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STRUCTURAL STUDIES ON ASCOPHYLLAN AND THE FUCOSE-CONTAINING COMPLEXES FROM THE BROWN ALGA *Ascophyllum nodosum*

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ABSTRACT

The two major "complexes" isolated from a dilute-acid extract of *Ascophyllum nodosum* were degraded by mild, acid hydrolysis to a fucan portion and an ascophyllan-like portion. Protein was enriched in the ascophyllan-like fraction. A time-course study of the acid hydrolysis, together with data on digestion by pronase and hydrolysis by base indicated that the complexes were formed by a fucan backbone to which varyious numbers of ascophyllan-like molecules were attached by an acid-labile linkage. The primary uronic acid in the ascophyllan-like portion of the complex was mannuronic acid, whereas the free ascophyllan isolated in this study contained a mixture of guluronic, mannuronic, and glucuronic acids*. In each of these uronic acid-rich materials, hydrolysis by base suggested a uronic acid backbone having relatively long, fucose-containing, side chains. Hydroxyproline was present in the complex, but did not appear to be part of the major carbohydrate–protein linkages. A protein-enriched fraction was obtained by treatment of the ascophyllan-like portion of the complex with oxalic acid. Amino acid analysis, before and after treatment with mild base suggested that serine and threonine were involved in the linkage of protein to carbohydrate.

INTRODUCTION

In the preceding paper[1] we have shown that about 80% of the fucose-containing sulfated polysaccharides from *A. nodosum*, isolated by extraction with dilute acid, may be accounted for in three electrophoretically pure fractions. Fraction 1 was electrophoretically identical to ascophyllan[2,3], a xylofucoglucuronan isolated by previous workers. Fractions 2 and 3 were considered to be "complexes" as, when subjected to mild, acid hydrolysis (0.02M hydrochloric acid, 1 h, 80°), they were changed into two new polymer fractions.

*See footnotes to preceding paper.

The present paper describes structural information on these complexes and reexamines some structural features of ascophyllan in light of the information obtained on the complex fractions.

RESULTS AND DISCUSSION

Each of the fractions studied (Fractions 1, 2 and 3, Table I, preceding paper[1]) contained small, but significant, proportions of protein. Larsen, *et al.*[2,3] suggested that protein was part of the ascophyllan molecule, but it also was found in the complexes. Fractions 2 and 3, when treated with 0.02M hydrochloric acid for 1 h at 80°, showed no indication of the original material on electrophoresis, but gave two new bands (Fig. 1). One was faster-moving and almost as intense as the original, and the other was slower-moving and less intense.

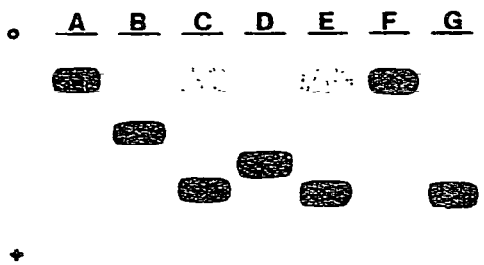

Fig. 1. Cellulose acetate electrophoresis, Toluidine Blue stain, at pH 7.5. A, ascophyllan; B, Fraction 2; C, Fraction 2, hydrolyzed; D, Fraction 3; E, Fraction 3, hydrolyzed; F, hydrolyzed Fraction 2, ethanol-insoluble fraction; G, hydrolyzed Fraction 2, ethanol-soluble fraction.

