Determination of Recombinant Mannitol-1-phosphate Dehydrogenase Activity from *Ectocarpus* sp.

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[Abstract] Brown algae belong to a phylogenetic lineage distantly related to green plants and animals, and are found predominantly, but not exclusively, in the intertidal zone, a harsh and frequently changing environment. Because of their unique evolutionary history and of their habitat, brown algae feature several peculiarities in their metabolism. One of these is the mannitol cycle, which plays a central role in their physiology, as mannitol acts as carbon storage, osmoprotectant, and antioxidant. This polyol is derived directly from the photoassimilate fructose-6-phosphate via the action of a mannitol-1-phosphate dehydrogenase (M1PDH, EC 1.1.1.17) and a mannitol-1-phosphatase (M1Pase, EC 3.1.3.22). This protocol describes the biochemical characterization of the recombinant catalytic domain of one of the three M1PDHs identified in *Ectocarpus* sp. This recombinant catalytic domain, named hereafter M1PDHcat, catalyzes the reversible conversion of fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P) using NAD(H) as a cofactor. M1PDHcat activity was assayed in both directions *i.e.*, F6P reduction and M1P oxidation (Figure 1).
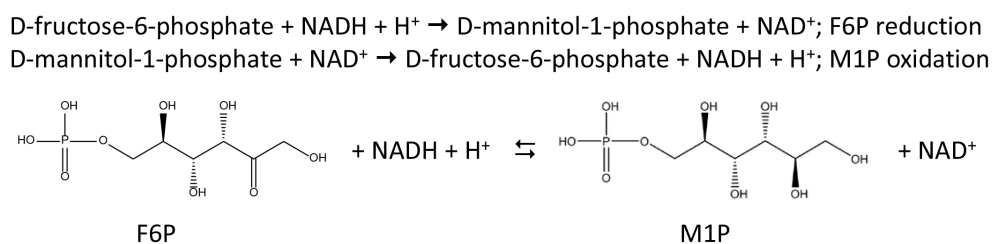
D-fructose-6-phosphate + NADH + H$^+$ ➜ D-mannitol-1-phosphate + NAD$^+$; F6P reduction
D-mannitol-1-phosphate + NAD$^+$ ➜ D-fructose-6-phosphate + NADH + H$^+$; M1P oxidation

Figure 1. Reversible reaction of mannitol-1-phosphate dehydrogenase

Materials and Reagents

1. UV-Star® PS microplate (96 well) (Greiner Bio One International, catalog number: 655801)
2. 0.22 µm filter
3. Purified recombinant His-tagged M1PDHcat
   Note: This protein was produced in Escherichia coli BL21 (DE3) containing the recombinant pFO4_M1PDHcat vector, as described by Groisillier et al. (2010). This recombinant protein was purified by affinity chromatography using a HisPrep FF 16/10 column (GE Healthcare) and then

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by gel filtration using a Superdex 200 (GE Healthcare) onto an Äkta avant system (GE Healthcare). The complete purification protocol is described in details in Bonin et al. (2015).

4. MilliQ water

5. Trizma® base (Sigma-Aldrich, catalog number: T1503)

6. 4-morpholineethane-sulfonic acid (MES) (Sigma-Aldrich, catalog number: M2933)

7. HEPES (Sigma-Aldrich, catalog number: H3375)

8. Bis-Tris propane (Sigma-Aldrich, catalog number: B6755)

9. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 71380)

10. Examples of chemicals to be tested to assess substrate and co-factor specificity:

    a. D-mannitol-1-phosphate (Sigma-Aldrich, catalog number: 92416)

    b. D-fructose-1-phosphate (Sigma-Aldrich, catalog number: F1127)

    c. α-D-glucose-1-phosphate disodium salt hydrate (Sigma-Aldrich, catalog number: G9380)

    d. D-mannose-6-phosphate sodium salt (Sigma-Aldrich, catalog number: M3655)

    e. D-glucose-6-phosphate sodium salt (Sigma-Aldrich, catalog number: G7879)

    f. D-fructose-6-phosphate disodium salt hydrate (Sigma-Aldrich, catalog number: F3627)

    g. β-NAD (Sigma-Aldrich, catalog number: N1636)

    h. β-NADP (Sigma-Aldrich, catalog number: N5755)

    i. β-NADH (Sigma-Aldrich, catalog number: N8129)

    j. β-NADPH (Sigma-Aldrich, catalog number: N5130)

