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Partial Characterization of 1-Aminocyclopropane-1-carboxylate Oxidase from Excised Mesocarp Tissue of Winter Squash Fruit

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Summary

1-Aminocyclopropane-1-carboxylate (ACC) oxidase, the enzyme that catalyzes the conversion of ACC to ethylene, the final step of ethylene biosynthesis was extracted from wounded mesocarp tissue of winter squash (*Cucurbita maxima* Duch. cv. Ebisu) fruit. The enzyme was characterized with respect to temperature optima, thermostability, stability in the presence of selected metal ions and alkylating agents, and K_m value for ACC.

ACC oxidase requires Fe^{2+} as a co-factor and maximum activity was achieved using Fe^{2+} at 20 μM in the reaction mixture. The enzyme was activated by CO_2 and inclusion of CO_2 in the reaction mixture increased the apparent K_m value of the enzyme with respect to ACC. The enzyme exhibited apparent K_m values for ACC of 147 μM in air and 454 μM in the presence of 5 % CO_2. ACC oxidase was partially inactivated by ACC during its catalytic action. The enzyme had maximum activity at 30 °C and its activity was almost completely lost in the presence of Ag^{+}, Co^{2+}, Cu^{2+} and Zn^{2+} ions. The alkylating agents, iodoacetamide and iodoacetic acid partially inhibited and almost completely abolished ACC oxidase activity respectively, thereby suggesting requirement of sulfhydryl groups for ACC oxidase activity. Following excision of the mesocarp tissue, the increase in *in vivo* ACC oxidase activity was accompanied by a concomitant increase in *in vitro* ACC oxidase activity.

The results support and extend previous observations and indicate that the solubilized enzyme is indeed ACC oxidase and resembles the physiological ACC oxidase in several aspects.

Introduction

The biosynthetic pathway for ethylene in higher plants has been established as L-methionine → S-adenosyl-L-methionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene[2]. The last step in this biosynthetic pathway is catalyzed by ACC oxidase (also known as ethylene-forming enzyme), which until recently had not been extracted in an active status in a cell-free state. Thus, ACC oxidase activity has been routinely determined *in vivo* by supplying saturating concentrations of ACC to tissues [12,27]. Furthermore, earlier investigations had led to the conclusion that ACC

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oxidase requires membrane integrity[12,13,18,27] and that its activity is completely lost upon tissue homogenization[12,13]. This loss in enzyme activity after tissue homogenization has however, been attributed to loss of essential co-factors such as Fe^{2+} [9].

Despite the technical problems previously associated with the isolation and characterization of ACC oxidase[13], a number of cell-free ethylene-forming systems have been described[1,3,20,25]. These systems are dependent on oxygen and except for intact protoplasts and vacuoles that possess native ACC oxidase properties[10], they lack high affinity for ACC, a characteristic of *in vivo* ACC oxidase, and display no stereospecificity towards 1-amino-2-ethylcyclopropane-1-carboxylic acid stereoisomers for the synthesis of 1-butene[25].

Recently, Ververidis and John[26] showed that authentic ACC oxidase activity from melon fruit can be fully recovered under extraction conditions that had been shown previously to preserve *in vitro* the activity of flavanone-3-hydroxylase of *Petunia hybrida* petals. Following this discovery, *in vitro* ACC oxidase has been isolated and partially characterized in apple[7,9,14], melon[23,24], and avocado[19]. Although the biochemical entity of the enzyme has not yet been well established, *in vitro* ACC oxidase activity requires Fe^{2+} and ascorbate as co-factors suggesting that the enzyme is a hydroxylase[9]. This notion is however, not new since it had previously been demonstrated that the conversion of ACC to ethylene in plant tissues is absolutely dependent on oxygen, suggesting that what had previously been known as ethylene-forming enzyme is either an oxidase or a hydroxylase[12].

The results presented here support and extend recent reports on some characteristics of *in vitro* ACC oxidase extracted from wounded mesocarp tissue of winter squash fruit.

