

LC/MSによる5-Enolpyruvylshikimate-3-phosphate Synthaseの酵素活性の測定

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Determination of Enzymatic Activity of 5-Enolpyruvylshikimate-3-phosphate Synthase by LC/MS

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A liquid chromatography-mass spectrometry (LC/MS) method for determining the enzymatic activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), an enzyme of the shikimate pathway, was developed. EPSP synthase catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S-3-P) and phosphoenolpyruvate (PEP) in microorganisms and plants. The enzymatic activity of EPSP synthase was assessed by the determination of EPSP after a 30-min incubation with S-3-P and PEP using the LC/MS system. EPSP synthase activity is given in terms of the produced EPSP (pmol/min/mg protein). Glyphosate (*N*-phosphonomethyl glycine)-tolerant EPSP synthase from the *Agrobacterium* sp. strain CP4 (CP4-EPSP synthase) in genetically modified soybeans (GM-soybeans) was found to have an enzymatic activity of 736 EPSP pmol/min/mg protein in the presence of 3 nmol of S-3-P. In contrast, the enzyme activity of non-GM-soybeans was 21 EPSP pmol/min/mg protein. The EPSP synthase activity was markedly decreased in the non-GM-soybeans by the addition of glyphosate, but the enzyme activity of the GM-soybeans was only slightly decreased with this treatment. This LC/MS system could also be applicable to the measurement of EPSP synthase activity in different plant species and the detection of herbicide-tolerant EPSP synthase in GM foods.

Key words: 5-enolpyruvylshikimate-3-phosphate synthase; LC/MS; genetically modified soybeans; shikimate-3-phosphate; glyphosate

Introduction

5-Enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EC 2.5.1.19), an enzyme of the shikimate pathway, catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S-3-P) and phosphoenolpyruvate (PEP)¹⁻³⁾. The shikimate pathway of aromatic amino acid biosynthesis is present in plants and microorganisms, but not in mammals, making it a good target for herbicidal attack⁴⁾. The herbicide glyphosate (*N*-phosphonomethyl glycine) is a specific inhibitor of EPSP synthase and does not affect other PEP-related enzymatic reactions^{5), 6)}.

Recently, a number of genetically modified (GM) crops have been introduced into the mainstream cultivation worldwide. Some genetically modified soybeans (GM-soybeans) contain bacterial genes such as epsps from the *Agrobacterium* sp. strain CP4 (CP4-epsps) that have been introduced to confer tolerance to glyphosate¹⁾.

We have already reported a detection method for the recombinant CP4-EPSP synthase DNA in GM-soybeans using a PCR technique⁷⁾. Recently, a variety of geneti-

cally modified soybeans which does not express CP4-EPSP synthase has been found, even though the CP4-EPSP synthase DNA was detected in the variety using the PCR method. Therefore, it is also important to investigate the activity of CP4-EPSP synthase. A radioactive labeling method and an HPLC method with UV detection have been reported for the measurement of EPSP synthase activity^{1), 8)}. However, the methods involve time-consuming steps because of many interfering peaks on the HPLC chromatogram or the potential hazard of radioactivity.

In the present report, we describe a novel and simple method based on LC/MS for the determination of the enzymatic activity of EPSP synthase without using complicated extraction steps or radioactive labeling. This LC/MS method was applied to measure the enzymatic activities of CP4-EPSP synthase expressed in the *E. coli* strain BL21 and GM-soybeans, and of EPSP synthase in non-GM-soybeans. This method allows the sensitive measurement of EPSP synthase activity in soybean crude extract, and should be applicable to the determination of EPSP synthase in different species of plants⁹⁾. We also compared the EPSP synthase ac-

tivities in the crude extracts of GM and non-GM-soybeans in the presence of glyphosate and confirmed the presence of herbicide-tolerant CP4-EPSP synthase.

