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Purification and Characterization of Arginine Decarboxylase from Soybean (*Glycine max*) Hypocotyls

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Arginine decarboxylase (EC 4.1.1.19) was purified from soybean, *Glycine max*, hypocotyls by a procedure which includes ammonium sulfate fractionation, acetone precipitation, gel filtration chromatography, and affinity chromatography. Using this procedure, ADC was purified to one band in non-denaturing PAGE. The purified ADC has an M_r of 240 kDa based on gel filtration chromatography and is a trimer of identical subunits which has an estimated M_r of 74 kDa based on SDS-PAGE. ADC is active between 30 and 50°C and has a K_m value of 46.1 μM. ADC is very sensitive to agmatine or putrescine but not to spermidine or spermine. In the presence of 0.5 mM agmatine (or putrescine), the enzyme activity was inhibited by 70%. However, at the same concentration of spermidine (or spermine), the enzyme activity was inhibited by only 10–20%.

Key words: Agmatine — Arginine decarboxylase (EC 4.1.1.19) — Molecular weight — Polyamines — Soybean (*Glycine max*) hypocotyls.

Polyamines are found in all life forms studied and are involved in various physiological functions (Evans and Malmberg 1989). The biosynthetic pathway of the three most abundant polyamines, putrescine, spermidine, and spermine, is well known (Galston 1983). In plants, putrescine is synthesized via two different pathways. In one, ornithine decarboxylase (ODC) converts ornithine directly to putrescine (ODC pathway). In the other, arginine decarboxylase (ADC) converts arginine to agmatine, then the agmatine is converted to putrescine by a stepwise reaction involving iminohydrolase and putrescine synthase (or *N*-carbamoylputrescine amidocarboxylase) (ADC pathway) (Slocum et al. 1984). The putrescine is then converted to spermidine and spermine by sequential addition of an aminopropyl group. In addition to its role as a precursor of spermidine and spermine, it is well known that the putrescine content of a plant increases when the plant is placed under various stressful conditions (Flores and Galston 1984, Galston and Kaur-Sawhney 1990, Young and Galston 1983, 1984). Even though the function of putrescine in these stressed plants still remains to be understood, it has been reported in various systems that this increase in putrescine content is due to an increase in ADC activity (Flores and Galston 1984, Foster and Walter 1991, Reggiani et al. 1990). These reports suggest that regulation of ADC activity is one of the key steps in regulating polyamine synthesis in plants, especially when the plant is under stress.

To understand the regulatory mechanism of the activity of an enzyme, both characterization of the purified enzyme and cloning of the gene encoding the enzyme are necessary. The biochemical properties of ADC have been studied in several plants including oat, *Lathyrus sativas, Oriza sativa*, avocado fruit, and *Vicia fava* (Smith 1979, Ramakrishina and Adiga 1975, Choudhuri and Ghosh 1982, Winer et al. 1984, Matsuda 1984). The characteristics of the purified ADCs appear to differ from species to species. For example, the ADC from *Lathyrus sativus* is a hexamer of six identical subunits with a M_r of 220 kDa. In contrast, the ADC from rice coleoptile is a trimer and has an estimated M_r of 176 kDa (Reggiani 1994). The purified ADCs have also shown different sensitivities to agmatine, the product of decarboxylation, and putrescine, the end product of the pathway. Even though the molecular compositions and some biochemical properties of these purified ADCs differ, they share some common characteristics. They all require thiol compound and pyridoxal phosphate for their activity and stability. ADC cDNAs have also been cloned from several species. In the oat, ADC cDNA encodes a protein of 66 kDa (Bell and Malmberg 1990), which is processed into two polypeptides with M_r of 42 and 24 kDa linked with a disulfide bond (Malmberg et al. 1992). ADC cDNAs from tomato and pea encode proteins of estimated M_r of 55 and 78.5 kDa, respectively (Rastogi et al. 1993, Perez-Amodor et al. 1995). However, purification of ADC from these species has not been reported yet.

