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Ammonia assimilation in *Vitis vinifera* L.:

I. Isolation and properties of leaf and root glutamate dehydrogenase

by

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Die Ammonium-Assimilation bei *Vitis vinifera* L.:

I. Isolierung und Eigenschaften der Glutamatdehydrogenase aus Blättern und Wurzeln

Zusammenfassung. — Bei der Rebsorte Chenin blanc (*Vitis vinifera* L.) wurde die Glutamatdehydrogenase- (GDH-)Aktivität der strukturierten und löslichen Fraktionen von Blatt- und Wurzelgeweben bestimmt. Die NADH-GDH-Aktivität von Blattextrakten verteilte sich zu ca. 66 % auf das nach Zentrifugation bei 10 000 *g* erhaltene Sediment und zu ca. 34 % auf das 23 500-*g*-Sediment, während die NADPH-GDH-Aktivität hauptsächlich an die lösliche Fraktion (23 500 *g*) gebunden war. Bei Wurzelextrakten waren ca. 53 % der NADH-GDH-Aktivität in der löslichen Fraktion und 43 % im 10 000-*g*-Sediment enthalten, während jeweils 47, 37 und 16 % der NADPH-GDH-Aktivität auf die lösliche Fraktion, das 10 000-*g*-Sediment bzw. das 23 500-*g*-Sediment entfielen. Die GDH aus dem 10 000-*g*-Sediment des Blattmaterials und aus der löslichen Fraktion der Wurzelextrakte unterschieden sich in ihrer Substrataffinität. Die K_m-Werte der Blatt- und Wurzel-GDH betrugen 3,9 ± 1,1 bzw. 0,7 ± 0,4 mM für α-Ketoglutarat, 35,7 ± 7,1 bzw. 61,3 ± 12,4 mM für NH_4Cl sowie 100,0 ± 7,4 bzw. 36,2 ± 4,4 µM für NADH. Die GDH aus Blatt- und Wurzelgeweben folgte mit allen Substraten der Michaelis-Menten-Kinetik, ausgenommen mit NH_4Cl; hier lag bei Wurzelextrakten eine sigmoide Beziehung vor. *In vitro* waren die optimalen Reaktionsbedingungen ein pH von 7,90—8,10, eine Inkubationstemperatur von 38—40 °C und eine Enzymmenge, die dem Gehalt von 80—110 mg Frischgewebe entsprach. Durch EDTA und L-Glutamat wurde das Enzym sowohl der Blätter als auch der Wurzeln inhibiert. Wurzel-GDH wurde durch Ca^{2+} stärker aktiviert als Blatt-GDH.

Introduction

Glutamate and glutamine are the primary products of inorganic nitrogen assimilation. Until recently, NH_4^+ assimilation in plants was considered to be mediated mainly by glutamate dehydrogenase (GDH; L-glutamate : NADP$^+$ oxidoreductase, deaminating, EC 1.3.1.2.). Since the characterization in plants of glutamate synthase (L-glutamate : ferredoxin oxidoreductase, transaminating, EC 1.4.7.1., or L-glutamate : NADP$^+$ oxidoreductase, transaminating, EC 1.4.1.13.), it has been generally accepted that the glutamine synthetase/glutamate synthase pathway has the main responsibility for NH_4^+ assimilation (MIFLIN and LEA 1980); however, this does not preclude GDH as an alternative pathway for NH_4^+ assimilation (MIFLIN and LEA 1980); however, this does not preclude GDH as an alternative pathway for NH_4^+ entry into the metabolism of plant cells.

Although there is considerable literature on the isolation and properties of the above-mentioned enzymes in annual plants (see review by STEWART *et al.* 1980), few studies have been done on perennial plant species. To a large extent this is probably

due to difficulties in extracting active enzymes from tissues rich in phenolic substances, which characterize most perennial plants. This communication provides information on the isolation, subcellular distribution, and properties of GDH from leaves and roots of grapevine, *Vitis vinifera* L. cv. Chenin blanc.

