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Purification and Properties of the Constitutive Arginase of *Evernia prunastri*[1]

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ANGELES MARTÍN-FALQUINA AND MARÍA ESTRELLA LEGAZ*
Department of Plant Physiology, The Lichen Team, Faculty of Biology, Complutense University, 28040 Madrid, Spain

ABSTRACT

Constitutive arginase (molecular weight 330,000) 920-fold purified from *Evernia prunastri* thallus, is activated by putrescine, L-ornithine, and agmatine with K_a values of 2.7, 1.1, and 5.8 millimolar, respectively. Constitutive arginase is also activated by endogenous L-arginine, reaching its maximum activity at 16 hours of incubation on Tris-HCl (pH 9.15) with a subsequent decrease. Urea behaves as a mixed inhibitor of the enzyme with a K_i value of 2.6 millimolar. Atranorin and evernic acid behave as *in vitro* activators of the enzyme; usnic acid does not have any significant effect as activator.

Free L-arginine accumulates in the thallus of many lichen species (18, 19) and it is the most abundant free amino acid in *Evernia prunastri* thalli (11), although seasonal variations in the arginine concentration have been reported.

L-Arginine can be hydrolyzed by arginase to produce L-ornithine and urea (10) or decarboxylated by L-arginine decarboxylase to produce agmatine (24), which is later hydrolyzed to give putrescine and urea (25). The main route in *E. prunastri* thallus is the hydrolytic one, since arginase has greater affinity for L-arginine than L-arginine decarboxylase.

Arginase synthesis is induced by L-arginine in *E. prunastri* thallus incubated in the dark (10). It has a mol wt of 180,000 and a pH optimum at 9.1. The K_m value has been estimated as 0.2 mM for L-arginine. L-Ornithine and putrescine behave as activators of the enzyme (K_m = 0.13 and 0.14 mM, respectively) (10). Agmatine is a noncompetitive inhibitor (K_i = 21.5 mM) and urea is an uncompetitive one (K_i = 2.6 mM) (10).

The enzyme is also induced in *Escherichia coli* (5), *Lactobacillus lactis* (1), rat kidney (9), and human liver (3). But, in *Neurospora crassa*, arginase activity is not dependent on protein synthesis (28, 29), being activated by the liberation of L-arginine to the cytosol.

In germinating seeds, arginase is dependent on the mobilization of reserve proteins with high content of L-arginine, as reported in *Cucurbita* (20), *Vicia faba* (2), and *Pisum sativum* (17).

A second form of arginase, the constitutive one, reported in *E. prunastri* thallus is an inactive and preexistent protein which is activated by the liberation of L-arginine to the cytosol. It has a mol wt of about 330,000 and a pH optimum at 6.5 (12).

When lichen thalli are floated on media containing the inducers of urease of *Lobaria pulmonaria* (21), *Parmelia roystonea* (22), *Cladonia verticillaris* (23), and *E. prunastri* (4), induction is followed by a loss of enzyme activity after a certain time of incubation. In the latter species, a decrease in activity of enzymes of the arginine catabolism—arginase, L-arginine decarboxylase, and agmatine amidinohydrolase—has been reported, which is parallel by the accumulation of both chloroatranorin and evernic acid (13). The regulation of both phenolics depends, not only on its concentration, but also on the enzyme involved.

This paper reports the purification and properties of the constitutive arginase in *E. prunastri* thallus, as well as the role that lichen phenolics could play on the arginase regulation, in a similar way to that described for the inducible arginase (13).

MATERIALS AND METHODS

E. prunastri (L.) Ach., growing on *Quercus pyrenaica* Lam. and collected in Valsain (Segovia, Spain) was used throughout this work. Thalli were stored in polyethylene bags at 7°C in the dark until required. Samples of 1.0 g of air-dried thallus were floated on 25 ml of 40 μM cycloheximide in 0.1 M Tris-HCl buffer (pH 9.15) for up to 18 h at 26°C in the dark. Where indicated, samples were floated on 40 mM L-arginine in the same buffer.

