Phytochemistry, Vol. 22, No. 9, pp. 1903–1908, 1983.
Printed in Great Britain.

0031 9422/83 $3.00 + 0.00

AN ETHYLENE-FORMING ENZYME IN *CITRUS UNSHIU* FRUITS

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(*Revised received* 3 *February* 1983)

Key Word Index—*Citrus unshiu*; Rutaceae; 1-aminocyclopropane-1-carboxylic acid; ethylene.

Abstract—An ethylene-forming enzyme from *Citrus unshiu* fruits was purified some 630-fold. The enzyme catalysed ethylene formation from 1-aminocyclopropane-1-carboxylic acid in the presence of pyridoxal phosphate, β-indoleacetic acid, Mn^{2+} and 2,4-dichlorophenol. It behaved as a protein of MW 40 000 on Sephacryl S-200 gel filtration, and gave one band corresponding to a MW of 25 000 on SDS-PAGE. It had a specific activity of 0.025 μmol/min·mg protein. It exhibited IAA oxidase activity and had no guaiacol peroxidase or NADH oxidase activity. Its K_m for ACC was 2.8 mM, and its pH optimum was 5.7. It was inhibited by potassium cyanide *n*-propyl gallate and Tiron. D-Mannose, histidine, iodoacetate, PCMB, dimethylfuran and superoxide dismutase showed no inhibition. β-Indoleacrylic acid against IAA competitively inhibited ethylene formation. Other IAA analogues, such as β-indolepropionic acid, β-indolecarboxylic acid and β-indolebutylic acid, slightly stimulated ethylene formation. β-Indoleacrylic acid against 1-aminocyclopropane-1-carboxylic acid non-competitively inhibited ethylene formation. Ascorbate was a potent inhibitor. The inhibitory effects, however, were not always reproduced *in vivo*. It is difficult to identify this enzyme system as a natural *in vivo* system from the above observations. Nevertheless, the possible *in vivo* participation of this *in vitro* enzyme system is discussed.

INTRODUCTION

Recently, ACC was suggested independently by Lurssen *et al.* [1] and Adams and Yang [2], as a possible intermediate between methionine and ethylene. Although many investigators are interested in the enzyme system catalysing the conversion of ACC to ethylene, little research has been carried out because of difficulties in the purification of an ethylene-forming enzyme. Konze and Kwiatkowski [3] purified such an enzyme nine-fold in its specific activity using ion exchange chromatography and ammonium sulphate fractionation.

Evidence from an *in vitro* system converting ACC to ethylene suggests the involvement of an *in vivo* peroxidase system and free radical reaction. Rohwer and Mader [4] concluded that peroxidase itself does not play a major role in ethylene formation from ACC, but that it might be involved in a more complex manner by providing radicals, hydrogen peroxide, or some unknown factors. This is in accordance with the data obtained with an unidentified enzyme by Konze and Kwiatkowski [3], isoperoxidase B1 [5] and IAA oxidase [6].

In the course of the purification of chlorophyllase from an acetone powder of *Citrus unshiu* fruits [7, 8], it was found that an ethylene-forming enzyme was contained in the acetone powder extracts. In this paper, purification of the ethylene-forming enzyme, various properties of the enzyme, and the problem of the participation of the enzyme system *in vivo* are discussed.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; DIECA, diethyldithiocarbamate; PCMB, *p*-chloromercuribenzoate; DCP, 2,4-dichloropnenol; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid.

RESULTS

Purification of the ethylene-forming enzyme

The purification of the ethylene-forming enzyme from 500 g of an acetone powder of fruit peel is summarized in Table 1. After DEAE ion exchange chromatography, a pooled fraction was obtained by Sephacryl S-200 gel filtration (Fig. 1). The ethylene-forming activity corresponded to IAA oxidase and did not coincide with guaiacol peroxidase or NADH oxidase. On the other hand, another peak of IAA oxidase corresponded to guaiacol peroxidase and NADH oxidase. However, this peak never corresponded to the ethylene-forming activity.

