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Purification and characterization of arginine decarboxylase from cucumber (*Cucumis sativus*) seedlings

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Abstract. A simple, reproducible and rapid protocol for the purification of arginine decarboxylase from *Cucumis sativus* seedlings has been standardised. The purification steps involved ion-exchange chromatography on diethylaminoethyl-cellulose followed by gel filtration on Sephadex G-l 50. The purified enzyme preparation migrated as a single stainable band on Polyacrylamide gels at both basic and acidic pH, but under denaturing and reducing conditions on sodium dodecyl sulphate-polyacrylamide gels resolved into polypeptides of molecular weight 48,000,44,000 and 15,000. However, in the absence of 2-mercaptoethanol on electrophoresis on sodium dodecyl sulphate-polyacrylamide gels, the enzyme moved as single band with a molecular weight of 150,000. Evidence was obtained to indicate that these three polypeptides were probably derived from a single larger molecular weight enzyme. On storage of the purified protein, the 48,000 species was preferentially degraded to smaller polypeptides. The preliminary data suggested that the 48,000 and 44,000 species shared many common tryptic peptides as revealed by finger printing of the [^{125}I]-labelled protein. The purified enzyme was a glycoprotein and had a K_m of 0·5 mM for arginine. Its activity was stimulated by dithiothrietol and pyridoxal phosphate. EDTA did not inhibit the enzyme activity. Mn^{2+} at 1 mM stimulated arginine decarboxylase activity but was inhibitory at higher concentration.

Keywords. Arginine decarboxylase; peptide mapping; purification.

Introduction

With the recognition that putrescine plays a pivotal role in polyamine biogenesis in all the organisms hitherto examined, the molecular and regulatory aspects of enzymes involved in the diamine elaboration have been the subject of intense research efforts in recent years (Bachrach, 1980; Canellakis *et al.,* 1979; Pegg and Williams-Ashman, 1981). In prokaryotes, unlike in mammals, both ornithine decarboxylase (EC 4.1.1.17) and arginine decarboxylase (EC 4.1.1.19) participate in putrescine production depending upon the culture conditions. Furthermore, in *Escherichia coli,* two distinct sets of enzymes (biosynthetic and degradative) of both ornithine decarboxylase and arginine decarboxylase have been purified and extensively characterized (Morris and Boeker, 1983). However, in higher plants (Smith, 1970; Ramakrishna and Adiga, 1974) arginine rather than ornithine seems to serve as the major precursor of putrescine production,

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Abbreviations used: PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; M_r molecular weight; DTT, dithiothrietol; 2-ME, 2-mercaptoethanol; PLP, pyridoxal 5'-phosphate; DEAE, diethylaminoethyl buffer 10 mM Tris-HCl pH 7·5 containing 2mM 2-ME and 10 μM PLP; PMSF, phenylmethyl sulphonyl fluoride.

although the existence and functional significance of ornithine decarboxylase in some specialized plant systems have been recognized recently (Altman *et al.,* 1982).

In plants, attempts to understand the enzymatic mechanisms involved in arginine → putrescine conversion have revealed that arginine decarboxylase in analogy with ornithine decarobxylase in animal cells, is the rate-limiting step subject to regulatory influence by several factors such as growth and development (Feirer *et al.,* 1984; Montague *et al.,* 1979; Palavan and Galston, 1982) hormonal stimuli (Dai *et al.,* 1982; Suresh *et al.,* 1978) and environmental conditions (Flores and Galston, 1982; Young and Galston, 1983; Smith, 1963). This plant enzyme has been purified from *Lathyrus sativus* (Ramakrishna and Adiga, 1975), oats (Smith, 1979) and rice seedlings (Choudhuri and Ghosh, 1982) and several of its molecular characteristics delineated. While the homogeneous enzyme from *L. sativus* is a homohexamer of identical subunits (Ramakrishna and Adiga, 1975), arginine decarboxylase activity associated with more than one molecular species has been reported in oat seedlings (Smith, 1979). This raises the distinct possibility that the various species of higher plants may contain different types of arginine decarboxylase with differing molecular characteristics. This paper deals with the purification and delineation of the molecular and regulatory features of arginine decarboxylase of cucumber seedlings and describes several novel features of this key enzyme of the polyamine biosynthetic sequence in this higher plant species.

