AA or Basic pH Causes in vitro and Nonenzymatic Cleavage of ACC to Ethylene

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ADDITIONAL INDEX WORDS. ACC oxidase, ethylene synthesis, fruit ripening

ABSTRACT. An in vitro assay was used to determine the effect of AA and pH on the enzymatic and nonenzymatic production of ethylene (C_2H_4) from ACC. We were interested in the effect of AA on C_2H_4 production from ACC because aldehydes, primarily AA, can accumulate in tissue as the result of ripening, storage under modified atmospheres, packaging, and stress. Using crude extracts of ACC oxidase from tomato (*Lycopersicon esculentum* Mill. 'Castlemart') and apple (*Malus domestica* Borkh. 'Golden delicious'), C_2H_4 production from ACC was shown to increase in response to an increase in pH above 7.2 and inclusion of 0.2 to 2 mM AA. Nonenzymatic C_2H_4 production from ACC also increased linearly with increasing AA concentrations in all the buffers tested. Removal of ascorbate or O_2 suppressed AA-induced nonenzymatic C_2H_4 production. Nonenzymatic AA-induced production of C_2H_4 from ACC appeared to be an ascorbate dependent oxidation, which was augmented by O_2 and was sensitive to minor pH fluctuation. The nonenzymatic AA-stimulated conversion of AEC to 1-butene lacked stereospecificity. Formaldehyde and propionaldehyde also stimulated C_2H_4 production from ACC. These data indicate that ACC oxidase assays or C_2H_4 measurements assessing physiological status can be seriously affected by the presence of aldehydes, such as AA. Chemical names used: AA, acetaldehyde; ACC, 1-aminocyclopropane-1-carboxylic acid; AEC, 1-amino-2-ethylcyclopropanecarboxylic acid; ADH, alcohol dehydrogenase; EtOH, ethanol.

Ethylene (C_2H_4) is a plant hormone that regulates many physiological aspects of plant growth, development, maturation, and senescence (Abeles et al., 1992). The rate of C_2H_4 production is often used as a measure of the physiological maturity of climacteric fruit or as a general indicator of the level of plant stress. The C_2H_4 biosynthetic pathway in higher plants is from methionine to S-adenosyl-L-methionine (SAM) to ACC to C_2H_4 (Adams and Yang, 1979). The enzymes catalyzing these reactions are SAM synthetase, ACC synthase, and ACC oxidase, respectively (Kende, 1989). The rate limiting step for C_2H_4 biosynthesis in vegetative tissue is ACC synthase, while ACC synthase and ACC oxidase contribute to the regulation of C_2H_4 biosynthesis in ripening fruit (Liu et al., 1985).

Measurements of the substrates and enzymes involved in C_2H_4 biosynthesis are sometimes performed after extraction or treatments where aldehyde impurities may exist (e.g., after treatment with AA or EtOH, or after anaerobic storage). For example, endogenous levels of AA and EtOH increase in ripening climacteric fruit (Fidler, 1968; Gustafson, 1934; Janes and Frenkel, 1978). Removal of astringency from persimmon (*Diospyros kaki* L.) fruit held under low O_2 atmospheres is correlated with AA accumulation (Pesis and Ben-Arie, 1984). Treatment with AA improves flavor and stimulates respiration in apple (*Malus domestica*), blueberry (*Vaccinium corymbosum* L.), orange [*Citrus sinensis* (L.) Osbeck], and strawberry (*Fragaria xananassa* Duch.) (Fidler, 1968; Janes, et al., 1978; Pesis and Avissar, 1989). Exposure to high AA or EtOH concentrations significantly de-

