

Integrity of a recombinant hemagglutinin protein of an avian influenza virus

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Abstract An open reading frame representing cDNA from a hemagglutinin (HA) encoding gene of a low pathogenic avian influenza virus (AIV) subtype H10N7 was cloned in the pNMT1-TOPO vector under the control of thiamine response promoter. This construct was designated as pNMT1-HA. The pNMT1-HA construct was transformed into *Schizosaccharomyces pombe* for expression of HA antigen. The correct expression of recombinant HA protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The level of expression of recombinant HA protein was approximately 0.2% of total soluble protein. Purified yeast-derived recombinant HA protein showed hemagglutination activity. The 2-D and 3-D scanning images of

recombinant HA protein were observed with an atomic force microscope (AFM). The structural integrity of the HA protein under AFM and hemagglutination activity provided support that the recombinant HA protein may be suitable for development of AIV subunit vaccine for mass administration to poultry.

Keywords Atomic force microscope · Avian influenza virus · Hemagglutinin · *Schizosaccharomyces pombe* · Vaccine

Introduction

Avian Influenza Virus (AIV) belongs to the family *Orthomyxoviridae* (Lamb 1989). Other members of the family include influenza viruses (IV) type B and C, which infect only humans. The virus has an envelope with a host-derived lipid bilayer and covered with about 500 projecting glycoprotein spikes with hemagglutinin (HA) and neuraminidase (NA) activities. The HA and NA surface glycoproteins are present as homotrimers and homotetramers, respectively. The HA is the most abundant protein which mediates the attachment of the virus to the terminal sialic acid-containing cell receptors and its fusion with the cell endosomal membrane (Carroll and Paulson 1985; Danniels et al. 1987). Subtypes of AIV affecting birds, pigs, horses, and humans are classified by the antigenic relationship of HA and NA proteins.

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AIVs are a significant threat to the poultry industry. The virus spreads by wild free flying birds, thus increasing the threat of a pandemic. Highly pathogenic AIVs (HPAIVs) belonging to H5 and H7 subtypes have caused respiratory disease with nearly 100% mortality in flocks (Wood and Robertson 2004). Accidental genetic reassortments of RNA segments from different subtypes of avian and human AIV are capable of inducing a global pandemic (Wood and Robertson 2004). The HPAI H5N1 was transmitted from birds to pigs and humans leading to significant mortality (Choi et al. 2005). Vaccination and eradication of infected animals have been used for limited control of AIV outbreaks (Ison and Hayden 2001). More effective vaccines are needed to control AIV spread in birds, animal, and humans.

Yeast cells are used as a probiotic component in commercial poultry, and are generally administered during the early stages of bird growth through the drinking water. *Saccharomyces cerevisiae* yeast have been used most extensively in live stock (Patterson and Burkholder 2003). Yeast cells are mixed with non-pathogenic bacteria to competitively exclude pathogenic bacteria. Addition of recombinant yeast producing HA protein as a probiotic component would be cost effective and an efficient way to immunize large number of commercial poultry. We have previously shown that a yeast-derived recombinant sigma C protein induced immunity against avian reovirus (ARV) when given orally to young chickens (Wu et al. 2005). Here we present a strategy for producing a recombinant HA protein expressed in *S. pombe* with hemagglutination activity and structural integrity comparable to normal HA protein, which could be used as subunit vaccine against AIV.

Materials and methods

Propagation and extraction of viral RNA

The AIV H10N7, A/Northern Shoreler/AL/26/2006 (Dormitorio et al. 2009) isolated from a wild duck in Alabama, was inoculated into 9-day-old specific pathogen free (SPF) embryonated chicken eggs via the allantoic cavity. Eggs were incubated at 37°C and candled daily. Embryos, which died within 24 h, were discarded. Allantoic fluid was collected and tested for AIV by the HA test (Thayer and Beard

1998). Positive for HA fluid, was collected and used for viral RNA extraction with a Trizol RNA extraction kit (Invitrogen, Carlsbad, CA).