Materials and methods

Materials

The following chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, USA: Sephacryl S-300, Sephadex G-150, reagents for electrophoresis, *p*-aminophenyl mercuric acetate, N-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin, chloramine-T. X-ray film was a product of Hindustan Photofilms Ltd., Ootacamand. [U-^{14}C]-Arginine (sp. activity 246 mCi/m mol) was purchased from Bhabha Atomic Research Centre, Bombay. Carrier-free N a [^{125} I] was purchased from Radiochemical Centre, Amersham, England. The sources of other chemicals and Cucumber seeds were same as referred to earlier (Ramakrishna and Adiga, 1975; Suresh *et al.,* 1978). All other chemicals were of analytical grade.

Methods

Germination and growth conditions for cucumber seedlings were as described earlier (Suresh *et al,* 1978).

Affinity chromatography on organomercuric acetate

Cyanogen bromide was prepared and Sepharose 4B was activated according to March *et al.* (1974). *p*-Aminophenylmercuric acetate was coupled to CNBr-activated Sepharose according to Sluyterman and Wijdenes (1974) as described by Srivenugopal and Adiga (1981).

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) at pH 8·3 under nondenaturing conditions was carried out using either 7·5 % or 5 % Polyacrylamide gels (Davis, 1964). PAGE at pH 4·3 on 5% gels was carried out as described by Reisfield *et al.* (1962).

Sodium dodecyl sulphate (*SDS*) *PAGE*: Gel electrophoresis in Tris-acrylamide system in the presence of SDS was carried out on slab gels according to Laemmeli (1970). A standard curve was constructed by plotting the relative migration *vs* log molecular weight (M_r) of the standard protein markers and from the distance of migration of the proteins, M_r of unknown samples was computed from the standard curve. Phosphorylase b (M_r 93,000), bovine serum albumin (68,000), ovalbumin (45,000), egg white chicken riboflavin carrier protein (38,000) (Murthy *et al.,* 1979), lactoglobulin (18,000) and lysozyme (14,000) were employed as standard protein markers.

Glycoprotein staining: After the electrophoresis, the gels were stained for glycoproteins and destained according to the procedure of Zacharius *et al.* (1969).

Analytical ultracentrifugation

Analytical ultracentrifugation was carried out in a Beckman L8-70 ultracentrifuge (Prepscan model) equipped with ultra-violet optics. About 1 $A_{274\ nm}$ unit of the purified protein sample was centrifuged at 42,000 rpm at 5°C. Boundary was recorded for every 8 min on the recorder and the distance of migration from the origin was calculated (Eason and Campbell, 1978). 'S' value was calculated from the slope of the plot of x (distance of migration) *vs* time and temperature correction was applied for the observed *S* value at 5°C to get $S_{20°w}$.

Protein estimation

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

Assay of arginine decarboxylase

Arginine decarboxylase activity was assayed by measuring $^{14}CO_2$ liberated from [U-^{14}C]-arginine in Warburg flasks as described earlier (Suresh *et al.,* 1978). The assay mixture consisted of 100 mM of Tris-HCl pH 8·2, 2 mM dithiothrietol (DTT), 50 μM pyridoxal 5'-phosphate (PLP) and 2×10^5 cpm (367 p mol) of [U-^{14}C]-arginine (sp. activity 246 mCi/m mol) .

Unit activity

One unit of enzyme activity is that catalyzing the liberation of 1 p mol of $^{14}CO_2$/2 h at 40°C. Specific activity is the number of units of enzyme activity/mg protein.

Enzyme purification

Preparation of crude enzyme extracts: Six-day-old cucumber seedlings were chosen as the starting material since preliminary experiments (data not given) showed that at this

stage of growth, the quality and quantity of the plant material was most suitable in terms of ease of processing and the yield of the total soluble protein obtained. The seedlings (150 g fresh wt.) were separated from roots, washed thoroughly and homogenized in a Waring blender at 4°C with equal volume of 20 mM Tris-HCI, pH 7·6, containing 5 mM 2-mercaptoethanol (2-ME) and 20 μM PLP. Unless otherwise stated all the operations were carried out at 4°C. The homogenate was filtered through 4 layers of cheese cloth and sedimentable cell debris was removed by centrifugation at 15,000 *g* for 15 min. The resultant supernatant was referred to as crude enzyme extract.

