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Application of Immunoaffinity Column as Cleanup Tool for an Enzyme Linked Immunosorbent Assay of Phosphinothricin-*N*-acetyltransferase Detection in Genetically Modified Maize and Rape

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We have developed a new immunoassay method to detect genetically modified (GM) maize and rape containing phosphinothricin-*N*-acetyltransferase (PAT). PAT encoded by Bialaphos resistance gene (*bar*) was highly expressed in soluble form in *Escherichia coli* BL21(DE3) and purified to homogeneity by Ni^{2+} affinity chromatography. A simple and efficient extraction and purification procedure of PAT from GM maize and rape was developed by means of the immunoaffinity column (IAC) as a cleanup tool. Purified polyclonal antibodies against PAT was produced and coupled covalently to CNBr-activated Sepharose 4B. Both the binding conditions and elution protocols were optimized. The IAC was successfully employed to isolate and purify the PAT from the various tissues of GM maize (Bt11 and Bt176) and rapes (MS1/RF1 and MS8/RF3). Enzyme linked immunosorbent assay (ELISA) procedures were established further on to measure the PAT protein. GM maize cannot be differentiated from non-GM maize by ELISA. But IAC-ELISA allowed 0.5% GMOs to be detected in MS1/RF1 and MS8/RF3 and 10% GMOs to be detected in Bt11 and Bt176, which makes this method an acceptable method to access PAT protein in GM rapes and maize.

KEYWORDS: Purification; PAT; GMO; detection; immunoaffinity column; enzyme linked immunosorbent assay

INTRODUCTION

Phosphinothricin-*N*-acetyltransferase (PAT) is an enzyme that inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. It was encoded by both the *pat* gene and the *bar* gene isolated from *Streptomomyces hygroscopicus* and *Streptomomyces viridochromogenes* (*1*). The *pat* gene is a homologue of the *bar* gene, and both had been evaluated as selectable markers in transgenic plants. The herbicide-resistant trait using *bar* and *pat* genes encoding PAT had been produced in several crops such as tobacco and were approved worldwide for cultivation and consumption (*2*−*7*). Bt maize and MS rapes were researched in this paper because they have *bar* or *pat* genes. As we known, MS rapes stand out among GM rapes, as Bt maize is the prominent GM maize and Roundup Ready soybean is essential to GM soybeans. However, since genetically modified organisms (GMOs) have entered the food chain, the scientific and public debate concerning their safety and the need for labeling information arose specially in the European Union. In the light of this, many countries and international organizations have been enforcing new labeling systems providing product information for the general public.

Methods proposed for GMO labeling are based on detection of either transgene(s) or their protein product(s). A variety of PCR-based methods (*8*, *9*) have been routinely used in most analytical laboratories to detect GMOs, including nestle PCR (*10*), competitive PCR (*11*), multiplex PCR (*12*), and PCR−ELISA techniques (*13*). Application of real-time PCR (*14*) has raised the possibility of more accurate quantification of GMOs. At the same time, immunoassay technologies are ideal for qualitative and quantitative detection of many types of proteins in complex matrixes. The assays on raw materials could be performed with immunological assay such as ELISA based on the specific binding between proteins and antibodies. Both monoclonal antibodies with high specificity and polyclonal antibodies with high sensitivity can be used depending on the amounts needed and the specificity of the detection system. The immunoassay methods were hardly reported for detection and quantitation of proteins expressed by most biotechnology-derived crops with the exception of the Bt Cry1Ab protein in MON810 corn (*15*), the CP4-EPSPS protein in Roundup Ready soybeans (*16*), and the neomycin phosphotransferase II enzyme (*npt* II) in some plant species (*17*).

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In this paper, we reported a new immunoassay method to detect GMOs containing PAT. Purified PAT was obtained using a recombinant expressed system based on the pET30a(+) vector and BL21(DE3) system. Polyclonal antibodies against PAT were produced and purified. A new sample pretreatment method based on an immunoaffinity column (IAC) for the plant samples was developed to clean up and enrich PAT derived from transgenic plants. The IAC showed specific affinity for PAT due to the high selectivity of the immunoaffinity reaction. Both the binding conditions and elution protocols were optimized. ELISA is a very rapid, specific, and economical means for determining antigens and haptens. This means has been developed into many kinds of new methods by fusing with other technologies. Four kinds of GMOs were detected efficiently by IAC−ELISA. This new method proved to be a simple, practical, and reliable sample pretreatment method for trace samples of GMOs.

MATERIALS AND METHODS

Reagents and Instruments. CNBr-activated Sepharose 4B was obtained from Pharmacia Biotech. pET30a(+), Ni−NTA His·Bind resins and His·Tag monoclonal antibody were purchased from Novagen. Peroxidase-conjugated goat anti-mouse IgG secondary antibody and protein A−Sepharose 4B were purchased from Sigma. Plasmid P3301 containing the *bar* gene was provided by Prof. Guoying Wang from China Agricultural University. Microplate Reader MK3 was from Finland LABSYSTEMS. All the solvents used were of analytical grade or better.

