The Expressed Protein in Glyphosate-Tolerant Soybean, 5-Enolypyruvylshikimate-3-Phosphate Synthase from *Agrobacterium* sp. Strain CP4, Is Rapidly Digested In Vitro and Is Not Toxic to Acutely Gavaged Mice[1,2]

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ABSTRACT The safety of 5-enolpyruvylshikimate-3-phosphate synthase enzyme derived from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) was assessed. CP4 EPSPS is the only protein introduced by genetic manipulation that is expressed in glyphosate-tolerant soybeans, which are being developed to provide new weed-control options for farmers. Expression of this protein in plants imparts high levels of glyphosate tolerance. The safety of CP4 EPSPS was ascertained by evaluating both physical and functional characteristics. CP4 EPSPS degrades readily in simulated gastric and intestinal fluids, suggesting that this protein will be degraded in the mammalian digestive tract upon ingestion as a component of food or feed. There were no deleterious effects due to the acute administration of CP4 EPSPS to mice by gavage at a high dosage of 572 mg/kg body wt, which exceeds 1000-fold the anticipated consumption level of food products potentially containing CP4 EPSPS protein. CP4 EPSPS does not pose any important allergen concerns because this protein does not possess characteristics typical of allergenic proteins. These data, in combination with seed compositional analysis and animal feeding studies, support the conclusion that glyphosate-tolerant soybeans are as safe and nutritious as traditional soybeans currently being marketed. J. Nutr. 126: 728–740, 1996.

INDEXING KEY WORDS:

- *soybean*
- *5-enolypyruvylshikimate-3-phosphate synthase*
- *glyphosate* • *mice*

Glyphosate-tolerant soybeans (GTS),[4,5] genetically modified to tolerate commercially relevant application rates of the nonselective, broad-spectrum herbicide glyphosate, promise to offer farmers a new, environmentally sound method of weed control in soybeans. Weed control in soybeans is critical to maintain a high yielding, high quality harvest, free of weed seeds. Following the consultation process established by the U.S. Food and Drug Administration (FDA) in the 1992 food policy (U.S. Food and Drug Administration 1992), we assessed the safety of GTS relative to that of soybeans currently consumed by animals and humans. The first paper in this series (Padgette et al. 1996b) presented extensive compositional data on GTS seeds and selected processing fractions, which led to the conclusion that GTS seeds are substantially equivalent to conventional soybean seeds. The following paper in this series (Hammond et al. 1996) presents the results of animal feeding studies using GTS seed or meal as a feed source. The current paper focuses on the safety assessment of the protein expressed in GTS that confers glyphosate tolerance, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) (Padgette et al. 1996a).

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[4] Abbreviations used: BSA, bovine serum albumin; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4; GTS, glyphosate-tolerant soybeans; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

[5] Glyphosate is the active ingredient of the broad-spectrum, nonselective herbicide Roundup®, and GTS are also denoted as Roundup Ready® soybeans (Monsanto Company, St. Louis, MO).

0022-3166/96 $3.00 © 1996 American Institute of Nutrition.
Manuscript received 7 February 1995. Initial review completed 1 May 1995. Revision accepted 27 November 1995.

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The development of glyphosate-tolerant crops has been ongoing since the early 1980s (Padgette et al. 1996a). The method of tolerance developed was the "target-site" approach, whereby a herbicide-insensitive target protein was identified and introduced into soybean by genetic modification techniques (Padgette et al. 1995). Glyphosate specifically binds to and blocks the activity of its enzyme target, EPSPS (Steinrucken and Amrhein 1980), an enzyme of the aromatic amino acid biosynthetic pathway (Haslam 1993). Glyphosate inhibition of EPSPS prevents the plant from synthesizing the aromatic amino acids essential for protein synthesis. 5-Enolpyruvylshikimate-3-phosphate synthase is the only physiological target of glyphosate in plants, and no other phosphoenolpyruvate-utilizing enzymes are inhibited by glyphosate (Steinrucken and Amrhein 1984). 5-Enolpyruvylshikimate-3-phosphate synthase is present in all plants, bacteria and fungi, but not in animals; animals do not make their own aromatic amino acids, but obtain them from plant, microbial or animal-derived foods. Thus, EPSPS is normally present in food and feeds derived from plant and microbial sources.

The GTS plant is unaffected by glyphosate treatment because the continued action of the introduced glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic amino acids and other compounds derived from this pathway. The CP4 EPSPS enzyme encoded by the gene expressed in GTS was identified from a screen of microorganism cell extracts as exhibiting a high degree of tolerance to inhibition by glyphosate, coupled with efficient interaction with the phosphoenolpyruvate substrate (Barry et al. 1992, Padgette et al. 1996a). The gene was cloned, sequenced and shown to encode a 47.6-kDa protein consisting of a single polypeptide of 455 amino acids (Padgette, S. R., Barry, G. F., Eichholtz, D. E. and Weldon, M. unpublished data).

Relatively large amounts of CP4 EPSPS were required to conduct the safety assessment experiments described herein, greater than could readily be isolated from GTS seeds due to the relatively low expression levels (Padgette et al. 1995). Therefore, the CP4 EPSPS protein was produced in an *Escherichia coli* expression system, purified to a high degree (Padgette, S. R., Heeren R. and Harrison L. A., unpublished data) and characterized relative to GTS-produced CP4 EPSPS. *E. coli*–produced CP4 EPSPS was shown to be a suitable substitute for the GTS-produced CP4 EPSPS, based on both structural and functional characteristics. We have compared the CP4 EPSPS produced in *E. coli* to the CP4 EPSPS expressed not only in GTS but also in glyphosate-tolerant canola and cotton. On the basis of results obtained, we have shown that the CP4 EPSPS expressed in several different transgenic crops is equivalent to the CP4 EPSPS produced in *E. coli*. Thus, the protein safety assessment described herein is applicable not only to GTS but also to other crops expressing CP4 EPSPS.

Our evaluation of the safety of CP4 EPSPS comprised three main lines of experimentation. First, the *E. coli*–expressed CP4 EPSPS test material was compared with plant-expressed CP4 EPSPS and characterized with respect to molecular weight, immunoreactivity, absence of glycosylation and enzymatic activity, to establish equivalence. Second, an acute oral toxicity study in mice was performed with the *E. coli*–expressed CP4 EPSPS test material. Acute administration was considered appropriate to assess the safety of CP4 EPSPS because toxic proteins act via acute mechanisms (Jones and Maryanski 1991, Pariza and Foster 1983, Sjoblad et al. 1992). Finally, digestive fate studies in simulated gastric and intestinal fluids were performed. In vitro studies with simulated digestive solutions are widely used to investigate the digestibility of plant and animal proteins (Marquez and Lajolo 1981, Nielson 1988, Zikakis et al. 1977), to assess protein quality (Akeson and Stahmann 1964), and for a variety of other animal digestion and pharmaceutical applications (Alam et al. 1980, C.A.B. International 1991, Doherty et al. 1991). Supplementary information relative to allergenic and toxic potential was acquired by comparing the amino acid sequence of CP4 EPSPS to sequences in a database of known allergens and toxins.

