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Characterization and purification of a hydrogenase from the eukaryotic green alga *Scenedesmus obliquus**

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Several catalytic properties of the hydrogenase from *Scenedesmus obliquus* have been examined to optimize the purification conditions. The K_m-value for H_2-evolution in the presence of the most effective electron mediator methylviologen is 0.66 mM. The pH-optimum is 6.3, the temperature-optimum is 50°C and the energy of activation is 38.4 ± 2 kJ·mol^{-1}. The soluble hydrogenase from the green alga, *Scenedesmus obliquus*, was purified 1290-fold to homogeneity. The enzyme consists of two subunits with molecular masses of 55 kDa and 36 kDa. The molecular weight of the native enzyme, determined by gel filtration, is 150 ± 5 kDa.

Eukaryotic hydrogenase; *Scenedesmus obliquus*

1. INTRODUCTION

Hydrogenases are key enzymes in hydrogen metabolism. Current research is focused on structure and catalytic function of hydrogenases under the aspect of formation of molecular hydrogen. Although the purification and biochemical properties of a number of bacterial hydrogenases have been reported (see [1,2] for review) no isolation and purification of a hydrogenase from an eukaryotic organism has so far been succeeded. About 50 years ago Gaffron discovered that the eukaryotic unicellular green alga, *Scenedesmus obliquus*, is able to evolve molecular hydrogen by means of a hydrogenase in the light under anaerobic conditions [3]. Thereafter, numerous physiological studies about this enzyme from *Scenedesmus* and other eukaryotic algae have been published [4–8].

The activity of eukaryotic hydrogenases is only observed after an anaerobic adaptation of the cells [3]. The active form of hydrogenase is extremely sensitive to oxygen [9]. While in vivo recovery of the hydrogenase activity is always possible by anaerobic re-adaptation, no activity could be reestablished in vitro [10].

Erbes et al. [11] and also Roessler and Lien [12] reported a partial purification of a hydrogenase from *Chlamydomonas reinhardtii*. However, these studies have neither been continued nor have they been confirmed. To our knowledge no purification of hydrogenases from eukaryotic algae to apparent homogeneity has so far been documented.

This study presents initial data about the properties of the hydrogenase from *Scenedesmus obliquus* in cell-free extracts, the purification to homogeneity and some biochemical characteristics of the isolated enzyme.

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*This paper is dedicated to Prof. Wolfhardt Rüdiger on the occasion of his 60th birthday.

2. MATERIALS AND METHODS

2.1. *Culture and adaptation conditions*

Cultures of the unicellular green alga, *Scenedesmus obliquus* strain D3 [13], were grown heterotrophically in a shaker at 30°C in darkness. Cells were inoculated in 500 ml Erlenmeyer flasks filled with 250 ml of a basal nitrate-medium [14] supplemented with glucose (0.5%) and yeast extract (0.1%). Three days after inoculation, i.e. at the end of their logarithmic growth phase, the cells were harvested by centrifugation at 1,500 × *g*. The cells were washed with bis-Tris-buffer (50 mM, pH 7.5) and adjusted to 500 µl packed cell volume·ml^{-1}. The algal suspension was placed in a 50 ml flask sealed with a serum stopper. The sample was repeatedly vacuum-degassed, flushed with nitrogen and finally shaken for 4 h at 30°C in the dark.

2.2. *Assay for hydrogenase activity*

Hydrogen production was measured at 25°C in 1.9 ml phosphate buffer (50 mM, pH 6.3) containing 10 mM Na-dithionite with a Clark-type electrode (Scientific Division, Yellow Springs, USA). Reduced methylviologen (10 mM) was used as the electron donor. The reaction was started by adding 0.1 ml of the sample.

2.3. *Native molecular weight*

The native molecular weight of the hydrogenase was determined under anaerobic conditions using gel filtration column chromatography on a Sephacryl S-200 column (2 × 54 cm). The column was equilibrated with bis-Tris-buffer (50 mM, pH 7.5). Different molecular weight standards were used (catalase 245 kDa; lactate dehydrogenase 146 kDa; ovotransferrin 77 kDa; carbonic anhydrase 30 kDa; and cytochrome *c* 13.5 kDa).

One milliliter of a cell-free extract was applied to the Sephacryl

S-200 column. The flow rate was 11 ml·h^{-1} and fractions of 1 ml were collected. Hydrogenase activity was assayed as described above.

2.4. *Protein determination*

Protein was quantified either by the method of Lowry [15] or the method of Bradford [16].

