

Properties and Partial Purification of 1-Aminocyclopropane-1-carboxylate Synthase¹

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ABSTRACT

We studied the regulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase activity in tomato (*Lycopersicon esculentum* Mill.) fruit tissue and attempted the purification of this enzyme. The increase of ACC synthase activity in wounded tomato pericarp was inhibited by cordycepin and cycloheximide. Density labeling studies showed a 0.75% increase in the buoyant density of ACC synthase isolated from tomato pericarp tissue that had been incubated on ²H₂O as compared to ACC synthase from H₂O-treated tissue. These data are consistent with the hypothesis that ACC synthase is synthesized *de novo* following wounding of tomato pericarp tissue. SDS-gel electrophoresis and fluorography showed that the pattern of incorporation of L-[³⁵S]methionine into protein changed with time after wounding of the tissue. Radioactive protein bands that were not detected 1 hour after wounding, became apparent 2 to 3 hours after wounding.

Gel filtration on Sephadex G-100 gave a molecular weight estimate for ACC synthase of 57,000 ± 1,500 daltons. Hydrophobic interaction chromatography on phenyl-Sepharose yielded a 60- to 70-fold purification of the enzyme. SDS-gel electrophoresis of this preparation indicated the presence of one intense band at 57,000 daltons and several less intense bands. Affinity chromatography was of limited usefulness in the purification of ACC synthase since the enzyme could not be eluted specifically from any of the affinity gels tried. Purification methods that involved pH changes led to a rapid loss of ACC synthase activity. ACC synthase was estimated to comprise less than 1% of the total protein in tomato pericarp tissue.

Ethylene biosynthesis can be induced by a variety of stresses, including wounding, drought or waterlogging, and noxious chemicals (1, 13). It has been shown that wound-induced ethylene biosynthesis in tomato fruits is controlled at the level of ACC² synthase (3, 8, 18), the enzyme that converts SAM to the immediate ethylene precursor ACC (4, 19). Both the activity of ACC synthase and the level of ACC rise after wounding. This indicates that the rate-limiting step in stress-ethylene synthesis is the formation of ACC. The wound-induced increase in ACC synthase activity can be inhibited by cycloheximide (8, 18). This observation indicates that wound-induced ACC synthase may be synthesized *de novo*. However, cycloheximide is also capable of perturbing processes other than protein synthesis, *e.g.* energy transfer reactions (15), and care should be taken in the interpretation of results obtained with this inhibitor. Therefore, the regulation of ACC synthase activity required further investigation. This and further

purification of ACC synthase were the aims of the experiments described in this publication.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill., breeding line 61-37, Fireball × Cornell 53-149) were grown in controlled environment chambers under a 16-h photoperiod (350 μE m⁻² s⁻¹) at day and night temperatures of 24 and 21°C, respectively. Six pericarp discs, 11.5 mm in diameter and trimmed to a thickness of 3 mm by removing tissue that originally faced the inside of the fruit, were used per treatment (3.00 ± 0.06 g fresh weight of tissue). Each disc was further divided into 12 sectors, and the sectors from six such discs were incubated in a Petri dish (60-mm diameter) on filter paper wetted with 1 ml of water or test solution at 28°C in darkness. For partial purification of ACC synthase, 40 to 60 g of pink (4) tomato tissue was sliced into sections (5 × 1 mm) and incubated in two Petri dishes (150-mm diameter) on filter paper wetted with 2 ml of water.

Chemicals. 2'-Deoxyadenosine was purchased from P-L Biochemicals Inc., Milwaukee, WI; L-[³⁵S]methionine was from Amer-sham Co., Arlington Heights, IL, ²H₂O was from Aldrich Chemicals, Milwaukee, WI, and Hepes buffer was from Research Organics Inc., Cleveland, OH. Column chromatographic material was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, except for the S-adenosylhomocysteine affinity gel which was purchased from Bethesda Research Laboratories Inc., Gaithersburg, MD. Catalase (EC 1.11.1.6) and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Extraction of ACC Synthase. Tissue was frozen in liquid N₂ at the end of the 3-h incubation period and stored at -20°C for a maximum of 14 h. Frozen tissue was powdered with a pestle in a mortar. The powder was homogenized in 100 mM Hepes-KOH buffer, pH 8.0, containing 4 mM DTT, 0.4 μM pyridoxal phosphate, and 5% (w/v) PVP 40,000 using a glass homogenizer with a motor-driven Teflon plunger. Homogenization buffer was used at a ratio of 1 ml/g fresh weight of tissue. The homogenate was centrifuged at 20,000g for 15 min. The supernatant was brought to 30% saturation with (NH₄)₂SO₄ and centrifuged at 20,000g for 15 min. The resulting supernatant was brought to 95% saturation with (NH₄)₂SO₄ and centrifuged again at 20,000g for 15 min. The precipitate was dissolved in 10 mM Hepes-KOH buffer, pH 8.0, containing 0.1 mM DTT and 0.2 μM pyridoxal phosphate and dialyzed overnight against three changes of this buffer. This buffer, with additions as specified, was also used as the elution buffer in column chromatography and will be referred to as either dialysis or elution buffer. The above operations were performed at 0 to 4°C.

