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Purification and characterization of hydrogenase from the marine green alga, *Chlorococcum littorale*

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Abstract Hydrogenase from the marine green alga, *Chlorococcum littorale*, was purified 1485-fold, resulting in a specific activity for hydrogen evolution of 75.7 μmol/min/mg of protein at 25°C, using reduced methyl viologen as an electron donor. The K_m value for methyl viologen was 0.5 mM. The purity of the enzyme was judged by native PAGE. The molecular weight was estimated to be 55 kDa by SDS-PAGE, and 57 kDa by gel filtration. The optimum temperature and pH value for hydrogen evolution were 50°C and 7.5, respectively. The partially purified hydrogenase catalyzed hydrogen evolution from ferredoxin that had been isolated from the same cells, but not from NADH or NADPH. The K_m value for ferredoxin was 0.68 μM. The enzyme was extremely oxygen sensitive, losing over 95% of its activity upon exposure to air within minutes, even at 4°C. Two peptide fragments were obtained from the hydrogenase protein digested enzymatically, and their amino acid sequences were determined. No significant homology was found to any other known sequences of hydrogenases.

Key words: Hydrogenase; Ferredoxin; Hydrogen production; *Chlorococcum littorale*

1. Introduction

Hydrogenase was first described by Stephenson and Stickland in 1931 [1] as a bacterial enzyme that catalyzed the reversible oxidation of hydrogen according to the following reaction: $2H + 2e^- \rightleftarrows H_2$.

Hydrogenase activity was subsequently found in a number of anaerobic and aerobic prokaryotes [2,3]. The physiological function of most prokaryotic hydrogenases is to oxidize hydrogen gas and reduce electron acceptors. Another function is to produce hydrogen that will maintain the intracellular pH and redox potential at suitable levels [2].

Hydrogenase is present in members of most major taxonomic groups of algae such as Chlorophyceae, Euglenophyceae, Phaeophyceae, and Rhodophyceae [4,5]. Green algae are the only eukaryotic organisms for which hydrogenase activity has been well established. The hydrogenases of green algae can be considered reversible enzymes in vivo, since green algae produce or consume hydrogen after anaerobic adaptation. Two green algal hydrogenases have been purified to homogeneity. *Chlamydomonas reinhardtii* hydrogenase seems to consist of a single subunit with an apparent molecular weight of 48 kDa whose N-terminal amino acid sequence shows no significant homology to any other hydrogenases [6]; *Scenedesmus obliquus* hydrogenase consists of two subunits with molecular weights of 55 kDa and 36 kDa [7]. The former hydrogenase is the Fe-only type, while the latter seems to contain nickel, in addition to iron [8]. These enzymes were both extremely sensitive to oxygen.

Chlorococcum littorale is a new species of unicellular marine green alga which can rapidly grow under an extremely high concentration of carbon dioxide [9,10]. There have been several physiological studies on *C. littorale* [11–13] dealing with photosynthetic activity. Hydrogen production by this alga has been observed in association with photochemical water-splitting under photosystem II [14] and decomposition of endogenous starch under dark anaerobic conditions [15]. However, the hydrogenase of this alga has not been studied at the protein level, and the physiological electron donor for hydrogen formation in this alga was not clear. In this paper, we report the purification of the hydrogenase from *C. littorale* and some biochemical characteristics of the enzyme.

2. Materials and methods

2.1. Chemicals and materials

Sodium dodecyl sulfate and methyl viologen were purchased from Sigma Chemie (Germany). The other chemicals were purchased from Wako Co. (Japan), except for Q-Sepharose FF, Sephacryl S-200, phenyl Superose, Mono-Q, and Superose 12 which were obtained from Amersham-Pharmacia (Sweden).

