RESEARCH ARTICLE

Characterization of Avian Influenza H5N1 Virosome

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A B S T R A C T

The purpose of this study was to prepare and characterize virosome containing envelope proteins of the avian influenza (H5N1) virus. The virosome was prepared by the solubilization of virus with octaethyleneglycol mono (n-dodecyl) ether ($C_{12}E_8$) followed by detergent removal with SM2 Bio-Beads. Biochemical analysis by SDS-PAGE and western blotting, indicated that avian influenza H5N1 virosome had similar characteristics to the parent virus and contained both the hemagglutinin (HA, 60-75 kDa) and neuraminidase (NA, 220 kDa) protein, with preserved biological activity, such as hemagglutination activity. The virosome structure was analyzed by negative stained transmission electron microscope (TEM) demonstrated that the spherical shapes of vesicles with surface glycoprotein spikes were harbored. In conclusion, the biophysical properties of the virosome were similar to the parent virus, and the use of octaethyleneglycol mono (n-dodecyl) ether to solubilize viral membrane, followed by removal of detergent using polymer beads adsorption (Bio-Beads SM2) was the preferable method for obtaining avian influenza virosome. The outcome of this study might be useful for further development veterinary virus vaccines.

INTRODUCTION

Avian influenza is a highly pathogenic infectious disease of poultry and other avian species. This disease is also recognized as a natural infection and disease of the upper respiratory tract of human and other mammals (Yin *et al.*, 2013). The disease is caused by the influenza virus A subtype H5N1. The influenza virus is an RNA virus in the Orthomyxoviridae family. Currently, the influenza virus has been subtyped into 16 hemagglutinin (H1-16) and 9 neuraminidase (N1-9) (Lung *et al.,* 2012). The first outbreak of highly pathogenic avian influenza (HPAI) in Thailand occurred in 2004. This outbreak caused the carcass condemnation of poultry and many avian species in Thailand of more than 60 million birds and 17 people of the 25 confirmed infected died (WHO, 2012). The World Organization for Animal Health (OIE, 2012) has categorized the HPAI virus as a notifiable disease, due to its virulence and ability to transmit from animal to animal. Furthermore, avian influenza can be transmitted from avian to man and can cause a severe clinical condition or

death in infected humans. Not only can Avian Influenza have an effect of humans and animal health but it can also affect the poultry industry and exportation. In 2002, the revenue from Thai poultry meat and production export was around 1200 million USD. After the HPAI outbreak in 2004, the poultry meat and production exportation was reduced by ninety percent.

The international measure for controlling HPAI is the eradication of infected flocks, the establishing of disease outbreak declaration zones, animal movement control, surveillance and monitoring in the infected zone, and other measures such as determining the vaccination zone to stop the disease from spreading. The advantage of vaccination is the reduction of virus spread to the environment and also vaccine can reduce virus replication and transmission. Virosomes are particles that originate by the re-arrangement of protein and lipid molecule in the components of viral envelopes. The new particle will have the same structure and component similarly to the prototype virus but no nucleoprotein or genetic material is present in the virosome structure resulting in the inability

of the virosome to replicate in the host cells (Huckrieda *et al.,* 2003; Wilschut, 2009). Anyhow, the virosome still has the property of stimulating the immune system (Gluck and Metcalfe, 2003). The virosome is expected to be an alternative way of developing vaccines for the prevention and control of avian influenza virus (AIV) in poultry. The objective of the experiment was to develop and characterize the virosome originating from AIV H5N1.

MATERIALS AND METHODS

Virus preparation and inactivation: Influenza virus A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) was used to inoculate the allantoic sac of 10-day-old specific antibody negative, embryonated chicken eggs. The eggs were observed for 24-72 h post inoculation. The allantoic fluid of the inoculated eggs was collected and centrifuged 1000 x g, 15 min. The supernatant was stored at -70°C until used. The AIV antigen for producing virosomes in this study had hemagglutination (HA) titer of 256 HA unit/50 µl. The virus was inactivated by treatment with 0.01 M BEI (pH 8.5) for 6 h and inoculated into 10-day-old chicken embryonic eggs for 2 passages that were intended to assure the inactivation efficacy (Sarachai *et al.,* 2010)

Virus purification by Sucrose gradient solutions: The sucrose gradient was prepared by a laying process of linear gradient (Griffith, 1986). The sediment of the virus was separated from the supernatant, dissolved in HNE buffer (150 mM NaCl, 0.1 mM EDTA and 5 mM HEPES adjust pH to 7.4) (Metsikko *et al.,* 1986) and centrifuged in 10-60% (w/v) sucrose gradient at 100,000 x g (Beckman, SW41Ti), 4°C for 16 h. The virus bands were collected and stored at -70°C until used for virosome preparation.