Materials. Bt11, Bt176, and non-GM maize flours were from Bayer Co., MS1/RF1, MS8/RF3 and non-GM rape seeds were from Syngenta Co.

Construction of Recombinant Plasmid pET30a-*bar* Containing the *bar* Gene. The *bar* gene (GenBank Accession No. X05822) was recovered from plasmid p3301 by PCR reaction. Two oligonucleotide primers were designed (5′CGG AAT TCA TGA GCC CAG AAC GAC GC3′ and 5′CCC AAG CTT ATC AAA TCT CGG TGA CGG GCA GG3′) which defined the sequence of *bar* gene and introduced *Eco*R I and *Hin*d III at the 5′ and 3′ end of the PCR product, respectively. The PCR product was digested with *Eco*R I and *Hin*d III and cloned into pET30a(+), resulting in pET30a-*bar*. PCR conditions were as follows: preincubation at 95 °C for 5 min, 40 cycles consisting of denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The sequence of the *bar* gene was verified by DNA sequencing (Shanghai Sangon Co. Ltd). Its encoding amino acid sequence was compared with the sequence reported by GeneBank.

Expression and Purification of PAT. To prevent protein degradation, cells and protein solutions were kept at 0−4 °C at all times. Expressions of PAT fusion proteins were done in 100−400 mL cultures of Luria-Bertain (LB) and incubated at 37 °C until an OD (600 nm) of 0.6 with 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 30 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 500 mM NaCl). Afterward they were treated with lysozyme (2 KU of lysozyme per gram cell paste) and sonication (40−50% duty for 15 bursts). The soluble protein fraction was recovered by centrifugation. The purification of PAT was performed by Ni−NTA metal-affinity chromatography. His_6 fusion proteins were isolated by binding to a Ni−NTA-agarose column preequilibrated with 20 mL of Ni−NTA bind buffer (50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole pH 8.5). The soluble protein fraction was allowed to bind to the column for 1 h at 4 °C, washed with 30 mL of Ni−NTA washing buffer (50 mM $NaHPO_4$, 300 mM NaCl, 20 mM imidazole pH 8.5) at 4 °C, and then eluted with 20 mL of Ni−NTA elution buffer (50 mM $NaHPO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.5). The fusion protein was incubated with an enterokinase (EK) for 18 h at 10 °C to cut out the His_6·Tag and at the same time dialyzed against Ni−NTA binding buffer to remove imidazole. Then the dialyzed liquids were again rebound to a Ni−NTA-agarose column to remove any remaining uncut protein. Purified PAT was acquired by collecting the eluate in a fresh tube, while His_6·Tag remained bound to the gel.

Identification of the Recombinant PAT Protein. The purified PAT was identified by SDS−PAGE, Western blot, and enzymic activity analysis. Samples containing purified PAT fusion protein were subjected to 12% SDS−PAGE and were transferred to a 0.45 μm nitrocellulose membrane (*18*). Protein concentrations were measured spectrophotometrically by using the Bradford assay (*19*). The emzymic activity was assayed by using an acetyl-coenzyme A to *N*-acetylate PPT method described by Thompson et al. (*1*). The reaction was carried out at 22 °C in a mix that contained Tris-HCl (50 mM, pH 7.5), 0.4 mg mL^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and acetyl-coenzyme A (0.1 mg mL^{-1}). After establishing a baseline rate of DTNB reduction, the reaction started by the addition of substrate (0.2 mM PPT). The rate of substrate-specific increase in absorption at 412 nm was divided by 13.6 to give the activity in micromoles of substrate acetylated per minute, and one unit of PAT is defined as 1 μmol of acetylated PPT min^{-1} mg^{-1} of protein at 37 °C.

Production and Purification of Polyclonal Antibodies. The polyclonal antibody against purified PAT protein was obtained from New Zealand White rabbits. In brief, the PAT dissolved in 0.9% NaCl was emulsified with an equal volume of Freund's complete adjuvant to give a final concentration of 1 mg mL^{-1}. This mixture was given in four intradermic injections. Boosting injections of Freund's incomplete adjuvant were made at 2-week intervals. Blood samples were collected 1-week after the third booster injections and tested for titer determination. Blood collected from rabbits was first allowed to stand overnight at 4 °C and then centrifuged at 200*g* for 15 min.

