Safety Assessment of 1-Aminocyclopropane-1-carboxylic Acid Deaminase Protein Expressed in Delayed Ripening Tomatoes

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Tomato plants with delayed fruit ripening have been produced by stable insertion of the gene encoding the 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) protein into the tomato chromosome. Two approaches were used to assess the safety of the ACCd protein for human consumption. Purified *Escherichia coli*-produced ACCd protein, which is chemically and functionally equivalent to the ACCd protein produced in delayed ripening tomato fruit, was used in these studies. First, the ACCd protein was readily degraded under simulated mammalian digestive conditions. Second, the ACCd protein did not have any deleterious effects when administered to mice by acute gavage at a dosage of up to 602 mg/kg of body weight. This dosage correlates to greater than a 5000- fold safety factor relative to the average daily consumption of tomatoes, assuming that all tomatoes consumed contain the ACCd protein. These results, in conjunction with previously published data, establish that ingestion of tomato fruit expressing the ACCd protein does not pose any safety concerns.

Keywords: *1-Aminocyclopropane-1-carboxylic acid deaminase; delayed ripening tomato; protein safety; in vitro digestion; acute gavage*

INTRODUCTION

The average annual *per capita* consumption of fresh tomatoes is 17 lb and is increasing approximately 2% per year (Florida Tomato Committee, 1991−1992). Sales of tomato, at the retail and food service level, have surpassed those of potato and lettuce. Annual sales of fresh tomatoes are valued at greater than $3.5 billion. However, while tomatoes have a large share of the U.S. produce market, tomatoes are considered by the consumer as having poor taste quality (Stevens, 1986). The poor quality tomato product can primarily be attributed to a production system based on harvesting fruit at the mature green stage of development. Mature green fruit are firmer and have the handling and market life attributes necessary for a national distribution system. However, mature green fruit are indistinguishable externally from immature green fruit and immature fruit do not develop full flavor qualities when ripened by exposure to exogenous ethylene (Grierson and Kader, 1986). To avoid contamination with inferior immature green fruit, many growers will harvest fruit showing color (color formation indicates the fruit has progressed beyond the immature stage). These fruit, which are referred to as vine-ripened, typically have good taste quality but a very short market life. To prolong the life of a vine-ripened or mature green fruit, the retailer and/or consumer may refrigerate the tomato, which has been shown to destroy tomato flavor (Kader et al., 1978; Buttery et al., 1987). Therefore, tomatoes with good taste quality can be produced commercially by control of the rate of ripening of the vine-ripened tomato.

Tomato plants with delayed fruit ripening have been produced by *Agrobacterium tumefaciens*-mediated transfer of a gene encoding 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) into the tomato genome (Reed et al., 1995; Klee et al., 1991). The ACCd protein catalyzes metabolism of ACC, an essential precursor for ethylene biosynthesis (Taiz and Zeiger, 1991), and removal of ACC in these tomato plants reduces ethylene production and delays fruit ripening (Reed et al., 1995; Klee et al., 1991). The delayed ripening trait extends both the time for fruit to ripen (Reed et al., 1995) and fruit shelf life (Klee et al., 1991). Therefore, expression of the ACCd protein in fresh market tomatoes will allow growers to harvest vine-ripened tomatoes with extended market life and to supply superior flavor tomatoes to the consumer nationwide.

This paper focuses on the food safety of the ACCd protein expressed in delayed ripening (DR) tomatoes. Studies were conducted to address two questions concerning food safety of the expressed ACCd protein. First, does the ACCd protein show any resistance to proteolytic degradation in the human gastrointestinal system and potentially pose a concern as an allergen? Second, is the ACCd protein inherently toxic? The fate of the ACCd protein during mammalian digestion was assessed using a simulated, *in vitro* digestion model of gastric and intestinal fluids. The *in vitro* studies with simulated digestive solutions are widely used as models of animal digestion and have been used to investigate the digestibility of plant and animal proteins (Nielson, 1988; Marquez and Lajolo, 1981; Zikakis et al., 1977). Results of this study established that the ACCd protein is readily degraded under these simulated digestion conditions, which is typical for most food proteins exposed to the proteases and acidic conditions of the mammalian digestive tract (Guyton, 1981).

