Determining whether transgenic and endogenous plant DNA and transgenic protein are detectable in muscle from swine fed Roundup Ready soybean meal[1,2,3]

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ABSTRACT: Questions regarding the digestive fate of DNA and protein from transgenic feed have been raised in regard to human consumption and commercial trade of animal products (e.g., meat, milk, and eggs) from farm animals fed transgenic crops. Using highly sensitive, well-characterized analytical methods, pork loin samples were analyzed for the presence of fragments of transgenic and endogenous plant DNA and transgenic protein from animals fed meal prepared from conventional or glyphosate-tolerant Roundup Ready (RR) soybeans. Pigs were fed diets containing 24, 19, and 14% RR or conventional soybean meal during grower, early-finisher, and late-finisher phases of growth, respectively, and longissimus muscle samples were collected (12 per treatment) after slaughter. Total DNA was extracted from the samples and analyzed by PCR, followed by Southern blot hybridization for the presence of a 272-bp fragment of the *cp4 epsps* coding region (encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4) and a 198-bp fragment of the endogenous soybean gene *le1* (encoding soy lectin). Using 1 μg of input DNA per reaction, none of the extracted samples was positive for *cp4 epsps* or *le1* at the limit of detection (LOD) for these PCR/Southern blot assays. The LOD for these assays was shown to be approximately one diploid genome equivalent of RR soybean DNA, even in the presence of 10 μg of pork genomic DNA. A 185-bp fragment of the porcine preprolactin (*prl*) gene, used as a positive control, was amplified from all samples showing that the DNA preparations were amenable to PCR amplification. Using a competitive immunoassay with an LOD of approximately 94 ng of CP4 EPSPS protein/g of pork muscle, neither the CP4 EPSPS protein nor the immunoreactive peptide fragments were detected in loin muscle homogenates from pigs fed RR soybean meal. Taken together, these results show that neither small fragments of transgenic DNA nor immunoreactive fragments of transgenic protein are detectable in loin muscle samples from pigs fed a diet containing RR soybean meal.

Key Words: Biotechnology, Enzyme-Linked Immunosorbent Assay, Pigs, Polymerase Chain Reaction, Soybeans

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Introduction

Approximately 68% of the soybeans grown in the United States in 2001 were Roundup Ready (**RR**; Faust, 2002), and animal feed use accounts for the majority of total soybean production since soybean meal is the preferred protein source for swine and poultry diets (NRC, 1994; 1998). Soybean plants tolerant to glyphosate herbicide (RR soybean event 40-3-2) were produced by inserting a gene expression cassette encoding a glyphosate-tolerant enzyme, 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium sp.* strain CP4 (**CP4 EPSPS**), into the soybean genome. Interest in the digestive fate of transgenic DNA and protein in animals fed RR soybean and other genetically modified crops has arisen because of questions over human consumption and commercial trade of products from these animals. A number of studies of the digestive fate of transgenic DNA and proteins have recently been reviewed (Flachowsky and Aulrich, 2001). Three studies (Klotz and Einspanier, 1998; Khumnirdpetch et al., 2001; Phipps et al., 2002) reported that fragments of

[1]Roundup Ready is a registered trademark of Monsanto Technology LLC.

[2]Roundup Ready soybean event 40-3-2.

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the *cp4 epsps* coding region of RR soybean were not detectable in blood and milk from dairy cows and a variety of samples from broiler chickens fed RR soybean meal. In addition, the CP4 EPSPS protein appears to be rapidly degraded under simulated gastric conditions (Harrison et al., 1996). Consistent with these in vitro results, a sensitive ELISA did not detect the CP4 EPSPS protein in whole eggs, egg whites, liver, or feces from laying hens fed RR soybean meal over their 7-wk productive life (Ash et al., 2000). However, the digestive fate of the CP4 EPSPS gene and protein is unknown for nonruminant mammals, such as swine.

The present study describes DNA and protein analytical methods optimized for high sensitivity to test for fragments of the *cp4 epsps* and *le1* (encoding soy lectin) genes and both the intact CP4 EPSPS protein and its immunoreactive fragments in loin muscle samples from finishing swine fed a diet containing RR soybean meal.

Materials and Methods

Samples and Processing

The swine from which tissues were obtained were from a feeding study to assess growth performance and carcass composition of pigs fed diets containing dehulled soybean meal prepared from RR and conventional soybeans (Cromwell et al., 2002). Pigs (five replications of barrows and five replications of gilts with five pigs per pen) were fed fortified corn-soybean meal diets containing 24, 19, or 14% RR or conventional soybean meal on an air-dry weight basis during grower (24 to 55 kg), early-finisher (55 to 87 kg), and late-finisher (87 to 111 kg) phases of growth, respectively. The identity of the soybeans used to formulate both the conventional and transgenic diets was confirmed by a standard sandwich ELISA for CP4 EPSPS protein prior to diet formulation. In addition, the meal used to formulate the diets was also tested by CP4 EPSPS immunoassay using a Traitcheck RUR lateral flow test (Strategic Diagnostics Inc., Newark, DE) to confirm the presence or absence of the transgenic protein in the test and control processed meals, respectively. At the termination of the experiment, the barrows were killed. A sample of the longissimus muscle was collected from two or three randomly selected barrows from each pen (12 per treatment) and placed in individual plastic bags with labels indicating each animal's ear notch number, sample collection date and tissue type, and then immediately frozen and shipped to the laboratory on dry ice. Animal selection was based on randomly selected ear notch numbers prior to sample collection, and gilts were retained for use as breeding stock.

Loin muscle samples from 12 pigs fed diets containing RR soybean meal and 12 pigs fed diets containing conventional soybean meal were subjected to DNA and protein analyses. The ear notch numbers were used to track tissue samples, resulting in a blinded sequence of samples that was maintained throughout the subsequent analyses. Frozen tissue samples were further processed in the laboratory using aseptic techniques as described by Jennings et al. (2003). All subsampling occurred in a laboratory where no PCR products or plasmids were handled. Subsamples were stored in a –80°C freezer until analyzed.