ACC Synthase Assay. ACC synthase activity was determined according to Boller *et al.* (4). ACC formed in this assay was measured as described by Yu *et al.* (19). Ethylene was determined with a Varian Aerograph 2400 gas chromatograph according to Kende and Hanson (9). One unit of ACC synthase activity is

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² Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosylmethionine; AOA, aminoxyacetic acid; AVG, aminoethoxyvinylglycine.

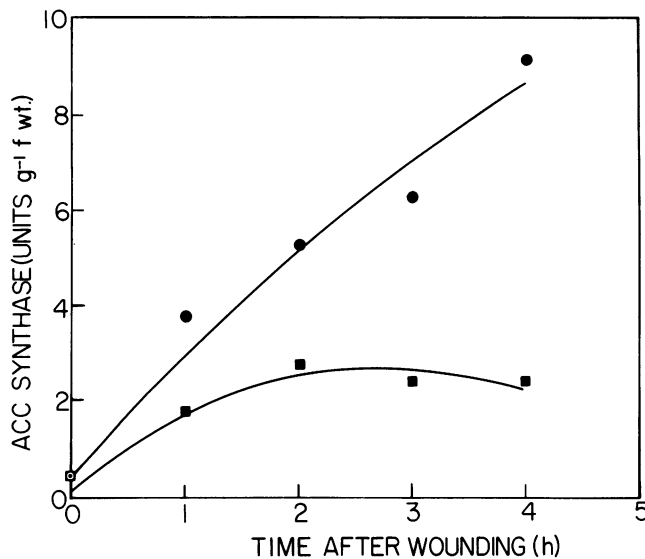


FIG. 1. Effect of cordycepin on ACC synthase activity in pink tomato pericarp tissue. Sectors were placed on filter paper wetted with 1 ml of H₂O (●) or a solution of 100 μg ml⁻¹ of cordycepin (■) in a Petri dish. They were vacuum infiltrated three times for 10 s each with the bathing medium and were incubated at 28°C in darkness for the times indicated. The tissue was extracted with homogenization buffer without PVP, centrifuged at 20,000g for 15 min, and dialyzed overnight against a total of 2 L of dialysis buffer (three buffer changes) prior to assaying for ACC synthase activity.

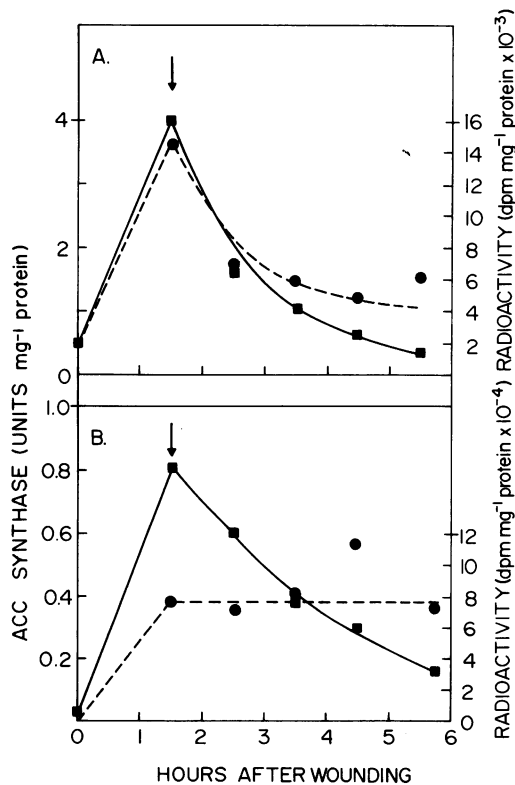


FIG. 2. Effects of cycloheximide on ACC synthase activity and on L-[³⁵S]methionine incorporation into protein in: A, pericarp tissue from pink fruit, and B, green fruit. Sectors of pericarp discs were incubated in 0.3 ml water in a Petri dish (60-mm diameter) for 90 min after which 0.9 ml of water containing 37.5 μg cycloheximide was added (indicated by the arrow). (■), ACC synthase activity; (●), radioactivity, both in the 30 to 95% saturated (NH₄)₂SO₄ fraction.

defined as the formation of 1 nmol of ACC/h at 30°C.

Density Labeling Studies. Density labeling studies were performed according to Zielke and Filner (20). Eighteen pericarp discs (9.12 ± 0.20 g fresh weight), subdivided into 12 sectors each, were incubated on filter paper wetted with 4 ml of H₂O or ²H₂O (99.8%) in Petri dishes (100-mm diameter) at 28°C in darkness for 5 h. The sectors were extracted, and the extracts were fractionated by precipitation with (NH₄)₂SO₄ and dialyzed as described above. Homogenization and dialysis in aqueous media eliminated carry-over of exchangeable ²H onto the density gradients (2). Each dialyzed preparation was divided into three 2-ml aliquots. These were thoroughly mixed in 4-ml nitrocellulose centrifuge tubes with 1 ml of a saturated CsCl solution (pH 8.0) containing 1,500 units of catalase as internal density marker. The contents of the centrifuge tubes were overlaid with mineral oil and centrifuged in a Beckman L-2 ultracentrifuge at 157,000g_{av} using a swinging bucket rotor (SW 56 at 40,000 rpm) for 72 h. After centrifugation, the tubes were punctured at the bottom, and fractions were collected. Alternate fractions were assayed for ACC synthase and catalase activities, respectively. The refractive index of every 10th fraction was determined with a Bausch and Lomb Abbe-32 refractometer. Refractive indices were converted to densities by reference to a standard curve. Catalase activity was assayed by the method of Sinha (16). One unit of catalase activity is defined as the decomposition of 1 μmol of H₂O₂/min at pH 7.0 and 25°C.

