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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE FROM BANANA FRUIT

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Key Word Index—*Musa* AAA group, Cavendish subgroup; Musaceae; banana; enzymology; 1-aminocyclopropane-1-carboxylate (ACC) oxidase.

Abstract—A novel method using PEG and ammonium sulphate was used to concentrate ACC oxidase from a crude extract prepared from the pulp of ripe fruit of banana (*Musa* AAA group, Cavendish subgroup). The 145-fold purification of the ACC oxidase to electrophoretic homogeneity was carried out in four chromatographic steps: hydrophobic interaction, anion exchange, chromatofocusing and gel filtration. The molecular mass of the purified enzyme was estimated to be 40 000 by gel filtration and 36 000 by SDS-PAGE, indicating that the enzyme is active as a monomer. The enzyme was recognized by a polyclonal antibody directed against a recombinant polypeptide derived from the tomato ACC oxidase, showing that the banana enzyme shares immunogenic epitopes with ACC oxidases from other fruits. An isoelectric point at pH 4.9 was estimated by chromatofocusing. The K_m with respect to ACC was found to be 56 μM. Ascorbate, Fe^{2+} and sodium bicarbonate were required for the activity of the enzyme, and the presence of ascorbate was found to be necessary to avoid loss of activity during the purification. In general, the properties of the ACC oxidase from this monocotyledonous source resemble those of the enzyme previously purified from apple fruit.

INTRODUCTION

Ethylene is a plant hormone involved in many aspects of plant growth and development, but it is especially important during the ripening of climacteric fruit [1]. The final step in ethylene biosynthesis is catalysed by the enzyme 1-aminocyclopropane-1-carboxylate (ACC) oxidase (formerly known as the ethylene-forming enzyme). The purification and subsequent characterization of this enzyme has only recently become possible with the discovery that *in vitro* activity requires ascorbate and Fe^{2+} [2]. The authenticity of this activity as ACC oxidase rather than in being a non-enzymatic free-radical attack on ACC, has been shown by its low K_m for ACC, and by stereodiscrimination towards stereoisomers of 1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC), an ACC analogue [2]. ACC oxidase activity has been studied in several dicotyledonous fruits including melon [3], avocado [4], apple [5, 6], Winter squash [7] and pear [8], but the enzyme has been purified to homogeneity only from apple [9–11].

During the course of ripening in the banana there is a large increase in the ACC oxidase activity extracted from both the peel and the pulp tissue [12]. This enzyme requires CO_2 as an essential cofactor, like the enzyme from melon [13] and apple [6, 9, 14]. In the present paper we describe a procedure for the purification of the banana pulp enzyme to electrophoretic homogeneity, and the properties of the purified enzyme.

RESULTS AND DISCUSSION

Purification

As with the ACC oxidase from other fruits [9, 15] that of banana was found to require glycerol in the extraction buffers for activity to be maintained *in vitro*. However, homogenization of the pulp from ripe bananas in a glycerol-containing medium yielded a homogenate from which it was difficult to precipitate proteins by conventional ammonium sulphate precipitation. Thus the novel technique described here using a combination of polyethyleneglycol (PEG) and ammonium sulphate was developed. Table 1 summarizes the purification procedure, which involved four chromatography steps: hydrophobic interaction on Phenyl Sepharose (Fig. 1A), anion exchange on Mono-Q (Fig. 1B), chromatofocusing on

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Table 1. Purification of the ACC oxidase from ripe banana fruit

Purification step	Total activity (pkat)	Total protein (g)	Specific activity (pkat mg^{-1} protein)	Purification (fold)	Recovery (%)
Crude extract	375.8	98700	3.8	—	100
PEG-$(NH_4)_2SO_4$	301.2	29020	10.4	2.7	80.2
Phenyl Sepharose	63.5	2460	25.8	6.8	16.9
Mono-Q	18.5	266	69.4	18.2	4.9
Mono-P	4.8	44	109.9	28.9	1.3
Superdex 75	4.4	7.9	550.6	145	1.2

The enzyme was purified from 60 g of banana pulp tissue as described in the text.