The enzyme preparation (fraction 6, see Fig. 1) was found to be heterogeneous by PAGE at pH 8.9; there were one major and two minor protein bands. The enzyme activity however, was associated with only the major protein band. SDS-PAGE of the enzyme gave one band corresponding to a MW of 25 000 cf. 40 000 by Sephacryl S-200 gel filtration. The MW of guaiacol peroxidase was 80 000.

pH optimum

This was found to be 5.7 with phosphate buffer. The pH values for half-maximal activity were 5.3 and 6.3, respectively. At pH values above 8.0, non-enzymatic ethylene formation was observed. The optimum pH for IAA oxidase was 5.0.

Cofactor requirements

Pyridoxal phosphate, IAA, DCP and Mn^{2+} were found to be required. Hydrogen peroxide induced an early onset of ethylene formation, but further formation was strongly

Table 1. Purification of ethylene-forming enzyme from *C. unshiu* fruits

Purification step	Total activity (μmol/min)	protein (mg)	Specific activity ($10^3 \times$ μmol/min·mg protein)	Yield (%)	Purification (-fold)
Acetone powder extract	0.56	13712.5	0.04	100	1
80% ammonium sulfate	0.46	421.2	1.1	83	28
DEAE Sephadex	0.33	25.6	12.9	59	323
Sephacryl S-200	0.17	6.8	25.4	31	635

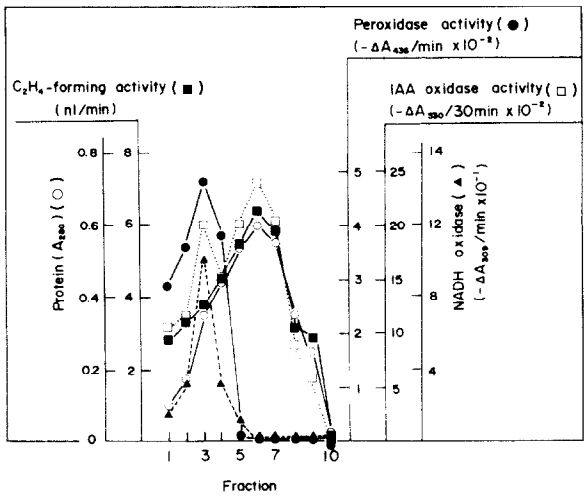

Fig. 1. Gel filtration of active DEAE fraction on Sephacryl S-200. (○) Protein; (■) ethylene forming activity; (□) IAA oxidase; (●) guaiacol peroxidase; (▲) NADH oxidase.

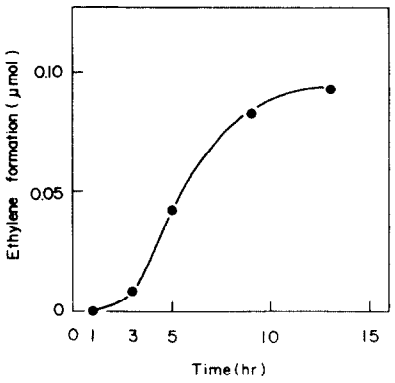

Fig. 2. Time course of ethylene formation from ACC by the ethylene-forming enzyme of *C. unshiu* fruits. The reactions were carried out under the standard conditions.

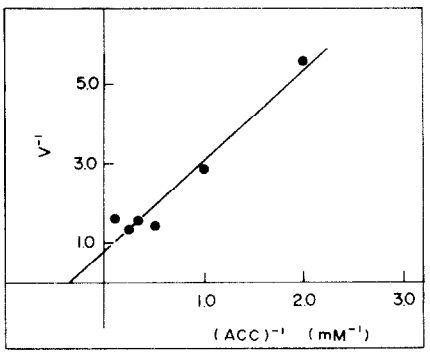

Fig. 3. Double reciprocal plots of ACC with respect to ethylene formation. The initial velocities were measured under the standard reaction conditions except that various amounts of ACC were used.

inhibited. When NADH was added in place of IAA as a hydrogen donor, ethylene formation could not be detected.