MATERIALS AND METHODS

Analytical methods. All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Protein was quantified by the method of Bradford (1976) using a commercially available reagent (Bio-Rad, Hercules, CA) adapted to a 96-well format according to manufacturer's instructions. SDS-PAGE was performed by the method of Laemmli (1970) using Coomassie blue stain. Gels were scanned by densitometry with an Ultrascan XL densitometer (Pharmacia LKB, Uppsala, Sweden). Carbohydrate moieties associated with proteins were detected by electrophoretically transferring the proteins from an SDS-PAGE gel onto polyvinylidene difluoride paper, followed by detection of associated carbohydrates with the Glycotrack kit (Oxford Systems, Rosedale, NY). Amino-terminal amino acid sequencing was by automated Edman degradation chemistry. A gas-phase sequencer (model 470A, Applied Biosystems, Foster City, CA) was employed for the degradations (Hunkapiller et al. 1983) using the standard sequencer cycle (03RPTH). The respective phenylthiohydantoin-amino acid derivatives were identified by reverse-phase HPLC analysis in an on-line fashion employing an PTH Analyzer (model 120A, Applied Biosystems) fitted with a Brownlee (Foster City, CA) 2.1-mm i.d. PTH-C_{18} column according to manufacturer's instructions.

Functionally active EPSPS was determined by either the HPLC radioassay or phosphate release assay, as de-

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scribed previously (Padgette et al. 1987 and 1988). For EPSPS, 1 unit is defined to be 1 μmol 5-enolpyruvyl-shikimate-3-phosphate produced/min at 25°C. The ELISA for CP4 EPSPS is also described elsewhere (Padgette et al. 1995).

Purification of CP4 EPSPS from seeds. Soybean-expressed CP4 EPSPS was purified from the seed of GTS line 61-67-1. The buffers used were an extraction buffer (100 mmol/L Tris Cl, 5 mm dithiothreitol, 1 mmol/L EDTA, 1 mmol/L benzamidine HCl, 5 mg/L bestatin, 10% glycerol, pH 7.5), a QA buffer (10 mmol/L Tris Cl, 2 mmol/L dithiothreitol, 1 mmol/L benzamidine HCl, 10% glycerol, pH 7.8), and a phenyl A buffer (100 mmol/L Tris Cl, 2 mmol/L dithiothreitol, 1 mol/L ammonium sulfate, 10% glycerol, pH 7.5). Seed from GTS line 61-67-1 (100 g) was powdered in liquid nitrogen contained in a Waring blender. After allowing the liquid nitrogen to evaporate, the remaining powder was blended with acetone (2 × 500 mL) and collected on a Buchner funnel, air-dried, then vacuum-dried to yield 69.2 g of yellow soybean seed acetone powder. The acetone powder was stirred with extraction buffer (1400 mL) on ice, homogenized and centrifuged. The supernatant was filtered through cheesecloth to yield soybean seed extract. The extract was treated with solid ammonium sulfate to 80% saturation, with stirring, and the 0–80% pellet was collected by centrifugation. CP4 EPSPS was purified from the seed protein extract by Q Sepharose Fast Flow (Pharmacia) (5 × 32 cm column, 260-min gradient elution with a 0.01–0.4 mol/L KCl gradient in QA buffer, 10 mL/min), Phenyl Sepharose (Pharmacia) (1.4 × 31 cm column, 350-min gradient elution with 1 to 0 mol/L ammonium sulfate in phenyl A buffer, 3 mL/min), Mono Q 10/10 (Pharmacia) (60-min gradient elution with 0–0.3 mol/L KCl in QA buffer, 3 mL/min) and Phenyl Superose 5/5 (Pharmacia) (60-min gradient elution with 1–0 mol/L ammonium sulfate in phenyl A buffer, 1 mL/min) column chromatography.

CP4 EPSPS protein was partially purified from glyphosate-tolerant canola line 17131-68 utilizing a method similar to that described for soybean seed, except for the elimination of the acetone precipitation step. Similar methodology was also used for the purification of CP4 EPSPS protein from glyphosate-tolerant cotton line 1698, except that an additional chromatography step, Reactive Blue 4 agarose, was added. Controls for transgenic canola and cotton lines were the corresponding parental lines (Westar and Coker 312, respectively).

CP4 EPSPS protein test substance. The CP4 EPSPS used in the safety assessment studies described here was microbially expressed CP4 EPSPS produced in *E. coli* strain GB100, transformed with plasmid pMON21104 (Padgette, S. R. and Kolacz, K. H. unpublished data). The protein was purified to greater than 90% purity, based on SDS-PAGE analysis (Padgette, S. R., Heeren, R. and Harrison, L. A. unpublished data). The purified protein was dialyzed into the vehicle control buffer [50 mmol/L sodium bicarbonate (Sigma S5761), 10 mmol/L cysteine (Sigma C8152 98%), 50 g/L sucrose (Sigma S1888, 99%), pH 8.5] and stored at −80°C.

Experimental animals and diet. CD-1 albino mice (50 males, 50 females) were obtained at 5.5 wk (males) or 7.0 wk (females) of age from Charles River Breeding Laboratory (Portage, MI). Weight ranges at the time of study were 25.2–29.8 g (males) and 22.7–27.2 g (females). Animals were housed individually in stainless steel cages in a room with a temperature of 22–25°C and a 12-h light:dark cycle (lights on from 0630 h). The mice were given free access to the nonpurified basal diet (Purina Certified Rodent Chow no. 5002) and water (St. Louis, MO, public water supply, zeolite-conditioned). Animal housing and husbandry were in accordance with the *Guide to the Care and Use of Laboratory Animals* (NRC 1985).

Choice of dosage levels. The dosage levels for the acute oral toxicity study in mice were chosen based on two criteria: *1*) the estimated exposure to CP4 EPSPS, summed over potential commercial food products expressing CP4 EPSPS under development by Monsanto and commercial partners, assuming a "worst-case scenario" including 100% consumption of all the food products considered, no CP4 EPSPS loss upon processing, and 100% market penetration; and *2*) a safety factor, which was selected to be approximately 1000-fold.

The potential human exposure to CP4 EPSPS resulting from consumption of potential commercial products that may express CP4 EPSPS was estimated using the Technical Assessment Systems (TAS) program Exposure 1® Chronic Dietary Exposure Analysis (Technical Assessment Systems 1993). First, the percentage of protein fresh weight was estimated in various soybean, potato, tomato and corn products. To estimate the concentration of CP4 EPSPS in these products, an expression level for CP4 EPSPS of 0.1% of the total protein was taken as a reasonable upper limit, based on soybean expression studies utilizing ELISA (Padgette et al. 1995). The information supporting the crop protein levels and the estimated dietary exposures utilized in setting the CP4 EPSPS dosages is available through an auxiliary publication.[6] These estimates allowed calculation of estimated concentrations of CP4 EPSPS in the products, which were then entered into the TAS computer program as the CP4 EPSPS expo-

[6] Supplementary information has been deposited with American Society for Information Science, National Auxiliary Publication Service (NAPS). See NAPS document no. 04949 for four pages of supplementary material. Order from NAPS, c/o Microfiche Publications, P.O. Box 3513, Grand Central Station, New York, NY 10163-3513. Remit with your order, not under separate cover, $7.75 (U.S. funds on a U.S. Bank only) for photocopies or $4.00 for microfiche. Outside the U.S. and some parts of Canada, add postage of $4.50 for photocopies, $1.75 for microfiche. Institutions and organizations may order by purchase order; however, there is a billing and handling charge for this service of $15, plus any applicable postage.

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sures in a variety of population groups for each crop, for both the 1977–1978 and 1987–1988 USDA surveys on which the program is based. To estimate the maximal exposure possible for CP4 EPSPS, the sum of the estimated exposures in each crop was calculated for the two population groups with the highest exposures for both USDA surveys. The sums of the exposures from soybean, potato, tomato and corn expressing CP4 EPSPS at 0.1% of the total protein were estimated to be 0.269 and 0.173 mg/(kg body wt·d) for those two population groups, based on the 1977–1978 USDA survey. For the 1987–1988 USDA survey, the calculated exposures were estimated to be 0.440 and 0.300 mg/(kg body wt·d) for the two highest groups. To obtain a safety factor of approximately 1000-fold based on the highest possible exposure (first group, 1987–1988 survey), the target dose was set at 400 mg/kg. Two further dilutions of that dose were also performed for feeding to evaluate any potential dose-related effects.

