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ETHYLENE FORMATION FROM α-KETO-γ-METHYLTHIOBUTYRATE BY TOMATO FRUIT EXTRACTS

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Abstract—Cell-free extracts prepared from tomato fruits (*Lycopersicon esculentum* Mill.) were not capable of catalyzing ethylene production from α-keto-γ-methylthiobutyrate, in the presence of Mn^{2+}, sulfite, and phenol, until an endogenous heat-stable and dializable inhibitor was removed. After its removal, the enzyme-catalyzed formation of ethylene was readily demonstrated. Many of the properties of this tomato enzyme system relevant to ethylene production were found to be similar to those of the horse-radish peroxidase system.

INTRODUCTION

ALTHOUGH enzymic formation of ethylene from methional has been demonstrated with extracts of cauliflower[1-2] and pea seedling,[3] some investigators have failed to show enzymic formation of ethylene by extracts from fruits.[1,4] It was assumed that the ethylene-producing mechanism was so labile that ethylene production by fruit tissue ceased when the cells were disintegrated. Recently, Yang has shown that ethylene is formed rapidly from methional[5] or from α-keto-γ-methylthiobutyrate[6] by horse-radish peroxidase in the presence of Mn^{2+}, sulfite, oxygen, and certain phenols.

We had found that a homogenate of tomato fruit did not catalyze the production of ethylene from α-keto-γ-methylthiobutyrate. However, when the supernatant of the tomato extract is fractionated with ammonium sulfate and dialyzed, enzymic production of ethylene is readily demonstrated, suggesting that some inhibitors are present in the crude extract. This paper describes the properties of the tomato enzyme and discusses the nature of the endogenous inhibitors.

RESULTS

Table 1 summarizes the results of purification on the enzyme activities in terms of *o*-dianisidine peroxidation and ethylene production. Although peroxidase activity was observed, and most of this activity was recovered through the stages of purification, there was no ethylene-producing activity in the crude homogenate. However, after the extract was fractionated with ammonium sulfate and extensively dialyzed, ethylene production was observed. Results similar to the dialysis were obtained by passing the homogenate through a Sephadex column.

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TABLE 1. SUMMARY OF THE PURIFICATION OF TOMATO ENZYME

Stage of purification	Protein (mg)	Total enzymic activity*		Specific activity	
		o-Dianisidine peroxidation (10^{-2} × units)	Ethylene production (μl/10 min)	*o*-Dianisidine peroxidation (unit/mg protein)	Ethylene production (μl/10 min/mg protein)
Homogenate	13·0	3·4	0	26	0
$(NH_4)_2SO_4$, 0 to 90% saturation	12·5	3·3	33	27	2·6
DEAE Fraction I	2·6	3·1	340	120	130
DEAE Fraction II	3·4	0·2	12	6	3·5

* The reaction mixture for assay of peroxidase activity consisted of 0·1 μmole of *o*-dianisidine, 3 μmoles of H_2O_2, 0·1 ml of diluted enzyme solution (0·01 to 1μg protein), and 30 μmoles of potassium phosphate buffer (pH 6·0) in a total volume of 3 ml. Incubation was at 25°. The reaction mixture for measurement of ethylene formation consisted of 0·5 μmole of α-keto-γ-methylthiobutyrate, 0·1 μmole of $MnSO_4$, 50 mμmoles of phenol, 2 μmoles of $NaHSO_3$, 50 mμmoles of EDTA, 50 μmoles of potassium phosphate (pH 7·8), and 0·05–0·5 ml enzyme solution (0·1–0·4 mg protein) in a total volume of 1 ml. Incubation was at 30°.

If the absence of ethylene-producing activity in crude fruit homogenate is due to the presence of inhibitor(s), one might expect that ethylene production by a pure peroxidase system would be inhibited by the fruit preparation. This possibility was tested by adding boiled tomato fruit homogenate to the horse-radish peroxidase system.[5] As shown in Table 2, the inhibitor was present in all stages of tomato fruit ripening; boiled homogenate, derived from

TABLE 2. EFFECT OF BOILED TOMATO FRUIT HOMOGENATE ON ETHYLENE PRODUCTION BY A HORSE-RADISH PEROXIDASE SYSTEM*

Nature of homogenate addition	Amount added† (mg)	Relative ethylene production
None	0	100
Preclimacteric fruit		
untreated	50	0
untreated	5	63
carbon-treated	50	110
dialysate	50	0
Climacteric fruit		
untreated	50	0
untreated	5	15
carbon-treated	50	107
dialysate	50	0
Post-climacteric fruit		
untreated	50	0
untreated	5	21
carbon-treated	50	120
dialysate	50	0

* The reaction mixtures are as in Table 1, except that 2 μg of horse-radish peroxidase (Sigma Co., type II, R.Z. 1·4) were substituted for tomato enzyme.

