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Purification and Properties of Arginase from Soybean, *Glycine max*, Axes

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ABSTRACT

Arginase (EC 3.5.3.1) was purified to homogeneity from cytosol of soybean, *Glycine max*, axes by chromatographic separations on Sephadex G-200, DEAE-sephacel, hydroxyapatite, and arginine-affinity columns. The molecular weight of the enzyme estimated by pore gradient gel electrophoresis was 240,000, while sodium dodecyl sulfate polyacrylamide gel electrophoresis gave a single band at the molecular weight of 60,000. The optimal pH for activity was 9.5 and the K_m value was 83 millimolar. The enzyme was stimulated by polyamines such as putrescine.

Earlier results (24) indicated that, in plants, putrescine is derived from arginine via agmatine, the product of arginine decarboxylase, but ornithine decarboxylase is not present in significant amounts. More recently, however, ornithine decarboxylase has been found to vary considerably according to the species, tissues, and physiological state (2, 7, 9, 12, 17, 21, 29). In the case of putrescine derived from ornithine, arginase (EC 3.5.3.1) which catalyzes the hydrolysis of L-arginine into L-ornithine and urea seems to be possibly responsive for the putrescine biosynthesis. In animals, much is known about this enzyme, especially in the urea cycle (5, 13, 20). In contrast, far less is known about the properties of the enzyme in higher plants (4, 6, 19, 27, 28). Although the enzyme has been isolated and characterized from cotyledons of soybean (*Glycine max*) (8), no properties of the purified enzyme from the axes of soybean have been reported.

The present paper describes the purification to apparent homogeneity of arginase from axes of soybean and reports some of the properties of the purified enzyme. The purification procedure for this enzyme now allows for further examination of other properties.

MATERIALS AND METHODS

Plant Materials

Seedlings of soybean (*Glycine max*) were grown in plastic trays at 25°C for 4 d in the dark. All plants were watered daily. Samples were taken at intervals throughout seedling development and the cotyledons and embryonic axes were manually separated. The embryonic axes contain hypocotyl hook, hypocotyl, and root.

Enzyme Assay

The assay for arginase was based on the formation of urea from arginine. The reaction mixture, consisting of 10 mM Tris (pH 9.5), 1 mM $MnCl_2$, 125 mM L-arginine (pH 9.5), and enzyme solution in a total volume of 1.0 mL, was incubated for 30 min at 37°C. The reaction was initiated by adding the enzyme and terminated by addition of 0.1 mL 50% TCA. Protein was removed by centrifugation, and the urea content in the supernatant was measured colorimetrically by the modified method of Archibald (20). One unit is defined as the amount of enzyme producing 1 μmol urea per min. The arginase activity was a linear function of both incubation time and concentration under these conditions. Boiled enzyme preparations were used as the control. In analyzing the effects of test compounds on arginase activity, assays were conducted in the presence of a given concentration of test compound and the results compared with control assays.

Protein Determination

The protein content of each fraction was determined by the method of Lowry *et al.* (16), with bovine serum albumin as the standard.

Enzyme Purification

Purification of the enzyme was carried out at 0 to 4°C. Plant axes (300 g) were harvested and immediately ground in a chilled electric mixer with 500 mL of 50 mM Tris (pH 7.5) containing 1 mM $MnCl_2$ and 10% glycerol. The homogenate was filtered through four layers of gauze and clarified by centrifugation (13,000 *g*, 30 min). The supernatant was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and stirred at 4°C for 5 h. The solution was then centrifuged and the pellet was discarded. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$ and treated as above, except that the pellet was retained. The pellet containing the enzyme was dissolved with 30 mL of 10 mM Tris (pH 7.5) containing 1 mM $MnCl_2$ and 10% glycerol and then dialyzed twice against 1 L of the same buffer. After centrifugation (13,000 *g*, 15 min), the supernatant was applied to a Sephadex G-200 column (2.5 × 80 cm) equilibrated with 10 mM Tris (pH 7.5) containing 1 mM $MnCl_2$ and 10% glycerol. The enzyme was eluted with the same buffer and the flow rate was 2 mL per 10 min. Active fractions were applied to a DEAE-Sephacel column (Sigma) (2.5 × 15 cm) previously equilibrated with 10 mM Tris (pH 7.5) containing 10% glycerol. The column was washed with the same buffer. Elution of the enzyme was achieved with 1 L of a linear gradient of 0 to 500 mM potassium chloride made up in the equilibration buffer. Active fractions were pooled and applied on a hydroxyapatite column (Bio-Rad) (2.5 × 5 cm) previously equilibrated with 10 mM Tris (pH 7.5) containing 10% glycerol. The column was