creases C_2H_4 production and inhibits tomato (*Lycopersicon esculentum*) fruit ripening (Beaulieu et al., 1997; Saltveit, 1989), while exposure to low AA concentrations stimulate ripening (Beaulieu and Saltveit, 1997). The reported effects of EtOH and AA as inhibitors and promoters of ripening in climacteric fruit appear inconsistent (Burdon et al., 1996; Paz et al., 1981; Pesis and Avissar, 1989; Pesis and Marinansky, 1993; Saltveit and Mencarelli, 1988). Application of AA to grape (*Vitis vinifera* L.) berry inhibits ACC oxidase activity (Pesis and Marinansky, 1992). AA affects the ripening of banana (*Musa AAA*) (Hewage et al., 1995) and mango (*Mangifera indica* L.) fruit (Burdon et al., 1996). It is possible that AA levels or pH changes could affect C_2H_4 production (perhaps through its effect on ACC oxidase activity), or they could affect other C_2H_4 -induced ripening related phenomena.

Although the assay solutions are buffered, we noticed that the pH of the assays can often shift significantly during the reaction. In vitro ACC oxidase activity is apparently pH- and buffer-dependent. Avocado ACC oxidase is inactive below pH 6.0 in various buffers, and has an optimum activity at pH 7.5 in Na-Mops, or 8.1 in Tris-HCl (McGarvey and Christoffersen, 1992). Kuai and Dilley (1992) found that the pH optimum for apple ACC oxidase was 7.2 with Tris-Mes buffer, and 7.6 with phosphate buffer. For melons (*Cucumis melo* L.), ACC oxidase has been assayed at pH 6.4 and 7.3 in Mops, and at pH 7.5 or 7.7 with Tricine (Smith and John, 1993).

Interactions among various extraction impurities and assay components could suppress or stimulate nonenzymatic and in vivo C_2H_4 production from ACC. ACC oxidase activity in homogenized apple cortex has an absolute requirement for CO_2 (Dong, et al., 1992a; Fernández-Maculeit et al., 1993) and the activation of ACC oxidase by CO_2 is pH-dependent (Fernández-Maculeit et al., 1993). Assays of substrates and enzymes involved in C_2H_4 biosynthesis are often done with minimal regard to stereospecificity of the reaction. Various oxidants and other compounds can produce C_2H_4 from ACC nonenzymatically (McRae et al., 1983; Neider et al., 1986; Venis, 1984; Yang and Hoffman, 1984).

As part of a larger study examining ripening-related effects of AA and EtOH on tomatoes, we investigated the in vitro effects of AA and pH on ACC oxidase activity and nonenzymatic C_2H_4

Received for publication 12 Sept. 1997. Accepted for publication 19 Feb. 1998. This research was partially supported by a grant from the United States-Israel Binational Agriculture Research Organization (BARD) number I-1787-90. This work was partially supported by grant number I-1787-90 from BARD; The United States-Israel Binational Agriculture Research Organization. The authors wish to thank Jian-Guo Dong, Juan Carlos Fernández-Maculeit, and Yee Yung Chang in the laboratory of Dr. Shang Fa Yang for their valuable advice and review and for supplying the AEC isomers. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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production. We present data indicating that the *in vitro*, *in vivo* and nonenzymatic production of C_2H_4 from ACC can be influenced by AA concentration and assay pH. These data indicate that ACC oxidase assays or C_2H_4 measurements assessing physiological status can be seriously affected by the presence of AA.

Materials and Methods

PLANT MATERIALS. Freshly harvested breaker, turning and light-red 'Castlemart' tomatoes were obtained from fields or greenhouses at the Univ. of California, Davis. Fruit were washed in dilute (20%; v/v) bleach (5.25% sodium hypochlorite), rinsed in sterile water and air dried under a laminar-flow hood. Discs were excised with a 1.4-cm-diameter cork borer, and trimmed of locular material to produce 4-mm-thick discs (Saltveit, 1989). The discs were rinsed in sterile deionized water, carefully blotted dry, and put, epidermis surface down, in 100×15 -mm sterile petri dishes. The dishes were held in a flow of ethylene-free, humidified air for 1 d at $20^\circ C$ to allow dissipation of wound-induced ethylene.

