C-PHYCOCYANIN FROM THE THERMOPHILIC BLUE-GREEN ALGA *MASTIGOCLADUS LAMINOSUS*. ISOLATION, CHARACTERIZATION AND SUBUNIT COMPOSITION

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Received 23 November 1971

1. Introduction

Phycocyanins and phycoerythrins have been extensively studied due to their involvement in photosynthesis in blue-green algae. The general classes of these phycobiliproteins have been based on the spectra of their chromophore–protein complexes [1–3]. Among other physical and chemical properties, minimum molecular weight [1, 4–7], amino acid composition [7–9] and end groups [1, 7] of the C-phycocyanins of different species have been determined.

The thermophilic blue-green alga, *Mastigocladus laminosus*, represents a phylogenetically primitive type of organism within the system of the blue-green algae [10, 11] and exhibits growth under varied thermal conditions, optimally at 55° (upper growth limit 60°) [10–12].

In this study, the C-phycocyanin of *M. laminosus* was isolated by gel filtration and ion exchange chromatography. The protein was characterized with respect to its amino acid composition, minimal molecular weight, amino-terminal end groups, subunit composition and spectral properties.

2. Materials and methods

Sephadex G-150 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala; corticotropin (1–24) tetracosapeptide (Synacthen®) from Ciba-Geigy, Basle; lysozyme, ribonuclease, insulin and trypsin from Serva, Heidelberg; and carboxypeptidase A from Worthington. All reagents were of analytical grade.

The thermophilic alga *M. laminosus* was cultured in the water of a hot spring near Reykyavik, Iceland [12]. The algae were collected, shipped at 4° and frozen upon receipt. No adverse effects were noted during normal shipment times of 2 days.

For the isolation of C-phycocyanin, the algae suspension was first centrifuged for 30 min at 10,000 *g* in a Sorvall centrifuge Model RC-2B to remove surplus water and the algal filaments were then homogenized in a mixer (MSE AtoMix) for 5 min in 0.05 M Tris buffer, pH 7.5, at a ratio of 8.5 ml buffer per g wet wt residue. Further cell disruption was accomplished by 2 passages through a Manton-Gaulin homogenizer Model 15M. The suspension of disrupted cells was centrifuged for 3 hr at 40,000 *g* in a Beckman ultracentrifuge Model L-2 and the supernatant transferred to a 10 × 100 cm column of Sephadex G-150, equilibrated with 0.05 M Tris buffer, pH 7.5. Elution was performed with the same buffer. Those fractions containing C-phycocyanin (absorption maximum at 615 nm) were pooled and dialyzed against 0.05 M phosphate buffer, pH 8.0. The dialyzed protein was applied to a 5 × 100 cm column of DEAE-Sephadex A-50 and chromatographed with NaCl gradient from 0.15–0.45 M in 0.05 M phosphate buffer, pH 8.0 (total elution vol, 2 l). Those fractions containing C-phycocyanin were pooled, dialyzed against water and lyophilized.

In disc electrophoretic experiments, the discontinu-

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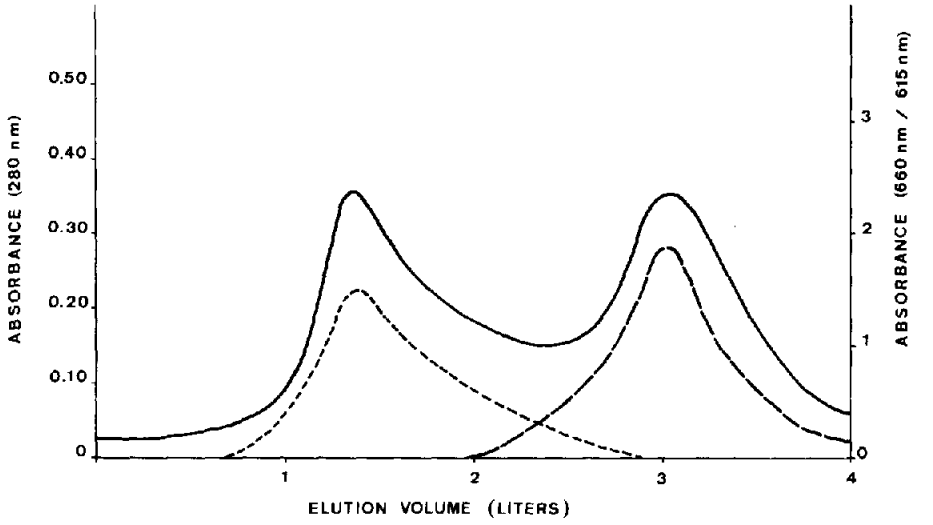

