Characterization of 1-Aminocyclopropane-1-carboxylate Oxidase Partially Purified from Cherimoya Fruit

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The enzyme 1-aminocyclopropane-1-carboxylate (ACC) oxidase was extracted in a soluble form from mesocarp of ripe cherimoya fruit. ACC oxidase purification to near homogeneity was carried out in three chromatographic steps: anion exchange, chromatofocusing, and gel filtration. The molecular mass of the purified enzyme was estimated to be 66 kDa by gel filtration, 62 kDa by native PAGE, and 35 kDa by SDS−PAGE, indicating that the enzyme could be active as a dimer. An isoelectric point at pH 4.35 was estimated by chromatofocusing. The activity of semipurified enzyme eluted from Mono Q columns required Fe^{2+}, sodium bicarbonate, and ascorbate (K_m = 6.5 mM). The pH optimum was at 7.4 and the apparent K_m with respect to ACC was 82 μM in the absence of added sodium bicarbonate (194 μM in the presence bicarbonate). The particular characteristics of the purified enzyme in relation to the primitive phylogenetic position of cherimoya are discussed.

Keywords: *Annona cherimola; ACC oxidase; enzyme; ethylene; cherimoya fruit*

INTRODUCTION

Fruit ripening involves a series of complex biochemical and physiological changes, and the process is under a strict genetic control (Tucker, 1993), with each kind of fruit having a specific ripening pattern that leads to the development of the color, aroma, flavor, and texture characteristic of that fruit (Sacher, 1973).

Cherimoya (*Annona cherimola* Mill.) is a climacteric tropical fruit crop that is mostly grown in Spain, South America, and California. The principal producer is considered to be Spain, with about 2583 ha planted and a harvest of 22 346 metric tonnes per year in 1992 (AEA, 1994). The most important problem in the marketing of cherimoya fruit is its high perishablility, with a shelf life of less than 6 days at normal temperatures in the crop areas (Palma et al., 1993). Unusual among climacteric fruit, cherimoyas show two sucessive rises in respiration rate, with ethylene production following the first respiratory peak (Brown et al., 1988). Ripening coincides with the onset of the second respiration rise (Alique et al., 1994). Postharvest technologies applied for prolonging the storage life of cherimoya fruit have a clear effect in preventing or delaying ethylene production (Lahoz et al., 1993; Alique and Oliveira, 1994). Thus, an understanding of ethylene biosynthesis is of fundamental as well as applied significance.

The final step in the biosynthesis of ethylene in higher plants is catalyzed by 1-aminocyclopropane-1-carboxylate (ACC) oxidase (previously ethylene-forming enzyme) (Kende, 1993). Following the discovery that *in vitro* activity requires, in addition to ACC, the presence of ascorbate and Fe^{2+} (Ververidis and John, 1991), the enzyme has been purified and characterized as a soluble protein from different fruit sources: melon (Smith et al., 1992), apple (Fernández-Maculet and Yang, 1992; Kuai and Dilley, 1992; Dong et al., 1992, Dupille et al., 1993; Pirrung et al., 1993), avocado (McGarvey and Christoffersen,1992), and banana (Moya-León and John, 1994, 1995). Recently, it has been found that ACC oxidase activity requires CO_2 as an essential activator (Dong et al., 1992; Fernández-Maculet et al., 1993; Smith and John, 1993; Pirrung et al., 1993).

It is generally observed that ACC synthase is rate limiting for ethylene biosynthesis and that ACC oxidase is a constitutive enzyme. However, it has recently become apparent that the activity of ACC oxidase also increases in some plants in response to internal or external factors that induce ethylene formation (Kende, 1993).

Against this background the present paper describes, for the first time, the characteristics and kinetics parameters of the ACC oxidase partially purified from ripe cherimoya fruit.

MATERIALS AND METHODS

Plant Material. Cherimoya (*A. cherimola* Mill. cv. Fino de Jete) fruits of uniform size and maturity stage (skin becoming yellowish green) were purchased from a commercial source in Madrid (Spain).

