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Immunodiagnostic methods for detection of 5-enolpyruvylshikimate-3-phosphate synthase in Roundup Ready® soybeans

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Abstract

We have developed immunological-based detection methods to support labeling of protein-containing food fractions derived from Roundup Ready® soybeans. Western blotting and enzyme linked immunosorbent assay (ELISA) procedures were developed to measure the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein derived from the *Agrobacterium* sp. strain CP4 in the major processed fractions derived from Roundup Ready soybean. Expression of the CP4 EPSPS protein confers tolerance to Roundup® herbicide. The western blotting method utilizes a polyclonal goat anti-CP4 EPSPS antibody which specifically binds to CP4 EPSPS followed by detection of bound goat antibody with biotinylated Protein-G. Detection of this complex is accomplished using horseradish-peroxidase (HRP) labeled NeutrAvidin™ and signal development by enhanced chemiluminesence. Data from western blot analyses of these fractions establish that stable epitopes remain after the seed has been subjected to processing conditions typically employed by the food industry, thereby enabling development of an ELISA method. The ELISA for measurement of CP4 EPSPS is a triple antibody sandwich procedure utilizing a monoclonal capture antibody and a polyclonal detection antibody followed by a third biotin labeled monoclonal anti-rabbit antibody. Sandwich formation is detected using HRP labeled NeutrAvidin™ with color development using TMB substrate. In the sandwich ELISA, the immunological activity of CP4 EPSPS was reduced by the extraction method required to solubilize CP4 EPSPS protein from processed fractions. Sensitivity of the CP4 EPSPS ELISA was sufficient to detect CP4 EPSPS protein in processed soybean fractions that contained 2% Roundup Ready soybean mixed with conventional processed soybean fractions, thereby making the ELISA an acceptable method to assess CP4 EPSPS protein in processed soybean fractions. Data on sensitivity, accuracy, precision and specificity, established that the western blot and ELISA methods are appropriate for compliance with the EC Novel Foods Regulation.

1. Introduction

Monsanto Company has genetically modified soybeans (Roundup Ready®) to tolerate the application of Roundup® herbicide. Roundup Ready soybeans were produced through the insertion of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene obtained from the soil bacterium *Agrobacterium* sp. strain CP4 into the soybean genome. Roundup Ready soybeans are equivalent to traditional soybeans with the exception of the presence of the CP4 EPSPS gene and gene product.

Two immunological based methods were developed for characterization and estimation of the levels of the newly expressed CP4 EPSPS protein: the western blot and the enzyme linked immunosorbent sandwich assays (ELISA). These methods have been routinely used during the development and safety assessment of Roundup Ready soybean plants and plant products. The western blot was primarily utilized for characterization of expressed protein and estimation of CP4 EPSPS protein levels in processed products. Unlike ELISA, western blots are not used as high throughput assays but are favored in situations where the protein of interest is difficult to extract, where multiple matrices are assayed or where the physical characteristics need to be evaluated. ELISA sandwich assays are the method of choice for accurate quantitation of protein expression levels in genetically modified plants. These methods are quantitative, robust, inexpensive, highly specific and readily automatable. Both techniques require little investment in equipment and are easily performed by trained technicians.

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Monsanto has developed these two immunological-based methods for detection of CP4 EPSPS protein in processed soybean fractions in anticipation of the labeling provisions for EC Regulation 1139/98. Western blotting and ELISA methods described in this report were initially developed and validated to support the development and safety evaluations for Roundup Ready soybean. These assays were subsequently modified to enable detection of CP4 EPSPS protein in processed soy fractions. Key modifications to both techniques involved extraction optimization and enhancement of assay sensitivity. Validation data establish that these methods exhibit sufficient accuracy, precision, sensitivity and specificity to enable enforcement of the EC Regulation.

2. Materials and methods

Antiserum production; *Western blot*. Antibodies specific to CP4 EPSPS were produced in goats by immunization of three goats with recombinant SDS-PAGE purified CP4 EPSPS from *E. coli*. Goats were initially immunized by multiple intermuscular injections with approximately 0.4 mg of CP4 EPSPS in 4 mL of Freunds complete adjuvant. Animals were immunized on monthly intervals with injections of approximately 0.4 mg of CP4 EPSPS in 4 mL of Freunds incomplete adjuvant. Antiserum was selected from a goat that displayed low non-specific binding to non-transformed soybean and high specific binding to CP4 EPSPS protein.

