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ALGAL PHYCOCYANINS PROMOTE GROWTH OF HUMAN CELLS IN CULTURE

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SUMMARY

The growth-promoting substances in a non-dialyzable extract of Synechococcus elongatus var. on RPMI 8226 cells (a human myeloma cell line) were separated by gel filtration and ion exchange chromatography. By gel filtration with Sepharose 4B, the dialyzate was separated into two fractions. One fraction was green-colored (P-1) and the other was blue-colored (P-2). The P-2 fraction had a higher growth-promoting activity than P-1. By ion exchange chromatography, the P-2 fraction was separated into two blue-colored fractions of phycocyanin and allophycocyanin. Both biliproteins promoted the growth of RPMI 8226 cells; however, allophycocyanin was more active than phycocyanin.

Key words: phycobiliproteins; phycocyanins; human myeloma cell; thermophilic blue-green alga; Synechococcus elongatus var.

INTRODUCTION

Our previous study demonstrated that the extract of a thermophilic blue-green alga, *Synechococcus elongatus Naegeli var. vestitus Copland (Syne. elongatus var.)* promoted the growth of human and mammalian cell lines in a serum-free media. One of the growth-promoting substances existed in the non-dialyzable extract (1). The dialyzate was also found to promote the growth of several kinds of human cell lines (2).

The present study deals with separation and identification of the growth-promoting substances in the dialyzate of *Syne. elongatus var.* on human cell lines in serum-free medium.

MATERIALS AND METHODS

Culture of Syne. elongatus var. and preparation of dialyzate. The culture of *Syne. elongatus var.* and the subsequent preparation of the dialyzate were carried out as described in a previous paper (2).

Human cells and cell culture. The cell line used was a myeloma cell line, RPMI 8226 cells. The RPMI 8226 cells cannot grow in RDF medium (2:1:1 mixture of RPMI 1640, Dulbecco's modified Eagle's Medium, and Ham's F12) (2). Cells were routinely cultured in RDF medium supplemented with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, MD) at 37° C in 60 mm or 100 mm plastic dishes (Falcon, Becton Dickinson & Co., CA) in a humidified atmosphere of 5% CO_2:95% air.

Assay of growth-promoting activity. As described in our previous paper (2), exponentially proliferating cells were collected by centrifugation and washed once with RDF medium without FCS. About 5×10^4 cells/ml were cultured in 2 ml RDF medium in 35 mm plastic dishes (Falcon). Some of the cultures were supplemented with fractions of the dialyzate which were separated by column chromatography. The supplements were added only at the time of the inoculation of cells. After culture for 4 days, the cell number was counted with a Sysmex micro cell counter CC-130A (Toaiyodensi Co. Ltd., Kobe, Japan).

The amounts of supplements added to RDF medium were expressed by protein concentration, which was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Fractionation of dialyzate. The dialyzate was first fractionated by gel filtration on a Sepharose 4B gel column (2.5 × 62 cm) equilibrated with phosphate buffered saline (PBS) and eluted with PBS at 5° C. Fractions (9 ml) were collected at a flow rate of 0.5 ml/min. The active fraction was pooled and dialyzed against 5 mM phosphate buffer (pH 7.0). The dialyzed sample was then applied to anion exchange chromatography on a DEAE-Sepharose CL-6B column (1.5 × 20 cm) equilibrated with 5 mM phosphate buffer (pH 7.0) and eluted with a gradient of 0.1-1.0 M NaCl in the same buffer. Fractions (5 ml) were collected at a flow rate of 1 ml/min.