Materials and Methods

Plant material

Winter squash (*Cucurbita maxima* Duch. cv. Ebisu) fruit from a commercial market in Okayama City were used for the preparation of the enzyme extracts.

Extraction of ACC oxidase

ACC oxidase was extracted as described by Fernandez-Maculet and Yang[9]. To obtain a high activity of the enzyme, cubes (5 mm) were excised from the mesocarp tissue, placed on a petri dish containing a moist filter paper and incubated at 25 °C for 10 to 15 h. The cubes were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle in the presence of 5 % (w/v) polyvinylpyrrolidone. The powder was then transferred to centrifuge tubes containing two volumes of an extraction buffer consisting of 0.1 M Tris-HCl (pH 7.2), 10 % (w/v) glycerol and 30 mM sodium ascorbate and the mixture was centrifuged at 15,000 × g for 20 min. The resulting supernatant was used as the enzyme source. The supernatant was passed through a membrane filter (Toyo, Cellulose Nitrate, 0.45 μm) and subjected to chromatography on a Sephadex G-25 column previously equilibrated with the extraction buffer. The column was eluted with the same buffer. The desalted macromolecular protein fraction was collected and used as the ACC oxidase preparation and for protein estimation. All steps were carried out at 0–4°C.

ACC oxidase assay

In vitro ACC oxidase activity was assayed in a reaction mixture containing 1.8 ml of the enzyme preparation, 2 mM ACC, and 20 μM $FeSO_4$ in the presence of 5 % CO_2 unless otherwise stated. The flasks were incubated at 30°C and the ethylene produced in 30 min was determined by withdrawing 1 ml of the headspace gas and injecting into a gas chromatograph equipped with an activated alumina column and a flame ionization detector. *In vitro* ACC oxidase activity was expressed as C_2H_4 (nl) produced per mg protein per hour.

In vivo ACC oxidase activity was measured as the rate of ethylene production by the mesocarp tissue in the presence of saturating concentration of ACC as previously described[17].

Protein determination

Protein contents in the enzyme preparations were determined by the method of Bradford[5] using bovine serum albumin as a standard.

Enzyme thermostability and temperature optima

Thermostability of the enzyme was determined by incubating the enzyme preparation at 0, 10, 25, 30 and 45 °C for up to 24 h. Samples were withdrawn at various time points and assayed for ACC oxidase activity.

The optimum assay temperature for the enzyme activity was determined by incubating the complete reaction mixture over a temperature range of 0 to 50 °C for 30 min and the

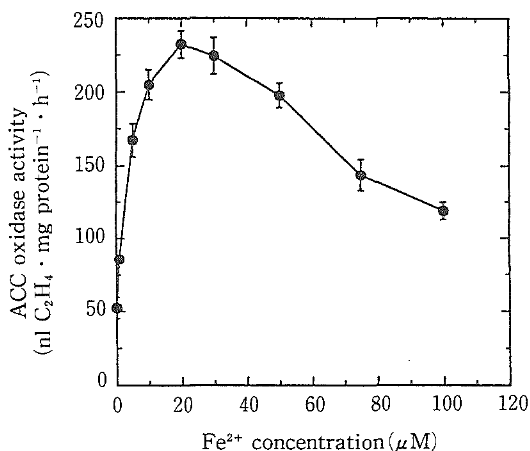

Fig. 1 Dependence of the ACC oxidase activity on Fe^{2+} concentration in the reaction mixture. The crude enzyme preparation was passed through a Sephadex G-25 column and aliquots (1200 μg protein) were assayed immediately by the standard assay procedure in the presence or absence of the indicated concentrations of Fe^{2+}. Vertical bars represent SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbol.