$MnCl_2$ treatment

The crude enzyme extract was adjusted to a final concentration of 7·5 mM $MnCl_2$ by the addition of 1 M $MnCl_2$ and stirred for 1 h to precipitate the nucleoproteins. The precipitate obtained was removed by centrifugation and the supernatant was saved.

Diethylaminoethyl (*DEAE*) *- cellulose chromatography*

The above supernatant (380 mg protein) was applied onto a DEAE-cellulose column (1·0 cm × 28 cm), pre-equilibrated with 10 mM Tris-HCI, buffer pH 7·4 containing 2mM 2-ME and 10μM PLP at a slow flow rate (15 ml/h), After washing off the unbound proteins with the buffer, the column was eluted with 400 ml of 0 → 0·4 M linear KCl gradient. Fractions (4 ml each) corresponding to the three major peaks (I,II, III) were pooled, dialyzed and scanned for the arginine decarboxylase activity. The enzyme activity was found to be associated only with the major protein peak II.

Ammonium sulphate precipitation

The active pooled fractions of peak II (fractions 56 72) from the above step was subjected to 0–90% saturation with respect to ammonium sulphate. This precipitation step was primarily employed to rapidly concentrate the protein rather than as a purification step.

Gel filtration on Sephadex G-150

The clarified ammonium sulphate protein fraction (about 2·0 ml, 45 mg) was loaded onto a Sephadex G-150 column (1·5 × 70 cm) pre-equilibrated with the buffer containing 0·2 M KCl. Fractions (2·0 ml) were collected and scanned for protein at 280 nm. Most of the protein was eluted in the void volume, which was well separated from the broad shallow protein peak representing the protein held on the column. The protein fractions corresponding to those that were excluded in the void volume as well as those that eluted subsequently were separately pooled, dialyzed extensively against buffer to remove the salt which otherwise interfered with the enzyme assay. Arginine decarboxylase activity was found to be exclusively associated with the protein that eluted in void volume, whereas the other fraction did not exhibit any detectable enzyme activity.

Results

Purification of arginine decarboxylase

The rather lower magnitude of fold purification (table 1) may be the consequence of considerable inactivation of the enzyme as the purification progressed and/or due to the action of protease actively associated with it. The gel filtration step could also be replaced by thiol affinity chromatography with the same ultimate result. Figure 1 shows the elution profile of arginine decarboxylase from organomercuric acetate affinity column obtained subsequent to the DEAE-cellulose step.

Since exclusion chromatography on Sephadex G-150 column was employed as the final purification step, the possibility was considered that some minor protein contaminants might have coeluted along with arginine decarboxylase which are undetectable by gel electrophoresis. However, gel filtration on Sephacryl S-300, with a much higher exclusion limit also yielded similar results (figure 2a,b).

Criteria of homogeneity

Analytical PAGE: The Sephadex G-150 excluded enzyme, Sephacryl S-300 eluate as well as that eluted from organomercurial affinity column exhibited a single stainable band on gel electrophoresis at pH 8·3 (figure 2a,b,c) and at pH 4·3 (data not shown).

SDS *gel electrophoresis* : When the purified protein was subjected to SDS-PAGE under reducing conditions, the protein resolved into 3 discrete bands of apparent M_r of 48,000, 44,000 and 15,000 respectively. In the absence of 2-ME, the enzyme exhibited a single slow moving band of M_r 150,000 (figure 3a,b,c). Based on the results obtained under reducing and non-reducing conditions, it would appear that all the three polypeptide species (M_r 48,000, 44,000 and 15,000) were held together by disulphide bonds in the final preparation. From the gel electrophoretic pattern it could be argued that the multiple bands obtained on SDS-PAGE may represent either unequal subunits or proteolytic degradation products of single subunit of the enzyme.

Evidences for proteolysis

A clue for the possibility that proteolytic degradation of the enzyme may be the principle cause for the above pattern, stems from the observation that when the purified

Table 1. Summary of the purification procedure for arginine decarboxylase from *Cucumis sativus* seedlings.

Purification step	Protein mg	Total enzyme units	Specific activity* units	Fold purification	Yield
Crude extract	1196	10764	9	1·0	100
$MnCl_2$ treatment	380	6080	16	1·8	56
DEAE-Cellulose chromatography	44	275	6	—	—
Sephadex G-150 chromatography	11	1938	173	19·2	18

* Specific activity unit: 1 p mol of CO_2 liberated/mg protein/2 h.

