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Enzymes of Krebs-Henseleit Cycle in *Vitis vinifera* L.

III. *IN VIVO* AND *IN VITRO* STUDIES OF ARGINASE[1, 2]

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KALLIOPI A. ROUBELAKIS[3] AND W. MARK KLIEWER
Department of Viticulture and Enology, University of California, Davis, California 95616

ABSTRACT

The presence of arginase (EC 3.5.3.7) in various tissues from *Vitis vinifera* L. cultivars was demonstrated by both *in vivo* and *in vitro* enzyme assays. Initial velocities determined by the two methods were in close agreement. Optimum conditions for maximum enzyme activity were 25 to 30 millimolar L-arginine, about 1 millimolar Mn^{2+} (pH 9.4 to 9.8), and incubation temperature of 37 to 38 C. L-Arginine hydrolysis was linear with increasing sliced fresh tissue up to 500 milligrams for *in vivo* assay, and with enzyme extract equivalent up to about 200 milligrams of fresh tissue for *in vitro*. Similarly, L-arginine hydrolysis was linear with incubation time for the first 45 minutes for *in vivo* assay and for the first 20 minutes for *in vitro*.

Arginase (EC 3.5.3.1) activity has been demonstrated in extracts from several annual plants (11), including bitter lupin seedlings (12, 14), *Lathyrus sativus* L. plants (2), and pumpkin seeds and seedlings (8). Evidence for arginase in apple trees was provided through studies in which ^{14}C-labeled L-arginine was administered to the trees and its subsequent metabolic products were determined (6).

The two previous publications (16, 17) in this series demonstrated the presence of ornithine carbamoyltransferase, arginosuccinate synthetase and lyase in *Vitis vinifera* L., along with some of their kinetic properties. Herein we present evidence for arginase in leaves, fruit, seeds, and seedlings of grapevines and thus for the first time have characterized all of the enzymes of the ornithine-urea cycle from a single plant source.

MATERIALS AND METHODS

PLANT MATERIAL

The plant material used was the same as described previously (15).

IN VIVO TISSUE SLICE TECHNIQUE

Mature leaves were used from Chenin blanc, Cabernet Sauvignon and, Gewürztraminer grapevines and Chenin blanc SG 3 seedlings.

Tissue Preparation. The plant material was sectioned into tissue strips of 300 to 400 μm onto ice-filled Petri dishes and then immediately dipped into ice-cold deionized H_2O. After the sectioning was completed, the sliced tissue was rapidly blotted dry between paper towels, weighed, and placed into prepared reaction mixtures kept on ice.

Reaction Mixture. The reaction mixture consisted of 100 mM Tris-HCl (pH 9.5), 25 mM L-arginine-HCl adjusted to pH 9.7 with 1 N KOH, 1 mM $MnCl_2 \cdot 5H_2O$, 200 mg of sliced fresh tissue, and deionized H_2O in a total volume of 10 ml. The controls used were reaction mixtures lacking enzyme source, and complete reaction mixtures to which 2 ml of 7% (v/v) perchloric acid was added at zero time. The reaction was initiated by incubating the vials containing the reaction components in a water bath at 38 C; the reaction proceeded for 15, 30, 45, and 60 min and was terminated by adding 2 ml of 7% (v/v) perchloric acid. Maceration of the tissue was omitted since it did not affect the results. After mixing, the reaction mixture was centrifuged at 6,000*g* for 20 min, and enzymic activity was determined on an aliquot of the supernatant.

ENZYME EXTRACTION PROCEDURE AND IN VITRO ASSAY

Arginase was extracted from germinating seeds, seedlings, mature leaves, mature grape berries from *V. vinifera* L. cv. Chenin blanc, and mature leaves from Cabernet Sauvignon and Gewürztraminer.

