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Gracilaria tikvahiae agglutinin. Partial purification and preliminary characterization of its carbohydrate specificity

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ABSTRACT

A potent agglutinin of rabbit and sheep red blood cells, obtained from the red alga *Gracilaria tikvahiae*, was purified by ammonium sulfate fractionation, ion exchange, gel filtration, and hydroxylapatite chromatography. Human A and B blood group erythrocytes were also agglutinated, whereas human O blood group erythrocytes were not agglutinated. The hemagglutination titer was not significantly affected by the addition of EDTA or the divalent cations Ca^{2+}, Mg^{2+}, or Mn^{2+}. The carbohydrate specificity was characterized by hemagglutination inhibition using various monosaccharides, glycoproteins, and glycopeptides. The results suggested that the agglutinin has affinity for *N*-acetylneuraminic acid as well as glycoconjugates containing *N*-acetylneuraminic acid.

INTRODUCTION

Lectins represent a large group of soluble and membrane-associated proteins which bind carbohydrates[1]. The binding of lectins to carbohydrates is reversible and, in many instances, can be inhibited by simple sugars. Because of their polyvalency with respect to carbohydrate binding, these proteins possess the ability to precipitate glycoconjugates and agglutinate a wide range of cells, such as animal erythrocytes. As a result, lectins have been employed to identify and purify glycoconjugates and aid in the elucidation of their glycan structures[2–5].

Lectins are widely distributed throughout higher plants and vertebrates, yet little information is available concerning similar proteins in lower plants such as marine macroalgae. Although many species of marine algae have been screened for the presence of hemagglutinins, the carbohydrate-binding specificity of these agglutinins is generally unknown[6–8]. Thus, only a small number of lectin or "lectin-like" proteins of known carbohydrate-binding specificity have been characterized from marine algae[9]. We describe herein the partial purification of an agglutinin from the red alga *Gracilaria tikvahiae*. The agglutination of animal erythrocytes is inhibited by *N*-acetylneuraminic acid and glycoconjugates containing *N*-acetylneuraminic acid.

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EXPERIMENTAL

Materials. — Diethylaminoethyl (DEAE)-trisacryl M was obtained from IBF Biotechnics (Fisher Scientific, Orlando, FL), Sephadex G-150 superfine from Pharmacia (Piscataway, NJ), and hydroxylapatite from Bio-Rad (Richmond, CA). Pronase E (Type XXV), electrophoresis reagents, monosaccharides, and glycoproteins were obtained from Sigma (St Louis, MO). Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline solution (PBS) was obtained from Gibco (Grand Island, NY). Rabbit and sheep erythrocytes were obtained from Colorado Serum Co. (Denver, CO), and human erythrocytes from Central Florida Blood Bank (Orlando, FL). *Gracilaria tikvahiae* Strain G-5 was obtained from outdoor culture tanks maintained at the Harbor Branch Oceanographic Institution and grown under nitrogen-supplemented culture conditions as described[10]. Glycopeptides were prepared from lactoferrin and fetuin by treatment with Pronase[11–13].

Hemagglutination assays and related methods. — The agglutinin was assayed in the presence and absence of monosaccharides and glycoproteins as described[8]. Rabbit erythrocytes were routinely used for the hemagglutinin assays and the results are presented as the minimum concentration (μg of protein/mL) of agglutinin which produced hemagglutination. Carbohydrate content was determined by the phenol–H_2SO_4 method[14] and protein concentration as described[15]. Poly(acrylamide) gel electrophoresis was performed according to the Laemmli method as modified[16]. Albumin (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000) were used as mol. wt. standards to determine M_r of the polypeptides associated with *G. tikvahiae* agglutinin activity.

