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Quantitation of CP4 5-Enolpyruvylshikimate-3-Phosphate Synthase in Soybean by Two-Dimensional Gel Electrophoresis

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Abstract Changes of CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) in the glyphosate-tolerant *Roundup Ready*® soybean were examined using purified CP4 EPSPS produced in cloned *Escherichia coli* as a control. CP4 EPSPS in genetically modified soybean was detected by two-dimensional gel electrophoresis (2-DE) and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) with databases. CP4 EPSPS in soybean products was resolved on 2-DE by first isoelectric focusing (IEF) based on its characteristic pI of 5.1, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on its molecular mass of 47.5 kDa. We quantified various percentages of soybean CP4 EPSPS. The quantitative analysis was performed using a 2D software program on artificial gels with spots varying in Gaussian volumes. These results suggested that 2-DE image analysis could be used for quantitative detection of GM soybean, unlike Western blotting.

Key words: CP4 5-enolpyruvylshikimate-3-phosphate synthase, GMO, soybean, two-dimensional gel electrophoresis, image analysis

Genetic modification of agricultural crops promises food products with more desirable traits, such as higher vitamin content or lower saturated fat content, reduced use of pesticides and other chemicals, and increased yields. To date, about 70 kinds of genetically modified organisms (GMOs) belonging to 15 different crops, such as soybean, corn, cotton, and potato, have been commercially available. The Monsanto Company (St. Louis, MO, U.S.A.) developed GM soybeans (*Roundup Ready*®) tolerant to the application of *Roundup*, glyphosate-based agricultural herbicides, by inserting the CP4 EPSPS gene from *Agrobacterium* sp. strain CP4, which confers tolerance to glyphosate, into *Roundup Ready*® [16].

Concern about the safety of GMOs has led to the development of methods for their detection [1]. PCR methods are generally used because of their high sensitivity and low detection limits (0.1%). However, PCR is expensive and primers are difficult to make. Furthermore, it is not effective for processed food products, since excessive heat and compounds present in foods such as proteins, fats, polysaccharides, and polyphenols can inhibit DNA polymerase [1]. As new types of GMOs are developed, much time and labor are required to prepare new primers. Western blotting and enzyme-linked immunosorbent assay (ELISA) are possible alternatives to PCR [10, 18, 22], and these immunological methods using antibodies could help detect GMOs at the protein level: ELISA has a high sensitivity, can simultaneously examine a large number of samples, and can produce results within hours. However, a protein-based method requires a specific antibody for each GM crop. In addition, it may not be applied to highly processed soy products, because of denaturation and degradation of soy proteins during food processing.

Two-dimensional gel electrophoresis (2-DE) is currently used for protein separation [14]. Peptide mass fingerprinting (PMF), using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [9] and electrospray ionization tandem mass spectrometry (ESI-MS/MS), plays a major role in protein identification; attributes are matched with those computed for every entry in the protein sequence database [5, 19]. Gel image patterns obtained from different samples can be compared either manually or using commercially available software packages to investigate different patterns of qualitative and quantitative protein expression [17]. Many researchers have used 2-DE to obtain crop protein profiles, and in turn used to detect GMOs by comparing 2-DE gel images. Furthermore, 2-DE quantitative data can be derived from image analysis with

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computer assistance. Therefore, in this study, we used 2-DE for protein-based detection and quantitation of CP4 EPSPS in GM soybeans.

MATERIALS AND METHODS

Materials

Roundup Ready® was acquired from the Korean Food and Drug Administration (Seoul, Korea). Antibodies for CP4 EPSPS were obtained from the Korea Food Research Institute (Sungnam, Korea). SDS, acrylamide, bis-acrylamide, TEMED, and ammonium persulfate were purchased from Sigma (St. Louis, MO, U.S.A.). DTT, urea, Tris-HCl buffer, glycine, glycerol, and CHAPS were purchased from Bio-Rad (Richmond, CA, U.S.A.) or USB (Cleveland, OH, U.S.A.). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, U.S.A.). Other chemicals and solvents were of analytical-reagent grade.

