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Production of Influenza Virus HA1 Harboring Native-Like Epitopes by *Pichia pastoris*

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Abstract The outbreak of the H5N1 highly pathogenic avian influenza which exhibits high variation had brought a serious threat to the safety of humanity. To overcome this high variation, hemagglutinin-based recombinant subunit vaccine with rational design has been considered as a substitute for traditional virion-based vaccine development. Here, we expressed HA1 part of the hemagglutinin protein using the *Pichia pastoris* expression system and attained a high yield of about 120 mg/L through the use of fed-batch scalable fermentation. HA1 protein in the culture supernatant was purified using two-step ion-exchange chromatography. The resultant HA1 protein was homogeneous in solution in a glycosylated form, as confirmed by endoglycosidase H treatment. Sedimentation velocity tests, silver staining of protein gels, and immunoblotting were used for verification. The native HA1 reacted well with conformational, cross-genotype, neutralizing monoclonal antibodies, whereas a loss of binding

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activity was noted with the denatured HA1 form. Moreover, the murine anti-HA1 serum exhibited a virus-capture capability in the hemagglutination inhibition assay, which suggests that HA1 harbors native-like epitopes. In conclusion, soluble HA1 was efficiently expressed and purified in this study. The functional glycosylated protein will be an alternative for the development of recombinant protein-based influenza vaccine.

Keywords Hemagglutinin · *Pichia pastoris* · Fed-batch fermentation · Monoclonal antibody · Native epitopes

Introduction

Recurrence of avian influenza outbreaks caused by the orthomyxoviridae influenza virus is of global concern, particularly for Asian countries mainly engaged in poultry trading and farming, in terms of the pandemic nature of the virus in posing immediate threat to human health. Breakthroughs in proteomics, including X-ray crystallography, cryo-EM, and structure analytical software, have identified that the HA glycoprotein contains the major surface epitopes of the influenza virus, with a trimetric arrangement comprising three homo-monomers encoded by the HA0 gene [1–3]. This HA0 monomer, synthesized by the infected host cells, must be cleaved into two parts—N-terminal HA1 and C-terminal HA2—to allow for the generation of functionally active viruses.

Traditional vaccines, including inactivated and live attenuated vaccines, have been developed extensively for protection against avian influenza [4, 5]. Structural determination has revealed that the high mutation rate on the globular head region of the HA protein (HA1) contributes to the strong virulence and high variability of influenza viruses [6, 7]. However, the ability to predict such mutational tendency remains low, and it is often not feasible to create a large-scale culture of a strain that is determined to likely have pandemic potential for traditional vaccine production. Despite these limitations, the current research points to the role of HA proteins as key targets for influenza virus infection and prevention. Genetically engineered vaccines comprising recombinant antigens are considered as ideal substitutes for traditional vaccines because of their controllability and stability. For flu vaccine, recombinant HAs that are later scaled up for industrial production are proposed to be used as a vaccine, such as Flublok [8]. Preclinical animal models, such as mouse or ferret, have also shown promising results [9–12], and yeast and insect cells have been widely used as recombinant protein factories to produce proteins of interest [13–15]. To date, it is known that several human recombinant vaccines have been successfully made with such systems, including the human papillomavirus vaccine (HPV) [16, 17] and the hepatitis B vaccine (HBV) [18, 19]. Therefore, there has been a tendency to produce native-like subunit antigens from recombinant system in place of traditional vaccine production methods.

Eukaryotic systems often come with a compromise of a much lower expression efficiency and production level, due to this complicated modification process. Fed-batch fermentation technology provides an alternative method to overcome this disadvantage. Indeed, high-density fermentation offers a key advantage to yeast, reaching a similar protein expression level as compared with that generated using bacterial systems such as *E. coli* [20, 21].

In this study, the HA1 gene construct of influenza virus H5N1 was made and expressed in *Pichia pastoris*. Using methods described by Yang et al. [22], the sequential scaling up of fermentative batches gives improved expression of rHA1. Here, we purified the excreted rHA1

protein from the *P. pastoris* culture supernatant with ion column chromatography and investigated the protein conformation and bioactivity by direct ELISA and mouse immunization. A hemagglutinin inhibition assay was also performed to evaluate the reactivity of the antisera produced with rHA1 to the native influenza viruses. The priority focus of this study was to develop an effective, stable, and practical method for batch process scale-up in *P. pastoris* and the purification of soluble and functionally active influenza antigens that could then be used as an alternative for development of vaccine or diagnostic reagent.

Materials and Methods

Strains and Plasmids

P. pastoris GS115 and *E. coli* DH5α were purchased from Invitrogen (Carlsbad, CA, USA). The HA1 gene (a.a. 1–338; GenBank GI 82053774), derived from avian influenza viruses A/CK/HK/Yu22/2002 (YU22, H5N1, clade 8), was kindly provided by the Department of Biology, University of Hong Kong. For expression of *P. pastoris* GS115, the HA1 was cloned into the expression vector pPIC9K (Invitrogen, USA), hereafter referred to as pPIC9K-rHA1.

