Plant Physiol. (1995) 108: 1241–1249

Purification and Characterization of 1-Aminocyclopropane-1-Carboxylic Acid *N*-Malonyltransferase from Tomato Fruit

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1-Aminocyclopropane-1-carboxylic acid (ACC) can be oxidized to ethylene or diverted to the conjugate 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) by an ACC *N*-malonyltransferase. We developed a facile assay for the ACC *N*-malonyltransferase that resolved [^{14}C]MACC from [^{14}C]ACC by thin-layer chromatography and detected and quantified them using a radioisotope-imaging system. Using this assay, we showed that ACC *N*-malonyltransferase activity has developmental and tissue-specific patterns of expression in tomato (*Lycopersicon esculentum*) fruit. In the pericarp, activity was elevated for several days postanthesis, subsequently declined to a basal level, increased 3-fold at the onset of ripening, and again declined in overripe fruit. In the seed, activity increased throughout embryogenesis, maturation, and desiccation. Treatment of fruit with ethylene increased activity 50- to 100-fold in the pericarp. ACC *N*-malonyltransferase was purified 22,000-fold to a specific activity of 22,000 nmol min^{-1} mg^{-1} protein using ammonium sulfate precipitation, DyeMatrex Green A affinity, anion-exchange, Cibacron Blue 3GA affinity, hydrophobic interaction, and molecular filtration chromatography. Native and sodium dodecyl sulfate-denatured enzyme showed molecular masses of 38 kD, indicating that the enzyme exists as a monomer. The enzyme exhibited a K_m for ACC of 500 μM, was not inhibited by D- or L-amino acids, and did not conjugate α-aminoisobutyric acid or L-amino acids.

The plant hormone, ethylene, is synthesized via the pathway: Met → *S*-adenosylmethionine → ACC → ethylene (Adams and Yang, 1979; Yang and Hoffman, 1984). In some cases (i.e. preclimacteric fruit, young flowers, and auxin-induced tissue), ACC synthesis appears to be the rate-limiting step in ethylene biosynthesis (Yu and Yang, 1979; Hoffman and Yang, 1980; Apelbaum and Yang, 1981; Mor et al., 1983; Yang and Hoffman, 1984). However, other tissues contain pools of ACC and a conjugated form of ACC, which in a few cases has been identified as MACC (Amrhein et al., 1981, 1982; Hoffman et al., 1982, 1983; Su et al., 1984). Similarly, applied ACC is oxidized by tissues such as climacteric fruit primarily to ethylene, whereas in other tissues it remains unmetabolized or is converted primarily to MACC by an ACC *N*-malonyltransferase (Amrhein et al., 1982).

MACC is found throughout the plant, including vegetative tissue, seeds, and ripening fruit (Amrhein et al., 1982; Su et al., 1984; Liu et al., 1985b), and ACC *N*-malonyltransferase has been described as a constitutive enzyme. However, the role of MACC in the plant and the factors that regulate its accumulation are incompletely understood. In tomato (*Lycopersicon esculentum*) fruit, the endogenous ACC and MACC levels, like the rates of ethylene biosynthesis, were reported to increase substantially with the onset of ripening (Su et al., 1984). Similarly, treatment of mature green tomato fruit with ethylene was reported to increase conversion of ACC to MACC and to increase extractable ACC *N*-malonyltransferase activity (Liu et al., 1985b). In both cases, malonylation of ACC may serve to temporarily or irreversibly sequester ACC and thus to reduce net ethylene biosynthesis. Similar arguments have been advanced for the role of malonylation in other tissues (Hoffman et al., 1983; Satoh and Esashi, 1983; Yang, 1986). Although malonylation of ACC may function as a regulator of ethylene biosynthesis, little is known about the enzyme responsible for the malonylation and how it is regulated.

An impediment to studying the malonylation of ACC has been the laborious methods used to measure MACC levels and the activity of the ACC *N*-malonyltransferase. ACC and MACC in each assay had to be separated by ion-exchange column chromatography prior to quantifying MACC by scintillation counting if radiolabeled ACC was used as the substrate (Kionka and Amrhein, 1984). If the MACC was not radiolabeled, it was quantified by measuring ethylene formation after acid hydrolysis of MACC to ACC, followed by chemical oxidation of ACC to ethylene (Lizada and Yang, 1979). McGaw et al. (1985) showed by selected ion-monitoring/isotope dilution MS that the method of Lizada and Yang appreciably underestimates ACC. The same or greater problems would be encountered in quantifying MACC.

In this paper, we report the development of a facile assay for ACC *N*-malonyltransferase activity. This assay was used to measure in vitro the developmental and tissue-specific patterns of activity of the enzyme in tomato fruit and to monitor activity during the purification of ACC *N*-malonyltransferase. We also report the purification and characterization of ACC *N*-malonyltransferase from the

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Abbreviations: AIB, α-aminoisobutyric acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid.

pericarp of ethylene-treated tomato fruit. We show that the ACC *N*-malonyltransferase from tomato fruit differs in several respects from the enzyme purified from mung bean (Guo et al., 1992, 1993).

MATERIALS AND METHODS

Plant Material

Fruit from tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig) were used throughout. Plants were grown in a greenhouse in the winter and in a greenhouse or in field plots during the summer. Fruit were harvested at the stage indicated for each experiment and treated as described below.

