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ISOLATION AND CHARACTERIZATION OF PIGMENT-PROTEIN PARTICLES FROM THE LIGHT-HARVESTING COMPLEX OF *PHAEODACTYLUM TRICORNUTUM*

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The light-harvesting complex of the marine diatom *Phaeodactylum tricornutum* was fractionated into two large pigment-protein particles. One pigment-protein particle, which was contained in a yellow fraction, has a molecular weight, determined by gel filtration, of approx. 230 000 and can be dissociated in sodium dodecyl sulfate/mercaptoethanol solution to apopolypeptides of approx. 15 000. Characterization of particles with regard to molecular weights, subunits, protein and pigments suggests approx. 12 subunits per particle. The other pigment-protein particle, which was found in a green fraction, of approx. 95 000 molecular weight also reduces to apopolypeptide subunits of approx. 15 kDa. The relative molar proportions of chlorophyll *a*, chlorophyll *c*, fucoxanthin and total other accessory pigments in the former fraction are 3:1.3:6:2, whereas the proportions in the latter fraction are 5:1:3:1.

Introduction

Accessory pigments and the bulk of the chlorophyll pigments of green algae and higher plants are associated with specific proteins and believed to comprise supramolecular matrices [1] whose molecular weights, in each photosynthetic unit, may range up to at least 1 000 000. The light-harvesting chlorophyll *a*/*b*-protein of green algae and higher plants [2], the phycobilipigment proteins of red and *cryptomonad* algae [3], and the peridinin, chlorophyll *a* proteins of *dinolflagellates* [4] have been characterized in some detail with respect to energy transfer from accessory pigments to chlorophyll *a* as well as with respect to protein and pigment compositions. Studies on light-harvesting complexes of brown algae and diatoms are more recent. Holdsworth and Arshad [5] reported separation of a metallo-pigment protein complex of 850 000 molecular weight from *Phaeodactylum tricornutum*. The complex was reported to have about 40 subunits protein, 40 mol chlorophyll *a*, 20 mol chlorophyll *c* and 20 mol fucoxanthin. Anderson and Barrett [6] also described separation of a pigment protein from brown algae that was rich in chlorophyll *c*. Separation of SDS-solubilized photosynthetic membranes of brown and diatomaceous algae into three pigment protein bands by gel electrophoresis was also shown by Alberte et al. [7]. These bands consisted of P-700 chlorophyll *a*-protein, chlorophyll *a*/*c*-protein and a chlorophyll *a*/fucoxanthin protein.

If the individual pigment-proteins obtained by SDS-solubilization of membrane material and subsequent separation by gel electrophoresis are indeed present in the photosynthetic unit in the form of supramolecular matrices, then perhaps the next point of investigation should be concerned with

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internal structures within the matrices and how these influence energy transfer. Presence of discoid morphological subunits were demonstrated in phycobilisomes from cyanobacteria [8,9]. The disc-shaped subunits of phycobilisomes from cyanobacteria are reported to be morphologically similar to phycobiliprotein hexamers [10,11] and to contain two polypeptide subunits. It would be of interest to determine if substructures of large pigment-protein complexes of other algae are similar to those of blue green algae and how this relates to energy transfer.

In a prior communication [12], a method was described for separation of a carotenoid-chlorophyll *c*-chlorophyll *a*-protein from *P. tricornutum* that involved disruption of cells by an abrasion process, minimal treatment of thylakoid material with sodium lauryl sarcosinate detergent and subsequent centrifugations. The resulting pigment-protein, which contained chlorophyll *a*, and the accessory pigments present in the initial cells showed good energy transfer from accessory pigments to chlorophyll *a*. The present report covers subsequent work concerned with refinement and characterization of this particle, as well as on a second particle that also contains all the pigments present in the initial cells.

