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# Development of a chemiluminometric immunosensor array for on-site monitoring of genetically modified organisms

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ABSTRACT

Genetically modified organisms (GMOs) have been mainly developed for mass production of agricultural plants; however, there are concerns that transgenic crops might cause side effects on ecosystems and human beings. Therefore, to quantitatively trace the genetically modified products, we constructed a chemiluminometric immunosensor array for the detection of recombinant marker proteins expressed in GMOs, i.e., 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), neomycin phosphotransferase II (NPT II), and phophinothricin acethyltransferase (PAT). Monoclonal and polyclonal antibodies specific to each marker were raised, and the specificities and immunoreactivities to the respective markers were characterized. The capture antibodies were immobilized on predetermined regions of a glass slide where the sandwich-type immunoassays were carried out. Photodiodes were located on the bottom of the slide in an aligned arrangement to the immobilized antibody sites such that the light signals resulting from the immunoassays could be detected in situ. Under optimal conditions, the immunosensors were able to detect 1% GMO marked with EPSPS, which was the minimum content over the total content, and 3% GMOs labeled with NPT II or PAT. The sensor array developed in this study would be useful for measuring a particular GMO in a specimen containing unidentified species.



## 1. Introduction

After the advent of the commercialized genetically modified (GM) tomato in 1994, various GM organisms (GMOs) such as soybean, corn, rice, cotton, and rape have been developed using recombinant DNA technology [1]. This technology has allowed us to fabricate plants that are resistant to herbicides or directly to insect pests, which has in turn increased the harvest yields of agricultural plants. Genetic recombination can also allow us to functionally enhance plant foods in regards to quality, taste, smell, and size [2,3].

Despite such advantages, concerns that GMOs might accompany unknown side effects on ecosystems and human beings have been raised since they carry new genes that have not been exposed to nature [4–6]. Since unstable recombinant DNA may undergo mutation and then be pollinated with another plant, an ecosystem may be genetically disordered. Many critics have also pointed out that an expansion of GMO cultivation may cause the spread of more resistant 'superweeds' or 'superpests' [7]. Moreover, if DNA is ingested with foods, it might trigger the production of an unwanted protein that may have a direct effect on human health such as allergies [8,9]. It has also been reported that the body may become resistant to antibiotics by consuming recombinant plants that contain an antibiotic marker, which was used for their selection when manufactured [10]. Since it is very difficult to predict the eventual effects of GMOs, particularly, after a lapse of several generations, monitoring the route of GMO food products would be a minimal necessity [11]. Furthermore, since some GMO-producing manufacturers do not use a reliable labeling system for GMOs, it would be necessary to develop methods that would allow us to let consumers know if a food product is a GMO.

Recombinant products have been traditionally measured and detected using two different well-known analytical methods: enzyme-linked immunosorbent assay (ELISA) [12,13] and real-time polymerase chain reaction (PCR) [14–16]. Nevertheless, the use of these techniques has been limited to laboratory environments because these approaches require a long assay time, large-scale instruments, and expert knowledge. Moreover, unlike the immunoassay, the PCR-based method may not be appropriate for monitoring the quantity of target recombinant protein expression [17]. Recently, the lateral-flow immunoassay was introduced for the rapid detection of a protein indicative of GMOs in the field

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(e.g., quarantine station in port and food warehouse) where the specimen was furnished. This assay, however, is still insufficient for wide spread use due to its relatively inferior analytical performances, i.e., low detection capability and it can only provide qualitative yes-or-no results [18].

In this study, we develop a chemiluminometric immunoanalytical system that is able to not only quantitatively detect GMOs in unknown samples with high sensitivity, but is also portable. The system consists of a glass slide as a solid matrix for antibody immobilization and miniatured sensors (e.g., photodiodes) located on the bottom, which are attached through physical contact. This configuration allows us to efficiently transfer the light signal produced from the capture antibody sites to the sensors because of the minimal distance. In addition, this design produces a device that is highly compact in size. The glass surfaces are arranged in an array format by immobilizing different capture antibodies in spatially separated sites such that GMOs can be identified in specimens with an unknown source. As target analytes, we select two markers related to herbicide tolerance [19], 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and phosphinotricin acetyltransferase (PAT), and a recombinant protein furnishing antibiotic resistance [10], neomycin phosphotransferase II (NPT II).

## 2. Materials and methods

### 2.1. Materials

GM and wild-type plants (soybean, red pepper, and rice leaves) were kindly supplied by Rural Development Administration (Suwon, Korea). ELISA kits for NPT II, EPSPS and PAT were purchased from Agdia (Elkhart, IN) and Envirologix (Portland, ME), respectively. Glass slides (76 mm × 26 mm) were obtained from Marienfeld-Superior (Lauda-Konigshofen, Germany). Phenylmethanesulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), Triton X-100, polyethyleneimine (PEI), glutaraldehyde, glycerol, dithiothreitol (DTT), Sephadex G-25, dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP) and streptavidin were supplied by Calbiochem (San Diego, CA). Insoluble chromogenic substrates for HRP (3,3′,5,5′-tetramethylbenzidine, TMB) were supplied by Moss (Pasadena, MD). SuperSignal West Femto Maximum Sensitivity luminogenic substrate for HRP and anti-rabbit goat IgG conjugated to HRP, N-hydroxy succinimide (NHS)-LC-LC-biotin, succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC), and N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) were purchased from Pierce (Rockford, IL). Other reagents used in this research were of analytical grade.

