehyde-fixed ghosts in the presence of 10 mM $CaCl_2$. After a 2-h incubation at room temperature, the mixture was centrifuged at 10000*g* for 15 min. The supernatant showed no hemagglutinating activity. The precipitate was washed 5 times with 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.15 M NaCl and suspended in 2 mL of 0.1 M Tris-HCl (pH 7.5) with 10 mM EDTA. It was incubated for 30 min at 4 °C and centrifuged at 10000*g* for 20 min. The ghosts were precipitated, while hemagglutinin was released in the supernatant. The supernatant was dialyzed against two changes of 10 volumes of 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1 mM $CaCl_2$.

Gel Electrophoresis. Electrophoresis on SDS–polyacrylamide slab gels was carried out by the method of Laemmli (1970) using a separating gel containing 15% acrylamide–0.45% bis(acrylamide). The protein sample was denatured at 80 °C for 2 min in a solution of 1% SDS with or without 1% 2-mercaptoethanol. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. The proteins used in SDS gel electrophoresis as molecular weight standards were bovine serum albumin (M_r 66000) (Wako Pure Chemicals, Tokyo), ovalbumin (M_r 45000) (Sigma), aldolase (M_r 40000) [Boehringer Mannheim Yamanouchi (BMY), Tokyo], soybean trypsin inhibitor (M_r 21500) (Sigma), and cytochrome *c* (M_r 12400) (BMY).

High-Performance Liquid Chromatography (HPLC) Gel Filtration. The purified hemagglutinin was dialyzed against 20 mM imidazole buffer (pH 7.0) with 1 mM $CaCl_2$ and 0.5 M NaCl (henceforth abbreviated as $CaCl_2$–NaCl buffer) or the same buffer with 0.5 mM EGTA and 0.5 M NaCl (EGTA–NaCl buffer). The resulting hemagglutinin solution was loaded on a HPLC column (TSK G4000 SW) equilibrated with either the $CaCl_2$–NaCl buffer or the EGTA–NaCl buffer. The column was eluted with either buffer at a flow rate of 0.7 mL/min at 25 °C. The Stokes radius of hemagglutinin was estimated by comparison of its partition coefficient (K_{av}) with those of standard proteins. The effective Stokes radii of standard proteins calculated from their diffusion coefficients (Smith, 1976) were 23.8 nm for myosin, 8.5 nm for thyroglobulin (Sigma), 6.0 nm for apoferritin (Sigma), 4.8 nm for catalase (BMY), 2.8 nm for the β-lactoglobulin (Sigma) dimer, and 2.1 nm for its monomer.

Analytical Ultracentrifugation. Sedimentation studies were done with a Beckman Spinco Model E analytical ultracentrifuge equipped with a UV photoelectric scanner. All measurements were taken at 280 nm. Sedimentation equilibrium experiments were carried out on the purified protein (0.15 mg/mL) either in the $CaCl_2$–NaCl buffer or in the EGTA–NaCl buffer at 5214 rpm and 18.6 °C. The hemagglutinin (<0.4 mg/mL) was also subjected to sedimentation velocity measurements at 32000 rpm in the presence and absence of Ca^{2+}.

Protein Assay. The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Electron Microscopy. The electron micrograph of the protein was taken with a Hitachi HU-11B electron microscope. The sample in 1 mM $CaCl_2$ containing 0.15 M NaCl and 20 mM imidazole (pH 7.0) was negatively stained with 3% uranyl acetate.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 N HCl for 24, 48, and 72 h, respectively, at 110 °C in vacuo and analyzed on a Hitachi 835 automatic amino acid analyzer. The results for serine and threonine were log-linearly extrapolated to zero time of hydrolysis. The contents of isoleucine and valine were taken from the results obtained for the sample hydrolyzed for 72 h. Cysteine was estimated as cysteic acid in performic acid oxidized samples subjected to HCl hydrolysis according to the method described by Hirs (1967) with a slight modification. Tryptophan was determined after hydrolysis in 3 N mercaptoethanesulfonic acid (Penke et al., 1974).

Carbohydrate Determination. Neutral sugar was determined by the phenol–sulfuric acid method (Dubois et al., 1956) with glucose as a standard. Sialic acid was determined by the direct Ehrlich reaction (Werner & Odin, 1952).

