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FREE RADICALS PLAY LITTLE ROLE IN THE CONVERSION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID TO ETHYLENE IN CARNATION MEMBRANE FRACTION

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The role of free radicals in the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by a membrane-bound enzyme from carnation petals was studied. The membrane preparation oxidized ACC more effectively than it oxidized cyclopropaneamine or 2-keto-4-methylthiobutyric acid (KMB). All these substrates were oxidized chemically by NaOCl to ethylene very effectively. Free radicals generated by the xanthine/xanthine oxidase system oxidized KMB far more effectively than it oxidized ACC; only 0.004% of the ACC included in the reaction mixture was oxidized in 1 h, compared with 0.9% of the KMB. Conversion of ACC to ethylene by the membrane-bound enzyme was inhibited by Co^{2+}, ATP and EDTA, while the inhibition of the oxidation of KMB by the same inhibitors was much less pronounced. These results suggest that ACC, the natural immediate precursor of ethylene, is specifically oxidized by the membrane-bound enzyme rather than through a nonspecific oxidation by free radicals.

KEY WORDS: Ethylene forming enzyme.

INTRODUCTION

Ethylene is a plant hormone which is involved in the regulation of many aspects of plant growth, development and senescence. The detailed biosynthetic pathway of ethylene in plants was established[1] and consists of the following steps:

Methionine ⟶ S-adenosylmethionine ⟶ 1-aminocyclopropane-1-carboxylic acid (ACC) ⟶ ethylene

The enzymatic activity responsible for the last step, namely the conversion of ACC to ethylene is still poorly understood. It has been characterized in many *in-vivo*, intact systems[1–4] and, also, several subcellular, isolated systems have an *in-vitro* capacity to produce ethylene from ACC[3,5–7]. However, most of the *in-vitro* systems have properties which differ, apparently, from those of the corresponding intact, *in-vivo* systems[1,3].

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A prevailing explanation for the existing differences was proposed by Yang and Hoffman[1]. They suggest that the physiological, ethylene forming enzyme (EFE), which expresses its activity only in intact cells or organelles, is catalyzing directly the conversion of ACC to ethylene. In contrast, enzymes in isolated, *in-vitro* systems are activating molecular oxygen to free radicals instead, which in turn react with ACC to produce ethylene non enzymatically. This proposition is based, essentially, on the following observations: a. Oxidants[8] and free radicals[9,10] can convert ACC to ethylene. b. Some of the *in-vitro* systems which produce ethylene from ACC also produce free radicals[11,12]. c. The conversion of ACC to ethylene is strongly inhibited by free radical scavengers[5,7,10].

Microsomal membranes from carnation petals is an *in-vitro* system which produces ethylene from ACC at a high rate and with a low Km[5,13–15]. In the present study we examined the role of free radicals in this conversion.

MATERIALS AND METHODS

Membrane fraction from petals of carnation flowers (*Dianthus caryophyllus* L. cv. White Sim) was prepared by differential centrifugation as previously described[13].

The reaction mixture for the assay of the enzyme converting ACC to ethylene consisted of 50 mM N-2-hydroxyethylpiperazine propane sulfonic acid (EPPS) pH 8.5, 5 μM $MnCl_2$, 25 μg protein and 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC), cyclopropaneamine (CA) or 2-keto-4-methylthiobutyric acid (KMB) in a total volume of 0.5 ml. The reaction was carried out in tubes of 1.7 ml capacity at 30° C for 30 min, after which the head space was sampled for ethylene by means of gas chromatography (Varian 2400).

Chemical oxidation by NaOCl of each of the three substrates was performed as described by Lizada and Yang[8], using 0.1 μM (final concentration) of ACC, CA or KMB in a total volume of 0.5 ml. Superoxide anions, H_2O_2 and hydroxyl radicals were generated using the xanthine/xanthine oxidase system, essentially as described by Diguiseppi and Fridovich[16]. The reaction mixture consisted of 50 mM sodium phosphate buffer pH 7.8, 0.1 mM xanthine, 0.012 units/ml xanthine oxidase, 0.1 mM EDTA, 50 μM $FeSO_4$ and 1 mM of ACC, CA or KMB in a total volume of 0.5 ml. The reaction conditions were as described for the enzyme activity assay except that the reaction was allowed to proceed for 15 min only.

All the chemicals were Sigma USA made and of the highest purity available.

