Ethylene Formation from Acrylic Acid by a Banana Pulp Extract†

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An enzyme extract from ripe banana pulp catalyzed the formation of ethylene in the presence of Mg^{2+} , TPP and acrylic acid under the gas phase of argon. Reaction conditions, cofactor requirement and optimum pH are presented.

Ethylene formation from acrylic acid catalyzed by TPP at pH 9.0 or by yeast decarboxylase was also demonstrated.

The following route was proposed as a biosynthetic pathway of ethylene in banana fruit;

In previous reports,^{1,2)} a pathway of ethylene formation from acetate-2-¹⁴C and propionate-2-¹⁴C in banana pulp slices and homogenates has been proposed.

However, direct proof on the final step of the proposed pathway—decarboxylation of acrylic acid—has not been obtained. The present paper describes the formation of ethylene from acrylic acid catalyzed by an enzyme extract obtained from banana pulp, non-enzymatic production of ethylene catalyzed by TPP and the evolution of ethylene catalyzed by yeast pyruvate decarboxylase.

MATERIALS AND METHODS

Materials. Unripe (green) banana fruits (*Musa*

sapientum L.) were obtained from Kyoka Co., Ltd. at the Central Market in Kyoto City. They were ripened by keeping them in desiccator at 20°C. Pulp of yellow banana (at the climacteric rise) was used as a material. Pyruvate decarboxylase was obtained from Boehringer, Mannheim, Germany. Acrylic acid was purchased from Nakarai Chemicals Co., Kyoto.

Assay conditions. Protein was determined by the method of Warburg and Christian.³⁾ Ethylene was determined by gas chromatography as shown in the previous report.¹⁾

Preparation of enzyme extract. Pulp of banana, yellow in their peel color, was cooled at 5°C for 2~3 hr, then ground in ice-cold 0.1M potassium phosphate buffer, pH 7.0 in a mortar. The homogenates were squeezed through gauze and centrifuged at 100,000 g, for 20 min, at 5°C. Table I shows the distribution of activity of ethylene formation from acrylic acid in the homogenate, supernatant and sediment. To the supernatant was added solid ammonium sulphate (390 mg/ml, 60%) and adjusted to pH 7.0. The protein precipitated was collected by centrifugation and dis-

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1) K. Shimokawa and Z. Kasai, *Agr. Biol. Chem.*, **34**, 1633 (1970).

2) K. Shimokawa and Z. Kasai, *ibid.*, **34**, 1640 (1970).

Abbreviation used in this paper: TPP, thiamine pyrophosphate.

3) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).

TABLE I. DISTRIBUTION OF ETHYLENE-FORMING ACTIVITY IN HOMOGENATES, SUPERNATANT AND SEDIMENTS

Fraction	C ₂ H ₄ (m μ l)	Specific activity (m μ l/mg protein)
Homogenates	122.4	5.05
Supernatant	72.0	4.50
Sediments	26.4	2.54

The reaction mixture contained 3 ml of either homogenates, supernatant or sediments (4.84, 3.20 and 2.08 mg protein per ml, respectively), 400 μ moles of potassium phosphate buffer, pH 7.0, 3 μ moles of acrylic acid, 100 μ moles of MgSO₄, 10 μ moles of TPP, in a total volume of 4 ml. Incubation time, 60 min; Incubation temp., 30°C; Gas phase: argon.

solved in 0.1 M potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer for 24 hr, with three changes of the buffer. Any precipitate formed during the dialysis was removed by centrifugation and the resulting supernatant was used as the enzyme extract.

Assay of enzyme activity. To test the activity of the enzyme extract to catalyze ethylene production, the following reaction mixture was used: 3 μ l (36 m μ moles) of acrylic acid, 100 μ moles of MgSO₄, 10 μ moles of thiamine pyrophosphate, 400 μ moles of potassium phosphate buffer, pH 7.0, and a suitable amount of the enzyme extract (4.6 mg protein/ml) in a total volume of 4 ml. The reaction was carried out in a 50 ml Erlenmeyer flask at 30°C. The reaction was started by injecting acrylic acid with microsyringe. Because of the instability of aqueous solution of acrylic acid, it was added directly without dilution. The flask was flowed by argon or by air, and was bubbled first in 20% KOH and secondly in 0.25 M mercuric perchlorate. After the incubation, the mercuric perchlorate adsorbed ethylene was transferred into the sealed flask, and ethylene was released by adding of 4 M lithium chloride. The atmosphere containing ethylene in each flask was sampled with a hypodermic syringe, and ethylene was determined by gas chromatography under the same conditions described previously.¹⁾

RESULTS

Identification of ethylene

After the incubation in the presence of

FIG. 1. Identification of Ethylene by Gas Chromatography.