Assay of Hemagglutinating Activity. Hemagglutinating activity was determined by serial 2-fold dilution in microtiter U-plates. The maximum dilution causing hemagglutination was taken as the hemagglutination titer. The samples to be tested were prepared, if not otherwise described, in 25 μL of 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1 mM $CaCl_2$, to which 25 μL of a 3% suspension (v/v) of erythrocytes washed 3 times with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl was added. The plates were incubated at room temperature for 1 h (Matsumoto & Osawa, 1969).

The inhibition assay was carried out in the same microtiter U-plates as follows. To each 25 μL of a 2-fold serial dilution of the sample solution to be tested was added an equal volume of hemagglutinin solution whose hemagglutination titer was determined to be 2^2 by the method described above. After incubation for 1 h at room temperature, 25 μL of human erythrocyte suspension was added. The mixture was kept at room temperature for 1 h and then examined for agglutination. Inhibitory activity was expressed as the minimum concentration of inhibitor that effected complete inhibition of hemagglutination.

Ca^{2+}–EGTA Buffer. Ca^{2+}–EGTA buffer was prepared according to Ogawa (1968) by addition of EGTA and $CaCl_2$ to 20 mM imidazole buffer (pH 7.0) containing 0.15 M NaCl. $CaCl_2$ was provided by addition of an appropriate quantity of HCl to $CaCO_3$ in the same buffer. $CaCO_3$ and EGTA were dried at 110 °C under evacuated conditions before use. Several buffer solutions with varying concentrations of free Ca^{2+} were prepared by changing the amount of EGTA and $CaCl_2$. The concentration of free Ca^{2+} was calculated by the equation:

$$[\mathrm{EGTA}]_{\mathrm{total}} = ([\mathrm{Ca}^{2+}]_{\mathrm{total}} - [\mathrm{Ca}^{2+}]_{\mathrm{free}})\frac{1 + K'[\mathrm{Ca}^{2+}]_{\mathrm{free}}}{K'[\mathrm{Ca}^{2+}]_{\mathrm{free}}}$$

$$K' = 5 \times 10^5\ \mathrm{M}^{-1}$$

where K' was the apparent binding constant of EGTA for Ca^{2+} at neutral pH (Ogawa, 1968). Glass-distilled water was used for the preparation of Ca^{2+}–EGTA buffer.

Effect of Ca^{2+} on the Hemagglutinating Activity. Sample solutions (0.2 mg/mL) were dialyzed overnight at 4 °C against 20 mM imidazole buffer with 0.15 M NaCl, whose free Ca^{2+} concentration was controlled as described above. The hemagglutinating activity was then measured by using the dialysis buffers for dilution and also for the suspension medium of erythrocytes.

Preparation of Tryptic Fragments from Human Erythrocyte Membranes. Soluble tryptic fragments from human erythrocyte membranes were prepared as described previously (Winzler et al., 1967) with a slight modification. Washed erythrocytes (100 mL) were added to 100 mL of 0.15 M NaCl–50 mM Tris-HCl (pH 7.5) containing 25 mg of trypsin. The cell suspension was incubated with shaking at 37 °C for 1 h and centrifuged at 2000*g* for 20 min in the cold, and the red-tinted supernatant was removed by suction. To this supernatant was added one-tenth volume of 50% trichloroacetic acid. The resulting precipitate was removed by centrifugation at 50000*g* for 20 min, and the supernatant fluid, which contained tryptic fragments, was neutralized with NaOH, dialyzed against water at 4 °C, and lyophilized.

Alkaline Borohydride Treatment of Peptide. The tryptic fragments of red cell membranes were incubated with 0.4 M sodium borohydride in 0.2 N NaOH for 36 h at room temperature under an N_2 atmosphere. The solution was then neutralized with 2 N acetic acid and applied to a Sephadex G-50 column (1.0 cm × 55 cm) equilibrated with water. Fractions were analyzed for hexose by the phenol–sulfuric acid test and for peptide by the absorbance at 226 nm. Glycopeptide and oligosaccharide fractions were separately recovered by lyophilization.

Sialidase Treatment of Tryptic Fragments of Erythrocyte Membranes. Tryptic fragments of erythrocyte membranes containing about 3 mg of sialic acid were incubated with 0.4 unit of sialidase in 0.5 mL of 0.1 M sodium acetate buffer (pH 5.5), supplemented with 0.2 mM $CaCl_2$ and 20 μM EDTA. After incubation at 37 °C for 5 h, the mixture was directly applied to a Sephadex G-25 column equilibrated with water. The peptide peak was lyophilized.