Fractionation of the hydrolyzate with ethanol allowed clean separation of the two new components (Fig. 1 and Table I). The insoluble (slower-moving) fraction was similar to ascophyllan and was greatly enriched in protein. The soluble (faster-moving) fraction was low in uronic acid, and rich in fucose. It approached the composition of a fucan and probably constitutes presents the "fucoidan" originaliy

TABLE I

FRACTIONS FROM MILD, ACID HYDROLYSIS OF *A. nodosum* COMPLEX

Fraction	*Yield*[a] (%)	*Protein* (%)	*Uronic acid* (%)	*Fucose* (%)	*Distribution of neutral sugars*[b] (%)				
					Fuc	*Xyl*	*Man*	*Gal*	*Glc*
2 (complex 1)		2.4	15.8	33	73	11	10	2	5
Insoluble	32	6.8	24.1	11	28	22	28	6	17
Soluble	68	1.6	4.1	42	77	8	8	1	6

[a]Based on recovered material only. [b]Calculated from gas chromatograms, considering the total area under the five peaks as 100%.

associated with this species. Alginic acid was not detected and, contrary to an earlier suggestion[3], does not appear to be part of the purified complexes from *Ascophyllum*.

Electrophoretic monitoring of the hydrolysis at 75° (Fig. 2) showed gradual increases in the mobility of the original band and a simultaneous, gradual buildup of the slower-moving component. Both fractions behaved similarly in this experiment; after 10 min, Fraction 2 had a mobility identical to that of Fraction 3.

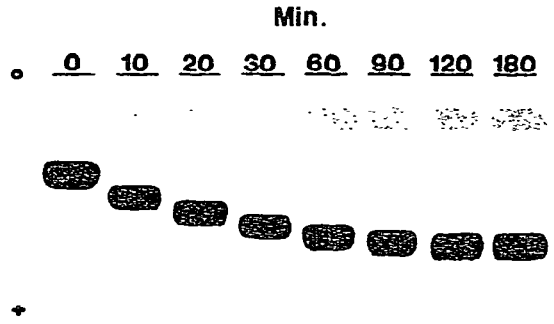

Fig. 2. Cellulose acetate electrophoresis, Toluidine Blue stain, at pH 7.5. Mild, acid hydrolysis (0.02M HCl, 75°) of Fraction 2.

These data suggest that both "complexes" are composed primarily of an ascophyllan-like component and a fucan. Fucan forms the backbone of the molecule, and the ascophyllan-like components are attached as branches by acid-labile linkages. The two complexes differ from each other only in the number of ascophyllan-like molecules attached to the fucan backbone.

Because of the accumulation of the protein in the ascophyllan-like fraction, it was considered that the two polysaccharide components in the complex might be linked via an acid-labile, protein bridge. After fraction 2 had been treated with pronase, the protein content of the product decreased from 2.4 to 2.0% without any change in electrophoretic mobility. Treatment with M sodium hydroxide decreased the protein content to 1%, but did not result in the formation of two new components similar to those from acid hydrolysis. These data indicate that the protein fraction is not a bridge, but is linked primarily to the ascophyllan portion of the complex.

Fractions 1 (ascophyllan-like) and 2(complex) were both hydrolyzed with base and the products analyzed, before and after dialysis, for uronic acid, unsaturated uronic acid, fucose, and total carbohydrate (Table II). For Fraction 1, treatment with base resulted in β-elimination, with loss of uronic acid and production of unsaturated uronic acid. However, 25% of the remaining uronic acid and 34% of the unsaturated uronic acid were found in the dialyzable fraction. In contrast, there was no loss of fucose on treatment with base, and 94% of the fucose was recovered in the non-dialyzable fraction. These data suggest that most of the fucose residues occur in relatively large fragments that do not contain uronic acid, whereas the uronic acid residues are degraded to give a significant proportion of dialyzable fragments. These

data are consistent with a uronic acid backbone having side chains where most of the fucose is located. This interpretation supports the conclusions of Larson, *et al.*[2,7], but differs from the data reported by Percival[5,6].

