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Carbohydrate Research 275 (1995) 193–200

CARBOHYDRATE RESEARCH

Note

Isolation and characterization of a new β-galactose-specific lectin from the sea worm *Chaetopterus variopedatus*

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Received 9 January 1995; accepted 27 March 1995

Keywords: Affinity chromatography; Carbohydrate-binding protein; Gal-specific lectin; *Chaetopterus variopedatus*

Lectins isolated from numerous plants, bacteria, and animals including marine invertebrates are now known to represent proteins and glycoproteins of the non-immune system. Owing to their unique properties to bind carbohydrates and sugar determinants of biopolymers with a higher avidity, lectins are now very widely used for studies of the structural and functional roles of glycoconjugates as well as for monitoring alterations on the surface of normal and neoplastic cells [1,2]. Lectins have been used for separation and purification of glycoconjugates [3] and in diagnosis of various diseases [4].

Marine organisms are attractive for lectinologists as new origins and sources of unusual lectins. We now report the isolation, purification and properties of a new lectin from sea worm *Chaetopterus variopedatus* (CVL) which showed β-galactose specificity. An attempt to clarify the mechanism of inhibition of the CVL hemagglutination with mono- and oligo-saccharides is made.

CVL was isolated from fresh marine worms *C. variopedatus* collected at the sublittoral of the Bay of Peter Great of the Sea of Japan. Marine animals harvested were homogenized and subjected to extraction with saline, followed by precipitation with ammonium sulphate. The fraction obtained was shown to possess hemagglutinating activity, which was inhibited with galactose and lactose. In this connection, a purifica-

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SSDI 0008-6215(95)00139-5

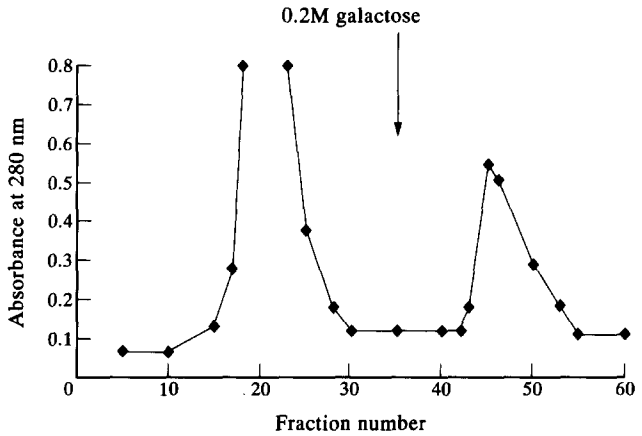

Fig. 1. Affinity chromatography of the ammonium sulfate precipitate on a lactosyl-Sepharose 4B column (1.5 × 20 cm) equilibrated with 0.1 M PBS (pH 7.5). Elution (↓) was carried out with 0.2 M galactose in PBS (V fractions 5 mL).

tion of CVL was achieved using affinity chromatography on lactosyl-Sepharose 4B (Fig. 1). The partly purified lectin fraction was subsequently separated by gel filtration on Sephadex G-75 (Fig. 2). On SDS-PAGE, the lectin ran as a single band at 30 kDa independent of the presence or absence of β-mercaptoethanol, thus indicating it is a monomer (Fig. 3).

CVL was found to agglutinate all types of the human erythrocytes together with sheep and rabbit erythrocytes. The concentrations of CVL giving hemagglutination with human, rabbit and sheep erythrocytes are presented in Table 1. Treatment of erythrocytes with trypsin was shown to enhance the hemagglutination.

An alteration of pH-values of the buffer solutes demonstrated that the activity of CVL is maintained in a range of pH 5–9. CVL showed a maximal activity at pH 8 (Fig. 4).

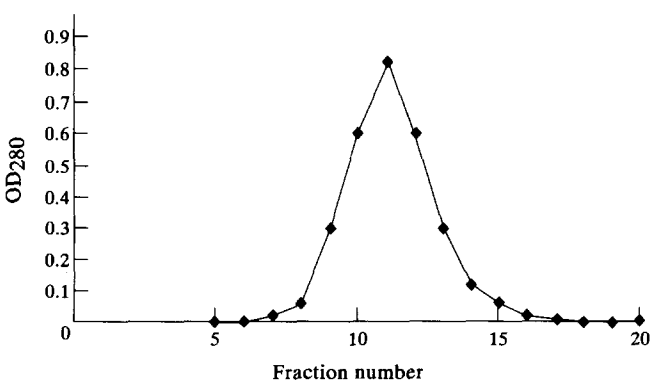

Fig. 2. Gel filtration of CVL on a column (50 × 1.5 cm) with Sephadex G-75. Elution was achieved with 0.01 M PBS (pH 7.3) with a rate of 18 mL/h (V fractions 3 mL).

