Synthesis and Degradation of 1-Aminocyclopropane-1-carboxylic Acid by *Penicillium citrinum*

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1-Aminocyclopropane-1-carboxylic acid (ACC), which is a precursor of ethylene in plants, has never been known to occur in microorganisms. We describe the synthesis of ACC by *Penicillium citrinum*, purification of ACC synthase [EC 4.4.1.14] and ACC deaminase [EC 4.1.99.4], and their properties. Analyses of *P. citrinum* culture showed occurrence of ACC in the culture broth and in the cell extract. ACC synthase was purified from cells grown in a medium containing 0.05% l-methionine and ACC deaminase was done from cells incubated in a medium containing 1% 2-aminoisobutyrate. The purified ACC synthase, with a specific activity of 327 milliunit/mg protein, showed a single band of M, 48,000 in SDS-polyacrylamide gel electrophoresis. The molecular mass of the native enzyme by gel filtration was 96,000 Da. The ACC synthase had the $K_m$ for S-adenosyl-l-methionine of 1.74 nm and $k_{cat}$ of 0.56 s$^{-1}$ per monomer. The purified ACC deaminase, with a specific activity of 4.7 unit/mg protein, showed one band in SDS-polyacrylamide gel electrophoresis of M, 41,000. The molecular mass of the native ACC deaminase was 68,000 Da by gel filtration. The enzyme had a $K_m$ for ACC of 4.8 nm and $k_{cat}$ of 3.52 s$^{-1}$. The presence of 7 mM Cu$^{2+}$ in alkaline buffer solution was effective for increasing the stability of the ACC deaminase in the process of purification.

**Key words:** 1-aminocyclopropane-1-carboxylic acid (ACC); ACC synthase; ACC deaminase; *Penicillium citrinum*

The cyclopropanoic amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC), is a precursor of the plant hormone ethylene, which controls various stages of plant growth and development. Biosynthesis of ethylene involves two unique enzymes. ACC synthase, which forms ACC from S-adenosyl-l-methionine (SAM), has been highly purified from various kinds of plants. ACC oxidase, which forms ethylene from ACC in the presence of ascorbate and ferrous ion, also has been purified from a few kinds of plants.

Microorganisms liberate ethylene through two routes: the formation from 2-oxoglutarate and that from 2-oxo-4-methylthiobutyrate, a deamination product of methionine. The enzyme forming ethylene by the former route was purified from *Penicillium digitatum* and *Pseudomonas syringae*, and the enzymatic basis of ethylene formation by the latter route was reported. These pathways do not involve ACC as the intermediate.

In plants ACC is converted into the N-malonyl derivative to reduce the concentrations of ACC and ethylene. In microorganisms ACC is degraded into ammonia and 2-oxobutyrate by ACC deaminase. This enzyme has been found and isolated from a few strains of *Pseudomonas* species and *Hansenula saturnus*. Expression of ACC deaminase gene in plant reduced ethylene liberation and delayed the fruit ripening. Thus, ACC has a distinct role in growth of higher plants. On the other hand, a bacterial strain causing plant disease was shown to synthesize 2-methyl and 2-ethyl derivatives of ACC, but ACC has not been found in microorganisms.

In this work, we show that *Penicillium citrinum* synthesizes ACC from SAM and degrades it by the ACC deaminase. As it has been clearly shown that *P. digitatum* forms ethylene from 2-oxoglutarate, fungi must have a different form of ACC metabolism from higher plants. This is the first report of an organism with both ACC synthase and ACC deaminase except for the transgenic plant.

**Materials and Methods**

Microorganisms and culture conditions. Strains of *Penicillium* used were from the Laboratory of Culture Collection of Microorganisms, Faculty of Agriculture, Hokkaido University (AHU) and Institute for Fermentation, Osaka (IFO).

For the studies of the formations of ethylene and ACC, *Penicillium* fungi were incubated in a medium (the first medium) composed of 2% glucose, 0.4% NH$_4$NO$_3$, 1.36% KH$_2$PO$_4$, 0.125% MgSO$_4$·7H$_2$O, 0.002% FeCl$_3$·6H$_2$O, and 0.1% yeast extract, under shaking at 28°C for 3 days.

