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Biochemical Characterization of 1-Aminocyclopropane-1-Carboxylate Oxidase in Mung Bean Hypocotyls

EonSeon Jin, Jae-Hyeok Lee, and Woo Taek Kim*

Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea

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The final step in ethylene biosynthesis is catalyzed by the enzyme 1-aminocyclopropane-1-carboxylate (ACC) oxidase. ACC oxidase was extracted from mung bean hypocotyls and its biochemical characteristics were determined. *In vitro* ACC oxidase activity required ascorbate and Fe^{2+}, and was enhanced by sodium bicarbonate. Maximum specific activity (approximately 20 nl ethylene h^{-1} mg protein^{-1}) was obtained in an assay medium containing 100 mM MOPS (pH 7.5), 25 μM $FeSO_4$, 6 mM sodium ascorbate, 1 mM ACC, 5 mM sodium bicarbonate and 10% glycerol. The apparent K_m for ACC was 80 ± 3 μM. Pretreating mung bean hypocotyls with ethylene increased *in vitro* ACC oxidase activity twofold. ACC oxidase activity was strongly inhibited by metal ions such as Co^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}, and by salicylic acid. Inactivation of ACC oxidase by salicylic acid could be overcome by increasing the Fe^{2+} concentration of the assay medium. The possible mode of inhibition of ACC oxidase activity by salicylic acid is discussed.

Keywords: 1-Aminocyclopropane-1-carboxylate (ACC) oxidase, Ethylene, Mung bean (*Vigna radiata* L.), Salicylic acid.

Introduction

The phytohormone ethylene plays an important role in regulating plant growth and development. Ethylene has been implicated as a factor controlling the timing of seed germination, rate and dimension of etiolated seedling growth, the initiation and progression of fruit ripening, and the expression of a number of stress-related responses in plants (Abeles *et al.*, 1992). The complete ethylene biosynthetic pathway has been elucidated in higher plants (Kende, 1993; Yang and Hoffman, 1984). Ethylene is generated from methionine (Met) via S-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylate (ACC) (Met → AdoMet → ACC → C_2H_4). ACC oxidase catalyzes the last step in ethylene biosynthesis. Until recently, attempts at *in vitro* characterization of ACC oxidase have been largely unsuccessful due to rapid loss of enzyme activity upon tissue homogenization. Cloning of ripening-related cDNAs in tomato led to the identification of clone encoding ACC oxidase polypeptide (Hamilton *et al.*, 1990). The tomato ACC oxidase cDNA pTOM13 showed homology to flavanone-3-hydroxylase. Subsequently, Ververidis and John (1991) were the first to show the complete recovery of ACC oxidase activity *in vitro* by extracting ACC oxidase from melon fruit and by using assay protocols established for flavanone-3-hydroxylase. Since this first report, other investigators have measured *in vitro* ACC oxidase activities in a variety of fruit tissues including apple (Dong *et al.*, 1992; Dupille *et al.*, 1993; Fernandez-Maculet and Yang, 1992; Kuai and Dilley, 1992; Pirrung *et al.*, 1993), avocado (McGarvey and Christoffersen, 1992), melon (Smith *et al.*, 1992), pear (Vioque and Castellano, 1994), *Citrus* (Dupille and Zacarias, 1996) and banana (Moya-Leon and John, 1995). Only in a few cases has an ACC oxidase activity been isolated from vegetative tissue (Finlayson and Reid, 1994; Mekhedov and Kende, 1996).

Recent molecular studies have shown that the expression of ACC oxidase gene is important for the regulation of ethylene biosynthesis in fruit tissues (for review, see Kende, 1993). While there are many molecular studies on the expression of ACC oxidase in fruit tissues, there have been few studies of its expression in vegetative tissues. Two ACC oxidase cDNAs (pVR-ACO1 and pVR-ACO2) which exhibit high homology in their coding regions have been isolated from mung bean hypocotyls (Kim and Yang, 1994). The deduced molecular mass of the protein encoded by pVR-ACO1 is 35.8 kDa which is

* To whom correspondence should be addressed. Tel: 82-2-361-2661; Fax: 82-2-312-5657; E-mail: wtkim@bubble.yonsei.ac.kr

similar to the apparent molecular mass of ACC oxidase purified from apple (Dong *et al.*, 1992; Kim and Yang, 1994). ACC oxidase transcripts were constitutively present in all parts of mung bean seedlings, but transcript levels increased markedly in response to exogenous ethylene and wounding in hypocotyl tissue. These results imply that ACC oxidase may play a role in regulating ethylene biosynthesis in vegetative tissue as has been shown in the fruit tissues. Although expression of ACC oxidase has been investigated in mung bean hypocotyls, the biochemical properties of the gene product have not been examined. Herein, we describe the isolation and *in vitro* characterization of ACC oxidase in mung bean hypocotyls. The effect of ethylene pretreatment and salicylic acid on *in vitro* ACC oxidase activity is also examined.

