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Photosynthetic Characteristics of Polyvinylalcohol-Immobilized Spinach Chloroplasts

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Polyvinylalcohol에 고정화한 시금치 엽록체의 광합성특성에 대한 연구

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ABSTRACT

Photosynthetic properties of polyvinylalcohol (PVA)-immobilized chloroplast especially regarded to stability of photosynthetic electron transport and the fluorescence induction pattern were studied. When isolated spinach chloroplasts were immobilized with PVA, it showed good preservation of photosynthetic electron transport activity, especially PS II activity, during storage at −15℃, 4℃ and 20℃. And immobilized chloroplasts revealed similar thermostability of whole chain electron transport to free chloroplasts. And the absorption peak of red band of chloroplasts showed the blue-shift of 2-4 nm after immobilization. F_v/F_m ratio of chlorophyll fluorescence slightly decreased after immobilization. White light pulse after continuous light do not induced the additional fluorescence rise. This means chlorophyll fluorescence at room temperature reached to F_{max} under continuous light in the immobilized chloroplasts. It seems that PVA may be a good candidate for immobilization matrix for the preservation of photosynthetic function of thylakoids and for the continuous use of chloroplast membranes of higher plants for solar energy storage and conversion.

INTRODUCTION

The conversion of visible light energy to chemical or electrical energy via the isolated photosynthetic apparatus of plants or bacteria as photobiocatalysts is an interesting and advancing field of solar energy bioconversion research. Biophotolysis of water, hydrogen production, photocurrent generation and ATP production with microalgal cells can be used for the direct bioconversion of solar energy and have been studied by numerous authors (Hall, 1976; Bennemann *et al.*, 1980; Muallem *et al.*, 1983; Rao *et al.*, 1990; Hall *et al.*, 1991; Park *et al.*, 1991). Isolated chloroplast membranes (thylakoids) are able to perform the photolysis of water and production of hydrogen (Cuendet and Grätzel, 1982; Rao *et al.*, 1976; Gisby and Hall, 1980; Rao *et al.*, 1982), but their instability is a major limitation to their continuous use as a biocatalyst (Morris *et al.*, 1982). Among the two photosystems, photosystem II is more susceptible to environmental condition. Therefore preservation of photosystem II activity is a major problem to use chloroplasts as a biocatalyst of solar energy conversion system. Thus it seems necessary to protect and to stabilize photochemical activities after isolation of organelles.

Recently immobilization processes have imerged as an important tool for increasing the longevity of enzymes, organelles and cells as biocatalysts (Chibata *et al.*, 1987). Immobilized enzymes, organelles, cells may offer certain specific advantages such as increased density per unit volume, no washout of cells or enzymes, high operational stability, and better control of the catalytic processes. And there is some evidence that immobilization of cells, organelles and enzymes may lead to increased yields in the biotransformation of natural products (Kumakura *et al.*,

1978; Thomasset *et al.*, 1984). Immobilized plant cells and photosynthetic apparatus in natural or synthetic polymers also may have a potential use as a simple and effective system for biotransformations and biosynthesis of chemicals and biological conversion of solar energy. So much attentions has been given to the design of immobilized photosynthetic systems capable of conversion, storage and usage of solar energy into chemicals, such as high energy compounds and valuable secondary metabolites (Hall, 1976; Park *et al.*, 1991; Kaetsu *et al.*, 1979; Kitajima and Butler, 1976; Hochkoeppler and Luici, 1991).

In this report we immobilized spinach chloroplasts with PVA and then tested the functional stability and fluorescence induction patterns during storage.

MATERIALS AND METHODS

Chloroplast preparation. Class II broken Chloroplasts were prepared by Park and Kwon (1986). Spinach leaves were homogenized with the STN buffer (0.3 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, 0.25% bovine serum albumin, pH 7.8) for 20 seconds and filtered through one layer of muslin cloth. The sap obtainedd was centrifuged at 500×g for 1 min and the resulting supernatant was centrifuged at 3,000×g for 10 min. The resulting pellet was suspended in the small aliquot of STN buffer. This suspension was regarded as broken chloroplast and used for other experiments.

Immobilization. A mixture of 15% PVA in STN buffer and broken chloroplasts suspension in a total volume 3 m*l* was spread on the glass plates and dried. After drying the glass plates were stored at various temperature conditions.