Materials and Methods

Growth conditions

The diatom *P. tricornutum* (642, Bohlin) was grown under conditions similar to those reported previously [12], in which ASP-2 medium of Provasoli et al. [13] was used according to the modifications of Mann and Myers [14]. In this work, however, the cells were grown at 18°C in 3 l bottles in batch processes. Bottles were fitted with one breather tube and two inlet tubes, one for admitting medium and the other for admitting 5% CO_2 in air for aeration. Continuous light for photosynthesis was provided by a single 15 W, cool-white fluorescent light; the light intensity at the front of the growth chamber was 10 W/m^2.

Harvesting

3 l of *P. tricornutum* cells was harvested during the late log phase growth ($6–9 \cdot 10^6$ cells/ml) and filtered through gauze. The total cell count was noted, because the amount of detergent added to disrupted cells is based on the total number of cells processed. Cells weighed, on the average, about 40 pg and contained approx. 12 pg (dry basis) of protein. All solvents and buffers used after harvesting cells were sonicated under vacuum to remove dissolved oxygen.

Cell disruption

A Braun model MSK cell homogenizer, cooled with CO_2, was used to disrupt cells at 0–5°C. Wet-packed cells obtained by centrifugation were transferred to a thick-walled glass Braun homogenizer bottle (45 ml cap) and made up to a 25 ml volume with 0.1 M Tris-H_2SO_4 buffer containing 0.25 M Na_2SO_4 at pH 7.2. To this was then added about 15 ml glass beads (1–2 mM in diameter). Cells were homogenized for 2-min periods for a total of 10 min; each time the bottle was observed to determine if the suspension was still in a fluid state.

Isolation of pigmented-protein particles

Contents of the homogenizer bottle were emptied into a 100 ml beaker imbedded in cracked ice, and the bottle was rinsed with 10–15 ml of cold 0.1 M Tris-H_2SO_4/0.25 M Na_2SO_4 (pH 7.2) buffer. The mixture was stirred to ensure uniform suspension of cell fragments. Fluid withdrawn by means of a Pasteur pipette was centrifuged at $270 \times g$ at 5°C for 15 min. The dark brown supernatant was withdrawn and a small amount of dark sediment was discarded. The supernatant was treated, while stirring in the dark and cold, with sodium lauryl sarcosinate, which was added as 1% solution in 0.1 M Tris-H_2SO_4/0.25 M Na_2SO_4 (pH 7.2) that had been filtered and sonicated. Amounts of detergent added to the supernatant were based on the amounts of protein present in the initial cells and were equivalent to 4–8 molecules per 10 kDa of protein. Detergent was added in 2 ml aliquots with stirring over a 2-h period. Detergent-treated cell fragments were centrifuged in 10 ml polycarbonate tubes at $90000 \times g$ for 1 h at 5°C. The resulting green supernatant contained a pigment-protein complex, and a second readily isolatable complex was in the pellet. About 1 ml of buffer (no detergent) per tube was added to the pellet and shaken to resuspend soft-packed

material, which was collected; the hard-packed nonresuspendable pellet was discarded. Resuspended soft-packed pellet was centrifuged at 750 $\times g$ for 15 min at 5 °C, and any pellet that sedimented was discarded. Color of the supernatant depended on the concentrations of the pigment-protein particles. Highly concentrated solutions, achieved by controlling the amounts of buffer used in resuspension, were dark brown in color.

Column chromatography

The dark brown supernatant was filtered through a 0.45 μm Millipore HA filter, and about 7 ml was injected onto a column (61 × 2.6 cm) packed with Pharmacia, Sepharose CL-4B resin at 5 °C in the dark. The column was equilibrated with 0.1 M Tris-H_2SO_4/0.25 M Na_2SO_4 buffer (pH 7.2) containing 0.06% sodium lauryl sarcosinate which was also used as the elution buffer. The rate of flow was 20 ml/h, and effluent was collected in 5 ml fractions. A major yellow band moved down the column followed by a minor green band. Absorbances at 670 nm, 480 nm and 280 nm were plotted against elution volumes.

Fractions totaling 30–40 ml, collected from both sides of the peak elution volume (265 ml) of the yellow band, were collected and noted as the yellow fraction. The fraction was dialyzed first against 0.1 M Tris-H_2SO_4 (pH 7.2) (in the dark at 5 °C) using a 3.5 kDa cutoff dialysis tubing, and then against 1% acetic acid; the latter solution caused precipitation of pigmented material. Contents of the tubing were freeze dried. Freeze-dried solid assaying 2–3 mg of protein was obtained from $(1–2) \cdot 10^{10}$ cells.

