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Ascidian–Algal Symbioses: I. Isolation and Characterization of Phycobiliproteins from Symbiotic Cyanophytes in Ascidians

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Abstract

Presented here is a comparative study of the phycoerythrins of the unicellular, red cyanophytic symbionts of the ascidians *Trididemnum tegulum*, *Didemnum viride* and *Didemnum* aff *sphaericum*.

This group of phycoerythrins has been divided into three types — CU-PE I, CU-PE II and CU-PE III. All three types have molecular weights of 39,000, and peptide subunits of 19,000 Da. The chromatographic properties on Bio-Rex 70 in acid urea are identical and amino acid compositions are similar. The major difference between the three types is spectroscopic. The presence or absence of the 498 nm peak or shoulder is attributed to the phycourobilin chromophore and the presence or absence of the 542 nm and 560 nm peaks or shoulders is attributed to the phycoerythrobilin chromophores. The ratio of phycourobilin :phycoerythrobilin is most probably under environmental control.

Keywords: Ascidians, *Trididemnum tegulum*, *Didemnum* aff *sphaericum*, *Didemnum viride*, phycoerythrins, phycourobilin, phycoerythrobilin

Abbreviations: PVB — Phycourobilin, PEB — Phycoerythrobilin, PE — Phycoerythrin, Dv — *Didemnum viride*, Ds — *Didemnum* aff *sphaericum*, Tt — *Trididemnum tegulum*

1. Introduction

The phycobiliproteins are the principal photoreceptors for photosynthesis in the divisions Rhodophyta, Cyanophyta and Cryptophyta (Larkum and Barrett, 1983). These algal biliproteins are differentiated into two principal groups on the basis of their coloration; phycoerythrins are intensely red and phycocyanins are blue. Phycoerythrins dominate in the Rhodophyta and phycocyanins in the Cyanophyta; however, phycoerythrins are known to dominate in some species of Cyanophyta (O'Carra and O'hEocha, 1976; Hirose et al., 1969).

The phycobiliproteins have a number of characteristics in common. They are each made up of 2 dissimilar polypeptide chains which carry the covalently bound tetrapyrrole prosthetic groups; have similar amino acid sequences, physical and immunological properties and intracellular organization (Glazer, 1981 and references therein). There are several types of phycoerythrins and phycocyanins each with characteristic absorption spectra (O'Carra and O'hEocha, 1976).

Three distinct groups of phycoerythrins have been identified. The C — phycoerythrins which have a single peak at 565 nm from the PEB (phycoerythrobilin) chromophore, with a subunit structure of $(\alpha\beta)_6$ (Hattori and Fujita, 1959). The B-phycoerythrins have peaks at 545, 563 nm and a shoulder at 498 nm, with a subunit structure $(\alpha\beta)_6\gamma$ (Glazer and Hixson, 1977). The R-phycoerythrins have peaks at 567, 538, and 498 nm, with a subunit structure of $(\alpha\beta)_{6\gamma}$ (Glazer, 1977). Within the B- and R-phycoerythrins there are a number of types each with characteristic absorption spectra (Glazer et al., 1982).

There have been reports within the class Ascidiacea (S.P. Tunicata) of several species of ascidians with symbiotic cyanophytes, both unicellular and filamentous,containing a red pigment (Olson, 1980; Symbesma et al., 1981; Lafargue and Duclaux, 1979; Lewin and Cheng, 1975). In none of these reports was the red pigment, a phycobiliprotein, identified. More recently the phycobiliproteins of a number of cyanophytic symbionts in ascidians have been studied and shown to be unusual phycoerythrins with a PUB (phycourobilin) chromophore (Parry, 1984; Cox et al., 1985; Larkum et al., 1987).

This paper reports the isolation and characterisation of phycoerythrins from unicellular symbiotic cyanophytes in the ascidians *Didemnum viride* and *Didemnum* aff *sphaericum* and further characterises the phycoerythrins from *Synechocystis trididemni*, the symbiont of the ascidian *Trididemnum tegulum.*

2. Materials and Methods

Collection

Three species of ascidians were collected from Heron Island, Great Barrier Reef, each of which contain a red, prokaryotic symbiont.

1. *Trididemnum tegulum*, Kott, 1984, was collected on the reef crest under boulders or clustered at the end of *Acropora* branches. The colonies are spherical and range in size up to 1 cm in their maximum dimension. The red, prokaryotic symbiont is embedded in the test of the ascidians.
2. *Didemnum viride*, Herdman, 1906, was collected at depth of 10–15 m where it forms enormous sheeting colonies covering areas of several m^2. The colony is packed with calcium carbonate spicules to a point where colonies are almost brittle. The red, prokaryotic symbionts are embedded in the test of the ascidian.
3. *Didemnum* aff *sphaericum*, Tokioka, 1967, was collected on the reef crest, under boulders. It is an encrusting species, packed with $CaCO_3$ spicules with the red, prokaryotic symbiont again embedded in the test.

Extraction of the algal pigments

In all three species the symbionts are embedded and could not be removed from the ascidian host. Therefore, the whole colonies or sections of colonies in the case of *D. viride* were extracted. They were extracted by homogenizing in cold 0.1 M phosphate buffer (pH 7.0) with an ultraturrax. The homogenate was then freeze thawed several times and centrifuged at 16,000 g for 20 min at 4°C.

This yielded a bright, fluorescent red-maroon extract from all three species. The residue was green.

