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Production of Recombinant Proteins as Immuno-Analytical Markers of Genetically-Modified Organisms (GMO)

HWANG, OK-HWA[1], HYUK-GU PARK[1], EUI-HWAN PAEK[3], SE-HWAN PAEK[1,2], AND WON-MOK PARK[1]*

[1]*Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*Department of Biotechnology, Korea University, Jochiwon 339-700, Korea*
[3]*BioDigit Laboratories Corp., Technology Incubation Center, Biotechnology Building, Korea University, Seoul 136-701, Korea*

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Abstract Marker proteins of genetically-modified organisms (GMO) and their antibodies were prepared and characterized as major components of an analytical system. We selected two GMO markers, neomycin phosphotransferase II and 5-enolpyruvylshikimate-3-phosphate synthase, and produced them from *E. coli* employing genetic recombination technology. After purification, their structural conformation and binding affinities to the respective antibodies were characterized. The results showed that the recombinant proteins were identical with commercially obtained reference proteins. We further used them as immunogens to raise polyclonal antibodies capable of discriminating GMO containing protein from non-GMO. Well-characterized marker proteins and antibodies will be valuable as immunoreagents in constructing analytical systems such as biosensors and biochips to measure quantities of GMO.

Key words: GMO markers, gene cloning, recombinant proteins, mass production, antibodies, protein chip

Genetic recombination of plants has been successful in adding new characteristics needed in such areas as agriculture, fruit growing, and floriculture [9]. As an outstanding milestone in this field, a genetically-modified (GM) tomato {'Flavr savr' by Calgene, [7]}, showing an improved preservative effect, was developed in 1994 and cultivated for mass production two years later. Recently, researches in such direction, especially for an increased yield of agricultural plants, have been accelerated. Particularly, GM plants such as soybean, maize, rice, oilseed rape, and cotton with a tolerance to a herbicide have been produced mainly by US companies [12] and their safety has been approved in many nations.

*Corresponding author
Phone: 82-2-3290-3412; Fax: 82-2-927-2797;
E-mail: wkpark@korea.ac.kr

Although the needs of GMO production have been generally accepted, many concerns over their potential side effects on human health have also been evoked. Two effects at the genetic and protein levels are quite conceivable. Firstly, an unstable recombinant DNA may undergo mutation, and the GMO may pollinate with other plants, thereby possibly causing a genetic disorder of an ecosystem [20]. Secondly, under such presumed circumstances, the DNA can trigger a resting DNA to an active form or interfere with the action of a normal DNA. Either case might produce an unwanted protein having a direct effect on the human health [21]. As evidence for such possibilities, allergy was caused by recombinant protein in persons who ate GM soybean [14], and a resistance to antibiotics was developed by taking the recombinant plants with an antibiotic marker for their selection [11, 22].

Since it is very difficult to predict the eventual effects of GMO on the human health, particularly after a lapse of several generations, the route of food provision should be monitored. To this end, recombinant products used in food processing can traditionally be measured, using either enzyme immunoassays [10, 13] or real-time polymerase chain reaction (PCR) [1]. Immunoassays may be appropriate for simultaneously performing multiple analyses employing a commercially available kit based on microtiter plate, however, their uses are limited only to before food processing which could denature proteins. Although such restriction can be overcome by a real-time DNA analytical method after PCR, this requires a costly instrumental system. Nevertheless, these are all laboratory methods that cannot be used for instantaneous tests at fields, such as quarantine stations in port and food warehouses where specimens can be furnished.

We investigated immuno-analytical systems suitable for on-site determination of GMO: a biochip with spatially-arranged antibodies on a glass slide and biosensor based on immunochromatography. They will be devised to offer

quantitative results regarding the content of different GMOs in a sample as well as qualitative identification. In the present study, biological constituents, including GMO marker proteins and antibodies, essential for the systems were prepared. The marker proteins were produced by genetic recombination [16, 17] and characterized for their immunological properties. Subsequently, they were used to raise antisera capable of specifically recognizing each target protein.