Antiserum production; *ELISA*. Polyclonal and monoclonal antibodies were produced by Strategic Diagnostics, (Newark DE, USA). Monoclonal antibodies were produced in mice immunized with CP4 EPSPS protein. Polyclonal antibodies were produced in New Zealand white rabbits. Purified IgG fractions from cell culture fluid or animal serum were used for development of the ELISA.

2.1. Western blot for CP4 EPSPS in processed soybean fractions

Extraction of CP4 EPSPS protein from processed soybean fractions. Food grade, processed soybean fractions were prepared at a pilot food processing facility. All raw and processed soybean samples were ground to a fine powder prior to extraction using a small blender or mortar and pestle. Samples were homogenized with a Omni-2000 hand held homogenizer (Omni Intl., Waterbury, CT) at instrument speed setting 4 to 5 for approximately 30 s using a 1:50 tissue to volume buffer ratio. Following homogenization, samples were centrifuged for 20 min at $20{,}000 \times g$ to pellet insoluble debris. Supernatants were saved for subsequent analysis.

Extraction efficiency was performed by repeatedly (3 times) extracting CP4 EPSPS from a toasted meal sample and determining the CP4 EPSPS content in the supernatant derived from the preceding sample pellet. Percent extraction efficiency was calculated by dividing the CP4 EPSPS content observed for the initial extract by the total CP4 EPSPS content and multiplying by 100.

Western blot detection procedure. Proteins were separated by SDS-PAGE using pre-cast 4–20% gradient or 10% Tris-glycine mini gels (NOVEX™, San Diego, CA) using the discontinuous buffer system of Laemmli (1970). Proteins were electrophoretically transferred to an Immobilon P membrane, obtained from Sigma, St. Louis, MO, using a Hoefer TE series Transphor electrophoresis unit. Nonspecific sites on the membrane were blocked for a minimum of 1 h at room temperature in 5% non-fat dried milk dissolved in Tris buffered saline supplemented with Tween 20 (TTBS). CP4 EPSPS protein bound to the membrane was probed using a 1:1,000 dilution of goat polyclonal antisera raised against purified CP4 EPSPS protein in 1% non-fat dried milk (NFDM) in TTBS for 1–2 h at room temperature. Goat antibody bound to the blot was subsequently detected using a 1:2,000 dilution of biotinylated Protein-G (Pierce P/N = 29989) in 1% NFDM in TTBS at room temperature for approximately 1 h. Finally, the membrane was incubated for approximately 1.0 h at room temperature with a 1:10,000 dilution of horseradish-peroxidase (HRP) conjugated NeutrAvidin (Pierce, P/N = 31001) in 1% NFDM in TTBS. Unbound antibodies or HRP-conjugated NeutrAvidin were removed between all steps by TTBS washes. Antibody reactions were visualized using the enhanced chemiluminescense system of Amersham and the results recorded by X-ray film.

Western blot validation: *Accuracy*, *precision*, *specificity*, *sensitivity*. The accuracy and precision of the western blot procedure was assessed using a blind analysis of 100 protein concentrate samples of defined composition ranging from 0% to 100% (wt.%) Roundup Ready soybean prepared by the mixing of protein concentrate from Roundup Ready and non-modified soybean. Extracts were prepared in 1X Laemmli buffer and analyzed for CP4 EPSPS content by western blot. The concentration of CP4 EPSPS protein in the sample extract was compared to a defined 2% Roundup Ready sample control prepared under identical conditions and analyzed concurrently. Accuracy was computed using the composite responses of four trained laboratory scientists.

The specificity was assessed by performing western blot analysis using EPSPS proteins derived from different plant sources to determine if the antibody produced to CP4 EPSPS protein recognized EPSPS proteins with amino acid sequence homology.

The sensitivity (least detectable dose) for the western blot was estimated by spiking CP4 EPSPS protein into the appropriate non-modified processed fraction. Visual comparisons were made to determine the level of CP4 EPSPS protein that could be distinguished from the

appropriate non-modified control sample analyzed on the same gel.

For all blots, the concentration of CP4 EPSPS in processed soybean fractions were based on the intensity and thickness of the CP4 EPSPS band in the respective processed fraction compared to the band produced by known concentrations of CP4 EPSPS spiked into the appropriate control matrix or contained in defined mixtures of Roundup Ready soybean and conventional non-modified soybean materials analyzed on the same gel.