Photosynthetic electron transport. Photosynthetic electron transport activity was determined by oxygen evolution or uptake rate at 20℃ with Clark type oxygen electrode (YSI 53, Yellow Springs Inst., U.S.A.) calibrated with air-saturated water. Reaction medium (total volume 2.4 m*l* of HSN buffer; 0.1 M sorbitol, 5 mM $MgCl_2$, 5 mM NaCl, 0.25% bovine serum albumin, 50 mM HEPES-KOH, pH 7.6) containing 100-200 μg chlorophyll was illuminated at a saturating intensity (2,000 $\mu E/m^2/sec$) by a halogen lamp and filtered through an orange filter. The electron donor, acceptor, electron transport inhibitor and uncoupler used were: 2 mM potassium ferricyanide (Fy), 50 μM methyviologen (MV), 0.1 mM p-phenylenediamine (p-PD), 0.1 mM dichlorophenolindophenol (DCPIP), 20 μM dichlorophenylmethylurea (DCMU), 5 mM ascorbate, 5 mM NaN_3 and 5 mM NH_4Cl (Park, 1987).

Measurement of chlorophyll fluorescene. Chlorophyll fluorescence measurements were carried out with PAM 101 chlorophyll fluoremeter (Walz Co., Germany). The fiberoptics were fixed in the surface of free chloroplast suspension or PVA-immobilized chloroplast film. And chlorophyll fluorescence was measured according to Chun *et al.* (1990).

Chlorophyll determination and absorption spectrum. Chlorophyll content was calculated from the absorbances at 663 nm and 645 nm after extraction with 80% acetone (Arnon, 1949). And absorption spectrum of free chloroplasts suspension and PVA-immobilized chloroplast films were measured using Shimadzu UV-240 spectrophotometer (Shimadzu Corp., Japan).

RESULTS

Ochiai *et al.* (1982) used PVA as a adhesive agent to spread thylakoids on transparent glass electrode (SnO_2) for the photocurrent generation. However, until there is no report about the photochemical properties of PVA-immobilized thylakoids. We immobilized the isolated spinach chloroplasts with alginate and PVA and tested the stabilities of photosynthetic electron transport activity during storage. Photosynthetic electron transport activity of alginate-immobilized chloroplasts was lower than that of the free chloroplasts (data not shown). However PVA-immobilized chloroplasts showed good preservation of photosynthetic electron transport activity. Table 1 shows the photosynthetic electron transport activity and F_v/F_m ratio of the free chloroplasts and PVA-immobilized chloroplasts after one day storage. Whole chain electron transport activity ($H_2O/Fy+NH_4Cl$) of free chloroplasts and immobilized chloroplasts decreased to 61% and 52% of

Table 1. Photosynthetic electron transport activities and F_v/F_m ratio of chlorophyll fluorescence of free and PVA-immobilized chloroplasts after one day at 4℃

	Free (%)	Immobilized (%)
$H_2O/Fy+NH_4Cl$	61.0	52.0
H_2O/Fy	32.0	144.0
$H_2O/p\text{-}PD+Fy$	82.0	94.0
$DCPIPH_2/MV$	200.0	174.0
$DCPIPH_2/MV+NH_4Cl$	98.0	86.0
F_v/F_{max} ratio	0.73	0.68

*100% of PS I+II (uncoupled), I+II (coupled), II, I (coupled), and I (uncoupled) electrontransport activities of free native chloroplasts are 142.5, 51.5, 113, 149.2 and 326.2 μmole O_2/mg chl/h, respectively.