Exaggerated doses of purified ACCd protein were administered by acute gavage to mice to directly assess any potential mammalian toxicity associated with the protein. Acute administration was selected to assess the safety of ACCd since proteins that are toxic act via acute mechanisms (Sjoblad et al., 1992; Pariza and Foster, 1983; Jones and Maryanski, 1991). There are no reports of proteins that are either mutagenic or

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carcinogenic (Pariza and Foster, 1983; Jones and Maryanski, 1991). Results of this study demonstrated that the ACCd protein is not toxic, consistent with rapid degradation of ACCd in the digestive tract. The ACCd protein was overproduced and purified from *Escherichia coli*, biochemically characterized, and demonstrated to be equivalent to the tomato-produced ACCd (Finn et al., 1996). There were no adverse effects in mice administered ACCd protein by oral gavage at doses up to 602 mg/kg. This dose represents greater than a 5000-fold safety margin relative to the highest potential human consumption of ACCd, assuming the protein is expressed in all tomato products and that there is no loss of ACCd due to processing.

MATERIALS AND METHODS

Dose Formulation of ACCd and Control Proteins. The ACCd protein used in the digestive fate and acute mouse gavage studies was produced in *E. coli*, purified, characterized, and shown to be chemically and functionally equivalent to the ACCd protein expressed in DR tomatoes (Finn et al., 1996). The protein was stored frozen at −80 °C in 0.1 M dibasic sodium phosphate, pH 7.5, 5.0 mM dithiothreitol (DTT), 1.0 mM ethylenediaminetetraacetate (EDTA), and 20% (v/v) glycerol at a concentration of 7.66 mg/mL, prior to use in the safety assessment studies. For the digestive fate studies, the enzyme was thawed and diluted with enzyme buffer [EB; 0.1 M Tris-HCl, pH 8.0, 5 mM DTT, 1 mM EDTA, 10% (v/v) glycerol] immediately prior to use. For the mouse acute gavage study, the ACCd protein was thawed and dialyzed against 50 mM sodium bicarbonate, pH 8.3. The dialyzed protein preparation was concentrated to approximately 45 mg/mL in an Amicon stirred cell (Amicon Inc., Beverly, MA) with an Omega Series 10K membrane (Filtron Technology Corp., MA). Immediately prior to gavage administration, the ACCd preparation was diluted to final dose concentrations of 16.7, 3.08, and 0.319 mg/mL in the 50 mM sodium bicarbonate dialysate. The control protein for the acute gavage study was bovine serum albumin (BSA), which was formulated in 50 mM sodium bicarbonate dialysate at a concentration of 15.9 mg/mL. All dose preparation steps described above were conducted at 4 °C.

Digestive Fluids for Metabolic Fate Studies. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in *The United States Pharmacopeia* (U.S. Pharmacopeial Convention, 1990). Control fluids included gastric (GB) and intestinal (IB) fluid buffers. Preparation of each control fluid was similar to the corresponding SGF and SIF, except they did not contain pepsin or pancreatin, respectively. Simulated digestion solutions in this study were used within 1 h of preparation and were stored at 4 °C before use. The digestive fluids were assayed for proteolytic activity prior to use in the digestion assays as described (Fuchs et al., 1993).

ACCd Digestion in Simulated Gastric Fluids. ACCd protein was added to 1 mL digestion solutions that were temperature-equilibrated to approximately 37 °C. The final concentration of ACCd in the solutions was 4 μg/mL. Digestion solutions were agitated in a Gyratory water bath shaker (Model G76, New Brunswick Scientific, Edison, NJ), with the exception that incubations of less than 2 min were manually agitated. The agitation was stopped periodically to remove 50 μL aliquots of the digestion solutions for analysis. Aliquots were removed from the gastric digests at specific time intervals and were immediately quenched by neutralization with 15 μL of 0.2 M sodium carbonate. ACCd concentrations in the aliquots were measured by Western blot, and the activity of the protein was measured by enzymatic assay. Sample aliquots analyzed by Western blot were diluted 1:1 (v/v) with 2× sample buffer [125 mM Tris-HCl, pH 6.8, 4% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue], heated at approximately 100 °C for 5 min, and stored at approximately −80 °C prior to analysis. Sample aliquots analyzed for enzyme activity were quenched as described above and placed on ice until analysis. For samples measuring ACCd levels and enzyme activity with no incubation in digestive fluids (i.e. $t = 0$), the protein was added to either SGF or GB after addition of quenching reagent. Recovery of ACCd from the digestive fluids was estimated by comparison of protein added to digestive fluid with no incubation and to protein added to enzyme buffer (EB), sampled, and quenched as described. An additional control was included to confirm that the 50 μL aliquot was representative of the whole incubation solution. For this control, the volume of the digestion incubation solution was reduced to 0.1 mL and the entire solution was quenched and analyzed.

