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Immunocharacterization of NADH-Glutamate Dehydrogenase from *Vitis vinifera* L.

C. A. Loulakakis and K. A. Roubelakis-Angelakis*

Department of Biology, University of Crete (C.A.L., K.A.R.-A.), and Institute of Molecular Biology and Biotechnology (C.A.L.), P. O. Box 1470, 711 10 Heraklion, Greece

ABSTRACT

Rabbit antiserum was raised against NADH-glutamate dehydrogenase (GDH) isoenzyme 1, purified from leaves of *Vitis vinifera* L. cv Soultanina and its specificity was tested. This antiserum was used for immunocharacterization of the GDH from leaf, shoot, and root tissues. The antiserum recognized the seven isoenzymes of NADH-GDH and precipitated all the enzyme activity from the three tissues tested. Western blot following SDS-PAGE revealed the same protein band for the three tissues, with a molecular mass of 42.5 kilodaltons corresponding to NADH-GDH subunit. Results, based on the immunological studies, revealed that NADH-GDH from leaf, shoot, and root tissues are closely related proteins. Furthermore, addition of ammonium ions to the culture medium of *in vitro* grown explants resulted in a significant increase in NADH-GDH activity in root, shoot, and leaf tissues.

The physiological role of large amounts of GDH[1] (EC 1.4.1.2.) present in the tissues of higher plants is still obscure. Possible conditions under which GDH may play a significant role in cell metabolism have not been fully resolved.

One function of mitochondrial GDH is to synthesize glutamate from some of the ammonia ions released within the mitochondrion by glycine decarboxylation during photorespiration (17, 18).

The enzyme present in different tissues may be different in its isoenzymic pattern, in its regulatory properties, and control mechanism. In *Medicago*, GDH organ-specific isoenzymes were reported (8) and in *Zea* root, callus, and leaves, different isoenzymes/conformers of GDH were found (6). By using immunochemical approach, antigenic homologies between GDH forms from pea seeds were detected (10). In addition, exogenous nitrogen source has been reported to affect the *in vivo* and *in vitro* GDH activity (6, 7, 15 and references therein); however, there is not yet enough information on the molecular mechanisms of GDH regulation in higher plants.

Recently, NADH-GDH from grapevine (*Vitis vinifera* L. cv Soultanina) was characterized and its major isoenzyme was purified 2050-fold to homogeneity. The molecular mass of the native enzyme was estimated to be 252 kD and it consisted of six identical 42.5 kD subunits. The amination reaction was fully activated by about 100 μM Ca^{2+} while the deamination reaction was not affected by the addition of Ca^{2+} (4).

[1] Abbreviation: GDH, glutamate dehydrogenase.

In this report we present further results on the preparation and characterization of GDH-specific antibodies raised against the NADH-GDH isoenzyme 1 from grapevine leaf tissue and on the immunological characteristics of leaf, shoot, and root GDH from the same plant species. In addition, we present information on the effect of nitrogen source on NADH-GDH in *in vitro* grown grapevine plantlets.

MATERIALS AND METHODS

Plant Material

Vitis vinifera L. cv Soultanina *in vitro* grown plants were used. One node explants from green shoots of *in vitro* grown vines were aseptically positioned for rooting into Roubelakis medium (13), and were kept in a tissue culture room at 25 ± 1°C, 16/8 photoperiod and total energy of 55 μE $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps. Leaf, shoot, and root tissues were collected from the newly developed plants and used.

For studying the effect of nitrogen source on NADH-GDH, explants were grown for one generation into culture medium (13) modified to contain 16 mM KNO_3, or 6 mM NH_4Cl, or both. At the end of a 42-d growth period, roots, shoots, and leaves were collected and used.

Standard Enzyme Extraction

Leaf, shoot, and root tissues were pulverized in liquid nitrogen with the aid of a mortar and pestle. The powder was suspended in 5 volumes (v/w) of ice-cold grinding medium consisting of 200 mM Tris-HCl (pH 8.0), 10 mM L-cysteine-HCl, 14 mM β-mercaptoethanol, 0.5 mM PMSF, and 2% (w/v) PVP-40 and was homogenized in an Omnimixer homogenizer 4 times, 12 s each. After filtration through four layers of cheesecloth, the filtrate was centrifuged at 10,000 *g* for 30 min.

The supernatant thus obtained was precipitated with solid ammonium sulfate. The fraction of 35 to 70% saturation was resuspended in a small volume of 10 mM Tris-HCl (pH 7.4) containing 14 mM β-mercaptoethanol and 0.5 mM PMSF. This preparation was dialyzed two times against the same buffer and used for enzyme assays and immunological studies. All steps were carried out at 4°C.