Periodate Oxidation of Desialyzed Tryptic Fragments. Desialyzed tryptic fragments were incubated with 0.015 M sodium metaperiodate (Nakarai Chemicals Ltd.) in 50 mM sodium acetate buffer (pH 4.5). After incubation at 0 °C in the dark for 63 h, the oxidation was terminated by adding an excess of ethylene glycol. The sample was extensively dialyzed first against distilled water and then against 0.15 M NaCl in 50 mM Tris-HCl (pH 7.5).

Tryptic Digestion of Glycophorin. Digestion of purified glycophorin with trypsin was done at an enzyme:substrate ratio of 1:100 and at a glycophorin concentration of 10 mg/mL in 50 mM Tris-HCl (pH 7.5), at 37 °C for 24 h. The reaction was terminated by adding 3 vol % of 1 mM PMSF (phenylmethanesulfonyl fluoride) in DMF (dimethylformamide). During the digestion period a precipitate usually formed, which was removed by centrifugation at 7000*g* for 10 min. Usually more than 95% glycophorin was digested under these conditions, judging from the elution pattern of the gel filtration of the digestion mixture on a G4000 SW column (Toyo Soda, Tokyo).

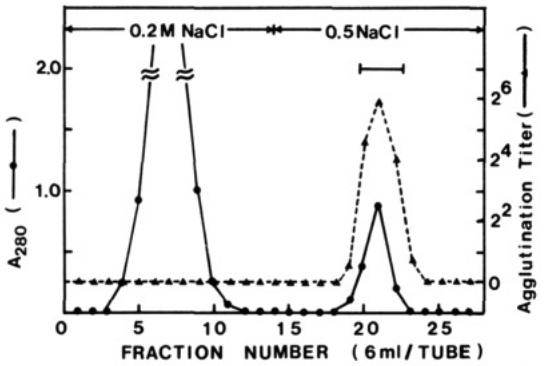

FIGURE 1: Elution pattern of DEAE-cellulose column chromatography of the whole coelomic fluid. The column (1.3 cm × 15 cm) was equilibrated with 50 mM Tris-HCl (pH 7.5). Elution was done first with 0.2 M NaCl and then 0.5 M NaCl in 50 mM Tris-HCl (pH 7.5). (●) Absorbance at 280 nm; (▲) hemagglutination titer against human erythrocytes. Fractions under the bar were collected.

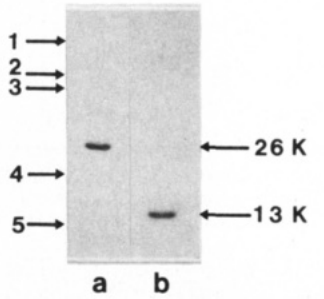

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the purified hemagglutinin (a) before and (b) after reduction with 1% 2-mercaptoethanol. The arrows show the molecular weights of the markers: (1) bovine serum albumin, M_r 66 000; (2) ovalbumin, M_r 45 000; (3) aldolase, M_r 40 000; (4) trypsin inhibitor, M_r 21 500; (5) cytochrome *c*, M_r 12 400. The estimated molecular weight of the hemagglutinin was 26 000 and 13 000 before and after reduction, respectively, both in the presence of SDS.

Results

Purification of Hemagglutinin. The coelomic fluid (150 mL) that was dialyzed against 50 mM Tris-HCl (pH 7.5) was applied to a DEAE-cellulose column (1.3 cm × 15 cm) equilibrated with the same solvent. The column was first washed with 50 mM Tris-HCl (pH 7.5) and then with 0.2 M and 0.5 M NaCl containing 50 mM Tris-HCl (pH 7.5) in successive steps. Figure 1 shows an elution pattern from the DEAE-cellulose column. The hemagglutinating activity toward human erythrocytes was observed only in fractions under the bar. These fractions were collected and further purification was performed by affinity adsorption to the glutaraldehyde-fixed ghosts, followed by elution with 10 mM EDTA (see Materials and Methods). The resulting solution was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1 mM $CaCl_2$. It had a hemagglutinating activity and showed only a single band in the SDS gel electrophoresis (Figure 2). The apparent molecular weight of the polypeptide was 13 000 when the sample was reduced with 2-mercapto-

Table I: Purification of Hemagglutinin

fraction	volume (mL)	total protein (mg)	sp act.[a]	recovered act. (%)
whole coelomic fluid	150	135	4.4	100
eluate from DEAE-cellulose column	18	10	57.1	96
purified hemagglutinin	2	0.5	1024	86

[a] Specific activity = titer/(mg/mL). (See the text for the definition of titer.)

