Partial Purification and Characterization of Arginine Decarboxylase from Avocado Fruit, A Thermostable Enzyme[1]

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ABSTRACT

A partially purified preparation of arginine decarboxylase (EC 4.1.1.19), a key enzyme in polyamine metabolism in plants, was isolated from avocado (*Persea americana* Mill. cv Fuerte) fruit. The preparation obtained from the crude extract after ammonium sulfate precipitation, dialysis, and heat treatment, had maximal activity between pH 8.0 and 9.0 at 60°C, in the presence of 1.2 millimolar $MnCl_2$, 2 millimolar dithiothreitol, and 0.06 millimolar pyridoxal phosphate. The K_m of arginine for the decarboxylation reaction was determined for enzymes prepared from the seed coat of both 4-week-old avocado fruitlet and fully developed fruit, and was found to have a value of 1.85 and 2.84 millimolar, respectively. The value of V^{app}_{max} of these enzymes was 1613 and 68 nanomoles of CO_2 produced per milligram of protein per hour for the fruitlet and the fully developed fruit, respectively. Spermine, an end product of polyamine metabolism, caused less than 5% inhibition of the enzyme from fully developed fruit and 65% inhibition of the enzyme from the seed coat of 4-week-old fruitlets at 1 millimolar under similar conditions. The effect of different inhibitors on the enzyme and the change in the nature of the enzyme during fruit development are discussed.

It is well established that the intracellular concentration of polyamines changes markedly in response to conditions which affect growth and development of plant tissue (11, 12). Hence, processes like cell division (7) or cell differentiation (4, 9) are deemed to be regulated by polyamines. The properties of the enzymes involved in the biosynthesis of polyamines and their degradation, and the mode by which the activities of these enzymes are regulated, may therefore contribute to our understanding of the overall processes of development and senescence of fruits.

Whereas in mammalian cells putrescine is synthesized from L-ornithine by ornithine decarboxylase (22), there have been a number of reports indicating that putrescine in plants is formed from L-arginine (19). Arginine is converted by ADC[2] to agmatine which is then converted to putrescine (1, 11, 12). In recent studies by Heimer *et al.* (10) and Cohen *et al.* (6) on tomato ovaries and fruits, it was observed that the activity of ODC changed during fruit development, whereas the relatively low

activity of arginine decarboxylase did not change as much under the same conditions. This was interpreted to mean that ODC rather than ADC is involved in polyamine metabolism in fruits in this system. It was previously suggested, however, that the presence of inhibitors of ADC in the crude extract from the tissue might prevent the measurement of the enzyme activity (14). Indeed, Ramakrishna and Adiga (17) found that ADC from *Lathyrus sativus* is inhibited by its end product, polyamines, and suggested that it may be a regulatory system *in vivo*.

In the present study we attempted to partially purify and characterize an arginine decarboxylase preparation from avocado fruit in order to be able to gain more information on its role in fruit development and ripening. It was demonstrated that special precautions have to be taken in order to separate the activity from inhibitors co-extracted with it. Although some properties of this enzyme are similar to those found in ADC from other sources, ADC from avocado fruit is unique in its thermostability.

MATERIALS AND METHODS

Fruit Material. Avocado fruits (*Persea americana* Mill. cv Fuerte) were harvested in a local plantation at various stages of development. The enzyme was extracted immediately after harvest, unless otherwise indicated.

Enzyme Extraction and Purification. Fruit mesocarp, 100 g, was grated and rapidly frozen in liquid nitrogen. It was then pulverized with a pestle in a chilled mortar and finally suspended in 300 ml of ice cold solution containing 50 mM Tris buffer at pH 9.0, 50 mM ascorbate and PVP (20.0 mg/1.0 g frozen tissue). The suspension was stirred for 30 min at 2 to 4°C and KOH (1 M) was added occasionally to keep the solution at pH 8.0 to 9.0. The brei was subsequently strained through four layers of cheesecloth and the filtrate was centrifuged at 8000g for 10 min.