Deoxyribonucleic Acid Analysis

Deoxyribonucleic Acid Extraction. Pork loin samples (2 to 9 g) were homogenized and treated with Proteinase K and RNase A as previously described (Jennings et al., 2003). Immediately following a 30-min incubation, 250 μL of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol) was added to each 600-μL homogenate and vortex mixed. The homogenate was then centrifuged at 12,000 × *g* to separate the phases, and the aqueous phase was transferred to a 1.5-mL tube (previously treated with UV light to destroy any potential DNA contamination). A one-third volume of 7.5 *M* ammonium acetate and one volume of 100% isopropanol were added to the aqueous phase. The solution was gently mixed by inversion for approximately 5 min, and then the DNA was precipitated by centrifugation at 12,000 to 14,000 × *g* for 15 to 20 min. DNA pellets were washed with 70% (vol/vol) ethanol, dried under vacuum, and then resuspended in 100 μL of 1× TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). To serve as a positive control for the extraction procedure, approximately 250 pg of purified genomic DNA from RR soybeans was spiked into tissue homogenates containing 120 to 150 mg of starting tissue immediately prior to the start of the DNA extraction procedure. A negative DNA extraction control, consisting of a 600-μL aliquot of extraction buffer subjected to the extraction procedure, was also included with each set of samples. Every pork loin sample was extracted in duplicate. Each extract was analyzed by PCR for *cp4 epsps* and *le1*.

Polymerase Chain Reaction and Southern Blot Analysis. Extreme caution was used when handling animal samples and DNA extracts so as not to contaminate them with previously amplified DNA, plasmids containing transgenes, or any other potential sources of plant and/or transgenic material (Jennings et al., 2003). Extracted DNA was quantitated using Hoefer's DyNA Quant 200 Fluorometer or Molecular Devices' *fmax* Fluorescence Microplate Reader with Hoechst dye. Prior to the initiation of these studies, a standard preparation of genomic DNA from RR soybean event 40-3-2 was extracted, purified, and characterized, and then used throughout these analyses as the reference standard for PCR. Experiments with the *cp4 epsps* and *le1* PCR assays were conducted with this purified RR soybean genomic DNA added to pork genomic DNA extract to determine that up to 10 μg of pork DNA could be used in both assays without detectable matrix effects. Therefore, the PCR assays were conducted with 1 μg of genomic DNA in a 50-μL total reaction volume. Pipetters dedicated for PCR setup were used with sterile, aerosol-

Table 1. Primers used in PCR analysis of swine muscle tissue from pigs fed Roundup Ready and conventional soybeans

Name[a]	Sequence (5′ to 3′)
cp4 epsps forward	GCG TCG CCG ATG AAG GTG CTG TC
cp4 epsps reverse	CGG TCC TTC ATG TTC GGC GGT CTC
le1 forward	CTG AAG CAA AGC AAT GG
le1 reverse	CCC GAG GAG GTC ACA ATA G
prl forward	TGC TTT TTA TAA CCT GCT CCA CTG C
prl reverse	AAA AAG CTA TAA AAC TAA AAG AAT C

[a]*cp4 epsps* = encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4; *lel* = encoding soy lectin; *prl* = encoding porcine preprolactin.

resistant tips. Each reaction was performed in a PTC-225 DNA Engine Tetrad thermocycler (MJ Research, Inc., Watertown, MA) and contained 2.5 m*M* $MgCl_2$, 0.2 μ*M* each primer, 1 μL of PCR Nucleotide Mix^{Plus} (Roche), 2 units of REDTaq DNA Polymerase (Sigma, St. Louis, MO), and 2 units of uracil-DNA glycosylase (Roche) in 1× reaction buffer. Uracil-DNA glycosylase and dUTP (instead of dTTP) were used to eliminate any amplicon contamination. The PCR cycling conditions for amplification of the *cp4 epsps* sequence fragment were: 1 cycle at 37°C for 20 min; 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 30 s, 65°C for 30 s, 72°C for 45 s; and 1 cycle at 72°C for 5 min. PCR cycling conditions for the endogenous soybean gene, *le1* (encoding soy lectin, GenBank Accession No. K00821), were: 1 cycle at 37°C for 20 min; 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; and 1 cycle at 72°C for 5 min. Primers for *cp4 epsps* and *le1* amplification are listed in Table 1. Each set of PCR assays included both positive and negative controls. The positive controls were reactions containing 100 ng of purified RR soybean genomic DNA. Additional positive controls were the DNA extracts from tissue samples spiked with RR soybean genomic DNA as described above. Negative PCR controls did not contain any template DNA. Additional negative controls were the buffer-only extracts described above.

Agarose gel electrophoresis and Southern blotting were performed as previously described (Jennings et al., 2003). Probe templates were purified amplicons of the 272-bp fragment of the *cp4 epsps* coding region or the 198-bp fragment of the *le1* gene. The blot was exposed to Kodak BioMax MS-2 film in conjunction with one BioMax MS intensifying screen (Sigma).

As described above, appropriate positive and negative controls were included in all of the analyses to ensure the sensitivity and reproducibility of the DNA extractions and PCR assays. In addition, all DNA extracts were analyzed by PCR for a 185-bp region of the porcine preprolactin (***prl***) gene (GenBank Accession No. X14068) to ensure the quality and suitability of the DNA extracts for PCR. Reaction conditions were the same as those used for *cp4 epsps* and *le1*, except omitting the uracil-DNA glycosylase and using 100 ng of DNA template instead of 1 μg. Cycling conditions for the *prl* assay were: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s; and 1 cycle at 72°C for 5 min. Primers for *prl* amplification are listed in Table 1. Products of *prl* amplification were visualized on an ethidium bromide-stained agarose gel. Limit of detection (**LOD**) studies were not conducted for the *prl* gene fragment because a relatively large amount of pig genomic DNA was used in each reaction. Assuming *prl* is a single-copy gene, then 100 ng of pig genomic DNA contains tens of thousands of copies of *prl*, and 1 μg of pig genomic DNA contains hundreds of thousands of copies of *prl*. Assays for *prl* ensured the quality of the DNA being tested.