Fig. 1. Chromatography of algae extract on Sephadex G-150. The extract from 30 g algae (wet wt) in 300 ml 0.05 M Tris buffer, pH 7.5, was applied to a 10 × 100 cm column of Sephadex G-150 by reverse flow pumping and elution carried out with the same buffer. Chlorophyll *a* and phycocyanin regions were identified according to their visible spectra. —— : A_{280}; - - - - - : A_{660}, absorption maximum of chlorophyll *a*; – – : A_{615}, absorption maximum of *C*-phycocyanin.

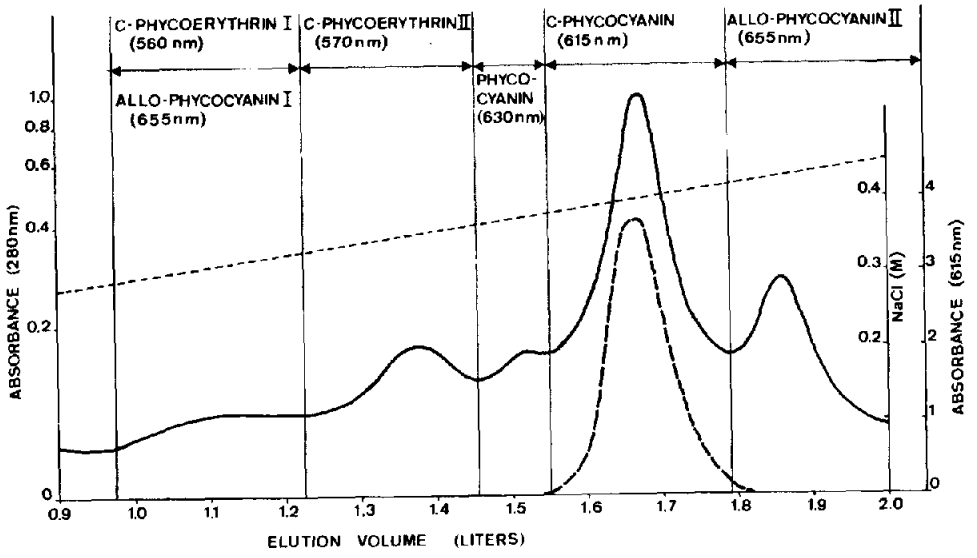

Fig. 2. Chromatography of the phycocyanin fraction obtained from Sephadex G-150 on DEAE-Sephadex A-50. Half of the phycocyanin pool from Sephadex G-150 (representing the phycocyanins from 15 g algae wet wt) were placed on a 5 × 100 cm column of DEAE-Sephadex A-50, which was equilibrated with 0.05 M phosphate buffer, pH 8.0. The column was developed with a linear gradient from 0.15–0.45 M NaCl (total vol, 2 l). The wave-lengths given for the different phycoerythrins and phycocyanins represent their respective absorption maxima. The designation I and II represent the order of elution of the 2 *C*-phycoerythrins and the 2 *Allo*-phycocyanins from the column. —— : A_{280}; – – : A_{615}.

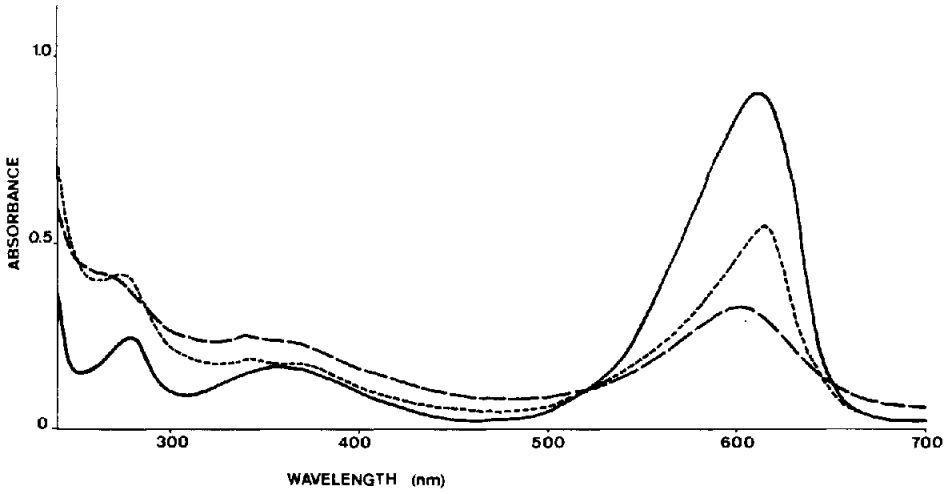

Fig. 3. Absorption spectra of native *C*-phycocyanin and the α-, and β-subunits in 0.05 M phosphate buffer, pH 8.0. ——— : *C*-phycocyanin: — — : α- subunit: - - - - - : β- subunit.

