Isolation of intact detergent-free phycobilisomes by trypsin

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Phycobilisomes have been detached from red algal thylakoids by means of trypsin and purified by sucrose-gradient centrifugation in the absence of detergents. These phycobilisomes have identical absorbance and fluorescence properties to phycobilisomes prepared with detergent. Their peptide composition determined by SDS–PAGE suggests that a lamellar peptide is cleaved by trypsin to release the intact phycobilisome. Subsequently trypsin attacks the 94 kDa peptide implicated [Redlinger, T. and Gantt, E. (1982) Proc. Natl. Acad. Sci. USA 79, 5542–5546] in the phycobilisome attachment.

Phycobilisome *Biliprotein* *Fluorescence* *Trypsin* (Griffithsia monilis) *(Rhodophyta)*

1. INTRODUCTION

In red algae and cyanobacteria most of the light energy for photosynthesis is harvested by the phycobiliproteins, phycoerythrin and phycocyanin and then trasnferred with high efficiency via allophycocyanin to chlorophyll *a*. The phycobiliproteins are organised into discrete bodies, the phycobilisomes which are readily apparent in electron micrographs of intact thylakoids. Phycobilisomes have been isolated from many organisms using a variety of detergents in buffers containing high concentrations (>0.5 M) of salt, especially potassium phosphate [1,2]. During recent studies (in preparation) of the inhibition of photosystem II by trypsin at intermediate salt concentrations there was an increased fluorescence at 77 K, probably from the phycobilisomes. This encouraged us to explore the use of trypsin at high salt concentrations to prepare intact phycobilisomes free of any detergents.

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2. MATERIALS AND METHODS

Griffithsia monilis Kunth. (Rhodophyta) was obtained initially from Athol's Wharf in Sydney Harbour and subsequently cultured axenically in Provasoli's enriched sea water [3,4]. The algae grew readily at 18°C with air bubbling under continuous illumination (Mazda Universal White fluorescent, $20\,\mu E.m^{-2}.s^{-1}$). Rhodoplasts were prepared by blending the algal mat in 0.5 M potassium phosphate plus 0.3 M potassium citrate (pH 7.2) [5]. The resulting brei was filtered through 2 layers of Miracloth and centrifuged at $1000 \times g$ for 5 min. After washing, the pellet was resuspended in the blending medium to give 200 μg chl.ml^{-1}. Trypsin (Sigma Type XI) was added to give 0.1 mg.ml^{-1} final conc. and the reaction was terminated by addition of excess (0.2 mg.ml^{-1} or 0.5 mg.ml^{-1}) trypsin inhibitor (Sigma Type II-T) as indicated.

Liberation of phycobilisomes was assessed by the increase in fluorescence emission at 685 nm (560 nm excitation) at 77 K and from the absorbance at 565 nm of a 10× diluted supernatant following centrifugation of the reaction mixture at $3000 \times g$ for 10 min. Phycobilisomes were iso-

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lated by centrifugation of samples in a linear gradient of 10–40% sucrose in 0.5 M potassium phosphate–0.3 M potassium citrate (pH 7.2) without detergent (Beckman SW 41 rotor for 16 h at 234000 × *g*). For comparison, similar rhodoplasts preparations were treated with 0.5% Triton X-100 and phycobilisomes isolated as above except for the addition of 0.1% (v/v) Triton to the sucrose gradients.

Fluorescence measurements were made on a Perkin Elmer MPF 44B spectrofluorometer in the ratio mode but not further corrected. Absorbance measurements were made on a Philips 8-200 spectrophotometer.

Polypeptides were analysed by SDS–PAGE essentially as in [6]. Phycobilisomes were dialysed against 0.01 M Tris–HCl (pH 8.3) transferred to 0.12 M Tris–SO_4 (pH 6.8) and then concentrated by centrifugation through Amicon CF 25 centriflo membrane cones. Concentrated samples were made to 2% SDS and 0.05 M DTT and boiled for 2 min immediately before application to the gel.

3. RESULTS

The fluorescence emission at 77 K of *Griffithsia* thylakoids with attached phycobilisomes excited by 560 nm light is characterised by peaks at 685 and 695 nm together with a shoulder at 710 nm (fig.1A). The peak at 685 nm is believed to originate principally from the terminal emitter of the phycobilisome. On treatment with trypsin, the fluorescence at 685 nm increases sharply whereas that at 695 nm disappears (fig.1B,C) but the latter may only be masked by the 685 nm fluorescence. The shoulder at 710 nm persists. The enhanced 685 nm emission caused by trypsin treatment still originates from light absorbed by R-phycoerythrin as shown by its excitation spectrum with peaks at 500 nm, 547 nm and 568 nm (fig.1D).

