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HIGH LEVELS OF PHENOLIC COMPOUNDS IN *PROCHLORON* SPECIES

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Key Word Index –*Prochloron*; Prochlorophyceae; Cyanophyceae; didemnids; symbiotic algae; protein extraction; phenolic compounds.

Abstract—Strains of the prokaryotic alga *Prochloron*, occurring internally in a variety of ascidian hosts in the South Pacific Ocean, were determined to have high intracellular concentrations of phenolic compounds, ranging from 1.8 to 7.1% of the cell dry weight. Only the externally occurring *Prochloron* from *Didemnum candidum*, a species found in the Gulf of California, exhibited low concentrations of phenolic compounds. Investigations with enzyme protectants and the lack of intracellular coagulation in *Prochloron* from *D. candidum* suggest that phenolic substances may be the cause of intracellular coagulation in other strains of *Prochloron*. This process has inhibited the extraction and study of enzymes from these unique algae.

INTRODUCTION

Prochloron species are phototrophic prokaryotes containing chlorophylls *a* and *b* and lacking phycobilisomes. They are symbionts of didemnids, a group of colony-forming ascidians [1], and have also been reported to occur on colonies of bryozoa [2]. A great amount of research interest has been focused on the prochlorophytes because they have been suggested as a possible ancestor of the chloroplasts of the Chlorophyceae and higher plants [3]. Some research has cast doubt on this hypothesis suggesting that phylogenetically *Prochloron* are members of the Cyanophyceae (blue-green algae) [4]. However, one of the best fits of existing data has been obtained by a phylogenetic tree placing the origin of *Prochloron* closer to the point of divergence of chloroplasts and cyanobacteria [5].

Very little is known about the biochemistry of *Prochloron*. Biochemical studies have been hampered by two factors: a lack of a method for culturing *Prochloron* in the laboratory [1]; and the difficulty in extracting soluble protein and active enzymes from these algae [6]. With regards to the latter problem, only superoxide dismutase [1], ribulose bisphosphate carboxylase [7], glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [6] have been detected in *Prochloron*. When *Prochloron* spp. are isolated from their host they appear to undergo rapidly an irreversible loss of cell fragility. This process, termed intracellular coagulation, is accompanied by a redistribution of thylakoids around the cell periphery, a loss of photosynthetic O_2 production, and a drastic decrease in the extractability of cell proteins [6].

The mechanism of intracellular coagulation and inextractability of protein in *Prochloron* is currently unknown. However, in some species of algae and many higher plants, difficulties with protein extraction are the result of a high concentration of phenolic compounds in the cells. Phenolic compounds combine reversibly with proteins through hydrogen bonding and irreversibly by oxidation of phenols to quinones with the resulting copolymerization of quinones and proteins [8]. The purpose of the research presented here was to determine whether phenolic compounds are present in *Prochloron*, and if present, to determine whether these compounds were the cause of the poor extractability of cellular proteins in *Prochloron*.

RESULTS

To determine whether *Prochloron* contains polyphenols, we used the Folin–Ciocalteau procedure developed by Jennings [9] to assay for total phenol content in plant extracts. Freeze-dried *Prochloron* samples were extracted under nitrogen with 80% ethanol at 100° for 2 hr and extractable phenolics assayed.

The concentrations of phenolics detected in this way, in *Prochloron* from various hosts and locations, are shown in Table 1. The concentration of phenols in *Prochloron* from the South Pacific Region was high, ranging from 1.8 to 7.1% of the dry weight. *Prochloron* from *Lissoclinum voeltzkowi* and *Diplosoma similis* had the highest levels of phenols. Concentrations of phenolic compounds from the same *Prochloron* spp. collected on different dates and from different locations were relatively similar. The phenol content of the host ascidians (without *Prochloron*) was very low. Samples of *L. patella* contained an average of only 0.3% phenolic compounds as dry weight. The only *Prochloron* sp. with a low level of phenolic compounds was the species found on *Didemnum candidum* from the Gulf of California. Phenolic concentrations in this *Prochloron* averaged 0.20% of the dry weight. In com-

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Table 1. Concentrations of phenolic compounds in *Prochloron* spp. from various locations

