Sterols and Polysaccharides in Freshwater Algae *Spirogyra* and *Mougeotia*

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Several species of freshwater green algae belonging to the order Zygnematales (*Spirogyra crassa* (Ktz.) Czurda, *S. condensata* (Vauch.) Czurda, *S. longata* (Vauch.) Ktz., *S. juergensii* Ktz., *S. olivascens* Rabenh. and *Mougeotia viridis* (Ktz.) Wittr.) were shown to have specific sterol content and characteristic monosaccharide composition of the biomass. Polysaccharide fractions were obtained by stepwise extraction of *S. condensata* and characterised by a determination of monosaccharides liberated after acid hydrolysis. Action of amyloglucosidase was used to prove the presence of starch in algal biomass. The main polysaccharide seems to be a complex mucilage composed of rhamnose, arabinose, xylose, galactose, and uronic acid, and the structure of this mucilage needs further investigation.

Introduction

Till now the chemical composition of the freshwater algae has been less extensively studied than that of marine algae. However, the chemical investigation of freshwater algae can be expected to provide some taxonomically and evolutionary significant results and ecologically important information, taking into account the correlation of chemical structures of algal biopolymers with algal systematics (Painter, 1983) as well as the adaptive dependence of lipid composition on the environment (Dembitsky *et al.*, 1992). In addition, large-scale cultivation of several freshwater algae may be used for the production of some biologically active secondary metabolites and valuable polysaccharides.

There are only scarce data on the chemical composition of the fresh water green algae belonging to the family Zygnemaceae (order Zygnematales). The most numerous representatives of this family in the Bulgarian flora are genera *Spirogyra, Mougeotia* and *Zygnema,* which inhabit rivers and ponds. Because of their specific sexual reproduction and absence of flagellate stage the algae from the family Zygnemaceae have a special position in the phylogenetic scheme of Chlorophyta, between Bryopsidales and Charophyta. Recently we found that a lipophylic extract from *Spirogyra crassa* possesses antibacterial activity (unpublished results). The classification of these algae was not related to their chemical composition. Now we present the preliminary results of chemical characterisation of polysaccharides and sterols in five *Spirogyra* species [*Spirogyra crassa* (Ktz.) Czurda, *S. condensata* (Vauch.) Czurda, *S. longata* (Vauch.) Ktz., *S. juergensii* Ktz. and *S. olivascens* Rabenh.] and in *Mougeotia viridis* (Ktz.) Wittr. Two samples of *S. crassa* collected at two different seasons were investigated.

Materials and Methods

Algal material

Sample of *M. viridis* was collected from a lake at south part of Vitosha mountain in May (21091996).

Samples of *S. crassa* were collected in November (07111996) and May (10051997) at the same place.

Sample of *S. olivascens* was collected in May in a pond in Sofia (09051997).

Sample of *S. condensata* was collected in November in a pond at northern part of Vitosha mountain (05111996).

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Samples of *S. longata* (03061995) and *S. jurgensii* (04061996) were collected in June in ponds at northern part of Vitosha mountain.

Voucher specimens were determined by Dr. St. Dimitrova-Konaklieva and deposited in the herbarium of the Pharmaceutical Faculty in the Medical University, Sofia.

All algal samples were investigated microscopically and bacterial or other cells, different from these of the investigated species, have not been found.

Isolation and identification of sterols

The fresh algal material (100–110 g fresh wt) was homogenised with chloroform-methanol (500 ml, 1:1, v/v) and refluxed for a few minutes in order to inactivate the enzymes. The extraction were repeated three times and the combined extracts were mixed with 400 ml water. After concentration of the lower layer a portion of the total lipophylic extract (35–40 mg) was treated with 15% HCl (4 ml) for 12 h at 55 °C. Then water (5 ml) was added and the mixture was extracted three times with 5 ml hexane (Bligh and Dyer, 1959). The extracts obtained were combined, concentrated and subjected to a preparative thin-layer chromatography on Kieselgel 60 GF_{254} (Merck, Germany) with hexane-diethyl ether (1:1 v/v). The spots were visualised by heating with 50% sulfuric acid. The total sterol mixture obtained was analysed by gas-liquid chromatography (GLC) on a Pye Unicam gas chromatograph equipped with a glass capillary column (15 m x 0.2 mm i.d., OV-17) at 270 °C. The individual sterols were identified by comparison with known samples (cholesterol and sitosterol from Merck, Germany; campesterol, brassicasterol, isofucosterol and stigmasterol were isolated earlier by us from marine organisms and characterised by RRT, mass and NMR spectra. Comparisons were based on RRT and mass spectra. The sterol composition of five samples of algal biomass is given in Table I.