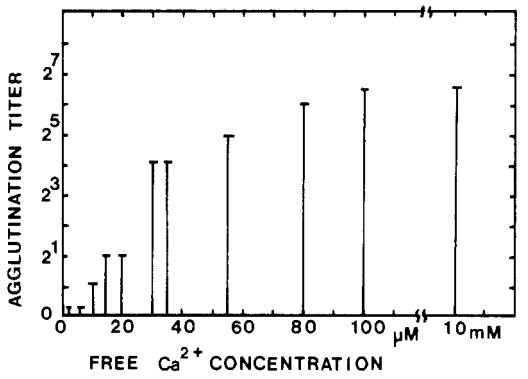

FIGURE 3: Effect of Ca^{2+} on hemagglutinating activity. The free Ca^{2+} concentration was controlled by Ca^{2+}–EGTA buffer (see Materials and Methods). The sample concentration was 0.2 mg/mL. The agglutination assay was done with human erythrocytes.

ethanol prior to the electrophoresis while it was 26000 without reduction, indicating that the 13K polypeptides were cross-linked with disulfide bonds to form dimers in the native hemagglutinin molecule.

The purification procedure of the hemagglutinin from the whole coelomic fluid resulted in about a 200-fold increase in the specific activity. The purification steps are summarized in Table I.

Effect of Divalent Cations on Hemagglutinating Activity. Ca^{2+} exerted a marked effect on the hemagglutinating activity of the purified protein, as shown in Figure 3. When Ca^{2+} was absent from the reaction mixture, no hemagglutinating activity was detected. The activity appeared as the concentration of Ca^{2+} was increased, and a half-maximal activity was observed at a Ca^{2+} concentration of 20 μM. Mg^{2+} showed no effect on the activity even when its concentration was 0.1 M.

Physicochemical Analysis of the Purified Protein. The molecular weight, sedimentation coefficient, and Stokes radius of the purified protein were determined in the presence or absence of Ca^{2+}.

Sedimentation equilibrium experiments gave linear plots of ln A_{280} vs. r^2, where r is the distance from the rotor center, both in the presence and in the absence of Ca^{2+}. The molecular weight was calculated as 300000 and 280000 in the presence and absence of Ca^{2+}, respectively, by using a partial specific volume of 0.70 cm^3/g (calculated from the amino acid composition and carbohydrate content as shown below).

Sedimentation velocity experiments showed that the purified hemagglutinin in the $CaCl_2$–NaCl buffer behaved as a single component with $s^0_{20,w}$ = 13.7 S (Figure 4a,c). In the EGTA–NaCl buffer it also behaved as a single component with $s^0_{20,w}$ of 11.4 S (Figure 4b,d).

The Stokes radius of the hemagglutinin in the presence and absence of Ca^{2+} was determined by the HPLC gel filtration experiment (TSK G4000 SW). In both cases the protein was eluted with a single symmetrical peak (Figure 5). From the elution positions of standard proteins with known Stokes radii, its Stokes radius was estimated to be 5.5 and 6.7 nm in the

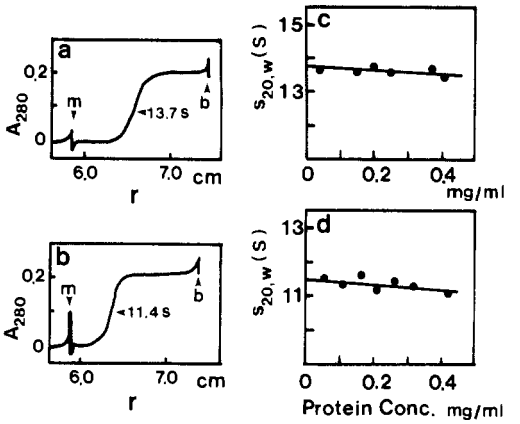

FIGURE 4: Sedimentation velocity analysis of the purified hemagglutinin. (a) Scanner trace of the sedimentation boundary of the purified hemagglutinin in 1 mM $CaCl_2$ and 0.5 M NaCl in 20 mM imidazole (pH 7.0). (b) Scanner trace of the sedimentation boundary of the purified hemagglutinin in 0.5 mM EGTA and 0.5 M NaCl in 20 mM imidazole (pH 7.0). m and b indicate the positions of the meniscus and the bottom of the centrifuge cell, respectively. r is the distance from the center of revolution. The sedimention boundaries were traced at 64 min after reaching the rotor speed of 32000 rpm. (c) Concentration dependence of the sedimentation coefficient ($s_{20,w}$) of hemagglutinin in 1 mM $CaCl_2$ and 0.5 M NaCl in 20 mM imidazole (pH 7.0). (d) Concentration dependence of the sedimentation coefficient ($s_{20,w}$) of hemagglutinin in 0.5 mM EGTA and 0.5 M NaCl in 20 mM imidazole (pH 7.0).

