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A MITOGENIC AGGLUTININ FROM THE RED ALGA *CARPOPELTIS FLABELLATA*

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Key Word Index—*Carpopeltis flabellata*; grateloupiaceae; agglutinin; mitogen; glycoprotein; isolation; sugar-binding specificity; biological activity.

Abstract—An agglutinin has been isolated from the marine red alga *Carpopeltis flabellata* by ammonium sulphate fractionation, affinity chromatography on a yeast mannan-Sepharose 4B column and gel permeation HPLC. This new protein, designated carnin, is a monomeric glycoprotein with a M_r of 25 000, and it contains large amounts of Gly and Asx. It strongly agglutinated untreated rabbit, mouse and horse erythrocytes, and very weakly untreated human erythrocytes, whereas it did not agglutinate untreated sheep and chicken erythrocytes. Treatment of the erythrocytes with trypsin affected their sensitivity to haemagglutination by the agglutinin. The haemagglutinating activity was inhibited only by glycoproteins with *N*-glycosidic sugar chains. The activity was not affected by divalent cations. Carnin also showed mitogenic activity for T lymphocytes from mouse spleen. It inhibited the normal embryonic development of marine invertebrates.

INTRODUCTION

The presence of agglutinins in marine algae was first demonstrated by Boyd *et al.* [1]. Since then, three groups have examined British, German and Japanese algae for haemagglutinins [2–6]. Out of 260 species ranging from green to red algae hitherto examined, *ca* 100 species have been found to possess haemagglutinating activity. Despite the many active species, agglutinins have been isolated and characterized only from nine species [7–15]. They are similar to agglutinins from terrestrial plants, except for a mucopolysaccharide isolated from the brown alga *Fucus vesiculosus* [12]. However, we found that agglutinins from seven Japanese algae are not only smaller in molecular size than terrestrial plant agglutinins but also have no affinity for monosaccharides [16]. We have isolated and characterized the glycoconjugate-specific agglutinins with low M_rs from the green alga *Boodlea coacta* [14] and the red alga *Hypnea japonica* [15], the major agglutinin from the latter species is a peptide with a very low M_r of 4200.

We have now isolated an agglutinin with a relatively low M_r, named carnin, from the red alga *Carpopeltis flabellata* (Holmes) Okamura. This paper describes its isolation and some of its properties.

RESULTS AND DISCUSSION

The agglutinin, carnin, of *C. flabellata* was quantitatively precipitated between 25 and 70% saturation with ammonium sulphate from a buffer extract. When the precipitate was placed on a column of yeast mannan coupled to Sepharose 4B, the adsorbed agglutinin was specifically eluted with 1 M D-mannose. The active fraction was purified by HPLC on a TSK G3000SW column, and showed one major and two minor peaks when monitored by absorption at 280 nm. However, the haemagglutinating activity was detected only in the major peak, and this was further purified by HPLC on the same column. The agglutinin thus obtained was homogeneous in SDS-polyacrylamide gel electrophoresis (PAGE). The yield of carnin was 4.3 mg of protein from 100 g dry material.

The M_r of carnin was estimated to be 25 000 from both gel filtration on a TSK G3000SW column and SDS-PAGE. It was positive to periodic acid-Schiff(PAS)-stainining after SDS-PAGE. Carnin is thus a small monomeric glycoprotein, similar to other agglutinins from marine algae [7, 8, 14, 15]. The amino acid composition of carnin (Table 1) shows that it is high in glycine and asparagine, as are other marine algal agglutinins [7, 9, 10, 14, 15] and terrestrial plant agglutinins [17–20].

Carnin strongly agglutinated untreated erythrocytes of rabbit, mouse and horse, and very weakly untreated ones of human A, B, O and AB groups irrespective of the blood group (Table 2). On the other hand, the agglutinin did not agglutinate untreated erythrocytes of chicken and sheep

Table 1. Amino acid composition of carnin

Amino acid	Mol %	Amino acid	Mol %
Asx	12.80	Ile	3.36
Thr	6.84	Leu	9.40
Ser	9.90	Tyr	1.56
Glx	8.03	Phe	1.82
Gly	12.98	Lys	4.50
Ala	6.08	His	3.21
Val	9.36	Arg	3.57
Cys	—*	Pro	3.08
Met	1.36	Trp	2.14

*Not detected.