Preclimacteric fruits were stored in the dark at 20 °C, and the ripening stage of each individual fruit was monitored. Ethylene production was measured by enclosing each fruit in a sealed glass jar. After 1 h, the ethylene produced in the head space was determined by a gas chromatograph (Pye Unicam Series 104), equipped with a flame ionization detector, using an activated alumina column (heated at 60 °C) and N_2 (40 mL min^{-1}) as the carrier gas. When ethylene production achieved a maximum, the fruit firmness was determined with a manual penetrometer and the soluble solids content in a sample of the pulp with an Atago refractometer. Pulp of ripe fruits was minced, frozen in liquid nitrogen, and stored at −80 °C until used. Seeds were discarded.

Enzyme Extraction and Partial Purification. Frozen pulp (125 g) was homogenized for 5 min with a Waring blender in 450 mL of 50 mM Tricine buffer (pH 7.5), 5% (v/v) glycerol, 1.5% (w/v) poly(vinylpyrrolidone) (PVP), and 3 mM dithiothreitol (DTT). All steps were carried out at 4 °C. After stirring for 15 min, the homogenate was filtered through two layers of muslin and centrifuged at 27000*g* for 30 min. The

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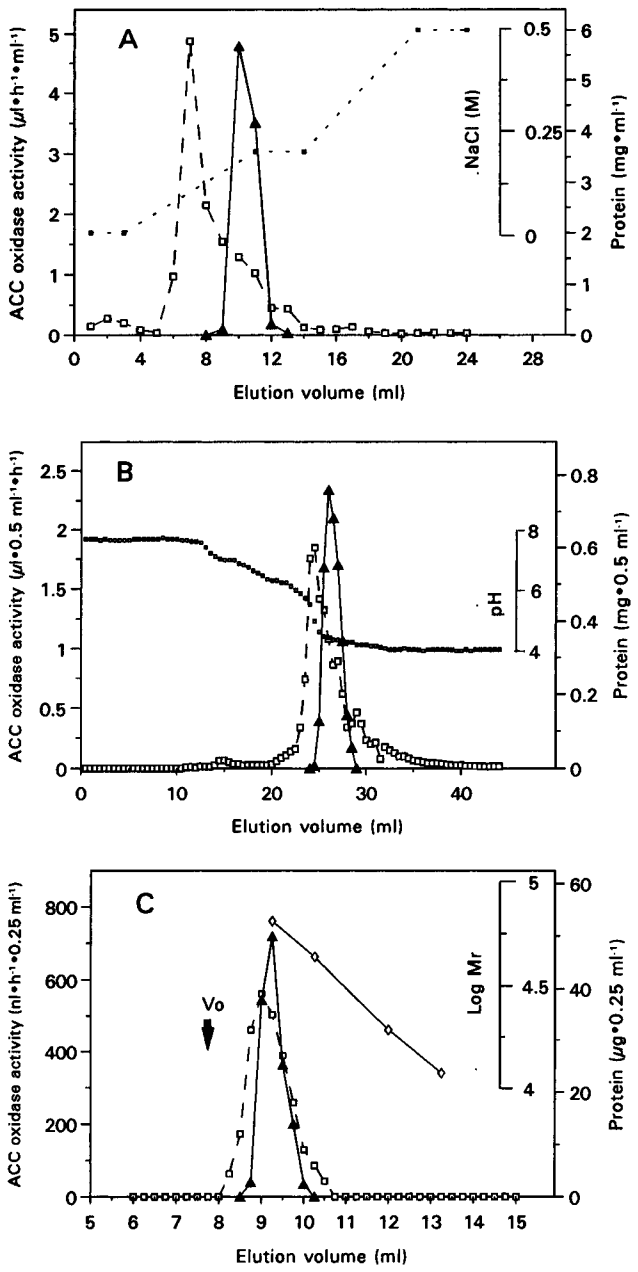

Figure 1. Elution profiles of the purification of ACC oxidase activity from cherimoya fruit after passage through (A) Mono Q anion exchange column, (B) Mono P chromatophocusing column, and (C) Sephadex G-75 gel filtration column (▲, ACC oxidase activity; □, protein).

supernatant was adjusted to 40% saturation with $(NH_4)_2SO_4$. After removal of precipitated material by centrifugation, the supernatant was brought to 80% saturation with $(NH_4)_2SO_4$ and centrifuged at 30000*g* for 30 min. The pellet was resuspended in 20 mM Tricine buffer (pH 7.5), 10% glycerol, and 3 mM DTT (buffer A) and desalted with a Sephadex G-25 column (PD-10, Pharmacia) equilibrated with buffer A.