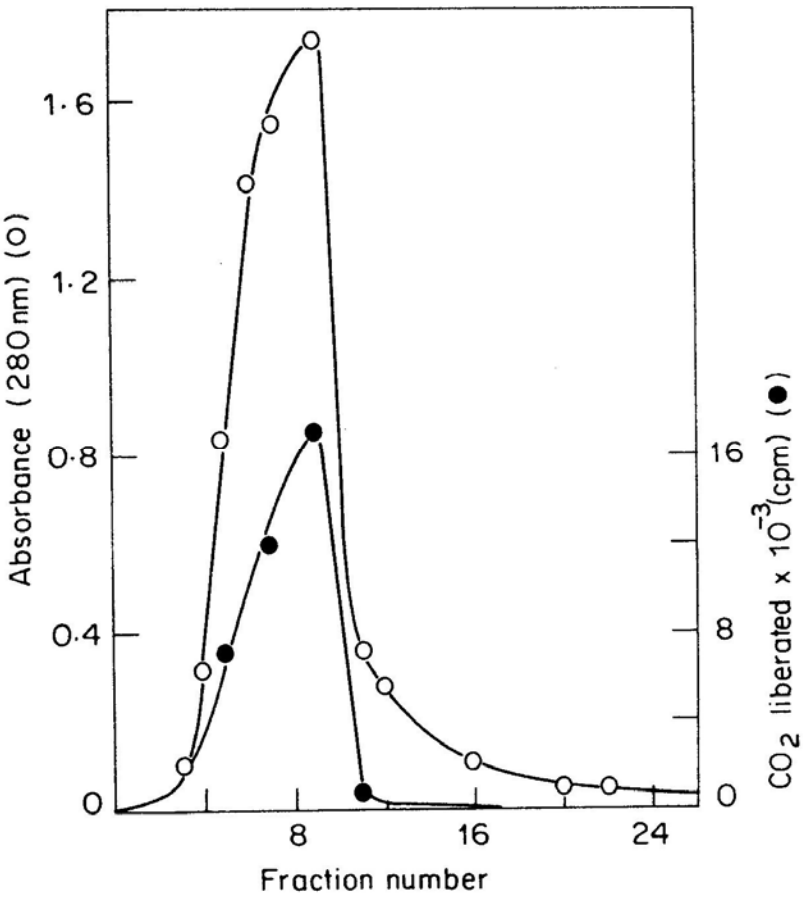

Figure 1. Elution of cucumber arginine decarboxylase from organomercurial Sepharose column.

The concentrated protein fraction (peak II of DEAE-Cellulose eluate) was dialysed against 10 mM Tris-HCl pH 7·6 containing 10 μM PLP and applied to an organomercurial column (1·0 × 8·0 cm). After washing the column with the above buffer, the column was eluted with a linear gradient (30 ml) of 0→10 mM 2-ME. Enzyme activity was monitored in the fractions (1·0 ml) as described in Materials and methods.

enzyme was stored for about 2 weeks at 4°C, the intensity of the band corresponding to protein species of M_r 48,000 specifically decreased with the concomitant appearance in higher amounts of lower M_r polypeptides < 44,000 (figure 4); the 44,000 protein band did not seem to be grossly affected under these conditions. Hence, it would appear that the arginine decarboxylase preparation eluted from the Sephadex G-150 column possesses an intrinsic or associated proteolytic activity.

Further evidence that these three polypeptides represent proteolytic degradation products of a common precursor is obtained from 2-dimensional peptide mapping of polypeptides of M_r 48,000 and 44,000 resolved on SDS gels (data not shown). A similar observation was made with regard to the tryptic peptide constituents of the protein species with M_r of 15,000 (data not shown).