Final fractionation was done by anion exchange chromatography on a TSK gel DEAE-5PW column, which was a prepacked column for HPLC. The active fraction of the chromatography on a DEAE-Sepharose CL-6B was

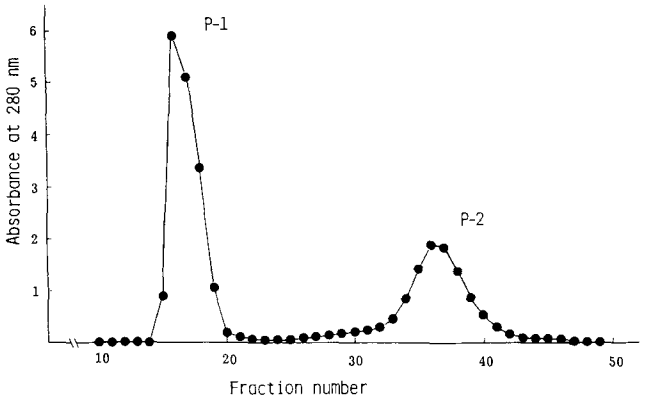

FIG. 1. Sepharose 4B Chromatography of the Dialyzate of *Syne. elongatus var.* The dialyzate of *Syne. elongatus var.* was charged on a Sepharose 4B column (2.5 × 62 cm) equilibrated with phosphate buffered saline (PBS) and eluted with the PBS at 5° C. At a flow rate of 0.5 ml/min, 9 ml fractions were collected. ●, absorbance at 280 nm.

pooled and redialyzed against 5 mM phosphate buffer (pH 7.0), and then charged on the column equilibrated with the same buffer. The column was developed with a linear gradient of 0.1-0.2 M NaCl in the same buffer. Fractions (1 ml) were collected at a flow rate of 0.5 ml/min. These elutions were monitored by the absorbance at 280 nm. The protein contents of elutents were measured by Bio-Rad Protein assay.

Visible absorption spectrum. Visible absorption spectra of fractions were measured with a Shimazu multipurpose spectrophotometer 5000 (Shimazu Seisakusho Ltd., Kyoto, Japan).

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli, using a 15% gel. Approximately 20 μg (as protein) of each sample was applied to slab gel (2 mm thick) and electrophoresis performed at pH 8.8 at a constant current of 15 mA. The gel was stained with a solution of 0.25% Coomassie Brilliant Blue R-250 in ethanol-acetic acid -water (9:2:9 by volume). The standard proteins kit for molecular weight calibration was purchased from Pharmacia (Uppsala, Sweden).

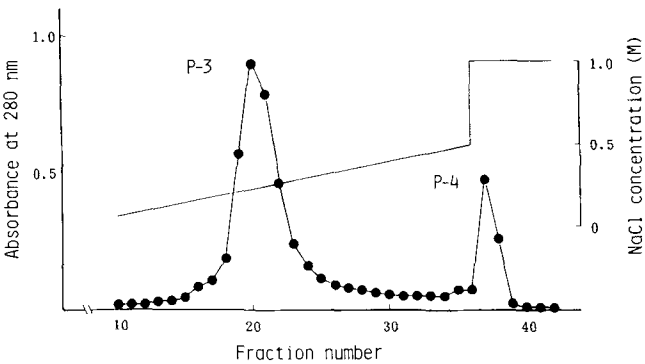

FIG. 2. DEAE-Sepharose CL-6B Chromatography of P-2. P-2 fraction was pooled and dialyzed against 5 mM phosphate buffer (pH 7.0). The dialyzed P-2 was charged on a DEAE-Sepharose CL-6B column (1.5 × 20 cm) equilibrated with 5 mM phosphate buffer (pH 7.0). The column was washed with one column volume of the same buffer and eluted with a gradient of 0.1 - 1.0 M NaCl in the same buffer. Fractions (5 ml) were collected at a flow rate of 1 ml/min. ●, absorbance at 280 nm; __, NaCl concentration.