Preparation of Protein Extracts

Roundup Ready® soybean powder was treated with cold acetone and homogenized at 11,000 rpm for 1 min. Supernatants were removed and the remaining white powder was vacuum-dried. Protein was extracted by following the modified method of Hird *et al.* [6]. Briefly, the soybean powder (0.15 g) was added to 1.2 ml of 50-mM sodium bicarbonate buffer, pH 9.2, containing 30% w/v tetramethylene sulfone, and the mixture was sonicated for 1 min and stirred at room temperature for 1 h. The sonicate was centrifuged at 10,000 $\times g$ for 10 min, the clarified extract was mixed with acetone at a ratio of 9:1 v/v, acetone:extract, and the protein was precipitated at −20°C for 30 min. After centrifugation at 10,000 $\times g$ for 10 min at 4°C, the protein solids were re-extracted at a ratio of 25 mg to 1 ml of extraction buffer containing 5 mM triethanolamine buffer, pH 8.5, containing 2% w/v tetramethylene sulfone and 2 mM EDTA. The proteins were precipitated with cold acetone and then dried.

Western Blotting

The extracted proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by following the method described by Laemmli [11], using a 12% acrylamide separating gel and a 5% stacking gel containing 0.1% SDS. After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes by following the modified method of Towbin *et al.* [23] at a constant amperage of 0.1 A for 90 min. The primary and secondary (HRP-labeled anti-mouse antibody) antibodies were diluted (1:1,000 and 1:5,000, respectively) with PBS-T buffer. The chemiluminescent method was used with Amersham ECL kit reagents (Amersham Biosciences, Uppsala, Sweden).

2-D Gel Electrophoresis

Isoelectric Focusing. Isoelectric focusing (IEF) was performed using 18-cm immobilized pH gradient (IPG) strips and an IPGphor focusing system (Amersham Bioscience). Samples containing 100−150 μg protein for analytical gels or up to 1 mg for micropreparative gels were diluted to 350 μl with rehydration solution containing 8 M urea, 2% CHAPS, 100 mM DTT, 0.5% v/v pH 4−7 IPG buffer (Amersham Biosciences), and trace levels of bromophenol blue. The samples were then applied to the strips by rehydration for 12 h at 50 V. The proteins were focused in succession for 1 h at 500 V, 1 h at 1,000 V, and 4 h at 8,000 V. All IEF steps were carried out at 20°C.

Equilibration. The gels were placed in equilibration buffer I containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a few grains of bromophenol blue, and 65 mM DTT. The gels were subsequently placed in equilibration buffer II containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a few grains of bromophenol blue, and 65 mM iodoacetamide for 15 min [4].

2-DE. Separation in the second dimension was carried out using Protean® II Xi electrophoresis equipment (Bio-Rad) and Tris-glycine buffer (25 mM Tris, 192 mM glycine) containing 0.1% SDS at 5 mA/gel for the first 1 h and 10 mA/gel thereafter. 2-DE was carried out until the bromophenol blue dye marker had reached the bottom of the gel. The gel was then stained with Coomassie Brilliant Blue R-250 and destained in a solution containing 10% methanol and 10% acetic acid.

Identification of Protein

Digestion of Proteins. Protein spots were excised from the gels after the Coomassie Brilliant Blue staining and destaining. They were washed with 50% acetonitrile in 0.1 M ammonium bicarbonate and dried in a SpeedVac evaporator. The gel particles were reduced for 45 min at 56°C using 10 mM DTT/0.1 M ammonium bicarbonate. After cooling, the DTT solution was immediately replaced with 55 mM iodoacetamide/0.1 M ammonium bicarbonate. After 30 min of incubation at room temperature in the dark, the gel particles were washed with 50% acetonitrile in 0.1 M ammonium bicarbonate and dried in a SpeedVac evaporator. The dried gel pieces were swollen in a minimum volume of 10 μl of digestion buffer containing 50 mM ammonium bicarbonate, 5 mM $CaCl_2$, and 12.5 ng/μl of trypsin in an ice-cold bath. After 45 min, the supernatant was removed and replaced with the same buffer, but without trypsin, to keep the gel pieces moist during enzymatic cleavage (at 37°C, 16−24 h). One volume of the solution, composed of acetonitrile and 5% formic acid (1:1), was mixed with the digestion mixture. The sample was then sonicated for 5 min and centrifuged for 2 min in a microcentrifuge [7].