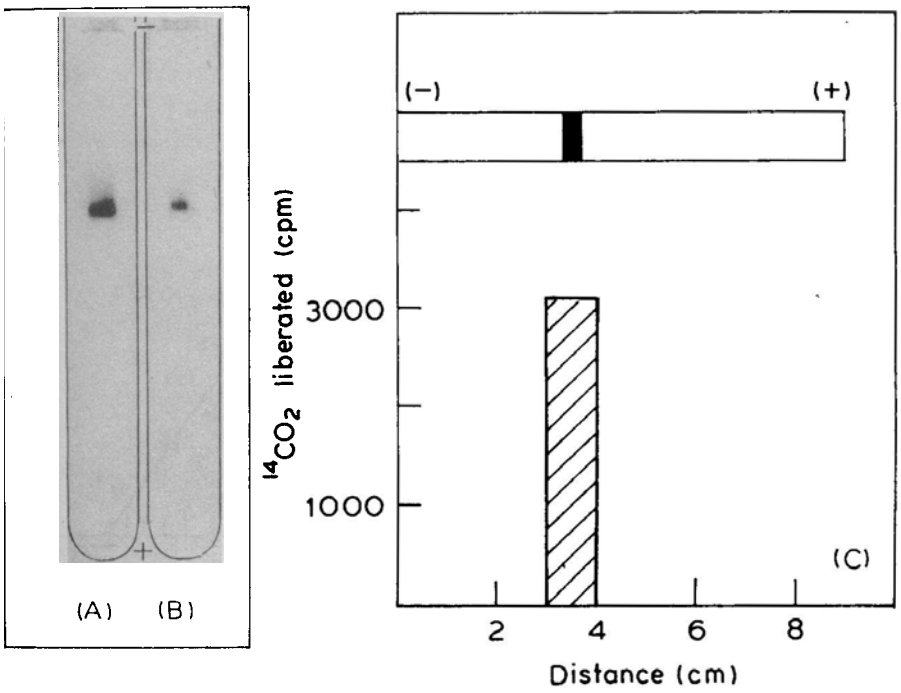

Figure 2. PAGE of cucumber arginine decarboxylase.

About 100 μg of protein is employed, in each case. Electrophoresis was performed at pH 8·3 under nondenaturing conditions on 5 acrylamide gels. (**A**)Sephadex G-150 eluate; (**B**) Sephacryl S-300 eluate;(**C**) Sephadex G-150 eluate (100 μg) was subjected to PAGE on 5 gel at 4°C. After the electrophoresis, the gel was sliced to 0·5 cm pieces, homogenized and then eluted at 4°C for 12 h with buffer B (for details see text). The enzyme activity was measured in the supernatants of the homogenate as described in Materials and methods.

General properties of arginine decarboxylase

The purified enzyme was unstable and susceptible to proteolytic degradation. The enzyme had a pH optimum of 8·2. Determination of the native M_r of the enzyme was hampered by the fact that the protein was excluded from the standard gel filtration columns employed, presumably due to aggregation. However, an estimate of its molecular size can be gauzed from sedimentation value of the protein which corresponded to 16 S (figure 5). The minimum M_r of the plant arginine decarboxylase preparation was about 150,000 as revealed by SDS-PAGE under denaturing conditions (*i.e.*, in the absence of 2-ME). As already mentioned, the constituent partially degraded products could not be separated either on Polyacrylamide gels or on gel filtration columns unless treated with SDS and 2-ME together. All these observations suggest that the protein exists as a large aggregate of partially degraded polypeptides. Repeated attempts to minimize the presumed proteolytic processing of the enzyme by inclusion of phenylmethylsulphonyl fluoride (PMSF) in the buffers have proved futile so far. Arginine decarboxylase of cucumber seedlings appears to be a glycoprotein as observed by periodic acid Schiff-staining of Polyacrylamide gels.