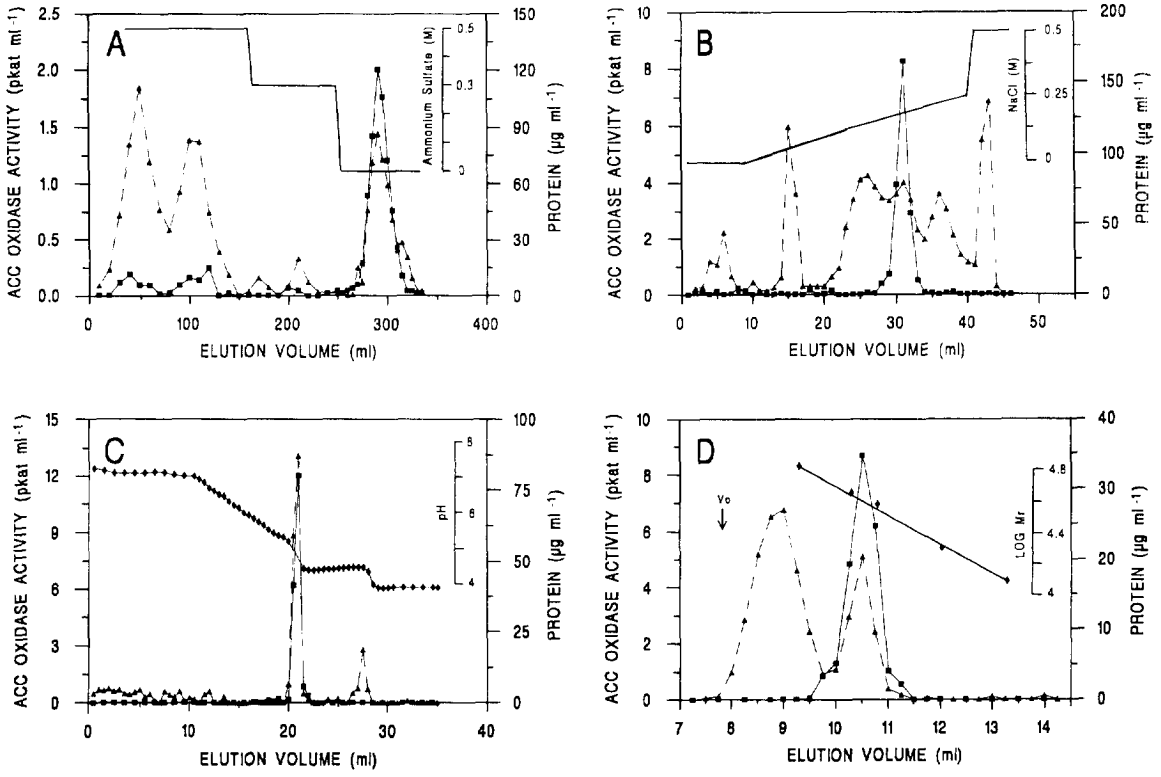

Fig. 1. Elution profiles of banana ACC oxidase during the purification of the enzyme. (A) Hydrophobic interaction chromatography (Phenyl Sepharose CL-4B) of concentrated crude extract; (B) anion exchange chromatography (Mono-Q/FPLC column); (C) chromatofocusing on Mono-P column (FPLC); (D) gel filtration chromatography on Superdex 75 column (FPLC). ACC oxidase activity, —■—; protein, —▲—.

