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LOW MOLECULAR WEIGHT CARBOHYDRATES IN *CYANIDIUM CALDARIUM* AND SOME RELATED ALGAE

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Abstract—Floridoside (2-*O*-glycerol-α-D-galactopyranoside) and a small amount of *iso*-floridoside (1-*O*-glycerol-α-D-galactopyranoside) were found in *Cyanidium caldarium*. Floridoside was also found in the red algae *Porphyridium cruentum* and *Porphyra yezoensis*, although in the latter *iso*-floridoside was the main component. Sucrose and glucose were found in the green algae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*, and also in a blue-green alga, *Anacystis nidulans*. Another blue-green alga, *Phormidium foveolarum*, contains mostly trehalose. From these results and from morphological considerations, it is suggested that *Cyanidium caldarium* belongs to the primitive Rhodophyta.

INTRODUCTION

Cyanidium caldarium, a unicellular alga, is widely distributed in acid hot springs of the world [1–3]. It appears to be a blue-green alga because it contains chlorophyll *a* and phycocyanins. It is, however, a primitive eukaryote having a nucleus, mitochondria and a chloroplast which contains a single-layered thylakoid [4]. Views about the systematic position of the alga are varied in spite of many studies on its ultrastructure, pigments and other chemical components. Thus it has been classified as a blue-green alga, a green alga, a red alga [5] or a transitional form between the blue-green and the red algae [6].

It was reported in a previous paper [7] that the study of the distribution of low MW carbohydrates in red algae is useful in the elucidation of the algal phylogeny. In the present paper, the distribution of low MW carbohydrates in *Cyanidium caldarium* and in six species of the typical algae is reported and the systematic position of the alga is discussed.

RESULTS

Floridoside (2-*O*-glycerol-α-D-galactopyranoside) [8] in the hot ethanolic extract of *Cyanidium* gave chromatographic data identical to those of authentic floridoside. The mass spectrum of its TMSi derivative showed the presence of low intensity ions of *m/e* 686 (M^+: $C_{27}H_{66}O_8 \cdot Si_6$), 671 ($M^+$–Me), 581 [$M^+ - HOSi(Me)_3$], 539, 491 and 451 and of high intensity ions of *m/e* (rel. int.) 217 (19), 204 (100), 147 (17), 103 (22) and 73 (61). The latter ions are also produced from the TMSi derivatives of galactose, glucose, methylglycosides [9, 10] and disaccharide alditols [11]. De Jongh *et al.* [9] reported that the intense peak at *m/e* 204 indicated the presence of the pyranose ring in a sugar molecule. The mass spectrum of the sample was identical with that of authentic floridoside. To confirm the structure of this compound, it was hydrolysed by acid and α-galactosidase (EC 3.2.1.22) [12]. The crude floridoside gave glycerol: galactose ratios (acid, 0.95; enzyme, 1.06) close to the expected value of 1. The same results were obtained from the acid- and the enzymatic hydrolyses of authentic floridoside.

An isomer of floridoside, *iso*-floridoside (1-*O*-glycerol-α-D-galactopyranoside) [13], was also found in the carbohydrate fraction of *Cyanidium*. On α-galactosidase treatment, the substrate, although it was a mixture of *iso*-floridoside (61.1%) and floridoside (37.3%), gave rise to nearly equal amounts of glycerol (40.4%) and galactose (44.1%).

The ethanolic extracts of *Porphyridium cruentum*, *Porphyra yezoensis*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Anacystis nidulans* and *Phormidium foveolarum* were also analysed for the presence of low MW carbohydrates. The results obtained are summarized in Table 1.

DISCUSSION

Neutral low MW carbohydrates, which are photoassimilatory reserve products, are known to vary considerably among the different algal phyla [14]. Green algae (Chlorophyta) contain glucose, fructose or sucrose, the latter being most widely distributed except in *Dunaliella* [15]. It was confirmed here that sucrose is the main reserve product in both *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Table 1). The soluble sugars of the Cyanophyta suggest that these algae may be divided into those containing glucose, fructose or sucrose, and those which contain trehalose. From this study (Table 1), *Anacystis nidulans* belongs to the former and *Phormidium foveolarum* to the latter.