Hydrophobic Interaction Chromatography. The resuspended 30 to 95% (NH₄)₂SO₄ fraction was brought to 10% saturation with (NH₄)₂SO₄ and loaded directly onto the top of a phenyl-Sepharose column (150 × 9 mm), which had been equilibrated previously

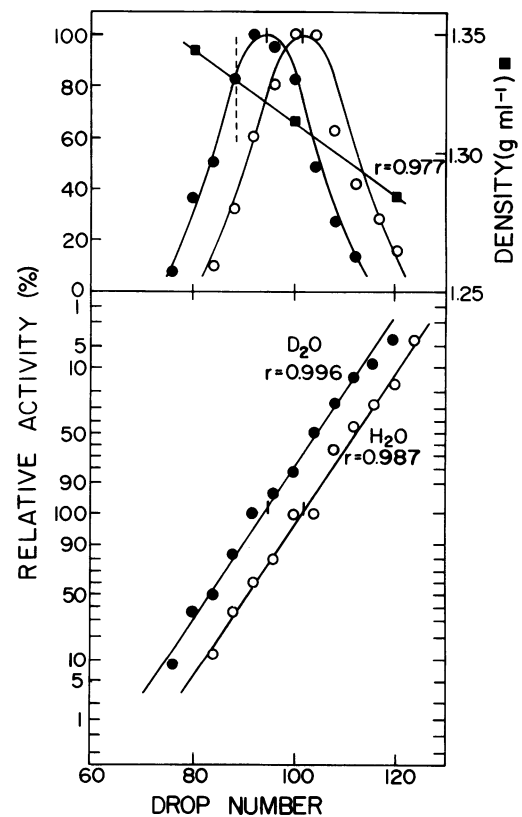


FIG. 3. Buoyant density of ACC synthase from pericarp sectors that had been incubated on either H₂O (○) or 99.8% ²H₂O (●). The centers of the enzyme activity peaks were determined on Gaussian graph paper (lower graph). The distribution curves of ACC synthase activity were aligned by superimposing the catalase peaks. The buoyant density of catalase in both gradients was 1.331 g ml⁻¹. The center of the catalase peaks is indicated by the broken, vertical line.

with 10% $(\text{NH}_4)_2\text{SO}_4$ in dialysis buffer. The column was eluted stepwise at a flow rate of 40 ml/h with 0.5 M NaCl in dialysis buffer, 0.1 M NaCl in dialysis buffer, and finally with dialysis buffer only. The absorbance of the effluent was monitored continuously at 280 nm. Fractions of 6 ml were collected.

Gel Filtration. The resuspended 30 to 95% $(\text{NH}_4)_2\text{SO}_4$ fraction was loaded onto a Sephadex G-100 column previously equilibrated with dialysis buffer. The column was eluted with more dialysis buffer at a pressure of 85 cm H_2O . The absorbance of the effluent was monitored continuously at 280 nm. Fractions of 6 ml were collected. The column was calibrated with BSA (mol wt 67,000), ovalbumin (mol wt 43,000), carbonic anhydrase (mol wt 30,000), and lysozyme (mol wt 14,000).

Gel Electrophoresis and Fluorography. One-dimensional SDS-gel electrophoresis of proteins on gradients of 5 to 15% (w/v) polyacrylamide was performed according to Laemmli (12). Proteins were stained in a solution of 0.1% (w/v) Coomassie blue, 50% (v/v) methanol, and 7% (v/v) glacial acetic acid. The gels were destained in 20% (v/v) methanol, 7% (v/v) glacial acetic acid, and 3% (v/v) glycerol. The destained gels were prepared for fluorography by soaking in EN³HANCE (New England Nuclear, Boston, MA), dried, and placed onto Kodak SB-5 x-ray film. The film was exposed at -76°C for 21 to 28 d.

Protein Determination. Protein was determined according to Bradford (5) using the Bio-Rad protein assay mixture (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Effects of Cordycepin. ACC synthase activity increased in wounded tomato pericarp of pink tomato fruits. This increase in enzyme activity was reduced by 56% in pericarp tissue that had been incubated in $100 \mu\text{g ml}^{-1}$ cordycepin for 2 h (Fig. 1). Cordycepin at $12.5 \mu\text{g ml}^{-1}$ inhibited the development of ACC synthase activity by 20% after 2 h of incubation. 2'-Deoxyadenosine, an inactive analog of cordycepin, did not affect the wound-induced increase in ACC synthase activity (results not shown).

Effects of Cycloheximide. Discs from both pink and green fruits showed the normal rise in ACC synthase activity as a result of wounding (Fig. 2), the pink fruits developing nearly five times as much enzyme activity as the green fruits. Following the addition

of cycloheximide, ACC synthase activity declined in both tissues as expected (8). Pericarp tissue from green fruits incorporated 10 times as much L-[³⁵S]methionine into protein as that from pink fruits. After the addition of cycloheximide, the radioactivity incorporated into protein of pink fruits declined in parallel with the activity of ACC synthase. The radioactivity incorporated into the protein of the green fruits remained essentially at the same level after the addition of cycloheximide.