Mono-P (Fig. 1C) and finally gel filtration on Superdex 75 (Fig. 1D). This purification procedure differs from those that have been used to purify the apple ACC oxidase [9–11]. We found that the banana enzyme required ascorbate to be present throughout the purification in order to retain activity. The ascorbate requirement could not be replaced by DTT. A requirement for ascorbate had not been reported for the apple enzyme, but flavanone 3-hydroxylase, a member with ACC oxidase of the 2-oxo-acid-dependent dioxygenases family [16], is stabilized during purification by the inclusion of 2-oxoglutarate [17], which acts as a co-substrate for the flavanone 3-hydroxylase just as ascorbate acts as a cosubstrate for the ACC oxidase [9]. In the present case ascorbate could be acting to help stabilize the banana ACC oxidase in its role as a co-substrate. Alternatively, it could be acting to help exclude oxygen or reduce enzyme-bound Fe^{2+}. During purification of the apple ACC oxidase, the enzyme was protected against Fe^{2+}-catalysed oxidative attack by including a Fe^{2+}-chelator in the purification media [10]. However, with the banana enzyme this ingenious tactic failed to have any significant effect, either in the presence or absence of ascorbate.

From the chromatofocusing step the isoelectric point of the enzyme was estimated to be at pH 4.9. In Fig. 1C there is a deflection of the pH curve presumably due to

the presence of ascorbate (pK_1 4.17) added to the buffer. When ascorbate was omitted from the buffer it was possible to estimate an isoelectric point at pH 4.9.

The banana ACC oxidase resembled the enzyme purified from apple [9–11] and other fruits [3, 4] in being active as a monomer with a molecular mass of about 40 000 estimated from gel filtration (Fig. 1D).

The progress of purification observed by SDS–PAGE is shown in Fig. 2A. The purified enzyme corresponded to a single band with a molecular mass of about 36 000, which was recognized, on Western blotting (Fig. 2B), by an antibody raised against a polypeptide fragment of the recombinant tomato enzyme. In these respects the banana enzyme resembled that of apple [10]. However, as shown in Fig. 2B, the antibody cross-reacted with more bands in the banana extract (lane b) than in a similar extract prepared from apple (lane a), probably because the epitope is more abundantly represented in the banana. Until purification on the Mono-Q the ACC oxidase could not be detected by Western blotting. This we attribute to the low concentration of ACC oxidase protein in banana compared with apple. When the rate of ethylene production by the whole fruit during the climacteric phase is compared, it can be seen that apples (80–100 nl g^{-1} fr. wt hr^{-1}, [18]) produce 20 times more ethylene than do bananas (4–5 nl g^{-1} fr. wt hr^{-1}, [19]). This difference is consistent with the higher concentration of ACC oxidase in apple compared to banana that is indicated by the Western blots of the crude extracts from the two fruits (Fig. 2B).