Saturated $(NH_4)_2SO_4$ was added to the resulting supernatant fraction (which was referred to as crude extract) to make 30% saturation, stirred slowly for 20 min, and centrifuged at 10,000g for 30 min. The pellet (0–30 p) was discarded and the supernatant fraction brought to 60% saturation by the addition of saturated $(NH_4)_2SO_4$ solution. Precipitated protein was sedimented as above. The pellet was dissolved in 10 ml of 20 mM ascorbate and 25 mM Tris buffer at pH 9.0. This suspension (which was referred to as 30–60 p) was dialyzed for 4 h in a 25-fold excess of the same buffer, with change after 2 h. The dialyzed solution (dialyzed fraction) was incubated for 10 min at 60°C and centrifuged at 10,000g for 10 min; the supernatant fraction was referred to as the heat-purified fraction. Purification of the enzyme was done at 2 to 4°C, except for the heat purification step.

Assay Conditions. Arginine decarboxylase was assayed by measuring $^{14}CO_2$ formation. An aliquot of the enzyme was added to a tube containing, in a final volume of 175 μl, 2 mM DTT, 30 mM buffer Tris, 30 mM buffer Caps (pH 9.0), 60 μM PLP and 0.2 to 0.3 mg of the protein. The reaction was started by the

[2] Abbreviations: ADC, arginine decarboxylase; PLP, pyridoxal 5'-phosphate; Caps (3-[Cyclohexylamino]-1-propane sulfonic acid; NEM, N-ethylmaleimide; ODC, ornithine decarboxylase.

addition of 7 mM L-[U-^{14}C]arginine (1-2 μCi/μmol, obtained from New England Nuclear Corp.). The plastic test tubes were then capped with rubber stoppers fitted with plastic center wells containing 100 μl of 1 M KOH, absorbed on a Whatman No. 1 paper wick (to trap the ^{14}CO$_2$ formed during the reaction), and placed in a shaking water bath at 60°C for 30 min. The reaction was stopped by the injection of 0.5 ml of 1 M H$_2$SO$_4$ to the reaction mix. Additional shaking at 60°C for 30 min was allowed to ensure the liberation of ^{14}CO$_2$ that was still trapped in the reaction mixture. The paper wicks were placed in scintillation vials with 0.5 ml H$_2$O and 5 ml Aqualuma-Plus scintillant (Lumac B.V., Holland). The samples were counted after equilibration overnight in the dark in a Kontron liquid scintillation counter.

Blank values were obtained by using boiled dialysis or heat-purified extract and adding acid before substrate.

The amount of ^{14}CO$_2$ produced was determined by the amount of ^{14}CO$_2$ trapped by the KOH on the paper wick. The specific activity of the enzyme is the amount of CO$_2$ produced per hour per milligram of protein.

Protein Determination. Protein concentrations were determined by the method of Bradford (5), using BSA as a standard.

Urease Assay. Urease was determined as described previously by Stutts and Fridovich (21).

RESULTS

Enzyme Isolation. Partial purification of ADC from mature avocado mesocarp is summarized in Table I. The heat treatment procedure resulted in a 6.7-fold purification of the enzyme with a specific activity of 140 nmol arginine converted per mg of protein per h and a yield of 144%. When the rate of the reaction was plotted *versus* protein concentration, deviation from linearity was observed frequently in all the fractions isolated except for the preparation obtained after heat treatment. This may be attributed to an inhibitor which is co-isolated with the enzyme and which is sedimented or inactivated by heating. The degree of this inhibition varied in the different preparations and was always maximal in the crude extract.

The routine use of L-[U-^{14}C]arginine as the substrate raised the possibility that the ^{14}CO$_2$ released could be at least in part attributed to the presence of arginase, giving rise to urea formation followed by urease activity which results in the release of ^{14}CO$_2$ from the guanido group, rather than from the carboxyl group. This sequence of reactions was observed in extracts from *Cucurbita* seedlings (13). However, no urease activity could be detected in the partially purified preparations from avocado fruits eliminating possible ^{14}CO$_2$ release other than through ADC activity.