Transformation and Expression

Recombinant plasmid pPIC9K-rHA1 was linearized by SalI digestion and transformed into *P. pastoris* GS115 by electroporation (Bio-Rad Gene Pulser; Bio-Rad, Hercules, CA) at 1.5 kV, 25 μF, and 2000 Ω. Transformants were grown on yeast extract peptone dextrose agar plates. After 3 days in culture, recombinant colonies were selected and screened for HA integration by colony PCR using forward and reverse AOX primers. PCR products were analyzed by 1 % agarose gel electrophoresis. The recombinant strains integrating the HA1 gene of interest were picked and incubated in yeast extract peptone dextrose medium until the optical density reached 0.8–1.3. The strains were then transferred to buffered glycerol-complex medium (BMGY) for expansion for 24 h at 28 °C and then transferred to buffered methanol-complex medium (BMMY) at 25 °C for induction. The sampling of supernatants were done every 12 h, and the culture medium was supplemented with 1 % (*v*/*v*) methanol every 24 h as a carbon source. The supernatant was collected to determine the expression level of the rHA1 by quantitative sandwich ELISA (coated antibody 13H8/1A6; enzyme-labeled antibody 3G4) [22]. The pPIC9K-transformed *P. pastoris* GS115 was used as a negative control.

Fed-Batch Fermentation

The rHA1-expressed recombinant strains were amplified in 1-L shaking flasks with 300-ml medium until $OD_{600\ nm}$ reached ~4. The recombinant strains were then transferred into a fermenter using fermentation culture medium as per the protocol from Invitrogen (Catalog #K171001). The oxygen level was maintained at 30 % by regulating the air flow and degree of agitation. The fed-batch fermentation process was divided into three phases: glycerol growth phase, glycerol fed-batch phase, and methanol fed-batch phase. In the glycerol growth phase, nutrients were consumed by the recombinant *P. pastoris* constantly and the biomass underwent expansion. After 12 h of culture, the initial glycerol in the batch was depleted, as determined by the abrupt increase in the dissolved oxygen levels. In the second phase, 50 % (*w*/*v*) glycerol

was fed at a rate of 15 ml/h for 1 h. When the $OD_{600\ nm}$ reached ~250, the glycerol feed rate was set to decrease linearly from 15 ml/h to 0. For the next 1 h, no new nutrients were supplemented; during this time, the dissolved oxygen levels began to increase rapidly. Following this, 40 ml methanol was injected into the fermentation culture system to make the *P. pastoris* adapt. This injection was performed three times until the dissolved oxygen levels decreased to 30 %. After the *P. pastoris* had adopted to the methanol, methanol was fed at a rate of 15 ml/h and programmed to increase every 3 h at the rate of 15 ml/h. During the third phase, the product of interest was expressed in the supernatant and this product was collected for subsequent analysis.

Purification of the HA1 Protein and Its Deglycosylation Assay

The products of interest in supernatant were eluted from cation ion column with PB 6.0 (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 6.0) containing 1 M NaCl and then flew through from the anionic column (buffer: PB 8.0). SDS-PAGE and western blotting were used to analyze the purity of rHA1. Furthermore, according to manufacturer's instructions, the purified HA1 was then treated by endoglycosidase H (EndoH) digestion (New England BioLabs, Ipswich, MA). Briefly, 200 ng HA1 protein was denatured with 5× reaction buffer and 2 % SDS in 100 °C water bath for 5 min and then treated with 2 μL EndoH at 37 °C for 1 h, prior to SDS-PAGE analysis with silver staining.

Analytical Ultracentrifuge (AUC) Assay

The AUC assay was performed using a Beckman XL-Analytical ultracentrifuge (Beckman Coulter, Fullerton, CA), as described elsewhere [23]. The sedimentation velocity (SV) was carried out at 20 °C with the diluted rHA1 (1 mg/ml) in PB 6.0. The rotor speed AN-60 Ti was set to 60,000 rpm, in accordance with the theoretical mass size of 37 kDa for the highest resolution. Data was collected using SEDFIT computer software [24], fitting multiple curves to calculate the sedimentation coefficient C (s) and the f/f0.

In sedimentation equilibrium (SE) experiments, rHA1 was diluted to 0.12 OD, 0.3 OD, and 0.6 OD in PB 6.0, raising the speed of the same rotor from 14,000 rpm gradually to 42,000 rpm. The equilibrium time at first was 24~48 h, followed by 18 h in the subsequent steps. The data from the SE experiment were analyzed and fit to a globular model using a nonlinear fit [25] by Origin (OriginLab, USA) and SEDPHAT [26] software, and the fitted molecular mass was acquired.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The mass spectra of the rHA1 sample was measured using the Reflex III MALDI-TOF mass spectrometer (Burker Daltonik, Bremen, Germany) with α-cyano-4-hydroxy cinnamic acid as a matrix prior to the MS analysis.