1-a. Gas chromatogram of standard acetylene, ethylene and ethane.

1-b. Gas chromatogram of gas sample obtained from reaction mixture in the presence of Mg²⁺, TPP, acrylic acid and the enzyme extract.

1-c. Gas chromatogram after absorbent-treatment of the mixture containing acetylene and the gas sample used in 1-b.

Gas chromatography was carried out as described previously.¹⁾

Mg²⁺, TPP, the enzyme extract and acrylic acid as a substrate, the gas sample of the reaction vessel was analyzed by gas chromatography. As shown in Fig. 1, ethylene was identified by comparison of the retention time with that of authentic ethylene and by co-chromatography with the standard ethylene (99.99%). This was also supported from the evidence that the gas in the reaction vessel was absorbed by the specific absorbent (Absorite-PM, Tokyo Kasei Co, Ltd.) for unsaturated hydrocarbons (Fig. 1-c). Any other hydrocarbons was not recognized in the space of the reaction vessel.

Co-factor requirement for ethylene formation from acrylic acid

For the ethylene formation from acrylic acid by the enzyme extract, the presence of TPP and gas phase of argon were required (Table II). When the complete system was incubated under air or oxygen, the formation of ethylene was not observed. Ethylene for-

TABLE II. CO-FACTOR REQUIREMENT FOR ETHYLENE FORMATION FROM ACRYLIC ACID BY THE ENZYME EXTRACT

Gas phase	System	Ethylene (m μ l)
Argon	Complete	47.5
	minus Mg ²⁺	37.5
	minus TPP	7.6
	minus Mg ²⁺ and TPP	5.0
	minus acrylic acid	3.5
	with boiled enzyme extract	<1.0
Oxygen	Complete	<1.0
Air	Complete	<1.0

The complete system contained 3 ml of the enzyme extract, 400 μ moles of potassium phosphate buffer, pH 7.0, 3 μ l of acrylic acid, 100 μ moles of MgSO₄, 10 μ moles of TPP in a total volume of 4 ml. Incubation time; 10 min. Incubation temp; 30°C.

mation was not detected in the case of the boiled enzyme extract, even in the presence of acrylic acid, Mg²⁺ and TPP. This fact indicates that the formation of ethylene from acrylic acid proceeds enzymatically. Requirement for an argon gas phase might be due to the protection of acrylic acid from its oxidation and polymerization.

Effect of pH

The optimum pH for the formation of acrylic acid from acrylic acid by the enzyme extract was 7.0 (Fig. 2).

Time course of ethylene formation

The relationship between ethylene formation and time was determined using the standard conditions described previously. Ethylene formation increased with time for 10 min, and after that the rate decreased. Ethylene was reproduced, however, by the further addition of acrylic acid, as shown in Fig. 3.

Effect of enzyme concentration

Figure 4 shows an observation indicating a

FIG. 2. Effect of pH on Ethylene Formation from Acrylic Acid by the Enzyme Extract.

The reaction mixture contained 3 ml (14 mg protein) of the enzyme extract, 400 μ moles of potassium phosphate buffer (pH as indicated), 3 μ moles of acrylic acid, 100 μ moles of MgSO₄, 10 μ moles of TPP in a total volume of 4 ml. Incubation time; 10 min. Incubation temp; 30°C.

FIG. 3. Time Course of Ethylene Formation from Acrylic Acid by the Enzyme Extract.

The assay conditions were described in Materials and Methods.

Arrows indicate that acrylic acid (3 μ l) was supplied in the reaction mixture at this time.

FIG. 4. Effect of the Enzyme Extract Concentration on Ethylene Formation from Acrylic Acid.

The assay conditions were described in Materials and Methods. One ml of the enzyme extract contained 4.6 mg protein. Reaction time; 10 min.

FIG. 5. Effect of Bovine Serum Albumin on Ethylene Formation from Acrylic Acid in Enzyme Extract.

