Purification and Properties of a Lectin in the Skin Mucus of the Dragonet *Repomucenus richardsonii*

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A lectin in the skin mucus of the dragonet *Repomucenus richardsonii* was purified to an electrophoretically pure state by affinity chromatography on Sepharose 4B and HPLC on Chemcosorb 300-7CM. The purified lectin was an acidic glycoprotein with an isoelectric point of 6.1. Its molecular weight was determined to be 48000 by HPLC on TSK-gel G3000SW or 12000 by SDS electrophoresis, suggesting that it exists as a tetramer of the same subunit. The amino acid composition was characterized by the abundance of glycine, acidic amino acids and hydroxy amino acids and the absence of sulfur-containing amino acids. Inhibition tests revealed that the purified lectin has high affinity for D-galactose related sugars, especially lactose, and two glycoproteins (asialofetuin and mucin).

Lectins, sugar-binding proteins or glycoproteins, have so far been found in various biological sources, mainly terrestrial plants. Because of their unique biological activities such as cell agglutination and mitogenic activity, they are frequently utilized as pharmacological reagents. The fish skin mucus also seems to be a rich source of lectins; lectins have been detected in the skin mucus of the catfish *Tachysurus australinus*,¹⁾ the Pacific hagfish *Eptatretus stoutii*,²⁾ the window-pane flounder *Lophopsetta maculata*,³⁾ the member of Ophidiidae *Genypterus blacodes*,⁴⁾ the Japanese eel *Anguilla japonica*,⁵⁾ and the Arabian Gulf catfish *Arius thalassinus*.⁶⁾

Recently, a wide distribution of lectins in the fish skin mucus was confirmed by our survey, in which 23 of 33 fish were shown to contain lectins in their skin mucus.⁷⁾ Furthermore, a galactose-binding lectin in the skin mucus of the conger eel *Conger myriaster* has already been purified and characterized.⁸⁾ Independently, properties of the conger eel lectin were also reported by Kamiya *et al.*⁹⁾ In this communication we describe the purification and properties of a lectin in the skin mucus of another positive species, the dragonet *Repomucenus richardsonii*.

Materials and Methods

Fish

Specimens of *R. richardsonii* were collected in

shallow waters of Lake Hamana and immediately frozen. They were transported frozen to our laboratory and stored at -20°C until use.

Extraction

The skin mucus was collected from half-thawed specimens by scraping with a spatula, and homogenized in 5 volumes of 0.01 M phosphate buffer (pH 6.0). The homogenate was centrifuged at 15000 rpm for 20 min and the supernatant obtained was used as crude extract.

Purification Procedure

The crude extract was applied to a Sepharose 4B column (Pharmacia Chem. Co., 2×55 cm) equilibrated with 0.01 M phosphate buffer (pH 6.0). The column was first eluted with the same buffer until the absorbance at 280 nm of the eluate was back to the background level, and then with 0.1 M lactose in the buffer. Fractions of 4 ml were collected at a flow rate of 15 ml/h. Active fractions were combined and subjected to high performance liquid chromatography (HPLC) on a Chemcosorb 300-7CM column (Chemco Sci. Co., 0.46×25 cm) equilibrated with 0.01 M phosphate buffer (pH 6.0). After washing with the buffer, the column was eluted by a linear gradient of NaCl (0.0–1.0 M) in the buffer, which was terminated in 15 min. The flow rate was maintained at 1 ml/min. Lectin-containing fractions were manually collected, dialyzed against distilled water and

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lyophilized.

Assay of Hemagglutinating Activity

Only rabbit erythrocytes were used for assay of hemagglutinating activity since they were most sensitive to the crude extract.⁷⁾ Hemagglutinating activity was determined by the method of Shiomi *et al.*¹⁰⁾ and expressed in terms of minimum active concentration ($\mu\text{g protein/ml}$) required for positive agglutination or in terms of titer value, the reciprocal of the highest dilution of sample giving positive agglutination.

Inhibition Assay by Sugars and Glycoproteins

Inhibition tests were performed according to the method of Shiomi *et al.*¹⁰⁾ A mixture of sample (titer 4) and sugar was assayed for hemagglutinating activity after being kept at 4°C for 1 h. The following sugars were used: D-galactose, D-galactosamine, N-acetyl-D-galactosamine, lactose, melibiose, raffinose, stachyose, D-glucose, D-glucosamine, N-acetyl-D-glucosamine, D-glucuronic acid, maltose, cellobiose, sucrose, D-arabinose, L-arabinose, D-fructose, D-fucose, L-fucose, D-mannose, inositol and sorbitol. N-Acetyl-D-galactosamine, D-fucose and D-glucuronic acid were purchased from Aldrich Chem. Co., and the other sugars from Wako Pure Chem. Ind. The following glycoproteins were also used: fetuin (type III, from fetal calf serum), asialofetuin (type I, from fetal calf serum), mucin (type I, from bovine submaxillary glands), orosomucoid (α_1 -acid glycoprotein, from human cohn fraction VI), ovalbumin (gel filtration calibration kit) and ovomucoid (trypsin inhibitor, type III-0, from chicken egg white). All glycoproteins were obtained from the Sigma Chem. Co., except ovalbumin from the Pharmacia Chem. Co.