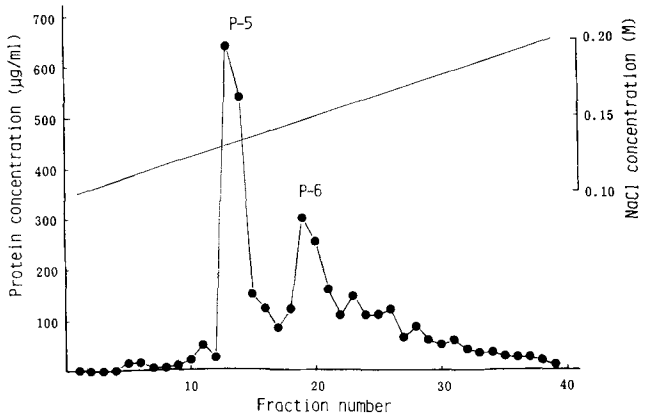

FIG. 3. TSK gel DEAE-5PW Chromatography of P-3. P-3 fraction was pooled and dialyzed against 5 mM phosphate buffer (pH 7.0). The dialyzed P-3 was charged on a TSK gel DEAE-5PW column (prepacked, 0.75 × 7.5 cm) equilibrated with 5 mM phosphate buffer (pH 7.0). After elution with one column volume of the same buffer, the column was developed with a linear gradient of 0.1 - 0.2 M NaCl in the same buffer. Fractions (1 ml) were collected at a flow rate of 0.5 ml/min. ●, protein concentration (μg/ml); __, NaCl concentration.

RESULTS

Sepharose 4B gel filtration of the dialyzate of Syne. elongatus var. The profile of gel filtration of the dialyzate on a Sepharose 4B column is shown in Figure 1. The dialyzate was mainly separated into two fractions (P-1 and P-2). P-1 was green-colored and was found to be a chlorophyll-rich fraction from the visible absorption spectrum, while P-2 was blue-colored and rich in phycobiliprotein.

The two fractions were filtered for sterilization with a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) and assayed for their growth-promoting activity on RPMI 8226 cells. Both fractions were found to promote the growth of RPMI 8226 cells. However, P-2 had a higher growth-promoting activity than P-1. The growth activity of the dialyzate was 4.1 at the optimum dose (23 μm/ml), and that of P-1 and P-2 were 2.6 (11 μg/ml) and 4.9 (87 μg/ml), respectively. These results suggest that the major part of the activity of the dialyzate exists in P-2.

Ion exchange chromatography of P-2. P-2 was then applied to anion exchange chromatography on a

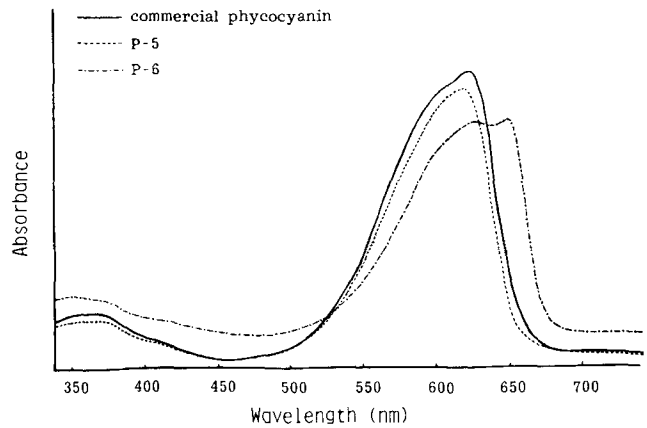

FIG. 4. Visible Absorption Spectra of P-5, P-6, and Commercial Phycocyanin.

TABLE 1

SUMMARY OF FRACTIONATION

	Dialyzate	P-2	P-3	P-5	P-6	Commercial phycocyanin
RPC3	5.4	8.8	16.9	38.5	10.8	41.0
GRmax	4.1	4.9	4.3	3.7	5.3	4.0
(dose μg/ml)	(23)	(87)	(72)	(90)	(70)	(120)

RPC3 is sample protein concentration in culture medium when the growth ratio was three. "GRmax" is growth ratio at optimum dose.

DEAE-Sepharose CL-6B column. The elution profile is shown in Figure 2. P-2 was separated into two fractions (P-3 and P-4). P-3 was blue-colored and rich in phycobiliprotein, while P-4 was colorless and had little protein. The growth-promoting activity on RPMI 8226 cells was mainly detected in P-3. The growth activity of fraction P-4 was smaller than that of P-2.

P-3 was further purified by anion exchange chromatography on a TSK gel DEAE-5PW column (Fig. 3). The elution profile gave two blue-colored fractions (P-5 and P-6).

