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Production of H5N1 Hemagglutinin in *Trichoplusia ni* Larvae by a Novel Bi-cistronic Baculovirus Expression Vector

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***Abstract*—**Highly pathogenic avian influenza (HPAI) H5N1 viruses have created demand for a cost-effective vaccine to prevent a pandemic of the disease. Here, we report that *Trichoplusia ni* (*T. ni*) larvae can act as a cost-effective bioreactor to produce recombinant HA5 (rH5HA) proteins as an potential effective vaccine for chickens. To facilitate the recombinant virus identification, virus titer determination and access the infected larvae, we employed the internal ribosome entry site (IRES) derived from *Perina nuda virus* (PnV, belongs to insect picorna like *Iflavirus* genus) to construct a bi-cistronic baculovirus expression vector that can express the rH5HA protein and enhanced green fluorescent protein (EGFP) simultaneously. Western blot analysis revealed that the 70 kDa rH5HA protein and partially cleaved products (40 kDa H5HA1) were generated in *T. ni* larvae infected with recombinant baculovirus carrying the H5HA gene. These data suggest that the baculovirus-larvae recombinant protein expression system could be a cost-effective platform for H5N1 vaccine production.

***Keywords*—**Avian Influenza; baculovirus; hemagglutinin; *Trichoplusia ni* larvae

I. Introduction

INFLUENZA is a acute respiratory disease caused by viruses belonging to the *Orthomyxoviridae* family. Influenza viruses are single-stranded ribonucleic acid (RNA) viruses spiked with two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), in a lipid-containing envelope. There are 16 subtypes, labeled according to an H number (for the type of hemagglutinin) and an N number (for the type of neuraminidase). They have 16 different H antigens (H1 to H16) and nine different N antigens (N1 to N9). Since 1997, H5N1 avian influenza viruses (AI) have caused more than 262 fatalities in humans [World Health Organization, WHO; http://www.who.int/en/, Ref. date 4 September 2009]. Prior to 2003, outbreaks of H5N1 viruses in poultry occurred in Asian countries such as China, Thailand, Vietnam and Indonesia. After 2005, H5N1 viruses were transferred via migratory birds or poultry transportation and spread from Asia to Europe and Africa [1], [2]. Thus, H5N1 has thus caused alarm for global human health and the poultry industry. Vaccines for H5N1 are critical for preventing, or at least limiting, potential H5N1 pandemic influenza outbreaks.

The use of licensed technologies such as inactivated [3] or attenuated viral vaccines [4] is the most popular approach to produce H5N1 vaccines. However, there are several practical and scientific challenges to the development of H5N1 vaccines via the traditional egg-based method [5]. These include not only the need of high containment facilities but the high pathogenicity of wild-type H5N1 influenza viruses also results in reduced yields of candidate vaccine viruses in fertilized eggs as compared to yields of human influenza viruses; both limited manufacturing capacity [5]. Therefore, alternative vaccine production strategies such as DNA vaccines, adenovirally expressed HA and recombinant antigens purified from baculovirus-infected insect cells have been explored [6]. Among these approaches, the baculovirus-insect cell expression system seems to be the most promising, as rHA influenza vaccines produced using this approach have been tested in several Phase I and Phase II human clinical trials and were found to be safe, immunogenic and efficient [5].

In previous studies, it had shown that baculovirus-infected insect cells can produce rH5HA vaccines that could protect chickens from lethal HPAI virus challenges [7]. However, the cost of producing rH5HA vaccines by insect culture system for poultry flocks was not economical. In the present study, we aimed to produce rHA vaccines in *Trichoplusia ni* (*T. ni*) larvae**.**

II. Materials and methods

Cells, viruses, and transfection

The *Spodoptera frugiperda* IPBL-Sf9 (Sf9) cell line was cultured in TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum [8]. Sf9 monolayers were used for virus propagation. All viral stocks were prepared and titers were determined according to standard protocols described by O'Reilly et al. [9]. For transfection, Cellfectin™ (Invitrogen) was used according to the protocols provided by the manufacturer.