Assay of ACC *N*-Malonyltransferase

The assays contained, in a volume of 50 μL, enzyme, 100 mM Tris-Cl, pH 8.0, 1 mM DTT, 1 mM EDTA, 2 mM malonyl-CoA, and 2 mM [2,3-^{14}C]ACC (7.4 kBq/assay). [2,3-^{14}C]ACC with a specific activity of 1.97 GBq/mmol was obtained from Research Products International[2] (Mount Prospect, IL). The reactions were initiated with [^{14}C]ACC, incubated for 15 to 120 min at 30°C, and terminated by the addition of 50 μL of absolute ethanol or by boiling for 5 min. If chromatographic separation was not performed immediately, the assays were stored at −20°C. Usually, 5 μL of each terminated assay were spotted onto a lane of an Analtech (Newark, DE) HPTLC-GHLF high-performance normal phase silica TLC plate (10 × 20 cm) and rapidly separated using a solvent system of 1-propanol:ammonia (6:4, v/v) to resolve [^{14}C]ACC and [^{14}C]MACC. Prior to chromatography, assays containing crude protein extracts were centrifuged in the same microcentrifuge tubes in which the assays were performed for 5 min at 13,000*g* to pellet protein and other debris. When crude protein extracts were used in the assays, chromatography was performed on J.T. Baker reversed-phase octadecyl (C_{18}) TLC plates in a solvent system of 1-propanol:water (8:2, v/v). This was necessary to resolve MACC from a second metabolite of ACC (M.N. Martin, J.D. Cohen, and R.A. Saftner, unpublished results). Product and substrate were detected, quantified, and imaged simultaneously using an Ambis (San Diego, CA) model 4000 Radioisotope Image Acquisition and Analysis System. One unit of enzyme activity is defined as the amount catalyzing the formation of 1 nmol MACC min^{-1}.

In Vitro Activity of ACC *N*-Malonyltransferase

Seeds or maternal tissue from fruit at each stage of development or ripening were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The ground tissue was extracted with buffer A, which contained 100 mM Tris-Cl, pH 8.0, 2 mM EDTA, 5 mM DTT, 1 mM benzamidine, 1 mM 6-amino-*n*-hexanoic acid, 1 mM PMSF, 250 μM *N*-α-*p*-tosyl-L-arginine methyl ester, 250 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 3 μM pepstatin, and 1 μM leupeptin. Two milliliters of buffer per gram of maternal tissue and 5 mL buffer/g seed were used to prepare crude extracts. Assays were performed as described above using the crude extracts directly or after centrifugation for 10 min at 10,000*g*.

Ethylene Treatment

For ethylene treatment, whole fruit at the stage indicated were sealed for 12 to 16 h in a 37-L jar containing 120 μL/L ethylene and 50 mL of 10% KOH in a beaker. Controls were harvested and maintained unenclosed or enclosed in a jar with 50 mL of 10% KOH and 0.25 M $Hg(ClO_4)_2$ each in separate beakers. All treatments were at 20°C in the dark.

Purification of ACC *N*-Malonyltransferase

Tomato fruit were harvested at the mature green stage and treated with 120 μL/L ethylene for 12 to 16 h as described above. After treatment, 2 kg of pericarp were harvested and frozen in liquid nitrogen. All subsequent steps were performed at 5°C. The frozen pericarp was ground to a powder in a Waring blender, and the powdered tissue was extracted in 4 L of buffer A containing 2 g of Polyclar AT powder (GAF Corp., Wayne, NJ). The extract was filtered through Miracloth (Calbiochem) and fractionated by ammonium sulfate precipitation. Following addition of ammonium sulfate to 20% saturation, proteins and cell debris were pelleted by centrifugation for 15 min at 10,000*g*, and the pellet was discarded. Ammonium sulfate in the supernatant was increased to 65% saturation, and precipitated proteins were pelleted by centrifugation at 10,000*g* for 15 min.

The pellet was dissolved in one-fourth-strength buffer A, dialyzed against the same buffer using a membrane with a 14,000 molecular weight cutoff, centrifuged for 30 min at 150,000*g* to eliminate particulate material, and applied at a flow rate of 0.5 mL/min to an Amicon (Beverly, MA) DyeMatrex Green A column (2.5 × 30 cm), which was equilibrated in buffer B containing 25 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 1 mM DTT. This and subsequent columns were interfaced with a Pharmacia fast protein liquid chromatography system. The column was washed with buffer B to remove unbound protein, and the ACC *N*-malonyltransferase activity was eluted with a 0 to 2.0 M gradient of KCl in buffer B. Fractions having ACC *N*-malonyltransferase activity were pooled, dialyzed against buffer B, and loaded at a flow rate of 0.5 mL/min onto a Pharmacia Mono Q HR 16/10 strong anion-exchange column equilibrated with buffer B. The enzyme was eluted with a linear gradient of 0 to 0.3 M NaCl in buffer B. Fractions with ACC *N*-malonyltransferase activity were pooled, dialyzed to buffer B, and loaded at a flow rate of 0.25 mL/min onto a Cibacron Blue 1000–3GA-agarose (Sigma) column (0.5 × 6 cm) equilibrated in buffer B. The column was washed with buffer B and 2 mM ACC in buffer B, and the ACC *N*-malonyltransferase activity was eluted with buffer B containing 2 mM

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that might be suitable.

ACC and 2 mM malonyl-CoA. Fractions with ACC *N*-malonyltransferase activity were pooled, and ammonium sulfate was added to a concentration of 1 M. The enzyme was applied at a flow rate of 0.25 mL/min to a Pharmacia phenyl Superose 5/5 column equilibrated in 1 M ammonium sulfate in buffer B and eluted with a gradient of 1.0 M to 0 ammonium sulfate in buffer B. Active fractions were pooled, concentrated in an Amicon Centricon-10 concentrator, and applied to Pharmacia Superose 12 HR 10/30 and Superdex 75 HR 10/30 columns arranged in series and equilibrated with 100 mM Tris-Cl, pH 8.0, containing 1 mM EDTA and 1 mM DTT. After elution from these columns, active fractions were stored frozen at −75°C if not used immediately. Except where otherwise noted, enzyme eluted from the phenyl Superose column and having a specific activity of 5000 nmol min^{-1} mg^{-1} protein was used for all further characterization of the ACC *N*-malonyltransferase.