Heat Activation. The dialyzed fraction which contained ADC activity was incubated at different temperatures for various periods of time (10–60 min). The heat-treated fraction was then centrifuged and ADC activity was assayed. As illustrated in Figure 1, maximal activity was obtained upon 10 min incubation at 60°C, where the specific activity of ADC increased by 3- to 4-fold. Prolonging the incubation times at 60°C to 20 min resulted in a decrease in enzyme activation. Preincubation at 55°C for 10 min increased the specific activity by about 2-fold, whereas at 45°C no effect was recorded.

Effect of pH and Temperature on the Activity of ADC. When ADC activity was assayed at different temperatures, maximal activity was obtained at 60°C which was constant for more than an hour. The presence of substrate in the incubation medium prevented the inactivation which was observed during preincubation periods longer than 10 min (Fig. 1). A nonlinear dependency was obtained when the logarithms of the specific rate of the reaction at any temperature were plotted against the reciprocal of the absolute temperature (Fig. 2). The transition temperature for this reaction, where the dependency of V_{max} changes from one rate-limiting step to another, was found to be 49.5°C. The sudden drop in the plot at temperatures higher than 60°C indicates enzyme inactivation. The Arrhenius energy of activation for the decarboxylation reaction, as calculated for the enzyme from fully developed fruit (Fig. 2), was 26.64 kcal/mol below the transition temperature and 7.74 kcal/mol at temperatures higher than 49.5°C. Similar results were obtained with an enzymic preparation from 4-week-old avocado fruitlets (not shown). For ADC from *Lathyrus sativus* seedlings a value of 7.32 kcal/mol for the energy of activation was reported (17).

When the rate of the decarboxylation reaction by the heat-treated fraction was plotted against the pH of the medium at 60°C, maximal activity was obtained between pH 8.0 and 9.0 (Fig. 3). A mixture of the two buffers, Tris-Cl and Caps, gave maximal rates at pH 9.0. The activity of the enzyme was inhibited by about 50% when phosphate buffer at pH 9.0 had been used. Both the pH profile for the activity of the enzyme and the inhibitory effect of phosphate were similar in the fully developed fruit and in 4-week-old avocado fruitlets.

Effect of Substrate Concentration and Other Additives. The enzyme isolated from fully developed fruit exhibited typical Michaelis-Menten kinetics with a K_m value of 2.85 mM and a V_{max} of 68 nmol of CO$_2$ liberated per mg of protein per h. Similar values were obtained for ripe and unripe mature fruits (Fig. 4A). When the enzyme was isolated from the seed coat of 4-week-old avocado fruitlets, a K_m value of 1.85 mM and a V_{max} of 1613 nmol of CO$_2$ liberated per mg of protein per h were obtained under the same conditions (Fig. 4B).

Addition of 10 μM PLP to the reaction mixture caused an increase in the activity of the enzyme of about 30%. DTT (2 mM) was added routinely to the reaction mixture in order to protect the enzyme against possible reducing reactions, even

Table I. *Purification of ADC from Mature Avocado*

Purification of ADC activity from 100 g of mesocarp from mature avocado was done as described under "Materials and Methods."

| Purification Step	Total Protein	Total Activity[a]	Specific Activity	Purification	Yield
	mg	*units*	*units/mg protein*	*-fold*	*%*
1. Crude extract	280.0	6,003	21	1.0	100
2. Ammonium sulfate fractionation 0–30 p	6.8	Undetectable	Undetectable	Undetectable	Undetectable
3. Ammonium sulfate fractionation 30–60 p	234.6	10,362	44	2.1	172
4. Dialysis	234.4	18,311	78	3.7	305
5. Heat purification	61.8	8,668	140	6.7	144

