Purification and Biochemical Comparison of 1-Aminocyclopropane-1-carboxylic Acid Deaminase Proteins Expressed in Delayed Ripening Tomato and *Escherichia coli*: Studies for a Food Safety Assessment

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Tomato plants with delayed fruit ripening have been developed by introduction of the gene encoding the 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) protein into the tomato genome. The ACCd enzyme delays fruit ripening by decreasing ethylene synthesis. To facilitate safety assessments of the ACCd protein, the identical coding sequence used for tomato transformation was introduced into *Escherichia coli* to produce gram quantities of the protein. A method that included hydrophobic interaction and anion exchange chromatography was developed to purify approximately 2.5 g of ACCd. The *E. coli*-produced ACCd protein was demonstrated to be chemically and functionally equivalent to the ACCd protein expressed in and purified from delayed ripening tomato fruit. The proteins from each source had comparable apparent molecular weights, immunoreactivities, primary amino acid sequences, and enzymatic activities, and both proteins lacked glycosylation. These data validate the use of *E. coli*-produced ACCd protein to assess the safety of ACCd produced in delayed ripening tomatoes.

Keywords: *Delayed ripening tomato; 1-aminocyclopropane-1-carboxylic acid deaminase; protein purification; protein equivalence*

INTRODUCTION

The biosynthetic pathway of the phytohormone ethylene and the stimulatory effect of ethylene on tomato fruit ripening have been well characterized (Taiz and Zeiger, 1991; Grierson and Kader, 1986). The amino acid methionine is converted to ethylene in a series of reactions involving the intermediates *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC). Tomato lines that exhibit delayed fruit ripening have been developed at Monsanto Co., St. Louis, MO. The gene encoding the 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) enzyme was isolated from the soil bacterium *Pseudomonas chloroaphis*, strain 6G5, and introduced into the genome of tomato cultivar UC82B (Klee et al., 1991). The ACCd enzyme catalyzes conversion of the cyclopropanoid amino acid ACC to α-ketobutyrate and ammonia (Honma and Shimomura, 1978). Removal of ACC in transformed plants reduces ethylene synthesis and delays fruit ripening (Klee et al., 1991; Klee, 1993; Reed et al., 1995). The delayed fruit ripening trait in tomato lines expressing the ACCd protein will allow growers to harvest fruit with ripening color and supply superior flavor quality fruit to the consumer.

In a companion paper, Reed et al. (1996) report results that support the safety of the ACCd protein produced in delayed ripening (DR) tomatoes. Safety assessments of the protein include a mouse gavage study performed with purified ACCd protein and digestive fate studies that show rapid degradation of the purified ACCd protein under simulated digestive conditions. The ACCd protein is expressed at low levels in DR tomatoes, accounting for approximately 0.002−0.004% of tomato fruit fresh weight (Reed et al., 1995). Therefore, it was not technically feasible to extract sufficient quantities of the ACCd protein from tomato tissue for safety assessments of the protein. The same ACCd coding sequence used for tomato transformation was introduced into *Escherichia coli* for overexpression of the protein. Microbial fermentation provided sufficient starting material for purification of gram quantities of the ACCd protein required for the safety assessment studies. The current paper describes purification of gram quantities of ACCd protein from *E. coli* as well as studies that establish biochemical equivalence of *E. coli*-produced ACCd protein to the ACCd protein expressed in DR tomato plants. Demonstration of equivalence of the ACCd protein from the two sources justifies use of the microbially produced ACCd for safety assessments of the ACCd protein produced in DR tomato plants.

MATERIALS AND METHODS

Expression and Fermentation. The microbial system used to produce ACCd was *E. coli* MM294 harboring a plasmid containing the nucleotide sequence for the *P. chloroaphis* ACCd gene, as reported by Klee et al. (1991). The ACCd gene encoding the 338 amino acid protein was cloned behind the *rec*A promoter and gene 10 leader ribosome binding site from phage T7 (Olins et al., 1988) for high-level expression. A single 100 L scale fermentation was performed in the presence of exogenous pyridoxal phosphate (0.025 g/L) with induction of the ACCd protein by addition of nalidixic acid at 50 ppm.

Purification of ACCd Produced in *E. coli*. All purification steps were performed at approximately 4 °C. *E. coli* cells from a single 100 L fermentation were collected by centrifugation, resuspended in disruption buffer [0.1 M sodium phosphate, pH 7.5, 5.0 mM dithiothreitol (DTT), 1.0 mM ethylene-