Protein concentrations were determined as described by Bradford (1976) using a Bio-Rad kit and BSA as a standard. Protein purity was determined by one-dimensional SDS-PAGE using gels with a 4 to 20% gradient of acrylamide (Laemmli, 1970). Proteins were detected by silver staining (Pharmacia). The molecular mass was estimated by gel filtration on a Pharmacia Superose 12 and a Superdex 75 column in series and calibrated with proteins of known molecular mass.

RESULTS

Assay of ACC *N*-Malonyltransferase Activity

We developed a protocol for the assay and quantification of ACC *N*-malonyltransferase activity that greatly reduced the time and labor required as compared to published procedures. The assay was performed in a volume of 50 μL and could be further scaled down, since only 5 μL of each assay were actually used for quantification. Up to 30 assays were spotted without further processing directly onto small, high-performance thin-layer silica gel plates and chromatographed in approximately 30 to 60 min to resolve the substrate, [^{14}C]ACC, and the product, [^{14}C]MACC. Both the ACC and MACC in 30 assays were imaged and quantified simultaneously in 30 to 120 min of detection time using an Ambis Radioisotope Image Acquisition and Analysis System.

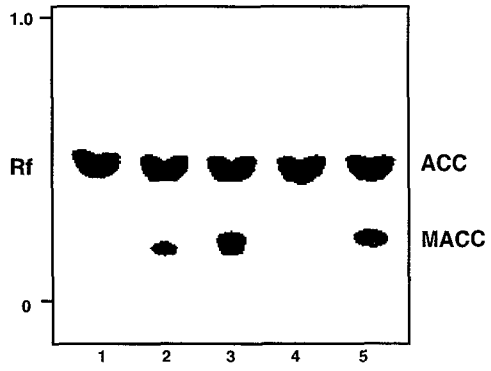

Figure 1. Radioisotope image showing the separation of [^{14}C]ACC and [^{14}C]MACC on a normal phase, high-performance, silica gel thin-layer chromatogram in a solvent system of 1-propanol:ammonia (6:4, v/v) following assays of ACC *N*-malonyltransferase activity in extracts from tomato seeds. Assays were performed as described in "Materials and Methods" with the components noted for each lane number: 1, heat-killed crude protein extract, ACC, and malonyl-CoA; 2, crude protein extract and ACC; 3, crude protein extract, ACC, and malonyl-CoA; 4, ammonium sulfate-precipitated protein and ACC; 5, ammonium sulfate-precipitated protein, ACC, and malonyl-CoA.

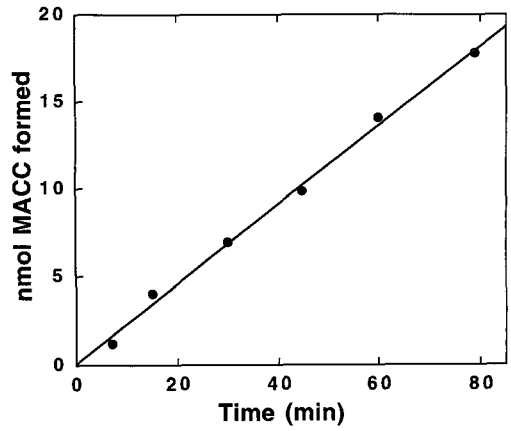

Figure 2. Time-dependent conversion of ACC to MACC. ACC *N*-malonyltransferase activity, assayed as described in "Materials and Methods," was linear for greater than 1 h. Enzyme eluted from the phenyl Superose column was used.

Figure 1 shows the resolution by high-performance TLC of [^{14}C]ACC and [^{14}C]MACC. Lane 1 shows that product formation did not occur with heat-denatured enzyme. Lanes 2 and 3 show the formation of [^{14}C]MACC by a crude tomato seed extract in the absence and presence, respectively, of malonyl-CoA. Product formation occurred without malonyl-CoA but was greater with malonyl-CoA. Lanes 4 and 5 show the formation of [^{14}C]MACC by dialyzed ammonium sulfate-precipitated protein in the absence and presence of malonyl-CoA; MACC formation, in this case, was absolutely dependent on malonyl-CoA. These results can be explained by the presence of a pool of malonyl-CoA in crude extracts, which is lost upon ammonium sulfate precipitation. However, addition of malonyl-CoA to assays containing ammonium sulfate-precipitated protein resulted in less than 50% recovery of activity. In fact, the apparent loss of ACC *N*-malonyltransferase activity is due to the removal of the substrate for a second ACC-conjugating activity (M.N. Martin, J.D. Cohen, and R.A. Saftner, unpublished results). Figure 2 shows that the activity measured by the new assay was linear with time for at least 1 h.