Virosome preparation: Virosome preparation was done with octaethyleneglycol mono (n-dodecyl) ether ($C_{12}E_8$) (Bron *et al.,* 1993). One mg of purified virus was diluted and centrifuged. The supernatant was removed and the sediment was added 100 mM $C_{12}E_8$ in 0.7 ml HNE buffer. The virus and $C_{12}E_8$ were mixed. The nucleocapsid proteins were separated from the solution by centrifugation. The $C_{12}E_8$ was washed from the nucleocapsid and removed by BioBead SM2 (BioRad, Hercules, CA). The suspension exhibited turbidity indicating virosome forming and then centrifuged in 10-40% discontinuous sucrose gradient at 130,000 x g (Beckman, TLS-55 SW), 4°C for 90 min. The virosome band was located between the sucrose layers.

Physiological characterization of virosomes
Morphological study: An electron microscope was used to study the morphology, structure and particle size of AIV and its virosomes. The negative-staining was followed Homhuan *et al.* (2004). Transmissible electron microscopy (TEM; JEM-2010) was used at 80 kV.

Viral protein component by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting: SDS-PAGE and Western blotting were used for the protein separation and identification

(Westermeier and Marouga, 2005). The SDS-polyacrylamide gel was transferred to nitrocellulose membranes (BioRad, Singapore) and blocked overnight. The specific antibody of AIV (National institute of animal health, Thailand) was diluted 1:1,000 and soaked in the nitrocellulose membrane for 1 h. The protein was detected by goat anti-chicken immunoglobulin G (IgG) labeling with horseradish peroxidase (KPL, USA) in TBS-3% nonfat milk in a ratio of 1:2,500 (v/v) for 2 h incubated with 0.6% 3,3'-diaminobenzidine carbonyl chloride in Tris-HCl (pH 7.6) with 0.03% hydrogen peroxide for 10-30 min until the color had developed.

Biological characterization of virosomes
Hemagglutination activity test: The HA assays were followed the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial animals (2012).

Reverse Transcriptase-Polymerase chain reaction (RT-PCR) analysis for nucleoprotein (NP) contamination: RT-PCR analysis was used for checking the NP contamination in virosome by the primers specific to NP. The viral RNA of AIV was extracted by QIAamp® viral Mini kit (Qiagen, USA). The first amplification reaction was carried out with one-step RT-PCR (AccessQuick[TM] RT-PCR system, Promega, USA) by the NP forward primer of 5'-TAGTCGACTCAGTGACTAT GAAGG-3' and the NP reverse primer of 5'-GACTCGA GTGGAGTCCATTGCTT-3' (Jin *et al.,* 2004). The PCR reaction was followed by reverse transcription reaction at 48°C for 60 min, 1 cycle; initial denaturation at 95°C for 2 min, 1 cycle; denaturation at 95°C for 30 sec; annealing at 55°C for 30 sec and extension at 72°C for 1 min. The PCR was processed for 40 cycles with the final extension step at 72°C for 10 min (Amonsin *et al.,* 2006). The amplification products were visualized by means of ethidium bromide staining after electrophoresis in a 1.5% agarose gel.

RESULTS

Virus purification and virosome preparation: AIV H5N1 was replicated by injection into the allantoic cavity of 9-11 embryonated eggs which were specifically antibody negative to AIV. Glycoprotein on the surface of virus was separated from the viral molecule using detergent ($C_{12}E_8$). $C_{12}E_8$ was used for breaking down the mentioned lipid structure for separating the nucleocapsid protein from the virus when the nucleocapsid was removed by centrifugation and the viral suspension could be clarified. Biobead (SM2) was added into the solution for absorbing $C_{12}E_8$ resulting in the arrangement of glycoprotein and phospholipid to form a new structure that was similar to the intact virus resulting in the turbidity of the solution. When detergent was absorbed from the solution, the virosome was separated from the solution by centrifugation by 10-40% discontinuous sucrose gradient at 130,000 x g. The virosome band appeared in a turbidity band between 10 and 40% sucrose concentration.