Table 2. Minimum concentration of carnin giving a positive haemagglutination reaction

Erythrocyte	Minimum concentration of carnin (μg protein/ml) Untreated	Trypsin-treated
Rabbit	0.5	5.8×10^{-8}
Mouse	15.6	3.0×10^{-5}
Horse	7.8	7.8
Chicken	—	125.0
Sheep	—	—
Human A	500.0	—
B	500.0	—
O	500.0	—
AB	500.0	—

Bars indicate no activity at 500 μg protein/ml.

even at a concentration of 500 μg protein/ml. The haemagglutinating activity of the agglutinin was increased toward trypsin-treated erythrocytes of rabbit and mouse, whereas the activity was not changed toward trypsin-treated horse erythrocytes. Chicken erythrocytes were also agglutinated after trypsin treatment of the erythrocytes. However, the agglutinin showed no agglutination toward trypsin-treated erythrocytes of sheep and human A, B, O and AB groups at a concentration of 500 μg protein/ml.

Carnin was not inhibited by any of mono-, oligo- and polysaccharides tested (Table 3). Many other algal agglutinins [7–9, 14–16] are not inhibited by monosaccharides whereas terrestrial plant agglutinins are [18–20]. However, carnin was inhibited by glycoproteins such as yeast mannan, transferrin, fetuin and α_1-acid glycoprotein which have *N*-glycosidic sugar chains, as well as *B. coacta* [14] and *H. japonica* [15] agglutinins which we have previously reported. Yeast mannan was most inhibitory, which supported the previous result that the crude extract of *C. flabellata* agglutinated yeasts [16]. The binding nature to yeast mannan was also supported by the fact that the agglutinin adsorbed on a yeast mannan-Sepharose 4B column. The binding was reversible by 1 M D-mannose, in contrast to the result obtained by a haemagglutination-inhibition test. On the other hand, ovomucoid and ovalbumin were not inhibitory. Desialylated fetuin (asialofetuin) showed an inhibitory activity about 30 times more than fetuin. The glycopeptide fraction prepared from fetuin also inhibited the activity, suggesting that the sugar moiety was responsible for the inhibition. The *N*-glycosidic glycopeptide from yeast mannan also inhibited the activity. However, the agglutinin was not inhibited by mannobiose, mannotriose and mannotetraose prepared by acetolysis of the *N*-glycosidic glycopeptide, or by *N*,*N*′-diacetylchitobiose, the common constituent of *N*-glycosidic sugar chains. Since acetolysis cleaves only the α-(1 → 6)-linkage in the backbone of the yeast mannan, the mannooligoses tested were composed of α-(1 → 2)- and α-(1 → 3)-linkages in the side chains [21]. Therefore, carnin recognizes the mannotriose moiety at the core in the *N*-glycosidic sugar chain and/or α-(1 → 6)-linked polymannose. Thus, carnin appear to be a useful reagent for elucidation of complex carbohydrate structures.

Table 3. Inhibition of haemagglutinating activity of carnin by sugars and the related compounds

Sugars and related compounds	Minimum inhibitory concentration (μg/ml)
Monosaccharides*	–
Oligosaccharides*	–
Polysaccharides*	–
Glycoprotein	
Transferrin	250.0
Fetuin	250.0
α_1-Acid glycoprotein	1000.0
Ovomucoid	–
Asialofetuin	7.8
Yeast mannan	3.9
Ovalbumin	–
Glycopeptide-fraction from fetuin	+
N-Glycosidic glycopeptide from yeast mannan	+
Mannooligose from yeast mannan	
Mannobiose	–
Mannotriose	–
Mannotetraose	–

Trypsin-treated rabbit erythrocytes were used. The minimum inhibitory concentration is that required to inhibit completely the haemagglutinating activity with a titre of 4 of carnin. + and – indicate positive and lack of inhibition, respectively, at a concentration of 100 mM in the case of mono- and oligosaccharides, and at 2 mg/ml in the case of polysaccharides, glycoproteins and the other related compounds.

*Mono-, oligo- and polysaccharides tested are given in the Experimental.

Carnin showed mitogenic activity for splenic lymphocytes from BALB/c mice at a concentration of 10.5 μg protein/ml in the assay using a glucose consumption method. T cell-depleted cells prepared by treatment of the splenic lymphocytes with alloantiserum to Thy-1·2 and complement were not reactive for the blastogenic responses by the agglutinin. These results indicate that the mitogenic activity is specific for the T lymphocytes. This is the first example of a marine algal agglutinin showing mitogenic activity.