Table 1 Dependence of ACC oxidase activity on carbon dioxide concentration

CO_2 concentration (%)	ACC oxidase activity (nl C_2H_4 · mg protein^{-1} · h^{-1})
Air (control)	56.2± 3.2
1	121.9± 4.7
3	173.8±10.3
5	242.2± 6.4
7	258.7± 9.2

The various concentrations of CO_2 were introduced into the incubation flasks containing a complete reaction mixture consisting of 1.8 ml of the enzyme preparation (900 μg protein), 2 mM ACC and 20 μM of $FeSO_4$. The flasks were incubated at 30 °C for 30 min and the ethylene produced was determined. Values are means±SE of three replications.

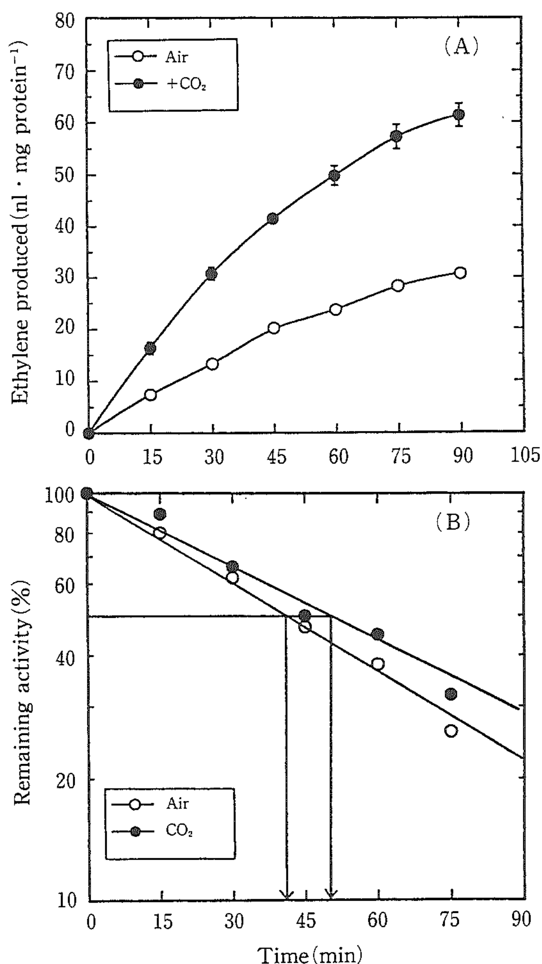

Fig. 2 Time courses of ethylene production by the ACC oxidase (A) and change in the remaining ACC oxidase activity (B) during incubation in air or in the presence of 5 % CO_2. A : Samples of the enzyme preparation were incubated in the standard reaction mixture at 30 °C and at 15-min intervals, the ethylene produced was determined. B : The remaining activity at a given incubation time was calculated from the data of (A) as the amount of ethylene produced during a subsequent 15-min incubation period and plotted on a log scale. The half life values of ACC oxidase were estimated from the straight lines. Vertical bars represent SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbol.

ethylene produced was determined.

Effects of metal ions and alkylating agents

The effects of selected cations on ACC oxidase activity in the complete reaction mixture were determined by adding the following metal ions at 50 μM final concentration : Ag^+, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Li^+, Mg^{2+}, Mn^{2+} and Zn^{2+}.

To examine the role of sulfhydryl groups in ACC oxidase activity, the alkylating agents, iodoacetamide ($IAcNH_2$) and iodoacetic acid (IHAc) at 20 mM final concentration were added to the complete reaction mixture to block the free sulfhydryl groups of the enzyme[8].

Enzyme kinetics

The Michaelis-Menten constant (K_m) was determined by supplying the enzyme preparation with various concentrations of ACC (0-5 mM) in air or in the presence of 5 % CO_2. Ethylene production rates were then determined after 30 min of incubation at 30 °C. Since ACC oxidase is a bi-substrate enzyme and changes in one substrate may affect the apparent K_m value of the other[28] all experiments were performed under 21 % O_2. From the measured ethylene production rates and the corresponding ACC concentrations, K_m values for ACC in air or in presence of 5 % CO_2 were estimated from a Lineweaver-Burk double reciprocal plot.

All experiments were repeated at least three times with similar trends being obtained despite variation between experiments. The data reported here were from typical experiments.