For the above purpose, we selected two GMO markers, neomycin phosphotransferase II {NPTII, [7]} and 5-enolpyruvylshikimate-3-phosphate synthase {EPSPS, [23]}, for their mass production for the use as immunogens. NPTII is an enzyme allowing an organism to survive in the presence of antibiotics such as neomycin and kanamycin, which make it possible to select a recombinant organism coding the nptII gene [6]. EPSPS is also a protein furnishing resistance to a herbicide, glyphosate, to GMO. Since these are markers that are currently being used for detecting genetically-modified plants, we produced, characterized, and utilized them for raising antibodies as binders.

Materials and Methods

Materials

Lysozyme (from chicken egg white), phenylmethylsulfonyl fluoride (PMSF), Freund's Adjuvant, Coomassie brilliant blue G-250, casein (sodium salt), and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO, U.S.A.). Goat anti-rabbit antibody (IgG fraction)-alkaline phosphate (AP) conjugate and nitroblue tetrazolium/bromochloroindolylphosphate (NBT/BCIP) substrate were purchased from Promega (Madison, WI, U.S.A.), goat anti-rabbit antibody (IgG fraction)-horseradish peroxidase (HRP) conjugate was from Pierce (Rockford, IL, U.S.A.), antibody binding to hexa-histidine was from Santacruze (Santa Cruze, CA, U.S.A.), pET28a vector, nickel (Ni)-bound nitrilotriacetic acid (NTA) column, and thrombin (from human plasma) were from Novagen (Madison, WI, U.S.A.), skim milk (non-fat) was from Difco (Franklin lakes, NJ, U.S.A.), and GMO detection kits for EPSPS and NPTII were from Agdia (Elkhart, IN, U.S.A.).

Production of NPTII and EPSPS as Recombinant Proteins

Cloning. DNA fragments, expressing NPTII and EPSPS proteins, were extracted from genetically-modified tobacco and soybean leaves. Polymerase chain reaction (PCR) was used to amplify the genes coding the marker proteins. The primers initiating PCR comprised of the following sequences:
for nptII gene
5'-GAGAGGATCCATGATTGAACAAGATGGAT-3'
5'-GAAGAACTGCTCAAGAAGTTCGAAGAGA-3'
and for epsps genes
5'-GAGAGGATCCATGATTGAACAAGATGGAT-3'
5'-AGAGAAAGCTTGAAGAACTCGTCAAGAAGCC-GATAGAA-3'

PCR solution was prepared by adding 2.5 mM $MgCl_2$, 200 mM dNTPs, 0.25 U *Taq* polymerase, and 25 pmol each of primer into 6.7 mM Tris-HCl, pH 8.8, containing 16 mM $(NH_4)_2SO_4$ and 0.01% Tween-20 (1 × PCR buffer). After adding 1 ml of the target DNA (10 ng/ml) into the PCR solution (total 50 ml), PCR was started by incubating at 95°C for 2 min. The reaction, for nptII gene, was then carried out at 95°C for 30 sec, at 65°C for 40 sec, and at 72°C for 1 min; for epsps genes, at 94°C for 40 sec, at 57°C for 30 sec, and at 72°C for 30 sec. After repeating each cycle 30 times, they were finally reacted at 72°C for 10 min. The products were confirmed by electrophoresis on 1% agarose gel and subsequent staining with ethidium bromide (EtBr).

The DNA amplified fragments were subsequently ligated to pET28a vector using T4 DNA ligase at 16°C for 21 h. These recombinant DNA plasmids were separately transfered into *E. coli* strain BL21 [25]. The cells were spread onto Luria-Bertani (LB) agar plates containing 100 mg/ml kanamycin. They were then incubated at 37°C for 16 h, and grown bacterial colonies were transferred into 5 ml LB broth containing kanamycin and cultivated under the same conditions. Plasmid DNA within the cultured cells was isolated by the alkaline-lysis extraction method [2]. The prepared DNA was digested with restriction enzymes, including endonucleases, *Bam*HI, *Hin*dIII, and *Eco*RI. The resulting DNA fragments were separated by electrophoresis on 1% agarose gel and visualized under ultraviolet light after staining the gels in an EtBr solution.