Materials and methods

Plant material Dry seeds of mung bean (*Vigna radiata* L.) were imbibed overnight in aerated tap water. The soaked seeds were sown on the agar plate (0.5%, 15 cm × 15 cm) and grown in darkness at 25°C for 3 d. Hypocotyl segments were cut at one-centimeter below the hook, immediately frozen in liquid nitrogen, and then stored at −80°C until used.

Ethylene treatment Three-day-old mung bean seedlings were enclosed in 3-l desiccators fitted with injector ports that were sealed with rubber serum vial caps. Ethylene (20 μl/l) was injected into the desiccators. Internal ethylene concentrations were confirmed by gas chromatography. After 12 h of treatment, hypocotyls were excised, and used for ACC oxidase assays or frozen in liquid nitrogen and stored at −80°C until used.

Extraction and assay of ACC oxidase For *in vitro* enzyme assays, mung bean hypocotyl tissues were pulverized in liquid nitrogen and homogenized in extraction buffer (0.5 ml extraction buffer/g fresh weight tissue) consisting of 100 mM MOPS (pH 7.5), 10% glycerol and 1 mM PMSF. The homogenate was centrifuged at 28,000 *g* for 25 min at 4°C. The supernatant was used for enzyme assay after passing through a Sephadex G-25 column (10 cm × 1 cm) equilibrated with 0.1 M MOPS (pH 7.5) and 10% glycerol. The standard assay medium contained 100 mM MOPS (pH 7.5), 10% glycerol, 1 mM ACC, 25 μM $FeSO_4$, 6 mM sodium ascorbate, 5 mM sodium bicarbonate plus an aliquot of desalted crude extract in a total volume of 2 ml. The assay was conducted at 35°C in a 12 ml test tube which was sealed with a rubber serum cap. After 40 min of incubation, 1 ml gas sample was withdrawn from the head space of the tube and analyzed for ethylene production by gas chromatography. ACC oxidase activity was expressed as nl ethylene h^{-1} mg $protein^{-1}$. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad assay reagent and BSA as a standard. *In vivo* ACC oxidase activity was assayed as described previously (Kim and Yang, 1994). All determinations were made in triplicate and the results are expressed as mean ± S. E.

Enzyme kinetics Apparent K_m values and standard errors were determined by nonlinear regression analysis using Grafit kinetics software (Erithicus Software, London).

Results and Discussion

ACC oxidase was extracted as a soluble enzyme from mung bean hypocotyls. In apple and pear fruits, ACC oxidase activity is associated with a pellet fraction which is solubilized using Triton X-100 (Fernandez-Maculet and Yang, 1992; Smith *et al.*, 1992; Mizutani *et al.*, 1995). In mung bean hypocotyls, the addition of Triton X-100 is not necessary for the improvement of extracting ACC oxidase. ACC oxidase activity measured in crude extracts of mung bean hypocotyls declined 60% during 4 h of incubation at 4°C in the presence of 1 mM PMSF. However, enzyme activity was more stable in the desalted crude extracts and maintained 80% of its activity during 24 h of incubation at 4°C in the presence of 1 mM PMSF. Presumably, this is due to the presence of inhibitory compounds in crude extracts. Desalted crude extracts were used for all subsequent experiments.