A given weight of tissue (usually 5 g of seedlings, 50 g of leaves, and 100 g of grape berries) was homogenized in 10 volumes (w/v) of grinding medium for 60 sec with an Omni-Mix homogenizer. The grinding medium consisted of 0.5 M Tris-HCl buffer (pH 8.7), 7 mM diethyl-dithiocarbamic acid-Na salt, 6 mM L-cysteine-HCl, 2.5 mM EDTA, and 7% (w/v) PEG 4000 (5). The homogenate was filtered through eight layers of cheesecloth. PEG 4000 was added to the filtrate to make a 40% (w/v) mixture. This mixture was then stirred for 60 min in a Gyrotory shaker at 140 rpm and centrifuged at 30,000*g* for 15 min in a refrigerated Sorvall centrifuge. The supernatant solution was discarded, and the pellet was suspended in 0.5 ml of homogenizing medium consisting of 10 mM Tris-HCl buffer (pH 8.0), and 1% (v/v) Triton X-100/g of fresh tissue. This suspension was recentrifuged at 19,500*g* for 10 min, and the resulting supernatant solution was saved and designated enzyme 1 (E 1). The pellet was redissolved in the same homogenizing medium as before and centrifuged at 10,000*g* for 10 min. This latter pellet was discarded, and the supernatant solution was saved and designated enzyme 2 (E 2).

All extraction steps were conducted at 0 to 4 C. Supernatants E 1 and E 2 were combined and used as the enzyme source. These fractions contained 90 to 92% of the total enzyme present in the tissue.

Reaction Mixture. The reaction mixture consisted of 100 mM Tris-HCl (pH 9.5), 25 mM L-arginine-HCl adjusted to pH 9.7 with 1 N KOH, 1 mM $MnCl_2 \cdot 5H_2O$, enzyme extract equivalent to about 100 g of fresh tissue, and deionized H_2O in a total volume of 2.5

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[3] Present address: Agricultural College of Athens, Iera odos 75, Votanikos, Athens 301-Greece.

ml. The controls were the same as described under the *in vivo* assay. The reaction, initiated by raising the temperature to 38 C, unless specified otherwise, was allowed to proceed for 15, 30, 45, and 60 min. The reaction was terminated by adding 2 ml of 7% (v/v) perchloric acid. The precipitated protein was centrifuged off, and enzyme activities were determined on a portion of the supernatant.

Enzyme activity in both assays was determined from the amount of ornithine present in the reaction mixture. L-Ornithine was determined colorimetrically by the method of Chinard (3) modified as follows. In Pyrex screw-cap culture tubes (13 × 100-mm), 3 ml of glacial acetic acid, 1 ml of the reaction mixture diluted to contain less than 0.25 μmol of L-ornithine, and 1 ml of a ninhydrin solution were added, mixed, and boiled in a covered water bath for 60 min. The ninhydrin solution consisted of glacial acetic acid, concentrated phosphoric acid, and distilled H_2O in a ratio of 3:1:1 (v/v/v), and ninhydrin at a concentration of 25 mg/ml of final solution. Ninhydrin was first dissolved in glacial acetic acid by agitating the mixture in a water bath at about 60 C. Then the other two components were added and the solution was chilled to 4 C before use. After the samples containing ornithine were boiled for 60 min, the tubes were cooled by immersing them for no longer than 10 min in a water bath kept in the dark, and absorbancies were read at 515 nm with a Bausch & Lomb Spectronic 21. Ornithine standards were run simultaneously with each series of unknowns, and a standard curve was prepared. Absorbancy was linear for ornithine concentrations between 0.0 and 60 μM.

To verify the results obtained by the colorimetric determination of ornithine the composition of the reaction mixture was determined with an amino acid autoanalyzer (Dept. of Food Science and Technology, UCD). An aliquot of the reaction mixture was treated with 5-sulfosalicylic acid, sequanal grade, 10% (w/v) in deionized H_2O, in a ratio 1:5 (v/v). Any precipitate formed was centrifuged off. A 0.30-ml portion was then used for amino acid determination. Results with this method were in good agreement with the colorimetric determinations.

