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DECARBOXYLATION OF HOMOARGININE AND LYSINE BY AN ENZYME FROM *LATHYRUS SATIVUS* SEEDLINGS

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Key Word Index—*Lathyrus sativus*; Leguminosae; chick pea; homoarginine; lysine; decarboxylase purification.

Abstract— Homoarginine decarboxylase has been purified *ca* 110-fold from *Lathyrus sativus* seedlings and resolved from arginine decarboxylase by DEAE-Sephadex column chromatography. The enzyme was less active than arginine decarboxylase and was highly labile. This preparation decarboxylated L-lysine in addition to L-homoarginine. The purified enzyme preparation had an absolute requirement for exogenous Mn^{2+} or Fe^{2+} for both the enzyme activities. The pH and temperature optima for decarboxylation of both homoarginine and lysine were the same viz. 8·4 and 41° respectively. The K_m value L-homoarginine was 3·33 mM and for L-lysine was 0·88 mM. Arginine and homoarginine decarboxylases appear to be different and separable entities having different physico-chemical characteristics, despite the fact that their respective guanido amino acid substrates undergo similar metabolic conversion to guanido- and diamines in this plant system.

INTRODUCTION

We have reported earlier that arginine and homoarginine undergo a similar sequence of metabolic conversions to guanido- and diamines and that the metabolites of the 2 guanido amino acids are elaborated in a concerted fashion during development of *Lathyrus sativus* seedlings [1-4]. However, the finding that the purified arginine decarboxylase from this plant source is specific for L-arginine and does not decarboxylate L-homoarginine under standard assay conditions [5], suggested that homoarginine decarboxylating activity is probably associated with a different enzyme protein. Prompted by this observation, attempts were made to detect and purify homoarginine decarboxylating activity from cell-free extracts of the seedlings. It became evident that homoarginine decarboxylase activity is extremely weak and labile thus imposing a severe limitation in carrying out a more detailed investigation on its physico-chemical and kinetic properties. However, the data recorded in this paper clearly indicate that arginine decarboxylase and homoarginine decarboxylase activities are separable and exhibit different properties. Of interest in this connection is the demonstration that homoarginine and lysine decarboxylating activities are associated with the same protein fraction.

RESULTS

The resolution of homoarginine (lysine) decarboxylase activity from arginine decarboxylase on a DEAE-Sephadex column is shown in Fig. 1. Homoarginine decarboxylating activity emerged from the column as a distinct peak (Peak III) well resolved from arginine decarboxylase (Peak V) which required a higher salt concentration (0·4 M KCl) in the elution buffer. Lysine decarboxylating activity was associated with two different protein fractions; one eluting with the buffer alone (Peak I) and the other corresponding to homoarginine decarboxylase fraction (Peak III). Further examination of Peak I lysine decarboxylase activity revealed that $^{14}CO_2$ release catalyzed by this peak fraction is accompanied by concomitant production of ammonia and that $^{14}CO_2$ evolution can be severely curtailed by the inclusion in the reaction of beef liver catalase. Similar results were

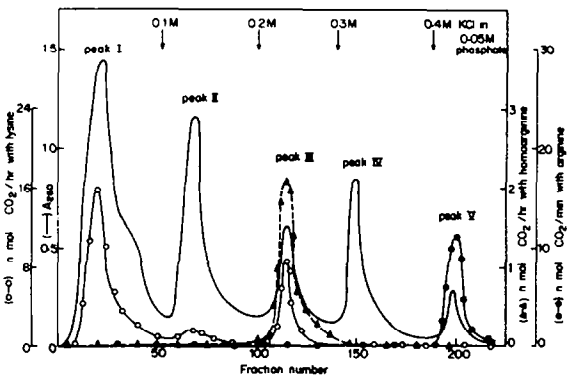

Fig. 1. Resolution of arginine, lysine and homoarginine decarboxylases on DEAE-Sephadex column. The concentrated step-5 fraction was applied on to a DEAE-Sephadex column (2·2 × 35 cm) pre-equilibrated with 50 mM Pi buffer. The column was washed with the same buffer till the *A* at 280 nm was ≤0·1. Proteins were eluted batchwise with *ca* 300 ml each of 0·1, 0·2, 0·3 and 0·4 M KCl in 50 mM Pi buffer. 6·4 ml fractions were collected. 0·4 ml of the fraction was used for testing lysine and homoarginine decarboxylating activity, while 0·2 ml for arginine decarboxylase activity. Enzyme activities were assayed as detailed under Experimental.

