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# ISOLATION OF HYDROGENASE FROM THE THERMOPHILIC CYANOBACTERIUM MASTIGOCLADUS LAMINOSUS

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SUMMARY: A soluble, cytoplasmic hydrogenase was detected and partially purified from Mastigocladus laminosus. It was found to be unstable but could be stabilized to some extent by $Mg^{++}$; 50% of the activity remained after one week in air at 4°C.

In recent years, the enzyme hydrogenase has become the subject of much investigation because of its relevance to $H_2$ evolving biophotolysis systems which contain immobilized chloroplasts, an electron mediator and hydrogenase (Rao et al., 1980). In this way solar radiation could be used to generate hydrogen from water. Unfortunately, many hydrogenases so far isolated from bacteria are unstable and $O_2$-sensitive. Hydrogenases have been detected in a number of algae and their use in hydrogen evolving systems been studied in several laboratories (Adams et al., 1981; Lambert and Smith, 1980). However, the complete purification of an algal hydrogenase has not yet been successful. The only purification thus far reported has been from the cyanobacterium Spirulina maxima (Llama et al., 1978a). As thermophilic organisms often contain very stable enzymes (Zuber, 1976) we have attempted to purify the hydrogenase from the thermophile Mastigocladus laminosus, a cyanobacterium which has recently received some attention due to the work of Ochiai et al. (1980) who used it in a "living electrode". This electrode consists of alginate-immobilized intact cells which are able to generate an electric current upon illumination and is thought suitable to replace unstable isolated chloroplasts in biophotolysis systems.

## MATERIALS AND METHODS

The cells of Mastigocladus laminosus were a gift from Dr. W. Sidler and Prof. H. Zuber, E.T.H. Zurich, Switzerland. They were collected in the hot springs (55-60°C) near Reykjavik in Iceland in Summer 1977 and kept frozen at -20°C. All chemicals were of the highest purity available and were purchased from BDH Chemicals, Poole, England. Sephadex G-100, Sephacryl S-200 and DEAE-Sephacel were from Pharmacia (London). Sephacryl S-200 was packed into a 1.6 x 90 cm column equilibrated with 50 mM Tris-HCl, pH 8.0, 0.1M NaCl, 20 mM $MgCl_2$, containing 2 µM Genticin (antibiotic from Nicholas Laboratories Ltd, Slough, England) and 0.02% sodium azide (respiratory inhibitor) and was run at a hydrostatic pressure of about 120 cm. For DEAE-Sephacel, a 2.2 x 27 cm column was equilibrated with 50 mM Tris-HCl, pH 8.0, and elution was with a gradient (300 ml) of 0 to 0.5 M NaCl in 50 mM Tris-HCl. Hydroxylapatite was prepared according to Tiselius et al. (1956) and used in a 1.1 x 7 cm column equilibrated with 20 mM phosphate, pH 7.0, and eluted with a gradient (40 ml) of 20 to 100 mM phosphate. All buffers were flushed with $N_2$. The Polytron blender (Kinematica GmbH, Luzern, Switzerland) was operated at setting 3. Sonication was performed with a Soniprobe (Dawe Instruments, London) at maximal output

(about 5 A). Before blending and sonication, 1 mM phenylmethylsulfonylfluoride (Sigma) was added to the cell suspensions to inhibit proteases. The hydrogenase activity was assayed at 30°C by measuring the $H_2$ evolution from 0.75 mM methyl viologen, reduced by 10 mM sodium dithionite, in a total volume of 2.0 ml using 20 mM phosphate pH 7 and 50 mM Tris pH 8 as buffer media. $H_2$ in the gas phase was determined by a gas chromatograph run with $N_2$ (Rao et al., 1976). The rate of $H_2$ evolution was calculated from the slope of straight lines in plots of peak height vs. time.

## RESULTS AND DISCUSSION

Partial purification of the hydrogenase activity: To 25 g wet weight of cells, frozen together with 75 ml of spring water, 100 ml of 50 mM Tris-HCl buffer, pH 8.0 was added and bubbled with $N_2$ during thawing. The filaments were disrupted using a Polytron blender for 3 periods of 20 secs. The suspension was sonicated for 5 min in an ice bath and centrifuged at 40,000 x g for 1 h. The pellet was resuspended in the above buffer and sonicated and centrifuged as above. The combined blue-green supernatants will be called "cell extract".