ACC oxidase activity increased *in vitro* was linear for up to 40 min, but declined thereafter (Fig. 1A). A similar loss of activity over time has been previously noted with ACC oxidase extracted from various fruit tissues (Smith *et al.*, 1992; Pirrung *et al.*, 1993; Mizutani *et al.*, 1995; Dupille and Zacarias, 1996) and carnation petals (Nijenhuis-De Vries *et al.*, 1994). It has been suggested that the loss of enzyme activity could be due to catalytic inactivation (Munoz De Rueda *et al.*, 1995; Dupille and Zacarias, 1996). The effect of pH on the ACC oxidase activity was tested between pH 6 and pH 8.5 using MES, MOPS, and Tris-HCl buffers. The pH optimum was found to be 7.5 in MOPS buffer (Fig. 1B). This optimum for ACC oxidase is similar to values reported for other plant species (Munoz De Rueda *et al.*, 1995; Dupille and Zacarias, 1996). The effect of temperature on ACC oxidase activity was measured in the range of 20–60°C (Fig. 1C). ACC oxidase activity exhibited a high temperature optimum of 45°C. The standard assay, however, was performed at 35°C which is thought to be more physiologically relevant. *Citrus* ACC oxidase exhibited two temperature optima of 35°C and 45°C (Dupille and Zacarias, 1996). Two similar temperature optima were also found for ACC oxidase from pear fruits and chick-pea seeds (Vioque and Castellano, 1994; Munoz De Rueda *et al.*, 1995). These reports suggest that two forms of ACC oxidase may be present in these tissues. McGarvey and Christoffersen (1992) found evidence for two ACC oxidases during ammonium sulfate fractions of avocado fruit. In mung bean hypocotyls, two ACC oxidase cDNA clones (pVRACO1 and pVRACO2) that exhibited 80% homology in the coding regions were isolated (Kim and Yang, 1994). These two genes were transcribed but the gene products have not yet been characterized. It is possible that more than one ACC oxidase may exist in mung bean hypocotyls. However, we did not observe more than one temperature optimum for ACC oxidase activity in the desalted fraction that we used to assay ACC oxidase.

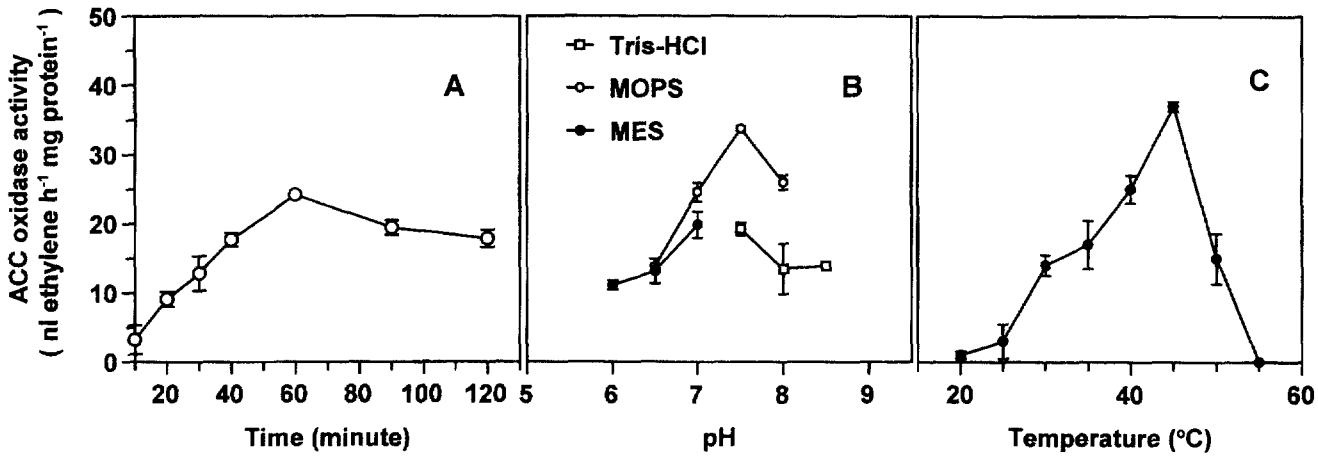

Fig. 1. Effects of duration (A), pH (B), and temperature (C) on ACC oxidase activity measured in desalted crude extracts from mung bean hypocotyls. The enzyme was assayed in the standard reaction medium (100 mM MOPS, pH 7.5, 25 μM $FeSO_4$, 6 mM sodium ascorbate, 1 mM ACC, 5 mM sodium bicarbonate and 10% glycerol).

