Purification and Characterization of 1-Aminocyclopropane-1-Carboxylate N-Malonyltransferase from Etiolated Mung Bean Hypocotyls

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ABSTRACT

1-Aminocyclopropane-1-carboxylate (ACC) N-malonyltransferase converts ACC, an immediate precursor of ethylene, to the presumably inactive product malonyl-ACC (MACC). This enzyme plays a role in ethylene production by reducing the level of free ACC in plant tissue. In this study, ACC N-malonyltransferase was purified 3660-fold from etiolated mung bean (Vigna radiata) hypocotyls, with a 6% overall recovery. The final specific activity was about 83,000 nmol of MACC formed mg⁻¹ protein h⁻¹. The five-step purification protocol consisted of polyethylene glycol fractionation, Cibacron blue 3GA-agarose chromatography using salt gradient elution, Sephadex G-100 gel filtration, MonoQ anion-exchange chromatography, and Cibacron blue 3GA-agarose chromatography using malonyl-CoA plus ACC for elution. The molecular mass of the native enzyme determined by Sephadex G-100 chromatography was 50 ± 3 kD. Protein from the final purification step showed one major band at 55 kD after sodium dodecyl sulfate polyacrylamide gel electrophoresis, indicating that ACC N-malonyltransferase is a monomer. The mung bean ACC N-malonyltransferase has a pH optimum of 8.0, an apparent Km of 0.5 mM for ACC and 0.2 mM for malonyl-coenzyme A, and an Arrhenius activation energy of 70.29 kJ mol⁻¹ degree⁻¹.

The biosynthetic pathway for the plant hormone ethylene has been established by Adams and Yang (1, 19) as Met → S-adenosylmethionine → ACC → ethylene. The immediate precursor of ethylene, ACC, can also be converted to MACC (3, 7). The enzyme catalyzing this latter conversion is suggested to be ACC N-malonyltransferase (8). Malonylation of ACC can decrease the free ACC level inside plant tissues and thus may play an important role in the regulation of ethylene production. ACC N-malonyltransferase has been found to be involved in autoinhibition of ethylene formation (12, 15) and seed germination (6).

ACC N-malonyltransferase is a constitutive and widespread enzyme (2, 8). Generally, the conversion of ACC to MACC is thought to be irreversible and that MACC is an inactive end product (8). Using crude or ammonium sulfate-precipitated protein fractions from etiolated mung bean (Vigna radiata) hypocotyls, ACC N-malonyltransferase has previously been partially characterized (8, 11, 18). To understand better how ACC N-malonyltransferase is involved in the regulation of ethylene biosynthesis, purification of ACC N-malonyltransferase was needed. In this article, we report a five-step purification protocol for ACC N-malonyltransferase and partial characterization of the enzyme.

MATERIALS AND METHODS

Plant Material

Mung bean (Vigna radiata Rwilcz cv Berken) seeds were soaked in distilled water under continual aeration for 20 h. The seeds were rinsed thoroughly with tap water to remove inhibitors exuded during imbibition. The seeds were then sown between two layers of moist Kimpak and grown at 28°C in the dark for 4 d. Seedlings were rinsed once daily with distilled water. After this period, the hypocotyl length was 7 to 10 cm. The entire hypocotyl region was used for enzyme purification.

Enzyme Purification

All purification steps were conducted at 4°C with the exception of FPLC, which was carried out at room temperature. Etiolated mung bean hypocotyls (2.5 kg) were homogenized in a blender for four 20-s intervals in an equal weight of 100 mM potassium phosphate buffer, pH 7.2, containing 0.4 mM DTE and 100 mM KCl (buffer A). The homogenate was squeezed through six layers of cheesecloth and centrifuged at 27,500g for 40 min. A 50% (w/v) PEG-8000 (Sigma Chemical Co.) solution was slowly added to the crude extract with continuous stirring to give a 12% (w/v) PEG solution. The suspension was allowed to stand for 2 h, and then it was centrifuged at 27,500g for 30 min. The supernatant fluid was adjusted to 25% PEG as described above, and after 2 h, it was centrifuged at 27,500g for 30 min. The supernatant fraction was discarded and the pellet was dissolved in 300 mL of 20 mM Tris-HCl, pH 8.0, at 4°C, containing 0.4 mM DTE (buffer B).