Floridoside and *iso*-floridoside are considered to be one of the phylogenic criteria of Rhodophyta [7]. The fact that floridoside and *iso*-floridoside are found in *Cyanidium caldarium* (Table 1) suggests that it is more closely related to red algae than to blue-green or green algae. It is of great interest that *iso*-floridoside, as well as sucrose and glucose,

Table 1. Distribution of neutral low MW carbohydrates in several algae

Algae	Dry wt (g)	Total* carbohydrate (mg)	(% of dry wt)	Amount (% total carbohydrate)								
				Flo†	Isf	Glc	Fru	Hex	Suc	Mal	Tre	US
Cyanidium caldarium	2.23	4.40	0.20	81.2	7.1	—	—	—	—	—	—	3.0
Rhodophyta												
Porphyridium cruentum	0.38	4.15	1.08	73.7	—	2.7	—	8.5	—	5.9	—	—
Porphyra yezoensis	0.40	2.08	0.51	11.1	50.8	—	—	—	—	—	—	20.1
Chlorophyta												
Chlorella pyrenoidosa	1.70	47.5	2.79	—	—	6.0	0.6	—	93.0	—	—	—
Scenedesmus obliquus	0.59	2.12	0.36	—	—	9.0	5.0	—	66.3	—	—	14.2
Cyanophyta												
Anacystis nidulans	0.38	8.74	2.28	—	—	16.7	—	—	35.0	—	—	36.7
Phormidium foveolarum	0.26	0.38	0.18	—	—	—	—	3.2	—	—	86.6	—

* Estimated as glucose equivalents by phenol–H_2SO_4 reagent.

† Flo, floridoside; Isf, *iso*-floridoside; Glc, glucose; Fru, fructose; Hex, hexitol; Suc, sucrose; Mal, maltose; Tre, trehalose; US, main unidentified substances.

is found in the Chrysophyta, *Ochromonas malhamensis* [16]. However, the alga is different from *Cyanidium* or red algae on many taxonomic criteria. Thus it can swim by the action of two unequal flagella and its chloroplast has triple layered thylakoids and contains large amounts of carotenoids but no biliproteins [17]. In contrast, the unicellular red alga *Porphyridium cruentum* has a biliprotein (phycoerythrin) and has one chloroplast containing single-layered parallel thylakoids [18]. Our findings and recent studies by Seckbach *et al.* [19, 20] on the morphology and lipid composition of *Cyanidium* support the idea proposed first by Hirose [5] that *Cyanidium* belongs to the primitive Rhodophyta.

A bibliography of *Cyanidium* studies has been prepared by Fukuda [21].

EXPERIMENTAL

Algal material. Cyanidium caldarium (Tilden) Geitler RK-1 was isolated by Fukuda [22] from Yumoto-spa, Nikko, Japan. *Anacystis nidulans* (P. Richt) Drouet & Deiley M-6, *Phormidium foveolarum* (Montagne) Gomont M-43, *Porphyridium cruentum* Nägeli R-3, *Chlorella pyrenoidosa* Chick C-28 and *Scenedesmus obliquus* (Turpin) Kützing C-72 were kindly provided from the algal collection of the Institute of Applied Microbiology, the University of Tokyo, Japan. *Porphyra yezoensis* Ueda was courteously supplied by Yamamoto Nori Kenkyu-sho, Tokyo, Japan.

Culture. Cyanidium was grown in inorganic medium [3] at 35° with continuous aeration (5% CO_2 in air) and illumination (fluorescent light, 3000 lux). *Chlorella* was grown in MC-medium [23] at 25° under the same culture conditions as *Cyanidium*. Other algae were grown in shake culture (media dispensed in 500 ml flasks) at 25° under fluorescent light (2000 lux). The inorganic culture media [23] used were: MDM for *Anacystis* and *Phormidium*, ASP for *Porphyridium* and MC for *Scenedesmus*.

Preparation of neutral low MW carbohydrates. After 2 weeks' cultivation, algal cells were harvested by centrifugation and washed with H_2O (×3). The yields (g fr. wt) of cells were: *Cyanidium*, 10.8; *Porphyridium*, 2.7; *Porphyra*, 5.0; *Chlorella*, 18.0; *Scendesmus*, 4.1; *Phormidium*, 15.5; *Anacystis*, 4.5. Carbohydrates were extracted from the cells by boiling with 80% EtOH for 15 min (×3). The EtOH-soluble fractions were combined, evapd to dryness at <40° and the lipids and pigments removed with Et_2O and petrol. The Et_2O-insoluble fractions were dissolved in H_2O and deionized by passage through the ion-exchange resins Amberlite IR-120 and IRA-410. The neutral fractions were filtered and the filtrates (these contain the neutral low MW carbohydrates) analysed by PC and GLC.

Chromatography. Two solvent mixtures were used for PC: solvent 1, *n*-BuOH–EtOH–H_2O (5:2:2); solvent 2 EtOAc-pyridine–H_2O (8:2:1). Papers were developed for 2 days at room temp. and the sugars detected [7] with $AgNO_3$ reagent for low MW carbohydrates, aniline phthalate reagent for reducing sugars, and resorcinol reagent for ketoses. The amounts of low MW carbohydrates were estimated as glucose equivalents with phenol–H_2SO_4 reagent [24]. Authentic floridoside was isolated and crystallized from the red alga *Serraticardia maxima* [25]. Authentic *iso*-floridoside was kindly provided by Dr. B. Lindberg, Sweden. $R_{glucose}$ (R_g) of standard samples in solvent 1 (solvent 2) were: glucose, 1.00 (1.00); glycerol, 2.92; fructose, 1.15 (1.45); mannitol, 1.07 (0.94); floridoside, 1.02 (0.67); galactose, 0.92 (0.80); *iso*-floridoside, 0.86 (0.48); sucrose, 0.73 (0.39); trehalose, 0.60 (0.19); maltose, 0.55 (0.31); inositol, 0.50 (0.19); lactose, 0.35 (0.16).