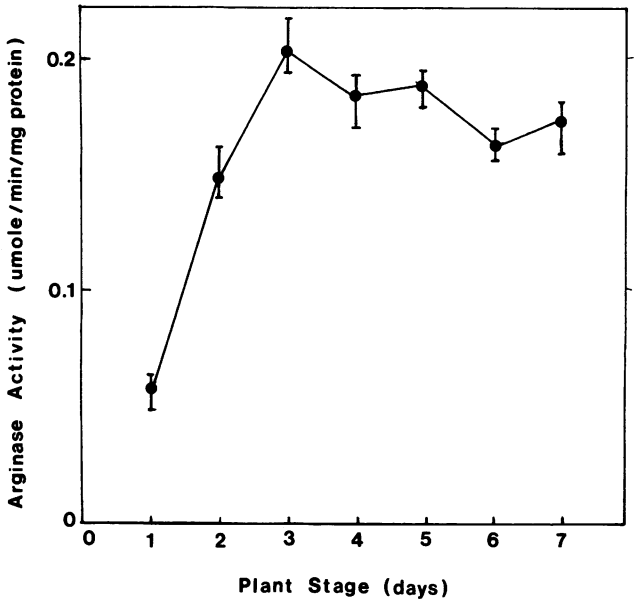

Figure 1. Change in arginase activity in soybean axes following imbibition in darkness.

washed with the same buffer. The enzyme was eluted with a 600 mL linear gradient of 0 to 300 mM potassium phosphate in the equilibration buffer. Active fractions were pooled and dialyzed overnight against the equilibration buffer. The dialysate applied on an arginine linked Sepharose 4B column (Pharmacia) (1.8 × 15 cm) was equilibrated with 10 mM Tris (pH 7.5) containing 1 mM $MnCl_2$ and 10% glycerol. The column was washed with the equilibration buffer. Finally, the enzyme was eluted by increasing the molarity of Tris (pH 7.5) from 10 to 500 mM. Active fractions were pooled and dialyzed against 10 mM Tris (pH 7.5) containing 1 mM $MnCl_2$ and 10% glycerol. The dialysate was concentrated by centricon (Amicon Centricon 30).

Mol Wt Determination

The mol wt of the purified enzyme was estimated by gel filtration with a Sephadex G-200 column (Sigma) (1.2 × 90 cm) that had previously been calibrated with BSA (66,000), γ-globulin (150,000), and catalase (240,000).

Electrophoresis

SDS-PAGE was performed as described by Laemmli (14). β-Lactoglobulin (18,000), carbonic anhydrase (29,000), ovalbumin (45,000), and BSA (66,000) served as mol wt markers. The purified enzyme was treated with 1% SDS and 2-mercaptoethanol for 10 min at 100°C and then subjected to SDS electrophoresis on 12% gels. The native purified enzyme was subjected to native pore gradient gel electrophoresis on 5% to 20% gels. The gels were stained with Coomassie blue R-250 (Sigma).

RESULTS AND DISCUSSION

Change of Arginase Activity during Seedling Growth

Figure 1 shows the changes in arginase activity during germination and early growth of the soybean. At the onset of germination, enzyme activity was very low; thereafter, activity increased rapidly. Subsequently, enzymatic activity declined slightly as germination progressed.

