Biochimica et Biophysica Acta, 637 (1981) 490–495
Elsevier/North-Holland Biomedical Press

BBA 48142

HYDROGEN EVOLUTION BY CO-IMMOBILIZED CHLOROPLASTS AND *CLOSTRIDIUM BUTYRICUM*

ISAO KARUBE, TADASHI MATSUNAGA, TOSHIAKI OTSUKA, HIROMICHI KAYANO and SHUICHI SUZUKI

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama 227 (Japan)

(Received December 23rd, 1980)
(Revised manuscript received May 18th, 1981)

Key words: Hydrogen evolution; Immobilized chloroplast; Immobilized bacteria; Hydrogenase; Water photolysis; (Cl. butyricum, Spinach)

Spinach chloroplasts and *Clostridium butyricum* cells were immobilized in 2% agar gel. Crude ferredoxin isolated from spinach and benzyl viologen were used as electron carriers. The optimum pH for both NADP reduction by immobilized chloroplasts and for hydrogen evolution by immobilized *Cl. butyricum* was 8.0. The optimum temperature was between 25 and 30°C for NADP reduction by immobilized chloroplasts, and 37°C for hydrogen evolution by immobilized cells. The total amount of hydrogen evolved in 6 h was 41 μmol/mg Chl for the immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* system, and 11 μmol/mg Chl for the immobilized chloroplast-ferredoxin-*Cl. butyricum* system. The systems evolved only a trace amount of hydrogen when dichlorophenyldimethylurea was added. The immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* system evolved hydrogen continuously for 6 h, and immobilized *Cl. butyricum* retained the initial hydrogenase activity. However, the photoreduction activity of chloroplasts decreased to 30% of the initial activity after 6 h of reaction.

Introduction

Biophotolysis of water by algae and the chloroplast is now attracting attention as one of the hydrogen-production systems. The coupling of spinach chloroplast PS I to a *Clostridium* hydrogenase with ferredoxin was noted by Arnon et al. [1]. Benemann et al. [2] reported that coupling of the photosynthetic system in plant chloroplasts with a hydrogenase could result in light-driven splitting of water into hydrogen and oxygen. Rao et al. [3] observed a rate of 94 μmol H_2/h per mg Chl using *Chenopodium album* chloroplasts and *Clostridium pasteurianum* hydrogenase. However, the lifetime of the isolated chloroplasts was very short. Moreover, hydrogenase is inactivated with oxygen produced by PS II in the chloroplasts. Therefore, this system lost 50% of its activity after 15 min of the reaction.

Immobilization techniques for living microorganisms and organelles have been developed in our laboratory. Chloroplasts were immobilized in polyacrylamide, agar gel, poly(vinyl alcohol) film or calcium alginate gel [4–7]. The immobilized chloroplasts were subjected to carbon dioxide fixation and NADP reduction. The lifetime of the immobilized chloroplasts was longer than that of isolated intact chloroplasts. Furthermore, the authors succeeded in stabilizing the hydrogenase system by immobilization of living whole cells [8]. Hydrogenase in immobilized cells was protected from the deleterious effects of oxygen.

In this study, spinach chloroplasts and *Clostridium butyricum* were immobilized in agar gel, and hydrogen evolution by photolysis of water was studied by the immobilized chloroplast-ferredoxin or benzyl viologen-immobilized *C. butyricum* systems.

Abbreviations: PS, photosystem; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Materials and Methods

Materials. Yeast extract was purchased from Difco Laboratories. Peptone (from casein) and agar were purchased from Kyokuto Pharmaceutical Co. Crude ferredoxin was isolated from spinach according to the procedure of San Pietro and Lang [9]. Other reagents were commercially available reagents or laboratory grade materials. Deionized water was used in all procedures.