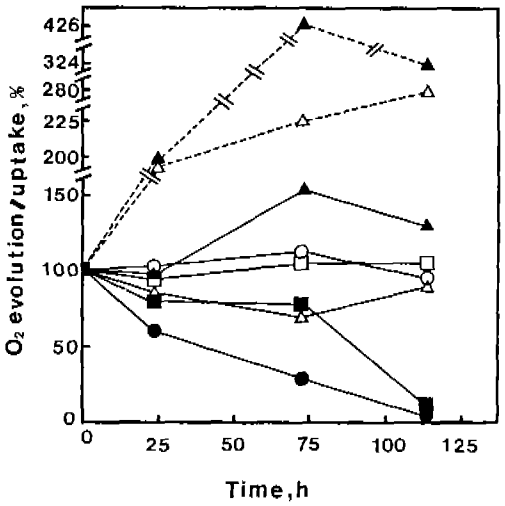

Fig. 1. Photosynthetic electron transport activity of free (closed) and PVA-immobilized (open) chloroplasts during storage at −15℃. H_2O/Fy+NH_4Cl (-○-), H_2O/p-PD+Fy (-□-), DCPIPH_2/MV (--△--), DCPIPH_2/MV+NH_4Cl (-△-).

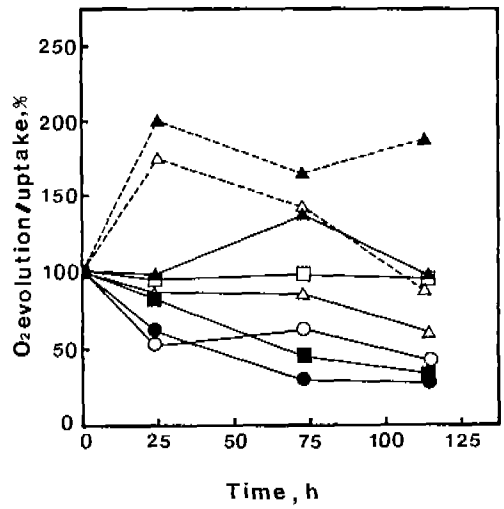

Fig. 2. Photosynthetic electron transport activity of free (closed) and PVA-immobilized (open) chloroplasts during storage at 4℃. H_2O/Fy+NH_4Cl (-○-), H_2O/p-PD+Fy (-□-), DCPIPH_2/MV (--△--), DCPIPH_2/MV+NH_4Cl (-△-)

initial activity under uncoupled condition, respectively. However, under coupled conditon, immobilized chloroplasts shows 144% of initial activity of electron transport, however, free chloroplasts shows 32% of initial activity. PS II activity of free and PVA-immobilized chloroplasts remained 82% and 94% of initial activity, respectively. PS I activity of free and immobilized chloroplasts increased to 200% and 174% of initial activity. This increase may be resulted from the coupling during storage or immobilization. So we treated the uncoupler, NH_4Cl, to reaction medium for PS I measurements, the activities were 98% and 86%. This means the possibility that immobilization process and storage may play a role of uncoupling on the thylakoids. When we measured the chlorophyll induction, the F_v/F_m ratio of immobilized chloroplasts decreased to 0.68 from 0.73 of free chloroplasts.

Fig. 1 shows the stability of photosynthetic electron transport activity of free and immobilized chloroplasts during storage at −15℃. When we measured the photosynthetic electron transport activities after thawing intervally during storage, the activity of free chloroplasts decreased to 10-15% of initial activity after 5 days. However immobilized chloroplasts maintained almost 100% of initial photosynthetic electron transport activity (H_2O/Fy+NH_4Cl). Moreover, even after one month, photosynthetic electron transport activity remained in the immobilized chloroplasts (data not shown). PS II (H_2O/p-PD+Fy) activity of immobilized chloroplasts was higher than that of free chloroplasts during storage. PS II activity of immobilized chloroplasts remained even after one month, but free chloroplasts lost activity after about 10 days. However PS I activity of free chloroplasts is higher than that

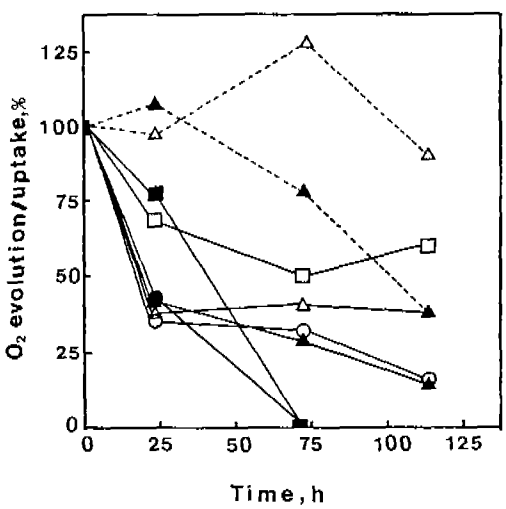

Fig. 3. Photosynthetic electron transport activity of free (closed) and PVA-immobilized (open) chloroplasts during storage at 20℃. H_2O/Fy+NH_4Cl (-○-), H_2O/p-PD+Fy (-□-), DCPIPH_2/MV (--△--), DCPIPH_2/MV+NH_4Cl (-△-)