Purification of NADH-GDH Isoenzyme 1

NADH-GDH isoenzyme 1 was purified to homogeneity from leaves of grapevine by the method described previously (4).

Immunization

Antibodies against purified grapevine NADH-GDH isoenzyme 1 were obtained by injecting subcutaneously a New Zealand white rabbit with a dose of the homogeneous GDH (800 μg) mixed (1:1) with Freund's complete adjuvant. The booster injection was done 3 weeks later at half enzyme concentration emulsified with the incomplete Freund's adjuvant at a ratio of 1:1. One week later serum was collected and titer was estimated by ELISA. Nonimmune serum was taken from the rabbit before immunization.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to Laemmli (2). Samples were treated at 100°C for 5 min with 3% SDS and 5% β-mercaptoethanol before electrophoresis.

Immunological Methods

Double immunodiffusion tests were performed by the method of Ouchterlony (9). The plates were prepared with 1% (w/v) agarose, in 0.05 M Tris-HCl (pH 8.2) and 0.02% (w/v) sodium azide. Then 10 μL each of samples were added to wells and the plates were incubated at 25°C for 15 h. Proteins were washed away and immunoprecipitin bands were stained with Coomassie brilliant blue R.

Immunoprecipitation was carried out by incubating the enzyme solution, adjusted to 0.9% NaCl, with various volumes of antiserum for 1 h at 25°C and 20 h at 4°C. The antigen-antibody complex was collected by centrifugation at 10,000*g* for 10 min and the enzyme activity was determined in the supernatants. The immunoprecipitate was washed two times with 20 mM Tris-HCl (pH 7.4), proteins were dissociated by incubating at 100°C for 5 min with SDS and β-mercaptoethanol, and subjected to SDS-PAGE.

Transfer of electrophoretically resolved proteins on SDS-polyacrylamide gels onto nitrocellulose filters (0.2 μm pore size, Schleicher and Schuell) was carried out essentially as described by Towbin *et al.* (16). The transfer buffer consisted of 25 mM Tris-192 mM glycine, 20% (w/v) methanol, and 0.02% (w/v) SDS using the LKB 2005 Transphor apparatus at 40 V overnight. Nitrocellulose blots were blocked with 2% BSA in PBS, washed, incubated with first and second antibodies, and developed by standard methods. Antibody dilutions were done in PBS containing 1% (w/v) BSA and 0.5% (w/v) Tween-20. Visualization of antigen-antibody complexes was performed by horseradish peroxidase conjugated second antibody and the 4-chloro-1-napthol color development reaction (1).

Enzyme Assay and Protein Determination

GDH activity was determined in the aminating direction by following the absorption change at 340 nm (12). One unit of GDH activity was defined as the oxidation of 1 μmol of NADH per min at 30°C. Protein concentration was determined following TCA precipitation by the method of Lowry (5).

RESULTS AND DISCUSSION

Specificity of Rabbit Anti-NADH-GDH Isoenzyme 1 Serum

The titer of the antiserum which was prepared against purified NADH-GDH-isoenzyme 1 from grapevine leaves was determined by ELISA and was found to be 1:32,000, suggesting the high concentration of antiserum in specific antibodies. Figure 1A shows an Ouchterlony double immunodiffusion test by using standard GDH preparation from leaves, and Figure 1B presents test with purified GDH isoenzyme 1. It is obvious that only one band was visualized even with standard enzyme extract. Also, high dilutions of the antiserum showed high reactivity. No contaminating bands were detected and nonimmune serum gave no immunoprecipitin band with the enzyme (Fig. 1B). Only 0.5 μL of antigrapevine leaf GDH serum could precipitate 0.35 units of enzyme, whereas Ratajczak *et al.* (11) using antilupine root GDH serum with a titer of 1:64, found that 200 μL were necessary to completely precipitate equivalent units of enzyme.

This prepared antiserum was used for further immunological studies on GDH from different grapevine tissues.

Immunological Relationship of NADH-GDH from Grapevine Leaf, Shoot, and Root Tissues

Quantitative immunoprecipitation was performed for studying the behavior of the enzyme from the tested tissues.

