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PURIFICATION AND PROPERTIES OF NAD-GLUTAMATE DEHYDROGENASE FROM TURNIP MITOCHONDRIA

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Abstract—NAD(H)-glutamate dehydrogenase (GDH) was isolated from turnip (*Brassica rapa*) and its properties examined. The enzyme was found to be associated with mitochondria and located entirely within the mitochondrial matrix compartment. The enzyme displayed both NAD(H)- and NADP(H)-dependent activity with NAD(H) being the preferred substrate, i.e. NAD(H)/NADP(H)-dependent activities were the order of 11 1. The pH optima for the amination and deamination reactions were 8 0 and 9.5 respectively The apparent K_m values for glutamate, 2-oxoglutarate, ammonia (as ammonium sulphate at pH 8.0), NAD and NADH were 28 6, 2.0, 22.2, 0.25, and 0 09 mM respectively under optimum pH conditions. The K_m values for the substrates varied depending upon the assay pH, however, the K_m for NAD(H) was not changed significantly. NAD(H)-GDH activity was activated by the bivalent cations Ca^{2+}, Mn^{2+} and Zn^{2+} Ca^{2+} was the most effective cation for activation. The deamination reaction was fully activated by 7–8 μM Ca^{2+}, however, 60 μM Ca^{2+} was required to fully activate the enzyme when catalysing the amination reaction The enzyme was completely inactivated by the addition of EGTA, but activity was fully restored by the addition of Ca^{2+} The enzyme was purified 346-fold and the M_r of the enzyme sub-unit was estimated to be 43 000 by SDS–PAGE

INTRODUCTION

It is well accepted that the glutamine synthetase (GS)/glutamate synthase (GOGAT) system is the major pathway for ammonium fixation in higher plants and that glutamate dehydrogenase (GDH) does not play a major role in nitrogen assimilation unless ammonium is in excess [1]. This conclusion is based on the apparent poor affinity of GDH for ammonium (K_m 5–70 mM) [2], and would suggest the principle function of this enzyme may be the deamination of glutamate

It has been reported that NAD(H)-GDH activity is regulated by bivalent cations [3–7]. Furthermore, Yamaya *et al.* [8] have recently reported that the levels of Ca^{2+} in corn shoot mitochondria are sufficient to fully activate mitochondrial GDH leading to significant rates of ammonium fixation.

There are a number of reports on the properties of NAD(P)-GDH from various plant tissues including leaves [9], epicotyls [10], roots [11], seeds [12], cotyledons [13], and nodules [14]. However, little is known about the enzymes involved in nitrogen metabolism in storage tissue In this paper we report on the general characteristics of NAD(H)-GDH isolated and purified from turnip storage root (*Brassica rapa* L.).

RESULTS

Enzyme localization

The intracellular localization of NAD(H)-GDH in turnip was determined by differential centrifugation Fumarase, catalase and phosphoenolpyruvate (PEP)-carboxylase were used as marker enzymes for mitochondria, peroxisomes and cytoplasm, respectively. The results given in Table 1 show that NADH-GDH activity closely followed the distribution of the mitochondrial enzyme, fumarase. The NADH-GDH activity in the 15 000 *g* supernatant could not be pelleted at 105 000 *g* indicating that this activity was not associated with any other cellular membrane fraction (data not shown). The small amount of activity) in the supernatant approximated that of fumarase and so could have been released from broken mitochondria

A comparison of NADH-GDH activities measured using intact turnip mitochondria versus detergent treated mitochondria is given in Table 2. NADH can permeate the outer membrane of plant mitochondria but cannot cross the inner mitochondrial membrane. Thus, the very low level of GDH activity observed with intact mitochondria compared to that obtained with a detergent treated fraction demonstrates that the enzyme is located almost entirely within the mitochondrial matrix compartment.