Generation of Anti-H5N1 Monoclonal Antibodies (mAbs) and Preparation of rHA1 Mice Sera

In this study, the designed experimental procedures were firstly approved by the Xiamen University Laboratory Animal Management Ethics Committee. All procedures were

conducted in compliance with animal ethics guidelines and approved protocols. Anti-H5 mAbs were produced using standard hybridoma technology [27]. Six-week-old female BALB/c mice were first immunized with formalin-inactivated YU22. At the following two-times strengthened immune four virus types—A/DK/VNM/S654/2005 (clade 1), A/DK/IDN/MS/2004 (clade 2.1), A/BH/Goose/QH/15C/2005 (clade 2.2) and A/DK/VNM/568/2005 (clade 2.3)—were selected to replace YU22 based on genetic analysis. The specific anti-H5N1 antibody was screened by HA inhibition assay against YU22. Limiting dilution analysis was carried out to dilute fused cells into monoclonal cells. The mAb-producing monoclonal cell was amplified and injected into BALB/c mice to generate anti-H5N1 mAbs. The anti-H5N1 monoclonal antibodies were purified from ascites fluid using protein A chromatography, as previously described [28].

The rHA1 protein immunization process was as follows. Six 6-week-old female BALB/c mice were chosen, and the serum was collected before immunization. Mice were immunized via the subcutaneous delivery of 100 μg HA1 protein absorbed onto Freund's incomplete adjuvant (Sigma-Aldrich, St Louis, MO). All mice were given two booster doses of HA1 at 2-week intervals. After 5 weeks, the serum of immunized mice was collected to test for HI activity and blocking ELISA analysis.

Blocking ELISA Assay

Individual wells of the ELISA plate were coated with 100 ng of two anti-H5N1 monoclonal antibodies (13H8/1A6) in PB 7.4 for 2 h at 37 °C. The same 4HA units of the virus YU22 were captured, prior to incubation with the tested serum. After washing, the plates were incubated with four anti-H5N1 IgGs (13D4, 10F7, 8H5, and 8G9 [29]) conjugated to HRP (Dingguo, China) and the absorbance was read (within 10 min) at 490 and 620 nm using an ELX800 Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT). Pre-bleed serum, HPV antibody 1D12 (produced in our lab), and phosphate-buffered saline (PBS) were used as negative controls.

Hemagglutination Inhibition (HI) Assay

The immunized mouse serum was firstly diluted 1:10 with PBS, and the same 4HA unit viruses was separately mixed with serial 2-fold dilutions of the 1:10 diluted sera for 30 min at room temperature. To this mix, 50 μL 1 % (*v*/*v*) chicken erythrocyte cells were added and incubated for a further 30 min at room temperature. The titer was taken as the highest dilution in which there was complete inhibition of hemagglutination. Wells containing PBS as substitute for sample were set as the negative control.

Results

Expression and Purification of Recombinant HA1

The globular head of influenza type A virus HA (encoded by HA1 gene) harbors key antigenic sites and receptor binding sites [30]. In this study, the gene of the full-length HA1 (a.a. 1–338) derived from influenza A virus H5N1 A/CK/HK/Yu22/02 was expressed using the pPIC9K vector (Fig. 1). High-density *Pichia* fermentation of rHA1 at the 10-L scale was achieved in four different batches with OD_{600} at ~300. The soluble rHA1 protein in the supernatant was

shown to be 123~140 mg/L (Table S1), implying a reproducible and robust fermentation process.

The purity of the final product was estimated at ~90 % in SDS-PAGE. However, rHA1 was detected as several bands of ~30 kDa by western blotting (Fig. 2a), indicating different levels of glycosylation [33, 34]. Deglycosylated rHA1 protein (after EndoH treatment) migrated as two bands, as detected by immunoblotting with anti-H5N1 rabbit polyclonal (p)Abs or mouse mAbs (Fig. 2b, lane 5). The deglycosylated rHA1 still exhibited different partially glycosaylated form and could be resolved in SDS-PAGE, which might be due to partial cleavage by EndoH enzyme. The two chromatography steps (described in the "Materials and Methods" section) removed effectively any impurities in the sample, yielding rHA1 protein of high purity.

A direct binding ELISA experiment was performed to characterize the conformation and bioactivity of the purified rHA1 using the polyclonal antibodies induced by H5N1 virus. As shown in Fig. 2c, there lots of rHA1 were produced with great antigenic consistency. Following denaturation, the reactivity between the denatured rHA1 and rabbit antisera showed a notable decrease in the OD_{450} value using ELISA assay as compared with that of the native rHA1 (Fig. 2d). This denaturation treatment disrupted some of the epitopes of *P. pastoris*-derived HA1, leading to lower binding reactivity of antisera to the surface-immobilized antigen.

Molecular Weight and Hydrodynamic Behavior of the Recombinant HA1

AUC was used to analyze the purified protein in the aqueous state to determine protein hydrodynamic behavior in solution. SV analysis showed that the target protein existed with a sedimentation coefficient about 3.07 S in solution (Fig. 3a). The frictional coefficient f/f0, which reflects the macromolecular shape in solution, had a fitting value 1.23 for rHA1, which reflects that of a globular protein (~1.2). Moreover, the molecular weight of the target protein was 36.872 ± 1.353 kDa, as calculated by the SE, and this result was consistent with the theoretical value of 38.000 kDa for monomeric HA1 (Fig. 3b). The molecular weight of rHA1 protein produced in the three batches was consistent, as determined by MALDI-TOF, with a mean molecular weight of 35.15 kDa (Table S1, Fig. 3c). Both of the fitting results showed that rHA1 existed as a monomer in solution.