The obtained antiserum was purified in two steps by first using a saturated ammonium sulfate method (*20*). The 20 mL of antiserum was purified by the saturated ammonium sulfate method. At last the precipitates were dissolved in 2 mL of PBS and dialyzed against PBS until no sulfate ion could be detected in the dialysis solution with 0.5 M $BaCl_2$ acidified with HCl. The obtained immunoglobulin (IgG) was purified in the second step by way of protein A−Sepharose 4B (*21*). The eluates (0.1 M glycine buffer pH 7.2, 0.5 mL) were collected in tubes containing 50 mL of 1 M Tris (pH 8.0) and mixed gently to bring the pH back to neutral. The IgG concentration in the obtained solution was calculated on the basis of the UV absorption difference between 280 and 260 nm (*22*). The formula used for the calculation was $C_{protein}$ (mg mL^{-1}) = $1.45A_{280nm} - 0.74A_{260nm}$. The purity of the purified IgG was checked on SDS−PAGE gel.

Antiserum Sensitivity and Specificity. The sensitivity and specificity of the antiserum were tested by ELISA. The sensitivity (least detectable dose) for the ELISA was estimated with 2-fold dilution of the purified PAT in carbonate buffer (pH 9.6) from 0.01 to 0.01 × 2^{-11} mg mL^{-1}.

The specificity (cross-reactivity) was assessed by performing ELISA using two kinds of potential interferents with structures relating to PAT from plant sources: phosphotransacetylase and chloramphenicol acetyltransferase. They were diluted to 0.1 mg mL^{-1} in carbonate buffer (pH 9.6) for ELISA.

Coupling Antibody to CNBr-Activated Sepharose (*23*). Purified antibodies against PAT were coupled to CNBr-activated Sepharose to generate the immunoaffinity column (IAC). The IgG solution was dialyzed against 0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, 0.2 M NaCl (pH 8.3) and was added to the Sepharose beads activated with 1 L of 1 mM HCl under constant rotation at 4 °C for overnight at a concentration of 2 mg mL^{-1} (IgG/Sepharose beads). Then the supernatant was removed and the beads were incubated with 1 M ethanolamine pH 9.0 to deactivate the remaining active sites for 2 h under constant rotation at 4 °C. Beads were washed with 0.1 M acetate (pH 4.0), 0.5 M NaCl and then with bead buffer (PBS pH 7.4) and stored in 20% suspension in bead buffer at 4 °C until further use.

Negative rabbit antiserum containing no antibody against PAT was applied to generate the blank column as a negative control.

Determination of the Optimal Eluting Conditions of PAT on the Immunoaffinity Column. To select the optimal pH condition for elution, a 40 μL solution of a mixture of antibody (against PAT)−Sepharose beads was washed with 2 mL of bead buffer. The matrix was incubated with 1 mL of purified PAT (1 mg mL^{-1}) and 1 mL of BSA (1 mg mL^{-1}) for 40 min under constant rotation at 4 °C. After washing seven times with the bead buffer (500 μL), the column was

eluted with 0.2 M glycine buffer (400 μL) under various pH conditions (pH 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5). The collected fractions were assayed by ELISA.

To further investigate the optimal time for elution, 320 μL of a mixture of antibody (against PAT)−Sepharose beads was washed with 8 mL of bead buffer, divided into eight equal shares, and incubated separately with the extract of 1 g of powders from GM maize (Bt11 and Bt176), 1 g of leaves from GM rape (MS1/RF1 and MS8/RF3), and their corresponding non-GM parents for 40 min under constant rotation at 4 °C. After washing nine times with bead buffer (500 μL), the column was eluted with 0.2 M glycine buffer (400 μL, pH 2.5) three times. The collected fractions were assayed by ELISA.

Sample Preparation from GM Plant Tissues. Lyophilized leaves of MS1/RF1 and MS3/RF8 and seeds of Bt11 and Bt176 were ground in a Retch ball-mill and sonicated on ice by using a microtip at 40−50% duty for 15−20 bursts. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins. Approximately 1.0 g of grounded tissue was extracted twice with 5 mL of PBS/0.01 M EDTA (pH 7.5) at room temperature and then the mixture was centrifuged for 30 min at 200*g* twice to remove insoluble debris. The supernatant was passed through a Whatman 3 MM filer twice. The clarified extracts were maintained on ice for short-term storage or frozen at −20 °C for further ELISA or Western blot experiments.

Application of Immunoaffinity Column Cleanup to GM Plants Samples. Forty microliters of antibody (against PAT)−Sepharose beads were washed with 1 mL of bead buffer (PBS pH 7.4). The matrix was incubated with 2 mL of PAT extracts in one tube (10 mL) for 30 min under constant rotation at 4 °C. Beads were washed nine times with 500 μL bead buffer followed by three washes with 400 μL of eluting buffer (0.2 M glycine pH 2.5). The pH of the eluting supernatants was adjusted to 7.5 with 0.1 M NaOH, and then the washing supernatants and the eluting supernatants were assayed for PAT by ELISA.

