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CARBOHYDRATE CONSTITUENTS OF THE MARINE ALGAE OF SRI LANKA. PART III. COMPOSITION OF THE CARBOHYDRATES EXTRACTED FROM THE BROWN SEAWEED *TURBINARIA CONOIDES*.

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Abstract: The brown seaweed *Turbinaria conoides* was subjected to sequential extraction with 80% ethanol, aqueous 2% calcium chloride, dilute hydrochloric acid and aqueous 3% sodium carbonate solution. Mannitol and eight polysaccharide fractions I - VIII were separated. The neutral sugar composition and the uronic acid content of each fraction was determined. Results indicate the presence of laminaran in fractions I, II and IV, while fractions III, V, VI and VII are rich in 'fucans.'

1. Introduction

The brown seaweed *Turbinaria conoides* is found in the coastal regions of Sri Lanka, India and Japan. Carbohydrates which have been isolated from the brown seaweeds include low molecular carbohydrates such as mannitol, while laminaran, 'fucans' and alginates comprise a characteristic range of polysaccharides which have been found in all species of brown seaweeds investigated.

The sugar alcohol mannitol acts as a food reserve carbohydrate[1,2] and also as a substrate for respiration.[5] Laminaran is a β—D—(1⟶3) linked glucan found in all brown algae and occasionally in the green algae.[7] It is the food reserve material of the brown seaweeds which, unlike red and green algae, do not synthesize starch-like polysaccharides. Fucose containing sulphated polysaccharides have been described under different names such as fucoidan, fucoidin, ascophyllan, sargassan, glucuronoxylofucans and fucans. These are polymers of fucose (6—deoxy—L—galactose) sulphate containing xylose, glucuronic acid and in some species galactose and / or mannose as the major constituents. 'Fucans' are water soluble and present in the intercellular tissue of brown seaweeds. It is also found in the mucilage which exudes from the surface of fronds. Alginic acid is a mucilaginous polyuronide which is an important cell wall constituent of the brown seaweeds, where it occurs as a mixed salt of sodium, calcium and magnesium.

Seasonal variations in the growth and contents of alginic acid and mannitol in *T. conoides* from the Gulf of Mannar (India) have been studied earlier.[1,3] In a previous report we have discussed the composition and sequence of uronate residues in alginates extracted from three species of brown algae including *T. conoides*.[8]

2. Experimental

2.1 Analysis of dried seaweeds

Fronds of *T. conoides* were collected at Mankumban in the Northern coast of Sri Lanka. These were washed in fresh water, sun dried and ground on a Wiley mill to pass a 1 mm screen. The contents of dry matter, ash and crude protein were analysed by standard methods.[4] The ground seaweed was extracted successively with 80% ethanol and chloroform in a Soxhlet apparatus and the residue analysed for starch,[3] Klason lignin,[2] uronic acids[16] and glycosyl composition[2] following acid hydrolysis.