At different times, the samples were washed with distilled H_2O and then macerated with sufficient volume (15 ml) of 0.1 M Tris-HCl buffer (pH 9.15) containing 0.5 mM manganese sulfate and 0.75 mM maleic acid (7). The homogenates were centrifuged at

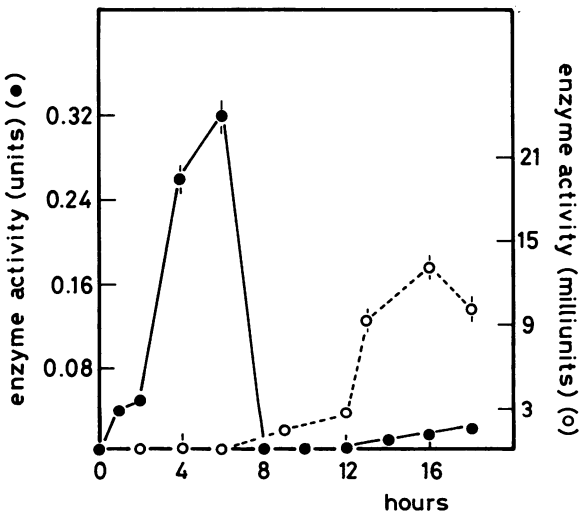

FIG. 1. Time course of inducible arginase activity (●) in *E. prunastri* thallus incubated on 40 mM L-arginine in 0.1 M Tris-HCl buffer (pH 9.15) and of constitutive arginase activity (○) in thallus floated on 40 μM cycloheximide in 0.1 M Tris-HCl buffer (pH 9.15). Values are the mean of three replicates. Vertical bars give SE where larger than the symbols.

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Table I. *Purification of the Constitutive Arginase of E. prunastri*

Step	Volume	Protein	Total Protein	Specific Activity	Total Activity	Yield	Purification
	ml	*mg·ml⁻¹*	*mg*	*milliunits*		%	*-fold*
Cell-free extract	95	6.3	598.5	2.5	1496	100	
Supernatant from the $(NH_4)_2SO_4$ precipitation	156	0.05	8.1	53.5	434	29	21
Fraction eluted from calcium phosphate gel with 0.14 M Tris-HCl buffer	50	0.01	0.5	799	399	26.7	320
Filtrate at 120 ml from Sephadex G-200 column	10	0.002	0.02	2,300	46	3.1	920

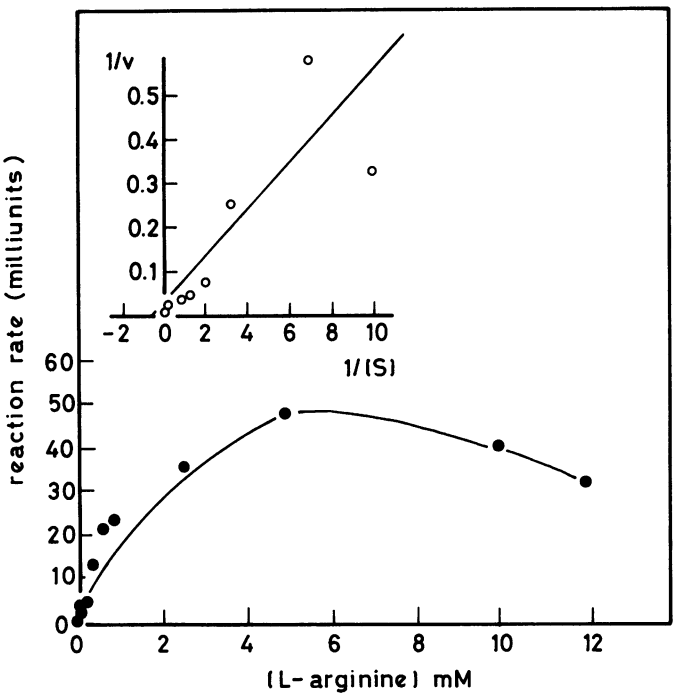

FIG. 2. Effect of substrate concentration on constitutive arginase activity. Regression equation, $y = 0.05x + 0.02$, $r = 0.86$. The interaction coefficient estimated by Hill's plot was fitted to a linear regression equation $y = 0.91x - 0.30$, $r = 0.93$, and $n_H \simeq 1$.