A Large-Scale Protein Expression. To produce recombinant proteins in a high level, the genetically-modified *E. coli* cells were inoculated into 500 ml LB broth, and grown at 37°C for 2 h and subsequently at 15°C for 6 h. The cells were harvested by centrifugation and lysed by sonication in 10 mM Tris buffer, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 1 mM lysozyme, and 1 mM PMSF. Soluble proteins were separated from the cell debris by centrifugation, and the target protein fused with hexa-histidine by genetic recombination was purified on a Ni-NTA column [19] using a protocol provided by the manufacturer. The bound fractions, including the target protein, was eluted in the presence of imidazole and then dialyzed against 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS). The histidine tag was enzymatically cleaved from the purified protein by treating with 0.5 U/mg thrombin at 25°C for 8 h on a shaker [4]. The tag was removed from the mixture by the affinity chromatography on a Ni-NTA column. The target protein was finally dialyzed against PBS and quantified by the Bradford method [3].

Production of Polyclonal Antibody

Antibodies against the above prepared recombinant proteins were produced by immunizing a New Zealand White rabbit with the proteins. Immunization was initially carried out by an intramuscular injection of the antigen (1 mg/ml PBS) diluted with the same volume of Freund's Complete Adjuvant. After 10 days, two more injections of the antigen diluted with Freund's Incomplete Adjuvant were accomplished by the same manner at a 10-day interval. Blood was finally drawn from the vein of an ear of the rabbit after 10 days [24], and blood cells were sedimented overnight at 4°C. The serum was then separated and stored as aliquotes at - 20°C after snap freezing.

Characterization of Recombinant Proteins

Preparation of Intact Reference Proteins. Intact proteins, NPTII and EPSPS, as references were prepared by extraction from genetically-identified GMOs plants. For NPTII, tobacco (*N. tabacum* cv. Samsun nn) leaves (total 1 g) were ground in a pestle bowl, and 1 ml PBS containing 1 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF was added [17, 26, 8]. After mixing at 4°C for 1 h on a shaker, it was spun down at 15,000 rpm for 1 h. The supernatant was then collected, aliquoted, snap-frozen, and stored at - 20°C for subsequent analyses. For EPSPS, it was extracted from soybean (Roundup Ready, Monsanto) using the same procedure as that for NPTII, except for the use of a 5 times larger volume of the extraction buffer.

Western Blotting. The proteins were separated by SDS-PAGE on a 12% gel [25] and transferred onto a nitrocellulose membrane [5]. The membrane was blocked in 10 mM Tris buffer, pH 8.0, containing 5% non-fat dried milk and 0.002% Tween-20 (Blocking buffer). The proteins bound to the membrane were probed using polyclonal rabbit antibodies binding to either hexa-histidine or the target proteins. The binding complexes were detected using a conjugate of a goat anti-rabbit antibody and AP, diluted with Blocking buffer. The unbound material was washed with 20 mM Tris buffer containing 0.1 M NaCl and 0.01% Tween-20, and the antibody reactions were finally visualized using the NBT/BCIP substrate.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was performed to characterize the recombinant proteins using GMO detection kits purchased from commercial sources. All steps were performed according to the protocol provided by the manufacturer. Briefly, each protein was transferred to the wells for reaction, and the complexes were detected by a secondary antibody-HRP conjugate. Each reaction was performed at room temperature for 2 h. A substrate solution containing TMB was added to produce signals, a stopping solution was added after 15 min, and the color signals were measured by absorbance at 450 nm.