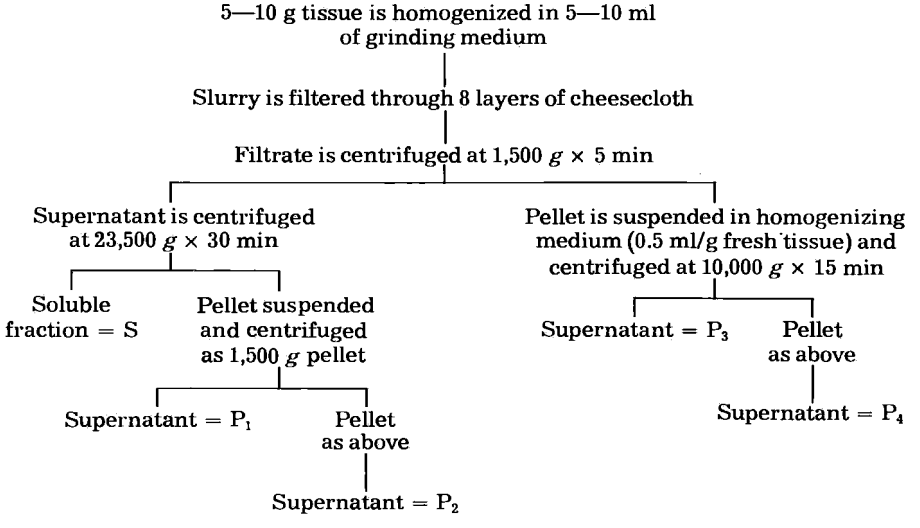
Materials and methods

GDH was extracted from leaf and root tissue of *Vitis vinifera* L. cv. Chenin blanc. Vines grown in the experimental vineyard of the University of California, Davis, were the sources of leaf samples; they were fertilized with NH_4NO_3 at a rate of 112 kg N/ha during the dormant season. Leaf samples were collected from the 4th basal node of annual shoots during the active growing season, placed in plastic bags, and kept on crushed ice until transferred to the laboratory. The central-apical portion of the lamina was then removed from each leaf, rinsed with deionized water, blotted dry between paper towels, weighed, and either used immediately or kept in plastic bags at −20 °C until used.

1- and 2-year-old roots, rootlets, and leaves were also obtained from potted Chenin blanc vines grown under field climatic conditions and irrigated with Hoagland No. 1 solution at 2-week intervals. Enzymes were extracted from roots in the same manner as from leaves.

Enzyme extraction: The diagram summarizes the procedure for extraction and partial purification of GDH. The grinding medium consisted of 100 mM Tris-HCl pH 8.0, 10 mM L-cysteine-HCl, and 4 % (w/v) Polyethylene glycol 4000 (ROUBELAKIS and KLIEWER 1978). The homogenizing medium consisted of 100 mM Tris-HCl pH 8.0, 10 mM L-cysteine-HCl, and 1 % (v/v) Triton X-100. All centrifugations were done in a Sorvall refrigerated ultracentrifuge, and all extraction steps were performed at 0—4 °C.

Flow diagram for isolation of glutamate dehydrogenase from leaf and root tissues of *Vitis vinifera* L. cv. Chenin blanc



Enzyme assay conditions: Leaf and root GDH activity was determined in the amination direction. The reaction mixture for leaf enzyme consisted of 100 mM Tris-HCl pH 8.0, 6.7 mM α-ketoglutarate, 200 μM NADH, 100 mM NH_4Cl, 5 mM $CaCl_2$, enzyme extract equivalent to about 100 mg of fresh tissue and deionized water to a total volume of 3 ml. For root enzyme, 5 mM α-ketoglutarate and 120 μM NADH or NADPH were used. All reagents were made up in 100 mM Tris-HCl pH 8.0 and neutralized if necessary. Complete reaction mixtures lacking NADH were allowed to equilibrate in a water bath at 38 °C for 5 min; then NADH or NADPH was added and the reaction was allowed to proceed. Enzyme activity was determined by observing the change in absorbance at 340 nm resulting from the oxidation of NADH. If necessary, corrections for nonspecific NADH oxidase were done by using controls lacking NH_4Cl and α-ketoglutarate.