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diaminetetraacetate (EDTA), 20% (v/v) glycerol], and lysed by two passes through a Gaulin homogenizer at 9000 psig. The crude extract was centrifuged at 13680*g* to remove insoluble cellular debris and then fractionated with ammonium sulfate. Material precipitating between 30 and 80% ammonium sulfate saturation was collected by centrifugation and solubilized in 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, and 10% (v/v) glycerol. Solid ammonium sulfate was added in steps to the protein solution to a 1.0 M concentration. The sample was loaded onto a phenyl-Sepharose column (90 mm × 150 mm column, Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, 10% (v/v) glycerol, and 1.0 M ammonium sulfate. After sample loading, the column was washed sequentially with equilibration buffer containing 1.0 and 0.25 M ammonium sulfate, respectively. The ACCd was eluted from the column by a linear 0−50% (v/v) ethylene glycol gradient in 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, and 10% (v/v) glycerol. The peak of ACCd activity eluted from the phenyl-Sepharose column was collected in two pools. Each pool was dialyzed against a Q-Sepharose equilibration buffer of 20 mM Tris, pH 8.5, 5.0 mM DTT, 1.0 mM EDTA, and 10% (v/v) glycerol. Each pool was chromatographed separately on a Q-Sepharose fast flow column (47 mm × 500 mm column, Pharmacia) equilibrated in the same buffer. The column was washed with equilibration buffer until the ultraviolet absorbance (A_{280}) reached baseline. ACCd was eluted from the column using a linear 0.0−0.5 M NaCl gradient. The active fractions were pooled and dialyzed versus storage buffer, 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, and 20% glycerol. Active ACCd pools from the two Q-Sepharose purifications were combined to make a single final enzyme preparation.

Purification of ACCd from Tomato Fruit. Red ripe fruit of DR tomato line 5673 were harvested from greenhouse-grown plants. Tomato line 5673, expressing the ACCd protein, has been fully characterized (Reed et al., 1995). Approximately 0.5 kg of line 5673 fruit was frozen in dry ice and ground to a fine frozen powder in a commercial grade Waring blender in the presence of liquid nitrogen. The fruit powder was homogenized (approximately 1 g of fruit tissue/1.5 mL of buffer) in extraction buffer [100 mM sodium phosphate, pH 7.5, 100 mM boric acid, 2.5 mM EDTA, 2.5 mg/mL ascorbic acid, 5.0 mM DTT, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), 10% (v/v) glycerol, 0.05% (v/v) Tween 20, containing the following protease inhibitors: aprotinin, leupeptin, and pepstatin A, each at 1 μg/mL] using a Polytron PT3000 homogenizer (Brinkman, Inc., Westbury, NY). The soluble tomato extract was collected by centrifugation, and the pellet was resuspended in tomato extraction buffer. The pellet suspension was homogenized again, followed by centrifugation. The two homogenate supernatants were pooled, and solid ammonium sulfate was added to 25% saturation. The ammonium sulfate soluble extract was clarified by centrifugation and increased to an 80% ammonium sulfate saturation. The precipitate was collected by centrifugation and resuspended in DEAE-Sepharose buffer [50 mM Tris, pH 8.0, 1.0 mM DTT, 10% (v/v) glycerol]. The sample was dialyzed versus the DEAE-Sepharose buffer and loaded onto a DEAE-Sepharose column (50 mm × 54 mm column, Pharmacia). The ACCd was eluted from the column with a linear 0.0−0.5 M NaCl gradient. The eluate was concentrated, dialyzed versus amikacin affinity buffer [25 mM Tris, pH 7.6, 50 mM potassium chloride, 2.5 mM DTT, 10% (v/v) glycerol], and loaded onto an amikacin affinity column. This step was included for purification of the NPTII protein present in the tomato fruit (Fuchs et al., 1993). The column flow through, which contained ACCd, was dialyzed versus phenyl-Sepharose buffer, 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, 10% (v/v) glycerol, and 1.0 M ammonium sulfate, and loaded onto a phenyl-Sepharose column (150 mm × 130 mm). The column was sequentially washed with the phenyl-Sepharose buffer containing 1.0 and 0.0 M ammonium sulfate, respectively. ACCd was eluted from the column by a linear 0−50% (v/v) ethylene glycol gradient as described previously for purification of the *E. coli*-produced protein. ACCd fractions were pooled, concentrated and dialyzed versus storage buffer containing 0.1 M sodium phosphate, pH 7.5, 5.0 mM DTT, 1.0 mM EDTA, and 20% (v/v) glycerol. Final purification of the ACCd protein for primary amino acid sequence analysis was achieved by preparative SDS−PAGE, electrotransfer to a poly(vinylidene difluoride) (PVDF) membrane, and excision of the ACCd band.

Tissue Extracts for ELISA and Western Blot Analyses. Red ripe fruit extracts were prepared from DR tomato line 5673 and control tomato line UC82B according to the method described by Reed et al. (1995). Frozen tissue powders were extracted in phosphate-buffered saline plus 0.05% Tween 20 (PBST) at a 1:40 tissue to buffer volume ratio, using a Polytron PT-3000 homogenizer (Brinkman). Extracts were centrifuged and supernatant samples stored at −80 °C prior to analysis by ELISA or Western blot.

Amino Acid Composition. The amino acid composition of purified *E. coli*-expressed ACCd was determined on triplicate samples of the protein. After hydrolysis in 6 N HCl at 115 °C for 24 h, amino acid composition was determined using a Beckman Model 6300 amino acid analyzer as modified from the procedure of Moore and Stein (1963).