The net increase in ornithine in the reaction mixture as a result of arginase activity was estimated, and initial velocity was computed and expressed as μmol of L-ornithine/g of fresh tissue · hr.

RESULTS AND DISCUSSION

The existence of arginase in *V. vinifera* mature leaves, mature fruit, germinating seeds, and seedlings was demonstrated by either *in vivo* or *in vitro* assay or both techniques (Table I).

Several *V. vinifera* cultivars have been classified into high, moderate, and low arginine accumulators (7). Of the three cultivars in this investigation, Cabernet Sauvignon and Chenin blanc are moderate arginine accumulators, whereas Gewürztraminer is a high arginine accumulator. Arginase activities in leaves were higher in Gewürztraminer than in the other cultivars (Fig. 1).

***In Vivo* Arginase Properties.** Initial velocities as a function of substrate concentration were plotted according to the Lineweaver-Burk equation (Fig. 1) and the Henri-Michaelis-Menten equation (Fig. 1, inset). Michaelis constants (K_m), calculated from the double reciprocal plot for Cabernet Sauvignon, Chenin blanc, and Gewürztraminer leaf arginase, were, respectively, 17, 10, and 22 mM. Optimum substrate concentration was about 25 to 30 mM for all tested cultivars (Fig. 1, inset). L-Arginine at concentrations greater than the optimum inhibited the enzyme activity.

Table I
In Vivo and In Vitro Arginase Activity in Various Tissues from V. vinifera Vines

Plant material	Arginase activity[1] μmoles (g fresh wt)$^{-1}$ hr^{-1}	
	In vivo	In vitro
Chenin blanc mature leaves	4.42 ± 0.96	4.71 ± 1.12
Chenin blanc mature fruit	--	2.69 ± 0.44
Chenin blanc SG 0 seeds	--	1.85 ± 0.50
Chenin blanc SG 3 seedlings	12.12 ± 2.07	14.48 ± 2.45
Cabernet Sauvignon mature leaves	4.69 ± 0.85	5.25 ± 0.78
Gewürztraminer mature leaves	6.67 ± 0.92	9.09 ± 1.05

[1]The values represent the means from 10 experiments, 5 replicates per experiment and 3 determinations of L-ornithine per replicate. Enzyme extraction and assay conditions were as described in the text.

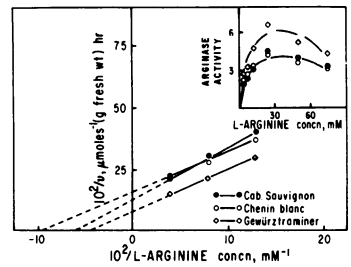

FIG. 1. Lineweaver-Burk and Henri-Michaelis-Menten (inset) plots for *in vivo* arginase in mature leaves from three *V. vinifera* cultivars. Reaction conditions were as described in the text.

Omission of Mn^{2+} from the reaction mixture resulted in an initial velocity about 10% of that obtained with 1 mM Mn^{2+} for Chenin blanc and Cabernet Sauvignon leaves, and only 5% for Gewürztraminer. Mn^{2+} concentrations higher than about 2 mM resulted in the formation of a yellow-brown precipitate and a reduction of the initial reaction velocities, which could be due to unavailability of Mn^{2+} or to enzyme inhibition by high Mn^{2+} levels.

Ornithine formation was linear with increasing amounts of incubated sliced fresh tissue up to about 500 mg. With greater weights of tissue, the amount of substrate available to the enzyme was probably the limiting factor, preventing further linearity. Although the response to increasing amounts of fresh tissue was similar for the three cultivars, Gewürztraminer showed a further slight increase in the amount of produce formed with sliced tissue up to 1,000 mg. The amount of ornithine formed was also linear with time for the first 45 min; and thereafter, it continued to increase for the next 15 hr.