Purification of the Phycobiliprotein

Each of the 3 phosphate buffer extracts were purified using the same procedure.

1. *Fractional precipitation:* The extract was saturated with 30-50% $(NH_4)_2SO_4$ and left to stand overnight in the dark at 4°C. The red precipitate was obtained by centrifugation at 20,000 g for 10 min at 4°C and the yellow supernatant discarded. The precipitate was then washed with a cold, saturated solution of $(NH_4)_2SO_4$ and again centrifuged.

2. *Chromatography*:

(a) Biogel-HTP: The red precipitate was redissolved in 0.001 M phosphate (pH 7.0) with 0.1 M NaCl and exhaustively dialysed against the same buffer in the dark at 4°C. The solution was then filtered through a 0.45 μm millipore filter and applied to a column of Biogel-HTP (2.6×38 cm). The phycobiliprotein was eluted with a linear gradient of phosphate buffer from 0.001 M to 0.1 M, pH 7.0 made 0.1 M in NaCl; at a flow rate of 1.0 ml/min.

The chromatogram was monitored at 542 nm and 498 nm using a HP 8450A diode array spectrophotometer.

(b) DEAE-sephacel: a 2.6×43 cm column of DEAE-sephacel was equilibrated with 0.025 M sodium acetate (pH 5.5). The phycobiliproteins were exhaustively dialysed against 0.025 M sodium acetate (pH 5.5) at 4°C in the dark. It was then filtered through a 0.45 μm millipore filter and applied to the column. The column was eluted with a linear gradient of sodium acetate from 0.025 M to 0.25 M (pH 5.5) at a flow rate of 0.85 ml/min. The eluent was monitored at 542 nm and 498 nm using the HP8450 A diode array spectrophotometer.

Characterization

Spectroscopy

UV/VIS spectra were measured using a HP-8450A diode array spectrophotometer or Varian DMS 90. Circular Dichroism (CD)spectra were recorded on a Jobin-Yvon Dichrograph III. Fluorescence spectra were obtained with an Aminco SPF-500 ratio spectrofluorimeter.

Chromophore content of the phycoerythrins

The phycoerythrins were exhaustively dialysed against 0.01 M acetate pH 5.5 at 4°C in the dark. The proteins were lyophilized and weighed into 2 ml volumetric flasks to which was added 8 M urea made 0.01 M with β-mercaptoethanol (pH 1.9). The absorption spectra were measured immediately using a HP8450 A diode array spectrophotometer. All calculations are based on the data of Glazer and Hixson (1975, 1977); and Bryant et al. (1981); i.e. phycoerythrobilin (ϵ_M at 495 nm= 13,400 M^{-1} cm-1; ϵ_M at 555 nm = 43,000 $M^{-1}cm^{-1}$ and phycourobilin ϵ_M at 495 nm = 104,000 M^{-1} cm^{-1}; ϵ_M at 555 nm = 0 M^{-1} cm^{-1}).

Chromatofocusing

A 1.6 cm× 18 cm column of PBE 94 (polybuffer exchanger, Pharmacia) was equilibrated with 0.025 M imidazole-HCL (pH 7.35). The phycobiliproteins were dialysed against 3 × 5 L volumes of 0.025 M Im-HCl (pH 7.35) at 4°C in the dark, filtered through a 0.45 μm millipore filter and applied to the column. The column was eluted with polybuffer 74 adjusted to pH 3.9 with HCL after dilution of the polybuffer 1:8 (polybuffer: dist. H_2O).

Isoelectric focusing

Isoelectric focusing was performed using a Pharmacia PhastSystem, with 5% homogeneous polyacrylamide gels covering the pH range 3 to 9. The standard was the Pharmacia Broad pI calibration kit. The gels were stained with Coomassie Blue.

Molecular weight determination

The phycoerythrins were chromatographed on a column of Sephadex G-200 Fine (2.6 cm×38 cm) which had been calibrated with molecular weight markers. The column was equilibrated with 0.05 M phosphate buffer (pH 7.0) and eluted with the same buffer at a constant flow rate of 1.0 ml/min. The apparent molecular weights were calculated from the elution volumes.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE (sodium dodecyl sulfate-PAGE) was performed using the method of Laemmli (1970) and the SDS microslab linear gradient gel system of Matsudaira and Burgess (1978).

Separation of subunits

Isolation of subunits was attempted using 2 methods of chromatography on Bio-Rex 70.

1. Glazer and Hixson (1975):

A Bio-Rex 70 (minus 400 mesh) column (1.6 × 16 cm) was pre-equilibrated with 0.4% V/V acetic acid (pH 3.0). The phycobiliprotein solution was dialysed to equilibrium against 0.1 M potassium phosphate (pH 7.0) in the dark at 4°C. The dialysed protein solution was then acidified with glacial acetic acid to pH 3.0 and β-mercaptoethanol added to a concentration of 10 mM. The protein solution was then filtered and applied to the column. The column was developed with 1 column volume of 2 M urea, 10 mM β-mercaptoethanol (pH 3.0). This was followed by stepwise elution with solution of increasing urea concentrating up to 9 M urea.

2. Modified method 1 — the phycobiliprotein was adjusted to pH 2.0 and applied to the Bio-Rex 70 (minus 400 mesh) column and eluted with a stepwise gradient from 2 M urea to 9 M urea (pH 2.0) all 0.01 M in β-mercaptoethanol. A further modification was the use of a linear gradient from 2 M urea to 9 M urea pH 2.0. All chromatograms were monitored at 555 nm and 496 nm with a HP 8450 Diode Array Spectrophotometer.