Kinetic properties

The general kinetic properties of NAD(H)-GDH were determined using enzyme extracted from isolated mitochondria. The pH optima were 8.0 and 9.5 for the amination and deamination reactions respectively (Fig. 1) The apparent K_m values for glutamate, 2-oxoglutarate, ammonia (as ammonium sulphate at pH 8.0), NAD, and NADH under various pH conditions are shown in Table 3. It can be seen that whilst the K_m values for glutamate, 2-oxoglutarate or ammonium sulphate varied depending upon the assay pH conditions (Table 3), the K_m values for NAD(H) did not The mitochondrial GDH enzyme also displayed some activity with NADP(H), however, acti-

Table 1 Distribution of NADH–GDH, fumarase, catalase and PEP–carboxylase in various subcellular fractions isolated from turnip root tissue using differential centrifugation

	Activity (%)			
	NADH–GDH	Fumarase	Catalase	PEP–carboxylase
Homogenate	100	100	100	100
15000 *g* sup	19	13	75	93
15000 *g* pellet	63	67	9	0 3

Table 2 Comparison of NADH–GDH activities measured using intact mitochondria versus detergent treated mitochondria

	NADH–GDH activity (μmol NADH ox /min/mg protein)
−Triton X-100	0 02
+Triton X-100	1 71

The NADH-GDH activity was measured in 0 4 M sucrose using standard reaction medium with or without 0 04% (w/v) Triton X-100

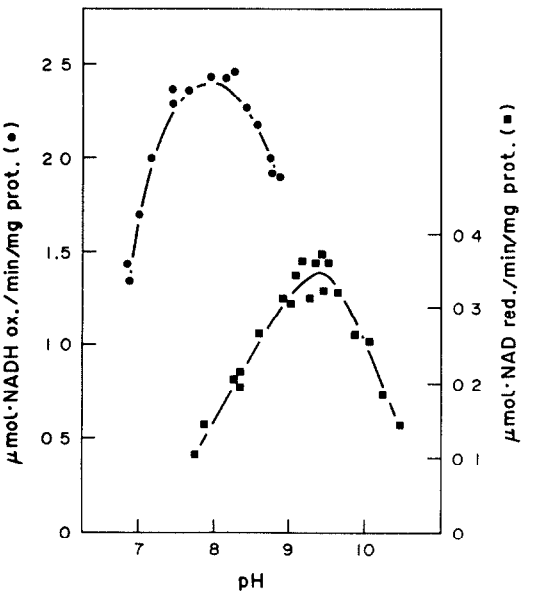

Fig. 1. pH optima of NAD(H)–GDH from isolated turnip mitochondria Assays were carried out as described in Experimental and adjusted to various pHs using KOH or HCl

vities were of the order of 11 times lower than those observed with NAD(H).

Effect of bivalent cations

NAD(H)–GDH was activated by the addition of $CaCl_2$, $MnCl_2$ and $ZnSO_4$ in both the amination and the deamination direction (Table 4), however, there was no effect of $MgCl_2$, KCl or NaCl The most effective cation was Ca^{2+} with lower concentrations required to fully activate the enzyme than that with Mn^{2+} or Zn^{2+} The relationship between Ca^{2+} concentration and NAD(H)–GDH activity is shown in Figs 2 and 3 The deamination activity of GDH was fully activated in the presence of 4 μM of added Ca^{2+} whereas 60 μM of added Ca^{2+} was required to give maximal enzyme activation in the amination direction It should be noted that the distilled water used in these experiments did contain a small amount of Ca^{2+} (*ca* 3 μM) and this may explain the significant amount of deamination activity observed in the absence of added Ca^{2+} solution (note complete inhibition of deamination activity by EGTA, Table 5). Therefore, maximum activity of the deamination reaction is observed with 7–8 μM Ca^{2+}

NAD(H)–GDH activity was strongly inhibited by the chelating agent, ethylene glycol bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), and this inactivation could be recovered completely by the addition of Ca^{2+} or Mn^{2+} (Table 5)

Properties of purified NAD(H)–GDH

Mitochondrial GDH was purified 346-fold using the steps outlined in Table 6 Initial treatment of the mitochondrial extract included incubation with the protease, trypsin GDH activity was found to be insensitive to trypsin treatment in contrast to other mitochondrial enzymes such as NADH-malate dehydrogenase (MDH) (Fig 4) It can be seen that trypsin treatment markedly reduced the protein concentration (Fig. 5 A), but there was no change in the GDH isozyme pattern (Fig. 5 B) It

Table 3. Influence of pH on the apparent K_m values of turnip NADH–GDH in the presence of 0 1 mM $CaCl_2$ for substrates of the amination and deamination reaction