2.2. Strain and cultivation

C. littorale was isolated in our laboratory in 1991 [9]. The strain was photoautotrophically grown at 25°C in a 5-l jar fermenter containing 4 l of MC medium [16] made from an equal amount of deionized water and aged seawater. The culture was continuously illuminated by eight fluorescent lamps around the cylindrical glass vessel and sparged with air containing 5% carbon dioxide for agitation and to supply the carbon source at a flow rate of 500 ml/min. The pH value was not regulated.

2.3. Preparation of the crude extract exhibiting hydrogenase activity

After 10 days of cultivation, cells in the linear growth phase were harvested by centrifugation (1600×*g*, 4°C, 10 min) and washed twice with a 67 mM phosphate buffer (pH 7.9) containing 1% NaCl. The resulting pellet was resuspended in an equal volume of the same buffer, then the cell suspension was flushed with oxygen-free argon gas under dark anaerobic conditions.

After 12 h of the anaerobic adaptation process, cells were harvested by centrifugation (1600×*g*, 4°C, 10 min) and resuspended in a basal buffer containing 50 mM sodium dithionite. The basal buffer consisted of 50 mM Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$ and 1 mM DTT. The suspension was put into a sealed steel beaker, glass beads (0.5 mm diameter) and 1 mM PMSF (v/v) were added, and the cells were broken by a vibrogen mill (VI-4, Johanna Otto, Germany) for 30 min at 4°C. The cell debris was removed by ultracentrifugation (100 000×*g*, 4°C, 60 min), and the resulting supernatant was used as

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid

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the crude extract. The crude extract was prepared under strictly anaerobic conditions at 4°C.

2.4. Purification of the hydrogenase and ferredoxin

Purification was conducted in an anaerobic box (Hirasawa Works Co., Japan) under nitrogen gas containing 10% hydrogen gas. Unless otherwise stated, all buffers and solutions contained 10 mM of sodium dithionite. The chromatographic elution pattern was monitored by a protein assay, since sodium dithionite itself has absorption at 280 nm.

The crude extract was diluted 3-fold with the basal buffer and loaded into an anion-exchange column of Q-Sepharose FF (16 mm diameter × 100 mm) that had been previously equilibrated with the basal buffer. Elution was performed with a linear gradient of KCl (0–500 mM) in the basal buffer at a flow rate of 3 ml/min. The fractions that showed hydrogenase activity were collected, to which the basal buffer containing ammonium sulfate to a final concentration of 0.5 M was added. The sample was loaded into a hydrophobic interaction column of phenyl Superose (0.5 mm diameter × 50 mm) that had been previously equilibrated with the basal buffer containing 0.5 M ammonium sulfate. Elution was performed with a linear gradient of ammonium sulfate (0.5–0 M) in the basal buffer at a flow rate of 0.5 ml/min. The active fractions were collected and applied to a gel filtration column of Sephacryl S-200 (16 mm diameter × 600 mm) that had been previously equilibrated with the basal buffer containing 150 mM KCl, and were eluted with the same buffer at a flow rate of 0.7 ml/min. The active fractions were collected and applied to an anion-exchange column of Mono-Q (0.5 mm diameter × 50 mm) that had been previously equilibrated with the basal buffer. Elution was performed with a linear gradient of KCl (0–300 mM) in the basal buffer at a flow rate of 1 ml/min. The active fractions were collected, diluted 3-fold with the basal buffer, and loaded again into an anion exchange column of Mono-Q. Elution was performed with a linear gradient of KCl (200–300 mM) in the basal buffer at a flow rate of 1 ml/min. The resulting purified hydrogenase was stored at −80°C under anaerobic conditions.

After the first anion-exchange chromatographic step with Q-Sepharose FF, the fractions with a brown color were collected, concentrated, and then subjected to gel filtration chromatography with Sephacryl S-200 to purify the ferredoxin. Elution was performed under the same conditions as those already described.

