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Duck hepatitis A virus structural proteins expressed in insect cells self-assemble into virus-like particles with strong immunogenicity in ducklings

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ABSTRACT

Duck hepatitis A virus (DHAV), a non-enveloped ssRNA virus, can cause a highly contagious disease in young ducklings. The three capsid proteins of VP0, VP1 and VP3 are translated within a single large open reading frame (ORF) and hydrolyzed by protease 3CD. However, little is known on whether the recombinant viral structural proteins (VPs) expressed in insect cells could spontaneously assemble into virus-like particles (VLPs) and whether these VLPs could induce protective immunity in young ducklings. To address these issues, the structural polyprotein precursor gene *P1* and the protease gene *3CD* were amplified by PCR, and the recombinant proteins were expressed in insect cells using a baculovirus expression system for the characterization of their structures and immunogenicity. The recombinant proteins expressed in Sf9 cells were detected by indirect immunofluorescence assay and Western blot analysis. Electron microscopy showed that the recombinant proteins spontaneously assembled into VLPs in insect cells. Western blot analysis of the purified VLPs revealed that the VLPs were composed with the three structural proteins. In addition, vaccination with the VLPs induced high humoral immune response and provided strong protection. Therefore, our findings may provide a framework for development of new vaccines for the prevention of duck viral hepatitis.

1. Introduction

Duck hepatitis A virus type 1 (DHAV-1) is the major causative agent of duck viral hepatitis, and it can cause an acute hepatitis in young ducklings less than 3 weeks of age characterized by liver necrosis and mortality up to 100% (Levine and Fabricant, 1950). Duck hepatitis A virus (DHAV), formally known as duck hepatitis virus type 1 (DHV-1), was originally classified as an enterovirus, and now is a member of the new genus *Avihepatovirus* in the family *picornaviridae* (Li et al., 2013; Wu et al., 2015). DHAV contains three distinct serotypes: DHAV-1 (the worldwide traditional serotype), DHAV-2 (serotype recently isolated in Taiwan), and DHAV-3 (serotype isolated in South Korea and China) (Kim et al., 2007), and there is no cross-neutralization among them (Tseng and Tsai, 2007).

The DHAV-1 genome is comprised of a single strand positive sense RNA of about 7690 nucleotides in length, which encoding a single large open reading frame (ORF) flanked by the 5′ and 3′ untranslated regions (UTRs). A large polyprotein can be translated from the ORF, and be hydrolyzed into 12 mature products by its own encoded protease, including three structural proteins of VP0, VP3 and VP1 (Kim et al., 2006; Tseng et al., 2007; Ding and Zhang, 2007). At present, the prevention and control of DHAV mainly rely on the attenuated live vaccine (Crighton and Woolcock, 1978; Rispens, 1969). Although it can induce strong immune response and provide adequate protection for young ducklings, there is still a risk of virulence reversion, which could lead to the spread of duck hepatitis virus (DHV) (Fu et al., 2012). Therefore, the development of one safe and efficient new genetic engineering vaccine to control the disease is a promising direction in the future.

Virus-like particles are hollow structures that contain no viral nucleic acids. Because of the structural similarity to the natural viral particles, VLPs have strong immunogenicity and biological activity, but lack of infectivity. VLP based vaccines have been proved to be one of the most safe and effective vaccines (Kushnir et al., 2012). Since the birth of the first genetic engineering VLP vaccine in 1986, the research and development of VLP vaccine has achieved brilliant results in the past thirty years, there are many types of VLPs generated from at least 30 different viruses that infected humans and animals (Crisci et al., 2012).

There are several expression systems used for VLPs production, baculovirus expression system (BES) has been demonstrated to be an efficient tool for investigating capsid formation of many viruses (Maranga et al., 2002), including papillomavirus (Rose et al., 1993),

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parvovirus (Ju et al., 2011), herpesvirus (Thomsen et al., 1994), rotavirus (Sabara et al., 1991), orbivirus (French et al., 1990), and picornavirus (Ku et al., 2013). The structural proteins of many picornavirus have been proved to have the ability to self-assemble into VLPs, such as Enterovirus 71 and Coxsackievirus A16 (Ku et al., 2014; Somasundaram et al., 2016). However, owing to the late development of molecular biology of DHAV, there is no report about DHAV-derived VLPs, and no information about whether the three structural proteins could self-assemble into VLPs. The object of this paper was to prepare DHAV-1 VLPs using the BES and evaluate the immunogenicity in young ducklings.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda (Sf9, Invitrogen, USA) cells were maintained at 27 °C in Sf-900 II serum-free medium (Gibco, USA). DHAV-1 strain SH (Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) was propagated in duck embryos as described previously (Song et al., 2012). The virus was stored at −80 °C until use.