Developmental and Tissue-Specific Pattern of ACC *N*-Malonyltransferase Activity

ACC *N*-malonyltransferase activity was measured in extracts of seed, pericarp, placenta, and locular fluid at several stages throughout the development and ripening of tomato fruit (Fig. 3). In the pericarp, ACC *N*-malonyltransferase activity was greater than 100 pmol min^{-1} g^{-1} fresh

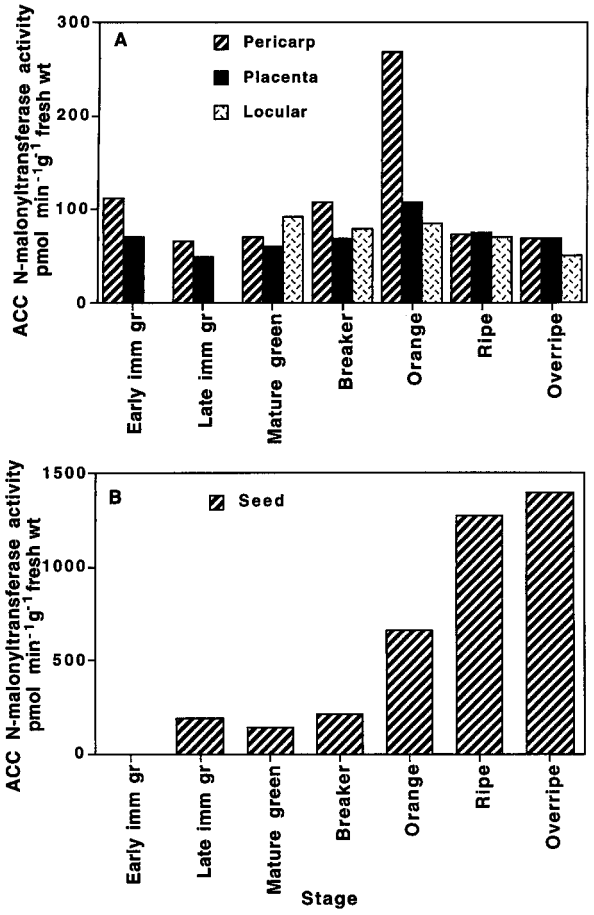

Figure 3. ACC *N*-malonyltransferase activity in developing and ripening tomato fruit. Maternal tissues (A) and seed (B) at each stage of development were extracted and assayed for ACC *N*-malonyltransferase activity as described in "Materials and Methods." Early immature green (imm gr) fruit were harvested 7 to 8 d postanthesis; late immature green fruit were harvested near the end of that developmental stage. Data shown were obtained using five fruit at each developmental stage and harvested at a single growing period. Each assay was performed in triplicate. Data from different growing seasons were obtained but not averaged.

weight in immature green fruit 7 to 9 d postanthesis but decreased to nearly one-half of that level and remained low until the onset of ripening (Fig. 3A). The level of activity began to increase with the onset of climacteric ethylene synthesis, reached a plateau at nearly 300 pmol min^{-1} g^{-1} fresh weight in orange fruit, and again declined in overripe fruit to the level found in green fruit. In the placenta and locular fluid, the level of ACC *N*-malonyltransferase activity was comparable to that in the pericarp of preclimacteric fruit and did not change appreciably in the course of fruit development and maturation (Fig. 3A). Activity in the seeds of immature green fruit was approximately 2-fold higher than the level in maternal tissues at the same developmental stage (Fig. 3B). However, soon after the onset of ripening, the level of activity increased dramatically to a plateau in the seeds of overripe fruit at a level 10-fold higher than the level found in the seeds of green fruit.

During the course of this investigation, the ACC *N*-malonyltransferase activity in seeds and all maternal tissues varied depending on growing conditions, but the developmental and spatial patterns of activity were maintained.

Effect of Ethylene on ACC *N*-Malonyltransferase Activity

Treatment of whole fruit (cv Ailsa Craig) with 120 μL/L ethylene for 12 to 16 h resulted in an increase in the ACC *N*-malonyltransferase activity by as much as 50- to 75-fold in immature green fruit and 100-fold in mature green fruit (Fig. 4). In contrast, stimulation of the ACC *N*-malonyltransferase activity was not observed in ethylene-treated climacteric fruit. Liu et al. (1985b) reported that treatment of mature green tomato fruit (cv T3) with 27 μL/L ethylene for 18 h resulted in a 4- to 5-fold increase in the in vivo conversion of ACC to MACC and a similar increase in the ACC *N*-malonyltransferase activity extractable from pericarp.

Purification of an ACC *N*-Malonyltransferase

Because ACC *N*-malonyltransferase activity is greatly increased in the pericarp of ethylene-treated green tomato fruit, this tissue was used for purification of the enzyme. Representative results are summarized in Table I for purification of approximately 10 μg of the enzyme with a final specific activity of 22,000 nmol min^{-1} mg^{-1} protein from 2 kg of ethylene-treated mature green pericarp.

More than 50% of the total soluble protein, the cell wall debris, and most of the pigments were pelleted from the pericarp extract and discarded after addition of ammonium

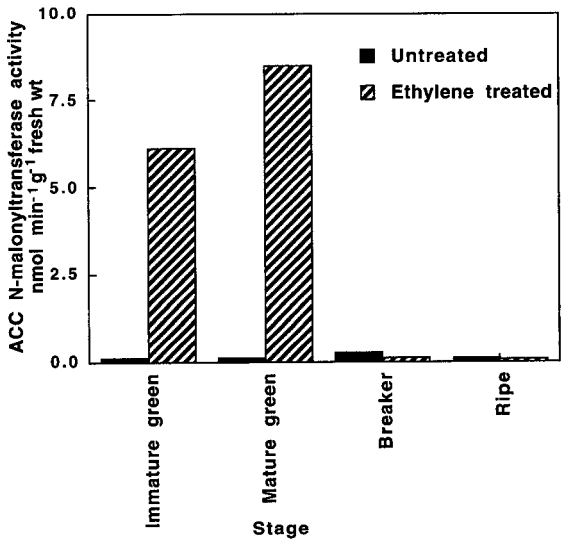

Figure 4. Effect of ethylene treatment on the ACC *N*-malonyltransferase activity. Whole fruit at each developmental stage were harvested and exposed to air or ethylene (120 μL/L) for 12 h. Pericarp was harvested, crude protein extracts prepared, and enzyme activity measured as described in "Materials and Methods." Data shown were obtained using five fruit at each developmental stage and harvested at a single growing period. Each assay was performed in triplicate. Data from different growing seasons were obtained but not averaged.