Desalted enzyme extract was loaded onto a Mono Q HR 5/5 anion exchange column (Pharmacia) attached to a Pharmacia FPLC system. The column was pre-equilibrated with buffer A. Samples of 21 mg of protein were loaded onto the column by five consecutive injections of 2 mL. Protein was eluted using a NaCl gradient (from 0 to 0.5 M) in buffer A as indicated in Figure 1A. A flow rate of 1 mL min^{-1} was used, and fractions of 1 mL were collected.

Fractions containing ACC oxidase activity from the Mono Q column were pooled, desalted on Sephadex G-25 columns, and loaded directly onto a Mono P HR 5/20 chromatofocusing column attached to a Pharmacia FPLC System. The pH gradient (7.4−4.0) was formed with 20 mM Tricine buffer (pH 7.4), 10% glycerol, and 2 mM DTT as starting buffer and with 1:8 dilution of Polybuffer 74−HCl (pH 4.0), 10% glycerol, and 2 mM DTT as elution buffer. Fractions of 0.5 mL were collected at a 0.7 mL min^{-1} flow rate.

The most active fractions were pooled, passed through a Sephadex column equilibrated with buffer A, and concentrated onto the Amicon Centricon 10 system. Samples of 200 μL were loaded onto a 1 cm × 30 cm Superdex 75 gel filtration column attached to a Pharmacia FPLC system. The column was pre-equilibrated with 50 mM Tricine (pH 7.5), 10% glycerol, 3 mM DTT, and 150 mM NaCl. The flow rate was 0.8 mL min^{-1}, and fractions of 250 μL were collected. The column was previously calibrated with BSA (M_r 66 000), ovalbumin (M_r 45 000), soybean trypsin inhibitor (M_r 20 100) and cytochrome *c* (M_r 12 400).

ACC Oxidase Enzymatic Assay. Enzyme activity was assayed in a standard reaction mixture containing 0.1 mM ferrous sulfate, 20 mM sodium ascorbate, 20 mM sodium bicarbonate, 1 mM ACC, 10−50 μL of enzyme extract, and buffer A [0.1 M Tricine buffer (pH 7.5), 10% glycerol, and 3 mM DTT] to a final volume of 1 mL. The reaction was carried out in 8.3 mL sealed vials. After 15 min of incubation at 30 °C, the ethylene produced in the head space of the vials was determined by gas chromatography (Mitchell et al., 1988).

For the determination of the pH optimun, aliquots of enzyme extract were filtered through Sephadex G-25 columns, equilibrated with different buffer solutions: 0.1 M Na-Mes (pH 6.5); 0.1 M Na-Mops (pH 7.5); 0.1 M Tricine (pH 7.5); and 0.1 M Tris-HCl (pH 7.5) (all buffer solutions contained 10% glycerol and 3 mM DTT). In the assay, buffer A was replaced by the corresponding buffer at different pH values. The reactions were carried out without sodium bicarbonate addition.

Three replicates were performed in each assay and the results expressed as means ± SE.

Protein Determination. Protein concentration was determined as described by Bradford (1976) using bovine serum albumin as standard.

Gel Electrophoresis. Denaturating electrophoresis was performed in 9% polyacrylamide gels in the presence of SDS according to the method of Laemmli (1970). Proteins were stained with $AgNO_3$. Molecular mass markers used were BSA (66 kDa), ovalbumin (45 kDa), rabbit glyceraldehyde 3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), and bovine trypsinogen (24 kDa).

Native electrophoresis was performed in 9% polyacrylamide gels under nondenaturing conditions (without either DTT or SDS or boiling the extracts). Proteins were stained with $AgNO_3$. Molecular mass markers used were BSA (66 kDa), ovalbumin (45 kDa), rabbit glyceraldehyde 3-phosphate dehydrogenase (36 kDa), and soybean trypsin inhibitor (20.1 kDa).

RESULTS AND DISCUSSION

The biosynthesis of ethylene is closely associated with the keeping quality of most horticultural crops. The characterization and the elucidation of the enzymes of ethylene biosynthesis have constituted the major research effort of recent years in this area. In cherimoya, the climacteric development of ethylene production can be regulated by an increase of ACC synthase and ACC oxidase activities (Martinez et al., 1993).