The green supernatant, described in the previous section, was reduced in volume to 10 ml by placing the solution in dialysis tubing and then burying it in granulated sugar at 5 °C in the dark. After dialysis against 0.1 M Tris – H_2SO_4/0.25 M Na_2SO_4 (pH 7.2) buffer, the 10 ml volume was chromatographed as described above. Absorbances of effluent at 670, 480 and 280 nm were plotted against elution volume; fractions amounting to 30 ml were collected at the peak elution volume (280 ml) and regarded as the green fraction. The fraction was dialyzed as described above. Acetic acid caused aggregation of pigmented material. Freeze-dried green solid analyzed 6–10 mg of protein. The column was calibrated with protein standards.

SDS gel electrophoresis

SDS-poly(acrylamide-co-*N*,*N'*-methylene-bis-acrylamide) gel electrophoresis with 2.6% cross-linker was used to analyze proteins in both the isolated yellow and green particles by the method of Laemmli [15] and by use of a Studier type apparatus [16]. 1 mg of protein was heated in 1 ml of SDS-mercaptoethanol reducing solution [17] for 5 min at 100 °C, which was then applied to 15% gels in 20–40 μl amounts.

Protein analyses

Protein analyses and amino acid assays were done as described previously [12].

Pigment analyses

Pigment analyses were carried out by HPLC using an analytical C_{18} reverse phase column described previously [12]. Pigments for calibration of the column were extracted from *P. tricornutum* and separated on a Waters preparative HPLC C_{18} reverse-phase column. Concentrations of pure pigments were calculated at specific wavelengths from absorption spectra obtained in appropriate solvents by means of a Cary 219 spectrophotometer. The equation of Jeffrey and Humphrey [18] was used to determine chlorophyll *a* and *c* concentrations, whereas an $E^{1\%}_{1cm}$ value of 800 at 480 nm was used to calculate the concentration of fucoxanthin in 90% acetone [12]. A coefficient reported by Strain et al. [19] was used to calculate the concentration of neofucoxanthin B, and one reported by Isler et al. [20] was used to calculate the concentration of β carotene.

Results

Cell-growth conditions were kept as constant as possible, because differences can cause variations in concentrations of pigments in cells. Previously, under high light intensity conditions and harvesting at lower cell counts, an HPLC elution pattern was obtained in which neofucoxanthin B had a peak area of about one-half that of fucoxanthin. In this work, in which cells were grown at lower light intensities and harvested at higher cell counts,

the neofucoxanthin B area was only about one- to two-tenths the area of fucoxanthin.

Chlorophyll *c*'s were the first pigments to elute from the C_{18} column; and, in the case of the yellow complex, these were followed by chlorophyllide *a*, presumably an artifact from chlorophyllase *a* activity. Although base-line separation did not occur, the area of chlorophyllide *a* was estimated and added to that of chlorophyll *a*, since spectra of the two are simular [21]. Molar ratios of chlorophyll *c* and *a* obtained by HPLC were cross-checked with those calculated from a spectrum of the pigments by use of equations of Jeffrey and Humphrey [18].

Isolation of the yellow and green fractions

Yellow supernatants from the resuspended soft-packed pellet described in the Materials and Methods section contained the energy-transferring complex reported earlier [12]. After the yellow supernatant was chromatographed on CL-4B Sepharose, plots of absorbances at 670 nm, 480 nm and 280 nm against ml of effluent showed fractions having uniform proportions of chlorophyll *a*, carotenoids and protein. A narrow band of fractions on both sides of the elution peak volume (265 ml) was combined, designated the yellow fraction, and characterized with regard to pigment composition and protein.

When the green supernatant was chromatographed on the Sepharose CL-4B column, plots of absorbances against effluent showed that proportions of carotenoid to chlorophyll *a* and protein were not as uniform as those of the yellow fraction. A narrow band of fractions favoring the right side of the elution peak volume (282 ml) from 280–300 ml was collected and designated as the green fraction.

