IMMOBILIZATION OF PHOTOCHEMICALLY-ACTIVE CHLOROPLASTS ONTO DIETHYLAMINOETHYL-CELLULOSE

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1. Introduction

The use of immobilized photosynthetic systems as multifunctional biocatalysts is a novel approach to the problem of photobiological energy conversion. The main advantages in the use of the immobilized photosystems are the increased stability and the flexibility in reactor design. Recently, reports have appeared which emphasize the increasing importance of research in the field [1 5]. Chloroplasts have been immobilized by entrapment within polyacrylamide gel [1], polyvinyl alcohol [2] and microcapsules [3]. However, high retention of photosynthetic activity was not achieved, in contrast to bacterial chromatophores immobilized by entrapment within polyacrylamide gel [4], because of the instability of photosystem II (PS II) activity of chloroplasts.

Enzyme immobilization by adsorption is simple, mild and reversible permitting reuse of both enzyme and the substratum. Thus, adsorption may be a suitable method for immobilization of chloroplasts. Diethylaminoethyl (DEAE)-cellulose, one of the anion exchangers, is commercially available and widely used as an ion exchanger for protein fractionation. It has already been used effectively as a substratum for various enzymes such as acid acylase [6], glucoamylase [7], glucose isomerase [8] and invertase [9]. We have investigated its application for preparation of immobilized chloroplasts with a high retention of photochemical activities.

Here we describe the preparation and properties of chloroplasts immobilized by adsorption to DEAE-cellulose. Some observations on its use in the continuous photoreduction of 2,6-dichloroindophenol (DCIP) as an indicator of PS II activity are also reported.

2. Materials and methods

2.1. *Chloroplast preparation*

Spinach chloroplasts were isolated by the method in [10]. Chlorophyll (chl.) was determined in 80% acetone extract using the absorption coefficients in [11].

2.2. *Chloroplast immobilization*

Chloroplast suspension (3.2 mg chl.) was added to a suspension of 1 g (dry wt) of precycled DEAE-cellulose (Whatman DE23) in 50 mM Tris–HCl buffer (pH 7.5) containing 0.4 M sucrose and 10 mM NaCl (TSN buffer) (final vol. 80 ml) and was stirred gently for 3 min in an ice–water bath. The chloroplast-adsorbed DEAE-cellulose was washed with 200 ml TSN buffer (pH 7.5) in a column with occasional stirring (the washings were collected to determine the effluent activity) and finally suspended in 40–80 ml of the same medium. The resulting suspension was used as the immobilized chloroplasts.

2.3. *Activity measurements*

The activity of photosystem I (PS I) was measured as methyl viologen (MV) photoreduction with reduced DCIP as the electron donor. Oxygen uptake was measured polarographically using an oxygen sensor (YSI 5331). The reaction mixture (6 ml) contained: TSN buffer, 240 μmol Tris–HCl, (pH 7.5); 1.5 μmol

MV; 10 μmol sodium ascorbate, 0.3 μmol DCIP, 10 nmol 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); 15 μmol sodium azide; and chloroplasts equivalent to 60 μg chl. The activity of PS II was assayed as DCIP photoreduction by water as in [10], except for continuous stirring in the sample cuvette. The standard reaction mixture contained: TSN buffer, 150 μmol Tris–HCl (pH 7.5); 0.2 μmol DCIP; and chloroplasts equivalent to 20 μg chl. in 3 ml total vol. Actinic light was supplied from a 300-W Elmo projector and was passed through a water layer (5 cm thick). The intensity of the actinic light was 16 mW $.cm^{-2}$.

2.4. *Continuous photoreduction of DCIP by DEAE-cellulose–chloroplasts in a stirred column*

A column (1.6 × 10 cm) containing 500 mg (dry wt) DEAE-cellulose immobilized chloroplasts (1.6 mg chlorophyll) was used as the tank reactor and continuously operated at 20°C. DCIP (A_{600} 0.94) in TSN buffer (pH 7.5) was pumped through the column at 18 ml/h flow rate. The reaction mixture was stirred constantly by a magnetic stirrer. After 1 h residence time, reaction was started by irradiation of white light (2.4 mW $.cm^{-2}$). The reduced DCIP solution from outlet of the reactor was collected at time intervals using a fraction collector and measured at 600 nm for the reduced DCIP concentration.

3. Results and discussion

3.1. *Preparation of immobilized chloroplasts*

In order to determine the maximum amount of chloroplasts and activities that DEAE-cellulose could hold, different concentrations of chloroplasts were added to the DEAE-cellulose by the methods in [8]. Figure 1 shows that the amount of activity retained onto the ion exchanger support varied depending on the chloroplasts added. At ≤ 3.2 mg chl./g substratum, the activity retained was proportional to the chloroplasts added. In this range DEAE-cellulose adsorbed 97–100% of the chloroplasts added and retained 95–100% of the PS II activity. Beyond this level of chloroplasts, the increase of activity became less and increasing added chloroplast concentration increased the activity in the washings. Under the present experimental conditions, the maximum level for the