Carnin inhibited the normal embryonic development of the sea urchin *Hemicentrotus pulcherrimus* at the stage of blastula when exposed to a concentration of 10 μg protein/ml. The agglutinin also inhibited the gasturulation at a concentration of 5 μg protein/ml and induced the abnormal gastrula in the case of starfish *Asterina pectinifera*. On the other hand, of seven terrestrial agglutinins that were tested, only concanavalin A and wheat germ agglutinin inhibited the normal embryonic development of the sea urchin at concentrations of 10 and 1 μg/ml, respectively; development was stopped at the stage of blastula. Concanavalin A also inhibited the gasturulation of the starfish embryos at a concentration of 10 μg/ml. The inhibition may be triggered by binding of their agglutinins to an unknown receptor in the cell membranes, though they have different sugar-binding specificity from each other. The results also suggest that

carnin may play some role in an interaction between macroalgae and other microorganism of the marine ecosystem.

The haemagglutinating activity was not affected by heating at 30° for 30 min, but was reduced by heating at 40° for 30 min. The activity was retained at a pH range between 7 and 10, but decreased at below pH 6. The activity was not affected either by treatment with EDTA or divalent cations such as Ca^{2+} and Mg^{2+}.

EXPERIMENTAL

Materials. Specimens of *C. flabellata* were collected on the coast of Wakayama, Japan, in June. The specimens were transferred on dry-ice to the laboratory and the whole parts of the alga were freeze-dried and ground to a powder.

Isolation. A 100 g portion of the powdered alga was stirred overnight at 4° with 1 l. of 0.02 M phosphate buffer (pH 7) containing 0.85% NaCl (PBS). After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant to attain a satn of 25% and the mixture kept overnight at 4°. To the supernatant, more solid $(NH_4)_2SO_4$ was slowly added to give a satn of 70%. The mixture was kept overnight at 4° and centrifuged at 6000 rpm for 30 min. The ppt was dissolved in PBS, dialysed against PBS and subjected to affinity chromatography on a yeast mannan-Sepharose 4B column (1 × 10 cm) equilibrated with PBS; the affinity gel was prepared by coupling yeast mannan to CNBr-activated Sepharose 4B according to the Pharmacia manual. It contained 2.2 mg of yeast mannan coupled to 1 ml of the gel. The column was washed with PBS until the washings showed no absorption at 280 nm, then eluted with 1 M NaCl followed by 1 M D-mannose in PBS. The eluate was monitored by absorption at 280 nm and haemagglutinating activity. Active fractions were pooled, dialysed against distilled water and lyophilized. The lyophilized sample was dissolved in 0.05 M phosphate buffer (pH 7) containing 0.3 M NaCl and applied to gel permeation HPLC on a TSK G3000SW column (7.5 × 600 mm) equilibrated with the same buffer. The elution was carried out at a flow rate of 0.5 ml/min with the buffer, and the eluate was monitored by absorption at 280 nm and haemagglutinating activity. Active peak was further purified on the same HPLC column.

M_r *of carnin* was determined by HPLC on a TSK G3000SW column with 0.05 M phosphate buffer (pH 7) containing 0.3 M NaCl. Bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 25 000) and ribonuclease A (M_r 13 700) were used as standard proteins.

SDS-PAGE was carried out as described by Laemmli [22] using a 10% polyacrylamide gel. The preparation was treated with 2% SDS and 5% 2-mercaptoetanol at 100° for 5 min. Ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 25 000) and ribonuclease A (M_r 13 700) were used as standard proteins. The gel were stained with Coomassie Brilliant Blue R-250 for proteins and PAS reagent for carbohydrates by the method of Zacharius *et al.* [23].

Amino acid composition was determined on an amino acid analyser after hydrolysis of the sample in an evacuated tube with 6 N HCl at 110° for 24, 48 and 72 hr, respectively. The contents of Thr and Ser were obtained by extrapolation at zero time. The values of 72 hr-hydrolysis were adapted for Ile and Leu, while that of 24 hr-hydrolysis for Tyr. Average values for the three periods of hydrolysis were used for other amino acids. Cys or Cys | was determined by the performic acid method [24] and Trp by the thioglycol–HCl method [25].

Protein contents were measured by the method of Lowry *et al.* [26] using bovine serum albumin as a standard.

Haemagglutinating activity was determined by the method described in our previous paper [16]. Trypsin-treated erythrocytes were prepared as follows; to a 2% erythrocyte suspension, 0.5% trypsin in PBS was added and the mixture incubated at 37° for 1 hr. After washing × 3, a 2% trypsin-treated erythrocyte suspension was prepared in PBS.