ACCd Digestion in Simulated Intestinal Fluids. Incubation conditions for ACCd in intestinal fluids were as described for digestion in gastric fluids. Fifty microliter sample aliquots were removed for Western blot analysis at specific time intervals. The reaction was immediately terminated by 1:1 (v/v) dilution with 2× sample buffer and the sample boiled at 100 °C for 5 min. Samples were stored at −80 °C prior to analysis. For enzyme activity analysis, 50 μL aliquots were removed from the incubation solutions at specific time intervals. The aliquots were diluted 1:1 (v/v) with ice-cold EB to quench the reaction and placed on ice until assayed. Controls for the intestinal fluid digests were as described for the gastric fluid digests and included zero time, ACCd recovery, and whole incubation samples.

Acute Gavage Study. Albino mice, strain CD-1, were obtained from the Charles River Breeding Laboratory, Portage, MI, and acclimatized for 14 days. Ten mice were randomly assigned to each of the treatment groups for males and females. Male mice (ranging from 27.2 to 30.6 g) were approximately 7 weeks old at the start of the study, and females (ranging from 23.9 to 27.9 g) were approximately 8 weeks old. Mice were individually ear tagged for identification. Designated doses of either ACCd protein or control solution were administered to mice by gavage, in single doses of 1.08 mL/30 g of mouse body weight. Treatments included three dose levels of ACCd (602, 111, and 11.5 mg of ACCd/kg of mouse body weight), a BSA protein control administered at 572 mg/kg of body weight, and a vehicle control of 50 mM sodium bicarbonate, pH 8.3. After gavage administration of ACCd protein and controls, mice were supplied with food (Purina Certified Rodent Chow 5002) and water, *ad libitum*.

Clinical observations, mortality, and moribundity were assessed twice daily during the study. Detailed observations for signs of toxicity were performed on day 7 postdosing. Body weights were measured pretest and 7 days postdosing. Food consumption was measured from 1 to 7 days postdosing. All males were sacrificed on day 8 and all females on day 9. All animals were necropsied. Internal cavities were opened and organs examined *in situ* and then removed. Hollow organs were opened and examined. Differences in body weights and food consumption between treatment groups were statistically analyzed using Dunnett's two-tailed test (Dunnett, 1955) at the 5% level of significance.

Western Blot Analysis. *ACCd Digestive Fate Studies.* Samples in 1× sample buffer were thawed and reheated at 100 °C and allowed to cool on ice. Sample proteins were separated by SDS−polyacrylamide gel electrophoresis (PAGE) and detected by Western blotting and enhanced chemiluminescence (ECL). The SDS−PAGE and Western blotting procedures were essentially as described (Finn et al., 1996). After SDS−PAGE, proteins were electrophoretically transferred to a Hybond-ECL nitrocellulose membrane (Amersham International, England) and probed with ACCd-specific goat antibody. Antibody bound to the blot was detected using rabbit anti-goat antibody coupled to horseradish peroxidase, ECL Western blot detection reagents (Amersham International, England), and exposure to X-ray film. ACCd protein recoveries and extents of degradation were estimated by visual comparison to relative intensities of standards on the same blot.

Acute Mouse Gavage Study. The concentration and integrity of the ACCd dose administered to mice in the gavage study

was confirmed by SDS−PAGE and Western blot procedures as described (Finn et al., 1996). Protein blots were probed with ACCd-specific goat antibody and detected with rabbit anti-goat antiserum conjugated to alkaline phosphatase.

