J. Microbiol. Biotechnol. (2007), **17**(4), 681–684

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Quantitative Analysis of Phosphinothricin-*N*-acetyltransferase in Genetically Modified Herbicide Tolerant Pepper by an Enzyme-Linked Immunosorbent Assay

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Received: September 19, 2006
Accepted: November 29, 2006

Abstract An immunoassay method was developed to quantitatively detect phosphinothricin-*N*-acetyltransferase (PAT) encoded by the Bialaphos resistance (*bar*) gene in genetically modified (GM) pepper. The histidine-tagged PAT was overexpressed in *Escherichia coli* M15 (pQE31-*bar*) and efficiently purified by Ni^{2+} affinity chromatography. A developed sandwich enzyme-linked immunosorbent assay (S-ELISA) method (detection limit: 0.01 μg/ml) was 100-fold more sensitive than a competitive indirect ELISA (CI-ELISA) method or Western blot analysis in detecting the recombinant PAT. In real sample tests, PAT in genetically modified herbicide-tolerant (GMHT) peppers was successfully quantified [4.9± 0.4 μg/g of sample (n=6)] by the S-ELISA method. The S-ELISA method developed here could be applied to other GMHT crops and vegetables producing PAT.

Keywords: Phosphinothricin-*N*-acetyltransferase, *bar* gene, GM pepper, sandwich ELISA

Over the years, the genetically modified (GM) crops developed by recombinant DNA techniques have increased, and they are increasingly permitted to be traded and consumed throughout the world [2]. Currently, PCR methods including competitive PCR, multiplex PCR, and real-time PCR have been most widely developed for the detection of transgenic DNA regions from GMOs [1, 3, 4, 11]. Recently, for the visual detection and sequence confirmation of GMO, a nanobiotechnological method using the first dry-reagent DNA biosensor in a disposable dipstick format, by hybridization within minutes, and application of 2-DE for protein-based detection and quantification of CP4EPSPS in GM soybeans has been reported [3, 5]. However, immunoassay methods for the detection of GMOs have rarely been developed, in spite of their economical benefit and high sensitivity [1]. Several papers on the immunoassay methods for the Bt Cry1Ab protein in the MON810 maize line, the CP4EPSPS protein in Roundup Ready soybeans, and the neomycin phosphotransferase II enzyme (npt II) in some plants have been published [9, 10, 14]. Genetically modified herbicide-tolerant (GMHT) red pepper was recently developed by the Rural Development Administration (RDA) and, based upon substantial equivalency, its safety has been assessed by analyzing the nutrients composition [7]. Furthermore, qualitative and quantitative analyses of recombinant DNA from GMHT were conducted by PCR, using the construct-specific primer pairs and amplifying the junction region of the *bar* gene [13]. In this study, a sensitive sandwich ELISA (S-ELISA) method was developed to detect PAT from GMHT pepper as a paradigm GM plant and the sensitivity was compared with those of other immunoassay methods.

To construct a recombinant expression plasmid, a 0.6-kb *bar* fragment digested from pMOG6-*bar* [8] was inserted into the SmaI site of the pQE31 expression vector (Fig. 1A). Plasmid pQE31-*bar* containing the *bar* gene was identified by sequence analysis for the SmaI junction site. *E. coli* M15 host strain containing both the expression (pQE31-*bar*) and the repressor (pREP4) plasmids can be used for the production of recombinant proteins. This plasmid pQE31-*bar* has a six-histidine tag at the amino-terminus of the recombinant protein for Ni-NTA-affinity chromatography.

This recombinant his-tagged PAT was overexpressed in *E. coli* M15 transformed by a chimeric DNA, pQE31-*bar*,

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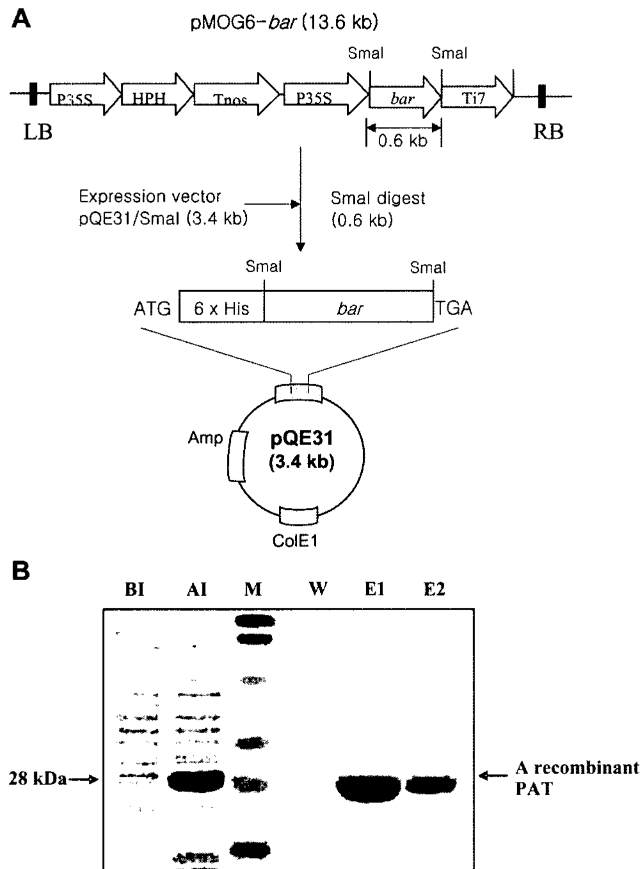