	K_m values (mM)				
pH	Glutamate	2-Oxoglutarate	$(NH_4)_2SO_4$	NAD	NADH
7.2	—	1 7	66 7	—	0 08
8 0	12 2	2 0	22 2	0 17	0 09
9 5	28 6	10 0	19 6	0 25	0 07

Table 4. Activation of NADH–GDH by divalent cations

	Addition Final conc (mM)	Relative activities (%) Amination reaction	Deamination reaction
	None	100	100
Ca^{2+}	0 1	809	182
	1 0	752	167
Mn^{2+}	0 1	114	103
	1 0	786	114
Zn^{2+}	0 1	94	102
	1 0	197	135
Mg^{2+}	0 1	94	92
	1 0	90	92

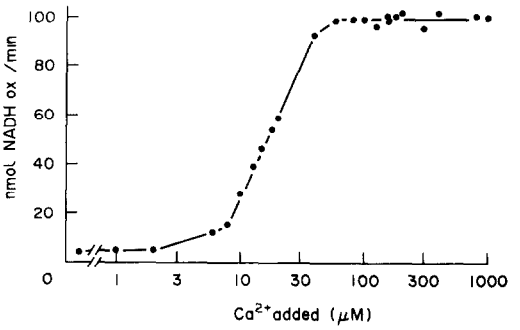

Fig 2. Activation of the amination reaction of NAD(H)–GDH from turnip mitochondria by Ca^{2+}. Assays were carried out as described in Experimental except for Ca^{2+} which was present at the concentrations shown above

should also be noted that at least 4 isozyme bands were observed (Fig. 5 B) as was the case for lupin nodules [14].

Non-denaturing PAGE of the purified enzyme revealed only one protein band which corresponded exactly with a GDH activity band as resolved by activity staining (Fig 6) Only one band was apparent using activity staining in contrast to the four isozyme bands of the

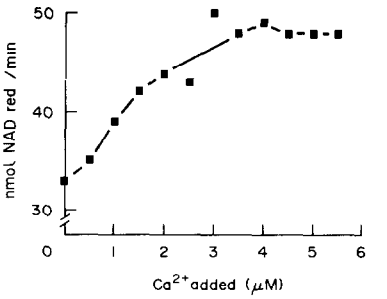

Fig 3. Activation of the deamination reaction of NAD(H)–GDH from turnip mitochondria by Ca^{2+} Assays were carried out as described in Fig. 2

mitochondrial extract (Fig. 5B). SDS-PAGE of the purified enzyme revealed the protein to consist of subunit with a M_r of 43 000 (Fig. 7). The apparent M_r of the native enzyme was estimated to be of the order of 300 000–310 000 using either Sephacryl S-300 or Superose 6 columns. Inclusion of 0 05% Triton X-100 detergent in the elution buffer (50 mM Tris–HCl, pH 8.0) for Sephacryl S-300 had no effect on the estimation of M_r. This does not allow us to clearly resolve the exact nature of the protein structure, i.e. hexameric, octomeric etc. and may indicate that the enzyme possess certain characteristics of shape or presence of non-protein components etc. which complicate determination of M_r by gel exclusion.

DISCUSSION

The results indicate that the NAD(H)–GDH enzyme of turnip storage root is located within the mitochondrion as with other plant tissues [1, 2]. Nauen and Hartmann [15] reported that NADH–GDH is located in the matrix. However, recently, Yamaya *et al.* [8] claimed that NAD(H)–GDH is loosely associated with the inner mitochondrial membrane. Our results show it to be located entirely within the mitochondrial matrix compartment, but do not permit any distinction to be made between the membrane and the matrix.

The enzyme displayed both NAD(H)- and NADP(H)-dependent activities with NAD(H) being the preferred substrate NAD(H)/NADP(H) dependent activity ratios were of the order of 11.1 This is similar to values for mitochondrial GDH isolated from lettuce leaf [9] and *Lemna minor* [3]

Table 5 Activity recovery experiment

Addition EGTA conc (mM)	Metal conc (mM)	Relative activity (%) Amination reaction Ca^{2+}	Mn^{2+}	Zn^{2+}	Deamination reaction Ca^{2+}	Mn^{2+}	Zn^{2+}
0	0	100	100	100	100	100	100
1 0	0	25	3	15	0	0	1.6
1 0	0 2	—	—	—	1 5	0	0
1 0	0 5	21	3	13	1 5	0	1 6
1 0	1 0	771	73	26	145	14	1 6
1 0	5 0	864	807	400	153	217	4.5