† The amount of boiled homogenate added was equivalent to the stated weights of fresh tomato fruit tissue.

50 mg of fresh tissue inhibited ethylene production completely, while that derived from 5 mg inhibited production by one-third to two-thirds, depending on the tissue from which the boiled homogenate was made. On the other hand, homogenates treated with activated carbon or by dialysis were without effect, showing that an inhibiting material was removed.

In the ethylene-producing system with horse-radish peroxidase,[5] monophenols and resorcinol were activators while *o*-dihydric phenols were very potent inhibitors. Similar

TABLE 3. EFFECT OF VARIOUS PHENOLIC COMPOUNDS ON ETHYLENE PRODUCTION*

Test substance	Ethylene production (mμl/10 min)
None	290
Phenol	790
Resorcinol	800
Tyrosine	575
p-Coumarate	825
Hydroquinone	210
3,4-Dihydroxyphenylalanine	20
Catechol	15
Caffeic acid	11
Chlorogenic acid	11
Quercetin	20

* The reaction mixtures contained, in a total volume of 1 ml, 0·5 μmole α-keto-γ-methylthiobutyrate as substrate, 0·1 μmole $MnSO_4$, 50 mμmoles EDTA, 2 μmoles $NaHSO_3$, 0·2 ml enzyme solution, 50 μmoles potassium phosphate (pH 7·8), and various phenolic compounds (50 mμmoles). Incubation at 30°.

TABLE 4. COFACTOR REQUIREMENT FOR ETHYLENE PRODUCTION*

Components	Ethylene production (mμl/10 min)
Complete*	790
Omit Mn^{2+}	350
Omit resorcinol	280
Omit sulfite	41
Omit substrate	0
Complete, but in N_2	33
Complete, but with boiled enzyme	5

* The reaction mixture and conditions are as described in Table 3 except that 50 mμmoles of resorcinol are used as the phenolic substance.

results are found in this tomato enzyme system. As shown in Table 3, resorcinol, phenol, and *p*-coumarate stimulate ethylene production by about three times, while catechol, 3,4-dihydroxyphenylalanine (dopa), caffeic acid, chlorogenic acid, and quercetin at concentrations of 50 μM inhibited ethylene production almost completely. Table 4 shows the cofactor requirements for this system. There is a definite requirement for enzyme, phenol, Mn^{2+}, sulfite ion, and oxygen. The Mn^{2+} requirement could be partially substituted by Co^{2+}, but not by other divalent cations such as Mg^{2+}, Fe^{2+}, and Ca^{2+}. In N_2, ethylene production is only 5 per cent of that in air. The optimum pH for enzyme activity under these assay conditions was about 7·8.

Figure 1 illustrates the rate of ethylene formation as a function of time and enzyme concentration. Ethylene production was linear with time to about 10 min for enzyme concentrations up to 0.05 ml containing 0·4 mg of protein per reaction mixture but, at higher enzyme concentrations, the reaction rate was no longer proportional to the enzyme concentration. It may also be noted in Fig. 1 that, when the enzyme preparation was boiled, ethylene pro-

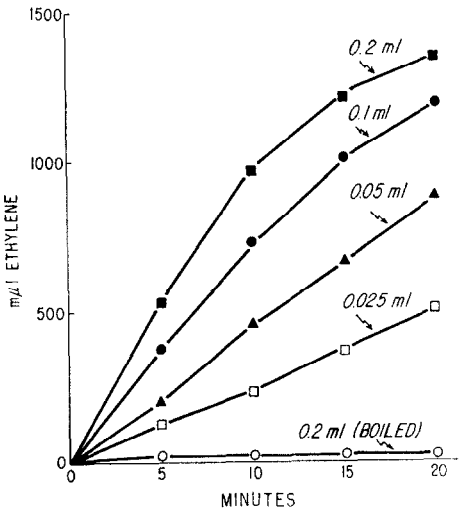

FIG. 1. TIME COURSE OF ETHYLENE FORMATION BY TOMATO ENZYME.
The reaction mixtures and conditions are as described in Table 3, except that various amounts of enzyme solution are used as indicated.