Quantitative Immunochemical Analysis of the Purified rHA1

A panel of 93 mouse mAbs (produced with a natural influenza virus in-house) was used to detect the conformation and bioactivity of rHA1. In this panel, 20 mAbs (including 13D4, 8H5, 8G9) were shown previously to cross-react with the H5N1 virus or hemagglutinin inhibition activity [29, 35]. The 93 mAbs exhibited different affinities to rHA1 based on their endpoint titers (Table S2). Since these mAbs were developed using inactivated viruses as immunogens and as screening antigens or viruses in HI assays, their reactivity to *P. pastoris*-derived rHA1 indicates rHA1 bears resemblance to H5 hemagglutinin in the authentic virus.

Eight mAbs with higher affinities to rHA1 (Table S2) were selected as binding antibodies to further investigate the conformational integrity of rHA1. The reactivity (OD_{450} value) between denatured rHA1 (mentioned in Fig. 2) and these mAbs were markedly decreased as compared with that of the native rHA1, as determined by direct ELISA (Fig. 4a, b). The binding affinities (reflected by EC_{50}) of these mAbs—with the exception of 3G4—were three orders of

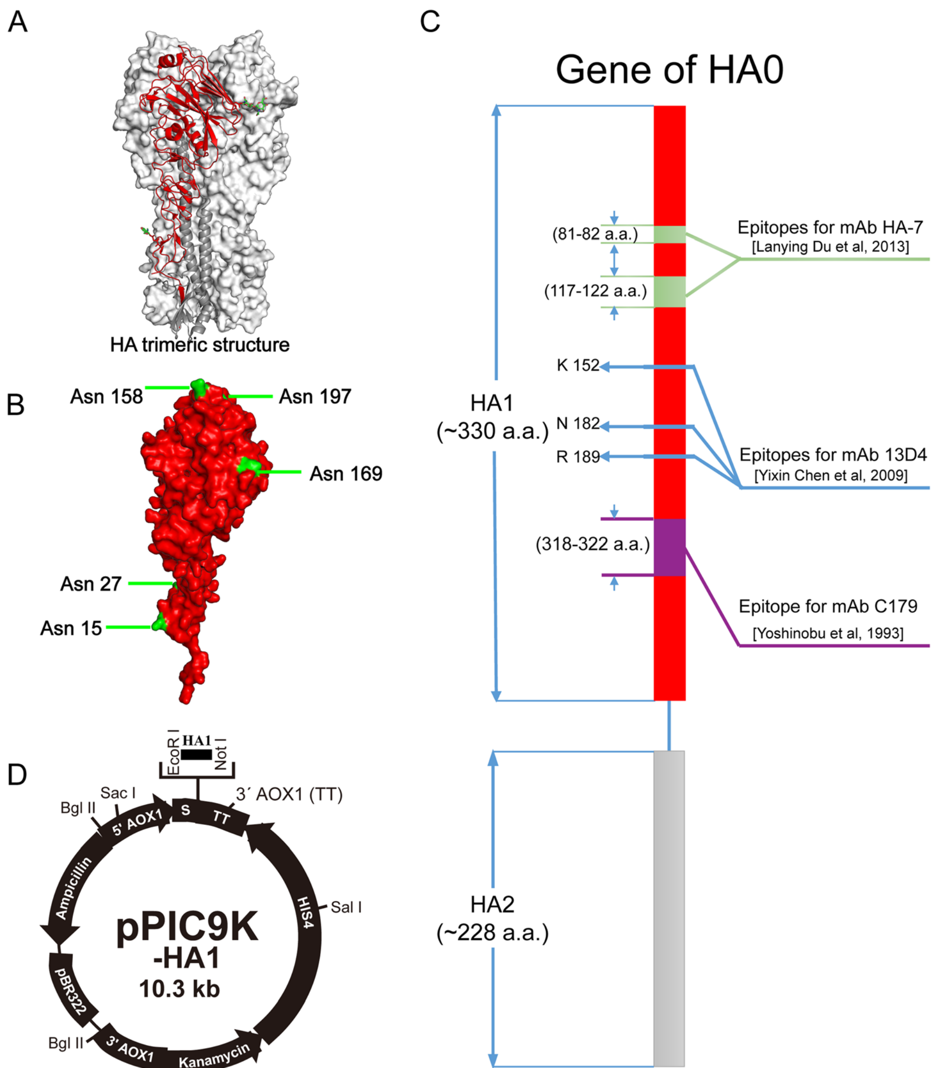


Fig. 1 Structure and epitopes of HA1, and cloning of HA1 gene for expression in *Pichia pastoris*. **a** Native hemagglutinin (HA) on the surface of influenza virus has a trimeric structure, as determined by X-ray crystallography (PDB No. 2IBX) (http://www.rcsb.org/pdb/home/home.do). **b** Five reported glycosylation sites on HA1 of hemagglutinin were shown and colored in *green*. **c** The HA1 head portion of the protein harbors key conserved epitopes of hemagglutinin, such as a.a. 81–82 and a.a. 117–122 epitopes for the broad neutralizing capacity of mAbs HA-7 [31]; a.a. 318–32 for mAb C179 [32]; or K152, N182, and R189 for mAb 13D4 [29]. **d** The full-length HA1 (a.a. 1–338) gene was inserted into the expression vector (pPIC9K) following the promoter AOX1 for overexpression in *P. pastoris* (color figure online)

magnitude lower following denaturation as compared with the native rHA1 (Table S3). Furthermore, three of the mAbs, 4A7, 8H5, and 17E6, were sensitive to denatured buffer treatment (with a 4300-, 4100-, and 1800-fold change, respectively). Other mAbs, including 8G9, 3C8, 10F7, and 13D4, showed high sensitivity to the conformational change, with a fold change of over 5000. These results imply that the *P. pastoris*-derived rHA1 possesses a certain conformation that is likely similar to the epitopes of the native virus.