Table 1. Summary of purification of homoarginine decarboxylase from *L. sativus* seedlings

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold purification	Yield (%)
1. Crude extract	24400	2580	0·10	1·0	100
2. $MnCl_2$ treatment	21000	2280	0·11	1·1	89
3. Ammonium sulphate fractionation	9610	1530	0·16	1·6	59
4 Acetone fractionation	3130	852	0·27	2·7	33
5. Positive adsorption on C-γ gel	840	406	0·48	4·8	16
6. DEAE-Sephadex chromatography	32	362	11·3	113	14

obtained when Peak I protein was purified to homogeneity (as revealed by analytical disc gel electrophoresis) and enzymatic release of $^{14}CO_2$ from ^{14}C-lysine was investigated [6]. Thus, it appears that CO_2 evolution catalyzed by Peak I protein is not due to enzymatic decarboxylation of L-lysine, but is due to L-lysine oxidase-type reaction [7]. In contrast to this, Peak III lysine decarboxylase activity, like the arginine and homoarginine decarboxylase activities, did not involve simultaneous release of ammonia. In view of this, further studies on lysine decarboxylase activity were carried out solely with the peak III protein fraction which also possessed homoarginine decarboxylase activity.

Table 1 summarizes the purification of homoarginine decarboxylase of *L. sativus* from 3 kg fresh seedlings. The enzyme was purified 113-fold from the crude extract with a recovery of 14% and a sp. act. of 11·3 nmol CO_2/hr/mg protein. Because of the deaminating lysine decarboxylase activity associated with Peak I (Fig. 1) it was not possible to evaluate the degree of purification achieved with respect to the lysine decarboxylase (Peak III). In view of the extreme lability and low yields of homoarginine- and lysine decarboxylase activities associated with Peak III protein fraction, no attempt was made to purify the enzyme further.

Characterization of the enzyme activities. Like arginine decarboxylase [5] the partially purified enzyme fraction also showed no activity below pH 7, and the optimal activity was *ca* pH 8·4 for both lysine and homoarginine decarboxylating activities. The optimum temperature for both lysine and homoarginine decarboxylating activities was the same (41°). At 37° and 45° activity was 75 and 63% respectively for homoarginine decarboxylase and 56 and 35% for lysine decarboxylase with respect to activity at 41° (100%). In the absence of metal ions both the decarboxylating activities were very low unlike arginine decarboxylase from the same source [5]. Addition of divalent metal ions like Mn^{2+} and Fe^{2+} enhanced the two enzyme activities to a similar degree with maximal stimulation at 1 mM Fe^{2+} or 2 mM Mn^{2+} (Table 2). Mg^{2+} was without effect on both lysine and homoarginine decarboxylating activities.

Effect of substrate concentrations

When L-homoarginine was used as the substrate, the partially purified enzyme fraction showed regular Michaelis–Menton kinetics with respect to the guanido amino acid. From the double reciprocal plots of velocity and substrate concentrations, a K_m value of 3·33 mM and V_{max} of 0·8 nmol/hr were obtained for homoarginine.

Table 2. Effect of divalent metal ions on homoarginine and lysine decarboxylase activities

Metal ion (mM)	Activity (units) Homoarginine decarboxylase	Lysine decarboxylase
Control	0·09	0·25
Mn^{2+} (1)	0·41	2·50
Mn^{2+} (2)	0·67	4·60
Mn^{2+} (5)	0·48	2·75
Fe^{2+} (1)	0·50	2·65
Fe^{2+} (3)	0·22	1·99
Mg^{2+} (5)	0·11	0·31

With lysine as substrate, a similar substrate saturation curve was obtained. The K_m value for lysine as substrate calculated from the Lineweaver-Burk plot was 0·88 mM i.e. *ca* 4 times lower than that obtained with homoarginine. The corresponding V_{max} for lysine was 16 times higher than that for homoarginine as the substrate (12·5 nmol/hr). In view of the presumptive evidence that the same enzyme decarboxylates both the substrates (S_1 & S_2) it was of interest to investigate the influence of fixed varying concentrations of one amino acid (unlabelled) on the substrate saturation kinetics of the other (labelled) amino acid. The double reciprocal plots were linear (Fig. 2a & 2b) as also the replots of the slope vs concentration of amino acids (unlabelled) indicating a linear competitive pattern of inhibition. The experimental data could be fitted well with the values calculated according to the general equation

$$V^1 = \frac{V_{max_1} S_1}{S_1 + K_{m1}\{1 + [(S_2)/K_{m2}]\}}$$

These data suggest strongly that a single enzyme form is involved in the binding and subsequent decarboxylation of both lysine and homoarginine.