Materials and Methods

Preparation of S-3-P and [³²P] S-3-P

S-3-P was prepared the following method of Akiyama *et al.*¹⁰ The reaction buffer contained 10 μ L (protein content 260 μ g) of shikimate kinase II (in destruction buffer of *E. coli* including the *aroL* expression vector), 10 mmol/L shikimic acid (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), 16 mmol/L ATP (Wako Pure Chemicals Industries, Ltd.), 16 mmol/L MgCl₂, 1 mmol/L (NH₄)₆Mo₇O₂₄, 10 mmol/L benzamidine HCl, 50 mmol/L KF and 50 mmol/L HEPES buffer (pH 7.0) in a 100 μ L total volume. The mixture was incubated at 37°C overnight. After deproteinization using a Tosoh Air Press-30 (Tosoh Co., Tokyo, Japan), the reaction mixture was subjected to the HPLC method described below. The S-3-P peak fraction was collected repeatedly and evaporated to dryness at 40°C. The residue was weighed to determine the amount of purified S-3-P.

When [³²P] ATP (2.0 \times 10⁸ dpm/64 nmol) was used for preparing [³²P] S-3-P, the hot reaction mixture was assayed under the radio-HPLC in the conditions described below. For the control experiments, reactions without shikimate kinase II were run in parallel.

HPLC conditions for preparing S-3-P

A Shimadzu LC-10AD pump and an SPD-10AV UV detector (Shimadzu Co., Kyoto, Japan) were used as the HPLC system. The column was a TSK gel CARBON-500 (Tosoh Co.). The column was maintained at 40°C with a flow rate of 1 mL/min. The mobile phase was 0.1% trifluoroacetic acid (TFA), and S-3-P was monitored at 215 nm. The injection volume was 100 μ L. The determination of S-3-P obtained by enzyme reaction (shikimate kinase II) was performed by LC/MS¹⁰.

HPLC conditions for analyzing EPSP

EPSP was analyzed according to the method reported by Washizu with some modifications⁸. A Shimadzu LC-10AD pump and an SPD-10AV UV detector (Shimadzu Co.) were used as the HPLC system. The column was a Senshu Pak NH₂-1251 (4.6 \times 250 mm i.d.; Senshu Scientific Co., Tokyo, Japan). The operating conditions were: column temperature, 40°C; flow rate, 1 mL/min; injection volume, 20 μ L; detector wavelength, 215 nm. The mobile phase was 200 mmol/L potassium dihydrogen phosphate (pH 7.0).

Radio-HPLC conditions for preparing [³²P] S-3-P and analyzing [³²P] EPSP

[³²P] S-3-P were prepared according to the method reported by Washizu with some modifications⁸. A Hitachi L-7100 pump, an L-7400 UV detector and L-7300 column oven (Hitachi Co., Tokyo, Japan) were used as the HPLC system. The column was a Senshu Pak NH₂-1251 (4.6 \times 250 mm i.d.; Senshu Scientific Co., Tokyo,

Japan). The operating conditions were: column temperature, 40°C; flow rate, 1 mL/min; injection volume, 20 μ L; detector wavelength, 215 nm. The mobile phase was 200 mmol/L potassium dihydrogen phosphate (pH 7.0). The eluted fractions (each 1 mL) were collected every minute. A 50 μ L volume of each fraction was added to 9 mL of liquid scintillation fluid (Perkin-Elmer, Inc., Wellesley, Massachusetts, USA) and the [³²P] radioactivity was detected using a liquid scintillation counter, SC-5100 (Aloka Co., Tokyo, Japan).