RESULTS AND DISCUSSION

Production of GMO-Marking Proteins

Genes coding the marker proteins were inserted into vectors, and each resulting recombinant DNA was then incorporated into *E. coli*. The proteins from such

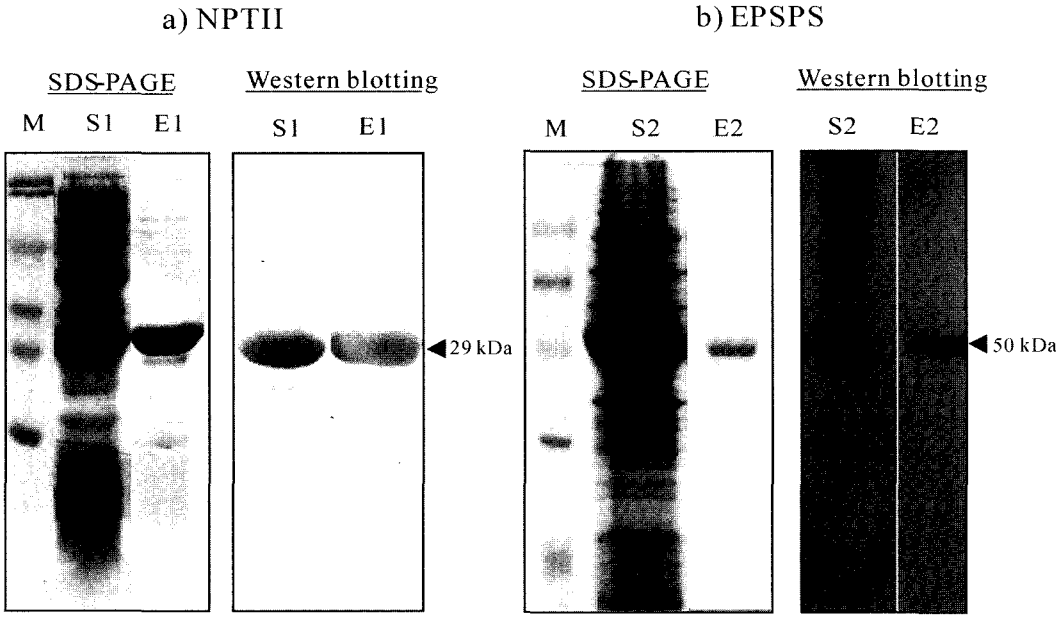

Fig. 1. PAGE analysis and Western blotting of the recombinant proteins, NPTII and EPSPS, before (S1, S2) and after (E1, E2) affinity purification.
M is a size marker with the range of 21 kDa to 96 kDa.

recombinant organisms were produced in large quantities by isopropyl-thiogalactopyranoside (IPTG) induction as soluble bodies in culture media. The products were subsequently purified by chromatography, using a Ni-NTA affinity column to histidine hexamer that was present on the protein molecules by genetic recombinant expression.

To identify the proteins produced in the culture, PAGE analysis and Western blotting with an antibody specific to the histidine hexamer were performed (Fig. 1). Although the recombinant *E. coli* contained a number of constitutive proteins (SDS-PAGE, S1 and S2 for NPTII and EPSPS, respectively), we were able to define each protein target, using the respective antibody, at designated sites of molecular weight; i.e., 29 kDa for NPTII and 50 kDa for EPSPS (Western blotting, S1 and S2). They were confirmed after the affinity purification (E1 and E2) which yielded each target in a high purity (higher than 95%, based on the area of protein bands stained).

Characterization

The recombinant proteins produced were examined as to whether they are present in proper molecular structures due to folding of each polypeptide chain. Structural conformation of a protein determined by a tertiary structure may be characteristic to a protein folding and be tested using a specific antibody [18].