MALDI-TOF-MS

MALDI-TOF-MS was performed with a Voyager-DE system (PerSeptive Biosystem, Framingham, MA, U.S.A.) using α-cyano-4-hydroxycinnamic acid (CHCA) for calibration. One μl of the purified sample and CHCA (10 mg/ml) was dropped onto a sample plate and thoroughly dried. The sample was then placed in a Voyager-DE Biospectrometry workstation. The workstation was operated at the acceleration voltage of 23 kV.

ESI-MS/MS

ESI-MS was performed using a Q-Tof II hybrid quadrupole/ orthogonal acceleration TOF spectrometer (Micromass, Inc., Beverly, MA, U.S.A.) equipped with an electrospray ion source. Samples were dissolved in acetonitrile/0.1% (v/v) formic acid and delivered to the electrospray at a flow-rate of 5 μl/min by a syringe infusion pump. The capillary voltage was set to 2,500–3,000 V, and data-dependent MS/MS acquisitions were performed on precursors with a charge of 2 or 3.

Database Searching

Data searching with the peptide mapping data was performed against the NCBInr using a peptide searching algorithm provided by the MS-Fit, Mascot search algorithm, and the SWISSPROT protein database [12].

Quantitative Analysis

Image Analysis. The gels were digitized into 256-level grayscale TIFF files using a UMAX Powerlook III scanner, and the images were analyzed with Image-Master 2D Elite Version 4.10 software (Amersham Biosciences). The spots on the gels were automatically detected in the images and matched to a reference gel. This procedure accounted for the differences between the gels and the differences in protein positions on the gels. All spots matched to reference gel spots were given a number, and the same number was used for all gels, making comparisons among gels possible. Comparison analysis was performed on the matched spot set, comparing the average and standard deviation of protein abundance [13] for a given spot between CP4 EPSPS and GM% level. The quantitative differences in target spots were evaluated [2].

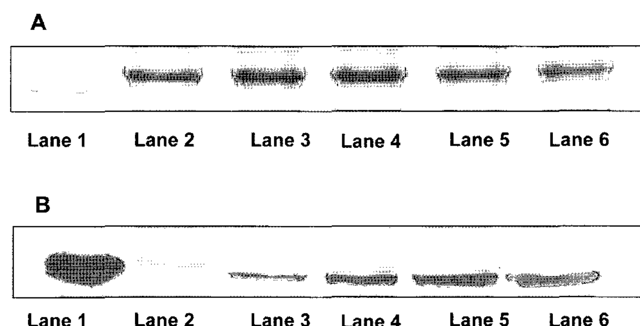


Fig. 1. CP4 EPSPS in GM soybeans detected by immunological method. SDS-PAGE (A) and Western blotting (B) of a 1-DE gel of proteins extracted from GM soybean.

Lane 1, Purified CP4 EPSPS produced in *E. coli*; lane 2, proteins from non-GM soybean; lanes 3, 4, 5, and 6: proteins from GM soybean 25%, 50%, 75%, and 100%, respectively.