2.5. Activity measurement

The hydrogenase activity was quantified by the amount of hydrogen evolved from methyl viologen which had been reduced by sodium dithionite. The hydrogen formation was determined by gas chromatography (GC-14A, Shimadzu Co., Japan) [15]. The assays were performed in seal-lock vials with equal volumes of the liquid and gas phase. The sample (0.1–0.25 ml) was injected into 10 ml of basal buffer containing 5 mM methyl viologen and 5 mM sodium dithionite and incubated in a shaker at 25°C for 30 min. One unit of activity is defined as the amount of hydrogenase evolving 1 μmol hydrogen gas per minute.

2.6. Determination of K_m values

Non-linear curve fitting was performed to evaluate the kinetic parameters for hydrogen formation with SYSTAT V5.2 software by a Macintosh computer.

2.7. Protein analysis

Protein concentration was quantified by the method of Bradford [17], using a Protein assay kit (Bio-Rad, USA). γ-Globulin was used as a standard.

2.8. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [18] and performed at a constant current of 20 mA for 3 h on a mini-gel apparatus with 10–20% gradient gel (Daiichi Co., Japan). Proteins were stained with Coomassie brilliant blue (CBB-1, Wako Co., Japan) and by the silver-staining method to determine the purity of the proteins.

The electrophoresis for activity staining was run without sodium dodecyl sulfate under anaerobic conditions. The lane for activity staining was cut out and soaked in the basal buffer containing 5 mM methyl viologen and 5 mM sodium dithionite under nitrogen gas containing approximately 10% hydrogen gas. After the methyl viologen had been reduced, the stain was fixed by adding 1 mg/ml of 2,3,5-triphenyltetrazolium chloride, then the gel was extensively washed with deionized water [19].

2.9. Molecular weight determination

The molecular weight of the purified hydrogenase was estimated by the electrophoretic method already described with a regression curve for low-range standard protein (Amersham-Pharmacia, Sweden) and by gel filtration chromatography of Superose 12 with a regression curve for standard protein (Boehringer, Germany).

2.10. Amino acid sequence

The purified hydrogenase was pyridylethylated and then digested with lysyl endopeptidase purchased from Wako (Japan). The resultant peptide fragments were separated by reversed-phase HPLC. The sequences of the fractionated peptide fragments were analyzed by a protein sequencer (G1000A, Hewlett Packard, USA) and analyzed by a PTH analyzer (model 1090, Hewlett Packard).

The purified ferredoxin was applied to SDS-PAGE, and then electroblotted on to a polyvinylidene difluoride membrane (0.2 μm; Bio-Rad, USA) by the wet blotting procedure (Mini-Transblot electrophoretic transfer cell, Bio-Rad) according to the manufacturer's instructions. The blotting was carried out at 50 V for 2 h at 4°C in a CAPS buffer (pH 11.0). Edman degradation of the sample was automatically performed by the amino acid sequencer just described.

3. Results and discussion

3.1. Induction of hydrogenase and its activity

Hydrogenases from green algae are induced when the cells are exposed to dark anaerobic conditions [6,7,20]. The time needed for this process is variable, ranging from a few minutes to several days, depending on the organism and adaptation conditions [21–23]. Hydrogenase of *C. littorale* was also induced during the anaerobic adaptation process, and its activity increased with incubation time. No activity was found in

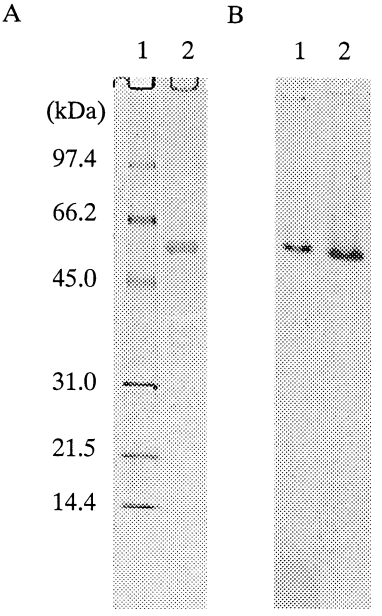

Fig. 1. Electrophoresis of the purified hydrogenase protein. A: SDS-PAGE analysis of purified hydrogenase. Lane 1: standard proteins; lane 2: purified hydrogenase. The standard proteins were purchased from Pharmacia (phosphorylase, MW 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 400). Coomassie brilliant blue was used for staining. B: Activity staining by native-PAGE. Lane 1: purified hydrogenase stained for protein; lane 2: purified hydrogenase stained for activity.

