

Full Length Research Paper

Photosynthetic properties of the protoplasts from *Bryopsis hypnoides* Lamouroux. during the early regeneration process

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The scanning electronic microphotograph (SEM), photosynthetic oxygen evolution rate and excitation spectra at liquid nitrogen temperature (77 K) of the protoplasts from *Bryopsis hypnoides* were determined at the early regeneration period (12 h). SEM revealed an existence of the tight aggregation; cell membrane and wall were about 10 nm, 6 and 12 h respectively, after the culture. Oxygen evolution rate decreased slowly during the whole process and a minus value appeared at 6 h. Peaks originated from photosystem II (PS II) and photosystem I (PS I) apparently shifted to shorter wavelength during the regeneration process in the excitation spectra. No peak from PS I was determined until in the spectrum of 12 h, which was excited by the wavelength of 436 nm; while the peak from PS I appeared in the spectrum of extruded protoplast excited by the wavelength of 660 nm and strengthened during the rest of the regeneration process.

Key words: Photosynthetic oxygen evolution rate, absorbance spectra, excitation spectra.

INTRODUCTION

Isolated protoplasts from single cells show their attractiveness by acting as a valuable system for plant breeding and for various fundamental studies, such as photosynthetic property query (Quick and Horton, 1986; Pier and Berkowitz, 1989; Matsue et al., 1993; Peng et al., 1996; Goh et al., 1997; Kao et al., 2002; Shiku et al., 2005).

Bryopsis is a coenocytic unicell giant alga whose protoplasts can swirl into sub-protoplasts and regenerate into new lives in seawater (Kim et al., 2001; Ye et al., 2005). Ye et al. (2005) suggested that both the total soluble proteins and the ratio of chlorophyll a to chlorophyll b between the regenerated *B. hypnoides* and the wild type were quite different. Shiku et al. (2005) investigated the respiration and photosynthesis activities as a function of the size of the protoplast from *Bryopsis plumosa* and their

results indicated that the respiration rate was linear to the cube of the sample radius, while the photosynthesis rate was linear to the square of the sample radius. It is suggested, therefore, the former was controlled by the volume of the protoplast, and the latter was controlled by the surface area of the protoplast.

The information about the photosynthetic properties of the protoplasts from *Bryopsis* is still limited. In this paper, the microphotograph, photosynthetic oxygen evolution rate and excitation spectra of the protoplasts from *B. hypnoides* were employed to evaluate the photosynthetic characteristics during their regeneration process.

MATERIALS AND METHODS

Plant material

Gametophytic specimens of *Bryopsis hypnoides* were collected from the intertidal zone (35.35°N, 119.30°E, 20 - 50 cm depth) of Zhangqiao Wharf, Qingdao, China. Seawater was sampled using a pump placed 2 m deep under the water surface, and filtered with

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nested plankton nets (20 µm). Collected water was autoclaved and made up into enriched seawater (ES) (McLachlan, 1979). In the laboratory, the thalli were examined and those that were intact were isolated, washed several times with sterile seawater, disinfested with 1% sodium hypochlorite (NaClO) for 2 min, then rinsed with autoclaved seawater. The prepared material was placed into a sterile aquarium (diameter = 40 cm, height = 30 cm) containing ES enriched seawater, and maintained at 20°C under 20 µmol photons m⁻²s⁻¹ provided 12 h.d⁻¹ by cool-white fluorescent tubes.

Preparation and culture of protoplasts and the thallus segments

For the regeneration investigations, *Bryopsis hypnoides* thalli were sterilized again with 1% sodium hypochlorite for 2 min and rinsed with autoclaved seawater, and the surface moisture was removed at once with 3 layers of absorbent paper. The clean algae were cut into segments (about 4 mm), and protoplasts were squeezed out with 8 layers of muslin into 10 ml Eppendorf tubes. The initially extruded protoplasts were cultured in 10 cm Petri dishes containing autoclaved ES enriched seawater at 20°C under 20 µmol photons m⁻²s⁻¹. Some of the protoplasts were used at intervals for the spectral analysis; some of them were used in the oxygen evolution determination; some of them were cultured in Petri dishes and fixed at intervals for SEM.