RESULTS AND DISCUSSION

Distinguishing Between GM and Non-GM Soybeans

Both the purified CP4 EPSPS and proteins in soybean extracts were visualized using SDS-PAGE and Western blotting analysis (Fig. 1). Although not possible with SDS-PAGE (Fig. 1A), GM and non-GM soybeans were clearly

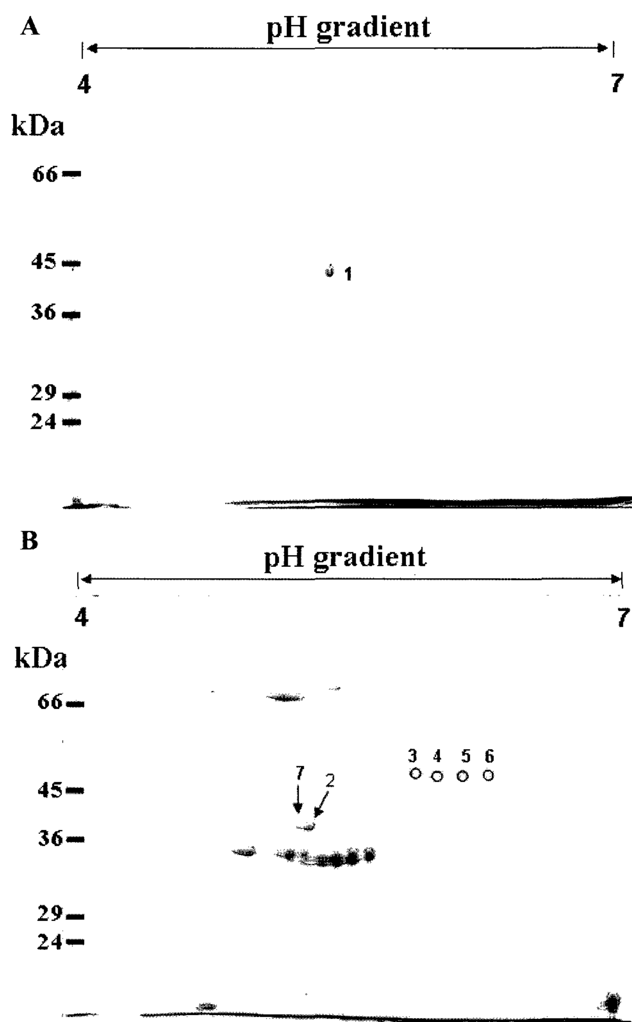


Fig. 2. Images of purified CP4 EPSPS produced in *E. coli* (A) and GM soybean proteins (B) (2-D gel of pH 4–7) separated by 2-DE.

Detection by Coomassie blue-staining. The targeting (CP4 EPSPS) spot is indicated as 1 and 2 by arrow. Spots 3, 4, 5, and 6 are isoforms of β-conglycinin.

distinguished by Western blotting (Fig. 1B), in agreement with Rogan *et al.* [18], who reported that the sensitivity (least detectable dose) for Western blot was estimated by spiking CP4 EPSPS protein into appropriate nonmodified processed fraction. The Western blotting procedure, however, could not be applied for quantitative detection, because the band intensity was not completely dependent on the amount of CP4 EPSPS (Fig. 1B) [18].

Detection of Purified CP4 EPSPS by 2-DE

The soybean proteins separated by 2-DE included groups of proteins with molecular mass of around 20–65 kDa. Most spots appeared to be concentrated at the acidic and neutral pH regions. This pI range is similar to that of tomato reported by Corpillo *et al.* [3]. The stained gel showed a number of clear spots (Fig. 2). Most proteins were distributed in the range of pH 4 to pH 7, and the pI for the target protein of CP4 EPSPS was 5.1, as confirmed by a pH strip gel (pH 4–7). As the pH 4.5–5.5 strip gel has a very narrow pH range, and 2D gels using these strip gels require large amounts of sample proteins and focusing time, we used the pH 4–7 strip gel for separation of the sample proteins [15].