Time course

Figure 2 shows the time course of ethylene formation from ACC. Ethylene formation started after a lag of *ca* 1 hr and then increased rapidly for 3 hr and reached a plateau after 10 hr. Under the conditions of the experiment, 0.9 μmol ACC was converted into 0.08 μmol ethylene after 13 hr. The conversion efficiency was 10%.

Substrate concentration

Ethylene formation increased linearly with increasing concentration of ACC. However, high concentrations, such as 10 mM, suppressed ethylene formation. This suggests the occurrence of substrate inhibition. The K_m value, estimated from a double reciprocal plot, was 2.8 mM for ACC (Fig. 3).

Inhibitor experiments

Potassium cyanide (3 mM) completely inhibited ethylene formation. Sodium azide (3 mM) inhibited it slightly (36% of control). Iodoacetate (3 mM) and PCMB (3 mM) were without effect. Sodium ascorbate completely inhibited ethylene formation at 0.01 mM. Inhibition of 50% was obtained with 0.03 mM *n*-propyl gallate. D-Mannose (0.6 mM) and histidine (0.6 mM), scavengers of hydroxyl radicals, and dimethylfuran (1 mM), a scavenger of singlet oxygen, had no effect on ethylene formation. The formation was completely inhibited by Tiron (3 mM), a superoxide scavenger. Superoxide dismutase (500 units) showed

no inhibition. To clarify the properties of the *Citrus* enzyme as a metalloenzyme, several metal chelators (1 mM) [9] were added to the reaction mixture. Ethylene formation was strongly inhibited by the copper chelator DIECA (63% of control) but much less by EDTA (5% of control). 110-Phenanthroline inhibited the formation by 57%. Another copper chelator, cuprizone, inhibited it by 10%. Bathophenanthroline was 32% effective for inhibiting the formation.

Effect of structural and hormonal analogues of IAA

Whether or not IAA can be replaced by structural and hormonal analogues was investigated. It was found that no analogues promoted the formation of ethylene. However, the addition of β-indolepropionic acid (0.12 mM), β-indolecarboxylic acid (0.12 mM) and β-indolebutylic acid (0.12 mM) to the reaction mixture containing IAA (0.12 mM) stimulated ethylene formation for 4 hr by 208, 129 and 155%, respectively. Of its analogues β-indoleacrylic acid against IAA competitively inhibited ethylene formation (Fig. 4). On the other hand, IAA oxidation itself was competitively inhibited by four analogues (data not shown). β-Indoleacrylic acid was found to be a non-competitive inhibitor with respect to ACC, as indicated by a K_i value of 0.08 mM (Fig. 5).

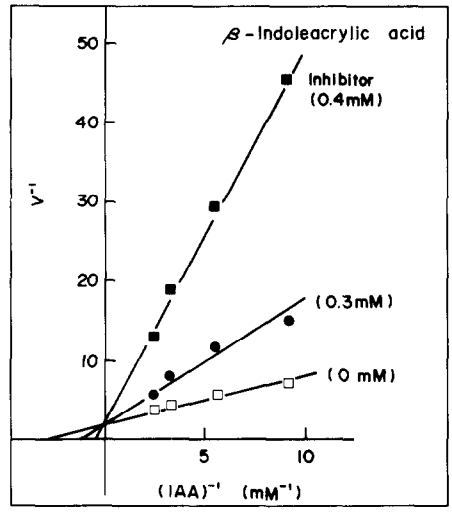

Fig. 4. Competitive inhibition of ethylene-forming enzyme by β-indoleacrylic acid against IAA. The reactions were carried out under the standard conditions except that various amounts of β-indoleacrylic acid and IAA were used.