'Golden Delicious' apples were obtained from a local orchard, and handled according to Fernández-Maculeit and Yang (1992). Apples were monitored for C_2H_4 production rates, and preclimacteric fruit were gassed with $70 \mu L \cdot L^{-1}$ C_2H_4 to enhance ACC oxidase activity.

BUFFER PREPARATIONS. Stock solutions (200 mM) of various enzymatic assay buffers (7.2 for Mops, 9.0 and 10.0 for Borax, and 11.0 for Na_2HPO_4) were prepared in 20% glycerol (v/v) according to Perrin and Dempsey (1974). A 1.0 M Pipes–glycylglycine buffer was prepared in 20% glycerol and adjusted to several pHs to test the standard assay reactions over a broad pH range (modified from Perrin and Dempsey, 1974). All buffers and final assay reaction pH's were measured with a gel-filled combination electrode.

ENZYME EXTRACTION. Pericarp tissue from freshly harvested tomatoes (free of locular jelly and seeds) was cut into 2-cm^2 segments. Tissue was homogenized at $1^\circ C$ for 1 min without buffer in the presence of 5% PVPP (w/w). The homogenate was squeezed through four layers of cheesecloth into a funnel contain-

ing another four layers of cheesecloth and the resulting solution was centrifuged at $27,000 g_n$ for 30 min at $0^\circ C$. Supernatants were collected, and pellets were resuspended in 0.5 mL 200 mM Mops (pH 7.2) buffer in 1.5-mL centrifuge tubes. An ACC oxidase assay demonstrated that, unlike apple extracts (Dong et al., 1992a; Fernández-Maculeit and Yang, 1992), the resuspended tomato pellet had almost no ACC oxidase activity and we concluded that tomato ACC oxidase activity was in the soluble fraction. The pellet was therefore discarded in subsequent experiments.

Apple ACC oxidase was obtained according to the procedure of Dong et al., (1992a) with the exception that dithiothreitol and Triton X-100 were not used in the resuspension buffer. Briefly, mesocarp tissue was homogenized in a 400 mM potassium phosphate buffer (pH 7.2) including 10 mM $NaHSO_3$, 4 mM mercaptoethanol, and 3 mM sodium-ascorbate. The homogenate was filtered and centrifuged at $28,000 g_n$ for 30 min, and pellets were collected and stored at $-20^\circ C$. Pellets were resuspended in 25 mM Mops (pH 7.2) in 30% glycerol with 3 mM Na-ascorbate, then passed through a DEAE-Sephacel column (equilibrated with resuspension buffer), and eluted with 100 mM $(NH_4)_2SO_4$. For all apple assays mentioned, purified ACC oxidase (DEAE-Sephacel) fractions were used.

ENZYMATIC ASSAYS. The standard *in vitro* ACC oxidase assays for tomato and apple samples contained 100 mM Mops (pH 7.2), 10% glycerol (v/v), 0.2 mM $FeSO_4$, 2 mM sodium ascorbate, 20 mM sodium bicarbonate or 6.7% CO_2 , and 2 or 4 mM ACC. The tomato crude supernatant or apple ACC oxidase was added to the 15-mL reaction tubes after the ACC. In assays testing the effect of AA, it was added last. The final volume was made to 1 mL with the addition of sterile water. In the apple ACC oxidase experiments, the same general assay was used with 125 mM Mops. Assay tubes were sealed with rubber serum stoppers, gently mixed for 20 sec, and incubated at $29^\circ C$ for 0.5 or 1.0 h.