Target Spot Analysis with MALDI-MS and ESI-MS/MS

The target spots from the 2-DE gels were excised and digested with trypsin, and the resulting peptides were extracted from the gel. Values for MW and pI estimated from the gels were used to search MS-fit and MASCOT to identify the proteins. The search was originally performed with a mass tolerance of 50 ppm. The results of CP4 EPSP from *Escherichia coli* are shown in Fig. 2, and Table 1 lists the measured matching peptide masses and information about the amino acid sequences of the matching peptides. The matched peptides were covered with 55% of the amino acid sequence of this protein. The PMF method is usually performed by MALDI-MS and is the most commonly used mass spectrometry identification method [24]. As known in Table 1, the soybean targeting spots were analyzed and the matching peptides were covered with 53% of the amino acid sequence of this protein. An NCBI BLAST search indicated that the spots 3, 4, 5, and 6 were isoforms with high similarity. These spots were determined to be β-conglycinin, in agreement with Shun-Tang *et al.* [20]. The spots 2 and 7 were identified as CP4 EPSPS, and the analysis indicated the existence of the protein with an apparent molecular mass significantly different from the theoretical molecular mass. Figure 2B shows a mass shift of the spots 2 and 7. Sickmann *et al.* [21] reported that modifications lead to a shift of pI and/or mass of the protein. The spot 7 had a different pI and mass resulting from the experimental modification. Kast *et al.* [8] reported that tandem mass spectrometry (MS/MS) is needed by a sequence of MALDI-MS to increase the confidence level of the identification of these proteins. Therefore, the second mass spectrometry analysis with ESI-MS/MS was carried out for the CP4 EPSPS spots in *E. coli* (spot 1) and soybeans (spot 2) (Table 2). ESI-MS/MS sequence coverage for the targeting proteins was 30% and 27%, respectively. Molecular mass and pI values for the protein spots on the 2-DE gels were estimated and compared with their theoretical masses and pI values (Fig. 2). Overall, gel-estimated and theoretical values of molecular mass matched well. The soluble protein of CP4 EPSPS

Table 1. CP4 EPSPS identified by tryptic mapping and a PMF database search.

(A) Purification of CP4 EPSPS in *E. coli*: matches (55%)

m/z submitted	MH^+ matched	Delta ppm	Start	End	Database sequence
948.4999	948.5155	−16	161	168	(K)TPTPITYR(V)
1,308.7088	1,308.7687	−46	128	138	(K)RPMGRVLNPLR(E)
1,357.7565	1,357.7076	36	146	157	(K)SEDDGDRLPVTLR(G)
359.7565	1,359.7232	24	354	366	(K)ESDRLSAVANGLK(L)
1,646.9120	1,646.8363	46	389	405	(K) LGNASGAAVATHLDHR (I)
182.254	2,183.1746	37	275	294	(R)TGLILTLQEMGADIEVINPR(L)
3,249.7505	3,249.6176	41	321	351	(R)APSMIDEYPILAVAAAFAEGATVMNGLEELR(V)

(B) Soybean: matches (53%)

m/z submitted	MH^+ matched	Delta ppm	Start	End	Database sequence
1,993.9581	1,993.9653	-3.6	206	224	(K)MLQGFGANLTVEDADGVR(T)
2,199.0845	2,199.1695	-39	275	294	(R)TGLILTLQEMGADIEVINPR(L)
2,450.2095	2,450.2165	-2.9	127	127	(R)LTMGLVGVYDFDSTFIGDASLTK(R)
3,249.6533	3,249.6176	11	351	351	(R)SMIDEYPILAVAAAFAEGATVMNGLEELR(V)

Acc. #: 8469107 Species: AGRSP.

Name: 3-phosphoshikimate-1-carboxyvinyltransferase (5-enolpyrovylshikimate-3-phosphate synthase, EPSP syntase, EPSPS).

Molecular weight: 47,589.

pI: 5.1.