11. 1 M Tris-HCl (see Recipes)

12. 5 M NaCl (see Recipes)

13. 10 mM NADH (see Recipes)

14. 10 mM NAD$^+$ (see Recipes)

Equipment

1. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, model: NanoDrop 2000)

2. Safire2 UV spectrophotometer microplate reader (Tecan Trading)

Software

1. Hyper 32 (Informer Technologies, model: hyper32)

2. Microsoft Excel

Procedure

1. The standard F6P reduction reaction mixture contains 50 mM Tris-HCl (pH 7.0), 1 mM F6P, 0.2 mM NADH and 0.1 to 10 µg of purified recombinant M1PDHcat, in a final volume of 100 µl. The standard M1P oxidation reaction mixture contains 50 mM Tris-HCl (pH 9.0), 1 mM M1P, 0.5 mM NAD^+ and 0.1 to 10 µg of purified recombinant M1PDHcat in 100 µl. Quantities of proteins are determined with NanoDrop at 280 nm based on the extinction coefficient of 37,525 M^{-1} cm^{-1} calculated for the protein of interest. The negative control corresponds to the reaction mixture in which substrate is substituted by MilliQ water (Table1).

Table 1. Composition of negative control and reaction mixture for determination of M1PDHcat activity in the direction of F6P reduction and M1P oxidation

Stock solutions	µl added in negative control	µl added in reaction mix (test)
F6P reduction		
Tris-HCl pH 7.0 (1 M)	5	5
NADH (10 mM)	2	2
F6P (25 mM)	0	4
M1PDHcat (at 0.01-1 µg/µl)	1	1
MilliQ water	92	88
M1P oxidation		
Tris-HCl pH 9.0 (1 M)	5	5
NAD^+ (10 mM)	5	5
M1P (25 mM)	0	4
M1PDHcat (at 0.01-1 µg/µl)	1	1
MilliQ water	89	85

2. The mix is prepared with water, Tris-HCl, NAD(H) and enzyme, then the reaction is started by adding the substrate and the activity is monitored by following changes in absorbance at 340 nm, which corresponds to the consumption (F6P → M1P) or production (M1P → F6P) of NADH, in a Safire2 UV spectrophotometer microplate reader. The plate is subjected to 10 sec of orbital shaking before measurements. Values are recorded every 13 sec approximately, and this interval of time can be modified according to the activity of the enzyme under consideration. The assay is performed at 30 °C for up to 20 min. Only the early linear part of the curves is used to calculate activity (Figures 2 and 3).

3. F6P reduction and M1P oxidation activities, based on consumption or production of NADH respectively, are calculated using the formula:

$$[(\Delta A340 \text{ nm test} - \Delta A340 \text{ nm negative control}) \times 0.0001/(t \times 6220 \times 0.3)]$$