[a] nmol of ^{14}CO$_2$ formed per h.
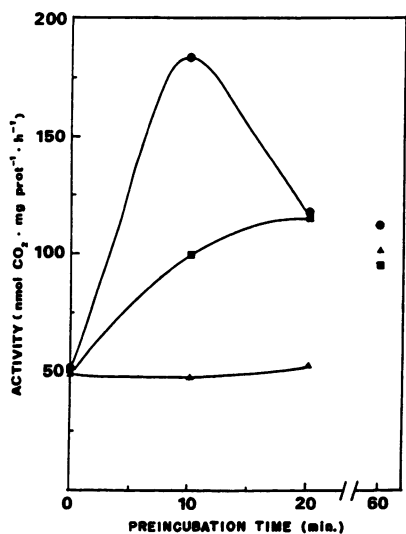
FIG. 1. Purification of ADC by heat treatment. The dialyzed 30 to 60 p (NH₄)2SO₄ preparation was preincubated in the dialysis medium at three different temperatures: (▲), 45°C; (■), 55°C; and (●), 60°C. The different samples were centrifuged at 10,000g for 10 min following the indicated preincubation times and protein concentration was determined in each of the supernatants. Protein from the supernatant (at a final concentration of 0.36–1.14 mg/ml) was added to the assay medium for the determination of ADC activity, as described under "Materials and Methods."
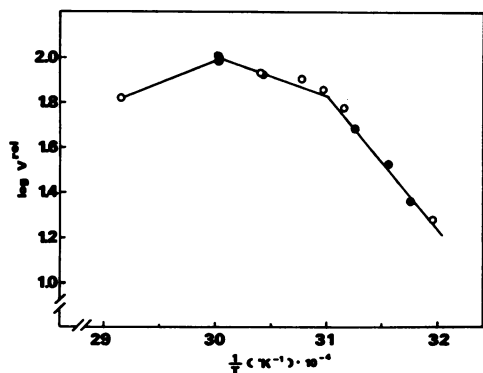

FIG. 2. Dependence of ADC reaction on temperature. Assay conditions were as described under "Materials and Methods." Data from three independent experiments are presented. Protein concentration in the assay medium was 1.1 to 1.8 mg/ml. Enzyme preparations were extracted from mature fruit. (O), MnCl₂ was omitted from the assay medium; (●), 1.2 mM MnCl₂ was present in the assay medium. V^{rel}, rate of ADC expressed as per cent of the maximal value obtained at 60°C. Maximal values were 60 and 45 nmol of CO₂ liberated per mg of protein per h in the presence of MnCl₂, and 8 nmol of CO₂ liberated per mg of protein per h in its absence.

though it did not significantly affect the activity of the enzyme.

The enzyme did not show an absolute requirement for metal ions. Low concentrations of Mn²⁺ (up to about 1.2 mM) enhanced the activity of the enzyme by more than 4-fold and higher concentrations were inhibitory (Table II). Mg²⁺ and Ca²⁺ did not have any effect on the activity of the enzyme at a concentration of 5 mM. Cu²⁺ (1 mM) inhibited the activity of the enzyme by 25% and 5 mM caused 94% inhibition (Table II).

Effect of Inhibitors. In order to test if −SH groups are involved in the activity of ADC extracted from mature avocado, the enzyme was assayed in the presence of NEM, which is known to bind to −SH groups. The enzyme was preincubated with various
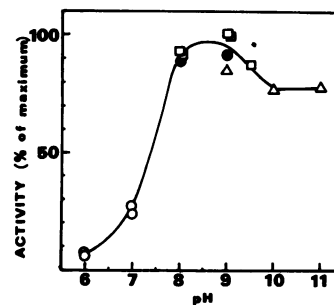

FIG. 3. Effect of pH on ADC activity. Assay medium was as described under "Materials and Methods." Protein concentration in each sample was 0.078 mg/ml. The enzyme was extracted from the seed coat of 4-week-old avocado fruitlets. The buffer added to the reaction mixture was 30 mM Pipes (O), 30 mM Tris (●), 30 mM Caps (Δ), 30 mM Tris + 30 mM Caps (□).
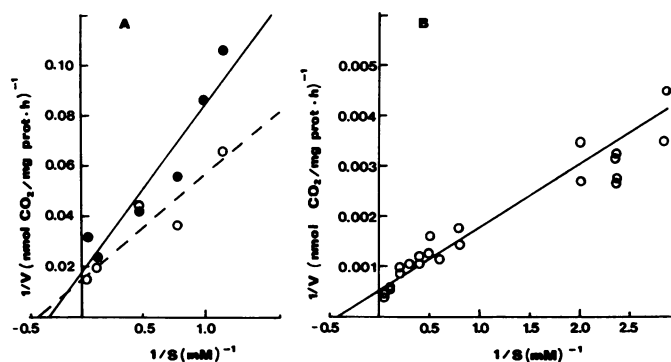