Enzyme Purification

The results are summarized in Table I. The enzyme fraction was freed of ammonium sulfate and low mol wt protein by passing the solution through a Sephadex G-200 column (Fig. 2, curve A). Arginase was absorbed on a DEAE-Sephacel column and eluted at about 300 mM KCl (Fig. 2, curve B). The enzyme was eluted at 100 mM phosphate on the hydroxyapatite column. $MnCl_2$ forms precipitate in Tris buffer containing phosphate, hence in this step we did not use $MnCl_2$. Finally, this enzyme was specifically bound on an arginine Sepharose 4B column and was eluted at a high molarity of Tris (Fig. 2, curve D). In this step, we obtained the overlapping protein profile of arginase and another protein that seems to have affinity for arginine when the bound enzyme was eluted with arginine. Our procedure resulted in a purification of more than 150-fold relative to the crude extract. This is 30-fold lower than that reported for the *Neurospora* enzyme (3). However, 1.2 mg of the total protein were obtained from crude extract. Arginase isozymes that differ from the liver enzyme in catalytic, molecular, and immunological properties, was present in the kidney, small intestine, and lactating mammary gland (13). Arginase isozymes have not been reported in Iris bulbs, *Vicial faba* L., *Pisum sativum*, and Jerusalem artichoke tubers, whereas arginase has been (4, 14, 25, 28). We could not find any evidence of the presence of arginase isozymes in purification steps and purity tests including pore gradient gel electrophoresis. Such results are in good agreement with the previous reports (4, 14, 25, 28). We suggest that our purification protocol is effective, compared with other methods, and it may be useful in studies where purified

Table I. *Purification of Arginase from the Axes of Soybean (G. max)*

Fraction	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	*units*	*units/mg*	%	*-fold*
Crude extract	4869	2880	0.59	100	
$(NH_4)_2SO_4$ (40–60%)	1077	1440	1.34	50	2.3
Sephadex G-200	261.5	545	2.08	18.9	3.5
DEAE-Sephacel	44.7	364	8.14	12.6	13.8
Hydroxyapatite	17.6	292	16.59	10.1	28.1
Arginine-Sepharose 4B	1.2	108	90.00	3.75	152.5

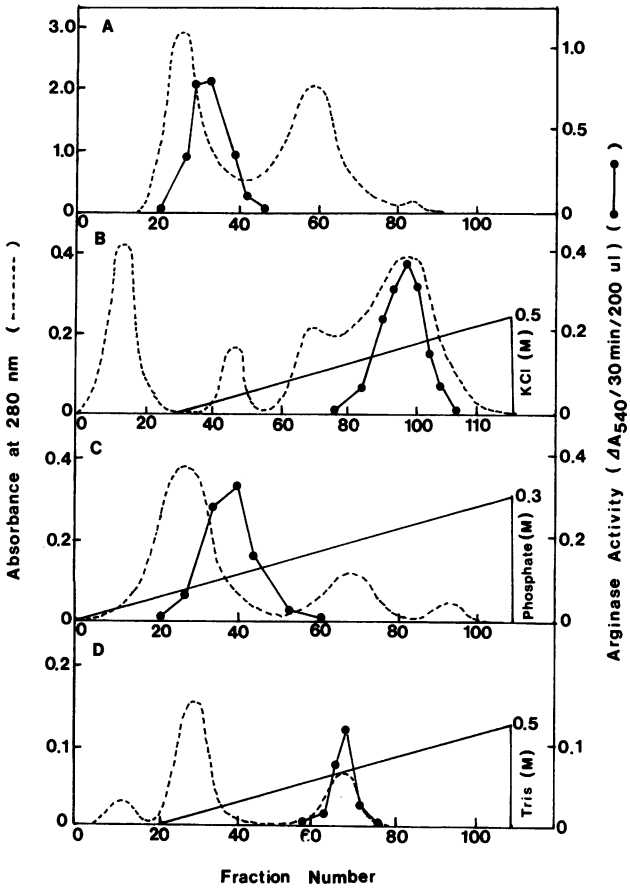

Figure 2. Profiles of protein and arginase activity from Sephadex G-200 (A), DEAE-Sephacel (B), hydroxyapatite (C), and arginine-Sepharose 4B (D) column chromatography.

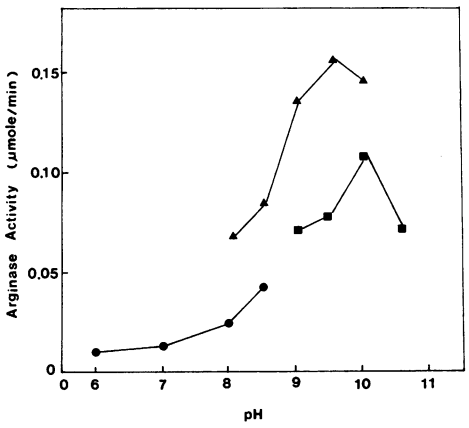

Figure 3. Determination of the optimum pH for arginase. The pH dependence of the enzymatic activity of arginase was determined by replacing the buffer used in the respective enzyme assays with 10 mM potassium phosphate buffer for pH values between 6.0 and 8.5 (●), 10 mM Tris buffer for pH values between 8.0 and 10.0 (▲), and 10 mM glycine buffer for pH values between 9.0 and 10.5 (■).