The optimum conditions for *in vitro* assays of ACC oxidase activity were determined to ensure that enzyme activity was measured under conditions where substrates or cofactors were not limiting (Fig. 2). As found in other plants, ACC oxidase in mung bean hypocotyls requires sodium ascorbate for activity with 6 mM being the optimum concentration (Fig. 2A). Higher concentration inhibited the enzyme activity. The optimum sodium ascorbate concentration for mung bean hypocotyls is similar to that reported for ACC oxidase activity in other plant tissues such as seeds, fruits, and flowers (Nijenhuis-De Vries *et al.*, 1994; Munoz De Rueda *et al.*, 1995; Dupille and Zacarias, 1996). Fe^{2+} was required for activity of mung bean ACC oxidase with an optimal concentration of about 25 μM (Fig. 2B). Several metal ions and divalent cations were examined for their ability to replace Fe^{2+} as a cofactor. ACC oxidase in the desalted crude extract was measured in the standard assay medium containing 25 μM Mn^{2-}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}, or Ca^{2+}. However, none of these ions were able to replace Fe^{2+} (data not shown). These results agree with the competition experiments which showed that Mn^{2+}, Cu^{2+}, Zn^{2+}, and Co^{2+} were inhibitory to enzyme activity (Table 1). Among the metal ions examined, Co^{2+} which is known to inhibit ACC oxidase *in vivo* (Yang and Hoffman, 1984), completely inhibited mung bean ACC oxidase at 5 μM. The inhibitory effect of these metal ions could be partially recovered by increasing the concentration of Fe^{2+} (Table 1). ACC oxidase has been classified as a member of an enzyme superfamily that requires Fe(II)-ascorbate for enzyme activity. This superfamily also includes flavanone-3-hydroxylase, hyoscyamine-6-hydroxylase and isopenicillin N synthase (Matsuda *et al.*, 1991; Tang *et al.*, 1993; Prescott and John, 1996). Phylogenetic analysis based on optimal alignment of amino acid sequences revealed that several amino acids are completely conserved among all members of the Fe(II)-ascorbate superfamily (Tang *et al.*, 1993). Three histidine residues (H-39, H-177, and H-234)

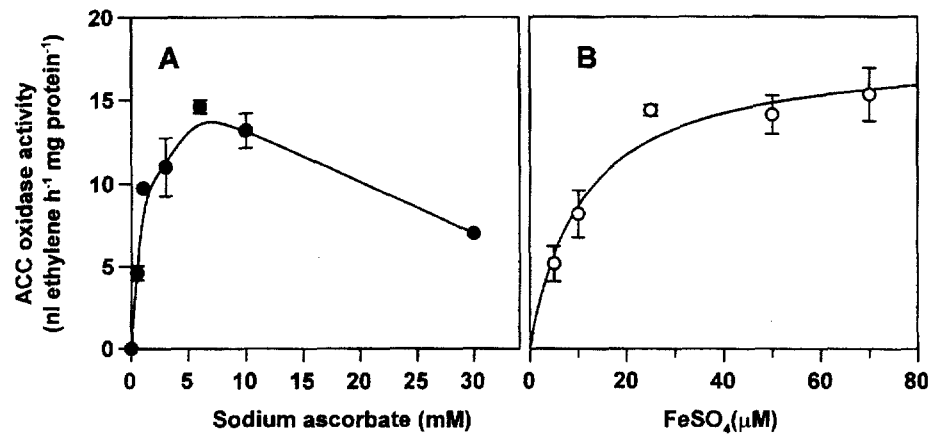

Fig. 2. Concentration-dependent effect of sodium ascorbate (A) and $FeSO_4$ (B) on ACC oxidase activity. Enzyme activity in desalted crude extracts was measured in the standard reaction medium as described in Fig. 1.

are found to be conserved in ACC oxidases from apple, tomato, and avocado. It has been proposed that the histidine residues may serve to bind Fe^{2+} in these enzymes (Tang *et al.*, 1991; Britsch *et al.*, 1993; Zhang *et al.*, 1995). The modification of histidines in ACC oxidase and flavanone-3-hydroxylase by diethylprocarbonate (DEPC) abolished enzyme activity, supporting their role as a Fe^{2+} ligand (Britsch *et al.*, 1993; Zhang *et al.*, 1995). Mung bean ACC oxidase also contained three conserved histidine residues that may play a role in ligating Fe^{2+} (Kim and Yang, 1994).

Table 1. Effect of ferrous iron alone or in combination with other metal ions on ACC oxidase activity. Concentration of metal ions was 25 μM except for Co^{2+} which was 5 μM.