The question of the operation of this system in vivo

Ascorbic acid is a potent inhibitor of the *in vitro* enzyme system. If this system also operates *in vivo*, ethylene formation should be inhibited by this reagent. *n*-Propyl gallate is also an inhibitor of *in vitro* ethylene formation. Whether or not the *in vitro* system of *C. unshiu* fruits operates *in vivo* can probably be determined using ascorbic acid and *n*-propyl gallate as inhibitor. *n*-Propyl gallate inhibited endogeneous ethylene production of

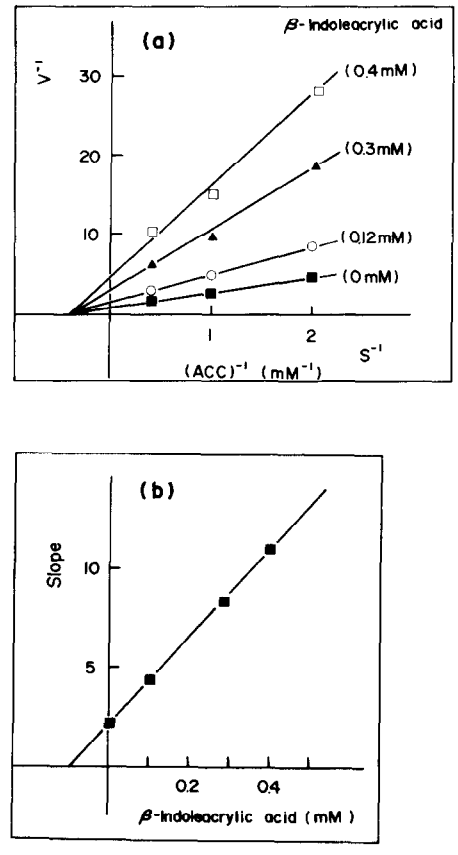

Fig. 5. Non-competitive inhibition of ethylene-forming enzyme by β-indoleacrylic acid against ACC. (a) Lineweaver–Burk plot. (b) Replot of slopes obtained from (a). The reactions were carried out under the standard conditions except that various amounts of β-indoleacrylic acid and ACC were used.

apple (68% of the control), tomato (73%) and banana peel (40%). On the other hand, ascorbic acid slightly stimulated ethylene formation from apple (120% of the control), tomato (110%) and banana peel (105%). *n*-Propyl gallate also inhibited IAA-induced ethylene production of bean sprouts (38% of the control), leak (27%), honewort leaves (52%) and radish seedlings (25%). Ascorbic acid strongly stimulated IAA-induced ethylene production of bean sprouts (150% of the control), leak (167%), honewort leaves (186%) and radish seedlings (150%).

Endogeneous ethylene production of apple tissues was not inhibited by β-indoleacrylic acid, a non-competitive inhibitor *in vitro*. Also, no inhibition with this IAA analogue was observed in IAA-induced ethylene formation of radish seedlings and honewort leaves.

Ethylene formation and ACC content in wounded peel discs from C. unshiu *fruits*

Wound-ethylene formation of cut peel discs from *C. unshiu* fruits increased gradually over a 7 hr lag phase after cutting (Fig. 6). Ascorbic acid strongly stimulated wound-ethylene formation (180% of the control). ACC concentration in the cut peel of *C. unshiu* fruits increased

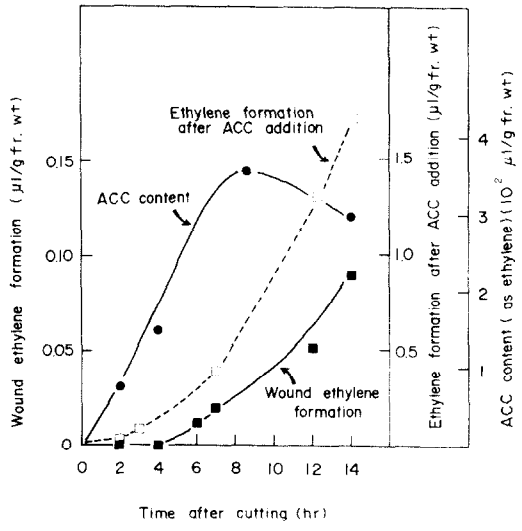

Fig. 6. Wound (endogeneous) ethylene formation by cut peel discs (■) ethylene formation after ACC addition (□) and ACC content (●) in *C. unshiu* fruits.

prior to ethylene formation (Fig. 6). Exogeneous ACC stimulated ethylene formation even when endogeneous ACC was not taken into account and endogeneous ethylene was not formed (Fig. 6).