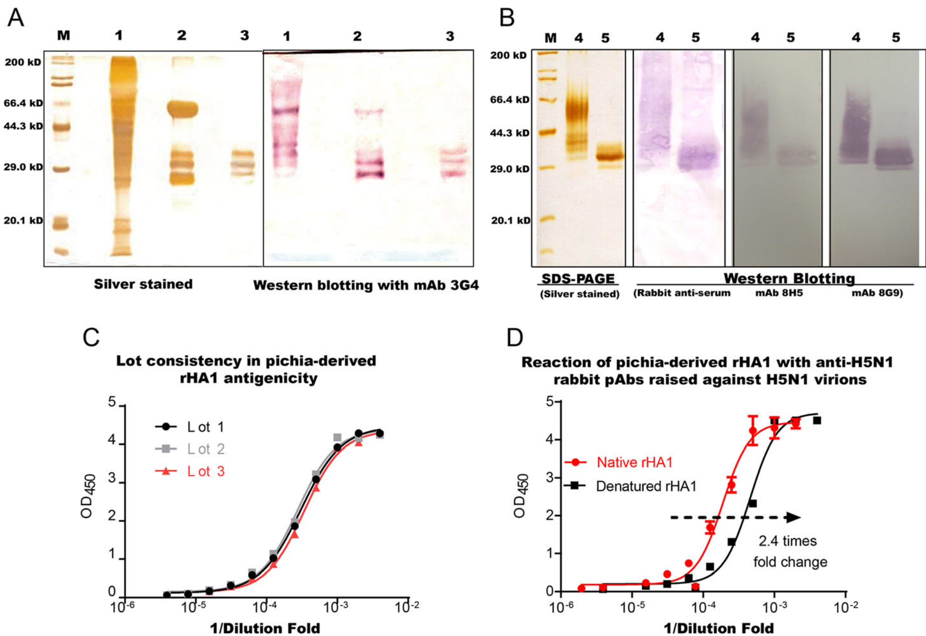


Fig. 2 Molecular and immunochemical characterization of recombinant HA1. The soluble rHA1 in the culture supernatant was purified in two steps using ion-exchange chromatography. Deglycosylation of rHA1 was examined through treatment with a deglycosylating enzyme, endoglycosidase H (EndoH). **a** *Left:* Silver-stained SDS-PAGE showing HA1 in culture supernatant, the purified intermediate, and the purified rHA1. *Right:* Western blot analysis using anti-H5N1 monoclonal antibody 3G4. *Lane M*: protein molecular weight marker; *lane 1*: culture supernatant; *lane 2*: rHA1 in eluent from the first step of the purification process (SPFF cation-exchange chromatography); *lane 3*: rHA1 in the penetration buffer after the second step of the purification process (DEAE anion-exchange chromatography). **b** Purified rHA1 was treated without (*lane 4*) or with (*lane 5*) EndoH and analyzed with immunoblotting for anti-H5N1 rabbit polyclonal antibody (immunized with A/CK/HK/Yu22/02 virus), or mouse mAb 8H5 or 8G9. **c** Antigenic consistency analysis of three lots of *Pichia pastoris*-derived rHA1 was measured by direct-binding ELISA (detecting antibody: rabbit polyclonal antibodies). **d** Serial dilutions of rabbit polyclonal antibodies were used to detect the antigenicity of native rHA1 and denatured rHA1 (treated by bicarbonate buffer, pH 10.5). There was a 2.4-fold reduction in binding affinity of the pAbs to the denatured rHA1 as compared with the native protein

Functional Analysis of Mouse Antisera Raised Against *P. pastoris*-derived rHA1

Here, we adopted some reported broadly neutralizing antibodies (bnAb), 8G9, 13D4, 8H5, and 10 F7, for blocking ELISA experiments to characterize the epitopes recognized by anti-rHA1 serum. As shown in Table 1, the blocking rates were 49.1 % for 13D4, 92.2 % for 10F7, 53 % for 8H5, and 78.2 % for 8G9. Most of the bnAbs showed a significant decrease in binding to the H5N1 virus, as caused by the anti-serum inhibition. The more than 50 % blocking rate suggested that the *P. pastoris*-derived HA1 could display native-like epitopes as the virion.