Detection of GM Plants by ELISA. Two transgenic maize (Bt11 and Bt176) and two transgenic rapes (MS1/RF1 and MS8/RF3) were applied to evaluate detection of the presence of PAT, while their non-GM parents were treated as negative controls.

For the ELISA assay, all the subsequent steps were performed in a volume of 0.4 mL of PAT extract from Bt11, Bt176, MS1/RF1, MS3/RF8, and their non-GM parents for ELISA (if not stated otherwise). PAT extracts (0.4 mL) were diluted in 0.4 mL of carbonate buffer (pH 9.6) and absorbed to the wells of ELISA plates overnight at 4 °C. The plates were blocked with 0.1 mL/well of blocking buffer (5% nonfat dried milk power in PBS) for 1 h at 37 °C. Wells were washed with PBST (PBS/0.05% Tween-20) four times, the purified antibody against PAT was diluted 1:1000 in incubation buffer (PBS/0.5% BSA/0.05% Tween-20) and added into each well, and the plates were incubated for 45 min at 37 °C. Wells were washed with PBST four times, alkaline phosphatase-conjugated streptavidin (1:1000 dilution) was added to each well, and the plates were incubated at 37 °C for 45 min. After washing with PBST, 1 mg mL^{-1} *p*-nitrophenyl phosphate dissolved in substrate buffer (10% diethanolamine, pH 9.8, containing 0.5 mM MgCl2) was added to each well. Absorbance at 405 nm was measured by a microplate reader.

To determine the detection limits of ELISA for the GM plants, different proportions from Bt11, Bt176, MS1/RF1, and MS8/RF3 (100%, 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0%) were applied for the extractions of PAT, immunoaffinity column cleanup, and ELISA analysis. Each proportion of sample was assayed for 10 times to assay its reproducibility.

RESULTS AND DISCUSSION

Construction of Recombinant Expressed Plasmid pET30a-*bar* Vectors. The *bar* gene (GenBank Accession No. X05822) was amplified by PCR from the plasmid p3301 and correctly inserted into recombinant expressed plasmid pET30(+) vectors at the *Eco*R I site and *Hin*d III site. The integrity of the *bar* gene was first verified by PCR amplification and restriction enzyme (**Figure 1**). DNA sequencing indicates that two nucleotide mutations were found, but the encoded amino acids were not changed, so the encoding amino acid sequences were identical to the sequence reported from GenBank Accession No. X05822 (**Figure 2**). The results indicated that the *bar* gene was inserted into the expression vector pET30(+) in-frame with the His·Tag·gene.

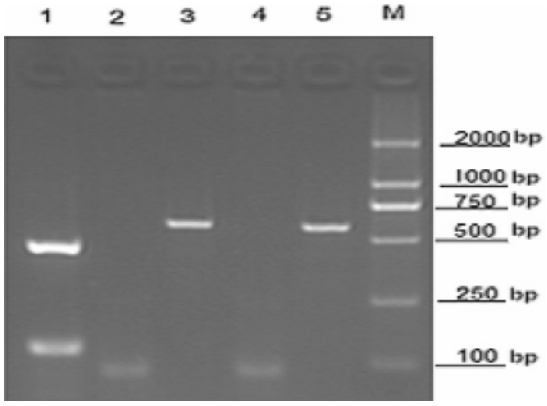

Figure 1. The identification by PCR amplification and restriction enzyme: (**1**) enzyme digestion with *Acc*III, (**2**) blank control, (**3**) recombinant expressed plasmid pET30a-*bar*, (**4**) pET30a(+) vector, (**5**) p3301 vector contained of *bar* gene, (**M**) DL2000 marker. The PCR amplification product of *bar* gene was completely digested into two segments, 132 bp and 426 bp, by the restriction enzyme *Acc*III (band 1). The 558 bp segment was amplified from both the recombinant expressed plasmid pET30a-*bar* and the p3301 vector, because they both contained *bar* gene (bands 3 and 5). Nothing was amplified from the pET30a(+) vector because the vector has no *bar* gene (band 4).

Expression and Purification of PAT. The PAT fusion protein was highly expressed in *Escherichia coli* BL21(DE3) in the presence of IPTG. Most products existed in a soluble form. After the lysozyme treatment and sonication, the soluble protein factions were recovered by centrifugation. PAT was mainly exported into the periplasm, which is a more favorable environment for folding and disulfide bond formation (**Figure 3**). The BL21(DE3) system can protect target protein from *lon* and *omp*T proteases and so more complete lengths of protein were obtained.