Protein components of AIV and the virosome: SDS-PAGE was used to analyze the component protein of the

virosome. The protein component of the virosome differed from the protein of prototype AIV. The band of molecular weight 10-50 kDa protein appeared only in AIV. Generally, the molecular weight at 10-50 kDA of AIV was composed of nucleoprotein (~56,000 Da), Matrix protein (~10,000-27,000 Da) and NS protein (~14,000-27,000 Da). The protein of molecular weight 60-220 kDa appeared on both kinds of AIV and virosome. These kinds of protein composed of glycoprotein hemagglutinin, polymerase protein and neuraminidase (Fig. 1A). Western blotting was used to study the protein components of AIV and the virosome. A specific antibody against AIV H5N1 was used. The result shows the protein component of virosome was 60-75 kDa and 220 kDa protein (Fig. 1B).

Morphological study by electron microscope: A negative-staining transmission electron micrograph of AIV H5N1 revealed a spherical viral particle with dimensions of 80-120 nm and a glycoprotein structure of hemagglutinin and neuraminidase. A negative-staining transmission electron micrograph of the virosome also found the structure was similar to the AIV H5N1 virus. (Fig. 2A and 2B).

Biological characterization of virosome and Contamination of nucleoprotein in virosome: The virosome originating from AIV H5N1 exhibited the property to agglutinate chicken erythrocytes similar to AIV H5N1. The result indicates that virosome particles inactivated by BEI still conserve the property of erythrocyte agglutination (Fig. 3). The nucleoprotein of 2 sets of virosome preparation was tested for nucleoprotein contamination. No contamination of the nucleoprotein was found in virosomes compared with AIV H5N1 indicating no nucleoprotein in virosomes for replication when administered in the animal experiment.

DISCUSSION

The virosome is a particle constructed by the arrangement of lipid and protein on viral envelopes to create a new structure and components similar to the prototype virus (Huckrieda *et al.,* 2003). The process of creating the virosome will destroy the lipid structure by various kinds of detergents that have the property of destroying and re-arranging lipid after the detergent has been removed (Bron *et al.,* 1993). The virosomal structure is similar to the prototype virus in respect of its morphology and ability to bind between the proteins on the virosome envelope and the specific position of the host cells and movement throughout the host cell. However, the virosome is a particle that does not contain genetic material so; the virosome cannot replicate and multiply in the host cells (Huckriede *et al.,* 2003). The protein structure of the virosomal particle still has the ability to elicit immunity (Huckrieda *et al.,* 2003)

The virosome preparation by dissolving lipid with various kinds of detergents such as Triton-X100, octylglycoside or octaethyleneglycol mono (n-dodecyl) ether ($C_{12}E_8$) found a residue of detergents so a process of separating the detergent was needed by absorption with styrene-divinylbenzene copolymer (Bio-Beads SM2).
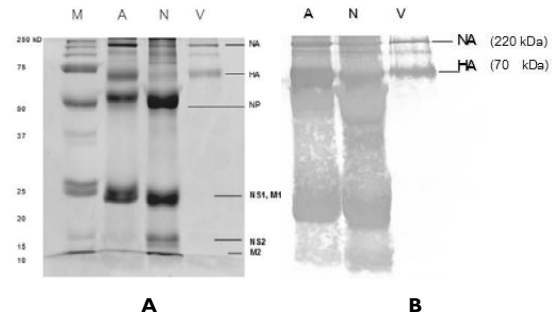

Fig. 1: The protein component of AIV H5N1 and the virosome were analyzed by SDS-PAGE. M = molecular weight marker, kDa, A= AIV H5N1, N = nucleocapsid protein, V = virosome protein (A). The protein component of AIV H5N1 and virosome by Western blotting. A = AIV H5N1, N = nucleocapsid, V = virosome (B).
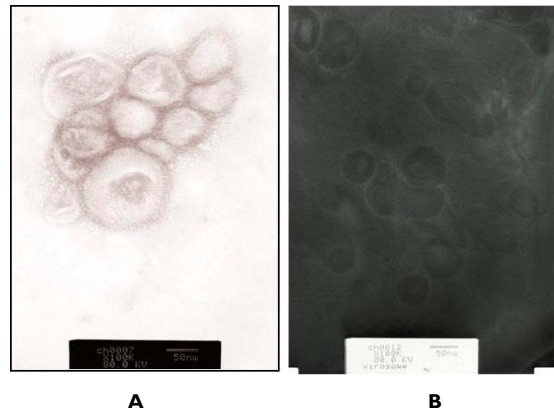

Fig. 2: Negative staining transmission electron micrograph (TEM) of AIV H5N1 (A) and virosome (B) (80,000x) showed the spherical vesicles with the glycoprotein structure of viral envelopes.
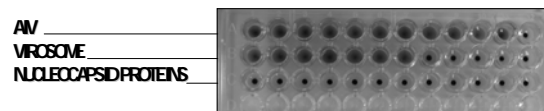

Fig. 3: The biological property of virosome prepared from AIV H5N1 by HA test.