sulfate to 20% saturation followed by centrifugation. Greater than 100% of the ACC *N*-malonyltransferase activity found in the crude extract was consistently recovered in the pellet after precipitation of proteins with 20 to 65% saturation of ammonium sulfate, suggesting that an inhibitor was present in the crude extract. Demonstration of complete recovery of activity after ammonium sulfate precipitation required chromatography of the assays using the reverse-phase C_{18} system. This is because of the presence of a second ACC-conjugating activity (M.N. Martin, J.D. Cohen, and R.A. Saftner, unpublished results).

DyeMatrex Green A dye-ligand chromatography was used for the second step of purification because of its ability to accommodate crude extracts and to bind proteins requiring CoA derivatives as substrates. The ACC *N*-malonyltransferase activity bound to this column, and 80 to 90% of the activity was consistently recovered with a 40-fold purification. Activity was found in the 0.8 to 1.2 M fractions upon elution with a 0 to 2.0 M linear gradient of KCl. Nearly 95% of the protein applied to this column failed to bind. Similar results were obtained with Amicon DyeMatrex Red A and DyeMatrex Blue A and Cibacron Blue 3GA-agarose. A mixture of protease inhibitors was included in the buffer until the enzyme was applied to the first column to reduce the probability of purifying a proteolytic fragment of the ACC *N*-malonyltransferase.

A Mono Q strong anion-exchange column was used for the third purification step. The ACC *N*-malonyltransferase eluted between 0.08 and 0.12 M NaCl in the 0 to 0.3 M gradient with an overall purification of 215-fold and a yield of 36%. More than 90% of the protein applied to this column either did not bind or eluted much later in the gradient of NaCl.

Cibacron Blue 1000–3GA-agarose was used for the fourth purification step. The fractions from the Mono Q column were applied after brief dialysis against buffer B and were eluted with 2 mM ACC plus 2 mM malonyl-CoA with a purification of 600-fold and an overall recovery of 16%. Elution was not possible with ACC alone. Elution of the enzyme from Cibacron Blue 3000–3GA, DyeMatrex Red A, and DyeMatrex Green A was not possible with the two substrates. Binding of the ACC *N*-malonyltransferase to Cibacron Blue 300- or 100–3GA was not quantitative.

A phenyl Superose HR 5/5 column was used for the fifth purification step. The ACC *N*-malonyltransferase activity from the Cibacron Blue column was pooled and applied directly in 1.0 M ammonium sulfate to this column and eluted at 0.5 M ammonium sulfate in a 1.0 to 0 M ammonium sulfate gradient. An overall purification of 5100-fold was obtained with an overall recovery of 13%. Most of the protein applied to this column did not bind.

The final purification step utilized molecular filtration on Superose 12 and Superdex 75 in series. The Superose 12 column alone provided little additional purification, but the two columns together separated the ACC *N*-malonyltransferase from approximately 90% of the protein applied to the column. At this stage the ACC *N*-malonyltransferase was purified at least 22,000-fold with a recovery of 4% overall and a specific activity of 22,000 nmol min^{-1} mg^{-1} protein.

Properties of ACC *N*-Malonyltransferases

Following purification (as summarized in Table I) the ACC *N*-malonyltransferase appeared as a major 38-kD band and a minor 32-kD band when analyzed by SDS-PAGE followed by silver staining (Fig. 5). By gel-filtration chromatography on Superose 12 and Superdex 75 columns, the molecular mass of the enzyme was estimated to be 38 kD (Fig. 6), suggesting that the enzyme is monomeric. In contrast, the ACC *N*-malonyltransferase from mung bean hypocotyl was reported to have a native and subunit molecular mass of 55 kD (Guo et al., 1992).

The ACC *N*-malonyltransferase exhibited a broad pH optimum between 8.0 and 8.5 (data not shown), similar to that reported for the ACC *N*-malonyltransferase from mung bean (Guo et al., 1992).

The activity of ACC *N*-malonyltransferase as a function of various ACC concentrations at a fixed malonyl-CoA concentration of 2 mM exhibited Michaelis-Menten kinetics with a K_m for ACC of 500 μM (Fig. 7). Figure 8A shows the activity of ACC *N*-malonyltransferase at varied malonyl-CoA concentrations and a fixed ACC concentration of 2 mM. At malonyl-CoA concentrations higher than 0.8 mM, activity was inhibited. From this plot, the K_m for malonyl-CoA was estimated to be 100 μM (Fig. 8A). Based on a

Table I. *Purification of an ACC N-malonyltransferase from tomato pericarp*

Purification Step	Total Protein	Total Activity	Specific Activity	Purification	Recovery[a]
	mg	*units*[b]	*units/mg*	*-fold*[c]	%
Crude extract	4,367	4,298	0.984	1	100
Ammonium sulfate (20–65%)	2,070	5,468	2.65	3	127(100)
Dye-Matrex Green A	115	4,423	38.6	39	81
Pharmacia Mono Q	9.37	1,985	211	215	36
Cibacron Blue 3GA	1.39	857	618	628	16
Phenyl Superose HR 5/5	0.14	713	5,093	5,180	13
Molecular filtration	0.01[d]	220	22,000	22,350	4

[a] Yield is based on activity in the ammonium sulfate precipitate, since that is greater than activity in the crude extract. [b] One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 nmol MACC min^{-1}. [c] Purification values are based on total activity in a crude extract prepared from 2 kg of ethylene-treated pericarp. [d] Protein was estimated based on A_{280}.