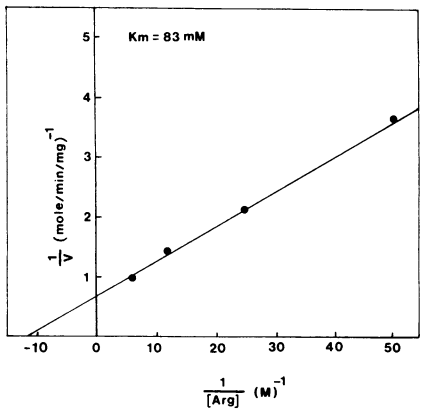

Figure 4. Lineweaver-Burk plot of initial reaction velocities (mol/min) for arginase measured at various concentrations of arginine.

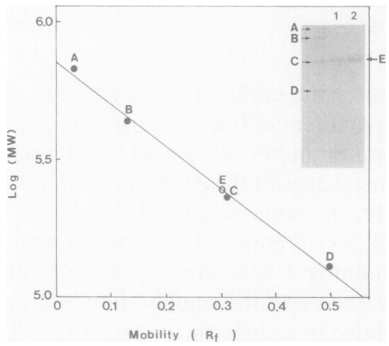

Figure 5. Determination of the mol wt of purified arginase by pore gradient gel electrophoresis. A, thyroglobulin (669,000); B, ferritin (440,000); C, catalase (232,000); D, lactate dehydrogenase (140,000); E, arginase. Lane 1, arginase (15 μg); lane 2, arginase (30 μg).

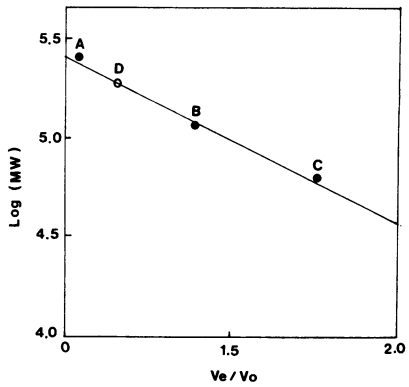

Figure 6. Determination of mol wt of soybean arginase by calibrated Sephadex G-200 gel filtration chromatography. A, catalase (240,000); B, γ-globulin (150,000); C, BSA (66,000); D, arginase.

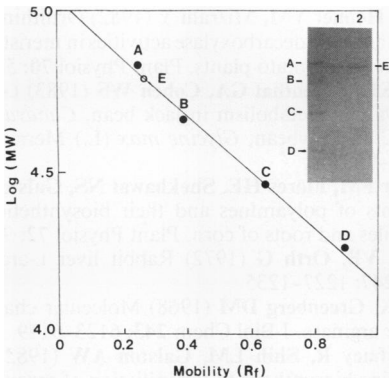

Figure 7. Subunit molecular mass for the purified soybean arginase was estimated by SDS-PAGE electrophoresis. A, BSA (66,000); B, ovalbumin (45,000); C, carbonic anhydrase (29,000); D, β-lactoglobulin (18,000); E, arginase. Lane 1, arginase (10 μg); lane 2, arginase (20 μg).

arginase is required for further enzyme studies, production of antibodies, etc.

Effect of pH

The activity of the purified arginase was determined as a function of pH by buffering the reaction mixture with potassium phosphate, Tris, and glycine from 6.0 to 10.5. The pH optimum for the enzyme was 9.5 (Fig. 3), that of *Evernia prunastri* is 9.1 (1).

Kinetic Properties

The effect of arginine concentration on the reaction velocity was determined (Fig. 4). The double reciprocal plot was turned out to be linear for all arginine concentrations. The enzyme possessed a K_m value of 83 mM, which is high when compared with those of rat liver (6 mM) and rabbit liver (1.3 mM), but low compared with that of *Neurospora crassa* (131 mM). In plants, the K_m value is high compared to those of *E. prustri* (2.5 mM), and soybean cotyledon (7–8 mM). In our pilot experiment, the method of ornithine determination was not suitable for arginase assay because an amount of ornithine was not in proportion to the increasing of the enzyme concentrations. But the release of urea from arginine by arginase was in a good proportion to an amount of the enzyme. Thus, the assay for arginase was carried out determining urea. The velocity versus substrate concentration curve was a hyperbola and K_m value of this plot was in a good agreement with that of Lineweaver-Burk plot.

