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ISOLATION AND PARTIAL CHARACTERIZATION OF A PROTEOGLYCAN FROM THE RED ALGA *LAURENCIA SPECTABILIS*

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Key Word Index—*Laurencia spectabilis*; Rhodophyceae; red alga; isolation; partial characterization; proteoglycan; hydroxyproline; galactose; uronic acids.

Abstract—A proteoglycan was isolated from the red alga *Laurencia spectabilis* Postels & Ruprecht (Ceramiales) by extraction in a dilute buffer–NaCl solution followed by gel and anion exchange chromatography. The molecule was composed of 92% carbohydrate and 8% protein. Galactose and uronic acids were the major monosaccharides. Ester sulfate was not detected. A small quantity of hydroxyproline was present in the protein component. The proteoglycan accounted for a very small portion (less than 1% by fr. wt) of the alga.

INTRODUCTION

A wide range of proteoglycans, as distinct from glycoproteins, are known from plants. Hydroxyproline-rich arabinogalactan-proteins have been isolated from wheat endosperm by extraction in water [1], from cell walls of *Chlamydomonas* using the chaotropic agent sodium perchlorate [2], from various plant cell walls by alkaline extraction [3–5], and from numerous higher plant sources after extraction in a dilute buffer–NaCl solution [6, 7]. Acid-soluble proteoglycans from brown algae have contained fucose, mannose, xylose and glucuronic acid as predominant monosaccharides with sometimes lesser amounts of galactose and glucose [8–11]. A proteoglycan from *Ascophyllum nodosum* contained a small amount (*ca* 0.2%) of hydroxyproline in the protein moiety [11]. Two different water-soluble extracellular proteoglycans have been isolated from the culture media of the unicellular red algae *Porphyridium cruentum* [12–14] and *Rhodella maculata* [15]. Both of these contained xylose (major sugar), galactose, glucose, uronic acids and sulfate as well as a protein moiety (1.5–16% by wt). Only the amino acid composition of *P. cruentum* was reported and it lacked hydroxyproline [14].

We report the isolation and partial characterization of a proteoglycan from another red alga, *Laurencia spectabilis*, in which galactose is the predominant monosaccharide and a small quantity of hydroxyproline is present in the protein component [16].

RESULTS

Purification of the proteoglycan

Figure 1 shows the carbohydrate and protein profile after the elution of *L. spectabilis* 100% saturated $(NH_4)_2SO_4$ supernatant from Sepharose 4B. Carbohydrate was located in 3 peaks (A, B, and C). Protein was detected in peaks B and C, but only peak B contained proteoglycan material. After re-chromatography of peak B, the fractions forming the single carbohydrate peak were pooled.

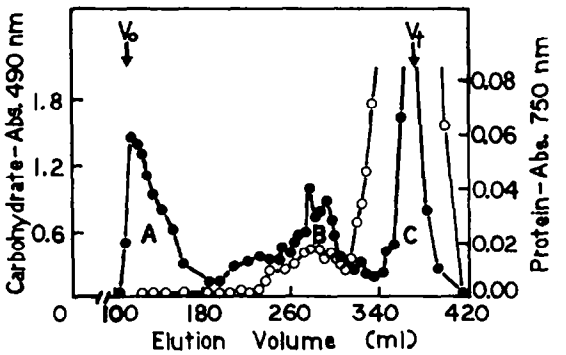

Fig. 1. Sepharose 4B gel chromatography of *L. spectabilis* 100% saturated $(NH_4)_2SO_4$ supernatant. A 15 ml sample of a *L. spectabilis* extract (see Experimental for preparation) was subject to chromatography on a Sepharose 4B column (50 × 3.4 cm). The running buffer was 10 mM K-Pi (pH 7.0) containing 1% (w/v) NaCl and 3 mM NaN_3. Carbohydrate (32; ●——●) and protein (34, 35; ○——○) were detected in the eluate.

The material was shown to contain at least 6 components when analysed by cellulose acetate strip electrophoresis (Fig. 2). All the substances reacted with alcian blue (polyanionic groups present), but only 2 (band Nos. 4 and 6) reacted with high iron diamine stain (sulfate groups present). A single, non-homogeneous carbohydrate peak was obtained from this mixture after ion exchange chromatography (Fig. 3). Electrophoretic examination revealed that fractions eluted at the front of the peak (Fig. 3, fraction Nos. 27–31) contained 2 alcian blue reactive substances, whereas later fractions contained only 1 component. The major component (band No. 2) throughout the peak corresponded to the proteoglycan. Only those fractions shown to be homogeneous by electrophoresis were used for the chemical analysis. The combined yield of the purified proteoglycan from 3 isolation procedures was *ca* 32 mg dry wt from 800 g fr. wt of alga.