Metal ions present (molar ratio)	Activity
	%
Fe^{2+}	100
Fe^{2+}/Cu^{2+}	
(1 : 1)	0
(2 : 1)	28.5
Fe^{2+}/Mn^{2+}	
(1 : 1)	0
(2 : 1)	41
Fe^{2+}/Zn^{2+}	
(1 : 1)	0
(2 : 1)	38
Fe^{2+}/Co^{2+}	
(1 : 0.2)	0
(2 : 0.2)	22

It was previously reported that ACC oxidase requires CO_2/HCO_3^- for activity (Dong *et al.*, 1992; Fernandez-Maculet *et al.*, 1993; Poneleit and Dilley, 1993; Smith and John, 1993; Nijenhuis-De Vries *et al.*, 1994; Munoz De Rueda *et al.*, 1995; Dupille and Zacarias, 1996). We found that addition of bicarbonate in the assay medium greatly stimulated ACC oxidase activity in both crude extracts and desalted crude extracts from mung bean hypocotyls (Fig. 3). However, the level of stimulation was much greater with the desalted crude extracts than the crude extracts. Furthermore, maximal stimulation of ACC oxidase was reached at a lower bicarbonate concentration with the desalted extracts (5 mM) than that with the crude extracts (10 mM). This may be due to the presence of inhibitory compounds in the crude extracts. A similar effect of bicarbonate addition was also observed with ACC oxidase extracted from melon (Smith and John, 1993). In mung bean hypocotyls, the optimum bicarbonate concentration for ACC oxidase was 5 mM and the concentration for the half maximal enzyme activity was estimated to be 0.76 mM in the desalted crude extracts (Fig. 3). The optimum bicarbonate concentration for ACC oxidase from mung bean is within the range of values reported for apple, *Citrus* peel, and tomato ACC oxidase that was overexpressed in *E. coli* (Pirrung *et al.*, 1993; Zhang *et al.*, 1995; Dupille and Zacarias, 1996). Dong *et al.* (1992) reported that apple ACC oxidase was activated by CO_2 with activity being saturated at 4% CO_2 in the gas phase, and half-maximal activity being observed at 0.5% CO_2 which corresponds to a 0.15 mM concentration of bicarbonate. It has been reported that raising the CO_2 levels from ambient to levels in the range of 5–20% increased the V_{max} as well as apparent K_m toward ACC (Hyodo *et al.*, 1993; Poneleit and Dilley, 1993; Smith and John, 1993; Finlayson and Reid, 1994; Mizutani *et al.*, 1995). In addition, raised CO_2 levels lowered the pH optimum of ACC oxidase (Fernandez-Maculet and Yang, 1992; Poneleit and Dilley, 1993).

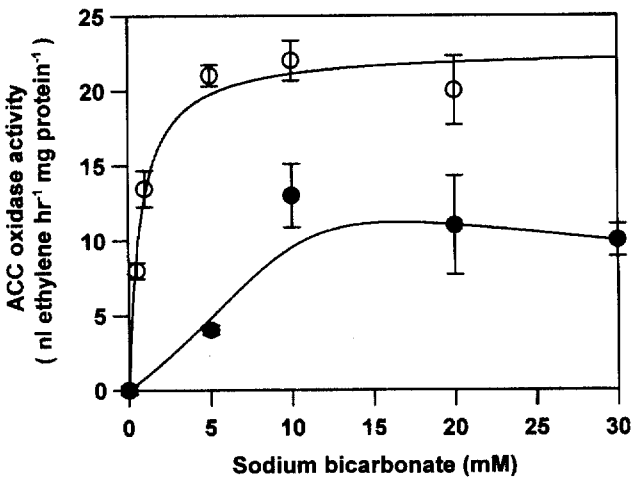

Fig. 3. Effect of sodium bicarbonate concentration on activity of ACC oxidase. Enzyme was assayed by incubation in the standard reaction medium using crude (●) or desalted extracts (○).

Under optimal bicarbonate concentration and pH optimum, apparent K_m for ACC was estimated to be 80 ± 3 μM (Fig. 4). This value is very close to those reported as 85 μM in melon (Ververidis and John, 1991) and 98 μM in root of sunflower seedlings (Finlayson and Reid, 1994), but less than those found in winter squash (175 μM) (Hyodo *et al.*, 1993) and *Citrus* peel (175 μM) (Dupille and Zacarias, 1996), and far greater than those found in apple (6–12 μM) (Kuai and Dilley, 1992; Pirrung *et al.*, 1993).