Any material that was not readily solubilized was removed by centrifugation at 45,440g for 10 min and discarded. The
supernatant fluid was applied to a Cibacron blue 3GA-agarose (Sigma Chemical Co.) column (2.5 × 20 cm) that had been equilibrated with buffer B. The column was then washed with 400 mL of buffer B at a flow rate of 40 mL/h, and the enzyme was eluted with a linear gradient of 0 to 1 M KCl in buffer B. Active fractions (10 mL) were pooled and concentrated using an Amicon PM-10 (Danvers, MA) ultrafiltration membrane.

Fifteen milliliters of concentrated protein was applied to a Sephadex G-100 column (2.5 × 95 cm) previously equilibrated with buffer B. The enzyme was then eluted at a flow rate of 15 mL/h, and active fractions (5 mL) were pooled and concentrated to 10 mL as described above.

The active fraction was then loaded onto a MonoQ anion-exchange column (0.5 × 5 cm) attached to a Pharmacia (Piscataway, NJ) FPLC system, and the enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in buffer B for 30 min at a flow rate of 1 mL/min.

Active fractions from the FPLC were pooled and loaded directly onto a 0.5-mL Cibacron blue 3GA-agarose column at a flow rate of 1 mL/min. The column was then washed with 10 bed volumes of 20 mM Tris-HCl, pH 8.0, at 4°C, containing 0.4 mM DTE and 0.2 M KCl (buffer C), to remove unbound protein. The enzyme was then eluted with 2 mM malonyl-CoA plus 2 mM ACC in buffer C at a flow rate of 0.2 mL/min.

The partially purified fractions from Sephadex G-100 and FPLC were divided into small aliquots and stored at −80°C until analysis. Activity was stable for more than 3 months at this temperature (data not shown). The partially purified Sephadex G-100 fraction was used for determination of pH optima and the effect of assay temperature on activity (Fig. 3). For the determination of $K_m$ values (Figs. 4 and 5), FPLC-purified protein was used.

**ACC N-Malonyltransferase Assay and Protein Determination**

The enzyme assay was conducted according to the method of Liu et al. (12) with slight modifications. The standard reaction mixture contained 0.1 M KCl, 0.1 M potassium phosphate buffer, pH 8, 1 mM malonyl-CoA, 1 mM ACC, and enzyme in a total volume of 125 μL. The reaction mixture was incubated at 35°C for 15 min. At the end of the reaction, the mixture was passed through a 0.2-mL Dowex 50 (H+ form) column. The eluent was then hydrolyzed with 6 N HCl at 105°C for 30 min. After neutralization with 12 M NaOH, the resulting ACC was assayed according to the method of Lizada and Yang (13). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmol MACC/h at 35°C. Protein concentrations were determined according to the method of Bradford (5), using BSA as standard.

**Determination of Enzyme Molecular Mass and Purity**

The relative molecular mass was estimated by gel filtration on a Sephadex G-100 column (1.5 × 100 cm) calibrated with a mixture of known molecular mass standards. The standard enzyme assay was used to detect ACC N-malonyltransferase activity in the different fractions.

Enzyme fractions were examined by one-dimensional SDS-PAGE 10% gels (9). Proteins were detected by silver staining (17).

**RESULTS**

**Purification of ACC N-Malonyltransferase**

The results of the protocol developed for the purification of ACC N-malonyltransferase are summarized in Table I. The purification procedure was repeated four times. For each purification, 2.5 kg of mung bean hypocotyl segments were extracted, yielding approximately 3,300 units (nmol/h) of purified enzyme with a specific activity of about 83,000 units/mg protein.