DISCUSSION

The existence in *L. sativus* of a decarboxylase for homoarginine distinctly different from arginine decarboxylase has been predicted on the basis of the substrate specificity of the latter enzyme and this has been largely borne out by the data presented in this paper. The demonstration that during purification through identical fractionation steps, the decarboxylase activities exhibit differences in both the increments in specific activities and the percentage yields, can be construed as the first indication of the nonidentity of the 2 enzyme activities. Further conclusive proof for this premise stems from the clear-cut resolution of these two activities achieved by column chromatography on DEAE-Sephadex (Fig. 1). A comparison of the available data on some of the properties of the two enzymes also provides additional support to these findings. Thus, while arginine decarboxylase does not exhibit an absolute requirement for an exogenously added metal cofactor, homoarginine decarboxylase shows an obligatory requirement for Fe^{2+} or Mn^{2+} for its catalytic function. In addition, despite similar profiles of pH dependency the two decarboxylases have clearly different temperature optima viz. 45° and 41°. Moreover, in terms of catalytic efficiency, arginine decarboxylase appears to be more active by several orders of magnitude than homoarginine decarboxylase as reflected in their respective specific activities at any given stage of purification.

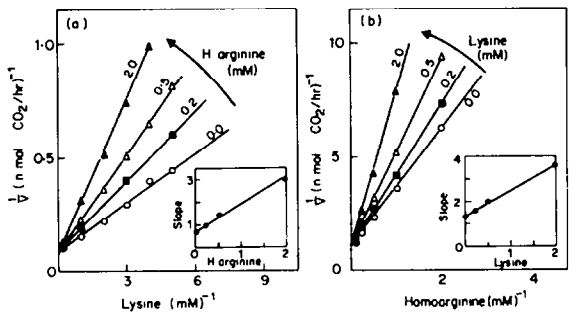

Fig. 2. Competitive kinetics with lysine and homoarginine (H. arginine) as substrates. (a) Labelled lysine vs fixed varying concentrations of unlabelled homoarginine. (b) Labelled homoarginine vs fixed varying concentrations of unlabelled lysine. The insets represent the replots of slope vs concentration of unlabelled aminoacids. The enzyme activities were assayed as described under Experimental.

Another notable feature of the present study is the demonstration that the partially purified homoarginine decarboxylase also catalyzes the decarboxylation of L-lysine. Based on the assumption that these 2 decarboxylase activities are associated with the same protein, lysine appears to be definitely a preferred substrate in view of the fact that the K_m value for the amino acid is 4 times lower than that for the guanido amino acid. While it is conceded that the enzyme preparation is heterogeneous (as revealed by polyacrylamide gel electrophoresis) several lines of evidence suggest that the two activities may be associated with the same protein molecules. These are (i) both the activities exhibit the same pH and temperature optima (Fig. 2), (ii) both have an obligatory requirement for a metal ion like Fe^{2+} or Mn^{2+} and both respond to graded amounts of these metal ions with similar degrees of enhancement of activities (iii) preliminary data on the distribution of enzyme activities on polyacrylamide gels following electrophoresis indicate that both decarboxylases band at the same region of the gels and (iv) the competitive kinetics between the two substrates show a linear competitive inhibition when assayed specifically for each substrate. Thus it would appear that the homoarginine decarboxylase of *L. sativus* is the same protein that also catalyzes the decarboxylation of L-lysine and differs from the arginine decarboxylase from this plant in one important respect viz. the guanido function in the substrate molecule is not an essential structural requirement for catalytic activity. What probably is more important is the C-4 side chain attached to the α-carbon atom of the amino acid.

We have reported earlier that arginine and homoarginine differ in another important aspect viz. the mode of their biosynthesis. Infiltration experiments showed that ^{14}C-ornithine is readily incorporated into arginine and then to agmatine etc. whereas ^{14}C-lysine is not converted to homoarginine and subsequently to homoarginine [2]. The data presented in this paper reveal that in terms of decarboxylation of the guanido amino acids the reverse situation exists viz. the decarboxylase specific for L-arginine does not attack L-ornithine [5], but that for L-homoarginine readily decarboxylates L-lysine.