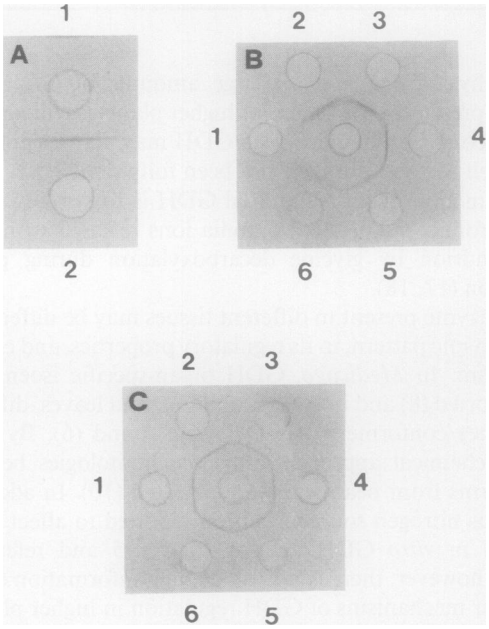

Figure 1. Double immunodiffusion tests. Ten μL of each sample were added to wells and the plates were incubated at 25°C for 16 h. Proteins were washed away and immunoprecipitin bands were stained with Coomassie brilliant blue-R. A, Well 1, standard leaf extract; well 2, anti-NADH-GDH isoenzyme 1 serum. B, Center well, purified leaf NADH-GDH isoenzyme 1 preparation; well 1, nonimmune serum; wells 2 to 6, serial dilutions of anti-GDH serum. C, Center well, anti-GDH isoenzyme 1 serum, wells 1 and 2, root extract; wells 3 and 4, leaf extract; wells 5 and 6, shoot extract.

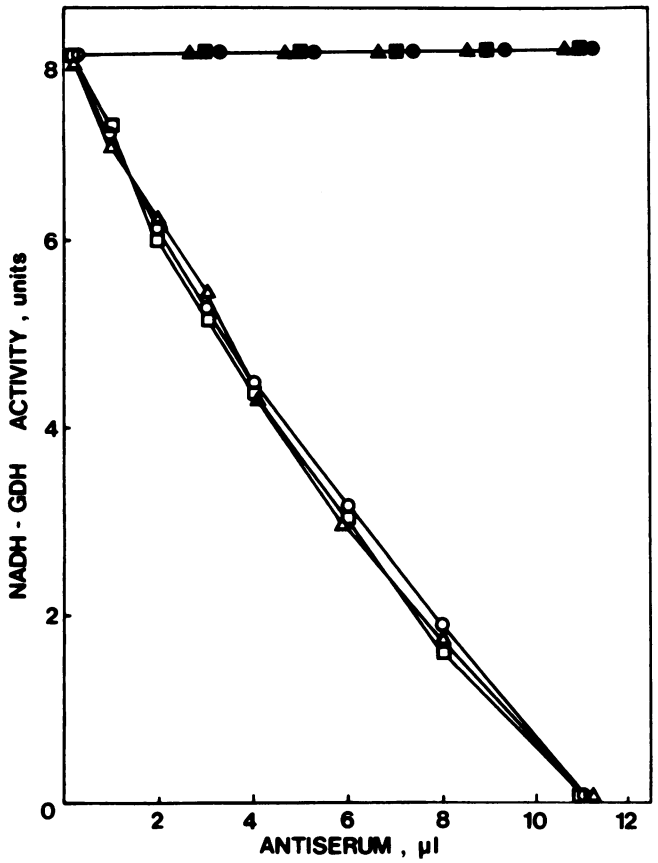

Figure 2. Immunotitration curves of GDH from different organs of grapevine. Eight units of enzyme were incubated with increasing volumes of antiserum raised against the NADH-GDH isoenzyme 1 from grapevine leaf tissue. The enzyme activity was assayed as described in "Materials and Methods." Activity of glutamate dehydrogenase from leaf (△), shoot (○), and root (□) tissues. Results using preimmune rabbit serum correspond to the enzyme from leaf (▲), shoot (●), and root (■) tissues.

Constant amounts of standard enzyme extract from leaf, shoot, and root tissues were incubated with increasing volumes of antiserum and enzyme activity was measured in supernatants following the removal of the immune complexes. A linear correlation between the decrease of enzyme activity and the increase in GDH-antiserum concentration was found. The immunotitration curves which were obtained were very similar (Fig. 2). At very low antiserum concentration a significant decrease in enzyme activity from all the tissues was noticed and finally with 11 µL antiserum the enzyme activity was completely precipitated. Nonimmune serum had no effect on GDH activity; all the activity was found in the supernatants (Fig. 2).

Ratajczak *et al.* (11) used antiserum raised against lupine root GDH; at low concentrations of antiserum a substantial reduction in the activity was observed and increase in the concentration of antiserum resulted to less dramatic effect.