Immobilization of chloroplasts. Spinach chloroplast preparations were made by homogenizing 30 g spinach leaves with 70 ml buffer (0.8 M sucrose, 0.02 M Tricine, 0.01 M NaCl, pH 8.0) in a Waring blender for 15 s at 4°C. After filtration through a gauze, the extract was centrifuged at $1\,000 \times g$ for 1 min. The pellets were discarded and the supernatant was centrifuged at $1\,500 \times g$ for 5 min. The chloroplasts (containing 4 mg Chl) were resuspended in 2 ml 0.1 M Tris-HCl buffer (pH 8.0), and used for immobilization. Chlorophyll contents were determined from the absorption coefficient according to the procedure of Arnon [10].

Spinach chloroplasts were immobilized in agar gel. Agar (0.24 g) was dissolved in 10 ml physiological saline in a flask at 100°C and cooled to 50°C. Then, 2 ml Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2 g chloroplasts (0.6–0.8 mg Chl) and ferredoxin (36–60 μM) or benzyl viologen (7.2 μM) were added to the flask, mixed, and immediately cooled to 30°C.

Immobilization of Cl. butyricum. An authentic culture of *Cl. butyricum* was maintained in a cooked-meat medium. The bacterial cells were grown under anaerobic conditions at 37°C, for 9 h in 1 l medium (pH 7.0) containing 10 g glucose, 4 g peptone, 4 g yeast extract, 2 g beef extract, 12.5 g K_2HPO_4 and 0.5 g $FeSO_4$. The cells were isolated by centrifugation at $8\,000 \times g$ for 10 min. The cells were washed twice 0.1 M phosphate buffer (pH 8.0, 4°C) at $8\,000 \times g$ for 10 min. For immobilization, 0.2 g of agar was dissolved in 8 ml Tris-HCl buffer (pH 8.0) in a flash at 100°C and cooled to 50°C. Then 2 ml Tris-HCl buffer (pH 8.0) containing 1 g wet cells were added to the flask and mixed. This flask was immediately cooled to 30°C.

Determination of chloroplast activity. The activity of chloroplasts was determined by measuring the initial rate of $NADP^+$ reduction in the Hill reaction. The reduction of $NADP^+$ was measured by a spectrometric method (the increase in absorbance at 340 nm). The reaction mixture, which consisted of 2 g immobilized chloroplasts and 5 ml Tris-HCl buffer (0.1 M, pH 8.0) containing ferredoxin (8 μM), $NADP^+$ (0.8 mM) and $NADP^+$-ferredoxin oxidoreductase, was placed in a flask and mixed. The absorbance of the buffer containing ferredoxin, $NADP^+$ and the enzyme was measured with a spectrometer (Shimadzu Seisakujo, UV-200) before mixing. The flask was evacuated with a vacuum pump and incubated for 5 min at 30°C under illumination provided by a 500 W reflector lamp (Toshiba Co., light intensity 15 000 lx). After illumination, the reaction mixture was filtered through a membrane filter (Toyo Roshi Co., Ltd., pore size 0.2 μm) and the absorbance was measured again. The activity of chloroplasts was calculated from the difference between absorbances obtained from both measurements.

Determination of hydrogenase activity in immobilized cells of Cl. butyricum. The activity of hydrogenase was determined by measuring the initial hydrogen evolution from reduced benzyl viologen. The reaction mixture, which consisted of 0.5 g immobilized bacteria and 5 ml phosphate buffer (0.1 M, pH 8.0) containing benzyl viologen (1.2 μM), was placed in a flask. Then, 5 mg sodium dithionite were added to the mixture, and the flask was evacuated with a vacuum pump and sealed immediately. The reaction mixture was incubated at 30°C for 2 h, and hydrogen was determined by a gas chromatograph (Shimadzu Seisakujo, Model GC 6AM) as described previously [8].

Hydrogen evolution by the immobilized chloroplast-Cl. butyricum system. Hydrogen evolution was assayed in a 50 ml shrenk flask stoppered with a cap. In the flask placed 2 g immobilized chloroplasts, 1 g immobilized cells of *Cl. butyricum* and 5 ml Tris-HCl buffer (0.1 M, pH 8.0) containing ferredoxin (8 μM) or benzyl viologen (1.2 μM). The flask were exhausted with a vacuum pump and sealed. The reaction mixture was incubated at 30°C under illumination provided by a 500 W reflector lamp (light intensity 15 000 lx). Hydrogen was determined by gas chromatography.

