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PROPERTIES OF AN ARGINASE FROM THE COTYLEDONS OF *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; seeds; cotyledons; arginase; germination; Mn^{2+}; metal ions; kinetics.

Abstract—In the cotyledons of *Phaseolus vulgaris*, the arginase activity reached a maximum at about 2–3 days of seed germination; thereafter, it began to decrease. The enzyme was partially purified and characterized. Canavanine was not a substrate and the K_m values for arginine were 45 ± 2 mM at the optimum pH of 9.6 and 93 ± 5 mM at pH 7.5. Several metal ions activated the enzyme to a different degree ($Mn^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+}$), but the K_m and K_i values were the same for all the metal activated enzymes. Reactivation of fully inactivated enzyme by Mn^{2+} followed hyperbolic kinetics with a K_d value of 0.47 ± 0.05 μM.

INTRODUCTION

Arginase (L-arginine urea amidino hydrolase, EC 3.5.3.1) catalyses the hydrolysis of L-arginine to L-ornithine and urea. This enzyme, which requires a bivalent metal ion, specially Mn^{2+}, for full activity and structural stabilization [1–3], serves several functions in living organisms [4–7]. In plants cells, it is mainly involved in the production of proline and glutamate [6] and also of polyamines [7], which are important in various physiological processes in these species [8, 9].

Although there is much information on arginases from animals [1–5, 10] and some microorganisms [11–15], much less is known about the enzyme from plants [16–19]. The metal ion requirement and the kinetic properties of the enzyme from the cotyledons of germinating seeds of *Phaseolus vulgaris*, were examined and compared with the properties of other arginases. The results are discussed in connection with the use of arginine as a source of nitrogen during seed germination [6].

RESULTS AND DISCUSSION

Changes in arginase activity during germination and isolation of the enzyme

As shown in Fig. 1, the arginase activity in the cotyledons of *P. vulgaris* reached a maximum at about two to three days of germination and thereafter, it began to decrease; at day 5, the arginase activity was similar to that determined at the first day of germination. Even though metal ions were not added during germination or preparation of the homogenates, significant arginase activity was detected in assays performed in the absence of added Mn^{2+}. It is, therefore, clear that the Mn^{2+} content of the seeds of *P. vulgaris* was enough to ensure a significant arginase activity, but not for full activation of the enzyme during germination. In agreement with this, when germination was performed on filter papers impregnated with a solution of 1 mM manganese chloride, the arginase activity was essentially the same when assayed in the absence or presence of added Mn^{2+}. On the other hand, the time course of activity was the same as that observed during germination in the absence of added Mn^{2+}. Arginase activity also increases during germination in *Glycine max* [18] and *Pisum sativum* [20], but in these cases the effects of Mn^{2+} were not examined.

In the light of the results shown in Fig. 1, arginase was partially purified from cotyledons obtained after three days of germination and, for simplicity, germination was performed in distilled water. Species differing in electrical charge or molecular size were not detected by the chromatographic procedures described in the Experimental, and the specific activity of the resulting partially purified preparation was 40 nkat mg^{-1} of protein. This preparation was used to characterize the enzyme. Recently, information of great potential physiological significance was obtained from studies of arginase in homogenates of mouse liver [10] and rat liver cytosols [21]. One interesting suggestion was that the sensitivity of arginase to activation by Mn^{2+} would be altered during purification of the enzyme [10]. It is, thus, important to note that the same properties described below were observed for arginase in the partially purified preparation and the homogenate of cotyledons of *P. vulgaris*.

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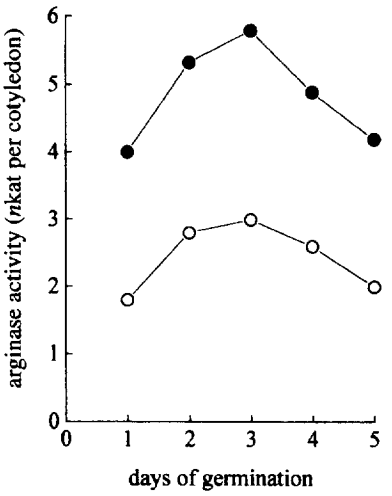

Fig. 1. Change in arginase activity in the cotyledons of germinating seeds of *Phaseolus vulgaris*. At the indicated times, the arginase activity in the supernatant of the homogenates, was determined both in the absence of added Mn^{2+}(○) and after activation with 2 mM $MnCl_2$ (●). Activation was performed as described in the Experimental. Values are the mean of duplicate assays in each of two separate experiments.