To prepare ACC synthase, *P. citrinum* was grown in a medium composed of 0.5% glucose, 0.4% polypeptone, 1.36% KH$_2$PO$_4$, 0.125% MgSO$_4$·7H$_2$O, 0.002%

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**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylic acid; FPLC, Fast Protein Liquid Chromatography; PLP, pyridoxal 5'-phosphate; PMSF, phenylmethylsulfonyl fluoride; PTC, phenylthiocarbamoyl; SAM, S-adenosyl-l-methionine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis
FeSO$_4$·7H$_2$O, 0.1% yeast extract and 0.05% L-methionine, under shaking at 27°C for 72 h.

To prepare ACC deaminase, cells which were grown in the first medium were harvested by filtration and washed with 0.05 M potassium phosphate, pH 7.5, and further incubated in the second medium containing 2% glucose, 0.55% KH$_2$PO$_4$, 0.104% K$_2$HPO$_4$, 0.125% MgSO$_4$·7H$_2$O, 0.002% FeCl$_3$·6H$_2$O, and 1% 2-aminoisobutyrate under shaking at 28°C for 72 h.

All incubations were done by using a 500-ml Erlenmeyer flask containing 100 ml of the medium.

**Assay of ethylene.** After the incubations of *Penicillium* fungi, 1 ml of each culture broth was transferred to a sterile test tube (15 mm diameter, 26 ml capacity). The test tube was sealed with a rubber stopper and then incubated on a reciprocal shaker at 28°C. After incubation, a gas sample (1 ml) was withdrawn through the stopper with a syringe and ethylene was measured with a gas chromatograph with FID under the following conditions: column size, 3 mm × 2 m; solid phase, active alumina; temperature, 70°C; carrier gas, nitrogen, at a flow rate of 30 ml/min.

**Extraction of intracellular ACC.** The cells were collected by filtration, suspended in five volumes of 6.75% perchloric acid, and frozen by liquid nitrogen, then thawed at room temperature. The processes of freezing and thawing were repeated. The filtrates were neutralized with a concentrated solution of potassium hydroxide and the supernatants were used to assay ACC.

**Assay of ACC.** The amount of ACC present was measured by the method of Lizada and Yang, in which ACC was fragmented to ethylene chemically and measured by gas chromatography. In an experiment to confirm the formation of ACC by *P. citrinum*, two other methods were used for estimation of the amount of ACC. One was amino acid analysis with a Derivatizer 420A (Applied Biosystems). Amino acids were converted to phenylisothiocarbamoyl (PTC) derivatives with phenylisothiocyanate and analyzed with reversed-phase chromatography on line. The other method was enzymatic analysis. ACC was incubated with 0.1 units of ACC deaminase purified from *Pseudomonas* sp. for 1 h at 30°C. The resultant 2-oxobutyrate was measured by the way of the ACC deaminase assay.

**Assay of enzyme activities.** To assay ACC synthase, the enzyme was incubated with 1 mM SAM in 0.1 ml of 0.2 M potassium pyrophosphate (pH 9.0) at 30°C. The reaction was stopped with 0.1 ml of ice cold 10 mM mercuric chloride. After adding 0.7 ml of ice cold water, the reaction tube was sealed with a serum cap and kept in an ice bath. The amount of ACC formed was measured. One millimicromol (mM) of ACC synthase indicates the activity forming 1 mmol of ACC per minute under these conditions.

Assay of ACC deaminase was done by measurement of 2-oxobutyrate generated by enzymatic deamination of 50 mM ACC as described by Honma and Shinomura. One unit (U) of the enzyme indicates the activity forming 1 μmol of 2-oxobutyrate per minute under these conditions.

**Assay protein.** Protein was measured by the method of Bradford with bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring protein in column effluents.

