ISOLATION AND CHARACTERIZATION OF THE HYDROGENASE ACTIVITY FROM THE NON-HETEROCYSTOUS CYANOBACTERIUM *SPIRULINA MAXIMA*

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1. Introduction

In the last five years great interest has been focussed on the purification and characterization of hydrogenases from different sources, not only for their intrinsic importance, but in order to use them in the chloroplast ferredoxin-hydrogenase [1] or other suitable in vitro systems, to produce molecular H_2 which is a potential fuel source.

Although the enzyme activity has been found in many bacteria and algae [2], homogeneous enzyme preparations have only been isolated from photosynthetic [3–5], aerobic [6] and anaerobic bacteria [7,8]. There are a few reports about hydrogenase activity in nitrogen-fixing, heterocystous Cyanobacteria [9–12], and recently a partial purification of the enzyme from *Mastigocladus laminosus* [13], *Spirulina maxima* [13] and *Anabaena cylindrica* [14] have been achieved.

Here we report the purification (to 110-fold) and properties of hydrogenase activity from the non-heterocystous cyanobacterium *S. maxima.* This enzyme, as is the case with hydrogenases from bacterial sources [2,15], is strongly inhibited by CO and its activity is not stimulated by the presence of ATP in vitro suggesting the activity is due to a hydrogenase and not due to nitrogenase.

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2. Materials and methods

Bovine serum albumin (BSA), *p*-hydroxymercuribenzoate (*p*-HMB), *N*-ethyl maleimide (NEM) and bathophenanthroline were purchased from Sigma Chemical Co. (London); molecular weight markers, cytochrome *c*, NADH, NADPH, FMNH and ATP were from Boehringer Corp. (London); DEAE-cellulose (DE 52) was from Whatman Biochem.; Sephacryl S-200 was from Pharmacia (London); CO was from Matheson Gas Products; other reagents were from BDH Chem. (England).

Fresh cells of *Spirulina maxima* grown in natural populations in the lake Texcoco, Mexico, were stored in liquid N_2 until they were needed. All the purification steps were performed at 4°C under strictly anaerobic conditions by flushing buffer (20 mM Tris–HCl (pH 8.0), 10 mM 2-mercaptoethanol) with N_2. Wet cells (50 g) were thawed and suspended in 250 ml buffer. After 1 h stirring the lysate was centrifuged for 20 min at 40 000 × *g*. The sediment was discarded and the supernatant was filtered through cheesecloth and the filtrate spun for 1 h at 100 000 × *g*. The blue supernatant was decanted and applied to a DE52 column (2.5 × 30 cm) equilibrated with buffer. The column was washed with 0.2 M KCl containing buffer until all the blue pigments were eluted. The hydrogenase activity eluted with 0.3 M KCl. Fractions (10 ml) were collected (fig.1). The active fractions were combined and solid ammonium sulphate added to 65% saturation. After stirring for 15 min the mixture was centrifuged for 30 min at 40 000 × *g*. The supernatant

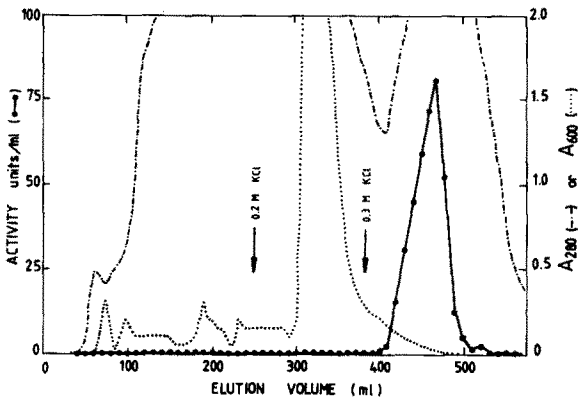

Fig.1. Elution pattern of *S. maxima* hydrogenase from DEAE-cellulose (DE52) column. The blue photosynthetic pigments (measured as A_{600}) eluted with 0.2 M KCl and the enzyme activity with 0.3 M KCl. Protein was monitored as A_{280}.

was saved and stored for ferredoxin purification. The active sediment was resuspended in a small volume of buffer and applied to a column (2.2 X 90 cm) of Sephacryl S-200 equilibrated and developed with 50 mM KCl containing buffer. Fractions (2 ml) were collected. The most active fractions were pooled and used for further studies.

Hydrogenase activity was assayed by H_2 evolution from methyl viologen (MV), reduced by sodium dithionite, at 30°C [16]. The activity was located in analytical 10% polyacrylamide gels by the method in [17]. The O_2-sensitive band of reduced MV was fixed by anaerobically incubating the gel in 2% (w/v) tetrazolium salt solution. The molecular weight of the enzyme was calculated by gel filtration on Sephacryl S-200 according to [18]. *Spirulina* and *Clostridium* ferredoxins were isolated by the method in [19] and [20], respectively. Protein concentration was measured by the method in [21] with crystalline BSA as standard.