Ni−NTA metal affinity chromatography was applied to purify the PAT-His·Tag fusion protein to near homogeneity in one step (**Figure 4**). The His·Tag sequence binds to Ni^{2+} cations, which were immobilized on the Ni−NTA His·Bind Resin. The unbound proteins were washed away, and the PAT fusion protein was recovered by elution with imidazole. The His·Tag was cut out from the PAT fusion protein by EK, and the PAT was released from the fusion protein and purified to homogeneity with another N^{i2+} affinity chromatography because the uncut fusion PAT and the His·Tag still bound to the resin.

Identification of Recombinant PAT. The purified PAT was identified by SDS−PAGE, Western blot, and enzymic activity analysis. To elucidate whether the expressed protein was the recombinant PAT-His·Tag fusion protein, SDS−PAGE and Western blot were performed. The results indicated that the PAT-His·Tag fusion proteins were expressed with the right size of 31 kDa (data not shown).

Both purified PAT (without His·Tag) and PAT fusion (with His·Tag) proteins had the bioactivities. However, the enzymic activity of the PAT fusion protein was present at low levels of 0.29 units/mg of protein. After the purification steps and cutting of His·Tag, the activity of the purified PAT increased to the high levels of 3.32 units/mg of protein. The results showed that the acetylation was catalyzed by the production of the bar gene. The fusion proteins of PAT also conferred bialaphos resistance

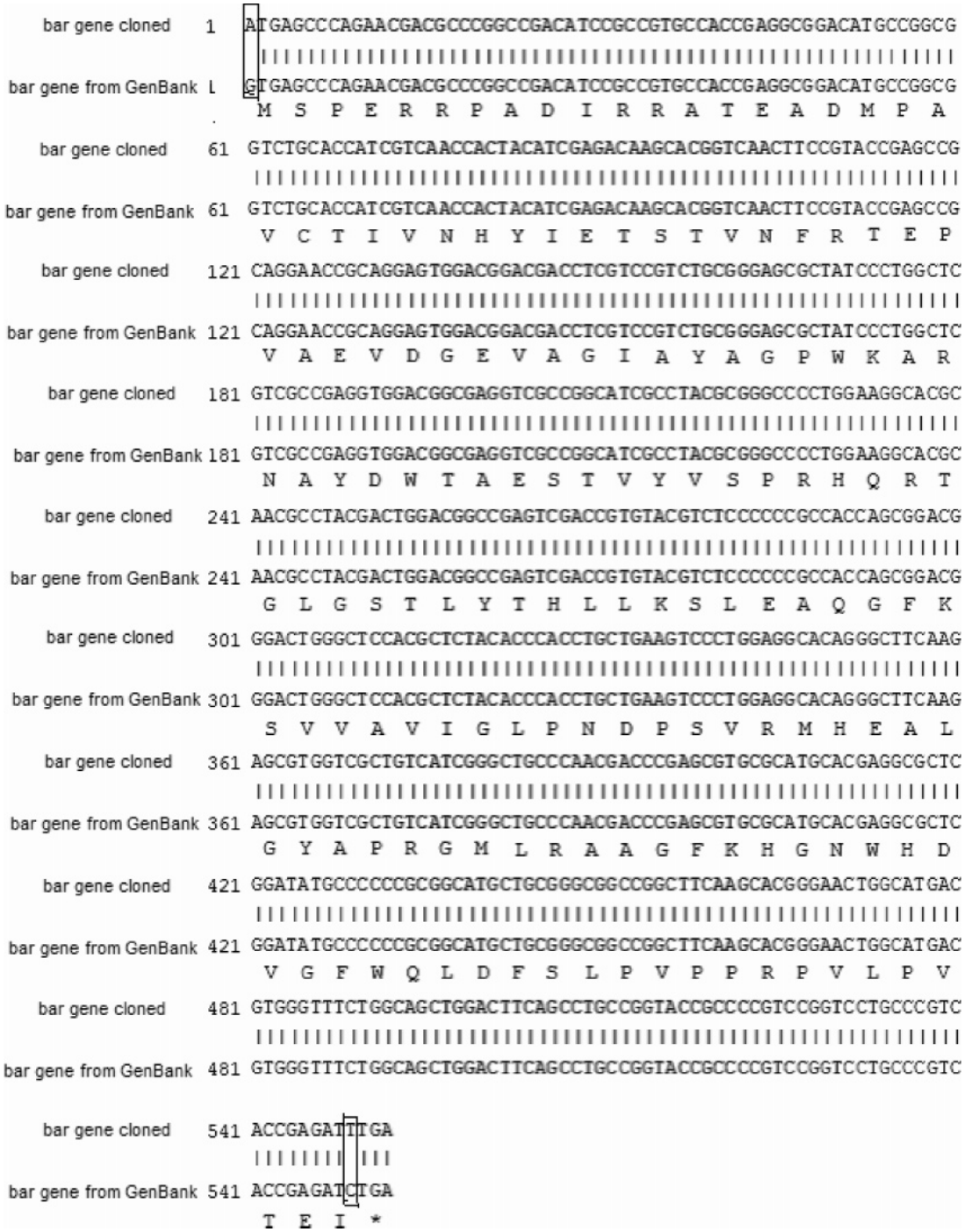

Figure 2. Alignment of DNA sequences of *bar* gene cloned and from GenBank (X05822) and their resulting amino acid sequence. Differences in DNA sequences from the other are shown by outline type.

so that it could be used to select for transfer and expression of genes fused to *bar*.