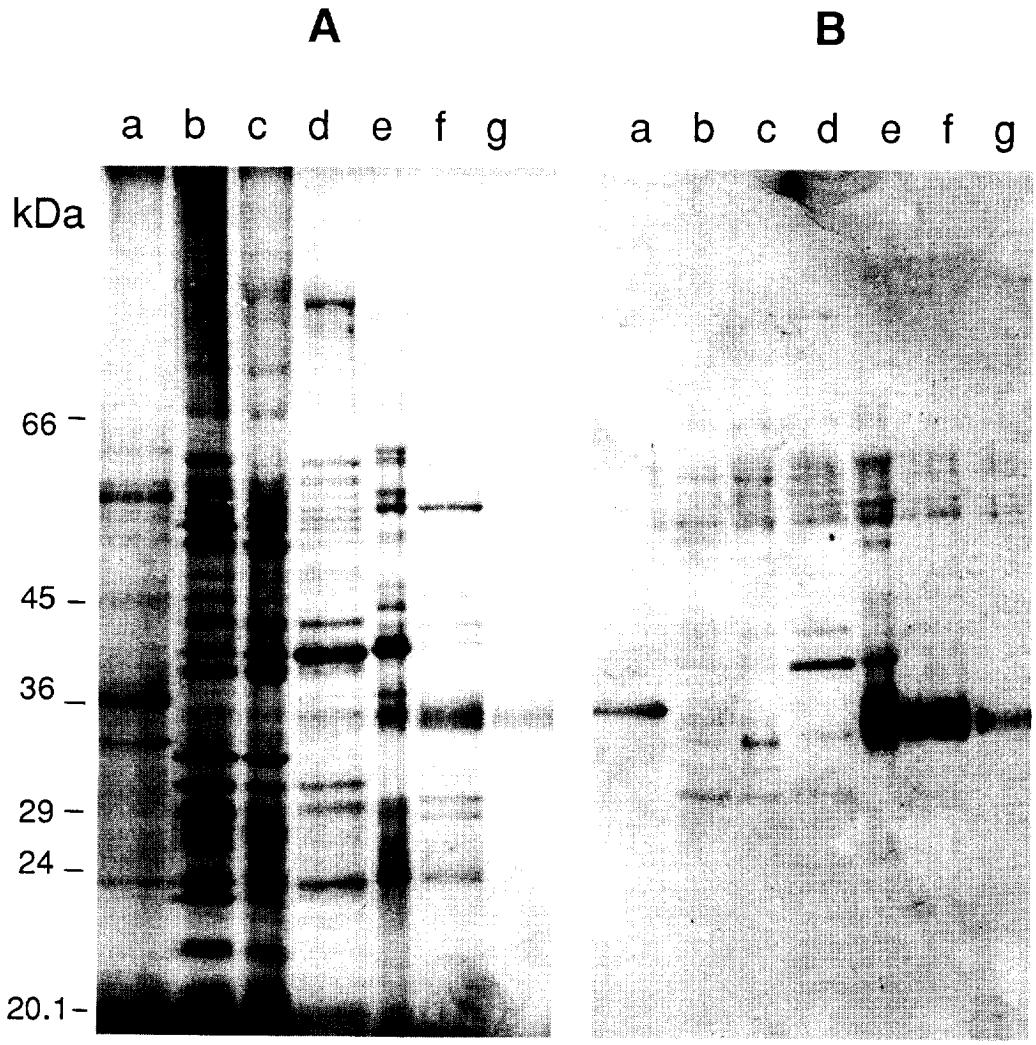

Fig. 2. (A) Silver stained SDS–PAGE documenting the progress of purification of banana ACC oxidase, and (B) Western blotting with an antibody raised against pTOM13 polypeptide. (Lane a) Apple crude extract (4 μg); (lanes b–g) fractions obtained during the purification procedure of banana ACC oxidase: (b) crude extract (4 μg); (c) PEG–ammonium sulphate concentrated extract (4 μg); (d) Phenyl Sepharose pool (3 μg); (e) Mono-Q pool (2 μg); (f) Mono-P pool (2 μg); (g) Superdex 75 (1 μg). Protein markers were: BSA, egg albumin, glyceraldehyde 3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen and soyabean trypsin inhibitor.

The purified enzyme had a specific activity of 551 pkat mg^{-1} protein (Table 1), which is comparable to the activities of 333 [9] and 300 [11] pkat mg^{-1} protein reported for the apple enzyme fully activated by carbon dioxide.

Characterization

The dependence of the banana ACC oxidase on Fe^{2+}, ascorbate and carbon dioxide (supplied as bicarbonate added to the reaction mixture) was determined using the enzyme obtained after passage through the Mono-Q column (Fig. 3). Half-maximal activity required 19 μM Fe^{2+} (Fig. 3A), 10 mM ascorbate (Fig. 3B) and 2.9 mM (Fig. 3C) bicarbonate.