Preparation of soluble protein extract. — All procedures were conducted at 4°. *G. tikvahiae* was harvested, cleaned, and rinsed with distilled water (dH_2O). The plant material ($\sim$ 1.1 kg) was homogenized in a Waring Blendor (1 min) containing 10mM Tris (1 L, pH 7.4), frozen, and then lyophilized. The dried algal material was resuspended in TBS [10mM Tris, 150mM NaCl, pH 7.4; 20 vols. (v/w)] and mixed for 18 h. After centrifugation (10 000 *g* for 20 min), the supernatant solution was filtered under vacuum (Whatman No. 4) and solid $(NH_4)_2SO_4$ was added to yield a 25% (w/v) solution. After 4 h, the insoluble material was removed by centrifugation at 10 000 *g* for 20 min, and the supernatant solution recovered and adjusted to a concentration of 75% (w/v) with $(NH_4)_2SO_4$. The mixture was stirred for 18 h and then centrifuged (10 000 *g*, 30 min). The pellet was resuspended in Buffer *A* (10mM Tris, pH 8.0) and extensively dialyzed against Buffer *A*. The insoluble material was subsequently removed by vacuum filtration (Gelman GF/A filter).

Partial purification of the G. tikvahiae *agglutinin.* — The concentration of the soluble protein extract, prepared as described above, was adjusted to 2.4 mg of protein/mL and applied to a DEAE-trisacryl M column (2.7 × 35 cm) at a flow rate of 35 mL·h^{-1} (Fig. 1). The column was washed with Buffer *A* (400 mL) and then eluted with a linear gradient of 1mM (800 mL) to 1M (800 mL) NaCl in Buffer *A*. Fractions were collected and assayed for hemagglutinin activity. The active fractions were pooled

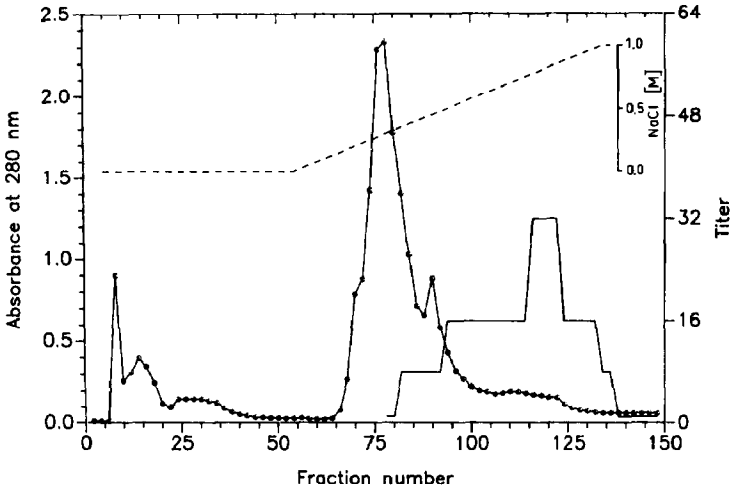

Fig. 1. DEAE-trisacryl M chromatography of the ammonium sulfate-fractionated protein extract. Protein elution was monitored by absorbance at 280 nm (-o-o-) before and during application of a NaCl gradient (---); ~ 60μL from each fraction (18 mL) was assayed for hemagglutination activity and is represented as titer (—).

(80–137), concentrated, and dialyzed against PBS. The active material (8 mL) was then loaded onto a Sephadex G-150 column (1.6 × 86 cm), equilibrated with PBS at a flow rate of 2 mL·hh^{-1} (Fig. 2). Fractions containing activity (20–29) were pooled, dialyzed against Buffer *B* (20mM K phosphate, pH 7.4) and applied to a hydroxylapatite column (1.6 × 20 cm) equilibrated with Buffer *B* (Fig. 3). The column was washed with Buffer *B* (400 mL) and the agglutinin eluted with a linear gradient of 20mM (150 mL) to 500mM (150 mL) K phosphate (pH 7.4) at a flow rate of 25 mL·h^{-1}. Active fractions (46–58) were pooled, concentrated, and stored at −70°.