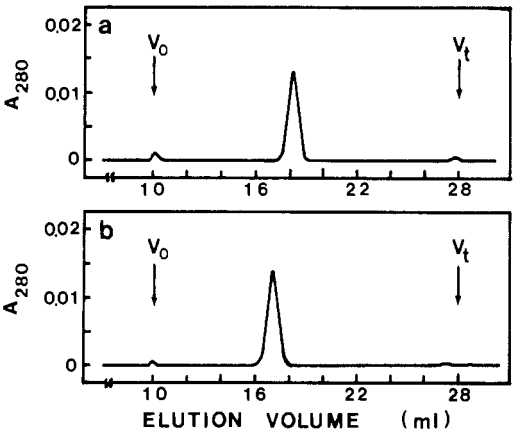

FIGURE 5: Elution profiles of the purified hemagglutinin from the TSK G4000 SW column. (a) Hemagglutinin in 1 mM $CaCl_2$ and 0.5 M NaCl in 20 mM imidazole (pH 7.0) was loaded on the column and eluted with the same solvent. (b) Hemagglutinin in 0.5 mM EGTA and 0.5 M NaCl in 20 mM imidazole (pH 7.0) was loaded on the column and eluted with the same solvent. Proteins were monitored by the absorbance at 280 nm.

presence and absence of Ca^{2+}, respectively.

Using the values of the sedimentation coefficient, $s^0_{20,w}$, the Stokes radius, R, and the partial specific volume, $\bar{v}$, we calculated the molecular weight of the protein in the $CaCl_2$–NaCl buffer as 290000 and that in the EGTA–NaCl buffer also as 290000, according to the equation $M_r = 6\pi\eta NRs^0_{20,w}/(1 - \bar{v}\rho)$, where η, N, and ρ are the viscosity, Avogadro's number, and density of the solvent. Molecular weights of the hemagglutinin in the presence and absence of Ca^{2+} were identical and consistent with those obtained by sedimentation equilibrium experiments. The results when combined with those of SDS gel electrophoresis indicated that both in the presence and in the absence of Ca^{2+} the hemagglutinin molecules consisted of 22 ± 2 polypeptides with an apparent molecular weight of 13000, which were linked by disulfide bonds in pairs.

Amino Acid Composition and Carbohydrate Content. The results of the amino acid analysis are given in Table II in terms

Table II: Amino Acid Composition of Sea Urchin Hemagglutinin

amino acid	mol %	amino acid	mol %	amino acid	mol %
Asp	11.3	Ala	5.7	Tyr	2.1
Thr[a]	7.6	$^1/_2$-Cys[b]	2.6	Phe	5.3
Ser[a]	15.4	Val[c]	4.0	Lys	1.8
Glu	11.4	Met	1.2	His	3.0
Pro	3.0	Ile[c]	2.9	Arg	2.5
Gly	13.3	Leu	4.5	Trp[d]	2.4

[a] Obtained by a log-linear extrapolation to zero time of hydrolysis. [b] Estimated as cysteic acid by performic acid oxidation. [c] Determined from the results obtained for the 72-h hydrolysis sample. [d] Determined by 3 N mercaptoethanesulfonic acid hydrolysis.

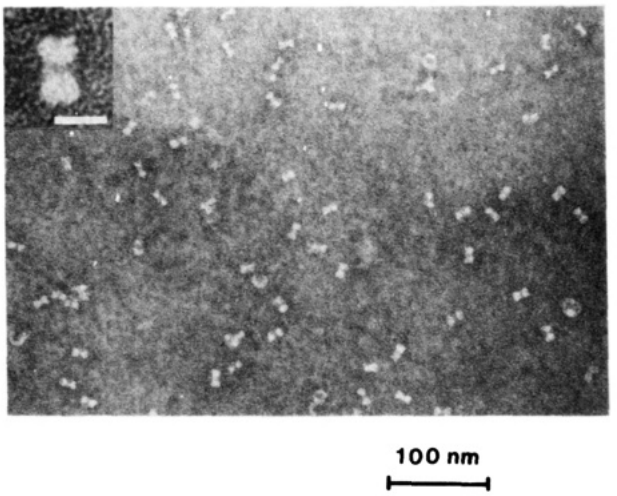

FIGURE 6: Electron micrograph of the purified hemagglutinin. The white bar in the inset shows 10 nm. The sample was negatively stained with 3% uranyl acetate.

