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Analytical Methods

Development of nanocolloidal gold based immunochromatographic assay for rapid detection of transgenic vegetative insecticidal protein in genetically modified crops

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ABSTRACT

Vegetative insecticidal protein (Vip) is now being used for transgenic expression in several crops; conferring resistance against lepidopteron pests. A rapid, single step, sensitive and specific immunochromatographic (IC) strip test for the detection of recombinant Vip-S protein in the transgenic samples was developed. Polyclonal rabbit anti-Vip-S IgG conjugated to nanocolloidal gold served as a probe to detect Vip protein in test samples. The detection limit for the developed IC strip was 100 ng/ml (100 ppb) and on addition of gold enhancer the sensitivity increased to 1 ng/ml (1 ppb) of Vip-S protein. The assay was validated with transgenic brinjal samples. The assay time was less than 10 min, suitable for rapid on-site testing. No cross-reactivity was observed with other transgenic plant proteins employed for pest and weed management, i.e. Cry1Ac, Cry1Ab, and CP4-EPSPS. This on-site test offers rapid screening for a genetically modified crops having relatively new transgene (*vip*) entering the global market.

1. Introduction

The insect pest infestation severally affects the productivity of several crops. The lepidopteran black cutworm (*Agrotis ipsilon*) is a worldwide pest that attacks more than 50 crops, including cereal grains (Rings, Arnold, Keaster, & Musick, 1974). The use of chemical pesticides has long-term detrimental effects, leading to environmental degradation. Several insect and mite species have developed resistance to one or more chemical insecticides. *Bacillus thuringiensis* (*Bt*) has proved to be a remarkable source of the insecticidal protein genes used for transgenic expression in several crops (Kumar, 2003). In 1994, Syngenta group company discovered vegetative insecticidal protein (Vip), an exotoxin derived from the soil bacterium *B. thuringiensis* (International Cotton Advisory Committee, 2003). Vip includes the binary toxins Vip1 and Vip2 with coleopteran specificity and Vip3 with lepidopteran specificity (Rang, Gil, Neisner, Van Rie, & Frutos, 2005). Vip3 has been sub-grouped into Vip3A and Vip3B. Vip-S (NCBI accession number: CAA76665), recently introduced in tobacco, cotton, and brinjal, is part of Vip3A subgroup. Vip3A does not show any similarity to δ-endotoxins (Cry family proteins) but its insecticidal activity is ~260-fold higher than δ-endotoxins (Cry1A) against *A. ipsilon* (Estruch et al., 1996). Vip3A shows insecticidal mechanism different from that of the other known *Bt* toxins (Lee, Miles, & Chen, 2006).

The cultivation of genetically modified (GM) crops having Vip3A type protein is likely to increase exponentially and reach the global market. Syngenta Seed Inc. has developed the transgenic cotton varieties (COT102, COT202, and VipCot™) and maize varieties (MIR 162, BT11 × MIR162, and BT11 × MIR162 × MIR604) carrying Vip3A protein. Some of these varieties have been launched in USA, Australia, Taiwan, and Brazil (AGBIOS, 2009). The transgenic crops cotton and brinjal carrying Vip-S protein are currently under field trial in India.

Many countries have implemented labelling thresholds for mixing of GM crops defined as 0.9% in the EU, 3% in Korea, 5% in Taiwan, 1% in Australia, New Zealand and Brazil, and 5% in Japan (Michelini, Simoni, Cevenini, Mezzanotte, & Roda, 2008). Therefore, large scale demand for detection tests for Vip protein/gene is expected soon. Such tests are mandatory for regulatory compliance of GM labelling. To date, no detection kits are available for screening of GM crops/produce carrying Vip protein.

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Though several test methods for qualitative and quantitative assessment of GM crops and produce are commercially available, the most often used test of choice has been immunochromatographic (IC) strip/dipstick test. The IC strip method provides convenience coupled to speed and can be done in field conditions. The IC strips are available for detection of transgenic proteins in several GM crops such as cotton, corn, soybean, rice, alfalfa, sugarbeets, and canola (AgDia Inc.: EnviroLogix Inc.). However, neither the field friendly IC strip nor the conventional quantitative tests, i.e. enzyme-linked immunosorbent assay (ELISA)/Polymerase Chain Reaction (PCR) are commercially available for Vip protein/transgene.

The goal of this study was to develop a rapid, simple, qualitative, and sensitive colloidal gold based sandwich IC strip assay for one step detection of transgenic Vip protein. This method has distinct advantage over traditional immunoassays – ease of procedure, rapid operation, low cost and immediate results. The test has immense potential for use as a farm/field test.

2. Materials and methods

2.1. Chemicals and reagents

Nitrocellulose membrane cast on polyester backing and three pads (conjugate, sample and absorbent pads) were purchased from Advance Microdevices (India). Goat anti-rabbit IgG and Ni-NTA CL column were purchased from Bangalore Genei (India). Gold tetrachloride ($HAuCl_4{\cdot}3H_2O$), complete and incomplete Freund's adjuvant, sodium citrate, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), bovine serum albumin (BSA), polyvinylpyrrolidone (PVP-40) and protease inhibitor cocktail were obtained from Sigma (USA). HiTrap Protein-A column was obtained from GE Healthcare (USA). The Bradford reagent was from Biorad (USA). All other chemicals and organic solvents were of analytical grade.