TABLE II

COMPOSITION OF FRACTIONS 1 AND 2 AFTER HYDROLYSIS BY BASE

	Total carbohydrate (mg/ml sample)	*Fucose (mg/ml sample)*	*Uronic acid (mg/ml sample)*	*Unsaturated uronic acid (mg/ml sample)*
Fraction 1				
Original	0.70	0.12	0.242	0.0
Base-hydrolyzed	0.76	0.13	0.206	0.042
Base-hydrolyzed and dialyzed	0.62	0.11	0.153	0.028
Fraction 2				
Original	0.44	0.22	0.128	0.0
Base-hydrolyzed	0.46	0.23	0.098	0.028
Base-hydrolyzed and dialyzed	0.38	0.20	0.067	0.017

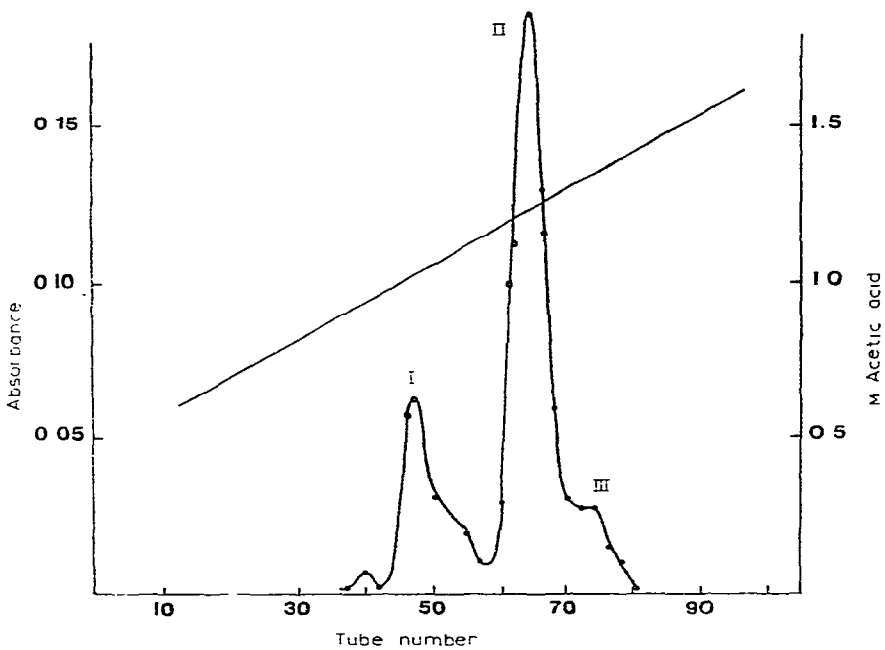

Fig. 3. Acetic acid gradient-elution pattern of the uronic acids from the ascophyllan-like portion of Fraction 2. Absorbance is from the phenol–sulfuric acid procedure.

The data (Table II) for Fraction 2 (complex) indicate that the ascophyllan-like portions of these molecules have the same type of structure as found in Fraction 1. In this instance, 31% of the remaining uronic acid and 39% of the unsaturated uronic acids were lost during dialysis, in contrast to a 9% loss of fucose.

The identity of the uronic acid present in Fraction 2 was determined by ion-exchange chromatography[9] and confirmed by paper electrophoresis[10]. The ion-exchange results for the portion remaining insoluble after mild, acid hydrolysis (Table I) are shown in Fig. 3. The preponderant uronic acid was mannuronic (Peak II), with a small proportion of guluronic (Peak I), and traces of glucuronic acid (Peak III). A similar result was obtained when Fraction 2 was itself subjected to the same analyses. The complex isolated from *A. nodosum* has, therefore mannuronic acid as its only significant uronic acid, and not glucuronic acid as reported by previous workers[2,3,5,8]. For this reason, we have referred to the major uronic acid portion of the complex as ascophyllan-like, as the data on general chemical analysis, electrophoretic mobility, and response to base clearly indicate its similarity to ascophyllan.