Enzyme activity was calculated from the initial velocity and expressed as units per g of fresh tissue. 1 unit is defined as the amount of enzyme required to oxidize NADH equivalent to 0.1 OD unit per h. Under the described experimental conditions this was equivalent to about 45 μM of NADH.

Table 1

In vitro glutamate dehydrogenase activity in grapevine leaf and root extracts

Die Glutamatdehydrogenase-Aktivität von Blatt- und Wurzelextrakten der Rebe *in vitro*

Plant tissue	Glutamate dehydrogenase activity[1] units · (g fresh tissue)$^{-1}$		
Leaf	NADH-dependent	35.4 ± 8.6	(10)[2]
	NADPH-dependent	23.2 ± 5.7	(10)
Root	NADH-dependent	56.9 ± 12.1	(4)
	NADPH-dependent	17.1 ± 4.9	(4)

[1]) Assay conditions were as described in the text.
[2]) Numbers in the brackets denote the performed extractions.

Results and discussion

Extracts from grapevine leaf and root tissues exhibited GDH activity as determined by the oxidation of NAD(P)H. Activity was absolutely dependent on the presence of both α-ketoglutarate and NH_4^+. GDH has been widely found in nearly all parts of many annual plant species (see review by STEWART *et al.* 1980); and in apple leaf and bark tissues (COOPER and HILL-COTTINGHAM 1974). GDH from grapevine roots showed higher activities (60 %) than enzyme from leaves did (Table 1), which agrees with findings on shoot and root GDH from rice seedlings (KANAMORI *et al.* 1972) and castor bean leaf and root enzyme (LEES and DENNIS 1981).

Oxidation of NADH was linear as amount of enzyme increased up to the equivalent 110 mg of fresh leaf and 80 mg of fresh root tissue. Linearity of oxidized NADH with time was dependent on the amount of added enzyme; linearity was maintained for about 25—30 min under the described experimental conditions (Fig. 1).

Table 2 shows the distribution of GDH activity among the subcellular fractions obtained by differential centrifugation. In leaf extracts, all NADH-dependent activity was associated with the particulate fractions. The 10,000 *g* pellets, which contained

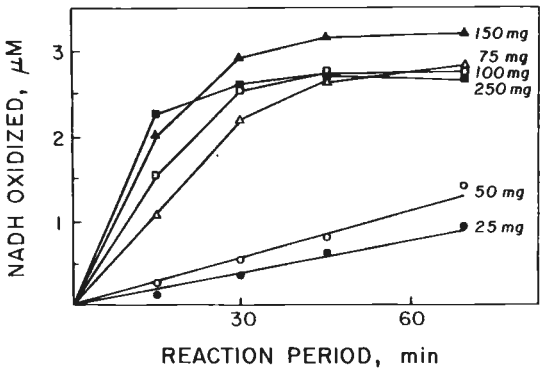


Fig. 1: NADH oxidation by grapevine leaf glutamate dehydrogenase as a function of incubation time and amount of enzyme added at 25, 50, 75, 100, 150 and 250 mg equivalent fresh tissue. Other reaction conditions were as described in the text.

Die Oxidation von NADH durch Glutamatdehydrogenase aus Rebenblättern in Abhängigkeit von Inkubationsdauer und zugesetzter Enzymmenge (Äquivalente von 25, 50, 75, 100, 150 und 250 mg frischem Blattgewebe). Übrige Reaktionsbedingungen s. Text.

mostly plastids, as preliminary work with marker enzymes indicated (ROUBELAKIS-ANGELAKIS, unpublished data), represented 66 ± 15 % of the total activity and 23,500 *g* pellets, which contained mostly mitochondria (ROUBELAKIS-ANGELAKIS, unpublished data) represented 34 ± 14 %. Leaf NADPH-dependent GDH activity was associated mainly with the 23,500 *g* soluble fraction (81 ± 15 %), and the remaining activity was in the mitochondrial fraction.