2.5. *Purification of the hydrogenase*

Unless otherwise stated all purification steps were carried out at room temperature in an anaerobic chamber (Coylab, MI, USA) under an atmosphere of 90% N_2 and 10% H_2.

2.5.1. Preparation of crude homogenate

The anaerobically adapted cells were disrupted in a vibrogen cell mill (Bühler, Tübingen, Germany) for 10 min at 4°C in a sealed steel beaker [17]. The glass beads (0 7 mm diameter) were filtered off.

2.5 2. Anion exchange chromatography on DEAE

The crude homogenate was centrifuged at 300,000 × *g* for 1 h at 4°C. The supernatant, containing all hydrogenase activity, was loaded onto a Fractogel TSK-DEAE column (3 × 20 cm) equilibrated with bis-Tris-buffer (50 mM, pH 7.5). If not otherwise stated this buffer was used for all purification steps. Unbound protein was washed from the column. The hydrogenase was eluted with a stepwise NaCl-gradient. Hydrogenase was eluted at a NaCl concentration of 200 mM. This fraction was used for further purification.

2.5.3. Gel filtration chromatography on Sephacryl S-200

The DEAE-fraction was concentrated by ultrafiltration using Microsep 30K (Filtron, Northborough, USA). The concentrated fraction was applied onto a Sephacryl S-200 column (1.5 × 55 cm), equilibrated with bis-Tris-buffer The flow rate was 11 ml·h^{-1} and active fractions were pooled for further purification.

2.5.4. Anion exchange chromatography on Mono-Q

The pooled S-200-fractions were loaded onto a Mono-Q column (HR 10/10, Pharmacia, Freiburg, Germany). The column was equilibrated with sterile-filtered bis-Tris-buffer. Hydrogenase was eluted with a linear NaCl gradient from 0 to 500 mM (100 ml total volume) in bis-Tris-buffer. The active fractions were pooled, diluted in the ratio 1:3 and again loaded onto a Mono-Q column. This time a linear NaCl gradient from 180 to 250 mM (60 ml total volume) in bis-Tris-buffer was used for elution.

2.6. *Non-denaturing gel electrophoresis*

Non-denaturing gel electrophoresis was performed according to the method of Davis [18] using a 9% acrylamide slab gel. The location of hydrogenase was determined by the method of Adams and Hall [19] using a solution of 2,3,5-triphenyltetrazoliumchloride (2.5%, w/v) and methylviologen (0.25%, w/v) in equal volumes.

2.7. *SDS-PAGE*

SDS-PAGE was performed using the buffer system of Laemmli [20] with a 15% acrylamide slab gel. Proteins were detected by the silver staining method of Blum et al. [21].

3. RESULTS AND DISCUSSION

3.1. *Characterization of hydrogenase in crude cell extracts*

In order to optimize the purification and assay conditions several properties of the soluble hydrogenase from *Scenedesmus obliquus* were investigated. Using a cell-free homogenate, prepared as described above, methylviologen was found to be the most effective electron donor for H_2-evolution (Table I). The redox potential of methylviologen, which is similar to that of ferredoxin, suggests that ferredoxin functions in vivo as the natural electron donor of the hydrogenase from *Scenedesmus obliquus*. This result is in accordance with findings made for a number of prokaryotic hydrogenases [22–24]. Using methylviologen in vitro as electron donor, pH optimum, temperature optimum, K_m-value for the hydrogenase-catalyzed oxidation of methylviologen coupled to the evolution of molecular hydrogen and, by using the Arrhenius-equation, the value for the energy of activation (E_{act}) was determined (Table II). The catalytic properties of the hydrogenase from *Scenedesmus obliquus*, i.e. substrate specificity, K_m-value, temperature optimum and pH optimum are very similar to these of the eukaryotic alga *Chlamydomonas reinhardtii* [11,13], but also to a number of prokaryotic hydrogenases [1,2,19,26–31].

Table I

Substrate specificity of the hydrogenase from *Scenedesmus obliquus*

Activity was determined as H_2-evolution as described in section 2.