Seventy percent of the original soluble protein was precipitated by 12% PEG; however, there was little ACC N-malonyltransferase activity in this fraction. Almost 90% of the total enzyme activity was obtained from the 12 to 25% PEG fraction, giving a 4-fold purification (Table I).

Dye-ligand chromatography was used as a second step because ACC N-malonyltransferase requires malonyl-coenzyme A as a substrate. We initially tested six dye-ligand matrices and found Cibacron blue 3GA-agarose to be the best. ACC N-malonyltransferase strongly bound to the blue agarose matrix, whereas more than 90% of the total soluble

<table>
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* One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmol MACC/h at 35°C.

Table I. Summary of Purification of ACC N-Malonyltransferase from Etiolated Mung Bean Hypocotyls (2.5 kg)
protein from the 12 to 25% PEG fraction did not. After all proteins that did not bind to the column had passed through the matrix, ACC N-malonyltransferase was eluted with a linear gradient of KCl. The active fractions (numbers 49-65), eluting at 550 to 700 mM KCl, were pooled, yielding a 22-fold purification with a 59% overall recovery (Table I).

The third purification step utilized Sephadex G-100 gel filtration chromatography. The pooled active fractions from the previous step were concentrated by ultrafiltration and loaded onto the sizing column. The major protein peak came out early in the elution profile, whereas the ACC N-malonyltransferase came out later. Active fractions (numbers 60-67) were pooled, yielding a 5.6-fold purification and a 45% overall recovery (Table I).

The fourth step of the purification procedure utilized FPLC with a MonoQ anion-exchange column. The active fraction from the previous step was injected onto the column and eluted with a linear, 0 to 0.4 M NaCl gradient. The elution profile is shown in Figure 1 and resulted in a 3.7-fold purification, with an 18% overall recovery. Fractions 8 to 10 were pooled and loaded on a second Cibacron blue 3GA-agarose column, and 2 mM malonyl-CoA plus 2 mM ACC in buffer C was used to elute ACC N-malonyltransferase.

After this five-step protocol, ACC N-malonyltransferase had a specific activity of about 83,000 units/mg protein and revealed one major, 55-kD band when analyzed by SDS-PAGE (Fig. 2).

Properties of Purified ACC N-Malonyltransferase

Using gel filtration chromatography, the relative molecular mass of ACC N-malonyltransferase was estimated to be 50 ± 3 kDa, indicating that ACC N-malonyltransferase is a monomer.

The effect of assay pH on ACC N-malonyltransferase activity using three different buffer systems revealed maximal activity at pH 8.0. The effect of assay temperature on ACC N-malonyltransferase activity is shown in Figure 3A. Measurements were carried out under saturating substrate conditions and at pH 8.0. Arrhenius activation energy was found to be 70.29 kJ mol⁻¹ degree⁻¹ over the range of 20 to 40°C. ACC N-malonyltransferase showed maximum activity at 50°C. The enzyme activity at 50°C was approximately 3 times that at 35°C. Enzyme activity decreased dramatically at 55°C. The effect of preincubation of the enzyme for 15 min at different temperatures before assay at 35°C is shown in Figure 3B. The enzyme was stable up to 35 to 40°C, after which there was a marked decrease in activity.

ACC N-malonyltransferase is an enzyme with two substrates, ACC and malonyl-CoA. The determination of apparent K_m values for each substrate was obtained from a series of activity assays with varied concentrations of one substrate at several fixed concentrations of the other (4). Figure 4 shows Lineweaver-Burk plots for ACC N-malonyltransferase with four malonyl-CoA concentrations varied at four ACC concentrations. The y intercepts (1/apparent V_max) for each ACC concentration were replotted in the inset, giving a K_m value for ACC of 0.5 mM. Figure 5 summarizes the Lineweaver-Burk plots for ACC N-malonyltransferase with ACC concentrations varied at several malonyl-CoA concentrations, and the inset is the secondary plot of y intercepts, giving an apparent K_m value for malonyl-CoA of 0.2 mM.