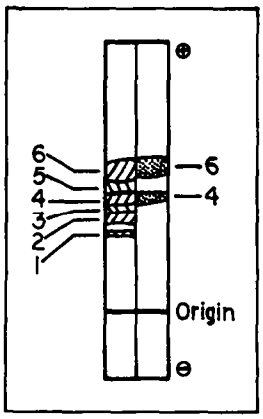

Fig. 2. Cellulose acetate strip electrophoresis of *L. spectabilis* proteoglycan after re-chromatography on Sepharose 4B. The proteoglycan after re-chromatography on Sepharose 4B was subject to electrophoresis and then stained with alcian blue ▨ and high iron diamine ⊡ (see Experimental for details). Band No. 2 is the proteoglycan investigated in this study.

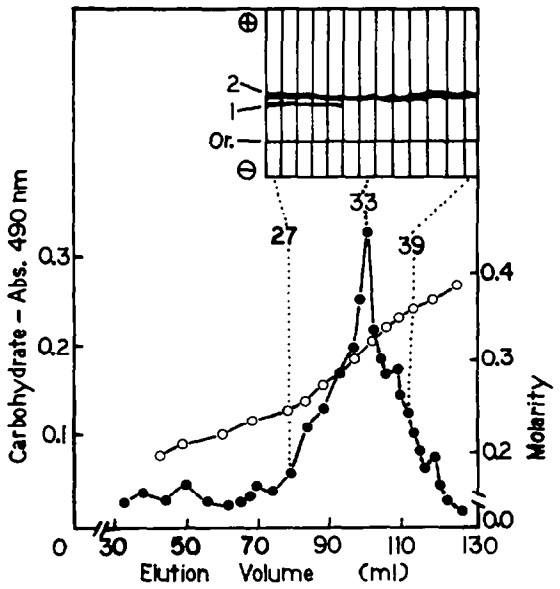

Fig. 3. Salt-gradient elution of *L. spectabilis* proteoglycan from DEAE-Sephadex A-50 and cellulose acetate strip electrophoresis of the proteoglycan-containing fractions. A proteoglycan sample purified by gel chromatography was applied to the anion exchange column (27 × 1.7 cm) and eluted by a salt gradient. The carbohydrate content (●——●) and the salt molarity (○——○) for each fraction were determined. Electrophoresis was performed on all carbohydrate-containing fractions followed by staining with alcian blue and high iron diamine. Details in Experimental. The drawing shows the results of the electrophoresis and staining with alcian blue of fraction Nos. 27–39. No substances reacted with high iron diamine. Band No. 2 is the proteoglycan investigated in this study. Or. = origin.

Chemical analysis of the proteoglycan

The composition of the apparently homogeneous proteoglycan is shown in Table 1. The preponderance of carbohydrate over protein confirmed the view that the compound isolated from *L. spectabilis* was a proteoglycan. Uronic acids accounted for *ca* 9% of the total composition. Sulfate was not detected by either the assay of Nader and Dietrich [17] or, after electrophoresis of the proteoglycan, reaction with high iron diamine stain (minimum detection limit of stain *ca* 20 μg of sulfate when determined with the assay of ref. [17]).

Table 1. Composition of isolated proteoglycan from *Laurencia spectabilis*

Carbohydrate	92%	Uronic acids	
Protein	8%	Total weight	8.4%
		Total carbohydrate	9.1%
Carbohydrate/ protein ratio	12:1	Sulfate	N.D.*

Results are the mean of 4 analyses for 1 preparation.
* N.D.—not detected; if present, less than 2.0% of total weight.

The neutral sugar compositions of two different proteoglycan preparations are shown in Table 2. The proteoglycan was apparently a galactan with small quantities of xylose and glucose in addition to the uronic acids. The arabinose detected in both preparations was present in trace quantities.

The amino acids and amino sugars recovered from the hydrolysates of two different preparations of the proteoglycan are given in Table 3. Aspartate, threonine, serine, and glutamate accounted for ca 60% of the recovered amino acids. Hydroxyproline and several other amino acids were detected in small (less than 1.5%)

Table 2. Neutral sugar composition of *Laurencia spectabilis* proteoglycan

	Preparation No. 1		Preparation No. 2.	
Arabinose	1.8%	(1.5%)*	2.3%	(1.9%)
Xylose	6.4	(5.4)	7.4	(6.2)
Galactose	84.6	(70.7)	84.6	(70.7)
Glucose	7.3	(6.1)	5.7	(4.8)

The results for each preparation are the average of 2 analyses.
* Results in parentheses are the proportions of the neutral sugars by total wt of the proteoglycan.