Protein Determination

Protein was determined by the method of Lowry *et al.*,¹¹⁾ using bovine serum albumin as a standard.

Analytical Electrophoresis

Analytical disc electrophoresis was performed as described by Davis¹²⁾ using a 7% polyacrylamide gel and Tris-glycine buffer (pH 8.3). After running, the gel was stained for proteins with Coomassie Brilliant Blue R-250 or for sugars with periodic acid-Schiff (PAS) reagent according to the method of Zacharius *et al.*¹³⁾

Molecular Weight Determination

The molecular weight of the purified lectin was

determined either by gel filtration or by sodium dodecyl sulfate (SDS) disc electrophoresis. Gel filtration was carried out by HPLC on a TSK-gel G3000SW column (Tosoh Co., 0.75 $\times$ 30 cm) with 0.2 M phosphate buffer (pH 6.8). The flow rate was maintained at 1 ml/min and the eluate monitored by absorbance at 280 nm. Bovine serum albumin (MW 67000), ovalbumin (MW 43000), ribonuclease A (MW 13700) and cytochrome c (MW 12500) were used to calibrate the column. SDS disc electrophoresis was performed as described by Weber and Osborn¹⁴⁾ using a 10% polyacrylamide gel and 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. After running, the gel was stained with Coomassie Brilliant Blue R-250. The reference proteins used were bovine serum albumin, ovalbumin, chymotrypsinogen A (MW 25000) and ribonuclease A.

Electrofocusing

Electrofocusing on a 7.5% polyacrylamide gel was carried out as described in our previous paper.⁸⁾ Colored pI marker proteins (Oriental Yeast Co.) were used to know the pH gradient formed.

Amino Acid Analysis

The purified lectin (100 μg) was hydrolyzed with 50 μl of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole in an evacuated tube at 110°C for 24 h. After neutralization with 3.5 N NaOH, the hydrolysate was applied to a Hitachi 835 amino acid analyzer.

Results and Discussion

Purification

Fig. 1 shows the elution profile of the crude extract from a Sepharose 4B column. Although almost all proteins contained in the crude extract were eluted with 0.01 M phosphate buffer (pH 6.0), no hemagglutinating activity was detected in the buffer eluate. Further elution with 0.1 M lactose in the buffer afforded a small protein peak with hemagglutinating activity, suggesting that the *R. richardsonii* lectin specifically binds to Sepharose 4B. When the active fraction was then subjected to HPLC on Chemcosorb 300-7CM, the lectin was separated from inactive proteins and eluted in a symmetrical protein peak (Fig. 2). The final preparation thus obtained was homogeneous as judged by analytical disc electrophoresis (Fig. 3). The purified lectin was designated as richardsonin. In a typical run, 0.5 mg of rich-

Fig. 1. Affinity chromatography of the *R. richardsonii* lectin.

The crude extract was applied to a Sepharose 4B column (2×55 cm) equilibrated with 0.01 M phosphate buffer (pH 6.0). After washing with the buffer, the column was eluted with 0.1 M lactose in the buffer (indicated by an arrow). Fractions of 4 ml were collected at a flow rate of 15 ml/h. A bar represents the lectin fraction.

Fig. 2. HPLC of the *R. richardsonii* lectin.

The lectin fraction obtained by affinity chromatography (Fig. 1) was subjected to HPLC using a Chemcosorb 300-7CM column (0.46×25 cm) equilibrated with 0.01 M phosphate buffer (pH 6.0). After washing with the same buffer, the column was eluted at a flow rate of 1 ml/min by a linear gradient of 0–1.0 M NaCl in the buffer. A bar represents the lectin fraction.

ardsonin was obtained from the skin mucus of 16 specimens. The minimum agglutinating concentration of richardsonin against rabbit erythrocytes was estimated to be 0.14 µg/ml.

Affinity chromatography on Sepharose 4B was very effective in purifying richardsonin. Previously epoxy-activated Sepharose 4B, to which D-galactose was coupled, was used as an adsorbent for the purification of the conger eel lectin.⁸⁾ As described below, richardsonin, like the conger eel lectin, binds to D-galactose related sugars. However, purification of richardsonin by affinity

Fig. 3. Analytical disc electrophoresis of the purified lectin.