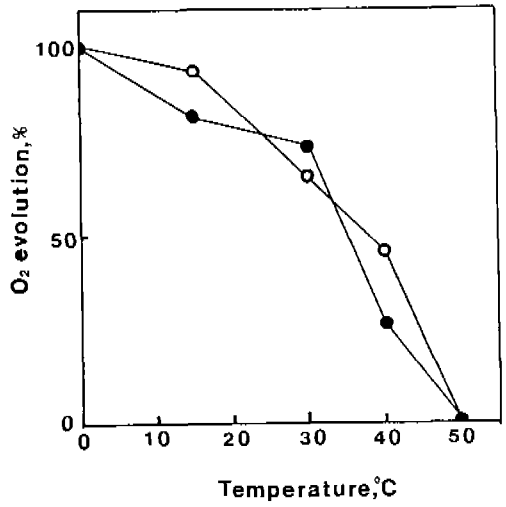

Fig. 4. Photosynthetic electron transport activity (H_2O/Fy+NH_4Cl) of free (closed) and PVA-immobilized (open) chloroplasts after 10 min of heat treatment.

of immobilized chloroplasts.

Fig. 2 shows the stability of photosynthetic electron transport during storage at 4℃. The activity of immobilized chloroplasts were remained about 10% after one month. But free chloroplasts rapidly loss photosynthetic electron transport activity. Especially, PS II activity of immobilized chloroplasts almost 100% remained after 5 days storage. However PS I activity of free chloroplasts is higher than that of immobilized chloroplasts.

Fig. 3 shows the activity of free and immobilized chloroplasts during storage at room temperature (20℃). The activity of PS I+II (H_2O/Fy+NH_4Cl) and PS II (H_2O/p-PD+Fy) of free chloroplasts diminished after 2 days. However, the activity of immobilized chloroplasts were maintained even after 5 days. In the case of PS I+II, even after one month of storage, about 10% of the initial photosynthetic activity (H_2O/Fy+NH_4Cl) was remained in immobilized chloroplasts. However PS I activity of free chloroplasts is higher than that of immobilized chloroplasts.

Fig. 4 shows the heat stability of photosynthetic electron transport in free and PVA-immobilized chloroplasts after heat treatment during 10 min. The thermostability of free and immobilized chloroplasts were almost same.

Fig. 5 shows the changes in red absorption band of free and immobilized chloroplasts during storage at room temperature (20℃) and low temperature (4℃). The peak of the red bands of free chloroplasts do not showed the blue-shift. However, blue-shift of 2-3 nm after one day

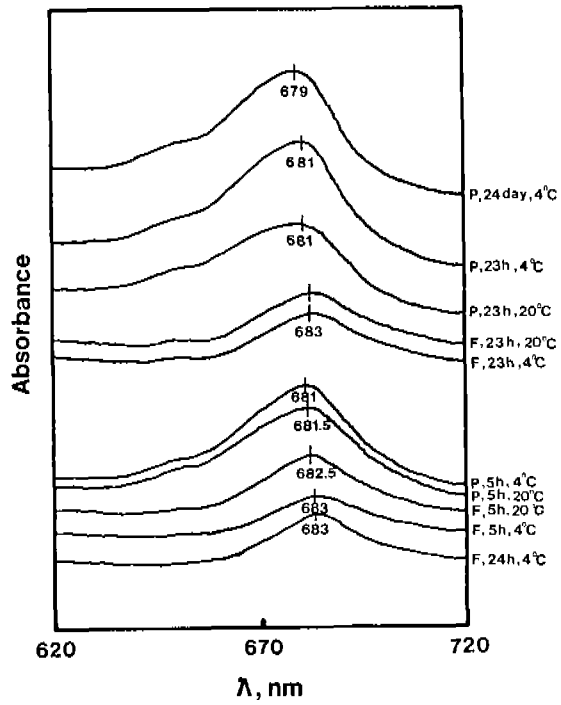

Fig. 5. Absorption spectrum of free (F) and PVA-immobilized (P) chloroplasts in red light region during storage.