3. Results

3.1. *Hydrogenase purification*

The results of a typical preparation are given in table 1. A purification of >100-fold with an 11% recovery could be obtained routinely. The electrophoretogram in polyacrylamide gels of one sample at this stage of purification showed a major band of protein (R_m 0.58) which coincided with a single band of activity, and several (4–7) minor contaminant bands of protein. Chromatography of the Sephacryl S-200 fraction on a second column of DE52 (equilibrated with 50 mM KCl containing buffer), washing with 0.2 M KCl and elution with a linear gradient (0.2–0.3 M KCl) led to a preparation with lesser number of undesirable proteins (as shown by polyacrylamide gel electrophoresis) but the specific activity decreased due to the extreme instability of the enzyme.

3.2. *Molecular weight determination*

The molecular weight of the purified hydrogenase estimated by chromatography on Sephacryl S-200 was 56 000 (± 2000). Aldolase, BSA, ovoalbumin and β-lactoglobulin were run as molecular weight markers (fig.2).

Table 1
Purification of hydrogenase from *Spirulina maxima*

Step	Total prot. (mg)	Total act. (units)	Spec. act. (Units/mg prot.)	Purification (-fold)	Yield (%)
Lysate	12800	12860	1.0	1.0	100
40 000 × *g* supernatant	1207	6265	5.2	5.2	48.7
100 000 × *g* supernatant	800	6027	7.5	7.5	46.8
DE52 eluate	107	3750	35.0	35.0	29.2
Sephacryl S-200	6.6	1453	112.4	112.0	11.3

Activity units are expressed as μmol H_2 evolved/h using the MV (1.5 mM)–dithionite (10 mM) system, at 30°C in 20 mM phosphate buffer (pH 7.0)

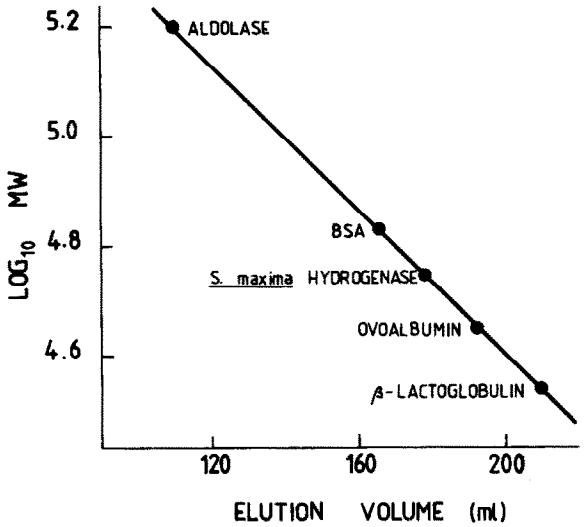

Fig.2. Estimation of the molecular weight of *S. maxima* hydrogenase by gel-filtration on Sephacryl S-200. The column (90 × 2.2 cm) was equilibrated with 50 mM KCl containing buffer. Fractions (2 ml) were collected. Flow rate was 30 ml/h.

3.3. *pH optimum*

The pH effect on hydrogenase activity was studied in the presence of Tris–HCl or potassium phosphate buffer (at 50 mM) in the range pH 6.0–9.0. An optimum pH 7.5–8.0 was found in both cases, However, a 25% activation could be observed in the case of phosphate buffer with respect to Tris–HCl.

3.4. *Electron donors and carriers*

Purified *S. maxima* hydrogenase was only able to evolve H_2 using viologens – MV or benzyl viologen (BV) – chemically reduced by dithionite. Activity using reduced BV was 45% of that obtained with reduced MV. No activity could be detected when *Spirulina* or *Clostridium* ferredoxin (at 0.04 mM), or horse heart cytochrome *c* (at 0.05 mM) were used instead of the viologens in the presence of dithionite. NADH, NADPH or FMNH (at 5 mM) alone or in combination were ineffective as well as electron donors.

3.5. *Substrate affinities*

Apparent K_m values were calculated for reduced MV and BV using Lineweaver-Burk plots. K_m values of 0.16 mM and 0.33 mM were found for MV and BV, respectively.

Table 2
Inhibitors of the H_2 evolution catalysed by *Spirulina maxima* hydrogenase using the MV-dithionite system

Inhibitor	K_i (mM)	Type of inhibition
Azide	3.4	Non-competitive
Bathophenanthroline	0.1	Non-competitive
Cyanide	0.85	Non-competitive
EDTA·Na_2	0.02	Competitive

The K_i value and the inhibition type were determined by using Dixon plots [22]

3.6. *Inhibitors*

The hydrogenase catalysed evolution of H_2 from dithionite-reduced MV was affected by the metal-complexing reagents azide, bathophenanthroline, cyanide and EDTA (table 2). The thiol-reagents *p*-HMB and NEM caused >80% inhibition at 0.1 mM.