Table 6 Purification of NADH-GDH

Purification Step	Total* activity (Unit)	Sp activity (Unit/mg protein)	Recovery (%)	Purification (fold)
Mitochondria	579	1 3	100	1
Partially purified enzyme	464	10 0	80	7 7
Sephacryl S-300	367	131	63	123
Hydroxyappatite	267	381	46	293
DEAE-Sephacel	54	450	9 4	346

* One unit is defined as the enzyme necessary to oxidize 1 μmol NADH/min at 28°

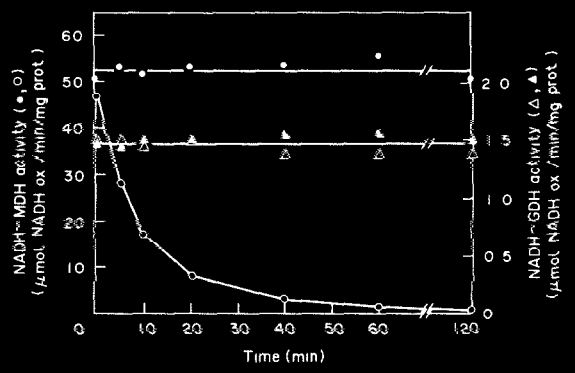

Fig 4 Comparative effect of Trypsin on NADH–malate dehydrogenase (MDH) and NADH–GDH Mitochondrial extracts were incubated in the presence (open symbols) or absence (closed symbols) of trypsin (0.2 mg trypsin/mg protein) in 50 mM Tris–HCl (pH 8 0) at 28°. Samples were withdrawn at various times and assayed for NADH–MDH and NADH–GDH as described in Experimental

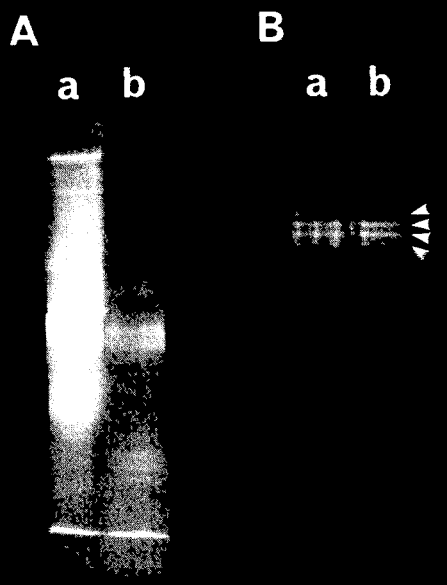

Fig. 5 Polypeptide and GDH isozyme patterns of turnip mitochondrial extract after trypsin treatment as resolved by non-denaturing PAGE. Mitochondria were extracted with 0.1% Triton X-100 and passed through a PD-10 column equilibrated with 50 mM Tris-HCl (pH 8 0) A. Lane a, mitochondrial extract (0 1 mg protein) at zero time with added trypsin The gel was stained with Coomassie Brilliant Blue R-250 and b, extract after incubation with trypsin for 120 min B Lane a, mitochondrial extract (0 4 mg protein) without trypsin treatment and b, after trypsin treatment for 120 min The gel was stained by activity staining as described in Experimental

The pH optima (Fig. 1), K_m values (Table 3) and the sub-unit M_r (Fig 7) of turnip root GDH fall within the ranges determined for this enzyme from other plant tissues [2, 12, 14, 16].

NAD(H)-GDH from turnip root was strongly activated by bivalent cations, particularly Ca^{2+} and inactivated by addition of the chelator, EGTA The amination reaction was activated by micromolar levels of Ca^{2+} as observed for this enzyme from other sources [2–8]. Furthermore, we have also been able to show for the first time that the deamination activity of this enzyme is also activated by Ca^{2+} (Fig 3)

Yamaya *et al* [8] have suggested that the Ca^{2+} concentration within the matrix of corn mitochondria is of the order of 52 to 56 mM. If this is also true of turnip mitochondria, GDH would be fully activated under all conditions and, thus, is unlikely to function in any regulatory capacity However if the turnip GDH is bound to the inner mitochondrial membrane [8], the effective concentration of Ca^{2+} within the immediate environment surrounding the enzyme may be much lower and may therefore play some role in the regulation of intramitochondrial GDH activity