Preparation of CP4-EPSP synthase in *E. coli*

The entire coding sequence of CP4-EPSP synthase was PCR-amplified using a set of overlapping oligonucleotide primers, PR01-5' (5'-TCCTTCGCAAGACCTT-CCTCTAT-3') and R1-EPSP synthase primer including *EcoRI* and *SacI* sites (5'-GCTGCCTGATGAGCTCGAA-TTCGA-3'), based on the reported GM-soybean genomic DNA sequence¹¹, using the GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA, USA). The reaction mixture (25 μ L) contained 0.625 U Taq polymerase, 0.3 μ g GM-soybean DNA, 3.5 μ L of PCR premix (Takara Shuzo Co., Ltd., Tokyo, Japan), and 500 nmol/L primers. The PCR reactions were carried out for 40 cycles (94°C: 30 sec, 62°C: 1 min, 74°C: 2.5 min for each cycle) after incubation at 94°C for 4 min, and final extension was done at 74°C for 10 min. The amplified fragment was purified by gel electrophoresis in 1.5% agarose (GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Co., Tokyo, Japan) and used as a template for the 2nd PCR using the F1-EPSP synthase primer with an *NdeI* site (5'-TTTCATCATGCTTCAC-GGTGCAAGCAGCCGG-3') and the R1-EPSP synthase primer. The PCR product was extracted with phenol/chloroform, precipitated with ethanol, and digested with restriction enzymes *NdeI* and *SacI* (Toyobo Co., Ltd., Osaka, Japan). The digested fragments were inserted between the *NdeI* and *SacI* sites of the pET23b (+) expression vector (Novagen, Inc., Madison, Wisconsin, USA) and cloned. Briefly, 55.5 ng of the insert DNA and 51 ng of pET23b(+) were ligated at 16°C for 24 hours with 7.5 μ L of Takara Ligation Kit Ver. 2 solution 1 (Takara Shuzo Co., Ltd., Tokyo, Japan). After subcloning of the inserted DNA (1,388 bp), the nucleotide sequence was determined by the dideoxynucleotide chain termination method¹². *E. coli* strain BL21 was transformed with the CP4-EPSP synthase cDNA expression vector pET23b(+) by the heat shock method. The cloned BL21 cells including CP4-EPSP synthase cDNA-pET23b(+) in 200 mL of NZCYM culture medium (NZ amine 10 g, Bacto yeast extract 5 g, NaCl 5 g, casamino acids 1 g, MgCl₂·7H₂O 2 g/L) (Sigma Chemical Co., St. Louis, MO, USA) were stimulated with 0.5 mmol/L isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, MO, USA) to induce the CP4-EPSP synthase protein expression and then suspended in disruption buffer for BL21 (5 mmol/L MgCl₂, 50 mmol/L Tris-HCl (pH 7.0), 0.37% KCl), and sonicated. The supernatant was collected and used as the CP4-

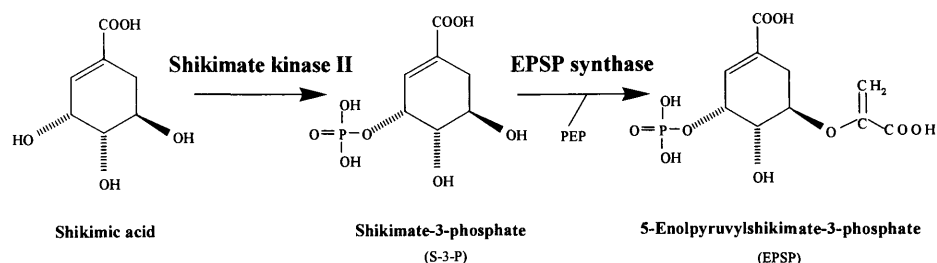


Fig. 1. Shikimic acid pathway and related enzymes
Shikimate kinase II catalyzes the phosphorylation of shikimic acid to shikimate-3-phosphate (S-3-P).
5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase catalyzes the reaction of S-3-P and phosphoenolpyruvate (PEP) to form EPSP and phosphate.

EPSP synthase from *E. coli*.

Extraction of EPSP synthase from GM- and non-GM-soybeans

Powders of 50 g of GM- and non-GM-soybeans, which were products from the state of Ohio, USA, and purchased from a Japanese trading company, respectively, were used. The soybean powder was prepared by the method of Teshima *et al.*¹³⁾ A 200-mL volume of acetone was added to the soybean powder and the mixture was stirred for 30 min at room temperature. The acetone solution was removed by filtration, and 100 mL of acetone was added to the precipitate again. The suspension was stirred for 20 min, then filtered and the precipitates were dried. Next, 250 mL of 50 mmol/L PBS (pH 7.4) with 0.05% sodium azide was added to the dried powder and the mixture was stirred overnight at 4°C. On the next day, it was centrifuged at 40,000 rpm for 1 hour at 4°C, and the supernatant was dialyzed against PBS for 2 days. The dialyzed supernatant was used as the extract for determining the EPSP synthase activity. The protein concentration of the extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the method of Bradford¹⁴⁾.