The *in vivo* activity of arginase depended on incubation temperature. Enzyme assays were conducted at 0, 20, 38, and 60 C. Optimum temperature for arginase activity was about 37 to 38 C. At 60 C the enzyme was still very active; the initial velocities obtained were only slightly lower at 60 C than at 38 C. *In vivo* hydrolysis of exogenous L-arginine by arginase in mature leaves from each of the three tested cultivars showed about the same response to different temperatures.

***In Vitro* Arginase Properties.** Arginase activity was detected in extracts from mature leaves of three *V. vinifera* cultivars and in Chenin blanc mature fruit, germinating seeds, and seedlings (Table I).

PEG 4000 in the grinding medium was indispensable for extracting active arginase. Enzyme extracts from lyophilized tissue or acetone powder had lower activities than extracts from fresh or frozen plant material. Triton X-100 in the homogenization medium helped solubilize the enzyme, giving higher activities. The arginase preparation from *Vicia faba* germinating seeds showed a 4- to 5-fold increase in enzyme activities in the presence of Triton X-100 (8).

Initial enzyme velocities as a function of L-arginine concentration from Chenin blanc SG 4 seedlings are shown in Figure 2. The estimated Michaelis constant was 5 mM for seedlings, 12 mM for leaves, and 41 mM for mature fruit. Table II gives the K_m value for arginase extracted from various mammalian and plant sources. Reported K_m values have been generally higher for enzyme extracted from animal tissues than for plant arginases. K_m values for *V. vinifera* tissues appear to be in good agreement with K_m values reported for other plant arginases.

Differences in K_m values for an enzyme extracted from different organs of the same plant, or from the same organ at different stages of its development, could be due to different degrees of enzyme purification. That was not the case, however, for plant arginases (9). Arginases of different origin showed immunological

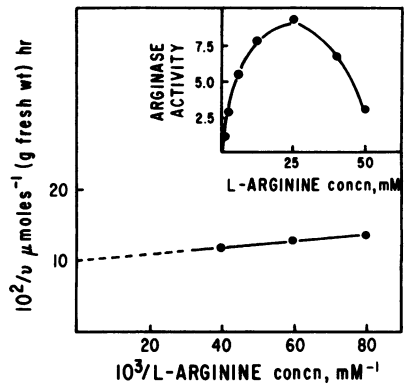

FIG. 2. Lineweaver-Burk and Henri-Michaelis-Menten (inset) plots for *in vitro* arginase activity from Chenin blanc SG 4 seedlings. Reaction conditions were as described in the text. For definition of stage of seedling growth see reference 15, Figure 1.

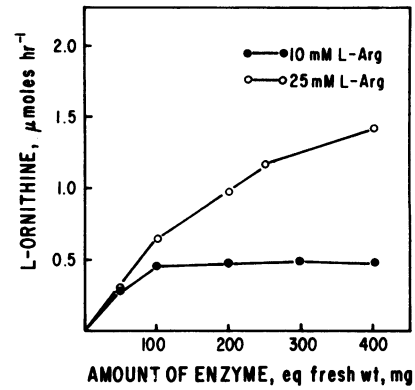

FIG. 3. Effect of endogenous substrate on *in vitro* arginase activity from Chenin blanc SG 2 seedlings at saturation (25 mM) and subsaturation (10 mM) exogenous substrate concentrations. Other reaction conditions were as described in the text. For definition of stage of seedling growth, see reference 15, Figure 1.