Thylakoids which had been exposed to trypsin were centrifuged and the resulting supernatant was the magenta colour characteristic of *Griffithsia* phycobilisome preparations. Purification of the supernatant on 10–40% linear sucrose gradients

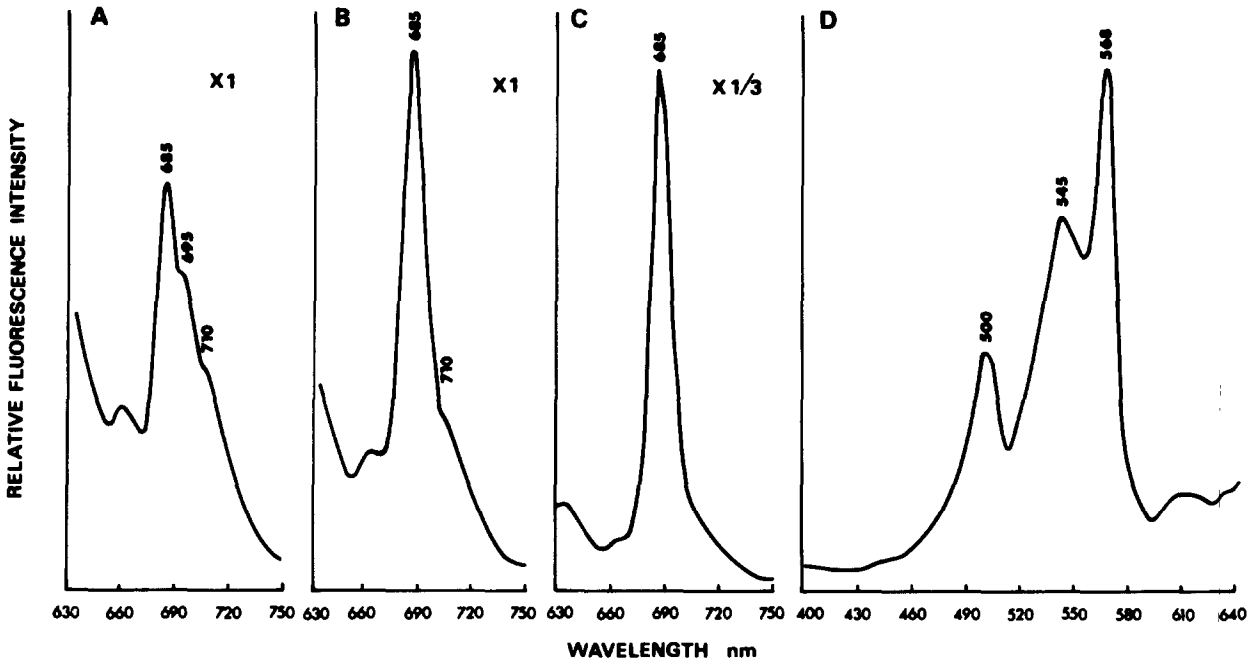

Fig.1. Effect of trypsin on fluorescence of *Griffithsia* thylakoids at 77 K: (A) emission before trypsin addition; (B) after 5 min exposure to trypsin; (C) after 40 min exposure to trypsin. The excitation wavelength was 560 nm; (D) excitation spectrum of the 685 nm emission after 40 min exposure to trypsin.

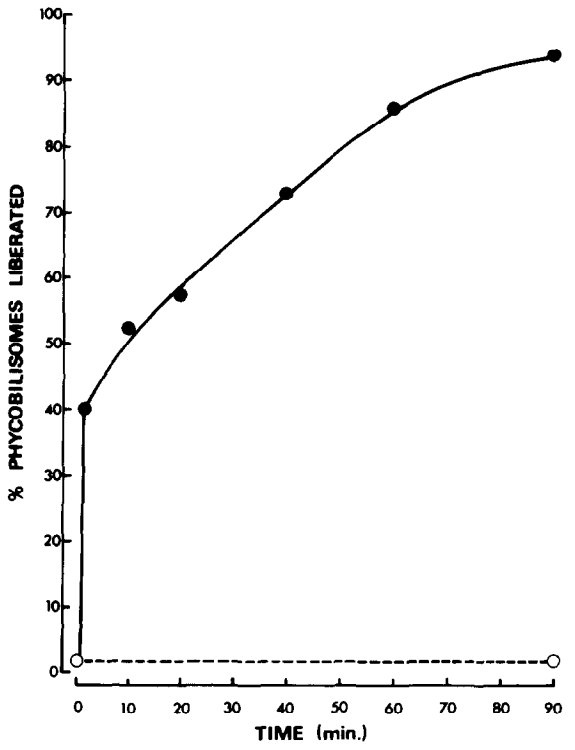

Fig.2. Time course of the percentage of the total phycobilisomes liberated from the thylakoids by trypsin.

gave an intense purple band which coincided exactly with that of phycobilisomes liberated by means of Triton X-100. A fraction containing chlorophyll *a* banded at 20% sucrose in the trypsin treatment which was absent from the Triton treatment.