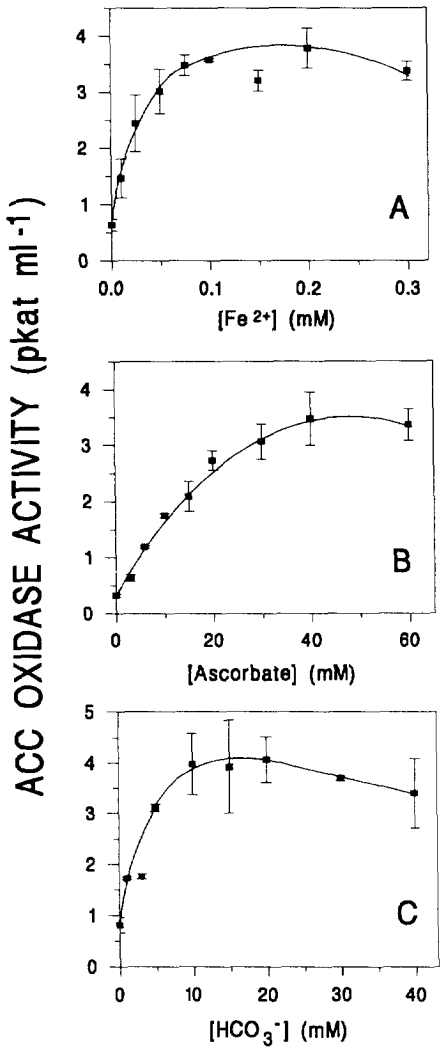

Fig. 3. Dependence of the activity of banana ACC oxidase on (a) $FeSO_4$, (b) sodium ascorbate, and (c) sodium bicarbonate. Partially purified enzyme obtained after the Mono-Q column was used, 20 mM sodium bicarbonate was added to the assay medium in (a) and (b).

The relationship between the bicarbonate concentration added and the carbon dioxide levels measured in the headspace under the conditions of the experiment shown in Fig. 3 was determined by direct measurements of the carbon dioxide at the end of the reaction period (A. D. Bauchot and P. John, unpublished data). From this relationship the carbon dioxide concentration at half-maximal activity was determined to be 0.3% (v/v). This value compares with those of 0.68% [6] and 0.5% [9, 14] for half-maximal activity of the apple ACC oxidase. The carbon dioxide concentration within a banana during the course of ripening has been estimated to be about 5% (v/v) [20]. Therefore, during ripening the banana ACC oxidase is probably fully activated by carbon dioxide.

The purified banana ACC oxidase showed Michaelis–Menten kinetics towards ACC, with an apparent K_m of 56 μM, which is comparable to the values of 121 μM [14] and 20 μM [6] found for the purified apple enzyme also assayed in the presence of saturating carbon dioxide levels.

EXPERIMENTAL

Plant material. Bananas (*Musa* AAA group, Cavendish subgroup) and apples (*Malus domestica* Borkh. cv Granny Smith) were purchased locally, allowed to ripen at room temperature, and the pulp was frozen in liquid nitrogen, ground with a mortar and pestle, and stored at − 70° until use.

Enzyme extraction and concentration. All operations were carried out at 4°. Frozen banana pulp (60 g) was homogenized in 300 ml of extraction buffer [0.1 M Tricine, pH 7.5, 10% (v/v) glycerol, 2 mM DTT and 30 mM sodium ascorbate] using a Waring Blender. The homogenate was filtered through a double layer of muslin and the crude extract was clarified by centrifugation at 10 000 *g* for 20 min. For apple, frozen pulp was homogenized in two volumes (v/w) of extraction medium consisting of 0.1 M Tricine (pH 8.0), 10% (v/v) glycerol, 1% (w/v) insoluble PVP (Polyclar AT), 0.1% (v/v) Triton X-100 and 5 mM DTT.

Banana crude extract was concd using a novel fractionation method which utilizes PEG 4000 and $(NH_4)_2SO_4$. To 300 ml of crude extract 15 g of PEG was added, and the mixture was stirred for 10 min. Then 117 g of $(NH_4)_2SO_4$ was added, and mixing was continued for a further 30 min. After centrifugation at 10 000 *g* for 20 min an upper white layer, containing the PEG was removed, extracted with 20 ml of extraction buffer using a hand-operated pestle fitting in a ground-glass tube. The concentrated enzyme fraction, obtained as a supernatant after a further centrifugation (20 000 *g*, 15 min), was passed through a pre-packed column of Sephadex G-25 M (PD-10, Pharmacia) pre-equilibrated with buffer A [20 mM Tricine, pH 7.5, 10% (v/v) glycerol, 2 mM DTT and 20 mM Na ascorbate] containing 0.5 M $(NH_4)_2SO_4$.