of mole percent. The most notable results were the high contents of serine, glycine, and acidic amino acids, which constituted about 50% of the total amino acid residues. The contents of methionine and basic amino acids were low. No hemagglutinin has been reported yet that has a similar amino acid composition to this one. The carbohydrate content of this protein was 3.2% of the total weight, determined by the phenol–sulfuric acid method.

Electron Microscopy. Figure 6 shows the electron microscopic images of the negatively stained hemagglutinin, which was incubated in 50 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and 0.15 M NaCl before staining. Images like "butterflies" with unfolded wings were the most prevalent in the field, and they were quite uniform in size, giving average dimensions of 13 nm × 8 nm. At least four semiglobular domains were visible in some of the molecules. Ringlike structures were occasionally seen, but their relation to the major ones was not clear. No structural change could be detected when the sample was incubated in the Ca^{2+}-free buffer before staining.

Hemagglutinating Activity and Inhibition Studies. The purified hemagglutinin agglutinated human erythrocytes with little specificity as to the ABO blood types or to the MN types. As for hemagglutinating activity on erythrocytes of other species, bovine and chicken erythrocytes were not agglutinated even at a high hemagglutinin concentration while those of sheep were agglutinated to the same extent as human erythrocytes.

The following sugars failed to give a significant inhibition against hemagglutination of human erythrocytes when tested up to a concentration of 200 mM: D-glucose, D-galactose, D-glucosamine, L-fucose, D-mannose, methyl α-D-glucoside, methyl α-D-mannoside, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, lactose, sucrose, lactosamine, *N*-acetylneuraminic acid, and thiodigalactoside. Glycoproteins such as ovalbumin and thyroglobulin also showed no inhibitory activity. Ryoyama (1974) also reported that the hemagglutinating activity of coelomic fluid against rabbit erythrocytes was not inhibited by various kinds of simple sugars.

Table III: Inhibitory Activity of Tryptic Fragments of Human Erythrocyte Membranes and Glycophorin

	mM amounts required[a] for inhibitory activity on	
	sea urchin hemagglutinin	red kidney bean hemagglutinin
tryptic fragments of erythrocyte membranes		
whole soluble fraction	2.0	3.2
residual glycopeptides after alkaline borohydride treatment	none[b]	1.8
released oligosaccharides after alkaline borohydride treatment	6.0	none[c]
sialidase-treated tryptic fragments	1.8	2.9
glycophorin		
solubilized form (whole)	0.18	0.3
trypsin-treated glycophorin	2.2	3.1

[a] Minimum amounts required to completely inhibit the titer four (2^2) of hemagglutination toward human erythrocytes, which were expressed as D-glucose equivalents. [b] Inhibitory activity was not observed even at concentrations of 12 mM. [c] Inhibitory activity was not observed even at concentrations of 10 mM.

In this study, however, we found that the tryptic fragments of human erythrocyte membranes showed an inhibitory effect against the hemagglutinating activity (Table III). It has been known that such fragments contain two types of oligosaccharide chains (Thomas & Winzler, 1969; Kornfeld & Kornfeld, 1970). One of them is O-glycosidically linked to serine and threonine residues and can be selectively released from the peptide backbone with an alkali treatment. The other one is N-glycosidically linked to asparagine residues and is alkali stable. To identify which type of oligosaccharide is recognized by the sea urchin hemagglutinin, we treated the tryptic fragments with alkaline borohydride and split off the O-glycosidically linked oligosaccharides. The digestion product was subjected to gel filtration on Sephadex G-50 for the separation of the residual glycopeptides from the released oligosaccharides. As shown in Table III, we found that the residual glycopeptides were not able to inhibit the hemagglutination of the sea urchin hemagglutinin even at a concentration of 12 mM as glucose equivalent. On the other hand, the activity of the red kidney bean *Phaseolus vulgaris* hemagglutinin, which was known to be inhibited by alkali-stable N-glycosidically linked oligosaccharide chains (Kornfeld & Kornfeld, 1970), was fully inhibited by 1.8 mM of the residual glycopeptides. The released oligosaccharides showed an inhibitory activity against the sea urchin hemagglutinin though the effect was rather weak. They showed no inhibitory activity toward the red kidney bean hemagglutinin. These results indicated that the sea urchin hemagglutinin mainly recognized O-glycosidically linked carbohydrate chains for its hemagglutinating activity and the role of N-glycosidically linked ones was insignificant.