The time course and extent of phycobilisome liberation by trypsin was assessed from the increase in phycoerythrin present in the supernatant after a 10-fold dilution with water. An exposure of only 1 min to trypsin resulted in 40% of the phycobilisomes being liberated. This high figure may in part be due to a slow action of trypsin inhibior (0.2 $mg.ml^{-1}$) at the high salt concentration employed and to thylakoids remaining in contact with the reaction mixture until the end of the experiment. Longer incubations up to 90 min resulted in liberation of 90% of the phycobilisomes (fig.2). In a subsequent experiment, the action of trypsin was terminated by the addition of 0.5 $mg.ml^{-1}$ trypsin inhibitor and samples were centrifuged 2 min later. After 10 min exposure to trypsin, 22% of the phycobilisomes had been released and this increased to 67% after 30 min, a similar value to that obtained in fig.3.

Phycobilisomes liberated by either trypsin or Triton X-100 were purified on sucrose gradients

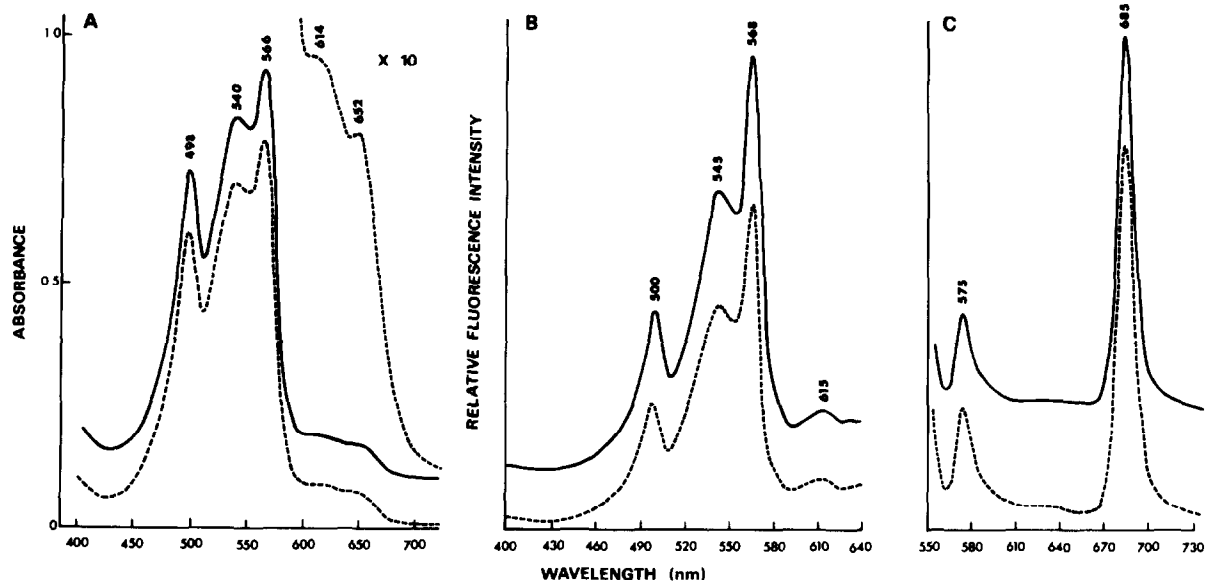

Fig.3. A comparison of the absorbance and fluorescence properties of purified phycobilisomes released by trypsin (– – –) and by Triton X-100 (——): (A) absorbance spectra; (B) fluorescence excitation spectra of 685 nm emission; (C) fluorescence emission spectrum of 500 nm excitation.