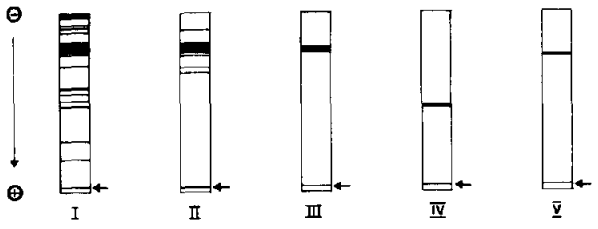

Fig. 4. Polyacrylamide gel electrophoresis of *C*-phycocyanin and the α- and β- subunits at the various stages of isolation. Electrophoresis was carried out in a 7.5% gel, pH 7.5, at 25 V/cm and 5 mA/gel. The arrows indicate the marker bromophenol blue. I: Soluble proteins after extraction and centrifugation. II: Phycocyanins from Sephadex G-150 chromatography. III: *C*-phycocyanin from DEAE-Sephadex chromatography. IV: α- Subunit of *C*-phycocyanin after separation by gel electrophoresis in 8 M urea and 0.1 M 2-mercaptoethanol. V: β- Subunit of *C*-phycocyanin after separation by gel electrophoresis in 8 M urea and 0.1 M 2-mercaptoethanol.

ous system of Ornstein and Davis [13, 14] was employed. Staining and destaining were done according to Weber and Osborn [15]. For electrophoresis in the absence of sodium dodecylsulfate (SDS), the Tris-Barbital buffer system, pH 7.5, of Williams and Reisfeld [16] was used. The concentrating gel was omitted. Disc electrophoresis in 0.1% SDS and 8 M urea was used for molecular weight determinations [17].

Table 1

Molecular weight determination by SDS gel electrophoresis.

Protein	R_f-value	Molecular weight
Carboxypeptidase A	0.324	34,600
Trypsin	0.375	23,300
Lysozyme	0.528	14,300
Ribonuclease	0.438	13,700
Insulin	0.645	5,700
Synacthen®	0.740	3,500
C-phycocyanin	0.477	14,000

Electrophoresis carried out on 10% acrylamide gels at 5 mA/gel and 15 V/cm in the Tris-phosphate gel system of Swank and Munkres [17] which included 0.1% SDS and 8 M urea. Results are derived from a logarithmic plot of known molecular weights versus R_f-values relative to an internal marker of bromophenol blue.

Preparative gel electrophoresis (Poly-Prep, Buchler Instruments, New Jersey) employing the Tris-glycine buffer system suggested by Buchler [18] was performed for isolation of the *C*-phycocyanin subunits. Electrophoresis was done in the absence of a concentration gel. The resolving gel buffer was 7 M in urea, 0.1 M in 2-mercaptoethanol, and adjusted to pH 7.5 rather than pH 8.9 as suggested by Buchler [18]. Ammonium persulfate was removed from the gel by prerunning for 6 hr.

For subunit separation, *C*-phycocyanin was dissolved in 0.05 M phosphate buffer, pH 8.0, containing