Based on the biochemical properties of the purified enzymes, the cloned cDNAs have been to study the regulation of ADC activity during various stress conditions. The results from these studies showed that the regulation mechanism of ADC activity is very complex and suggested that the mechanism might differ depending on the species. These results also suggest that it may be necessary to have both the purified ADC and the cloned gene from one species to understand the regulation mechanism in that particular species. However, up to now, there has been only one

Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase.

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such case, the oat, in which both purification of ADC and cloning of the gene have been achieved in the same species (Smith 1979, Bell and Malmberg 1990).

We have reported cloning of ADC cDNA from soybean, *Glycine max*, hypocotyls (GenBank U35367) and expression of the gene during early development and acid stress (submitted for publication). In this paper, we report the purification and characterization of the ADC.

Materials and Methods

Plant growth conditions—Dry soybean (*Glycine max*) seeds were germinated and grown in trays overlaing with three layers of moistened gauze at 25°C in the dark. For acid treatment, 5-d-old hypocotyls were incubated in 5 mM potassium phosphate solution (pH 3.0) with shaking for various periods at 26°C.

Preparation of crude extract—Five-day-old soybean hypocotyls were incubated in the acidic potassium phosphate solution for 2 h. The hypocotyls were washed with distilled water and homogenized in ice-cold Buffer A (50 mM potassium phosphate, pH 7.5, 5 mM EDTA, 0.3 mM 5'-pyridoxal phosphate (PLP), 5 mM DTT). After centrifugation (4,000 × *g*, 20 min, 4°C), the supernatant was recovered and recentrifuged at 15,000 × *g* with a Beckman JA20 rotor at 4°C for 30 min. The final supernatant was saved and used for further study. When examining the effect of pH on the enzyme activity, soybean hypocotyls were homogenized in buffer systems with different pHs. Na-citrate buffer (50 mM Na-citrate, 5 mM EDTA, 0.3 mM PLP, 5 mM DTT) was used between pH 4–6. Potassium phosphate buffer (50 mM potassium phosphate, 5 mM EDTA, 0.3 mM PLP, 5 mM DTT) was used between pH 6–9. Tris-HCl buffer (50 mM Tris-HCl, 5 mM EDTA, 0.3 mM PLP, 5 mM DTT) was used between pH 7–10.

Determination of ADC activity—Enzyme assay was carried out (Young and Galston 1983) in center well flasks containing filter paper (Whatman No. 2, 1 × 1.5 cm) soaked in 2 M KOH. All of the enzyme assays were carried out under the following conditions, except when examining the effects of pH. For the assay, 200 μl of the enzyme source was added to flasks containing 300 μl of enzyme reaction buffer (12.5 mM potassium phosphate pH 7.5, 1.25 mM EDTA, 2 mM DTT, 75 μM PLP) with 3.7 kBq L-[U-^{14}C] arginine (12 GBq mmol^{-1}) and 40 μM L-arginine. After 1 h at 37°C, the reaction was stopped by addition of 200 μl of 5% perchloric acid (PCA), and the reaction mixture was further incubated for 1 h at the same temperature to allow liberated $^{14}CO_2$ to be absorbed into the filter paper. The amount of $^{14}CO_2$ was determined by scintillation counting (Beckman LS6500). One unit of the ADC activity was determined as 1 nmol CO_2 h^{-1} (mg protein)$^{-1}$. The amount of protein in the enzyme source was determined by the method of Bradford (1976) using bovine serum albumin as a standard. When we examined the effect of pH on the enzyme activity, we used crude extract prepared with appropriate buffers (see above).

PAGE—For nondenaturing gel electrophoresis, the samples were concentrated to a small volume using the Centricon (Millipore) and separated on 7.5% gels. For SDS-PAGE, 5 volumes of pre-chilled (−20°C) acetone was added to each sample. After centrifugation, the pellet was washed with EtOH : ether (1 : 1) and resuspended in SDS sample buffer (Hames 1981).