Primary Amino Acid Sequence. Automated Edman degradation chemistry was used to determine the N-terminal amino acid sequences of the ACCd proteins isolated from *E. coli* and DR tomato fruit, as described by Hunkapiller (1983). An Applied Biosystems, Inc. (Foster City, CA), Model 470A gas phase sequencer was used for the degradations using the standard sequencer cycle, 03RPTH. The PTH-amino acid derivatives were identified by an on-line RP-HPLC analyzer, Model 120A PRTH, from Applied Biosystems fitted with a Brownlee PT-C_{18} analytical column. The *E. coli*-expressed ACCd was loaded directly onto the sequencer. In addition, the *E. coli*-produced ACCd was digested *in situ* using cyanogen bromide (CNBr) following the procedure of Wadsworth et al. (1992). The mixture of peptide fragments generated from the CNBr cleavage was loaded directly onto the gas phase sequencer. To obtain sequence of ACCd from DR tomato fruit, the partially purified ACCd protein was further purified on SDS−PAGE, blotted onto PVDF as described by Matsudaira (1987), and digested *in situ* using CNBr. The mixture of peptide fragments generated from this digest was loaded onto the sequencer for determination of N-terminal amino acid sequence of each fragment.

ACCd Enzymatic Assay. The enzymatic activities of the *E. coli*-produced ACCd and the DR tomato-produced ACCd were measured using a modification of the spectrophotometric assay described by Honma and Shimomura (1978). ACCd enzyme diluted in storage buffer was reacted at 37 °C with the substrate, ACC, to produce α-ketobutyrate and ammonia. The α-ketobutyrate product formed was reacted with 2,4-dinitrophenylhydrazine to generate a dinitrophenylhydrazone adduct. The absorbance of the dinitrophenylhydrazone adduct was measured at 520 nm and compared to an α-ketobutyrate standard curve. A unit (U) of ACCd activity was defined as micromoles of α-ketobutyrate produced per minute. ACCd specific activity was determined as units per milligram of protein.

SDS−PAGE and Western Blot. SDS−polyacrylamide electrophoresis was performed according to the method of Laemmli (1970). Protein bands were visualized by staining with colloidal Coomassie brilliant blue G-250 (Neuhoff et al., 1988). Protein bands were scanned and quantified using the BioImage Visage 2000 (Millipore, Ann Arbor, MI). The molecular weight of the ACCd protein was estimated by extrapolation from a plot of molecular weight versus average relative migration of the protein standards. For immunological detection of the ACCd protein after SDS−PAGE separation, proteins were electrophoretically transferred to PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA). Protein blots were probed with a 1:1000 dilution of ACCd specific goat antibody [production and purification of the ACCd antibody have been described by Reed et al. (1995)]. Goat antibody bound to the blot was detected using a 1:7500 dilution of rabbit anti-goat antiserum conjugated to alkaline phosphatase (Pierce, Rockford, IL) and nitro blue tetrazolium/5-bromo-4-chloro-3-

indolyl phosphate (NBT/BCIP) color development (Promega, Madison, WI).

Carbohydrate Detection. SDS−PAGE and electrophoretic transfer of separated proteins to a PVDF membrane was as described for Western blot analysis. Carbohydrate moieties associated with the transferred protein bands were detected using the Glycotrack kit (Oxford Systems, Rosedale, NY) according to the manufacturer's instructions.

ELISA. The ACCd ELISA is a validated, direct double-antibody sandwich assay, specific for the ACCd protein (Reed et al., 1995). A protein G-purified polyclonal goat anti-ACCd antibody was used for antigen capture, and the same goat antibody, conjugated to alkaline phosphatase, was used for detection. ACCd ELISA dose−response curves were generated from serial dilutions of crude tomato extracts, using purified *E. coli*-produced ACCd protein as the standard. The shape and slope of the dose−response curves for the ACCd produced in DR tomatoes were compared to those for the *E. coli*-expressed ACCd to demonstrate dilution equivalence.

Protein Assay. Protein was quantified using the method described by Bradford (1976), using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Elemental Analysis. A sample of the ACCd protein purified from *E. coli* was dialyzed against H_2O and lyophilized. A sample of the lyophilized powder was analyzed for percentage of carbon, hydrogen, and nitrogen (CHN) using a Perkin-Elmer 2400 elemental analyzer (Perkin-Elmer, Norwalk, CT).

Moisture Analysis. The water content of the lyophilized ACCd protein purified from *E. coli* was evaluated using the Karl Fischer coulometric titration method (Mitchell and Smith, 1980).