Production and Purification of Polyclonal Antibodies. The polyclonal antibody against PAT was purified through a saturated ammonium sulfate and a protein A−Sepharose 4B affinity column. This two-step purification using 20 mL of antiserum resulted in 70 mg of pure antibody. The antibody purity was checked by SDS−PAGE, which showed one strong band (the heavy chain) and one weak band (the light chain) under reducing conditions (data not shown).

Two types of antibodies were used in immunoaffinity chromatography. The most usable antibody was the polyclonal antibody (*24*−*27*). Polyclonal antibodies were obtained by immunizing a rabbit or goat and purifying the immunoglobulin fraction from the resulting serum. One can use quite pure antigen to avoid raising unwanted antibodies to minor impurities in the protein preparation. They are relatively easy to produce but not completely reproducible from one immunized animal to another, so it is difficult to obtain large quantities of consistent material. Monoclonal antibodies (mAbs), on the other hand, can be produced with smaller quantities of immunogen that need not be pure. Once a hybridoma line is established, it can be used to produce a potentially unlimited supply of antibody with reproducible properties, but it is more difficult and expensive to produce the mAbs.

Antiserum Sensitivity and Cross-Reactivity. The sensitivity and cross-reactivity of polyclonal antibody has been demonstrated in an ELISA test. The antiserum appears to reach a very

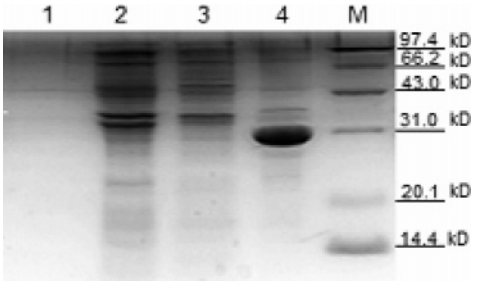

Figure 3. Analysis of expression product by SDS–PAGE: (**1**) lysis supernatant of pET30a-*bar* without induction of IPTG, (**2**) medium of pET30a-*bar* under induction of IPTG, (**3**) lysis supernatant of pET30a(+) under induction of IPTG, (**4**) lysis supernatant of pET30a-*bar* under induction of IPTG, (**5**) marker proteins. The proteins were hardly expressed in *Escherichia coli* BL21(DE3) without IPTG (band 1). There was little PAT fusion protein in the medium (band 2) and most active, soluble protein was mainly exported into the periplasm (band 4). Because the pET30a vector does not contain *bar* gene, no PAT fusion protein was expressed (band 3).

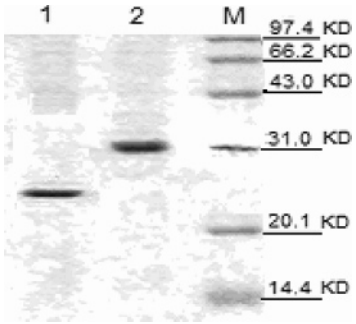

Figure 4. Cleavage of purified PAT fusion protein by enterokinase: (**1**) homogeneous PAT, (**2**) PAT fusion protein, (**M**) marker proteins. The molecule mass of PAT fusion protein is 30 800 Da and the molecule mass of homogeneous PAT is 23 428 D. After cleavage of purified PAT fusion protein by EK, the about 7400 kDa fusion segment was cut out from the PAT fusion protein.

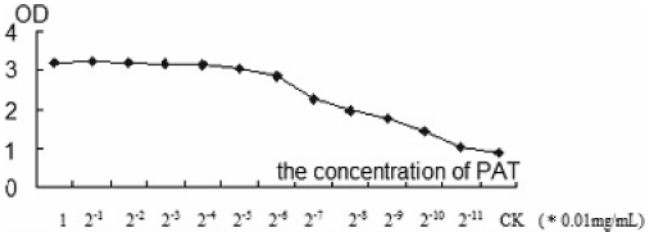

Figure 5. The sensitivity of anti-PAT antiserum. The result of the ELISA for antiserum sensitivity was 2×10^{-5} (0.01×2^{-9}) mg mL^{-1} and the antiserum appears to reach a very high degree of sensitivity to PAT protein.

high degree of sensitivity to recombinant PAT protein. The result of the ELISA for antiserum sensitivity was about 2×10^{-5} (0.01×2^{-9}) mg mL^{-1} (**Figure 5**). As the PAT has no isoenzymes or analogues, two kinds of potential interferents from the plants which have similar structures to PAT were included in the assay: phosphotransacetylase and chloramphenicol acetyltransferase. The cross-reactivity determined experimentally showed a high degree of specificity for the antiserum used, because values were all less than 0.1%. The purified polyclonal anti-PAT antiserum showed a very high degree of sensitivity and specificity, which was important to differentiate the PAT from other unrelated impurities in samples.