Table 3. Amino acids and amino sugars recovered from hydrolysates of *Laurencia spectabilis* proteoglycan

	Prep. No. 1	Prep. No. 2		Prep. No. 1	Prep. No. 2
Asx	23.9%	25.5%	Lys	1.7%	2.7%
Thr	9.9	9.5	His	0.6	0.5
Ser	8.8	8.0	Arg	0.8	1.0
Glx	15.4	17.7			
Pro	11.2	11.7			
Gly	5.1	6.5	GlcN	4.9	2.1
Ala	6.6	5.9	GalN	2.3	0.5
Cys	0	0			
Val	5.0	4.3			
Met	0.6	0.5	Hyp*	0.3	0.3
Ile	0.8	0.7			
Leu	1.2	1.2			
Tyr	0.3	0.3			
Phe	0.8	1.2			

The results for each preparation are the average of 2 analyses.
* Determined spectrophotometrically.

quantities, while neither preparation contained detectable cyst(e)ine. Glucosamine and galactosamine were present in both preparations, although the recovery of both amino sugars was lower in preparation No. 2.

DISCUSSION

A dilute buffer–NaCl solution was used for extraction since this mild condition, compared with more rigorous acid or base extraction conditions, was more likely to leave proteoglycans intact. One major proteoglycan fraction (Fig. 1, peak B) was obtained from *L. spectabilis* The substances in peak A did not contain any protein, while the material in peak C consisted of substances that were eluted at the total volume (V_t) of the Sepharose 4B column. Removal of the 'contaminant' component from the proteoglycan (Fig. 3) was achieved only when a 'slow' continuous salt gradient (total vol. 600 ml) formed part of the ion exchange chromatography procedure. Earlier attempts using a step-wise or a 'steep' continuous gradient (total vol. less than 300 ml) did not provide resolution of the two substances. This 'contaminant' component (band No. 1, Figs. 2 and 3) appeared to be a polysaccharide composed mainly of uronic acids, arabinose and glucosamine, but without any detectable protein (according to electrophoretic, sugar, and amino acid analyses of fraction Nos. 27–31 in Fig. 3). The 4 additional substances detected by electrophoresis (band Nos. 3, 4, 5 and 6 in Fig. 2) were not recovered after ion exchange chromatography, even when the molarity of the elution buffer was raised to 2 M.

The two proteoglycans reported previously from *Porphyridium cruentum* [12–14] and *Rhodella maculata* [15] were water-soluble mucilage components. The carbohydrate compositions of both proteoglycans were similar, but only the amount of uronic acids was determined quantitatively for both molecules (8.5% by wt for *P. cruentum* and 12% for *R. maculata*). The carbohydrate portion of the *L. spectabilis* proteoglycan (Tables 1 and 2) had sugars and uronic acid content similar to the other 2 molecules, except galactose, not xylose, was the major neutral sugar and a trace amount of arabinose was present. The same neutral sugars (galactose and glucose, plus trace amounts of arabinose and xylose) were the main constituents of the carbohydrate portions of glycoproteins from the red algae *Phyllophora nervosa* [18–20] and *Furcellaria fastigiata* [21].

Protein accounted for *ca* 1.5–7% (by wt) of the *Porphyridium cruentum* proteoglycan [12–14] and for *ca* 16% of the *Rhodella maculata* proteoglycan [15]. The published amino acid composition of the *P. cruentum* proteoglycan does not report analysis of hydroxyproline. The *L. spectabilis* proteoglycan contained 8% (by wt) protein and hydroxyproline was present. Our report of hydroxyproline is based upon results from a more sensitive assay [22] than used previously [23–25] as well as upon analysis of a purified substance rather than a whole plant extract. Either or both factors could account for the disagreement with the results of Gotelli and Cleland [25] for other red algae. The low amount of hydroxyproline detected in *L. spectabilis* suggests that any role which it may have in cell wall growth or structure differs at least quantitatively from the roles suggested for hydroxyproline in the hydroxyproline-rich proteoglycans from higher plants [26, 27] or green algae [4, 28, 29].

The presence of sulfate in red algal polysaccharides has been documented [30], although some non-sulfated polysaccharides are known, for example, that from the mucilage of *Batrachospermum* sp. [31]. The acidic proteoglycan from *Porphyridium cruentum* contained 9–10% (by wt) ester sulfate [12–14], while that from *Rhodella maculata* had 10% sulfate [15]. Sulfate was absent from the acidic *L. spectabilis* proteoglycan; thus, the uronic acids in the molecule accounted for its reaction with alcian blue stain and its behaviour during both ion exchange chromatography and cellulose acetate strip electrophoresis.

EXPERIMENTAL

Laurencia spectabilis Postels & Ruprecht was collected at Botany Beach near Port Renfrew, B.C., Canada in April and November 1976. The fresh material was kept on ice in plastic bags filled with seawater during transportation to the laboratory. The material was cleaned, blotted dry and stored at −15°.