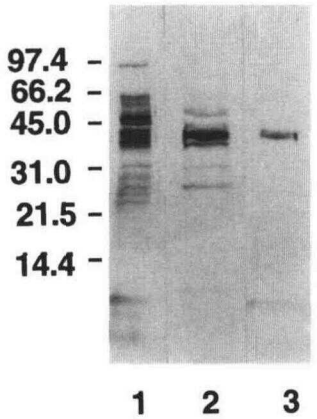

Figure 5. Silver-stained SDS-PAGE of ACC *N*-malonyltransferase at steps during the purification scheme. Fractions containing ACC *N*-malonyltransferase activity after Cibacron Blue (lane 1), phenyl Superose (lane 2), and molecular filtration (lane 3). Lanes 1, 2, and 3 contained 750, 250, and 50 ng of protein, respectively. Molecular masses in kD of standard proteins are designated at the left.

Lineweaver-Burk plot of the same data (Fig. 8B), the K_m was estimated to be 200 μM.

Requirements for Activity

The partially purified ACC *N*-malonyltransferase from mung bean hypocotyl was reported to be stimulated 2- to 4-fold by the salts listed in Table II at a concentration of 0.1 M (Kionka and Amrhein, 1984). In contrast, the ACC *N*-malonyltransferase from tomato fruit was inhibited by these salts (KCl, KNO_3, KBr, NaCl, and $CaCl_2$) at concentrations of 0.1 M. These salts also were inhibitory when included in assays using crude protein extracts. $MnCl_2$ at 1 mM and $ZnCl_2$ at 0.1 mM also were inhibitory, probably because of binding of the divalent metal to reduced sulfhydryl groups. This is in agreement with the report by Su et al. (1985) that the partially purified enzyme from mung bean was inhibited by heavy metals and several reagents known to inactivate sulfhydryl groups.

DTT and EDTA were included at all stages in the purification and in the assay. Omission of DTT and EDTA during the initial extraction resulted in recovery of less than 25% of the activity obtained with DTT and EDTA

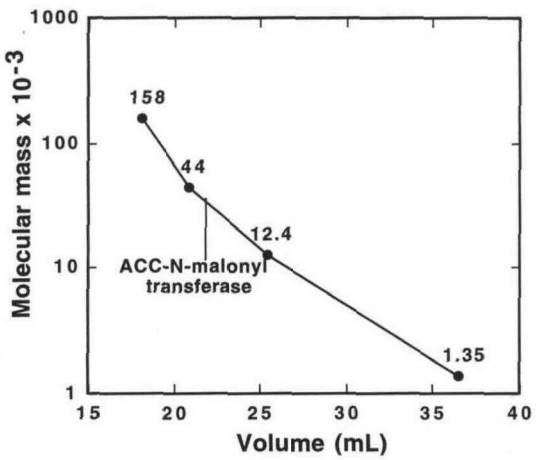

Figure 6. Molecular mass of ACC-*N*-malonyltransferase determined on Pharmacia Superose 12 HR 10/30 and Superdex 75 HR 10/30 columns in series. Elution volumes of proteins of known molecular mass (monitored by A_{280}) are indicated.

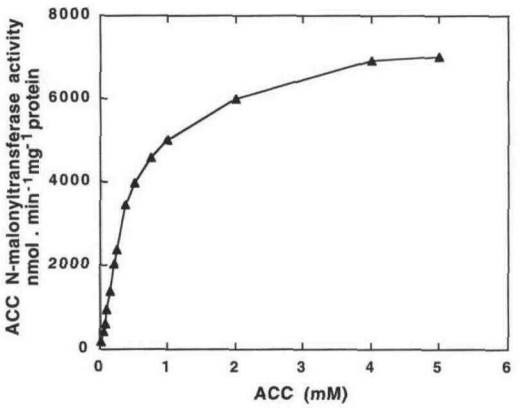

Figure 7. ACC *N*-malonyltransferase activity as a function of ACC concentration. Thirty-minute assays were performed as described in "Materials and Methods" with a malonyl-CoA concentration of 2.0 mM.

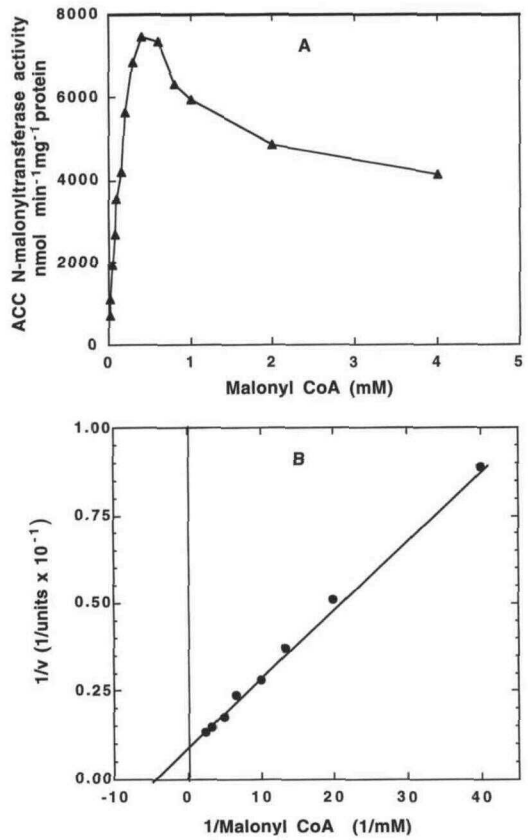

Figure 8. ACC *N*-malonyltransferase activity as a function of malonyl CoA concentration. A Michaelis-Menten plot of the data is shown in A and a Lineweaver-Burk plot of the same data is shown in B. Thirty-minute assays were performed as described in "Materials and Methods" with an ACC concentration of 2 mM.