To analyze further the functional activity of rHA1, we investigated the binding of mouse anti-rHA1 serum and native influenza virus using an HA inhibition assay. As a negative control, the pre-bleed contained no anti-H5N1 antibody, and thus allowed demonstration of the HA reaction of the erythrocytes with the influenza virus. Six different clades of influenza viruses were selected and were all captured by anti-rHA1 serum. The HI titer against A/CK/HK/Yu22/02 virus was highest (1280; Fig. 5a), but this is not surprising, given that the rHA1 gene was derived from the YU22 virus. Against the other five influenza viruses—A/CK/HK/

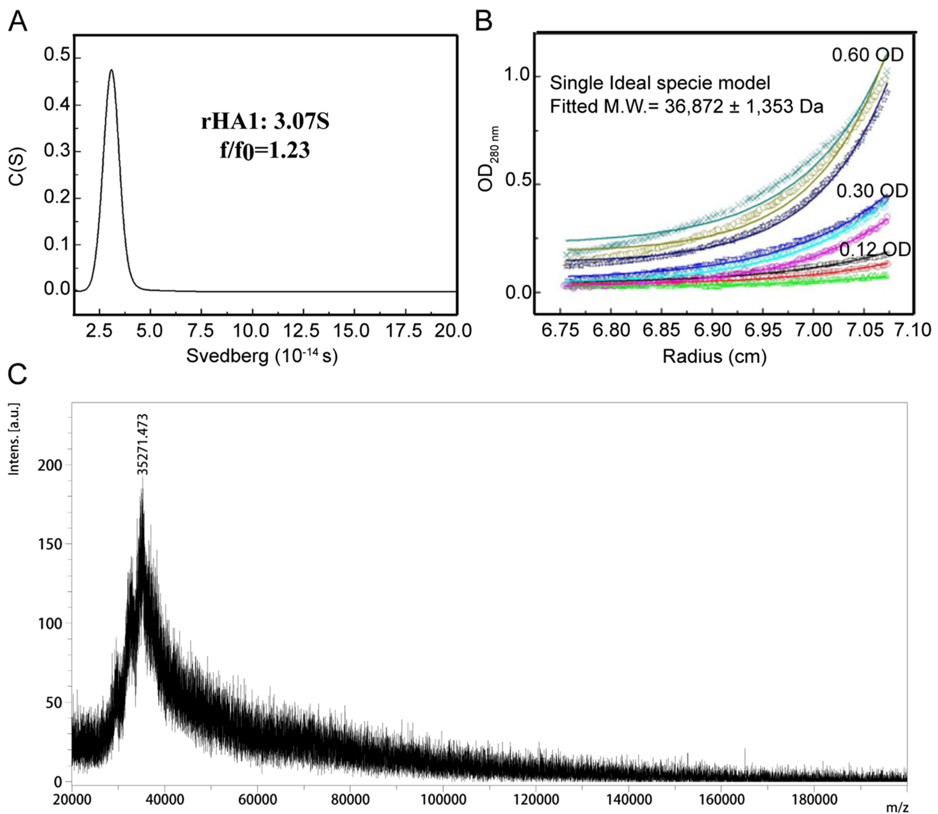


Fig. 3 Molecular size of recombinant HA1 in solution, as analyzed with analytical ultracentrifugation (AUC) and MALDI-TOF MS. **a** The sedimentation velocity analysis (*left*) of the rHA1 showed that it behaved as a single component with a sedimentation coefficient of 3.07 S and f/f0, a hydrated friction ratio, of 1.23, similar to that of a globular protein (1.2). **b** Sedimentation equilibrium (*right*) analysis indicated the rHA1 exists in monomeric form with a molecular weight of 36.872 ± 1.353 kDa, in good agreement with the theoretical value of 38.002 kDa. **c** MALDI-TOF-MS spectrum of rHA1 showed single major peak of molecular mass of about 35 kDa

213/03 strain from clade 1, A/VNM/1194/04 strain from clade 1, A/CK/GX/2439/04 strain from clade 5, A/CK/HK/Yu324/03 strain from clade 2.1, and A/Indonesia/5/05 strain from clade 2.1—the anti-rHA1 serum showed varied binding activities, with HAI titers of 320, 640, 320, 160, and 160, respectively (Fig. 5b). These results suggest that the rHA1 produced by *P. pastoris* maintained native-like epitopes and could induce antibodies with good reactivity to native virions.

Discussion

Avian influenza is drawing increasing attention because of its high mortality rates. Prophylactic vaccines from influenza viruses cultured in embryonic chicken eggs would provide the most effective method against avian influenza. However, the avian influenza virus is an RNA virus with high variability, and levels of a virus-based vaccine produced through this method