Results

Immobilization conditions

Spinach chloroplasts and *Cl. butyricum* were immobilized in agar gel. Fig. 1 shows the effect of agar concentration on the activities of the photosystem in chloroplasts and hydrogenase in *Cl. butyricum*. The activity of the photosystem in chloroplasts decreased with increasing agar concentration. The activity of hydrogenase in *Cl. butyricum* also decreased with increasing agar concentration. The maximum activities of both the photosystem and hydrogenase were observed at 1% gel. However, this gel preparation was too weak to use. Therefore, 2% gel preparations were employed for the following experiments.

Optimum reaction conditions

Fig. 2 shows the effect of pH on the photoreduction activity of immobilized chloroplasts and the hydrogenase activity of immobilized *Cl. butyricum*. Immobilized chloroplasts lost their activity below pH

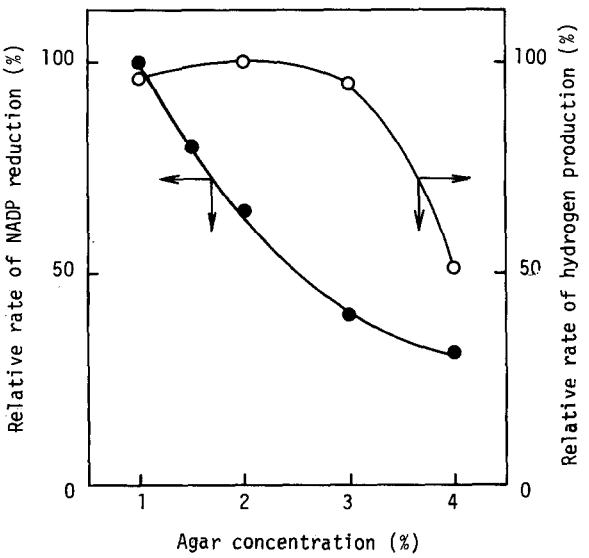

Fig. 1. Effect of agar concentration on the photoreduction activity of immobilized chloroplasts (●——●) and the hydrogenase activity of immobilized *Cl. butyricum* (○——○). Reactions were carried out at 30°C and pH 8.0. For details, see the text. The maximum rates of NADP reduction and hydrogen production are defined as 100% in Figs. 1–3. In this figure, the NADP reduction rate in 1% agar gel was 53 μmol/h per mg Chl and that of hydrogen evolution in 2% agar gel was 185 μmol/h per g wet cells.

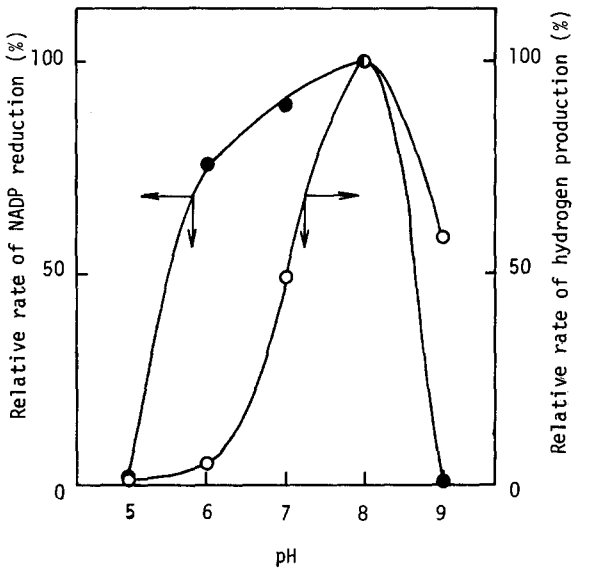

Fig. 2. Effect of pH on the photoreduction activity of immobilized chloroplasts (●——●) and the hydrogenase activity of immobilized *Cl. butyricum* (○——○). Chloroplasts and *Cl. butyricum* immobilized in 2% agar gel were employed. Reactions were carried out at 30°C. The NADP reduction and hydrogen evolution rates at pH 8.0 were 35 μmol/h per mg Chl and 185 μmol/h per g wet cells, respectively.

