MARINE BIOPOLYMERS WITH CELL SPECIFICITY—III—AGGLUTININS IN THE RED ALGA *CYSTOCLONIUM PURPUREUM*: ISOLATION AND CHARACTERIZATION

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ABSTRACT.—The red alga, *Cystoclonium purpureum*, contains agglutinins which selectively agglutinate mouse leukemia cells L5178Y but not L1210. It also agglutinated erythrocytes, lymphocytes, and a marine bacterium. The major component was a glycoprotein having a molecular weight of 12,500 daltons. It contained 5.6% carbohydrates and consisted of two subunits.

Agglutinins, which are capable of agglutinating erythrocytes or other types of cells, are widely found in nature. Some, particularly those found in the seeds of plants, have been isolated in the purified form and are now being widely used as research tools in cell biology, immunology, and cancer research (1–4). However, very little is known about the nature of agglutinins in seaweeds. In searching for agglutinins in the North Atlantic marine algae, we have found some species highly active against various kinds of animal erythrocytes and have recently isolated and characterized a unique agglutinin from the red alga, *Agardhiella tenera* (5). Another member of the Rhodophyta, *Cystoclonium purpureum*, which was previously reported to be negative against human erythrocytes (6) or active only by the sensitive method (7), was found to contain agglutinins. They can strongly agglutinate mouse leukemia cells, various kinds of animal erythrocytes, and lymphocytes. We report here the isolation and characterization of agglutinins in *C. purpureum*.

The activity of *Cystoclonium* agglutinins was fairly stable and unchanged over a wide range of pH values between 4 and 10. Activity was unaffected by heating at 50° for 30 min, but was reduced by heating at 60–90° for 30 min. No dependency upon metal ions such as Ca^{2+} and Mg^{2+} was recognized.

The *Cystoclonium* agglutinins were precipitated from the crude extract between 25 and 70% saturation of $(NH_4)_2SO_4$ and the precipitate was purified by gel-filtration with Sephadex G–100. The active fraction was further separated by DEAE–Sephadex column chromatography. The major active fraction, representing about 70% of the total activity, was apparently unadsorbed by a DEAE–Sephadex column. Two minor active components were eluted by a linear gradient method. The unadsorbed active fraction was then applied to preparative disc electrophoresis. The active fraction (CPA–1) was finally passed through a column of Bio-Gel P–2 and obtained in pure form as judged by analytical disc electrophoresis. In a typical run, 100 g of dried algal powder yielded 3.5 mg of CPA–1.

CPA–1 showed identical activity against various types of cells as the crude extract indicating CPA–1 represents the activity of *Cystoclonium* agglutinins. The minimum active concentration of purified agglutinin against various cells is listed in table 1. The most sensitive cells were guinea pig erythrocytes which were agglutinated at a concentration of 0.6 μg/ml. Moreover, it can distinguish between two different types of mouse leukemia cells; it reacted with L5178Y at a concentration of 30 μg/ml but not with L1210 even at a concentration of

TABLE 1. Minimum active concentration (μg/ml) of the *Cystoclonium* agglutinin.

Erythrocytes		Mouse Leukemia Cells	
Guinea pig	0.6	L5178Y	30
Rabbit	2.5	L1210	—[a]
Mouse	10	Marine bacteria	
Horse	80	*Microcyclus marinus*	80
Sheep	—	*Vibrio alginolyticus*	—[a]
Human A	40	*Pseudomonas* sp.	—[a]
B	40	Marine yeast	
O	20	*Metschnikowia reukaufii*	—[a]
Lymphocytes		Marine fungi	
Guinea pig	18	*Dendryphiella salina*	—[a]
Mouse	10	Common yeast	
		Candida albicans	—[a]

[a]Activity was checked at a concentration of 1000 μg/ml.

1000 μg/ml. *Agardhiella* agglutinins also showed similar selectivity (5), and it may be a common property of Rhodophyta agglutinins that they can distinguish L5178Y from L1210. Because of this interesting selectivity, agglutinins from red algae will be a useful tool in examining the differences of membrane structure between those two types of leukemia cells. CPA–1 also strongly agglutinated guinea pig and mouse lymphocytes but weakly agglutinated the marine bacterium *Microcyclus marinus*.

Although most lectin-type agglutinins are known to be inhibited by certain sugars such as galactose, glucose, and fucose, the *Cystoclonium* agglutinin was not affected by available common sugars. This unusual result may indicate that the carbohydrate binding site of *Cystoclonium* agglutinins is specific to a more extended structure than the simple sugars tested, or that this algal agglutinin acts by a completely different mechanism.