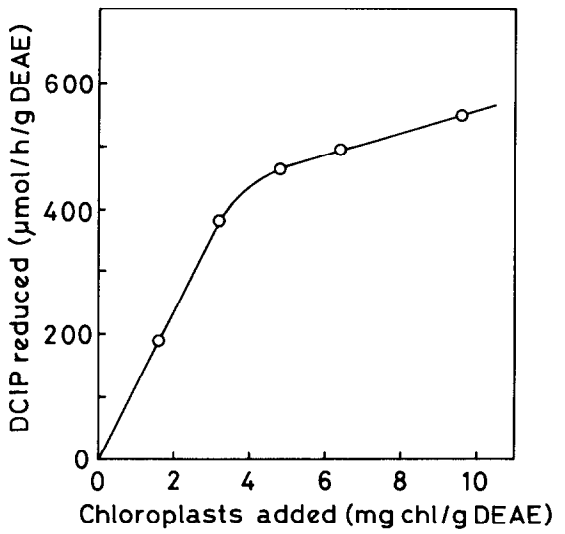

Fig.1. Retention of PS II activity after the immobilization of chloroplasts onto DEAE-cellulose. Different concentrations of chloroplasts (1.6–9.6 mg chl.) were added to the suspension of 1 g (dry wt) of precycled DEAE-cellulose. The chloroplast-adsorbed DEAE-cellulose was washed with 200 ml TSN buffer (pH 7.5) and suspended in 80 ml of the same medium. Details for the preparation of immobilized chloroplasts and assay conditions are in section 2.

adsorption of chloroplasts was not found. This may be due to the short incubation period used to avoid the destruction of chloroplast structure and solubilization of loosely bound chloroplast components.

The specific activity of PS II remained constant (~130 μmol DCIP reduced/mg chl./h) throughout the concentration range studied in fig.1. Furthermore, the specific activities of the immobilized chloroplasts were higher than those of the free chloroplasts (table 1). This increase of the specific activity was similar to the observation with glucoamylase [7] and glucose isomerase [8] immobilized on DEAE-cellulose. However, it may be asked whether the higher activity observed in our immobilized chloroplasts was mainly caused by uncoupling of photophosphorylation. In order to obtain further insight into this question, we examined the effects of uncouplers on the activities of PS I and PS II catalyzed by the free and the immobilized chloroplasts (table 1). NH_4Cl and methylamine–HCl accelerated the rate of the Hill reaction with DCIP and MV in the free chloroplasts. In the

Table 1
Effects of uncouplers on the photochemical activities in free or immobilized chloroplasts

Addition	MV photoreduction (μmol O_2/mg chl./h)		DCIP photoreduction (μmol DCIP/mg chl./h)	
	Free	Immobilized	Free	Immobilized
None	256.6 (100%)	441.4 (100%)	120.4 (100%)	137.5 (100%)
5 mM NH_4Cl	558.4 (218)	536.5 (122)	314.9 (262)	141.8 (103)
20 mM methylamine–HCl	701.8 (274)	543.3 (123)	340.4 (283)	145.9 (106)

Uncoupler was added to the reaction mixture to the final concentrations. Assay conditions are in section 2

immobilized chloroplasts, however, both reactions were insensitive to the uncouplers, indicating that the immobilized chloroplasts have already been uncoupled.

3.2. *Effect of pH*

Figure 2 shows the pH profiles for PS I and PS II activities in the free and immobilized chloroplasts.

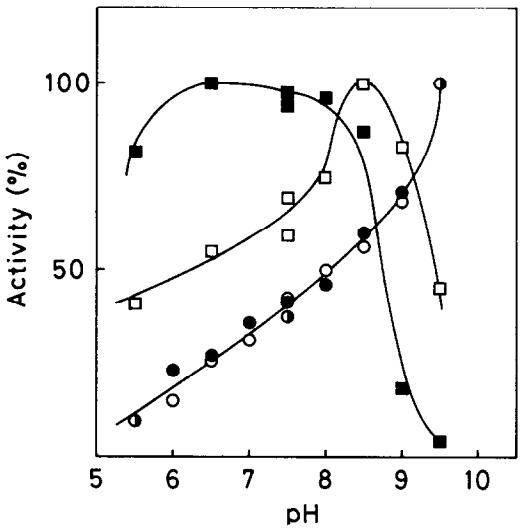

Fig.2. Effect of pH on photochemical activities in free and immobilized chloroplasts. The activity at the pH optimum was arbitrarily set at 100%. Assay conditions are described in the text, except that potassium phosphate (pH 5.5–7.5) or Tris–HCl (pH 7.5–9.5) buffer was used. Absorption coefficient of DCIP was corrected at each pH according to [13]. MV photoreduction (PS I) : (○) free; (●) immobilized. DCIP photoreduction (PS II) : (□) free; (■) immobilized.

Similar pH profiles were observed for PS I activity in both preparations. The pH optimum for PS II activity in the free chloroplasts was 8.5. The curve for the immobilized chloroplasts had a rather flat shape compared to that of the native chloroplasts. The optimum pH was also shifted to acidic side by ~0.5 pH unit. This shift may be caused by the uncoupling of phosphorylation, as indicated in the pH activity profiles of uncoupled NaBr chloroplast particles [12].