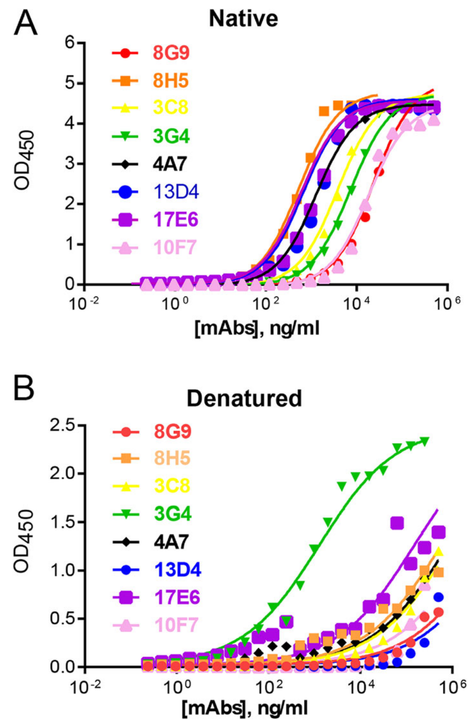


Fig. 4 Reduced binding of anti-H5N1 antibodies to denatured rHA1 as compared to native rHA1 in ELISA. Comparison of the antigenicity of rHA1 diluted in PBS buffer (native, **a**) and in bicarbonate buffer, pH 10.5 (denatured, **b**) with monoclonal antibodies. A panel of eight mAbs (refer to Table S2) with high affinity to rHA1 were selected for binding analyses. Data shown are the direct ELISA coated with native and denatured rHA1. Serial dilutions of the mAbs were used to bind to the coated rHA1, and the reaction was detected with the HRP-conjugated goat anti-mouse polyclonal antibody (GAM-HRP). The *line* represents the calculated line for a given data set in a four-parameter logistic fit to determine the EC_{50} value (Table S3) using GraphPad Prism software

are low, with reports of ~20–40 mg/200 eggs [36]. As a new type of vaccine, recombinant subunit vaccines are shown to be safe, easy to produce and with good production yield, and these vaccines have been used in humans successfully to date, with the advent of the recombinant HBV, HPV, and hepatitis E (HEV) vaccines [37]. Against influenza, the HA

Table 1 Blocking ELISA analysis of the presence of anti-H5N1 antibody in serum from mice immunized with rHA1

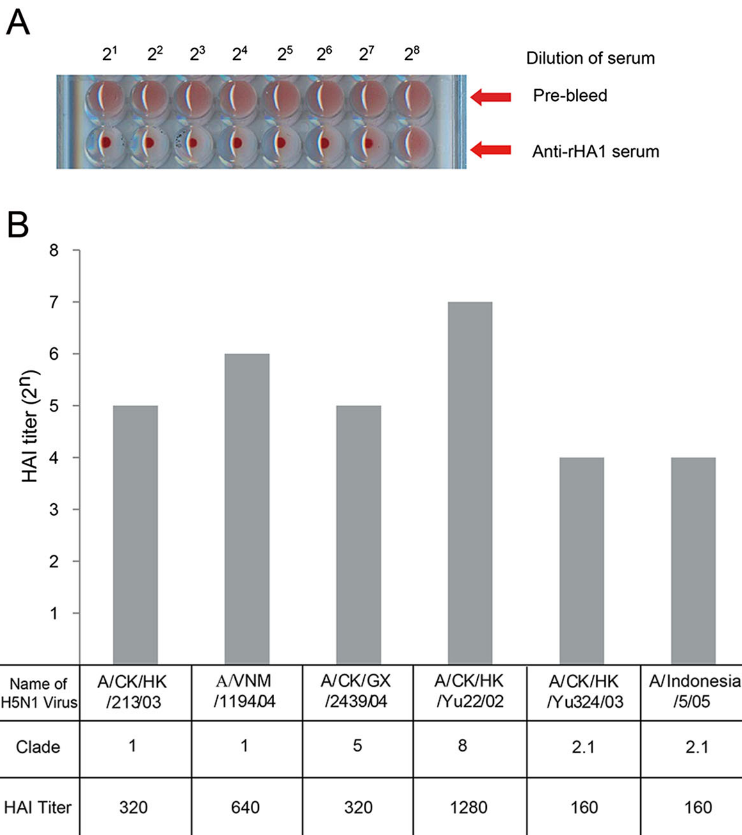
	Anti-H5 antibody labeled with HRP			
Poly-mAb/mono-mAb	13D4-HRP	10F7-HRP	8H5-HRP	8G9-HRP
Blank[a]	1.843[d]	1.434	2.232	1.289
HPV antibody (1D12)[b]	1.765	1.343	1.677	0.938
Anti-rHA1 serum	0.805	0.145	0.933	0.233
Pre-bleed	1.583	1.873	2.012	1.023
Percentage of blocking[c]	49.1 %	92.2 %	53 %	78.2 %

[a] Reaction with no antibody or serum blocking the HRP-conjugated antibody

[b] HPV-related antibody was used as the nonblocking interactant

[c] The percentage of blocking was calculated by the following formula: Percentage of blocking $= 1 - \frac{OD_{450(\text{anti-rHA1 serum})}}{OD_{450(\text{pre-bleed})}} \times 100\%$

[d] ELISA plates were coated with anti-H5N1 antibodies 1A6 (2 μg/mg) and 13H8 (2 μg/mg) to capture the influenza virus A/CK/HK/Yu22/02/H5N1. Plates were then incubated with saturating levels of anti-rHA1 serum (diluted 4-fold in phosphate-buffered saline) prior to the addition of HRP-conjugated antibodies (1 μg/ml). Data shown are the OD_{450} values



Name of H5N1 Virus	A/CK/HK /213/03	A/VNM /1194/04	A/CK/GX /2439/04	A/CK/HK /Yu22/02	A/CK/HK /Yu324/03	A/Indonesia /5/05
Clade	1	1	5	8	2.1	2.1
HAI Titer	320	640	320	1280	160	160