The kinetic constants were determined for ACC oxidase isolated from ethylene-treated mung bean hypocotyls (Table 2). The V_{max} for ACC oxidase from ethylene-treated tissue is twofold higher than that of control. However, the apparent K_m toward ACC was not significantly changed by

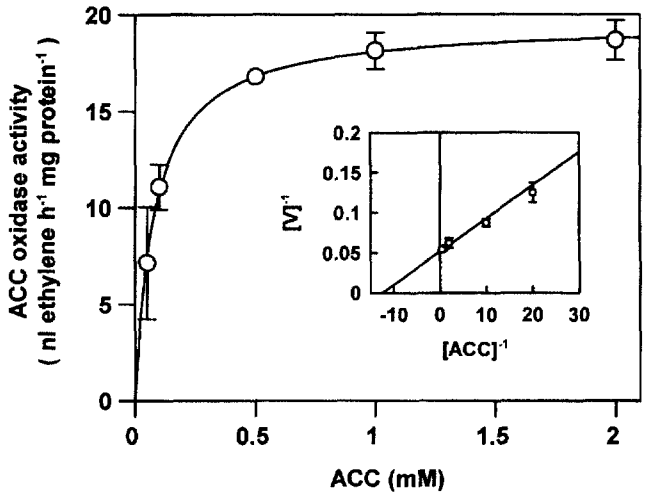

Fig. 4. Effect of various concentrations of ACC on the activity of ACC oxidase. Inset panel is the Lineweaver-Burk plot for the determination of the apparent K_m for ACC. The enzyme was assayed in the standard reaction medium as described in Fig. 1.

Table 2. Kinetic constants for ACC oxidase isolated from ethylene-treated and control tissues.

	V_{max} (nl ethylene h^{-1} mg $protein^{-1}$)	K_m (μM)
ethylene-treated	38 ± 2.5	90 ± 10
control	19 ± 1.9	80 ± 3.0

ethylene treatment. Similar degree of increase in the *in vivo* ACC oxidase activity was exhibited in ethylene-pretreated mung bean hypocotyls (Kim and Yang, 1994). The relevant question to the present study is how ethylene enhances ACC oxidase activity. It was previously reported that ethylene induced the accumulation of transcript of ACC oxidase in mung bean hypocotyls (Kim and Yang, 1994; Kim *et al.*, 1997) and also increased the amount of ACC oxidase protein when probed with mung bean ACC oxidase antibody (manuscript in preparation). The data from the current and previous reports suggest that increasing ACC oxidase activity in the response to ethylene is due to the induction of transcript of ACC oxidase which is followed by the increase in the ACC oxidase protein, not to the change in kinetic properties of this enzyme such as a substrate affinity.

Salicylic acid inhibited ACC oxidase activity in mung bean hypocotyls (Fig. 5). Previously, salicylic acid has been investigated at the view of the elicitor effect in plants (Raskin, 1992), as has been inhibitory effect on the ethylene biosynthesis (Leslie and Romani, 1986, 1988). Inhibition by salicylic acid in ethylene biosynthesis on the conversion from ACC to ethylene was reported recently (Munoz De Rueda, 1995; Fan *et al.*, 1996). However, the mechanism whereby salicylic acid inhibits ACC oxidase activity is not known. The concentration for the inhibition of 50% enzyme activity is estimated to be 0.4 mM from both the *in vivo* and *in vitro* assays (Fig. 5). When various concentrations of Fe^{2+} were added to the reaction mixture with the various salicylic acid concentrations, inhibitory effect of salicylic acid was gradually recovered by increasing concentration of Fe^{2+} (Fig. 6). Inactivation of ACC oxidase by salicylic acid could not be reversed by the addition of high level of sodium bicarbonate or ascorbate (data not shown).

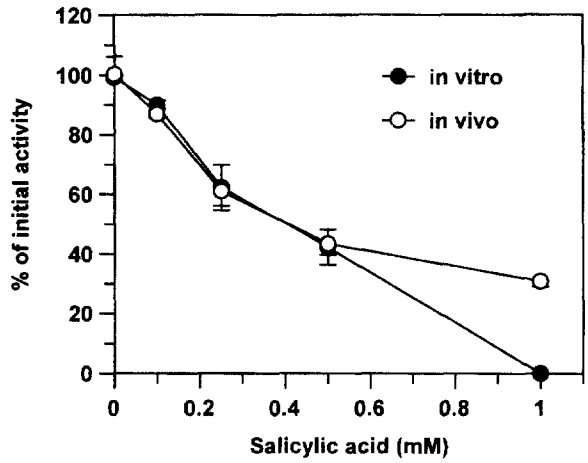

Fig. 5. Inhibition of ACC oxidase activity by salicylic acid. For *in vitro* assays of ethylene production (●), the enzyme was assayed in the standard reaction medium containing various concentration of salicylic acid. For *in vivo* enzyme assays (○), mung bean hypocotyls were incubated for 1 h at 25°C in a medium containing 50 mM MES (pH 6.2), 2% (w/v) sucrose, 1 mM $CaCl_2$, and 1 mM ACC. Ethylene production was measured by GC.