3.3. *Effect of temperature*

The thermal stability of the photochemical activities in the free and immobilized chloroplasts is shown in fig.3. At least for the 10 min periods studied, there is no difference between the preparations. Thus, we have found no improvement in thermal stability upon adsorption to DEAE-cellulose in contrast with the chloroplasts entraped within polyacrylamide gel [1] or within microcapsules [3]. A similar observation has been made with glucose isomerase immobilized on DEAE-cellulose [8].

3.4. *Continuous photoreduction of DCIP*

Figure 4 illustrates the continuous photoreduction of DCIP catalyzed by immobilized chloroplasts with water as the electron donor. Concentration of reduced DCIP in the effluent increased rapidly after the onset of illumination, reaching a plateau after 10 min. The maximum level was sustained for ~2 h. Then, the activity decreased during continuous operation, reaching 50% of the initial activity after 4.5 h. This decline seems to be mainly due to a decrease in the activity of the chloroplasts as indicated in other

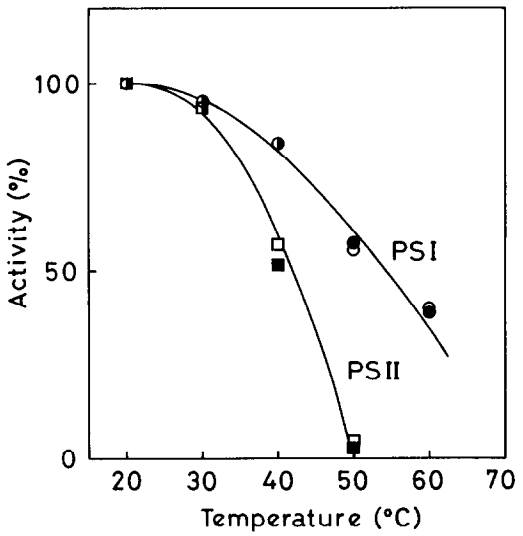

Fig.3. Thermal stability of photochemical activities. Chloroplast suspension or immobilized chloroplast suspension in TSN buffer (pH 7.5) were preincubated for 10 min at the indicated temperature, rapidly cooled, and the remaining activities determined at 25°C. PS I and PS II activities were measured by MV and DCIP photoreduction, respectively. The reaction mixture contained 20 mM methylamine–HCl. Open symbols, free; solid symbols, immobilized.

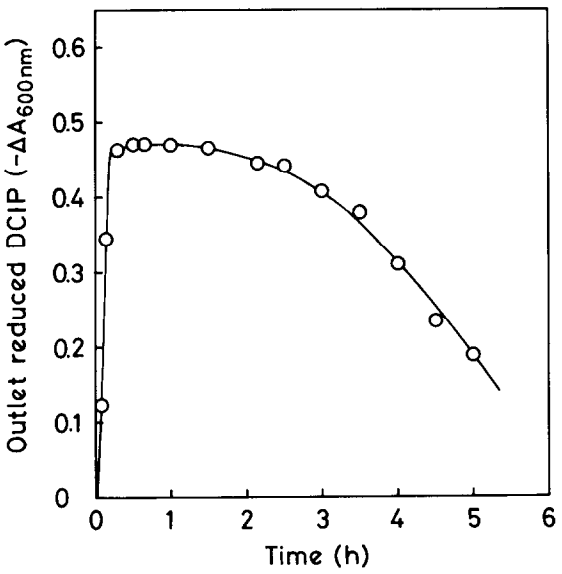

Fig.4. Continuous photoreduction of DCIP by immobilized chloroplasts in a stirred column. Reactions were carried out at 18 ml/h flow rate at 20°C. Light intensity: 2.4 mW .cm^{-2}. DCIP concentration: A_{600} 0.94. Other conditions and details are in the text.

studies [1,14,15]. It cannot be ascribed to the desorption of chloroplasts during operation, because after 5 h the amount of chloroplasts remained the same as in the initial stage. When the log of the activity retained was plotted against time for operation, it gave non-linear relationship, indicating complicated reaction kinetics of the inactivation process. Thus, the mechanism for the inactivation process is not understood at present.

In a preliminary study of factors controlling inactivation during continuous operation, we examined the use of intermittent light as the actinic light source. The purpose is to shorten the exposure time to illumination, based on the time delay of the reactions between light and electron transport, preventing generation of superoxide anion radical by photosensitization [15]. No pronounced effect was observed, however. In the presence of nitrogen gas bubbling through the reaction mixture during continuous operation, the stability of the immobilized chloroplasts was slightly improved, increasing the decay half-time to ~6 h. More detailed studies will be necessary to improve the stability during continuous operation.

We demonstrated the immobilization of chloroplasts with a high retention of the PS II activity. The chloroplasts thus immobilized may be useful as a multifunctional biocatalyst for hydrogen production system [16] and for production of useful compounds such as NADPH. We are now studing immobilized chloroplasts capable of producing NADPH with water as the electron donor.

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