DISCUSSION

Purification of ACC N-malonyltransferase has been difficult, both due to the low abundance of this enzyme in plant
tissue and the tedious assay method. According to our results (Table I), ACC N-malonyltransferase is approximately 0.02% of the total soluble protein in etiolated mung bean hypocotyls. This tissue is a good source of plant material for the purification of ACC N-malonyltransferase because it is very easy to obtain large amounts of uniform tissue with high specific activity. In mung bean hypocotyl extracts, the specific activity of ACC N-malonyltransferase is about 21 units/mg, compared to 4.1 in green tomato pericarp, 4.5 in red tomato, and 0.9 units/mg in grapefruit flavedo discs (10). However, treatment of tomato fruits and grapefruit flavedo discs with ethylene increases the specific activity of ACC N-malonyltransferase to about 30 units/mg (10, 12). In this article, we report a five-step protocol to purify ACC N-malonyltransferase 3660-fold with a 6% overall recovery. PEG fractionation was found to be the best initial purification step, and after this step, ACC N-malonyltransferase was more stable than in the crude extract. Both our results and other reports (18) indicate that (NH₄)₂SO₄ is detrimental to ACC N-malonyltransferase.

Sandermann et al. (16) reported that the native molecular mass of N-malonyltransferase in cultured soybean cells is 48 kD. This N-malonyltransferase can use xenobiotics such as 3,4-dichloroaniline as substrates. Matern et al. (14) reported that in peanut seedlings, there are four different N-malonyltransferases. Each of these enzymes has its own substrate specificity, and their molecular masses ranged from 38 to 54 kD. In this article, we report that ACC N-malonyltransferase in etiolated mung bean seedlings is a monomer with a molecular mass of approximately 55 kD. Whether our ACC N-malonyltransferase can use xenobiotics or other chemicals as substrate remains to be explored.

Using crude extracts or (NH₄)₂SO₄ precipitated ACC N-malonyltransferase, Kionka and Amrhein (8) and Su et al. (18) reported apparent $K_m$ values for the enzyme of 0.17 mM for ACC and 0.25 mM for malonyl-CoA. In this report, we show that the $K_m$ of ACC N-malonyltransferase is 0.5 mM and 0.2 mM for ACC and malonyl-CoA, respectively. Kionka and Amrhein (8) used only one fixed concentration of one substrate while varying the other, and the ACC concentration used in their assay was nonsaturating, as indicated in their article. In addition, other researchers (8, 18) used different assay conditions and a crude enzyme source for the characterization of ACC N-malonyltransferase, which could have contributed to these slight differences in apparent $K_m$ values.

In our experiments, ACC N-malonyltransferase showed maximum activity at 50°C (Fig. 3A), although the enzyme

![Figure 3](https://example.com/figure3.png)

**Figure 3.** A. Effect of assay temperature on the activity of ACC N-malonyltransferase. B. Effect of preincubation of ACC N-malonyltransferase for 15 min at different temperatures before assay at 35°C. Enzyme purified through the Sephadex G-100 step was used.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Lineweaver-Burk plot for ACC N-malonyltransferase with malonyl-CoA concentrations varied at several ACC concentrations. The inset is a secondary plot of $y$ intercepts versus the reciprocal of the concentration of ACC in the primary plots. □, 0.15 mM ACC; ▲, 0.2 mM ACC; ○, 0.25 mM ACC; ●, 0.30 mM ACC. Enzyme purified through the FPLC step was used.
was not stable above 35 to 40°C (Fig. 3B). To avoid complications that might arise from possible temperature inactivation at higher temperatures, all our routine assays were performed at 35°C. The optimum pH reported by Kionka and Amrhein (8) agrees with the optimum pH of 8.0 reported in this article.

LITERATURE CITED