Acceptance criteria for a "true positive" and "true negative" PCR result are described in Jennings et al. (2003) with the following modifications: each extract in this study was analyzed only once by each PCR assay (instead of by duplicate assays for each extract), and there was only one spiked extraction control per set of analyses (instead of spiked controls at two concentrations per set). In addition, each set of extractions and assays in this study included a buffer-only extract as described above. These changes, coupled to the testing scheme shown in Figure 1, helped to streamline the analysis process while maintaining rigorous and comprehensive controls.

Protein Analysis

Protein Extraction. Tissue samples were lyophilized, disrupted, extracted for protein, and then stored as previously described (Jennings et al., 2003), except that 20 volumes of extraction buffer were used for each extraction rather than 10 volumes.

Competitive Immunoassay. Analysis of tissue samples for the presence of CP4 EPSPS protein was performed using a competitive ELISA. Recombinant CP4 EPSPS protein, used in the ELISA analyses described below, was expressed in *E. coli* and purified from batch culture. The identity and purity of this CP4 EPSPS standard was confirmed prior to its use in these studies. CP4 EPSPS was diluted to 0.8 μg/mL in coating buffer (50 m*M* carbonate buffer, pH 9.6) and 100 μL per well was dispensed into 96-well microtiter plates. The CP4 EPSPS protein was immobilized to the microtiter plates by incubation overnight at 4°C. After immobilization, each well was aspirated and washed three times with PBST washing buffer [10 m*M* phosphate buffer, pH 7.4, 0.8% (wt/vol) NaCl, 0.05% (vol/vol) Tween-20] and then blocked with 1% (wt/vol) nonfat dry milk in PBST washing buffer overnight at 4°C. Sera from goats that were immunized with purified, recombinant CP4 EPSPS protein were purified on a CP4 EPSPS immunoaffinity column (Pierce, Rockford, IL) to obtain antibodies specific for CP4 EPSPS protein. The specificity of these antibodies was confirmed by Western blot analysis of purified CP4 EPSPS protein standard (data not shown). The CP4 EPSPS-specific, immunoaffinity purified anti-

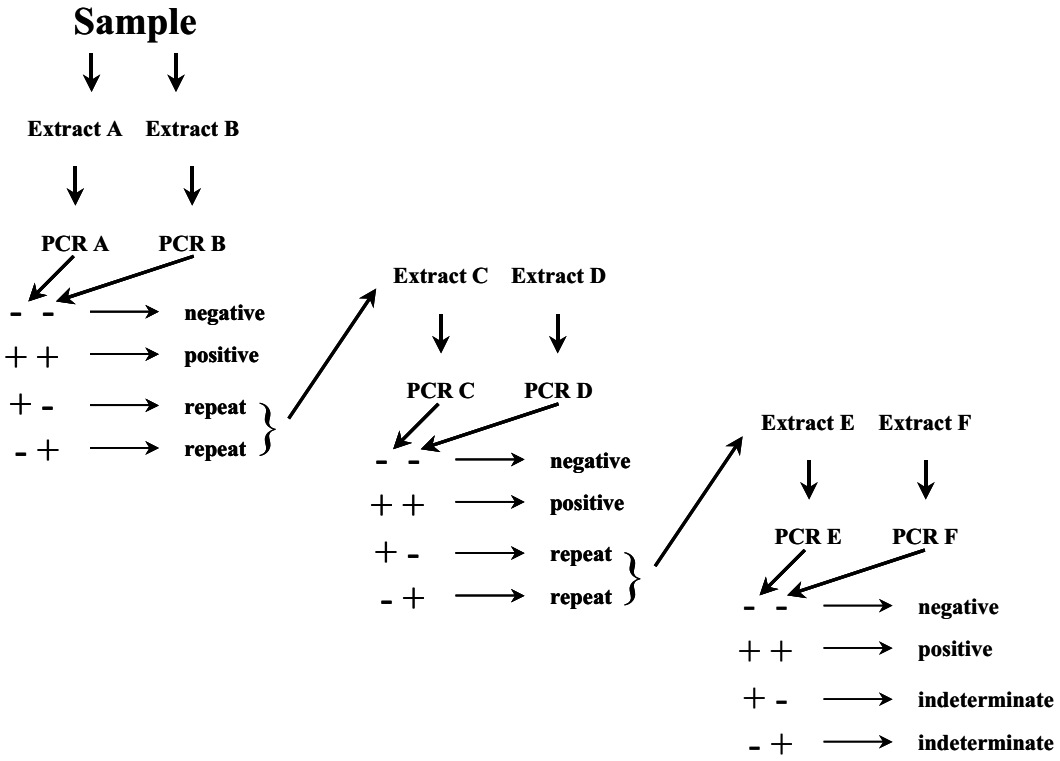

Figure 1. Testing scheme for analysis of pork samples for transgenic and plant DNA. Each sample was extracted for DNA in duplicate then analyzed by PCR for *cp4 epsps* and *le1* gene fragments (*cp4 epsps* = encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4; *lel* = encoding soy lectin). A "true positive" or "true negative" required that both extracts and PCR gave the same result. If discordant results were obtained after three rounds of extraction and PCR, a sample was called "indeterminate." No indeterminate results were actually obtained.

bodies were preincubated overnight at 4°C at a concentration of 0.05 μg/mL with either: 1) tissue sample extracts or 2) protein standards in the presence of an equivalent concentration of tissue extract. Into each well of the CP4 EPSPS protein-coated microtiter plate was transferred 100 μL of the antibody/extract mixture.