NONENZYMATIC ASSAYS. Initially, the standard ACC oxidation assay included 100 mM Mops (pH 7.2), 10% glycerol (v/v), 0.2 mM $FeSO_4$, 2 mM sodium ascorbate, 20 mM sodium bicarbonate, and 2 mM ACC. AA (0.2 mM) was added to the assays immediately after the addition of ACC. The final volume was made to 1 mL with the addition of sterile water. No ACC oxidase was included in any of these assays. Assays were incubated at $29^\circ C$ in sealed 15-mL

Fig. 1. Effects of AA and pH on C_2H_4 production by apple and tomato ACC oxidase. The pH's evaluated were 7.2 (Mops), 9 and 10 (Borax), and 11 (Na_2HPO_4). Final (1 mL) reaction concentrations were 100 mM buffers, 10% glycerol (v/v), 0.2 mM $FeSO_4$, 2 mM ascorbate, 2 mM ACC, and 20 mM bicarbonate. Assays contained crude tomato ACC oxidase (200 μL) in Mops buffer or DEAE-Sephacel purified apple ACC oxidase (20 μL) in 100 mM $(NH_4)_2SO_4$, with or without 0.2 mM AA. Each point represents the average of three replications with error bars indicating the SD about the mean.

Fig. 2. Effects of AA concentration on C_2H_4 production in the standard assay with 20 μL apple ACC oxidase and without enzyme (nonenzymatic). The 0.5 mL reaction final concentrations were 125 mM Mops (pH 7.2), 10% glycerol (v/v), 0.2 mM Fe, 4 mM Asc, 4 mM ACC, 6.7% CO_2 , and AA. Data points are means of three replications with error bars indicating the SD about the mean.

Table 1. Effects of AA and ACC on the in vivo C₂H₄ production by 4-mm-thick light-red tomato discs. Discs were prepared as described in the Materials and Methods.

Treatments	C ₂ H ₄ production nmol·g ⁻¹ ·h ⁻¹
Control (Mops buffer)	3.6 ± 1.8 ²
107 μmol AA	2.0 ± 1.6
4 μmol ACC	13.5 ± 3.4
+ 37 μmol AA	22.0 ± 5.7
+ 107 μmol AA	20.1 ± 3.8
+ 356 μmol AA	15.5 ± 4.6

²Data points are means of three replicates ±SD about the mean.

culture tubes as before. During our investigations, Fernández-Maculet et al. (1993) determined that CO₂ was required for ACC oxidase activity. Therefore, assay conditions were modified to include injections of CO₂ (6.7% v/v) into the reaction tubes in lieu of sodium bicarbonate (Fernández-Maculet et al., 1993). The final volume in these sealed 15-mL culture tubes was 0.5 mL, and CO₂ and/or O₂ (0% or 20%) was added after purging the sealed reaction tube with N₂. Tubes were then mixed for 20 s, and incubated at 29 °C.

STEREOSPECIFICITY. To determine if nonenzymatic C₂H₄ production was stereospecific, ACC stereoisomer analogs (i.e., AEC) were analyzed for their ability to produce 1-butene. Both AEC diastereomer pairs, (±) coronamic acid and (±) allocoronamic acid, were used at 50 mM in the previous ACC oxidation assays.

GAS CHROMATOGRAPH DETERMINATIONS. Ethylene concentration was determined by injecting a 1-mL head space gas sample into a gas chromatograph (GC) as previously described (Saltveit and Yang, 1987). The same column was used to determine the concentrations of 1-butene. Authenticity and concentration were based on GC response to known standards.

STATISTICS. Each experiment was designed and analyzed as a completely randomized design. All experiments contained at least three replicates and were repeated at least once with similar results. Data are usually presented as means and standard deviations from representative experiments.

Results

AA AND pH EFFECTS ON ACC OXIDASE ACTIVITY. Production of C₂H₄ from assays using crude tomato ACC oxidase was slight after 3 h (0.009 ± 0.004 nmol) and showed no statistical difference among the ripeness stages. Incorporation of 0.2 mM AA in the assay stimulated C₂H₄ production so that C₂H₄ levels after 3 h were 0.143 ± 0.005, 0.105 ± 0.007, and 0.158 ± 0.011 nmol for assays containing extract from breaker, turning and light-red fruit, respectively. Rates of C₂H₄ production were linear over the 3 h assay period. When supplemented with 0.2 mM AA, C₂H₄ produced in the assay was apparently nonenzymatic.