Table II
Comparison of the Published Michaelis Constants for Arginases from Different Mammalian and Plant Sources with those Found for Various V. vinifera Tissues[1]

Enzyme	Reference	Km, mM
Rat liver	10	20-40
Chicken liver	10	100-200
Chicken liver	14	200
Ox liver	14	29
Lupin seedlings	13	36
Pumpkin seedlings	17	0.026
Lupin seedlings	14	55
Lathyrus sativus seedlings	2	5.5
Chenin blanc seedlings	this report	5-18
Chenin blanc mature leaves	this report	12
Chenin blanc mature fruit	this report	41

[1]Reaction conditions were as described in the text

Table III
Some Properties of Arginase from Chenin blanc Leaves Determined by In Vivo and In Vitro Assay Methods[1]

	Assay Method In Vivo	In Vitro
Michaelis constant, Km	10 mM	12 mM
Initial velocity, υ	4.4 units[2]	4.7 units
Optimum substrate concn	25 mM	25 mM
Substrate inhibition	above 30-35 mM	above 25-30 mM
Optimum manganese concn	1-2 mM	0.5-1.0 mM
Manganese inhibition	above 2 mM	above 1 mM
Incubation period for linear product formation	45 min	20 min
Reaction duration	15 hrs	10 hrs
Amount of enzyme for linear product formation	up to 500 mg fresh tissue	up to 200 mg eq fresh wt
Temperature response	$\upsilon_{38\ C} \simeq \upsilon_{60\ C}$	$\upsilon_{38\ C} > \upsilon_{60\ C}$

[1]Reaction conditions were as described in the text
[2]One unit is defined as 1 μmole per g fresh wt per hour

and electrophoretical differences (1), various rates of inhibition by certain amino acids (10), and varied capacity for hydrolyzing L-arginine derivatives (9). A noncompetitive arginase inhibitor was isolated and purified from lupin seedlings (12, 13). The K_i for the inhibitor was metal activator-dependent.

The optimum substrate concentration for *in vitro* arginase activity was about 25 mM L-arginine. Higher substrate concentrations resulted in arginase inhibition (Fig. 2, inset). The authors know of no previous information on substrate inhibition of arginase. L-Arginine concentrations used by other investigators ranged from 0.2 to 225 mM (3, 9, 12, 17). In the present investigation substrate inhibition of arginase was found for both the *in vivo* and the *in vitro* assay conditions when concentrations of L-arginine exceeded 25 to 30 mM. Enzyme inhibition, however, was greater *in vitro* than *in vivo*. Reduced penetration of arginine to the enzyme site would be more likely *in vivo* than *in vitro*, thus possibly accounting for the difference in substrate inhibition between the two assay methods.

Mn was the most effective of several divalent metal activators tested. Enzyme velocities were maximum at Mn^{2+} concentrations ranging from 0.5 to 1.0 mM. At Mn^{2+} concentrations greater than 1.5 mM a precipitate was formed and reaction velocities significantly decreased. The amount of ornithine formed was linear with amount of enzyme present in the mixture up to about 200 mg equivalent of fresh tissue.

Arginase activity was strongly affected by the pH of the reaction mixture. The optimum pH was in the range of 9.4 to 9.8. Arginase preparations from different *V. vinifera* plant tissues showed the same optimum pH range. For both mammalian and plant arginases the pH optimum occurs between 9.3 and 10.0 (4, 9, 10).

The temperature response of arginase *in vitro* was very close to that of the *in vivo* assay procedure. At temperatures higher than about 40 C, however, the extracted enzyme was more readily inactivated than that in intact tissue, giving lower initial velocities.

Arginine present in the enzyme preparation could have affected the kinetic properties of the enzyme. To check whether endogenous substrate was present in appreciable amounts, increasing quantities of arginase were assayed under saturated (25 mM) and subsaturated (10 mM) concentrations of exogenous substrate. The data in Figure 3 indicate no effect of endogenous arginine on arginase activity. We concluded that the properties of arginase discussed previously and its kinetic behavior were not affected by the minute amount of arginine that might be present in the plant extract.

L-Ornithine formation by arginase was linear with incubation time for the first 20 min. Ornithine continued to increase up to about 10 hr and then leveled off. The formation of L-ornithine under *in vivo* assay conditions was linear with incubation time for the first 45 min, and equilibrium was reached in about 15 hr.

Some properties of leaf arginase determined by the tissue slice technique and the *in vitro* assay method are compared in Table III. These data indicate that both methods gave comparable results.

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