2.2. ELISA for detection of CP4 EPSPS in processed food fractions

ELISA procedure. Levels of CP4 EPSPS protein in processed soy fractions were quantitated using an indirect ELISA triple antibody sandwich assay. The assay was performed using the following steps:

(1) 100 ng of monoclonal anti-CP4 EPSPS antibody was diluted in 100 μl of 0.05 M carbonate–bicarbonate buffer pH 9.6 and absorbed to the wells of microtiter plates overnight at 4°C.

(2) CP4 EPSPS extracts, standards and quality control samples or buffer were added and the plate was incubated at 37°C for 2 h.

(3) Secondary antibody (purified rabbit anti-CP4), was added to the plate and the plates was allowed to incubate at 37°C for 1 h.

(4) Biotin-conjugated Mab anti-rabbit IgG (Sigma, St.Louis, MO) was added to each well (1:40,000 dilution) and incubated at 37°C for 30 min.

(5) NeutrAvidin conjugated to HRP (Pierce) was diluted (1:10,000 dilution) using StabilZyme® HRP-stabilizer (SurModics, Eden Prairie, MN) and incubated at 37°C for 15 min.

(6) TMB substrate (Kirkegaard and Perry, Gaithersburg, MD) was added for 10 min and the reaction was quenched using 3 M phosphoric acid.

All plates were washed three times between each step using PBS, 0.05% Tween, pH 7.4. Wash buffer and ELISA buffer was purchased from Boehringer Mannheim (P/N 1666 789) and supplemented with 0.05% Tween-20 [Sigma, St. Louis (P/N P1379)]. Assay buffer was also supplemented with 0.2% bovine serum albumin protein [Sigma, St. Louis (P/N A7188)], 0.05% Tween-20, pH 7.4. All plates were read on a Bio-Rad (Richmond, CA) Model 3550 96-well microtiter plate reader at 450 nm using a reference wavelength of 650 nm.

Extraction of CP4 EPSPS from processed soybean fractions for ELISA. The extraction of protein from plant tissues for quantitative analyses for ELISA has typically utilized aqueous non-denaturing conditions. These conditions are more conducive to ELISA and have shown excellent extraction efficiency for target proteins from the raw agricultural commodity (Rogan et al., 1992). Preliminary studies showed that extraction procedures employing aqueous buffers were not appropriate for efficient extraction of CP4 EPSPS protein from processed fractions due to protein precipitation and denaturation that occurs throughout soybean processing. Several extraction conditions and buffers were evaluated. Efficient extraction could only be achieved through the use of detergent and heating.

The optimal extraction conditions for CP4 EPSPS from processed soybean utilized detergent (SDS) in an aqueous buffer (PBS), mechanical tissue disruption and heat. The extraction buffer consisted of: 0.138 M NaCl, 0.081 M Na_2HPO_4, 0.015 M KH_2PO_4, 0.027 M KCl, 2% SDS, pH 7.4. The following extraction conditions were found to be optimal: samples were weighed and extraction buffers were added, samples were homogenized with a Polytron® (Brinkman Instrument Company, Westbury, NY, model PT 3000 fitted with a PT-DA 3012/2TS generator) using one 30 s burst, samples were boiled and cooled to room temperature. Following extraction, samples were centrifuged for 10 min at approximately $21{,}000 \times g$ to pellet insoluble debris. Samples were aliquoted and subjected to ELISA analysis or stored at or below −20°C. For ELISA analysis, samples were thawed and warmed to approximately 40°C for 5 min to completely solubilize precipitates that formed during storage.

The extraction efficiency for CP4 EPSPS protein was estimated as described by Rogan et al. (1992). Tissue was extracted with extraction buffer using a 1:20 tissue to volume extraction buffer ratio. Percent extraction efficiency was calculated by estimating the percent of CP4 EPSPS extracted in the initial extract compared to the total amount of CP4 EPSPS in all of the extracts.

ELISA validation: *Accuracy*, *precision*, *sensitivity*, *stability*. Accuracy was assessed by conducting a parallelism study to determine whether bacterially-expressed CP4 EPSPS was immunologically and conformationally equivalent to plant-expressed CP4 EPSPS solubilized from the processed fractions and to assess if the assay was free from matrix effects. Dilutions of CP4 EPSPS and fraction extracts were prepared in ELISA assay buffer. Recombinant CP4 EPSPS (produced and purified from *E. coli*) was used in the calibration curve to estimate the concentration of the enzyme in various extracts. Amounts of extract corresponding to 4–0.031 μl of extract were assayed in triplicate in the ELISA. The concentration in individual replicates was determined. The log of the ng observed at each dilution was plotted versus the log of the volume assayed and a linear regression performed using selected volumes. This experiment was performed for all fractions. Accuracy was further evaluated by spiking native CP4 EPSPS protein into extracts prepared from processed fractions and determining the amount (% recovery) of CP4 EPSPS protein using the ELISA.