The molecular weight of CPA–1 was determined to be 12,500 daltons by gel-filtration with Sephadex G–75. In sodium dodecyl sulfonate (SDS) electrophoresis, a single band was detected between those of ribonuclease and insulin used as reference proteins, and calculated to be 6,000 daltons. The ratio of the subunit to the intact protein in molecular weight was close to 2. In *N*-terminal amino acid determination, two kinds of amino acids, lysine and alanine, were detected. These results indicate that CPA–1 consists of two subunits with equal molecular weight. It is noteworthy that the molecular weight of CPA–1 is comparable with that of *A. tenera* agglutinin, which is smaller than any agglu-

TABLE 2. Amino acid composition of CPA–1.

Amino acid	Mol.%	Amino acid	Mol.%
Asp	10.3	Met	1.4
Thr	7.1	Ile	2.9
Ser	14.0	Leu	3.5
Glu	6.3	Tyr	2.9
Pro	10.1	Phe	3.3
Gly	17.2	Try[a]	1.6
Ala	11.2	Lys	1.9
Cys (half)	0	His	0
		Arg	0

[a]Tryptophan was determined on the unhydrolyzed sample by the spectrometric method (10).

tinins reported from other sources (5). In isoelectric focusing, CPA–1 gave a single band and the isoelectric point was determined to be 5.6.

Neutral sugar content was estimated to be 5.6%. Gas chromatography analysis gave D-mannose as a major component. D-galactose was also detected, but its content was *ca* one-tenth of D-mannose. The amino acid composition was listed in table 2. Lectin-type agglutinins are usually high in acidic and hydroxy amino acids and low in half cystine or cysteine and methionine. CPA–1 showed a similar inclination; the content of the hydroxy amino acids, threonine and serine, was detected to be more than 20% of the total residue, and only a small amount of lysine was observed. Half cystine, arginine, and histidine were not detected.

EXPERIMENTAL[1]

COLLECTION OF SEAWEED.—The red alga, *Cystoclonium purpureum*, was collected at Conanicut Island, Rhode Island, in June, 1976, and 1977. The specimens were sun-dried or freeze-dried and ground to powder. The voucher specimen is kept at the Department of Pharmacognosy, College of Pharmacy, University of Rhode Island.

TEST FOR AGGLUTINATING ACTIVITY.—Agglutinating activity was determined as follows. A serial two-fold dilution of the test solution was made in a final volume of 50 µl saline in microtest plates. To each well was added an equal volume of one cell suspension. After allowing the mixture to stand at room temperature for 2 hr, the maximum titer value was read visually in the case of erythrocytes or microscopically for other types of cell. In the case of lymphocytes and leukemia cells, agglutination of more than 50% of the cells was considered to be a positive result. Agglutinating activity was expressed in terms of titer value or the minimum concentration of test solution required for agglutination. The bloods used were of human groups A, B, and O (Ortho Diagnostics, Inc.) guinea pig, sheep, horse (Grand Island Biological Co.), rabbit and mouse. Mouse and guinea pig lymphocytes were isolated from the spleen by use of the Ficoll-Hypaque technique (Pharmacia Fine Chemicals). Each lymphocyte suspension was made in RPMI 1640 (Grand Island Biological Co.) at a concentration of 5 x 10^6 cells/ml. Two types of mouse leukemia cells, L5178Y and L1210, in Fisher's medium containing 10% of horse serum were used. After washing, each cell suspension was prepared in saline at a concentration of 2 x 10^6 cells/ml. Marine bacteria, *Vibrio alginolyticus, Microcyclus marinus* ATCC No. 25205, and *Pseudomonas* sp., marine yeast *Metschnikowia reukaufii* and *Dendryhiella salina*, and a common yeast *Candida albicans* were suspended in sterilized sea water or saline.

ISOLATION OF AGGLUTININ.—Dried algal powder (100 g) was extracted with 1 liter of 0.05 M phosphate buffer, pH 7.0. The mixture was centrifuged at 10,000 rpm for 30 min. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant at 4° to give a final concentration of 25% saturation. After centrifugation, more solid $(NH_4)_2SO_4$ was slowly added to the supernatant to give a final concentration of 70% saturation. After keeping the supernatant at 4° overnight, it was centrifuged. The precipitate (390 mg) was dissolved in distilled water, dialyzed and lyophilized. The lyophilized sample was dissolved in 0.05 M phosphate buffer, pH 7.0, applied to a Sephadex G–100 column (4 x 95 cm), and eluted with the same buffer. The active fraction obtained was subjected to rechromatography on the same column. Active fractions were combined, dialyzed and lyophilized. The preparation (156 mg) was subjected to a DEAE-Sephadex column (2.5 x 45 cm) equilibrated with 0.01 M phosphate buffer. The column was eluted first with 400 ml of the buffer and subsequently by a linear gradient method between the buffer (750 ml) and the buffer containing 1.0 M NaCl (750 ml). The unadsorbed fraction (20 mg) was purified by preparative disc electrophoresis. About 2 mg of the sample was put onto each gel column (14 x 95 mm). After running for 4.5 hr at a constant current of 8 mA/tube, the sections corresponding to the active band (CPA–1) were extracted with 0.01 M phosphate buffered saline of pH 7.0. The preparation was applied to a Bio-Gel P–2 column (1 x 57 cm) equilibrated with glass distilled water. The active fractions were combined, dialyzed and lyophilized (3.5 mg).

