Identification of a unique isoform of 1-aminocyclopropane-1-carboxylic acid synthase by monoclonal antibody

(ethylene/1-aminocyclopropane-1-carboxylic acid synthase/hormones/*Lycopersicon esculentum* L.)

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ABSTRACT 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (EC 4.4.1.14) is a key enzyme regulating ethylene biosynthesis in higher plants. A monoclonal antibody (mAb T20C) that immunoprecipitates the ACC synthase activity from tomato pericarp tissue extracts revealed that mAb T20C immunodecorates an $\approx$67-kDa polypeptide. On isoelectric focusing gels, ACC synthase activity in cell-free preparations was resolved into three distinct activity peaks with pI values 5.3, 7, and 9. mAb T20C specifically recognized the pI 7 form of the enzyme on electrophoretic transfer (Western) blots. When analyzed by sodium dodecyl sulfate gel electrophoresis under reducing conditions, the eluted pI 7 form was confirmed to migrate as a polypeptide of 67 kDa. The 67-kDa pI 7 isoform is a previously undescribed form of ACC synthase.

Ethylene is considered a plant hormone influencing many aspects of growth, development, and senescence of higher plants (1, 2). The biosynthesis of ethylene from methionine occurs by way of the following metabolic sequence: methionine → *S*-adenosyl-L-methionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene (for review see ref. 2). In addition to being developmentally regulated, ethylene biosynthesis is also promoted by a variety of environmental, chemical, and physicomechanical stimuli (1, 3). A common endogenous site where the effect of these stimuli is more often realized is the step of conversion of AdoMet to ACC catalyzed by the enzyme ACC synthase (EC 4.4.1.14) (2, 4). The mechanisms that underly induction or repression of this enzyme during ripening and senescence of plant tissues remain to be elucidated.

The regulation of ACC synthase is of considerable interest in plant hormone research. However, its low abundance and labile nature have impeded molecular studies that can be done with a purified protein. Furthermore, reports of its subunit molecular mass range from 50 kDa in tomato fruit (5) to 72 kDa in potato (6) and 84 kDa in squash (7). These varied reports on the size of the protein have not been reconciled. By using protease inhibitors in the extraction and purification buffers and a different purification procedure, we have partially purified a form of ACC synthase not described in the literature and raised monoclonal antibody (mAb) to it. This unique ACC synthase protein is shown to have an apparent molecular mass of 67 kDa on NaDodSO$_4$/PAGE and a pI of 7 on isoelectric focusing (IEF) gels. The denatured and undenatured enzyme form are recognized by the mAb.

MATERIALS AND METHODS

Plant Material. Fruits from greenhouse-grown or field-grown tomato plants (*Lycopersicon esculentum* cv. Pik-Red) were harvested at the early red stage, surface-sterilized with 70% ethanol, and thoroughly rinsed with sterile water. The fruits were sliced, seeds were removed, and the pericarp tissue was incubated for 8 hr to induce ACC synthase activity prior to homogenization as described (8) except that, in addition, the homogenization buffer contained 0.8 mM phenylmethylsulfonyl fluoride and 100 units of aprotinin per ml.

ACC Synthase Assay. Aliquots of the enzyme preparation (5–200 μg of protein) were incubated for 1 hr at 30°C in 50 mM *N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulfonic acid (EPPS)/KOH, pH 8.5, containing 50 μM AdoMet and 5 μM pyridoxal phosphate in a final volume of 1 ml. The enzyme reaction was stopped with the addition of 100 μl of 10 mM mercuric chloride. ACC formed was chemically converted to ethylene, which was then quantified by gas chromatography (9). One unit of enzyme activity is defined as the production of 1 nmol of ACC per hr at 30°C.

