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BIOCHEMICAL STUDIES OF ANTARCTIC ALGAE: DETECTION OF FERREDOXIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: Two clones of *Chlorella* (A1 and A2) were isolated from an algal mat collected from a fresh-water pond, Lake Yukidori, Langhovde, and were cultured in the laboratory. Ferredoxins were isolated from these *Chlorella* cells and analyzed by high performance liquid chromatography (HPLC) using a TSK-gel Phenyl-5PW column for hydrophobic chromatography and a DEAE-5PW column for ion-exchange chromatography. *Chlorella* A1 cells contained two distinct ferredoxins separable by hydrophobic but not by ion-exchange chromatography. *Chlorella* A2 cells also contained two distinct ferredoxins whose retention times in the hydrophobic HPLC analysis were different from these of ferredoxins in A1 cells.

Ferredoxins were also isolated directly from an algal mat collected at another fresh-water pond, Lake Skallen Ôike, Skallen. Cyanobacterial and non-cyanobacterial ferredoxins were separately extracted from the algal mat. Cyanobacterial ferredoxins extracted by sonication of the mat in 35% saturated ammonium sulfate were separated into four peaks by the hydrophobic chromatography. Non-cyanobacterial ferredoxins, extracted from acetone powder of the mat debris remaining after extraction of cyanobacterial ferredoxins, were separated into two peaks. This result suggests that the algal mat contains at least two cyanobacterial and one non-cyanobacterial species.

1. Introduction

Despite much efforts in the research on Antarctic organisms, investigations in biochemical aspects have remained to be done. The present work was undertaken in an attempt to promote such biochemical studies on Antarctic algae. In this study, our attention was focused on ferredoxin, an iron-sulfur protein with a low molecular weight. It has been well known that ferredoxin is distributed in all photosynthetic organisms from blue-green algae to higher plants and plays the role of a key substance of biochemical events in photosynthesis. Although no information has been obtained about ferredoxin in Antarctic algae, it must be certain that ferredoxin is involved in the pathway of photosynthesis in these organisms also. In this work, we detected ferredoxins by an analytical method using an HPLC* system in the cultured cells of Antarctic *Chlorella*

* **HPLC**: High performance liquid chromatography.

and also in an algal mat piled up at the bottom of a fresh-water pond in the Antarctic. This is the first information about ferredoxin in Antarctic organisms and the presence of ferredoxin in the algal mat provides evidence that some organisms are actually performing photochemical events in the extremely cold climate of the Antarctic.

2. Materials and Methods

2.1. Algal materials

2.1.1. Antarctic *Chlorella* A1 and A2

These *Chlorella* were isolated from the algal mat, which was collected at Lake Yukidori, Langhovde, in January 1983, by Prof. Y. OHYAMA and was brought back to the National Institute of Polar Research, Tokyo, in a frozen state (at −20°C). The KRATZ-MYERS' culture medium C (KRATZ and MYERS, 1955), 50 m*l*, was added to a portion of the collected algal mat in a small flask (100 m*l*) and the mixture was kept at 10°C under illumination. Green algae proliferated in the supernatant were then transferred onto an agar plate and well-grown colonies were selected for a large scale culture. The large scale culture was performed at 24°C in 1.5*l* shake flasks containing KRATZ-MYERS' medium C. The cells were aerated with air containing 3% CO_2. Continuous illumination was provided by fluorescent lamps (120 $\mu Em^{-2}s^{-1}$). After one week of culture, cells were harvested by centrifugation at 3000×g for 5 min and subjected to the ferredoxin analysis.

2.1.2. Algal mat

The algal mat was collected at Lake Skallen Ôike, Skallen, in January 1984, by E. TAKAHASHI and was brought back in a frozen state (at −20°C).