24,000*g* for 20 min at 4°C. Supernatants were filtered through Millipore GS Filters (0.22 μm pore diameter).

Arginase was assayed according to Greenberg's method (7) modified by Legaz and Vicente (10). Reaction mixtures contained 0.1 M Tris-HCl buffer (pH 6.5), 0.4 μmol L-arginine, 8.1 mg urease, 7.5 μmol maleic acid, and 1.0 mg protein in a final volume 2.9 ml. A unit of specific activity is 1.0 μmol urea produced·mg^{-1} protein·min^{-1}.

Protein was estimated in the cell-free extracts by the method of Potty (15) using BSA as a standard.

Purification of the constitutive arginase was carried out as follows: samples of 10.0 g of thallus were floated on 250 ml of 40 μM cycloheximide in 10 mM Tris-HCl buffer (pH 9.15) for 16 h in the dark at 26°C. The thallus was washed, centrifuged, and filtered as above and the supernatant was subjected to 70% $(NH_4)_2SO_4$ fractionation at 4°C and kept for 2 h, after which the precipitate recovered by centrifugation at 43,000*g* for 1 h was discarded. The supernatant containing the greater enzyme activity, was dialyzed for 20 h against 2.0 l of 10 mM Tris-HCl buffer (pH 6.5) at 4°C. Protein in the supernatant was then adsorbed on calcium phosphate gel (75 mg dry weight of gel·mg^{-1} protein.

Table II. *Range of Effector Concentrations on Constitutive Arginase Activity of E. prunastri*

Effector	Concn. of the Effector	Per cent Activation/ Inhibition
	mM	%
L-Ornithine	1.0	213.0
	2.4	254.4
	3.0	269.4
Agmatine	1.0	133.0
	3.0	266.9
	4.0	333.8
Putrescine	1.0	164.5
	2.4	240.6
	3.0	630.5
Urea	0.4	97.2
	1.0	97.6
	1.4	99.4
	1.8	100.0

The mixture was vigorously shaken and then centrifuged at 12,000*g* for 5 min at 4°C. Protein was eluted from the pellet by increasing the molarity of the Tris-HCl buffer (pH 6.5) from 10 to 180 mM. The eluate from 140 mM Tris-HCl buffer was filtered through a Sephadex G-200 column (21.0 × 3.0 cm), which had been equilibrated with the same buffer. Fractions of 10 ml were collected until the wash-liquid contained only traces of protein. The procedure of purification was similar to that described for the inducible arginase (10).

To identify lichen acids, samples of thallus were superficially washed with acetone to remove the cortical phenols and then macerated with 12 ml methanol for HPLC (Scharlau, Spain). The homogenates were centrifuged at 24,000*g* for 20 min and the supernatants were used for the phenolic determination.

Lichen phenolics were extracted from the cell-free extracts with benzene (25:10, v/v). the benzene-phase containing lichen phenolics was removed, after 2 h of vigorous shaking, from the aqueous phase and then lyophylized. The dry residue was dissolved in pure methanol (0.1 mg/ml). The quantitative determination of lichen acids was achieved by reverse-phase HPLC by the method of Legaz and Vicente (13).