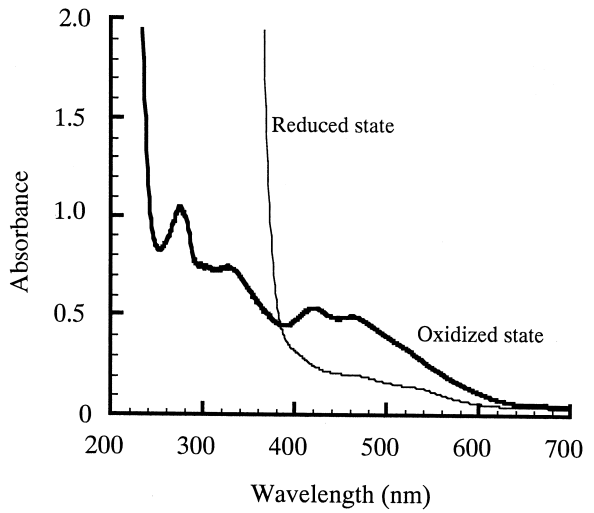

Fig. 2. Absorption spectra of the purified ferredoxin. The spectrum in the air-oxidized state and that in the reduced state were measured in a quartz cuvette (1-cm path length) containing 0.6 mg/ml of ferredoxin in the basal buffer with a spectrophotometer (DU640, Beckman, USA). The spectrum in the reduced state was taken after adding Na-dithionite to the cuvette.

cells without anaerobic adaptation, while the maximum activity of 0.05 U/mg protein was obtained after approximately 11 h of anaerobic adaptation.

The optimum temperature and pH value for hydrogen evolution from reduced methyl viologen were 50°C and 7.5, respectively. These values are similar to those for the green algae *Chlamydomonas reinhardtii* [6] and *Scenedesmus obliquus* [7], and are also similar to those for a number of prokaryotic hydrogenases [24–26]. The K_m value of the hydrogenase reaction in the crude extract of *C. littorale* for methyl viologen was estimated to be 0.5 mM (data not shown). This value indicates that the affinity of the enzyme for the substrate was lower than that of cyanobacterial hydrogenase so far reported, but higher than the hydrogenases from *C. reinhardtii* [6] and *S. obliquus* [7].

The addition of sodium chloride to the reaction buffer stimulated hydrogen evolution, and the relative activity was enhanced with increasing of sodium chloride concentration, approximately 3-fold enhancement being obtained with 1.7 M sodium chloride. The sodium ion seems to have affected the activity, since no enhancing effect was observed with the addition of potassium chloride. Although the mechanism for the stimulation has not been clarified, sodium chloride stimulation of hydrogen production from dithionite with methyl viologen has been reported for the rumen bacterium *Megasphaera elsdenii* [27].

3.2. Purification of the hydrogenase

The enzyme was rapidly inactivated in the absence of sodium dithionite, even under strictly anaerobic circumstances, suggesting that the coexistence of the reducing agent governed the stability of the enzyme throughout the purification process. Inactivation of hydrogenase due to absence of reducing agent has also been reported in *C. reinhardtii* [6]. The hydrogenase was purified approximately 1485-fold with 12% recovery of total activity from the crude extract (Table 1). The specific activity in the final chromatographic step was 75.7 μmol/min/mg protein. Both the hydrophobic interaction and gel filtration chromatographic steps brought about over 7-fold purification with the loss of 17% and 23% of activity, respectively. The purified protein gave a single band by a native PAGE analysis and was visible in the activity staining procedure (Fig. 1B). Approximately 0.05 mg of hydrogenase was obtained from 630 mg of total protein in the crude extract.