Density Labeling. Density labeling was used to distinguish between activation of an ACC synthase precursor and *de novo* synthesis of this enzyme (7). Wounded pericarp tissue was incubated on $^2\text{H}_2\text{O}$ or H_2O , and the buoyant density of ACC synthase was determined by isopycnic equilibrium centrifugation in CsCl. Catalase was added to each centrifuge tube as an internal density marker. The centers of the ACC synthase and catalase peaks were determined by plotting the distribution of the enzyme activities on Gaussian graph paper. The activity distribution curves of ACC synthase were aligned by superimposing the catalase peaks of the respective gradients. The buoyant density of ACC synthase isolated from H_2O -treated pericarp discs was $1.316 \pm 0.001 \text{ g ml}^{-1}$ compared with $1.326 \pm 0.002 \text{ g ml}^{-1}$ for ACC synthase from $^2\text{H}_2\text{O}$ -treated pericarp discs (Fig. 3). The average density increase of [²H] ACC synthase was 0.75%.

Affinity Chromatography. A variety of Sepharose derivatives were prepared using the ACC synthase inhibitor AOA as ligand. AOA was linked either through its $-\text{NH}_2$ group or its $-\text{COOH}$ group to CNBr-activated Sepharose 4B. Linkage through the $-\text{NH}_2$ group was either directly onto the activated Sepharose or via a linear five-carbon spacer arm (ϵ -amino-*n*-caproic acid). Linkage through the $-\text{COOH}$ group was via a linear six-carbon spacer arm (1,6-hexenediamine). An affinity gel using AVG as the ligand was also synthesized. AVG was linked to the CNBr-activated Sepharose through its $-\text{COOH}$ group via a six-carbon spacer arm. An *S*-adenosylhomocysteine affinity gel was also tested. ACC synthase bound only to the *S*-adenosylhomocysteine gel and to those gels with 1,6-hexenediamine spacer arms. Once bound, ACC synthase was not eluted with dialysis buffer containing $50 \mu\text{M}$ SAM but was released from the column by elution with 0.5 M NaCl in dialysis buffer. The enzyme eluted along with 40% of the protein and only a 2- to 3-fold purification over the original sample was obtained. Overall recovery of protein was 90%, and recovery of enzyme

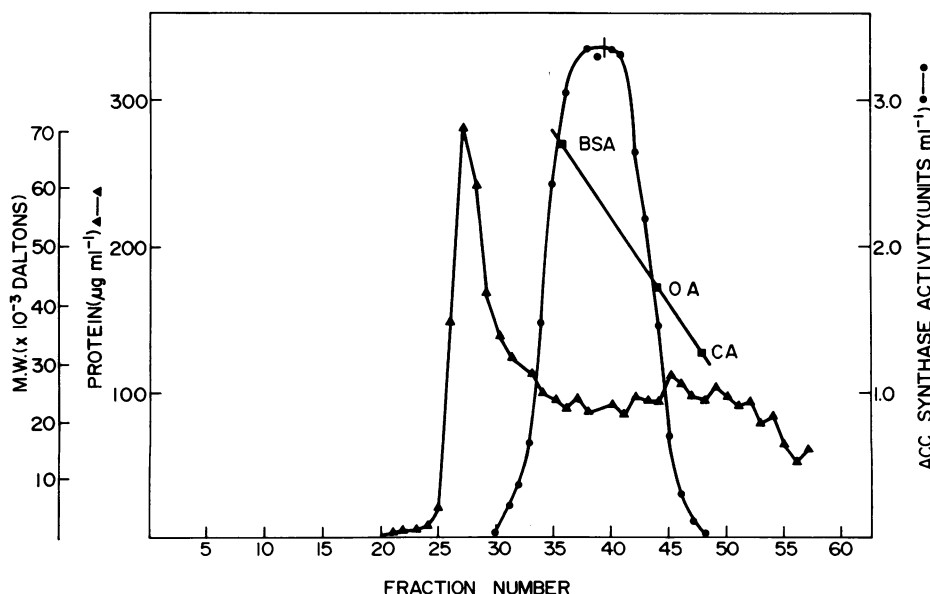


FIG. 4. Elution profile from a Sephadex G-100 column that had been calibrated with BSA (mol wt 67,000), ovalbumin (OA, mol wt 43,000), and carbonic anhydrase (CA, mol wt 30,000). Twenty mg of protein in 4 ml of dialysis buffer were loaded onto a Sephadex G-100 column and eluted with dialysis buffer.