**Molecular mass measurement.** The molecular mass was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% acrylamide and gel filtration with Superose 12 HR 10/30 or Superdex 200 HR 10/30 column (Pharmacia). In the analysis of SDS-PAGE, molecular mass markers used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (22 kDa), and lysozyme (14 kDa). On the gel filtration, the standard proteins used were immunoglobulin M (150 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and cytochrome c (13 kDa).

**Purification of ACC synthase from *P. citrinum*.** The buffer solution used at all steps of purification contained 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.01 mM pyridoxal 5'-phosphate (PLP).

**Extraction.** After the incubation, *P. citrinum* cells were collected from the medium by filtration and suspended in 0.05 M potassium pyrophosphate (pH 9.0), then homogenized by Polytron (Kinematica) and ruptured by the high-pressure extrusion method with a French Press (Ohtake Works). The disrupted sample was centrifuged at 19,000 × g at 4°C for 30 min. The supernatant was used as the cell extract in the next step of purification.

**DEAE-Sepharose CL-6B column chromatography.** The cell extract solution was dialyzed against 0.05 M potassium pyrophosphate (pH 9.0) and poured into a column of DEAE-Sepharose CL-6B (4.6 × 20 cm) equilibrated with the same buffer. A linear gradient elution was done with zero to 0.5 M potassium chloride. The fractions containing the ACC synthase were pooled and dialyzed overnight against 0.01 M potassium pyrophosphate (pH 9.0).

**Aminohexyl-Sepharose column chromatography.** The dialyzed sample was put on a column of aminohexyl-Sepharose (2 × 32 cm) equilibrated with 0.01 M potassium pyrophosphate (pH 9.0) and a linear gradient elution was done with zero to 0.8 M potassium chloride. The active fractions were combined and concentrated by ultrafiltration, then dialyzed overnight against 0.02 M Tris-chloride (pH 8.0).

**Mono Q HR 5/5 column chromatography.** The dialyzed sample was put on a column of Mono Q HR 5/5 (0.5 × 5 cm) equilibrated with 0.02 M Tris-chloride (pH
8.0) in a Fast Protein Liquid Chromatography (FPLC, Pharmacia) and eluted with the concentration gradients of potassium chloride.

**Purification of ACC deaminase from P. citrinum.** The buffer solution used at all steps of purification contained 1 mM 2-mercaptoethanol and 0.01 mM PLP.

**Extraction.** After the induction of ACC deaminase by the second culture, cells were extracted by the same method as described for ACC synthase, except for the change of the buffer solution to 0.05 M potassium phosphate (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF).

**DEAE-cellulose column chromatography.** The cell extract was put on a column of DEAE-cellulose (4.5 x 20 cm) equilibrated with 0.05 M potassium phosphate (pH 7.5). A linear gradient elution was done with zero to 0.5 M potassium chloride.

**Aminoethyl-Sepharose column chromatography.** The ACC deaminase fraction was dialyzed overnight against 0.02 M potassium carbonate (pH 10) containing 7 mM copper sulfate, 0.05 M Tris, and 20% glycerol, and poured on a column of aminoethyl-Sepharose (1.5 x 10 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of potassium chloride in the concentration of zero to 0.4 M.

**DEAE-Sepharose column chromatography.** The fractions of ACC deaminase were combined, dialyzed overnight against 0.02 M potassium carbonate (pH 9.0) containing 7 mM copper sulfate, 0.05 M Tris, and 20% glycerol, and put on a DEAE-Sepharose column (1.5 x 10 cm) equilibrated with the same buffer. A linear gradient elution was done with zero to 1 M potassium chloride.

**Sephadex G-150 column chromatography.** Fractions corresponding to the peak of ACC deaminase activity were collected and concentrated by seamless cellulose tubing and polyethylene glycol #20,000. The concentrated sample was passed through a Sephadex G-150 column (2 x 50 cm) with 0.02 M potassium carbonate, pH 10.0, containing 7 mM copper sulfate, 0.05 M Tris, and 20% glycerol. The active fraction was concentrated and rechromatographed by the Sephadex G-150 column.