Ammonium sulphate was added to the cell extract to 35% saturation, and after centrifugation at 40,000 x g for 20 min, the pellet containing the chlorophyll was discarded. More ammonium sulphate was added to the blue supernatant to 55% saturation. After centrifugation, the pellet was resuspended in a small volume of 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 20 mM $MgCl_2$. Data on the specific activities are given in Table 1.

Release of the activity: When the thawed cells were centrifuged at 40,000 x g for 30 min (no addition of buffer or prior homogenization), only about 10% of the total activity normally found in the sonicated cells was detected in the supernatant whose blue-green hue suggested breakage of a small fraction of the cells, probably caused by freezing and thawing. An additional 17% of the total activity was released by incubation of the pellet in distilled water at 30°C for 15 min. Thorough sonication as described above was necessary to release all of the hydrogenase. Detergents (up to 1% Triton X-100 or deoxycholate) did not increase the yield. From these findings, we conclude that the hydrogenase is neither an integral membrane protein nor periplasmic, but soluble and located in the cytoplasm.

When the pH of the sonication medium was varied between 7 and 9, no difference in activity (measured at pH 7) was observed in the cell extracts, whereas at pH 6 only 60% and at pH 5 no activity at all was released.

Table 1: Partial purification of hydrogenase from Mastigocladus laminosus

Activity units are expressed as µmol of $H_2$ evolved/h

| Fraction | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg protein) | Activity yield (%) |
|---|---|---|---|---|---|
| Sonicated cells | 192 | 740 | 140 | 0.19 | 100 |
| Cell extract | 238 | 490 | 140 | 0.29 | 100 |
| 35-55%-satd. $(NH_4)_2SO_4$ | 4.2 | 105 | 76 | 0.72 | 54 |
| Sample loaded on G-100 | 4 | 16.7[a] | 12 | 0.72[b] | 54 |
| Active fractions from G-100 | 20 | 2.7[c] | 26.5 | 10 | 120 |

[a] not determined, but calculated from [b]
[b] assumed to be equal to value for 35-55%-satd. $(NH_4)_2SO_4$ fraction
[c] calculated from [a] and from $A_{280}$ measurements given in Fig. 2.

Stability of the hydrogenase: In sonicated cells, we measured a hydrogenase activity of about 16 µmol $H_2$/h/mg chlorophyll a or 6 µmol $H_2$/h/g wet cells. This is of the same order of magnitude as that found in several other cyanobacteria and in green algae, but compared to a number of bacterial hydrogenases it is rather low. In Desulfovibrio desulfuricans (Norway strain) a more than 3,000 times higher activity is found. Therefore, it was imperative to find the optimal conditions to preserve this inherently unstable activity. Even if frozen at -20°C, an average of 10% of the activity was lost every day in the cell extract; partly, this can be explained by the detrimental effect of freezing and thawing. This was investigated by freezing and thawing a sample of cell extract 7 times within 8 hours; 20% of the hydrogenase activity was lost.

Fig. 1 shows that at 4°C, the activity measured in the cell extract decreased steadily at a rate of about 25% per day when stored under air, but only about 8% per day when stored under $N_2$. This indicates that $O_2$ is responsible for part of the inactivation. The data of the upper trace in Fig. 1A suggests that the presence of 20 mM $Mg^{++}$ ions during storage reverses this $O_2$-inactivation, but Fig. 1B reveals not only a stabilizing, but an activating effect of $Mg^{++}$. The initial steady level of activity in the upper curve of Fig. 1A must therefore be composed of the rise in activity during the first 2 days brought about by $Mg^{++}$ under $N_2$ (Fig. 1B, upper trace) and the steep decrease due to $O_2$-inactivation (Fig. 1A, lower trace). Note that no activation was observed on day 0, about 2 hours after the addition of $MgCl_2$. NaCl (0.5 M) showed a

similar stabilization but did not show any activation under nitrogen; under air this effect was less pronounced.

