Purification and Physicochemical Properties of a Hemagglutinin (GVA-1) in the Red Alga *Gracilaria verrucosa*

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The red alga *Gracilaria verrucosa* was found to possess hemagglutinins against various kinds of animal erythrocytes; among these the horse, guinea pig, and chicken erythrocytes were strongly agglutinated. The hemagglutinins were very stable between pH 4 and 12 and also relatively stable to heat. Their activity was not affected by the divalent metal cations tested. One hemagglutinin component (GVA-1) was purified by successive column chromatographies on DEAE-cellulose, Sephadex G-75, Hydroxyapatite, and Sephadex G-75 again. GVA-1 was a tetramer which was comprised of two kinds of subunits (MW 12,000 and 10,500); its molecular weight was about 41,000. It contained high amounts of glycine and hydroxy amino acids (threonine and serine) but no half cysteine. The content of acidic amino acids (aspartic acid and glutamic acid) was fairly larger than that of basic amino acids (lysine, histidine and arginine); it coincided with the low isoelectric point, 4.8, of GVA-1. None of the sugars used inhibited the activity of GVA-1.

As the previous screenings done with human erythrocytes clarified that some marine algae contained hemagglutinins but the activity was weak,^{1,2)} no particular attentions have been so far paid to hemagglutinins from marine algae. However, SHIOMI *et al.*³⁾ recently pointed out that the use of animal erythrocytes was more effective in studying hemagglutinins of marine algae than that of human erythrocytes, and moreover succeeded in the isolation and characterization of hemagglutinins from two species of red algae *Agrdhiella tenera*³⁾ and *Cystoclonium purpureum*.⁴⁾ Significantly, these algal hemagglutinins could distinguish between two mouse leukemia cells. Independently, BLUNDEN *et al.*^{2,5-7)} detected hemagglutinins highly specific for human B cells in the red alga *Prilota plumosa* and examined their biochemical properties. Thus, like the seeds of higher plants, marine algae seem to be good sources for new hemagglutinins which would become useful biochemical reagents in immunology, cancer research, and cell biology.

In the course of our screening for hemagglutinins in Japanese marine algae, many species were found to be active against various animal erythrocytes. Some biochemical properties of hemagglutinins in the red alga *Serraticardia maxima* which is one of active species confirmed by our screening, have been reported previously.⁸⁾ In this paper we describe the purification and physicochemical properties of a hemagglutinin component (GVA-1) in another active red alga *Gracilaria verrucosa*

which is very important species in Japan as a food or a raw material of agar-agar. This alga was previously reported to be inactive against human erythrocytes²⁾ or active only by the sensitive method.⁵⁾

Materials and Methods

Seaweed

Samples of the red alga *G. verrucosa* were collected at Kominato, Chiba Prefecture, in November, 1980. Immediately after shipping to our laboratory, they were lyophilized and ground to powder. The powdered alga (12 g) obtained from 150 g of wet samples was used for the following purification.

Purification of a hemagglutinin (GVA-1)

Horse erythrocytes were used for the estimation of hemagglutinating activity throughout the purification procedure. The powdered alga was extracted three times with 25-folds its volume of buffered saline (0.15 M NaCl in 0.01 M phosphate buffer, pH 7.0). To the crude extract was added slowly solid $(\text{NH}_4)_2\text{SO}_4$ at 4°C to give a final concentration of 70% saturation. After keeping the solution at 4°C for 2 h, it was centrifuged at 10,000 rpm for 10 min. The precipitate dissolved in 300 ml of 0.01 M phosphate buffer (pH 7.5) was applied to a DEAE-cellulose column (2×35 cm) equilibrated with the same buffer. The column was washed first with 300 ml of the buffer and sub-

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sequently eluted by a linear gradient method between the buffer (750 ml) and 1.0 M NaCl in the buffer (750 ml). In DEAE-cellulose column chromatography two active fractions (Frs. I and II) were obtained (Fig. 1). Each active fraction was dialyzed against distilled water and lyophilized. Only Fr. I was further subjected to a Sephadex G-75 column (2 × 55 cm). The column was eluted with buffered saline. Active fractions obtained were directly applied to a Hydroxyapatite column (Seikagaku Kogyo, 2 × 35 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). After washing the column with the starting buffer, it was eluted by a linear gradient method of 1.5 l of 0.01–0.4 M phosphate buffer (pH 7.0). The active fractions were combined, dialyzed against distilled water, lyophilized and dissolved in a small volume of buffered saline. The active component (GVA-1) was finally purified by Sephadex G-75 column chromatography with the same conditions as described above.