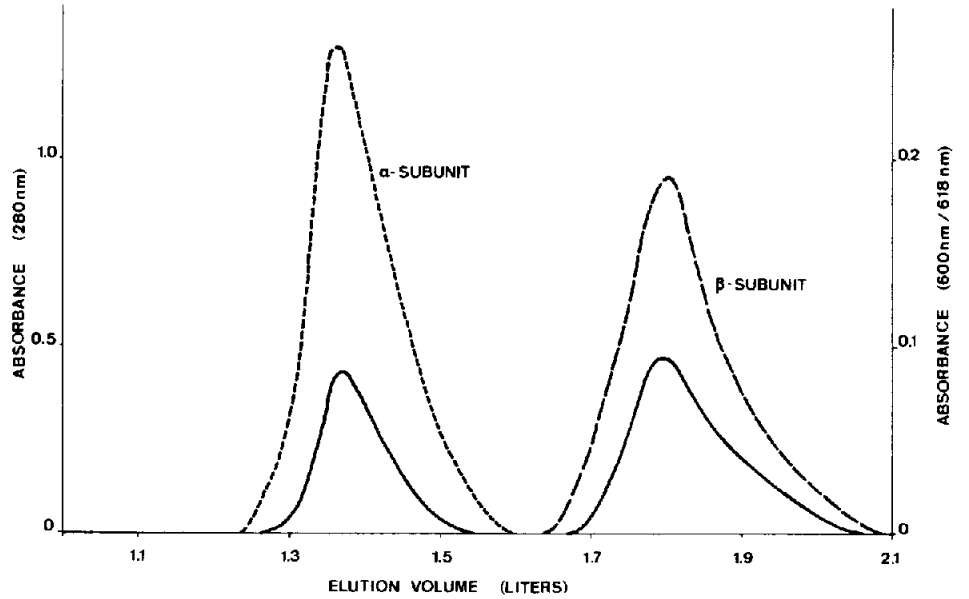

Fig. 5. Separation of the α- and β- subunits of *C*-phycocyanin by preparative polyacrylamide gel electrophoresis. 10 mg of *C*-phycocyanin, initially dissolved in 8 M urea, and 0.1 M in 2-mercaptoethanol, was applied to a 5 cm polyacrylamide column (7.5% acrylamide, pH 7.5, upper buffer pH 8.9) and electrophoresis carried out at 240 V and 50 mA for 12 hr. ——— : A_{280}; - - - - - : A_{600}, absorption maximum of the α-subunit; – – : A_{618}, absorption maximum of the β-subunit.

8 M urea and 0.1 M 2-mercaptoethanol. Electrophoresis was carried out at 240 V and 50 mA for 12 hr.

For determination of the amino terminal residues, 1 ml of a saturated solution of dansylchloride (DNS-Cl) in acetone was added to 1 ml of the protein solution (1 mg protein per ml 0.2 M $NaHCO_3$ containing 0.25% SDS). After further addition of acetone to dissolve the precipitate, the reaction was allowed to proceed for 16 hr at room temp in the dark. The reaction mixture was lyophilized, washed with acetone and with water, and hydrolyzed in 6 N HCl for 24 hr at 110°. After drying, the hydrolysate was dissolved in 50% (v/v) pyridine, and chromatographed on both polyamide sheets [19] and silica gel-G plates [20]. The DNS–amino acids were identified by comparison with DNS–amino acid standards.

Amino acid analyses were performed as described by Moore and Stein [21] on a Beckman-Spinco automatic amino acid analyzer Model 121. Samples were hydrolyzed in 6 N HCl at 110° under vacuum for 24, 48 or 72 hr.

All visible and UV-spectra were measured on a Beckman spectrophotometer Acta V at room temp.

3. Results and discussion

As shown in fig. 1, the phycocyanins of *M. laminosus* are readily separated from the chlorophyll–protein fractions on Sephadex G-150. Of the extractable phycocyanins, *C*-phycocyanin appears to be present in the greatest quantity as indicated by the absorption maximum at 615 nm. Complete purification of the *C*-phycocyanin fraction was achieved by ion exchange chromatography on DEAE-Sephadex A-50 (fig. 2). Four phycocyanins and 2 phycoerythrins were identified by their absorption maxima [1–3]. The absorption spectrum of *C*-phycocyanin is given in fig. 3 and corresponds with previously published results [2]. The progress of purification was followed by disc electrophoresis and the results are illustrated in fig. 4 (I, II, III). As fig. 4, III indicates, the protein preparation was homogeneous after ion exchange chromatography.

Molecular weight determinations in SDS of different preparations of *C*-phycocyanin resulted in an apparent minimum molecular weight of 14,000 (table 1). This molecular weight is in agreement with Glazer et al.

Table 2
Amino acid compositions of native *C*-phycocyanin and the α- and β-subunits