**Mono Q HR 5/5 column chromatography.** The enzyme solution obtained from gel filtration was put on a column of Mono Q HR 5/5 (0.5 x 5 cm) equilibrated with 0.02 M potassium carbonate (pH 10.0) in FPLC and eluted with the concentration gradients of potassium chloride.

**Miscellaneous.** Aminoethyl-Sepharose was prepared from Sepharose 4B and diaminohexane by the method of Shaltiel et al. ACC was prepared according to the description of Burroughs. dl-Coronamic acid and dl-allo coronamic acid were given to us by Dr. A. Ichihara, Hokkaido University.

**Results**

**Formation of Ethylene and ACC by Penicillium fungi.** Ethylene has been reported to be a metabolic product of many fungi. We preliminarily tested 13 strains of *Penicillium* fungi for ethylene and ACC formation and found that *P. digitatum*, which was an excellent strain for production of ethylene, had only a small amount of intracellular ACC, and that *P. citrinum*, which was a poor strain for ethylene production, synthesized the highest amount of intracellular ACC. In further experiments *P. citrinum* AHU 8443 was used.

**Formation and Degradation of ACC by P. citrinum**

The ACC that was produced at the initial growth stage of *P. citrinum* was discharged into medium (Fig. 1). Around the stationary phase of mycelial growth, the ACC in the culture medium began to decrease and went to almost zero finally. After mycelial growth reached the

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**Fig. 1.** Formation of ACC by *P. citrinum.*

*P. citrinum* were cultured in 100 ml of the medium at 28°C with shaking. The cells were harvested and fresh weight (△), ACC in cell extract (●), and ACC in medium (○) were measured as described in Materials and Methods. A, in the absence of L-methionine; B, in the presence of L-methionine. Each point shows the mean of four measurements.
maximum, the intracellular ACC also reached the maximum value of about 200 nmol per 100 ml of the culture and began to decrease (Fig. 1A). Addition of L-methionine in the medium stimulated accumulation of ACC, which reached about 300 nmol per 100 ml of the culture (Fig. 1B).

To confirm the formation of ACC, the extract (340 ml) from mycelial cells grown in the medium containing 0.05% methionine (Fig. 1B) was put on a column (1 x 25 cm) of Dowex 50W-X8 equilibrated with 0.02 N hydrochloric acid, and separated into amino acid fractions by a linear gradient of 0.02 to 1.5 N hydrochloric acid (600 ml). The fractions of ACC that were measured by the method of Lizada and Yang were collected, evaporated to dryness, and analyzed for ACC. Amino acid analysis of the ACC fraction with the Derivatizer 420A showed a chromatogram having an identical band to the authentic ACC (Fig. 2). Results of ACC assay with this fraction were 4.63 μmol by the amino acid analysis, 4.42 μmol by the enzymatic analysis, and 4.43 μmol by the method of Lizada and Yang. Thus these different methods resulted in good approximations to one other.

**Purification of ACC synthase**

ACC synthase was purified from mycelial cells grown on the medium containing 0.05% L-methionine. The purification procedure was summarized in Table 1. For the purification of ACC synthase, 2150 ml of crude extract was made from 430 g of *P. citrinum* cells, which was obtained from 6 liters of culture medium, to yield approximately 73 μU of ACC synthase with a specific activity of 0.08 μU/mg protein. After DEAE-Sepharose column chromatography, the enzyme activity was recovered in 436% yield overall, at a specific activity of 6.6 μU/mg. The passing and washing fractions from the DEAE-Sepharose column inhibited the ACC synthase activity of the eluate from this column (data not shown).

After aminohexyl-Sepharose column and Mono Q column chromatographies, the specific activity was increased to 327 μU/mg. The enzyme showed a single band in SDS-PAGE, and the molecular mass was estimated to be 48,000 Da. The molecular mass of the native form of the enzyme was calibrated to 96,000 Da from the enzyme’s elution volume from a Superdex 200 HR 10/30 filtration column compared with the elution volume of a series of standard proteins.

**Properties of ACC synthase**

The ACC synthase was found to have maximal activity at pH 9.6, with relative activity of 72% and 2.9% at pH 8.5 and 10.5, respectively (Fig. 3A). The effect of pH on the stability of the ACC synthase was studied by using the purified enzyme. The enzyme was stable at pH 6.5 to 10.5 at 4°C for 24 h (Fig. 3B).