Enzyme Purification and Properties. The ACC oxidase was extracted as a soluble enzyme from cherimoya fruits (Table 1). *In vitro* activity was enhanced by the inclusion of DTT and PVP in the extraction medium, but Triton X-100 or ascorbate was not required. In these respects the cherimoya enzyme differs from the apple enzyme, which required Triton X-100 for complete solubilization (Fernández-Maculet and Yang, 1992), and banana enzyme, which was stimulated by inclusion of ascorbate in the extraction medium (Moya-León and John, 1994, 1995).

Table 1. Requirements for the Extraction of ACC Oxidase Activity from Cherimoya Fruit[a]

extraction	μL of ethylene h^{-1} (g of FW)$^{-1}$	
	original supernatant	resuspended pellet
0.1 M Tricine pH 7.5 + 10% glycerol (A)	0.06 ± 0.005[b]	0.05 ± 0.006
(A) + 3 mM DTT	1.02 ± 0.006	0.20 ± 0.010
(A) + 1% PVPP	1.10 ± 0.001	0.06 ± 0.003
(A) + 3 mM DTT + 1% PVPP (B)	2.31 ± 0.090	0.19 ± 0.004
(B) + 0.1% Triton X-100	2.11 ± 0.090	0.16 ± 0.010
(B) + 20 mM ascorbate	2.22 ± 0.050	0.23 ± 0.004
(B) + 0.1% Triton X-100 + 20 mM ascorbate	2.06 ± 0.050	0.15 ± 0.014

[a] One gram of frozen pulp was homogenized in 5 mL of extraction medium and separated into pellet and supernatant fraction by centrifugation at 27000*g* for 30 min. Pellets were resuspended in buffer A in a volume equivalent to that of the original supernatant. Supernatants were desalted by passage through a Shephadex G-25 column equilibrated with buffer A. ACC oxidase activity was assayed inmediately as described under Materials and Methods. [b] Means ± SE.

Table 2. Purification of ACC Oxidase from Mesocarp Tissue of Ripe Cherimoya Fruit

purification step	total activity (nL of ethylene h^{-1})	total protein (mg)	specific activity (nL of ethylene h^{-1} mg^{-1})	recovery (%)	purification (fold)
crude extract	63973	172.9	370	100	
ammonium sulfate precipitation (40−80%)	49162	85.5	575	76.8	1.6
Mono Q	33209	11.0	3019	51.9	8.2
Mono P	9317	1.7	5481	14.6	15.2
Superdex 75	1825	0.1	18250	2.9	41.4

The results of the purification steps are summarized in Table 2 and Figure 1. The first step involved $(NH_4)_2SO_4$ precipitation between 40 and 80% saturation. Besides a great reduction of volume, it allowed some purification (1.5-fold) with 77% recovery. In the subsequent chromatographic fractionation steps, activity was always recovered as a single peak (Figure 1). Purification of the enzyme was taken no further than indicated because of problems of enzyme stability. These problems have been observed with other members of the 2-oxoacid-dependent dioxygenase family of enzymes [for examples see Britsch (1993)] to which ACC oxidase belongs (Prescott, 1993). The partially purified enzyme preparation reported here was purified 41-fold to give a specific activity of 18 μL of ethylene h^{-1} (mg of protein)$^{-1}$ [190 pkat (mg of protein)$^{-1}$]. This activity is comparable to the activities of 333 (Dong et al., 1992) and 300 (Pirrung et al., 1993) pkat (mg of protein)$^{-1}$ reported for the apple enzyme when assayed under conditions which it is fully activated by carbon dioxide.

The p*I* of ACC oxidase protein extracted from cherimoya was 4.35 (Figure 1B). This value is lower than the p*I* (4.99, 5.09, and 6.14) predicted for the proteins encoded by the three genomic clones of ACC oxidase from tomato (Bouzayen et al., 1993). It is also lower than the p*I* (4.90) of the ACC oxidase purified from banana (Moya-León and John, 1995).

The apparent molecular mass of the native protein from cherimoya estimated by gel filtration on a Superdex 75 column was 66 kDa (Figure 1C). After protein analysis by native gel electrophoresis, we observed two main bands at 52 and 62 kDa and a number of minor bands (Figure 2A). The protein of the second main band has a molecular mass similar to that estimated by gel filtration (Figure 1C). However, on SDS−PAGE the 62 kDa protein band disappeared and a new band at 35 kDa appeared (Figure 2B).