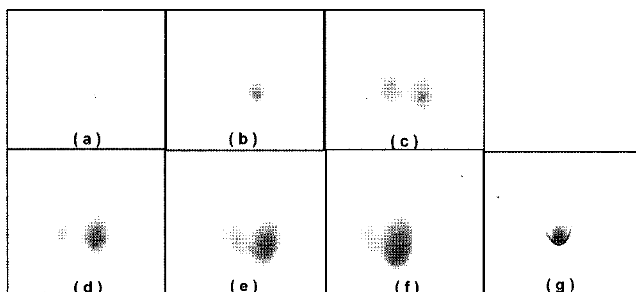
Table 2. Summary of database search using the derived partial sequence of the CP4 EPSPS in soybeans by ESI-MS/MS.

Proteins description	NCBI GI identifier	Mass (Da)
3-phosphoshikimate-1-carboxyvinyltransferase (5-enolpyruvylshikimate-3-phosphate synthase)	gi|8469107	47,558 (27%)
Species with homology region	**NCBI GI identifier**	**Mass (Da)**
Putative 3-phosphoshikimate-1-carboxyvinyltranserase protein [*Sinorhizobium meliloti*]	gi|15964006	47,585
3-phosphoshikimate-1-carboxyvinyltransferase [*Mesorhizobium loti*]	gi|13474346	47,425
5-enolpyruvylshikimate-3-phosphate synthase [*Brucella melitensis biovar Abortus*]	gi|13487156	50,603

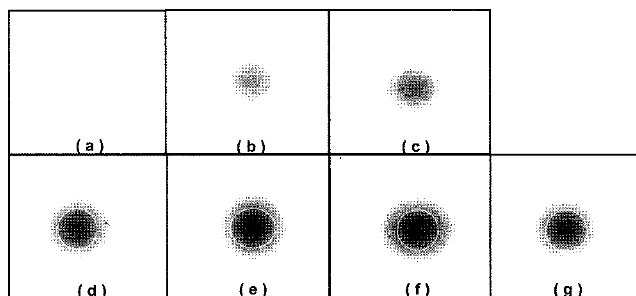
was resolved on 2-DE according to its characteristic pI of 5.1 by IEF and molecular mass of 47.5 kDa by SDS-PAGE (Fig. 2, Table 1).

Quantitative Analysis by 2-DE

Samples of varying proportions of GM soybeans were prepared by combining 100% GM soybean (*Roundup Ready*®) and 0% GM soybean (domestic goods). The extracted soluble proteins of samples containing 1–100% GM soybean were detected by 2-DE. A gel for purified CP4 EPSPS from cloned *E. coli* was used as a reference. Various percentages of CP4 EPSPS in soybean were quantified by a 2D software program. Gel-to-gel matching was carried out by spot detection, gel matching, and finally spot quantitation. Spot detection and quantitation were performed with the same samples using image software packages, i.e., ImageMaster 2D Elite (Amersham Biosciences) and PDQuest (Bio-Rad). By using different packages for



A



B

Fig. 3. Detection of various GM soybean percentages of CP4 EPSPS.
The volume ratio of the center spot in gels (a) 1%, (b) 3%, (c) 5%, (d) 25%, (e) 50%, (f) 100% as compared with the center spot in gel (g) CP4 EPSPS in *E. coli*. A Detection by 2-DE. B. Gaussian volume by PDQuest.