TABLE 5. EFFECT OF VARIOUS INHIBITORS ON ETHYLENE PRODUCTION*

Addition	Ethylene production (mμl/10 min)
None	800
Catalase (400 units)	585
Diethyldithiocarbamate (1 μmole)	24
Diethyldithiocarbamate (0·1 μmole)	480
KCN (1 μmole)	40
KCN (0·1 μmole)	200
Cu^{2+} (0·4 μmole)	458
Cu^{2+} (0·4 μmole, without EDTA)	120

* The reaction mixtures are as in Table 3, except that various inhibitors are used where indicated.

duction was greatly reduced, indicating that the reaction was indeed enzymatic. The effect of various inhibitors on ethylene production is shown in Table 5. Catalase (400 units per flask) inhibits ethylene production about 30 per cent, and diethyldithiocarbamate, KCN, and Cu^{2+} were also found to be very potent inhibitors. At a concentration of 1 mM, each inhibited ethylene production more than 90 per cent.

Various possible substrates for ethylene production were tested with this system. Only α-keto-γ-methylthiobutyrate and β-methylthiopropionaldehyde (methional) were found to

be active. Although methionine and ethionine are active substrates for ethylene production in the FMN-light[7] and Cu^{2+}-ascorbate[8] model systems, they were not active in this enzyme system. The following compounds were also tested and found to be inactive: DL-methionine sulfoxide, DL-methionine sulfone, DL-homocysteine, DL-homocystine, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, β-alanine, DL-homoserine, fumarate, and acrylate.

TABLE 6. ETHYLENE PRODUCTION FROM VARIOUS SUBSTRATES*

Substrate	Ethylene production (mμl/15 min)	
	With enzyme	With boiled enzyme
DL-Methionine	15	15
α-Keto-γ-methylthiobutyrate	990	5·5
β-Methylthiopropionaldehyde (methional)	1060	10
DL-Ethionine	30	30

* Reaction mixtures and conditions are as described in Table 3 except that 0·5 μmole of various substrates as listed are substituted for α-keto-γ-methylthiobutyrate.

DISCUSSION

Processes for the isolation of enzymes from tissues inevitably require disruption of the cells, causing mixing of enzymes and other substances that could be rigidly compartmentalized in the living cells. As a result, a potential enzyme activity in the homogenate could be inhibited due to the mixing of enzyme with inhibitors. In this work, we have shown that previous failures to demonstrate the enzymic formation of ethylene with tomato extracts were due to the presence of endogenous inhibitors in the crude extracts, rather than to the lability of the enzyme itself. Just as in the horse-radish peroxidase system,[5] *o*-diphenols are potent inhibitors of ethylene production by the tomato enzyme extract (Table 3). Phenolic compounds are known to be widely distributed in fruit tissues,[9–11] so the heat-stable and dialyzable inhibitors we observed could be polyphenolic substances, such as quercetin and caffeic acid derivatives, which have been identified in tomatoes.[11–12] A more definite conclusion as to the nature of the endogenous inhibitors awaits further purification and characterization.

The cofactors required in the tomato system (Tables 3 and 4) were essentially those of the horse-radish peroxidase system,[5] and both systems are inhibited by *o*-dihydric phenols (Table 3), KCN, Cu^{2+}, and diethyldithiocarbamate (Table 5). Furthermore, in this tomato enzyme system, the ethylene-producing enzyme migrates concomitantly with peroxidase on a DEAE-cellulose column, as assayed by the peroxidation of *o*-dianisidine[13] (Table 1). Therefore we suggest that the ethylene-producing enzyme in this tomato system may be a peroxidase.

Other enzyme systems catalyzing ethylene production from methional have been extracted from cauliflower florets[1] and pea seedlings.[3] These systems require both enzyme and cofactor

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fractions.[1,6] Ethylene formation from methional by peroxidase was first described by Yang[5,6] in 1967. The system comprises pure horse-radish preoxidase, sulfite, monophenol, Mn^{2+}, and oxygen. Hydrogen peroxide can replace Mn^{2+} and oxygen. The resemblance of this horse-radish system to the cauliflower and pea seedling systems led him to speculate that both the cauliflower and pea seedling systems may be peroxidases, and the cofactors may be a monophenol and sulfite ion or compounds related to them. Since then, Mapson and his coworkers[2,14] have re-examined and further characterized their cauliflower enzyme system and have found that their enzyme may in fact be a peroxidase. It is interesting to note that the cofactors isolated from the cauliflower systems are monophenol and methanesulfinic acid; sulfite is effective in replacing the methanesulfinic acid.