Of key importance in considering the metabolic function of this enzyme in turnip root is the apparent poor affinity of the enzyme for the key substrates ammonia and glutamate In photosynthetic tissue, ammonia is released within the mitochondrion by glycine decarboxylation under photorespiratory conditions. It has been suggested that in the presence of these high intramitochondrial ammonia concentration mitochondrial GDH may have an ancillary role to the GS/GOGAT system in ammonia

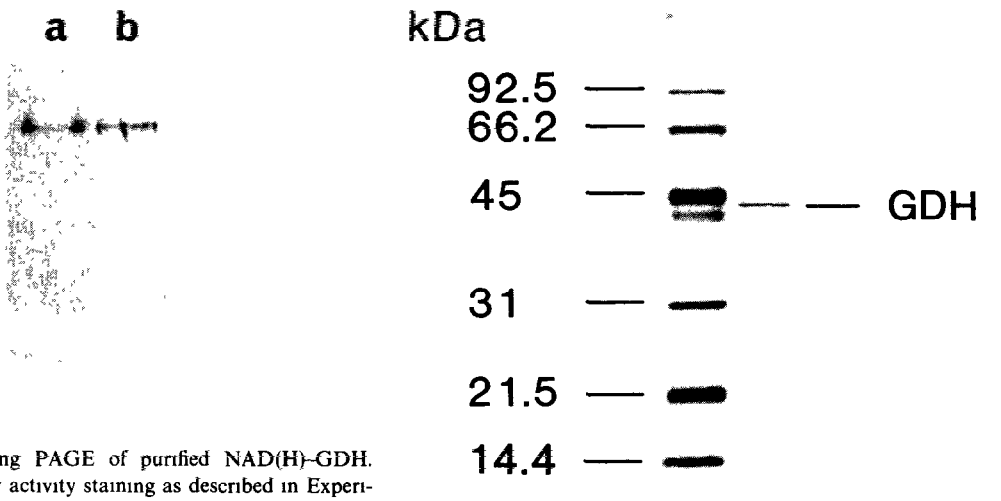

Fig. 6 Non-denaturing PAGE of purified NAD(H)-GDH. Lane a, gel stained by activity staining as described in Experimental and b, the gel was stained with silver as described in Experimental

refixation [8]. However, similar high levels of ammonia are unlikely to be present in the mitochondria of storage tissue to support any significant amination activity. Furthermore, while the affinity of the enzyme for glutamate is far superior to ammonia in the pH range found in the matrix of energised mitochondria, the maximal activity of this enzyme in the deamination direction is very much reduced at this pH Further investigations are required to determine the role of this enzyme in nitrogen metabolism of storage root tissue

EXPERIMENTAL

Preparation of mitochondria Fresh white turnip was purchased locally and mitochondria isolated according to Day and Wiskich [17].

Differential centrifugation For cell-fractionation studies the turnip homogenate prepared for mitochondrial isolation was centrifuged at 15 000 *g* for 15 min The pellet was resuspended in a small vol of 0 4 M sucrose The suspension and the original supernatant were incubated with 0 04% (w/v) Triton X-100 for 5 min and passed through a Pharmacia PD-10 column equibrated with 50 mM Tris(hydroxymethyl) aminomethane (Tris)–HCl (pH 8 0)

Assay procedures. NAD(H)-GDH was assayed essentially according to [11] The standard amination reaction medium contained 0 133 M Tris–HCl (pH 8 0), 13 mM 2-oxoglutarate, 0 166 mM NADH, 0.213 M $(NH_4)_2SO_4$, 0 4 mM $CaCl_2$ and 0 04% (w/v) Triton X-100. The standard deamination reaction medium contained 0.1 M Tris–HCl (pH 9 5), 100 mM glutamate, 0 4 mM NAD, 0 1 mM $CaCl_2$ and 0 04% (w/v) Triton X-100 All assays were performed at 28° The absorption change at 340 nm was measured using a Philips UV/VIS 88 000 spectrophotometer.