Fig. 1. **A.** Schematic diagram of the construction of pQE31-*bar*. LB, left border; RB, right border; P35S, CaMV 35S promoter; HPH, hygromycin phosphotransferase; Tnos: 3' signal of nopaline synthase; Ti7: terminator; 6×His: 6×His affinity taq. **B.** Purification of a recombinant PAT from an *E. coli* cell lysate.
The cell lysate was prepared under native condition and PAT protein was purified by Ni-NTA metal-affinity chromatography (Qiagen Co., Hilden, Germany) according to the manufacturer's instructions. M, broad-range SDS-PAGE standards (Bio-Rad Co., Hercules, CA, U.S.A.); BI, cell lysate before IPTG induction; AI, cell lysate after induction; W, wash; E1, E2, eluents.

and purified by Ni-NTA metal-affinity chromatography. Briefly, overnight culture of the recombinant *E. coli* M15 cells was subcultured in 100 ml of LB broth, containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml), until 0.6 of OD_{600} without induction, and the PAT was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated for an additional 5 h, and the cells were harvested by centrifugation at 4,000 ×*g* for 20 min. The cell pellets were stored overnight at -20°C, thawed on ice, and resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). One mg/ml of lysozyme (Sigma, St. Louis, MO, U.S.A.) was added to the suspension and left on ice for 30 min. After sonication and centrifugation, 1 ml of the 50% Ni-NTA slurry was added to 4 ml of the cleared lysate, and they were mixed gently by shaking at 4°C for 1 h. The lysate-Ni-NTA resin mixture was loaded onto a column and washed twice with 4 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the target protein was eluted 4 times with 0.5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions containing the PAT were pooled, dialyzed against pure water, freeze-dried, and stored at -20°C until use. The concentration of the purified PAT was measured by a Bio-Rad Protein Assay Kit (Hercules, CA, U.S.A.) and the purity was confirmed by SDS-PAGE.

As shown in Fig. 1B, a recombinant PAT (~28 kDa) was successfully overexpressed as a soluble form in the *E. coli* cytoplasm and the PAT was purified (20 mg from 100 ml of culture) in a large quantity, with a high purity (see lanes E1, E2), indicating that this system for the production of a recombinant PAT is well-designed.

For the production of polyclonal antibodies (pAbs) against the purified recombinant PAT, 1 ml of the PAT, which was adjusted to 1.0 mg/ml in 0.01 M phosphate-buffered saline (PBS) and mixed with Freund's complete adjuvant (1:1, v/v), was intradermally injected into New Zealand White rabbits (20 weeks old). Boosting injections with Freund's incomplete adjuvant were performed 4 times at 2-week intervals. Blood samples were collected 1 week after boosting injections, and antisera were obtained by standing on a 4°C chamber and centrifuging at 3,000 ×*g* for 20 min. The purification of immunoglobulin G (IgG) typed antibodies was performed by a T-Gel purification kit and a gel filtration chromatography (Sephadex G-25, Sigma, St. Louis, MO, U.S.A.). The eluted IgG fractions were pooled and desalted with a Sephadex G-25 column (250×15 mm, Sigma, MO, U.S.A.) equilibrated with PBS. The buffer for the IgG was changed to 0.2 M sodium carbonate-bicarbonate buffer (pH 9.4) to conjugate the antibodies with horseradish peroxidase (HRP). The pAb-HRP conjugates were purified by a gel (Sephadex G-25, Sigma, MO, U.S.A.) filtration chromatography and stored at 4°C until use.