Sensitivity (least detectable dose) was defined as the lowest standard used in the ELISA (2.5 pg/well). Specificity was assessed by assaying non-transformed extracts from each corresponding processed fraction in the ELISA.

Interassay precision was evaluated by running quality control samples on every assay plate. The quality control samples consisted of two different pools of extract prepared from Roundup Ready soybean seed and conventional soybean seed. The pool was aliquoted and frozen in single use portions and a aliquot thawed and run on every plate. The value (ng/well) was determined and the variation in these reported as percent coefficient of variation (%CV). Intra assay variation was evaluated by determining the variation between triplicates assayed at various concentrations on the standard curve. A precision profile was created from several runs of the assay by plotting the standard curve concentration versus the variation (%CV) for each assay standard. Overall variation in the method was evaluated by assaying for CP4 EPSPS content in several processing runs of protein isolate created over several years.

Analyte stability data were developed during the development and validation process which involved the assessment of CP4 EPSPS levels in stored extracts and tissue samples.

All data were analyzed with the aid of Microplate Manager® ELISA analysis software from Bio-Rad or using Microsoft Excel®. Curve fitting was accomplished using the log–log function with quadratic regression. The amount of CP4 EPSPS present in the tissue was expressed as μg CP4 EPSPS per gram weight of processed tissue or as a percent of the CP4 EPSPS protein measured in unprocessed soybean seed.

3. Results and discussion

Western blot and ELISA assays were developed and validated for accurate and precise measurement of CP4 EPSPS protein in major protein-containing fractions derived from Roundup Ready soybean. These fractions include: protein isolate, protein concentrate and toasted meal. CP4 EPSPS protein in processed soybean fractions is readily detectable by western blot and can be quantitated by ELISA. Both techniques were validated as potential methods to be used to support labeling of foods derived from Roundup Ready soybean under the EC Novel Food regulation. The major advantage of the ELISA is the ease of performance, whereas, the western blot is less susceptible to matrix effects and protein denaturation. However, both methods are viable options for measurement of CP4 EPSPS protein in processed soybean fractions.

Western blot validation. Validation data for the western blot established that the method is specific and sufficiently accurate and sensitive to enable detection of newly expressed proteins in food ingredients under conditions where detection involves an established threshold level of genetically modified product. The western blot was rapidly adapted for use in the measurement of CP4 EPSPS protein because protein extraction for western blot involves denaturation of samples using heat, denaturing agents and detergent which is relatively efficient. The results from the extraction optimization experiments showed that Laemmli extraction buffer or Tris extraction buffer supplemented with CHAPS detergent and guanidine hydrochloride provided the most efficient extraction buffers for highly processed fractions like toasted soybean meal (Fig. 1). As expected, the most highly processed