Purification of ACC Synthase. Routinely, crude homogenates prepared as described above were frozen in liquid nitrogen, lyophilized, and stored at −80°C. The lyophilized powder was suspended in buffer A (1:5, wt/vol), which contained 2 mM EPPS/KOH, pH 8.5, 0.4 mM dithiothreitol, and 5 μM pyridoxal phosphate in addition to the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 1 μg of pepstatin A per ml. The suspension was clarified at 11,000 × *g* for 5 min. To the supernatant, 0.5 vol of cold ethanol (−20°C) was added and the mixture was stirred gently at −5°C for 15 min. The precipitates were centrifuged at 10,000 × *g* for 15 min and discarded. More ethanol (at −20°C) was added to the supernatant to obtain 50% saturation. The resulting precipitate, containing most of the enzyme activity, was collected by centrifugation and dialyzed overnight against 2 liters of buffer A with four changes. The dialysate was applied to a Sephadex G-100 column (bed volume, 276 ml) previously equilibrated with buffer A. The fractions containing enzyme activity (specific activity, 12.1 units/mg of protein) were pooled and applied to an ethyl agarose (Miles) column (bed volume, 30 ml) equilibrated as above. Under these conditions the enzyme binds to the column (8). The column was washed with 10 vol of buffer A before applying a linear gradient of 10–300 mM NaCl in buffer A to elute bound proteins. The enzyme eluted between 80 and 170 mM NaCl. The active enzyme fractions (specific activity, 117 units/mg of protein) were pooled and gel filtered on a Sephadex G-100 column (bed volume, 276 ml). The gel-filtered active fractions (specific activity, 805 units/mg of protein) were concentrated by lyophilization and stored at −20°C. All enzyme purification steps were carried out in the cold room (4–8°C).

Protein Analysis and NaDodSO₄/PAGE. Protein content was measured by the Bradford method (10) using Bio-Rad reagent according to the supplier's manual. Proteins were solubilized in sample application buffer (11), fractionated by NaDodSO₄/PAGE according to Laemmli (12) using 5% stacking gel and 10–20% gradient or 12% polyacrylamide gel for separation, and then stained with either Coomassie blue or silver (13, 14). Molecular masses (kDa) were determined by comparison to ^{14}C-labeled molecular mass (Amersham) and high molecular mass range protein (Bethesda Research Laboratories) standards.

Immunization and Preparation of Hybridomas. A 12-week-old pristane-primed (primed 10 days before the first injection with 0.5 ml of 2,6,10,14-tetramethylpentadecane) female BALB/c mouse was injected i.p. with 30 μg of purified ACC synthase (15 μg of native and 15 μg of NaDodSO₄/denatured protein) emulsified with Freund's complete adjuvant. Each subsequent injection was given after emulsifying the antigen in Freund's incomplete adjuvant. The mouse was reinjected with 1:1 native/denatured antigen (total, 30 μg) 10 days after the first injection, with 50 μg of native antigen on day 21, and with 3:1 native/denatured antigen (40 μg; without the adjuvant) on day 32. The spleen was removed on day 36. Mouse myeloma cell line P3/NS1/1-Ag4-1 was maintained in RPMI 1640 medium containing 13% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 25 μg of gentamycin per ml (RPMI medium). Dissociated spleen cells from the immunized mouse were fused with NS1 myeloma cells at a 5:1 ratio using 45% (wt/vol) polyethylene glycol 4000 (Sigma) essentially as described by Oi and Herzenberg (15). Hybrid cell lines were plated at low cell density and initially maintained in RPMI medium containing 20% myeloma-conditioned medium (spent culture supernatant from 48-hr myeloma cultures; filtered), 6% Nu-serum (Collaborative Research, Waltham, MA), 6 mM Hepes, 0.018 mM 2-mercaptoethanol, 0.1 mM hypoxanthine, 0.016 mM thymidine, and 0.004 μM aminopterine (fusion plating medium). Once established, hybridomas were grown in fusion plating medium without aminopterin.

Production and Selection of mAbs. Hybridomas secreting antibodies against ACC synthase were selected by testing the culture supernatants using an indirect ELISA, immunodot blot, and immunoprecipitation assay. Antigen-specific, antibody-secreting hybridomas were cloned by limiting dilution. Antibody-containing ascites fluid was produced by injecting 10^7 hybridoma cells i.p. into 12- to 20-week-old BALB/c mice primed 10–20 days previously with 0.5 ml of pristane. mAbs were concentrated from ascites fluid by sequential (25% and 50%) ammonium sulfate fractionation (retaining the 25–50% fraction; ref. 16). Antibodies were further purified by protein A-Sepharose chromatography essentially as described by Ey *et al.* (17). Antibody class and subclass were determined by Ouchterlony double-diffusion using isotype-specific rabbit antisera (Litton Bionetics).