Substrate	Concentration (nM)	Activity (%)	E'_0 (mV)
Methylviologen	1.0	100	−446
Phenosafranine	1.0	80	−252
Benzylviologen	1.0	50	−359
Janus-green	1.0	43	−96
Methylene-blue	1.0	40	+11
Na-dithionite	1.0	0	–

3.2. *Purification of hydrogenase*

Severe precautions must be taken during the purification of hydrogenase from *Scenedesmus obliquus*. Since the hydrogenase is irreversibly inactivated by traces of oxygen [9] strict anaerobicity must be maintained throughout the entire isolation and purification processes. The soluble hydrogenase from *Scenedesmus obliquus* was purified 1290-fold to apparent homogeneity with a yield of 8% and aspecific activity (H_2-evolution) of about 2700 U·mg^{-1}, following the purification steps described in section 2. The results of the purification procedure are summarized in Table III. The most effec-

Table II

Catalytic properties of the hydrogenase from *Scenedesmus obliquus*

H_2-evolution was measured polarographical using a Clark electrode The K_m-value was determined for methylviologen as electron donor.

	Scenedesmus obliquus	*Chlamydomonas reinhardtii* [11,25]
pH-Optimum	6.3	6 8
T-Optimum [°C]	50.0	55.0
K_m-value [mM]	0.66	0 85
E_{act} [kJ·mol^{-1}]	38.4	40.8

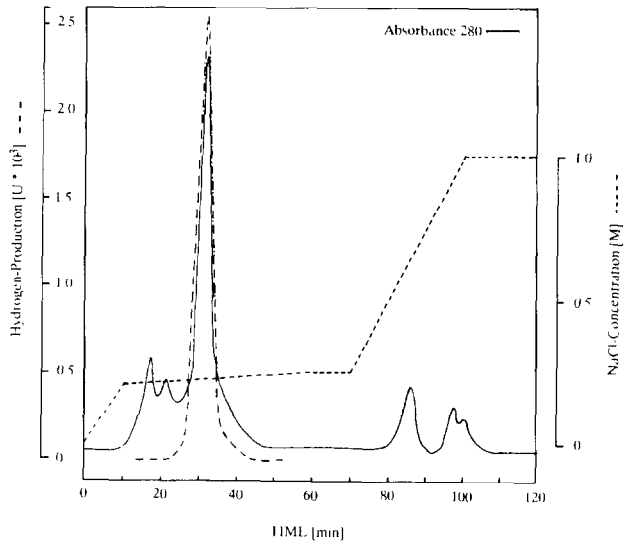

Fig. 1. Final separation of the hydrogenase active fraction from *Scenedesmus obliquus* with FPLC anion exchange chromatography on the second Mono-Q column. After removal of unbound proteins, hydrogenase was eluted with a linear salt gradient from 150 to 250 mM NaCl in bis-Tris-buffer. Fractions of 1 ml were collected and assayed for hydrogenase activity as described above.

tive purification step is anion exchange chromatography on Mono-Q. The hydrogenase eluted as a sharp peak at a NaCl-concentration of about 200 mM (Fig. 1). It must be pointed out that the specific activity of the purified hydrogenase from *Scenedesmus obliquus* is much higher than those reported for prokaryotic hydrogenases [32,33]. Analysis of the purified enzyme by non-denaturing PAGE (Fig. 2) revealed two protein bands with hydrogenase activity as demonstrated by coupled

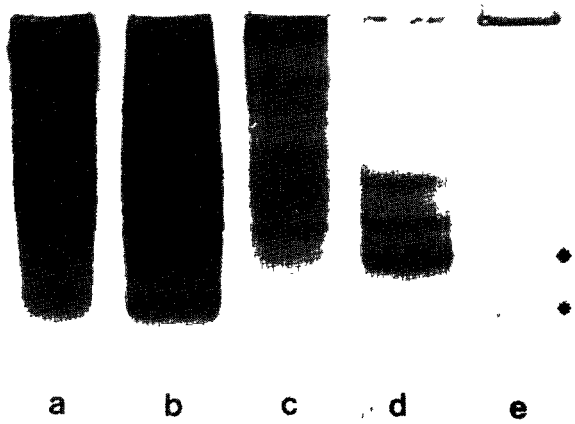

Fig. 2. Non-denaturing gel electrophoresis of proteins from all purification steps. Lane a = cell-free extract; lane b = active fraction from the DEAE column; lane c = active fraction the Sephacryl S-200 column; lane d = active fraction from the first Mono-Q column; lane e = active fraction the second Mono-Q column. Protein was stained according to the method of Blum et al. [21]; hydrogenase (*) was stained for activity as described in section 2.