DISCUSSION

The need for oxygen in ethylene biosynthesis led to considering the participation of some oxidase and free radicals of oxygen species. However, the data obtained did not indicate definitely the participation of known oxidases (peroxidase and IAA oxidase). Vioque *et al.* [6] reported that ethylene formation from ACC is catalysed by IAA oxidase *in vitro*. A parallel was observed by these authors between the inhibitors of both IAA oxidation and ethylene formation in the IAA oxidase system. They concluded that their IAA oxidase system is operative *in vivo* in the last step of ethylene biosynthesis. However, the actual participation of the IAA oxidase system *in vivo* should be supported with results obtained with ascorbic acid, a potent inhibitor of IAA oxidase.

For a long time, peroxidase has been investigated *in vitro* as a possible ethylene-forming enzyme. However, evidence obtained with the peroxidase system has indicated that there are great differences in the properties of the *in vivo* and *in vitro* systems. Peroxidase isoenzyme B1, purified by Machackova and Zmrhal [5], oxidized IAA in the presence of phenolic cofactor and Mn^{2+}. However, isoenzyme B1 could not catalyse the *in vitro* conversion of ACC to ethylene, even in the presence of pyridoxal phosphate and the cofactor system for IAA oxidation [5]. These authors concluded that peroxidase is not directly involved in ethylene biosynthesis. There is, however, still the possibility that it might be involved in a more complex way, such as providing radicals and other oxygen species [4].

The present enzyme preparation of *Citrus* fruits contains ethylene—forming activity and IAA oxidase activity, but not guaiacol peroxidase and NADH oxidase activity. The other enzyme fraction exhibited guaiacol peroxidase and NADH oxidase activity, and did not catalyse ethylene formation. Evidently there are two isoenzymes of IAA oxidase and only one of them stimulates ethylene production.

Ethylene formation from ACC occurred specificially in the fraction containing IAA oxidase which is known to form IAA hydroperoxide, but not O_2^- [10]. NADH oxidase which forms O_2^- does not participate in ethylene formation. These facts indicate no involvement of O_2^- in the present enzyme system. This is also supported from the results for superoxide dismutase. IAA hydroperoxide should be considered a true oxygen species in ethylene formation by the *Citrus* enzyme. Inhibition with Tiron would be due to its action as a quinone.

When the structural and hormonal analogues of IAA were added to the reaction mixture containing IAA, ethylene formation was promoted. These analogues, however, could not replace IAA. Therefore, it seems that this promotion by these analogues in the presence of IAA is a secondary effect. However, only β-indoleacrylic acid competitively inhibited ethylene formation. IAA oxidation by the *Citrus* enzyme was inhibited competitively by these IAA analogues. These results suggest that IAA oxidase does not catalyse ethylene formation. All the analogues, except β-indoleacrylic acid, could be displacing IAA from the oxidase to some site where it induces ethylene formation. β-Indoleacrylic acid may be unable to form a free radical because of its molecular structure.

Inhibition with potassium cyanide indicates the involvement of a metalloenzyme in ethylene formation. The inhibitory effects of chelating agents is in accordance with the data obtained *in vivo* by Apelbaum *et al.* [9]. They suggested that a copper enzyme, perhaps a copper peroxidase, may also be involved in the present enzyme preparation. From the results with the other inhibitors, the involvement of singlet oxygen, hydroxy radical and sulphydryl enzymes are uncertain. However, inhibition with *n*-propyl gallate suggests the participation of some free radicals. $RO_2\cdot$ and RO_2H of IAA could be postulated as candidates of the free radicals, in view of the potent inhibition with ascorbic acid.