Antibodies not reactive to ACC synthase were used as negative controls. These included a mAb (an IgG1; desig-

nated SC 4G4GS) reactive to a cell membrane protein on the plant pathogenic corn stunt spiroplasma (*Spiroplasma kunkeli*) (18) and a mAb (an IgM; designated BYMV-IC N7C3) reactive to the large subunit of ribulose bisphosphate carboxylase (R.L.J., unpublished).

Dot blot analysis (19) was used as the initial screen for selecting cell lines secreting ACC synthase-specific antibodies.

Immunoprecipitation Assay and Electrophoretic Transfer (Western) Blotting. The ability of antisera or antibodies to inhibit tomato ACC synthase activity was determined by incubating 5–25 units of partially purified enzyme in 250-μl aliquots with increasing amounts of antibody diluted in Tris-buffered saline (pH 8.0) for 4–16 hr at 4°C. The precipitate obtained was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was assayed for residual enzyme activity. Control tubes contained preimmune serum or purified antibodies to unrelated antigens. Fifty microliters of protein A-Sepharose CL-4B (Pharmacia) or 25 μl of goat anti-mouse IgG immunobeads (Bio-Rad, 10 mg/ml) was added to each tube and incubated at 25°C for 2 hr. The precipitates were centrifuged and the supernatant was assayed for any residual enzyme activity. For Western blot analysis, proteins were transferred from 10–20% gradient or 12% polyacrylamide gels onto nitrocellulose paper (Schleicher & Schuell) and probed with antisera according to Roberts *et al.* (20) using goat anti-mouse alkaline phosphatase conjugate or ^{125}I-labeled protein A (DuPont/NEN) as the secondary antibody.

IEF and Western Blotting. IEF of the homogenates was performed according to the Pharmacia technical manual using 6.5% Ampholine/agarose gels (pH 3.5–10), with a focusing time of 1800–2000 V hr. After completion of the run, the pH gradient was determined by comparing migration of known isogel pI standard markers as described in the FMC Bioproducts technical bulletin. The gels were fixed for 10 min in 20% trichloroacetic acid, stained for 15 min with Serva blue W, and destained in water. Identical gels were transferred to nitrocellulose papers by passive diffusion for 2 hr under a pressure of 1 kg weight. Following the transfer, the nitrocellulose papers were air dried for 1 hr and then treated with antiserum and detected with ^{125}I-labeled protein A. For eluting proteins from agarose gels, the gels were sliced into 40 strips, each 5 mm. The strips were incubated overnight with buffer A at 4°C with gentle shaking. The eluted proteins were gel-filtered using Sephadex G-25 spun column chromatography (21) before determining ACC synthase activity, protein content, and NaDodSO₄/PAGE pattern.

RESULTS

Purification of ACC Synthase. ACC synthase was purified 900-fold from tomato pericarp extracts following a protocol (described in *Materials and Methods*) that includes a number of protease inhibitors in all buffers, ethanol precipitation, hydrophobic chromatography, and gel filtration. Data from the various purification steps are summarized in Table 1. The purified protein had a specific activity of 805 nmol/hr per mg

Table 1. Summary of purification of ACC synthase from tomato fruit

Purification step	Total units	% recovery	Specific activity, nmol/hr per mg of protein	Fold purification
Homogenate	262	100	0.91	1
Ethanol precipitate	125	48	0.65	0.8
Sephadex G-100 gel filtration (I)	44	17	12.1	13
Ethyl agarose eluate	63	24	117	130
Sephadex G-100 gel filtration (II)	22	8	805*	900