Table 2 Residual ACC oxidase activity after preincubation with 5 mM ACC and/or 5 mM salicylic acid

Treatment ACC	Treatment Salicylic acid	Remaining ACC oxidase activity (nl C_2H_4 · mg protein^{-1} · h^{-1})	Relative activity (%)
−	−	195.8± 6.5	100
−	+	182.1±10.5	93
+	−	88.6± 6.4	45
+	+	100.7± 7.8	51

The crude enzyme extract was passed through a Sephadex G-25 column and immediately incubated with or without 5 mM ACC and/or 5 mM salicylic acid at 30 ℃. After 2 h of incubation the mixture was again passed through a Sephadex G-25 column to remove small molecules and the remaining enzyme activity was assayed by the standard assay procedure. The enzyme activity before incubation was 193.9±2.8 nl C_2H_4 · mg protein^{-1} · h^{-1}. Values are means±SE of three replications.

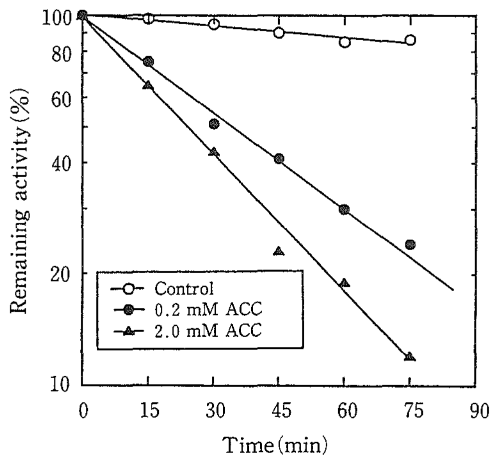

Fig. 3 Dependence of the remaining ACC oxidase activity on incubation time. The complete reaction mixtures containing 1.8 ml of the enzyme preparation (1110 μg protein) and 0.2 mM or 2.0 mM ACC were incubated at 30 ℃ and the ethylene produced in 15 min intervals was measured and the remaining activity was calculated. Another sample was incubated at 30 ℃ without ACC (control) and aliquots were withdrawn at 15 min intervals and the remaining ACC oxidase activity was measured by the standard assay method. Each point is the mean of triplicate determinations.

Results and Discussion

Effects of Fe^{2+} and CO_2 concentrations and incubation time

It has previously been demonstrated that *in vivo* ACC oxidase[4] and *in vitro* ACC oxidase[6,9,26] have an absolute requirement for Fe^{2+} as a cofactor and that CO_2 activates ACC-dependent ethylene production[6,27]. This observation was reconfirmed in the present study using ACC oxidase extracted from excised mesocarp tissue of winter squash fruit. Maximum ACC oxidase activity was obtained using Fe^{2+} at 20 μM in the standard reaction mixture (Fig. 1) and 5 % CO_2 was sufficient for the activation of ACC oxidase (Table 1). These results are consistent with those of Dong et al.[6] who recently reported that Fe^{2+} at concentrations as low as 10 μM and 4 % CO_2 are sufficient to maintain the maximum activity of ACC oxidase extracted from apple fruit.

The enzyme showed a non-linear time course during incubation, both in air or in the presence of CO_2 (Fig. 2A). The cause of this lack of linearity is not yet known and although CO_2 activated ACC oxidase, it had little effect on the inactivation of the enzyme during incubation. This was further confirmed by the observation that the presence of CO_2 had little effect on the half-life of the enzyme (Fig. 2B) (42 min in air compared to