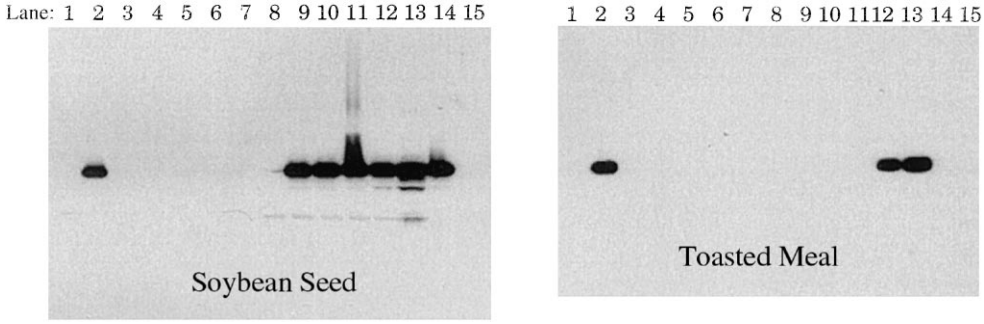

Fig. 1. Effect of different buffers on the extraction of CP4 EPSPS protein from Roundup Ready ® soybean seed and toasted meal samples. The data clearly show that solubilization of the denatured protein in highly processed fractions such as toasted meal requires anionic detergents or chaotrophic salts such as guanidine-HCl in combination with a zwitterionic detergent such as CHAPS. Lane loadings are identical in both panels except that the control and transgenic sample extracts were prepared from soybean seed and toasted meal, respectively. Lanes 1 and 15 were loaded with high range color markers (Amersham, RPN 756). Lane 2 was loaded with 5 ng of a CP4 EPSPS purified standard produced in *E. coli.* Lanes 3, 4, 5, 6, 7, and 8 represent control samples (3 μl loaded/well) extracted in buffer 1, 2, 3, 4, 5 and 6, respectively. Lanes 9, 10, 11, 12, 13 and 14 represent transgenic samples (3 μl loaded/well) extracted in buffer 1, 2, 3, 4, 5 and 6, respectively. Buffer 1 was 100 mM Tris–Cl, pH 7.5, 1 mM benzamidine-HCl, 5mM DTT, 2.5 mM EDTA and 1.0 mM PMSF. Buffer 2 was buffer 1 with 150 mM KCl. Buffer 3 was buffer 1 with 150 mM KCl and 10 mM CHAPS. Buffer 4 was buffer 1 with 10 mM CHAPS and 6M guanidine-HCl. Buffer 5 was 1 X Laemmli buffer [62.5 mM Tris–Cl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.05% (v/v) bromophenol blue]. Buffer 6 was buffer number 1 with 1% (v/v) Tween-20. All samples were extracted at a ratio of 80 mg tissue to 4.0 mL of buffer and prepared as 1 X Laemmli samples before analysis. Extracts prepared in buffer 4 were desalted into buffer 1 before further analysis.

soybean fraction (toasted meal) showed the lowest CP4 EPSPS content (30% of the level observed for unprocessed seed), whereas levels in protein concentrate and protein isolate were not reduced from that observed for unprocessed soybean seed.

The western blot also showed excellent extraction efficiency. Approximately 70% of the CP4 EPSPS protein that could be extracted was extracted in the initial extract prepared from toasted meal. This experiment was not performed for the other fractions since toasted meal is the most highly processed fraction and represents the most challenging matrix for extraction of CP4 EPSPS protein.

The accuracy and precision of the western blot was evaluated using a blind comparison test. The results from this test showed that trained laboratory personnel consistently and correctly identified samples that contained more or less than 2% Roundup Ready soybean (Table 1). The threshold value of 2% Roundup Ready soybean was arbitrarily selected since no threshold has yet been determined. A representative western blot used for the blind test is shown in Fig. 2. As described by Altman (1991), the positive predictive value (e.g., the number of times a sample was positive and rated as positive) for the evaluation was 0.97 and the negative predictive value (e.g., the number of times a sample was negative and rated as negative) was 0.90.

Anti-CP4 EPSPS protein antisera used for the western blot showed almost no reactivity with similar EPSPS proteins derived from different plant sources as evidenced by the very minor cross reactivity at a molecular weight equivalent to CP4 EPSPS protein (Fig. 3). The minor cross reactive bands observed are insignificant considering that the plant-derived EPSPS proteins were assayed at 10-fold greater levels on this blot than the CP4 EPSPS reference standard. Inherently low expression of endogenous EPSPS protein in processed soybean seed would result in negligible binding as evidenced by the western blot presented in Fig. 2, lane 6. Furthermore, the antiserum showed only minor cross reactivity with other proteins present in soybean extracts as evidenced by the low incidence of cross reacting bands observed in lanes where non-genetically modified processed fractions were analyzed (Fig. 2, lane 6).