RESULTS

The results presented in Table I confirm the findings of Lizada and Yang[8] that ACC and CA can be chemically oxidized to ethylene by NaOCl. However, in contrast to their report, the table also shows that KMB can be similarly oxidized. The overall theoretical efficiency for the oxidation of all of the three substrates was relatively high (70, 64 and 44% of ACC, CA and KMB respectively were converted to ethylene).

The xanthine/xanthine oxidase reaction might serve as a useful simulation system to test the explanation offered by Yang and Hoffman[1] for the mechanism by which enzymes convert ACC to ethylene *in-vitro*. The xanthine/xanthine oxidase reaction generates superoxide anions and H_2O_2 and in the presence of EDTA and $FeSO_4$, also

TABLE I

Substrate	Ethylene (nl)	Efficiency (%)
ACC	0.79 ± 0.06	70
CA	0.72 ± 0.03	64
KMB	0.49 ± 0.04	44

Chemical oxidation of ACC, CA and KMB to ethylene. Each substrate at the amount of 0.05 nmol was oxidized to ethylene by NaOCl according to Lizada and Yang[8]. Means of three replicates ± SE are presented

hydroxyl radicals[16]. Accordingly, the free radicals produced may react non enzymatically with ACC to form ethylene. Upon the introduction of ACC, CA or KMB to the xanthine/xanthine oxidase system, ethylene was produced from each of these substrates (Table II). However, the extent of oxidation of KMB to ethylene was much higher than that of ACC or CA (0.9% of the KMB was oxidized in comparison with only 0.004% of the ACC during the 1st hour of reaction, a difference of more than 200 fold).

Each of the three substrates, when incubated with the membrane preparation, was converted to ethylene. However, there were marked differences in the extent of conversion depending on the substrate used (Table III). The enzyme's specific activity was highest for ACC; for KMB it was 6.4 and for CA only 1.2% of the specific activity for ACC.

Cobalt ion, a known inhibitor of the *in-vivo* conversion of ACC to ethylene, inhibited the *in-vitro* conversion of both ACC and KMB to ethylene (Table IV). $CoCl_2$ at 10 μM inhibited ACC oxidation by 76% while the oxidation of KMB was inhibited only by 19%. ATP at 0.25 mM inhibited ACC conversion by 80%, and KMB conversion by 35%. EDTA at a concentration of 2 mM inhibited the oxidation of ACC by the membrane fraction almost totally (98%), while only 48% inhibition was achieved using KMB as substrate.

TABLE II

Substrate	Ethylene (nl/h)	Efficiency (%)
ACC	0.48 ± 0.20	0.004
CA	3.08 ± 0.17	0.027
KMB	99.92 ± 3.98	0.899

Xanthine/xanthine oxidase mediated conversion of ACC, CA and KMB to ethylene. The reaction mixture consisted of 50 mM NaPi pH 7.8, 0.1 mM xanthine, 0.012 units/ml xanthine oxidase, 0.1 mM EDTA, 50 μM $FeSO_4$ and 1 mM ACC, CA or KMB in a total volume of 0.5 ml. Means of four replicates ± SE are presented

TABLE III

Substrate	Ethylene (nl/mg protein/h)	Efficiency (%)
ACC	160.74 ± 12.19	0.717
CA	1.88 ± 0.23	0.008
KMB	10.25 ± 0.70	0.046

Conversion of ACC, CA and KMB to ethylene by microsomal membranes of carnation petals. The reaction mixture consisted of 50 mM EPPS pH 8.5, 5 μM $MnCl_2$, 25 μg protein and 2 mM of ACC, CA or KMB in a total volume of 0.5 ml. Means of five replicates ± SE are presented

TABLE IV

Effect of $CoCl_2$, ATP and EDTA on conversion of ACC or KMB to ethylene by membrane fraction. Two mM ACC or KMB were incubated with the standard reaction mixture for determination of enzymatic activity in the presence or absence of 10 μM $CoCl_2$, 0.25 mM ATP or 2 mM EDTA. Ethylene production was determined and inhibition was calculated. Means of three replicates ± SE are presented

Substrate	Inhibitor	Ethylene (nl/mg protein/h)	Inhibition (%)
ACC		155.58 ± 10.66	0
	$CoCl_2$ (10 μM)	37.74 ± 9.34	76
	ATP (0.25 mM)	30.47 ± 4.09	80
	EDTA (2 mM)	3.22 ± 0.25	98
KMB		13.38 ± 0.00	0
	$CoCl_2$ (10 μM)	10.84 ± 0.60	19
	ATP (0.25 mM)	8.67 ± 0.49	35
	EDTA (2 mM)	6.94 ± 0.65	48