Ascorbic acid stimulated not only endogeneous ethylene formation but also IAA-induced ethylene formation. This is an unexpected result in view of the properties of the enzyme system of *Citrus* fruits. However, ascorbic acid is expected to be involved in *in vivo* ethylene formation as a reducing agent for preserving the ethylene forming system in a reduced state, as a substrate in providing free radicals, or as a quencher of free radicals. It is known that methionine is non-enzymatically converted to ethylene in a Cu^{2+}–hydrogen peroxide–ascorbate system [11]. For the last 17 years, attention has not been directed to this model system by ethylene biologists. However, the stimulatory effects of ascorbic acid in ethylene production *in vivo* may now arouse interest in this system. Since the contradictory effects of ascorbic acid *in vivo* and *in vitro* are confusing further data are needed.

Wound-ethylene formation was detectable in the peel slices of *C. unshiu* fruits over a 7 hr lag phase after cutting. In support of this fact, the ACC level was very low or not taken into account during the lag phase. This is in agreement with the conclusion obtained by Yang [12] that ethylene is not synthesized in plants containing no ACC. However, following the addition of exogeneous ACC, ethylene formation could be detected in both the lag phase and an *in vitro* enzyme system obtained from *Citrus*

fruits containing no ACC. This suggests that the amount of ACC is the limiting factor in ethylene biosynthesis.

The following explanation is given for these results. There are two steps in ethylene biosynthesis: ACC biosynthesis and free radical generation which is independent of ethylene biosynthesis under ordinary conditions. Ethylene formation probably begins by the confluence of these two steps followed by disorganization resulting from internal and external factors, such as auxins, various chemicals, physical wounding and senescence. It may be that ethylene formation occurs through free radicals just as in the *in vitro* system. The microsomal membrane system [13], IAA oxidation system [6] and the enzyme system in this communication warrant consideration as possible sources of free radical generation.

In a recent paper, McKeon *et al.* [14] compared the stereospecificity of the conversion of 1-amino-2-ethylcyclopropanecarboxylic acid isomers to 1-butene in pea epicotyls and pea epicotyl homogenates. They concluded that the model systems catalysed the activation of oxygen to free radicals and that systems such as the *Citrus* enzyme system are of no physiological significance [15]. Indeed, the conversion of ACC to ethylene is low, as found in earlier *in vitro* studies [3, 4, 6]. Recently, Hoffman *et al.* [16] discovered *N*-malonyl-ACC as an end product of ACC in plant tissues. *N*-Malonyl-ACC formation, however, could be excluded in the *Citrus* enzyme system because this is an *in vitro* system devoid of any malonyl source. The fate of the unconsumed ACC remains obscure.

EXPERIMENTAL

Purification of ethylene forming enzyme. Fruits of *C. unshiu* were obtained from a local farm in Nov. *ca* 170 days following anthesis. The peel slices were homogenized with a blender in cold (−20°) Me_2CO for 3 min. The homogenate was allowed to stand for 6 hr at 4° in the dark, then filtered through a Buchner funnel. The residue was dried *in vacuo* and stored in a desiccator under red. pres. at −20°. The Me_2CO powder (500 g) was finely ground with a mill and extracted with 8 l. 20 mM Pi, pH 7.0 at 25° for 30 min. The extracts were filtered through cloth. The filtrate was centrifuged at 12 000 *g* for 15 min. $(NH_4)_2SO_4$ was added to the supernatant (6 l.) to give 60% satn. The ppt was removed. $(NH_4)_2SO_4$ was added to the supernatant to give 80% satn. The ppt was collected by centrifugation and dissolved in and dialysed against 50 mM Tris–HCl buffer, pH 8.0, for 24 hr with two changes of buffer. The dialysed enzyme was clarified by centrifugation (10 000 *g*, 15 min) and adsorbed onto a column of DEAE cellulose (3.2 × 40 cm) equilibrated with 50 mM Tris–HCl buffer, pH 8.0. The column was then eluted with a linear gradient of NaCl (0–0.5 M, total vol. 1 l. 50 mM Tris–HCl buffer). The active fractions (46 ml) were pooled and concd to *ca* 10 ml by ultrafiltration. The concd enzyme was dialysed against 20 mM KPi, pH 7.0. The dialysed enzyme was filtered on a column of Sephacryl S-200 (1 × 40 cm) equilibrated with the same buffer. Active fractions were stored at −20°. Fraction 6 (Fig. 1) was used as the enzyme preparation.