Fumarase activity was measured in a coupled assay system with NADP-malic enzyme [18] The reaction mixture contained 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)–KOH (pH 7 5), 0 04% (w/v) Triton X-100, 5 mM KH_2PO_4, 4 mM $MgCl_2$, 0 4 mM NADP and 0.2 unit of NADP-malic enzyme in a total of 1 ml The reaction was initiated with 10 mM fumarate and NADPH production was followed at 340 nm

Catalase was assayed using the method of Lück [19]. The reaction medium consisted of 0.1 M Pi buffer (pH 7 0), 0.05% (w/v) H_2O_2 and 0 04% (w/v) Triton X-100. The reaction was initiated by the addition of sample and the decrease in absorbance at 240 nm was measured

PEP-carboxylase was measured in a coupled assay with NADH–MDH The reaction mixture contained 30 mM HEPES–KOH (pH 8 0), 6 mM $MgCl_2$, 1 mM glucose-6-phosphate, 4 mM dithiothreitol, 8 mM $NaHCO_3$, 0 2 mM NADH and 1 6 unit of NADH–MDH. The reaction was initiated with 1 6 mM PEP NAD production was followed at 340 nm

NADH–MDH was measured at 340 nm by following NADH oxidation in 200 mM *N*-tris(hydroxymethyl)-methyl-2-aminino-ethanesulphonic acid (TES)–KOH (pH 7 5) and 0 2 mM NADH The reaction was initiated with 6 mM oxaloacetate

Purification of GDH Mitochondria were treated with 0.5% (w/v) Triton X-100 and centrifuged at 30 000 *g* for 15 min Trypsin (from bovine pancreas; Boehringer Mannheim Pty. Ltd) was added to the supernatant (protein:trypsin; 5) and incubated at 28° for 1 hr followed by a heat treatment of 60° for 5 min [16]. Solid $(NH_4)_2SO_4$ was then added and the fraction between 30 and 75% saturation was collected as a partially purified enzyme preparation. This preparation was diluted in a minimum amount of 50 mM Tris–HCl (pH 8 0) and undissolved materials were removed by centrifugation at 96 000 *g* for 20 min.

The supernatant fraction was loaded onto a Sephacryl S-300 column (2 6 × 70 cm) and eluted at a flow rate of 0 34 ml/min using an elution medium containing 50 mM Tris–HCl (pH 8.0). The NADH–GDH fractions were then loaded onto a hydroxyapatite column (2 6 × 7 cm, 0 34 ml/min) and eluted with a 50–300 mM Pi buffer gradient (pH 7.2) at a flow ratio of 0 34 ml/min The NADH–GDH fraction was charged onto a DEAE-Sephacel column (2 6 × 8 cm) and eluted with a NaCl gradient (50–300 mM) at flow rate of 0 34 ml/min. Proteins of standard M_r were purchased from Pharmacia Fine Chemicals.

Protein measurement The protein concentration was determined using the Bio-Rad reagent (Coomassie dye-binding method) with bovine serumn albumin as standard.

Fig 7 SDS–PAGE electrophoresis of purified NAD(H)–GDH The molecular mass standards were phosphorylase B (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400) The gel was stained with silver as described in Experimental

PAGE Non-denaturing PAGE was performed essentially according to Laemmli [20] but in the absence of SDS using a 3% acrylamide stacking gel, a 6% acrylamide separation gel and buffer containing 25 mM Tris and 192 mM glycine at 4°. At the completion of electrophoresis, NAD-GDH activity was detected in the gel with a modified tetrazolium assay [21] using 100 mM Tris–HCl (pH 9.5), 65 mM glutamate, 0.1 mM phenazine methosulphate, 0.1 mM nitro-blue tetrazolium, 0.4 mM NAD and 0.4 mM $CaCl_2$ at 28°. To detect peptides, the gel was stained by 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7% HOAc for 15 min. The gel was then destained in 5%MeOH and 7% HOAc overnight.

SDS-PAGE was performed as above, but with 0.1% SDS in all gels and buffers and with a 12% acrylamide separation gel. Samples were incubated in 3% SDS and 5% mercaptoethanol for 30 min at room temp. Following electrophoretic migration, the gel was stained with silver [22]. The molecular size of GDH sub-unit polypeptide was determined using a Bio-Rad low-M_r protein standards kit for SDS-PAGE.

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