Amino acid analysis

All phycobiliprotein samples were exhaustively dialysed against distilled water (Milli-Q) at 4° C in the dark. One ml of each containing approx. 0.5 mg of protein were pipetted into hydrolysis tubes and lyophilized. The proteins were hydrolysed *in vacuo* at 108°C, 48 hr with 1 ml 6 N constant boiling HCl (Pierce).

The amino acid analysis was carried out using a Waters amino acid HPLC system with post column detection of the OPA (orthophthalaldehyde) adducts. The Pierce-H amino acid standard was used. Amino acid compositions were calculated to give the best fit to the monomer molecular weights.

Tryptophan was determined using the method of Simpson et al. (1976). All samples were hydrolysed *in vacuo* at 115°C, 22 hr with 1 ml 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl indole) (Pierce). The amino acid analysis was performed as for HCl hydrolysates.

Half-cystine was measured as cysteic acid after oxidation of the proteins with performic acid (Moore, 1963). After oxidation, the excess performic acid was removed by reduction with 48% hydrobromic acid. Hydrolysis (for 20 hr) was carried out as described previously.

Disulfide analysis

The method of Anderson and Wetlaufer (1975) was used to determine disulfide groups in the proteins. The assay involves alkaline cleavage of the disulfide bonds and the thiol resulting from the cleavage is estimated colorimetrically with 5,5′-dithio-bis(2-nitrobenzoic acid). The absorption is measured at 412 nm.

3. Results and Discussion

The symbiont from *T. tegulum* has been identified as *Synechocystis trididemni* and a "C-PE" from this species has been identified (Cox et al., 1985). The symbiont from *D. viride* is also *S. trididemni* (Cox, 1986). *Didemnum* aff *sphaericum* has symbionts very similar to *S. trididemni.*

All three phosphate buffer extracts were red-maroon in colour and all exhibited maximal absorption at 498 nm. This 498 nm maximum is indicative of the presence of the PUB chromophore (O'Carra et al., 1964). There is a difference in the spectra of *T. tegulum* extracts and the *D. viride* and *Didemnum* aff *sphaericum* extracts with the *T. tegulum* extract having a peak at 560 nm whereas *D. viride* and *Didemnum* aff *sphaericum* have peaks at 542 nm and only shoulders at 560 nm (Fig. 1). The peak at 612 nm is due to phycocyanins (Hirose et al., 1969; O'Carra and O'hEocha, 1976). The fluorescence emission spectra of these extracts all consisted of single sharp bands at 570–572 nm.

Chromatography on Biogel-HTP resulted in 2 major bands for each of the extracts from *T. tegulum* and *Didemnum* aff *sphaericum* but, 4 bands from *D. viride*. In *T. tegulum* and *Didemnum* aff *sphaericum* the first band (Tt-PE I and Ds-PE I respectively) elutes with approximately 0.008 M phosphate and the second with approximately 0.05 M phosphate (Tt-PE II and Ds-PE II respectively). In *D. viride* the bands elute with 0.008 M, 0.016 M, 0.05 M and 0.1 M phosphate (Dv-PE I, Dv-PE II, Dv-PE III, and Dv-PE IV respectively) (Fig. 2). The first band from all 3 species has a 560 nm peak and a distinct shoulder at 542 nm (Fig. 3). Tt-PE II, Ds-PE II, Dv-PE III and Dv-PE IV have peaks at 498 nm and 542 nm with almost negligible shoulders at 560 nm (Fig. 4). The spectroscopic data are presented in Table 1.

The fluorescence spectra consist of single sharp emission bands (Table 1). The 570–572 nm band is emitted solely by the 560 nm type PEB. The 558–562 nm band emitted by Dv-PE III, Dv-PE IV and Ds-PE II is evidently emitted by a 542 nm PEB. The fluorescence excitation spectra of each of the phycoerythrins follow closely the visible absorption spectra; indicating that light quanta absorbed by these phycoerythrins is transferred very efficiently from the PUB (498 nm) to the 542 nm and 560 nm type PEB.

The presence of a number of bands prompted the use of DEAE-sephacel to check that the bands were not the result of some breakdown of the phycobiliprotein on the Biogel-HTP or an artifact formation.

A similar separation was obtained with the DEAE-sephacel i.e. 2 bands were seen for *T. tegulum* and *Didemnum* aff *sphaericum*; with *D. viride* giving 4 distinct bands. The visible spectra of the bands were identical to the spectra of the bands from Biogel-HTP.