A number of buffers were used in the standard ACC oxidase assay to assess the effect of pH and AA on C₂H₄ production. Without added AA, C₂H₄ production was either unaffected (tomato) or declined (apple) as the pH increased from 7.2 to 11.0 (Fig. 1). In the presence of 0.2 mM AA, C₂H₄ production using tomato ACC oxidase was consistently higher than without it at all pHs, and showed a pronounced peak at pH 9. In contrast, production of C₂H₄ using apple ACC oxidase decreased as pH increased in the absence of AA, but remained constant or increased as the pH increased when AA was added. A slight peak in C₂H₄ production from the apple extract occurred at pH 10 in the presence of 0.2 mM AA.

The influence and magnitude of various AA concentrations in the standard assay was studied using the apple extract in Mops (pH 7.2). In these assays, direct injections of CO₂ (6.7% v/v) were made into the reaction tubes in lieu of sodium bicarbonate (Fernández-Maculet et al., 1993). Ethylene production increased linearly with increasing AA concentration (Fig. 2, ACC oxidase). After 0.5 h, the C₂H₄ concentration (nmol) equaled 0.075 + 0.136 × AA concentration with an *r*² of 0.99; while after 4 h, the C₂H₄ concentration equaled 0.096 + 0.454 × AA concentration with an *r*² of 1.00. All assays were slightly acidified due to dissolved CO₂, however, there were no significant differences in the final reaction pH (data not shown).

Tomato discs were used to confirm that AA affected ACC oxidase and C₂H₄ production in vivo. Applying 107 μmol AA to freshly prepared light-red tomato discs decreased rates of C₂H₄ production by 44%. In contrast, applying 4 μmol ACC to discs increased their C₂H₄ production by 3.7-fold (Table 1). Application of 37, 107, and 356 μmoles of AA to discs with 4 μmol of ACC promoted C₂H₄ production by 63%, 49%, and 15% respectively, over that of discs with just ACC added.

AA AND pH EFFECTS ON NONENZYMATIC C₂H₄ PRODUCTION FROM ACC. The pH and AA concentration had profound effects on C₂H₄ production from the standard ACC oxidase assay with tomato and apple ACC oxidase (Figs. 1 and 2), and in assays without ACC oxidase (Fig. 3). Assays run in Mops buffer at pH 7.2 had the lowest rate of C₂H₄ production. AA (0.2 mM) facilitated nonenzymatic cleavage of ACC to C₂H₄ in all buffers used. At pH 11, however, the AA-induced stimulation was no longer significantly different from the control (i.e., non-AA assays).

To eliminate possible effects of the buffers themselves, the standard assay was performed without the enzyme in a 500 mM Pipes–glycylglycine buffer adjusted to several pHs (Fig. 4). Again, 0.2 mM AA and increased pH stimulated C₂H₄ production. Although it was difficult to conclude from these data if AA-stimulated nonenzymatic C₂H₄ production was additive, promotive, or directly related to pH changes, it was nonetheless shown that nonenzymatic cleavage of ACC was facilitated by AA and pH above 7.2.

Other aldehydes also stimulated nonenzymatic C₂H₄ production in the standard assay. After an initial rapid increase in C₂H₄ produc-

Fig. 3. Effects of AA and pHs on nonenzymatic C₂H₄ production. The buffers were Mops (pH 7.2), Borax (pH 9 and 10), and Na₂HPO₄ (pH 11). The assays contained 100 mM buffers, 0.2 mM FeSO₄, 2 mM ascorbate, 2 mM ACC, and 20 mM bicarbonate. The assays were run for 1 h without (control) or with 0.2 mM AA. Data points are means of three replications with error bars indicating the SD about the mean.