The distribution of GDH activity in root extracts differed somewhat from that in leaf extracts in that 53 ± 12 % of NADH-dependent GDH activity was associated with the 23,500 *g* soluble fraction, and the remaining activity was found in the 10,000 *g* pellets. NADH-dependent GDH activity was detected in the 23,500 *g* pellets in only a few

Table 2

Subcellular distribution of glutamate dehydrogenase activity in leaf and root tissues from *Vitis vinifera* L. cv. Chenin blanc[1])

Die subcelluläre Verteilung der Glutamatdehydrogenase-Aktivität in Blatt- und Wurzelgewebe der Rebsorte Chenin blanc (*Vitis vinifera* L.)

Subcellular fraction[2])	Glutamate dehydrogenase activity, %			
	Leaf		Root	
	NADH-GDH	NADPH-GDH	NADH-GDH	NADPH-GDH
P_1	21.8 ± 8.2	11.3 ± 3.3	2.1 ± 1.9	10.2 ± 1.9
P_2	11.9 ± 5.1	6.6 ± 2.1	1.8 ± 1.5	5.6 ± 1.6
P_3	61.0 ± 13.7	0.9 ± 0.6	27.4 ± 4.3	26.4 ± 4.7
P_4	5.3 ± 1.4	0.0	15.6 ± 4.6	11.0 ± 2.1
S	0.0	81.2 ± 14.1	53.1 ± 12.1	46.8 ± 3.4

[1]) Assay conditions were as described in the text.
[2]) The subcellular fractions are defined in the flow diagram, p. 203.

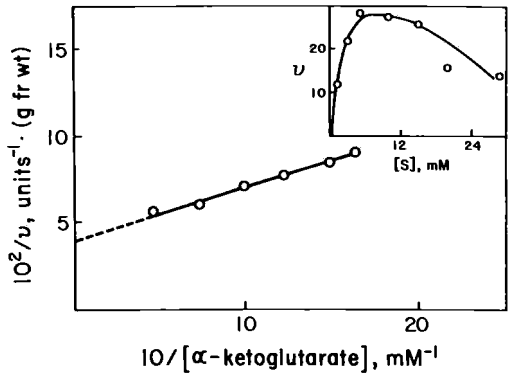


Fig. 2: Effect of α-ketoglutarate concentration on grapevine leaf glutamate dehydrogenase activity. Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions were as described in the text.

Einfluß der α-Ketoglutarat-Konzentration auf die Glutamatdehydrogenase-Aktivität von Rebenblattmaterial; Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen s. Text.

preparations; however, this never exceeded about 4 % of the total activity. NAD(P)H-dependent activity in root extracts averaged 47 ± 3 % in the 23,500 *g* soluble fraction and 37 ± 7 % in plastids, and the remaining activity was in the mitochondrial fraction (Table 2). Mitochondrial GDH has been reported to be more active with NADPH than with NADH (CHOU and SPLITTSTOESSER 1972), in agreement with our results with grapevine roots.

GDH activity was found in both the supernatant and the mitochondrial fractions in pea roots (PAHLICH and JOY 1971), in mung bean seedlings (LEA and THURMAN 1972) and in pea seedlings (DAVIS and TEIXEIRA 1975), when the fractionation was performed by differential centrifugation. Similar findings were obtained when the separation of subcellular fractions was done by sucrose density gradient (DALLING *et al.* 1972, LEA and THURMAN 1972). GDH activity in the supernatant fraction was considered to originate from broken mitochondria. In addition, LEECH and KIRK (1968) and LEA and THURMAN (1972) found that GDH activity in *Vicia faba* and *Lactuca sativa* leaves was associated with the mitochondrial and the chloroplastid fractions, whereas in developing castor bean endosperm only with the mitochondria (LEES and DENNIS 1981).

In the present study, extraction and cell fractionation procedures were identical for leaf and root tissues. Leakage of enzyme from ruptured organelles may have altered the distribution of GDH activity among the subcellular fractions; however, some NADH-GDH activity should have been in the soluble fraction of leaves, which never occurred. Evidence that the two enzymes, leaf chloroplastid and root-soluble GDH, may be different is based on their kinetic properties. These results confirm findings of CHOU and SPLITTSTOESSER (1972), who found that soluble and mitochondrial GDH from pumpkin cotyledons differed in some kinetic properties.