Formulation of CP4 EPSPS dose. The high, medium and low target dose levels were 400, 100 and 40 mg/(kg body wt·d), respectively. Three dosing solutions were prepared using thawed CP4 EPSPS test material with the vehicle buffer as diluent. The three solutions had target CP4 EPSPS concentrations of 12.0, 3.0 and 1.2 g/L. The assumptions used in the dosage formulation were 0.03 kg/mouse at time of gavage, with 20 mice/dosage level and a target dose volume of 1 mL/mouse. On a per-mouse basis, therefore, the 400 mg CP4 EPSPS/(kg body wt·d) dose corresponded to 12 mg CP4 EPSPS/(mouse·d), the 100 mg CP4 EPSPS/(kg body wt·d) dose corresponded to 3 mg CP4 EPSPS/(mouse·d), and the 40 mg CP4 EPSPS/(kg body wt·d) dose corresponded to 1.2 mg CP4 EPSPS/(mouse·d).

Formulation of bovine serum albumin control dose. A bovine serum albumin (BSA) control was prepared by dissolving 306 mg of dry BSA (catalogue no. A4378, lot no. 50H9300, 98% pure; Sigma Chemical) in vehicle buffer to a final volume of 25 mL. Therefore, the 400 mg BSA/(kg body wt·d) dose corresponded to 12 mg BSA/(mouse·d). For a 1-mL dose, the desired BSA concentration was 12 g BSA/L. Therefore, for the 98% pure BSA utilized, the weight of sample utilized for a 25-mL volume was 306 mg.

Dosage protocol. The CP4 EPSPS protein was administered once in a 1-mL volume by gavage to groups of 10 CD-1 mice/sex at target dosages of 40, 100 and 400 mg/kg. A vehicle control group of 10 mice/sex was administered the vehicle buffer at a dosage of 33.33 mL/kg. A protein control group of 10 mice/sex was administered BSA at a target dosage of 400 mg/kg body wt (actual dose was 363 mg/kg body wt). All doses were true solutions and were kept on ice until administered. Aliquots from each dose were taken before and after the mouse gavage to be used for the validation of the doses.

Validation of doses. Aliquots removed from the dose before and after gavage were analyzed. One-dimensional SDS-PAGE gels and Western blots were used to assess apparent molecular weight and immunological response; ELISA (Padgette et al. 1995) was also used to validate the concentration of each dose. The EPSPS specific activity of each sample (in units per milligram) was calculated based on data from the HPLC-based enzymatic activity assay and the protein assay as described above.

Evaluation of test animals. Mice were observed twice daily for signs of toxicity, and food consumption was recorded daily. Body weights were recorded before test and on d 7 after dosing. All animals were anesthetized by CO_2 and then killed by exsanguination on either d 8 or 9 after dosing and subjected to a gross necropsy. External and internal examinations were made visually. Internal cavities were opened, and organs were examined in situ and then removed. Hollow organs were opened and examined. Tissues were retained in 10% (v/v) buffered neutral formalin except for eyes, which were retained in 5% (v/v) buffered neutral formalin with 0.5% (v/v) glutaraldehyde.

Simulated digestive fluids. Reagents used for the in vitro digestive fate study were pepsin (P-7000), pancreatin (P-1500), and hemoglobin (H-2625) (Sigma Chemical). Resorufin-labeled casein (1080733) was obtained from Boehringer Mannheim (Indianapolis, IN). Simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) were prepared as described in the USP (1990) and assayed for activity as described previously (Fuchs et al. 1993).

Assessment of in vitro digestion by Western blot analysis. CP4 EPSPS protein diluted with buffer [50 mmol/L sodium bicarbonate (pH 8.5), 10 mmol/L cysteine, 5 g/L sucrose] was added to either 0.1 (t = 0) or 1 mL of temperature-equilibrated (37°C) digestion solutions to final concentrations of 2 and 50 mg/L for SGF and SIF, respectively. Digestion solutions with SGF were agitated by hand at each sampling interval. Digestion solutions with SIF were agitated continuously except for brief intervals for sampling. Aliquots (50 μL) were removed from the digestion solutions at specific intervals, and the reaction was immediately quenched. Samples incubated in SGF were quenched by neutralization with 15 μL of 0.2 mol/L sodium carbonate per 50 μL of SGF. Samples incubated in SIF were quenched by heating to 100°C for 5 min. Quenched samples were diluted 1:1 in 2× Laemmli SDS-PAGE sample buffer (Laemmli 1970), incubated at 100°C for 5 min and stored at −20°C. To allow an assessment of the recovery of CP4 EPSPS protein from the digestion fluids, CP4 EPSPS was added to 1 mL of buffer and then sampled and quenched as above. An additional control was prepared to determine whether the 50-μL aliquot was representative of the entire digestion incubation solution. For this sample, the digestion incubation volume was reduced to 0.1 mL, and the entire sample was quenched.

Assessment of in vitro digestion by enzymatic activity assay. CP4 EPSPS was added to SGF or SIF to a

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final concentration of 50 μL, incubated, and then quenched as described previously, with the following exceptions: *1*) total digestion solution volumes were 0.5 mL instead of 1 mL, *2*) digestion samples were not agitated during incubation, *3*) no 0.1-mL incubations (as described above) for $t = 0$ and whole-sample incubations were performed. Digestions in SGF were quenched as described above. Digestions in SIF were quenched by immediate dilution of 10-μL aliquots with 40 μL of EPSPS enzymatic activity assay reagents. For $t = 0$ treatments, the CP4 EPSPS was added to chilled (4°C) SIF, mixed, and immediately added to enzymatic assay reagents.

Homology searches using the CP4 EPSPS amino acid sequence. All of the protein comparisons were assisted by the Genetics Computer Group of Madison, WI, sequence-analysis software package (version 7.1, March 1992) (University of Wisconsin Genetics Computer Group 1991). The amino acid sequence of the CP4 EPSPS mature protein utilized was identical to the amino acid sequence of the CP4 EPSPS protein encoded by pMON21104 (Padgette, S. R. and Smith, C. unpublished data). Files containing lists of known allergen and toxin proteins were developed for the strings "allergen" and "toxin." The three available protein databases, SwissProt (Bairoch and Boeckmann 1994), Pir (Protein Information Resource, National Biomedical Research Foundation, Washington, DC) and Genpept (National Center for Biotechnology Information, Bethesda, MD), were analyzed separately using the logicals described in an auxiliary publication.[6] The total number of retrieved proteins was 121 for the keyword "allergen" and 1935 for the keyword "toxin." CP4 EPSPS was compared with the two separate databases using the FASTa program (Gibskov and Devereux 1992, Pearson 1990, Pearson and Lipman 1988, Wilbur and Lipman 1983). A chemical similarity in amino acids or mutational similarity was considered when a match was assigned, using the PAM250 (MDM_{78}) matrix (Dayhoff 1978, George et al. 1990) derived from the amino acid replacement analysis among related proteins.

Statistical analysis. Significant differences between treated animals and their respective controls were determined by Dunnett's multiple comparison test (two-tailed, after ANOVA) (Dixon and Massey 1969, Health Science Computing Facility 1977) for the live body weights, cumulative weight gain, and food consumption. Terminal body weights were evaluated by decision-tree statistical analysis procedures (**Fig. 1**), which, depending on the results of tests for normality (Dixon and Massey 1969) and homogeneity of variances (Bartlett's test) (Dixon and Massey 1969), utilized either parametric (Dunnett's test and linear regression) (Dunnett 1955, Draper and Smith 1966) or nonparametric (Kruskal-Wallis, Jonckheere's and/or Mann-Whitney tests) (Breslow 1970, Hollander and Wolfe 1973, Mann and Whitney 1947) routines to detect group differences and analyze for trend.