Results

Purification of ADC—Five-day-old soybean hypocotyls treated with acid (5 mM potassium phosphate, pH 3) for 2 h were chosen as the source of ADC because of the high activity of the enzyme in this tissue (data not shown). The purification procedure of ADC from this tissue is summarized in Table 1.

Crude extract (Materials and Methods) was fractionated with solid ammonium sulfate. The protein precipitating at 15–30% saturation was recovered by centrifugation (15,000 × *g*, 30 min at 4°C) then resuspended in Buffer A. Pre-chilled acetone (−20°C) was gently added to the protein solution. The precipitate at 40–60% acetone (v/v) was recovered by centrifugation (5,000 × *g*, 15 min at 4°C) and resuspended in Buffer B (1% Tween-80 in Buffer A). After dialysis against Buffer B, the acetone precipitate was loaded on Sephadex G-200 (2.1 × 90 cm) equilibrated with Buffer B and eluted at 4 ml h^{-1} with the same buffer (Fig. 1a). The fractions with ADC activity (fraction No. 31–42) were pooled, concentrated, and further fractionated by a Sephacryl S-300 (2.0 × 114 cm) equilibrated with Buffer B with a flow rate of 4 ml h^{-1} (Fig. 1b). The active fractions were pooled (Fraction No. 30–36) and loaded onto an Arginine Sepharose CL-4B affinity column (1.5 × 10 cm, Pharmacia). The loaded column was washed twice, first

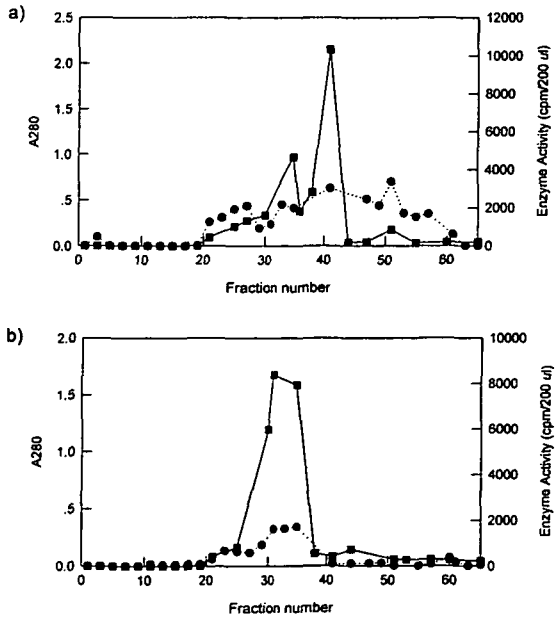

Fig. 1 Elution profile of soybean ADC activity on gel filtration chromatography. After acetone precipitation, the sample was loaded onto a Sephadex G-200 column and eluted. The fractions with enzyme activity were pooled, concentrated, and loaded onto a Sephacryl S-300 column (see text). (a) Sephadex G-200. (b) Sephacryl S-300. Symbols: ●, absorbance at 280 nm; ■, enzyme activity.

Table 1 Purification steps for ADC

Step	Total protein (mg)	Specific activity (units (mg protein)$^{-1}$)	Total activity (units)	Purification (fold)	Recovery (%)
Crude extract	2,680	1.4	3,830	1	100
$(NH_4)_2SO_4$ precipitation 15–30%	137	9.1	1,240	6.3	32.4
Acetone precipitation 40–60%	18.6	30.9	573	21.6	14.9
Sephadex G-200	3.87	98.4	391	68.8	9.9
Sephacryl S-300	1.54	93.8	145	65.6	3.8
Arginine-Sepharose CL-4B	0.092	575	57.6	402	1.5

with Buffer B, and then with Buffer B containing 0.1 M KCl. No ADC activity was detected in these washing steps. To elute the ADC from the affinity column, the column was washed with Buffer B containing 0.1 M KCl and 50