The position of the bands in the CD spectra agree reasonably well with those of the absorption bands, although in some cases there are small shifts relative to the absorption peaks (Fig. 5). The CD spectra of these phycoery-

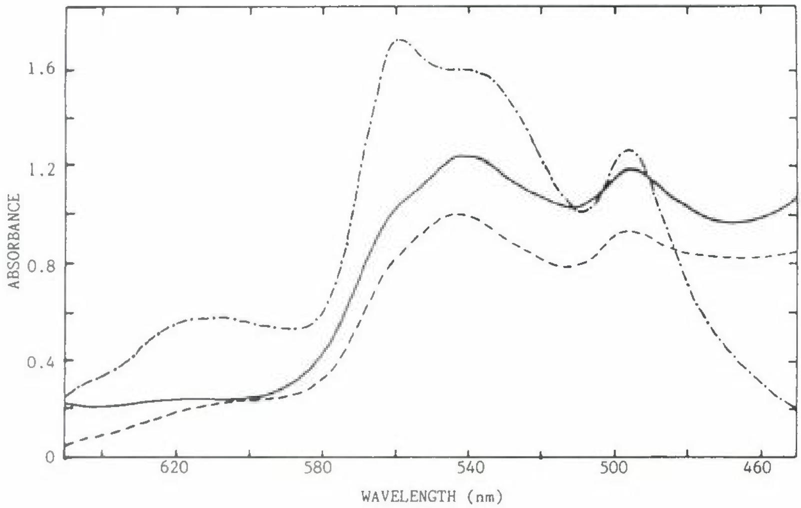


Figure 1. Absorption spectra of 0.1 M phosphate buffer (pH 7.0) extracts of *T. tegulum* (- . - . -), *D. viride* (————), and *D.* aff *sphaericum* (- - -).

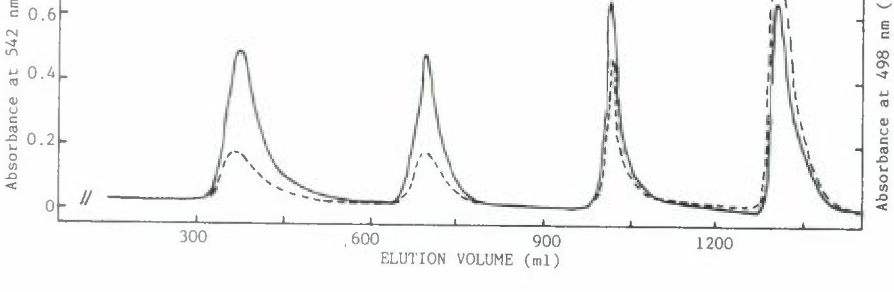


Figure 2. Chromatography of *D. viride* phycoerythrins on Bio-Gel HTP with a linear gradient of phosphate buffer (0.001 M–0.1 M) pH 7.0

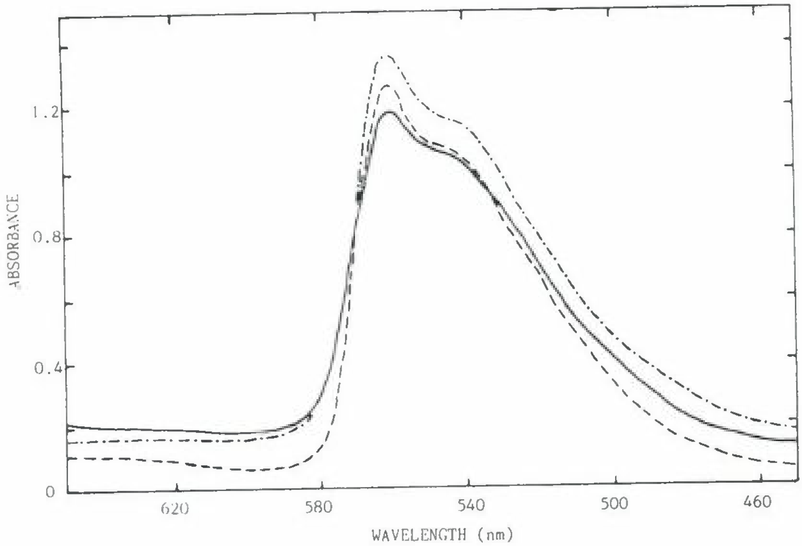


Figure 3. Absorption spectra of Tt-PE I (– . – . –), Dv-PE I (————), and Ds-PE I (– – –) in phosphate buffer (pH 7.0).

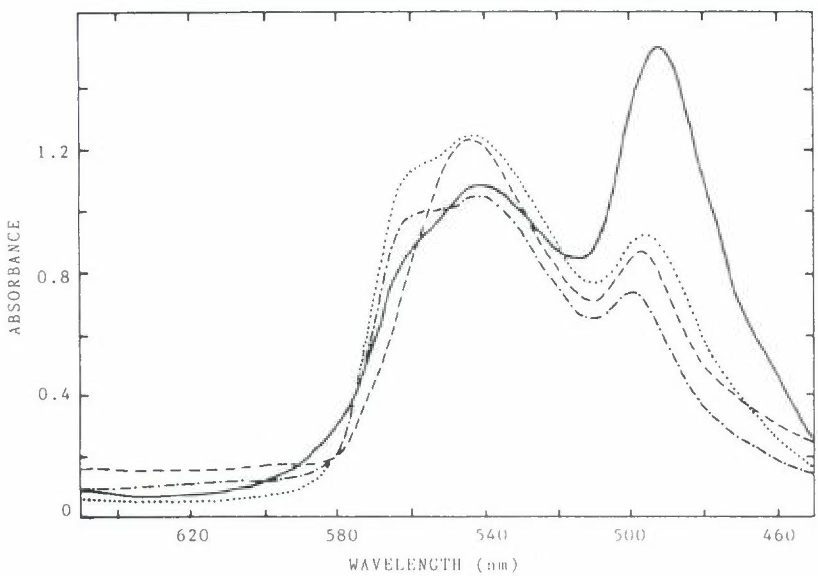


Figure 4. Absorption spectra of Tt-PE II (– . – . –), Dv-PE III (............), Dv-PE IV (————), and Ds-PE II (– – –) in phosphate buffer (pH 7.0).