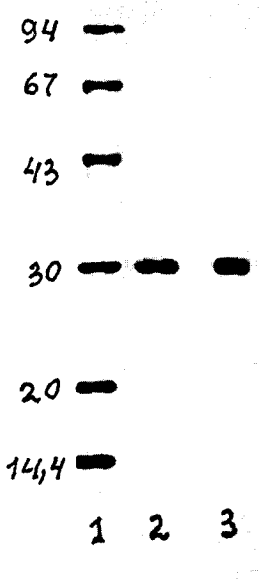

Fig. 3. SDS-PAGE of non-reduced CVL (15 μg) (2) and CVL (10 μg), reduced with 2-mercaptoethanol (3), molecular mass (in kDa). Marker proteins phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin (1).

Binding reactions were not dependent on divalent cations. Neither repeated dialysis of CVL against EDTA nor addition of Ca^{2+} and Mg^{2+} changed lectin activity. A significant loss of activity occurred during heating above 40°C, and activity was completely lost at 50°C.

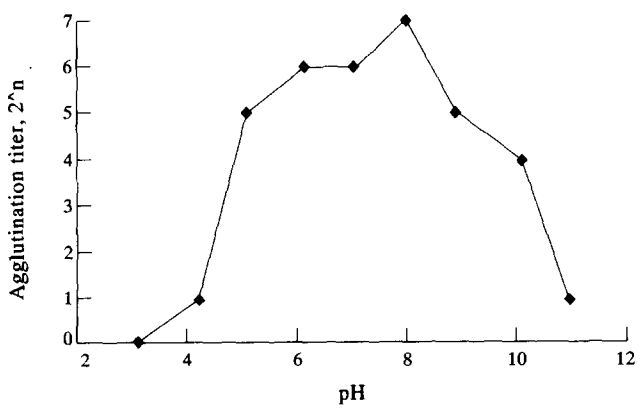

Fig. 4. Effect of pH on agglutinating activity of CVL. Agglutination titre was determined following incubation of samples at different pH values for 3 h. Subsequent dialysis against 0.1 M PBS (pH 7.3).

Table 1
Agglutination of erythrocytes by CVL

Type of erythrocytes	Minimal concentration of CVL (μg/mL)	
Human		
O	60	0.9 [a]
A	30	0.9 [a]
B	15	0.45 [a]
AB	15	0.22 [a]
Rabbit	3	
Sheep	60	

[a] Trypsin-treated erythrocytes were used for agglutination by CVL.

Table 2
Inhibition of hemagglutination by mono- and oligo-saccharides, glycoproteins

Carbohydrate	Concentration (mM)
D-Galactose	1.16
Methyl α-D-galactopyranoside	1.07
Methyl β-D-galactopyranoside	0.54
Methyl 2-*O*-methyl-β-D-galactopyranoside	0.50
Methyl 3-*O*-methyl-β-D-galactopyranoside	80.1
Methyl 4-*O*-methyl-β-D-galactopyranoside	0.50
Methyl 6-*O*-methyl-β-D-galactopyranoside	0.33
Methyl 2,6-di-*O*-methyl-β-D-galactopyranoside	0.47
Methyl 2,4,6-tri-*O*-methyl-β-D-galactopyranoside	> 14.1
Methyl 2-deoxy-α-D-galactopyranoside	4.69
Methyl 3-deoxy-β-D-galactopyranoside	> 18.7
Methyl 4-deoxy-β-D-galactopyranoside	> 18.7
D-Fucose	3.39
1,5-Anhydro-2-deoxy-D-galactitol	5.63
D-Galacturonic acid	4.30
Methyl (methyl β-D-galactopyranosid)uronate	1.88
Methyl (methyl 2-*O*-methyl-β-D-galactopyranosid)uronate	0.88
Methyl (methyl 4-*O*-methyl-β-D-galactopyranosid)uronate	14.1
1,6-Anhydro-β-D-galactopyranose	> 20.6
D-Talose	9.25
Methyl β-L-arabinopyranoside	10.2
Methyl α-L-arabinopyranoside	5.08
Methyl β-D-glucopyranoside	> 85.9
Phenyl β-D-galactopyranoside	0.20
D-Galactal	> 22.8
N-Acetyl-D-galactosamine	1.86
D-Lactose	0.58
D-Raffinose	1.65
BSM	0.06 [a]
Ds BSM	0.06 [a]
Fetuin	0.5 [a]
Ds fetuin	0.015 [a]
α_1-Acid glycoprotein	> 5 [a]
Ds α_1-Acid glycoprotein	0.125 [a]

[a] Concentration in mg/mL.