RESULTS

When lichen thalli were incubated on 40 μM cycloheximide in buffer, constitutive arginase activity was undetectable for up to 9 h; then, a low activity appears that reached its maximum value at 16 h incubation. However, when 40 mM L-arginine and no

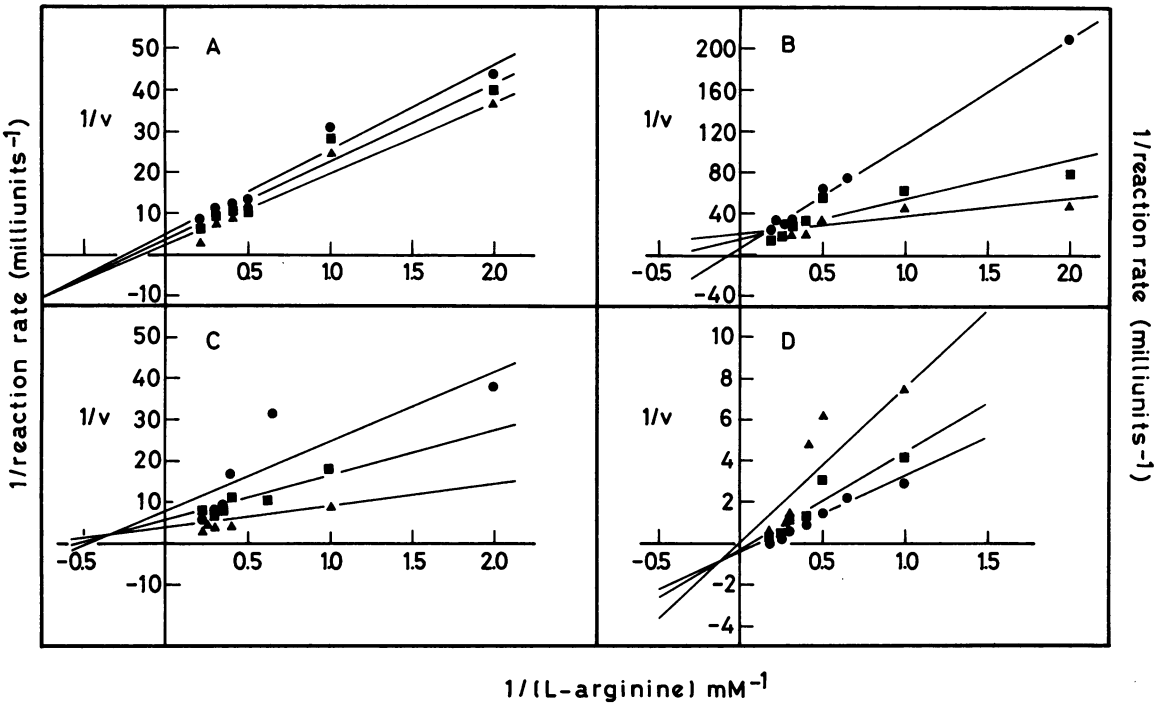

FIG. 3. Effects of (A) L-ornithine: regression equations were (●) 1.0 mM, $y = 20.70x + 5.17$, $r = 0.98$; (■) 2.4 mM, $y = 18.82x + 3.93$, $r = 0.97$; (▲) 3.0 mM, $y = 18.02x + 1.89$, $r = 0.99$. (B) Agmatine: regression equations were (●) 1.0 mM, $y = 111.73x + 4.19$, $r = 0.98$; (■) 3.0 mM, $y = 37.72x + 15.09$, $r = 0.87$; (▲) 4.0 mM, $y = 17.29x + 20.95$, $r = 0.83$. (C) Putrescine: regression equations were (●) 1.0 mM, $y = 17.07x + 7.92$, $r = 0.84$; (■) 2.4 mM, $y = 10.98x + 5.74$, $r = 0.93$; (▲) 3.0 mM, $y = 7.94x + 1.79$, $r = 0.98$. (D) Urea: regression equations were (●) 0.4 mM, $y = 3.63x - 0.37$, $r = 0.99$ (■) 1.0 nM, $y = 4.72x - 0.29$, $r = 0.94$; (▲) 1.4 mM, $y = 7.72x - 0.04$, $r = 0.80$ on constitutive arginase activity.

protein inhibitor was included in the media, inducible arginase activity, in contrast to the constitutive one, had its maximum at 4 to 6 h of incubation (Fig. 1).