Where,

ΔA340 nm = variation of absorbance during the duration of incubation,

T = duration of incubation (min),

6220 = extinction coefficient for NADH (L mol^{-1} cm^{-1}),

0.3 = optical path (cm). This value depends of the plate/cuvette used,

0.0001 = assay volume (L),

One unit (U) of activity, F6P reduction or M1P oxidation, corresponds to 1 µmole of co-factor oxidized or reduced per min respectively.
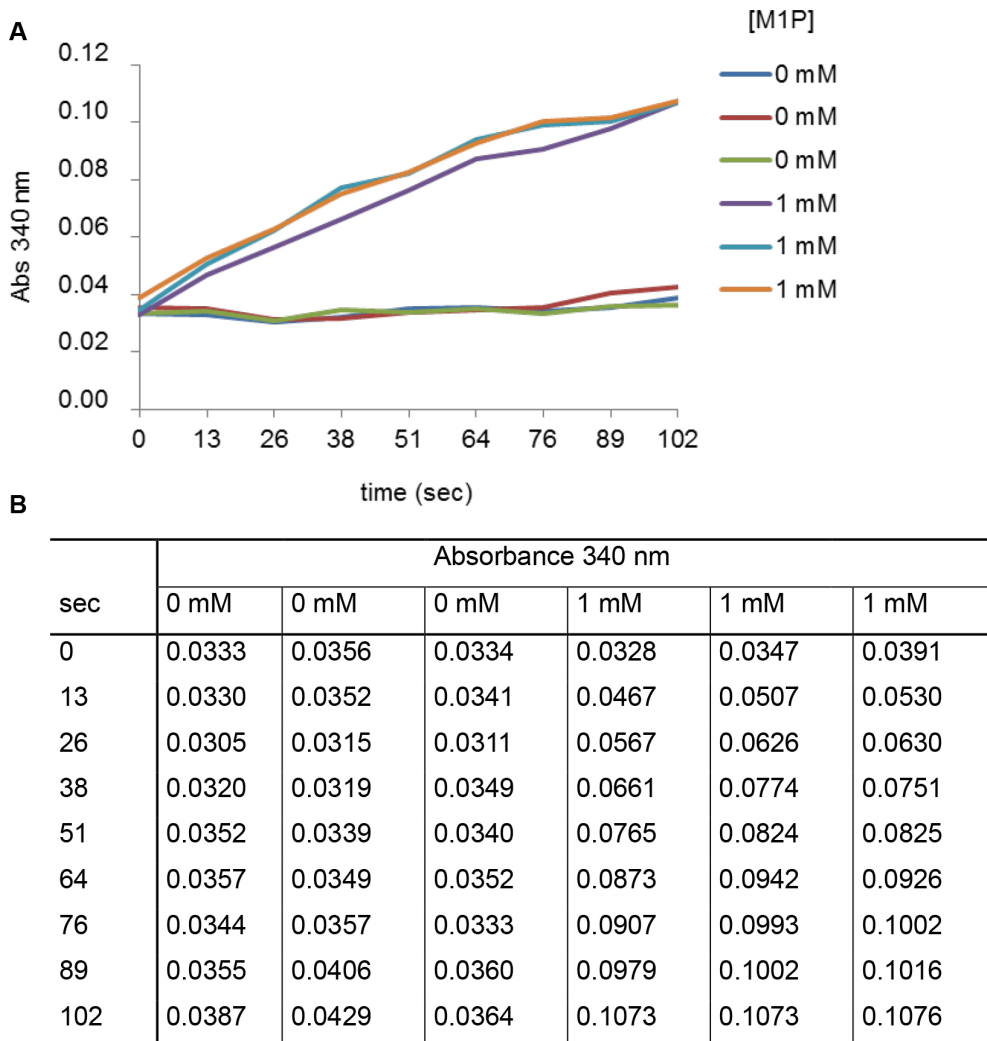

Figure 2. Measurement of absorbance (340 nm) monitored as a function of time (sec) in presence or in absence of M1P. A. The curves represent two series of triplicates obtained in absence or in presence of 1 mM of M1P and 0.2 µg of enzyme. B. The table contains values of absorbance measured under both conditions tested and at different times of experiment.