FIG. 4. Double reciprocal plots of ADC activity versus arginine concentration. ADC activity in partially purified extracts from (A), soft (O) and hard (●) mature avocado fruit; and (B), seed coat of 4-week-old avocado fruitlets (O). Measurement of decarboxylation of arginine was as described in "Materials and Methods." Final concentration of protein was 1.4 mg/ml in (A) and 0.02 mg/ml in (B).

Table II. Effect of Metal Ions on ADC Activity

Assay conditions were as described under "Materials and Methods." Protein concentration was 1.4 to 1.6 mg/ml. Control values were 42 nmol ¹⁴CO₂ released per mg of protein per h.

Addition	Cation Concentration	Activity
	mM	% of control
MnCl₂	0.05	235
	0.50	360
	1.14	420
	5.00	12
CuCl₂	1.00	75
	5.00	6
MgCl₂	5.00	95
CaCl₂	5.00	88

concentrations of NEM (0.5–8.5 mM) for 2 min. DTT was then added to the assay mixture to reduce excess NEM in the medium prior to the addition of substrate. It was found (Fig. 5) that NEM partially inhibited the specific activity of the enzyme (65% inhibition at a concentration of 3 mM).

Fluorescein isothiocyanate, which was suggested to bind to lysine residues with high affinity (16), caused a 40% inhibition when added at a concentration of 90 μM.

Addition of ornithine, diamines, or polyamine to the assay mixture resulted in inhibition of ADC activity (Fig. 6). In case of ADC extracted from the seed coat of 4-week-old fruitlets,
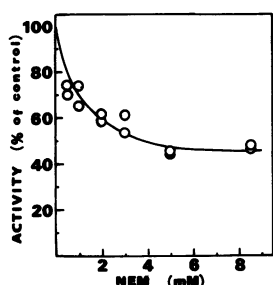
FIG. 5. Effect of NEM on ADC activity. Heat-purified fraction of the enzyme extracted from seed coat of avocado fruitlets at a final protein concentration of 0.058 mg/ml. NEM was added to the incubation medium containing the components of the assay medium, except arginine and DTT, for 2 min at 60°C. DTT was added at a final concentration of 60% of that of NEM just before the addition of the substrate (7 mM arginine).
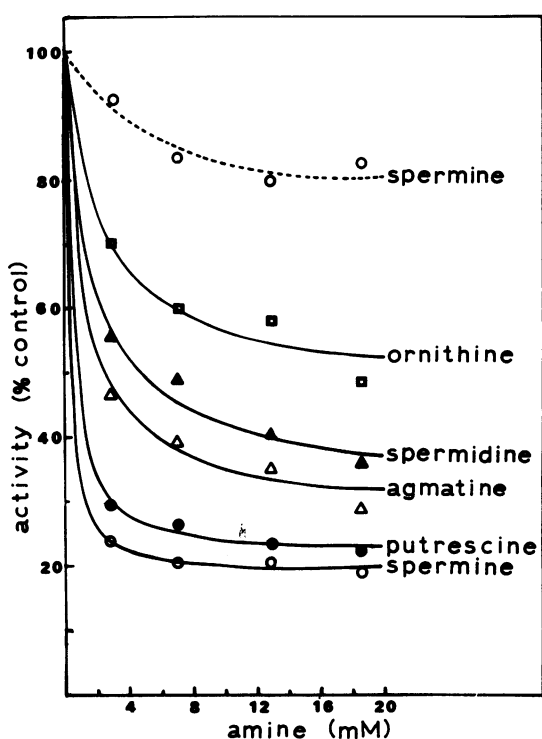