2.2. Measurement of optimal temperature for the growth of Chlorella

Temperature dependence of the growth of *Chlorella* cells was examined using a Toyo Temperature Gradient Biophotorecorder, model TN-112E (Toyo Co., Tokyo). Cells were grown in L-shaped test tubes containing 10 m*l* each of KRATZ-MYERS' medium C under illumination with fluorescent lamps (80 $\mu Em^{-2}s^{-1}$). The cells were aerated with air containing 3% CO_2. Ten tubes containing algal cells were placed on an aluminum block whose temperature was kept at 6.5° to 36°C as a gradient. The temperature of each cell suspension was measured by a thermocouple implanted in the aluminum block. The Biophotorecorder recorded the growth of cells at each temperature as changes in turbidity of the cell suspension.

2.3. Extraction and isolation of ferredoxins from Antarctic algae

2.3.1. *Chlorella* A1 and A2

Chlorella cells (12.2 g in wet weight) were suspended in 80% acetone chilled previously in a deep freezer and centrifuged. The precipitates were washed twice with chilled acetone and dried in air. The acetone powder was suspended in 25 m*l* of an extraction medium (20 mM Tris-HCl buffer, pH 7.5, containing 35% saturated ammonium sulfate) and was sonicated with an ultra sonic disruptor (UV-200P, Tomy Seiko Co., Ltd., Tokyo) at output level 7 for 10 min in an ice bath. Then the suspension was centrifuged at 18000×g for 20 min. The precipitated cell debris was resuspended in 10 m*l* of the same

extraction medium, sonicated, and centrifuged. The first and second supernatants were combined and supplemented with ammonium sulfate to 75% saturation. The resultant green precipitate was removed by centrifugation at 18000×g for 20 min and the supernatant was loaded on a TSK-gel Toyopearl HW-65C column (2×4 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 75% saturated ammonium sulfate. Ferredoxins were adsorbed at the upper portion of the column. After the column was washed with the same Tris-HCl buffer containing 50% saturated ammonium sulfate, ferredoxin was eluted with the Tris buffer containing 35% saturated ammonium sulfate. The eluate was supplemented with ammonium sulfate to 60% saturation and was loaded on a Butyl-Toyopearl 650 M column (1×3 cm) equilibrated with the Tris buffer containing 60% saturated ammonium sulfate. After the column was washed with the same buffer, the adsorbed ferredoxin was eluted with 0.1 M Tris-HCl buffer, pH 7.3, containing 0.8 M NaCl, and was subjected to analysis by HPLC.

2.3.2. Algal mat

1) Extraction of cyanobacterial ferredoxins. 200 g of algal mat was homogenized with the extraction medium (used above) using a mixer and then sonicated in the same manner as for *Chlorella*. The extracts were supplemented with ammonium sulfate to 60% and centrifuged to remove precipitates. The supernatant containing ferredoxin was loaded on a Toyopearl HW-65C column equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 60% saturated ammonium sulfate. After the column was washed with the same buffer, the adsorbed ferredoxin was eluted with the Tris buffer containing 20% saturated ammonium sulfate. The ferredoxin fraction was further concentrated with a small Butyl-Toyopearl column and was analyzed by the hydrophobic HPLC.

2) Extraction of non-cyanobacterial ferredoxins. Since non-cyanobacterial ferredoxins in the algal mat were not extractable by extraction method 1), the debris of the algal mat was treated with acetone and ferredoxins were extracted from the acetone powder in the same manner as for *Chlorella* ferredoxin.