Table 1. Effect of metal ions on arginase from the cotyledons of *Phaseolus vulgaris**

Enzyme species and metal ion added	Enzyme activity (%)
(*A*) *Native enzyme*	
Mn^{2+}	100
Co^{2+}	80
Ni^{2+}	40
Cd^{2+}	35
Zn^{2+}	0
(*B*) *Inactivated enzyme*	
No addition	0
Mn^{2+}	100
Co^{2+}	70
Ni^{2+}	30
Cd^{2+}	25

*The enzymes were incubated for 10 min at 37° with the metal ions at a concentration of 2 mM (except for Zn^{2+}, used at 1 mM) in 10 mM Tris–HCl (pH 7.5), and then assayed for enzymatic activity. Enzymes activated in this manner expressed maximal activities. In both experiments, 100% represents the activity of the corresponding Mn^{2+}-activated enzyme species.

Optimum pH

The optimum pH of the enzyme was 9.6 and 50% of the maximum activity was observed at pH 8.7. This optimum pH is very similar to those reported for other arginases, which are in the range 9–10 [16, 19, 22, 23].

Effects of metal ions on arginase activity

The enzyme was fully inactivated by incubation with 50 mM EDTA for 30 min at 37°, followed by dialysis for 24 hr against 5 mM Tris–HCl (pH 7.5) and then reactivation by several metal ions was attempted. As shown in Table 1, the order of decreasing catalytic efficiency was $Mn^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+}$. Even though all these metal ions have been considered as potential activators of arginase [1, 4], most of the studies have examined the effects on manganese-activated arginase, making it difficult to clarify whether a particular metal ion has any activating effect on the enzyme. As an example, Kaysen and Strecker [4] concluded that Co^{2+}, Cd^{2+} and Ni^{2+} all decreased the enzymatic activity of rat kidney arginase. From the results shown in Table 1, we may conclude that the decreased activity is the result of the replacement of Mn^{2+} by the other, less potent activators of the enzyme.

All the metal-activated species were completely inhibited by 1 mM Zn^{2+}. This metal ion, which is also inhibitory to several arginases [4, 11, 21], is required for the maintenance of the quaternary structure of *Saccharomyces cerevisiae* arginase [13], but this characteristic is apparently unique for the yeast enzyme.

Kinetics of Mn^{2+} interaction with arginase

The activation of the fully inactivated enzyme by Mn^{2+} followed hyperbolic kinetics, indicating the absence of binding with different affinities. From the data obtained, a K_d value of 0.47 ± 0.05 μM was calculated. Most of the studies reported to date have used total and not free Mn^{2+} concentrations, and therefore a direct comparison with our results is not possible. In any case, even though relatively few comparable data are available, they are also in the micromolar range [2, 10, 11, 21, 24]. Examples are the K_d values of 0.08 μM for mouse liver arginase [10], and 0.1 μM for the enzyme from the bivalve *Semele solida* [24]. Unfortunately, there is no information with respect to other plant arginases.

Kinetic studies of the arginase reaction

At all the pH values examined (pH 7.5–10), Michaelis–Menten kinetics was observed. The K_m values for arginine were 45 ± 2 mM and 93 ± 5 mM at pH 9.6 and 7.5, respectively. These K_m values are considerably higher than values reported for most arginases [2, 4, 10, 21], including the enzymes from *Evernia prunastri* [25] and the cotyledons of *Glycine max* and *Canavalia ensiformis* [26]. Exceptions are the K_m values of 131 mM for arginase from *Neurospora crassa* [15] and 83 mM for the enzyme from the axes of *Glycine max* [18]. Of special interest, in connection with our present studies, is the report that the K_m value of the enzyme from the cotyledons of *Canavalia lineata* increases from 30 mM at pH 9 to 82 mM at pH 8 [19].

Canavanine, the guanidooxy structural analogue of arginine, was not hydrolysed by *P. vulgaris* arginase and, as occurs with other arginases [4, 27], lysine was

a competitive inhibitor with respect to arginine. The inhibition was found to be linear competitive, with a K_i value of 27 ± 2.5 mM at pH 9.6. Of the other amino acids tested, only modest inhibitory effects were produced by proline and branched chain amino acids, which are potent inhibitors of the enzyme from other sources [5, 28]. For this reason, a detailed analysis of the inhibition by these amino acids, was not performed.