The ACC synthase of plants was reported to be inactivated by the reaction with the substrate. The purified ACC synthase (0.04 μU) was incubated for various periods of time with 2 mm SAM then the ACC formed was measured. The formation of ACC was almost linear with the reaction time up to 120 min.

A plot of reaction rate versus SAM concentration showed a simple saturation curve and $K_m$ and $k_{cat}$ were calculated to be 1.74 μM and 0.56 s⁻¹ per monomer respectively (Fig. 4A).

The absorption spectrum of purified ACC synthase

<table>
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<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
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<tr>
<td>Cell extract</td>
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<td>0.00008</td>
<td>100</td>
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<tr>
<td>DEAE-Sepharose</td>
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<td>Aminohexyl-Sepharose</td>
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<td>0.183</td>
<td>0.327</td>
<td>251</td>
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<tr>
<td>ACC deaminase</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell extract</td>
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<td>0.072</td>
<td>100</td>
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<td>DEAE-cellulose</td>
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<tr>
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<td>1.7</td>
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Fig. 3. Effects of pH on the ACC Synthase and ACC Deaminase.

Buffers used were: 0.1 M potassium acetate (pH 4.0–4.5), 0.1 M potassium phosphate-0.05 M potassium pyrophosphate (pH 5.0–9.0), 0.1 M potassium carbonate (pH 9.5–11.0), and 0.1 M potassium phosphate-KOH (pH 11.5–13.0).

A: the activity of ACC synthase. ACC synthase was incubated with 1 mM SAM in the various buffers at 30°C for 1 h.

B: the stability of ACC synthase. ACC synthase solution was incubated with the buffers (1:1.5, v/v) at 4°C for 24 h, then 10 μl of the mixture was used for enzyme reaction. The activity was represented as the percent of the activity at zero time.

C: the activity of ACC deaminase. The mixture of 50 μl of ACC deaminase solution and 100 μl of 100 mM ACC was added to 150 μl of buffers and incubated at 30°C for 60 min, then the reaction was stopped by adding 1.7 ml of 0.59 N HCl.

D: the stability of ACC deaminase. ACC deaminase solution was incubated with buffers (1:10, v/v) at 4°C for 24 h, then 30 μl of this solution was used for an assay of enzyme activity. The activity was represented as percent of the activity at zero time.

Each point in figures A to D shows the mean of four measurements.

preparation has a maximum at about 420 nm, indicating that the enzyme contains bound PLP as a cofactor (Fig. 5A).

The ACC synthase of P. citrinum showed a stringent substrate specificity for SAM and was inactive for S-adenosyl-L-homocysteine, S-methyl-L-methionine, L-methionine sulfoxide, and L-methionine. But these analogs inhibited the enzyme reaction toward SAM, especially S-adenosyl-L-homocysteine did strongly (Table 2).

The effect of various metal ions on ACC synthase reaction was examined and the results are summarized in Table 2. The enzyme reaction was not affected by Ca²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺ and Zn²⁺, but was inhibited by Hg²⁺.

**Purification of ACC deaminase**

As described in the cases of *Pseudomonas* [42] and *Hansenula saturnus*, [27] ACC deaminase was induced by 2-aminoisobutyrate in *P. citrinum*. After induction of ACC deaminase by the second culture, 300 g of *P. citrinum* cells was collected by filtration and extracted by the methods described in Materials and Methods, to yield 118 units of ACC deaminase with a specific activity of 0.072 unit/mg protein. Typical results of purification are summarized in Table 1. Since ACC deaminase activity was unstable in the process of purification, the effects of pH and metal ion on the stability of ACC deaminase was examined using the enzyme fraction from DEAE-cellulose column chromatography. The results indicated that *P. citrinum* ACC deaminase was more stable in alkaline solution than in the neutral, and that Hg²⁺ ion and Cu²⁺ ion increased the stability of ACC deaminase. Because of the drastic toxicity of Hg²⁺ ion and the inhibition of the ACC deaminase reaction (Table 2), 7 mm Cu²⁺ ion was added in the alkaline buffer solution to increase the stability of ACC deaminase in the process of purification except for the final step. After overall purification, the yield of ACC deaminase was 7% and the specific activity was increased to 4.7 unit/mg. The enzyme showed one band in SDS-PAGE, the molecular mass was estimated to be 41,000 Da. Using a Superoxer 12 HR 10/30 gel filtration column, the molecular mass of the native form of the enzyme was estimated to be 68,000 Da. Unlike the ACC deaminase from *Pseudomonas* which exists as a trimer, [42] ACC deaminase from *P. citrinum* probably exists as a dimer and the molecular mass values are approximate to those of the enzyme from yeast. [27]