Longer gametophytic filaments were selected and cut into 0.5 mm long segments using a sapphire knife and cultured in 6 cm Petri dishes containing enriched ES seawater. The segments were cultured two days for healing before being used for spectra analysis. The culture conditions were as follows: 20°C, 20 µmol photons m⁻² s⁻¹, 12 h.d⁻¹. Scanning electron microscope (SEM)

Some of the initially extruded protoplasts were cultured and fixed at intervals for SEM. Cultures were mixed for 1 h at 4°C with an equal volume of a fixative containing 5% glutaraldehyde (all the chemicals used were products of Sagon, Shanghai, China) and 2% osmium tetroxide dissolved in culture medium. After fixation, samples were placed on poly-L-lysine-coated glass plates, rinsed, dehydrated with ethanol, and then substituted with isoamyl acetate before critical point drying with a Hitachi HCP-2 (Hitachi Koki Co. Ltd., Tokyo, Japan). Imaging was carried out on a KYKY 2800B scanning electron microscope (KYKY Technology Development Ltd., Beijing, China) at an acceleration voltage of 25 kV. The chamber was kept in low vacuum mode under a pressure of 59.6 Pa at an ambient temperature (21°C).

Oxygen evolution of the protoplast/sub-protoplasts

Photosynthetic O₂ evolution in the light was monitored at 20°C polarographically using a Clark type O₂ electrode (Control Box 980321, Hansatech, King's Lynn, UK). Thermostated water of 20°C was circulated through the outer jacket of the reaction chamber (TB-85, Shimadzu, Japan). Prior to the measurement, samples were kept under the executive light condition for 5 min for adaptation, then 10 µl of samples were introduced into the chamber with the stirrer bar at the level of 3. The reaction seawater of 1 ml for the assay of protoplast photosynthesis contained 1 mM NaHCO₃ for optimal CO₂ at pH 8.0 and the protoplasts equivalent to 1 µg chlorophyll (Chl). Illumination of 300 µmol m⁻² s⁻¹ was provided by a 35 mm slide projector (halogen lamp, 224 V/500W), which was adjusted by the distance.

Spectral properties determination

Fluorescence excitation spectra (77K) of samples at different stages were taken and measured with a spectrofluorometer

(Hitachi).

RESULTS

The regeneration process of the protoplasts

The extruded protoplasts swirled into aggregation soon after being placed into autoclaved seawater (Figure 1 - 1). As light microscope revealed, protoplasts turned into compact aggregations 10 min later with the newly formed primary envelope, however, no primary envelope was found hereon because of the destructive treatment of the sample preparation for SEM (Figure 1 and 2). Protoplasts in microphotographs of 6 h (Figure 1, C 3) and 12 h (Figure 1D) were all covered by certain substances and there was no exposed organelle could be found any more.

Oxygen evolution of the protoplasts

Figure 2 shows the total oxygen content changes in the chamber with twelve selected oxygen evolution rates during the early regeneration period. The content of the oxygen increased slightly at the very start and gradually reached a maximum value at about 6 h, then began a decreasing all the rest of the time. However, the oxygen evolution rate decreased slower during the whole process and the minus value appeared 6 h later.

Spectral properties of the protoplasts and the thalli segments

Fluorescence spectra excited by the wavelengths of 436 and 660 nm were determined at liquid nitrogen temperature (77K). The result showed that emission peaks in both spectra moved toward shorter wavelength distinctly. Peaks of PS II were at 730 nm in the spectrum of 10 min, while it moved to 694 nm in the spectrum of 12 h (Figure 3a). Peaks attributed to PS I excited by the wavelength of 436 nm could not be found till in the spectrum of 12 h (Figure 3a), while weak peaks of PS I presented in all of the spectra excited by wavelength of 660 nm (Figure 3b).