These results differ from those obtained with ACC oxidases previously isolated from fruit (Smith et al., 1992; Dong et al., 1992; Dupille et al., 1993; Moya-León and John, 1994, 1995), which have consistently been shown to be active as monomers of about 40 kDa as determined by gel filtration, and with molecular masses of 35−40 kDa by SDS−PAGE. The cherimoya ACC oxidase activity that eluted at 66 kDa from the gel filtration column could be due to a dimer formed from two subunits with M_r values of about 35 kDa. Alternatively, there may be specific or unspecific binding to an enzymatically active monomer, which would account for the higher M_r under nondenaturing conditions. Further work is required to distinguish between these possibilities.

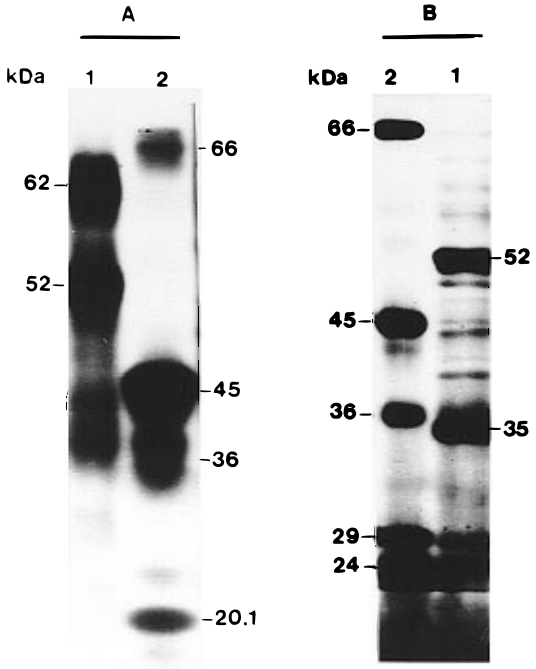

Figure 2. Silver-stained native (A) and SDS−polyacrylamide (B) gels: (lane 1) active fraction eluted from chromatography on Superdex 75 (3 μg of protein); (lane 2) protein standards.

Western blotting was carried out, as in Moya-León and John (1995), with the proteins separated by native PAGE and SDS−PAGE, using a rabbit polyclonal antibody raised against a recombinant polypeptide derived from the pTOM13 cDNA (Dupille et al., 1993) which encodes the tomato ACC oxidase (Hamilton et al., 1990). However, there was no cross-reaction with the cherimoya ACC oxidase (data not shown). This antibody reacts strongly with the apple (Dupille et al., 1993) and banana (Moya-León and John, 1995) ACC oxidases.