TMSi derivatives [26] of carbohydrate fractions were analysed by GLC: FID; glass column (2 m × 3 mm) packed with 1.5% SE-30, 3% OV-17 or 5% SE-52 on Gaschrom-Z; temp. program, 140–270° at 4°/min; N_2 flow rate, 50 ml/min; injector temp., 270°. RR_is of the TMSi derivatives of standards on 1.5% SE-30 (3% OV-17) were: inositol, 1.00 (1.00); glycerol, 0.049 (0.061); fructose, 0.57 (0.61); glucose 0.70 and 0.87 (0.78 and 0.93); galactose, 0.60, 0.66 and 0.73 (0.71 and 0.78); mannitol, 0.82 (0.72); floridoside, 1.29 (1.36); *iso*-floridoside, 1.34 (1.41); lactose, 1.65 and 1.78; sucrose, 1.81 (1.87); maltose, 1.92 and 1.99 (1.96 and 2.00); trehalose, 1.97 (2.00).

GC–MS (70 eV): a glass column (2 m × 3 mm) packed with 2% OV-17 on Gaschrom-Z; isothermal, 200°; ion source, 270°, molecular separator, 230°; He flow rate, 30 ml/min.

Cyanidium caldarium. Floridoside in the ethanolic extracts was identified by PC, GLC and GC–MS. Crude floridoside, prepared by prep. PC, was hydrolysed by 1 N H_2SO_4 at 100° for 3 hr and the hydrolysate neutralized with $Ba(OH)_2$. Authentic floridoside was acid-hydrolysed by the same method. The crude floridoside from *Cyanidium* or authentic floridoside was also hydrolysed by α-galactosidase (EC 3.2.1.22) from the mould *Mortierella vinacea* [12], which was kindly provided by Dr. T. Yoshikawa, Kitasato University, Tokyo. Reaction mixture: 0.1 ml sample, 0.1 ml 0.1 M NaOAc buffer (pH 4.5), 0.1 ml 0.1% α-galactosidase soln. The mixture was incubated at 30° for 1 hr, after which time it was boiled for 2 min and then directly analysed by GLC. *Iso*-floridoside was also detected in the alga by PC and GLC. Crude *iso*-floridoside, prepared by preparative PC, was hydrolysed by α-galactosidase under the same conditions as those used for the hydrolysis of floridoside. Other substances were present in the alga, one of which constituted 3.0% of the fraction and had an R_g value of 0.52 in solvent 1. The RR_is of its TMSi derivative were 1.10 (5% SE-52), 1.16 (1.5% SE-30) and 1.17 (3% OV-17). These values were slightly higher than those of inositol.

Porphyridium cruentum. The carbohydrate fraction was analysed by PC and GLC. The hydrolysate (1 N H_2SO_4 at 100° for 3 hr) of the separated floridoside was analysed by PC and GLC. Maltose in the fraction was then hydrolysed by α-glucosidase (EC 3.2.1.20) from *Aspergillus niger*. Reaction mixture: 0.1 ml sugar fraction, 0.1 ml 0.1 M citrate–Pi buffer (pH 6.4), 0.1 ml enzyme soln (2.5 mg/ml). After 1 hr at 37°, it was found by PC and GLC that maltose had decreased and glucose was present in the fraction. Identical results were obtained with authentic maltose.

Porphyra yezoensis. *Iso*-floridoside, floridoside and other non-reducing substances were detected in the carbohydrate fraction by PC and GLC.

Chlorella pyrenoidosa *and* Scenedesmus obliquus. Sucrose, glucose and fructose were detected in the sugar fractions by PC and GLC. On spraying with resorcinol, sucrose and fructose produced red coloured spots characteristic of ketohexoses. Sucrose in the fractions was hydrolysed by invertase (EC 3.2.1.26). Reaction mixture: 0.1 ml sugar fraction, 0.1 ml 0.1 M NaOAc buffer (pH 4.5), 0.1 ml enzyme soln (2.5 mg/ml). After 1 hr at 37° it was found by PC and GLC that all the sucrose had been utlized and glucose and fructose were present.

Anacystis nidulans. Sucrose, glucose and an oligosaccharide were detected in the carbohydrate fraction by PC and GLC.

Phormidium foveolarum. α,α-Trehalose and a kind of hexitol were detected in the carbohydrate fraction by PC and GLC.

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