Leaf and root GDH activities were similarly affected by the concentration of α-ketoglutarate in the reaction mixture. Both exhibited normal Michaelis-Menten kinetics as concentrations of the substrate increased from 0 to 30 mM; however, at concentrations greater than about 6 mM (root GDH) and 15 mM (leaf GDH), a progressively increasing inhibitory effect was observed (Fig. 2, inset). At 30 mM there was a 64 % and 75 % inhibition of GDH from leaves and roots, respectively. JOY (1973)

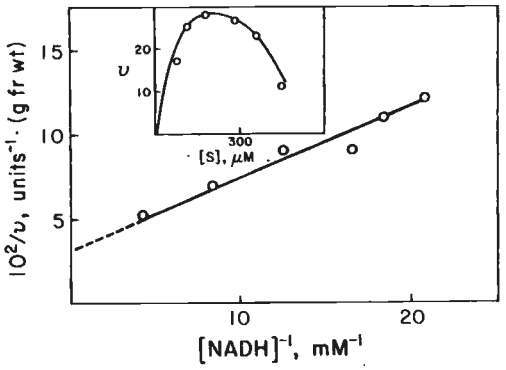


Fig. 3: Effect of NADH concentration on grapevine leaf glutamate dehydrogenase activity. Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions were as described in the text.

Einfluß der NADH-Konzentration auf die Glutamatdehydrogenase-Aktivität von Rebenblattmaterial; Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen s. Text.

reported on pea root GDH inhibition at high α-ketoglutarate concentrations. K_m for α-ketoglutarate calculated from the double reciprocal plots were 3.9 ± 0.4 mM for leaf and 0.8 ± 0.1 mM for root GDH. K_m values for this substrate reported in the literature for plant GDH ranged from 0.62 to 4.00 mM (STEWART *et al.* 1980).

GDH from grapevine leaves and roots also showed a Michaelis-Menten response to NADH concentration. The enzyme was inhibited at concentrations greater than about 100 μM (root GDH) and about 250 μM (leaf GDH, Fig. 3, inset). K_m values for NADH using leaf GDH was nearly 3-fold greater than K_m values using root GDH, the values being 100 ± 10 and 36 ± 5 μM, respectively.

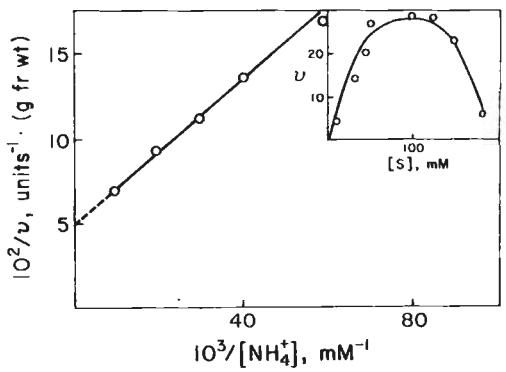


Fig. 4: Effect of NH_4Cl concentration on grapevine leaf glutamate dehydrogenase activity. Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions were as described in the text.

Einfluß der NH_4Cl-Konzentration auf die Glutamatdehydrogenase-Aktivität von Rebenblattmaterial; Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen s. Text.

GDH from grapevine leaves and roots responded to NH_4Cl differently. Leaf GDH showed a Michaelis-Menten response with respect to increasing NH_4Cl concentrations (Fig. 4), whereas root enzyme showed a sigmoid relationship. K_m value for leaf enzyme was 36 ± 4 mM. Reported K_m values for plant GDHs for ammonium ions ranged from 5.2 to 70.0 mM (STEWART *et al.* 1980). Concentrations greater than about 120 mM inhibited leaf GDH, in agreement with the results of JOY (1973) and CHOU and SPLITTSTOESSER (1972). A sigmoid relationship between pea GDH and α-ketoglutarate has been reported (PAHLICH and JOY 1971).

The addition of Ca^{2+} to the reaction mixture increased the activity of root GDH by about 70 %, but increased leaf GDH activity by only 11 %. CHOU and SPLITTSTOESSER (1972) also found that Ca^{2+} activated the amination activity of soluble GDH from pumpkin cotyledons but had not effect on this enzyme in the particulate fraction.