The purified enzyme could be stored for several weeks under anaerobic conditions at −80°C in the presence of 10 mM sodium dithionite.

3.3. Molecular properties of the hydrogenase

The molecular weight of the purified hydrogenase was estimated to be 57 kDa by gel filtration chromatography. The hydrogenase seemed to be monomeric in structure, since the results of the SDS-PAGE analysis showed a single protein band corresponding to a molecular weight of 55 kDa (Fig. 1A). This molecular weight is in the same order of magnitude as that of hydrogenases of bacterial origin [28,29].

The hydrogenase of *C. littorale* may be an Fe-only type, since the enzyme protein had a single subunit. Nickel-containing hydrogenases are generally oxygen stable, and most of the Ni-Fe hydrogenases characterized so far consist of at least two subunits, although four monomeric hydrogenase preparations have been reported to contain nickel at the reactive center [24,25,30]. It has been suggested that those monomeric nickel-containing hydrogenases might be a large subunit of the heterodimeric Ni-Fe hydrogenase, and that the large subunit alone appears to be active [24].

The hydrogenase was enzymatically digested into peptide fragments in order to determine its internal amino acid sequence. Two peptides were sequenced as AEALYAK and SATVPDGT. However, no identical sequences could be found among the known sequences of hydrogenases. The N-terminal amino acid sequence of the hydrogenase protein from *C. reinhardtii* also did not show any significant homol-

Table 1
Purification of hydrogenase from *C. littorale*

Sample	Total activity (U)	Total protein (mg)	Yield (%)	Specific activity (U/mg protein)	Purification factor (-fold)
Crude extract	32.1	629.6	100.0	0.05	1
First anion-exchange chromatography (Q-Sepharose)	23.9	148.7	74.5	0.16	3
Hydrophobic interaction chromatography (phenyl Superose)	19.0	16.30	59.1	1.17	23
Gel filtration chromatography (Sephacryl S-200)	14.7	1.51	46.0	9.79	192
Second anion-exchange chromatography (Mono-Q)	6.1	0.11	19.0	53.9	1057
Third anion-exchange chromatography (Mono-Q)	3.9	0.05	12.3	75.7	1485

1 U is defined as the amount of hydrogenase evolving 1 μmol H_2/min.

Table 2
Electron donor specificity for hydrogen evolution

Electron donor	Concentration	Relative activity (%)	
		Crude extract	Partially purified hydrogenase
Methyl viologen	5 mM	100.0	100.0
NADH	50 μM	14.7	0.1
NADPH	50 μM	14.8	0.1
Only Na-dithionite	5 mM	1.4	0.1
Ferredoxin from *Chlorella*	0.02 μg/ml	53.1	32.0
Ferredoxin from *C. littorale*	0.02 μg/ml	not determined	93.9

The relative activity for hydrogen evolution was determined using a crude extract containing 1.6 mg/ml protein, and 0.5 mg/ml of partially purified hydrogenase which was obtained after gel filtration chromatography (Table 1). Electron donors were reduced by adding Na-dithionite before the determination. Measurements were carried out as described in Section 2.

ogy to the known sequences of anaerobes, purple bacteria, and cyanobacteria [6].

3.4. General properties of ferredoxin

Ferredoxin was purified to homogeneity by gel filtration chromatography (Sephacryl S-200) after the first anion-exchange chromatographic step (Q-Sepharose FF). The scheme already described enabled approximately 2.7 mg of ferredoxin to be obtained from 630 mg of total protein in the crude extract.

The molecular weight was estimated to be approximately 16.2 kDa by SDS-PAGE analysis, this value being slightly larger than those from higher plants or prokaryotes so far characterized. Aggregation of the protein during the purification steps might have occurred, although the following typical characteristics of ferredoxin were observed: the absorption spectrum of the protein showed significant similarity to a typical chloroplast type of ferredoxin, with an absorption peak at 424 nm to indicate that this protein had an iron-sulfur cluster at the reaction center (Fig. 2); when the solution was reduced by adding sodium dithionite, the absorbance around 424 nm decreased.

