Production of Polyclonal Antibodies for Detection of *Nucleopolyhedrovirus* Infecting *Helicoverpa armigera**

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Abstract

Polyclonal antibodies were raised against purified polyhedrin [(polyocclusion body (POB)] protein preparations of Helicoverpa armigera nucleopolyhedrovirus *(HaNPV) and were used to develop a direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) to detect* HaNPV. *The sensitivity of the DAC-ELISA was 15 ng/ml of partially purified viral protein or 30 ng/ml POBs from the* HaNPV *infected larval extracts. The antibodies were highly specific to polyhedrin protein of* HaNPV *and were useful to diagnose NPV at early stages of larval infection and also for the quantification of the NPVs during production of viral insecticides.*

Keywords: Polyclonal antibodies, *Helicoverpa armigera,* nucleopolyhedrovirus, ELISA

Introduction

Helicoverpa armigera (Hubner), commonly known as legume pod borer or cotton bollworm, is a polyphagous lepidopteran pest attacking more than 182 plant species with global crop losses exceeding US$ 5 billion per annum, despite the use of US$ 1 billion worth of pesticides (Sharma, 2001). The insect is most difficult to control, as it has developed resistance to several commonly used insecticides.

Helicoverpa armigera nucleopolyhedrovirus (*Ha*NPV; family *Baculoviridae* and genus *Nucleopolyhedrovirus*) is a natural pathogen of *H. armigera,* which is being widely used as a viral insecticide under integrated pest management programs (Ranga Rao *et al.,* 2006). However, many *Ha*NPV products produced were characterized as weak, with poor efficacy and questionable quality control (Kern and Vaagt, 1996).

It is necessary to have an efficient strategy for virus production and specific quality control tools to develop successful microbial control products (Shieh, 1989). Although NPV insecticide production methods have been well established in many developing countries, the microscopic counting procedure used to screen the larvae for NPV infection and quality control of the viral insecticide lots has low-detection efficiency, unknown specificity and laborious (Wigley, 1976). Enzyme-linked immunosorbent assay (ELISA)-based procedures have been developed by different investigators to detect the NPV in infected larvae (Zang and Kaupp, 1988 and Lu *et al.,* 1995). This study was undertaken with an objective to produce polyclonal antibodies to polyhedrin [polyocclusion body (POB)] protein of *Ha*NPV and develop ELISA for the detection and quantification of *Ha*NPV.

Materials and methods

*Ha*NPV source and its propagation

The *H. arimigera* strain used for this study was a mixture of populations collected from pigeonpea and chickpea crops grown at International Crops Research Institute for the Semi Arid tropics (ICRISAT), Patancheru, India, and subsequently maintained in the laboratory. The *Ha*NPV strain (Patancheru isolate) was isolated from field infected larvae and multiplied in the laboratory reared *H. armigera* larvae on artificial diets (Shorey and Hale, 1965). The virus infection was attained by diet surface contamination method with the purified virus suspension at 10^8 POB/ml (Evans and Shapiro, 1997).

Purification of polyhedral protein and antibody production

POBs were purified from *Ha*NPV infected insect cadavers

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as per the procedure given in Christian *et al.*, (2001). The polyhedrin protein of POBs was purified as per method described by Quant *et al.*, (1984). The purified protein concentration was estimated by Bradford method and the purity and integrity of the polyhedrin protein preparation was analyzed in 12% SDS-PAGE gel as described in Kumar and Waliyar (2007). The protein molecular weight standards (MBI Fermentas Cat# SM0441) were used as size markers. The polyhedrin protein (29 kDa molecular weight, Fig. 1A) eluted from the 12% SDS-PAGE gels as per the method described in Harlow and Lane (1998). Five hundred µg protein was emulsified with Freund's complete adjuvant and used for immunization to a New Zealand White inbreed rabbit through intramuscular route. Five injections were given at weekly intervals, and two weeks after the 5th injection, animal was bled for polyclonal antiserum (Harlow and Lane, 1998). The titer and specificity of the antiserum was determined by direct antigen coating ELISA and western immuno-blotting techniques as described in Kumar and Waliyar (2007).