*This partially purified enzyme preparation had K_m values of 14.5 and 0.35 μM for AdoMet and pyridoxal phosphate, respectively. It was inhibited by aminoethoxyvinylglycine with a K_i of 2.2 μM.
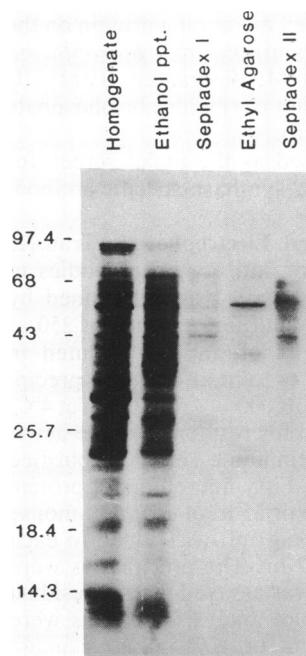
FIG. 1. NaDodSO₄/PAGE pattern of proteins at different stages of purification of ACC synthase (Table 1). Twenty-five micrograms of protein of the homogenate and the ethanol precipitate (ppt.) and 5 μg of protein of the remaining fractions were subjected to electrophoresis under denaturing conditions on 10–20% gradient NaDodSO₄/polyacrylamide gels. After fixation, the gels were stained with silver. The positions of the molecular mass protein standards are indicated in kDa.

of protein. However, we consistently observed that the enzyme is slowly inactivated upon isolation and during purification; therefore, the observed specific activity and purification level are underestimates. The NaDodSO₄/PAGE profile of proteins during each stage of enzyme purification is shown in Fig. 1. The 900-fold purified fraction was enriched in three or four major components with molecular masses of 43, 53, 67, and 94 kDa (Fig. 1, Sephadex II; Fig. 3A, lane 1). One or more of these polypeptides could be either bona fide subunits of ACC synthase, degradation products of the enzyme, or contaminants. Gel filtration on Sephadex G-100 or on Superose columns indicated a native molecular mass of ≈65 kDa for the active enzyme. Therefore, the 67-kDa

protein detected in NaDodSO₄/polyacrylamide gels was considered a prime candidate for a monomeric ACC synthase.

Immunochemical Characterization of ACC Synthase. mAbs were raised against the Sephadex II fraction. Fifty positive hybridoma culture supernatants were assayed for secretion of antibodies that inhibited and immunoprecipitated the activity of ACC synthase from partially purified preparations. Seven stable hybridoma cell lines secreting antibodies were selected. One hybridoma was subcloned and antibodies from its ascites fluid were purified. This mAb was found to be IgG1 by immunodiffusion assay and was designated mAb T20C. Immunoprecipitation of ACC synthase activity by mAb T20C followed saturation kinetics (Fig. 2). Normal mouse serum, ascites fluid from the myeloma cell line, and antibodies purified from ascites fluid containing mAbs to an unrelated plant pathogen membrane protein (Fig. 2, control mAb SC 4G4GS) did not inhibit or immunoprecipitate the enzyme activity. The material immunoprecipitated by mAb T20C was devoid of any ACC synthase activity. Also, it was noted that mAb T20C immunoprecipitated between 78% and 92% of the enzyme activity from partially purified preparations and only 47% and 63% of that in cell-free extracts.