ACCd Enzymatic Assay. ACCd enzyme activity was measured at time intervals during incubation in either simulated gastric and intestinal fluids or the respective digestive fluid control buffers. Aliquots removed from the gastric or intestinal digests were quenched prior to enzyme activity assay, as previously described. In addition, ACCd enzyme activity was measured before and after dose administration in the mouse acute gavage study, as part of dose confirmation and integrity analysis. The ACCd assay was a modification of the method described by Honma and Shimomura (1978). Briefly, ACCd was reacted at 37 °C with substrate ACC. The α-ketobutyrate product formed was reacted with 2,4-dinitrophenylhydrazine to generate a dinitrophenylhydrazone adduct. The absorbance of the adduct was measured at 520 nm, and product was quantified from an α-ketobutyrate standard curve. The specific activity of ACCd was defined as micromoles of α-ketobutyrate produced per minute per milligram of protein.

Protein Assay. Protein concentrations of the ACCd doses were determined according to the method described by Bradford (1976), using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Reagents. Pepsin (porcine), pancreatin (porcine), hemoglobin (bovine), and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade from commercial sources.

RESULTS

ACCd Degrades in Simulated Digestive Fluids. The susceptibility of the ACCd protein to proteolytic degradation under *in vitro* mammalian digestion conditions was evaluated in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Degradation was assessed by both Western blot, which detects protein epitopes recognized by an ACCd-specific polyclonal antibody, and enzymatic activity, which is dependent on intact protein tertiary structure. ACCd degrades extremely rapidly in SGF. No protein was detected by Western blot at the first incubation time point of 15 s (Figure 1A). In contrast, there was relatively minor degradation of the ACCd protein in the gastric fluid buffer (GB) control. This buffer control does not contain pepsin. Approximately 30% of the ACCd protein was degraded after incubation in GB for 20 min (Figure 1B). The ACCd protein degrades in SIF but at a slower rate compared to degradation in SGF. More than 50% of the ACCd protein was degraded in SIF in less than 4 h. No protein was detected by Western blot after 22 h of incubation in SIF (Figure 2A). Compared to ACCd degradation in SIF, there was relatively minor degradation of ACCd in the intestinal fluid buffer (IB) control. This buffer control does not contain pancreatin. Approximately 40% of the ACCd protein was degraded after 22 h of incubation in IB (Figure 2B), as determined by Western blot. These results show that degradation of ACCd in SGF and SIF is a result of proteolytic activity in the simulated digestive fluids and not due to any proteolytic activity inherently associated with the ACCd protein.

The SGF and SIF did not produce bands that interfere with Western blot analysis of digestive fate of the ACCd protein (lanes 5 and 6 in Figure 1A; lanes 11 and 12 in Figure 2A). The recovery of ACCd protein from the digestive fluids was estimated by comparison of protein added to digestive fluid without incubation and to protein added to enzyme buffer with no digestive fluid. Recovery of ACCd from both SGF and SIF was approximately 100% (compare lanes 4 and 7 in Figure 1A for SGF; compare lanes 5 and 6 in Figure 2A for SIF).

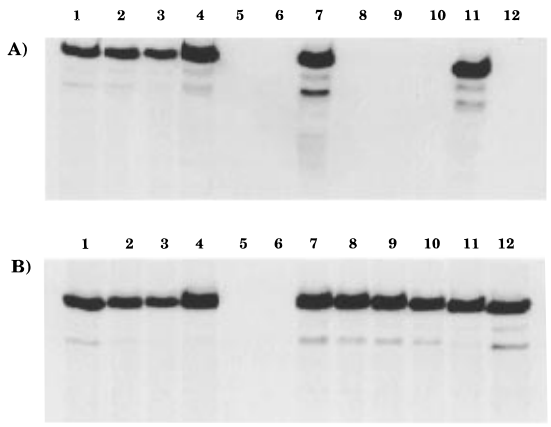

Figure 1. Degradation of ACCd protein in (A) SGF and (B) GB as analyzed by Western blot. The Western blot data shown are representative of three replicate digestions in either SGF or GB. Lane identifications are the same in both (A) and (B). Reference standards are ACCd protein in EB, loaded at 10, 5, and 2 ng (lanes 1, 2, and 3, respectively). Lane 4 is an aliquot from ACCd in EB. Lanes 5 and 6 are enzyme buffer in SGF or GB, incubated for 0 and 20 min, respectively. Lane 7 is a t = 0 sample, ACCd in SGF or GB. Lanes 8, 9, and 10 are ACCd incubated in SGF or GB at 15 s, 1 min, and 20 min, respectively. Lane 11 is ACCd in a total volume of 0.1 mL of EB, a dilution control for the whole incubation solution. Lane 12 is a whole incubation solution of ACCd in 0.1 mL of SGF or GB incubated for 20 min.