The K_m value of soybean axes arginase is not similar to that of cotyledon enzyme. It is not clear that isozyme exists in soybean because there is no further information on the cotyledon enzyme except K_m value determined using the method of ornithine determination (8). We tried to see if there is any isozyme in soybean axes during the purification, but there was a absence of the enzyme.

Stability

The purified enzyme was stable at 4°C for at least 1 month. No loss of activity of the purified enzyme occurred for 10 min at 37°C. However, after 10 min at 70°C, the enzyme activity was reduced to 50%, and the enzyme was completely inactivated at 80°C after 10 min. Pumpkin cotyledon enzyme was quite resistant to heating and could be heated for 5 min at 60°C with little loss of activity (23), while soybean arginase activity did not lose for 10 min at 60°C. Thus, the soybean enzyme also has a resistance for heat.

Mol Wt and Subunits

The apparent mol wt of the native enzyme was determined to be 240,000 and 220,000 by pore gradient gel electrophoresis (Fig. 5) and Sephadex G-200 gel filtration respectively (Fig. 6). This value is large, compared with those of rat liver (mol wt = 118,000 [11]), rabbit liver (mol wt = 110,000 [10]), but smaller than those of *N. crassa* (mol wt = 266,000 [3]) and *E. prunastri* (mol wt = 330,000 [1]). SDS gel electrophoresis showed a single band indicating a single mol wt for the subunit (Fig. 7). From these data, it is suggested that native soybean axis arginase is composed of four identical subunits.

Effect of Metal Ions

Table II presents the effects of various metal ions on the enzyme activity. Mn^{2+} is an absolute requirement for enzyme activity with 1 mM being optimal for enzyme activity. Mg^{2+}, Cu^{2+}, and Zn^{2+} cannot replace Mn^{2+}, even at concentrations in the range of 0.01 to 10 mM. Co^{2+} was somewhat better, giving 30% of the activity of Mn^{2+}. This manganese ion effect

Table II. *Effect of Metal Ions on Soybean Arginase*

Metal Ions	Concentration	Relative Activity
	mM	%
None		8
$MnCl_2$	1	100
$MgCl_2$	1	6
$CuCl_2$	1	3
$CoCl_2$	1	26
$FeCl_2$	1	1
$ZnCl_2$	1	4

Table III. *Effect of Polyamine on Soybean Arginase Activity*

Arginase was assayed in the presence of various concentrations of polyamine. Arginase is presented as percentage of control activity (no added polyamine).

Polyamine	Concentration	Relative Activity
	mM	%
Putrescine	0.1	104
	1	115
	10	146
Spermidine	0.1	119
	1	120
	10	127
Spermine	0.1	103
	1	113
	10	115

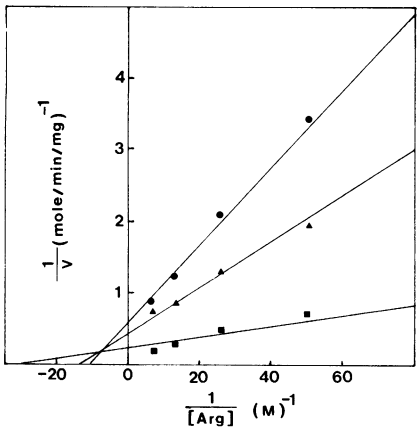

Figure 8. Double reciprocal plot for reaction of arginase with arginine and putrescine. Putrescine concentrations (mM) were: 0 (●), 1.0 (▲) and 10 (■).

on the enzyme is similar to that of the plant and animal enzymes (1, 11).

Effect of Polyamines

The effect of a polyamine on the arginase has previously been reported (1). In our experiment, putrescine increased the enzyme activity to 46% as the putrescine concentration increased (Table III). Especially, in the presence of putrescine, it decreased K_m value for arginine and increased V_{max} (Fig. 8). Polyamines have been shown to stimulate or inhibit the activities of several enzymes including activation of phosphorylase kinase (22) and protein kinase (18). Thus, the effects of these polyamines are not specific only for arginase. Although the exact mechanism by which putrescine acts on the enzyme is unclear, activation of arginase activity by putrescine may play a certain role of arginase activity.

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