Host	Location of collection	Date of collection	Phenol content (% dry wt)
Didemnum candidum	Puerto Penasco, Mexico	April 1983	0.2
D. molle	Palau	March 1983	1.8
Diplosoma similis	Palau	Feb. 1982	4.3
D. similis	Palau	March 1983	6.3
Lissoclinum patella	Great Barrier Reef	April 1983	2.3
L. patella	Great Barrier Reef	July 1983	3.1
L. patella	Palau	Feb. 1982	3.4
L. patella	Palau	March 1983	3.4
L. patella	Solomon Islands	March 1983	4.4
L. patella (without *Prochloron*)	Palau	Feb. 1979	0.4
L. patella (without *Prochloron*)	Palau	Feb. 1982	0.2
L. voeltzkowi	Palau	Feb. 1982	7.1
L. voeltzkowi	Palau	Feb. 1983	5.1
Trididemnum cyclops	Palau	Feb. 1982	2.1
T. cyclops	Palau	March 1983	3.8
Unidentified host	Solomon Island	March 1983	4.7

Table 2. Concentrations of phenolic compounds in selected blue-green algae

Blue-green algal species	Phenol content (% dry wt)
Anabaena flos-aquae (UTEX 1444)	0.92
A. cylindrica (UTEX LB1661)	0.91
A. inequalis (UTEX LB3817)	0.90
A. spirodes (UTEX 1552)	0.58
Nostoc muscorum (UTEX 387)	1.05
N. commune (UTEX 584)	0.34
Microcoleus vaginatus (UTEX 1815)	0.62
Gleocapsa sp. (UTEX LB795)	0.03
Synechoccus leopoliensis (UTEX 625)	0.83

parison with *Prochloron*, phenolic concentrations in other blue-green algae were generally less than 1% of their dry weight (Table 2).

In order to establish that material detected in the colorimetric assay was actually phenolic, we used a chromatographic procedure followed by detection by fluorescence and/or colour development [10]. Folin-positive spots were noted and chromatograms were then examined under fluorescent light and then developed with Gibbs reagent or diazotized *p*-nitroaniline. The R_f values and developed colours of the phenolic compounds from the *Prochloron* spp. are shown in Table 3. The R_f values and colours of the phenolic substances in *Prochloron* from *L. patella* obtained from Palau, the Solomon Islands and Australia were all very similar. The data also indicate that *L. bistratum Prochloron* and *Trididemnum cyclops Prochloron* appear to possess a similar phenolic compound. *Didemnum punctatum Prochloron*, *Diplosoma virens Prochloron* and *L. voeltzkowi Prochloron* all have phenolic compounds with R_fs of 0.73 in solvent B but the phenols have different colours with the various sprays employed in this study. *L. voeltzkowi* also appears to have two dominant phenolic compounds. None of the developed colours or R_f values exactly matched those of the compounds we used as standards or those reported by Van Sumere *et al.* [10].

In order to determine whether proteins in *Prochloron* were binding with phenolic compounds, several enzyme protectants were evaluated to analyse their contribution to soluble-protein recovery from the cells. PVPP with ascorbic acid exhibited the greatest amount of soluble protein released from the *Prochloron* cells (Table 4). The extract with these protectants contained almost six times as much soluble protein as the control and three times more than the next best treatment, PVP with ascorbic acid. Treatments with PVP and sodium pyruvate or sodium bisulphite exhibited no significant increase in soluble protein released over the control. Despite the increase in soluble protein in the PVPP extract, no enzyme activity was detected in the supernatant for the four enzymes evaluated.

DISCUSSION

Most of the *Prochloron* from the various hosts evaluated in the study possessed high levels of phenolic compounds. These concentrations are similar to those reported in brown and red algae [11, 12] and much higher than the concentrations generally found in blue-green algae. The polyphenolic substances found in brown algae are known to interfere with the extraction of soluble proteins from their cells. The concentrations of phenolic compounds reported in this study are probably minimal values because we were not extracting from 'fresh material', and various biochemical processes may have reduced the amount of phenols or inhibited their extraction. Alkaline or enzymatic hydrolysis may release additional phenolic compounds chemically linked to proteins.