Characterization of proteins

A comparison of elution volume peaks to those for protein standards indicated that the yellow fraction consisted of 230 kDa particles, whereas the green fraction consisted of 95 kDa particles. Although it is reasonable to expect particles of the size found in the green fraction to occur in the supernatant, it is not obvious why particles found in the yellow fraction should sediment at 90 000 × *g* during an earlier step in the preparation. However,

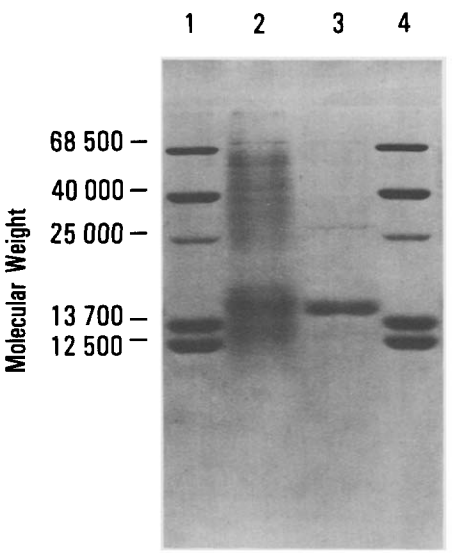

Fig. 1. SDS-poly(acrylamide-co-*N*,*N*′-methylene-bis-acrylamide) gel electrophoresis using 2.6% crosslinker in 15% gel of standard proteins and reduced yellow and green fractions. 1 and 4, standard proteins; 2, reduced green fraction; 3, reduced yellow fraction.

no pellet formed when yellow fraction effluent was centrifuged at 90 000 × *g*; all color remained in the supernatant.

Both freeze-dried yellow and green fraction material were treated with SDS and mercaptoethanol to produce reduced polypeptides for subsequent characterization by SDS-poly(acrylamide-co-*N*,*N*′-methylene-bis-acrylamide) gel electrophoresis on a 15% gel (Fig. 1) containing 2.6% crosslinker. Essentially only one size apopolypeptide of approx. 15 kDa make up the yellow particle; however traces of protein of approx. 30 kDa were noted. Reduced green particles also show a preponderance of protein in the same size range as reduced yellow particles. However, higher and lower molecular weight proteins are also present. In both cases, pigment separated in the gel and moved ahead of the protein.

Although the yellow complex may contain several different subunits of the same size, the amino acid assay (Table I) serves as an index to distinguish it from other complexes in the same or different organisms. Tryptophan was not measured, but its presence was demonstrated through fluorescent experiments on effluent (yellow fraction) collected from the Sepharose column. Excita-

TABLE I

MOLAR FRACTIONS OF AMINO ACIDS IN THE YELLOW FRACTION

Tryptophan was not determined.

Amino acid	Molar fraction
Aspartic acid	0.099
Theronine	0.054
Serine	0.077
Glutamic Acid	0.108
Proline	0.047
Glycine	0.114
Alanine	0.100
Valine	0.062
Cystine	$2.06 \cdot 10^{-3}$
Methionine	0.011
Isoleucine	0.054
Leucine	0.086
Tyrosine	0.026
Phenylalanine	0.057
Lysine	0.038
Histidine	0.016
Arginine	0.043
Ornithine	$5.26 \cdot 10^{-3}$

tion of solutions diluted with 0.1 M Tris-H_2SO_4 (pH 7.2) buffer at 280 nm gave emission peaks at 320 nm, which were attributed to energy transfer from phenylalanine and tyrosine to tryptophan [22,23].

Analysis of the yellow fraction with respect to pigment and protein is tabulated in Table II. 25 ml was found to contain 1.65 mg pigment and 5.35 mg protein, indicating that the particle contains 23.5% pigment.

TABLE II

PIGMENT PROTEIN COMPOSITION OF YELLOW FRACTION

Analyses were performed on 25 ml of yellow fraction. Pigments were determined by HPLC.