Hydrophobic interaction chromatography. A column (25 ml) of Phenyl Sepharose (CL-4B, Pharmacia) was pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. The concd extract was loaded onto the column, and after washing with about 4 column vols of the same buffer, the enzyme was eluted with buffers containing decreasing concentrations of $(NH_4)_2SO_4$. The flow rate was adjusted manually to 1 ml min^{-1}, and fractions of 5 ml were collected. All procedures were carried out at 4°. Each fraction was assayed for ACC oxidase activity and protein [21] using BSA as a standard.

Anion exchange chromatography. A Mono-Q HR 5/5 column (Pharmacia FPLC) of 1 ml was pre-equilibrated at room temp. with buffer A. Pooled fractions from the Phenyl Sepharose were over-loaded onto the column, by applying each time 5 ml and then washing with 5 ml of buffer A to remove unbound proteins. Proteins were eluted using a NaCl gradient at a flow rate of 1 ml min^{-1}, and fractions of 1 ml were collected and assayed. Fractions containing ACC activity were pooled and desalted by passage through a Sephadex G-25 M column to remove salt.

Chromatofocusing. A Mono-P HR 5/20 column (Pharmacia FPLC) of 4 ml was pre-equilibrated with buffer containing 25 mM imidazole–HCl (pH 7.4), 10% (v/v) glycerol, 10 mM sodium ascorbate and 2 mM DTT; and the desalted, pooled fractions from the Mono-Q column were loaded. The pH gradient was generated in the column during the passage of a solution of Polybuffer 74 (1:10, Sigma) pH 4.0, containing 10 mM sodium ascorbate and 2 mM DTT. The elution rate was 0.7 ml min^{-1}, and fractions of 0.5 ml were collected. The fractions containing ACC oxidase activity were pooled and concd by passage through a Centricon-10 filter (Amicon).

Gel filtration chromatography. A Superdex 75 HR 10/35 column (Pharmacia FPLC) was pre-equilibrated with a buffer containing 50 mM Tricine (pH 7.5), 150 mM KCl, 20 mM sodium ascorbate and 2 mM DTT. The pooled and concentrated fractions from the Mono-P column were applied in 200 μl to the column. Protein was eluted using the same buffer at a flow rate of 0.8 ml min^{-1}, and fractions of 0.25 ml were collected. The column was previously calibrated with the following relative molecular weight markers (M_r): BSA (66 000), egg albumin (45 000), soyabean trypsin inhibitor (20 100) and cytochrome *c* (12 400).

SDS–PAGE. SDS–PAGE was performed according to ref. [22]. Proteins in the gel (10%) were stained with $AgNO_3$, following the method reported in ref. [23].

Western blotting. After SDS–PAGE, proteins were electrotransferred from the gel to a nitrocellulose membrane of 0.45 μm using the method of ref. [24]. Free binding sites of the nitrocellulose membrane were blocked by incubation with 3% (w/v) low-fat dried milk prepared in a buffer containing 10 mM Tris–HCl (pH 7.4), 0.14 M NaCl and 0.1% (v/v) Tween-20. The nitrocellulose membrane was incubated with a fresh solution (1:4000 dilution) of a rabbit polyclonal antibody raised against a recombinant polypeptide derived from the pTOM13 tomato cDNA [10], which encodes the tomato ACC oxidase [25]. The membrane was then incubated with a fresh solution (1:5000 dilution) of an anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma). The alkaline phosphatase was detected using the chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium [23].

ACC oxidase activity. Enzyme activity was assayed by measuring the ethylene produced [2] after incubation of the enzyme for 15 min at 30° in a standard reaction mixture containing 0.1 M Tricine (pH 7.5), 10% (v/v) glycerol, 1 mM ACC, 30 mM sodium ascorbate, 0.1 mM ferrous sulphate and 20 mM $NaHCO_3$. All determinations were made in triplicate and, where indicated, the results are expressed as means ± s.e.

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