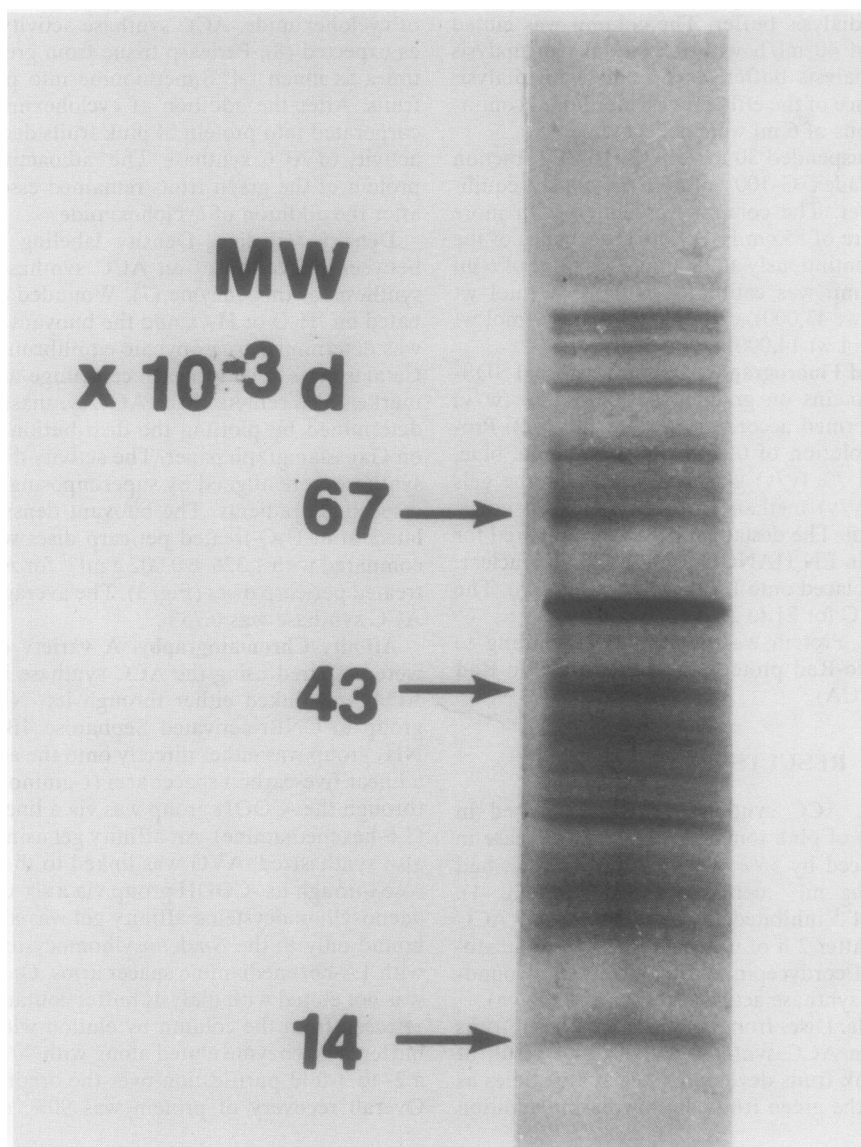


FIG. 5. SDS-gel electrophoretic profile of the Sephadex G-100 eluent. Fractions 39 to 42 (Fig. 4) were pooled, and the protein was precipitated with TCA. The precipitate was centrifuged at 10,000g for 10 min, and the resulting pellet was washed with acetone. The pellet was resuspended in 0.6 ml 0.1 M NaOH prior to the addition of 1.2 ml SDS sample buffer. The proteins were separated on a 5 to 15% polyacrylamide gradient gel and were stained with Coomassie blue.

activity was 95 to 105%. As ACC synthase could not be specifically eluted from these affinity columns by its substrate, binding might have been due to nonspecific interaction of ACC synthase with the spacer arms. This view was strengthened by the fact that a column consisting of 1,6-hexenediamine coupled to Sepharose 4B without the addition of a specific ligand was as effective an 'affinity column' as were those with bound AOA and AVG.

Gel Filtration. ACC synthase eluted from a Sepharose G-100 column after a large peak of excluded proteins (Fig. 4). The center of the peak was located by plotting the distribution of the enzyme activity on Gaussian graph paper. The mol wt of ACC synthase was estimated to be $57,000 \pm 1,500$ daltons. The specific enzyme activity of fractions 37 to 42 was 35 units mg^{-1} protein, which represented a 15-fold purification over the crude homogenate. Fractions 34 to 45 accounted for 29% of the protein and 150% of the enzyme activity loaded onto the column. The proteins in fractions 39 to 42 were separated by electrophoresis on an SDS gel (Fig. 5).

Hydrophobic Interaction Chromatography. By far the best pu-

rification of ACC synthase was obtained using hydrophobic interaction chromatography on phenyl-Sepharose CL-4B. ACC synthase did not bind to phenyl-Sepharose unless 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$ was added to the preparation to enhance hydrophobic interaction between the enzyme and the gel. Much of the protein eluted from the column with buffer containing 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$. Elution with 0.5 M NaCl in elution buffer released loosely bound proteins but no ACC synthase (Fig. 6). ACC synthase was eluted by 0.1 M NaCl in elution buffer and was associated with very little protein. The specific activity of ACC synthase in the 0.1 M NaCl fraction was 110 units mg^{-1} protein, which represented a 44-fold purification over the crude homogenate. There were other, presumably more hydrophobic, proteins that were eluted only by elution buffer without NaCl. Elution buffer containing 50% (v/v) ethylene glycol released some proteins that were not eluted by dialysis buffer only. No ACC synthase activity was associated with the 50% ethylene glycol fractions. The enzyme activity that was eluted with 0.1 M NaCl accounted for 86% of the activity loaded onto the column. Overall recovery of