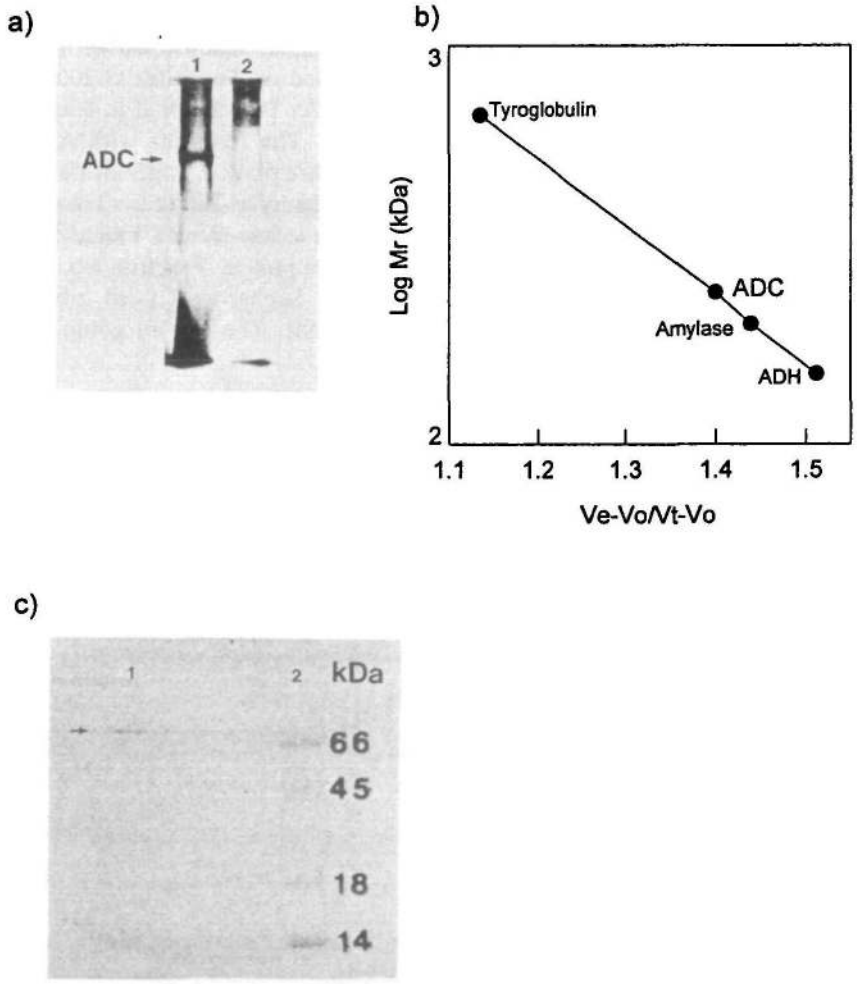

Fig. 2 Non-denaturing PAGE and determination of molecular weight of soybean ADC. (a) After arginine CL-4B affinity chromatography, the fractions with ADC activity (lane 1) and without ADC activity (lane 2) were pooled and concentrated (see text). 20 μl of the each concentrated sample was fractionated by 7.5% PAGE. The protein band was visualized by silver staining. (b) Molecular weight of the ADC was determined based on the elution profile of the Sephacryl S-300 gel filtration chromatography. The column was calibrated with marker proteins of known molecular weights. Marker proteins; tyroglobulin (669 kDa), amylase (200 kDa), alcohol dehydrogenase (ADH, 150 kDa). (c) SDS-PAGE of the purified soybean ADC. Lane 1, 0.2 μg of purified ADC. Lane 2, molecular weight markers, BSA (66 kDa), ovalbumin (45 kDa), lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The gel was fixed and stained with Coomassi-Brilliant Blue G.

mM arginine, and the eluates were separated into three fractions according to the order of elution: fraction A (7 ml), B (14 ml) and C (28 ml). The three fractions were dialyzed separately in Buffer B to remove the excessive arginine, and then concentrated. The ADC activity was detected only in fraction B, and a single band was observed when this fraction was analyzed by 7.5% nondenaturing gel electrophoresis (Fig. 2a). By this procedure, the ADC was purified 402 fold, and the specific activity was increased to 575 units (mg protein)$^{-1}$ with a yield of 1.5%.