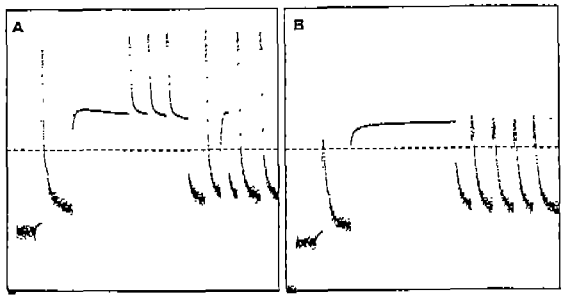

Fig. 6. Chlorophyll fluorescence induction pattern of one-day old free (A) chloroplasts and PVA-immobilized (B) chloroplasts after one day.

and 4 nm after one month occurred after immobilization. This means that immobilization process may induce the change of structural organization of pigments of thylakoid membranes.

Usually white light pulse induce the fluoresence spike to F_{max} in free chloroplasts and leaf tissues (Licthenthaler, 1988) during the steady state under actinic light. Fig. 6A shows the fluorescence spikes by the white light pulses were shown in one day-old free choroplasts. However, in the case of PVA-immobilized chloroplasts (Fig.

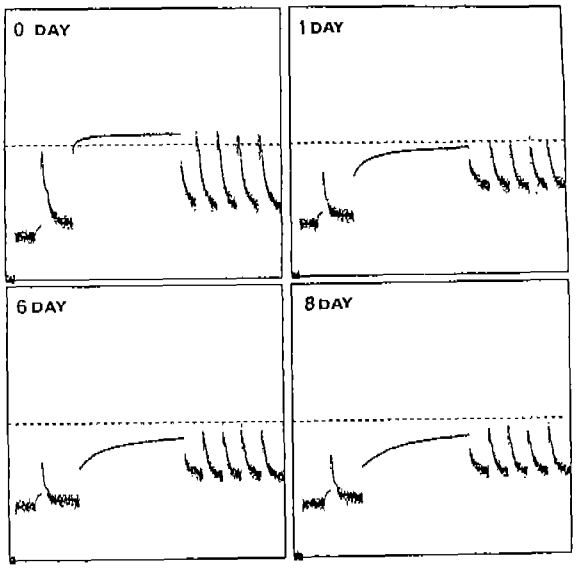

Fig. 7. Chlorophyll fluorescence induction curve of PVA-immobilized chloroplasts during storage.

6B), white light pulse do not induced the fluoresence rise. When we treated DCMU on one day-old free chloroplasts, the fluorescence rise pattern is similiar to that of the immobilized chloroplats. Probably this may be resulted from the dissipation of excited energy or blocking of PS II electron donor side.

Fig. 7 shows the chlorophyll fluorescence induction curve of immobilized chloroplasts during storage. After actinic light illumination, the fluorescence rise to steady-state was delayed gardually according to the duration of storage. According to the storage time, fluorescence induction patterns were changed in immobilized chloroplasts. As shown in Fig. 7, the fluorescence rise time to F_{max} from F_0 is increased gradually. The reason of delay of fluorescence rise time require further research in detail.

DISCUSSION

Sometimes immobilized photosynthetic cells exhibit higher physiological activity than that of free cells (Brouers *et al.*, 1988; Gisby and Hall, 1980; Park *et al.*, 1991). With regard to the changes in metabolic behavior of immobilized cell, there were several suggestions. From studies on mammalian cell adsorption, it was concluded that a primary reversible interaction between the cell and solid surface must somehow induce a secondary irreversible interaction (Carter *et al.*, 1981). And Mattiason and Hahn-Hargerdal (1982) proposed a model in which decreased water activity results in changed metabolic activity and product formation of immobilized cells. The changed water activity would be due to the high polymer concentration of the microenvironment; these macromolcules "organize" water and thereby decrease the amount of water available to the cells. The work of Holeberg and Margalith (1981) and Kraube *et al.* (1980) is in agreement with this hypothesis and shows that the presence of polymer even at a low level has substantial effects on biochemical reactions which are water-dependent. In our experiments, there may be a possibility that immobilization process may induce the uncoupling of the thylakoids. Therefore it can be inferred that in our case the PVA polymer has an effect on water activity and as a consequence on the function of the thylakoids.