After immunoprecipitation antigen-antibody complexes were dissociated and subjected to SDS-PAGE. Three major protein bands were obtained with molecular masses of 55, 42.5, and 25 kD, corresponding, respectively, to the heavy chain of the antibodies, the NADH-GDH subunit (4) and the light chain of the antibodies.

The purified leaf NADH-GDH isoenzyme 1 and proteins from standard leaf extract were resolved by SDS-PAGE and electroblotted onto nitrocellulose membrane, probing with anti-NADH-GDH isoenzyme 1 serum. As Figure 3 shows only a single protein band was visualized. By calibration with the standard molecular mass proteins, the molecular mass of this polypeptide chain was estimated to be 42.5 kD, which is in agreement with the subunit molecular mass of the purified NADH-GDH from grapevine leaves (4). Nonimmune serum did not give any protein band (Fig. 3A), whereas increased concentrations of extract resulted in broader protein bands suggesting a linear relationship between NADH-GDH and antibody (Fig. 3C).

In grapevine tissues 7 anodal migrating NADH-GDH isoenzymes were revealed by activity staining after native polyacrylamide gel electrophoresis which result from random association of two subunits into hexamer complexes (4). These isoenzymes from leaves were separated by DEAE-cellulose column chromatography and subjected to SDS-PAGE followed by Western blot (Fig. 4A). Only one band of the same molecular mass appeared in the samples of the different isoenzymes indicating that the prepared anti-NADH-GDH

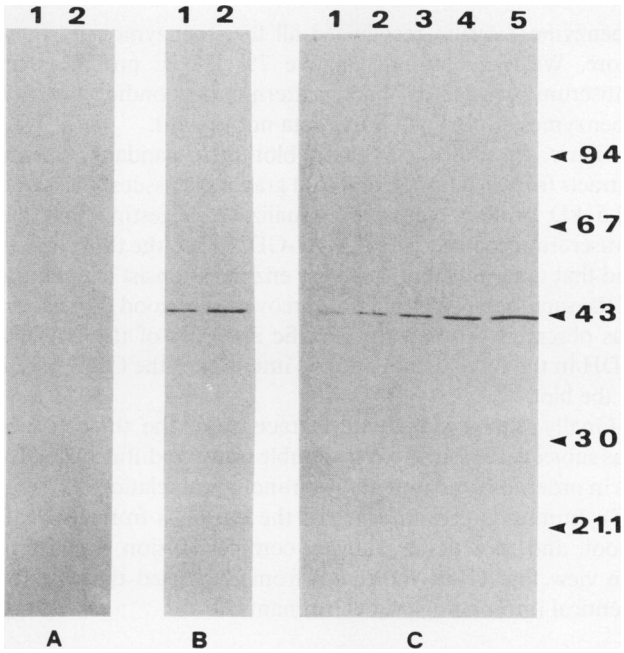

Figure 3. Western blot analysis of grapevine NADH-GDH. Purified NADH-GDH isoenzyme 1 or standard GDH leaf extract were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed for 1 h with 1:10,000 dilution of preimmune serum (panel A) or anti-GDH isoenzyme 1 serum (panels B and C). A, Lane 1, 60 ng purified GDH isoenzyme 1; lane 2, 20 µg standard GDH leaf extract. B, Lanes 1 and 2, 30 and 60 ng of purified NADH-GDH isoenzyme 1. C, Lanes 1 to 5, 5, 10, 20, 30, and 40 µg protein, respectively, from standard leaf GDH extract.

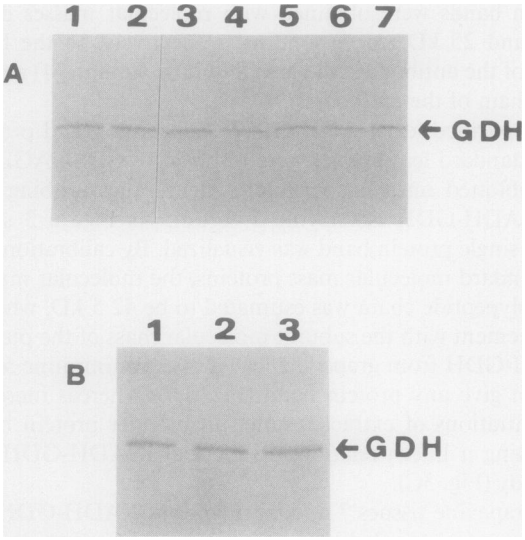

Figure 4. Western blot analysis of grapevine NADH-GDH from different tissues. Proteins were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed for 1 h with 1:10,000 dilution of anti-GDH isoenzyme 1 serum. A, Separated GDH isoenzymes from leaf tissue; lanes 1 to 7, 16 m units from the isoenzymes 1 to 7, respectively. B, Standard enzyme extract (20 μg protein) from leaf (lane 1), shoot (lane 2), and root (lane 3) grapevine tissues.

isoenzyme 1 serum recognized all the isoenzymes. Furthermore, Western blot after native 7% PAGE probing with antiserum revealed a 7-band pattern corresponding to the 7 isoenzymes of NADH-GDH (data not shown).