We tested several additives for their ability to prevent the condensation of proteins in *Prochloron* extracts. They were (1) polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP) to bind phenolics, (2) sodium bisul-

Table 3. R_f and developed colours* of the phenolic compounds in *Prochloron* spp. from various hosts

Prochloron†	Folin-positive spots	R_f $CHCl_3$-HOAc H_2O (4:1:1)	R_f MeOH HOAc H_2O (4:1:1)	Colour with diazotized nitroaniline	Colour with Gibbs reagent	Fluorescence in UV light (253 nm) Before NaOH	After NaOH
LPP (Palau)	a	0.77	0.82	Raw sienna	Grey	Light blue	Yellow
	b		0.74	Van Dyke brown	Grey	Absorbs	Absorbs
LPP (Australia)	a	0.76	0.82	Raw sienna	Grey	Light blue	Yellow
	b		0.74	Van Dyke brown	Grey	Absorbs	Absorbs
LPP (Solomon Islands)	a	0.76	0.84	Raw sienna	Grey	Light blue	Yellow
	b	-	0.74	Van Dyke brown	Grey	Absorbs	Absrobs
DCP (Mexico)	n.d.‡	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DPP (Palau)	a	0	0.73	Van Dyke brown	Brown	Dark orange	Orange brown
DVP (Palau)	a	0	0.73	Purple lake	Blue grey	Dark orange	Yellow
LVP (Palau)	a	0	0.74	Yellow ochre	Van Dyke brown umber	Orange	Yellow
	b	0	0.85	Raw umber	Brown	Yellow	Yellow
LBP (Palau)	a	0	0.85	Raw sienna	Brown	Absorbs	Absorbs
TCP (Palau)	a	0	0.83	Raw sienna	Brown	Absorbs	Absorbs

*Colours were matched against Windsor & Newton artist oil colours (Windsor & Newton, Secaucus, NJ 07094) and Liquitex artist watercolours (Binney & Smith, Easton, PA 18042). Reference phenolic compounds employed: 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, 7-hydroxycoumarin, *p*-coumaric acid, 3-hydroxyphenylproponic acid, *p*-hydroxyphenylacetic acid, 2,3-dibromo-4,5-dihydroxybenzyl alcohol and vanillin.

†LPP, *Lissoclinum patella Prochloron*; DCP, *Didemum candidum Prochloron*; DPP, *Didemnum punctatum Prochloron*; DVP, *Diplosoma virens Prochloron*; LVP, *Lissoclinum voeltzkowi Prochloron*; LBP, *Lissoclinum bistratum Prochloron*; TCP, *Trididemnum cyclops Prochloron*.

‡n.d., None detected.

phite to inhibit polyphenoloxidase and trap aldehydes, (3) sodium pyruvate to trap H_2O_2, and (4) sodium ascorbate to act as a reducing agent for any quinones that were formed. Of these agents, only PVPP appeared to be effective in increasing the amount of soluble protein obtained from *Prochloron* cells. Loomis and Battaile [8] have reported that PVPP is one of the most effective agents for preventing the hydrogen bonding of proteins by phenolic compounds. PVPP contains a large number of groups similar to the peptide linkage so the phenolics bind to PVPP rather than to proteins. Extracts of *Prochloron* containing PVPP showed a six-fold increase in soluble protein over the control. The extraction efficiency for soluble proteins could possibly be increased in future studies of *Prochloron* by utilizing extraction buffers with a lower pH. All of the extracts were in Tris-buffered seawater at pH 9.0. PVPP is more effective at pHs < 7.2 because ionization of the phenols is suppressed and they can bind more easily with PVPP [7]. Ionized phenols are also oxidized more readily to quinones than unionized forms.