Production and determination of EPSP

According to the reports of Padgett *et al.*^{9), 11)}, we examined the conditions for the determination of EPSP synthase activity. Among various conditions examined, the following conditions were concluded to be optimal for the determination of EPSP synthase activity using LC/MS. The reaction mixture (100 μ L) containing 500 μ M PEP, 3.2 mmol/L MgCl₂, 10 μ L of 3 nmol S-3-P, 100 mmol/L KCl, 50 mmol/L KF, 5 mmol/L HEPES buffer (pH 7.0) and 1 μ L of crude extract of the *E. coli* expressing CP4-EPSP synthase (protein content 1.5 μ g) or soybeans was incubated at 30°C for 30 min. The reaction tube was put on ice to stop the reaction after incubation since EPSP synthase activity is sensitive to temperature. The reaction mixture was applied to the LC/MS system. In advance, we had prepared an EPSP standard solution (10 ng/mL) to determine the extent of conversion of PEP to EPSP during the CP4-EPSP synthase reaction using the HPLC method for analyzing EPSP. The relationship between PEP reduc-

tion and EPSP production was linear up to about 25% conversion using the HPLC method with UV detection. EPSP was determined with the LC/MS system described below using the standard solution. Control reactions lacking CP4-EPSP synthase were also run in parallel. EPSP synthase activity is given in terms of produced EPSP (pmol/min/mg protein) at 30°C.

LC/MS conditions

The LC/MS system consisted of an Alliance 2790 HPLC system, 996 PDA detector and ZQ MS detector (Waters Co., MA, USA). A Develosil C30 column (4.6 mm i.d. \times 250 mm, Nomura Chemical, Tokyo, Japan) was used as the separation column and was maintained at 50°C. The mobile phase was 3% methanol solution containing 0.1% TFA at the flow rate of 0.7 mL/min. The cone voltage was 30 V. The ionization mode was negative ion mode of electrospray ionization (ESI) was used. The injection volume was 5 μ L.

Results and Discussion

Assay for enzymatic activity of CP4-EPSP synthase expressed in *E. coli*

To develop an assay method for the enzymatic activity of EPSP synthase by LC/MS analysis, CP4-EPSP synthase was over-expressed in *E. coli* for use as the positive control^{11), 15)}. Since S-3-P, which is a substrate for measuring the enzymatic activity of EPSP synthase, was not commercially available, shikimate kinase II (S-3-P synthase), which biosynthetically produces S-3-P (Fig. 1), was expressed in *E. coli* as previously described¹⁰⁾. The molecular weights of the expressed shikimate kinase II and expressed CP4-EPSP synthase were determined by SDS-PAGE to be 19 kDa and 44 kDa, respectively (data not shown), similar to the molecular mass of the enzyme predicted from the DNA sequence¹⁶⁾⁻¹⁸⁾. The complete reaction mixture containing CP4-EPSP synthase (protein content 1.5 μ g) and S-3-P (3 nmol) was subjected to HPLC method reported by Washizu with some modifications; an amino-silica column (Senshu Pak NH₂-1251) was eluted with 200 mmol/L KH₂PO₄ (pH 7.0) as shown in Fig. 2⁸⁾. The chromatogram showed an additional peak indicated by the arrow at 13 min (Fig. 2A), which was not obtained from the sample solution without CP4-EPSP synthase (Fig. 2B). Thus, the 13-min peak appears to correspond to EPSP.

Detection of [^{32}P] S-3-P and [^{32}P] EPSP on radio-HPLC

To further confirm that the peak detected on HPLC described above was EPSP, we used a radio-HPLC procedure as described in Materials and Methods section⁸⁾. We added [$\gamma\text{-}^{32}\text{P}$] ATP (2.0×10^8 dpm/64 nmol) to an enzyme reaction mixture for synthesis of [^{32}P] S-3-P. A single radioactive peak appeared at the retention time of 6 min (Fig. 3a), whereas the control solution reacted without shikimate kinase II showed no radioactive peak at this position (Fig. 3b). Typically, a 95% conversion

of [^{32}P] ATP to [^{32}P] S-3-P was achieved after 24 hours of incubation at 37°C. The peak fraction of [^{32}P] S-3-P was collected, added to a reaction mixture containing CP4-EPSP synthase from *E. coli* and incubated at 30°C for 30 min. The radio-chromatogram of the incubation mixture is shown in Fig. 3c. After the peak of [^{32}P] S-3-P was eluted, a new radioactive peak corresponding to [^{32}P] EPSP appeared at 10 min (Fig. 3c), which was not found in the reaction mixture without CP4-EPSP synthase (Fig. 3d). Though the retention time (10 min) of [^{32}P] EPSP was different from that (13 min) of EPSP detected above (Fig. 2a), the difference of the retention time was thought to be due to the difference of HPLC systems used.