Molecular mass of ADC—Molecular mass of the ADC was 240 kDa, based on the elution profile of the Sephacryl S-300 gel filtration chromatography (Fig. 2b). To examine the composition of the soybean ADC, fraction B from the affinity chromatography was analyzed by SDS-PAGE. In the gel electrophoresis, one band with an estimated M_r of 74 kDa was observed (Fig. 2c).

Effect of substrate concentration—The soybean ADC exhibited typical Michaelis-Menten kinetics with a K_m value of 46.1 μM and a V_{max} of 13.3 nmol CO_2 liberated h^{-1} (mg protein)$^{-1}$ (Fig. 3). This K_m value is higher than that of oat ADC (30 μM, Smith 1979) but lower than that of *Oriza sativa* (0.28 mM, Choudhuri and Ghosh 1982), avocado fruit (1.9 mM, Winer et al. 1984), or *Lathyrus sativus* (1.7 mM, Ramakrishina and Adiga 1975) ADC.

Effect of temperature and pH—The soybean ADC displayed a broad range of optimal temperatures (30–50°C, data not shown) and was stable for 30 min at 50°C. However, the enzyme activity was decreased by 50% in 10 min at 60°C and by 95% in 5 min at 70°C (Fig. 4a). Depending on the buffer system used in the preparation of crude extract, ADC showed different optimum pHs (Fig. 4b). In the standard assay condition (Buffer A), ADC showed maximal activity at pH 7 and 7.5. In Tris-HCl buffer, the maximum activity was observed at pH 8. In Na-citrate buffer (pH 4–6), the enzyme activity decreased below 40% of the control activity.

Effect of various amines—It has been known that ADC is inhibited by various amines in vitro but the degree of inihibition varied greatly, depending on the source of the enzyme and on the amines treated (Smith 1979, Matsuda 1984, Ramakrishina and Adiga 1975, Suresh et al. 1978). Both agmatine and putrescine failed to inhibit soybean ADC at 0.01 mM (Fig. 5). In the presence of 0.1, 0.5, and 1.0 mM agmatine, however, the ADC activity was inhibited by 25, 70, and 80%, respectively. In the presence of 0.1, 0.5, and 1 mM of putrescine, the enzyme was inhibited by 25, 65, and 75%, respectively. In the presence of 5 mM agmatine, the enzyme activity decreased to 10% of that of the control.

Unlike agmatine and putrescine, spermidine and spermine did not inhibit the ADC activity significantly at low concentrations (Fig. 5). The enzyme was not inhibited by 0.1 mM of spermidine or spermine. In the presence of 0.5,

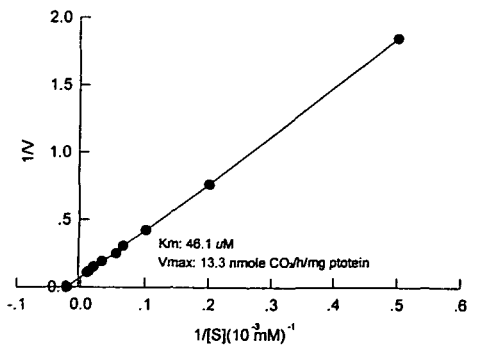

Fig. 3 Determination of K_m and V_{max} of the soybean ADC. The activity of soybean ADC partially purified by acetone precipitation was measured with various concentrations of L-arginine.