None of the R_f values or developed colours of the phenolic compounds from the *Prochloron* spp. closely matched the standards employed in this study or those reported by Van Sumere *et al.* [10]. The closest similarity was obtained between the phenolic substance in LVP and 2,3-dibromo-4,5-dihydroxybenzyl alcohol. The only difference noted between these compounds was that the phenolic substance in LVP had a R_f value of zero in solvent A while R_f of our standard, 2,3-dibromo-4,5-dihydroxybenzyl alcohol, was 0.17 in the same solvent system. All other R_f values and developed colours for these compounds were similar. Brominated phenols have been reported in other marine blue-green algae [13] and

Table 4. The effect of various enzyme protectants on the extractability of soluble protein from *Lissoclinum patella Prochloron*

Enzyme protectants*	Soluble protein ($\mu g \times 10^6$ cells $^{-1}$)
PVP† + NaCl	106.1
PVP + Na bisulphite	138.1
PVP + Na ascorbate	206.0
PVP + Na pyruvate	101.3
PVPP‡ + Na ascorbate	587.8

*Extraction solutions contained 35 mg/ml PVP or PVPP, 0.5 mg/ml EDTA and 27 mg/ml NaCl. Sodium bisulphite, sodium ascorbate and sodium pyruvate concentrations were 2.5 mg/ml.

†PVP, Polyvinylpyrolidone.

‡PVPP, Polyvinylpolypyrrolidone.

in red algae [14]. Recommended procedures for identification of naturally occurring phenolic compounds includes gas chromatography/mass spectrometry of methylated and trimethylsilylated derivatives [12]. We were unable to complete these procedures because only a few milligrams of each *Prochloron* spp. was available for all our analyses.

The lowest concentration of phenolic compounds in any of the *Prochloron* spp. tested in this study was found in *Prochloron* from *Didemnum candidum*. This *Prochloron* and its didemnid host are found in the Gulf of California [15]. They were the only *Prochloron* spp. that live externally on ascidians evaluated in this study. This is also the only *Prochloron* strain noted to date which does not exhibit intracellular coagulation. The high concentrations of phenolic compounds in the internally occurring *Prochloron* spp. may suggest that the phenolic substances serve as a sort of 'protective compound' in much the same manner as they do in many higher plants [16, 17]. The phenolic compounds could serve to inhibit the phagocytosis and digestion of *Prochloron* cells by the ascidians. However, Cox [18] has produced serial-section electron micrographs which suggest that phagocytosis of *Prochloron* cells by amoebocytes occurs in the ascidian *Lissoclinum voeltzkowi*. The phenolic content of many more externally occurring *Prochloron* needs to be quantified before this hypothesis can be tested.

It has recently been reported that methanol extracts of *D. molle* containing *Prochloron* exhibit cytostatic activity when tested in cultures of L5178$_y$ mouse lymphoma cells [19]. It could be possible that the phenolic compounds in *Prochloron* are the source of this activity.

The similar R_f values and developed colours of the phenolic substances in LLP from three distinct locations, Australia, Palau and the Solomon Islands, offers additional evidence that they are all the same species of *Prochloron*, as suggested by recent DNA-DNA reassociation studies [20]. The differences in R_f values and colours in the other *Prochloron* spp. evaluated may be the result of different precursor compounds supplied by the various host ascidians. Transfer of photosynthate from *Prochloron* to the ascidians has been reported [21, 22], but transfer of compounds in the opposite direction remains to be demonstrated.

In conclusion, the PVP/PVPP experiments and the correlation between the lack of intracellular coagulation and low phenolic concentration in *Prochloron* from *D. candidum* suggest that phenolic compounds are involved in the intracellular coagulation of some *Prochloron* spp. It was not determined whether these phenols were the same compounds as those isolated by the thin-layer chromatographic procedures. Further studies are needed to determine which of the presumptive phenolic compounds in *Prochloron* are possible tannins, phenols which react with proteins. The biochemical role of these phenolic compounds in *Prochloron* also remains to be determined. However, the apparent chemical diversity of these compounds adds to the complicated biological picture that has been developed for these unique algae.

EXPERIMENTAL

Chemicals. Reagent-grade chemicals were used whenever possible. Phenolic compounds for use as standards were obtained from Sigma, Aldrich Chemicals and ICN Pharmaceuticals.