RESULTS

Comparison of plant-expressed and E. coli–expressed CP4 EPSPS. CP4 EPSPS expressed in glyphosate-tolerant crops consisting of soybean seed (line 61-67-1), canola seed (line 17131-68) and cotton seed (line 1698) were purified in milligram quantities from seed extracts. The GTS line 61-67-1 was transformed with a plasmid that encodes a CP4 EPSPS gene identical to that found in the lead soybean line being pursued for commercialization, which is denoted 40-3-2 (Padgette et al. 1995). The only gene expressed from the DNA introduced into GTS line 40-3-2 is the preprotein consisting of the petunia EPSPS chloroplast transit peptide and the CP4 EPSPS (Padgette et al. 1995). Both the glyphosate-tolerant canola and cotton plants express a CP4 EPSPS gene product identical to the glyphosate-tolerant soybean CP4 EPSPS, except that the transit peptide sequence is from arabidopsis EPSPS (Klee et al. 1987). The EPSPS-specific activities in the purified samples of CP4 EPSPS from *E. coli* (pMON21104), soybean, canola and cotton were determined to be 3.0, 3.9, 3.6, (corrected for 25% purity) and 2.8 U/mg, respectively. Dilutions of the *E. coli*– and plant-expressed CP4 EPSPS proteins were analyzed on SDS-PAGE gels. Protein bands were visualized by both Coomassie blue stain (**Fig. 2***A*) and Western blot (Fig. 2*B* and **Fig. 3**). All CP4 EPSPS protein samples analyzed by Western blots contained a single protein band cross-reacting with anti-CP4 EPSPS polyclonal antibodies, which migrated consistent with the predicted apparent molecular weight of 47.6 kDa (Padgette, S. R. and Barry, G. F., unpublished data) (Fig. 2*B* and 3).

Because the CP4 EPSPS genes were engineered for plant expression by fusing the 5′-end of the gene to chloroplast-transit peptide sequences derived from either petunia EPSPS (for soybeans) (Padgette et al. 1995) or arabidopsis EPSPS (for cotton and canola), these results indicate that the chloroplast-transit peptides were cleaved from the "pre-CP4 EPSPS" as expected. The petunia EPSPS and arabidopsis EPSPS transit peptides fused to CP4 EPSPS have predicted molecular masses of 7.9 kDa (della-Cioppa et al. 1986) and 8.2 kDa (Klee et al. 1987), giving 55.5 and 55.8 kDa, respectively, as the predicted masses of the preproteins. No species of these molecular masses were identified in any of the Western blots performed. In addition, the amino-terminal amino acid sequences of the CP4 EPSPS purified from glyphosate-tolerant soybean, canola and cotton were determined, along with that of the *E. coli*–produced CP4 EPSPS from pMON21104 (**Table 1**). The sequences of the plant-expressed CP4 EPSPS matched the *E. coli*–produced enzyme with the exception that the CP4 EPSPS purified from *E. coli* pMON21104 retains the initiating methionine required for bacterial expression, whereas the plant-produced CP4 EPSPS lacks this methionine.

Another variable assessed was the presence or ab-

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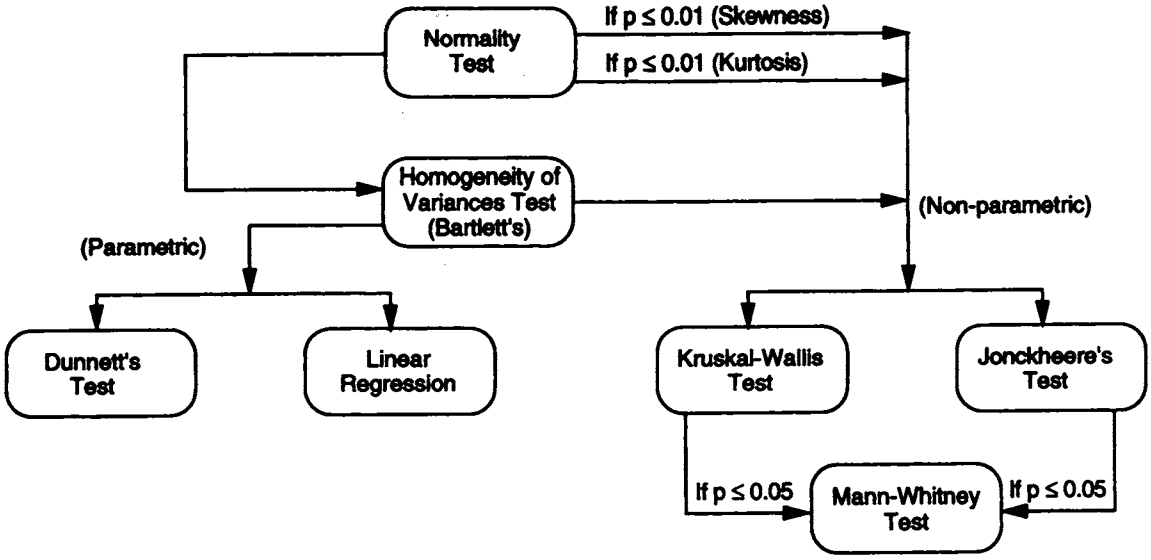

FIGURE 1 Decision tree statistical analysis procedure used for evaluation of terminal body weights in the acute oral toxicity study in mice. Categorical data were analyzed with an uncorrected chi-square test (Snedecor and Cochran 1967). Dunnett's (Dunnett 1994) and Mann-Whitney (Mann and Whitney 1947) tests to detect group differences were performed two tailed.

sence of glycosylation, whereby carbohydrate moieties associated with the blotted protein bands can be visualized using specific reagents. The glycoprotein standards used as positive controls, horseradish peroxidase and transferrin, both exhibited strong signals by glycosylation staining (Fig. 4). Neither the *E. coli*–expressed nor the soybean-expressed CP4 EPSPS protein stained positively for glycosylation with this detection system. Negative staining was observed due to the large amount of material applied (Fig. 4). The lack of carbohydrates detected in the *E. coli*–expressed EPSPS was not surprising, because proteins expressed in prokaryotes are not glycosylated (Slater 1988). These results indicate that, within the limits of sensitivity of the method used, the soybean-expressed CP4 EPSPS exhibits no evidence of glycosylation. Similar results were