RESULTS

Expression, Fermentation, and Purification of ACCd Produced in *E. coli*. Approximately 108 kg of *E. coli* cell mass was generated from a single 100 L fermentation. ACCd was expressed in *E. coli* as a soluble enzymatically active protein using the *rec*A gene 10L expression cassette previously described (Olins et al., 1988). Pyridoxal phosphate, a cofactor for ACCd, was added exogenously to ensure production of active enzyme. The ACCd protein was expressed in *E. coli* at approximately 80 mg/L of fermentation broth. Fermentation performance and ACCd expression at the 100 L scale were comparable to that of fermentations at the pilot 10 L scale.

Using differential ammonium sulfate precipitation and phenyl-Sepharose and Q-Sepharose column chromatography separations, approximately 2.5 g of highly purified ACCd protein was generated from 50 L of fermentation broth (Figure 1). After 30−80% ammonium sulfate precipitation, ACCd was approximately 20% of total protein. After phenyl-Sepharose chromatography, ACCd increased to approximately 58% of total protein. ACCd eluted from the phenyl-Sepharose column in gradient fractions corresponding to a 45% ethylene glycol concentration. Final purification to near homogeneity was achieved on the Q-Sepharose column. This step was carried out in two Q-Sepharose column runs. The ACCd fractions from each run were combined since pools from each run were comparable in purity and specific activity. The final purified protein solution in pH 7.5 storage buffer was yellow in color, consistent with a protein containing pyridoxal phosphate. The ammonium sulfate precipitation and two column chromatography steps afforded an approximate 11-fold purification of ACCd, and yield of the enzyme was approximately 63% of ACCd present in the initial fermentation broth.

Characterization of the Purified ACCd Protein. The purified *E. coli*-produced ACCd was characterized

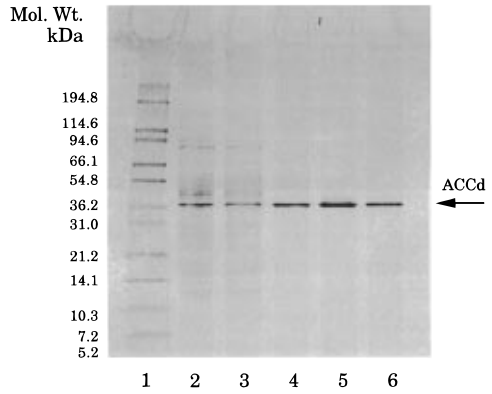

Figure 1. Purification summary of *E. coli*-produced ACCd protein. Samples (1 μg each) from each step of the purification were analyzed by 4−20% SDS−PAGE with Coomassie staining. Lane 1, molecular weight markers; lane 2, initial extract; lane 3, 30−80% ammonium sulfate precipitate; lane 4, phenyl-Sepharose pool; lane 5, final combined product from Q-Sepharose chromatography; lane 6, ACCd standard.

Table 1. Amino Terminal Sequences Obtained for ACCd Proteins

Source	Amino Terminal Residues Detected[1]
Predicted from ACCd gene[2]	M N L N R F E R Y P L T F G P
E. coli	M N L N R F E R Y P L T F G P
DR Tomato	[M] N L N X F E (R) Y P L T (F) (G) P

[1] Amino acid in parentheses denotes a tenuous assignment of residue identity due to low yield and/or high background. X refers to undesignated amino acids. [M] denotes N-terminal methionine suggested by the CNBr digestion product. [2] Klee et al. (1991).

by several analytical methods prior to use as a test substance in safety assessment studies described by Reed et al. (1996). The amino acid composition of the purified ACCd protein was determined. The analysis (data not presented) confirmed the theoretical amino acid composition deduced from the DNA sequence of the ACCd gene (Klee et al., 1991). A lyophilized preparation of the protein was shown to contain 6% moisture. The CHN elemental analysis of the preparation was corrected for water content and shown to be consistent with the amino acid composition of the protein (data not presented). These analyses showed that the lyophilized powder of *E. coli*-produced ACCd contained highly purified ACCd protein with 6% moisture.

The amino-terminal sequence of the ACCd protein was obtained through the first 15 residues (Table 1). The observed sequence matched the theoretical amino acid sequence deduced from the coding sequence of the ACCd gene (Klee et al., 1991). The molecular mass of the ACCd protein was estimated at 36.5 kDa by SDS−PAGE (Figures 1 and 2), which is in excellent agreement with the theoretical value of 36.8 kDa (calculated from the amino acid composition of ACCd; Klee et al., 1991). Densitometric scanning of Coomassie-stained SDS−PAGE bands established a purity of >90% for the ACCd protein. Western blot analysis (Figure 3) of the *E. coli*-produced ACCd protein confirmed the estimated molecular weight and demonstrated the immunoreactivity of the protein to ACCd-specific antibodies. A faint, smaller molecular weight, immunoreactive band was also observed, which is likely a degradation product of ACCd. The enzymatic activity of purified *E. coli*-produced ACCd was 3.24 units/mg of protein, which is similar to activities reported for ACCd from other