	α-subunit[1]		β-subunit[1]		Difference between α- and β-subunits (α−β)	Total α- and β-subunits (α + β)	Native[2] *C*-phycocyanin integer
	average	integer	average	integer			
Lysine	4.6	5	4.3	4	+1	9	9
Histidine	0.1	0	1.5	2	−2	2	2
Arginine	6.1	6	4.9	5	+1	11	11
Aspartic acid	14.9	15	15.0	15	0	30	30
Threonine[3]	8.3	8	2.5	10	−2	18	17
Serine[3]	11.4	11	10.2	10	+1	21	19
Glutamic acid	11.0	11	11.1	11	0	22	22
Proline	3.9	4	4.8	5	−1	9	9
Glycine	18.8	19	20.0	20	−1	39	37
Alanine	29.7	30	32.0	32	−3	62	68
Valine[4]	10.4	10	7.2	7	+3	17	16
Methionine	2.1	2	0.4	1	+1	3	3
Isoleucine[4]	9.1	9	8.7	9	0	18	18
Leucine	11.5	12	11.5	12	0	24	23
Tyrosine	3.2	3	4.8	5	−2	8	9
Phenylalanine	2.7	3	2.5	3	0	6	5
Tryptophan[5]							1
Cysteic acid[6]							3
Total		148		151		299	301

[1] Amino acid residues calculated per 14,000 molecular weight after 24 hr hydrolysis.
[2] Amino acid residues calculated per 28,000 molecular weight after 24, 48 and 72 hr hydrolysis in the presence of thioglycolic acid [24].
[3] Extrapolated to zero time of hydrolysis.
[4] Extrapolated to infinite time of hydrolysis.
[5] Tryptophan presence in either α- or β-subunit, not determined by amino acid analysis but preliminary results from difference spectroscopy indicates tryptophan present in the β-subunit.
[6] Cysteic acid determined after performic acid oxidation according to Hirs [25].

[22], Bennett et al. [23] and Hattori et al. [6] but not with the results of Kao et al. [5] who found a value of 30,000. These discrepancies in apparent minimal molecular weights, the range of 14–18,000 or 28–32,000, must be due to either the protein stability under the denaturating conditions employed or actual molecular weight differences of *C*-phycocyanins from different genera.

After treatment with 8 M urea and 0.1 M 2-mercaptoethanol, *C*-phycocyanin could be separated into 2 components by preparative gel electrophoresis in the presence of 7 M urea and 0.1 M 2-mercaptoethanol (fig. 5). As indicated in fig. 4 (IV, V), the preparative electrophoretic separation resulted in no detectable cross-contamination between the 2 subunits. The first subunit eluted from the gel, designated α, differs from the second, β, in its amino acid composition, amino terminal residue and its absorption spectrum (see following results). Previous results by analytical gel electrophoresis have indicated the presence of 2 subunits in other *C*-phycocyanins [22, 23].

The amino acid compositions of *C*-phycocyanin and the α- and β-subunits are given in table 2. A similarity between the content of any amino acid residue in the *C*-phycocyanin of the thermophilic blue-green alga *M. laminosus* and the *C*-phycocyanins previously reported [7–9] is not evident. However, the high alanine content and a high ratio of acidic to basic

residues appear to be common to *C*-phycocyanins. The difference in content of any amino acid between the α-subunit and the β-subunit is not greater than 3 residues, and the overall compositions of the 2 subunits do not differ significantly. Noteworthy differences between the 2 subunits exist only in the histidyl-, methionyl- and tyrosyl-residues. The average value for each amino acid residue was determined from analyses of 4 different preparations of the α- and β-subunits. The combined total of the composition of the α- and β-subunits is in agreement with the composition of the *C*-phycocyanin if a 1:1 ratio for the 2 subunits is assumed. Therefore, the minimum molecular weight of *C*-phycocyanin would be 28,000.

Further evidence for the non-identical nature of the α- and β-subunits was provided by amino-terminal analysis. As isolated, the *C*-phycocyanin was found to have the amino-terminal residues alanine and valine. Analysis of the separated subunits identified alanine as the amino-terminus of the α-, and the valine as the amino-terminus of the β-subunit. Therefore, each subunit seems to be composed of one polypeptide chain only. Of the amino-terminal end groups thus far identified in the *C*-phycocyanins of other algae [1, 7], this is the first report of terminal alanine and valine residues.

A comparison of the absorption spectra (fig. 3) of *C*-phycocyanin and the α- and β-subunits indicates different ratios between the visible and the UV-maxima, as well as shifts in the wavelengths of maximum absorptions.

With this relatively simple procedure for isolating *M. laminosus* *C*-phycocyanin and its α- and β-subunits, further characterization of these components will be possible. Work along these lines is currently in progress in this laboratory.

Acknowledgements

Sincerest gratitude is expressed to Miss Hanni Schneider for excellent technical assistance. The support for the field studies in Iceland, by the Swiss Foundation of Alpine Research is gratefully acknowledged. Laboratory studies were supported by the Schweizerische Nationalfonds (Project No. 4883 and 3.379.70).

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