After running on a 7% polyacrylamide gel with Tris-glycine buffer (pH 8.3), the lectin was visualized with Coomassie Brilliant Blue R-250.

chromatography on epoxy-activated Sepharose 4B, to which D-galactose or lactose was coupled, was unsuccessful. When the crude extract was applied to an epoxy-activated Sepharose 4B column, richardsonin was nearly passed through the column due to the non-specific binding of a large amounts of inactive proteins to the column.

Physicochemical Properties

Richardsonin was heat labile; its hemagglutinating activity was reduced to one-half by heating at 60°C for 10 min and completely lost by heating at 70°C for 10 min. Since the activity was neither reduced nor enhanced both upon dialysis against 50 mM EDTA and in the presence of 10 mM divalent cations (Ba^{2+} , Ca^{2+} , Co^{2+} , Mg^{2+} , and Mn^{2+}), the lectin appeared to be independent of divalent cations for its activity.

Richardsonin was positive to PAS staining, suggesting that it is a glycoprotein, like the ophidiid fish lectin⁴⁾ but unlike the windowpane flounder⁸⁾ and conger eel^{8,9)} lectins. It gave only one symmetrical peak in HPLC on TSK-gel G3000SW and gave a single band in SDS disc electrophoresis, supporting again its homogeneity. The molecular weight was determined to be 48000 by HPLC and 12000 by SDS electrophoresis, which indicated that richardsonin exists as a tetramer of the same

Table 1. Amino acid composition of richardsonin

Amino acid	Mol %	Amino acid	Mol %
Asp	9.9	Met	0.0
Thr	7.1	Ile	5.6
Ser	9.7	Leu	5.5
Glu	12.5	Tyr	5.4
Pro	5.4	Trp	0.5
Gly	13.2	Phe	3.9
Ala	5.4	Lys	5.4
Val	5.4	His	2.5
Cys	0.0	Arg	2.8

Table 2. Inhibition of hemagglutinating activity of richardsonin by sugars and glycoproteins

Sugars and glycoproteins	Minimum inhibitory concentration*
Sugars	
D-Galactose	25 (mM)
D-Galactosamine	50
N-Acetyl-D-galactosamine	100
Lactose	2.5
Melibiose	25
Raffinose	25
Stachyose	10
Glycoproteins	
Fetuin	>1 (mg/ml)
Asialofetuin	0.1
Mucin	0.75
Orosomucoid	>1
Ovalbumin	>1
Ovomucoid	>1

* The minimum inhibitory concentration is that required to inhibit completely the agglutinating activity (titer 4) against rabbit erythrocytes. The sugars showing no inhibition at 100 mM are not listed.

subunit with a molecular weight of 12000. Such a subunit structure is known for the windowpane flounder,³⁾ ophidiid fish⁴⁾ and conger eel^{8,9)} lectins. The isoelectric point of richardsonin was estimated to be 6.1 by electrofocusing. Differing from the windowpane flounder³⁾ and conger eel^{8,9)} lectins, richardsonin afforded a single band in electrofocusing, suggesting the absence of isolectins. The amino acid composition is summarized in Table 1. It was characterized by the abundance of acidic amino acids (aspartic acid and glutamic acid), hydroxy amino acids (threonine and serine) and glycine but the absence of sulfur-containing amino acids (half-cystine and methionine). The absence of half-cystine implies that the disulfide bonding is not involved in the association of subunits.

Sugar Inhibition

As shown in Table 2, the hemagglutinating activity of richardsonin was inhibited by D-galactose, its derivatives (D-galactosamine and N-acetyl-D-galactosamine) and its containing sugars (lactose, melibiose, raffinose and stachyose); lactose was the most potent inhibitor. These results are consistent with those obtained with the crude extract.⁷⁾ Although none of the glycoproteins tested showed any inhibitory effect on the crude extract,⁷⁾ the purified lectin was inhibited by two glycoproteins, asialofetuin and mucin.

In our previous paper,⁷⁾ the lectins in the skin mucus of eight fish species were tested for their sugar specificity. As a result, as many as seven lectins were found to bind to D-galactose and its related sugars. The Arabian Gulf catfish lectin was also reported to be inhibited by D-galactose related sugars.⁶⁾ Therefore, it is likely that many lectins in the fish skin mucus are highly specific for D-galactose related sugars. Richardsonin is obviously one of those lectins. However, there is a distinct difference in sugar specificity among these D-galactose binding lectins. For example, richardsonin is specific for all of the D-galactose related sugars tested, while the Arabian Gulf catfish lectin is not inhibited by raffinose and stachyose even at a concentration of 100 mM.⁶⁾ Moreover, the conger eel lectin is also inhibited by sugars (D-, L-arabinose and D-mannose) other than D-galactose related sugars.⁸⁾ Further study is needed to elucidate the sugar structure recognized by each lectin.

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