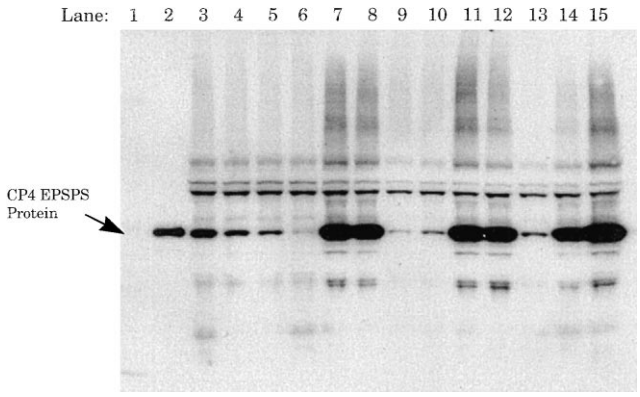

Fig. 2. Blind test. Representative blot used to estimate the accuracy of the CP4 EPSPS western blot analytical procedure. Samples of defined composition ranging from 0 to 100 wt% Roundup Ready soybean were prepared by the mixing of non-transgenic (Line A5403) and transgenic protein concentrate materials. The 1 X Laemmli extracts were analyzed (blindly) and compared to defined wt.% transgenic standards prepared under the same conditions. Trained personnel were asked to determine which samples were greater than 2 wt.% transgenic. Lane 1 was loaded with high range color markers (Amersham, RPN 756, 1.5 μg/band). Lane 2 was laoded with 5 ng of a CP4 EPSPS purified standard produced in *E. coli*. Lanes 3, 4, and 5 were loaded with 3 μl of 5, 2 and 1 wt.% transgenic standard extracts, respectively. Lane 6 was loaded with 3 μl of a non-transgenic control extract. Lanes 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 were loaded with 3 μl of extract prepared from blind samples containing 56, 45, 0.29, 0.97, 80, 49, 1.36, 23 and 21 wt.% transgenic samples, respectively.

Table 1
Summary for the blind analysis of 100 protein concentrate samples[a]

Assay[b]	True sample status		Total
	Positive	Negative	
Positive	276[c]	9[d]	285
Negative	12[e]	103[f]	115
Total	288	112	400

Positive predictive value $[a/(a+b)] = 0.97$, Negative predictive value $[d/(c+d)] = 0.90$.

[a] Samples varied from 0 to 100 wt% Roundup Ready soybean. Trained analysts were asked to classify samples as greater than or less than a 2% Roundup Ready reference standard analyzed concurrently.

[b] Data represents the pooled observation of four analysts.

[c] True positives.

[d] False positives.

[e] False negatives.

[f] True negatives.

Since the western blot is not a strictly quantitative method, the western blot would be most effectively utilized to determine if a sample contained CP4 EPSPS protein equal to or above a pre-determined level (threshold detection). Implementation could be essentially as described in the blind study described in this report. Laboratories would be supplied with a set of prediluted certified processed reference standards. Samples would be assayed on the same blot with the appropriate reference standard and scored using visual comparisons to the reference standard.

ELISA validation. Validation data for the ELISA established that the ELISA is an appropriate technique for quantitation of CP4 EPSPS levels in processed fractions prepared from Roundup Ready soybean. Optimization of

The detection limit for the for the western blot was 100 pg of CP4 EPSPS protein. The detection limit (expressed as the lowest dilution of Roundup Ready soybean seed that could be detected when Roundup Ready seed would be mixed with non-modified soybean seed) in the various processed fractions ranged from 0.25% to 1% for the various Roundup Ready soybean fractions (Table 2).

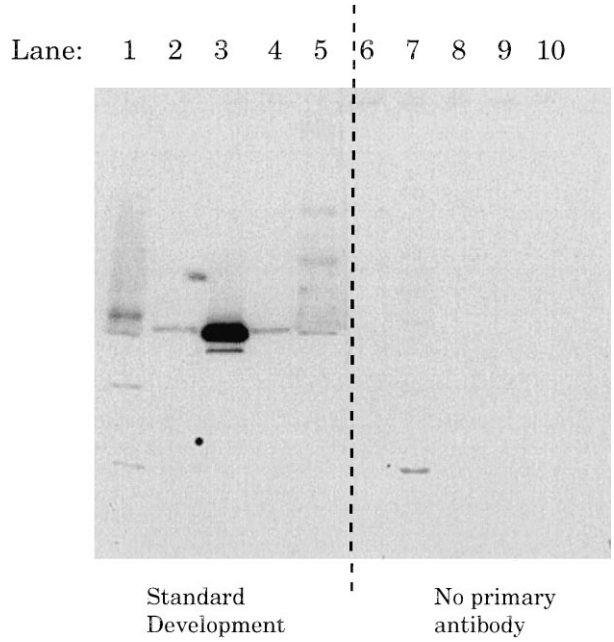

Fig. 3. Specificity of the CP4 EPSPS western blot analytical method for CP4 EPSPS compared to other EPSPS proteins. All EPSPS proteins were expressed in *E. coli* and purified to near homogeneity. Note that maize and petunia EPSPS were loaded at 10 times the level of CP4 EPSPS. These data show that the detection system is highly specific for CP4 EPSPS compared to other EPSPS proteins. Lanes 1 and 7 were loaded with Promega mid-range MW markers. Lanes 2 and 8 were loaded with 50 ng of petunia EPSPS. Lanes 3 and 9 were loaded with 5 ng of CP4 EPSPS. Lanes 4 and 10 were loaded with 50 ng of maize EPSPS. Lanes 5 and 6 were loaded with high range color markers (Amershnam, RPN 756).