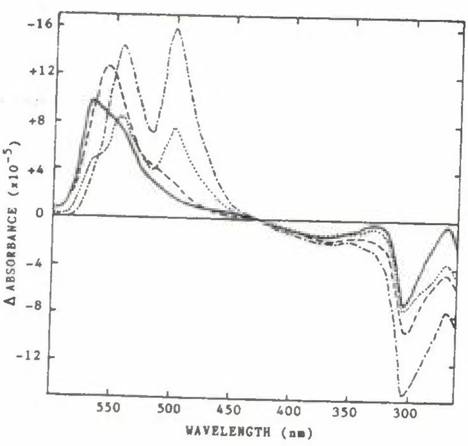
Table 1. Spectroscopic properties of the phycoerythrins

Phycoerythrin	Major visible absorption maxima (nm)	Fluorescence maxima (nm)[a]
Tt-PE I	542 < 560	570
Tt-PE II	498 ≤ 542 > 560(s)[b]	570
Dv-PE I	542(s) < 560	572
Dv-PE II	498(s) << 542 > 560(s)	570
Dv-PE III	498 < 542 > 560(s)[c]	562
Dv-PE IV	498 > 542 > 560(s)[c]	560
Ds-PE I	542(s) < 560	570
Ds-PE II	498 < 542	558

a. excitation at 500 nm.

b. 560 nm(s) occasionally appears as a distinct peak.

c. 560 nm(s) very weak, in some preparations it is not observed.



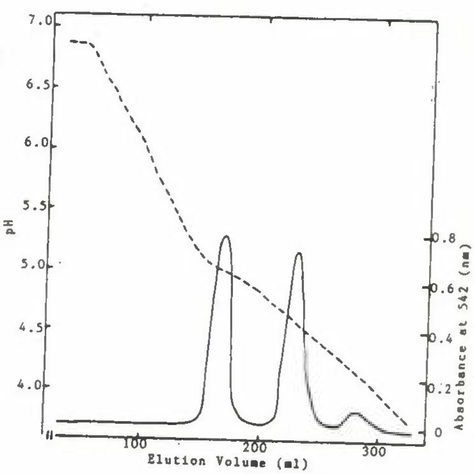


Figure 5. CD spectra of Dv-PE I (————), Dv-PE II (– – –), Dv-PE III (.............), and Dv-PE IV (– . – . –) in 0.01 M acetate (pH 5.5).

Figure 6. Chromatography of *Didemnum* aff *sphaericum* phycoerythrins on PBE 94; pH profile (– – –).

Table 2. pI values of the phycoerythrins determined by chromatofocusing

Phycoerythrins	Apparent pI
Tt-PE I	5.0
Tt-PE II	4.6
Dv-PE I	5.0
Dv-PE II	4.8
Dv-PE III	4.5
Dv-PE IV	4.3
Ds-PE I	5.0
Ds-PE II	4.6

thrins are similar to the CD spectra of R-PE, which have 3 positive bands at 572 nm, 536 nm and 496 nm (Pecci and Fujimori, 1969). In B-PE the 565 nm band exhibits splitting of its CD band, having one positive band at 572 nm and one negative band at 558 nm; with positive bands at 537 nm and 505 nm. This splitting is indicative of the interaction between chromophores (Pecci and Fujimori, 1969). C-PE shows a negative Cotton effect at 570 nm and a positive Cotton effect at 549 nm and has no 498 nm band (Kursar et al., 1981).

The apparent isoelectric points of the phycoerythrins were determined using PBE 94. The pI values for the phycoerythrins are shown in Table 2 and a chromatogram is shown in Figure 6. The blue phycocyanin eluted at pI 4.2. Most algal biliproteins have isoelectric points in the range of 4.3–4.9 (O'Carra and O'hEocha, 1976).

Two bands were obtained for each of the phycoerythrins at pI 4.55 and pI 4.85 on isoelectric focusing. This may be due to the dissociation of the phycoerythrins into their α and β subunits (Fig. 7). This has been observed in other phycobiliproteins (Glazer and Hixson, 1975).

SDS-PAGE shows the presence of 1 band, visible as a sharp red band prior to staining with coomassie blue, at approx. 19 kDa for all bands form *T. tegulum*, *D. viride* and *Didemnum* aff *sphaericum* and unlike R-PE have no γ peptide unit at approx. 30 kDa (Fig. 8). A weak second band was occasionally observed at approx. 39 kDa in Tt-PE I and Tt-PE II. On gel filtration on Sephadex G-200, all the native phycoerythrins gave homogeneous bands with apparent molecular weights of approx. 39,000 (Fig. 9).