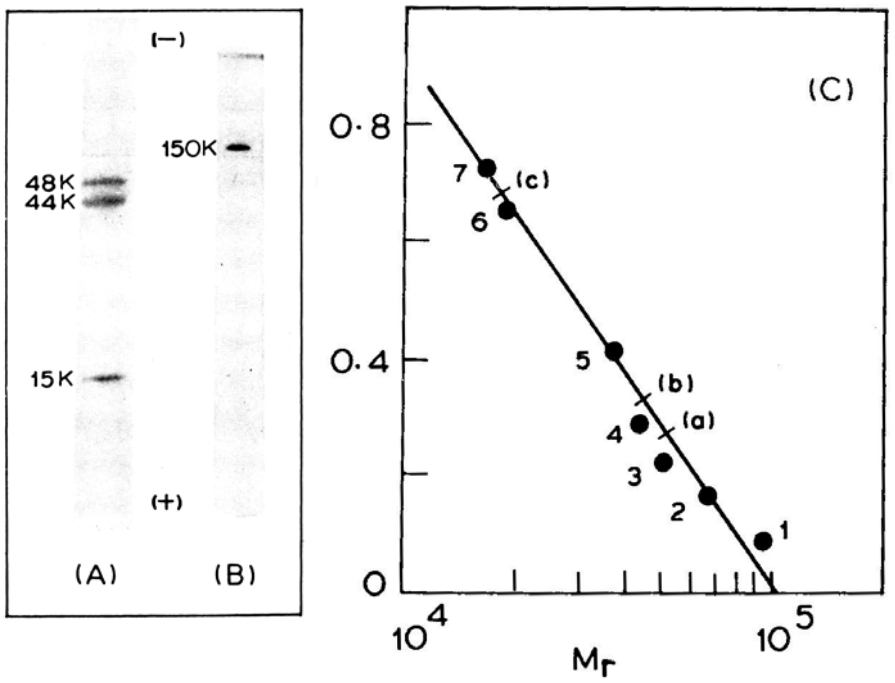

Figure 3. M_r determination of cucumber arginine decarboxylase on SDS-PAGE.
SDS-PAGE was performed on 10 % gels with (A) and without (B) 2-ME respectively. A single band was obtained on PAGE at pH 4·3 (data not shown). Arrow indicates origin. For details see Materials and methods. (C), M_r markers used are (1) Phosphorylase b; (2) bovine serum albumin; (3) immunoglobulin heavy chain; (4) ovalbumin; (5) chicken riboflavin carrier protein; (6) lactoglobulin; (7) lysozyme. a, b and c correspond to polypeptides of M_r 48,000, 44,000 and 15,000 respectively.

Kinetic properties

The enzyme activity was linear upto 200 μg of purified protein and with time upto 2 h. This plant arginine decarboxylase followed linear Michaelis-Menten kinetics with increasing substrate concentration and had a K_m of 0·5 mM as estimated by Lineweaver-Burk plot (figure 6).

Effect of DTT and PLP

The purified enzyme showed an increase in specific activity when additional amounts of DTT and PLP were added (table 2). With 5 mM DTT, the enzyme showed lesser activity than that with 2·5 mM but the activity was still higher than that in the absence of added DTT. On adding 100 and 200 μM PLP, the enzyme showed significantly enhanced activity.

Effect of EDTA, Mn^{2+} and Mg^{2+}

In the presence of 5 mM EDTA, 87 % of arginine decarboxylase activity could be still recovered. Lower concentrations of Mn^{2+} (< 1 mM) were stimulatory but at higher

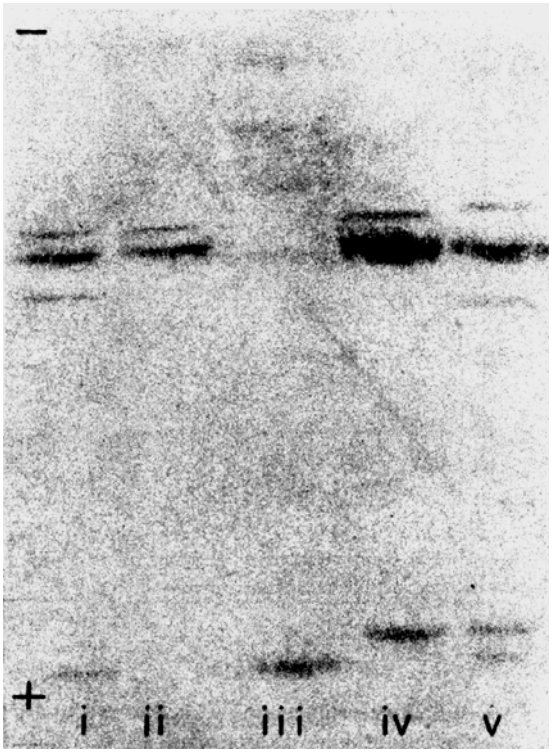

Figure 4. *In vitro* proteolytic degradation of cucumber arginine decarboxylase.