Relatively, a rapid decline in Hill reaction during *in vitro* ageing of chloroplasts could possibly be attributed to the release and accumulation of free fatty acids which are known to be toxic to photochemical reaction of thylakoid membranes (Harnischefeger, 1972; Siegenthaler and Rawler, 1977). In our experiments, PVA-immobilized chloroplasts showed a good longevity of photosynthetic function. So PVA may have an effect of preservation of molecular microenvironment of the thylakoids. There are several reports that uncouplers induce the increasement of light-dependent oxygen uptake in the leaf and chloroplasts (Asada and Takahashi, 1987). As shown in Table 1 and Fig. 1-3, PS I+II and PS I activity increased by uncoupler treatment. Therefore there may be a possibility that immobilization process play a role of coupling the photosynthetic electron transport chain.

The yield of chlorophyll fluorescence is influenced in multiple ways by the state of the photosynthetic apparatus. It directly participates in the primary events of energy adsorption, transfer and transformation, and indirectly senses the secondary changes induced at the membrane level in the course of photosynthetic reaction. In our experiments, white light pulse induced the fluorescence yield rise to F_{max} in free chloroplasts, however, in PVA-immobilized chloroplasts, white light pulse could not induced the additional rise to F_{max} (Figs. 6 and 7). This means that the fluorescence already may reached to F_{max} in immobilized chloroplasts under actinic light.

Variable fluorescence ratio (F_v/F_m) is proportional to the quantum yield of photochemistry. Values of F_v/F_m are usually between 0.6 and 0.8 and the difference between these value and the quantum yield of photochemistry is accounted for by the non-photochemical quenching of excitation in closed reaction centers. A decline in F_v/F_m

is symptomatic of the effect of various environmental stresses, and such changes indicate a loss of photochemical efficiency. However, it is important to distinguish increases in F_o from decreases in F_v. An increases in F_o is characteristics of PS II inactivation whereas a decline in F_v may indicate the increase in a non-photochemical quenching process at or close to the reaction center (Baker and Horton, 1987). In our experiments, immobilization induced the decrease of F_v/F_m ratio from 0.73 to 0.68. There are two possible explanations about F_v/F_m ratio decreasement after immobilization such as PS II inactivation or non-photochemical quenching process at /or close to the reaction center. Among them, non-photochemical quenching is more reliable than PS II inactivation, because the oxygen evolution activity is reamined. However it seems that further research in detail required to illustrate the mechanism of preservation of activity after immobilization of chloroplasts.

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적 요

시금치에서 분리한 엽록체를 폴리비닐알콜에 고정화한 후 광합성특성을 조사하였다. 고정화한 엽록체의 경우 고정화하지 않은 엽록체에 비하여 광합성전자전달활성의 안정성이 크게 증가하였는데 4℃에서 보관할 경우 1개월 후까지 전자전달활성이 보존되었으며, 열처리에 대한 내성은 유사하였다. 고정화한 경우 엽록체의 흡수스펙트럼의 적색피크가 청색쪽으로 2-4 nm 전이하였다. 그러나 이와 같은 엽록체의 구조적인 변화가 고정화 후의 전자전달활성이 대조구에 비하여 큰 저하를 일으키지 않는 것으로 보아 전자전달계의 직접적인 활성변화를 유발하는 구조적변화는 아닌 것으로 생각된다. 고정화한 엽록체의 엽록소 형광패턴은 백색광에 의한 spike전이가 일어나지 않아 이미 actinic 광하에서 F_{max}로의 전이가 일어난 것으로 생각된다. 또한 actinic 광하에서 F_{max}로의 전이시간이 길어짐을 볼 수 있었다. 이와 같은 현상은 고정화한 엽록체의 광계에서의 여기에너지의 열발산이나 광계 II의 전자공여부위의 저해에 의하여 나타날 수 있다고 보여지나 이에 대한 보다 자세한 연구가 필요하다고 사료된다.

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