Preparation of recombinant baculovirus with hemagglutinin gene

The A/duck/China/E319–2/03 virus was isolated from smuggled Muscovy ducks in Kinmen Island [10]. The virulence of this virus in chickens indicates that it is highly pathogenic. The genotype of the A/duck/China/E319-2/03

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virus was classified as being in clade 2 [11] and was found to be similar to the Z subtype, which has dominated avian influenza outbreaks in China since 2003 [10]. The entire hemagglutinin (HA) coding region, including nucleotides 29 to 1,765 (GenBank accession number: AY518362) with a deletion of the stop codon, was amplified from the A/duck/China/E319–2/03 virus and cloned into the pENTR/D-TOPO vector (Invitrogen). Target gene HA (E319-HA5/pENTR D TOPO) was cloned into vector pBac-DsRed-Pnv339-EGFP by replace DsRed segment. Then, the HA gene was PCR amplified with forward primer 5'CGC GCT AGC CCA CCA TGG AGA AAA CAG TGC TTC (5'NheI) and reverse primer 3'CGC CAA TTG TCA ATG GTG ATG GTG ATG ATG AAT GCA AAT TCT GCA TTG (3'MunI) and the restriction sites are underline.

Insect larvae and virus inoculation

Fourth instar *Trichoplusia ni* (*T. ni*) were used to express rH5HA to generate vaccines against H5N1 influenza in chickens. *T. ni* larva were reared under level-2 biosafety conditions and were provided by the Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture (Taichung, Taiwan). *T. ni* larva were infected with $1x10^9$ pfu/ml of recombinant baculovirus vAc-HA5 using the aerosol method as reported previously [12]. After infection, larvae were fed regularly with a fresh non-contaminated diet. The infected larvae were collected at 96 h post-infection (hpi) and frozen at –20°C until being used. Frozen insect larvae were homogenized using an extraction buffer containing phosphate-buffered saline (PBS) with a pH of 7.2. The homogenate was centrifuged at 10,000 ×*g* for 15 min at 4°C and the supernatant was then further clarified using a 0.22 μm filter membrane. The total soluble proteins were obtained from infected larvae and stored at -80°C. The identity of the samples was determined by SDS-PAGE and Western blot analysis.

Western blot

The homogenate expressed from the *T. ni*. larvae system and the supernatant collected from infected cell cultures were analyzed for the presence of the expected proteins via 8 % SDS-PAGE gradient electrophoresis and followed by trans-blotting onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and antigens were detected by monoclonal antibodies, YY1. The YY1 antibody is an H5N1-specific antibody that possesses hemagglutination inhibition (HI) and virus neutralizing activities. The recognition of rH5HA by YY1 is conformation-dependent; therefore, samples prepared for SDS-PAGE should be maintained under non-reducing conditions. Anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Labs, West Grove, PA, U.S.A.) and color development with NBT/BCIP reagent (Pierce, Rockford, IL, U.S.A.) was then applied.

Purification HA protein

Ni-affinity chromatography was used to enrich HA proteins that expressed in insect cell line. In this study, we used Ni-NTA His-Bind Resins kit (Novagen) and elute with 500 mM imidazole.

III. Results and Discussion

There were studies indicated that coexpression of two proteins, one being a selectable marker, a cell-surface antigen or a reporter gene, and the other the gene of interest, is often a valuable tool in biotechnological applications [13]. The homologous recombination is a rare event in insect cells (typically only about 0.1-1%) and has always led to a background of wild type virus [14]. So, it has necessitated multiple virus isolation steps to avoid eventual outgrowth by the wild type virus. In addition, either preparing viral stocks or when carrying out infections for protein production, it is very important to know the titer of a recombinant baculovirus [15]. Thus, the use of baculovirus expression vector system is hampered by slow and tedious procedures for the recombinant virus isolation [16]. Previous studies had indicated the green fluorescence protein is an ideal reporter molecule for the rapid selection of recombinant baculoviruses and for the virus titer determination [15]. Thus, we first constructed the recombinant baculovirus vAc-HA_5-PnIR-EGFP (Fig. 1A), which contains the hemagglutinin (H5HA) gene derived from an H5N1 strain (A/duck/China/E319-2/03) and the green fluorescent protein EGFP. In vAc-HA_5-PnIR-EGFP infected Sf21 cells, the transcription of H5HA and EGFP is controlled by the very late strong polyhedron promoter and the translation of rH5HA is cap-dependent but the translation of EGFP is via the PnV IRES [17]. We first identified the virus that can infected Sf21 cells and revealed the green fluorescence under fluorescence microscope (Fig. 1B).