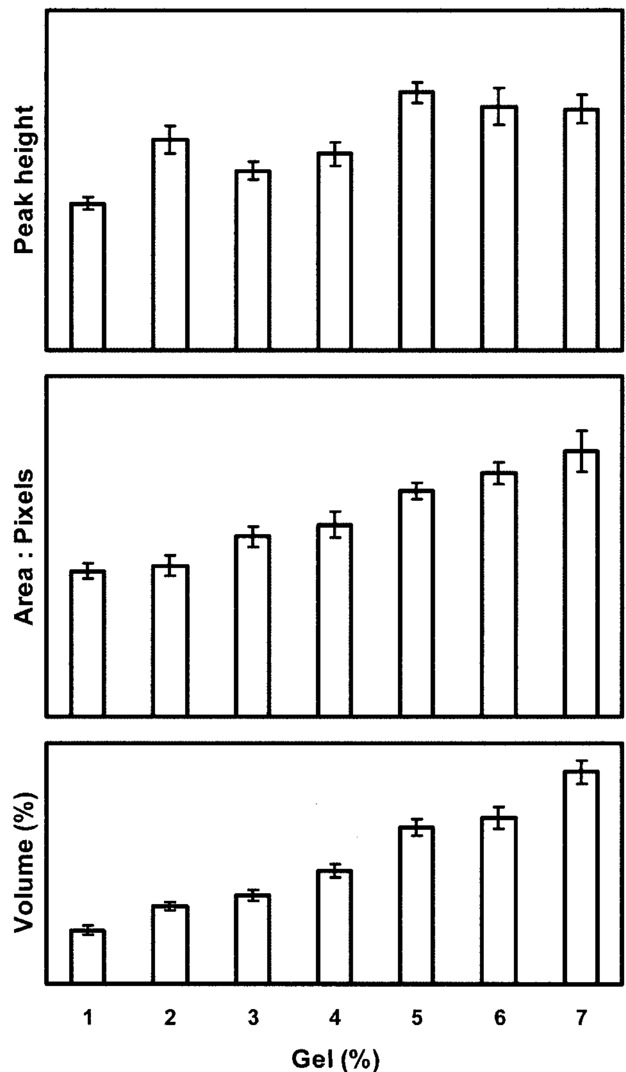


Fig. 4. Quantitative image analysis: comparison of spot ratio analyzed with ImageMaster 2D Elite program.
1, 1%; 2, 3%; 3, 5%; 4, CP4 EPSPS in *E. coli*; 5, 25%; 6, 50%; 7, 100%.

Table 3. Quantitation of various GM soybean percentages of CP4 EPSPS.

Gel (%)	Peak height	Area: pixels	Volume (%)
1 (1%)	43.038	512	10,034.654
2 (3%)	61.962	532	14,577.885
3 (5%)	52.779	635	16,489.070
4 (CP4 EPSPS in *E. coli*)	57.686	676	21,041.384
5 (25%)	75.716	796	29,139.968
6 (50%)	71.637	859	30,919.078
7 (100%)	70.752	934	39,573.857

different steps in the image analysis, 2-DE analysis could be more accurate. To evaluate spot quantitation efficiency, we generated a series of artificial gel images with "ideal" Gaussian-shaped spots (spot-area in pixels×pixel gray-level) of known volume using PDQuest (Fig. 3). Figure 4 shows the results from our quantitative comparison study. Volume refers to the variations in the optical density (OD) of the observed area or integrated optical density (IOD) of individual spots on 2-DE. In Table 3, relative volume ratios (%vol) of CP4 EPSPS are obtained by following the equation $vol\ (IOD)=\Sigma_{x,y\varepsilon spot} I(x, y)$, $\%vol=(vol/\Sigma^{n}_{s=1} vol_s)\times 100$ using ImageMaster 2D Elite. This program was used to identify the proteins expressed at different levels by quantifying the spot volume (Table 3). Our data showed that the targeted spot volume was increased from a low percentage to a high percentage through an analysis software program for 2-DE image analysis (Fig. 4). Volume continued to increase with increased GM percentage, but it was not directly proportional to the increase, because of the gel running and staining system [2]. The resulting figures are satisfactory for quantitative detection using 2-DE, and the detection limit (1%) of this method is suitable for distinguishing between GM soybean and non-GM soybean.

In conclusion, the detection limit for GM soybean was about 1% on the protein basis, suggesting that this method was suitable for distinguishing between GMOs and non-GMOs. Additionally, unlike the Western blotting procedure, it could be used for quantitative detection of GM soybean, because of its dose-dependency. Although the quantitation of CP4 EPSPS in various GM foods has not yet been completed, this method is precise and sufficiently sensitive for detection. If 2-DE maps were made for other GM products, a method for detection and relative quantitation of GM proteins without primers or antibodies could be developed.

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