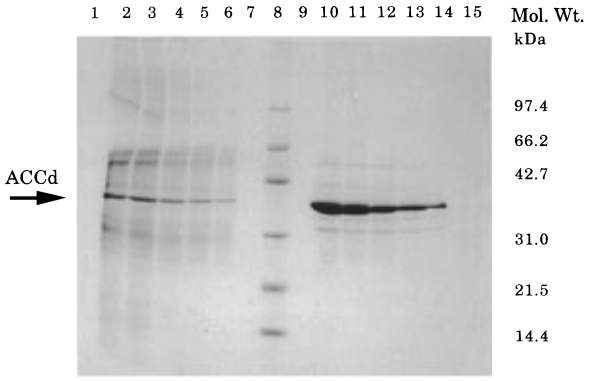

Figure 2. ACCd proteins from *E. coli* and DR tomato show equivalent apparent molecular weights by SDS−PAGE. Lanes 2, 3, 4, 5, and 6, partially purified DR tomato ACCd at 24, 16, 8, 4, and 2 μg of total protein loaded, respectively; lane 8, molecular weight markers; lanes 10, 11, 12, 13, and 14, *E. coli*-produced ACCd at 17, 11, 6, 3, and 1 μg of total protein loaded, respectively; lanes 1, 7, 9, and 15, buffer controls.

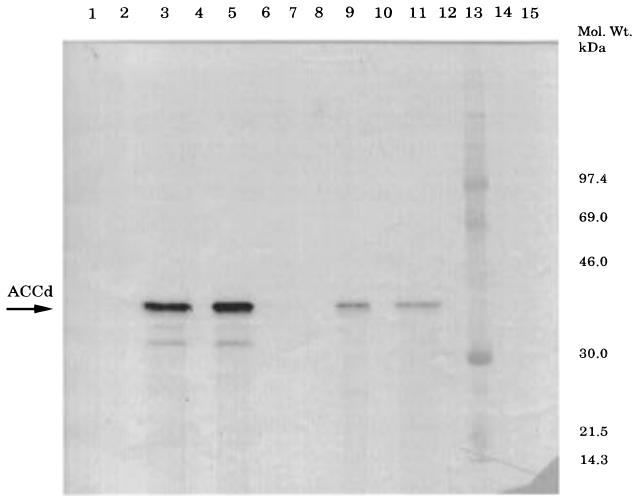

Figure 3. ACCd protein purified from *E. coli* shows equivalent apparent molecular weight and immunoreactivity to DR tomato-produced ACCd, by Western blot analysis. Lane 3, 5.7 ng of *E. coli*-produced ACCd; lane 5, 5.7 ng of *E. coli*-produced ACCd in control (UC82B) tomato extract; lane 7, control tomato extract; lane 9, 5.5 ng of crude DR tomato ACCd extract; lane 11, 0.2 ng of partially purified DR tomato ACCd; lane 13, molecular weight markers; lanes 1, 2, 4, 6, 8, 10, 12, 14, and 15, buffer controls.

microbial sources (Honma and Shimomura, 1978). The final purified ACCd solution was stored at −80 °C in pH 7.5 storage buffer at approximately 8 mg/mL (as determined by Bio-Rad assay). The protein was stable under these conditions for at least 6 months as assessed by ELISA, SDS−PAGE, Western blot, and specific activity assays (data not presented). The protein was readily soluble at a concentration of 45 mg/mL in sodium bicarbonate buffer, pH 8.3 (Reed et al., 1996).

Partial Purification of ACCd from DR Tomatoes. An enzymatically active, partially purified preparation of tomato-expressed ACCd was obtained using ammonium sulfate precipitation and conventional column chromatography. Total protein and ACCd enzyme activity at different stages of the purification protocol are shown in Table 2. Enzyme activity measurements on the crude tomato extract were unreliable because of pigment interference in the assay, and data are not presented. The ammonium sulfate precipitation steps were effective in removing most of the pigments from the preparation. The DEAE-Sepharose step removed

Table 2. Purification of ACCd from Delayed Ripening Tomatoes

fraction	total protein (mg)	total activity[a] (units)	specific activity (units/mg)	purifn (fold)
DEAE-Sepharose load	446	8.6	0.019	1.0
amikacin load	38	1.3	0.033	1.7
phenyl-Sepharose load	20	0.82	0.040	2.1
phenyl-Sepharose pool	1.7	0.60	0.36	19

[a] Conversion of ACC to α-ketobutyrate, μmol/min at 37 °C. ACCd activity measurements on fractions prior to the DEAE-Sepharose load were unreliable because of assay interferences from tomato pigments.