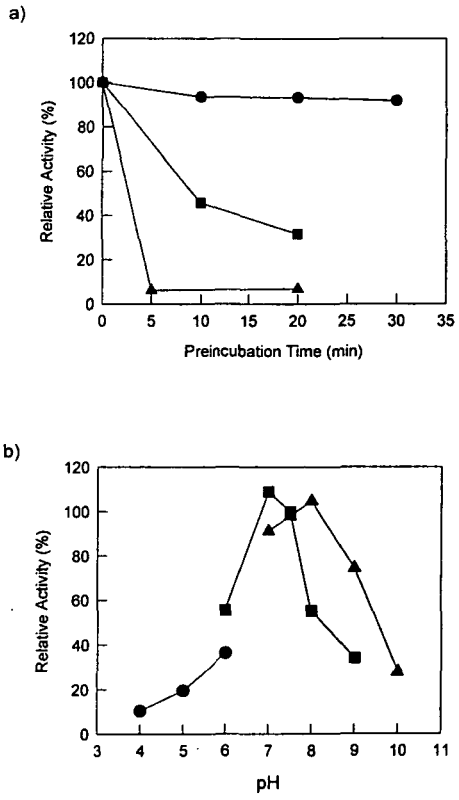

Fig. 4 (a) Effect of temperature on the soybean ADC. Soybean ADC partially purified by acetone precipitation was incubated at different temperatures for up to 30 min. After the preincubation, the enzyme activity assay was carried out at 37°C in potassium phosphate buffer (pH 7.5) as described in Materials and Methods. The enzyme activity at 37°C without the preincubation was used as a control (100%). (b) Effect of pH on the ADC activity. Crude extracts were prepared with different buffers, and the ADC activity at different pHs was determined. The ADC activity at pH 7.0 in 50 mM potassium phosphate buffer was used as a control (100%). ●, 50 mM Na-citrate buffer; ■, 50 mM potassium phosphate buffer; ▲, 50 mM Tris-HCl buffer.

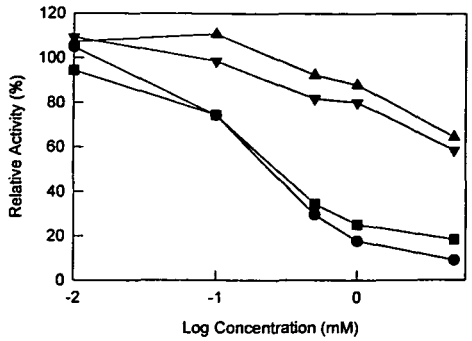

Fig. 5 Soybean ADC is sensitive to agmatine and putrescine. The enzyme assay was carried out at 37°C in potassium phosphate buffer (pH 7.5) containing the amines. The enzyme activity without any amines was used as a control (100%). ●, agmatine; ■, putrescine; ▼, spermidine; ▲, spermine.

1, and 5 mM of spermidine, the enzyme activity was inhibited by 5, 10, and 25%, respectively. The effect of spermine on the ADC activity was similar to that of the spermidine.

Effects of cations—It has been shown that cations generally inhibit ADCs from various sources. We examined the effect of various cations on the soybean ADC activity (Table 2), and found that ADC activity was inhibited in their presence. This inhibitory effect was more evident when divalent cations were added into the reaction mixture. Monovalent cations (Na^+ and K^+) had no effect on the ADC activity at low concentrations (<1 mM). The ADC lost its activity by 20 and 50% in the presence of 10 and 100 mM of the monovalent cations, respectively. At low concentrations (<1 mM), divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) did not inhibit the enzyme significantly either. However, the ADC activity decreased below 60 and 10% of the control when the divalent cations were added to 10 mM and 100 mM, respectively. Among the three cations tested, Mn^{2+} had the most significant inhibitory effect on the enzyme.