Fraction 1 (preceding paper[1]) was also analyzed for uronic acid composition and the results are shown in Fig. 4. Peak I contained guluronic acid plus another component tentatively identified as galacturonic acid. Peak II was mannuronic acid and Peak III was glucuronic acid. The extraction and fractionation procedure used in this study was also used to isolate Fraction 1 from the same algal sample used in the

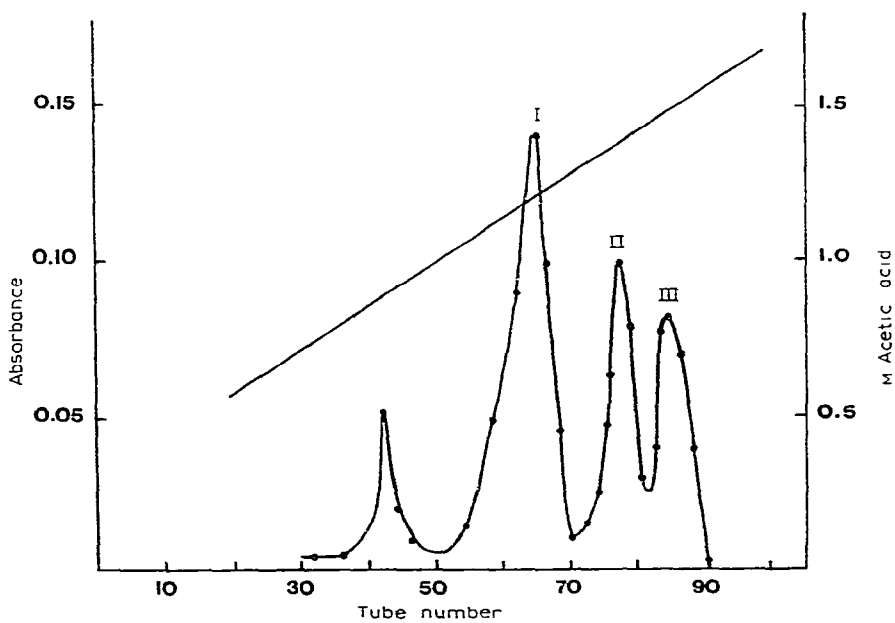

Fig. 4. Acetic acid gradient-elution pattern of the uronic acids from ascophyllan. Absorbance is from the phenol–sulfuric acid procedure.

original work on ascophyllan[2]. Analysis for uronic acids generally gave the same results as already reported here. The explanation for the difference between the native ascophyllan-like molecules in the present study and the originally reported ascophyllan molecules is unknown. It was also surprising to find that the ascophyllan-like portion of the complex contained only mannuronic acid. These data, plus the original isolation of only glucuronic acid, suggest that there may be several types of ascophyllan-like molecules in this species, each having the same fundamental structure, but differing in the relative amounts of each uronic acid.

Hydroxyproline is a common component of plant glycoproteins, and 0.012% of it was present in the protein-enriched ascophyllan-like component from Fraction 2 (Table I). However, this percentage did not increase in a base-treated sample that had lost about 50% of its original protein; in fact it decreased. As hydroxyproline–carbohydrate linkages are generally base-stable[11], it seems unlikely that the protein–carbohydrate linkage in this sample involved hydroxyproline.

The ascophyllan-like component (Insoluble, Table I) was treated with oxalic acid; the precipitate formed amounted to only 3% of the starting material, but its protein content was 18.1%. Amino acid analyses on this sample, before and after treatment with base[12], are shown in Table III. Significant proportions of serine and threonine were observed and the base-treated product showed a 29% loss of serine and a 23% loss of threonine. However, the expected[12], concomitant increase in alanine content, was not observed. These data suggest a possible role for serine and threonine in the carbohydrate–protein linkage.

TABLE III

AMINO ACID ANALYSIS OF PROTEIN-ENRICHED FRACTION FROM *A. nodosum*

Amino acid	*μmol/100 mg of sample*	
	Original	*After treatment with base*
Asp	1.72	1.59
Thr[a]	0.96	0.74
Ser[a]	1.04	0.74
Glu	1.35	1.43
Pro	0.62	0.63
Gly	1.40	1.50
Ala	1.04	0.94
Val	0.99	0.90
Ile	0.63	0.69
Leu	0.68	0.65
Met	0.16	0.18
Tyr	0.19	0.20
Phe	0.54	0.53
Lys	0.18	N.D.[b]
His	0.05	N.D.
Arg	0.42	N.D.