DISCUSSION

Extruded protoplasts of the coenocytic green alga *Bryopsis hypnoides* aggregate spontaneously in ES enriched seawater and some of the aggregations can grow into new plants (Ye et al., 2005). The regeneration process of the protoplasts from *B. hypnoides* revealed by the SEM (Figure 1) indicates that the new lives are derived from entirely disturbed protoplasts from the giant 'mother' cells and the regeneration of the protoplasts was quickly. It is reported that most the primary organelles and about 15% of the original cell membrane of *B. plumosa* are recycled to make the body of new individuals (Kim et al., 2005).

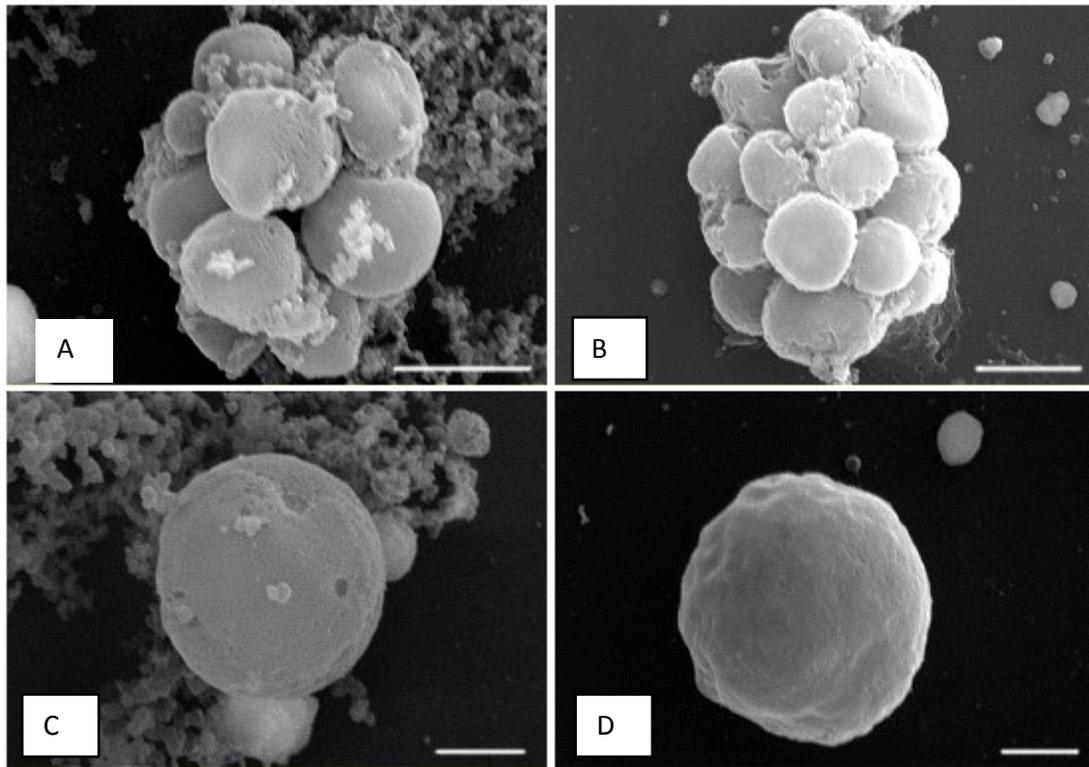


Figure 1. The regeneration process of protoplasts of *Bryopsis hypnoides*. Scale bars, 5 µm. (A) the aggregation of the protoplasts 10 seconds after the culture; (B) the aggregation of the protoplasts 10 min after the culture; and (C and D), aggregations being covered with some substances, which were fixed after 6 and 12 h after the culture respectively.