2.2. Cloning and construction of recombinant baculoviruses

Viral RNA was extracted from the viral suspension according to the Trizol R protocols (Invitrogen) and the first strand cDNA synthesized by RT-PCR with M-MLV reverse transcriptase (Promega, USA) was taken as template. The DHAV P1 gene between nucleotides 590 and 2779 (GenBank accession no. FJ157178) was amplified by PCR using primers 5′- gtagcggccgcaccATGGATACTCTTACTAAAAC-′3 and 5′-gtactgcagTTA TTCAATTTCCAGATTGAGTTC-′3 (*Not* I and *Pst* I sites are underlined; uppercases indicate the P1 coding sequence). A start codon (ATG) and a stop codon (TAA) were included in the primers, and a Kozak sequence was introduced in front of the start codon to increase the P1 protein translation. The amplified P1 gene was cut with *Not* I and *Pst* I and inserted into pFastBacDual (Invitrogen), the recombinant plasmid was then named pFB-P1. The DHAV 3CD gene between nucleotides 5432 and 7336 was amplified by PCR using primers 5′-gtactcgagaccATGAGCGGGCGGGTGAATTTCAG-′3 and 5′-gtagctagcTCA GATCATCATGCAAGCTGTG-′3 (*Xho* I and *Nhe* I sites are underlined; uppercases indicate the P1 coding sequence), the primers also contained a start codon (ATG), a stop codon (TGA), and a Kozak sequence. The amplified 3CD gene was then cloned into *Xho* I and *Nhe* I sites of pFB-P1, and the resultant plasmid was named pFB-P13CD. The recombinant vector was transformed into DH10Bac bacteria, and the recombinant bacmids containing the P1 and 3CD genes were prepared from the transformed DH10Bac cells, and were used to transfect Sf9 cells using the Bac-to-Bac Baculovirus Expression System (Catalog no. 10359-016, Invitrogen), according to the manufacturer's recommendations. The rescued recombinant baculovirus rBacP13CD was propagated and titered on Sf9 cells using plaque test.

2.3. Indirect immunofluorescence assay (IFA)

Sf9 cells were seeded in 24-well plates (6×10^5 cells/well) and infected with the recombinant baculoviruses at a multiplicity of infection (MOI) of 1 and incubated at 27 °C for 3 days. After incubation, the cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde at room temperature for 20 min. Probing of proteins was conducted with mouse anti-VP0, anti-VP1, anti-VP3, anti-3C, or anti-3D (1: 200, maintained in our lab) polyclonal antibodies. All the polyclonal antibodies were prepared through immunizing the mouse with the purified recombinant proteins VP1, VP0, VP3, 3C, or 3D expressed in *Escherichia coli*. Detection of antibody was performed using goat anti-mouse immunoglobulin G labeled with fluorescein isothiocyanate (Jackson ImmunoResearch, USA) at a dilution of 1:500, and examined under a fluorescence microscope equipped with a video documentation system.

2.4. SDS-PAGE and western blot analysis

Sf9 cells infected with the recombinant baculoviruses were lysed in 1 × sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF, F. Hoffmann-La Roche Ltd., Grenzacherstrasse, Switzerland). The membranes were blocked with 10% skimmed milk in PBS overnight and incubated with mouse anti-VP1 polyclonal antibodies (1: 100) for 2 h at room temperature respectively. The membranes were then incubated with a secondary anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Jackson ImmunoResearch) at a dilution of 1:2000. The protein bands were visualized by 3,3′-diaminobenzidine (DAB) tetrahydrochloride staining.

2.5. Production and purification of VLPs

Sf9 cells at a density of 2×10^6 cells/ml in suspension culture were infected with the recombinant baculoviruses at a MOI of 5. About 72 h later, Sf9 cells were collected by centrifugation and lysed by repeated freezing and thawing from −80 °C to 37 °C 3 times. The supernatant of the cell lysate was extracted once with chloroform, and the upper phase was loaded on sucrose gradients (10–60%) and ultracentrifuged at 30,000*g* for 2 h at 4 °C. The precipitation was resuspended in PBS and stored at −80 °C.

2.6. VLPs characterization

The purified viruses were identified by electron microscopy (EM). Briefly, the samples were mounted on a carbon-coated paper grid at room temperature for 10 min, and carefully drained with a filter paper. After being negatively strained with 2% phosphotumgstic acid (pH7.4) for 1 min, the samples were observed under a transmission electron microscope (TEM) (Tecnai 12, FEI, USA). To demonstrate the composition of the VLPs, ten microliters of the purified VLPs were lysed in 1 × SDS sample buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane for Western blot analysis. The procedure was the same as above, by using the anti-VP1 (1:100), anti-VP3 (1:100), or anti-VP0 (1:100) polyclonal antibody respectively, and a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch) at a dilution of 1:2000. To determine the amount of VP1 protein in the VLP preparations, we performed a Western blot followed by densitometric analysis, using the purified recombinant VP1 proteins of known concentration as a standard. The VP1 gene was amplified and subcloned into pET32a (+), and the recombinant VP1 fusion proteins were purified by Ni^{2+} affinity chromatography His-Bind Resin. The procedure of Western blot analysis was the same as above, by using the anti-VP1 polyclonal antibody (1:100) and a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2000).

2.7. Immunization of ducklings and virus challenge

The animal experiments were performed in accordance with the animal study protocol 15-08 of the Institutional Animal Care and Use Committee guidelines set by Jiangsu Agri-animal Husbandry Vocational College. One day ducklings were obtained from the National Waterfowl Germplasm Resource Pool of Jiangsu Sci-tech Demonstration Garden of Modern Animal Husbandry, China. The ducklings were randomly assigned to four groups (six ducklings each group), each group was housed in a specific pathogen-free facility with free access to water and food. The first experiment group was immunized one time with VLPs at a dose of 20 μg based on VP1 content in PBS intramuscularly (i.m.). The