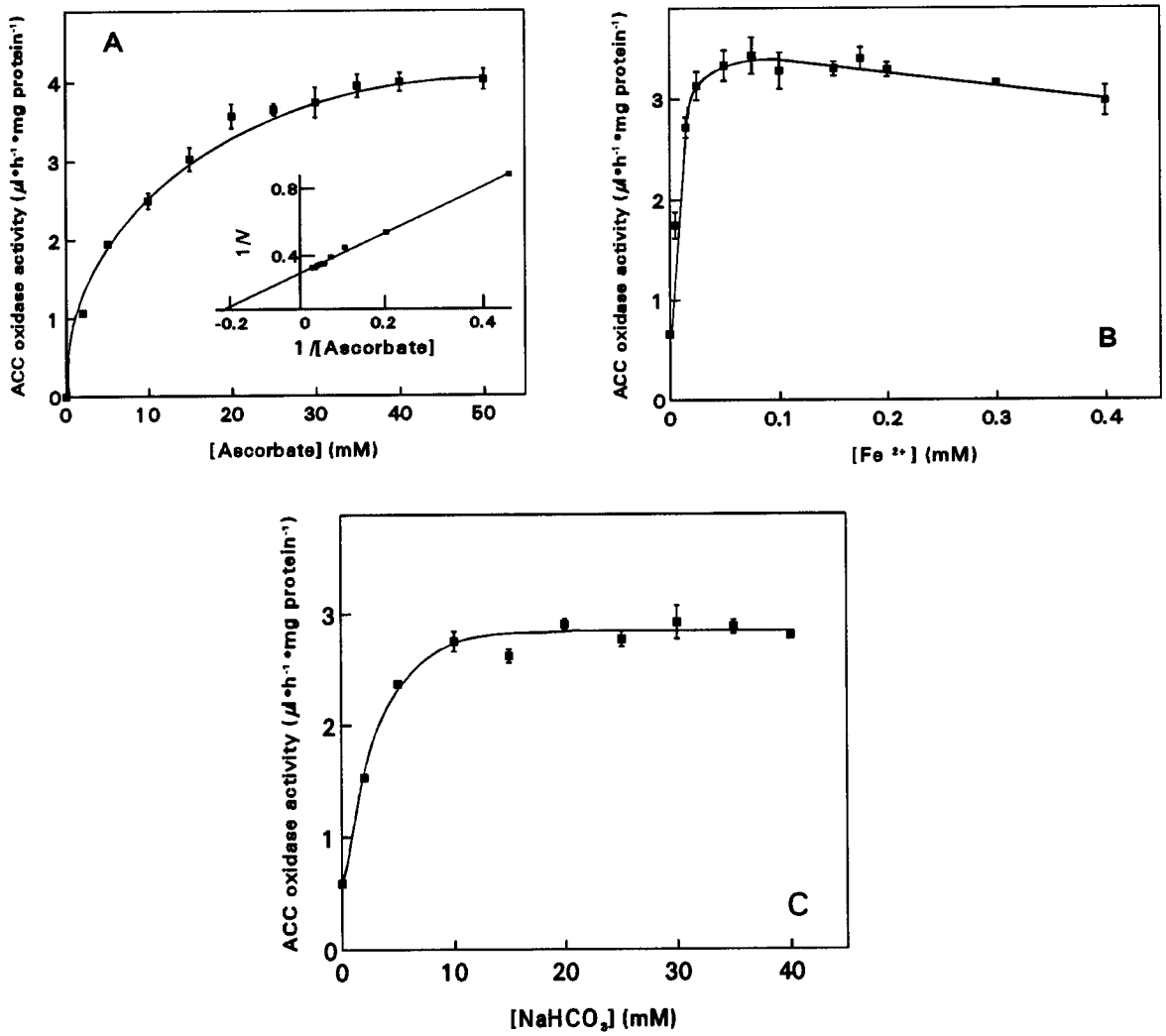

Figure 3. Effect of sodium ascorbate (A), ferrous sulfate (B), and sodium bicarbonate (C) on the activity of the partially purified ACC oxidase. The enzyme preparation (7.2 μg of protein) was added to a standard reaction mixture minus the specific compound to be tested. Vertical bars indicate ± SE.

The absence of a cross-reaction with the cherimoya enzyme may be because of the absence of the antigenic determinant or because the determinant is blocked.

Properties of the Partially Purified ACC Oxidase. Because of the instability of ACC oxidase, we have characterized the semipurified enzyme that eluted from Mono Q columns (Table 2). The specific activity of different preparations varied from 3 to 4 μL of ethylene h^{-1} (mg of protein)$^{-1}$.

In the absence of ascorbate, no activity was observed (Figure 3A). Increasing ascorbate concentrations stimulated activity, with the apparent K_m for ascorbate being 6.5 mM. The requirement for Fe^{2+} (Figure 3B) is presumably due to the loss of Fe^{2+} from the enzyme during extraction. Maximum activity was observed with the addition of about 50 μM. Carbon dioxide (supplied as bicarbonate added to the reaction medium) stimulated activity about 5-fold, maximum activity being attained with about 10 mM sodium bicarbonate (Figure 3C). In all of these respects, the ACC oxidase from cherimoya resembles the enzyme studied in other fruits (McGarvey and Christoffersen, 1992; Smith et al., 1992; Smith and John, 1993; Pirrung et al., 1993; Fernández-Maculet et al., 1993; Moya-León and John, 1995).

Figure 4A shows that the rate of ethylene synthesis declined during the course of the reaction. An approximation to linearity was obtained over the initial period of about 10 min, and this allowed the kinetics parameters to be measured. Nonlinearity of ACC oxidase catalyzed reactions has been observed previously (Smith et al., 1992) and has been attributed to a catalytic inactivation of the enzyme (Smith et al., 1994).

