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Isolation and characterization of envelope membranes of cyanelles from *Cyanophora paradoxa*

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Introduction

Cyanophora paradoxa, a glaucocystophyte, is one of the three eukaryotic descendants which have aquired photosynthesis by primary symbiosis. The photosynthetic machinery of *C. paradoxa*, the cyanelle, preserves some aspects which are common to those of cyanobacteria such as phycobilisomes, caroxysomes and peptidoglycan wall between outer and inner envelope membranes (Löfferhardt et al. 1997). Thus, *C. paradoxa* is considered to be the most primitive photosyntheitc eukaryote. According to the current hypothesis that chloroplasts are acquired by symbiosis of progenitor of cyanobacteria, envelope mebranes of cyanelles should be composed of three membanes; the outermost membranes derived from plasma membranes of the host, middle and innermost membranes from outer and plasma membranes of cyanobacteria, respectively. However, cyanelles of extant *C. paradoxa* contains only two membranes. Judging from the fact that peptidoglycan wall is present between the two envelope membranes, it is assumed that one of the two outer membranes was disappeared or two membranes were fused during the evolutionary process.

In the present study, we have isolated envelope membranes from cyanelles of *C. paradoxa* and partially characterized them.

Materials and Methods

Cyanelles of *C. paradoxa* was prepared as described by Koike et al. (2000). They were suspended in sucrose-containing HEM buffer (0.6 M sucrose, 50 mM HEPES-NaOH (pH7.5), 1 mM EDTA, 2 mM EGTA and 1 mM $MgCl_2$) and treated with lysozyme at 0.1 mg/ml for 15 min at 15 °C and passed through a chilled French Pressure cell twice. The homogenate was centrifuged at 5,000×*g* for 10 min to remove unbroken cyanelles. The concentration of sucrose of the supernatant was adjusted to 55% (w/v) by 90%-sucrose-containing HEM buffer and placed onto 60%- sucrose-containing buffer. A linear sucrose gradient (53 to 4%) was constructed above the sample. The sample was centrifuged for 16 h at 120,000×*g* to separate membrane fractions. The separated membranes were fractionated as described by Koike et al. (1998). The outer membranes were separated by solubilizing the thylakoid fraction with 0.5% dodecylmaltoside (DM) at 0.5 mg Chl/ml for 20 min at 0 °C. The homogenate was centrifuged at 39,000×*g* for 20 min. Precipitate was further treated with 0.5% DM and centrifuged again. The outer envelope membranes obtained as a precipitate was resuspended in HEM buffer.

Protein compositions of each fractions were analyzed by SDS-PAGE as described by Kashino et al. (2001). Pigment compositions were analyzed by HPLC as described by Kashino et al. (1998).

Results and Discussion

Figure 1 shows a separation profile of membranes of cyanelles after floatation centrifugation. A large amount of phycobiliproteins was found in fractions 4 to 9 as monitored at 630 nm. These fractions corresponded to the position where the sample was placed. The thylakoid membranes as monitored at 678 nm floated to a lower density region peaking at fraction 9. The buoyant density of the peak was 1.19 g/cm^3 which was close to that of *Anacystis nidulans* thylakoids of 1.18 g/cm^3 (Omata and Murata 1983). The asymmetric separation profile suggests that the thylakoids were still on the way of floatation after 16 h of centrifugation. In addition to the dense colored bands, two yellow bands were separated in the middle and top of the tube. They were fractionated centering at fraction 20 and at fraction 35, respectively. The buoyant densities of the fractions were 1.11 and ~1.00 g/cm^3 and were designated as Y2 and Y1, respectively.