2.2 Sequential extraction of dried seaweeds

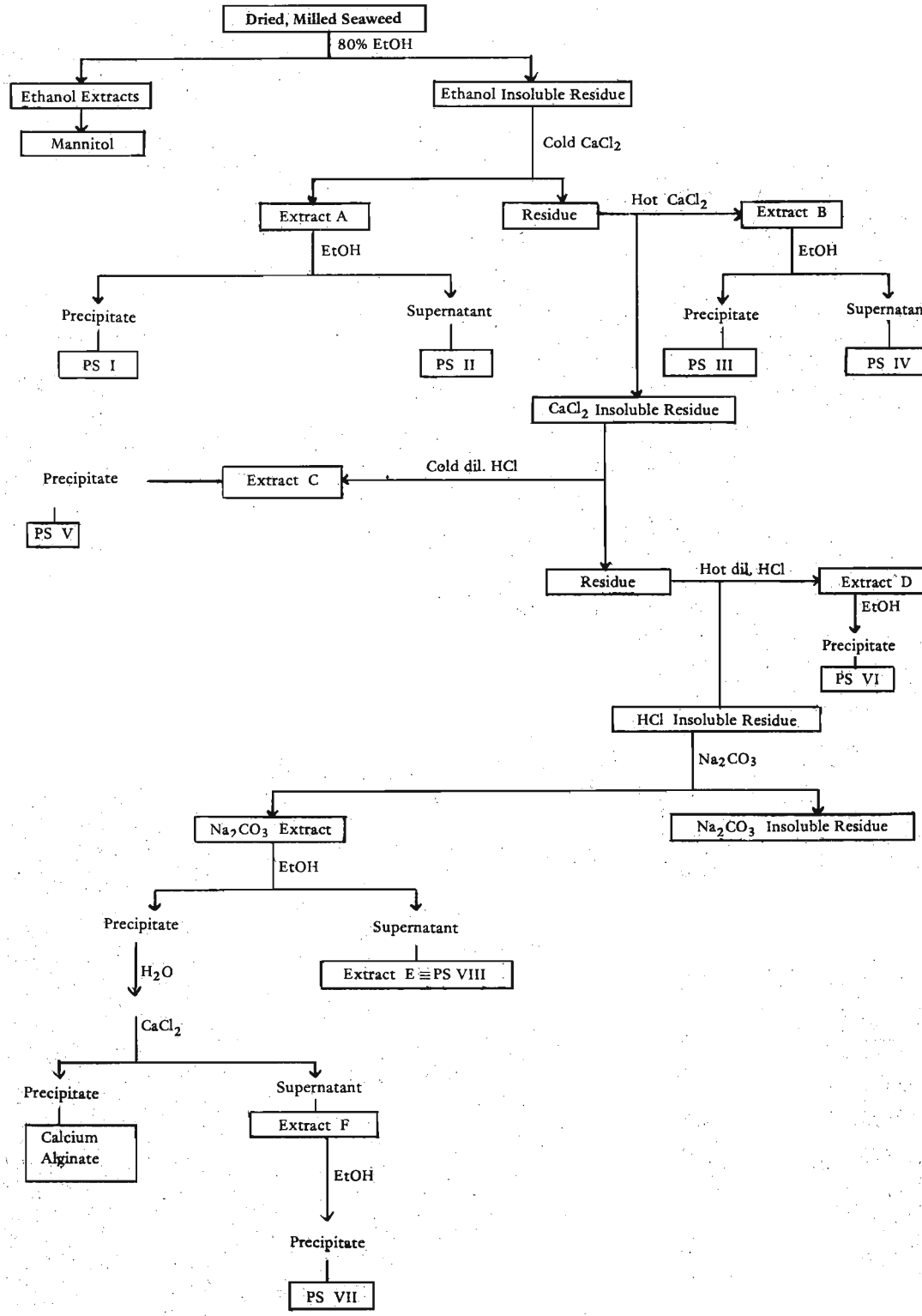
Dried and milled seaweeds (200 g, 85.2% dry matter) were extracted sequentially with (a) aqueous 80% ethanol, (b) aqueous 2% $CaCl_2$, (c) dilute HCl (pH 2, 0.01M) and (d) aqueous 3% Na_2CO_3 as shown in Scheme 1.

2.2.1 Ethanol extraction

Dried and milled seaweeds were extracted with aqueous 80% ethanol (2 x 100ml) for 2 x 6h at room temperature. The extracts obtained were combined and concentrated to give a precipitate and an ethanolic solution. The ethanolic solution was partitioned between toluene—n—butanol—water (1:1:1) and the ethanol water solution was poured into ethanol to give a white precipitate and an ethanol—water layer (Scheme 1). The residual seaweed was extracted with aqueous 80% ethanol (2 x 1000ml) for 2 x 4h at 70^0C and the extract was again treated as described above. The precipitates obtained were recrystallized from ethanol—water giving a white crystalline substance, m.p. 166^0C. (lit.[17] m.p. of mannitol 166^0C). The identity of mannitol was confirmed by paper chromatography and comparison with an authentic sample of mannitol. Paper chromatography was carried out on Whatman No. 1 chromatographic paper using the solvent systems *n*-butanol-ethanol-water (40:11:19) and ethyl acetic acid-water (3:1:1) with aniline hydrogen phthalate as the spray reagent.

2.2.2 Aqueous $CaCl_2$ extraction

The residue obtained after ethanol extraction was air dried and pre-treated with 40% formaldehyde solution overnight. The air dried pre-treated residue was extracted with aqueous 2% $CaCl_2$ (1000ml) at room temperature for 8h. The extract on centrifugation gave a supernatant which was dialysed and freeze dried to give the dry extract A. The residual seaweed was re-extracted with aqueous 2% $CaCl_2$ (1000ml) for 8h at 70^0C and treated similarly to yield dry extract B.