5.0 and above pH 9.0. The optimum pH was 8.0. The optimum pH of hydrogenase in immobilized whole cells was also 8.0. Therefore, the coupled reaction of immobilized chloroplasts and bacteria was carried out at pH 8.0.

The effects of temperature on the activities of the photosystem in immobilized chloroplasts and hydrogenase in immobilized cells are shown in Fig. 3. The optimum temperature for immobilized chloroplasts was between 25 and 30°C. Immobilized chloroplasts were not stable above 30°C. On the other hand, the optimum temperature for hydrogenase in immobilized whole cells was 37°C, and the activity decreased with decreasing temperature. Therefore, further experiments were performed at 30°C.

Hydrogen evolution by the immobilized chloroplast-Cl. butyricum system

Table I shows the amount of hydrogen evolved under various conditions. The systems were incubated for 6 h and the total amounts of hydrogen evolved by the various systems were determined. When electron

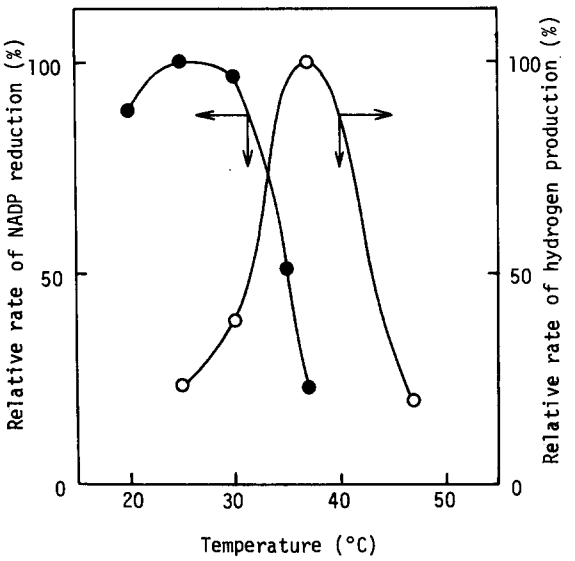

Fig. 3. Effect of temperature on the photoreduction activity of immobilized chloroplasts (●———●) and the hydrogenase activity of immobilized *Cl. butyricum* (○———○). Chloroplasts and *Cl. butyricum* immobilized in 2% agar gel were employed. Reactions were carried out at pH 8.0. The NADP reduction rate at 25°C was 36 μmol/h per mg Chl and that of hydrogen evolution at 37°C was 480 μmol/h per g wet cells.

TABLE I

HYDROGEN EVOLUTION UNDER VARIOUS CONDITIONS

Im., immobilized.

Conditions	Total amount of hydrogen evolved in 6 h (μmol/mg Chl)
Im. chloroplasts + Im. *Cl. butyricum*	0
Im. chloroplasts + Im. *Cl. butyricum* + ferredoxin	11.0
Im. chloroplasts + Im. *Cl. butyricum* + benzyl viologen	41.2
Im. chloroplasts + benzyl viologen	0
Im. *Cl. butyricum* + benzyl viologen	6.3
Im. chloroplasts + Im. *Cl. butyricum* + benzyl viologen (in the dark)	1.5
Im. chloroplasts + Im. *Cl. butyricum* + benzyl viologen + DCMU	2.3

carriers such as ferredoxin and benzyl viologen were not added to the system, no hydrogen was evolved. This fact shows that electrons are not transferred from immobilized chloroplasts to immobilized bacteria without electron carriers. The total amounts of hydrogen evolved in 6 h were 41.2 μmol/mg Chl for the immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* system, and 11.0 μmol/mg Chl for the immobilized chloroplast-ferredoxin-immobilized *Cl. butyricum* system. However, the immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* system evolved only a very small amount of hydrogen in the dark.