Fig. 4. Effect of AA and pH on nonenzymatic C_2H_4 production. Assays contained 500 mM Pipes–glycylglycine, 10% glycerol (v/v), 0.2 mM $FeSO_4$, 2 mM ascorbate, 2 mM ACC, and 20 mM bicarbonate. Assays were run for 4 h. Data points are means of four replications with error bars indicating the SD about the mean.

tion that lasted ≈ 30 min and was proportional among the aldehydes to their final values, the rate of C_2H_4 production remained constant from 0.5 to 4 h. The concentration of C_2H_4 in the reaction tubes at the end of the 4 h experiment was around 0.016 nmol for the control, while it was 0.52, 0.70, and 1.06 nmol for 4.0 mM formaldehyde (formalin), propionaldehyde (propanal), and AA, respectively.

To ascertain the influence and magnitude that AA exerted on the standard assay protocol, nonenzymatic assays were performed in Mops (pH 7.2) with 0.0 to 4.0 mM AA (Fig. 2). Ethylene production increased linearly with increasing AA concentration. The production of C_2H_4 within these given experimental parameters is given by the equation: $nmol\ C_2H_4 = 0.0964 \times AA\ concentration \times hours$. All assays, including the controls, had slight acidification from the initial pH of 7.2 due to dissolved CO_2 , however, there were no significant differences in the final assay reaction pH of 7.01 ± 0.03 . These data demonstrated that the nonenzymatic, AA-induced conversion of ACC to C_2H_4 is linear with AA concentration and time for up to 4 h.

EFFECT OF CO_2 ON NONENZYMATIC CLEAVAGE OF ACC. In preliminary experiments, CO_2 appeared to be required for the nonenzymatic cleavage of ACC similar to the bicarbonate-induced C_2H_4 produced in the Fenton reaction (McRae et al., 1983) (Table 2). However, addition of CO_2 caused acidification of the assay mixture which should have actually depressed C_2H_4 production. Assays run in the absence of CO_2 maintained a pH of 7.26 ± 0.01 whereas, the pH dropped to 7.10 in assays with CO_2 added. These data could not clearly separate the effects of CO_2 -induced acidification from the final assay pH.

If nonenzymatic C_2H_4 production was CO_2 -dependent, C_2H_4 production should increase as CO_2 concentration increased, however, this did not occur. As pH decreased in the standard assay with 120 mM Mops buffer from around 7.25 to 7.12, or from 7.12 to 6.87 as the CO_2 concentration in the reaction vessel was increased from 0.7% to 2.0% to 6.7% to 20%, C_2H_4 production actually decreased in a linear fashion from an average of 0.44 to 0.30 $nmol \cdot h^{-1}$. Purging the reaction tube with N_2 (thereby removing CO_2 and O_2) almost eliminated C_2H_4 production, even in the presence of 4.0 mM AA. Therefore, the origin of AA-induced nonenzymatic C_2H_4 production from ACC, in this specific buffering system, appears to require O_2 and to be highly sensitive to minor pH fluctuations.

EFFECT OF REMOVAL OF ASSAY COMPONENTS ON NONENZYMATIC

C_2H_4 PRODUCTION. Assays were run in the presence of 4.0 mM AA with successive removal of assay constituents to determine which factors influenced AA-induced nonenzymatic C_2H_4 production from ACC. Without added AA or ACC, C_2H_4 production was undetectable in all nonenzymatic assays (Table 2). Removal of $FeSO_4$ decreased C_2H_4 production 69% after incubation for either 0.5 or 1 h (Table 2). $FeSO_4$ is required to protect ascorbate from oxidation in ascorbate dependent oxidases and enzymatic assays (Bouzayen et al., 1991; England and Seifter, 1986), and it appeared influential in nonenzymatic assays as well. Ascorbate protects against numerous peroxidase activities and Ververidis and John (1991) established that melon ACC oxidase required ascorbate.

Removal of ascorbate suppressed AA-induced nonenzymatic C_2H_4 production completely at 0.5 h and by 98% after 1 h (Table 2). Ascorbate is a strong O_2 -dependent reducing agent and the oxidized form of ascorbic acid, L-dehydroascorbic acid, can undergo further oxidation in the presence of metal complexes in alkaline solution and, ascorbate or L-dehydroascorbic acid can also be oxidized by transition metal complexes independent of O_2 (e.g., as Fe^{+3} is reduced to Fe^{+2}) (Dong et al., 1992a). However, such oxidations involve free radical intermediaries.