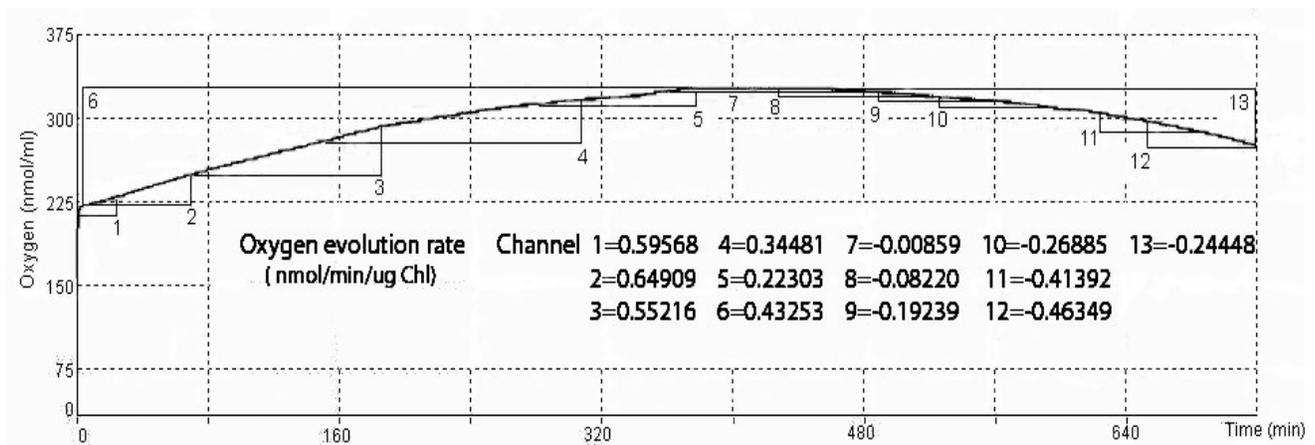


Figure 2. The oxygen content and the evolution rates of the protoplasts during the regeneration period of the experiment. 13 Channels were selected and the oxygen evolution rates were calculated using the software Oxygen Graph V 2.32. Chlorophyll was estimated from the fixed leaves. The method of chlorophyll estimation was that of Arnon (1949). The total chlorophyll was calculated according to the formula given by Arnon (1949).

The protoplasts swirled into tight globes within 10 min and started to form new cell membrane and wall within 12 h, which were consistent with the fluorescence dye results from Kim et al. (2001). According to the regenera-

tion process, three stages of the protoplasts (10 min, 6 h and 12 h after the culture) were selected for the comparison of thalli segments for the photosynthetic property analysis.