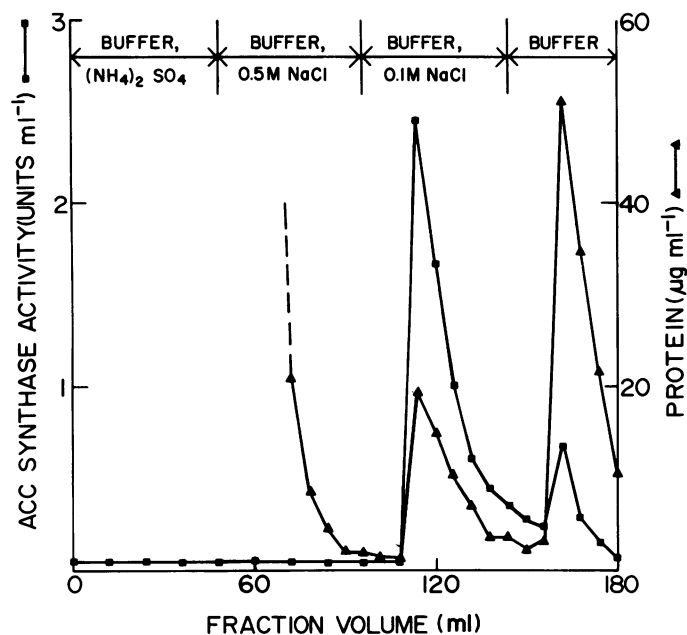


FIG. 6. Elution profile from a phenyl-Sepharose column. Pink tomato pericarp (40 g) was wounded, incubated for 3 h, and extracted. The 30 to 95% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was resuspended in 4 ml dialysis buffer containing 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$. Two ml of this was loaded directly onto a phenyl-Sepharose column and eluted stepwise with dialysis buffer containing 10% $(\text{NH}_4)_2\text{SO}_4$ (48 ml), 0.5 M NaCl (48 ml), 0.1 M NaCl (48 ml), and dialysis buffer alone (36 ml).

enzyme activity was 130% of that originally loaded onto the column, and overall recovery of protein was 95%. The SDS-polyacrylamide gel electrophoresis pattern of the proteins in the fractions eluted by 0.1 M NaCl is shown in Figure 7. Besides a very intense band at 57,000 D, there were up to 20 other, much less intense bands. Specific activities as high as 170 units mg^{-1} protein were obtained in later experiments using hydrophobic interaction chromatography. This represented a 60- to 70-fold purification over the crude homogenate.

Radiolabeling Studies. Wounded tomato pericarp tissue was incubated on L - ^{35}S methionine for 1 h during the first, second, or third h after wounding. The incorporation of radioactivity into protein was examined by SDS-polyacrylamide gel electrophoresis combined with fluorography. There were no obvious differences in the distribution pattern of proteins isolated from tissues 1, 2, and 3 h after wounding (Fig. 8A). The corresponding fluorograms showed that very few proteins became significantly radiolabeled in wounded tissue (Fig. 8B). After the first and second h of wounding, only four protein bands showed detectable radioactivity. During the third h after wounding, two new radioactive bands appeared with mol wt of 38,000 and 25,000 D.

DISCUSSION

The following results are consistent with the hypothesis that the enhancement of ACC synthase activity in wounded tomato pericarp tissue requires the synthesis of mRNA and is based on *de novo* synthesis of the enzyme. (a) Cordycepin, an inhibitor of post-transcriptional RNA processing (6), also inhibited the wound-induced increase in ACC synthase activity (Fig. 1). (b) The development of wound-induced ACC synthase activity was inhibited by cycloheximide (8, 18). (c) The density-labeling experiments showed a 0.75% increase in the buoyant density of ACC synthase isolated from wounded pericarp tissue that had been incubated on $^2\text{H}_2\text{O}$ instead of H_2O (Fig. 3). The fact that no broadening of the ACC synthase peak from $^2\text{H}_2\text{O}$ -treated tissue

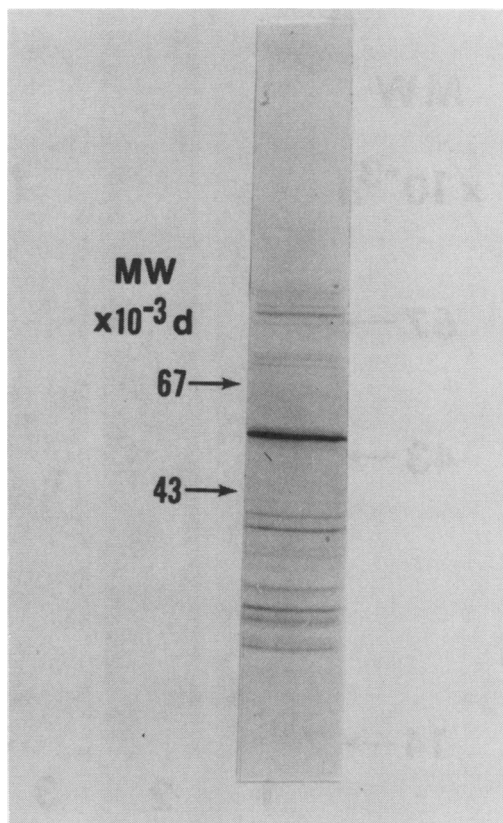


FIG. 7. SDS-gel electrophoresis of proteins eluted with 0.1 M NaCl from a phenyl-Sepharose column. The proteins in the 0.1 M NaCl eluate (Fig. 6) were pooled and concentrated for electrophoresis as before (see legend, Fig. 5).

was observed indicated that all ACC synthase molecules were equally labeled with ^2H and that the activity peak contained only negligible amounts of pre-existing ACC synthase, if any at all.