DCMU is known as an inhibitor of electron transport from PS II to plastoquinone. The amount of hydrogen decreased significantly when DCMU was added to the system (total amount of hydrogen evolved in 6 h, 2.3 μmol/mg Chl). The benzyl viologen-immobilized *Cl. butyricum* system also evolved only a small amount of hydrogen. These results indicate that the photosystem in immobilized chloroplasts contributes to hydrogen evolution.

Time course of hydrogen evolution

Fig. 4 shows the time course of hydrogen evolution by the immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum*, native chloroplast-benzyl viologen-immobilized *Cl. butyricum* and native chloroplast-benzyl viologen-native *Cl. butyricum* systems under illumination or in the dark. The native chloroplast-benzyl viologen-immobilized bacteria system evolved only a trace amount of hydrogen. The native chloroplast-benzyl viologen-native bacteria system also did not evolve hydrogen. During the initial 2 h, only a small amount of hydrogen was evolved by the immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* system. This is a result of the diffusion limitation of benzyl viologen. However, hydrogen production increased and the maximum rate was attained during 3–4 h. The rate of hydrogen evolution decreased gradually after 5 h. This system evolved hydrogen for 6 h. After 6 h incubation under illumination, the activities of the photosystem in immobilized chloroplasts and hydrogenase in immobilized *Cl. butyricum* were determined. Immobilized *Cl. butyricum* retained the initial hydrogenase activity. However, the photoreduction activity of immobilized chloroplasts decreased to 30% of the initial

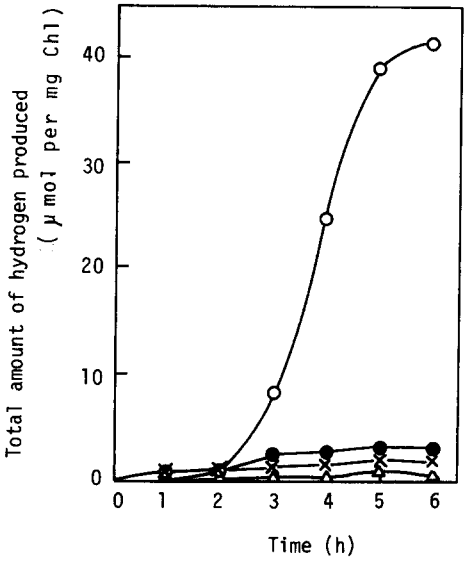

Fig. 4. Time course of hydrogen evolution by immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* (○———○), native chloroplast-benzyl viologen-immobilized *Cl. butyricum* (●———●), native chloroplast-benzyl viologen-native *Cl. butyricum* (△———△), and immobilized chloroplast-benzyl viologen-immobilized *Cl. butyricum* (X———X) in the dark. Reactions were carried out under optimum conditions. For details, see the text.

activity. Therefore, the decrease in the hydrogen evolution rate is attributed to inactivation of the photosystem in chloroplasts.

Discussion

As shown in Fig. 5, electron carriers such as benzyl viologen and ferredoxin are reduced by PS I and PS II in chloroplasts under illumination. Hydrogen is evolved through oxidation of this reduced benzyl viologen or ferredoxin by hydrogenase in *Cl. butyricum*. At the same time, oxygen is evolved by PS II in chloroplasts. The major problem of the coupling system of chloroplasts and hydrogenase is the oxygen sensitivity of hydrogenase. Benemann et al. [2] used glucose, glucose oxidase and catalase as an oxygen-scavenger system. As a result, the amount of hydrogen evolved increased 7-fold. However, glucose is used up with oxygen consumption. Rao et al. [3] employed oxygen-stable hydrogenases isolated from *Chromatium viridis* and *Rhodospirillum rubrum* [3].

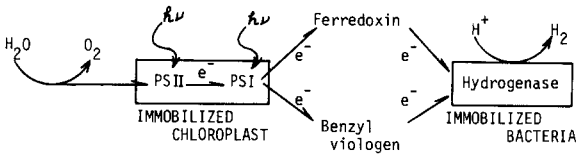

Fig. 5. Principle of hydrogen evolution by immobilized chloroplast-*Cl. butyricum* system.