the extraction process was the key challenge for application of the ELISA detection method due to the variable extent of protein denaturation in different processed fractions. Industrial soybean processing involves a careful balance of processing temperature, time, pressure and moisture content to produce the desired soybean fraction. During this process, the biological activity and solubility of soybean protein are reduced and proteins are denatured (Liu, 1997). Extraction optimization was required to consistently solubilize the CP4 EPSPS protein present after the various processing steps. Extraction optimization focused on the toasted meal soybean fraction since it represented the most extensively processed fraction (Liu, 1997). In contrast, CP4 EPSPS protein was relatively easy to extract from protein isolate and protein concentrate. Approximately 96%, 96% and 54% of the CP4 EPSPS protein that could be solubilized was solubilized in the first extract for protein isolate, protein concentrate and toasted meal, respectively, using the optimized extraction process.

The optimized process involved the use of heat and detergent to solubilize CP4 EPSPS from processed soybean fractions. However, these same conditions also decreased the immunological reactivity of CP4 EPSPS protein with the antibodies used in the sandwich ELISA. For instance, when the optimized extraction process was used for extraction of CP4 EPSPS from the unprocessed seed, approximately 96% of the expected immunological activity was lost in comparison to that observed when CP4 EPSPS was extracted using phosphate buffered-saline with −0.05% Tween −20, pH 7.4 and mechanical shearing without heating. The observed loss in immune reactivity is likely due to protein denaturation since the CP4 EPSPS protein would be expected to remain soluble due to the presence of detergents in the extraction buffer. As expected, no CP4 EPSPS protein was detected in extracts prepared from non-modified soybean or processed fractions derived from non-modified soybean seed. The difference in CP4 EPSPS levels in the various Roundup Ready soybean processed fractions can be attributed to the different protein content of each processed fraction (Liu, 1997) and different extraction efficiencies. These data establish that the optimized extraction conditions cause reduction in the epitopes available for ELISA assay that is consistent and reproducible across the various processed soybean fractions.

The epitopes of the CP4 EPSPS protein remaining in these extracts are immunologically equivalent to those present in the native CP4 EPSPS protein produced and purified from *E. coli* and used as the reference standard in the ELISA and western blot. As stated by Rodbard et al. (1978), a plot of the μl assayed versus ng observed should yield a straight line with an intercept of zero if parallelism exists. The mean intercept derived from titrations of

Table 2
Detection limits (expressed as % dilution) for western blot and ELISA in unprocessed soybean seed and processed soybean fractions

Fraction	Detection limit (western blot) (%)[a]	Detection limit (ELISA) (%)[b]
Seed	0.25	0.25
Protein isolate	0.6	0.5
Protein concentrate	0.8	0.25
Toasted meal	1.0	1.4

[a] The limit of detection for CP4 EPSPS in the western blot was 100 pg. The maximum amount of sample assayed was 10 μg of soluble protein.
[b] The detection limit was determined using the concentration of CP4 EPSPS in the respective processed fraction (determined using native CP4 EPSPS as the reference standard). The maximum amount of tissue fresh weight that could be assayed in the ELISA was (50 μg). The least detectable dose for native CP4 EPSPS was 2.5 pg/well.

extracts prepared from protein isolate, protein concentrate and toasted meal was −0.7 and mean correlation coefficient (R squared) was 0.99. Therefore, the combination of stable linear epitopes and conformational equivalence in CP4 EPSPS protein results in equivalent dose-response curves produced by CP4 EPSPS protein extracted from processed Roundup Ready soybean or from the CP4 EPSPS protein reference standard (Fig. 4). In addition, the processed CP4 EPSPS protein is indistinguishable from the CP4 EPSPS reference standard when analyzed by western blot.