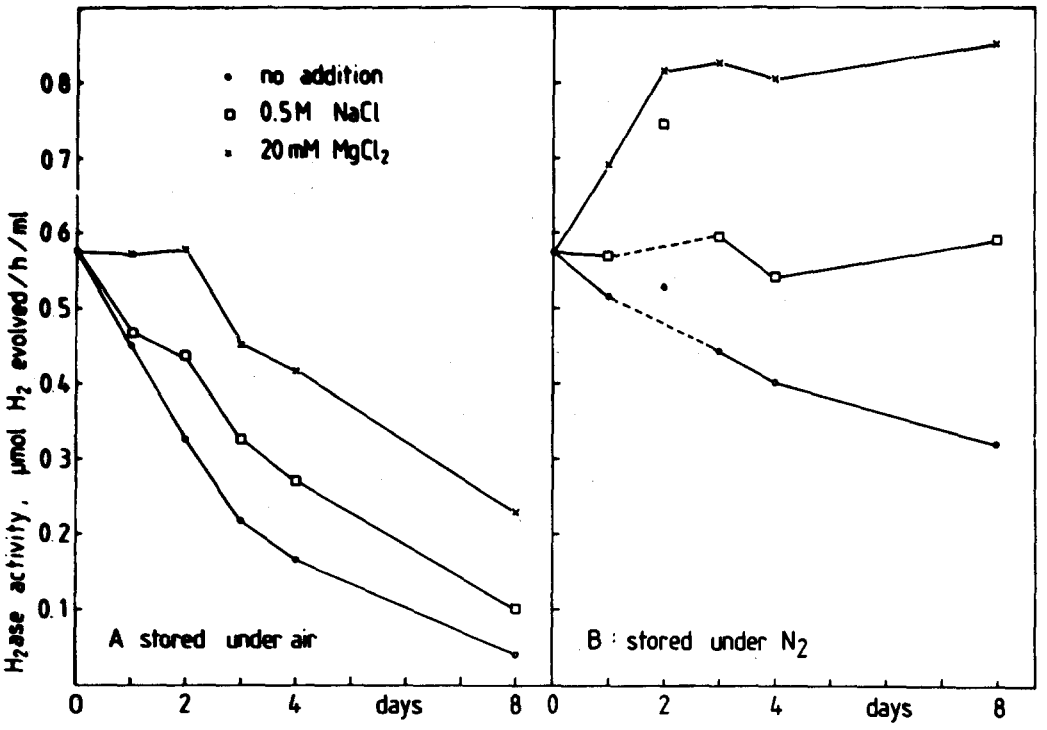


Fig. 1. Effect of $Mg^{++}$ and high ionic strength on the stability of hydrogenase activity. The cell extracts were stored at 4°C in 50 mM Tris-HCl, pH 8.0 containing 2 mM Streptomycin sulfate; additions (as indicated) were made after sonication, i.e. before the first assay (day 0). Measurements were done in duplicate with 1.0 ml of cell extract each. Thus, the actual concentrations in the assay mixture were half those indicated.

Further purification by chromatographic techniques: The most difficult problem in the isolation of hydrogenase from Mastigocladus laminosus was to separate the activity from the blue pigments released in large amounts by sonication. The blue phycobiliproteins aggregate into particles very similar in size and charge to the hydrogenase (Llama et al., 1978b). Whereas the chlorophyll could be removed satisfactorily by ammonium sulphate fractionation, the phycobiliproteins were co-purified. We tried chromatography on Sephadex G-100, Sephacryl S-200, DEAE-Sephacel and hydroxylapatite to purify the hydrogenase from the 35-55% saturated ammonium sulphate fraction. Fig. 2 shows our most successful result obtained on Sephadex G-100. A 2.2-fold increase in the total activity was observed, and the specific activity was increased considerably. However, the activity in the eluate stored at

$4^{o}C$ under $N_2$ decreased rapidly and had disappeared entirely after one week. Bacterial or fungal growth could not be ruled out in this experiment, since it was only in subsequent experiments that bacteriostatic agents (2 mM Streptomycin or 2 µM Genticin) and 0.02% sodium azide were included in the buffer solutions. On the other hand, the purified enzyme might well be much less stable than in a less purified state, a fact observed earlier by Llama et al. (1978b). In all of our subsequent purification attempts by gel filtration, mostly done on a larger scale using Sephacryl S-200 for the sake of a higher flow rate, the activity yield in the eluate was rather poor (typically $^{1}/_{3}$ of the loaded activity), and at least $^{1}/_{3}$ of the phycobiliproteins overlapped into the active fractions. An even less efficient separation was obtained by ion exchange chromatography on DEAE-Sephacel and the use of hydroxylapatite chromatography.