The CP4 EPSPS competitive immunoassay was initiated by incubating 100 μL of standard or test sample in each well of a plate for 1 h at 37°C. After aspiration and washing each well three times with PBST washing buffer (~300 μL/well), 100 μL of a 1:3,000 dilution of rabbit anti-goat polyclonal antibody, conjugated to horseradish peroxidase in stabilizing buffer (Stabilzyme HRP Conjugate Stabilizer, SurModics, Eden Prairie, MN), was incubated in each well of the assay plate for 1 h at 37°C. Following aspiration and washing of each well three times with PBST washing buffer (~300 μL/well), 100 μL of the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD) was added per well and allowed to develop for approximately 10 min at room temperature. The enzymatic reaction was terminated by the addition of 100 μL per well of 3 *M* H_3PO_4. Immediately after stopping the enzymatic reaction, absorbance readings were determined using a dual wavelength plate reader at 450 nm with a reference wavelength of 650 nm. The concentration of CP4 EPSPS protein was determined in each sample by interpolation against a serially diluted eight-point standard curve, with values that ranged from 0.25 to 4.0 ng/mL. The standard curve was determined by a quadratic curve-fitting model (Microsoft Excel). The LOD for this assay was determined to be approximately 94 ng of CP4 EPSPS protein/g of pork loin muscle tissue (~94 ppb).

Protein data from conventional soybean-fed animals were compared to data from RR soybean-fed animals using Microsoft Excel (*t*-test: paired two sample for means analysis). All samples were below the LOD for the assay. However, there was some variance around the level of the inhibition. Therefore, to determine if there was a statistically significant difference between the mean of the two populations, samples were analyzed using a Student's *t*-test, which assumes that the means of both data sets are equal; it is also referred to as a homoscedastic *t*-test. The test and control populations consisted of seven samples each. For the hypothesized mean difference, a value of zero was entered to indicate that the sample means are hypothesized to be equal. The alpha value used for the test was 0.05 with a 95% confidence level. There was no statistically sig-

nificant difference between the test and control sample populations at a 95% confidence level.

Intact CP4 EPSPS protein was subjected to simulated in vitro gastric conditions using a U.S. Pharmacopia (**USP**) method consisting of approximately 15 activity units/mL of pepsin (Sigma) in a pH 1.3 solution of simulated gastric fluid at approximately 18°C as previously described (Harrison et al., 1996). The pepsin enzyme was inactivated after 20 s by adding 100 μL of the digested sample to 200 μL of carbonate-bicarbonate buffer, pH 9.6 (Sigma). After pepsin enzyme was inactivated, one volume of 2× Laemmli buffer (Sigma) was added to the sample, and the sample was incubated in a boiling water bath for 2 min. Sample was then analyzed by SDS-PAGE on a 4 to 12% (wt/vol) gradient gel using a mini gel system (Bio-Rad, Hercules, CA). Electrophoresis was conducted at 150 V for about 1 h, and the gel was then stained with Brilliant Blue R (Sigma) and destained in 40% (vol/vol) methanol. Undigested CP4 EPSPS standard and prestained molecular weight markers (Novex MultiMark Multi-Colored Protein Standard, Invitrogen, Carlsbad, CA) were loaded onto the gel so the approximate molecular weight of the peptides could be confirmed. To demonstrate the ability of the method to detect peptide fragments of CP4 EPSPS, the partially digested protein was compared to intact CP4 EPSPS in the competitive ELISA.

Results

Testing for cp4 epsps and le1 DNA Fragments. PCR followed by Southern blot hybridization was used to analyze DNA extracted from pork loin muscle samples for a 272-bp fragment of the *cp4 epsps* coding region and a 198-bp region of the endogenous soybean gene *le1* (encoding soy lectin). Inherent to coupling sensitive PCR methods with Southern blotting is the increased risk of both false positives (i.e., a result due to contamination of the extract or reaction with template DNA) and false negatives (i.e., the presence of a PCR inhibitor in the extract). Therefore, all analyses were conducted in a sample-blinded manner following a detailed testing scheme (Figure 1) and applying a well-defined set of criteria for accepting "true positive" and "true negative" PCR results. Before a sample could be confirmed as having either detectable or undetectable levels of plant or transgenic DNA, the criteria described in Jennings et al. (2003) had to be met. First, samples were always extracted in at least duplicate and each extract was analyzed by PCR. For a Southern blot to be considered valid, both positive and negative control reactions had to produce the expected results. As a positive control for both extraction and PCR, the spiked sample had to produce a positive result on the Southern blot. As a negative control for both extraction and PCR, the buffer-only extract had to produce a negative result on the Southern blot. Lastly, to ensure that the DNA was amenable to PCR and did not contain significant inhibitors, each tissue extract was used in a PCR to amplify an endogenous gene fragment from *prl*. In addition, the results had to be consistent between duplicate extracts/reactions for a sample to be called negative or positive (Figure 1). As illustrated, if testing results were not consistent after three rounds of extraction and PCR, the results for that sample were termed "indeterminate."

Both assays were shown to be capable of detecting as little as 1 pg of purified genomic DNA from RR soybeans in the presence of 10 μg of swine genomic DNA (Figures 2 and 3). Routinely, however, the LOD was approximately 2.5 pg, or roughly one diploid genome equivalent of soybean DNA (Arumuganathan and Earle, 1991). Figures 2 and 3 show that it is difficult to visualize the low amounts of PCR product generated from picogram amounts of genomic template using merely ethidium bromide staining of an agarose gel. For example, PCR product from the *le1* assay is not visible on a stained gel unless more than 25 pg of purified template DNA is used in the reaction and, in fact, the gene-specific, 198-bp band is much weaker than the nonspecific amplicons that arise in the presence of pork matrix (Figure 3). However, when coupled to Southern blot analysis with specific ^{32}P-labeled probes, the presence of amplicon is unequivocal for both *cp4 epsps* and *le1* assays (Figures 2 and 3). The sensitivity of the Southern method was so great, as measured by the specific binding of radiolabeled probe, that exposures of less than 1 h were routinely sufficient to visualize a PCR product. Blots, however, were purposefully overexposed to film (typically twice the optimal exposure) for higher sensitivity. Not only is the sensitivity of each assay increased by Southern blotting, but also the clear bands obtained on an autoradiograph alleviate some of the uncertainty inherent in the interpretation of a gel result, such as the nonspecific amplicons that are evident in the presence of pork matrix with the *le1* PCR assay (Figure 3). As the number of cycles used in PCR is increased to achieve greater assay sensitivity, or when the assay is conducted in the presence of a relatively large amount of animal DNA, the possibility of producing non-specific or questionable PCR amplification products increases.