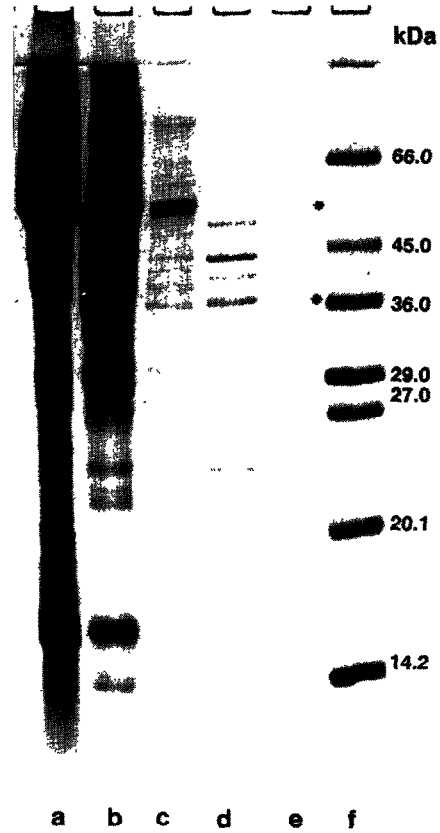

Fig. 3. SDS-PAGE of the purified hydrogenase from *Scenedesmus obliquus*. Lane a = cell-free extract; lane b = DEAE eluate; lane c = Sephacryl S-200 eluate; lane d = active fraction from the first Mono-Q column; lane e = active fraction from the second Mono-Q column; lane f = molecular weight standards.

reduction of 2,3,5-triphenyltetrazoliumchloride and methylviologen in the presence of H_2 and subsequent protein staining. The existence of isomeric forms of the hydrogenase after the final purification step could be excluded by SDS-PAGE of the two protein bands. Both proteins showed an identical subunit composition. We

Table III

Purification of the hydrogenase from *Scenedesmus obliquus*

All purification steps were carried out under strict anaerobiosis. Enzyme activity is expressed as H_2-evolution measured polarographical using a Clark electrode.

Purification step	Total protein (mg)	U_{spec}	U_{total}	Enrichment factor	Yield (%)
Crude homogenate	3134.0	2.1	4074	1.0	100
Supernatant	489.0	6.7	3276	3.2	80
DEAE	74.4	26.5	1970	12.6	48
Sephacryl S-200	10.2	97.0	989	46.2	24
(1) Mono-Q	0.5	1630.0	815	776.0	20
(2) Mono-Q	0.063	2710.0	340	1290.0	8

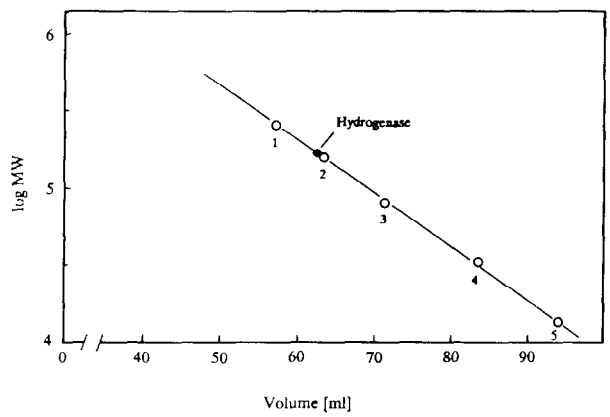

Fig. 4. Native molecular weight of the hydrogenase from *Scenedesmus obliquus*. A Sephacryl S-200 column was used. The column was calibrated with different molecular weight standards: (a) catalase; (b) lactate dehydrogenase; (c) ovotransferrin; (d) carbonic anhydrase; (e) cytochrome *c*.

agree with Nakos and Mortenson who suggest that the multiple forms of hydrogenase from *Clostridium pasteurianum* were artifacts, resulting from protein–protein interaction [34]. Analysis of the purified hydrogenase by SDS-PAGE showed that the hydrogenase consists of two subunits with molecular masses of 55 kDa and 36 kDa, respectively (Fig. 3). This result is in agreement with the subunit composition of a number of well-characterized prokaryotic (see [1,2] for review; [32,33,35,36]).

3.3. *Native molecular weight*

The native molecular weight of the hydrogenase was estimated by gel filtration chromatography on Sephacryl S-200 as described in section 2 using a cell-free extract. Hydrogenase eluted in only one single fraction. This result supports our suggestion that multiple forms of hydrogenase, as pointed out by non-denaturing polyacrylamide gel electrophoresis, result from protein–protein interaction during electrophoresis. Using this chromatography technique, a native molecular weight of 150,000 ± 5,000 was estimated (Fig. 4). By SDS-PAGE an apparent molecular weight of 91,000 was determined. This result indicates that either the native hydrogenase appears as a dimer of the two subunits do not occur in equimolar relations. This phenomenon has to be investigated in detail in further studies.

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