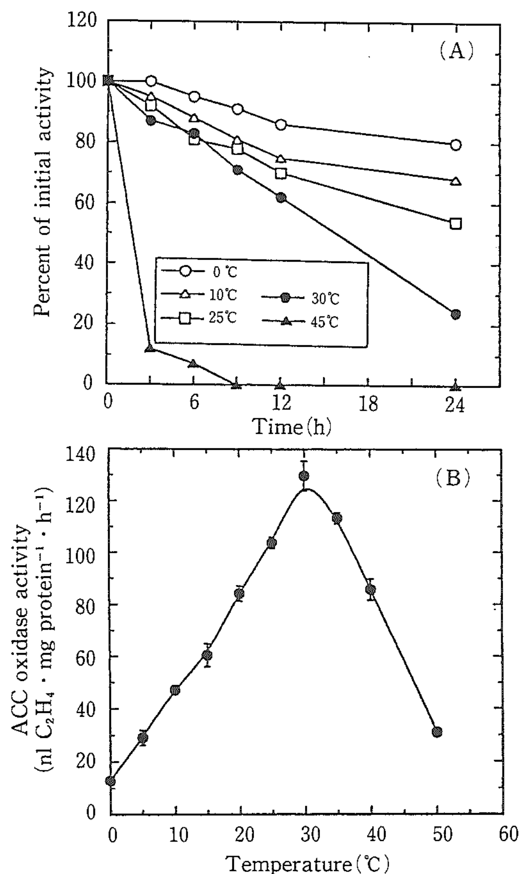

Fig. 4 Thermostability of the ACC oxidase during incubation at various temperatures (A) and activities of the ACC oxidase as a function of assay temperature (B). A : The enzyme preparation was incubated at the indicated temperature for 24 h. At the indicated time points, aliquots were withdrawn and assayed for ACC oxidase activity in the standard assay procedure. B : The standard reaction mixtures were incubated at the indicated temperatures for 30 min and the ethylene formed was determined. Vertical bars represent SE of the mean of three replications.

50 min in the presence of 5 % CO_2). Further work is needed to establish the mechanism through which CO_2 activates ACC oxidase. When this work was completed, Smith and John[23] reported that ACC oxidase from melon fruit is also activated by CO_2 and that inclusion of HCO_3^- in the reaction mixture has little effect on the catalytic inactivation of the ACC oxidase.

Possibility of ACC inactivation of ACC oxidase

Salicylic acid (SA) is an inhibitor of ACC oxidase activity[16] and at 5 mM final concentration, it inhibited ACC oxidase activity by 88 % (data not shown). To examine the possibility of ACC inactivation of ACC oxidase, the enzyme preparation was incubated with 5 mM ACC and/or 5 mM SA at 30 ℃ for 2 h and then subjected to gel filtration on a Sephadex G-25 column to separete protein. ACC at 5 mM final concentration caused a loss of 49 % and 55 % of the enzyme activity in the presence and absence of SA respectively, whereas, in the absence of ACC, SA had little effect on the residual ACC oxidase activity (Table 2). No loss in enzyme activity was observed in the absence of ACC. In addition, enzyme activity was followed during incubation of the enzyme with or without ACC. Figure 3 illustrates the changes in the remaining ACC oxidase activity during incubation with or without 0.2 mM or 2.0 mM ACC plotted on a log scale as a function of incubation time. We defined the remaining enzyme activity at a given time during the incubation period as the amount of ethylene produced during the subsequent 15-min period. The plot gave straight lines, indicating that the inactivation of the enzyme during incubation follows pseudo first-order kinetics and is time-dependent. Little enzyme inactivation was observed in the absence of ACC. A similar non-linear time course has been observed for *in vitro* ACC oxidase from melon[24] and avocado[19] fruits. Substrate inactivation has been reported for ACC synthase, the enzyme that catalyses

Table 3 Effect of metal ions on ACC oxidase activity

Ion	ACC oxidase activity ($nl\ C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$)	Inhibition (%)
Fe^{2+}	220.2±4.7	—
Li^{+}/Fe^{2+}	217.5±4.7	1
Ba^{2+}/Fe^{2+}	214.2±6.1	3
Ca^{2+}/Fe^{2+}	204.1±7.0	7
Mg^{2+}/Fe^{2+}	194.4±4.8	12
Mn^{2+}/Fe^{2+}	138.6±4.2	37
Co^{2+}/Fe^{2+}	31.3±0.7	86
Zn^{2+}/Fe^{2+}	18.1±0.9	92
Ag^{+}/Fe^{2+}	10.5±0.6	95
Cu^{2+}/Fe^{2+}	4.7±0.4	98