The Detection of Transgenic Plants by ELISA. The detections of transgenic plants were evaluated with two transgenic maize (Bt11 and Bt176) and two transgenic rapes (MS1/RF1 and MS8/RF3), while their non-GM parents were treated as controls. It was not typical to detect the transgenic maize (Bt11 and Bt176) by ELISA, but the transgenic rapes (MS1/RF1 and MS8/RF3) could be distinguished by ELISA (**Figure 6**). There were almost 2-fold differences between the OD values of MS1/RF1, MS8/RF3, and their non-GM parents, but there were almost no differences between the OD values of Bt11, Bt176, and their non-GM parents.

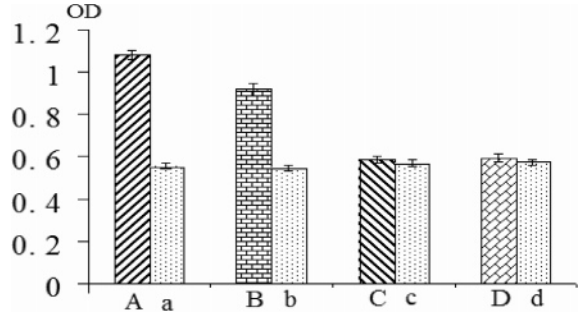

Figure 6. The assay of transgenic rapes MS1/RF1 and MS8/RF3 by ELISA: (**A**) MS1/RF1, (**a**) MS1/RF1 non-GM parent, (**B**) MS8/RF3, (**b**) MS8/RF3 non-GM parent, (**C**) Bt11, (**c**) Bt11 non-GM parent, (**D**) Bt176, (**d**) Bt176 non-GM parent.

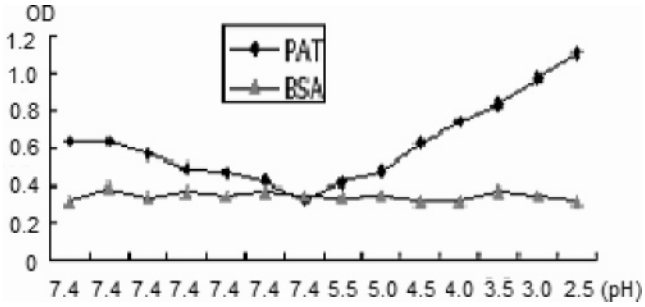

Figure 7. pH-reducing elution of antibody (against PAT)–Sepharose beads.

Different GM plants, which contain *pat* gene or *bar* gene, have different amounts of PAT. In PAT analysis, lack of an effective enriching and cleanup method greatly affects the sensitivity of many detection methods. Up to now, no literature was introduced to detect the transgenic maize (Bt11 and Bt176) by ELISA and Western blot. It could be the reason that only the trace PAT was extracted from these transgenic plants. So the results of ELISA lie to a considerable degree on the extraction and enrichment of the PAT.

Blank Column. A 1-mL blank column was generated as a negative control by negative rabbit antiserum containing no antibody against PAT. The blank column was loaded with PAT-spiked water using the modified immunoaffinity protocol as the above. The amount of washing and elution fractions was the same as that of columns containing anti-PAT antibodies. The results demonstrated that the retention of PAT was primarily due to the PAT antibody. The presence of PAT in the blank column eluate indicated that the PAT was only slightly retained by the blank column. This might possibly be due to insufficient blocking of active sites or might indicate that the wash volume was not adequate.

Optimization of the Eluting Conditions of PAT on the Immunoaffinity Column. **Figure 7** showed that the antibody (against PAT)−Sepharose beads were specifically combined with PAT. The PAT without binding to antibody (against PAT)−Sepharose beads was washed away with the PBS (pH

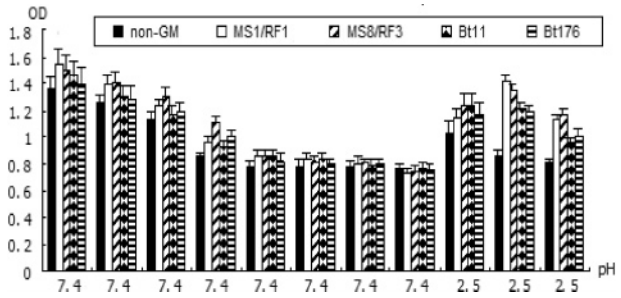

Figure 8. The detection of leaves of MS1/RF1 and MS8/RF3 and of seeds of Bt11 and Bt176.