### 2.2. Preparation of analytical components

#### 2.2.1. Production of antibodies

The three GMO markers, EPSPS, NPT II, and PAT, were produced as DNA recombinant proteins from microorganisms as described in the previous reports [4,20]. Monoclonal antibodies specific to NPT II or PAT were raised using a standard protocol described elsewhere [20] except for the use of recombinant proteins as immunogens. Polyclonal antibodies specific to each GMO marker protein were raised from rabbit as previously described [21]. Briefly, immunization was carried out by an intramuscular injection of the immunogen (500 μg) and the same amount of antigen was injected two additional times (i.e., boosting) at intervals of two weeks. Three days after the final injection of the immunogen, blood was drawn from the heart of the rabbit and the blood corpuscles were sedimented at 4 °C overnight. The serum was then affinity-purified on a protein G column (1 mL, HiTrap; Amersham Biosciences, Piscataway, NJ) and then stored as aliquots in a liquid nitrogen tank.

#### 2.2.2. Synthesis of labeled antibodies

The raised anti-EPSPS polyclonal antibody was coupled to HRP as the signal generator via chemical cross-linking. The antibody (400 μg) was first reacted with 20-fold molar excess of SPDP at room temperature for 1 h and then reduced with 10 mM DTT at 37 °C for 1 h. The excess reagents were removed on a Sephadex G-25 gel filtration column (10 mL volume). HRP was also activated with a 50-fold molar excess of SMCC at 4 °C for 4 h and the excess reagent was immediately removed via gel filtration. The modified antibody was then combined with a 5-fold molar excess of the activated HRP and the conjugation process was carried out at 4 °C overnight. The synthesized antibody–HRP conjugates were characterized and stored at 4 °C in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS), 1% BSA, and 0.05% thimerosal as a preservative.

#### 2.2.3. Characterization

The specificity and reactivity of the prepared antibody reagents were characterized using the sandwich ELISA [22]. Analytes were extracted by grinding each selected crop (total 1 g) in a pestle bowl, and PBS (1 mL) containing 1 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF was added. After mixing on a shaker at 4 °C for 1 h, 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. In the case of the EPSPS analysis, the polyclonal antibody (1 μg/mL; 100 μL) was coated as the capture binder on the inner surfaces of the microtiter plate wells and incubated at 37 °C for 1 h. The wells were then washed out three times using deionized water. The same incubation and washing procedures were conducted in the following steps unless stated otherwise. After antibody immobilization, the residual surfaces were treated with 100 mM Tris–HCl, pH 7.6, containing 0.5% casein and 0.1% Tween 20 (Tris–casein–Tween; 200 μL). The GMO and non-GMO (wild type) samples prepared via serial dilution (∞, 1:100, 1:25, and 1:10) in Tris–casein–Tween (100 μL) were added in the respective well. The detection antibody labeled with HRP (1 μg/mL) in the same medium was sequentially incubated in the wells. The colorimetric signal was generated from the enzyme by transferring a soluble substrate [13] containing TMB (200 μL) for 15 min. The reaction was stopped by adding 2 M sulfuric acid (50 μL) and then the signal was detected by measuring the absorbance at 450 nm using a microtiter plate reader (VERSAmax; Molecular Device, Chicago, IL).

For analysis of NPT II and PAT, the monoclonal antibodies (1 μg/mL) specific to each analyte were used as the capture binders and the polyclonal antibodies (1:1000 dilution) from rabbit were used as the detection binders. The analytical protocol was the same as that for EPSPS except that, prior to signal generation, a secondary antibody, i.e., anti-rabbit goat IgG, conjugated to HRP (1:5000 dilution; 100 μL) was reacted with the sandwich complexes.

### 2.3. Optimization of analytical conditions

#### 2.3.1. Chemical modification of glass

A glass slide was cleaned with a mixture of methanol and hydrochloric acid (1:1 ratio), and successively with concentrated sulfuric acid as described elsewhere [23]. After thorough washing with deionized water, the slide was dried under a stream of nitrogen gas and the surfaces were treated by immersion in 10 mM borate buffer, pH 7.0, containing 1% (w/v) PEI at room temperature for 4 h. The slide was washed again and then activated by incubating in a 2.5% glutaraldehyde solution under the same conditions. After thoroughly washing the slide, streptavidin (25 μg/mL) dissolved in