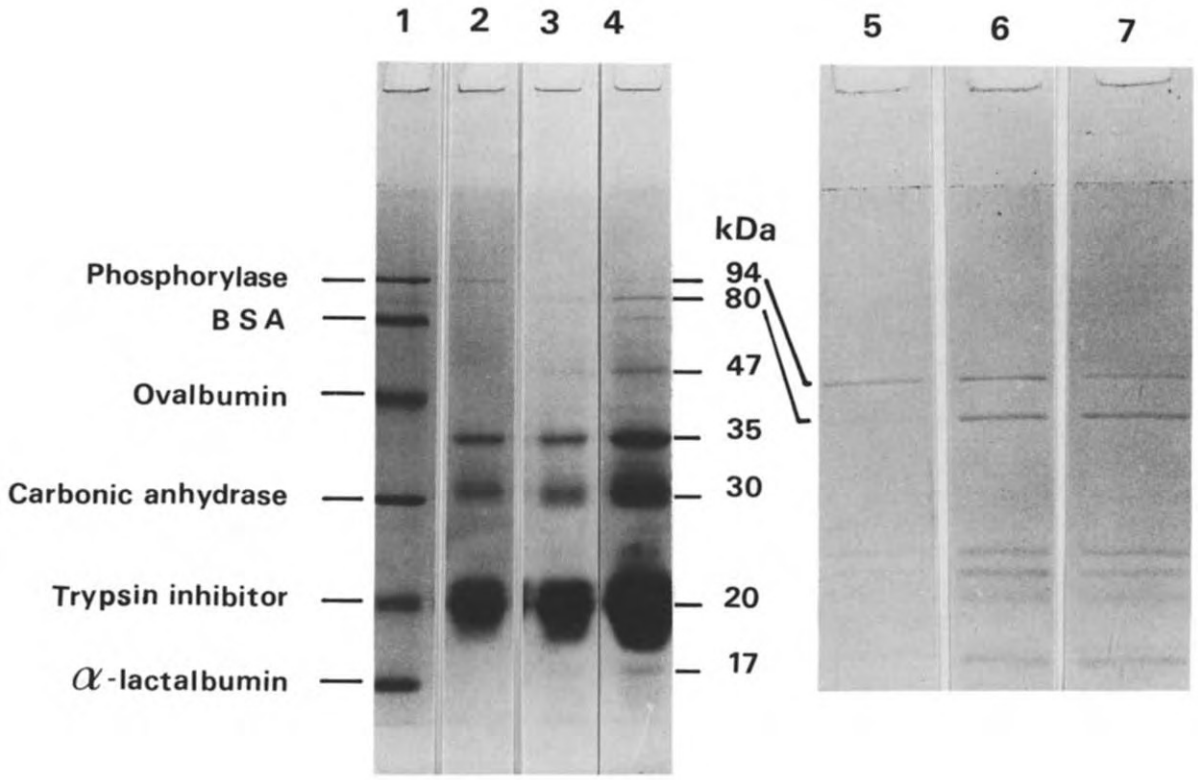

Fig.4. Separation of phycobilisome polypeptides by SDS–PAGE: (1) standard polypeptides – phosphorylase *b* (94 kDa), serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.4 kDa); (2) Triton X-100 phycobilisomes; (3) trypsin phycobilisomes (3 h trypsin); (4) trypsin phycobilisomes (3× loading); (5–7) enlarged 80–94 kDa region of gels of phycobilisomes released by exposure to trypsin for 10, 30 and 150 min, respectively; (2,3) loaded with equal amounts of phycoerythrin.

and their absorbance and fluorescence properties compared. As shown in fig.3(A–C), these are identical.

The polypeptide compositions of the two phycobilisome preparations were examined by SDS–PAGE (fig.4). By comparison with previously published phycobilisome polypeptide patterns we assign in the Triton phycobilisomes (track 2), the faint band at 94 kDa to the possible attachment polypeptide [7,8], that at 35 kDa to the j subunit of phycoerythrin, the heavily stained region at 20 kDa to the α and β subunits of phycocyanin and phycoerythrin. Trypsin phycobilisomes (tracks 3,4) show very similar polypeptide patterns except for the diminution in the 94 kDa band, appearance of an 80 kDa band and another band at 47 kDa. There is also some spreading of the 30 kDa band into lower molecular masses. Tracks 5–7 show an enlargement of the 94 and 80 kDa regions of the gels for phycobilisomes isolated after exposure to trypsin for 10, 30 and 150 min, respectively. At the shorter times the 94 kDa band is especially prominent.

4. DISCUSSION

Much recent work has centred on the peptides involved in the attachment of the phycobilisomes to the thylakoid. Peptides, present in relatively low amounts, in the M_r range of 75–95 kDa have been strongly implicated [7–9]. Evidence for this includes retention of allophycocyanin and the high-M_r polypeptides when thylakoids are washed in dilute salt solutions, isolation of the high-M_r peptides with attached chromophores showing long wavelength fluorescence emission and simultaneous disappearance of phycobilisomes and the high M_r peptides under nitrogen starvation. Our results

would tend to support this since the 94 kDa peptide is much reduced in the trypsin phycobilisomes. However, it has been shown that this peptide is preferentially attacked and converted to an 80 kDa peptide by trypsin in phycobilisomes prepared by means of detergent [10]. Since the 94 kDa peptide is always present in low amounts in our trypsin phycobilisomes and predominates after short times of exposure to trypsin we suggest that the attachment site includes another peptide normally associated with the thylakoid fraction and this is cleaved by trypsin. If this is the case we would expect to observe new peptides appearing in the composition of phycobilisomes liberated by trypsin, such as the 47 kDa peptide. However, a small peptide might not be noticed or be lost during the isolation procedures. The use of trypsin to isolate intact phycobilisomes free of detergent may well be a useful tool in further studies of the molecular architecture of the red algae and cyanobacterial photosynthetic apparatus.

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