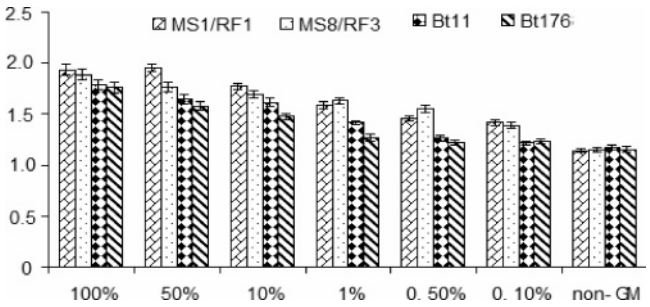

Figure 9. The detecting limits of leaves of MS1/RF1 and MS8/RF3 and of seeds of Bt176 and Bt11 by IAC–ELISA.

7.4) seven times and almost no unbound PAT remained on the IAC on the seventh elution with the PBS (pH 7.4). Then the eluting amount of PAT increased with the decrease of eluting pH. If the eluting pH was too low, the antibody (against PAT)−Sepharose beads were unstable, which affected the combination between antibody and antigen, so the prime pH of elution buffer was selected as pH 2.5.

According to the **Figure 8**, Bt11, Bt176, MS1/RF1, and MS8/RF3 could be easily detected by IAC−ELISA. The impurities and unbound PAT in the PAT extract of MS1/RF1, MS8/RF3, Bt11, and Bt176 were washed away with the PBS (pH 7.4) and almost no impurities and unbound PAT remain on the IAC on the seventh and eighth elution with the PBS (pH 7.4). There were maximum differences between the GM plants and their non-GM parents in the second elution with glycine buffer (pH 2.5), and the second elution was the prime one, resulting in the best results. This can be explained by realizing that the elution buffer for the first elution at pH 2.5 was neutralized by the remaining washing buffer (pH 7.4), and the impurity and PAT not tightly coupled with IgG−Sepharose beads were eluted out. Then the elution buffer for the second elution releases the most PAT from the IgG−Sepharose beads.

The Detecting Limits of Transgenic Plants (Bt11, Bt176, MS1/RF1, and MS8/RF3). The PAT in the transgenic plants was extracted and enriched by IAC and then was assayed by ELISA (**Figure 9**). The OD values decreased with the reduction of proportion of transgenic plants (Bt11, Bt176, MS1/RF1, and MS8/RF3). To reduce the error, the result was deemed as positive when its value of OD was 0.3 more than the value of OD of its non-GM parent. According to the standard, the detecting limit of Bt11 and Bt176 is 10%, and the detecting limit of MS1/RF1 and MS8/RF3 is 0.5%. Each proportion of sample was assayed for 10 times to assay its reproducibility, and results indicated that their P values were all below 0.3%.

The detecting limits of transgenic plants (Bt11, Bt176, MS1/RF1, and MS8/RF3) were different because the amounts of PAT are different in various kinds of transgenic plants.

Immunoaffinity chromatography has become a very efficient tool for enriching and purifying the compounds in a single step for subsequent analyses. IAC has proven extremely useful for both biochemical laboratory scale and larger scale protein purification (*28*−*30*).

In the present study, results from the present study indicate that the sensitivity of ELISA improves considerably after IAC enriching and cleanup. We found that the IAC could be used as an effective cleanup tool for PAT. The IAC in the present study has high capacity binding to PAT and effective removal of interferences by washing repeatedly with PBS.

The IAC−ELISA could be used for both determination of a given sample above or below a given threshold and computation of the percentage of genetically modified plants in the sample, i.e., to measure GMO concentration. Then the method was assessed of accuracy and precision. In the present study, the amount of antibody (against PAT) coupled to the CNBr-activated Sepharose beads was only 40 μL for one sample. The detecting limits of transgenic plants could be decreased with the increase of antibody (against PAT) coupled to the CNBr-activated Sepharose beads, but the cost of the examination may be increased. This matrix can be regenerated by the method: 5-fold elution buffer (pH 2.5) was used to clear the remaining PAT out of the matrix (IgG−Sepharose beads) and about 5-fold buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, 0.2 M NaCl, pH 8.3) was used to balance the matrix. In theory, the column can be reused at least five times, but the regenerated column has lower efficiency than the new column. Reusability was affected by many factors, including sample matrix, elution solvents, antibody properties, supporting matrix, storage conditions, and column capacity. Because the PAT in the generically modified plants was very minute and the decrease of affinity would greatly affect the detecting limits of transgenic plants, we suggest that the column be used only once in the assay of PAT.

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