Constitutive arginase was purified 920-fold from *E. prunastri* thallus (Table I). The enzyme prepared by standard procedure was used for enzyme analysis. The mol wt for constitutive arginase was estimated by gel filtration on Sephadex G-200 (equilibrated as above). The void volume V_o was determined from the elution volume V_e of dextran blue. Mol wt was estimated from the standard plot of log mol wt *versus* V_e/V_o. Molecular standards were: glutamic acid dehydrogenase ($V_e/V_o = 1.42$), urease ($V_e/V_o = 2.05$), phosphorylase *a* ($V_e/V_o = 3.11$), catalase ($V_e/V_o = 3.36$), alcohol dehydrogenase ($V_e/V_o = 3.45$) and glyceraldehyde 3-P dehydrogenase ($V_e/V_o = 4.02$). The most active fraction eluted at 120 ml ($V_e/V_o = 2.73$), which implies an approximate mol wt of 330,000 (data not shown).

The relation of the reaction rate *versus* substrate concentration showed a typical Michaelis-Menten relationship (Fig. 2). The K_m value for L-arginine was 2.5 mM with a V_{max} value of 48 nmol·min^{-1}. From 5 mM, a slight inhibition can be observed.

To elucidate the role of the different metabolites on the constitutive arginase activity, their actions were studied by using 920-fold purified enzyme. The concentrations of the effectors used, as well as per cent of activation or inhibition, are shown in Table II. Reaction mixtures were the same as for the enzyme assays but including the mentioned concentration of the effector. L-Ornithine, agmatine, and putrescine behave as nonessential activators of the enzyme (Fig. 3, A, B, and C). Apparent K_a values had been estimated from Dixon's plot (data not shown) as 1.1 mM for L-ornithine, 5.88 mM for agmatine, and 2.7 mM for putrescine. Urea is a mixed inhibitor with a K_i value, estimated from Dixon's plot of 2.6 mM (Fig. 3D).

The enzyme shows an optimal reaction time of 25 min and is dependent on Mn^{2+}.

Concentrations of lichen phenols retained in the thallus were calculated assuming that the density of the samples changes from 0.36 g·cm^{-3} (dry thallus) to 0.20 g·cm^{-3} (thallus over 16 or 18 h incubation) as shown in Figure 4. Evernic acid, atranorin, and chloroatranorin decrease in concentration for the first 4 h of incubation, but show a maximum at 8 h to decrease later. Usnic acid reached values 10 times higher than those observed for the other lichen phenolics, with two maxima at 4 and 12 h (Fig. 4). The concentration of phenolics present in the cell-free extracts is 1,000 times lower than those obtained in the thalli. Chloroatranorin and evernic acid were not detected in dry thalli, while atranorin and usnic acid were present in dry thalli and their concentrations drastically diminished up to 4 h, having a maximum at 8 h (Fig. 5).

In vitro assays were performed to examine whether the concentrations of lichen phenolics retained in the thallus or extracted in the buffer could be related to the activity of constitutive arginase. Reaction mixtures were the same as for the enzyme assays, but including the indicated concentration of the lichen phenol (Table III). Constitutive arginase is activated by evernic acid and atranorin at the concentrations measured in the cell-free extracts, as well as by the mixtures of them (Table IIIA). Lichen phenolics, at the concentration found in the thallus, also activated the constitutive arginase (Table IIIB).

DISCUSSION

In contrast to the inducible (light) arginase, *E. prunastri* has a constitutive (heavy) form of the enzyme (12). This fact is in agreement with that reported by Weiss and Davis (28, 29) for a preexistent arginase in *Neurospora crassa*. The presence of inducible arginase is excluded from thallus samples in this work because incubations are carried out in the presence of cycloheximide.