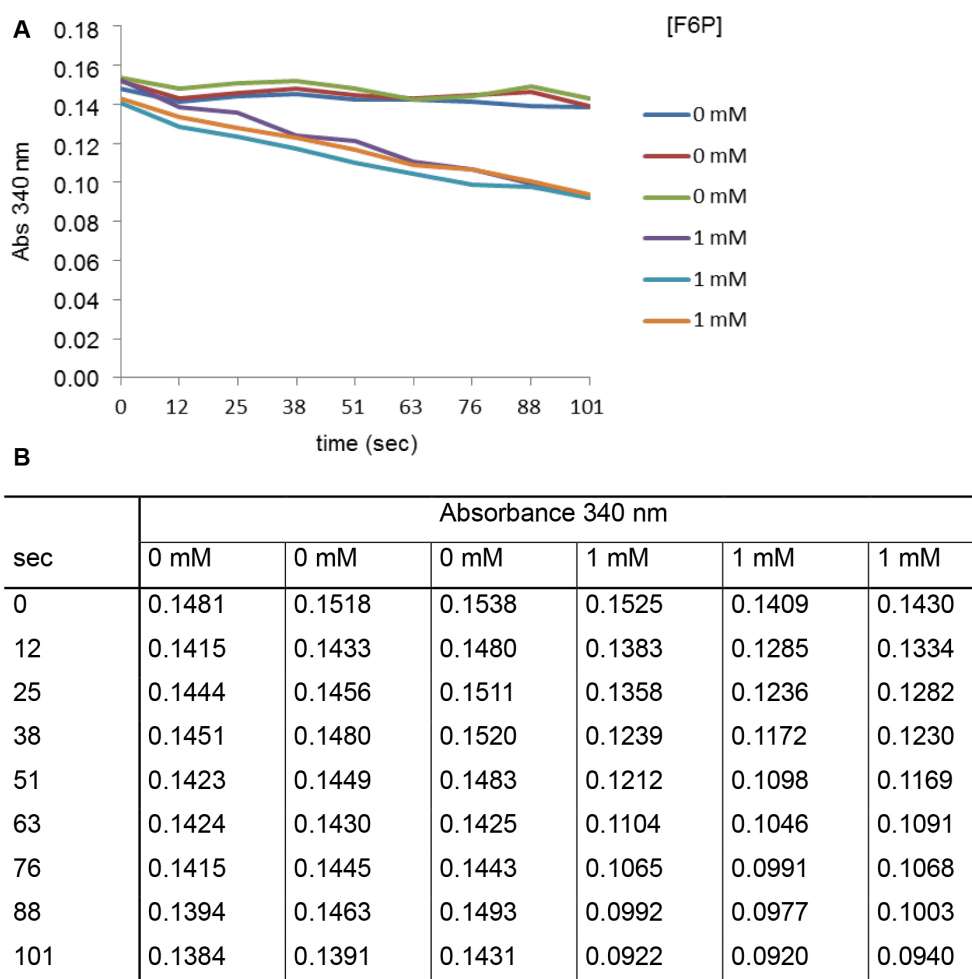| sec	Absorbance 340 nm					
	0 mM	0 mM	0 mM	1 mM	1 mM	1 mM
0	0.0333	0.0356	0.0334	0.0328	0.0347	0.0391
13	0.0330	0.0352	0.0341	0.0467	0.0507	0.0530
26	0.0305	0.0315	0.0311	0.0567	0.0626	0.0630
38	0.0320	0.0319	0.0349	0.0661	0.0774	0.0751
51	0.0352	0.0339	0.0340	0.0765	0.0824	0.0825
64	0.0357	0.0349	0.0352	0.0873	0.0942	0.0926
76	0.0344	0.0357	0.0333	0.0907	0.0993	0.1002
89	0.0355	0.0406	0.0360	0.0979	0.1002	0.1016
102	0.0387	0.0429	0.0364	0.1073	0.1073	0.1076

Figure 3. Measurement of absorbance (340 nm) monitored as a function of time (sec) in presence or in absence of F6P. A. The curves represent two series of triplicates obtained in absence or in presence of 1 mM of F6P and 0.04 µg of enzyme. B. The table contains values of absorbance measured under both conditions tested and at different times of experiment.

	Absorbance 340 nm					
sec	0 mM	0 mM	0 mM	1 mM	1 mM	1 mM
0	0.1481	0.1518	0.1538	0.1525	0.1409	0.1430
12	0.1415	0.1433	0.1480	0.1383	0.1285	0.1334
25	0.1444	0.1456	0.1511	0.1358	0.1236	0.1282
38	0.1451	0.1480	0.1520	0.1239	0.1172	0.1230
51	0.1423	0.1449	0.1483	0.1212	0.1098	0.1169
63	0.1424	0.1430	0.1425	0.1104	0.1046	0.1091
76	0.1415	0.1445	0.1443	0.1065	0.0991	0.1068
88	0.1394	0.1463	0.1493	0.0992	0.0977	0.1003
101	0.1384	0.1391	0.1431	0.0922	0.0920	0.0940

4. To calculate specific activities, divide the values obtained in the equation above by the quantity of M1PDHcat proteins present in the sample. Perform three replicates for each condition, and use the average calculated in absence and in presence of substrate to determine the specific activity of each reaction. This applies also to the experiments described below. From values of Figures 2 and 3, specific activities of M1PDHcat are 11.3 U/mg in presence of 1 mM M1P and 39.0 U/mg in presence of 1 mM F6P.

5. To determine substrate specificity, test M1PDHcat activity in the presence of each substrate listed in the 'Materials and Reagents' section, using concentration ranging from 1 mM to at least 50 mM, at room temperature.

6. To determine the optimal temperature, incubate the reaction mixtures used in step 1 at temperatures ranging, for instance, from 10 °C to 50 °C, with incremental of 5 °C or 10 °C. The experiments described below are performed at the optimal temperature of 30 °C.

7. To determine the optimal pH, replace the 50 mM Tris-HCl pH 7.0 or pH 9.0 buffer used in step 1 by other buffers prepared at different pHs. As an indication, it is possible to use:

    a. 50 mM MES for pH 5.5, 6, 6.5

    b. 50 mM Bis-Tris propane for pH 6.5, 7, 7.5, 8, 8.5, 9, 9.5

    c. 50 mM Tris-HCl for pH 7, 7.5, 8, 8.5, 9

    d. 50 mM Tris-acetate for 6, 6.5, 7

    e. 50 mM HEPES for 7, 7.5, 8, 8.5

8. To examine the influence of NaCl, add NaCl to the reaction mixture described in step 1 to obtain final concentrations ranging from 0 to 1 M.