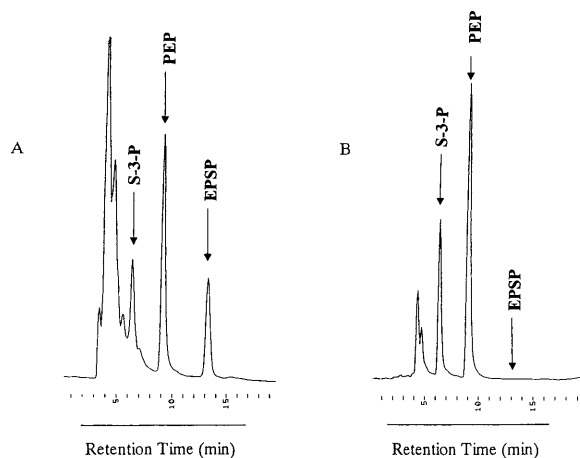


Fig. 2. Detection of EPSP using the HPLC method
HPLC chromatograms of the reaction mixtures with CP4-EPSP synthase from *E. coli* (A) and without CP4-EPSP synthase (B). The column was a Senshu Pak NH₂-1251 (4.6 × 250 mm i.d.). The operating conditions were as follows: column temperature, 40°C; flow rate, 1 mL/min; injection volume, 20 μL ; detector wavelength, 215 nm. The mobile phase was 200 mmol/L potassium dihydrogen phosphate (pH 7.0).

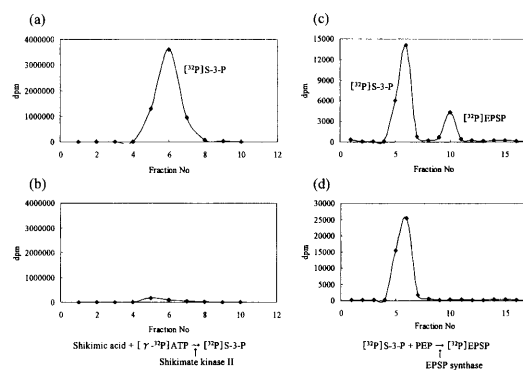


Fig. 3. Detection of [^{32}P] S-3-P and [^{32}P] EPSP using the radio-HPLC method
HPLC chromatograms of the reaction mixtures with shikimate kinase II (a) and without shikimate kinase II (b). Chromatograms of the reaction mixtures containing [^{32}P] S-3-P obtained (a) with CP4-EPSP synthase from *E. coli* (c) and without CP4-EPSP synthase (d).