The separation of subunits was attempted using Bio-Rex 70 chromatography. When preparing the phycoerythrins for application to the column

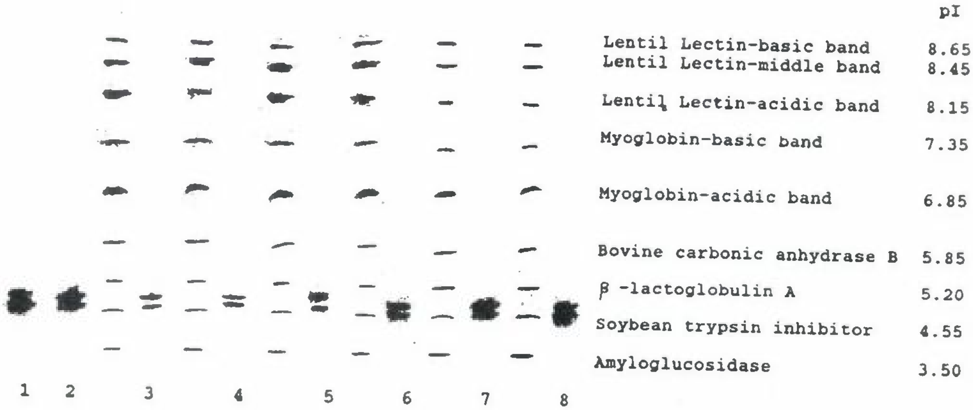


Figure 7. Isoelectric focusing on Pharmacia PhastGel IEF 3–9: Tt-PE I, Tt-PE II, Dv-PE I, Dv-PE II, Dv-PE III, Dv-PE IV, Ds-PE I and Ds-PE II (lanes 1,2,3,4,5,6,7 & 8 respectively); after staining with Coomassie blue.

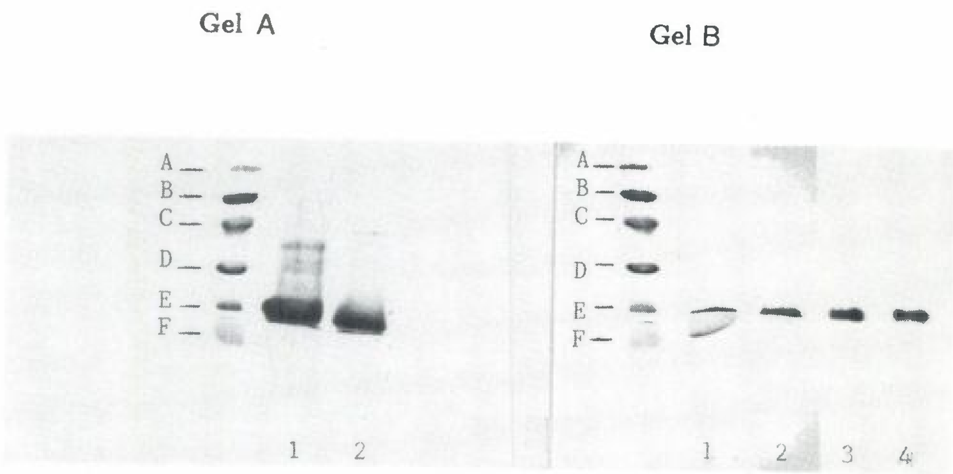


Figure 8. Electrophoresis of phycoerythrins on SDS- PAGE –
Gel A: Tt-PE I, and Tt-PE II (Lanes 1 & 2 respectively);
Gel B: Dv-PE I, Dv-PE II, Dv-PE III, and Dv-PE IV (Lanes 1, 2, 3, & 4 respectively). After staining with Coomassie brilliant blue. Standards: A- phosphorylase b; B- BSA; C- ovalbumin; D- carbonic anhydrase; E- soybean trypsin inhibitor, F- α- lactalbumin.

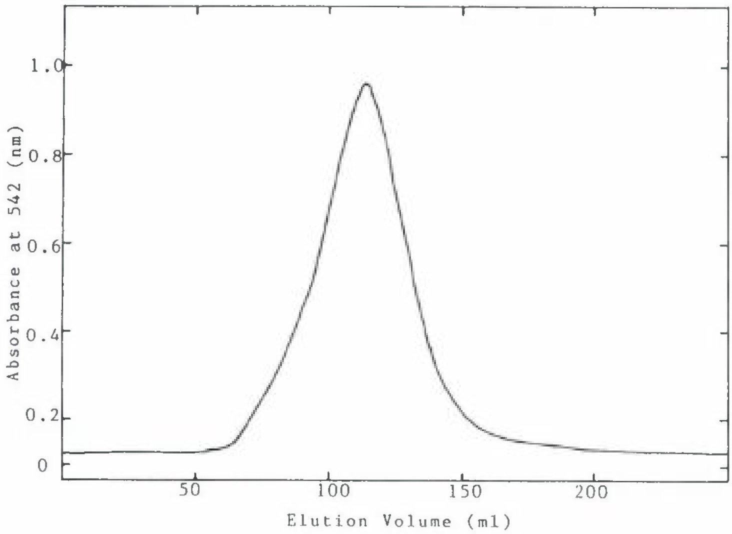
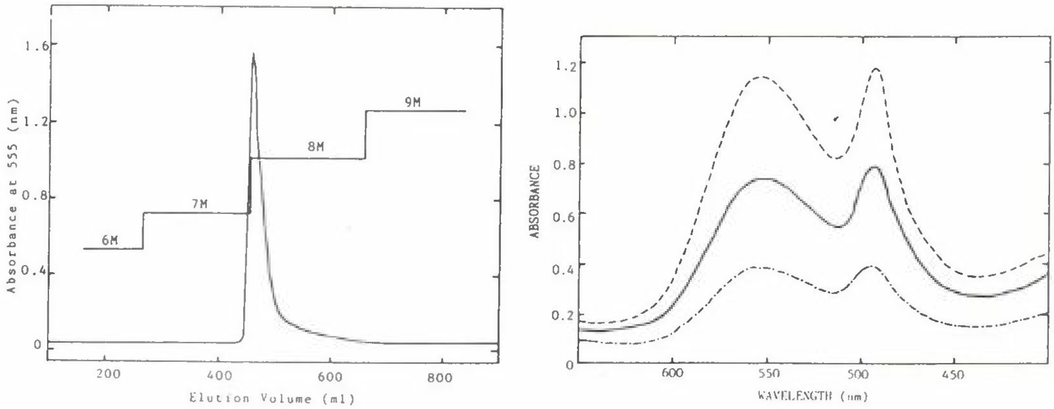