Fresh and stored preparations of purified arginine decarboxylase (Sephadex G-150 eluate (iv) and Sephadryl S-300 (i, ii)) were employed. Lanes (ii) and (iv) contained freshly isolated protein while (i) and (v) had 'aged' protein preparation. Lane (iii) had standard protein markers. Note the additional bands in the aged protein preparations. Lanes (iii) contained 50 μg of protein each while lanes (iv), (v) contained 100μg each.

concentrations, the metal ion exerted opposite influence on the enzyme activity. Mg^{2+} at 2·0 mM concentration had only marginal stimulatory effect (table 3) on the enzyme.

Discussion

The above investigations represent one of the few detailed studies hitherto carried out on the purification and molecular parameters of arginine decarboxylase from higher plant systems; the other three arginine decarboxylase enzymes earlier purified and characterized were derived from *L. sativus* (Ramakrishna and Adiga, 1975), oat (Smith, 1979) and rice (Choudhuri and Ghosh, 1982) seedlings. A simple and reproducible 3-step purification protocol was devised for arginine decarboxylase isolation from cucumber seedlings unlike the case with *L. sativus* enzyme which required a more protracted 7-step procedure (Ramakrishna and Adiga, 1975). Although the enzyme protein thus obtained appeared homogeneous by the criteria of analytical ultra-centrifugation and gel electrophoresis at pH 8·3 and pH 4·3, the final preparation was quite unstable and hence the fold purification (19 %) as well as the per cent yield (18 %)

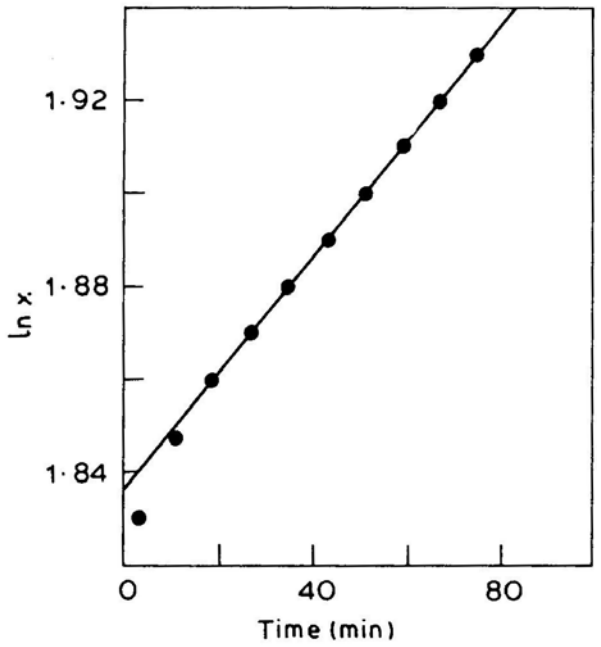

Figure 5. Analytical ultracentrifugation of *Cucumis sativus* arginine decarboxylase.
One $A_{274\ nm}$ unit of pure arginine decarboxylase was centrifuged at 42,000 rpm at 5°C in a Beckman L8-70 analytical centrifuge (prep, scan) provided with ultra-violet optics. Boundary was recorded at regular time intervals. 'x' value was calculated as described by Eason and Campbell (1978). From the slope of the line S value was calculated.

achieved were relatively modest. A serious impediment encountered in achieving higher degree of activity was the inherent instability of the enzyme as the purification progressed, presumably due to proteolytic cleavage, as is evident by the complex pattern of protein components resolved by SDS-PAGE (figure 3c). This observation also precluded a clear-cut enunciation of quaternary structure of the protein. The remarkable propensity of the purified enzyme to exist as aggregates of high M_r was clearly evident from its exclusion from Sephadex G-150 and Sephacryl S-300 columns and its higher sedimentation value ($S_{20°w}$ 16) as calculated by ultracentrifugation (figure 5). Since during gel electrophoresis in the presence of SDS, but under non-reducing conditions, the enzyme exhibited a M_r of 150,000, it is reasonable to conclude that this represents the minimal molecular size of the protein as isolated. The finding that on resolution on SDS-PAGE under reducing conditions, the protein dissociates into multiple bands of unequal size, (48,000,45,000 and 15,000) would in the first place argue against its being a homooligomeric protein unlike the arginine decarboxylase from either *L. sativus* (Ramakrishna and Adiga, 1975) or *E. coli* (Morris and Boeker, 1983). Data of figure 4 clearly indicates that the enzyme undergoes proteolytic cleavage upon storage. Although the exact quantitation of each of these polypeptides in fresh and stored preparations was not done, the 48,000 polypeptide appears to be preferentially attacked by the associated proteolytic activity. Both the 48,000 and 44,000 polypeptides share many common tryptic peptides (data not shown). Moreover,