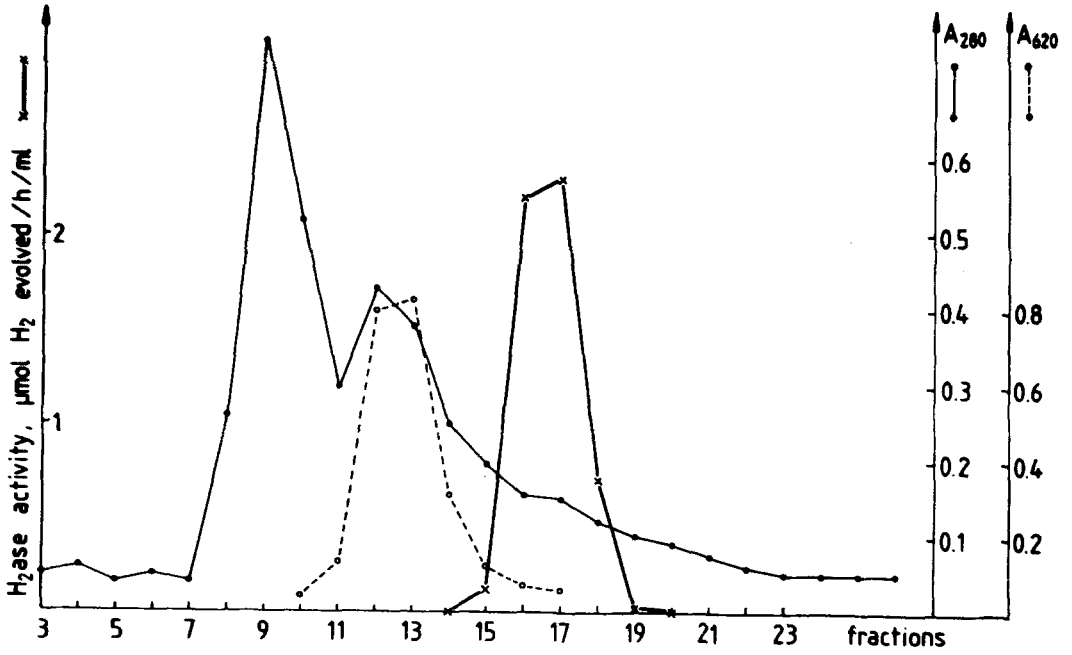


Fig. 2. Gel filtration of a 40-60% sat. $(NH_4)_2SO_4$ fraction on Sephadex G-100. A 2.2 x 90 cm column was equilibrated with 20 mM Tris pH 8.0, 0.1 M NaCl, 10 mM $MgCl_2$ and run on a hydrostatic pressure of about 80 cm. The fraction size was 100 drops (5-6 ml). The total activity in the loaded sample (4 ml) was 12 Units, whereas a total of 26.5 Units were recovered in fractions 15 to 18.

## CONCLUSION

We have succeeded in isolating a soluble cytoplasmic hydrogenase in the thermophile Mastigocladus. However, our partially purified preparation could not be stabilized to the degree necessary for purification of the active enzyme to homogeneity. Again this appears to be a problem which has been encountered in previous attempts to isolate hydrogenases from algae. As yet we cannot determine why algal hydrogenases are so unstable in vitro. However, because of the potential usefulness of algal hydrogenases and their possible diversity and unknown properties we will continue in our attempts to isolate, characterize and stabilize algal hydrogenases. At present for practical purposes, the use of intact cyanobacterial cells seems to be much more useful, and further research needs to be done on the use of immobilized or otherwise stabilized whole organisms (Ochiai et al., 1980; Lambert et al., 1979).

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