After complete absorption, the phospholipid accumulated and merged to create cell membrane structure and glycoprotein component of hemagglutinin and neuraminidase on the viral envelopes. The process could be observed through the turning of a clear to a turbid solution after adding Bio-Beads SM2 to the virus-detergent solution. Turbidity increased in the solution when the virosome structure was formed (Bron *et al.,* 1993). Test of the physical properties of the virosome particle revealed that the virosome was similar to the prototype virus. The morphology of the virosome was spherical, diameter 80-120 nm and its glycoprotein protruded from the virosome envelopes (Bron *et al.,* 1993). For analysis of the protein component of the virosome and AIV H5N1 by SDS-PAGE, the result revealed the protein components of the virosome had a molecular weight of 10 kDa, 15 kDa, 25 kDa, 50-55 kDa, 60-75 kDa, 80-90 kDa and 200-220 kDa. The molecular weight of the protein component of the virosome was similar to that of the AIV H5N1 of the Matrix protein, nucleoprotein, hemagglutinin glycoprotein, polymerase

and neuraminidase protein, respectively. The results accorded to the report of Lamb and Krug (2001). When compared with the virosome particle, the molecular weight of protein appeared at 60-75 kDa and 200-220 kDa indicating that nucleocapsid was eliminated during the virosome preparation. Normally the protein remaining in the virosome was located in the viral envelope such as hemagglutinin and neuraminidase (Bron *et al.,* 1993). The result accorded with Wang *et al.* (2006) that HA monomer of influenza virus A/New Caledonia/20/99 composed of 547 amino acids with a theoretical molecular weight of 63,156.43 kDa. The largest molecular weight was 70 kDa composed of hemagglutinin HA_1 and HA_2 at the molecular weight of 50 kDa and 28 kDa, respectively. Crawford *et al.* (1999) reported that non-clevaged hemagglutinin (HA_0) of subgroup H5 and H7 has the highest molecular weight of 69,000 Da (69 kDa) similar to 74 kDa of glycoprotein hemagglutinin of subgroup H5. Qiao *et al.* (2003) reported the molecular weight of neuraminidase was 220 kDa which was similar to the molecular weight of neuraminidase reported by Johansson and Kilbourne (1996). For Western blotting, the relation of protein component of virosome and AIV by specific polyclonal antibody against AIV H5N1 was confirmed. The protein components at a molecular weight of 60-75 kDa and 200-220 kDa were found and they were hemagglutinin and neuraminidase glycoprotein indicating that some proteins in the AIV were detached during virosome preparation.

The biological properties of virosome were similar to the prototype virus (Huckrieda *et al.,* 2003). The properties were tested by the hemagglutination activity between glycoprotein hemagglutinin and erythrocytes. The virosome particle processed by BEI inactivation still showed the same ability to agglutinate with chicken erythrocytes as the intact AIV. This result indicated that glycoprotein the hemagglutinin of virosome could bind to sialic acid on the receptors of chicken erythrocytes. The binding was related to the protein property, virosome morphology and the ratio between hemagglutinin and neuraminidase. The detergent used in the lysis process of lipid in viral envelopes might affect the efficacy of the protein. The result revealed that the HA titer of virosome was less than the HA titer of the intact AIV according to Homhuan *et al.* (2004). Moreover, the hemagglutination properties relate to the amount of glycoprotein hemagglutinin located on the surface of virosome particles (Huckrieda *et al.,* 2003). The RT-PCR could not detect the nucleoprotein of virosome confirming that the virosome did not have the ability to replicate and multiply in experimental animals.

Conclusion: The biophysical properties of the virosome were similar to the parent virus, and the use of octaethyleneglycol mono (n-dodecyl) ether to solubilize viral membrane, followed by removal of detergent using polymer beads adsorption (Bio-Beads SM2) was the preferable method for obtaining avian influenza virosome. The outcome of this study might be useful for further development veterinary virus vaccines.

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