DISCUSSION

Membrane fractions from different plant tissues have been shown to be capable of converting ACC to ethylene[5,7,10]. In all of these cases, the reaction was inhibited by free-radical scavengers. The production of superoxide by carnation and pea membrane preparations has also been reported[10,12]. These findings, together with the observation that ACC can be oxidized to ethylene by chemical means[8] including exposure to free radicals[9], led to the suggestion that in these membrane systems an enzymatic reaction produces free radicals which in turn react non enzymatically with ACC to form ethylene[1].

To test this hypothesis we have made use of three compounds which were reported before to be oxidized to ethylene: ACC, which is the natural immediate precursor of ethylene in plants[1], CA, analog of ACC, which degrades to ethylene upon the action of NaOCl[8] and KMB, a highly susceptible molecule to free radical attack, which results in ethylene production[16]. These compounds were incorporated into three oxidizing systems: NaOCl reagent which reacts with cyclopropylamines and degrades them to ethylene[8], xanthine/xanthine oxidase which produce H_2O_2, superoxide and hydroxyl radicals[16,17] and microsomal membranes of carnation petals which are capable of producing ethylene from ACC[5,13–15], and also superoxide radicals[12].

When each of the substrates ACC, CA and KMB was incubated with the free radical — generating system of xanthine/xanthine oxidase, it was oxidized to ethylene, with KMB being by far the most susceptible to free radical attack (Table II). Only 0.004% of the ACC is oxidized to ethylene in this system while the efficiency of KMB oxidation is 0.9%. The low values measured in our study are in agreement with those reported elsewhere for ACC[9] and KMB[16].

Another free-radical generating system capable of converting ACC to ethylene was recently described[11,18]; lipoxygenase, using linoleic or linolenic acid as substrates, released ethylene in the presence of ACC. However, the estimated conversion efficiency in that system was very low, comparable with that of the xanthine/xanthine oxidase system.

From our results it thus appears that KMB is more susceptible to free radical attack than the other substrates studied. If free radical attack is indeed the mechanism by

which ACC is converted to ethylene by membranes, then KMB when added to a membrane preparation should yield the highest ethylene production values. Our results, however, do not support this expectation. There are marked differences in the extent to which the membrane system converted each of the three substrates to ethylene (Table III): the rate of conversion for ACC was about 200 fold higher than those for KMB and CA. It is interesting to compare our findings with those on the pea homogenate, one of the most extensively studied *in-vitro* systems[3,6]. In the latter system KMB and ACC are oxidized equally and at low efficiency compared with the membrane system used in this study. Since KMB is relatively easily oxidized to ethylene by free radicals (Table II), the conversion of KMB by membranes might reflect the non-specific oxidation mediated by free radicals generated in the membrane fraction[12].

Following incubation in a strong chemical oxidation system, each of the three substrates was oxidized to about the same extent and with higher efficiency than in the xanthine/xanthine oxidase system (Table I). These findings support, in part, the results of Lizada and Yang[8]. However, in our experiments KMB also produced ethylene, not supporting the claims for the high specificity of this reaction toward substituted cyclopropylamines[8].

If both KMB and ACC are oxidized non enzymatically by free radicals generated by the membrane fraction, they should be inhibited equally by different inhibitors. However, on the introduction of three different inhibitors into the membrane system, each of them inhibited the oxidation of ACC more effectively than that of KMB (Table IV). Co^{2+}, which is known to be a very potent inhibitor of the EFE in plants[1,4], inhibited the conversion of ACC to ethylene by the membrane fraction but was much less effective in inhibiting the conversion of KMB. Similar trends were observed with ATP and EDTA, both of which inhibit the conversion reaction of ACC in membrane preparations as was shown before[7,15], and to a lesser degree, the conversion of KMB.

Our main findings can thus be summarized as follows: a. KMB is oxidized much more effectively than ACC by free radicals; b. The efficiency of ACC conversion by free radicals generated by xanthine oxidase is extremely low; and c. ACC is oxidized much more effectively than KMB by the membrane preparation. These findings suggest that in carnation petals, ACC is specifically oxidized by an enzyme associated with the membrane fraction rather than through a non specific oxidation by free radicals.

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