Structural Conformation. Antibody recognizes the reaction partner, antigen, in an extremely specific binding mechanism, provided it was properly raised and selected. Such characteristics of an immune reaction allows us to identify a tertiary structure of protein folding by employing an antibody. Since antibodies specific to each target protein are commercially obtainable, we used them for the confirmation of structural arrangement of the polypeptide chains by comparing to native intact materials (Fig. 2). Reference NPTII and EPSPS, purchased from commercial sources, were analyzed by PAGE and Western blotting using the respective antibody (Figs. 2a, 2b Reference). PAGE revealed that both preparations contained a number of proteins as constituents of genetically-modified plants. As shown by the Western blotting, the antibodies specifically bound to each target protein located at regions of 29 kDa and 50 kDa. The same procedures were also applied for structural identification of the proteins produced by recombination (Figs. 2a, 2b Recombinant): They also reacted with each specific antibody, which indicated that the epitopes of each recombinant or structural conformations were identical with those intact.

Binding Affinity. To test protein folding, we measured binding affinity of the recombinant proteins as antigen to specific antibodies. To carry out the affinity test, enzyme immunoassay kits for NPTII and EPSPS were purchased from commercial sources. Dose-response curves to each standard analyte provided by the manufacturers were obtained and compared with those of the recombinants. Regardless of the sources of analytes, the patterns of dose-response curves for each recombinant protein were similar to the standards: the limit of detection for NPTII was approximately 10 ng/ml, while that of EPSPS was 10 μg/ml. The results indicated equal binding affinities of the

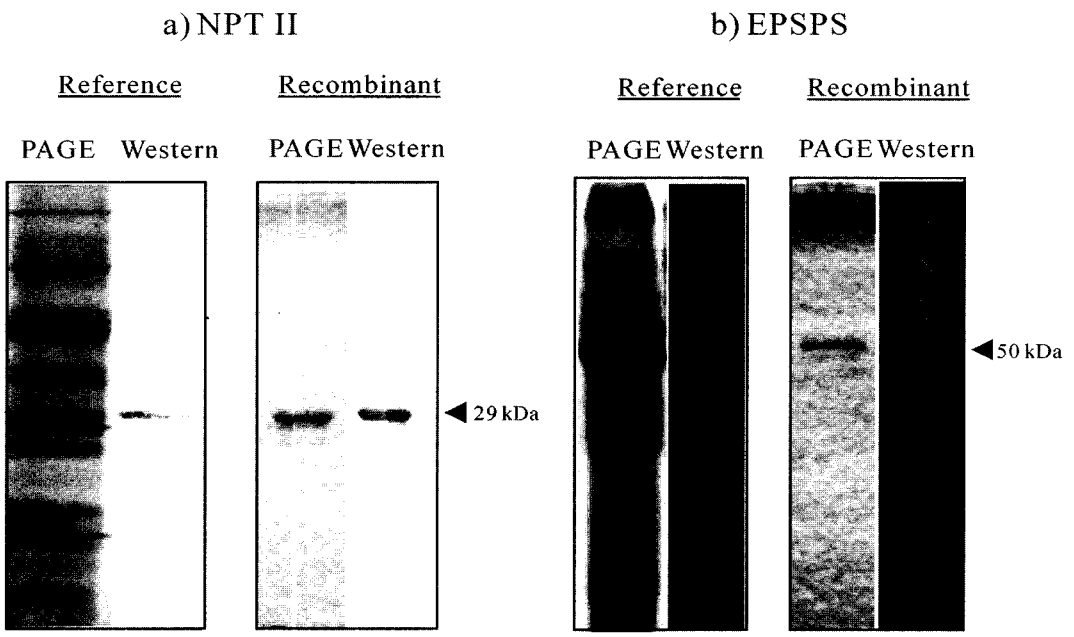

Fig. 2. Confirmation of structural folding status of recombinant proteins, employing antigen-antibody reactions.
The recombinant antigens of NPTII and EPSPS were produced from *E. coli*. The antigens for references were the proteins extracted from the GM plants; *N. tabacum* cv. Samsun nn for NPTII and Roundup Ready soybean for EPSPS. The antibodies to NPTII and EPSPS were commercially obtained.