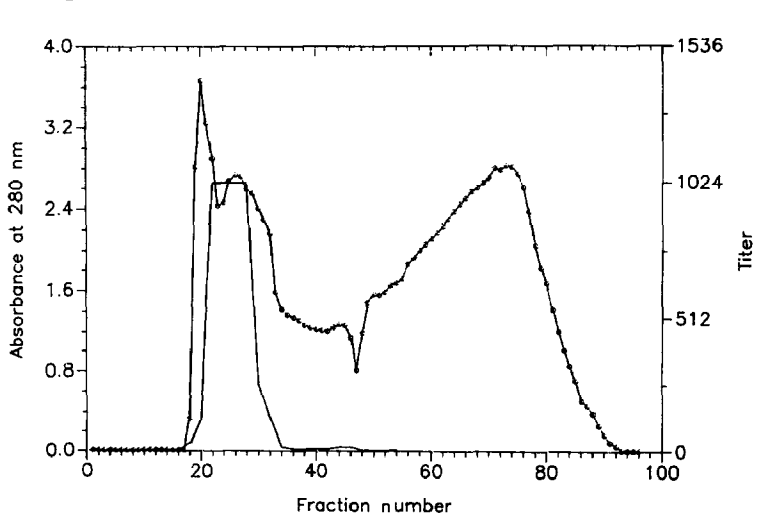

Fig. 2. Sephadex G-150 (superfine) chromatography of *G. tikvahiae* agglutinin activity. Protein elution was monitored by absorbance at 280 nm (-o-o-) and 10 μL from each fraction (1.5 mL) was assayed for hemagglutination activity and is represented as titer (—).

RESULTS AND DISCUSSION

Partial purification of the G. tikvahiae *agglutinin.* — Data representing the recovery of *G. tikvahiae* agglutinin during purification are presented in Table I. By use of a semiquantitative hemagglutination assay, which employed rabbit erythrocytes, the specific activity of the *G. tikvahiae* agglutinin was increased to ~3.6 hemaglutinin units/μg of protein with an overall recovery of 1.1%, and ~710 μg of protein was recovered after hydroxylapatite chromatography (Fig. 3). The polypeptides associated with agglutinin activity were analyzed by poly(acrylamide) gel electrophoresis. Two major, Coomassie Blue-stained bands were observed corresponding to M_r 29 700 and

TABLE I

Recovery of *G. tikvahiae* agglutinin activity during purification

Fraction	*Protein*	*Activity recovered* [a]			*Purification factor*
	(mg)	*(Units)*	*(Units/mg)*	*(%)*	*(-fold)*
Ammonium sulfate fractionation (25–75%)	422	239 773	568	100	1
DEAE-trisacryl[b]	160	145 454	909	61	1.6
Sephadex G-150[b]	15.3	21 571	1410	9	2.5
Hydroxylapatite[b]	0.71	2578	3631	1.1	6.4

[a] One hemagglutinin unit (H.U) is defined as the minimum concentration of protein (μg/mL) that produced agglutination after 1 h. [b] After each chromatographic step, the active fractions were pooled and the hemagglutination titer determined as described in the Experimental section.

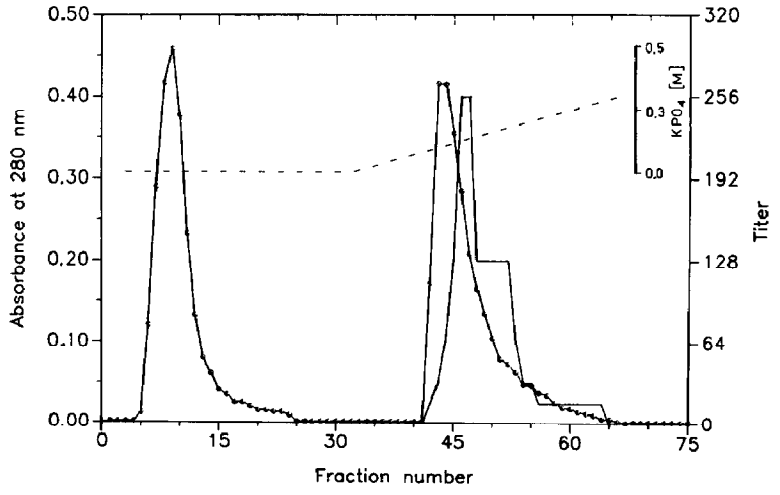

Fig. 3. Hydroxylapatite chromatography of *G. tikvahiae* agglutinin activity. Protein elution was determined by absorbance at 280 nm (-o-o-) before and during application of a K phosphate gradient (---); ~10μL from each fraction (4 mL) was assayed for hemagglutination activity and is represented as titer (—).