To see whether or not the sialic acids as the terminal residues of O-glycosidically linked oligosaccharides constituted the recognition sites by the sea urchin hemagglutinin, we treated tryptic fragments of erythrocyte membranes with sialidase. After the treatment, more than 90% of the sialic acids were released from the tryptic fragments as judged from mea-

surements by the direct Ehrlich reaction, but the inhibitory activity of such tryptic fragments remained unchanged against both the sea urchin hemagglutinin and the red kidney bean hemagglutinin. When these desialyzed tryptic fragments were treated with weak periodate to destroy the terminal galactose, they showed no inhibitory activity against the sea urchin hemagglutinin.

The purified human glycophorin showed a remarkable inhibitory activity toward the sea urchin hemagglutinin, as shown in Table III. This result is significant since glycophorin is one of the major glycoproteins of human erythrocyte membranes and has both O-glycosidically linked and N-glycosidically linked carbohydrate chains (Tomita & Marchesi, 1975). When glycophorin was digested with trypsin, the inhibitory activity toward these hemagglutinins decreased to 10% of the native glycophorin, in agreement with results shown above.

Binding Experiment of the Hemagglutinin to Phosphocholine. Recently, hemagglutinin in the hemolymph of a horseshoe crab, *Limulus polyphemus*, has been identified as a "C-reactive protein" (CRP) since it has the ability to precipitate C-polysaccharides and to bind to the immobilized phosphocholine in the presence of Ca^{2+} (Robey & Liu, 1981). CRP is one of the acute phase proteins found in man and other mammals and has been identified in the serum of several lower vertebrates such as plaice, *Pleuronectes platessa* L. (Pepys et al., 1978), and dog fish, *Mustelus canis* (Robey et al., 1983). They usually have a high molecular weight and are composed of many subunits with a molecular weight of 20 000–25 000. Since the newly purified sea urchin hemagglutinin has similar structural characteristics, we applied it to a phosphocholine–Sepharose affinity column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.15 M NaCl. The hemagglutinin was not retained by the column, indicating that the sea urchin hemagglutinin has no binding site for phosphocholine.

Discussion

Most significant features of the hemagglutinin purified in this work reside in its unique quaternary structure and its ability to recognize complex carbohydrate moieties. The native molecule probably has a fixed number of polypeptide chains between 22 and 24 that are disulfide bonded in pairs. The constituent polypeptides are uniform in size with an apparent molecular weight of 13 000. Hydrodynamic and electron microscopic studies of the active molecule showed that the subunits were arranged into a rather asymmetric structure (f/f_0 = 1.32 from $s^0_{20,w}$ and 1.26 from R), which looked quite different from the electron micrographs of limulin (Marchalonis & Edelman, 1968b) at least under the given conditions. Depletion of Ca^{2+} by the chelating agent EGTA destroyed the hemagglutinating activity of the molecule without changing its molecular weight. The hydrodynamic measurement, however, recorded a structural change that reduced the molecule into a more asymmetric conformation (f/f_0 = 1.59 from $s^0_{20,w}$ and 1.53 from R). Unfortunately, it is not known whether the structural change or the absence of Ca^{2+} was responsible for the loss of hemagglutinating activity. As reported by Ubaghs (1969), the Ca^{2+} concentration of sea urchin coelomic fluid can be as high as a few millimolar, so the hemagglutinin studied in this work is expected to be active in the natural environment.

In such gross structural features as stated above, the sea urchin hemagglutinin can be compared with limulin, a hemagglutinin of *Limulus polyphemus*, which has a molecular weight of 400 000 and 18 polypeptide chains of a uniform size (Marchalonis & Edelman, 1968a). Limulin has been reported to undergo a Ca^{2+}-dependent structural change that is characterized by the decrease of sedimentation coefficient from 13.5 to 10.6 S by the depletion of Ca^{2+}, a very similar change to the one we observed for the sea urchin hemagglutinin. Limulin, however, has been characterized as a C-reactive protein (Robey & Liu, 1981) and looks like ring-shaped molecules under the electron microscope, which are two quite different properties from the sea urchin hemagglutinin.