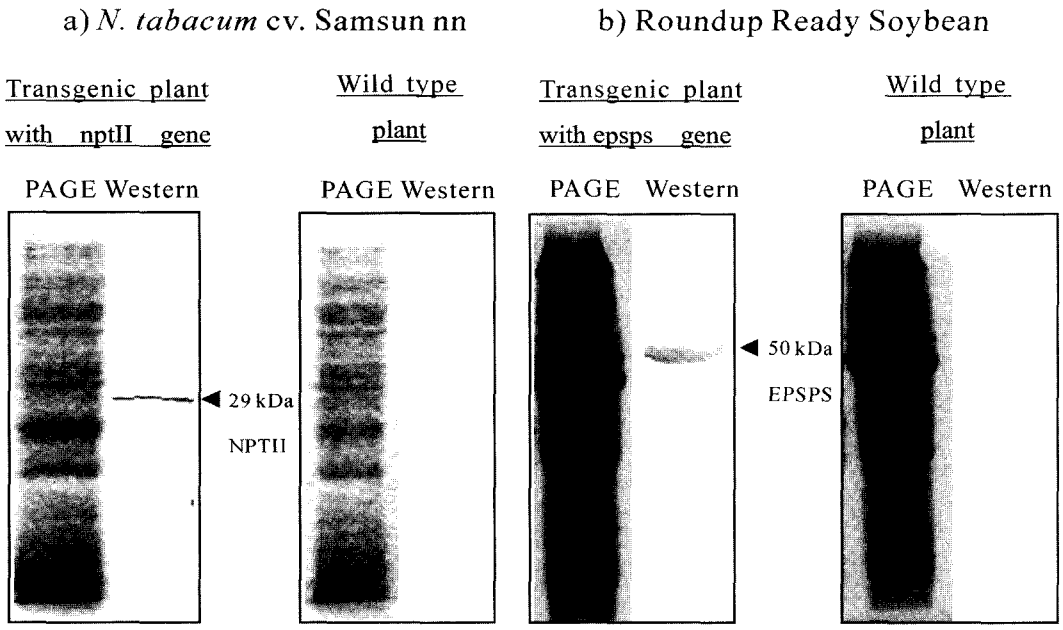

Fig. 3. Efficacy tests of antibodies, produced using the recombinant proteins, for specific detection of GMO.
To this end, two transgenic plant species retaining either nptII gene (a) or epsps gene (b) were selected and analyzed by PAGE and Western blotting using each specific antibody. These results were compared to those with wild-types of each species.

recombinants and standards to the antibodies (data not shown).

Induction of Specific Antibodies

Since the above described results showed that the present recombinant proteins were identical to those commercially available in terms of protein folding, we used them for immunizing animal (e.g., rabbit) for antibody production. Then, the antisera collected from the animals were tested for their abilities of discrimination between GMO and non-GMO within the species. To this end, the constitutive proteins contained in each species (*N. tabacum* cv. Samsun nn for NPTII and Roundup Ready soybean for EPSPS) were prepared in this laboratory and then analyzed by PAGE and Western blotting employing the antisera (Fig. 3). Although the protein profiles for the selected plant species were similar regardless of genetic modification (PAGE data for each species, Fig. 3), we were able to specifically detect transgenic plant, i.e., GMO, using the antiserum containing antibodies that reacted with the target marker (Western blot, Fig. 3).

In conclusion, we produced GMO marker proteins using a genetic recombination technology and subsequently used them as immunogens for the production of antibodies that selectively discriminated GMO from the non-modified in the same species. Such prepared immunoreagents would be valuable as essential constituents in constructing sensitive analytical systems for field tests without a laboratory. We are presently in a process of developing an enzyme immunosensor, based on membrane strip chromatography, suitable for a convenient, rapid determination of analyte concentration with minimum reagent handling. Another approach, particularly useful for the simultaneous detection of multiple GMO markers, could be an investigation of an immunosensor array, employing a solid substrate such as glass or silicon, which is also in progress in our laboratories.

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