Catalase stimulated enzyme activity when it was added at relatively high concentrations (Figure 4B) and was increasingly effective during the course of purification (Table 3). Stimulation by catalase has been observed previously with ACC oxidase (Smith et al., 1994) and with other members of the 2-oxoacid-dependent dioxygenase family (Blanchard et al., 1982).

When the enzyme was assayed in the absence of added bicarbonate, the pH optimum was found to be at about pH 7.4 (Figure 5), as found for other ACC oxidases assayed under these conditions (McGarvey and Christoffersen, 1992; Kuai and Dilley, 1992; Smith et al., 1992; Dupille et al., 1993). The strong buffer effect seen here is consistent with previous similar observations with ACC oxidase (Smith et al., 1992).

The apparent K_m for ACC obtained for the semipurified ACC oxidase was determined to be 82 μM [with a V_{max} of 0.8 μL of ethylene h^{-1} (mg of protein)$^{-1}$] (Figure 6) in the absence of added bicarbonate, in close agreement with the apparent K_m determined for ACC oxidases from melon fruit (60 μM; Ververidis and John,

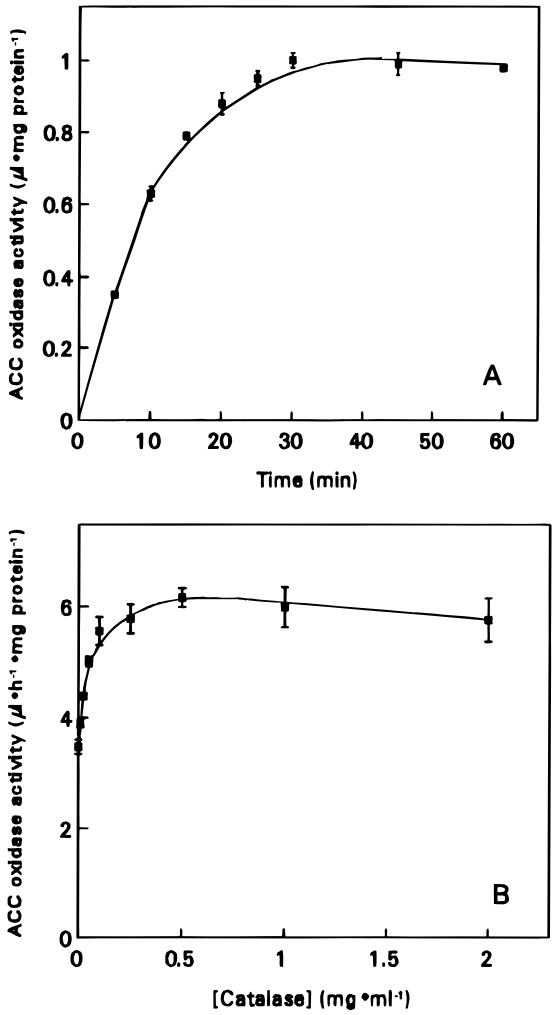

Figure 4. Time course of partially purified ACC oxidase activity (A) and effect of catalase on the partially purified ACC oxidase activity (B). The enzyme preparation (7.2 μg of protein) was added to a standard reaction mixture except where indicated. Vertical bars indicate ± SE.

Table 3. Effect of Catalase (1 mg mL^{-1}) Addition on the Activity of Cherimoya ACC Oxidase during Purification

purification step	% activation
crude extract	66 ± 1.3[a]
Mono Q extract	75 ± 2.5
Mono P extract	80 ± 1.9
Superdex 75 extract	153 ± 2.7

[a] Mean ± SE.

1991) and significantly higher than the values of 20 μM (Dupille et al., 1993), 17 μM (Fernández-Maculet and Yang, 1992), 12 μM (Pirrung et al., 1993), and 6.4 μM (Kuai and Dilley, 1992) for the enzyme from apple fruit or 32 μM from avocado fruit (McGarvey and Christoffersen, 1992). The addition of bicarbonate increased the apparent K_m of the enzyme with respect to ACC to 194 μM and stimulated the V_{max} of ACC oxidase at 3.5 μL of ethylene h^{-1} (mg of protein)$^{-1}$ (Figure 6). In agreement with previous findings with the enzyme from apple and melon fruits (Fernández-Maculet et al., 1993; Smith and John, 1993), the presence of the activator decreased the affinity of the enzyme toward its substrate, ACC. Fernández-Maculet et al. (1993) suggested that the enzyme binds to both ACC and activator randomly but the binding of CO_2 increases the dissociation constant for the enzyme−ACC complex at saturating activator concentrations.