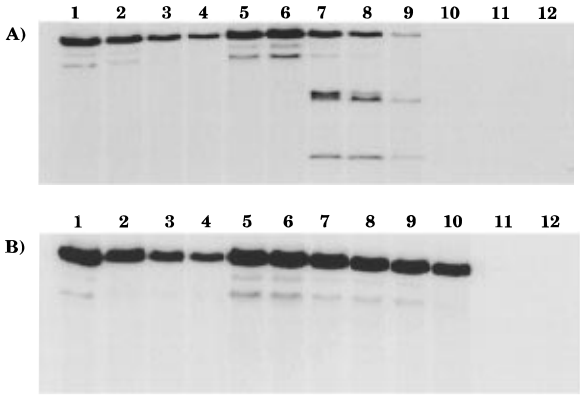

Figure 2. Degradation of ACCd protein in (A) SIF and (B) IB as analyzed by Western blot. The Western blot data shown are representative of three replicate digestions in either SIF or IB. Lane identifications are the same in both (A) and (B). Reference standards are ACCd protein in EB, loaded at 10, 5, 2, and 1 ng (lanes 1, 2, 3, and 4, respectively). Lane 5 is an aliquot from ACCd in EB. Lane 6 is a t = 0 sample, ACCd in SIF or IB. Lanes 7, 8, 9, and 10 are ACCd incubated in SIF or IB at 2, 4, 8, and 22 h, respectively. Lanes 11 and 12 are intestinal fluid controls, EB in SIF or IB, incubated for 0 and 22 h.

An additional control was included in these studies to assess whether the 50 μL aliquots removed from the 1 mL incubation solutions were representative of the entire incubation solution. If the ACCd protein precipitated during incubation in the digestive fluids, it is conceivable that the protein was not sampled in the 50 μL aliquot, and apparent ACCd degradation was an artifact of the assay method. This was addressed by adding protein quenching agent to the entire incubation solution, and results were compared to those from an aliquot of the incubation solution. The Western blot results were similar for aliquot and whole-sample treatments for both SGF and SIF (an example is shown in Figure 1A). These results show that ACCd degrada-

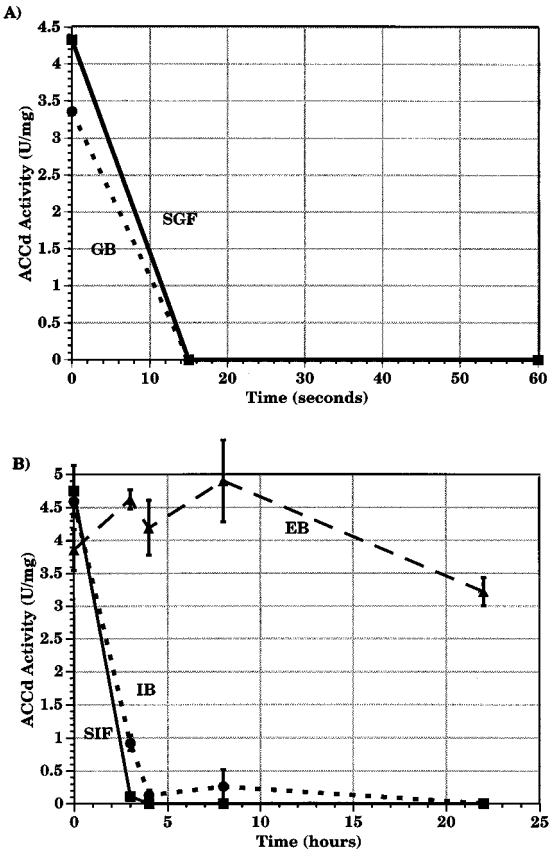

Figure 3. Loss of ACCd enzymatic activity in (A) SGF and in GB and (B) in SIF, in IB, and in EB. One unit is that amount of ACCd activity that catalyzes 1 μmol of ACCd-dependent α-ketobutyrate production/min at 37 °C. ACCd activity values reported are the mean values of three replicate treatments. Bars around points represent ± standard error. Where bars are not shown, points were larger than the standard error. ACCd activity in enzyme buffer (EB) was 4.04 units/mg at the zero time point of gastric fluid digestions in Figure 3A.

tion data from aliquot samples are representative of the whole incubated digestive fluids.