Extraction. Plant material (*ca* 300 g fr. wt) was thawed and homogenized in small portions in a Waring blendor in *ca* 800 ml of 10 mM K-Pi buffer (pH 7) containing 1% (w/v) NaCl and 3 mM NaN_3. Additional buffer (500–700 ml) was added and the slurry stirred for 2–6 hr at 5° then filtered through 4 layers of cheesecloth. The initial extract was stored at 5° and the residue was re-extracted in K-Pi buffer containing 5% (w/v) NaCl and 3 mM NaN_3, and filtered . The 2 extracts were combined. $(NH_4)_2SO_4$ was added stepwise to 40, 70 and 100% satn at room temp., and insoluble material was removed after each addition by centrifugation at 4800 *g* for 15 min. The resulting 100% $(NH_4)_2SO_4$ satd supernatant was dialysed against running tap H_2O for 48 hr and concd, but not dried, by rotary evaporation at 45°. It was important that complete drying of the sample be avoided here and at later isolation steps because dried proteoglycan was substantially insoluble in H_2O.

Gel and ion exchange chromatography. Samples (10–20 ml) were applied to a column (50 × 3.4 cm) of Sepharose 4B equilibrated with K-Pi buffer containing 1% (w/v) NaCl and 3 mM NaN_3. The column was eluted with the same buffer. The eluate was monitored for carbohydrate [32] and for protein [34, 35]. Fractions from the second carbohydrate-containing peak (which contained the proteoglycan) were pooled, dialysed against running tap H_2O for 24 hr, concd by rotary evaporation, and rechromatographed on Sepharose 4B. The fractions containing carbohydrate and protein were pooled, desalted on a column (50 × 2.3 cm) of Sephadex G-25 and concd by rotary evaporation. Samples (2–5 ml) in deionized H_2O were applied to a column (25–27 × 1.7 cm) of DEAE-Sephadex A-50 equilibrated with 200 mM Na-Pi buffer (pH 8.0) containing 3 mM NaN_3. The column was eluted (15–20 ml/hr) by a linear salt gradient formed by mixing 300 ml of Na-Pi buffer with 300 ml Na-Pi buffer containing 1.8 M NaCl. The molarity of the eluate was monitored using a conductivity meter. Column elution was stopped when the eluate molarity reached 0.5 M. Eluate samples were monitored for carbohydrate [32]. Usually, only one peak of carbohydrate activity was detected. The homogeneity of the carbohydrate-containing fractions was monitored routinely by cellulose acetate strip electrophoresis.

Cellulose acetate strip electrophoresis. Samples (5–10 μl) of the carbohydrate-containing fractions from the ion exchange column were applied to Sephaphore III cellulose polyacetate strips (15.2 × 2.5 cm; Gelman Instrument Co., Ann Arbor, MI). Electrophoresis was performed at 300 V for 40 min at room temp. using 60 mM Tris–barbital–Na barbital buffer (pH 8.8). The strips were cut in half and were stained with alcian blue (method a of [33]) to detect polyanionic substances or with high iron diamine [33] to detect half sulfate esters. The fractions which contained one band of similar electrophoretic and staining behaviour were combined, desalted by passage through Sephadex G-25, and lyophilized prior to chemical analysis.

General chemical analysis. Total carbohydrate was determined by a PhOH–H_2SO_4 acid method [32] using galactose as standard. Total protein was determined by modification [35] of the Folin–Lowry procedure [34] using BSA as standard. Total uronic acids were determined using the method of ref. [36] with a 1:1 mixture of galacturonic and glucuronic acids as standard. Total sulfate was determined using the method of ref. [17].

Neutral sugar analysis. Samples (1.5–4 mg) were hydrolysed with 1 ml 2 N TFA in sealed tubes at 110° for 1 hr. The neutral sugars were analysed as alditol acetate derivatives [37] by GLC using a dual FID instrument. The flow rates of N_2 and H_2 were 25 ml/min and of air 250 ml/min. The ss columns (1.8 m × 2 mm i.d.) contained 5% (w/w) Silar 10C on Gas Chrom Q and were temp. programmed from 120 to 260° at 2°/min (injector 210°; detector 275°). PC using the 3 solvent systems of ref. [38] confirmed the identity of the parent sugars.

Amino acid and amino sugar analyses. Samples (1–4 mg) were hydrolysed *in vacuo* with *ca* 0.5 ml of 6 N HCl at 110° for 20 hr. Hydrolysates were dried *in vacuo* and analysed using an amino acid analyser [39, 40]. Hydroxyproline was determined using the method of ref. [22].

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