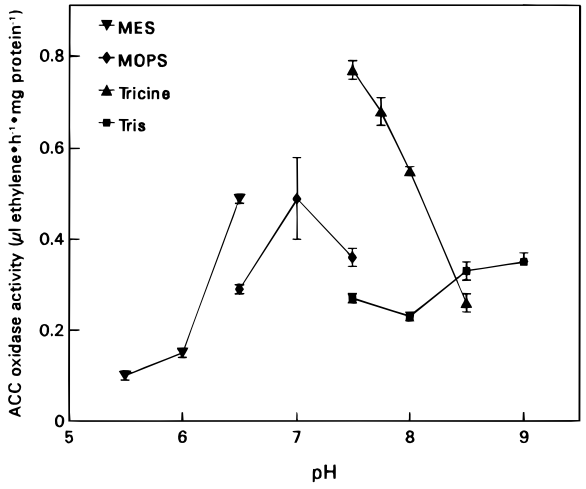

Figure 5. pH dependence of partially purified ACC oxidase activity. In addition to the buffers specified (0.1 M), the enzyme preparation (7.2 μg of protein) was assayed in a standard reaction mixture in the absence of sodium bicarbonate. Vertical bars indicate ± SE.

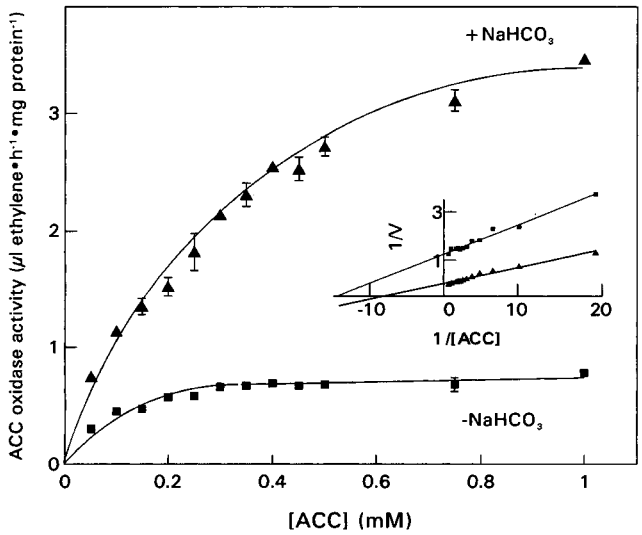

Figure 6. Dependence on ACC concentration of the partially purified ACC oxidase activity and effect of sodium bicarbonate on the enzyme affinity. The enzyme preparation (7.2 μg of protein) was added to a standard reaction mixture containing the sodium bicarbonate and ACC concentrations indicated. Vertical bars indicate ± SE.

Conclusions. The ACC oxidase from cherimoya resembles other ACC oxidases in its requirements for substrates and cofactors but differs in its lower p*I* value and apparently larger M_r, as determined by gel fractionation and on nondenaturing PAGE. Cherimoya belongs to the family Annonaceae, which is allied to the Magnoliaceae, and is counted among the most primitive of flowering plant families [see Heywood (1993)]. The many unique features of postharvest physiology shown by the cherimoya fruits (Paull, 1982; Palma et al., 1993; Alique et al., 1994; Gutierrez et al., 1994; Escribano and Merodio, 1994) and the apparently larger M_r shown here may be related to the primitive phylogenetic position of cherimoya. However, the affinity of the cherimoya enzyme with that of other ACC oxidases will only be revealed when its full molecular sequence becomes known. Monoclonal antibodies are currently being prepared, and these should help in understanding the role of the ACC oxidase in regulating ethylene synthesis during ripening in cherimoya.

ACKNOWLEDGMENT

We are grateful to J. C. Pech (Toulouse) for the generous gift of the antibody.

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Received for review July 10, 1995. Accepted November 20, 1995.[⊗] This research was sypported by grants from Comisión Interministerial de Ciencia y Tecnología de Alimentos (ALI92-1272 and STD3 92/1448) and from the European Community.

JF950426A

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1996.