[a]Corrected by 10 and 5%, respectively, for hydrolytic decomposition. [b]N.D. = not determined.

EXPERIMENTAL

General analytical procedures. — Analyses for nitrogen, uronic acid, fucose, and ratio of neutral sugars were performed as described in the preceding paper[1]. The procedures for total carbohydrate (phenol–sulfuric acid)[3], and unsaturated uronic acid[13,14], have also been described previously.

Electrophoresis. — Free-boundary and cellulose acetate electrophoresis were performed as described in the preceding paper[1].

Mild hydrolysis and fractionation of complexes. — A 1% solution of Fraction 2 (9 vol) was diluted with 1 vol 0.2M hydrochloric acid, and heated in a water bath for 60 min at 80°. The solution was neutralized, dialyzed, and concentrated to its original volume. An equal volume of 0.1M magnesium chloride was added, followed by ethanol to a concentration of 50%. The insoluble fraction was separated by centrifugation and redissolved in water. Both soluble and insoluble fractions were isolated, after dialysis, by freeze-drying (Table I). Total material recovery was generally about 60%. In small-scale reactions for electrophoretic analysis with cellulose acetate, 5-mg samples were used. The temperature of the water bath was varied, and aliquots were removed directly from the solution by using a micro-pipette and were spotted for electrophoresis.

Pronase digestion. — Fraction 2 (25 mg) was dissolved in 2.5 ml 0.1M Tris–acetate buffer at pH 7.8 and 0.25 mg of pronase was added. The mixture was incubated[15] for 72 h at 37° with a further 0.13 mg of pronase being added after 24 and 48 h. At the end of 72 h, the incubation mixture was dialyzed for 48 h against distilled water and then adjusted to 5 ml total volume. The polysaccharide was precipitated with 1 ml of 5% cetyltrimethylammonium bromide and redissolved in 4M sodium chloride. The polymer was precipitated with ethanol, redissolved in 2M sodium chloride, and reprecipitated with ethanol. The precipitate was dissolved in water, dialyzed, and freeze dried; yield, 13.5 mg.

Hydrolysis with base. — Samples of Fractions 1 and 2 were dissolved in M sodium hydroxide (1%) and an equal amount of potassium borohydride was added to minimize oxidative degradation[2]. Aliquots for analyses of total carbohydrate, uronic acid, unsaturated uronic acid, and fucose were removed before and after heating under reflux for 12 h at 80°. The remaining solutions were neutralized with M hydrochloric acid and dialyzed exhaustively against distilled water. After concentration to their original volume under diminished pressure, a further aliquot was removed for analysis, and the remaining solution freeze-dried.

Identification of uronic acids. — Polymer samples were hydrolyzed with 80% sulfuric acid and the uronic acids separated by ion-exchange chromatography as described by Larsen and Haug[9,16]. Identification of fractions was verified by paper electrophoresis[10] of appropriately pooled eluates.

Characterization of the protein. — A protein-enriched fraction was isolated from an ascophyllan-like sample (Insoluble, Table I) by treatment with 0.5M oxalic acid for 1 h in a boiling-water bath[2]. After 1 h, the precipitate that formed at 50 min

was isolated by centrifugation and dissolved in M sodium hydroxide. After neutralization with M hydrochloric acid, the solution was dialyzed and freeze-dried. Hydroxyproline was determined by the method of Kivirikko and Liesmaa[17]. Amino acid composition was determined after hydrolysis with 6M hydrochloric acid in a sealed tube for 24 h at 110°, by using a Beckman Amino Acid Analyzer. Treatment with mild base for identification of possible serine and threonine linkages to carbohydrate was performed as described by Katzman and Jeanloz[18].

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