DAC-ELISA and Western immuno assay for polyhedrin detection

Healthy 5th instar *H. armigera* larvae and *Ha*NPV infected larvae were grounded in 0.1 g/ml homogenization buffer (0.01M Tris pH 8.2, 0.15 M NaCl and 0.1mM phenyl methylsulfonyl fluoride) containing the 1M Na_2CO_3 and 0.5M NaCl. Homogenates were incubated for 10 min at 56°C, and then centrifuged at 26,000 rpm for 45 min in a Beckman SW28 rotor at 4°C. The supernatant fraction was collected and protein concentration was estimated by the Bradford method. The protein concentration was adjusted to 5 µg/ml and analyzed in DAC-ELISA as per the procedure described by Hobbs *et al.* (1987). Briefly, 100 µl (5 µg/ml protein) of the test sample was loaded into wells of ELISA plates (Nunc MaxiSorb, Denmark) and *Ha*NPV-antiserum was used at 1: 5,000 dilution. Alkalinephosphatase (ALP)-labelled anti-rabbit IgGs (Sigma, USA) were used at 1: 4,000 dilution to detect antigen-antibody complex, and paranitrophenyl phosphate was used at 0.5 mg/ml in 10% (v/v) diethanolamine buffer, pH 9.8. Optical density values at 405nm were measured in a Titertek Multiskan ELISA reader after 60 min of substrate reaction time. Readings were considered virus positive if the absorbance values of a sample differed by three-folds than those given by the healthy insect control. Proteins separated in 12% SDS-PAGE gels were electrophoretically transferred to a nitrocellulose filter and the membrane was probed with *Ha*NPV-antiserum at 1: 5000 dilution and the antigen-antibody reaction was visualized by colorimetric reaction using ALP-labelled anti-rabbit IgG and BCIP-NBT substrate as described in Kumar and Waliyar (2007).

Results and discussion

The method used for polyhedrin protein purification resulted in yields of about 15-20 mg/ml protein from 20 ml POB preparations (10^8 POB/ml). The purified polyhedrin preparations in PAGE gels revealed single protein band of estimated molecular weight 29 kDa (Fig. 1A, Lane 2). In addition, preparations also contained low molecular weight peptides of about 14-20 kDa and a high molecular weight 70 kDa fragment (Fig. 1A, Lane 2), which could be degraded peptides or dimers of the 30 kDa protein. The 29 kDa protein eluted from PAGE gels used for immunization has resulted in *Ha*NPV-antiserum with an ELISA titer of 1:5000 for the detection of 15 ng/ml polyhedrin protein. In western immunoassays, the antibodies specifically reactivated with polyhedrin protein, but not with the healthy proteins of *H. armigera* (Fig. 1B). To estimate the polyhedrin content in ELISA, purified polyhedrin, healthy larval extracts spiked with purified polyhedrin protein and healthy larval extracts were assayed by coating variable concentrations from 1000