Construction of yeast expression vector with cDNA encoding HA and expression of recombinant HA protein in yeast

Full-length HA coding sequence was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using sense primer Bm-HA-(TATTCGTCTCA GGGAGCAAAAGCAGGGG) and antisense primer Bm-NS-890R (ATATCGTCTCGTATTAGTAGAAA CAAGGGTGTTTT). RT-PCR was carried out following the manufacturer's protocol (Invitrogen, Carlsbad, CA) using the following program; 50°C for 30 min, followed by 34 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 1 min each cycle, and one cycle of 10 min at 72°C. The PCR product was examined by 1% agarose gel electrophoresis and purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA). A 1.8 kb amplified fragment was cloned into a 6 kb yeast expression vector, pNMT1-TOPO (Invitrogen, Carlsbad, CA). The construct was a 7.8 kb plasmid designated as pNMT1-HA. The pNMT1-HA construct was mobilized into competent *Schizosaccharomyces pombe* cells (Invitrogen, Carlsbad, CA). Transformed yeast was selected on plates containing thiamine on EMM medium (Invitrogen, Carlsbad, CA).

A single isolated colony of *S. pombe* containing pNMT1-HA construct was used to inoculate 50 ml EMM thiamine medium and grown overnight at 30°C with continuous shaking. Cells were harvested by centrifugation at 1,500×g for 5 min and washed twice with EMM medium and suspended in 10 ml EMM + thiamine. A quantity of 500 µl aliquots of washed cells were used to inoculate fresh 50 ml EMM medium + thiamine and grown for 18 h at 30°C with constant shaking. Cells were harvested by centrifugation at 1,500×g for 5 min at 4°C and washed with 10 ml TE buffer (Tris EDTA pH 8.0) containing 100 mM NaCl. Cells were centrifuged at 1,500×g for 5 min at 4°C and the pellet suspended in 1 ml TE + 100 mM NaCl in a microfuge tube. The tubes were centrifuged for 2 min at top speed in the microcentrifuge. The supernatant was removed and 400 µl of acid-washed glass beads were added to the yeast cells. Cells were broken apart using a Bead Beater (Scientific Industry Co., Bohemia, NY) at a

maximum speed for 45 s. Tubes were placed on ice for 5 min and this procedure repeated five times. Cells were centrifuged in a microcentrifuge for 2 min at maximum speed, and the supernatants were collected in a fresh tube. Protein concentration was determined by the Bradford method.

Purifications of recombinant HA protein

The recombinant HA protein was purified using ProBond purification system (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Eight milliliters of supernatant was added to the column. The protein in the supernatant was bound for 30 min followed by centrifugation at $800\times g$ for 5 min. The column was washed with 8 ml native buffer (Invitrogen, Carlsbad, CA) and centrifuged at $800\times g$ for 5 min. The column was washed three more times as previously indicated. The column was clamped in a vertical position and the cover at the lower end removed. The protein, bound to the column, was eluted with 8 ml native elution buffer (Invitrogen, Carlsbad, CA). Eluted fractions were analyzed with SDS-PAGE and Western blot.

SDS-PAGE and Western blot

Purified protein and cell lysates were electrophoresed using SDS-PAGE on precast 10–20% gradient mini-gel from Bio-Rad. The MW was determined by plotting the R_f value against the standard curve generated from the migration of the MW standards. Proteins from the gel were transferred onto the PVDF membrane using Bio-Rad electroblotter. Protein on PVDF membrane was probed with anti-HA monoclonal antibody followed by goat-anti-mouse HRP-conjugated secondary antibody. Antibody was detected using a chemiluminescent substrate (Amersham Pharmacia Biotech Inc.). Image of the band on PVDF membrane was developed using X-ray film.

Assay of hemagglutination activity

Hemagglutination activity assays were carried out on chicken red blood cells with purified yeast derived recombinant HA protein in V-bottomed microwell plastic plates by two-fold serial dilution method (Thayer and Beard 1998).