Since the nonenzymatic reactions could be attributed to free radicals and therefore not be O_2 -dependent per se, we incorporated the free radical scavenger, n-propyl gallate (3,4,5-trihydroxybenzoic acid propyl ester) in the nonenzymatic assay. In reactions containing 4 mM AA and 40 mM n-propyl gallate, C_2H_4 production was undetectable when $FeSO_4$ or ascorbate were absent, and only occurred at nominal rates when $FeSO_4$ and ascorbate were present (C_2H_4 production was 0.034 $nmol \cdot h^{-1}$ in the presence of O_2 , and 0.030 $nmol \cdot h^{-1}$ in the absence of O_2). When the reaction was run with ascorbate, but without $FeSO_4$, removal of O_2 reduced C_2H_4 production by 69%. It appears that the addition of n-propyl gallate removed the majority of potential agents capable of oxidizing ACC. Therefore, nonenzymatic cleavage of ACC in this system appeared to be ascorbate-dependent, and augmented by O_2 . Presumably ascorbate is required in nonenzymatic C_2H_4 generating reactions because it acts as a strong reducing agent which facilitates ACC oxidation (i.e., ascorbate provides a reversible oxidation–reduction system).

Since aldehydes can be oxidized to form H_2O_2 (McRae et al., 1983) in the presence of ascorbic acid, metal complexes (e.g., Fe^{+2}) and O_2 , catalase was added to the assay to consume any peroxides generated during the reaction. The standard nonenzymatic oxida-

Table 2. Effect of the sequential removal of assay components on the nonenzymatic production of C_2H_4 in the standard assay. All assays, except where indicate otherwise, were run in 100 mM Mops with 10% glycerol (v/v), 4 mM ACC, 0.2 mM $FeSO_4$, 4 mM ascorbate, 4 mM AA, 20% O_2 , and 6.7% CO_2 .

Assay conditions	C_2H_4 production (nmol) $\pm$ SD	
	0.5 h	1.0 h
Control (all components)	0.398 ± 0.043^z	0.667 ± 0.056
– AA	0.000 ± 0.000	0.000 ± 0.000
– ACC	0.000 ± 0.000	0.000 ± 0.000
– $FeSO_4$	0.122 ± 0.001	0.208 ± 0.003
– ascorbate	0.008 ± 0.000	0.016 ± 0.001
– CO_2 , + O_2	0.456 ± 0.050	ND ^y
– CO_2 and O_2	0.023 ± 0.010	0.035 ± 0.011
– $FeSO_4$ and ascorbate	0.000 ± 0.000	0.000 ± 0.000
– $FeSO_4$, ascorbate, and CO_2	0.000 ± 0.000	0.000 ± 0.000

^zData points are means of three replications $\pm$ SD about the mean.

^yND = not determined.

tion assays plus AA produced C_2H_4 at the rate of 1.508 ± 0.006 nmol·h⁻¹. Inclusion of catalase at 1 mg·mL⁻¹, reduced production by only 12%, suggesting that AA-induced ACC cleavage reaction is not due to peroxidation by H₂O₂. In contrast, Smith et al. (1994) concluded that H₂O₂ generated by autooxidation of ascorbate was the species responsible for lost ACC oxidase enzyme activity when ACC oxidase was incubated in ascorbate. Tuma et al. (1984) found that ascorbic acid increased covalent binding of AA to proteins and increased unstable (short-term) and stable (long-term) adduct formation. Our findings indicate that ascorbate may act as an electron acceptor to facilitate AA-mediated unstable AA-adduct formation. Perhaps as with bovine serum albumin (Fleming and Bensch, 1983; Tuma et al., 1984), ascorbate conferred a conformational change on ACC, which facilitated formation of an unstable AA-adduct that was subject to rapid oxidation in the reaction medium.