present (data not shown). Transfer of the enzyme to buffer lacking DTT and EDTA demonstrated that activity was stimulated nearly 2-fold by the presence of 10 mM DTT, suggesting that reduced sulfhydryl groups are required for activity. The activity also was stimulated 2.5-fold by 1 mM EDTA, suggesting that metals may be binding to the enzyme and are inhibitory. Exhaustive dialysis to buffer lacking DTT and EDTA resulted in complete loss of activity, which was not recovered by prolonged incubation with DTT and EDTA.

Substrate Specificity

In addition to malonyl-CoA, succinyl-CoA served as a substrate for conjugation of ACC at a rate 40% of that observed with malonyl-CoA. Acetyl-CoA and *n*-butyryl-CoA did not serve as substrates for conjugation of ACC.

Reports regarding the amino acid specificity of amino acid *N*-malonyltransferases from plants are contradictory. Since ACC is a neutral, optically inactive amino acid, we examined the amino acid specificity of the ACC *N*-malonyltransferase that we purified from tomato pericarp. Eighteen D-amino acids (Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, and His) were tested for inhibition of ACC *N*-malonyltransferase activity. Although the concentration of each D-amino acid (1.0 mM) was 2-fold higher than the concentration of ACC in the assay, the percentage of ACC-dependent activity remaining was between 78 and 100%. Representative examples are shown in Table III. No significant inhibition was observed with seven L-amino acids (Phe, Trp, Met, Ala, His, Leu, and Pro). Again, representative results are shown in Table III. Gly and the putative ACC analog, AIB, both of which lack asymmetric carbons, failed to inhibit MACC formation by the ACC *N*-malonyltransferase. When the purified ACC *N*-malonyltransferase was incubated with ^{14}C-labeled AIB, L-Phe, L-Ala, L-Met, or L-Glu, no product was detected.

Table II. *Effect of salts of alkali and divalent metals on the activity of ACC N-malonyltransferase*

Assays were performed as described in "Materials and Methods" with DTT and EDTA omitted from the assay buffer and with the additions noted below. The enzyme was stored in buffer B, which contained 1 mM of DTT and EDTA; thus final concentration of both DTT and EDTA when diluted into the assays was 0.1 mM.

Addition	Percentage ACC *N*-Malonyltransferase Activity
None (control)	100
KCl (0.1 M)	77
KCl (10 mM)	99
KNO_3 (0.1 M)	33
KBr (0.1 M)	50
$CaCl_2$ (0.1 M)	25
$CaCl_2$ (10 mM)	70
NaCl (0.1 M)	77
$MgCl_2$ (0.1 M)	40
$MgCl_2$ (10 mM)	101
$MnCl_2$ (1 mM)	73
$ZnCl_2$ (0.1 mM)	4

Table III. *Effect of other amino acids on the activity of ACC N-malonyltransferase*

Assays were performed as described in "Materials and Methods" except the unlabeled amino acid was added 5 min prior to addition of [^{14}C]ACC and both ACC and malonyl-CoA concentrations were reduced to 0.5 mM.

Amino Acid (1 mM)	Percentage ACC *N*-Malonyltransferase Activity
None	100
D-Phe	78
D-Trp	96
D-Met	85
D-Ala	90
D-His	86
L-Phe	108
L-Trp	102
L-Met	100
L-Ala	94
L-His	89
AIB	98
Gly	98

^{14}C-labeled D-amino acids are not commercially available and thus were not tested.

DISCUSSION

In this paper, we describe a facile method for quantifying the ACC *N*-malonyltransferase activity that eliminates much of the time and labor associated with published protocols (Lizada and Yang, 1979; Kionka and Amrhein, 1984). We also describe (a) the purification of an ethylene-regulated ACC *N*-malonyltransferase from fruit; (b) an ACC *N*-malonyltransferase with specificity for ACC rather than D- or L-amino acids; and (c) developmental and tissue-specific patterns of expression of an ACC *N*-malonyltransferase, an enzyme that previously has been described as constitutive (Amrhein et al., 1982; Kionka and Amrhein, 1984).

Tomato fruit proved to be a good source of the enzyme for large-scale purification if the appropriate tissue and stage of development were chosen (Fig. 3). However, we encountered difficulty in selecting a large number of fruit at just the breaker to orange stage, when activity is highest, and we encountered considerable seasonal variation in activity. For these reasons, we chose to use pericarp from ethylene-treated green fruit, in which the activity was reproducibly stimulated 50- to 100-fold (Fig. 4). Even in that tissue, we estimate that the ACC *N*-malonyltransferase represented no more than 0.006% of the total soluble protein. Ammonium sulfate precipitation not only served as a very effective first purification and concentration step but removal of the enzyme from the crude extract actually stimulated activity. In contrast, other researchers reported loss of malonyltransferase activity from mung bean extracts upon ammonium sulfate precipitation (Su et al., 1985; Guo et al., 1992). Instead, Guo et al. (1992) effectively used PEG as a first fractionation step. We did not obtain effective fractionation and purification of the ACC *N*-malonyltransferase activity from tomato fruit extracts with

PEG. The inhibition by ammonium sulfate reported by other researchers is likely the result of removal of the substrate for a second ACC-conjugating activity from the crude extract (M.N. Martin, J.D. Cohen, and R.A. Saftner, unpublished results).