Figure 4B shows a Western blot with standard enzyme extracts from leaf, shoot, and root grapevine tissues. The same 42.5 kD protein band was visualized, suggesting that the antiserum recognized the NADH-GDH from the three tissues and that the leaf, shoot, and root enzymes consist of subunits of the same molecular mass. Moreover, very good correlation was observed between the specific activities of the NADH-GDH in the three tissues and the intensity of the GDH bands in the blot.

Finally, standard enzyme extracts from the three tissues was subjected to Ouchterlony double immunodiffusion analysis in order to compare their immunological relationship (Fig. 1C). Immunoprecipitin lines of the enzymes from the leaf, shoot, and root tissues showed complete fusion supporting the view that GDH molecules from the tested tissues have identical immunological determinants.

Effects of Nitrogen Source on NADH-GDH from *in Vitro* Grown Plants

The activity of GDH in plants is affected by several endogenous and exogenous factors including the type of nitrogen source (for review see Srivastava and Singh [15]). One-node grapevine explants grown in culture medium containing NH_4^+ as the sole nitrogen source failed to exhibit morphogenetic responses and to develop plants. In Table I NADH-GDH activities are presented for leaf, shoot, and root enzyme from *in vitro* grown plants receiving only nitrates (16 mM KNO_3) or nitrates plus ammonia (16 mM KNO_3 + 6 mM NH_4Cl). In the presence of 16 mM KNO_3, NADH-GDH activity was higher in the root and shoot than in the leaf tissue. The addition of 6 mM NH_4Cl caused an increase in NADH-GDH activity of 66.6, 39.1, and 43.2% for root, shoot, and leaf enzyme, respectively. Always, root NADH-GDH exhibited significantly higher activities compared to leaf and shoot enzyme. Immunoblotting revealed similar results.

Ammonium may increase GDH activity by increasing the amount of enzyme protein and/or by modifying the activity of existing enzyme molecules. Our results using immunochemical approaches indicate an increase in GDH protein and activity in ammonium grown plants. Suggestions for *de novo* synthesis of GDH enzyme protein have been derived from time course studies, use of protein inhibitors, density and radioactive labeling techniques (15 and references therein), and in one case from immunocharacterization of newly synthesized protein (3). Also, ammonium has been reported to cause an activation of GDH (14). Our results tend to support the *de novo* GDH protein synthesis rather than the activation of preexisting enzyme protein. However, the possibility that the increase in GDH-protein is due to the decrease in the degradation of the enzyme cannot be excluded. To answer this question work is in progress for determining the rate of synthesis and degradation of the enzyme and the level of its translatable mRNA under varying *in vitro* culture conditions.

CONCLUSION

Information in the literature on the immunoaffinity of GDH from various plant organs is lacking. This would be of interest because differences in the isoenzymic pattern and kinetic characteristics of leaf, shoot, and root GDH have been reported (15). The antiserum raised against one grapevine leaf NADH-GDH isoenzyme recognized all the isoenzymes as it was evidenced by Western blot and immunoprecipitation tests. Also NADH-GDH enzyme molecule from leaf, shoot, and root tissues of *in vitro* grown grapevine plants had both identical immunological determinants and a similar mol wt of the polypeptide chain. On the immunological basis it is likely that NADH-dependent GDH activities in these tissues

Table I. *Effect of Nitrate and Ammonium Ions on NADH-GDH Activity in Vitis vinifera L. Tissues*

Plants were developed *in vitro* from one-node explants on Roubelakis medium (13) modified to contain only nitrates or ammonium or both. Different subscript letters indicate statistical difference at 5% level.

Treatment	NADH-Glutamate Dehydrogenase		
	Leaf	Shoot	Root
	munits·mg protein^{-1}		
16 mM KNO_3	370_a	460_c	450_c
16 mM KNO_3 + 6 mM NH_4Cl	530_b	640_d	750_e

are carried out by closely related protein molecules, with similar structural conformation.

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