Figure 9. Gel filtration of Tt-PE II on Sephadex G-200 eluted with 0.05 M phosphate buffer (pH 7.0).

nearly all the protein precipitated irreversibly. The small amount that did not precipitate was applied to the column but did not elute even at 9 M urea (pH 3.0). It was found that as the pH was lowered the phycoerythrin began to spread out over the column at 9 M urea (pH 2.5); but, when pH 2.0 was reached it eluted as a sharp band.

As a result of this observation the method was modified (see modified method 1). The use of this method ensured that the entire protein sample was applied to the column in a soluble form. The Bio-Rex 70 column was eluted with urea solutions at pH 2.0. The use of this method resulted in the complete elution of the protein as one sharp band with 7 M urea (pH 2.0) (Fig. 10a). No α and β subunits were separated using this stepwise elution procedure. Each of the eight phycoerythrins exhibited identical behavior, eluting as single sharp bands with 7 M urea (pH 2.0). A linear gradient was tried (Muckle and Rudiger, 1977); but still only one sharp band eluted at 7 M urea (pH 2.0). The visible spectra of fractions taken across the 7 M urea band for each Tt-PE I, Dv-PE I, Dv-PE III, Dv-PE IV, Ds-PE I and Ds-PE II were identical (Fig. 10b). However spectra of fractions taken across the 7 M urea band for Tt-PE II and Dv-PE II showed changes in the ratio of 495:555 (Fig. 11). This would indicate that the α and β subunits in these 2 phycoerythrins have different chromophore contents.



a b

Figure 10a. Chromatography of Dv-PE III on Bio-Rex 70 (minus 400 mesh) in urea at pH 2.0.

Figure 10b. Absorption spectra of fractions from the 7 M urea band from Dv-PE III (pH 2.0): Front(———), Peak (– – –), Tail (– . – . –).

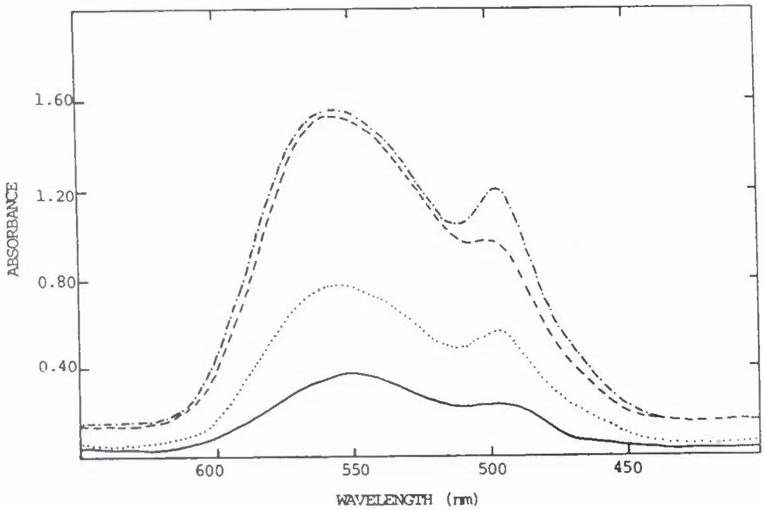


Figure 11. Sequential absorption spectra of the 7 M urea band of Tt-PE II: 1 (———); 2 (– – –); 3 (– . – . –); 4 (............).

As the molecular weights of the native phycoerythrins were calculated to be 39,000 it is most probable that the 19 kDa band represents 2 identical subunits. Cox et al. (1985) report the presence of α and β subunits from a phycoerythrin isolated from *T. tegulum*.

The disulfide analysis was negative for all the phycoerythrins from the cyanophytes of all 3 species. The amino acid analysis (Table 3) indicates a great deal of homology between the phycoerythrin proteins of the 3 symbionts. Acidic amino acids predominate over the basic residues and there is a relatively high proportion of amino acid residues with hydrophobic side chains. The complete amino acid sequences of these phycoerythrins will be necessary to establish whether they are equivalent.

The major spectroscopic differences between these phycoerythrins is in the presence (or absence) of the 498 nm peak or shoulder. This peak represents the contribution of the PUB groups on the polypeptide chain. The other differences between the absorption and CD spectra could be consequences of the differences in the aggregate states and the associated chromophore-chromophore interactions among the bilin moieties. These phycoerythrins are carrying one, or in most cases, two chemically distinct types of bilin prosthetic groups on 2 similar polypeptide chains. The diversity in the spectroscopic properties therefore appears to be a consequence of varying ratios of the two bilin chromophores (see Table 4 and Table 5). Tt-PE I, Dv-PE I and Ds-PE I have no PUB, only 6 PEB groups. The PUB:PEB ranges from 1:6 up to 1:1 for Dv-PE IV.