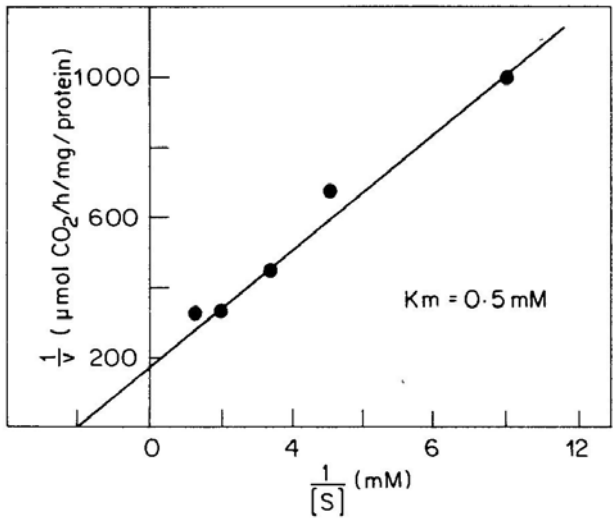

Figure 6. Effect of substrate concentration on cucumber arginine decarboxylase.

The enzyme activity was assayed as described in Materials and methods. K_m was calculated by Lineweaver-Burk plot.

Table 2. Effect of DTT and PLP on arginine decarboxylase activity from cucumber seedlings.

Additions (mM)	Activity units/mg protein
Nil (Control)	47
DTT (2·5)	84
DTT (5·0)	54
PLP (0·1)	50
PLP (0·2)	66

DTT and PLP were added at the beginning of the assay, in addition to those already present in standard assay mixture (2·0 mM DTT and 0·1 mM PLP).

our unpublished data suggest that the 48,000 and 44,000 polypeptides are generated from a larger polypeptide due to in *vivo* age-dependent specific proteolysis of the enzyme. This in turn would support the notion that this arginine decarboxylase protein consists of identical basic units. Furthermore, the demonstration that these constituent polypeptides can be resolved into individual species only in the presence of both SDS and sulphydryl agent but not of 8 M urea, shows that their association in the native state of arginine decarboxylase does not invovle mere non-covalent interactions, but is reinforced by disulphide bridges. It is obvious therefore that any attempt to visualize the exact spatial arrangement of the basic constituents of the arginine decarboxylase molecule at this stage should remain speculative and needs further study directed

Table 3. Effect of EDTA and metal ions on arginine decarboxylase activity from cucumber seedlings.

Additions (mM)	Activity units/mg protein
Nil (Control)	40
EDTA (5)	35 (87·5)[a]
Mn^{2+} (0·5)	52 (130)[a]
(1·0)	60 (150)[a]
(2·0)	30 (75)[a]
Mg^{2+} (2·0)	46 (115)[a]

[a] Values in parentheses are percent activity of control. EDTA, Mn^{2+} and Mg^{2+} at indicated concentrations were includedin the standard assay mixture. Assay was started by the addition of enzyme.

towards eliminating *in vivo* proteolytic degradation of the protein *in toto.* The physiological significance of extensive proteolysis of this plant arginine decarboxylase as observed in the present study is currently unknown. Of relevance in this context, is the observation that arginine decarboxylase from oat seedlings can be resolved into two active species of M_r 195,000 and 118,000 (Smith, 1979), but the interrelationship between these two molecular entities, including possible precursor–product relationship due to proteolytic modification, are currently unknown. It is pertinent to mention in this context that arginine decarboxylase isolated from K^+-deficient oat seedlings (Flores *et al.,* 1984) differs significantly from that described by Smith (1979). This preparation seems to resemble its counterpart in *L. sativus* (Ramakrishna and Adiga, 1975) in that it migrates as a single protein species of M_r 39,000 upon SDS-PAGE. The enzyme activity is stimulated by both pyridoxal phosphate and thiol reagents but inhibited by D-arginine. However, repeated attempts to find more than one molecular species of arginine decarboxylase in cucumber, that are separable by any of the physicochemical methods described above have not revealed any gross heterogeneity in arginine decarboxylase preparations from this plant system.

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