A summary of all *cp4 epsps* and *le1* analyses is shown in Table 2. The table shows that none of the extracted DNA from pork loin muscle samples was positive for *cp4 epsps* or *le1* according to the testing scheme in Figure 1 and criteria described above and, in fact, all samples met the criteria for being true negative results. No results were indeterminate for any tested sample.

Testing for CP4 EPSPS Protein. An immunoassay for CP4 EPSPS protein has been described (Lipp et al., 2000) that involves capture of the protein by immobilized antibody followed by detection of the captured protein with a second CP4 EPSPS antibody preparation (i.e., a sandwich ELISA). However, a critical component of the safety assessment of proteins introduced into agricultural biotech products approved for food and feed use is the demonstration that these introduced proteins are readily digestible in simulated gastric models (Met-

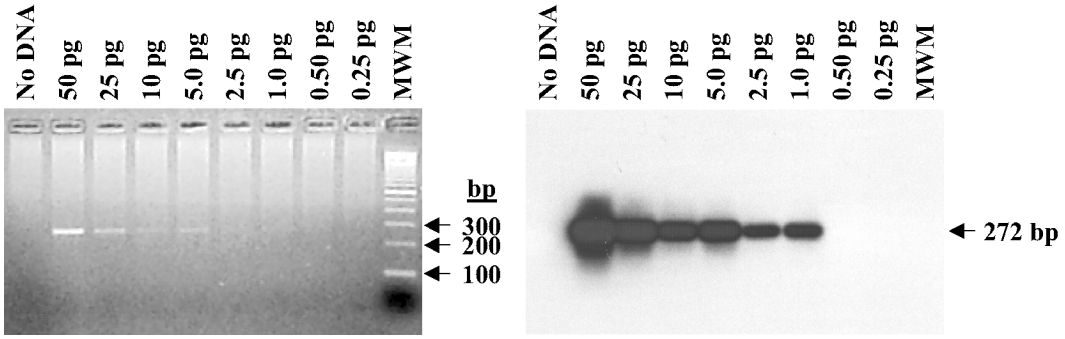

Figure 2. Polymerase chain reaction amplification and Southern blot analysis of genomic DNA from Roundup Ready soybeans for the *cp4 epsps* gene fragment (*cp4 epsps* = encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4). Purified genomic DNA from Roundup Ready soybean was used in PCR assays designed to amplify a 272-bp fragment of the *cp4 epsps* coding region. Included as a negative PCR control was a reaction without template DNA (No DNA). These assays were conducted to determine the limit of detection for the *cp4 epsps* assay in the presence of pork matrix. Therefore, each reaction also contained 10 μg of pig genomic DNA. From each 50-μL reaction, 20 μL were subjected to agarose gel electrophoresis, blotted onto a nylon membrane, and probed with ^{32}P-labeled *cp4 epsps* amplicon. The ethidium bromide-stained gel under UV illumination is shown on the left, and the corresponding autoradiograph of the Southern blot is shown on the right. The amount of template DNA in each reaction is listed at the top of each figure. The molecular weight marker (MWM) was 100-bp DNA Ladder (Life Technologies, Rockville, MD).

calfe et al., 1996). Based on the safety assessment of CP4 EPSPS protein, it was highly unlikely that intact CP4 EPSPS protein would be found in samples of muscle tissue from pigs fed RR soybean meal. A competitive ELISA was therefore developed that had the potential to detect partially digested forms of CP4 EPSPS protein in addition to intact protein. In the competitive ELISA, incubation of CP4 EPSPS protein and its immunoreactive fragments with anti-CP4 EPSPS antibodies in solution allows a binding equilibrium to be reached between the protein in solution with the CP4 EPSPS protein immobilized to the assay plate. Fragments of partially digested CP4 EPSPS protein that can bind to anti-CP4 EPSPS antibodies will be detected in this assay configuration, whereas some of these fragments would not be detected in a sandwich assay configuration due to the