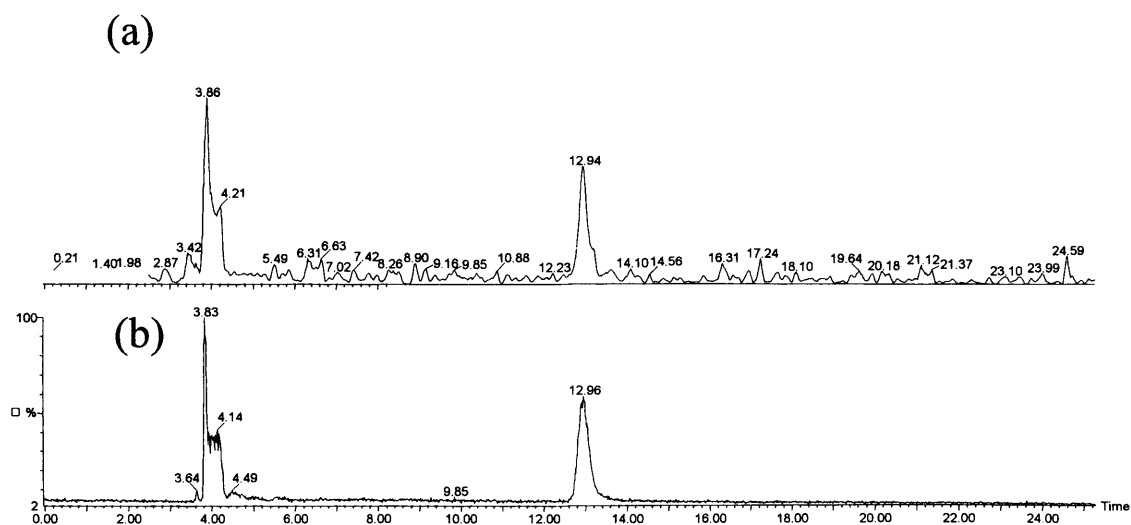


Fig. 4. Total ion chromatogram (a) and selected ion (m/z 323) chromatogram (b) for the LC/MS analysis of EPSP produced by the reaction mixture including GM-soybean crude extract
The LC/MS conditions were as follows. A devesil C30 column was used as the separation column and was maintained at 50°C. The mobile phase was 3% methanol solution containing 0.1% TFA at the flow rate of 0.7 mL/min. The cone voltage was 30 V. The ionization mode was negative ion electrospray ionization (ESI). The injection volume was 5 μL .

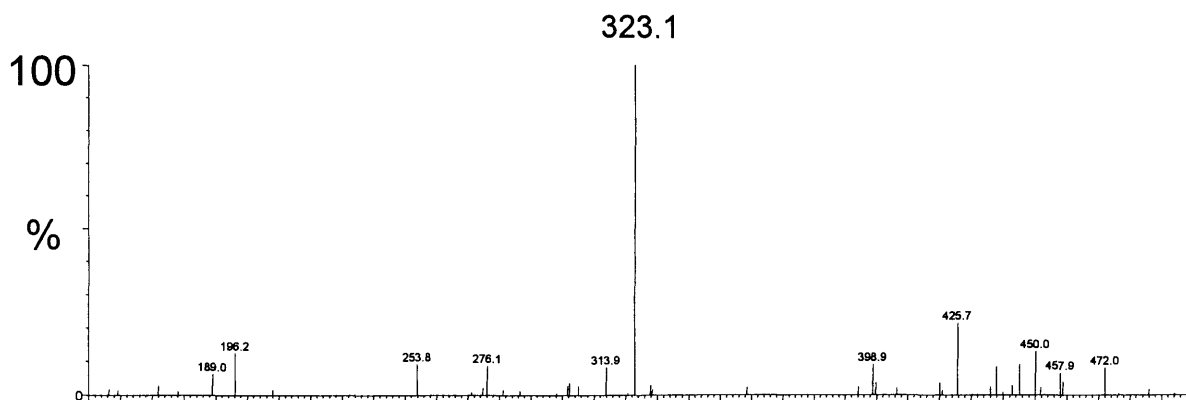


Fig. 5. ESI mass spectrum for the EPSP peak detected by the LC/MS analysis of EPSP produced by the reaction mixture including GM-soybean crude extract

Determination of EPSP using LC/MS system

To easily determine EPSP without using radio-HPLC, we developed an LC/MS method. Among the several reverse-phase columns examined, Develosil C30 showed the best retention of EPSP in combination with the volatile mobile phase described in the Materials and Methods section. The established HPLC separation system was adapted to the LC/MS system. Negative ion ESI produced deprotonated molecules $[M-H]^-$ (m/z 323) of EPSP without any fragmentation. Furthermore, the selected ion monitoring (SIM) mode unambiguously identified EPSP. Figures 4 and 5 show representative MS chromatograms and an ESI mass spectrum of EPSP, respectively. The relative standard deviation was less than 5% (3 ng/mL of EPSP) after six repeated injections. EPSP could be determined quantitatively in the range of 0.3–5 ng/mL. The detection limit was 0.1 ng/mL. This LC/MS method was used to analyze the EPSP synthase activity.

Determination of EPSP synthase activity in GM-soybean and non-GM-soybean crude extracts by LC/MS

We extracted proteins from GM-soybeans and attempted to detect the EPSP synthase enzymatic activity using the LC/MS method as described above. The enzymatic activity was calculated from the EPSP determination. The crude extract of the GM-soybeans (diluted 10, 50, 100, 500 and 1,000 times) was incubated with reaction mixture containing S-3-P at 30°C for 30 min. We confirmed the correlation between the dilution of the soybean crude extract and the production of EPSP.