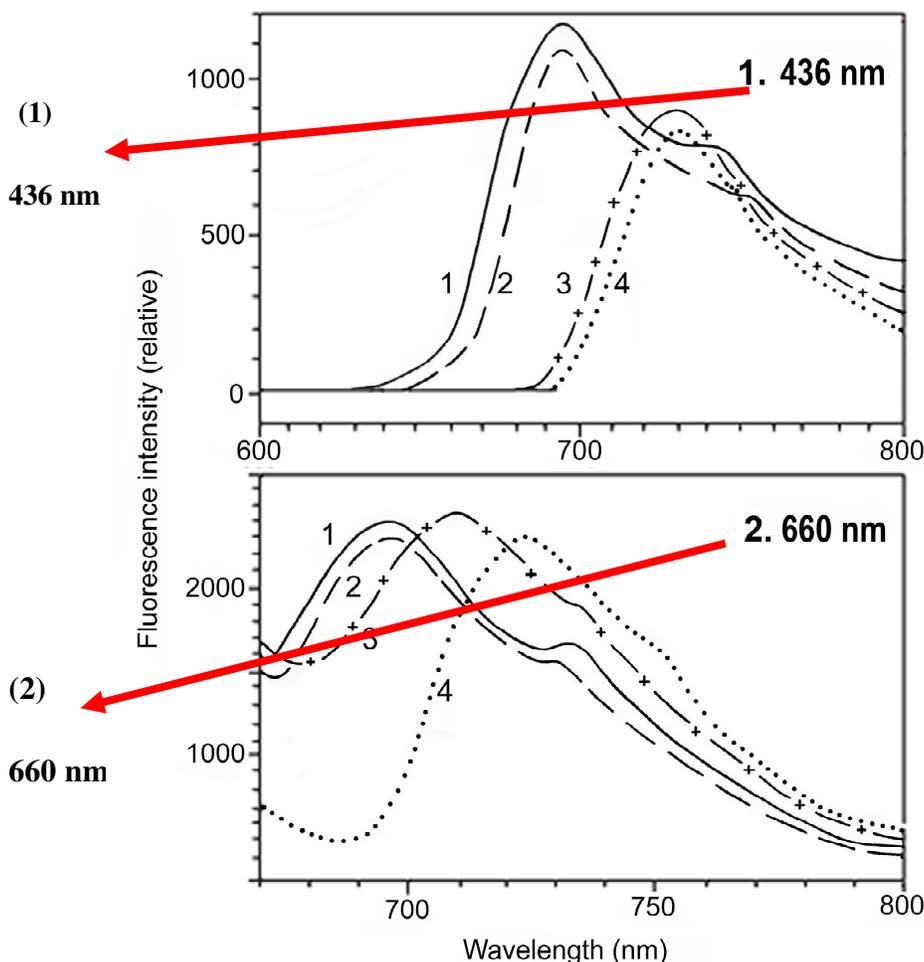


Figure 3. Fluorescence excitation spectra (77 K) of the protoplasts and thalli segments at different regeneration stages. 1, fluorescence excitation spectra excited by the wavelength of 436 nm; 2, fluorescence excitation spectra excited by the wavelength of 660 nm. —, Spectra of thalli segments; ---, Spectra of protoplasts 12 h after the culture; — + —, Spectra of protoplasts 6 min after the culture; Spectra of protoplasts 10 min after the culture.

SEM indicates that the regeneration of the protoplast from *B. hypnoides* is an absolute recombination process of the organelles (Figure 1); the excitation spectra (Figure 3) also suggest that the regeneration of protoplasts from *B. hypnoides* comprises a recombination or renaissance procedure of the organelles, including some important systems, such as photosystem I (PS I), which may be the main reason leading to the decline of the net oxygen evolution for energy consumption. The changes in the photosynthetic capacity during the cell cycle of *Scenedesmus quadricauda* are explained by the heterogeneity of photosystem II (PS II) (Kaftan et al., 1999). Kaftan et al. (1999) found that the decline in the photosynthetic oxygen evolution during the cell cycle mainly corresponded to an increase in inactive centers of PS II (PS II_x). Some studies also indicate that the energy storage (ES) and the yield of oxygen evolution rapidly increase during the first 3 - 4 h of the cell cycle, but in the second half of

the cycle, ES almost keeps a constant value, whereas the yield of oxygen steadily decreases (Szurkowski et al., 2001).

Previous studies revealed that PS I and PS II of red algae are evolutionarily in different positions: PS I is closer to that of higher plants whereas PS II is more similar to that of cyanobacteria (Szurkowski et al., 2001). Xiong et al (1998) suggested that PS I and PS II were independent to each other in their evolutionary history. It is thought that the emergence of individuality during the unicellular-multicellular transition is based on the evolution of cells that differentiate and specialize in reproductive and survival-enhancing vegetative functions (Michod et al., 2006). Taking the regeneration process of the protoplasts from *B. hypnoides* as a condensed evolution history, the result of this experiment reflects homologous cases: 1) chlorophyll b and PS I are closer to those of higher plants than chlorophyll a and PS II because the

latter take action earlier than the former; 2) chlorophyll a and b, as well as PS I and PS II are independent to each other in the evolutionary course because PS II and chlorophyll a can take action independently.

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