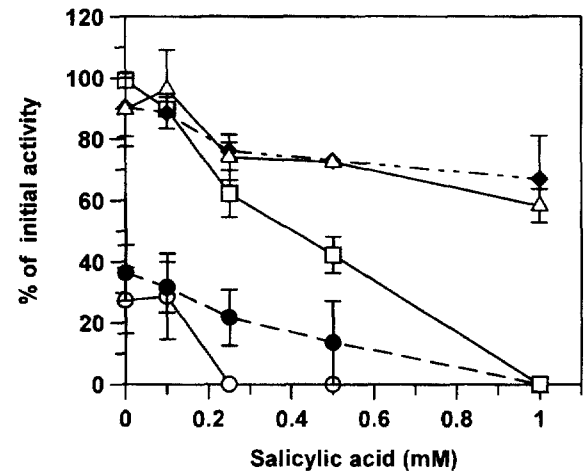

Fig. 6. Effect of various concentrations of $FeSO_4$ on the inhibition of ACC oxidase by salicylic acid. $FeSO_4$ was added to the standard reaction medium at the following concentrations: 5 μM (○), 10 μM (●), 25 μM (□), 50 μM (◆), 75 μM (Δ).

Based on the results of this study and previous studies, two possible modes of inhibition of ACC oxidase activity by salicylic acid are postulated. First, salicylic acid may directly chelate Fe^{2+}. Since salicylic acid is known to be a bidentate ligand (McDevitt *et al.*, 1990), it could contribute to phenolate and carboxylate coordination with iron atom. A recent report by Fan *et al.* (1996), however, indicated that inhibition of apple fruit ACC oxidase by acetylsalicylic acid was not due to the chelation of Fe^{2+} because the addition of Fe^{2+} to *in vitro* assay medium did not restore the ACC oxidase activity. In contrast, our results clearly showed that inactivation of ACC oxidase activity by salicylic acid could be overcome to the level which was over 70% of initial activity by gradual increment of Fe^{2+} (Fig. 6). Secondly, salicylic acid may bind to the ACC oxidase to impair the activity, presumably modifies the site which contributes to the ligand for Fe^{2+}. The study by Sanchez-Casas and Klessig (1994) revealed that the mode of action of salicylic acid in plant defense is to bind catalase and inhibits its activity. They suggested that catalase was the only salicylic acid receptor or the sole binding component in the plant cell. These implications led to the conclusion that salicylic acid is a signal transducer (Chen *et al.*, 1993) or an endogenous second messenger (Sanchez-Casas and Klessig, 1994). These speculations, however, were reevaluated by Ruffer *et al.* (1995) who demonstrated that salicylic acid bound to heme and non-heme iron enzymes so that the bound salicylic acid impaired catalytic activity. These studies indicate that the salicylic acid binds the iron-containing proteins to impair the enzymatic activities. ACC oxidase is known to be a non-heme iron protein (Pirrung *et al.*, 1993). Therefore, we may not exclude the possibility that salicylic acid binds to modify the site that may provide the ligand for Fe^{2+} in ACC oxidase. At this time it is not known the exact molecular association of ACC oxidase and Fe^{2+}. Binding assay by using labeled salicylic acid with the purified ACC oxidase will be useful to clarity these speculations.

In conclusion, the optimal conditions for *in vitro* ACC oxidase activity in mung bean hypocotyls were characterized in our study. The properties of this enzyme were similar to those previously reported ACC oxidases isolated from diverse fruit tissues. Our present data have clearly shown that ACC oxidase in mung bean hypocotyls is a Fe^{2+}-dependent enzyme, and that inhibition of ACC oxidase activity by salicylic acid could be reversed by increasing the concentration of Fe^{2+}. In addition, it has been shown that ethylene-induced ACC oxidase activity is not due to the change in the substrate affinity, but likely due to the increased level of ACC oxidase protein.

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