The degradation of ACCd protein in SGF and SIF as determined by Western blot was confirmed by loss of ACCd functional enzyme activity in these simulated digestive fluids. There was total dissipation of ACCd enzymatic activity after 15 s of incubation in SGF (Figure 3A) and after 4 h in SIF (Figure 3B). ACCd enzyme activity was also lost within 15 s in GB (Figure 3A), which suggests that functional activity of the protein is rapidly destroyed by the acid pH and temperature conditions of the gastric fluid buffer. Enzyme activity was dissipated at a slower rate in IB than in SIF (Figure 3B). This suggests that loss of ACCd enzyme activity in SIF is a result of both proteolytic degradation and instability of the protein under incubation conditions of the intestinal fluid digestive assay.

In summary, results of these studies establish that the ACCd protein and associated enzymatic activity are readily degraded in both gastric and intestinal digestion models. Therefore, it is expected that the ACCd protein and enzyme activity will readily degrade in the mammalian digestive tract upon ingestion of DR tomatoes containing the ACCd protein.

ACCd Protein Causes No Deleterious Effects in an Acute Gavage Mouse Study. Doses of 602, 111, 11.5, and 0 mg/kg of body weight of purified ACCd were each administered by gavage to 10 male and 10 female mice to further assess the safety of the ACCd protein.

Table 1. Summary of Food Consumption of Mice Administered ACCd Protein, BSA, or the Vehicle Control

	mean food consumption[b,c] (g/day)	
test group[a]	male	female
vehicle control	5.4 (0.3)	6.2 (1.7)
572 mg/kg BSA	5.4 (0.4)	5.4 (0.5)
11.5 mg/kg ACCd	5.6 (0.4)	5.6 (0.6)
111 mg/kg ACCd	5.4 (0.4)	5.5 (0.5)
602 mg/kg ACCd	5.3 (0.7)	6.5 (2.1)

[a] Each male or female test group consisted of 10 randomized mice. [b] Numbers in parentheses are standard deviations. There were no statistically significant differences ($p < 0.05$) between same sex control and treatment groups. [c] Mean food consumption was calculated from daily measurements from days 1 to 7 of the study.

On the basis of mean daily human consumption of tomatoes and tomato products (TAS, 1992) and ACCd expression levels in tomato fruit (Reed et al., 1995), the highest dosage tested was at least 5000 times higher than the maximum estimated daily human exposure to ACCd protein from consuming DR tomatoes. Another group of mice were gavaged with a dose of 572 mg/kg of body weight of bovine serum albumin (BSA) and served as the dosing protein control group. The dose rate of the BSA control was targeted to be equivalent to the high ACCd dose rate. However, after dose administration, we determined that the high ACCd dose was more concentrated than originally estimated. Because no treatment-related effects were observed in animals in this study, the concentration difference between BSA control and high ACCd dose was not significant. The dosing vehicle control was 50 mM sodium bicarbonate buffer, pH 8.2. Control and ACCd doses were administered in single doses of 1.08 mL/30 g of mouse body weight. The concentration and integrity of the ACCd doses were shown to be stable over the administration period by protein assay, SDS−PAGE, Western blot, and enzyme activity assay (data not presented). Mice were administered the appropriate dose on day 1 of the study and supplied diet and water for the remainder of the study.

There was no mortality, moribundity, or adverse clinical observations in any of the control or ACCd-treated groups during the study. There were no differences in cage-side behavior between the control or treatment groups during the course of this study. No statistical significance between the controls and ACCd protein treated groups was found in the group mean food consumption (Table 1), terminal body weights (Figure 4), or cumulative weight gain (Figure 4). A few incidental pathologic findings were observed at necropsy which were randomly distributed among all groups and are commonly observed in the strain of mice used in these studies. None of these findings were considered to be related to treatment.