Phycobiliproteins are bile pigment-protein complexes which function as light-harvesting pigments in the photosynthetic apparatus of the red (Rhodophyta), blue-green (Cyanophyta or cyanobacteria) and cryptomonad (Cryptophyta) algae (3,4). The principal phycobiliproteins are allophycocyanin (λmax 565 nm). All red and blue-green algae produce allophycocyanin and phycocyanin, but some of these organisms do not produce phycoerythrin (5). In *Syne. elongatus var.*, phycocyanin was easily found to be predominant from the visible absorption spectrum of the dialyzate.

The absorption spectrum of P-5 agreed closely with that of a commercial phycocyanin prepared form *Spirulina platensis* (Sigma, St. Louis, MO). The spectrum of P-6 had two peaks at approximately 620 and 650 nm (Fig. 4). The spectrum of allophycocyanin is reported to contain a peak or shoulder at near 620 nm (6,7,8). These results suggest that P-5 and P-6 are phycocyanin and allophycocyanin, respectively.

These two fractions and a commercial phycocyanin from *Spirulina platensis* were analyzed for their growth-promoting activity on RPMI 8226 cells. All three samples showed activity (shown in Table 1).

SDS-polyacrylamide gel electrophoresis of P-5 and P-6. The purity of P-5 and P-6 was checked by SDS polyacrylamide gel electrophoresis, compared with P-3 and a commercial phycocyanin prepared from *Spilurina platensis*. All phycobiliproteins are made of two dissimilar subunits, α and β, present in equal amounts. The molecular weight of the subunits differ depending on the organismic source of the protein, but in general, lie in the range of 14.0 to 20.0 K daltons (3,9,10).

P-5 had two bands (19.5, 22.0 K daltons), which agreed with those of a commercial phycocyanin. P-6 had four bands (17.5, 19.5, 21.0, 24.5 K daltons). Allophycocyanin in common with all other cyanobacterial biliprotein is made of two dissimilar subunits (8,11). However, the allophycocyanin of *Synechococcus sp.* 6301 was reported to have four bands (12), but their molecular weights slightly differed from those of the bands of P-6.

The activity of the fractions at every fractionation step was shown in Table 1. We assumed that "RPC3" is a sample protein concentration in culture medium when the growth ratio was three. Therefore, the smaller the RPC3 of a sample, the higher is its specific activity. We compared the activity of samples by the value of their RPC3 and the growth ratio at optimum dose (GRmax).

The RPC3 of the dialyzate was smaller than those of P-2 and P-3. In addition, the GRmax of the dialyzate was

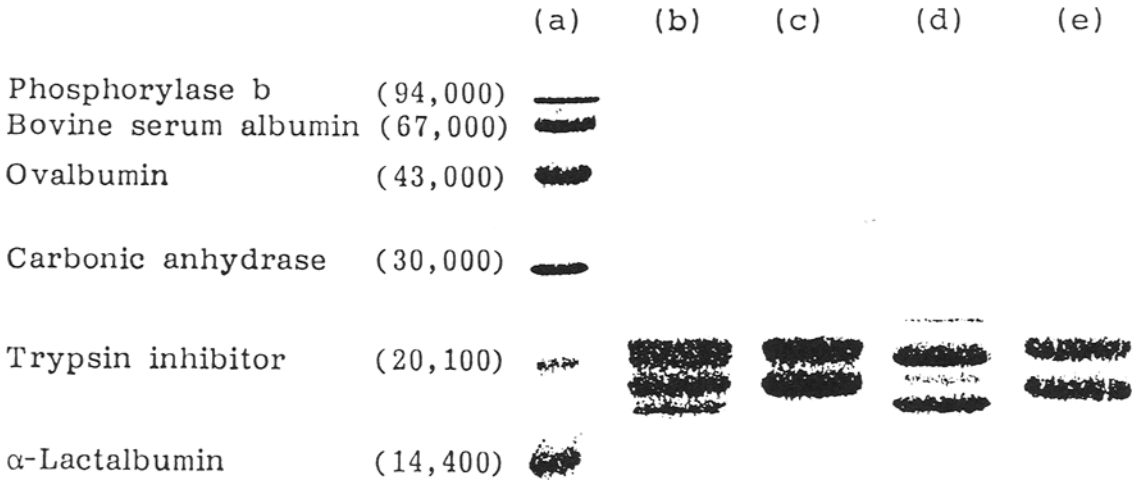

FIG. 5. SDS-Polyacrylamide Gel Electrophoresis of the Fractions and Commercial Phycocyanin. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli. (a) standard proteins, (b) P-3, (c) P-5, (d) P-6, (e) commercial phycocyanin.