a significant amount of protein from the ACCd preparation, but only a 2-fold increase in ACCd specific activity was obtained. There may have been incomplete elution of ACCd from the DEAE-Sepharose, or some enzyme denaturation may have occurred during purification. Recombinant neomycin phosphotransferase (NPTII) protein, which is also present in the DR tomatoes, coeluted with ACCd (approximately 0.25 M NaCl) from the DEAE-Sepharose column. The pool containing ACCd and NPTII proteins from the DEAE-Sepharose column was loaded onto an amikacin affinity column for purification of NPTII following a procedure similar to that described by Fuchs et al. (1993). As expected, ACCd did not bind to the column and there was no significant ACCd purification. The phenyl-Sepharose column step resulted in significant purification of ACCd, as the majority of the 280 nm absorbing material eluted in the column flow through and 0.0 M ammonium sulfate wash. ACCd enzymatic activity eluted from the phenyl-Sepharose column in gradient fractions corresponding to 45% ethylene glycol concentration. These fractions were greatly enriched in ACCd protein as shown by the 9-fold increase in specific activity of the protein by this purification step (Table 2). The tomato-produced ACCd was approximately 12% pure following the purification protocol described above (based on SDS−PAGE separation and densitometry quantitation, shown in Figure 2). ACCd concentration in the crude tomato extract was estimated at 0.05% of total protein by ELISA (our unpublished data). Therefore, the ACCd protein was purified approximately 240-fold from DR tomatoes by this protocol. The partially purified active enzyme preparation provided protein of sufficient quantity (approximately 200 μg from 500 g of DR tomato) and purity for equivalence studies involving SDS−PAGE, Western blot, carbohydrate staining, and biological activity assays. Further purification of the protein by preparative SDS−PAGE and electrotransfer to a PVDF membrane were necessary for determination of the N-terminal amino acid sequence of plant-produced ACCd.

ACCd Proteins from *E. coli* and DR Tomatoes Exhibit Equivalent Apparent Molecular Masses and Antigenic Activities. The apparent molecular mass of the ACCd protein produced in *E. coli* was equivalent to that produced in DR tomato, based on SDS−PAGE and Western blot analyses (Figures 2 and 3, respectively). ACCd from *E. coli*, from the partially purified DR tomato extract, and from the crude extract of DR tomato migrated with the same apparent molecular mass of approximately 37 kDa in these analyses.

Immunoreactivities of *E. coli*-produced ACCd and partially purified DR tomato-produced ACCd were

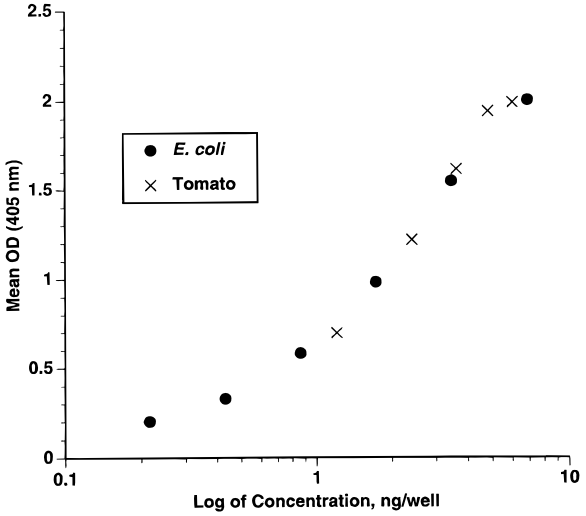

Figure 4. ACCd protein from DR tomato fruit shows equivalent dose−response in the ELISA compared to the *E. coli*-produced ACCd protein. The graph shows optical density (OD) at 405 nm plotted against the log of the ACCd protein concentration determined by ELISA.

equivalent in the Western blot assay on the basis of protein staining intensities and relative amounts of protein loaded (Figure 3). The band intensity of ACCd from the crude DR tomato extract was significantly less than that of *E. coli*-produced ACCd in the Western blot (Figure 3). The level of ACCd in the crude DR tomato extract was estimated by ELISA. Because the ELISA gives a higher estimate of ACCd levels than the Western blot (our unpublished data), differences in ACCd band intensities between these two sources of the enzyme were probably due to differences in amounts of protein loaded in the Western blot. Also, Western blot analysis shows that *E. coli*-produced ACCd spiked into an extract of the control tomato line (UC82B) is indistinguishable from the same protein in storage buffer (Figure 3). Therefore, the tomato extract does not affect ACCd immunoreactivity in the Western blot. The specificity of the antiserum for the ACCd protein was demonstrated by including an extract of the parental control tomato line in the Western blot. This tomato line does not contain the ACCd protein, and no immunoreactive bands were present in the lane loaded with this control tomato extract (Figure 3).

Equivalent immunoreactivities of the microbial and plant-produced ACCd proteins were confirmed by an ELISA. Dose equivalence was determined by plotting the optical density of the ELISA against the log of ACCd concentration for the two sources of the enzyme (Figure 4), as described by Rodbard et al. (1978). Similar slopes are predicted for ACCd from different sources if the proteins are immunologically equivalent. Comparable dose−response curves were observed for ACCd produced in *E. coli* and in DR tomato (Figure 4), as expected for immunologically equivalent proteins.