Guo et al. (1992) reported obtaining a 55-kD ACC *N*-malonyltransferase purified to homogeneity after elution of the enzyme from a Cibacron Blue 3GA-agarose column with the substrates ACC and malonyl-CoA as the final purification step. In contrast, we found that the ACC *N*-malonyltransferase from tomato represented only 1% of the protein eluted from a Cibacron Blue column with the two substrates. The two subsequent purification steps each provided nearly 10-fold additional purification and yielded a protein with a native and subunit molecular mass of 38 kD.

The purified ACC *N*-malonyltransferase from tomato fruit showed K_m values for ACC and malonyl-CoA (500 and 200 μM, respectively) identical to those reported for the purified enzyme from mung bean hypocotyls, but the specific activity was nearly 20-fold higher than that reported for the purified enzyme from mung bean. Unlike the enzyme from mung bean, the ACC *N*-malonyltransferase from tomato was not stimulated or stabilized by salts.

During the past 30 years, endogenous *N*-malonyl conjugates of ACC and D-amino acids have been isolated from a number of plant tissues, especially seeds (Good and Andreae, 1957; Zenk and Scherf, 1963; Keglevic et al., 1968; Rosa and Neish, 1968; Ladesic et al., 1971; Fukuda et al., 1973; Amrhein et al., 1981, 1982; Hoffman et al., 1982, 1983; Kawasaki et al., 1982; Liu et al., 1983). Whether a single *N*-malonyltransferase catalyzes the reactions with all D-amino acids and ACC is unknown. However, there are several reports that tissue extracts that malonylate ACC also malonylate D-amino acids, and D-amino acids competitively inhibit the malonylation of ACC (Liu et al., 1983, 1984, 1985a, 1985b; Kionka and Amrhein, 1984; Su et al., 1985). Likewise, ethylene treatment of mature green tomato fruit, which resulted in an increased in vivo level of MACC and increased in vitro ACC *N*-malonyltransferase activity, also resulted in increased in vivo conversion of [^{14}C]D-Phe and [^{14}C]D-Met to *N*-malonyl conjugates and increased in vitro conversion of AIB to the *N*-malonyl conjugate (Liu et al., 1985b). These observations led to the suggestion that a single enzyme is responsible for both activities (Liu et al., 1985b). Recently Guo et al. (1993) reported that D-Phe *N*-malonyltransferase and ACC *N*-malonyltransferase from mung bean hypocotyl co-purify to near homogeneity as a single, 55-kD protein and that both activities are similarly inhibited by other D-amino acids. They further reported that the K_m for D-Phe was 10-fold lower than the K_m for ACC (48 versus 500 μM). However, Silverstone et al. (1992) reported partial purification of distinct D-Trp and ACC *N*-malonyltransferases from peanut cultures. The purified ACC *N*-malonyltransferase from the pericarp of ethylene-treated tomato fruit is specific for ACC. It is not inhibited by a 2-fold excess of L- or D-amino acids (Table III), whereas the ACC *N*-malonyltransferase from mung bean was inhibited as much as 80% when ACC and D-amino acid concentrations were equal (Guo et al., 1993). Likewise, with the purified enzyme we failed to see malonylation of AIB, unlike what was reported by Liu et al. (1985b) for crude protein extracts of ethylene-treated tomato pericarp.

Establishing the substrate specificity of amino acid *N*-malonyltransferases is further complicated by recent reports by Sakagami et al. (1993a, 1993b) that the absolute stereochemistry of the Trp and 4-chlorotryptophan found in endogenous *N*-malonyl-4-chlorotryptophan and *N*-malonyltryptophan should be reassigned to the L configuration. They further reported that the ratio of L to D configuration of the *N*-malonyltryptophan varied depending on the tissue source. Similarly, Liu et al. (1984) reported that mung bean hypocotyls and tissue extracts of hypocotyls malonylated all four stereoisomers of the ACC analog, 1-amino-2-ethylcyclopropane-1-carboxylic acid. However, the two isomers having an *R* configuration as D-amino acids exhibited lower K_m values and were preferentially malonylated. In contrast, the purified ACC *N*-malonyltransferase from tomato was unable to conjugate neutral L-amino acids.

ACC *N*-malonyltransferase activity in tomato fruit was found primarily in the pericarp and seeds. In both cases the pattern of expression was clearly developmentally regulated. Activity in the pericarp mirrored the developmental pattern of ethylene evolution by the whole fruit. Activity was elevated several days postanthesis, declined, increased dramatically with the onset of ripening, and again declined in the ripe fruit. Similarly, treatment of whole green fruit with ethylene dramatically stimulated the activity of the ACC *N*-malonyltransferase in the pericarp. Whether the increase in ACC *N*-malonyltransferase activity in the seed of ripe fruit also can be correlated with increased ethylene biosynthesis in the seed remains to be determined. Activity in the seed reached a plateau well after the climacteric burst of ethylene had declined in the pericarp.

Further research is needed to define the pattern of expression of ACC *N*-malonyltransferase in tomato. It is very likely that the complexity of the regulation of ACC *N*-malonyltransferase in tomato fruit is as great as that of ACC synthase and ACC oxidase. Both ACC synthase and ACC oxidase are encoded by multigene families that are differentially activated by developmental, environmental, and hormonal factors (Van Der Straeten et al., 1990; Yip et al., 1992; Nadeau et al., 1993).

ACKNOWLEDGMENTS

The authors wish to thank Drs. Jerry Cohen and Janet Slovin for critically reading the manuscript.

Received December 27, 1994; accepted April 10, 1995.

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