The concentrations of various monosaccharides, oligosaccharides and glycoproteins required to completely inhibit agglutination were determined. The data obtained are listed in Table 2. The monosaccharides D-glucose, D-mannose and *N*-acetyl-D-glucosamine did not inhibit hemagglutination at 80 mM concentration.

As may be seen in Table 2, methyl β-D-galactopyranoside is twice as powerful an inhibitor as the α-anomer. Replacement of each hydrogen atom of hydroxyl groups at C-2, C-4 or C-6 of methyl β-D-galactopyranoside by a methyl group failed to influence inhibitory activity of the sugar, while replacement of the hydrogen atom of the hydroxyl group at C-3 by a methyl group reduced inhibitory ability of galactoside substantially. While the 2,6-di-*O*-methyl ether and the parent methyl β-galactoside have inhibitory ability, the 2,4,6-tri-*O*-methyl ether of methyl β-D-galactoside failed as an inhibitor of hemagglutination at a concentration less then 14.1 mM. This is in accord with the low inhibitory activity of the 4-*O*-methyl ether of uronate in comparison with the parent compound, probably due to unfavourable substituents at C-4 and C-6 in this molecule.

Methyl 2-deoxy-α-D-galactopyranoside was approximately fourfold less active as an inhibitor than the parent methyl α-D-galactopyranoside. In addition, 3-and 4-deoxy derivatives of methyl β-D-galactopyranoside failed to inhibit agglutination at concentrations less than 14.1 mM. These data indicated that O-atoms at C-2, and C-4 in the molecule of galactose appeared to participate in interactions with the binding site of the lectin. The α- and β-anomers of methyl L-arabinoside were 10-fold less active than the β- and α-anomers of methyl D-galactopyranoside, respectively. The inhibitory activity of D-fucose and D-galacturonic acid was 3- and 4-fold respectively less compared with D-galactose, indicating the participation of the substituent at C-5 in binding the lectin.

1,5-Anhydro-2-deoxy-galactitol showed similar inhibitory ability to methyl 2-deoxy-α-D-galactopyranoside thus indicating that the O-atom at C-1 in the α-anomer appeared not to significantly influence binding of CVL. It is interesting that D-galactal failed to inhibit agglutination even at a concentration of 22.8 mM, probably owing to differences in conformation. The inhibitory ability of lactose was found to be equal to that of methyl β-D-galactopyranoside and was 3-fold greater than raffinose possibly due to the anomeric configuration of the galactose: β in lactose, α in raffinose.

As may be seen from Table 2, some glycoproteins possessed inhibitory activity. Bovine submaxillary mucin (BSM), fetuin and α_1-acid glycoprotein were found to be effective inhibitors of agglutination. It is noteworthy that desialylation of fetuin and α_1-acid glycoprotein is accompanied by a substantial increase of inhibitory activity while the desialylated mucin showed the same inhibition as the parent glycoprotein. The neuraminic acid residues occur at C-6 of the subterminal galactose residue in BSM [5], while the C-3 atom is involved additionally in linkages with the neuraminic acid residues in both the other glycoproteins. This phenomenon demonstrated that the hydroxyl group at C-6 failed to influence binding of the galactose residues of glycoproteins with CVL while the hydroxyl group at C-3 appeared to be very significant for binding glycoproteins with the lectin.

Thus, CVL proved to show a specificity to β-D-galactose residues. The data reported here also emphasize the importance of studying and specifying such parameters as pH and temperature of the lectin isolation as well as configuration and conformation of galactose residues during interaction of the lectin with polysaccharides and glycoconjugates.

1. Experimental

Materials.—Mono- and oligo-saccharides were obtained from Merck (USA). The synthesis of 3-deoxy and 4-deoxy derivatives of methyl β-D-galactopyranoside will be reported elsewhere. Other monosaccharide derivatives were obtained by known methods: methyl ethers of methyl β-D-galactopyranoside [6], methyl ethers of methyl β-D-galacturonate methyl ester [7], methyl β-D-glucoside and methyl β-D-galactoside [8], methyl α- and β-L-arabinoside [9], methyl 2-deoxy-α-D-galactopyranoside [10], D-galactal and 1,5-anhydro-2-deoxy-D-galactitol [11], 1,6-anhydro-β-D-galactose [12], phenyl β-D-galactoside [13].

Fetuin, asialofetuin, mucin from bovine submaxillary glands were purchased from Sigma Chemical Co. (USA). Human α_1-acid glycoprotein was isolated by the method of Gahmberg [14]. Fresh human erythrocytes (type A,B,O or AB) were obtained from the Centre of Blood Utilization (Vladivostok). Fresh erythrocytes from other species were prepared as needed by bleeding animals directly into Alswer's solution.