Atomic force microscopy imaging

The 2-D and 3-D scanning pictures of purified recombinant protein was observed using AFM. Purified protein was dialyzed against double-distilled H_2O , lyophilized and dissolved in water to give 1 mg protein/ml. One microliter purified recombinant HA protein and 49 μ l water were deposited onto a microscope slide and air-dried for 30 min under a clean dry airflow until the surface was dry. The sample was examined under AFM (Nanoscope R2, Pacific Nanotechnology, Santa Clara, CA). The contact mode and standard silicon cantilevers ($450 \times 20 \mu$ m) were employed for imaging. The cantilever oscillation frequency was tuned to the resonance frequency of 256 kHz. Set point voltage was adjusted for optimum image quality. Both height and phase information were recorded at the scan rate of 0.5 Hz and in 512×512 pixel format. AFM images, containing HA protein in a large scanning area, were processed using NanoRole software (Pacific Nanotechnology, Santa Clara, CA).

Results

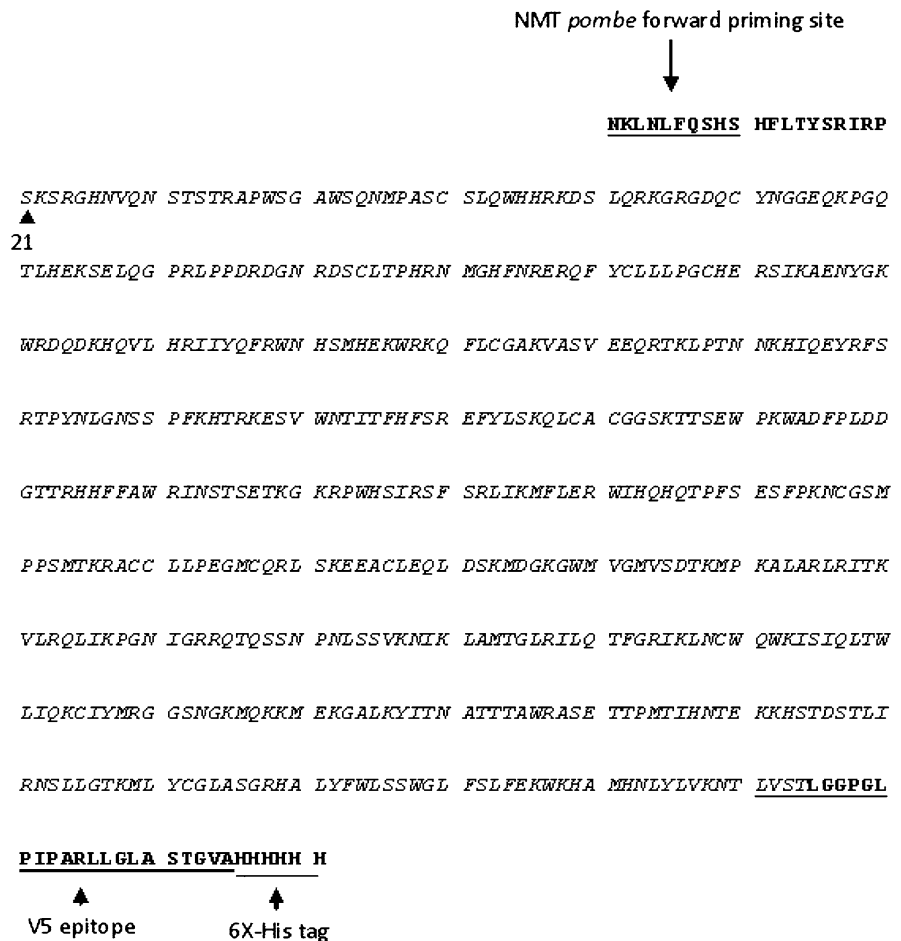
Construction of yeast expression vector pNMT1-HA

The cDNA encoding HA protein was amplified and yielded an 1,807 bp product, which was cloned into the yeast expression plasmid. The restriction endonuclease digestion pattern showed that the gene was inserted in correct orientation into the pNMT1 vector. The DNA sequence analysis showed an in-frame fusion within the expression cassette (data not shown). The deduced amino acid sequence of insert is shown in Fig. 1. Following transformation of pNMT1-HA plasmid into *S. pombe*, Three randomly selected colonies containing pNMT1-HA were screened for HA cDNA insertion. All three clones produced expected PCR amplicon 1,807 bp.