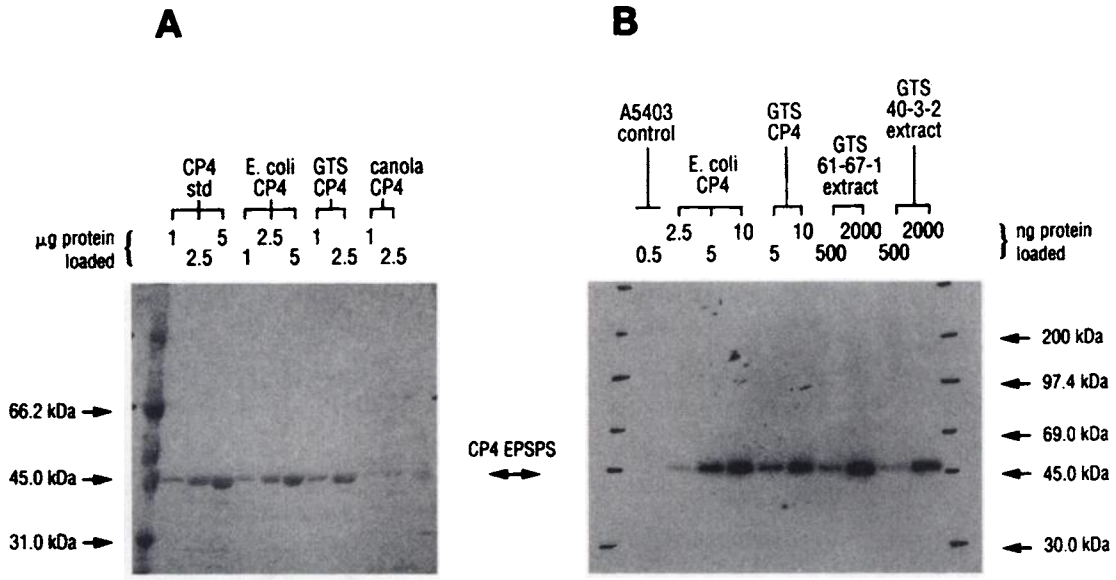

FIGURE 2 Comparison of *E. coli*–produced CP4 EPSPS to glyphosate tolerant soybean (GTS)-expressed CP4 EPSPS. SDS-PAGE gels (1.0 mm thick, 4 to 20% acrylamide gradient) were loaded with CP4 EPSPS protein from soybean (line 61-67-1), canola (line 17131-68) and *E. coli* test material and analyzed according to the method of Laemmli (1970). *A*. Coomassie-stained SDS-PAGE. The CP4 EPSPS standard was purified from pMON17101-expressing *E. coli* (Padgette, S. R. unpublished data). *B*. Western blot. SDS-PAGE gels were electrophoretically transferred to Fluorotrans polyvinylidene difluoride membrane (VWR 28152-454), a specific anti-CP4 EPSPS polyclonal antibody was hybridized to the blot, and the blot was then reacted with ^{125}I-labeled protein G and developed with autoradiography for 18 h. CP4 EPSPS from *E. coli* was spiked into 500 ng of protein from line A5403 (control) to account for possible matrix effects. Blotted molecular weight markers were marked on the blot for visualization on the autoradiogram.

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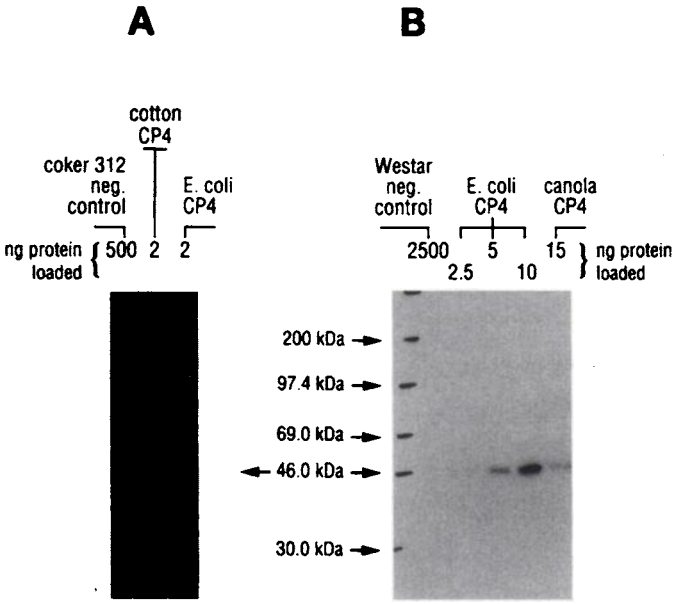

FIGURE 3 Western blot comparison of E. coli–produced CP4 EPSPS to canola-and cotton-expressed CP4 EPSPS. The Western blot procedure was as described in the legend to Figure 2B. The *E. coli*–produced CP4 EPSPS was the test material for the safety assessment studies, from pMON21104 (Padgette, S. R. and Kolacz, K. H. unpublished data). *A:* Cotton. The cotton-produced CP4 EPSPS was from line 1698. Cotton and *E. coli* CP4 EPSPS were spiked into 500 ng of protein from the Coker 312 control to account for possible matrix effects. *B:* Canola. The canola-produced CP4 EPSPS was from line 17131-68. CP4 EPSPS from *E. coli* was spiked into 1000 ng of protein from the Westar control.

obtained for the cotton- and canola-expressed CP4 EPSPS (data not shown). Therefore, on the bases of the enzymatic activity, apparent molecular weight, immunoreactivity, amino-terminal sequence and lack of glycosylation, we conclude that the *E. coli*–produced CP4 EPSPS was an appropriate test material for the acute mouse gavage and digestive fate studies.

Preparation and confirmation of doses. Values obtained from the protein assay of the thawed test material and the initial ELISA value for CP4 EPSPS content were used for the calculation of the CP4 EPSPS test material final dosage concentration. The solution was determined to contain 18.75 g/L total protein, at 88.6% purity (based on ELISA), yielding a CP4 EPSPS concentration of 16.62 g/L in the stock test material solution. High, medium and low doses were prepared using the 16.62 g/L CP4 EPSPS solution. Dilutions of each CP4 EPSPS dose were analyzed, before and after gavage, using the CP4 EPSPS ELISA for quantification. The results are summarized in **Table 2**; in every case, the analytical dose (actual) was somewhat higher than the theoretical dose. On the basis of these ELISA results, the actual doses of CP4 EPSPS gavaged were 572, 154 and 49 mg CP4 EPSPS/(kg body wt·d) for the high, medium and low doses, respectively, based on 1 mL dosing solution/mouse and 0.03 kg body wt/mouse. The amount of functionally active CP4 EPSPS in each dose was determined before and after gavage, based on protein concentration (g/L) and CP4 EPSPS activity (U/L), to yield EPSPS specific activities (U/mg protein) (Table 2).

The amounts of total protein determined by the Bradford protein assay in the before-dosing and after-dosing BSA solutions were 10.88 and 11.52 g/L, respectively. The BSA dosage amount was therefore 363 mg/(kg body wt·d), based on 10.88 g BSA/(L·d), 1 mL dosing solution/mouse, and 0.03 kg body wt/mouse.

SDS-PAGE was performed to evaluate the purity and apparent molecular weights of the test, control and reference substances. Dilutions of the doses before and after gavage were analyzed by SDS-PAGE stained with Coomassie blue (**Fig. 5**). Both the test and reference CP4 EPSPS protein samples migrated as a single band and with the same apparent molecular weight. In addition, there was no visible difference in the Western blots of protein before gavage and after gavage (data not shown). Thus, there was no evidence of substantial chemical or functional degradation of the CP4 EPSPS protein in any of the doses, based on SDS-PAGE, Western blot, enzymatic assay and ELISA (data not shown). The vehicle control buffer also had a faint immunoreactive band at the same apparent molecular weight as CP4 EPSPS. This can be explained by the fact that the vehicle control was the dialysate of the test material

TABLE 1

Amino-terminal amino acid sequence analysis of CP4 EPSP isolated from soybean, canola and cotton relative to the predicted sequence

CP4 EPSPS[1]	Amino-terminal amino acid sequence																
pMON21104, P[2]	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S...		
pMON21104, D	(M)	L	(H)	G	A	S	(S)	(R)	P	A	T	A	R	K	S		
Plant-expressed, P[3]	..	L	H	G	A	S	S	R	P	A	T	A	R	K	S...		
Soybean-expressed, D		(L)	H	G	A	S	S	R	P	A	T	A	R	K	S	S	
Canola-expressed, D		X	X	X	(A)	S	S	R	P	A	T	(A)	R	K	(S)	X	G
Cotton-expressed, D		L	H	X	A	S	(S)	R	P	A	(T)	A	R	K	S	S	

[1] P = predicted; D = determined.
[2] Padgette, S. R. and Kolacz, K. H., unpublished data.
[3] Padgette, S. R., Barry, G. F. and Eichholtz, D. E., unpublished data; the initial dotted line indicates that a chloroplast transit peptide was engineered onto the CP4 EPSPS gene for plant expression.