### Table 2. Effects of Various Metal Compounds and Substrate Analogs on the Activity of ACC Synthase and ACC Deaminase

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<th>Compound</th>
<th>ACC deaminase Relative activity (%)</th>
<th>ACC synthase Relative activity (%)</th>
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<td>None</td>
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<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
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<td>92</td>
<td>113</td>
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<td>L-Methionine sulfoxide</td>
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<td>S-Methyl-L-methionine</td>
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<td>L-Methionine</td>
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<tr>
<td>S-Adenosyl-L-homocysteine</td>
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</table>

Properties of ACC deaminase

Partially purified ACC deaminase was incubated with 66.7 mM ACC at various pHs from 4.0 to 13.0 at 30°C for 60 min. The results indicated that ACC deaminase activity occurred as a peak with an optimum at pH 8.5 (Fig. 3C). The pH optimum for the enzyme was identical to the value found by Honma and Shimomura [28] for ACC deaminase from a *Pseudomonas* sp. strain ACP.
The purified ACC deaminase from *P. citrinum* was stable at pH 6.0 to 11.0 at 4°C for 24 h (Fig. 3D).

The temperature optimum for ACC deaminase activity was found by incubating purified enzyme with 50 mM ACC in 0.2 ml of 0.1 M Tris-0.045 M KH₂PO₄, pH 8.5, at different temperatures between 10 to 60°C for 10 min. ACC deaminase relative activity increased from 10% at 10°C to 100% at 35°C. At temperatures above 35°C, the relative activity decreased until around to 60°C, at which no activity was observed. So the temperature optimum of ACC deaminase from *P. citrinum* was approximately 35°C under these assay conditions.

The Michaelis constant *Kₘ* and catalytic constant *kₘₐₜ* of ACC deaminase for ACC were calculated to be 4.8 mM and 3.52 s⁻¹ per monomer at pH 8.5 in 0.1 M Tris-phosphate buffer (Fig. 4B).

Isolation of cyclop propane amino acids, ACC₃ and several kinds of 2-alkyl-ACC from metabolites of *P. citrinum* was reported. Some of these amino acids were used for examination of the substrate stereospecificity of ACC deaminase. dl-Coronamic acid showed 3.6% of ACC activity in the reaction of ACC deaminase for 30 min. No activity was detected with dl-allo coronamic acid, (S)-2,2-dimethyl-ACC, or (R)-2,2-dimethyl-ACC. The activity of the ACC deaminase to L-serine was not detected. But D-serine is an active form to the enzyme. The relative activity was calculated to be 0.7% of ACC activity.

As a pyridoxal phosphate enzyme, the absorption spectrum of purified ACC deaminase includes peaks at about 420 nm and 330 nm at pH 7.5 (Fig. 5B), just the same as that of the enzyme from *Pseudomonas* sp. ACP reported previously. The peak at 420 nm decreased with increasing the pH and almost disappeared at pH 9.0, while the 330 nm peak changed conversely.