Algae. Prochloron material from Palau and the Solomon Islands was collected during the 7th and 8th International Prochlorophyte Expeditions (Feb. 1982 and March 1983). *Prochloron* from *Lissoclinum patella* in Australia was collected in March and June 1983. *Prochloron* from *Didemnum candidum* in Puerto Penasco, Mexico was collected by the authors in May 1983. In all cases the *Prochloron* spp. were squeezed from the hosts into filtered, buffered seawater, centrifuged and then freeze-dried.

Quantification of phenolic compounds. The phenolic compounds were extracted in 80% EtOH under N_2 for 2 hr at 100°. They were quantified with Folin-Ciocalteau reagent according to the procedure of Jennings [9] and reported as % dry wt (salt-free) of *Prochloron* cells. For comparative purposes, several cultures of blue-green algae were obtained from the University of Texas culture collection. The blue-green algae were *Anabaena flos-aquae* (UTEX 1444), *A. cylindrica* (UTEX LB1611), *A. inequalis* (UTEX LB3817), *A. spirodes* (UTEX 1552), *Nostoc muscorum* (UTEX 387), *N. commune* (UTEX 584), *Microcoleus vaginatus* (UTEX 1815), *Gleocapsa* sp. (UTEX LB795) and *Synechoccus leopoliensis* (UTEX 625). The blue-green algae were grown in the following medium (concn/l.): 360 mg KNO_3; 100 mg $MgSO_4 \cdot H_2O$; 32 mg $CaCl_2 \cdot 2H_2O$; 160 mg $K_2HPO_4 \cdot 3H_2O$ 16 ml 0.2 M borate buffer (pH 8.0); and 1 ml of a trace metal soln containing (per l.): 2.85 mg H_3BO_3; 1.80 mg $MnCl_2 \cdot 4H_2O$; 1.3 mg $FeSO_4$; 1.77 mg Na tartrate; 0.27 mg $CuCl_2$; 0.21 mg $ZnCl_2$; 0.41 mg $CoCl_2 \cdot 2H_2O$; and 0.25 mg $Na_2MO_4 \cdot 2H_2O$. The cells were grown at 22° under fluorescent lights and harvested during exponential growth and their phenol content was determined as outlined above.

Paper chromatography. For PC, the phenols were extracted as described previously and then heated at 100° for 1 hr in N_2 in 2 M NaOH to hydrolyse any glycosidic linkages. The extraction solns were then acidified to pH 1.0, the phenols partitioned into Et_2O and spotted on Whatman 5681 chromatography paper. Development of the chromatograms was carried out at room temp. in one of the following solvent systems: (A) $CHCl_3$-HOAc-H_2O (4:1:1); (B) MeOH-HOAc-H_2O (4:1:1); or (C) 2% HCO_2H. The chromatograms were removed after the solvent had ascended 10 cm from the origin. Development time was approximately 1 hr in these solvent systems.

Detection of the compounds was carried out by the following procedures: (1) examination of the chromatograms under UV light (253 nm) both before and after spraying with 2 M NaOH; and (2) by spraying the chromatograms with diazotized *p*-nitroaniline [10], Folin reagent [23], or Gibbs reagent [23]. Not

enough *Prochloron* material was available to identify the phenolic compounds by GC/MS.

Protein extraction. In Palau, *Prochloron* spp. from *Lissoclinum patella* were squeezed into buffered seawater (pH 8.0) containing PVP (70 mg/ml) and EDTA (1 mg/ml) and one of the following protectants at a concn of 5 mg/ml: Na bisulphite, Na ascorbate, Na pyruvate, or NaCl (as a control). In one additional treatment with Na ascorbate and EDTA at the above concn, PVPP was substituted for PVP.

The cell solns were then immediately frozen and transported back to the United States. After defrosting, the solns were passed twice through a French Press (20000 psi). The pressate was centrifuged (25000 *g*) for 30 min and the resulting supernatant was analysed for soluble protein by the Coomassie blue method [24]. The supernatant was also analysed for activity of the following enzymes: catalase, phenoloxidase, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphoglucanate dehydrogenase (6PGD). Catalase was analysed with an oxygen electrode. Activity of phenoloxidase was quantified by the method of Baldry *et al.* [25]. G6PD and 6PGD, dehydrogenases known to be present in blue-green algae [26], were assayed at pH 7.4, otherwise as described previously [27].

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