2.4. Analysis of ferredoxins by HPLC

2.4.1. Analysis by hydrophobic chromatography (hydrophobic analysis)

Analysis of ferredoxins by hydrophobic HPLC was performed as described previously (SAKIHAMA *et al.*, 1986); 0.1 m*l* of ferredoxin preparation was mixed with 0.2 m*l* of 20 mM Tris-HCl buffer, pH 7.5, containing 3 M ammonium sulfate, and 0.1 m*l* aliquot containing about 3 to 10 μg of ferredoxin was loaded on a TSK-gel Phenyl-5PW column (0.75×7.5 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 2.0 M ammonium sulfate. The column was eluted with a 40-min linear gradient of ammonium sulfate (2 to 1 M) in 20 mM Tris-HCl buffer, pH 7.5, at a flow rate of 1 m*l* per min by using a Toyo Soda HPLC system, HLC-803D equipped with a GE-4 gradient unit. Ferredoxin was detected by measuring its absorbance at 420 nm with a UV-8000 UV-visible spectrophotometer. Detection was performed also by measuring its NADPH*-cytochrome *c* reducing activity. 0.5 m*l* of ferredoxin fraction was mixed with the solution containing the following in 0.5 m*l*; 80.8 μM cytochrome *c*, 464 nM ferredoxin-NADP* reductase and 50 mM Tris-HCl buffer, pH 7.5. The reaction was started by

* NADP and NADPH: Oxidized and reduced nicotinamide adenine dinucleotide phosphate.

adding 10μl of 10mM NADPH and monitored by measuring the increase in absorbance at 550nm.

2.4.2. Analysis by ion-exchange chromatography (ion-exchange analysis)

Analysis of ferredoxins by ion-exchange HPLC was performed as follows: Ferredoxin preparation was passed through a small Sephadex G-25 column equilibrated with 20mM Tris-HCl buffer, pH 7.5, containing 0.2M NaCl, and 0.1ml aliquot was loaded on a TSK-gel DEAE-5PW column (0.75×7.5cm) equilibrated with the same buffer. The column was eluted with a 30-min linear gradient of NaCl (0.2 to 0.5M) in 20mM Tris-HCl buffer, pH 7.5, at a flow rate of 1ml per min, and ferredoxin was detected in the same manner with the hydrophobic analysis.

3. Results and Discussion

3.1. Culture of Antarctic Chlorella

Two clones of Antarctic *Chlorella* (*Chlorella* A1 and A2) were selected from agar plates to obtain pure culture of *Chlorella* from which ferredoxins were isolated. A1 and A2 cells were not distinguishable morphologically from each other under a microscope. Figure 1 shows the temperature dependence of the growth of *Chlorella* A1 (curve (1)) and *Chlorella vulgaris*, a species living in the temperate zone (curve (2)). The growth of *Chlorella* A2 cells showed temperature dependence similar to A1 cells (data not shown). Unexpectedly, the optimal temperature for growth of A1 cells was 25°C, which was only slightly lower than that for *C. vulgaris*. A1 cells, however, could grow slowly around 10°C where *C. vulgaris* could not. On the other hand, A1 cells died out at 29°C where *C. vulgaris* could still grow.

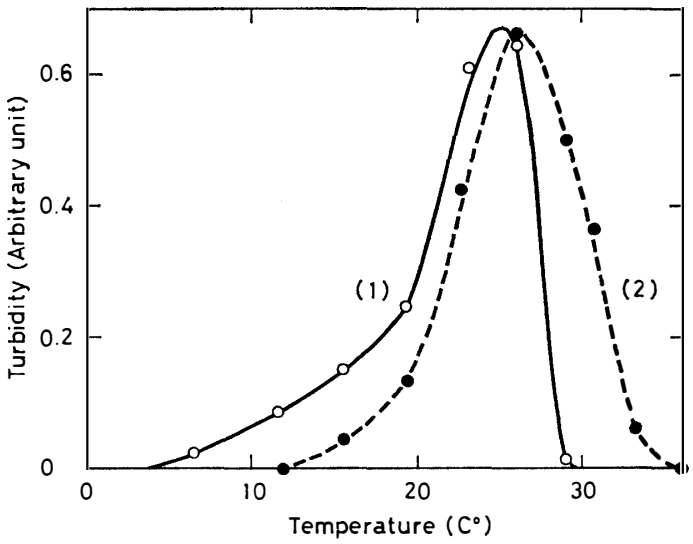

Fig. 1. Effect of temperature on the growth of Antarctic Chlorella. *Cells were grown for 5 days at various temperatures indicated under the conditions described in the text. The turbidity of cell cultures was measured as a function of the cell growth with a Toyo Temperature Gradient Biophotorecorder, model TN-112E.* (*1*) Chlorella *A1*, (*2*) Chlorella vulgaris.