Carbohydrate composition of algal biomass

Dried samples of defatted biomass (10–20 mg) were heated with 2 M CF_3COOH (1 ml containing 0.9 mg of myo-inositol as an internal standard) for 8 h at 100 °C. The neutral monosaccharides released were converted into acetylated alditols by reduction with $NaBH_4$ followed by acetylation with acetic anhydride – pyridine mixture (Sloneker, 1972). The alditol acetates obtained were analysed by GLC on a Hewlett-Packard 5890A gas chromatograph equipped with a flame-ionisation detector, HP Ultra-2 capillary column and HP 3393A recording integrator. Temperature was programmed to hold for 1 min at 175 °C, increase at 10°/min to 290 °C, and hold for 3 min. Individual alditol acetates were identified by comparison of their retention times with those of authentic samples. Peak areas relative to that of myo-inositol acetate were used for quantitative determinations (Sloneker, 1972). Monosaccharide composition of six samples of algal biomass is given in Table II.

An aliquot of the hydrolyzate of *S. condensata* biomass (0.2 ml) was taken off to determine the uronic acid content by colorimetric reaction with 3,5-dimethylphenol in sulfuric acid (Usov *et al.*, 1995), a glucuronic acid (GlcA) solution being used for calibration. The cellulose content of this biomass was determined according to the procedure of Updegraff (1969).

Extraction of polysaccharides

The powdered defatted biomass of *S. condensata* (5.5 g dry wt) was treated with 0.1 M HCl (35 ml), then water (20 ml) was added and the mixture was stirred for 6 h at 15°C. The viscous extract was separated by centrifugation, and the residue was treated as above with 0.1 M HCl (100 ml) and then with water (100 ml). The three extracts obtained were combined, neutralised with NaOH, dialysed, and lyophilised to obtain fraction 1 (0.4 g). The algal residue was extracted three times with 50 mM phosphate buffer pH 7 (150 ml) for 4 h at 85 °C, the combined extracts were dialysed and lyophilised to obtain fraction 2 (0.6 g). Similar treatment with 3% sodium carbonate solution was used to obtain fraction 3 (1.08 g). Extraction of the residue with 1 M NaOH under the same conditions gave rise to fraction 4 (0.8 g). The algal residue was washed several times with water to remove alkali, then washed with acetone and dried *in vacuo* to afford fraction 5 (0.62 g). The neutral monosaccharide composition of fractions determined after hydrolysis using GLC, as described above, is given in Table III.

Amylolysis of fraction 4

Fraction 4 (0.7 g) was stirred with 0.1 M acetate buffer pH 4.9 (100 ml) at 55 °C (the material did not dissolve completely). An amyloglucosidase preparation (Koch-Light, 50 mg) was added to the suspension, and the mixture was left at 55 °C for 24 h. Then the mixture was heated for 15 min at 100 °C, cooled, filtered, an aliquot (2 ml) was taken off to determine free glucose content (using GLC without chemical hydrolysis), which corresponded to 45.75 mg, or 70% of glucose content of the starting material. The residual solution was dialysed, filtered, and lyophilised to afford fraction 4-A. In addition to neutral monosaccharides listed in Table III, fraction 4-A contained 7.45% of uronic acid (as GlcA).

Results and Discussion

The sterol composition of all investigated samples with the exception of *S. crassa* appeared to be similar to that of terrestrial higher plants, C_{29}-sterols being the main sterol components (Table I). Two sterols predominate in *S. crassa*: cholesterol and sitosterol (stereochemistry at C-24 was not established). Their ratio depends on the collection season. High cholesterol content is characteristic for animals, while in plants it was found almost exclusively in red algae. *S. crassa* differs from the other *Spirogyra* species by its unusually thick cell membrane. Cholesterol stabilises the plant cell membranes better than sitosterol (Kniper, 1984) and probably this is the reason for its high concentration in the cell membranes of *S. crassa*. It should be also taken into account that only *S. crassa* among the investigated by us *Spirogyra* species inhabits alkaline waters (pH = 7.5–8.5). *Mougeotia viridis* sterols differ from those of *Spirogyra* species by the higher concentration of sitosterol. The total amounts of C_{29}-sterols in all investigated algae with the exception of *S. crassa* are similar.