FIG. 6. Inhibition of ADC activity by amines. The heat-purified fraction of the enzyme extracted from two sources: seed coat tissue of 4-week-old avocado fruitlets (——) and mesocarp tissue of preclimacteric mature avocado fruit (- - -). The assay medium was as described under "Materials and Methods," and the protein concentration in each sample was 0.078 mg/ml for the mixture from seed coats and 0.62 mg/ml for the mixture from the mesocarp. The enzyme activity was assayed in the presence of the inhibitor at the concentrations indicated.

ornithine at about 10 mM inhibited the activity of 40% whereas the diamines and polyamines caused 60 to 80% inhibition. ADC extracted from the mesocarp of mature fruits was less affected by the presence of polyamines in the assay mix; spermine at about 10 mM inhibited the activity by 20% (Fig. 6), whereas spermidine, putrescine, and agmatine were slightly less effective (not shown). Ornithine up to 20 mM had no effect on the activity of ADC extracted from mature avocado fruit mesocarp.

ADC extracted from avocado fruit was relatively stable on storage at −20°C. About 70% of the original activity was retained after 60 d of storage.

DISCUSSION

In the present investigation, partial purification of L-arginine decarboxylase from avocado fruit is described. Some resemblance was found between this enzyme and ADC isolated from *L. sativus* (17). The high pH optimum (8.0–9.0) and the requirements of PLP as a cofactor are similar in the two enzymes. A K_m value of 1.8 to 2.8 mM arginine was obtained for ADC from avocado. A slightly lower value of 1.3 mM was reported for the K_m of arginine for ADC from *L. sativus*. These two enzymes differ from each other in other properties. ADC from avocado fruit requires Mn^{2+} ions for maximal activity and has a uniquely high optimal temperature of 60°C. No other ADC studied so far exhibits such high thermostability. In this study, the heat stability of the partially purified enzyme has been used in the processes of purification. It should be noted, however, that thermostability of ADC is improved in the presence of its substrate, arginine. The exact factors which imparts thermostability to an enzyme is not completely understood. Although some explanations have been suggested (2, 3, 15, 20), a more detailed study of the molecular nature of ADC from avocado fruit is required in order to elucidate the nature of the thermostability property of this enzyme.

The possibility that polyamines participate in the processes of fruit growth and development was previously suggested (8, 10). ADC was considered to be a key enzyme in the biosynthesis of polyamines in plant tissue (14, 17, 18). However, it has recently been reported (6, 8, 10) that the activity of ADC found in crude extracts from tomato ovaries and potato tissue was lower than that of ODC and did not seem to change much during fruit development (6, 10). This was interpreted to mean that ODC is the key enzyme in the development of tomato fruit. In the present study we reported high activity of ADC in partially purified extracts from avocado fruit during its growth, development, and ripening whereas very low activity of this enzyme was observed in crude extracts of the same tissue. It should be noted that during the enzyme extraction some inhibitory components might be released into the medium and cause inhibition of enzyme activity during the assay of the crude enzyme. In our present study, these components are at least partially inactivated or eliminated by dialysis and heat treatment.

The inhibitory effect of the various polyamines on ADC might indicate that there is a regulatory feedback mechanism which enables the inhibition of ADC by its end product—polyamines. The inhibitory effect of polyamines on ADC may also explain the lower activity of the enzyme isolated from mature fruit as compared with that isolated from fruitlets (Fig. 4, A and B, respectively). The enzyme isolated from mature fruit may have a tightly bound polyamine which occupies the polyamine-binding site that might also be the regulatory site on the enzyme. This would result in both a lower apparent V_{max} of ADC from mature fruit and a lower sensitivity of the enzyme to the presence of polyamines in the assay medium, due to the fact that the polyamine-binding site is already occupied (Fig. 6).

These findings support the suggestion that avocado ADC, the first enzyme is the pathway of polyamine synthesis, is regulated by its end product—polyamines.

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