Sephadex G-75 was purchased from Pharmacia Fine Chemicals (Sweden). Lactosyl-Sepharose 4B, the standard proteins used for M_r estimation by SDS-PAGE and trypsin were obtained from Sigma Chemical Co. (USA).

Purification of CVL.—Samples of 100 g (fresh wt) of *Chaetopterus variopedatus* bodies were homogenized with 300 mL of saline in a blender and the mixture obtained was centrifuged. Pulverized $(NH_4)_2SO_4$ was added to the supernatant up to 70% saturation. The precipitate obtained was collected by centrifugation at 13000 *g*, dissolved in 0.1 M phosphate buffer (pH 7.5) containing 0.05 M NaCl (PBS) and dialysed against PBS. The dialysate was passed through a column (1 × 10 cm) of SP-Sephadex G-50. The eluate collected was applied to the lactosyl-Sepharose 4B column (1.5 × 27 cm) equilibrated with PBS. The affinity column was washed with PBS until absorbance of the effluent at 280 nm was zero. The galactose-specific protein bound to column was eluted with PBS containing 0.2 M galactose. A fraction eluted with galactose was dialysed against water and the solution obtained was lyophilized to yield a protein. The purified protein obtained was further fractionated on a column (1.5 × 50 cm) of Sephadex G-75, equilibrated with PBS (pH 7.3). A sharp symmetric peak of hemagglutinating activity exactly coincident with protein contents was seen. Fractions corresponding to this peak were collected, the combined material was dialysed against water and the solute obtained lyophilized to yield CVL.

Assays of hemagglutination and its inhibition.—CVL was serially 2-fold diluted with PBS in microtitre U-plates. To the sample (25 μL) in each well was added an equal volume of 2% suspension of human erythrocytes and the mixture agitated. The hemagglutination was visually evaluated after 30 min.

For the hemagglutination inhibition assay, the aqueous solutions of various substances to be tested were 2-fold serially diluted with PBS. To each sample (25 μL), CVL (25 μL, four doses of agglutination) and 2% human blood group B trypsin-treated erythrocyte suspension (25 μL) were added successively, the mixture obtained was stirred and kept for 1 h. The concentration of each substance required for complete inhibition was visually determined.

Protein and carbohydrate quantitation.—The protein contents of samples were determined according to the method of Lowry et al. [15], using crystalline bovine serum

albumin as the standard protein. Sugar contents were calculated by the phenol–sulfuric acid method, using D-glucose as standard [16].

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).—SDS-PAGE of the purified sample was carried out according to the procedure of Laemmli [17], using 9–25% polyacrylamide as separating gel and 5% polyacrylamide as stacking gel. A molecular weight (M_r) of CVL studied was determined by SDS-PAGE in the presence and absence of 2-mercaptoethanol. Reduction of CVL was performed by heating at 100°C for 5 min in sample buffer containing 2% SDS and 2.5% 2-mercaptoethanol. Gels were calibrated using the following standard proteins: phosphorylase B (M_r 97400), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000) and α-lactalbumin (M_r 14200). Gels were fixed and stained with Coomassie Blue G-250 (Serva).

pH and heat stability.—The purified lectin was dialysed against various buffers of the following pH values: 0.01 M sodium acetate/acetic acid (pH 4–5.4), 0.01 M sodium phosphate/HCl (pH 6–7), 0.01 M Tris/HCl (pH 7.5–8.5) and 0.01 M glycine/NaOH (pH 9–10.6). The effect of pH was checked by testing the hemagglutinating activity of dialysed lectin samples. To test stability of the lectin at higher temperatures the samples of CVL in PBS were incubated at different temperatures for 20 min and the hemagglutination assay performed after cooling.

Effect of divalent cations.—CVL was dialysed for 24 h against Tris-buffered saline (pH 7.3) containing 50 mM EDTA · Na_2 or 50 mM $CaCl_2$ + 50 mM $MgCl_2$. Human trypsin-treated erythrocytes (B type) were used as indicator cells.

Digestion of erythrocytes with trypsin.—A 10% suspension of erythrocytes in PBS (10 mL) was treated with trypsin (2 mg) for 3 h at 37°C. The material obtained was washed three times with a buffer, which was used for titration. The trypsinized erythrocytes were suspended at concentration of 2% in the same buffer.

Preparation of asialoglycoproteins.—Mucin and α_1-acid glycoprotein were incubated (1.5 h, 80°C) in diluted formic acid (pH 2.1). The mixtures obtained were cooled and dialysed against water. The solutes obtained were lyophilized to yield the corresponding asialoglycoproteins.

Acknowledgements

We thank Dr Alexander A. Kurika for useful discussions and Tatyana A. Fenik for typing this manuscript. This work was supported in parts by Research Grant 94-04-22013 from RBRF.

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