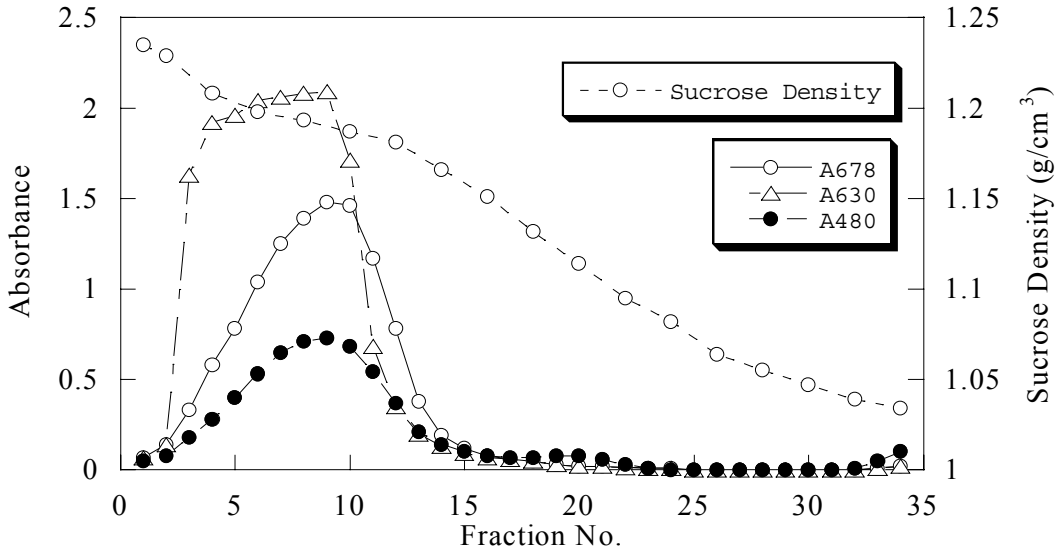

Fig. 1 Fractionation profile of thylakoid and envelope membanes after floation centrifugation. Separated membranes were collected from the bottom by 1 ml.

An absorption spectrum of Y1 showed peaks at 435 and 455 nm with a shoulder at 490 nm, while that of Y2 showed peaks at 445, 478, and 520 nm, respectively (Fig. 2). Both fractions contained practically no chlorophylls. Pigment composition of Y1 analyzed by HPLC indicated that ß-carotene was a major component, and a small amount of zeaxanthin was also found. The ratio of zeaxanthin to ß-carotene was 0.41. On the other hand, zeaxanthin was a major component in fraction Y2 and the content of ß-carotene was negligibly small; their ratio was 21.5. The carotenoid composition of fraction Y2 coincided with that of cyanobacterial plasma membranes such as *A. nidulans* (Omata and Murata 1983) in the point that the content of zeaxanthin is far higher than that of ß-carotene. Judging from the buoyant density (1.11 g/cm^3) and carotenoid composition, it is highly possible that the fraction Y2 is the inner envelope membrane of *C. paradoxa* cyanelles. It should also be noted that the buoyant density of Y2 coincided well with that of inner envelope membranes of spinach (Koike et al. 1998) or pea (Cline et al. 1981) chloroplasts.

The buoyant density of fraction Y1 was far lower than that of outer envelope membranes of chloroplast (Koike et al. 1998) or cyanobacterial outer membranes (Omata and Murata 1983). When sucrose was decreased to null in the top of the sucrose gradient, the fraction was still recovered in the top fraction. This indicates the density of the fraction is close or even lower than that of water. Electron microscopic analysis by negative staining indicated that the Y1 fraction shows some particular structure, while Y2 fraction, as well as thylakoid membranes, possesses membranous structure (data not shown). It was thus concluded that fraction Y1 is not the outer envelope membranes of cyanelles.