In the production of pAbs from the immunized rabbits, there were no significant changes in pAb titers of rabbits after the 1st immunization, whereas the titers increased remarkably from the 2nd immunization. After the 5th immunization, one of the rabbits showed the highest titer (antiserum diluted 15,000 times with PBST) level of pAb and the serum of the rabbit was used for HRP conjugates (data not shown). The pAb from the 5th antiserum was purified through a T-Gel column, and was found to be an IgG antibody (data not shown).

To extract a recombinant protein (PAT) from the samples, the GMHT and non-GMHT peppers were ground to a fine powder under liquid nitrogen using a mortar and pestle. A portion (0.5–1.0 g) of ground powder was transferred to 2.0-ml microtubes and resuspended in 5.0–10 ml of cold

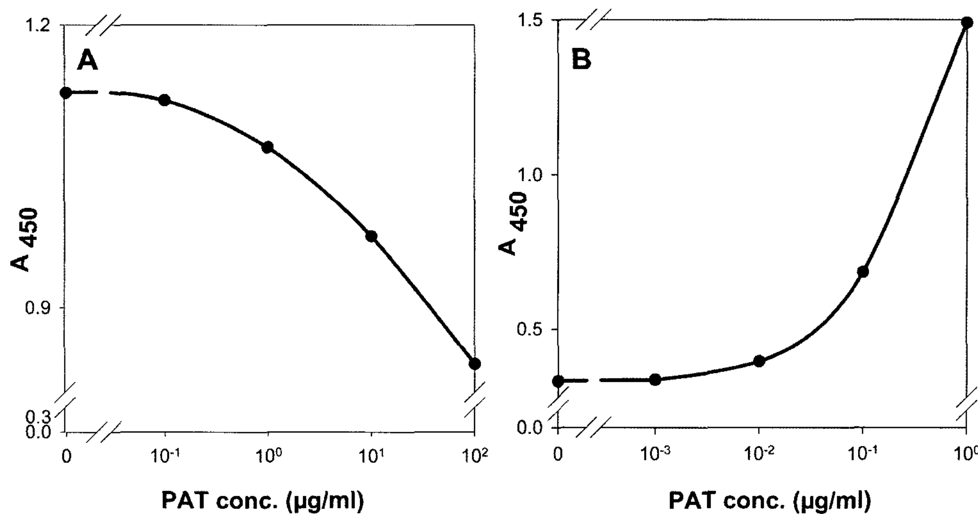

Fig. 2. Standard curves for determination of the concentration of PAT by ELISA methods.
A. Competitive indirect ELISA. **B.** Sandwich ELISA.

acetone. After vortexing for 30 s, the suspension was centrifuged (10,000 ×*g*, 3 min, 4°C); and the resulting pellet was washed once with acetone and rinsed 3 times with cold 10% (w/v) trichloroacetic acid (TCA) in acetone or until the supernatant was colourless. After centrifugation, the pellets were washed sequentially, twice with 10% (w/v) TCA in acetone, once with 10% (w/v) TCA, and finally, twice with 80% (v/v) acetone. This pellet was air-dried, and the dry powder was resuspended completely by vortexing and then centrifuged as above. The final pellet was dried at room temperature and stored at 80°C. Protein was quantified by the Bio-Rad Protein Assay Kit (Hercules, CA, U.S.A.) with BSA as standard. In order to cross-check the presence of a recombinant protein in GM peppers, flow lateral strip (Strategic Diagnostics Inc., Newark, DE, U.S.A.) tests were simultaneously performed. In the case of S-ELISA, 1.0 g of pepper was mixed with 9 ml of PBST and homogenized by a homogenizer at 11,000 ×*g* for 5 min. One ml of the extract was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was diluted 10^2–10^3 folds with PBST for the quantification of PAT by an S-ELISA method. For Western-blot analysis, the proteins extracted from peppers were resolved on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane by a Mini Trans-Blot Module (Bio-Rad Co., Hercules, CA, U.S.A.). The blot was exposed to the pAb-HRP conjugates, and the bound conjugates were detected by ECL Western Blotting Detection Reagents (Amersham Biosciences Co., Piscataway, NJ, U.S.A.).

As shown in Figs. 2A and 2B, the standard curves show the detection limit and ranges of the competitive indirect method and sandwich method using the pAb: the detection limit by S-ELISA was 0.01 μg/ml, whereas the CI-ELISA was 1.0 μg/ml. In the review by Kwak *et al.* [6] and Shim *et al.* [12], similar detection limits have been reported by using a pAb and mAb.