**Discussion**

By the analysis of ACC in *P. citrinum* culture and the purification of ACC synthase in this study, we found that this microorganism synthesized ACC through the same reaction as that of higher plants. Fractionation of amino acids in the mycelial extract by the cation exchanger made the identification of ACC in amino acid analysis easier (Fig. 2). The ACC fraction from the cation exchange chromatography of mycelial extract was analyzed for amount of ACC by three methods with different processes, chemical fragmentation to ethylene, enzymatic deamination to 2-oxobutyrate, and amino acid analysis of PTC derivative. The good approximation among three values from different methods confirmed the occurrence of ACC in the cell extract.

ACC synthase was purified from *P. citrinum* with the specific activity of 327 mU/mg. Several plants have provided sources for purification of ACC synthase, but there is no report that ACC synthase was purified from a microorganism up to now. The characterization of ACC synthase from *P. citrinum*, such as effects of pH on activity and stability, approximated to those of the enzyme from higher plants, but the Michaelis constant, *Kₘ* for SAM was 1.7 µM and *kₐₚ₃ₜ* was 0.56 s⁻¹ per monomer at 30°C, indicating lower substrate affinity and lower activity than that of the higher plants. Kinetic constants for the winter squash and apple enzymes expressed in **E. coli** were reported to be *Kₘ* 21 µM; *kₐₚ₃ₜ* 21 s⁻¹ and *Kₘ* 12 µM; *kₐₚ₃ₜ* 9 s⁻¹, respectively. *P. citrinum* ACC synthase seem to be a dimer on the basis of molecular weights of the enzyme protein estimated by
SDS-PAGE and a gel filtration column chromatography. It was shown that ACC synthase was closely related to a group of aspartate aminotransferases from sequence profiles. Aminotransferase is a homodimer, in which the active site is shared between the subunits. The quaternary structure of plant enzyme is ambiguous but apple and winter squash ACC synthase expressed in E. coli are dimeric. The result for P. citrinum is consistent with these facts.

We purified the ACC deaminase from P. citrinum induced by 2-aminoisobutyrate. The ACC deaminase was so labile in the purification process that the activity had a half-life of a week in crude extract with the presence of some stabilizers such as 2-mercaptoethanol, PMSF, and glycerol. It was found that the enzyme was stable in the presence of 7 mM copper ion in alkaline solution (pH 10). But the purified ACC deaminase was stable in a range of pH 6.0 to 11.0 without copper ion. It is not clear why copper ion is effective for stabilization of this enzyme. The purified ACC deaminase showed the same stereospecificity as the Pseudomonas enzyme. Relative activities of P. citrinum ACC deaminase to dL-cornameric acid, D-serine, and dimethyl-ACC (3.6, 0.7 and 0% of ACC activity) were lower than those of Pseudomonas enzyme (23, 3.3 and 1.1%) and of the Hansena saturnus one (15.4, 2.9 and 0.15%), indicating higher specificity for ACC relative to the other two enzymes. The Ka for ACC with the P. citrinum enzyme (4.8 mM) was higher than those of Pseudomonas enzyme (1.6 mM) and of the Hansena saturnus one (2.6 mM). This indicates that the P. citrinum enzyme has a lower substrate affinity than the other two enzymes, while the specific activity is almost similar among these three enzymes.

These results show the possibility that P. citrinum synthesizes ACC from SAM and metabolizes ACC to ammonia and 2-oxobutyrate, not to ethylene. Addition of ACC in the medium did not stimulate the formation of ethylene by P. citrinum (data not shown). Chernys and Kende described the presence of ACC synthase and the apparent absence or very low in-vivo activity of ACC oxidase in the semi-aquatic ferns. Although the metabolic pathway of ACC in these ferns has not been discovered, the interesting similarity between the semi-aquatic ferns, lower plants, and the fungi is the synthesis of ACC that is not converted to ethylene. So far, there is no evidence about physiological significance of the synthesis and deamination of ACC in microorganisms, except for Glick’s works in which the plant growth promoting role of bacterial ACC deaminase is described. There are questions which remain to be solved in P. citrinum: whether ACC accumulated in intracellular spaces induces the ACC deaminase, and whether P. citrinum has an ACC oxidase.

References
22. Amrhein, N., Schneebuck, D., Skorupka, H., and Tophof, S., Identification of a major metabolite of the ethylene precursor 1-