Expression of recombinant HA protein in *S. pombe*

A protein with 69 kDa was visualized on SDS-PAGE from yeast lysates (not shown) and Western blot showed a positive cross reaction of this protein with

Fig. 1 Deduced amino acid sequence of recombinant HA protein cloned in pNMT1-HA construct. HA (21–555) of H10N7 was fused in-frame with NMT *S. pombe* forward primer site at the N-terminus, and V5 epitope and 6-His tag at the C-terminus



anti-AIV HA monoclonal antibody (Fig. 2). The ratio of recombinant protein to total soluble protein expressed in *S. pombe* was estimated to be 0.2% as determined previously (Wu et al. 2004a).

Detection of hemagglutination activity

Three randomly selected clones showed expression of recombinant HA protein with high hemagglutination activity (Fig. 3). The hemagglutination activity of positive clones was determined to be in the range of $2\text{--}3 \times 10^5$ units per mg total purified protein. No hemagglutination activity was observed in the negative control containing extract of *S. pombe* without pNMT1-HA. Variation of the hemagglutination activity between the different clones may reflect the differences in HA protein expression level or its stability after extraction.

Scanning images of HA protein using atomic force microscope

The 2-D and 3-D scanning topology of recombinant HA protein dissolved in water was examined under AFM. Figures 4 and 5 shows recombinant HA protein has two subunits linked together showing different dynamic water contact angles (DCA). DCA for peak 1 was 0.82° , and for peak 2 it was 27.78° . DCA values show hydrophilicity or hydrophobicity of protein surfaces, with lower values indicating higher hydrophilicity (Teraya et al. 1990). The subunits of recombinant HA protein showed rough surfaces in 3-D images with a root mean square roughness of 4.7 nm. The projected size of the protein using a scan rate of 0.5 Hz indicated that the protein is ~ 69 kDa, where line 1 measured a distance of 3.2 nm and line 2 measured 1.9 μm .

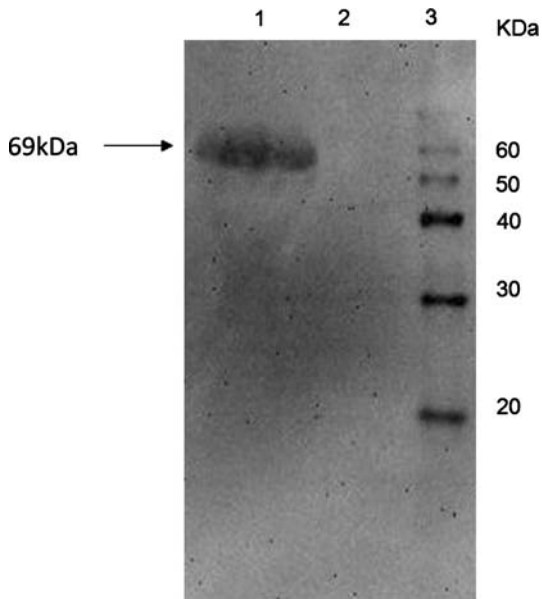


Fig. 2 Identification of *S. pombe* expressed H protein using Western blot. Lane 1 shows recombinant H protein from *S. pombe* cell lysate containing pNMT1-HA; lane 2 shows the *S. pombe* cell lysate containing empty vector pNMT1-TOPO; and lane 3 the Magic marker (Invitrogen, Carlsbad, CA)

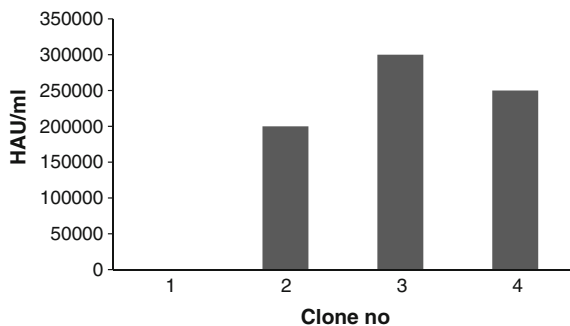


Fig. 3 HA activity of *S. pombe* expressed protein. Culture supernatants of pNMT1-HA clones expressed in *S. pombe* were assayed for HA activity. The experiment was done in triplicate. The unit of HA activity was the average value and standard deviation of the measurement is typically ± 0.0002 . Clone 1 was the negative control *S. pombe* containing the pNMT1 empty vector. HAU represent the unit of hemagglutinating activity