STEREOSPECIFICITY OF THE NONENZYMATIC REACTION. In the nonenzymatic assay, 4 mM AA stimulated the conversion of 50 mM AEC to 1-butene at a rate of 0.631 ± 0.049 nmol·h⁻¹, the conversion of (±) allocoronamic and (±) coronamic acids were only 0.102 ± 0.016 and 0.133 ± 0.005 nmol·h⁻¹, respectively. As a general criterion, only (+) allocoronamic acid (1R, 2S) will be preferentially converted by ACC oxidase into 1-butene in stereospecific reactions (Hoffman et al., 1982; McKeon and Yang, 1984). When both pairs of diastereomers are equally converted by ACC oxidase to 1-butene, the oxidation is considered nonselective. Therefore, researchers may discriminate whether AA-induced C_2H_4 is enzymatic or artifactually produced by using these ACC isomers. Our results indicated that nonenzymatic, AA-stimulated conversion of AEC to 1-butene was nonselective.

Discussion

Ethylene production from the standard ACC oxidase assay using DEAE-purified apple and crude tomato ACC oxidase was stimulated by inclusion of 0.2 to 2.0 mM AA, but reduced by 4 mM AA. Higher AA concentrations (200 mM) added to apple ACC oxidase standard assays completely inhibited C_2H_4 production (Fernández-Maculet, 1994, personal communication).

The addition of aldehydes stimulated C_2H_4 production maximally under basic pHs in assay mixtures, yet the nonenzymatic cleavage of ACC lacked stereospecificity. When a comparison between the nonenzymatic rates of C_2H_4 production and production with the ACC oxidase control suggests that AA-stimulated *in vitro* C_2H_4 production was not by an enzymatic pathway but was artifactually produced by a nondiscriminating chemical reaction.

AA has two highly reactive pairs of electrons on the carbonyl oxygen atom which can form covalent bonds to free amino groups. The highly reactive carbonyl group of the aldehydes could attack the unprotonated amino group of ACC, forming an unstable Schiff base. This reaction is favored by high pH (Donohue et al., 1983) and could render ACC more readily oxidized; subsequently yielding nonenzymatic C_2H_4 through ACC fragmentation. Any compound or protein with free amino groups has the potential to be affected by AA; especially if its active site contains significant amino-containing moieties. ACC and ACC oxidase are likely candidates for interactions with AA because ACC has a primary amino group and ACC oxidase contains 28 lysine residues (Dong et al., 1992b).

Many physiological disorders, biochemical modifications, and detrimental effects attributed to EtOH in humans and animals are caused by AA or AA-adduct formations with certain proteins (Israel et al., 1986; Lieber, 1988; Tuma et al., 1984). AA forms

nonspecific unstable adducts (Schiff bases) with carrot (*Daucus carota* L.) proteins (Perata et al., 1992). Researchers using ACC oxidase assays and C_2H_4 measurements as an estimation of physiological maturity or stress should be aware that the presence of AA (aldehydes) could affect their results.

The anomalous effect of AA on promoting or inhibiting tomato ripening could be explained on the basis of differential effects of low and high concentrations of AA on enzymatic and nonenzymatic C_2H_4 production from ACC. Low concentration of AA vapor in the presence of ascorbate and O₂ may stimulate nonenzymatic C_2H_4 production by either ascorbate-dependent or direct oxidation of ACC which could potentially stimulate autocatalytic C_2H_4 production. Short-term elevation of endogenous AA (vapor treatments or anaerobiosis induction) may therefore generate nonenzymatic C_2H_4 which autocatalytically promotes ripening, whereas long-term exposure to higher AA concentrations appear to reversibly impair C_2H_4 biosynthesis (Beaulieu et al., 1997), presumably via interaction with ACC oxidase. Since AA can be rapidly metabolized in plant tissue (Oppenheim and Castelfranco, 1967), a sufficiently high concentration or duration of exposure appears to be required to affect *in vivo* C_2H_4 biosynthesis which elicits a threshold promotion or inhibition response within a given tissue.

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