The comparison of both activities (Fig. 1) pointed out a

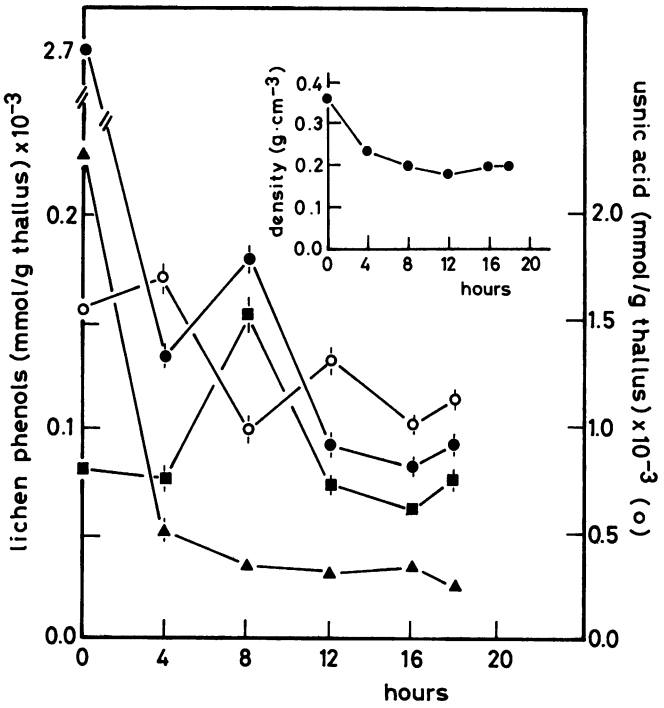

FIG. 4. Time course of lichen phenolics retained in the thallus of *E. prunastri* incubated on 40 μM cycloheximide in 0.1 M Tris-HCl buffer (pH 9.15). (●), Evernic acid; (○), usnic acid; (▲), atranorin; (■), chloroatranorin. The insert shows the time course of density changes of thallus samples floated in the same conditions as above. Values are the mean of three replicates. Vertical bars give SE where larger than the symbols.

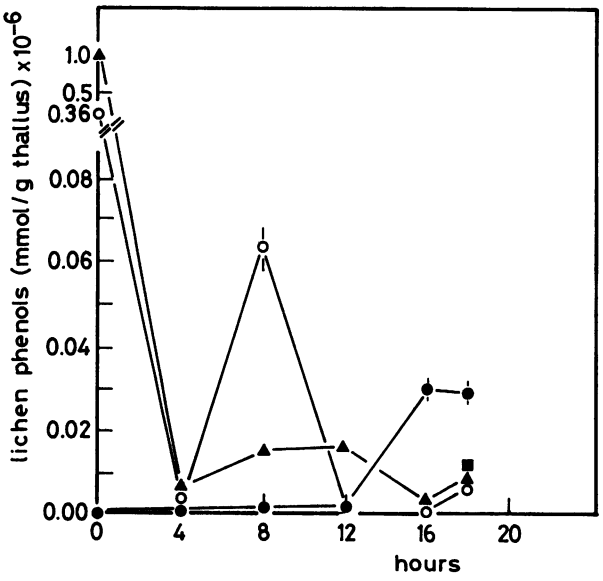

FIG. 5. Time course of lichen phenolics in cell-free extracts of *E. prunastri* incubated on 40 μM cycloheximide in 0.1 M Tris-HCl buffer (pH 9.15). Symbols are the same as in Figure 4. Values are the mean of three replicates. Vertical bars give SE where larger than the symbols.

rhythmic and reverse behavior of both enzymes. When inducible arginase reaches its maximum value of activity (at 6 h), the constitutive form is still undetectable; moreover, the decrease in the inducible enzyme is accompanied by the appearance of the constitutive one. It is important to emphasize that, in all the cases, inducible arginase shows a level of activity 20-fold higher than that obtained for the preexistent form. It could be possible that both enzymes were interconvertible forms of one another.