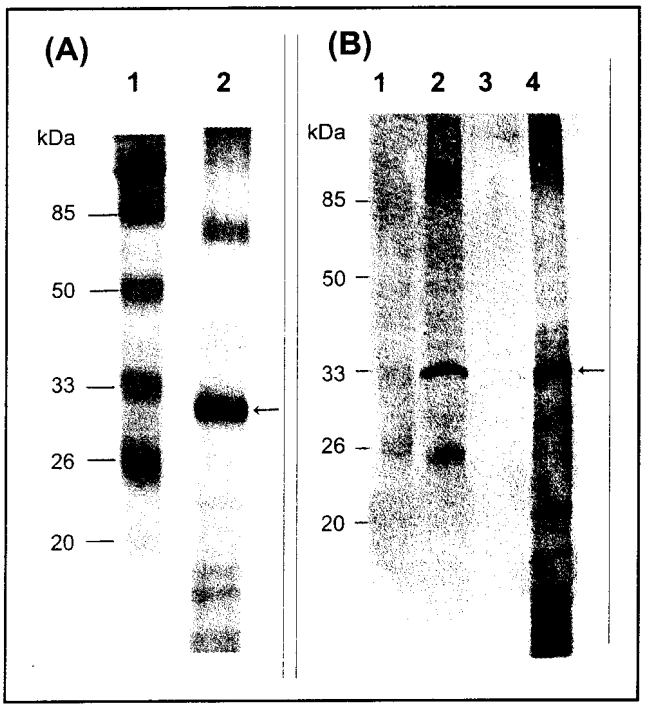

Figure 1. (A): Resolution of purified polyhedrin protein preparations of *Ha*NPV (Lane 2) in 12% SDS-PAGE. Molecular weight markers in Lane 1. Gel was silver stained. The 30 kDa polyhedrin protein is indicated with arrow. (B): Western-immunoassay of polyhedrin proteins from *Ha*NPV with *Ha*NPV antibodies. Lanes 1: Protein molecular weight markers; Lane 2: *Ha*NPV infected *H. armigera* larval extract; Lane 3: Healthy *H. armigera* larval extract; and Lane 4: Purified polyhedrin protein from *H. arimigera*. The 30 kDa polyhedrin protein is indicated with arrow.

to 3.9 ng/ml (Fig. 2). Antibodies detected up to 15 ng/ml of purified polyhedrin and up to 30 ng/ml of polyhedrin spiked in 5 µg/ml healthy larval extract. There was no cross-reaction between antibodies and healthy larval extracts (Fig. 2).

Virions of *Ha*NPVs are occluded in large protein crystals mainly comprised of polyhedrin protein. The occlusion bodies protect the virus particles, enable infection process and maintain virion viability for many years outside the insect host (Shieh, 1989). Polyhedrin protein is one of the highly conserved proteins among NPVs. Antibodies produced against polyhedrin protein can be used for the detection of POBs as well as virions. In this study antibodies produced against *Ha*NPV (Patancheru isolate) are highly specific and sensitive, which can detect the protein concentration as low as 15 ng/ml. These antibodies would be useful for monitoring production of the viral insecticides of *Ha*NPV.

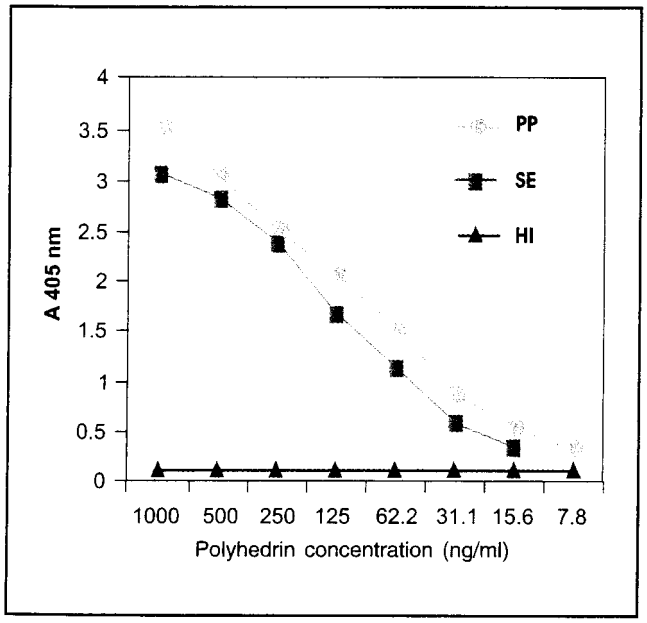

Figure 2. Detection of *Ha*NPV polyhedrin by DAC-ELISA using *Ha*NPV polyclonal antibodies. Polyhedrin concentrations given on x-axis and corresponding A405 nm values on y-axis. PP: Purified *Ha*NPV polyhedrin protein; SE: Healthy *H. armigera* larval extract (5 µg/ml) spiked with purified *Ha*NPV polyhderin protein; HI: Healthy *H. armigera* larval extract.

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