3.7. *Inhibition by CO*

CO was a powerful inhibitor of hydrogenase activity. At a partial pressure of 50 Torr, >85% inhibition could be observed (fig.3). The inhibition

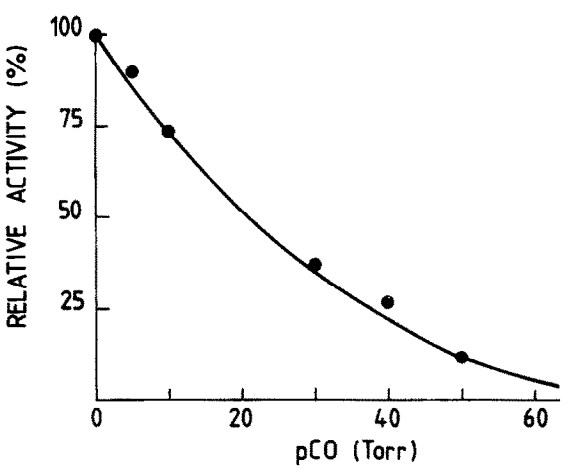

Fig.3. Inhibition of purified *S. maxima* hydrogenase by CO. The activity was assayed by the standard procedure except that the N_2 atmosphere was substituted by N_2–CO mixtures at the indicated concentrations. The CO concentrations (pCO, partial pressure of CO) are expressed in Torr.

was total at 100 Torr. A K_i value of 20 Torr was found by a Dixon plot [22]. Greater than 95% of the inhibition could be reversed by the removal of CO by flushing for 5 min with N_2.

3.8. *Effect of ATP and Mg^{2+}*

The presence of ATP (at 5 mM) and Mg^{2+} (at 5 mM) did not show any activation effect in the H_2 evolution and a strong inhibition could be observed.

3.9. *Effect of metal ions*

The effect of different divalent cations (at 1 mM) were investigated in 50 mM Tris–HCl buffer (pH 7.5). Cu^{2+} and Zn^{2+} caused total inhibition. Fe^{2+} activated >120%. Finally, Mg^{2+}, Mn^{2+} and Ca^{2+} showed a 60% activation.

4. Discussion

The main problems in the isolation of hydrogenase from *S. maxima* are:

(i) The separation of the enzyme activity from the blue pigments phycocyanins and phycoerythrins which have similar chemical and molecular properties.

(ii) The extreme instability of the enzyme.

We were able to remove completely the blue material by using stepwise elution on a DEAE-cellulose column (fig.1). Cell disaggregation by osmotic lysis and ultracentrifugation of lysate were absolutely necessary in order to eliminate chlorophillous materials and other fragments which reduced the column flow-rate. However, we have not had success in our efforts to stabilize the activity. The enzyme is extremely unstable under all the conditions tested, in an O_2-free atmosphere. Greater than 50% of original activity was lost when stored overnight at room temperature, 4°C, −20°C or even in liquid N_2. Addition of dithionite, 2-mercaptoethanol or MV to the enzyme did not improve the stability to any considerable extent. With the procedure described we have obtained 112-fold purification with an acceptable (11%) yield of initial activity (table 1).

In contrast with the results reported for hydrogenases from other Cyanobacteria [11,14], most of the enzyme activity from *S. maxima* is easily extractable without mechanical disruption or the use of detergents. Sonication in the presence of 2% (w/v) deoxycholate strongly inhibited the hydrogenase activity (data not shown).

The calculate molecular weight of the purified enzyme (56 000) is the same order of other hydrogenases characterized from bacterial sources [5,7,8] but much lower than that reported (230 000) for *Anabaena cylindrica* hydrogenase [14].

In a review on H_2 metabolism in Cyanobacteria [23] it was stated that the H_2-formation by intact cells is exclusively due to nitrogenase activity, its rate being ATP-dependent and CO-insensitive. We present here some evidence that purified *S. maxima* hydrogenase was able to evolve H_2 *in vitro* using non-physiological mediators. The enzyme acts as a true bacterial-type hydrogenase, the activity being highly sensitive to CO and being inhibited by ATP. As is the case with hydrogenases from photosynthetic bacteria [3–5] the enzyme was not able to evolve H_2 using chemically-reduced *Spirulina* or *Clostridium* ferredoxins or other natural mediators in vitro. The physiological electron carrier (if it exists) is not yet known.

Acknowledgements

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