3.2. *Analysis of ferredoxins from Antarctic Chlorella*

Ferredoxin was isolated from Antarctic *Chlorella* A1 and analyzed by hydrophobic and ion-exchange HPLC as shown in Fig. 2. The peaks indicated by arrows in the figure showed ferredoxin activity in the NADPH-cytochrome *c* reducing system. In the hydrophobic analysis A1 ferredoxin was separated into two components, a major and a minor, in a ratio of 91 to 9 as shown in curve (1). Their retention times were 36.8 and 42.2 min. On the other hand, the two components were eluted together at 22.9 min in the ion-exchange analysis (curve (2)). The analysis clearly indicates that A1 cells contain two distinct molecular species of ferredoxin. We have recently reported that all higher plants and algae so far examined contained two distinct ferredoxins (SHIN and SAKIHAMA, 1986; SAKIHAMA *et al.*, 1986). The present result further supports our speculation that a pair of ferredoxins in one species of plant is universal in the plant kingdom. Ferredoxins were also extracted from A2 cells and analyzed by hydrophobic chromatography as shown in Table 1. Although the A1 and A2 cells were not distinguishable morphologically from each other under a microscope, their ferredoxins were eluted at different retention times. As chromatographic behavior of proteins depends on their molecular structure, the present results indicate that each *Chlorella* clone possesses its own ferredoxins with characteristic molecular structure. Table 1 also indicates that retention times of a pair of ferredoxins from *C. vulgaris* were distinct from those measured with Antarctic *Chlorella*. So far as we have examined, ferredoxins from different plant species were eluted at distinct retention times (SAKIHAMA *et al.*, 1986; SHIN and SAKIHAMA, 1986). Consequently, *Chlorella* A1 and A2 are supposed to be different species. We expect that the HPLC analysis of ferredoxin facilitates classification of Antarctic *Chlorella* as well as other plant species.

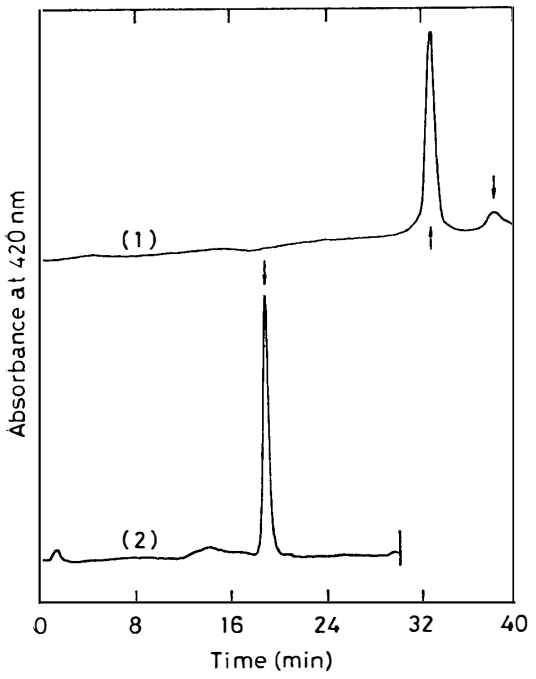

Fig. 2. HPLC analysis of ferredoxins from Antarctic Chlorella. *Ferredoxins extracted from* Chlorella *A1 were analyzed by HPLC. (1) Hydrophobic analysis. Ferredoxin solution containing 2M ammonium sulfate was loaded on a Phenyl-5PW column (0.75×7.5 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 2 M ammonium sulfate, and was eluted with a 40-min linear gradient of ammonium sulfate (2 to 1 M) in the Tris buffer at the flow rate of 1 ml per min. (2) Ion-exchange analysis. Ferredoxin sample containing 0.2 M NaCl was loaded on a DEAE-5PW column (0.75×7.5cm) equilibrated with 20mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl, and was eluted with 30-min linear gradient of NaCl (0.2 to 0.5 M) in the Tris buffer. Arrows in the figure indicate fractions with the NADPH-cytochrome c reducing activity.*