smaller than those of P-2 and P-3. In short, a large amount of P-1 showed a growth suppressing effect. P-5 demonstrated as much growth-promoting activity as that of commercial phycocyanin. The GRmax of P-6 was the highest of all.

Discussion

The present study revealed that one of the growth-promoting substances in the dialyzate from *Syne. elongatus var.* on a human myeloma cell line, RPMI 8226 cells is phycobiliprotein. The phycobiliproteins are one of the major light-harvesting pigments in most photosynthetic organisms and can be generally divided into three types based on their spectral properties: phycoerythrin, phycocyanin and allophycocyanin. *Syne. elongatus var.* belongs to blue-green algae (Cyanobacterium) and its C-phycobiliproteins consist of phycocyanin and allophycocyanin. It contains no phycoerythrin. Both phycocyanins promoted the growth of RPMI 8226 cells when they were cultured in the RDF basal medium. The activity of allophycocyanin was higher than that of phycocyanin. Commercially available phycocyanin from *Spirulina platensis*, which may be a mixture of phycocyanin and allophycocyanin, also had growth-promoting activity on RPMI 8226 cells.

Through this study, we used a human myeloma cell line, RPMI 8226 cells, as an indicator cell line to identify the principle factors in the dialyzate from *Syne. elongatus var.* It is also expected that the phycobiliproteins have the ability to promote the growth of other lymphocyte cell lines such as Molt-4 and HSB-2 (acute T-lymphatic leukemia cell lines), and HMY-2 and HO-323 (B-lymphoblastoid cell lines). The P-2 fraction of dialyzate from *Syne. elongatus var.* (crude phycobiliproteins) is found to promote the growth of these cell lines (13). These facts suggest that the phycobiliproteins from all kinds of blue-green algae can be growth stimulatory for human myeloma cell lines. The P-2 fraction also allows RPMI 8226 cells to grow in the RDF medium for 2 weeks without any addition of well-known growth factors (13). In this case, 1.75×10^4 cells/ml of RPMI 8226 cells at inoculation reached 1.1×10^6 cells/ml after culture for 2 weeks. In addition, the P-2 fraction is stable against heating at 100° C for 15 min or storage for about 8 months at 4° C. The culture of blue-green algae is easy and a large amount of fresh algae can be obtained easily and inexpensively with reproducibility when necessary. Separation of phycobiliproteins from algae extract is not complicated. These pigments are water soluble and are also used as cell stains. From these observations, phycobiliproteins seem to be useful growth factors for human myeloma cells. The combination of phycobiliproteins and well-known growth factors, such as insulin, transferrin, ethanolamine and so on, may be expected to promote the growth of human or animal cell lines more significantly.

The chromophore of phycobiliproteins is tetrapyrrole bound noncovalently to polypeptides. It is quite interesting that these structures resemble that of bile pigments (biliverdin), the chromophore of which is tetrapuran. If these chromophores were the active principle of the growth-promoting action, there may be a high possibility that biliverdin or another phycobiliprotein, phycoerythrin may have growth-promoting activity.

Phycobiliproteins are the first growth factors on human or animal cell lines identified from plant origins. The previous and present studies suggest that algae contain several kinds of growth-promoting substances as well as growth-inhibiting substances. Separation and identification of these substances may greatly contribute on the establishment of serum-free culture of human or animal cells.

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EDITOR'S STATEMENT

This report that phycobiliprotein from algae is capable of stimulating animal cell growth is unique, and raises the possibility that related compounds such as biliverdin might also be similarly active. Some puzzling aspects, such as the lack of increase in specific activity during purification, as well as the possibility that the activity might be due to a contaminant, remain to be resolved.