The assay conditions were the same as that described in Materials and Methods, except the addition of 4 mg bovine serum albumin.

Incubation time: 10 min.

lack of linearity between the rate of formation and the enzyme concentration. This fact suggests that there was a damage of the enzyme protein by acrylic acid, for example, as observed

by Cavins,⁴⁾ through the reaction with the SH-group of the enzyme. This was supported by the observation that the linearity was obtained when bovine serum albumin was added to the reaction mixture (Fig. 5).

Effect of acrylic acid concentration

Ethylene formation increased with increasing concentration of acrylic acid up to 3 μl in the reaction mixture (Fig. 6). High concentration

FIG. 6. Effect of Substrate Concentration on Ethylene Formation in the Enzyme Extract.

The assay conditions were described in Materials and Methods. The enzyme extract in the reaction mixture contained 12 mg protein.

of acrylic acid caused reduction in rate of ethylene formation. Approximately K_m for acrylic acid is $7 \times 10^{-3} M$. The failure⁵⁾ to enhance ethylene formation with acrylic acid, may result from the toxicity for the enzyme protein at high concentration.

Non-enzymatic formation of ethylene from acrylic acid in the presence of Mg^{2+} and TPP

As described above, it is possible that ethylene was formed by decarboxylation of acrylic acid. The occurrence of non-enzymatic

4) J. F. Cavins and M. Friedman, *J. Biol. Chem.*, **243**, 3357 (1968).

5) M. Meheriuk and M. Spencer, *Phytochem.*, **6**, 551 (1967).

TABLE III. NON-ENZYMATIC FORMATION OF ETHYLENE FROM ACRYLIC ACID IN THE PRESENCE OF Mg^{2+} AND TPP

System	Ethylene (m μ l)
Complete	27
minus acrylic acid	0
minus Mg^{2+}	0
minus TPP	0
minus Mg^{2+} and TPP	0

The complete system contained 200 μ moles of sodium borate buffer, pH 9.0, 3 μ l of acrylic acid, 100 μ moles of $MgSO_4$, 10 μ moles of TPP in a total volume of 2 ml. Incubation time; 60 min. Incubation temp; 37°C. The gas phase was argon.

decarboxylation of pyruvate in the presence of TPP has been well known.⁶⁾ This non-enzymatic reaction should assist in understanding the biological ethylene formation in the presence of Mg^{2+} and TPP. Therefore, an attempt to demonstrate the non-enzymatic formation in a model system containing TPP and acrylic acid was carried out. Table III shows that at pH 9 ethylene was formed from acrylic acid in the presence of TPP without any enzyme extract. For non-enzymatic formation a high pH was required, while the pH optimum of the enzymatic formation by banana pulp extracts was found to be pH 7.0 as shown previously. It is believed that this is another evidence to support participation of decarboxylation in the final step of ethylene formation from acrylic acid.

Ethylene formation from acrylic acid by yeast pyruvate decarboxylase

The decarboxylation of acrylic acid presented above has similar properties to the decarboxylation of pyruvate in the requirement for TPP.⁶⁾ Therefore, it is expected that pyruvate decarboxylase also catalyzes the decarboxyla-

TABLE IV. ETHYLENE FORMATION FROM ACRYLIC ACID BY YEAST PYRUVATE DECARBOXYLASE

System	Ethylene (m μ l)
Complete	12
minus acrylic acid	0
minus TPP	9
minus Mg^{2+}	0
minus enzyme	0
with boiled enzyme	0

The complete system contained 20 μ g of yeast pyruvate decarboxylase which catalyzes the decarboxylation of 0.4 μ moles of pyruvate at 25°C, 200 μ mole of potassium phosphate buffer, pH 7.0, 100 μ mole of $MgSO_4$, 10 μ moles of TPP, 3 μ l of acrylic acid and 2 mg of bovine serum albumin in a total volume of 3 ml. Incubation time; 10 min. Incubation temp 30°C. The gas phase was argon.

tion of acrylic acid. Table IV shows the formation of ethylene from acrylic acid by yeast pyruvate decarboxylase in the presence of Mg^{2+} and TPP. Without the enzyme ethylene was not formed at neutral pH employed in this experiment by using the enzyme extract from the banana pulp.