The addition of EDTA at a concentration of $2 \cdot 10^{-6}$ M inhibited leaf GDH activity by 38 % and root enzyme activity by 32 %. The addition of Ca^{2+} partly reversed the inhibitory effect of EDTA, which agrees with previous reports (STEWART *et al.* 1980).

The stability of leaf GDH was tested by incubating the enzyme at 22, 38, and 45 °C and determining its activity at various times. During the first 3.5 h there was a general linear decline of GDH activity at each incubation temperature, after which activity decreased sharply (Fig. 5). The optimum temperature for *in vitro* assay of leaf and root enzyme was 38—40 °C; and optimum pH was 7.9—8.1.

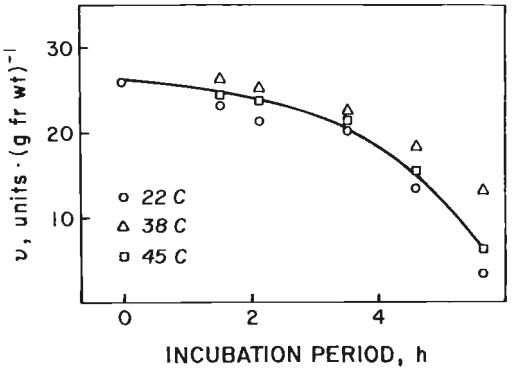


Fig. 5: Influence of temperature on *in vitro* grapevine leaf glutamate dehydrogenase inactivation. Other reaction conditions were as described in the text.

Einfluß der Temperatur auf die Inaktivierung der Glutamatdehydrogenase-Aktivität von Rebenblattmaterial *in vitro*. Übrige Reaktionsbedingungen s. Text.

From the data presented herein and by assuming that *in vivo* enzyme activities are similar to those *in vitro*, then NH_4^+ assimilation via α-ketoglutarate amination should be greater in roots than in leaves, as indicated by the higher root GDH activity. In grapevine xylem sap, glutamine was the predominant nitrogen constituent and glutamic acid was the predominant amino acid; nitrates constituted 16—27 % of the total soluble nitrogen (ROUBELAKIS-ANGELAKIS and KLIEWER 1979). This may suggest that in grapevines most of the nitrates taken up by roots are reduced therein, and the NH_4^+ formed is incorporated into either glutamate or glutamine, which then translocate to the aerial vine parts. The relative levels of glutamine to glutamate in grapevine xylem sap was about 20 : 1 (ROUBELAKIS-ANGELAKIS and KLIEWER 1979).

Summary

Glutamate dehydrogenase (GDH) activity in *Vitis vinifera* L. cv. Chenin blanc leaf and root tissues was associated with the particulate and the soluble fractions. In leaf extracts, about 66 % of the NADH-GDH activity was in the 10,000 *g* pellet and about 34 % was in the 23,500 *g* pellet fractions, whereas NADPH-GDH activity was associated mainly with the soluble fraction (23,500 *g*). In root extracts, about 53 % of the NADH-GDH activity was in the soluble and 43 % in the 10,000 *g* pellet fractions, whereas about 47, 37, and 16 % of NADPH-GDH activity were in the soluble, 10,000 *g* and 23,500 *g* pellet fractions, respectively. GDH from the 10,000 *g* pellet of leaf and the soluble fraction of root extracts differed in their affinities to substrates. The K_m values of leaf and root GDH were, respectively, 3.9 ± 1.1 and 0.7 ± 0.4 mM for α-ketoglutarate; 35.7 ± 7.1 and 61.3 ± 12.4 mM for NH_4Cl; and 100.0 ± 7.4 and 36.2 ± 4.4 μM for NADH. GDH from leaf and root tissues showed Michaelis-Menten kinetics with all substrates except NH_4Cl, which exhibited a sigmoid relationship in roots. Optimum *in vitro* reaction conditions were pH 7.90—8.10, incubation temperature of 38—40 °C, and amount of enzyme equivalent to 80—110 mg of fresh tissue. Enzyme from both leaves and roots was inhibited by EDTA and L-glutamate. Activation with Ca^{2+} was more pronounced in root GDH than in leaf GDH.

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