Although this group of phycoerythrins has similarities to the R-PE, B-PE and C-PE groups, they are more correctly designated as a group of CU-PE's. Using this nomenclature three types can be identified:

Tt-PE I, Dv-PE I, Ds-PE I = CU-PE I
Tt-PE II, Dv-PE II = CU-PE II
Dv-PE III, Dv-PE IV, Ds-PE II = CU-PE III

The wide variations in ratios can be understood when one considers the role of these phycoerythrins and the habitat occupied by the cyanophytes. The main role of the PUB chromophore attached to the phycoerythrins is to enhance the light harvesting capability of the phycobilisome in the region between 460 and 500 nm. This absorbance range coincides exactly with the maximum light transmittance of tropical seawater (Jerlov, 1976). These variations in chromophore content may be a result of chromatic adaption,

Table 3. Amino acid composition of phycoerythrins[a]

Amino Acid	Tt-PE I	Tt-PE II	Dv-PE I	Dv-PE II	Dv-PE III	Dv-PE IV	Ds-PE I	Ds-PE II
Asp	38	34	38	34	43	39	33	37
Thr	15	17	16	18	17	17	19	18
Ser	19	22	24	28	27	25	29	24
Glu	29	27	32	35	36	37	33	38
Pro	10	11	10	9	8	10	7	8
Gly	26	27	31	32	35	29	31	30
Ala	38	35	34	29	32	26	32	29
Half-[b] Cys	3	3	2	2	2	3	2	2
Val	15	14	12	13	11	16	15	16
Met	7	8	6	6	6	5	7	6
Ile	9	11	9	11	10	12	11	11
Leu	24	25	23	21	21	19	20	21
Tyr	12	12	10	11	9	8	10	8
Phe	7	8	8	9	8	9	9	9
His	3	2	5	5	3	4	5	5
Lys	16	14	14	14	14	11	11	10
Arg	15	17	15	12	11	15	14	13
Trp[c]	1	1	nd[d]	nd	1	1	nd	1

a. calculations based on the assumption that each phycoerythrin has a molecular weight of 39,000 with 6–7 bilin chromophores.

b. determined by method of Moore (1963)

c. determined by method of Simpson et al. (1976)

d. nd = not detected

Table 4. Calculated absorbance ratios $A_{495}:A_{555}$ from various mixtures of PUB and PEB chromophores and the observed ratios $A_{495}:A_{555}$ for the phycoerythrins in 8 M urea pH 1.9.

PUB:PEB	Calc. $A_{495}:A_{555}^{a}$	Phycoerythrins	Obs. $A_{495}:A_{555}$
0 (pure PEB)	0.32		
		Tt-PE I	0.37
1:1	2.71		
		Tt-PE II	0.69[b]
1:2	1.51		0.80
			0.91
1:3	1.11		
		Dv-PE I	0.39
1:4	0.91		
		Dv-PE II	0.61[b]
1:5	0.79		0.71
1:6	0.71	Dv-PE III	0.91[b]
			1.06
1:7	0.65		1.18
			1.28
		Dv-PE IV	1.41[b]
			1.50
			1.51
			2.55
		Ds-PE I	0.35
		Ds-PE II	1.32

a. Values taken from Bryant et al. (1981).

b. range of ratios from different extracts i.e. ascidians collected at different times.

Table 5. Bilin chromophore content of the phycoerythrins in 8 M urea, 0.01 M β-mercaptoethanol at pH 1.9

Protein	No. of determinations	PEB Content[a]	PUB Content[b]
Tt-PE I	3	6	
Tt-PE II	2	6	1
	2	5	1
	2	4	1
Dv-PE I	2	6	
Dv-PE II	3	6	1
Dv-PE III	2	4	2
	2	5	2
Dv-PE IV	3	4	2
	1	3	3
Ds-PE I	2	6	1
Ds-PE II	2	5	2

a. PEB content calculated from $\epsilon_{M}^{555\ nm}$ of $4.3 \times 10^{4} M^{-1}\ cm^{-1}$; $\epsilon_{M}^{495\ nm}$ of $1.34 \times 10^{4} M^{-1}\ cm^{-1}$ for polypeptide-bound PEB.

b. PUB calculated from ϵ_{M}^{495} of $10.4 \times 10^{4} M^{-1} cm^{-1}$ for polypeptide-bound PUB. (Bryant et al., 1981).

a phenomenon known in red algae and cyanobacteria (Glazer, 1977; Stanier and Cohen-Bazire, 1977). Yu et al. (1981) reported that the relative amounts of PEB and PUB in the R-phycoerythrin of the red alga *Callithamnion* are under environmental control, PUB being substituted for PEB at low light intensities. It is therefore difficult to determine the chromophore content accurately; it is governed by the quantity and quality of the light (Parry, 1988).

The presence of the PUB chromophore in these symbiotic cyanophytes obviously extends the habitat range, particularly of *D. viride*, which has an extremely wide range, from full sun conditions in only a few meters of water to depths of 18 m under ledges where the irradiance is extremely low (Parry, 1988). When one considers the fact that these cyanophytes are not free living, that they are symbionts, one could reasonably expect these variations in pigment composition. Indeed the variations that are exhibited by these symbionts are ideal for the habitats that they occupy. As a result it would be unwise to make direct comparisons with the phycobiliproteins from free living algae or algal cultures.

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