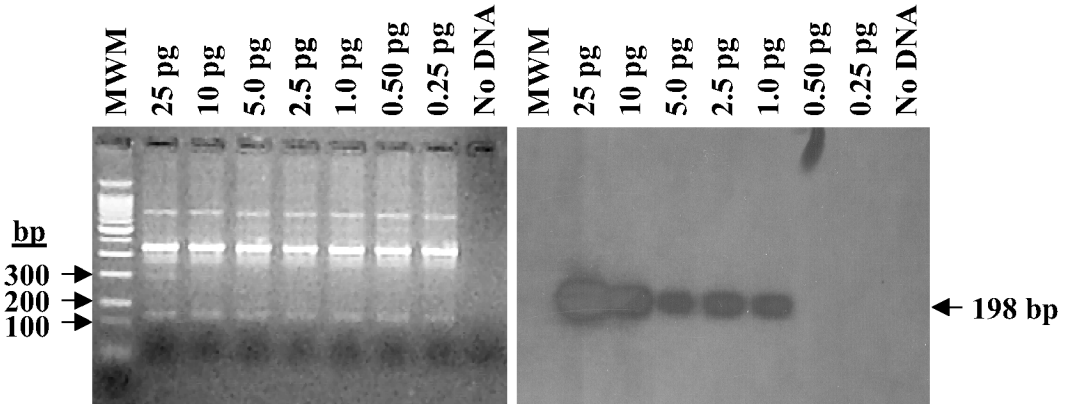

Figure 3. Polymerase chain reaction amplification and Southern blot analysis of genomic DNA from Roundup Ready soybeans for the *le1* gene fragment (*lel* = encoding soy lectin). Purified genomic DNA from Roundup Ready soybean was used in PCR assays designed to amplify a 198-bp fragment of the *le1* coding region. Included as a negative PCR control was a reaction without template DNA (No DNA). These assays were conducted to determine the limit of detection for the *le1* assay in the presence of pork matrix. Therefore, each reaction also contained 10 μg of pig genomic DNA. From each 50-μL reaction, 20 μL were subjected to agarose gel electrophoresis, blotted onto a nylon membrane, and probed with ^{32}P-labeled *le1* amplicon. The ethidium bromide-stained gel under UV illumination is shown on the left, and the corresponding autoradiograph of the Southern blot is shown on the right. The amount of template DNA in each reaction is listed at the top of each figure. The molecular weight marker (MWM) was 100-bp DNA Ladder (Life Technologies, Rockville, MD).

Table 2. Analysis of muscle tissue samples from pigs fed Roundup Ready or conventional soybean meal for fragments of the *cp4 epsps* and *le1* genes and the CP4 EPSPS protein

	PCR/Southern Blot		Competitive ELISA
Soybean meal	*cp4 epsps*	*le1*	CP4 EPSPS
Roundup Ready	ND[a]	ND[a]	ND[b]
Conventional	ND[a]	ND[a]	ND[b]

[a]No *cp4 epsps* or *le1* gene fragments were detected by PCR coupled to Southern blotting. *cp4 epsps* = encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4; *lel* = encoding soy lectin.

[b]There was no difference ($P < 0.05$) in measurements between homogenates of loin muscle samples derived from pigs fed Roundup Ready or conventional soybean meal. The presence or absence of CP4 EPSPS protein was confirmed by immunoassay for the Roundup Ready and conventional soybean meal, respectively, before diet formulation.

requirement of at least two accessible antibody epitopes being present on the peptide. The ability of the competitive assay to detect fragments of protein was demonstrated by digesting CP4 EPSPS protein in USP simulated gastric fluid for approximately 20 s (Figure 4), and then analyzing the digested protein in both the sandwich and competitive ELISA. The sandwich ELISA demonstrated no reactivity with the digested CP4 EPSPS protein up to a concentration of 16 ng/mL (i.e., all optical densities in sample wells were less than or equal to the optical densities of buffer-only wells; data not shown). However, the competitive assay successfully detected partially digested CP4 EPSPS protein in a manner very similar to intact CP4 EPSPS (Figure 4). It is important to note, however, that fully digested CP4 EPSPS is no longer immunoreactive and would not be detectable by any ELISA. Digestion of the CP4 EPSPS protein in simulated gastric fluid for times greater than 20 s results in increased degradation of the protein. Presumably, a point of degradation would be reached where the competitive ELISA could no longer detect CP4 EPSPS protein fragments.

The concentration of CP4 EPSPS protein was determined in test samples by interpolation against a serially diluted eight-point standard curve (Figure 5), with values that ranged from 0.25 to 4.0 ng/mL in the presence of 10% (vol/vol) pork tissue extract. To normalize for the possible impact that tissue matrix could have on binding of antibody to CP4 EPSPS protein fragments in sample extracts, the standard curve was generated in the same concentration of tissue homogenate as the test samples. The curve in Figure 5 was generated using a quadratic curve-fitting model. The LOD for this assay was determined to be approximately 94 ng of CP4 EPSPS protein/g of pork muscle tissue. There was no statistical difference ($P < 0.05$) in measurements between homogenates of pork loin samples derived from animals fed RR or conventional soybean meal when analyzed by the competitive immunoassay (Table 2).

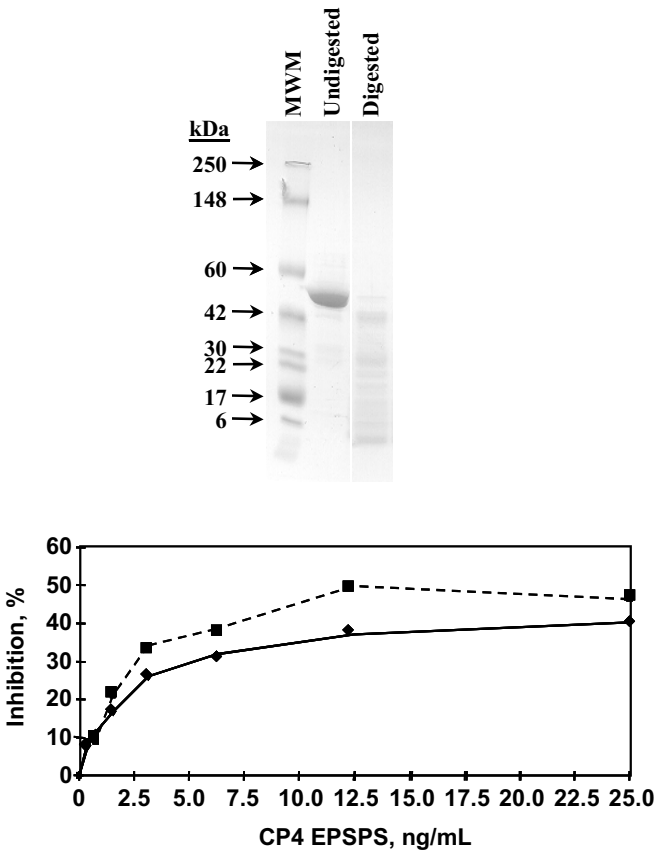

Figure 4. Pepsin digestion of CP4 EPSPS protein and sensitivity of the competitive ELISA for partially digested CP4 EPSPS. Highly purified CP4 EPSPS protein was subjected to low pH pepsin digestion in simulated gastric fluid for 20 s and then separated by SDS-PAGE and stained (top). The molecular weight marker (MWM) was Novex MultiMark Multi-Colored Protein Standard (Invitrogen, Carlsbad, CA). The indicated concentration of CP4 EPSPS protein (0.39 to 25 ng/mL), either undigested (—◆—) or partially digested (--■--), was then analyzed in the competitive CP4 EPSPS ELISA (bottom).