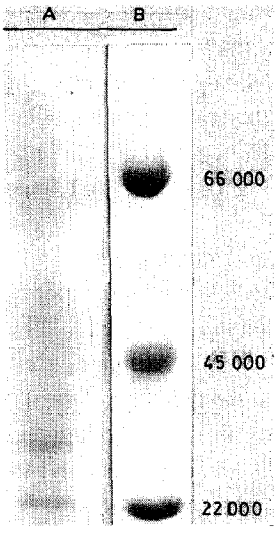

Fig. 4. Poly(acrylamide) gel electrophoresis of the *G. tikvahiae* agglutinin. The agglutinin (10 μg) obtained after hydroxylapatite chromatography was applied to a denaturing 7.5% discontinuous poly(acrylamide) gel (Lane A). The molecular-weight markers, albumin, ovalbumin, and carbonic anhydrase, are shown in Lane B.

24 900 (Fig. 4, Lane A). In contrast, the elution profile of the *G. tikvahiae* agglutinin after gel filtration suggested a molecular size in excess of 150 000 (Fig. 2); however, it is unclear whether this represents a native or an aggregated form of one or both M_r 29 700 and 24 900 polypeptides.

Physiochemical properties. — A significant lose of activity occurred during purification and storage of the agglutinin at 4.0°; this may account for the small increase (6.4-fold) in specific activity after ammonium sulfate fractionation. Storage of the protein preparation at −70° with repeated freeze–thawing did not reduce the hemagglutination titer. The *G. tikvahiae* agglutinin strongly agglutinated rabbit and sheep

TABLE II

Agglutination of erythrocytes by the *G. tikvahiae* agglutinin

Erythrocyte	*Minimum concentration*[a] (μg/mL)
Human type	
A blood group	8.5
B blood group	4.3
O blood group	[b]
Animal type	
Rabbit	0.2
Sheep	4.3

[a] Concentration of protein required to produce agglutination. [b] No observable agglutination at concentration ranging from 136 to 4.3 μg/mL.

red blood cells at a concentration of 230 ng and 4.3 μg of protein/mL, respectively (Table II). Human A and B blood group erythrocytes were agglutinated at a concentration of 8.5 and 4.3 μg of protein/mL, respectively; the *G. tikvahiae* agglutinin was not active at the concentrations tested for human O blood group erythrocytes. Ethylenediaminetetraacetic acid (EDTA) or the divalent cations Ca^{2+}, Mg^{2+}, or Mn^{2+} did not significantly affect hemagglutinin titer at concentrations ranging from 0.1 to 1.0mM (data not shown).

Hemagglutinin inhibition assays. — The specificity of the *G. tikvahiae* agglutinin was characterized initially by carbohydrate inhibition analysis. To this end, a number of monosaccharides were examined as potential inhibitors of hemagglutination. The results and minimum concentrations required for complete inhibition of hemagglutination are presented in Table III. Within the concentrations tested (320 to 2.4mM), most of the monosaccharides were not effective inhibitors (*i.e.*, L-fucose, D-galactose, D-glucose, D-mannose, D-glucuronic acid, D-galacturonic acid, D-xylose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-mannosamine); however, *N*-acetylneuraminic acid and *N*-acetylneuraminic acid-(2→3)-lactose were inhibitory at a concentration of 80 and 10mM, respectively. Sialidase-treatment of rabbit erythrocytes reduced the agglutination titer by 75% in comparison to untreated erythrocytes (data not shown). As indicated above, the material obtained after Sephadex G-150 chromatography was examined for binding to a variety of monosaccharide-Sepharose columns.

TABLE III

Inhibition of the *G. tikvahiae* agglutinin by various monosaccharides and glycoproteins

Inhibitors	*Minimum concentration (μg/mL)*
Monosaccharide	
N-Acetylneuraminic acid	80[a]
Glycoprotein	
α-Acid glycoprotein	333
Carbonic anhydrase	[b]
Asialofetuin	168
Fetuin	84
Lactoferrin	1.7
Ovalbumin	[b]
Ovomucoid	700
Ribonuclease B	[b]
Asialo bovine submaxillary mucin	1000
Bovine submaxillary mucin	42
Human transferrin	666
Glycopeptides	
Fetuin glycopeptide A	490
glycopeptide B	>>236[c]
Lactoferrin glycopeptide I	14
glycopeptide II	44

[a] Concentration in mM. [b] No inhibition at a concentration of 2 mg of protein/mL. [c] No inhibition was observed at this maximum concentration.