Discussion

Expression of foreign proteins in a heterologous system provides an alternative for production of viral antigens. Since *S. pombe* is a eukaryotic cell, which is unicellular, it allows for proper folding and post-translational modification of recombinant proteins in

large amounts and is comparatively simpler than mammalian or insect cell expression (Wu et al. 2005).

Despite mass culling of birds and use of vaccines, AIV remains a major problem in poultry industry and a threat to human health. Vaccines that can be injected into the birds are not suitable for commercial poultry, except in the hatchery. However, vaccines produced in yeast could be propagated and given in mass via the drinking water to commercial poultry.

The HA glycoprotein is the major antigenic determinant of the influenza virion and has both the receptor-binding and fusion activities. The recombinant HA protein is 69 kDa in size (Fig. 2). This is about 6 kDa larger than the expected native HA protein, because of the presence of a ~ 6 kDa His-tag and V5 epitope at the end. The detection of hemagglutination activity of the recombinant protein confirmed an important aspect of HA protein function. Influenza virus HA protein is synthesized as a precursor, where HA₀ is cleaved to the disulfide-linked subunits HA₁ and HA₂ to become biologically active (Choppin et al. 1975; Klenk et al. 1975; Lazarowitz and Choppin 1975).

Atomic force microscope has been used to obtain high-resolution images of virus that have been immobilized on a variety of surfaces such as glass, mica, silicon, and Lang-muir-Blodgett films (Kuznetsov et al. 2001; Ikai et al. 1993). AFM permits discrimination among viruses based on their shape and size. High-resolution AFM images of the viral capsomer packing patterns and triangulation numbers have been obtained (Kuznetsov et al. 2001). This facilitates discrimination between viruses of similar sizes. AFM images have been extensively used to study biological materials at different levels of structural organization. AFM of proteins absorbed on surfaces has been hampered by the mobility of absorbed molecules and their inherent flexibility. A locally rigid protein system will have stability and provide a means for assessing resolution limits of the AFM for biological systems. Image of the surface structure of the yeast expressed HA protein dissolved in water and immobilized on microslide was obtained at high resolution. It is known that the precursor of HA protein (HA₀) must be processed to disulfide-linked biologically active subunits HA₁ and HA₂. The HA₁ subunit is an important antigenic epitope which is located at the distal end of the membrane receptor binding pocket, whereas the