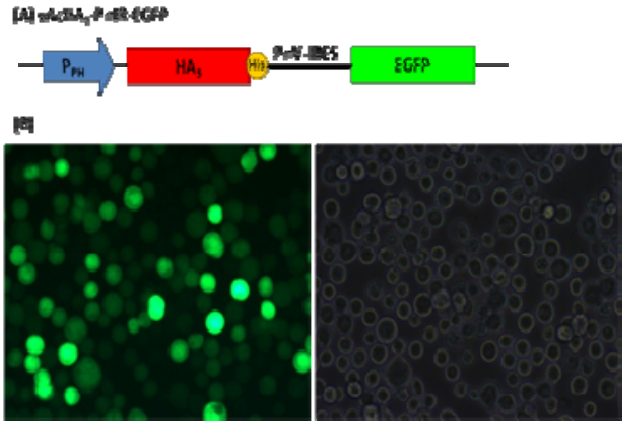

Fig. 1 Bi-cistronic baculovirus expression vector and infected Sf21 cells. (A)The construct of the recombinant virus vAc-HA5-PnIR-EGFP. P_{PH}, the polyhedrin promoter; HA_5, the H5HA gene; His, the six His tag; PnV-IRES, the element of PnV IRES; EGFP, the enhanced green fluorescent protein gene. The stop indicated the translational termination signal. (B) Sf21 cells (2×10^5 seeding in 24 wells) were infected with vAc-HA5-PnIR-EGFP (moi=1) and observed at 3 days after inoculation under fluorescence microscope (Nickon). Pictures were taken in the same field with conventional FITC channel (left) and bright field (right).

By the guide of fluorescence, we identified 6 strains of recombinant virus and using the HA-specific monoclonal antibody YY-1 as probe to conduct Western blot to confirm the simultaneously expression of rH5HA protein with the EGFP

protein. Fig. 2 shows that the co-expression of the rH5HA protein with the EGFP protein. Furthermore, the expression quantity of the EGFP protein and the rH5HA protein was nearly parallel. This result not only demonstrated the membrane protein (rH5HA) can co-express with the cytosolic protein (EGFP) and also confirmed the PnIR-EGFP module (Fig. 1A) can be employed to identify recombinant baculovirus under fluorescent microscope through the expression of EGFP. Furthermore, the virus titer can also be determined easily by fluorescent microscope. This will facilitate the recombinant proteins production using the baculovirus expression system for the titer determination and the proportion of virus infected cells can be monitored through the expression of green fluorescent protein.

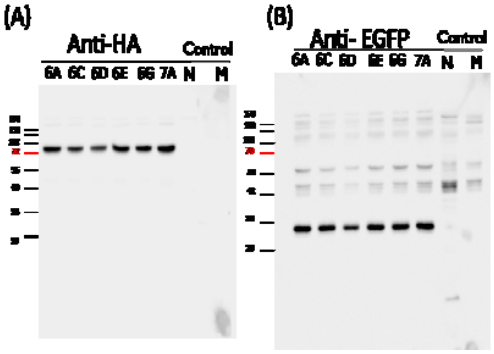

Fig. 2 Western blot analysis of vAc-HA5-PnIR-EGFP infected Sf21 cells. Six strains of vAc-HA5-PnIR-EGFP named 6A, 6C, 6D, 6E, 6G, and 7A infected Sf21cells with M.O.I=5 were harvest at 3 days after infection, loading 14 ug protein, and probed with YY-1 antibody (A) or EGFP-specific antibody (B). N, mock infected iSf21 cells l; M, Sf21 cells infected with wild type baculovirus.

To verify the His tag is functional in this PnV IRES based bi-cistronic vector, a Nickel column was used to purify the rH5HA protein in vAc-HA$_5$-PnIR-EGFP infected Sf21 cells lysates. 1ml HA protein was collected after cells lysis (300 ul/flask) and centrifuge at 4^{0}C, 30mins. Most of rH5HA proteins were purified efficiently although have some rH5HA proteins went through the column (Fig. 3). The second and third step of elution were collected more HA protein than the first one (Fig. 3). Thus, this novel bi-cistronic baculovirus expression vector can be used to produce rH5HA protein in Sf21 cells and easily enriched by Nickel affinity chromatography. Although cultured insect cells have been recognized as an excellent platform for the production of recombinant proteins, limitations associated with culturing insect cells on a large scale present the major bottle-neck for the commercial production of recombinant proteins. In addition, for production of vaccines for the poultry industry, economic factors are also important issues. The insect larvae of baculovirus hosts such as the