Table 1. Hydrophobic HPLC analysis of Chlorella *ferredoxins.*

	Retention time (min)	
	major	minor
Antarctic *Chlorella* A1	36.8	42.2
Antarctic *Chlorella* A2	35.7	37.6
Chlorella vulgaris	36.7	42.1

Ferredoxins were isolated and analyzed as in Fig. 2, (1).

3.3. Analysis of ferredoxin from algal mat

Collected algal mat was likely to contain a variety of algae, including cyanobacterial and other algal species. We have found that ferredoxins in cyanobacteria were easily extracted by sonication in a Tris-HCl buffer, pH 7.5, containing 35% saturated ammonium sulfate (SHIN *et al.*, 1984). On the other hand, extraction of ferredoxin from green algae, such as *C. vulgaris*, required acetone treatment of algal cells prior to sonication. Therefore, we attempted to separately extract cyanobacterial and non-cyanobacterial ferredoxins from the algal mat. Cyanobacterial ferredoxins were extracted first by directly sonicating the cell in 35% saturated ammonium sulfate. When the cyanobacterial ferredoxin preparation was subjected to the hydrophobic HPLC analysis on a Phenyl-5PW column, it was separated into four peaks with the NADPH-cytochrome *c* reducing activity as indicated by arrows in Fig. 3, curve (1). Their retention times were 28.8, 32.6, 35.6 and 39.2 min.

In order to extract non-cyanobacterial ferredoxins, the algal mat debris remaining after extraction of cyanobacterial ferredoxins was treated with acetone to prepare acetone powder. Non-cyanobacterial ferredoxins extracted from the acetone powder was separated into two peaks with the ferredoxin activity as indicated in Fig. 3, curve (2). The two peaks were eluted at 33.4 and 35.5 min. Since one species of plant always possessed two ferredoxins (SHIN and SAKIHAMA, 1986), the presence of four ferredoxin peaks in the cyanobacterial preparation and two in the non-cyanobacterial preparation suggests

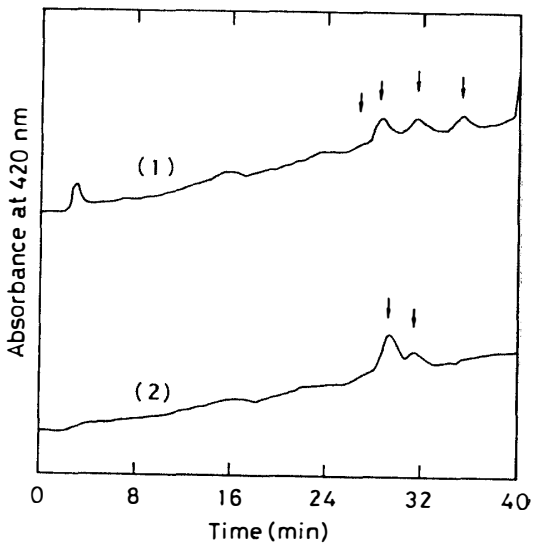

Fig. 3. Separation of ferredoxins from antarctic algal mat by hydrophobic HPLC. Cyanobacterial and non-cyanobacterial ferredoxins were extracted separately from algal mat as described in the text. Hydrophobic HPLC analysis was performed as in Fig. 2, (1). (1) Cyanobacterial ferredoxin. (2) Non-cyanobacterial ferredoxin. Arrows in the figure indicate fractions with the NADPH-cytochrome c reducing activity.

that at least two cyanobacterial and one non-cyanobacterial species were present in the algal mat.

Acknowledgments

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