Though these hydrogenases were reported to retain their activity during storage in air, their ability to evolve hydrogen continuously in the presence of oxygen has not yet been demonstrated. Moreover, these hydrogenases did not react with reduced ferredoxin. Another possibility of overcoming oxygen inactivation of hydrogenases is to immobilize hydrogenase. Several immobilization methods have been employed for hydrogenase [3,11,12]. Rao et al. [3] linked ferredoxin to AH Sepharose 4B and then bound hydrogenase on the immobilized ferredoxin. This immobilized ferredoxin-hydrogenase was effective in water-splitting hydrogen evolution [3]. But, the activity and stability of the immobilized hydrogenase were not studied in the presence of oxygen. The hydrogenase immobilized on glass beads was also inactivated by oxygen [11].

Recently, the authors used immobilized whole cells of *Cl. butyricum* for heterolytic hydrogen evolution [10]. We found that hydrogenase in immobilized cells was protected from the deleterious effect of oxygen. Therefore, immobilized cells of *Cl. butyricum* were employed for light-induced hydrogen evolution from water. Hydrogenase in immobilized whole cells was not inactivated by oxygen. Immobilized cells retained initial hydrogenase activity after 6 h of reaction.

Chloroplasts were also immobilized in agar gel. In previous studies, immobilized chloroplasts were used for carbon dioxide fixation [4] and NADP reduction [5]. The lifetime of the immobilized chloroplasts was 3-times longer than that of native chloroplasts. In this study, the immobilized chloroplast-*Cl. butyricum* system could evolve 41 μmol/mg Chl of hydrogen in 6 h, whereas the native chloroplast system evolved only 0.1 μmol/h per mg Chl of hydrogen. Thus, the lifetime of immobilized chloroplasts was prolonged as compared with native chloroplasts. However, the photoreduction activity of immobilized chloroplasts

decreased to 30% of the initial activity after 6 h of reaction. Further stabilization of chloroplasts is needed for light-splitting hydrogen production. In this study, ferredoxin and benzyl viologen were used as electron carriers. The molecular weight of benzyl viologen is lower than that of ferredoxin, and hence it permeates through the gel matrix and cell walls of bacteria. As a result, a higher amount of hydrogen was evolved when benzyl viologen was employed. However, benzyl viologen is unstable in the presence of oxygen. Furthermore, benzyl viologen cannot be used for many oxidation and reduction cycles. A decrease in hydrogen production of the system is also caused by this fact. Further developmental studies in the laboratory are now being directed toward stabilizing chloroplasts and electron carriers.

References

1 Arnon, D.I., Mitsui, A. and Paneque, A. (1961) Science 134, 1425

2 Benemann, J.R., Berenson, J.A., Kaplan, N.O. and Kamen, M.D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2317–2320

3 Rao, K.K., Gogotov, D.O. and Hall, D.O. (1978) Biochimie 60, 291–296

4 Karube, I., Aizawa, K., Ikeda, S. and Suzuki, S. (1979) Biotechnol. Bioeng. 21, 253–260

5 Karube, I., Otsuka, T., Kayano, H., Matsunaga, T. and Suzuki, S. (1980) Biotechnol. Bioeng. 22, 2655–2665

6 Ochiai, H., Shibata, H., Fujishima, A. and Honda, K. (1979) Agric. Biol. Chem. 43, 881–883

7 Gisby, P.E. and Hall, D.O. (1980) Nature 287, 251–253

8 Karube, I., Matsunaga, T., Tsuru, S. and Suzuki, S. (1976) Biochim. Biophys. Acta 444, 338–343

9 San Pietro, A. and Lang, H.M. (1958) J. Biol. Chem. 231, 211–229

10 Arnon, D.O. (1949) Plant. Physiol. 24, 1–15

11 Lappi, D.A., Stolzenbach, F.E., Kaplan, N.O. and Kamen, M.D. (1976) Biochem. Biophys. Res. Commun. 69, 878–884

12 Klibanov, A.M., Kaplan, N.O. and Kamen, M.D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3640–3643