Table III. *Effect of Lichen Acids in the Cell-Free Extracts (a) or in the Thallus (b) on the Activity of the Constitutive Arginase of E. prunastri*

Lichen Acid	Reaction Rate	V^+/V^-
	$\mu mol \cdot min^{-1}$	ratio[a]
(a)	0.13 ± 0.02	
Evernic acid (1.6 nM)	0.15 ± 0.04	1.14
Usnic acid (1.4 nM)	0.14 ± 0.02	1.04
Atranorin (1.46 nM)	0.16 ± 0.03	1.20
Evernic acid (1.6 nM) + usnic acid (1.4 nM) + atranorin (1.46 nM)	0.21 ± 0.06	1.57
(b)		
Evernic acid (17.2 μM) + usnic acid (214.0 μM) + atranorin (6.6 μM) + chloroatranorin (12.0 nM)	0.19 ± 0.05	1.50
Evernic acid (19.4 μM) + usnic acid (226.0 μM) + atranorin (5.2 μM) + chloroatranorin (14.8 μM)	0.26 ± 0.07	2.00

[a] The ratio between reaction rate in the presence (V^+) and in the absence (V^-) of lichen acids.

In this way, they could regulate the production of urea in the lichen. This fact, which needs to be confirmed, is symptomatic because the mol wt of 330,000, estimated for the heavy arginase, is approximately twice that of 180,000 reported for the light arginase by Legaz and Vicente (12).

The K_m value of 2.5 mM estimated for the constitutive arginase is 12 times higher than the K_m value described for the inducible form (10). This implies that the constitutive arginase has 12 times less affinity for its substrate. There are certain similarities and differences between the two forms of the enzyme, in addition to the different optimal pH values and mol wt previously described. For instance, agmatine behaves as a noncompetitive inhibitor of the inducible arginase (26), whereas it is an activator of the constitutive enzyme. In contrast, both forms of the enzyme are activated by putrescine and L-ornithine and inhibited by urea, although the latter is a competitive inhibitor of the inducible arginase and a mixed inhibitor of the constitutive form. As has been demonstrated by Vicente *et al.* (27), the carbamyl group of urea behaves as a very strong inhibitor of many hydrolases of L-arginine catabolism. Thus, when this function is removed from the molecule, products of arginase action, such as L-ornithine or even putrescine, do not inhibit these enzymes. These substances would activate arginase to make the hydrolytic pathway more active than the decarboxylating one. This mechanism could also explain the importance of urea production by any of the enzymes. When L-arginine decarboxylase acts, both agmatine and putrescine are produced and could regulate arginase. Another difference, even more significant, is the fact that phenolics acids promote inactivation of inducible arginase (13), whereas these substances behave as activators of the constitutive arginase (Table III). Although these phenolics have been mainly described as inactivators of several enzymes (14, 16), sometimes phenolics behave as activators of others. This phenomenon has been reported for laccase activity of *Agaricus bisporus* and *Pleorotus ostreatus* (6). Furthermore, the activation or the inactivation of laccase activity is dependent on the phenolic concentration. Lichen phenolics are activators of the constitutive arginase at the concentration found in buffered extracts which contain this enzyme activity. Thus, it appears that the decrease of constitutive arginase activity at 18 h incubation (Fig. 1) can not be explained by the accumulation of those compounds, as has been reported for inducible arginase (13).

It seems clear, however, that *E. prunastri* thallus contains two

different forms of arginase, being that the regulation of each is completely distinct. Whereas the inducible enzyme has a K_m value 12 times lower and is inhibited by phenolics, the constitutive form shows less affinity for its substrate and it is activated by lichen phenols. It is posible that the decrease in the enzyme activity found up to 18 h incubation may be due to a product inhibition by urea or to the accumulation of monocyclic precursors of lichen phenolics, an action which would be different from that of the bicyclic units, although this hypothesis needs to be confirmed.

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