The specificity of the mAb was demonstrated by Western blot analysis. Fig. 3A, lane 2, shows that mAb T20C immunodecorates a polypeptide that coelectrophoreses with the 67-kDa polypeptide in the enzyme preparation used as an
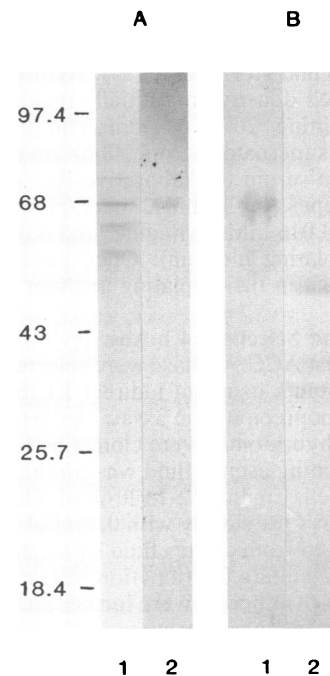
FIG. 3. (A) Immunodetection of 67-kDa protein by mAb T20C on Western blot (lane 2) of Sephadex II fraction (lane 1, silver stained). Five microgram protein equivalents of Sephadex II fraction was resolved by NaDodSO₄/PAGE. One part of the gel was transferred onto nitrocellulose paper. The nitrocellulose blot was incubated with mAb T20C and the antigen–antibody complex was detected by using goat anti-mouse alkaline phosphatase conjugate (lane 2). The other half of the gel was stained with silver (lane 1). Molecular masses are given in kDa. (B) Detection of low molecular mass protein products (indicated in kDa) on immunoblots. Cell-free extracts were prepared in a buffer containing 50 mM Tris·HCl (pH 8.5), 20 μM magnesium sulfate, 1 mM EDTA, 5 μM pyridoxal phosphate, 5 mM 2-mercaptoethanol, 10 μM leupeptin, 1 μg of pepstatin A per ml, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol with (lane 2) and without (lane 1) 50 mM NaCl. Twenty-five micrograms of protein of each extract was fractionated by NaDodSO₄/PAGE, transferred onto nitrocellulose paper, and probed with mAb T20C.
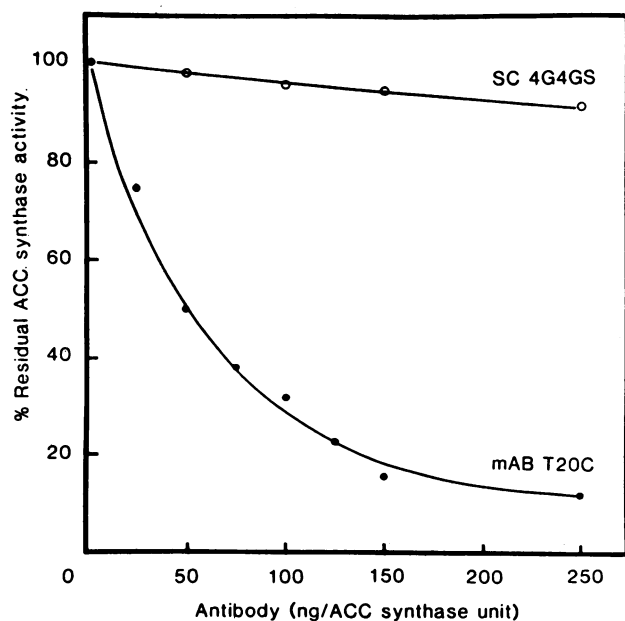
FIG. 2. Immunotitration of ACC synthase activity in Sephadex I fraction (Table 1). The residual enzyme activity was determined after addition of various amounts of mAb T20C (●) or mAb SC 4G4GS prepared against an unrelated protein (○). The enzyme activity of the untreated control was 1.9 units/ml. The titer of the antibody was found to be 25 μg/unit of ACC synthase activity.

immunogen. A 67-kDa polypeptide present in tomato cell-free extracts was similarly identified by mAb T20C (Fig. 3*B*, lane 1). However, when cell-free extracts were prepared in buffers containing salt (Fig. 3*B*, lane 2) or repeatedly frozen and thawed prior to electrophoresis and Western blotting (data not shown), several low molecular mass polypeptides (47 kDa, 38.5 kDa, 37-kDa doublet, and 31 kDa) reacting with the antibody were revealed (compare Fig. 3*B*, lane 2, with lane 1). This immunodecoration of low molecular mass polypeptides apparently occurred at the expense of the 67-kDa protein band, suggesting its labile nature. However, the identity of the smaller polypeptides as breakdown products of the parent 67-kDa protein remains to be elucidated.