Ethylene forming activity. The reaction mixture contained 20 mM Pi buffer, pH 5.7, 2.0 mM ACC, 0.3 mM $MnCl_2$, 0.3 mM DCP, 0.2 mM IAA, 0.4 mM pyridoxal phosphate and enzyme (36.6 μg) in a total vol. of 5.0 ml. The reaction mixture was incubated in a 20 ml vial capped with a serum cap at 25° in the dark. C_2H_4 content of the head space of the vial was determined by according to the method of ref. [17].

Peroxidase activity. Guaiacol was used as the substrate. Its oxidation was followed spectrophotometrically at 436 nm.

IAA oxidase. IAA oxidation was determined spectrophotometrically by two methods. (1) The reaction mixture contained 20 mM KPi, pH 5.0, 3 mM IAA, 1.5 mM DCP, 1.5 mM Mn^{2+} and enzyme in a total vol. of 1.0 ml incubated at 30° for 15 min. At the end of the incubation, Salkowski reagent was added and after 30 min the *A* of the soln at 530 nm was determined spectrophotometrically. (2) IAA oxidation was followed spectrophotometrically at 247 nm. The reaction mixture contained the same constituents mentioned above. This method was used only for expts in which the effects of IAA analogues on the IAA oxidation were determined.

NADH oxidase. NADH oxidation was followed spectrophotometrically at 309 nm. The reaction mixture contained 20 mM KPi, pH 5.0 3 mM NADH, 0.5 mM DCP, 0.5 mM Mn^{2+} and enzyme in a total vol. of 3.5 ml.

PAGE, SDS-PAGE and gel filtration for MW determination. Performed by the procedure described in ref. [7].

Inhibitor experiments. Inhibitors were dissolved in 20 mM Pi buffer, pH 5.7. Final concns were as indicated in the text. After 4 hr incubation, C_2H_4 was determined.

Endogeneous and IAA-induced C_2H_4 formation of plant tissue slices. Plant materials were purchased from a local market. After slicing, the tissue slices were immersed in 5 mM ascorbate or 5 mM *n*-propyl gallate for 30 min at room temp. After removal of excess soln by filter paper, the slices were incubated in flasks at 25° in the dark. In expts on IAA-induced C_2H_4 production, the tissue slices were preincubated in an IAA soln (10 mM) for 3 hr. The slices were further immersed in ascorbate or *n*-propyl gallate soln for 30 min. The slices, after removal of excess soln, were incubated in flasks at 25° in the dark. The head spaces of these flasks were analysed by GC for C_2H_4 content.

C_2H_4 formation of wounded peel discs from C. unshiu *fruits.* Fruits of *C. unshiu* were purchased from a local market. After peeling, peel discs (*ca* 0.5 × 1 cm) were obtained by slicing. In expts on C_2H_4 formation after ACC addition, ACC soln (0.72 μmol) was dropped with a micropipette onto the discs (10 g) which were then incubated in flasks (210 ml) at 25° in the dark. The head spaces of the flasks were analysed by GC for C_2H_4 content.

Determination of ACC concn. ACC concn was determined according to the method of ref. [18].

Acknowledgement—I am very grateful to Mrs. T. Kinoshita for her excellent technical assistance.

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