The residue obtained after extraction of algal biomass with chloroform and methanol contained mainly polysaccharides and proteins. Hydrolysis of this material under controlled conditions produced the monosaccharide constituents of readily hydrolyzable polysaccharides (cellulose is not splitted), and their qualitative and quantitative analysis was performed by gas-liquid chromatography (GLC). It is evident from Table II that all the species investigated have qualitatively similar monosaccharide composition, but can be separated into three groups according to the quantitative results: (i) *M. viridis*, (ii) *S. crassa*, and (iii) other *Spirogyra* species. Mannose was found to be the main monosaccharide in the hydrolyzate of *M. viridis* biomass, whereas high content of rhamnose, arabinose, xylose, and galactose was detected in three species of *Spirogyra*, namely, in *S. condensata*, *S. olivascens* and *S. juergensii*. The monosaccharide composition of *S. crassa* appeared to be intermediate between these three species and *M.viridis*. It should be noted that glucose content of hydrolyzates (reflecting mainly the amount of starch in the algal biomass, see below) may depend on the physiological status of the alga at the moment of harvesting (compare two samples of *S. crassa* collected during different seasons) and probably has no taxonomical significance.

To obtain more evidence on the polysaccharide composition, a sample of *S. condensata*, (in addition to the data of Table II the material was shown to contain 8.5% of uronic acid as GlcA and 10.75% of cellulose) was subjected to the procedure of fractional extraction, as described in

Table I. Sterol composition of genera *Spirogyra* and *Mougeotia* (% from total sterols).

□ Steroids	RRT	*Sp. condensata*	*Sp. crassa* November	*Sp. crassa* May	*Sp. longata*	*M. viridis*
Cholesterol	1.00	3 %	28 %	90 %	7 %	5 %
Brassicasterol	1.07	4 %	1 %	1 %	2 %	4.5 %
Campesterol	1.15	10 %	1 %	tr	8 %	10.6 %
Stigmasterol	1.24	25 %	2 %	1 %	30 %	5.6 %
Sitosterol	1.31	33 %	59 %	8 %	34 %	74.4 %
Isofucosterol	1.44	12 %	tr %	–	9 %	–

RRT of Cholesterol=1

Table II. Monosaccharide composition of *Spirogyra* and *Mougeotia* biomass after acidic hydrolysis (% of defatted dry biomass).

	Sp. condensata	*Sp. juergensii*	*Sp. crassa* November	*Sp. crassa* May	*Sp. olivascens*	*M. viridis*
Rhamnose	3 %	1.2 %	0.8 %	0.7 %	3.2 %	0.4 %
Arabinose	6.1 %	3.5 %	1.9 %	2.7 %	4.4 %	1.1 %
Xylose	5.3 %	1.9 %	0.9 %	1.6 %	5.8 %	0.7 %
Mannose	0.1 %	0.2 %	1.1 %	0.3 %	0.1 %	5.7 %
Glucose	3.2 %	5.1 %	8.2 %	4.7 %	13.1 %	7 %
Galactose	3.7 %	3.6 %	1.8 %	2.3 %	4.5 %	1 %

Materials and Methods. Fractions solubilized in slightly acidic solution at room temperature and then by heating with neutral buffer, sodium carbonate and sodium hydroxide solutions, respectively, were obtained and characterised by monosaccharide composition. In addition to the data of Table III, fractions 1 and 2 were shown to contain about 10% of uronic acids. Treatment of fraction 4, which had the highest glucose content, with amyloglucosidase resulted in liberation of free glucose, indicating that starch is the source of the main part of this monosaccharide in hydrolyzates of biomass. The algal residue (fraction 5) appeared to be composed mainly of cellulose, rather small amounts of other than glucose neutral monosaccharides being the result of incomplete extraction. But the main water-soluble polysaccharide (fractions 1–3) seems to be a complex mucilage composed of rhamnose, arabinose, xylose, galactose and uronic acid. Its structure and properties evidently need further investigation.

We can conclude that representatives of the order Zygnematales may differ in the metabolism of sugars and sterols. Both groups of compounds show characteristic patterns in the species of *Mougeotia* and *Spirogyra* investigated, and this observation may be used in the chemical taxonomy of these freshwater algae.

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Table III. Yields (% of defatted dry biomass) and monosaccharide composition (%, w/w) of polysaccharide fractions obtained from *S. condensata*.

Fraction	Yield	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose	Total
1	7.3	11.9	21.5	17.1	0.2	5.0	7.2	63.0
2	10.9	9.7	13.4	13.8	-	3.9	5.9	46.7
3	19.6	5.9	11.6	9.9	0.2	3.6	6.2	37.4
4	14.5	0.6	4.4	2.8	-	9.7	4.1	21.6
4-A	14.3*	1.4	11.4	6.7	-	8.7	9.5	37.7
5	11.3	0.2	2.2	1.8	-	0.4	1.7	6.3

* The yield of fraction 4-A as the result of amylolysis of fraction 4 (see Materials and Methods).

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