The crude enzyme extract was passed through a Sephadex G-25 column and 1.8 ml of the enzyme preparation (1011 μg protein) was assayed by the standard assay method in the presence of 50 μM of the various metal ions. The ethylene formed after incubation at 30 °C for 30 min was determined. ACC oxidase activity in the absence of Fe^{2+} was 42.8±3.7 nl $C_2H_4 \cdot$ mg protein$^{-1} \cdot h^{-1}$. Values are means± SE of three replications.

Table 4 Effect of alkylating agents on ACC oxidase activity

Treatment	ACC oxidase activity ($nl\ C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$)	Relative activity (%)
None (control)	194.8±9.7	100
Iodoacetamide	97.9±4.9	50
Iodoacetic acid	1.3±0.1	0.7

The crude enzyme extract was passed through a Sephadex G-25 column and 1.8 ml of the enzyme preparation (1110 μg protein) was assayed for ACC oxidase activity by the standard assay procedure in the presence or absence of 20 mM iodoacetamide or iodoacetic acid. Values are means±SE of three replications.

Table 5 Comparison between *in vivo* and *in vitro* activities of ACC oxidase upon excision of winter squash mesocarp tissue

Time after cutting(h)	ACC oxidase activity			
	In vivo ($nl\ C_2H_4 \cdot g^{-1} \cdot h^{-1}$)		*In vitro* ($nl\ C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$)	
	Air	+5% CO_2	Air	+5% CO_2
0	1.9±0.3	2.6±0.3	2.1±0.2	9.4±0.5
3	9.4±1.1	17.0±1.8	9.6±0.9	34.7±0.8
6	46.0±2.3	53.3±4.8	33.1±1.8	146.6±7.2
9	48.4±3.1	63.6±3.7	54.3±0.8	257.7±5.1
24	121.8±2.3	137.2±0.5	119.3±2.3	1151.7±9.2

Cubes (5 mm) were prepared from the mesocarp tissue of winter squash fruit and incubated at 25 °C in a petri dish containing a wet filter paper. At the indicated time points, samples were taken and assayed for *in vivo* and *in vitro* ACC oxidase activities in air or in the presence of 5 % CO_2. Values are means± SE of three replications.

the conversion of AdoMet to ACC[22]. The enzyme preparation used here was however, not purified and could therefore have contained other compounds which might also contribute to the inactivation of the enzyme. Moreover, very little reduction in enzyme activity was observed during incubation of the enzyme preparation without ACC (Table 2 and Fig. 3). From the above data we propose that the non-linearity is, at least in part, due to partial inactivation of the enzyme by ACC during its catalytic action.

Enzyme thermostability and temperature optima

The enzyme was stable at 0 °C for more than 24 h and lost most of its activity at 45 °C within 3 h of incubation (Fig. 4A). When the enzyme preparation was assayed at temperatures between 0 and 50 °C, ACC oxidase activity increased as a function of assay temperature from 0 to 30 °C and then started to decline (Fig. 4B). The optimum assay temperature for the *in vitro* ACC oxidase activity was achieved at 30 °C, which is similar to the optimum temperature reported for the *in vivo* assay of the enzyme[30]. Our observation is in agreement with that of McGarvey and Christoffersen[19], who, using extracts from avocado fruit observed maximum ACC oxidase activity at 25-30 °C, and the activity dropped to half maximal at 15 °C and 40 °C. The thermostability data indicate that the enzyme is not stable long above its optimum temperature.