Inhibition test was carried out with trypsin-treated rabbit erythrocytes according to the method described previously [16]. The sugars and glycoproteins used were; D-Glc, D-GlcN, D-GlcNAc, D-GlcA, Me-α-D-Glc, D-Gal, L-Gal, D-GalN, D-GalNAc, D-GalA, D-Man, D-ManN, Me-α-D-Man, D-Man-6-P, mannitol, inositol, L-Rha, L-Fuc, D-Xyl, D-Rib, 2-dD-Rib, D-Ara, L-Ara, NeuNAc, sucrose, maltose, lactose, β-gentibiose, raffinose, *N,N'*-diacetylchitobiose, gum arabic, laminarin, soluble starch, heparin, transferrin (human), fetuin (calf), asialofetuin (calf), α_1-acid glycoprotein (human), ovomucoid, ovalbumin and yeast mannan (*Saccharomyces cerevisiae*). A glycopeptide-fraction from fetuin was prepared according to the method of Spiro [27], as described previously [16]. The *N*-glycosidic glycopeptide from yeast mannan (*S. cerevisiae*) was prepared by β-elimination according to the method of Nakajima and Ballou [28], as described previously [14]. Mannobiose, mannotriose and mannotetraose were obtained by gel filtration of the acetolysis-products of the *N*-glycosidic glycopeptide, according to the method of Nakajima and Ballou [21]. These glycopeptides and mannooligoses were qualitatively examined at the concn of 2 mg/ml for inhibitory activity.

Mitogenic activity was determined by a modification of the glucose consumption method described by De Cock *et al.* [29]. Spleen cells were obtained from BALB/c mice in the same manner as described previously [30]. Finally, the cells were resuspended in Dulbecco's modified eagle medium supplemented with 10% foetal calf serum to give 2×10^7 cells/ml. One hundred μl of the cell suspension were placed into each well of a 96-well flat-bottomed culture plate and an equal volume of carnin (1.25–21.0 μg protein/ml in final concn) were added. The cultures were set up in triplicate and incubated at 37° in a 5% CO_2 incubator for 7 days. At 24 hr intervals, the glucose concn of supernatant in each well was estimated by the glucose oxidase method (glucose B test, Wako Pure Chem., Japan). The glucose consumption, as a indicator for blastogenesis of lymphocytes, was calculated from a following formula; glucose consumption (%) = [control culture (mg/dl) − stimulated culture (mg/dl)]/control culture (mg/dl) × 100. To obtain T cell-depleted spleen cells, on the other hand, spleen cells were treated with alloantiserum specific for Thy-1·2 (Cedalane Lab. Ltd., U.S.A.) and Low-tox-M rabbit complement (Cedarlane Lab. Ltd., U.S.A.). More than 95% of Thy-1·2 positive lymphocytes in the spleen cells were died by this treatment. The T cell-depleted cell suspension was used for a study of lymphocyte specificity in the carnin-induced blastogenesis.

Effect of carnin on the development of sea-urchin H. pulcherimus *and starfish* A. pectinifera *eggs* was examined according to the method of Ikegami [31]. The eggs were obtained as follows. The starfish ovaries were removed from the animal and washed by decanting with natural seawater. To the egg suspension was added a few drops of 10^{-5} M 1-methyladenine in saline solution. After germinal vesicle disappeared, the eggs were filtered through a gauze and suspended again in seawater, to which the sperm was added. In the case of the sea-urchin, eggs and sperm were collected from the animals by addition of 0.05 M KCl [32], and the sperm was added to the washed eggs. The fertilized eggs were pipetted into each sample well of a multi-dish that contained a test solution. The concn of the agglutinin after addition of eggs were adjusted to 100, 50, 20, 5 and 1 μg protein/ml. As references, seven agglutinins from terrestrial plant

sources [concanavalin A, peanut agglutinin, *Ricinus communis* agglutinin I, soybean agglutinin, *Dolichos biflorus* agglutinin, *Ulex europaeus* agglutinin I and wheat germ agglutinin (Vector Lab., Inc.)] were examined in a similar manner.

The effect of pH, *heat and divalent cations on haemagglutinating activity* was examined according to the method in our previous paper [15]. In this examination, trypsin-treated rabbit erythrocytes and PBS-soln of the agglutinin with a haemagglutination titre of 1024 were used.

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