The major part of the hemagglutinin activity did not bind D-mannose-, L-fucose-, D-xylose-, or D-galactose-Sepharose, as judged by the quantitative recovery of hemagglutinin units in the unbound column fractions. A significant amount of activity irreversibly bound to sialic acid-Sepharose; (data not shown), however, because only 15% of the applied hemagglutinin activity was recovered, sialic acid-Sepharose could not be employed for purification of the agglutinin.

In view of these findings, we investigated whether sialo- or asialo-glycoconjugates inhibited the *G. tikvahiae* agglutinin (Table III). Initially, the nonglycosylated protein, carbonic anhydrase, and glycoproteins devoid of sialic acid, such as ovomucoid, ovalbumin, and ribonuclease B, were examined. Although ovomucoid was inhibitory at a concentration >700 μg of protein/mL, the remaining asialoglycoproteins did not inhibit hemagglutination. In contrast, there was a strong correlation between inhibition of the *G. tikvahiae* agglutinin and glycoproteins containing terminal *N*-acetylneuraminyl groups. Although the minimum concentration of protein required to produce inhibition varied, each sialoglycoprotein examined was inhibitory. α_1-Acid glycoprotein[17] and human transferrin[18] were the least effective inhibitors, whereas bovine submaxillary mucin[19] and lactoferrin[20] were the most potent inhibitors at a concentration of 42 and 1.7 $\mu g \cdot mL^{-1}$, respectively.

The inhibition by submaxillary mucin is similar to that observed for other sialic acid-binding lectins. For example, wheat germ agglutinin (WGA) was inhibited by a variety of different submaxillary mucins at concentrations ranging from 0.32 to 0.65μM. The strong inhibition by mucins, with respect to sialic acid-binding lectins, has been attributed to their high content of clustered *O*-glycosyl-linked sialyloligosaccharides. On the other hand, lactoferrin, which was the strongest sialoglycoprotein inhibitor, contains both high-mannose and complex-type oligosaccharides[13,17,20].

To determine whether the oligosaccharide portion of the sialoglycoproteins was inhibitory in absence of an intact polypeptide, glycopeptides obtained from lactoferrin and fetuin were examined for hemagglutination inhibition. Both lactoferrin sialoglycopeptides I and II were inhibitory at a concentration of 14 and 44 $\mu g \cdot mL^{-1}$; however, it was not possible to compare the extent of inhibition by these glycopeptides with the parent lactoferrin because their molecular weights are unknown[20]. Fetuin sialoglycopeptide A was inhibitory at a concentration of 490 $ng \cdot mL^{-1}$, whereas glycopeptide B produced no detectable inhibition.

Removal of *N*-acetylneuraminyl groups from fetuin and submaxillary mucin by acid hydrolysis increased the concentration of glycoprotein required to produce a similar inhibition by 2- and 24-fold, respectively. If the *G. tikvahiae* agglutinin only recognizes *N*-acetylneuraminic acid, then a total loss of inhibition by the desialylated glycoproteins would be expected. Because this was not observed, the possibility was raised that the *G. tikvahiae* agglutinin may recognize additional oligosaccharide sugar determinants, rather than just *N*-acetylneuraminic acid (*i.e.*, assuming that the desialylation by acid hydrolysis was complete). If additional glycan determinants are involved, this may explain the inhibition seen with ovomucoid as well as the high inhibitory concentration required for *N*-acetylneuraminic acid.

In summary, the data presented herein suggested that the interaction between cell surface glycoconjugates and the *G. tikvahiae* agglutinin is mediated, in part, by *N*-acetylneuraminic acid. In addition, it is possible that other sugars, perhaps internal oligosaccharide hexosamine units, may be involved. Although the limited carbohyrate-inhibition analysis of the *G. tikvahiae* agglutinin suggested a role for *N*-acetylneuraminic acid, futher studies are needed to define precisely the carbohydrate specificity.

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