Whether ethylene production from α-keto-γ-methylthiobutyrate, which could be derived from methionine by transamination, is the physiological process *in vivo* has yet to be established. The findings that methionine stimulates ethylene production and is readily converted to ethylene in fruit[15–16] and vegetative[16] tissues strongly support this pathway.

EXPERIMENTAL

All procedures were carried out at 0–4°. Mature green tomato fruits (*Lycopersicon esculentum* Mill., cv. C65 T205) were cut into sections, and the seeds and highly acid locular contents were removed. 100 g of fruit wall tissue were then homogenized in a Waring Blendor with 25 ml of 0·1 M K_2HPO_4 containing 2 mM EDTA, squeezed through cheesecloth, and centrifuged at 30,000 × *g* for 20 min. The supernatant was brought to 90 per cent saturation with $(NH_4)_2SO_4$. The protein was collected by centrifugation, redissolved in 20 ml of 50 mM Tris-HCl buffer solution (pH 7·6), and dialyzed against 5 mM Tris-HCl (pH 8·0) for 18 hr with five changes of buffer. Any precipitate formed during the dialysis was removed by centrifugation, and the resulting supernatant was then fractionated with a DEAE-cellulose column (1·5 × 20 cm) previously equilibrated with 5 mM Tris-HCl buffer at pH 8·0. The proteins were eluted successively with 30-ml portions of 5 mM Tris-HCl (pH 8·0) containing the following sequence of concentrations of NaCl: 0, 0.025, 0·05, 0·075, 0·1, 0·5, and 1 M. The effluent was collected in 10-ml fractions and dialyzed against 5 mM Tris-HCl at pH 8·0. The protein fraction eluted by 0·075 M NaCl (Fraction I) showed the highest activity in ethylene production. Another peak was found in the fraction eluted with 0·5 M NaCl (Fraction II), and both fractions showed peroxidase activity. Protein content was determined by the method of Lowry *et al.*[17]

Assay of Enzyme Activity

Peroxidase activity was measured by following the change in absorbance at 460 nm due to the oxidation of *o*-dianisidine in the presence of H_2O_2.[13] One unit of peroxidase activity is that amount of enzyme decomposing 1 μmole of peroxide per min at 25°, assuming that the molar absorbancy of oxidized *o*-dianisidine is equal to $1{\cdot}13 \times 10^4$ cm^{-1} at 460 nm. To test the ability of the enzyme to catalyze ethylene production, the following incubation mixture was used: 0·5 μmole of substrate, 0·1 μmole of $MnSO_4$, 50 mμmoles of phenol, 2 μmoles of $NaHSO_3$, 50 mμmoles of EDTA, 50 μmoles of potassium phosphate (pH 7·8), and a suitable amount of enzyme solution (0·1 to 0·4 mg protein) in a total volume of 1 ml in a 25 ml Erlenmeyer flask fitted with a rubber serum cap. The reaction was started by injecting a mixture of enzyme-Mn^{2+} solution with a hypodermic syringe, and the flasks were incubated in a shaker at 30°. The atmosphere of each flask was sampled with a hypodermic syringe, and ethylene was determined by gas chromatography, as previously described.[7] Except as otherwise indicated, the enzyme source used was DEAE Fraction I.

Substrates

α-Keto-γ-methylthiobutyrate was prepared from L-methionine by L-amino acid oxidase, as described by Meister.[18] Methional (β-methylthiopropionaldehyde) was prepared by the method of Pierson *et al.*[19]

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Demonstration of Inhibitors

Three stages of fruit ripeness were tested for the presence of enzyme inhibitors by preparing "boiled homogenates". The fruit tissue (100 g) was placed in 100 ml of water, brought to boiling and held for 15 min, and then homogenized in a Waring Blendor at high speed for 2 min. After centrifugation at 30,000 × *g* for 20 min, the supernatant was used in proportional amounts which would be equivalent to the stated original fresh weight of tomato tissue (Table 2). The inhibiting substances could be removed from these extracts by dialysis or by treatment with activated carbon. Dialyzed homogenate was prepared by dialysis of 50 ml of boiled homogenate against 50 ml of water for 1–2 hr. Carbon-treated homogenate was prepared by mixing 0·5 g of activated carbon with 50 ml of boiled homogenate, stirring for 0·5 hr at 25°, and centrifuging; the supernatant was used.

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