The *N*-terminal amino acid sequence was determined for 40 residues (FQVTLKTPSGDKTIECSPDTYILDAAEEAGLDLPYSCRAG). This sequence has a long stretch at the N-terminus similar to those of other green algal ferredoxins that have been investigated so far. The purification procedure described enabled only one kind of ferredoxin to be isolated, although more than two ferredoxin iso-proteins have been observed in several higher plants and cyanobacteria [31,32].

3.5. Electron donor specificity and hydrogen evolution for the isolated ferredoxin

Hydrogen evolution by the crude extract was observed when NADH, NADPH, or ferredoxin from *Chlorella* (purchased from Wako Co., Japan) was used as the electron donor, although the relative activity was lower than that for methyl viologen. In contrast, NADH and NADPH were not used for hydrogen evolution by the partially purified hydrogenase fraction which was obtained after the second anion-exchange chromatography and contained minor proteins other than the hydrogenase (Table 2). This indicates that ferredoxin was the physiological electron donor for hydrogen evolution. NAD(P)H:ferredoxin oxidoreductase contained in the crude extract must have been involved in the electron donation from NAD(P)H to ferredoxin.

When the purified ferredoxin was treated as an electron donor, the relationship between the hydrogen evolution rate and the ferredoxin concentration could be expressed by the Michaelis-Menten type of kinetics, giving a K_m value of 0.68 μM (Fig. 3). This high affinity strongly indicates that the hydrogenase could react most efficiently via ferredoxin from the same algal cells compared with the ferredoxin from *Chlorella* (Table 2). There have been few reports about the reaction of algal hydrogenase and its in vivo electron donor. We confirm here hydrogen evolution coupled with hydrogenase and ferredoxin from the same origin.

The rate of hydrogen evolution from the purified ferredoxin by the highly purified hydrogenase was drastically decreased by the presence of sodium chloride (data not shown). A similar phenomenon was observed with *Megasphaera elsdenii* [27]. It has been suggested that a complex between the hydrogenase and electron carrier is disrupted by a high concentration of salt [27], as has been observed for the protein-protein complex of ferredoxin-$NADP^+$ reductase and ferredoxin [33].

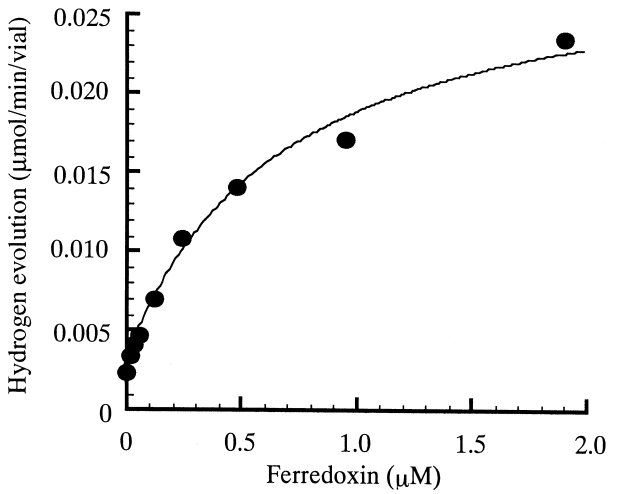

Fig. 3. Dependence of the ferredoxin concentration on hydrogen evolution. The partially purified hydrogenase fraction obtained after the second anion-exchange chromatographic step containing 0.08 mg/ml protein (0.1 ml) was injected into 5 ml of basal buffer containing the indicated concentrations of ferredoxin and 5 mM sodium dithionite. The ferredoxin concentration was calculated according to the protein concentration and molecular weight determined by SDS-PAGE (16.2 kDa).

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