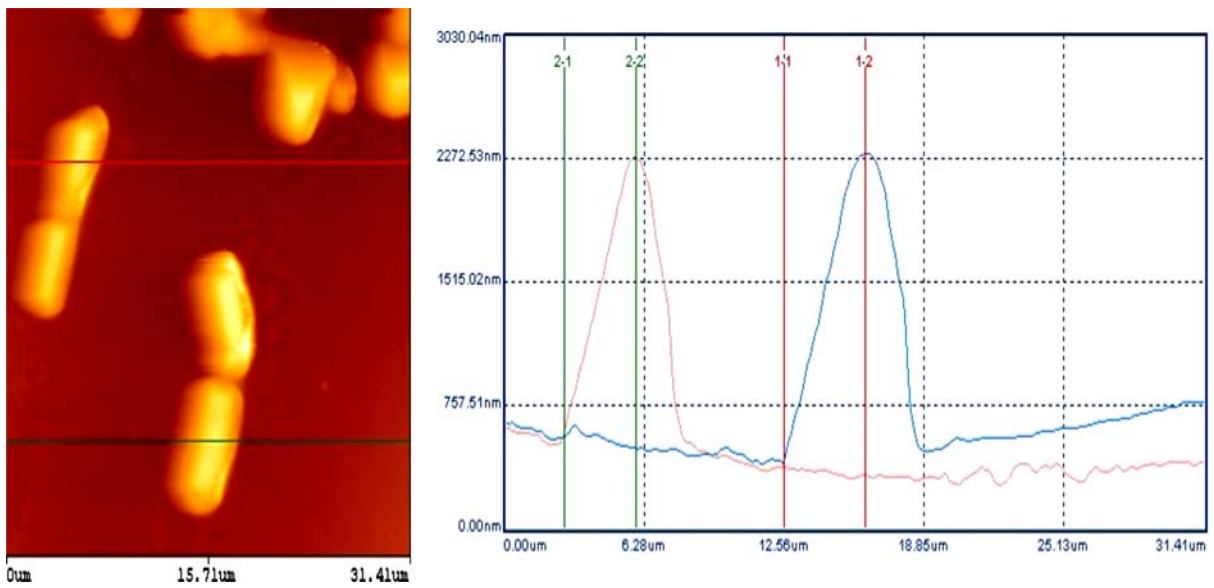


Fig. 4 2-D scanning image of recombinant HA protein observed under AFM. The image was scanned under 450 μm in length, 20 μm in widths and 256 kHz, the line profile

showed that the two prominent peak in the 2-D structure occurs at 6.28 and 16.28 nm. Supplementary information regarding this figure is given as supplementary Table 1

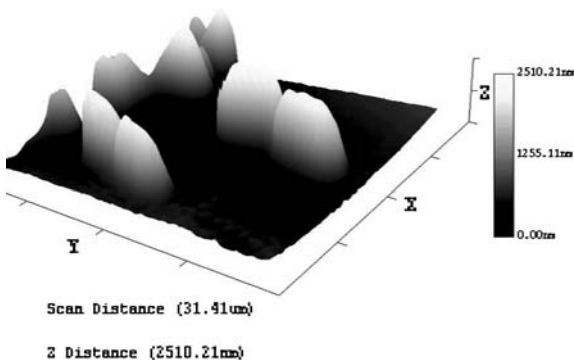


Fig. 5 3-D scanning picture of recombinant HA protein observed with AFM. The image was scanned under 450 μm in length, 20 μm in widths and 256 kHz, scanning distance was 31.41 μm and Z distance was 2510.21 nm

HA₂ subunit has hydrophobic N-terminal fusion peptide anchored into lipid bilayer by its C-terminal transmembrane domain (Choppin et al. 1975; Klenk et al. 1975; Lazarowitz and Choppin 1975; Wang et al. 2007). The AFM image of the recombinant HA protein shown in Figs. 4 and 5 compares well with previous structural studies on HA protein (Choppin et al. 1975; Klenk et al. 1975; Lazarowitz and Choppin 1975; Wang et al. 2007). The AFM scanning images of the purified recombinant HA protein showed that the two processed HA₁ and HA₂ subunits are linked

(Figs. 4, 5). The DCA value of peak 1 is low whereas peak 2 has high DCA value. This would suggest that peak 1 is hydrophilic and likely HA₁ subunit and peak 2 is hydrophobic and HA₂ subunit, respectively. The recombinant HA protein showed roughness on its surface as shown in Fig. 5. The surface roughness represents short lengths of amino acid chains projecting on the surface (Hoshi et al. 2008). Our result demonstrates that *S. pombe* cells are capable of producing required cleavage of the HA₀ precursor protein to produce HA₁ and HA₂ subunits.

These observations confirmed the authentic expression of recombinant HA protein and its correct processing in *S. pombe*. We have previously demonstrated that oral administration of recombinant VP2 protein against infectious bursal disease virus of poultry (Wu et al. 2004b) and recombinant sigma C protein against ARV (Wu et al. 2005) have induced immune responses in chickens. Our results suggest that the development of a recombinant vaccine against AIV in yeast for poultry is a sound idea and is likely to be successful. We are in the process of going to the next level where oral immunization of chickens with recombinant HA protein and/or use of transgenic yeast producing HA protein will be performed. This will be followed by the determination of immune responses as measured by antibody titer in the serum of immunized

birds as well as resistance to challenge with live virus. These results may lead toward the development of a commercial recombinant vaccine against AIV.

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