DISCUSSION

Typical eukaryotic cells, including cells from food products derived from plants and animals, contain 5000 or more different polypeptides (Kessler et al., 1992) that must be degraded by the digestive system into amino acids necessary for growth. Hundreds of thousands of proteins are consumed daily in the human diet. The gastrointestinal tract functions to catabolize ingested dietary proteins by digesting the protein into amino acids and small peptides which are then absorbed by the intestinal mucosa. It is widely recognized that most

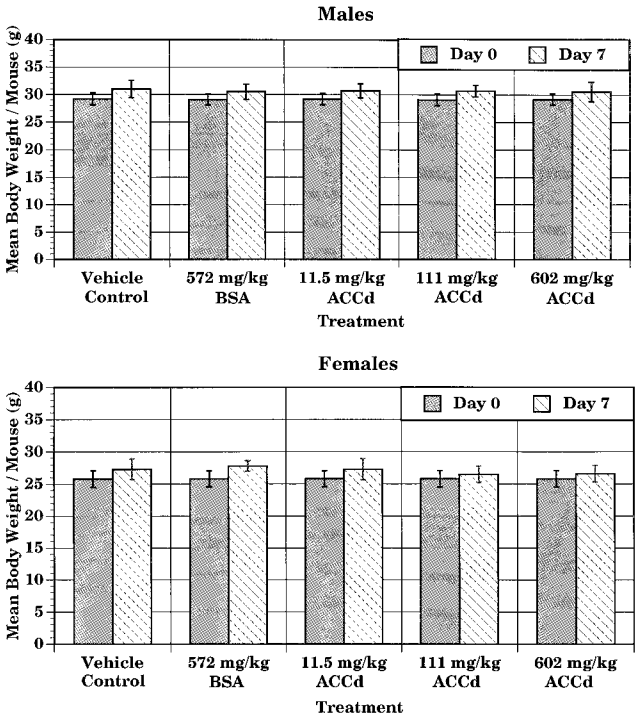

Figure 4. Summary of pretest (day 0) and terminal (day 7) body weights of mice administered ACCd protein, BSA, or the vehicle control. Each male or female test group consisted of 10 randomized mice. Vertical lines around the bar data points represent ± standard deviations. There were no statistically significant differences ($p < 0.05$) in pretest and terminal body weights between same sex control and treatment groups.

proteins rapidly degrade upon consumption and exposure to the mammalian digestive tract (Calgene, 1990; Nap et al., 1992; Pariza and Foster, 1983; Akeson and Stahmann, 1964). *In vitro*, simulated mammalian gastric and intestinal digestive mixtures were used to assess the susceptibility of ACCd to proteolytic digestion.

In vitro studies with simulated digestive solutions are widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (Nielson, 1988; Marquez and Lajolo, 1981), animal proteins (Zikakis et al., 1977), and food additives (Tilch and Elias, 1984); to assess protein quality (Akeson and Stahmann, 1964); to study digestion in pigs and poultry (Fuller, 1991); to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (Alam et al., 1980); and to investigate the controlled release of experimental pharmaceuticals (Doherty et al., 1991). Results of the current study show that ACCd is rapidly degraded by the components of the mammalian digestive system, as expected for other dietary proteins. The data demonstrated a half-life for ACCd of less than 15 s in the gastric system and approximately 2−4 h in the intestinal system, on the basis of Western blot analysis.