Comparison of Primary Amino Acid Sequences for ACCd Proteins Produced in *E. coli* and DR Tomato. The partially purified ACCd from DR tomatoes was further purified by electroblotting from SDS−PAGE prior to sequencing. The first 15 residues of the N-terminal amino acid sequence of ACCd from *E. coli* and DR tomato are identical and are as predicted from the nucleotide sequence of the ACCd gene (Klee et al., 1991), as shown in Table 1. The sequence for residues 2−15 of DR tomato-produced ACCd was obtained following *in situ* CNBr digestion of the blotted protein.

Table 3. Amino Acid Sequences for ACCd CNBr Fragments

Source		Amino Terminal Residues Detected[1]
Residues 93-108		
Predicted from ACCd gene[2]	M93	K C V L V Q E N W V N Y S D A
E. coli		K X (V) L (V) (Q) E N (W) V N Y S D (A)
DR Tomato		X X (V) L V (Q) X N (W) V N Y S D A
Residues 122-135		
Predicted from ACCd gene	M122	G A D V R L D A A G F D I
E. coli		G A D (V) R L (D) A A (G) (F) D I
DR Tomato		G A X (V) X L (D) A A X (F) D I
Residues 145-160		
Predicted from ACCd gene	M145	S D V V E Q G G K P F P G C S
E. coli		S X (V) (V) E (Q) G (G) K P (F) P (G) X X
DR Tomato		S X (V) (V) E (Q) G (G) K P (F) P (G) X (S)
Residues 206-221		
Predicted from ACCd gene	M206	V V G F A A D G R S K N V I G
E. coli		(V) (V) G F A A (D) (G) R (S) (K) N V I G
DR Tomato		V X X F A A (D) (G) (R) (S) (K) N X I G

[1] Amino acid in parentheses denotes a tenuous assignment of residue identity due to low yield and/or high background. X refers to undesignated amino acids. [2] Klee et al. (1991).

This result suggests that the first N-terminal residue of DR tomato ACCd is a modified methionine. Modification of the N-terminal residue is not uncommon among eukaryotic proteins (Jornvall, 1975; Brown and Roberts, 1976; Cammue et al., 1991; Huynh et al., 1992). The primary amino acid sequences of the ACCd proteins produced in *E. coli* and DR tomato were further compared by analysis of other peptide fragments generated from CNBr digestion of the protein. Four CNBr peptide fragments were obtained. The amino acid sequences of the peptides from *E. coli*-produced and DR tomato-produced ACCd are the same and correspond to the predicted sequence of CNBr cleavage products of ACCd (Table 3).

ACCd Proteins from *E. coli* and DR Tomatoes Are Not Glycosylated. Typically, proteins produced recombinantly in prokaryotes, such as *E. coli*, are not glycosylated (Slater, 1988). Proteins produced in plants are not typically glycosylated unless the protein is targeted through the secretory pathway (Taiz and Zeiger, 1991). The plant-expressed ACCd protein was not targeted for secretion, and no carbohydrates were detected with either *E. coli*-produced or DR tomato-produced ACCd using an amplified periodic acid Schiff method (Figure 5). Glycosylated proteins were detected in the partially purified DR tomato sample. The band corresponding to ACCd was observed as a negative staining band and therefore was not glycosylated. Biotinylated molecular weight reference standards were included on the blot. Two exogenous glycosylated protein controls, horseradish peroxidase and transferrin, were included as carbohydrate-positive controls. Positive staining bands were clearly observed at loadings of 500 ng for each of the glycosylated control proteins. The lower limits of carbohydrate detection were approximately 100 and 20 ng for the horseradish peroxidase and transferrin proteins, respectively. Since no carbohydrate-associated staining was observed at protein loadings greater than 500 ng of ACCd protein, we

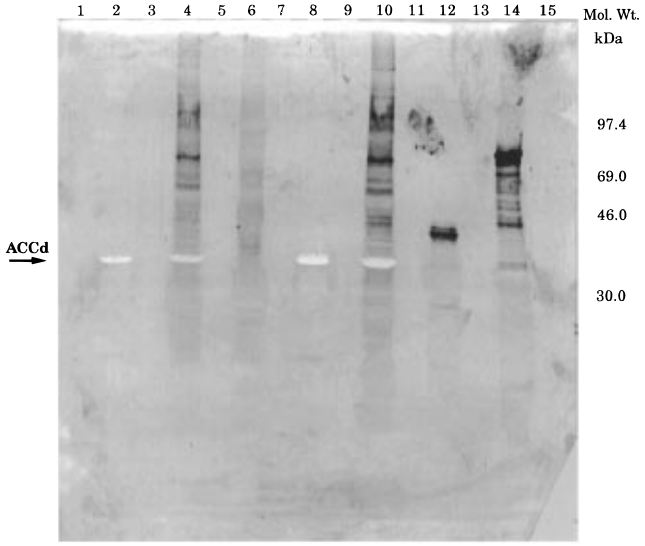

Figure 5. Glycosylation analysis shows no carbohydrate moieties on ACCd protein from either DR tomato or *E. coli*. Samples were run on SDS−PAGE gels, electrophoretically transferred to PVDF paper, and probed for carbohydrate moieties. The ACCd protein in samples is indicated by a negative staining band. Lane 2, 1.5 μg of *E. coli*-produced ACCd; lane 4, 1.8 μg of ACCd from partially purified DR tomato; lane 6, molecular weight markers; lane 8, 3.0 μg of *E. coli*-produced ACCd; lane 10, 3.6 μg of ACCd from partially purified DR tomato; lane 12, 0.5 μg of horseradish peroxidase; lane 14, 0.5 μg of transferrin; lanes 1, 3, 5, 7, 9, 11, 13, and 15, buffer controls.

conclude that neither *E. coli*-produced nor DR tomato-produced ACCd is glycosylated.