Effects of metal ions, alkylating agents and wounding

It is well documented that cobalt ions inhibit the oxidative deamination of ACC to ethylene in *in vivo* systems[12,29] and in *in vitro* systems[14,26]. We confirmed that Co^{2+} at 50 μM concentration inhibited *in vitro* ACC oxidase activity by 86 % (Table 3). This inhibition of ACC oxidase activity by Co^{2+} supports the view that the *in vitro* ACC oxidase activity thus demonstrated indeed represents the native ACC oxidase that functions in the tissue. Besides Co^{2+}, ACC oxidase activity was effectively inhibited by Ag^{+}, Cu^{2+} and Zn^{2+} whereas, Ba^{2+}, Ca^{2+}, Li^{+} and Mg^{2+} had little effect. It has been proposed that these inhibitory metals may act by replacing Fe^{2+} and forming inactive enzyme-metal complexes and that the metals which abolish almost completely ACC oxidase activity may associate with greater affinity than Fe^{2+} [19]. Similar observations have been reported for *in vitro* ACC oxidase from apple[15], avocado[19] and melon[24] fruits.

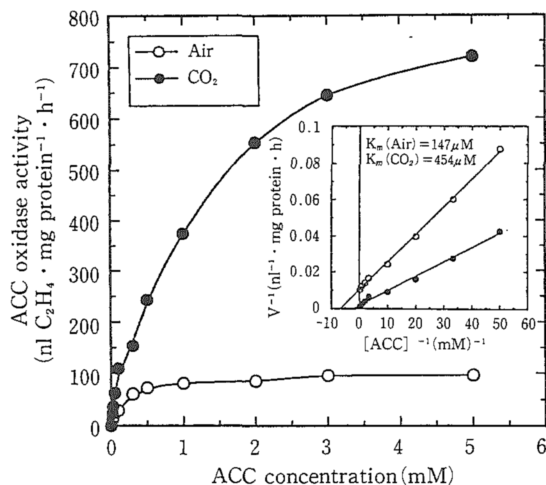

Fig. 5 Dependence of the ACC oxidase activity on ACC concentration. Inset is Lineweaver-Burk double reciprocal plot showing apparent K_m values of 147 μM in air and 454 μM in the presence of 5 % CO_2.

In vivo studies with metal ions have suggested that ACC oxidase requires free sulfhydryl groups for its activity[29]. Therefore, if free sulfhydryl groups are indeed required, it is anticipated that treatment of the enzyme preparation with alkylating agents which block the sulfhydryl

groups should inhibit *in vitro* ACC oxidase activity. Indeed, $IAcNH_2$ and IHAc at 20 mM inhibited *in vitro* ACC oxidase activity by 50 % and 99.3 % respectively (Table 4). This indicates that *in vitro* ACC oxidase like *in vivo* ACC oxidase requires free sulfhydryl groups for its activity. Iodoacetic acid was however, more effective in reducing ACC oxidase activity than $IAcNH_2$. This suggests that the target sulfhydryl group is not near a negatively charged residue that reduces by charge repulsion the rate of reaction between free sulfhydryl groups and IHAc[8]. Sulfhydryl reagents *p*-chloromercuriphenylsulphonate and N-ethylmaleimide[24] and 5, 5̃-dithiobis-(nitrobenzoate)[15] have been shown to inhibit *in vitro* ACC oxidase activity from melon and apple fruits respectively, suggesting a role for sulfhydryl groups. Based on the amino acid sequence for ACC oxidase recently reported by Dong et al.[7], the alkylation data indicate that the reactive center for ACC oxidase includes cysteine that is required for activity.

Wounding induces ethylene production in winter squash mesocarp tissue[11]. Upon excision of the mesocarp tissue, the extractable *in vitro* ACC oxidase activity increased in parallel with increase in *in vivo* ACC oxidase activity (Table 5), confirming further that the solubilized enzyme indeed represents the physiological ACC oxidase. Carbon dioxide however, had little effect on *in vivo* ACC oxidase activity suggesting that such levels already existed in the tissue, presumably due to the fact that wounding is known to cause an increase in respiration hence an accumulation of CO_2.