We think it is interesting to recognize that hemagglutinins of invertebrates, in a few cases, established quite elaborate quaternary structures of fixed subunit stoichiometries of as large as 20. It was probably a necessary step to enforce the action of such molecules, which could not be quite successfully performed by smaller aggregates. Such a tendency may have been an important factor in the molecular evolution of some proteins.

In other cases of invertebrate hemagglutinins, much lower molecular weights have been reported. Examples are hemagglutinins from sponge *Axinella polypoides* (M_r 15 000 and 21 000) (Bretting & Kabat, 1976), sponge *Geodia cydonium* (M_r 36 500) (Müller et al., 1983), snail *Helix pomatia* (M_r 100 000) (Hammarström & Kabat, 1969), snail *Biomphalaria glabrata* (M_r 67 000) (Bretting et al., 1983), shellfish *Saxidomus purpuratus* (M_r 40 000) (Tatsumi et al., 1982), and flesh fly *Sarcophaga peregrina* (M_r 190 000) (Komano et al., 1980).

Since no simple carbohydrates were found to inhibit the activity of the sea urchin hemagglutinin, we adopted a simple method for the affinity purification of the protein that uses adsorption of hemagglutinin to glutaraldehyde-fixed ghosts of human erythrocytes and subsequent elution with 10 mM EDTA. This method resulted in a 200-fold increase in the specific activity and gave an electrophoretically pure protein. We believe this method is generally applicable to the purification of proteins that show a Ca^{2+}-dependent hemagglutinating activity.

The erythroagglutination by hemagglutinin is generally inhibited by monosaccharides that presumably are a part of or are closely related to saccharide receptor sites on the erythrocyte surface. By the use of the inhibition assay with monosaccharides, the saccharide specificity of many hemagglutinins has been established. However, for some hemagglutinins, no monosaccharide that inhibits their agglutinating activity has been found (Sage & Vazquez, 1967; Presant & Kornfeld, 1972; Kawaguchi et al., 1974; Prigent & Bourrillon, 1976). The idea that these hemagglutinins required complex carbohydrate structures for an efficient interaction was confirmed for some of them (Irimura et al., 1975; Kaifu & Osawa, 1979; Duk et al., 1982). Although the sea urchin hemagglutinin was not inhibited by simple sugars, we showed that its activity was inhibited by tryptic fragments released from human erythrocyte membranes. Our study using such fragments suggested that the receptor sites for the sea urchin hemagglutinin are mainly composed of alkali-labile carbohydrate chains with tetrasaccharides such as AcNeuα2→3Galβ1→3(AcNeuα2→6)GalNAc→serine (or threonine) (Thomas & Winzler, 1969). Moreover, the result that the removal of terminal sialic acid residues from the tryptic fragments hardly affected their inhibitory activity indicates that the O-glycosidically linked disaccharide with the structure Galβ1→3GalNAc→serine (or threonine) is important for the receptor activity. This inference was confirmed by the fact that the desialyzed tryptic fragments whose terminal galactose was destroyed by the periodate oxidation showed no inhibitory activity.

Glycophorin is a major glycoprotein in the erythrocyte membrane and has many O-glycosidically linked tetrasaccharides as described above, together with N-glycosidically linked oligosaccharides (Tomita & Marchesi, 1975; Furthmayr et al., 1975). Thus, it was expected that this protein would show an inhibitory effect on the hemagglutininating activity of the sea urchin protein. In fact, we found that it was a potent inhibitor of the sea urchin hemagglutinin. Since tryptic digestion of glycophorin reduced its inhibitory effect, it seemed that the integrity of the polypeptide chains of glycophorin played a significant role in holding the O-glycosidically linked oligosaccharide chains in a favorable arrangement for interaction with hemagglutinin (Kornfeld & Kornfeld, 1970; Duk et al., 1982).

Finally, we must acknowledge that we have little idea as to the physiological role and location of the newly purified hemagglutinin, though the hemagglutinating activity is a common characteristic of the coelomic fluid of many sea urchins (Yeaton, 1981a), including *Araesoma owstoni* and *Diadema setosum* (Giga & Ikai, unpublished observation). Since in this work the coelomic fluid was collected after clottable materials were discarded together with coelomic cells, it is equally possible either that the protein was originally in some of the coelomic cells that were ruptured upon coagulation or that it was a natural constituent of the coelomic fluid.

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