The quantitative interpretation of density labeling results obtained with $^2\text{H}_2\text{O}$ is difficult since the expected level of ^2H incorporation into nonexchangeable positions of a given protein usually cannot be calculated. Nonexchangeable ^2H is incorporated into amino acids via two main routes: new synthesis and reversible transamination of amino acids. The first mechanism would result in uniformly deuterated amino acids, the second in amino acids with ^2H bound to the α -carbon. The maximal level of ^2H that could be incorporated into all stable positions of ACC synthase could be calculated only if the amino acid composition of ACC synthase were known. Transamination of each amino acid in the presence of 100% $^2\text{H}_2\text{O}$ would result in about a 1% density increase of the newly formed protein, assuming that the average amino acid has a mol wt of 100 which would be increased to 101 by substitution of one H atom with one ^2H atom. The observed 0.75% average density increase for ACC synthase isolated from $^2\text{H}_2\text{O}$ -treated tissue is reasonable considering that ripening tomato pericarp tissue has a high H_2O content which causes substantial dilution of the $^2\text{H}_2\text{O}$ taken up from the incubation medium. Also, our incubations were performed for a relatively short period of time using a tissue with a high rate of protein breakdown where incorporation of newly synthesized amino acids into protein was probably quite low. By way of comparison, a 0.3% increase in the buoyant density of BA-induced nitrate reductase was obtained in embryos of *Agrostemma githago* which were incubated in 50% $^2\text{H}_2\text{O}$ for 5 h (10). Higher levels of $^2\text{H}_2\text{O}$ incorporation into protein were found by Longo (14) who reported a density increase of 5.5% for isocitratase isolated from peanut cotyledons which had been excised from dry seeds and placed on 100% $^2\text{H}_2\text{O}$ for 5 d.