Analytical methods

According to the method of SHIOMI *et al.*,⁸⁾ agglutinating activity was determined using each 2% erythrocyte suspension prepared from horse, cow, sheep, rabbit, guinea pig, chicken and carp blood. The results were expressed by the minimum active concentration (μ g protein/ml) required for positive agglutination or by the titer value which was reciprocal of the highest dilution of sample that gave positive agglutination. Protein was estimated by the method of LOWRY *et al.*⁹⁾ with bovine serum albumin as the standard. Effects of pH, heat, metal ions and simple sugars on hemagglutinating activity against horse erythrocytes were examined as described in our previous paper.⁸⁾

Analytical disc electrophoresis was performed on a 7% polyacrylamide gel (5 × 50 mm) with Tris-glycine buffer (pH 8.3).¹⁰⁾ Proteins were visualized with Amido Black 10 B and glycoproteins or polysaccharides by the staining method of ZACHARIUS *et al.*¹¹⁾ An unstained gel was cut into 5 mm slices and each slice was used for the measurement of agglutinating activity against horse erythrocytes after extraction with 0.5 ml of buffered saline. Sodium dodecyl sulfate (SDS) disc electrophoresis was carried out as described by WEBER and OSBORN¹²⁾ using a 12.5% polyacrylamide gel (5 × 60 mm) and 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. Before electrophoresis the preparation was treated with 1% SDS and 2% 2-mercaptoethanol at room temperature overnight.

Bovine serum albumin (MW 67,000), ovalbumin (MW 43,000) and ribonuclease A (MW 13,700) were adopted as reference proteins. Electrofocusing on a 5% polyacrylamide gel (5 × 100 mm) was performed at 200 V for 6 h using 0.02 M phosphoric acid for an anode solution and 1.0 M NaOH for a cathode solution. Bio-lyte 3/10 (Bio-Rad Laboratories) was used as carrier ampholyte at a concentration of 2%. Gels were stained with Coomassie Brilliant Blue G-250. Six colored proteins (cytochrome c from horse and its acetylated derivatives) with known pI values (4.1, 4.9, 6.4, 8.3, 9.7 and 10.6) were purchased from Oriental Yeast Co., Ltd.

The molecular weight determination of native GVA-1 was done on a Sephadex G-75 column (1 × 50 cm) with buffered saline. Bovine serum albumin, ovalbumin, chymotrypsinogen A (MW 25,000) and ribonuclease A were used as standard proteins.

The amino acid composition of GVA-1 was measured on a Hitachi 835 amino acid analyzer after hydrolysis with 6 N HCl at 110°C for 24 h. In order to compensate for destruction by acid hydrolysis, threonine and serine were increased by 5 and 10%, respectively. Tryptophan was estimated spectrometrically by the method of GOODWIN and MORTON.¹³⁾

Results and Discussion

The *G. verrucosa* agglutinins against human erythrocytes were previously detected only by the sensitive method,⁸⁾ in which bovine albumen medium or enzyme-treated erythrocytes were used. However, when checked with animal erythrocytes, the crude extract was found to be active against all erythrocytes tested (Table 1). Of the erythrocytes, horse, guinea pig and chicken erythrocytes were strongly agglutinated by the crude extract while cow and carp erythrocytes very weakly agglutinated. Such high specificity against chicken erythrocytes has not been observed among other algal hemagglutinins.^{8,4,8)}

When checked with horse erythrocytes, the activity of crude extract was stable over a wide range of pH values between 4 and 12 but about 50% of the original activity was lost below pH 3. Activity was also unaffected by heating at 40°C for 30 min and was gradually reduced as the heating temperature was increased. However, even when heated at 90°C for 30 min, about 25% of the original activity remained. Divalent metal cations, Ca^{2+} ,

Mg^{2+} and Mn^{2+} , exhibited no acceleration on the activity of *G. verrucosa* hemagglutinins. The same results were reported for other algal hemagglutinins.^{8, 4, 8)}

Purification of GVA-1

In DEAE-cellulose column chromatography (Fig. 1) the *G. verrucosa* hemagglutinins, which were adsorbed by the column, were separated into two fractions, Fr. I (fractions 59 to 74 in Fig. 1) and Fr. II (fractions 75 to 110). As shown in Table 1, specificities of both fractions against animal erythrocytes were remarkably different from each other. Fr. I was highly active against horse erythrocytes and less active against chicken

erythrocytes. On the other hand, the highest activity of Fr. II was obtained with chicken erythrocytes. In addition, it was also noticeable that Fr. II was fairly less active against rabbit erythrocytes than Fr. I. Further purification of Fr. I was done on a Sephadex G-75 column. This step was effective to remove large molecules such as pigment proteins. In Hydroxyapatite column chromatography a single peak with activity appeared (Fig. 2). Gel filtration on Sephadex G-75 was adopted for the final purification step, in which GVA-1 was eluted as a symmetrical peak when detected either by the absorbance at 280 nm or by the agglutinating activity against horse erythrocytes (Fig. 3). The yields of protein and the agglutinat-

Fig. 1. DEAE-cellulose column chromatography of 70% saturated $(NH_4)_2SO_4$ precipitate. Column size, 2×35 cm. Fractions of 15 ml were collected at a flow rate of 75 ml/h. ●—●, absorbance at 280 nm; ○—○, hemagglutinin titer against horse erythrocytes; - · - · -, NaCl concentration in 0.01 M phosphate buffer (pH 7.5).