9. To estimate the kinetic parameters of the enzyme for a selected substrate S, run individual enzyme reactions in the presence of at least five different concentrations of this substrate (for example 0.1 mM, 0.25 mM, 1 mM, 4 mM, 20 mM final concentration), and a fixed concentration of NAD^+ or NADH. Determine the initial reaction rate for each reaction and plot $1/V$ versus $1/[S]$ to obtain a Lineweaver-Burk plot, from which K_m and V_m for S can be calculated (Figure 4) using the hyper 32 software.
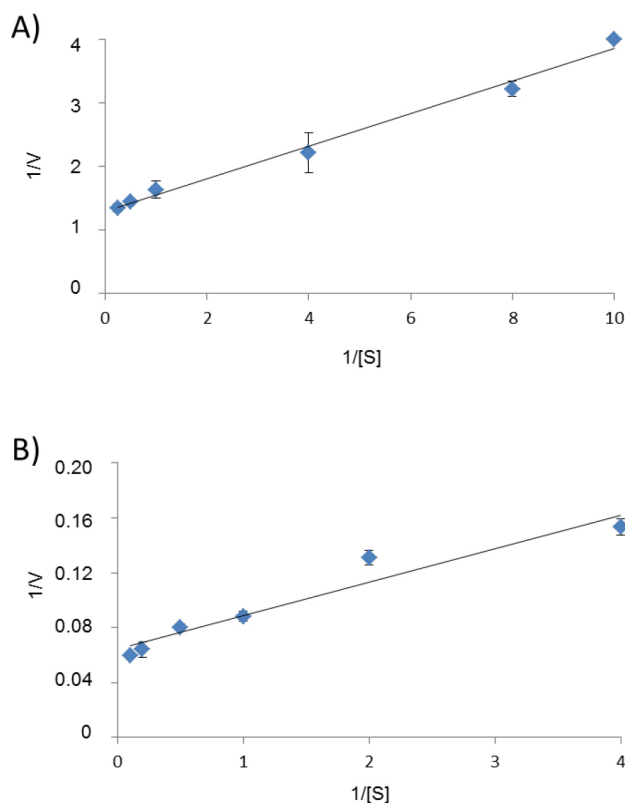

Figure 4. Lineweaver-Burk plots used to determine in A) the K_m (0.19 mM) and V_m (46 U/mg) of M1PDHcat for F6P and in B) the K_m (0.38 mM) and V_m (15.6 U/mg) of M1PDHcat for the M1P. [S] is the substrate concentration (in mM) and V is the reaction rate (in U/mg of protein). Three replicates were performed for each assay.

Notes

1. Before determining K_m and V_m for a given substrate S, be sure that cofactor is in excess, *i.e.*, that V does not increase with increasing quantities of cofactor in the reaction mixture; in the same vein, before determining K_m and V_m for the cofactor, be sure that S is in excess, *i.e.*, that V does not increase with increasing quantities of S in the reaction mixture. The theory is that this saturating concentration is equivalent to $100K_m$, but $10K_m$ is usually enough (Bisswanger, 2014). It is then necessary to adjust the amount of M1PDHcat and the incubation time to obtain a linear variation of absorbance at 340 nm, *i.e.*, a linear change of NADH quantity.

Recipes

1. 1 M Tris-HCl
   Dissolve 121.14 g of Trizma® base in about 800 ml of MilliQ water, adjust pH with HCl then complete to 1 liter. Filter through a 0.22 μm filter and store at room temperature.
2. 5 M NaCl
   Dissolve 95.21 mg of NaCl in 10 ml of MilliQ water. Filter through a 0.22 μm filter and store at room temperature.
3. 10 mM NADH
   Dissolve 6.63 mg of NADH in 1 ml of MilliQ water. Filter through a 0.22 μm filter and store at room temperature. Prepare fresh on the day of use.
4. 10 mM NAD^+
   Dissolve 6.63 mg of NAD^+ in 1 ml of MilliQ water. Filter through a 0.22 μm filter and store at room temperature. Prepare fresh on the week of use and store at -20 °C.

Acknowledgments

References

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