2.2. Preparation of recombinant Vip-S protein

The recombinant Vip-S protein was expressed and isolated from *Escherichia coli* M15 with recombinant Vip-S expression plasmid pQE30. The plasmid pQE30 construct has 6× His-tag coding sequences at the N-terminus of the protein sequences (789 amino acids). The procedure of protein purification was followed as described previously by Selvapandiyan et al. (2001). In brief, recombinant *E. coli* was cultured in Luria Bertani (LB) broth to exponential phase, and expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 8000 rpm for 10 min, the bacterial pellet was isolated. The pellet was suspended in lysis buffer (50 mM Tris–HCl pH 8.0, 0.3 M NaCl, 2 mg/ml lysozyme) and subjected to sonication. The solubilised protein mixture was then put on the Ni His-tag column (Bed volume: 1 ml) and the column bound protein was eluted with 50–400 mM imidazole gradient. The imidazole in the selected fraction containing Vip-S protein was removed and concentrated by 10 kDa cut-off Amicon Ultra-4 centrifugal filter (Millipore, USA). The aliquots were stored at 4 °C. The protein was quantified by Bradford method (Bilodeau, Dda, Sauvageau, & Martineau, 1991). The apparent purity of affinity purified Vip-S protein was verified by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Production of polyclonal antibodies

Two New Zealand white male rabbits (~1.5 kg) and three Balb/c female mice (~25 g) were procured from animal facility of Indian Institute of Toxicology Research. Approximately 50 μg (mouse) to 100 μg (rabbit) of the recombinant Vip-S protein mixed with Freund's complete adjuvant (1:1 ratio) was injected intramuscularly (rabbit) or intraperitoneally (mouse) to each animal. After 28 days, first booster dose was given with Freund's incomplete adjuvant. The second, third, and fourth booster doses were given at each succeeding 15 days interval. The fifth booster dose was given 4 weeks later the fourth booster and the animals bled, drawing blood from ear vein (rabbit) or by puncturing orbital plexus (mouse), 10 days later. The sera samples were collected and the antibodies titre was checked by dot blot. The high titre antibodies were pooled and affinity purified using HiTrap Protein-A column (Bed volume: 1 ml). The eluted immunoglobulin fractions were dialysed against PBS and its protein content was assessed by measuring absorbance at 280 nm using NanoDrop spectrophotometer (NanoDrop Technologies, USA). The specificities of antibodies were confirmed by Western blot.

2.4. Preparation of nanocolloidal gold particles

The nanocolloidal gold particles were prepared according to Frens (1973). About 50 ml of 0.01% gold solution ($HAuCl_4{\cdot}3H_2O$) was heated to vigorous boiling. To the boiling solution, 1 ml of 1% sodium citrate was instantaneously added under constant stirring. The colour of the solution changed from yellow purple to red within 1 min. The solution was allowed to boil for another 10 min. After cooling, in dark, the solution volume made up to 50 ml with Milli-Q water. The particle size of resultant colloidal gold was measured by a Zetasizer particle analyser (Malvern Instruments Ltd., UK). The colloidal gold solution was then scanned (300–700 nm) on a UV–visible spectrophotometer (GE Healthcare, USA) for assessment of approximate particle size and stability. The solution was stored at 4 °C.

2.5. Formation of colloidal gold-conjugated probe

The optimal antibody concentration and pH value for conjugation was determined by treating gold-conjugated antibody mixture with 10% NaCl solution as described by Sun, Zhao, Tang, Zhou, and Chu (2005). To 1 ml of colloidal gold, variable antibody concentration; 0–100 μg anti-Vip-S IgG was added, over a range of pH 5.5–9.0, in separate microcentrifuge tubes, left for over 1 h for maximum conjugation to occur. To each test tube, 100 μl of 10% NaCl was added and shaken for 5 min. The change in colour and absorption at 521–580 nm was recorded 10 min later. Subsequent to determination of optimal assay conditions, the pH of 1 ml colloidal gold solution was adjusted to 8.5 with freshly prepared 1% K_2CO_3 solution and about 35 μg rabbit anti-Vip-S IgG added. The mixture was incubated for 1 h at room temperature and BSA added to a final concentration of 0.1%. After 30 min stabilisation, the mixture was centrifuged at 10,000 rpm for 30 min at 4 °C. A loose pellet laden conjugated antibody was dispersed in 500 μl of phosphate-buffered saline (10 mM PBS, pH 7.4) and spun again to remove free unconjugated antibody and colloidal gold particles. The resultant pellet was redissolved in 100 μl conjugate storage buffer (10 mM PBS containing 0.1% BSA, pH 7.4) and stored at 4 °C.

2.6. Assembly of IC strip and immobilisation of antibodies on strip

The IC strip comprised of a nitrocellulose membrane (NC, 5 μm pore size) and three pads, i.e. sample, absorbent, and conjugate pads. The long plastic backing with precoated NC membrane (laminate) was cut into 7.5 × 0.5 cm strips. On each strip, two lines – a test line and a control line were assigned in the middle of the NC membrane separating 1 cm from each other. The mouse anti-Vip-S IgG (1 μl/line, 1 mg/ml), mixed with 3% methanol, was immobilised on test line closer to sample pad side while goat anti-rabbit IgG (1 μl/line, 1 mg/ml) was dispensed onto control line nearer