EXPERIMENTAL

Materials. L. sativus seeds were supplied by the Plant Breeding Section of Indian Council of Agricultural Research, New Delhi. U-^{14}C-lysine (sp. act. 99 mCi/nmol) was obtained from Bhabha Atomic Research Centre, Bombay. (1–5)-^{14}C-Homoarginine was prepared from U-^{14}C-lysine by guanidation as described in ref. [2]. Alumina C-γ gel was prepared by the method of ref. [8].

Germination and seedling growth. Seeds were germinated after sterilization in dark at 25–28° in an incubator as described in ref. [1].

Purification of the enzyme. Up to step-5, the purification procedure was similar to that adopted for purification of arginine decarboxylase [5]. Briefly the procedure consisted of homogenization of fr. 5-day old seedlings in a chilled pestle and mortar with one vol. of 50 mM Na_2HPO_4 containing 2 mM mercaptoethanol, and 50 μM pyridoxal phosphate. The extract filtered through a cheese cloth, and clarified by centrifugation, represented the crude extract (Step 1). Unless otherwise specified, all the operations were carried at 0–2° and centrifugations at 25000 *g* for 30 min. The crude extract was brought to 7·5 mM with respect to $MnCl_2$ with the addition of M $MnCl_2$, and the precipitated nucleoproteins were removed by centrifugation (Step 2). The $MnCl_2$ treated supernatant fraction was adjusted to pH 7 and solid $(NH_4)_2SO_4$ was added to 25% saturation. The ppt. was removed by centrifugation and discarded. To the supernatant additional $(NH_4)_2SO_4$ was added to 55% saturation (Step 3). The ppt. was collected by centrifugation and dissolved in 5 mM Pi buffer (Pi buffer used in this investigation was NaPi buffer (pH 7·5) containing 2 mM β-mercaptoethanol and 20 μM pyridoxal-5-phosphate). This 25–55% $(NH_4)_2SO_4$ fraction was further subjected to Me_2CO fractionation at −10° and was collected by centrifugation at −10° at 15000 *g* for 10 min and after removing traces of Me_2CO (Step 4) was dissolved in 5 mM Pi buffer. The 30–50% Me_2CO fractionated enzyme was dialyzed against 5 mM Pi buffer and adsorbed on alumina C-γ at a protein to gel ratio of 1:6. The C-γ gel was washed by centrifugation with 5 mM Pi buffer and the enzyme activity was eluted by centrifugation with 50 mM Pi buffer from the gel. The fractions with good activity were pooled (Step 5), concentrated over aquacide, and dialysed against 50 mM Pi buffer. The concentrated C-γ gel fraction was loaded on to a DEAE-Sephadex column, washed first with 50 mM Pi buffer and the proteins were then eluted batchwise successively with 0·1, 0·2, 0·3 and 0·4 M KCl in 50 mM Pi buffer. The 0·2 M KCl eluate (peak III, Fig. 2) was concentrated over aquacide, dialyzed against 3 changes of 200 vol. each of 5 mM Pi buffer and used in the characterization study.

Assay. The homoarginine and lysine decarboxylating activities were assayed in Warburg flasks by measuring $^{14}CO_2$ released from the respective ^{14}C-amino acids. The reaction mixture contained 0·1 M Tris–HCl buffer (pH 8·4), 2 mM dithiothreitol, 50 μM pyridoxal phosphate, 1 mM $MnCl_2$, 4 mM substrate (sp. act. $4·4 \times 10^5$ cpm per μmol) and appropriate amounts of enzyme protein (100–500 μg) in a total vol of 1 ml and was incubated at 41° for 4–6 hr. The reaction was initiated with the addition of substrate and terminated by tipping the acid (0.6 ml of 2M H_2SO_4) contained in the side arm and incubated for a further period of 2 hr at room temp. to release CO_2 from the acidified medium. $^{14}CO_2$ evolved was trapped in 0·2 ml of M KOH present in the centre well. Aliquots of 0·1 ml of KOH from the centre well were mixed with 10 ml portions of scintillation fluid (0·5% PPO in methyl cellosolve–toluene, 1:1) and counted. Quenching correction, calculated by the internal standard method was applied to the values obtained. In every expt two non-enzymic controls were always included and these contained the standard incubation mixture with appropriate additions, but enzyme was replaced by H_2O. One unit of homoarginine- or lysine decarboxylase is defined as the amount of enzyme required to liberate 1 nmol $^{14}CO_2$ per hr at 41°. Arginine decarboxylase assay was identical to that employed for homoarginine

decarboxylase except that the incubation was carried out for 1 hr at 45° and the buffer was pH 8·5.

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