The assay showed a slight bias due to matrix effects present in the processed fractions. For instance, the percent recovery of native CP4 EPSPS protein spiked into extracts prepared from protein concentrate was 80% whereas percent recovery was 111% when CP4 EPSPS protein was spiked into extraction buffer. Similar recoveries were observed for all of the matrices (including non-modified unprocessed seed). These matrix effects are easily corrected by spiking the standard curve with matrix prepared from non-modified soybean tissue extract.

For the ELISA, precision was evaluated in several ways: inter, intra assay precision and overall precision in CP4 EPSPS concentration in several different processing runs for protein isolate. Mean intra-assay precision (%CV) in CP4 EPSPS standards ranged from 5% to 8% based on the absorbance values compiled from 10 separate runs of the assay performed on separate days. Interassay variation was 28% coefficient of variation for the quality control sample. These data were developed using 14 separate runs of the assay performed during the validation of the assay. Furthermore, protein isolate from three different processing runs for Roundup Ready soybean was available to evaluate variability in CP4 EPSPS protein content in the processed soybean fractions. These data showed minor variation in CP4 EPSPS content (<25% CV) between processing runs developed over several years at different locations.

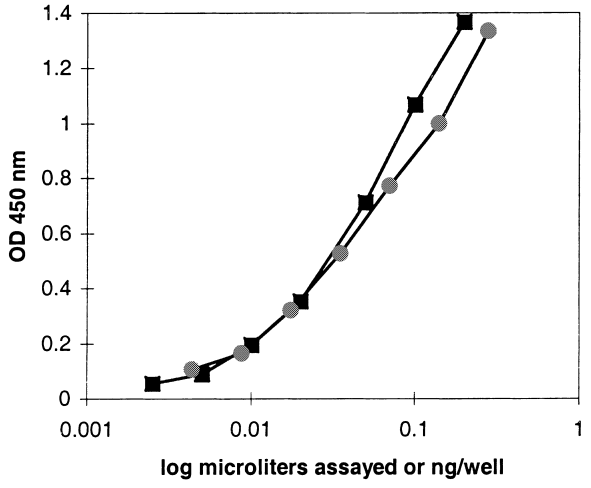

Fig. 4. Dose-response curves produced by native CP4-EPSPS protein or CP4-EPSPS protein extracted from toasted soybean meal. The ELISA was performed as described in the text. The dose-response curve produced by native CP4-EPSPS protein reference standard (squares) or by CP4-EPSPS protein extracted from toasted soybean meal (circles) are similar in shape, thus establishing that CP4-EPSPS protein obtained from either source is immunologically equivalent.

The detection limit for the ELISA was expressed as the lowest dilution of Roundup Ready soybean seed that could be detected when Roundup Ready seed would be mixed with non-modified soybean seed. The detection limit ranged from 0.5% to 1.4% Roundup Ready soybean for the various processed soybean fractions (Table 2).

The CP4 EPSPS protein was relatively stable when samples and extracts were stored frozen at or below −20°C. For instance, no degradation was observed for the concentration of CP4 EPSPS in the quality control standards assayed during the development of this assay. Furthermore, the protein retains stable immunoreactivity when stored in processed protein fractions as evidenced by similar levels of CP4 EPSPS in processed soybean fractions that had been stored for over six years.

Due to the decreased immunological activity after the harsh extraction conditions, quantitation of CP4 EPSPS protein using the sandwich ELISA requires the use of processed Roundup Ready soybean for valid reference standards. Certified reference standards of each respective processed fraction must be prepared at appropriate mixtures and used to generate a standard curve. Results must be presented as a percent of Roundup Ready soybean present in each processed fraction.

To develop appropriate certified reference standards, the level of CP4 EPSPS protein must be relatively consistent across various Roundup Ready soybean varieties. Using the initial ELISA developed for Roundup Ready soybean seed, an estimate of the variation in expression levels in Roundup Ready soybean seed was performed using several different commercial varieties of soybean. Mean CP4 EPSPS expression was 210 μg/g tissue fresh weight and ranged from 195 to 220 μg/g tissue fresh weight. This resulted in an overall variation of less than 15% CV.

4. Conclusion

Western blot and ELISA assays have been developed for detection of CP4 EPSPS protein in processed soybean fractions. These methods have been validated and are sufficiently accurate, precise, specific and sensitive to allow for detection and quantitation of CP4 EPSPS protein in processed soybean fractions. Implementation of these methods for use by the food industry and regulatory agencies will require additional validation to verify the performance characteristics of the assays in different laboratories.

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