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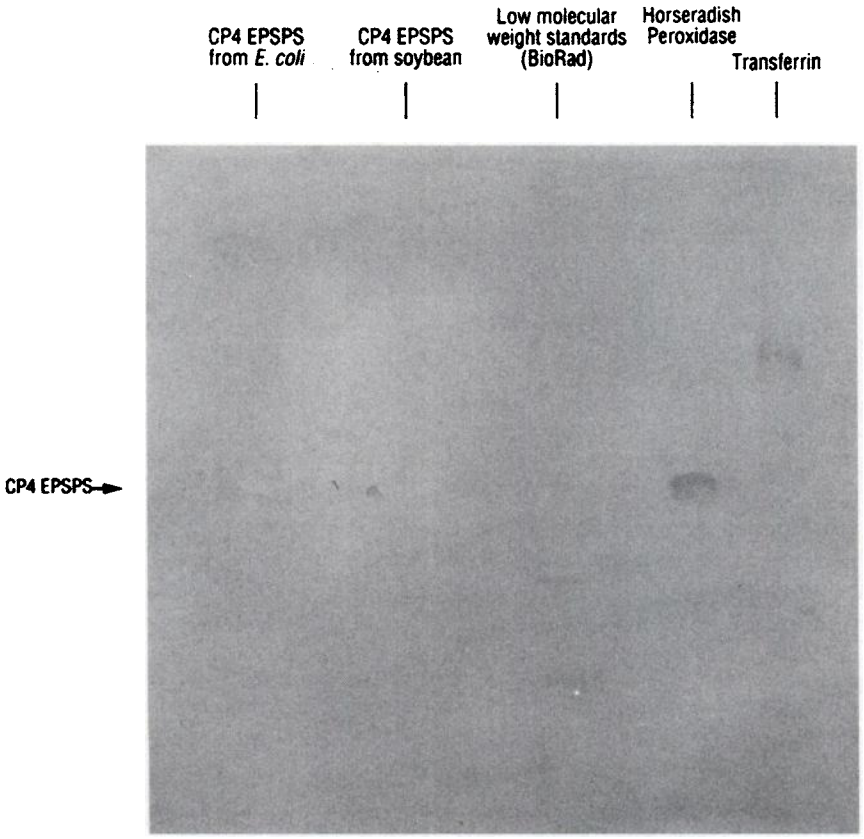

FIGURE 4 Glycosylation assessment of soybean and *E. coli*–derived CP4 EPSPS. SDS-PAGE of *E. coli*– and GTS-expressed CP4 EPSPS and controls was performed, followed by electrophoretic transfer of proteins onto polyvinylidene difluoride membrane and detection of carbohydrate moieties, as described in Materials and Methods.

prior to gavage. The presence of trace amounts of CP4 EPSPS in the buffer is most likely due to dialysis membrane molecular-weight cutoff inefficiency during dialysis. The concentration of CP4 EPSPS in the vehicle control was diluted by approximately 1:24,000 relative to the concentration of the high dose solution.

Acute mouse gavage in-life observations and gross pathology. No treatment-related adverse effects were observed in animals dosed with CP4 EPSPS protein. There were no significant differences in body weight (**Table 3**), cumulative body weight, or food consumption (data not shown) between the vehicle or protein control groups and CP4 EPSPS protein–treated groups. A few minor pathological findings in female mice (such as corneal opacity, kidney and pituitary lesions, and hydrometra of the uterus) were observed at necropsy

TABLE 2

CP4 EPSPS dosage calculations and enzymatic activities

Dosage level	Target CD4 EPSPS concentration	Actual CP4 EPSPS concentration by ELISA	Specific activity[1]	Target dose	Actual dose
	g/L		*U/mg protein*	*mg/(kg body wt·d)*	
Before dosing					
High	12.00	17.16	3.59	400	572
Medium	3.00	4.63	3.37	100	154
Low	1.20	1.48	2.81	40	49
After dosing					
High	12.00	19.71	3.19	—	—
Medium	3.00	3.73	3.10	—	—
Low	1.20	1.51	3.25	—	—

[1] Specific activity was calculated based on protein concentration (g/L) as determined by protein assay (three replications) and EPSPS phosphate release enzymatic assay (two replications).

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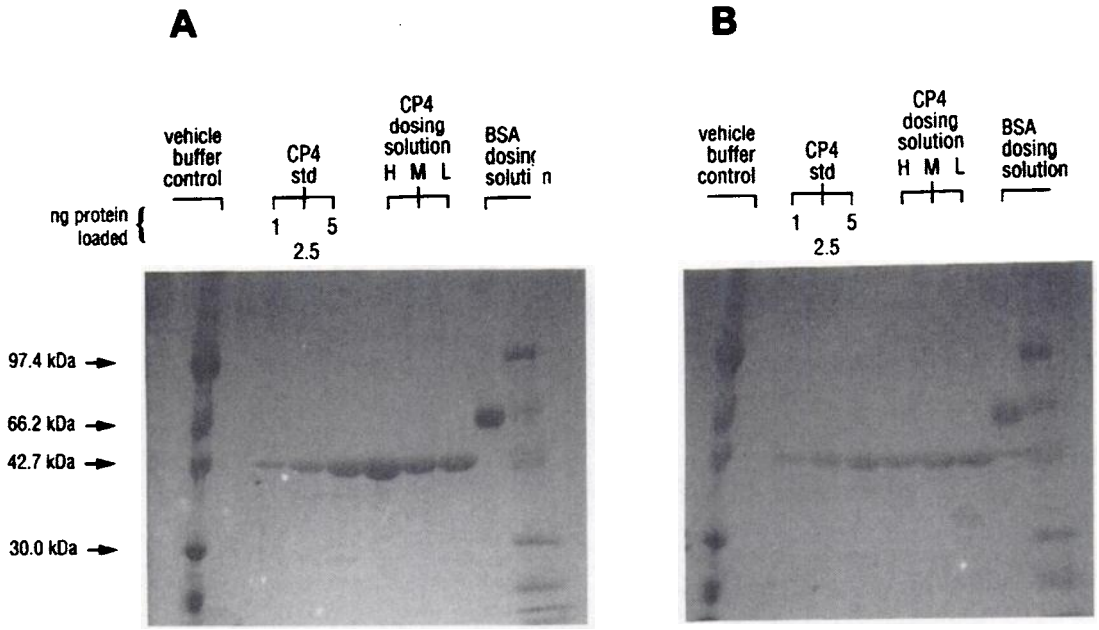

FIGURE 5 Coomassie blue–stained SDS-PAGE gels confirming integrity and apparent molecular weight of the doses. Gels were 1.0-mm-thick gradient gels, 4 to 20% acrylamide; H = high dose, M = medium dose, L = low dose. Approximately 5 μg of the high, medium, low and bovine serum albumin (BSA) dosing solutions was loaded per lane. *A:* Prior to dosing. *B:* After dosing.

to be randomly distributed among all groups and are commonly seen in the strain of mice used by the testing laboratory. No findings were noted in the male mice. None of these findings were considered treatment related.