DISCUSSION

In the previous report, a possible pathway of ethylene formation in banana pulp slices and homogenates was proposed. In this pathway, the final step is a decarboxylation of acrylic acid. The decarboxylation of acrylic acid as the biosynthetic sequence of ethylene has been proposed by Wang *et al.*⁷⁾ and Spencer *et al.*⁵⁾ Wang *et al.*⁷⁾ observed the incorporation of C-2 and C-3 of acrylic acid, but not C-1, into ethylene using specific labeled acrylic acid in *Penicillium digitatum*. Spencer *et al.*⁵⁾ also proposed the participation of acrylic acid decarboxylation to the formation of ethylene from their results indicating that ethylene formation from β -alanine was

6) S. Mizuhara, R. Tamura and H. Arata, *Proc. Japan Acad.*, **27**, 302 (1951); S. Mizuhara and K. Ōno, *ibid.*, **27**, 705 (1951); E. Yatco-Manzo, F. Roddy, R. G. Yount and D. E. Metzler, *J. Biol. Chem.*, **234**, 733 (1959).

7) C. H. E. Wang, A. Persyn and J. Krackov, *Nature*, **195**, 1306 (1962); D. W. Jacobsen and C. H. Wang, *Plant Physiol.*, **40** (suppl), xix (1965).

enhanced in the enzyme extract obtained from bean cotyledons. But they showed that acrylic acid was inhibitory in endogeneous ethylene formation from tomato tissues. Under these circumstances, the present paper is a first demonstration of enzymatic formation of ethylene from acrylic acid. The failure of the enhancement of ethylene formation by adding acrylic acid by Spencer *et al.*⁵⁾ may resulted from the oxidation of the acid by oxygen and a damage of the enzyme by the acid because the gas phase in their experiment was air.

Mapson and Wardale⁸⁾ found that ethylene formation from methional by an enzyme system from cauliflower florets was optimum at pH 6.6. Shimokawa and Kasai⁹⁾ found pH 6.0 was optimum for the formation of ethylene from pyruvate by a subcellular fraction from apple tissues, and Yang¹⁰⁾ found a pH range from 7.3 to 8.3 was optimum for the conversion of methional to ethylene by horseradish peroxidase. More recently, Stinson and Spencer¹²⁾ recognized the presence of two peaks, at pH 7.0 and 8.5, for optimum ethylene formation from β -alanine in the presence of TPP by an enzyme extract from bean cotyledons. Endogeneous formation of ethylene from apple tissues was also recognized at

high pH.¹³⁾ For the enzymatic formation of ethylene from acrylic acid by the banana enzyme extract, pH 7.0 was optimum. In contrast to this, the non-enzymatic formation of ethylene in the presence of TPP was observed at high pH. The ethylene formation at the high pH reported by Stinson and Spencer suggests the participation of non-enzymatic ethylene formation catalyzed by TPP, as found in the present experiments.

The physiological significance of the ethylene formation by yeast pyruvate decarboxylase remains to be evaluated. However, it should be attended that the activity of pyruvate decarboxylase rise with ripening of some fruits after harvest and with its rising of the activity ethylene formation increase as recognized in various fruits.^{14,15)}

No ethylene formation from acrylic acid was found in the presence of horseradish peroxidase instead of banana enzyme extract. This fact indicates that the present biosynthesis sequence is to be different from ethylene biosynthesis by oxidative degradation of methionine.¹⁶⁾

Finally we wish to propose the following route as a biosynthetic pathway of ethylene in banana fruits.

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9) K. Shimokawa and Z. Kasai, *Plant Cell Physiol.*, **8**, 227 (1967).

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11) T. Takeo and M. Lieberman, *Biochem. Biophys. Acta*, **178**, 235 (1969).

12) A. Stinson and M. Spencer, *Plant Physiol.*, **44**, 1217 (1969).

13) M. Liberman, A. T. Kunishi, L. W. Mapson and D. A. Wardale, *Plant Physiol.*, **41**, 376 (1966).

14) A. C. Hulme, J. D. Jones and L. S. C. Wooltorton, *Proc. Roy. Soc. Lond. Ser. B*, **158**, 519 (1963).

15) K. Shimokawa and Y. Ueda, Unpublished data.

16) L. W. Mapson, *Biol. Rev.*, **44**, 155 (1969).