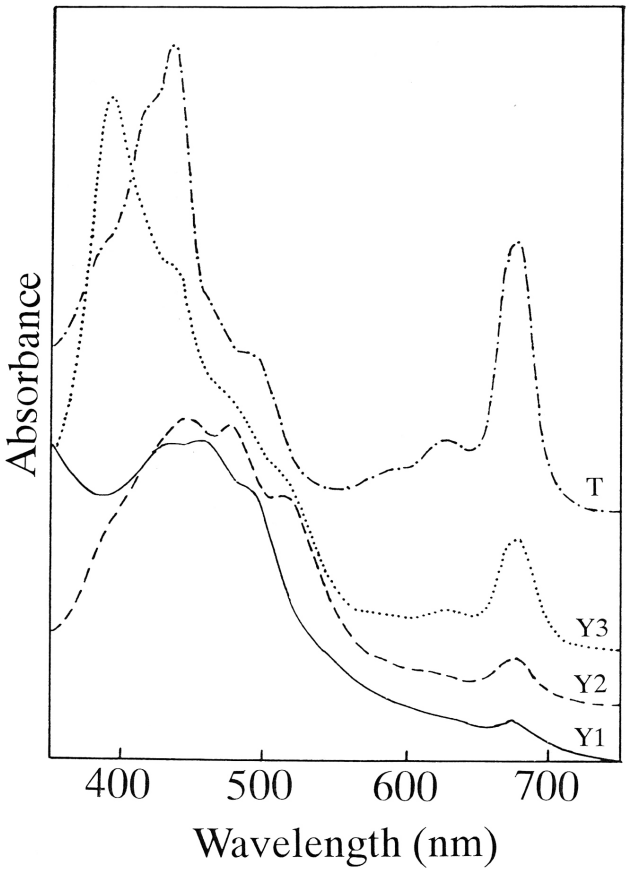

Fig. 2 Absorption spectra of fractions Y1, Y2 and Y3, and thylakoid membranes (T).

The cyanelles preserves a ludimentary but distinct peptidoglycan wall between outer and innver envelope membranes. The buoyant density of outer membranes of cyanobacteria (1.22 g/cm^3) is higher than that of thylakoid membranes (1.18 g/cm^3) due to the peptidoglycan layer adhered to the outer envelope membranes (Omata and Murata 1983). In the present study, the fractions with higher density than that of thylakoid membranes are not separated, so there is a possibility that the outer envelope membranes have a density similar to that of thylakoids. When the thylakoid fraction was solubilized with DM, a yellow precipitate (fraction Y3) was obtained after centrifugation. This coincided with the characteristics of outer membranes of cyanobacteria (Wilhelm and Trick 1995). The buoyant density was not determined since lipids and proteins might be removed by the detergent treatement.

The absorption spectrum showed a distinct peak at 390 nm and shoulders at 435, 480 and 510 nm indicative of higher content of carotenoids (Fig. 2). A shoulder at 435 nm is due to the Soret band of chlorophyll contaminated in the fraction. A similar spectroscopic feature is also reported in outer membranes of *A. nidulans* (Murata et al. 1981) and plasma membranes of *Prochloron didemnii* (Omata et al. 1985). HPLC analysis showed that the fraction Y3 was rich in zeaxanthin. The ratio of zeaxanthin to ß-carotene was close to 30, which was not very much different from that of inner envelope membranes of cyanelles (fraction Y2). However, the spectral feature was rather different between them. The absorption peak at 390 nm of outer membranes of *A. nidulans* is also reported to be due to zeaxanthin (Omata and Murata 1983). This might be due to difference in configuration of zeaxanthin bound to the proteins.

The two yellow fractions obtained in the present study (fraction Y2 and Y3) are highly possible to be inner and outer envelope membranes of cyanelles, respectively. The buoyant density of inner envelope membranes was close to that of plasma membranes of cyanobacteria or inner envelope membranes of chloroplasts, while that of outer envelope membranes were close to that of outer membranes of cyanobacteria probably due to peptidoglycan adhered to the membranes. The ratio between zeaxanthin and β-carotene in inner envelope membranes was similar to that of plasma membranes of cyanobacteria. However, the ratio in outer envelope membranes was far lower than that of cyanobacterial outer membanes, while it was much higher than that of chloroplast outer envelope membranes. Analyses of protein and lipid compositions are now in progress.

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