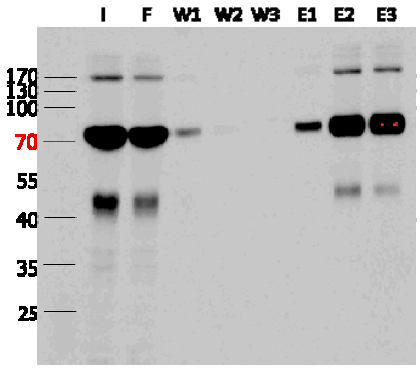

Fig.3. Enrichment of rH5HA proteins by Ni-affinity chromatography. Cell lysates from the vAc-HA5-PnIR-EGFP infected Sf21 cells (with M.O.I=1 in 3x10^6 cell/ml in 3 T25 flask) were harvest. I: cell lysates, F: flow through, W1, W2, W3: first, second, third time wash collection, E1, E2, E3: Elution of first, second, third fractionation.

cabbage looper *T. ni* may be explored as an alternative to cell culture for cost-effective recombinant protein production. Thus, we tried to explore this vAc-HA$_5$-PnIR-EGFP recombinant baculovirus to infect insect larvae to produce rH5HA protein.

Fig. 4A shows that the vAc-HA$_5$-PnIR-EGFP infected larvae revealed the green fluorescence under UV 340 nm illumination.

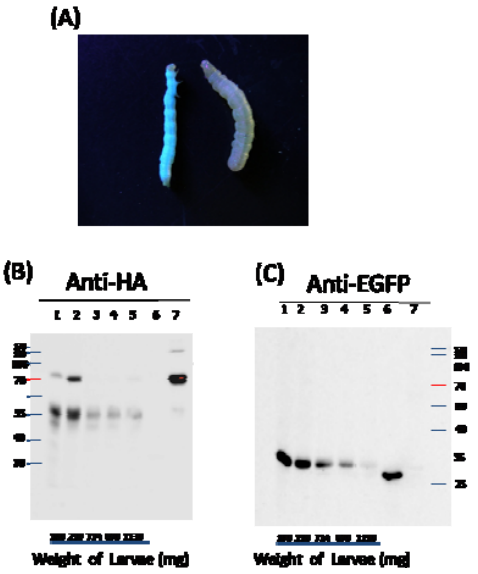

Fig. 4. Recombinant rH5HA proteins expression in *T. ni* larvae. (A) vAc-HA5-PnIR-EGFP infected larvae revealed the green fluorescence under UV 340 nm illumination. (B) Western blot analysis of rH5HA protein. Lane 1, 2, 3, 4, 5, loading 30 ug quantity of protein of insect larvae infected with vAc-HA5-PnIR-EGFP in comparison to 30 ug quantity of its in insect cells (lane 7, positive control) and lane 6 is a mock infection larvae. (C) (B) Western blot analysis of EGFP protein. Lane 1, 2, 3, 4, 5, loading 30 ug quantity of protein of insect larvae infected with vAc-HA5-PnIR-EGFP in comparison to 30 ug quantity of EGFP protein (lane 6, positive control) and lane 7 is a mock infection larvae.

Thus, the infected *T. ni* larvae can be easily identified through the green fluorescence and to distinguish from the

un-infected larvae. Besides, the “green” infected larvae seemed to be slimmer than the un-infected larvae (Fig. 4A). To further confirm the green larvae indeed expressed the rH5HA protein as well as the green fluorescent protein EGFP, Western blot probed with HA-specific monoclonal antibody YY-1 and EGFP-specific antibody, respectively. We found that the *T. ni* larvae infected with vAc-HA_5-PnIR-EGFP can simultaneously produce the rH5HA protein (Fig. 4B) as well as the EGFP protein (Fig. 4C). However, most of the larvae produced rH5HA protein were cleavage into HA1 (with molecular weight about 55 kDa), unlike the intact HA protein expressed in Sf21 cell (with molecular weight about 70 kDa). Interestingly, we also found that the infected larvae with weight about 200 mg could produce more abundant rH5HA protein and EGFP protein (Fig. 4 B and C). Thus, by this bi-cistronic baculovirus expression vector and the green fluorescence as a reporter as well as the body weight of infected larvae can facilitate to develop the baculovirus-larvae recombinant protein expression system as a cost-effective platform for vaccine production for poultry.

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