Enzyme kinetics

The effect of various ACC concentrations on ACC oxidase activity with Lineweaver-Burk double reciprocal plot (inset) is depicted in Fig. 5. The enzyme followed typical Michaelis-Menten kinetics and exhibited K_m values for ACC of 147μM and 454 μM in air and in the presence of 5 % CO_2 respectively. *In vivo* ACC oxidase has a high affinity for ACC although the apparent K_m value of *in vivo* ACC oxidase is difficult to determine accurately because of uncertainites regarding the compartmentation of both ACC oxidase and ACC[28]. However, for various plant tissues the K_m value for ACC based on *in vivo* studies has been estimated to be 8 μM in apple[28], 61 μM in pea leaf vacuoles[10] and as high as 200 μM in cocklebur cotyledon[21]. The value for air observed in this study is higher than 85 μM and 60 μM reported for ACC oxidase from melon fruit[23,25] and 17 μM and 6.4 μM reported for ACC oxidase from apple fruit[9,14]. This wide range is, at least in part, due to the fact that *in vitro* ACC oxidase activity and its K_m value as observed in the present study and recently reported[7,23] depends on CO_2 concentration, and therefore any observed K_m value will depend entirely on the CO_2 concentration in the reaction flask. K_m values for ACC of 175 μM and 435 μM in air and in the presence of 4.4 % CO_2 respectively, have been observed for ACC oxidase from wounded mesocarp tissue of winter squash fruit (H. Hyodo, personal communication). Despite the apparent lowering of the affinity of the enzyme towards ACC by CO_2, the rate of the reaction was greater in the presence of CO_2 than in air over the whole range of ACC concentrations tested. When this work was completed, Smith and John[23] reported that addition of HCO_3^- to the reaction mixture increased the apparent K_m value for ACC of ACC oxidase from melon fruit from 24 μM to 250 μM.

In conclusion, the enzyme preparation reported here resembles the *in vivo* system in that, it is heat denaturable, it has a high affinity for ACC (apparent K_m of 147 μM), its activity is inhibited by Co^{2+} and is activated by CO_2, it has temperature optima of 30 °C and following excision *in vitro* ACC oxidase activity increased in parallel with increase in *in vivo* ACC oxidase activity. We have further demonstrated that *in vitro* ACC oxidase activity, like the *in vivo* system, requires free sulfhydryl groups for its activity and that the non-linear time course during incubation is presumably, at least in part, due to substrate inactivation of the enzyme during its catalytic action.

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傷害処理したカボチャ果肉組織から抽出した ACC 酸化酵素の2，3の性質

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切断処理したカボチャ果肉組織から，エチレン生合成の最終段階を触媒する ACC (1-aminocyclopropane-1-carboxylic acid) 酸化酵素を抽出し，*in vitro* で，その性質を検討した.

抽出した ACC 酸化酵素は補助因子として Fe^{2+}を必要とし，その最適濃度は20μM であった. 反応液に炭酸ガス処理すると活性が顕著に増加し，ACC に対するみかけの Km 値も上昇した. 5％炭酸ガス存在下での ACC に対する Km 値は454μM であった. ACC 酸化酵素は触媒反応中に基質である ACC の存在によって部分的に不活性化された. 本酵素の活性は30℃で最大になり，それ以上の温度では顕著に低下した. Ag^{+}, Co^{2+}, Cu^{2+}及び Zn^{2+}イオンはほぼ完全に酵素を失活させた. また，アルキル化剤であるヨードアセトアミドは部分的に，ヨード酢酸は完全に酵素を失活させた. したがって，酵素活性の発現に SH 基が重要な役割を果たしていることが示唆された.

カボチャ組織の切断後，経時的に果肉サンプルを採取し，ACC 酸化酵素活性を *in vitro* と *in vivo* 両方の方法で測定したところ，両者は類似の変化様相を示した.

以上の結果から，今回 *in vitro* 抽出した酵素の性質は，従来の *in vivo* 測定により蓄積された知見とよく一致し，可溶化した酵素は実際のエチレン生合成の一環をなすものと思われた.