Finally, we also compared the kinetic properties of the various enzyme–metal complexes of arginase from *P. vulgaris*. In spite of important differences in maximum velocities, the values of K_m for arginine and of K_i for lysine inhibition were independent of the type of metal ion bound to the enzyme. This agrees with our previous suggestion, derived from studies of the enzyme from the sea mollusc *S. solida*, that the main function of the metal ion is in the hydrolysis of the enzyme-bound substrate and not in binding of arginine to arginase [24]. In this connection, that the environment in the coordination sphere of the manganese is not significantly altered by binding of arginine to the enzyme, is also indicated by the observation that the enhancement factor (ε) of proton relaxation rates is only slightly reduced (from 8 to 7.7) by binding of arginine to the enzyme–manganese complex of rat liver arginase [1].

Our results would be of importance for the understanding of the role played by arginase during seed germination in *P. vulgaris*. Thus, the increased levels of arginase activity and the high K_m value of the enzyme for the substrate would be advantageous for the use of arginine as a source of nitrogen, which is known to be an important event during this physiological process [29–31]. In fact, it is known that arginine is a major form of storage N in many plants, constituting up to 40% of the N in seed protein [29]. On the other hand, that arginine is incorporated into seed protein and also metabolized via the arginase reaction, has been shown by tracer kinetic studies of developing seed cotyledons (8). In this connection, it is known that the activity of ornithine aminotransferase, a key enzyme in glutamate production from arginine, also increases in cotyledons of germinating seeds [6]. Because of the high K_m value for arginine, the arginase activity would be largely regulated by changes in substrate concentration in germinating seeds of *P. vulgaris*.

EXPERIMENTAL

Chemicals and plant materials. Chemicals were obtained from commercial sources (most from Sigma, St Louis, MO, USA) and were the purest available. Seeds of *P. vulgaris* were obtained from local farmers.

Germination and partial purification of arginase. Dry seeds of *P. vulgaris* were washed thoroughly with H_2O and then transferred to plastic trays for germination in the dark at 37°, on filter papers impregnated with H_2O or 1 mM $MnCl_2$. The period between the beginning of germination and the moment the cotyledons were used for the experiments, is referred to as 'days of germination'. Cotyledons were homogenized with 10 mM Tris–HCl (pH 7.5), containing 100 mM KCl and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and, after centrifugation at 27000 *g* for 15 min, the supernatants were retained.

To obtain a partially purified enzyme, the supernatant was fractionated with $(NH_4)_2SO_4$ (35–70% satn). After centrifugation at 27000 *g* for 15 min, the ppt was dissolved in 5 mM Tris–HCl (pH 7.5), dialysed against the same buffer soln and then chromatographed on DEAE-Sephadex. The column was equilibrated with 5 mM Tris–HCl (pH 7.5) containing 2 mM $MnCl_2$ and eluted with a linear gradient of KCl (0–0.5 M). A single peak of activity, eluting at about 0.15 M KCl, was detected. Active fractions were pooled and concd with $(NH_4)_2SO_4$ (70% satn). After centrifugation at 27000 *g* for 15 min, the ppt was dissolved in 25 mM Tris–HCl (pH 8), containing 100 mM KCl and 2 mM $MnCl_2$ and finally chromatographed on a Sephadex G-200 column equilibrated and eluted with the same buffer soln. Pooled fractions, corresponding to the single peak of arginase activity, were concd with polyethyleneglycol (20% (w/v) in 5 mM Tris–HCl buffer pH 7.5).

Enzyme and protein assays. Arginase activity was determined by measuring the formation of urea [32] from arginine and protein by the method of Ref. [33]. Routinely, the enzyme activity was assayed in 50 mM glycine–NaOH (pH 9.6). Buffers used in kinetic studies were 50 mM Tris–HCl (pH 7.0–8.7) and 50 mM glycine–NaOH (pH 8.7–10.0). For the assay in the presence of added Mn^{2+}, the homogenate or the partially purified enzyme were pre-incubated with 2 mM $MnCl_2$ in 10 mM Tris–HCl (pH 7.5) for 10 min at 37° and the same metal ion concn was maintained during the incubation with the substrate. Kinetic data were analysed by double reciprocal plots and computer-fitted to the appropriate rate equations.

Affinity of Mn^{2+} binding. Fully inactivated arginase, obtained as described in the Results and Discussion section, was incubated with varied concns of free Mn^{2+} in 10 mM Tris-HCl (pH 7.5), containing 100 mM KCl and 10 mM nitrilotriacetic acid (NTA) as a metal ion buffer [34]. After equilibration for 10 min at 37°, arginase activity was determined and the K_d value for Mn^{2+} interaction with arginase was obtained from a double reciprocal plot of initial velocity versus free Mn^{2+} concns. Free Mn^{2+} concns were calculated with the aid of a computer program written in BASIC; data needed for the calculations (dissociation constant for the NTA–Mn complex and pK_{a_3} for NTA) were obtained from the literature [35].

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