ELECTROPHORESIS.—Analytical disc electrophoresis was performed on a 7% polyacrylamide gel (5 x 50 mm) using Tris-glycine buffer, pH 8.3 according to the method of Davis (8). Pre-

[1]The uv spectrum was taken on a Perkin-Elmer spectrophotometer Model 134. The amino acid composition was determined on a Durrum amino acid analyzer Model D–500. Gas chromatography was carried out on a Varian 1200 using a .1% OV–17 glass column. Polyacrylamide gels were stained with coomassie brilliant blue R–250 and were scanned with an ISCO gel scanner Model 1310 using a 540 nm filter.

parative disc electrophoresis was done under the same conditions using a bigger gel (14 x 95 mm). Sodium dodecyl sulfate (SDS) disc electrophoresis was carried out as described by Weber and Osborn (9) using 15% polyacrylamide gel (5 x 60 mm) and 0.1 M phosphate buffer, pH 7.2, containing 0.1% SDS. The preparation was treated with 1% SDS and 2% 2-mercaptoethanol at room temperature overnight. Bovine serum albumin (Mr=68,000, egg albumin (Mr=43,000), myoglobin (Mr=17,200) ribonuclease (Mr=13,600) and bovine insulin (Mr=6,000) were used as reference proteins. Electrofocusing on a 7% polyacrylamide gel (5 x 100 cm) was carried out at 200 V for 18 hr. Bio-Lyte 3/10 (Bio-Rad Laboratories) was used as carrier ampholite at a concentration of 2%.

CHEMICAL ANALYSIS OF CPA-1.—The amino acid composition was determined after the sample (200 μg) was hydrolized in an evacuated tube with 6 N HCl at 100°C for 24 hrs. In order to compensate for destruction by acid hydrolysis, serine and threonine were increased by 10 and 5% respectively. Tryptophane was estimated on the unhydrolyzed sample by the method of Goodwin and Morton (10). Neutral sugar content was measured by the anthrone method (11) using D-mannose as a standard. For analysis of the sugar composition a sample (500 μg) was hydrolyzed with 2 N H_2SO_4 at 100°C for 5 hr. After addition of $BaCO_3$ (100 mg) the reaction mixture was passed through anion and cation ion exchangers. The resultant neutral sugars were reduced with $NaBH_4$ to give alditols. The excess amount of $NaBH_4$ was consumed by adding a small volume of aqueous acetic acid. The mixture was dried and then acetylated with pyridine-acetic anhydride at 120° for 30 min (12). The resulting alditol acetates were subjected to gas chromatography. For N-terminal amino acid analysis, CPA-1 (100 μg) was dansylated according to the method of Gray (13). It was hydrolyzed with 1 N HCl in an evacuated tube for 18 hr at 105°C. The dansylated amino acid was identified by two dimensional thin-layer chromatography using precoated polyamide sheets (Schleicher and Schuell) with three different solvent systems: 1.5% formic acid in initial vector and benzene-acetic acid (9:1, v/v) and ethyl acetate-methanol-acetic acid (20:1:1, v/v), 90° vector, under a long wave ultraviolet lamp.

INHIBITION ASSAY.—The inhibition assay by simple sugars was carried out with the active fraction from a DEAE-Sephadex column. After incubation of 25 μl of saline solution containing 4 units of agglutinating activity and various amounts of simple sugars at room temperature for 2 hr, 50 μl of a 2% guinea pig erythrocytes was added to each mixture. The results were estimated after 2 hr incubation at room temperature. The following sugars were tested: D-glucose, D-glucosamine, D-glucose-1-phosphate, N-acetyl-D-glucosamine, D-galactose, D-galactosamine, N-acetyl-D-galactosamine, D-mannose, L-fucose, D-arabinose, L-arabinose, L-rhamnose, D-trehalose, D-xylose, D-ribose, D-sorbitol, D-maltose, D-fructose, i-inositol, D-lactose, N-acetylneuraminic acid, salicin, raffinose, and cellubiose.

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