In the present study, pAb was also used to determine the IgG-binding protein bands using Western blot analysis (Figs. 3A, 3B). Upon visualizing the IgG-binding protein bands at the level of 7.50, 3.75, 1.88, 0.94, and 0.48 μg, the recombinant PAT from cultivated *E. coli* M15 was detected at the levels of 0.94 μg, being about 100 times less sensitive than S-ELISA. Although no protein band was visible on SDS-PAGE, the IgG-binding proteins derived from the extracts of GMHT pepper were clearly detected at 28 kDa. As shown in Fig. 3, PAT in GMHT peppers was detectable at the levels of 3.5 μg and 7.5 μg when the PAT-HRP conjugates reacted with the proteins on the blot.

To evaluate the feasibility of the S-ELISA method developed, real sample tests were performed using six groups of non-GMHT and GMHT peppers. The result of S-ELISAs showed that PAT was detected in all GMHT

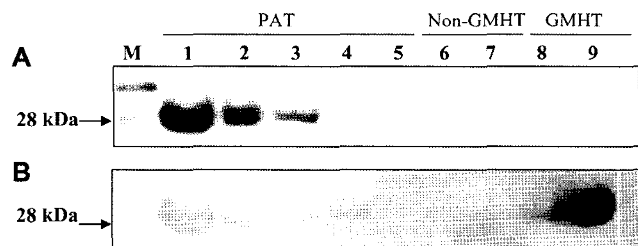

Fig. 3. Detection of PAT by Western blot analysis.
Blotted membrane was exposed to anti-PAT polyclonal antibody-HRP conjugates and visualized by an ECL kit. **A.** SDS-PAGE. **B.** Western blot: M, broad-range SDS-PAGE standards; lane 1, loading of 7.50 μg of purified recombinant PAT protein; lane 2, 3.75 μg ; lane 3, 1.88 μg; lane 4. 0.94 μg; lane 5, 0.48 μg; lane 6; 3.5 μg of PAT purified from non-GMHT pepper; lane 7, 7.5 μg; lane 8; 3.5 μg from GMHT pepper; lane 9; 7.5 μg.

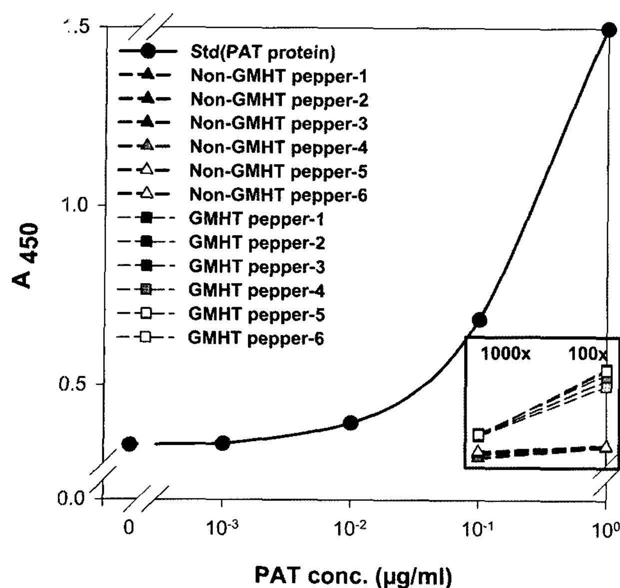

Fig. 4. Detection of PAT from genetically modified herbicide-tolerant peppers by a sandwich ELISA.

Peppers were extracted with PBST buffer (see Materials and Methods), and diluents (1:100 and 1:1,000) were used for the sandwich ELISA.

peppers (100 times and 1,000 times diluted) at the level of 4.9±0.4 μg/g of pepper and 3.6±0.7 μg/g (n=6) (data not shown), respectively, which was confirmed by the strip tests (data not shown). These results indicated that combination of the capture antibody (anti-PAT pAb) and the detecting Ab (anti-PAT-HRP) was most sensitive, suggesting that the S-ELISA method developed in this study could efficiently be applied to detect other PAT-producing GMHT plants.

From these results, it is clear that the detection limits of S-ELISA and CI-ELISA were 0.01 μg/ml and 1.0 μg/ml, respectively, indicating that S-ELISA was more sensitive than CI-ELISA. Furthermore, the present result is in good agreement with the detection limit (0.01 μg/ml) of CP4EPSPS protein derived from glyphosate-tolerant soybean against monoclonal antibody [6]. Therefore, the above results suggest that S-ELISA using the pAb produced could be applied to quantify the recombinant proteins in genetically modified (GM) crops and vegetables. Furthermore, because the ELISA methods have several benefits such as low cost and suitability for field test when compared with DNA-based methods such as PCR [1], these methods could be useful to quantify targeted proteins in various agri-products and could also be used as initial screening tools along with more accurate methods.

Acknowledgment

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration (RDA), Korea.

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