ACCd Proteins from *E. coli* and DR Tomatoes Have Comparable Biological Activities. The ACCd enzymes from *E. coli* and from DR tomato catalyzed deamination of the substrate, ACC, to α-ketobutyrate and ammonia, as described by Honma and Shimomura (1978). The specific activities of the enzyme from the two sources were measured for equivalence evaluation. The specific activity of partially purified ACCd from DR tomatoes was 0.301 unit/mg of total protein. Since partially purified ACCd from DR tomatoes was approximately 12% of total protein in this preparation (estimated by SDS−PAGE and densitometry), the specific activity of plant ACCd is equivalent to 2.51 units/mg of ACCd protein after correction for purity of the preparation. The specific activity of the purified *E. coli*-produced ACCd in the same assay was 3.24 units/mg of protein. Therefore, the specific activities of *E. coli*-produced and DR tomato-produced ACCd were similar.

DISCUSSION

The same *accd* gene coding sequence present in DR tomatoes was introduced into *E. coli*, and the ACCd protein was overexpressed and purified in gram quantities for food safety assessment studies that are described in a companion paper (Reed et al., 1996). Because ACCd expression in DR tomatoes is low (Reed et al., 1995), purification of large quantities of the plant-produced protein was not feasible. The *E. coli*-produced protein was an appropriate alternative source for safety studies. Approximately 2.5 g of highly purified *E. coli*-expressed ACCd was obtained by a method of ammonium sulfate precipitation, anion exchange, and hydrophobic interaction chromatography. The identity, strength, purity, solubility, and stability characteristics of *E. coli*-expressed ACCd were determined to establish that the protein was appropriate for safety assessment studies.

The purpose of the current study was to demonstrate the biochemical equivalence of *E. coli*-produced and DR tomato-produced ACCd, to justify the use of the microbial enzyme in food safety assessment studies of DR tomato. The DR tomato-produced ACCd protein was partially purified from tomato fruit by ammonium sulfate precipitation and column chromatography separations. The ACCd proteins from the partially purified tomato preparation and from a crude DR tomato extract were used in the equivalence studies. We conclude from these studies that ACCd produced in *E. coli* is chemically and functionally equivalent to ACCd expressed in DR tomatoes. As defined by Fuchs et al. (1993), chemical and functional equivalence means there are no significant differences between the microbial-produced and plant-produced ACCd proteins and does not mean that the proteins are identical. The equivalence criteria used in this study are similar to those used by the U.S. Food and Drug Administration to assess the equivalence of recombinant and calf-derived chymosins (Food and Drug Administration, 1993) and identical to those used for comparison of NPTII proteins from microbial and plant sources (Fuchs et al., 1993). The current studies establish that the ACCd protein produced in *E. coli* has comparable apparent molecular weight, immunological identity (Western blot), and immunological epitopes (ELISA) to ACCd produced in DR tomato fruit. In addition, ACCd from both sources had comparable biological activities and the same amino acid sequence for N-terminal residues and for CNBr peptide fragments, and both lacked post-translational glycosylation. Additionally, ACCd from both sources displayed the same binding and release characteristics on column chromatography during purification. The

Table 4. Summary of Protein Equivalence Studies: ACCd Proteins Expressed in DR Tomato and *E. coli* Are Chemically and Functionally Equivalent

equivalence criterion	test system	result
molecular weight	SDS−PAGE	ACCd proteins migrated with the same apparent molecular weights
	Western blot	
immunoreactivity	Western blot	ACCd-specific antibody recognized all blotted ACCd protein bands
	ELISA	specific dose−responses produced
epitope structure, overall conformation	ELISA	comparable dose−response curves
primary amino	N-terminal sequencing	identical sequences confirmed for 15 N-terminal residues and for CNBr peptide fragments; theoretical match
glycosylation	detection of carbohydrate associated with blotted protein	carbohydrate not detected at positions corresponding to ACCd protein bands for *E. coli* or plant samples
bioactivity	spectrophotometric ACCd enzymatic activity	specific activities of plant- and *E. coli*-derived ACCd proteins were equivalent

biochemical comparisons of the proteins from the two sources are summarized in Table 4. These studies justify the use of purified *E. coli*-produced ACCd protein as an appropriate substitute for the ACCd protein produced in DR tomatoes for food safety assessment studies.

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