Isoforms of ACC Synthase. Preliminary observations on the presence of isoforms of ACC synthase (22) together with varied reports (see Introduction) on the size of this enzyme prompted us to check if mAb T20C would recognize one or more of these isoforms. Cell-free extracts were fractionated on IEF agarose gels. The IEF gels were sliced and proteins eluted from them were assayed for ACC synthase activity. In this way, the cell-free extracts were resolved into three distinct enzyme activity peaks focusing at pI values 5.3, 7, and 9 (Fig. 4). The predominance of the pI 7 isoform was also apparent in pH-dependent binding assays (23). Identical but unsliced IEF agarose gels were blotted onto nitrocellulose paper and immunodecorated with mAb T20C. A minor
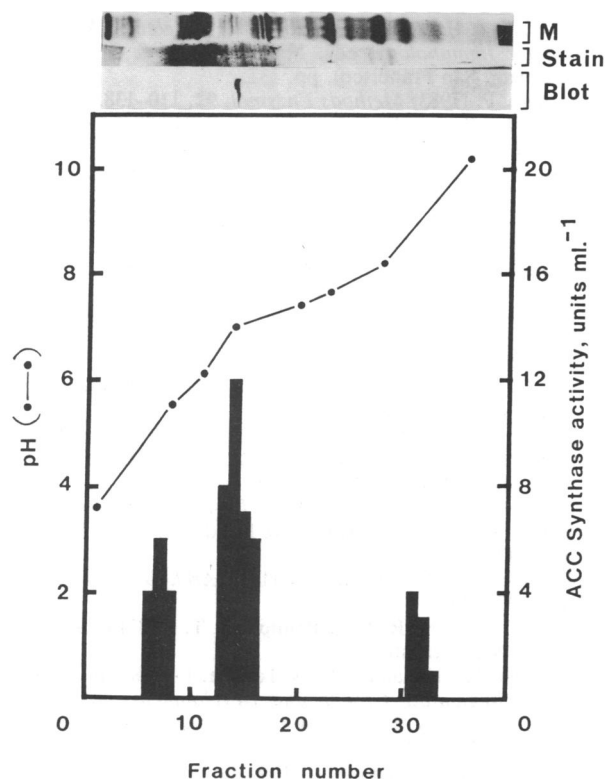

FIG. 4. Multiple molecular forms of ACC synthase in tomato fruit extracts and selective reactivity of pI 7 form with mAb T20C. Two hundred micrograms of protein of the cell-free extracts was fractionated under native conditions on IEF agarose gels with a pH gradient between 3.5 and 10. One part of the gel was stained with Serva blue W (Stain) and the second part was transferred to nitrocellulose paper and immunodecorated with mAb T20C (Blot). The remainder of the gel was sliced into 5-mm strips, proteins were eluted by an overnight incubation at 4°C with buffer A, and the eluates were assayed for ACC synthase activity. Bars represent the enzyme activity. The separation of protein standards (M) on the IEF gel is also shown. Three isoforms of enzyme activity apparent at pI values 5.3, 7, and 9 comprise 30%, 53%, and 17%, respectively, of total activity.
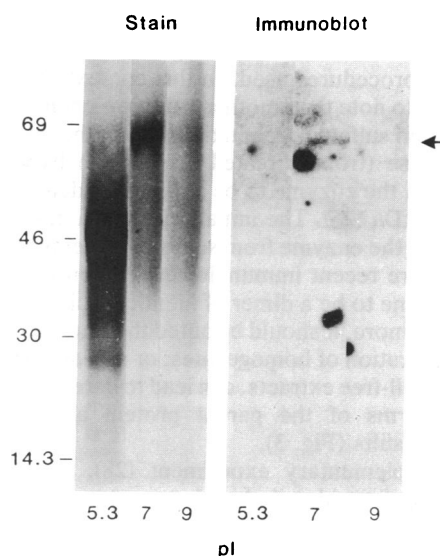

FIG. 5. NaDodSO$_4$/PAGE pattern and Western blot analysis of protein fractions with pI values 5.3, 7, and 9. Proteins eluted from IEF gels at the indicated pI values were separated on 10–20% gradient NaDodSO$_4$/polyacrylamide gel in duplicate. One part was stained with silver (Stain); the remaining part was transferred to nitrocellulose paper and immunodecorated with mAb T20C (Immunoblot) using ^{125}I-labeled protein A. Positions of ^{14}C-labeled protein molecular mass standards (indicated in kDa) and the 67-kDa protein (arrow) are given.

protein (see stained part of the gel) that cofocused with the pI 7 enzyme activity peak was revealed on the Western blot (Fig. 4). To further ascertain the relationship between the pI 7 form of ACC synthase and the 67-kDa protein, proteins in fractions that focused in IEF gels at pI values 5.3, 7, and 9 were subjected in duplicate to second-dimension NaDodSO$_4$/PAGE. One part was silver stained, whereas the other half was blotted onto nitrocellulose paper and challenged with mAb T20C. The results, shown in Fig. 5, indicate that the pI 7 isoform of ACC synthase resolves into a 67-kDa protein that is recognized on Western blots by mAb T20C.