Dried, Milled Seaweed
80% EtOH
Ethanol Extracts
Mannitol
Ethanol Insoluble Residue
Cold $CaCl_2$
Extract A
Residue
Hot $CaCl_2$
Extract B
EtOH
EtOH
Precipitate
Supernatant
Precipitate
Supernatant
PS I
PS II
PS III
PS IV
$CaCl_2$ Insoluble Residue
Precipitate
Extract C
Cold dil. HCl
PS V
Residue
Hot dil. HCl
Extract D
EtOH
Precipitate
PS VI
HCl Insoluble Residue
Na_2CO_3
Na_2CO_3 Extract
Na_2CO_3 Insoluble Residue
EtOH
Precipitate
Supernatant
H_2O
Extract E ≡ PS VIII
$CaCl_2$
Precipitate
Supernatant
Calcium Alginate
Extract F
EtOH
Precipitate
PS VII

Scheme I.

2.2.3 Dilute HCL extraction

The residue after $CaCl_2$ extraction was air dried, extracted with dil. HCl (pH 2, 0.01M, 1000ml) at room temperature for 8h and centrifuged. The supernatant was dialysed and freeze dried to give dry extract C. The residual seaweed was re-extracted with dilute HCl (pH 2, 0.01M, 1000ml) at 70^0C for 8h, then centrifuged, dialysed and freeze dried as above to give dry extract D.

2.2.4 Aqueous Na_2CO_3 extraction

The residue obtained after acid extraction was air dried and extracted with aqueous 3%. Na_2CO_3 (1000ml) at 50^0C for 6h and the extract was poured into ethanol giving a precipitate and a supernatant (extract E). Extract E is referred to as polysaccharide fraction VIII in Table 2. The precipitate was dissolved in water and treated with aqueous 2% $CaCl_2$ solution when calcium alginate was precipitated. The supernatant gave extract F which on precipitation with ethanol yielded polysaccharide fraction VII.

2.3 Isolation of polysaccharides

The dried extracts A, B, C, D and F were dissolved in water and precipitated with ethanol. The precipitates obtained were redissolved in water and freeze dried to obtain the crude polysaccharide fractions I, III, V, VI and VII. The fraction obtained after the precipitation of alginic acid was analysed directly and is referred to as polysaccharide fraction VIII.

2.4 Analysis of the crude polysaccharides

The polysaccharide fractions I — VIII were analysed as their alditol acetates by g. l. c.[1] to obtain the glycosyl composition. Uronic acid content was determined by the decarboxylation method.[16] The sulphate content was obtained in ug/ml using a spectrophotometric method.[10] The results are reported as percentage starting material in Table 2.

3. Results and Discussion

Compositional analysis of the washed, dried and milled fronds of *T. conoides* collected at Mankumban, showed the presence of a high content of ethanol and chloroform soluble extractives and non-starch polysaccharides (see Table 1). Uronic acids constituted 20.89% of the non-starch polysaccharides while neutral sugars were present in minor amounts (Table 1). The ash content was observed to be very high and is probably due to the presence of salts in the fronds.

Table 1. Chemical Composition of the dried fronds of *Turbinaria conoides* (% dry matter)

Ethanol and chloroform soluble extractives	38.06
Crude protein (N x 6.25)	8.60
Non–starch polysaccharides	31.83
Fucose	1.45
Ribose	0.09
Arabinose	0.14
Xylose	0.45
Mannose	1.66
Galactose	1.25
Glucose	5.90
Uronic acids	20.89
Klason lignin	19.27
Ash	34.30

The ethanol extracts yielded mannitol (m. p. 165^{0}C) in 9.3% yield. The identity of mannitol was confirmed by paper chromatography and comparison with an authentic sample. The sugar alcohol mannitol has been isolated from all brown seaweeds investigated[6,14] except *Dictyopteris plagiogramma.*[15] Higher amounts of mannitol (25%) have been extracted from other brown seaweeds such as *Laminaria* spp. where the mannitol content showed marked seasonal variation.[14] Other low molecular carbohydrates found in brown algae include the seven carbon polyol volemitol, 1—*O*—D—mannitol—β—D—glucopyranoside and 1,6—*O*—D—mannitol—di—β—D—glucopyranoside, laminitol and *C*—methyl inositol.[11]

Brown seaweeds contain a wide range of polysaccharides. Sequential methods of extraction utilizing differences in solubility of the various types of polysaccharides have been found to be the most effective method for separation of these polysaccharides. 'Soluble' laminaran may be extracted with cold water while 'insoluble' laminaran is extracted with hot water. These aqueous extracts may, however, be contaminated with fucans and alginates. Extraction of brown seaweeds with aqueous 2% calcium chloride converts the alginic acid in the fronds to calcium alginate thus preventing it from being extracted with dilute hydrochloric acid. Hence it is possible to get a good yield of alginates when the seaweed residue is later extracted with sodium carbonate. Laminarans may be extracted by aqueous 2% calcium chloride together with some 'fucans' while dilute hydrochloric acid extracts only the 'fucans'. Some 'fucans' may also be extracted into the sodium carbonate solution. The sequential extraction procedure used by Mian and Percival[9] and more recently by Venegas Jara[19] was used in the present study (Scheme 1).