Fig. 5 Native-like immune response of rHA1 as reflected by hemagglutination inhibition (HI) activity of anti-rHA1 serum. The serum was developed using *Pichia pastoris*-derived rHA1 as the immunogen against different H5N1 strains. **a** An example of an HI activity experiment. The anti-rHA1 mouse serum was evaluated for the presence of anti-H5N1 antibody by the virus A/CK/HK/Yu22/02, which was captured to prevent hemagglutination. The HI titer of the anti-rHA1 serum was calculated as 1280. The control group, using the preimmunized serum without H5N1 antibody, displayed normal hemagglutination. **b** Six different subtypes of H5N1 viruses (A/CK/HK/213/03, A/VNM/1194/04, A/CK/GX/2439/04, A/CK/HK/Yu22/02, A/CK/HK/Yu324/03, A/Indonesia/5/05) were tested in the HI experiment and shows the broad spectrum of antisera raised against *P. pastoris*-derived rHA1

protein is considered as a main target for the design of recombinant protein-based vaccines. Indeed, most studies have shown that a HA-based subunit vaccine could induce antibodies to protect animals from viral challenge [10, 38, 39]. More recently, Flublok—composed of three different subtypes of recombinant HA (H1, H3, and B viral strains) and expressed in insect cells—was developed as a seasonal influenza vaccine and approved for commercial production and marketing by the US Food and Drug Administration [8, 40]. The antigenic determinants of HA are reportedly exhibited mainly on the HA1 portion. Others have shown no difference in the production of polyclonal neutralizing antibodies by immunizing mice with recombinant HA1 or recombinant HA proteins [41]. In recent structure investigation, a few anti-H5 neutralizing mAbs are found targeting to receptor-binding sites, which is not directly associated with the trimeric structure [42]. It implied that if head region of HA1 harboring native-like epitopes share similar or even identical conformation when it is in the trimeric HA, trimerization of HA seems not be prerequisite on vaccine efficacy. Therefore, the choice of

HA1 for studying expression and functional epitopes for this virus is appropriate for the development of the H5N1 vaccine.

The insect cell-derived HA protein was reported in a trimeric form, similar to the native viral protein; however, due to its high production cost, the insect cell system has been deemed to be not an optimal system for vaccine development; furthermore, the technology required to scale-up the culture system has been considered to be too complicated and costly. Another research about bacterial expression of hemagglutinin was reported by Khurana with high yield [43, 44]. However, this hemagglutinin is lack of glycosylation. To our knowledge, there were no reports about bacteria-derived hemagglutinin for flu vaccine candidate, so such glycan-absent hemagglutinin might induce lower level of immunogenicity if compared with that of natural ones. In contrast, yeast systems have often been used to express various exogenous recombinant glycoproteins, and this system can be scaled up with high-density fermentation [45]. Therefore, eukaryotic yeast cells offer certain advantages in terms of the production of recombinant antigens. Here, we successfully expressed approximately 120 mg/mL of rHA1 protein using high-density fermentation of *P. pastoris*. The yield of rHA1 from *P. pastoris* was dramatically higher than up to the yield of 20 mg/L rHA expressed in baculo/insect cells systems for Flublok vaccine [46]. Considering the practical application of fed-batch fermentation, this technology is valuable in terms of batch consistency and production volume.

To date, it is still unclear what role glycosylation of HA has in the induction of the antibody response. As a glycoprotein, HA expressed in yeast is likely to encounter excessive glycosylation. Studies have shown that there are five potential glycosylation sites on the influenza HA1 sequence, and some of the nonglycosylated amino acids in the HA protein are unable to produce protective neutralizing antibodies, thus resulting a lower antibody response [47]. The over-glycosylation has a serious impact on glycoprotein identification, causing a smear when separated by SDS-PAGE. In this study, rHA1 pretreatment with a deglycosylation enzyme caused the smear to disappear, suggesting that the rHA1 was glycosylated in *P. pastoris*. This phenomenon was similar to that described by Shehata and his colleagues [48]. In the present study, we adopted *P. pastoris* to express rHA1 with native-like epitopes. The approach demonstrated remarkable advantages, such as high yield, simplified purification, and scaled-up strategy. It may be an alternative means to generate more glycoproteins related to other viruses.

In summary, this study showed the successful production of the H5N1 rHA1 in the supernatant of *P. pastoris* using fed-batch expression. Mass spectrometry verified the reproducibility and stability of the produced protein. The rHA1 in the supernatant was purified to over 90 % and the presence of virion-like epitopes on rHA1 were demonstrated with anti-H5N1 virus monoclonal antibodies. Blocking ELISA and HI tests further validated the native-like epitopes existed in *P. pastoris*-derived rHA1, indicating that the rHA1 mimics the native conformation of viral HA. Conceivably, the *P. pastoris*-derived rHA1 with a readily scaled-up and reproducible process could be used to develop a broad spectrum vaccine or a diagnostic agent.

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Contributors Conceived and designed the experiments: SL NX. Performed the experiments: QL KY FH JJ TL ZC RL. Analyzed the data: QL YC QZ SL. Contributed reagents/materials/ analysis tools: YC SL NX. Wrote the paper: QL QZ SL.

Compliance with Ethical Standards In this study, the designed experimental procedures were firstly approved by the Xiamen University Laboratory Animal Management Ethics Committee.

Conflict of Interest The authors declare that they have no conflict of interest.

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