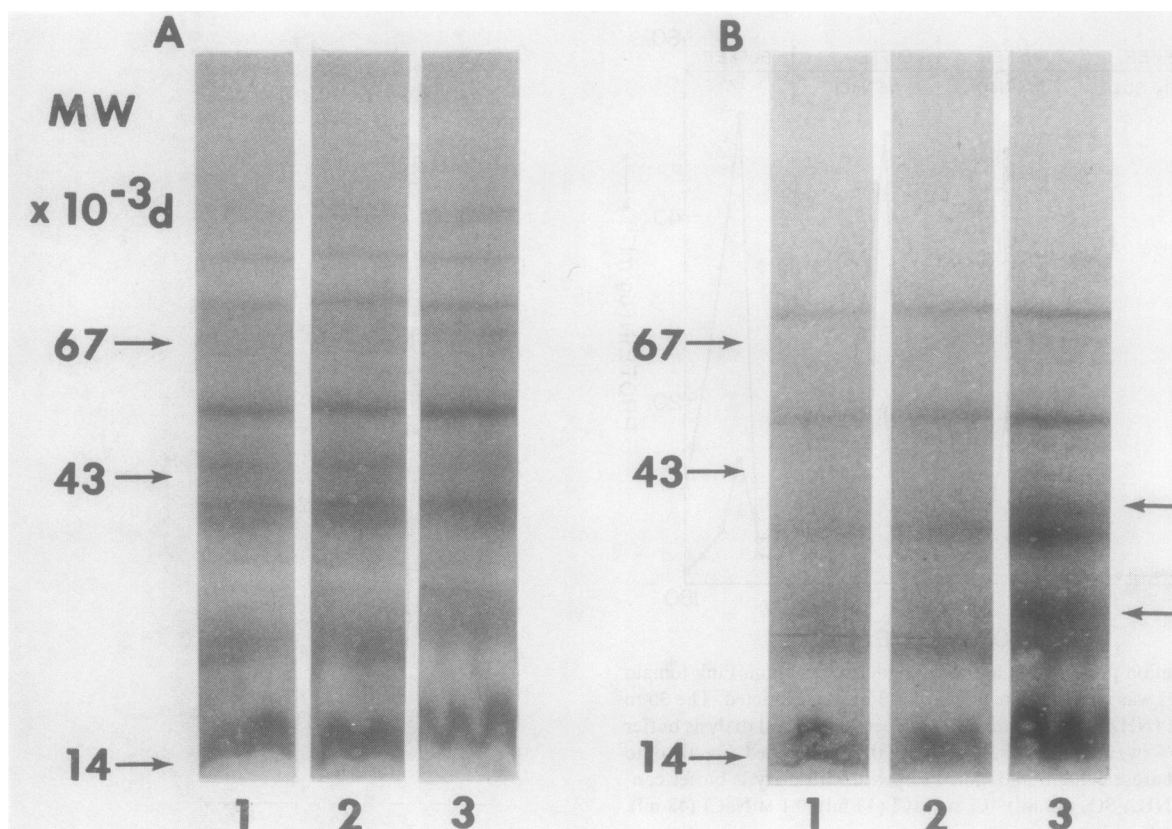


FIG. 8. Time course of incorporation of L-[³⁵S]methionine into protein of wounded tomato pericarp tissue. A, Coomassie blue-stained SDS gel; B, fluorogram of the same SDS gel. The tissue was prepared by incubating the sectors in 0.3 ml H₂O in a Petri dish (60-mm diameter). After 0, 1, and 2 h, samples were vacuum infiltrated with 60 μ Ci of L-[³⁵S]methionine and incubated for a further hour before freezing in liquid N₂ and extraction.

The turnover studies performed with cycloheximide confirmed that the activity of ACC synthase in wounded green and pink pericarp tissue declined rapidly following addition of cycloheximide (8). However, significant differences were found in protein turnover and enzyme activities between green and pink tomatoes. In green tomatoes, the level of radioactivity in protein remained constant after addition of cycloheximide, while in pink fruits, the decay of ACC synthase activity was paralleled by the disappearance of radioactivity from protein (Fig. 2). Further, the level of ACC synthase activity in wounded green tomato fruits was only one-fifth that found in wounded pink fruits. At the same time, green fruits incorporated 10 times more L-[³⁵S]methionine into many more proteins than did pink fruits (results not shown). The above results point to serious difficulties in using turnover, *i.e.* the rapid appearance and disappearance of labeled ACC synthase, as a tool for the identification of this enzyme on SDS gels. Pink tomatoes have higher ACC synthase activities, but the enzyme is turned over together with the bulk of recently synthesized proteins. Therefore, one cannot expect to observe the disappearance of one specific radioactive band corresponding to ACC synthase following addition of cycloheximide. Green tomatoes show more specific turnover of ACC synthase, but the enzyme activity is low, and the background incorporation of L-[³⁵S]methionine into proteins is very high.

The purification of ACC synthase proved to be very difficult. Techniques that involve pH changes, such as isoelectrofocusing or ion-exchange chromatography, caused a rapid loss of enzyme activity. ACC synthase was unstable even during storage at -20°C . Initially, affinity chromatography appeared to be a promising isolation technique because *S*-adenosylmethionine:protein carboxyl *O*-methyltransferase had been purified on an *S*-adenosylhomocysteine affinity column (11). However, affinity chromatography was not suitable for the purification of ACC synthase

because this enzyme could not be eluted specifically from those gels to which it had bound.

Both gel filtration and hydrophobic interaction chromatography gave useful separations of ACC synthase from other proteins. With both methods, more ACC synthase activity was recovered in the eluates than originally loaded onto the column. This may indicate the presence of an inhibitor in the original preparation. Gel filtration yielded a mol wt estimate of $57,000 \pm 1,500$ D for ACC synthase (Fig. 4). This estimate is similar to that of 55,000 D mentioned by Yang (17). The greatest purification of ACC synthase, namely a 44- to 70-fold increase in specific activity over that of the crude extract, was achieved by hydrophobic interaction chromatography using phenyl-Sepharose. Unfortunately, attempts to combine gel filtration and hydrophobic interaction chromatography resulted in a loss of enzyme activity. SDS-gel electrophoresis of the proteins that had been eluted from the phenyl-Sepharose column by 0.1 M NaCl showed a prominent band at 57,000 D and up to 20 other protein bands (Fig. 7). This fraction contained about 1% of the total pericarp protein. Should the 57,000-D protein band turn out to be ACC synthase, then ACC synthase represents about 0.5% of the total protein in tomato pericarp tissue. However, the 57,000-D protein may not be ACC synthase inasmuch as a protein of this mol wt was also present in homogenates of nonwounded tissue. Therefore, ACC synthase probably represents less than 0.5% of the total pericarp protein.

In our radiolabeling studies, we faced the problem that proteins could not be labeled without wounding the tissue. Therefore, we lacked the proper control consisting of labeled protein from nonwounded tissue. As a possible solution to this dilemma, we labeled proteins during the first, second, and third h after wounding. Since there was a lag of about 20 min between wounding and appearance of ACC synthase activity (3), we hoped to find a protein band on the SDS gels which became increasingly labeled

with time after wounding. One of the few proteins which became labeled during the first h after wounding had a mol wt of 57,000 D (Fig. 8). Two other radioactive bands with mol wt of 38,000 and 25,000 D appeared 2 h after wounding. The identity of these proteins could not be determined.

Our results indicate that RNA and protein synthesis are required for the induction of ACC synthase activity in wounded tomato pericarp. The density labeling experiments offer the strongest proof yet that wound-induced ACC synthase is synthesized *de novo* in this tissue. However, conclusive proof that the enhancement of ACC synthase activity is based on induction of enzyme synthesis will require the purification of ACC synthase and the application of immunological techniques. Our results also indicate that ACC synthase is a very minor fraction of the total protein in tomato pericarp tissue. This and the lability of the enzyme will have to be taken into account when further purification of ACC synthase is attempted.

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