Discussion

It is accepted that ingested DNA and protein (endogenous or transgenic) cannot be assimilated to any significant extent in animal tissues, including meat, milk, or eggs (Beever and Kemp, 2000). However, limited data exist on whether highly sensitive PCR and ELISA analytical methods can detect fragments of ingested DNA or protein, respectively, in animal tissues. In addition, there is a need for well-characterized PCR and immunoassay methods suitable for analysis of animal tissue samples for diet-derived plant DNA and protein.

The present study was conducted to assess whether a characterized DNA detection method, highly optimized for sensitivity, could detect fragments of transgenic DNA from RR soybean meal in muscle from pigs fed this genetically enhanced product. Highly sensitive methods are needed because the amount of transgenic

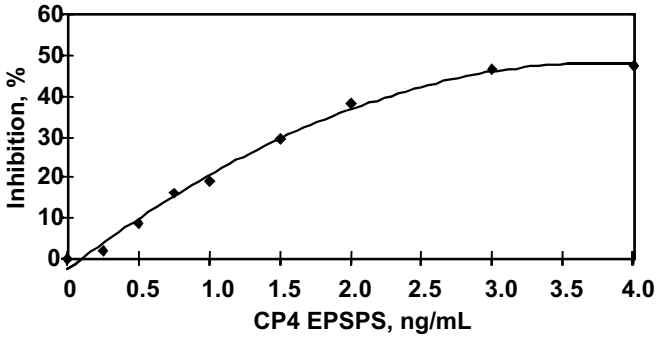

Figure 5. Standard curve for the competitive ELISA to detect CP4 EPSPS protein in the presence of pork muscle extract. The concentration of CP4 EPSPS protein in pork muscle extract was measured by interpolation against an eight-point standard curve with values ranging from 0.25 to 4.0 ng/mL of CP4 EPSPS protein in the presence of 10% (vol/vol) pork extract. The standard curve shown in this figure was generated using a quadratic curve-fitting model. The limit of detection for this assay is about 94 ng of CP4 EPSPS protein/g of pork muscle tissue (~94 ppb).

DNA that an animal consumes per day is very small, especially compared to the total amount of DNA consumed. For example, it has been calculated that a 600-kg dairy cow fed a diet enriched in insect-protected corn containing a Cry protein derived from *Bacillus thuringiensis* (Bt corn), with 40% of the diet as Bt grain and 20% as Bt corn silage, will consume about 600 mg of total plant DNA each day, with less than 3 μg being transgenic DNA (Beever and Kemp, 2000).

In the current study, relatively small DNA fragments (272 and 198 bp) of the glyphosate-tolerance transgene (*cp4 epsps*) and an endogenous soybean gene (*le1*) were not detected by PCR methods involving a relatively high level of input DNA followed by Southern blot hybridization of the amplified product. The routine LOD for the present PCR/Southern blot detection of *cp4 epsps* and *le1* gene fragments was approximately 2.5 pg of purified RR soybean genomic DNA per reaction. The LOD for these PCR/Southern methods is essentially at the theoretical limit of the assays by detecting a single diploid genome equivalent of soybean DNA (Arumuganathan and Earle, 1991). The appropriate positive and negative DNA extraction and PCR controls, including a control reaction for an endogenous pork gene (*prl*), data acceptance criteria, and testing scheme, are included in the present study to characterize and verify the results.

The present results parallel data on the fate of the *cp4 epsps* gene in other animal species. Klotz and Einspanier (1998) showed that a 200-bp portion of the *cp4 epsps* coding region was not detected by PCR and Southern blot analysis in blood or milk from dairy cows fed RR soybeans. However, it was reported that a fragment of a highly abundant chloroplast gene was detectable in the white blood cells, but not in the milk of these dairy cows. A simple explanation of these results is that the chloroplast gene fragment was detected because of its significantly greater abundance in plant cells compared to the single haploid copy of the coding region for *cp4 epsps* in RR soybeans.

Our results also are similar to the data obtained from a study with broiler chickens that were fed RR soybean meal (Khumnirdpetch et al., 2001). In that study, real-time PCR was used to test whether a fragment of transgenic DNA was detectable in meat, skin, duodenal, or liver samples from broilers fed RR soybean meal over the entire life of the birds. The investigators found that all samples from the 7-wk study were negative for transgenic DNA.

Just as the DNA detection methods were optimized for detection of small fragments of transgenic DNA in pork loin samples, the present study also described a protein detection immunoassay method that was specifically developed to determine whether intact CP4 EPSPS protein, or immunoreactive fragments of this protein, could be detected in loin samples from pigs fed RR soybean meal. The CP4 EPSPS protein is readily degraded under simulated gastric digestion conditions, with this protein being almost totally degraded within 30 s (Harrison et al., 1996). Therefore, it is highly unlikely that intact CP4 EPSPS protein would be present in loin tissue samples from pigs fed RR soybean meal. The present study demonstrated that an in vitro-digested preparation of CP4 EPSPS protein was detectable with a competitive ELISA configuration at concentrations similar to the intact protein (≤4 ng/mL), whereas a more conventional sandwich ELISA demonstrated no reactivity with the partially digested CP4 EPSPS preparation up to 16 ng/mL. The CP4 EPSPS competitive immunoassay had an LOD of approximately 94 ng of CP4 EPSPS protein/g of pork loin tissue extract (~94 ppb), and yet neither intact CP4 EPSPS nor immunoreactive fragments of the protein were detected in the loin homogenates from pigs fed RR soybean meal. It is important to note, however, that fully digested CP4 EPSPS would not be detected in this assay. Similar to the present results, it has been reported that the CP4 EPSPS protein is not detectable in a number of samples from laying hens fed RR soybean meal (Ash et al., 2000).