Sequential extraction of the dried fronds of *T. conoides* resulted in the separation of several polysaccharide fractions. In the present study the sugar composition of each fraction was analysed by g.l.c. of the derived alditol acetates.

The seaweed residue after extraction with ethanol was treated with aqueous 40% formaldehyde to polymerise phenolic constituents which may otherwise contaminate the polysaccharide fractions.[9] Pre-treatment with formaldehyde was followed by extraction with aqueous 2% $CaCl_2$ to give extract A, which on precipitation with ethanol gave polysaccharide fractions I and II (Scheme 1). Fraction I was found to contain fucose, glucose, galactose and uronic acids as the major constituents with small amounts of the other sugars (Table 2). Fraction II also contained fucose, glucose and galactose as the major sugar constituents but contents of rhamnose and mannose were higher than in Fraction 1. The sugar composition of fractions I and II suggest that the two fractions are probably mixtures of laminarans and fucans.

Table 2. Percentage composition of the crude polysaccharide fractions I — VIII isolated from *Turbinaria conoides*

	I	II	III	VI	V	VI	VII	VIII
Yield(%)	0.12	0.07	1.47	0.03	0.07	0.20	0.15	2.80
Non starch polysaccharides	35.4	52.0	43.1	27.2	43.3	52.0	46.0	8.8
Rhamnose	1.8	3.6	–	0.9	3.1	–	0.5	0.1
Fucose	9.9	18.6	27.4	1.2	21.9	25.1	14.0	2.5
Arabinose	0.2	0.3	0.2	17.3	0.2	0.2	0.2	0.1
Xylose	1.2	1.8	1.0	0.3	1.7	3.0	3.2	0.4
Mannose	1.5	2.5	1.3	0.5	2.6	2.8	5.1	1.0
Galactose	4.4	8.5	8.3	0.7	10.9	10.7	6.6	1.5
Glucose	8.4	13.2	2.0	6.3	2.2	2.1	2.4	0.2
Uronic acids	8.0	3.5	2.9	–	0.7	8.1	14.0	3.0
Crude protein	16.5	*	5.9	*	3.7	2.8	3.6	1.1
Sulphate$(SO_4)^{2-}$	15.3	*	60.9	*	34.8	38.4	38.8	*

*Not determined: – Not detected

Extraction of the seaweed residue with aqueous $CaCl_2$ at 70°C gave extract B which on precipitation with ethanol yielded polysaccharide fractions III and IV. Fraction III was composed mainly of fucose and galactose

(Table 2) with small amounts of the other sugars including uronic acids. Hence fraction III consists mainly of fucans. Aqueous calcium chloride is known to extract both fucans and laminaran. Fraction IV was rich in arabinose and contained little of fucose and galactose. Therefore this fraction contained a lower amount of fucans. The glucose content of fraction IV is significant and indicates the presence of some laminaran. The high content of sulphate in fraction III is probably an overestimation.

Extraction of the seaweed residue after $CaCl_2$ extraction with dilute hydrochloric acid at room temperature and at 70^0C gave extracts C and D respectively. Precipitation of extract C with ethanol gave fraction V which was composed mainly of fucose and galactose with small amounts of rhamnose, mannose and glucose. Fraction VI obtained by precipitation of extract D had a high content of fucose and substantial amounts of glucose and galactose. Rhamnose was found to be completely absent while the uronic acid content was quite high. The sulphate content of both fractions V and VI was high. Fractions III, V and VI had a low content of glucose suggesting that these fractions had little laminaran.

The seaweed residue after the acid extraction was extracted with aqueous 3% sodium carbonate solution. The sodium alginate obtained (Scheme 1) was converted to calcium alginate by treatment with 2% calcium chloride. The alcohol supernatant gave extract E which is referred to as fraction VIII in Table 2. Fraction VIII had a very low content of non—starch polysaccharides of which 3.0% was uronic acids. The supernatant obtained after the precipitation of calcium alginate gave extract F which on precipitation with ethanol yielded polysaccharide fraction VII. Fraction VII had high contents of uronic acid, fucose, galactose and sulphate. The neutral sugar composition of 'fucans' is known to vary from species to species.

4. Conclusion

Compositional analysis of the polysaccharide fractions I—VIII indicate the presence of laminaran in fractions I, II and IV while fractions III, V, VI, and and VII are rich in 'fucans'. Analysis of the alginate fraction has been reported elsewhere.[8]

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