Assessment of CP4 EPSPS digestion by Western blot. CP4 EPSPS protein degradation in SGF was assessed by Western blot analysis (**Fig. 6*A***). No CP4 EPSPS protein was detected after 15 s of incubation, the earliest time interval evaluated. Simulated gastric fluid alone did not contain bands that may interfere with the assessment of CP4 EPSPS protein levels at either $t = 0$ s or after 120 s of incubation. The recovery of CP4 EPSPS spiked into SGF was estimated to be approximately 100% by visual comparison of the band intensities of CP4 EPSPS in buffer alone and in SGF at $t = 0$ (unincubated). The dose of CP4 EPSPS to the SGF was confirmed by assessment of CP4 EPSPS detected by Western blot at $t = 0$ (10 ng CP4 EPSPS/lane) compared with the expected level if the final CP4 EPSPS protein added to SGF were 2 mg/L (10 ng/lane). We therefore conclude that the half-life of CP4 EPSPS in SGF is less than 15 s.

CP4 EPSPS degradation in SIF was also assessed by Western blot analysis (Fig. 6*B*). More than 50% of the CP4 EPSPS was degraded after a 10-min incubation in SIF at 37°C as compared with the level detected in the $t = 0$ sample by visual assessment of Western blots.

TABLE 3

Summary of body weight data from the acute oral toxicity study in mice[1]

		Body weight			
		Male		Female	
Group[2]	Target dose	Pre-test	Final	Pre-test	Final
		g			
Vehicle buffer control	33.3 mL/kg	28.0 ± 0.48	29.3 ± 0.57	24.8 ± 0.38	25.2 ± 0.52
BSA control	33.3 mL/kg	28.1 ± 0.43	29.9 ± 0.38	24.8 ± 0.38	25.0 ± 0.28
Low dose CP4 EPSPS	49.0 mg/kg	28.0 ± 0.42	30.2 ± 0.34	24.6 ± 0.39	24.8 ± 0.48
Medium dose CP4 EPSPS	154.0 mg/kg	28.0 ± 0.42	29.5 ± 0.53	24.6 ± 0.35	25.0 ± 0.33
High dose CP4 EPSPS	572.0 mg/kg	28.1 ± 0.41	29.5 ± 0.40	24.6 ± 0.40	24.7 ± 0.56

[1] No significant difference in body weights, Dunnett's test (two-tailed) ($P \leq 0.01$). Values are mean body weights ± SD, $n = 10$. BSA = bovine serum albumin.

[2] No significant difference among variances of the different groups, Bartlett's test ($P \leq 0.01$).

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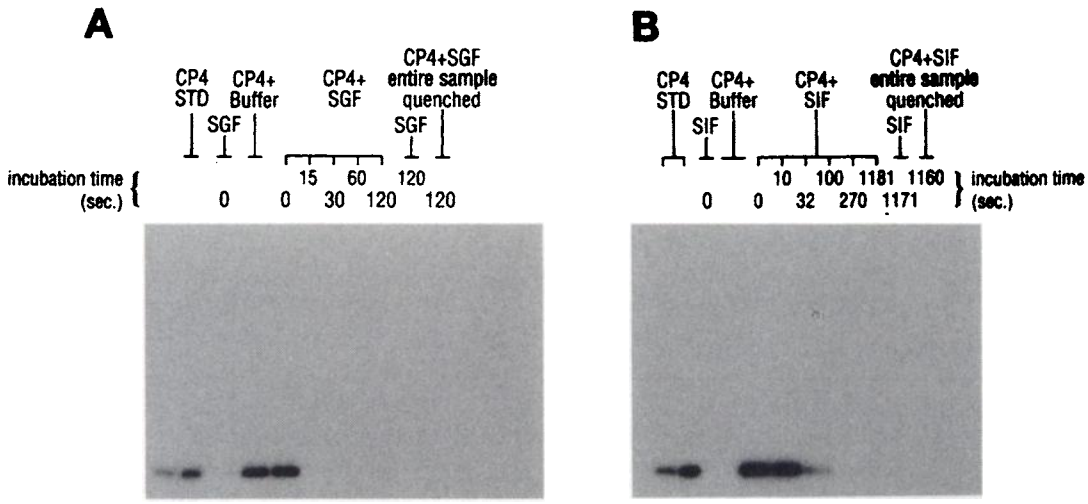

FIGURE 6 Digestive fate assessment of CP4 EPSPS. Each of the Western blots shown is one of three replicate digestions, performed as described in Materials and Methods. Two different amounts of CP4 EPSPS standard (5 and 10 ng) were loaded per lane. *A:* Simulated gastric fluid (SGF). Western blot analysis of CP4 EPSPS incubated in SGF for 0, 15, 30, 60 and 120 s. *B:* Simulated intestinal fluid (SIF). Western blot analysis of CP4 EPSPS incubated in SIF for 0, 10, 32, 100, 270 and 1181 min.

This assessment was corroborated by scanning densitometry (data not shown). No CP4 EPSPS protein was detected after 100 min of incubation in SIF. Simulated intestinal fluid alone did not contain bands that may interfere with the assessment of CP4 EPSPS protein levels at either $t = 0$ min or after 19.5 h of incubation. The recovery of CP4 EPSPS spiked into SIF was estimated to be approximately 100% by visual comparison of the band intensities of CP4 EPSPS in buffer alone and in SGF at $t = 0$ (unincubated). The dose of CP4 EPSPS to the SIF was confirmed by assessment of CP4 EPSPS detected by Western blot at $t = 0$ (10 ng/lane) compared with the expected level if the final CP4 EPSPS protein added to SIF were 50 mg/L (10 mg/L, after dilution). The visual assessment of CP4 EPSPS protein levels by Western blot analysis was compared with levels quantified by densitometry, and assessments were found to be comparable. We therefore conclude that the half-life of CP4 EPSPS in SIF is less than 10 min.

Assessment of digestion by enzymatic activity. The degree to which SGF and SIF interfere with the determination of CP4 EPSPS protein enzymatic activity was investigated. No substantial interference was observed: CP4 EPSPS activity was measured in the presence of both quenched SGF and SIF at the level (10 μL/assay) used for subsequent digestion assays (data not shown). No turnover of phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate was observed when SGF and SIF were incubated in assay reagents without added CP4 EPSPS or when SGF and SIF were added with CP4 EPSPS but without the substrate, shikimate-3-phosphate (data not shown). These results support the appropriateness of this assay to assess the degradation of CP4 EPSPS enzymatic activity in digestion solutions.

No CP4 EPSPS enzymatic activity was detected after a 2-min incubation in SGF ($n = 3$); the limit of detection of the assay corresponded to approximately 16% of the added CP4 EPSPS enzymatic activity. Two experiments were performed to assess CP4 EPSPS activity degradation in SIF (**Table 4**). In both experiments, 93–95% of added CP4 EPSPS was still present after a 10-min incubation in SIF. CP4 EPSPS activity had decreased to <9% and <6% of the initial level after incubations of 285 and 270 min, respectively.

Amino acid sequence homology searches. CP4 EPSPS was compared with the generated databases of known allergens and toxins using the FASTa algorithm for rapid database comparison. The results of the FASTa comparison (using *ktup* = 2) for each of the protein databases are listed in the supplemental material.[6] The PAM250 scores used for ranking the sequences (*initn*) ranged from 38 to 9 for the allergen database. A range was not generated for the toxin database because only the top 50 scores for each of the protein databases are reported. The data generated comparing CP4 EPSPS to allergenic and toxic proteins indicated that there are no significant stretches of homology between CP4 EPSPS and any of the allergens or toxins within the databases. The low *initn* scores from the top 10 FASTa scores did not contain any small peptides from which a 100% identity may generate a "low" *initn* score. Each alignment or FASTa output within the allergen database and the toxin database was examined, and, in all cases, there was no indication of significant homology between CP4 EPSPS and the database of allergenic or toxic proteins.