DISCUSSION

By using a mAb we have identified a previously undescribed form of ACC synthase present in ripe tomato fruit. The antibody immunoprecipitated the enzyme activity from cell-free extracts and recognized a single protein band of 67 kDa on Western blots. Gel filtration of the partially purified, native tomato enzyme revealed a molecular mass of about 65 kDa. These data in conjunction with the NaDodSO$_4$/polyacrylamide gel analysis under denaturing conditions indicate ACC synthase to be a monomeric enzyme of about 67 kDa. The purification procedure used in our study was designed to minimize \proteolytic degradation that may occur during enzyme isolation and avoid the use of ammonium sulfate for precipitation/concentration of the enzyme. In our hands, greater losses and enzyme inactivation were commonly observed whenever ammonium sulfate was used to salt out the enzyme. Recently Bleecker *et al.* (5) reported a mAb that inhibited ACC synthase activity in cell-free extracts. When an affinity column with this antibody was used to bind to tomato fruit proteins, a protein of 50 kDa was eluted from such a column under denaturing conditions (5, 24). However, their mAb could not be used on immunoblots. By a different radiolabeling approach, another study implicated a 50-kDa protein as a putative ACC synthase (25). Both studies used ammonium sulfate precipitation as an initial step in their purification protocols.

It is possible that the differences in the molecular masses reported from several laboratories are due to the different purification procedures used. In this context, it is of particular interest to note that another study which did not employ an ammonium sulfate precipitation step in the purification of ACC synthase (from etiolated mung bean hypocotyl segments) found the enzyme to be a dimer of identical subunits of about 65 kDa (26). The initial report of a molecular mass of 84 kDa for the enzyme from squash (7) seems to have been in error. More recent immunological evidence suggests the squash enzyme to be a dimer of identical subunits of 60 kDa (27). Furthermore, it should be noted that the presence of salt during preparation of homogenates, or repeated freezing and thawing of cell-free extracts, can lead to detection of possibly degraded forms of the parent protein and therefore to artifactual results (Fig. 3).

In a complementary experiment (28), we utilized the requirement of pyridoxal phosphate and its association with ACC synthase as a cofactor (2) to radiolabel the enzyme with sodium [^{3}H]borohydride by reduction of the Schiff base formed between the enzyme and the cofactor (25, 29). In these experiments as well, a 67-kDa protein was labeled. The ^{3}H-labeled 67-kDa protein coelectrophoresed with the enriched 67-kDa protein band in the purified ACC synthase fraction and was recognized by mAb T20C. Thus, the pyridoxal phosphate data support the contention that the 67-kDa protein reported here is ACC synthase.

On IEF agarose gels, tomato fruit extract was resolved into three isoforms of ACC synthase having pI values 5.3, 7, and 9. The pI 7 and pI 9 forms as major enzyme activity peaks were previously reported by us (22). The pI 5.3 form of ACC synthase may be related to a pI 4.5 form that was recently reported (25). The mAb T20C recognized the pI 7 form on Western blots of the IEF agarose gels. In addition, the pI 7 form was resolved into a 67-kDa protein on NaDodSO$_4$/polyacrylamide gels that reacted with mAb T20C on Western blots. Therefore, mAb T20C recognizes the native as well as the denatured form of pI 7 ACC synthase but, within the detection limits, not any other isoform or their denatured monomers. These data suggest that this mAb recognizes an epitope on ACC synthase protein which is probably not shared by other forms of the enzyme. It needs to be determined if mAb T20C can recognize related proteins from different plant sources.

The demonstration of different forms of ACC synthase raises several interesting questions concerning the developmental and environmental regulation of the enzyme. Are the different isoforms products of different genes? Are these isoforms interconvertible? Do different isoforms arise in response to different stimuli? For instance, is wound-inducible ACC synthase different from the developmentally regulated enzyme? It may be pertinent to point out that of the genes whose transcription is induced by ethylene—e.g., cellulase (30), polygalacturonase (31), and chitinase (32)—all are present either as multiple gene families or have isoenzymic forms. It will be interesting to know if the genes that encode the mRNAs for the biosynthetic enzymes for ethylene—for instance, ACC synthase—also belong to multiple families. To start answering these questions, the mAb can be used to analyze ACC synthase expression at the gene level and isolate the cognate gene. Such an approach should help in defining the relationship between the different ACC synthase isoforms and the molecular regulation of their expression.

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