Fig. 2. Hydroxyapatite column chromatography of active fractions obtained in gel filtration on Sephadex G-75. Column size, 2×35 cm. Fractions of 15 ml were collected at a flow rate of 60 ml/h. ●—●, absorbance at 230 nm; ○—○, hemagglutinin titer against horse erythrocytes; - · - · -, concentration of phosphate buffer (pH 7.0).

Table 1. Purification summary of the *G. verrucosa* hemagglutinins

Purification step	Protein (mg)	Minimum active concentration (μg protein/ml)						
		Horse	Cow	Sheep	Rabbit	Guinea pig	Chicken	Carp
Crude extract	459	38	600	150	75	38	38	600
Salting-out	200	26						
DEAE-cellulose Fr. I	41	10		40	20	20	80	
Fr. II	37	46		116	925	116	14	
Sephadex G-75	11	2.3						
Hydroxyapatite	4.5	1.3						
Sephadex G-75	2.0	0.8		3.2	1.6	1.6	6.4

Fig. 3. Gel filtration on Sephadex G-75 of active fractions obtained in Hydroxyapatite column chromatography. Column size, 2×55 cm; solvent, 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). Fractions of 3 ml were collected at a flow rate of 30 ml/h. Symbols as in Fig. 1.

ing activity in each purification step are given in Table 1. Based on the minimum active concentration, a 47.5-fold purification was achieved.

Physicochemical properties of GVA-1

No inhibition on the activity of GVA-1 was observed with the following simple sugars: D-glucose, D-glucosamine, N-acetyl-D-glucosamine, D-galactose, N-acetyl-D-galactosamine, D-mannose, L-fucose, D-arabinose, L-arabinose, D-ribose, D-sorbitol, *D*-inositol, D-lactose and cellobiose. The same results were reported for other algal hemagglutinins.^{8,4,8} In general the activity of hemagglutinins is inhibited by such simple sugars as used in this study. Accordingly, algal hemagglutinins, unlike those from other sources, may commonly bind more complicated carbohydrates than simple sugars.

The purified hemagglutinin (GVA-1) was shown

to be homogeneous from the view-point of protein chemistry by the following criteria: chromatography on Sephadex G-75, analytical disc electrophoresis and electrofocusing. As shown in Fig. 4a, a single, though relatively broad, protein band, which was in good accord with the agglutinating activity against horse erythrocytes (Fig. 4c), was detected in analytical disc electrophoresis. However, it was simultaneously found by the sugar staining that the purified hemagglutinin was contaminated with a polysaccharide (Fig. 4b). Since GVA-1 was negative to the sugar staining, it seemed to be a simple protein or a glycoprotein with a low content of sugars. The purified hemagglutinin gave a single protein band in electrofocusing and its isoelectric point was estimated to be 4.8. The value was rather low compared with

Fig. 4. Analytical disc electrophoresis of the purified *G. verrucosa* hemagglutinin.

Gels were stained with Amido Black 10 B (a) or by the method of ZACHARIUS *et al.*¹¹ (b). An unstained gel was cut into 5 mm slices and each slice was used for the estimation of agglutinating activity against horse erythrocytes after extraction with 0.5 ml of buffered saline (c).

Fig. 5. Molecular weight determination of GVA-1 by gel filtration on Sephadex G-75 (a) and SDS disc electrophoresis (b).

those of other algal hemagglutinins.^{3,4)}

The molecular weight of GVA-1 was estimated to be 41,000 by gel filtration on Sephadex G-75 (Fig. 5a). This value was larger than those reported for *A. tenera*,³⁾ *C. purpureum*⁴⁾ and *S. maxima*⁵⁾ but smaller than that for *P. plumosa*.⁷⁾ On the other hand, SDS polyacrylamide gel electrophoresis showed that two kinds of subunits with molecular weights of 12,000 and 10,500 were present in GVA-1 (Fig. 5b). No distinct difference in the intensity of protein staining was observed between the both subunits, suggesting that the both subunits exist in GVA-1 in the identical ratio. Therefore, judging from the molecular weight of native GVA-1 determined by gel filtration, GVA-1 was considered to be a tetramer which was comprised of each two of two kinds of subunits.

Table 2. Amino acid composition of the purified *G. verrucosa* hemagglutinin (GVA-1)

Amino acid	Mole %	Amino acid	Mole %
Asp	8.4	Met	1.7
Thr	6.3	Ile	4.7
Ser	16.9	Leu	4.0
Glu	9.6	Tyr	3.3
Pro	4.8	Phe	3.2
Gly	14.5	Try	2.3
Ala	5.7	Lys	2.9
Val	6.2	His	1.4
Cys (half)	0.0	Arg	4.2

The amino acid composition of GVA-1 is listed in Table 2. GVA-1 was characterized by the high content of glycine, and acidic and hydroxy amino acids, and also by the low content of sulfur-containing and basic amino acids. The total amount of hydroxy amino acids, threonine and serine, was as much as 23.2% of the total amino acid residues. The low pI value of GVA-1 may be explained by

the fact that the content of acidic amino acids, aspartic acid and glutamic acid, is fairly larger than that of basic amino acids, lysine, histidine and arginine. The absence of half cystine or cysteine was also noteworthy; it indicated that disulfide bonding is not involved for the association of subunits.

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