The relevance of the alignments was tested using the command "SHUFFLE" to randomize the CP4 EPSPS sequence while keeping the amino acid composition identical (Needleman and Wunsch, 1970). The shuffled protein was compared with the identical databases of

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TABLE 4

Dissipation of CP4 EPSPS enzymatic activity in simulated intestinal fluid[1]

Incubation time	Experiment 1		Experiment 2	
	CP4 EPSPS activity[2]	Activity remaining	CP4 EPSPS activity[2]	Activity remaining
min		%		%
0	21.3 ± 0.8	100	32.2 ± 0.5	100
10	20.3 ± 0.6	95	30.0 ± 0.7	93
270	—	—	<2.0 ± 1.1	<6
285	<2.0 ± 0.1	9	—	—

[1] Values are means ± SEM, $n = 3$.
[2] CP4 EPSPS activity = % turnover of 1 mmol [^{14}C]phosphoenolpyruvate to [^{14}C]EPSP in the assay.

allergenic and toxin proteins using the same word size (*ktup* = 2) as for the CP4 EPSPS sequence. The results of the FASTa comparison with the randomized CP4 EPSPS showed that the *initn* scores from the allergen database and the toxin database resembled the *initn* scores generated with the unrandomized CP4 EPSPS. This indicates that the generated alignments between CP4 EPSPS and the databases of allergen and toxin proteins were determined by random distribution of amino acids in a peptide sequence. The results from one randomization of the CP4 EPSPS peptide sequence clearly indicate that CP4 EPSPS has no relationship, other than a random distribution of its amino acids, to the database of allergens and toxins.

DISCUSSION

There is no a priori reason to expect any toxicity to be associated with the EPSPS protein from *Agrobacterium* sp. strain CP4. CP4 EPSPS is functionally similar to the EPSPS proteins typically present in foods and feeds derived from plant and microbial sources, based on the reaction catalyzed. With respect to amino acid sequence, CP4 EPSPS is 26% identical to soybean EPSPS. However, there is considerable divergence among known EPSPSs (Padgette et al. 1996a). For instance, the amino acid sequence of CP4 EPSPS is 41% identical at the amino acid level to *Bacillus subtilis* EPSPS, whereas the soybean EPSPS is 30% identical to *Bacillus subtilis* EPSPS. The divergence of the CP4 EPSPS amino acid sequence from typical food EPSPS sequences is therefore on the same order as the divergence among food EPSPS themselves. In addition, CP4 EPSPS shares no meaningful amino acid sequence homology with the 1935 proteins listed as "toxins" in the SwissProt, Pir and Genpept sequence databases.

Although no toxicity was anticipated for CP4 EPSPS, we performed several experiments to further assess the safety of CP4 EPSPS. Mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg experienced no adverse effects. This dose represents a safety margin of approximately 1300-fold relative to the highest potential human consumption of CP4 EPSPS if the protein was expressed in soybean, corn, tomato and potato (assuming no loss of CP4 EPSPS due to processing). There were no significant differences in body weight, cumulative body weight, or food consumption between the vehicle or BSA protein control groups and CP4 EPSPS protein–treated groups.

Simulated mammalian gastric and intestinal digestive mixtures were prepared and used to assess the susceptibility of CP4 EPSPS to proteolytic digestion. CP4 EPSPS was rapidly degraded by the simulated components of the mammalian digestive system, greatly minimizing any potential for this protein to be absorbed by the intestinal mucosa. The data demonstrated a half-life of CP4 EPSPS of less than 15 s in the gastric system and less than 10 min in the intestinal system, based on Western blot analysis. Enzymatic activity of CP4 EPSPS was not detected in the gastric system after 2 min, and less than 9% was detected after 285 min in the intestinal system. The intestinal system results suggest that the antigenic sites on the CP4 EPSPS protein for the particular antibody used in this study are more sensitive to proteolytic degradation under these conditions than is the functional active site of CP4 EPSPS. To put the rapid degradation of the CP4 EPSPS protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by approximately 50% in 2 h, whereas liquid empties 50% in approximately 25 min (McCallum 1989). If some of the CP4 EPSPS protein did survive the gastric system, it would degrade rapidly in the intestine. Time for the degradation of CP4 EPSPS compares with transit times through the intestine (for radiolabeled chromate, which is not absorbed) of 4 to 10 h for the first products to appear in the feces and 68 to 1654 h for the last to be detected (Davenport 1971). Thus, using both systems, CP4 EPSPS is predicted, as expected, to be readily digested in the mammalian digestive tract.

In addition, the allergenic potential of CP4 EPSPS is

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minimal. The EPSPS proteins are a diverse set of related proteins typically present in foods and feeds derived from plants and microbes and are not known to be commonly allergenic. The low allergenic potential of GTS was further supported from two perspectives. First, the endogenous protein allergens and their levels present in commercially available soybeans were compared with those present in GTS and shown to be similar (Burkes and Fuchs 1996). Second, the biochemical properties of known allergenic proteins were compared with those of the CP4 EPSPS protein as an approach to assess the allergenic potential for the CP4 EPSPS.

Although there are no predictive assays available to assess the allergenic potential of proteins (U.S. Food and Drug Administration 1992), the biochemical profile of the CP4 EPSPS protein provides a basis for allergenic assessment when compared with known protein allergens. Allergenic proteins are often, though not always, glycosylated proteins between 10 and 70 kDa in size. Moreover, protein allergens must be stable to the peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa. Another important factor contributing to the allergenicity of proteins is their high concentrations in foods that elicit an allergenic response (Taylor et al. 1987 and 1992).

CP4 EPSPS at 47.6 kDa fits the mass criterion of 10 to 70 kDa, as do most proteins, but possesses none of the other characteristics common to protein allergens. CP4 EPSPS is not heat stable, and all detectable functional activity and ELISA reactivity are lost after the processing and toasting procedure (Padgette, S. R., and Taylor, M. L., unpublished data; Padgette et al. 1996b). Importantly, CP4 EPSPS is degraded by proteases present in the mammalian digestive system, as described herein, which supports the prediction that the CP4 EPSPS protein will not survive the peptic and tryptic conditions of the mammalian digestive system.

Because most protein allergens are glycosylated, the CP4 EPSPS, as purified from GTS seed (and glyphosate-tolerant cotton and canola seed), was examined for glycosylation and shown not to be glycosylated. In addition, CP4 EPSPS showed no significant homology to any of the 121 amino acid sequences reported for the allergens in the three current protein databases. Native CP4 EPSPS showed no greater homology to any of the 121 amino acid sequences for the allergenic proteins than did a scrambled sequence of the same amino acids that compose the CP4 EPSPS.

Finally, most allergens are present as major protein components in the specific food (see references in Padgette et al. 1996a). In contrast, CP4 EPSPS is present in soybean seed at low levels of approximately 0.03% of the fresh weight of the soybean seed and approximately 0.08% of the total protein (Padgette et al., 1995). The low levels of CP4 EPSPS in soybeans, combined with the thermal (Padgette, S. R., and Taylor, M. L., unpublished data; Padgette et al. 1996b) and digestive lability of this protein relative to that for known food allergens, suggest a very low probability of CP4 EPSPS surviving to be absorbed via the intestinal mucosa during consumption and thus potentially triggering production of antibodies, including the immunoglobulin E antibodies responsible for allergenicity.

The data presented herein confirm the safety of the CP4 EPSPS protein and, when taken in context with the compositional analysis (Padgette et al. 1996b) and animal feeding studies (Hammond et al. 1996) performed on GTS, provide a key element of the information supporting the conclusion that GTS is as safe and nutritious as traditional soybeans currently being marketed.

ACKNOWLEDGMENTS

We would like to thank C. Smith and M. Jennings for protein sequencing, L. Holden for statistical analysis, the Monsanto Environmental Health Lab for animal work, and all of our colleagues at Monsanto for their efforts in support of this study.

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