Next, we compared the enzyme activity of CP4-EPSP synthase produced by *E. coli* and the soybean crude extracts. The enzymatic activity of EPSP synthase in the *E. coli* and GM- and non-GM-soybean crude extracts was $63,267.7 \pm 4,933.3$ EPSP pmol/min/mg protein, 735.6 ± 67.8 EPSP pmol/min/mg protein and 21.1 ± 10.3 EPSP pmol/min/mg protein, respectively. Each value represents the mean \pm SD from triplicate analyses.

CP4-EPSP synthase in the GM-soybeans is glyphosate-resistant. To measure only the glyphosate-resistant EPSP synthase in the GM-soybeans, we incubated S-3-P,

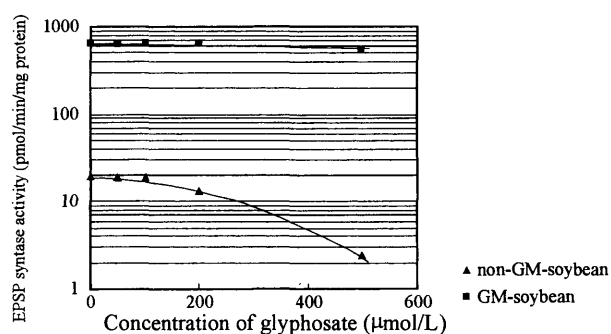


Fig. 6. EPSP synthase activity of GM- and non-GM-soybean extracts in the presence of glyphosate. EPSP synthase activity of GM- or non-GM-soybean crude extracts was assayed in the presence of 0–500 $\mu\text{mol/L}$ of glyphosate. EPSP produced by the reaction was measured by LC/MS analysis. The protein concentration of GM- and non-GM-soybean extract was 19 mg/mL. Each value represents the mean \pm SD from triplicate analyses.

PEP and the crude extracts of the GM-soybeans in the presence of glyphosate (0–500 $\mu\text{mol/L}$) and analyzed these using the LC/MS method as described above. The crude extract from non-GM-soybeans was examined in parallel. Figure 6 represents the EPSP synthase enzyme activity in the GM- and non-GM-soybean crude extracts for several concentrations of glyphosate. The EPSP synthase activity of the GM-soybeans was unchanged or only slightly reduced in the presence of 50–500 $\mu\text{mol/L}$ of glyphosate, whereas the EPSP synthase activity in the non-GM-soybeans was dose-dependently inhibited by the glyphosate. The concentration of glyphosate which caused 50% inhibition of the EPSP synthase activity of the non-GM-soybeans (IC_{50}) was 258 $\mu\text{mol/L}$, and 90% of the EPSP synthase activity was inhibited at 500 $\mu\text{mol/L}$ of glyphosate. The IC_{50} value obtained with our system was similar to that reported previously^(6), 8), 19). Therefore, the GM-soybeans show herbicide-resistant EPSP synthase activity.

Padgett *et al.*⁽¹⁾ reported a radio-HPLC assay for the determination of EPSP synthase activity using ^{14}C label. However, this poses a potential hazard. Moreover, Washizu also reported an HPLC method using an

amino-silica column for the determination of EPSP synthase⁸⁾. The method involved time-consuming steps because of many interfering peaks on the HPLC chromatogram. It also seems to be difficult to identify the EPSP peak reliably because of the rapid decline in the quality of the amino-silica column. Therefore, we used a reverse-phase C30 column to retain EPSP, because this should be more durable than the amino-silica column. The separation system of the HPLC was adapted to the LC/MS system for the qualitative and quantitative analysis of EPSP. The established LC/MS method for EPSP analysis proved to be more sensitive and precise than the HPLC method with UV detection. It provides a simple procedure for the determination of CP4-EPSP synthase activity or EPSP synthase activity by LC/MS without using any radioactive label.

Furthermore, we confirmed that the glyphosate-resistant enzymatic activity of CP4-EPSP synthase from enzyme reaction mixtures containing S-3-P and PEP could be detected using the LC/MS method in the presence of 500 $\mu\text{mol/L}$ glyphosate. This method should be applicable to the detection of other glyphosate-resistant EPSP synthase species, which have been introduced into various grains or rapeseeds.

Acknowledgments

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