Table 2 Effect of various cations on the ADC activity

Cation	Relative activity of ADC (%)[a]		
	1 mM	10 mM	100 mM
Na^+	95.8±2.5	84.8±6.4	50.0±8.2
K^+	73.9±8.7	79.9±3.2	51.8±2.2
Mg^{2+}	76.3±5.7	58.2±4.7	12.7±4.2
Ca^{2+}	90.1±1.6	51.8±5.3	4.2±0.3
Mn^{2+}	77.2±7.5	31.5±3.8	3.2±1.1

[a] Data represent an average of two sets of independent experiments.
The enzyme activity without any cations was used as a control (100%).

Discussion

Soybean ADC was purified from acid treated 5-day-old hypocotyls to homogeniety by a procedure which includes ammonium sulfate fractionation, acetone precipitation, gel filtration chromatography, and affinity chromatography (Table 1). In this procedure, we used Buffer B, which contains 1% Tween-80, to stabilize the enzyme after the acetone precipitation step (Barnett and Kazarinoff 1984, Miyamoto et al. 1989). Under these conditions, the enzyme activity was stable up to 6 months if stored at −80°C. By this procedure, we purified ADC 402 fold and visualized a single band by non-denaturing PAGE (Fig. 2a).

The optimal temperature and pH of the soybean ADC are similar to those of other reported ADCs except avocado ADC which is thermostable (Winer et al. 1984) (Fig. 4a, b). The soybean ADC activity was inhibited in the presence of high concentrations of metal ions (Table 2), but none of the five examined metal ions showed specific inhibition. These results suggest that the inhibitory effect of these ions might be a result of increased ionic strength. It has been reported that Mn^{2+} (<0.1 mM) promoted ADC from *Lathyrus sativas* (Ramakrishna and Adiga 1975) and that ADC from *E. coli* (Wu and Morris 1972) required Mg^{2+} as a cofactor with PLP. However, these divalent cations had no effect on the soybean ADC at low concentrations. These results suggest that the soybean ADC may not require these metal ions for its activity.

The estimated molecular weight of the purified enzyme was 240 kDa (Fig. 2b). This is similar to that of *Lathyrus sativas* ADC but is greater than that of *Avena sativa*, at 195 kDa (Smith 1979), and *Oriza sativa*, at 176 kDa (Reggiani 1994). When the purified enzyme was fractionated on SDS-PAGE, we observed one band with an estimated M_r of 74 kDa (Fig. 2c). This is very close to the calculated M_r of the soybean cDNA clone, 74.5 kDa (Nam et al. 1996). When the ADC cDNA was transcribed and translated in vitro, we observed one major band with a M_r of 75 kDa (Nam et al. 1997). This in vitro translated product has ADC activity which is specifically inhibited by α-difluoromethylarginine (DFMA). These results suggest that the soybean ADC is a trimer composed of one subunit. Trimeric composition was also suggested for the rice coleoptile ADC and oat ADC (Reggiani 1994, Malmberg et al. 1992).

It has been reported that oat ADC is proteolytically processed into two fragments which are linked by a disulfide bond and that this processing step is required to make the enzyme active (Malmberg and Cellino 1994). Our results suggest that such proteolytic processing does not occur in the soybean ADC. However, we can not rule out the possibility that the proteolytic processing might occur at

either end of the enzyme.

Inhibition of ADC activity by amines (agmatine, putrescine, spermidine, and spermine) has been reported in various systems. Based on these data, a feedback regulation of the ADC activity has been proposed. However, the effective concentrations of the amines in vitro were very high in these reported ADCs. For example, ADCs from oat and rice were inhibited only when a high concentration of agmatine (>10 mM) was present in the assay (Smith 1979, Choudhuri and Ghosh 1982). Unlike these ADCs, the soybean ADC is much more sensitive to agmatine and putrescine (70% inhibition of the ADC activity at 0.5 mM) than are the oat and rice ADC but relatively insensitive to the same concentration of spermidine and spermine. These results suggest that the soybean ADC activity could be regulated by agmatine and/or putrescine in vivo.

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