The present results showing the absence of detectable levels of fragments of either transgenic DNA or protein are consistent with the general understanding that these macromolecules undergo rapid and effective degradation in animal digestive tracts (Beever and Kemp, 2000). In addition, the safety of DNA and protein introduced into genetically enhanced agricultural products is based on strong scientific principles and premarket regulatory assessments (FAO/WHO, 1991; 1996; OECD, 1998; 2000). It is important to remember, nevertheless, that even if transgenic DNA is detected by a future technology, scientific evidence and opinion concludes that ingested transgenic DNA would not be dif-

ferent from ingestion of DNA already in foods, which is clearly safe (Jonas et al., 2001).

To summarize, previous studies and the present study show that fragments of the *cp4 epsps* transgene are not detectable in a variety of tissue samples from pigs, dairy cows, and broiler chickens fed a diet that includes RR soybeans or soybean meal.

Implications

Using highly sensitive polymerase chain reaction assays coupled to Southern blot detection of reaction products, small fragments of the *cp4 epsps* transgene (encoding 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium sp.* strain CP4) and *le1* gene (encoding soy lectin) were not detected in loin muscle samples from pigs fed a diet containing Roundup Ready soybean meal. Additionally, neither intact nor immunoreactive fragments of the CP4 EPSPS protein were detected by a sensitive, well-characterized competitive enzyme-linked immunosorbent assay in the pork tissue samples. Although it is conceivable that a more sensitive deoxyribonucleic acid or protein detection technology may someday allow for the detection of a fragment of a low-abundance transgene or protein in animal tissues, this is highly improbable given that the present assays were optimized to operate at or near the theoretical limits of detection for these analytical methods.

Literature Cited

Arumuganathan, K., and E. D. Earle. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Reporter 9:208–218.

Ash, J. A., E. Scheideler, and C. L. Novak. 2000. The fate of genetically modified protein from Roundup Ready® soybeans in the laying hen. Poult. Sci. 79(Suppl. 1):26. (Abstr.)

Beever, D. E., and C. F. Kemp. 2000. Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. Nutr. Abstr. Rev., Ser. B: Livest. Feeds Feeding 70:175–182.

Cromwell, G. L., M. D. Lindemann, J. H. Randolph, G. R. Parker, R. D. Coffey, K. M. Laurent, C. L. Armstrong, W. B. Mikel, E. P. Stanisiewski, and G. F. Hartnell. 2002. Soybean meal from Roundup Ready or conventional soybeans in diets for growing-finishing swine. J. Anim. Sci. 80:708–715.

FAO/WHO. 1991. Strategies for Assessing the Safety of Foods Processed by Biotechnology. Report of a joint FAO/WHO consultation. WHO, Geneva, Switzerland.

FAO/WHO. 1996. Biotechnology and Food Safety. Report of a joint FAO/WHO consultation. FAO, Food and Nutr. Paper 61. WHO, Geneva, Switzerland.

Faust, M. A. 2002. New feeds from genetically modified plants: The U.S. approach to safety for animals and the food chain. Livest. Prod. Sci. 74:239–254.

Flachowsky, G., and K. Aulrich. 2001. The use of genetically modified organisms (GMOs) in animal nutrition. Surveys Anim. Nutr. 29:45–79.

Harrison, L. A., M. R. Bailey, M. W. Naylor, J. E. Ream, B. G. Hammond, D. L. Nida, B. L. Burnette, T. E. Nickson, T. A. Mitsky, M. L. Taylor, R. L. Fuchs, and S. R. Padgette. 1996. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3 phosphate synthase from *Agrobacterium sp.* strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. J. Nutr. 126:728–740.

Jennings, J. C., L. D. Albee, D. C. Kolwyck, J. B. Surber, M. L. Taylor, G. F. Hartnell, R. P. Lirette, and K. C. Glenn. 2003. Attempts to detect transgenic and endogenous plant DNA and transgenic protein in muscle from broilers fed YieldGard Corn Borer corn. Poult. Sci. 82:371–380.

Jonas, D. A., I. Elmadfa, K.-H. Engel, K. J. Heller, G. Kozianowski, A. König, D. Müller, J. F. Narbonne, W. Wackernagel, and J. Kleiner. 2001. Safety considerations of DNA in food. Ann. Nutr. Metab. 45:235–254.

Khumnirdpetch, V., U. Intarachote, S. Treemanee, S. Tragoonroong, and S. Thummabood. 2001. Detection of GMOs in the broilers that utilized genetically modified soybeanmeals as a feed ingredient. Plant Anim. Genome IX Conf., San Diego, CA. (Abstr. 585).

Klotz, A., and R. Einspanier. 1998. Detection of "novel-feed" in animals? Injury of consumers of meat or milk is not expected. Mais 3:109–111.

Lipp, M., E. Anklam, and J. W. Stave. 2000. Validation of an immunoasssay for detection and quantitation of a genetically modified soybean in food and food fractions using reference materials: Interlaboratory study. J. AOAC Int. 83:919–927.

Metcalfe, D. D., J. D. Astwood, R. Townsend, H. A. Sampson, S. L. Taylor, and R. L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Crit. Rev. Food Sci. Nutr. 36:165–186.

NRC. 1994. Nutritional Requirements of Poultry. 9th ed. Natl. Acad. Press, Washington, DC.

NRC. 1998. Nutritional Requirements of Swine. 10th ed. Natl. Acad. Press, Washington, DC.

OECD. 1998. Report of the OECD Workshop on the Toxicological and Nutritional Testing of Novel Foods. Paris, France.

OECD. 2000. Report of the OECD Task Force for the Safety of Novel Foods and Feeds. Okinawa, Japan.

Phipps, R. H., D. E. Beever, and D. J. Humphries. 2002. Detection of transgenic DNA in milk from cows receiving herbicide tolerant (CP4EPSPS) soyabean meal. Livest. Prod. Sci. 74:269–273.