Rapid degradation of the ACCd protein in simulated mammalian digestive fluids suggests that consumption of DR tomatoes containing ACCd should not pose any allergenic concerns. Several studies have shown that one of the critical characteristics of major allergenic food proteins is their resistance to proteolytic degradation during digestion (Taylor et al., 1987, 1992). For a food protein to elicit an allergenic response, the protein must survive the acidic and proteolytic environment of the gastrointestinal tract to reach and be absorbed by the intestinal mucosa and initiate an IgE-mediated series of responses. The very rapid degradation of the ACCd protein in SGF (loss of detectable protein in <15 s) as measured by both Western blot analysis and enzymatic activity strongly suggests that this protein will be readily degraded in the stomach when ingested by mammals. To put the rapid degradation of the ACCd protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by about 50% in 2 h, while liquid empties 50% in approximately 25 min (Sleisenger and Fordtran, 1989). Therefore, the ACCd protein should not survive gastric digestion and not reach the intestine. If some of the ACCd protein did survive the gastric system, it would be degraded in the intestine. Greater than 50% of ACCd protein was degraded in the simulated intestinal system in less than 4 h (Western blot analysis). This compares with transit times through the intestine (for radiolabeled chromate, which is not absorbed) of 4−10 h for the first products to appear in the feces and 68−165 h for the last to be detected (Davenport, 1971). Therefore, the potential for ACCd to reach the intestinal mucosa and initiate an IgE-mediated response is extremely minimal.

Further support for the lack of allergenic potential of the ACCd protein is obtained by comparison of the biochemical properties and characteristics of known allergenic proteins (Taylor, 1992; Taylor et al., 1987, 1992) to those of ACCd. Known food allergens are typically heat and pH stable, are glycosylated, and are abundant in the food product. The ACCd protein is not heat and pH stable, and enzyme functional activity is lost on processing DR tomatoes to commercial tomato products (T. Rangwala, Monsanto Co., personal communication). The ACCd protein is not glycosylated (Finn et al., 1996), is not a major protein component in tomato fruit [0.004% of fruit fresh weight, as reported by Reed et al. (1995)], and shows no significant amino acid sequence homology to any known allergens contained in several computer data bases (unpublished data). Therefore, ACCd does not possess any of the characteristics common to known allergenic proteins.

To address the question of the potential toxicity of the ACCd protein itself, mice were administered exaggerated doses of the ACCd protein by acute gavage. An acute study was selected since proteins known to be toxic to mammals manifest toxicity in an acute exposure (Sjoblad et al., 1992; Pariza and Foster, 1983; Jones and Maryanski, 1991). The ACCd protein used in the acute gavage study was purified from *E. coli* and shown to be chemically and biologically equivalent to the ACCd produced in DR tomato fruit (Finn et al., 1996). The ACCd protein was administered to mice at three different dose rates, the highest of which was 602 mg/kg of body weight. This dose represents an approximate 5000-fold safety margin relative to the highest potential human consumption of ACCd in fresh tomatoes and processed tomato products. This is equivalent to an average human consuming, in 1 day, approximately 6000 fresh tomatoes expressing ACCd at levels previously reported (Reed et al., 1995). There was no treatment-related mortality or morbundity and no differences in weight gain or food consumption between the treatment groups; no abnormal behavioral or clinical signs were observed. There were no grossly observable pathologic changes in mice considered to be related to administration of the ACCd protein. These results were expected since ACCd protein is readily digested in simulated gastric and intestinal fluids. Also, the ACCd

protein shows no significant amino acid sequence homology to sequences of 1935 known protein toxins contained in several computer data bases (unpublished data). Also, the products of ACC deamination, NH_3 and α-ketobutyrate, are natural plant metabolites.

A whole fruit feeding study performed for a longer duration of 28 days using DR and parental control tomatoes also showed no treatment-related effects (unpublished data). Rats were fed 1−2 fresh tomatoes every 2−3 days for 28 days, and rodent chow was provided *ad libitum*. This consumption rate is equivalent to an average human consuming approximately 60 tomato fruit per day. There were no statistically significant differences in body weight gain or food consumption between the groups fed DR tomatoes and those fed control tomatoes. No treatment-related abnormal clinical signs were observed, and all animals appeared to be healthy. These data were generated using lower doses of the ACCd protein than those used in the mouse acute gavage study but using exaggerated food consumption levels compared to the average human daily consumption of tomatoes (TAS, 1992).

The design and conduct of the current studies were consistent with those recommended by the U.S. Food and Drug Administration (1992) for the safety assessment of foods derived from new plant varieties. We conclude that the ACCd protein produced in DR tomato plants poses no discernible food safety concerns. The ACCd protein is readily degraded in mammalian digestive fluids like other ordinary dietary proteins, does not possess the attributes of known protein food allergens, is not toxic to mammals, and, hence, presents no risks for human or animal consumption.

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