Components	μgms	μmoles
Chl *a*	526.71	0.591
Chl *c*	158.0	0.259
Fucoxanthin	729.6	1.154
Neofucoxanthin B	101.2	0.175
Unknown [a]	108.8	0.158
Carotene	29.3	0.055
Protein [b]	5350.0	

[a] Calculated as neofucoxanthin B.

[b] Amino acid assay was conducted on the solution and does not include tryptophan.

The procedure used for extraction of pigments from the green fraction was the same as that for the yellow fraction. More than 90% of chlorophyll *a* had been converted to chlorophyllide *a*; however, since absorption of the two pigments in the region of 670 nm is the same [21], the equation of Jeffrey and Humphrey [18] was used to determine the amounts of chlorophyll *a* and *c*. HPLC was used to determine the amounts of fucoxanthin and other accessory carotenoid pigments. The relative molar ratios of chlorophyll *a*, chlorophyll *c*, fucoxanthin and other accessory pigments in the green fraction were calculated to be 5 : 1 : 3 : 1.

Discussion

Treatment of disrupted cells with minimal amounts of sodium lauryl sarcosinate does not give 100% liberation of photosynthetic units from the thylakoid membranes. Earlier unpublished work in this laboratory showed that the pellet contained lipid (determined as methyl esters of fatty acids by gas chromatography) that amounted to about 10% of the pellet protein. The presence of an unresuspendable hard-packed portion of the pellet (and Gugliemelli, L.A., unpublished data) suggests that some or parts of the photosynthetic units may still be bound to membrane material, whereas the resuspendable soft-packed portion of the pellet indicates treatment was sufficient to disrupt some of the large array of pigmented protein to free particles of uniform sizes. Perhaps these particles are representative of those that give structure to the light-harvesting pigment-protein complex.

Calculations based on the weight of pigment and protein in the yellow fraction (Table II), as well as molecular weights of the yellow particles (estimated from the Sepharose column by filtration) and of the average subunit, were used to estimate the number of subunits per complex. Presence of essentially one size apopolypeptide indicates absence of nonpigment-related protein and that calculations showing presence of 23.5% pigment in the complex are reasonable. Further, one size subunit (15 kDa) from a parent complex containing 23.5% pigment indicates that the aver-

age pigmented subunit is 19.6 kDa and the estimated number of subunits per complex is 12.

Perhaps success in isolating the yellow particle from disrupted cells is due to its sedimentation as large aggregates during centrifugation at 90 000 × *g*, thus permitting separation from lower molecular weight protein in the supernatant. Presumably, subsequent chromatography on Sepharose in the presence of 0.06% sodium lauryl sarcosinate causes dissociation to lower molecular weight particles that do not sediment at 90 000 × *g*. Yellow, sedimented particles undoubtedly have molecular weights considerably greater than 230 000.

The green supernatant seems to contain some protein not related to pigmented protein. A comparison of absorbancy plots of green fraction effulent at 280 nm with those at 480 and 670 nm suggested the possible presence of some nonpigmented proteins that elute in the same size range as pigmented proteins during chromatography on Sepharose. SDS-poly(acrylamide-co-*N*, *N'*-methylene-bis-acrylamide) gel electrophoresis of reduced green fraction particles (Fig. 1) shows presence of a preponderance of protein in the same size range as was separated from reduced yellow particles. However, other proteins are also present. Conceivably some of these may be subunits of the green complex, but it is suspected that many are not. Conclusions regarding the number of subunits must await application of a method that will give better separation of the complex from unwanted protein.

Differences in pigment compositions between the two complexes were also noted. Molar proportions of chl *a*, chl *c*, fucoxanthin, and total other accessory pigments in the yellow complex are 3 : 1.3 : 6 : 2, whereas those in the green are 5 : 1 : 3 : 1. The green has nearly twice as much chl *a* as fucoxanthin, whereas the yellow has twice as much fucoxanthin as chl *a*.

Futher investigation is required before it can be determined if the yellow particles have substructures similar to those reported for the blue-green algae.

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