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## Avian influenza A/H5N1 neuraminidase expressed in yeast with a functional head domain

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### ABSTRACT

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The study reports heterologous expression in *Pichia pastoris* of active neuraminidase derived from avian influenza virus A/Viet Nam/DT-036/2005(H5N1). A gene encoding the neuraminidase N1 head domain (residues 63–449) was fused directly in-frame with the *Saccharomyces cerevisiae*  $\alpha$ -factor secretion signal in pPICZ(A vector). Recombinant N1 neuraminidase was expressed in *P. pastoris* as a 72 kDa secreted, soluble protein. Glycopeptidase F treatment generated a 45 kDa product, indicating that the secreted recombinant N1 neuraminidase is an N-linked glycoprotein. Kinetic studies and inhibition tests with oseltamivir carboxylate demonstrated that the recombinant N1 neuraminidase has similar  $K_m$  and  $K_i$  values to those of the viral N1 neuraminidase. This yeast-based heterologous expression system provided functionally active recombinant N1 neuraminidase that should be useful in anti-influenza drug screening, and also as a potential protein-based vaccine.

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### 1. Introduction

The pathogenic avian influenza A (H5N1) virus is highly contagious among birds including poultry in several continents, and poses a serious threat to human health, evidenced by increasing numbers of H5N1 virus-infected patients (Abdel-Ghafar et al., 2008; Capua and Alexander, 2002; de Jong and Hien, 2006; Joseph and Subbarao, 2005; Ligon, 2005; Yuen and Wong, 2005). To date, more than 380 people worldwide have been infected with H5N1 virus with a cumulative case-fatality rate approaching 63% (WHO, 2008). Owing to its virulence with high lethality, endemic presence, increasingly large host reservoir, and significant ongoing mutation, H5N1 virus has the potential of becoming a significant pandemic threat.

Effective control of H5N1 epidemics includes both effective vaccination and antiviral-drug treatment. However, influenza vaccines are not efficient because of antigenic variation. Therefore, during the early infection period, prevention and treatment of patients

with H5N1 infection must rely mainly on antiviral drug treatments with oral oseltamivir (Tamiflu<sup>®</sup>) and inhaled zanamivir (Relenza<sup>®</sup>) (Bardsley-Elliott and Noble, 1999; Colman, 2005; Doucette and Aoki, 2001; Leneva et al., 2000; McKimm-Breschkin, 2002; Moscona, 2008; Woodhead et al., 2000). Both drugs target the type II integral membrane glycoprotein, neuraminidase (NA), found on the virion surface.

NA removes terminal sialic acid residues from glycoconjugates, allowing release of viral progeny from infected cells and preventing viral aggregation (Burnet, 1979; Gottschalk, 1958; Griffin et al., 1983; Liu et al., 1995). Inhibition of NA activity limits the spread of viral infection, thereby suppressing the onset of disease (Colman, 2002). Oseltamivir is recommended by WHO for early treatment of confirmed or strongly suspected H5N1 infection and is now being stockpiled in many countries for treatment of pandemic influenza (Schunemann et al., 2007).

While the prophylactic and therapeutic efficacy of oseltamivir is well established in uncomplicated seasonal human-influenza infection, the clinical effectiveness of oseltamivir for human H5N1 infection remains somewhat limited (Crusat and de Jong, 2007). In addition, accumulating studies have shown that some particular strains of viruses can develop resistance to oseltamivir

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and surveillance for NA inhibitor-resistance is on-going (Aoki et al., 2007; Escuret et al., 2008; Ferraris and Lina, 2008; Handel et al., 2007; Hayden et al., 2005; Le et al., 2005; McKimm-Breschkin, 2000; Mendel and Sidwell, 1998; Moscona, 2005; Reece, 2007; Yen et al., 2007). Recently, oseltamivir-resistant H5N1 isolates carrying a critical mutation at NA His274Tyr have been identified in patients who have died of the infection, despite early initiation of oseltamivir therapy (de Jong et al., 2005; Le et al., 2005), raising a major concern that oseltamivir-resistant H5N1 variants may become more widespread. Hence, it is of utmost importance to prepare for the emergence of oseltamivir-resistant H5N1 viruses and the advent of a pandemic by developing novel NA inhibitors.

Screening for novel inhibitors of H5N1 NA requires an adequate amount of pure NA which is also active catalytically. NA is prepared conventionally by proteolytic cleavage or detergent-treatment of the viral envelope followed by subsequent purifications to obtain the catalytic NA head domain (França de Barros et al., 2003; Gallagher et al., 1984; Hocart et al., 1995; Kilbourne et al., 1968; Russell et al., 2006; Seto and Rott, 1966; Takimoto et al., 1992; Ward et al., 1982). Using such methods, head domain of N1 NA was extracted from viral particles in sufficient amounts for studies of three-dimensional structure (Russell et al., 2006). However, large-scale production of viral particles requires inoculation of embryonated chicken eggs, a procedure restricted to a limited number of laboratories and confounded by inadequate yield and purity, and by virus adaptation.

A heterologous expression system provides an alternative means for viral protein production. Compared with heterologous recombinant protein expression using mammalian or insect cell lines, the methylotrophic yeast *P. pastoris* is considered the simplest organism capable of post-translational modification for production of proteins in large amounts, either intracellularly or extracellularly (Bretthauer and Castellino, 1999; Cereghino et al., 2002; Cereghino and Cregg, 2000; Cregg et al., 2000). In addition, *P. pastoris* is potentially adaptable for safe and inexpensive large-scale fermentation. *P. pastoris* expression system has been used to produce a number of viral proteins, including hepatitis virus proteins (Chen, 2007; Han et al., 2006), HIV-1 envelope glycoprotein (Scorer et al., 1993), viral capsid antigen of Epstein-Barr virus (Hu et al., 2007), influenza virus RNA polymerase (Hwang et al., 2000), SARS coronavirus nucleocapsid protein (Liu et al., 2005), dengue virus envelope protein (Valdés et al., 2007), rubella virus glycoprotein E1 (Wen and Wang, 2005), and hemagglutinins of avian influenza virus H5, H7, and H9 subtypes (Wang et al., 2007; Xu et al., 2006).

In this study, the first successful heterologous expression is reported of avian influenza virus A/Viet Nam/DT-036/2005(H5N1) NA head domain using *P. pastoris*. The construction of the expression vector pPICZ $\alpha$ A-NA and its application to produce recombinant N1 NA as a secreted N-linked glycoprotein are described. In addition, it is shown that the recombinant N1 NA is functionally active with similar kinetic properties to that of viral H5N1 NA.

## 2. Materials and methods

### 2.1. Viruses and inhibitor

H5, N1, and N3 genes (GenBank accession nos. **DQ497729.1**, **DQ094291**, and **AY207510** respectively) derived from avian influenza viruses A/Vietnam/CL2009/2005 (H5N1), A/Viet Nam/DT-036/2005(H5N1), and A/duck/Singapore/3/1997(H5N3) respectively, were obtained from recombinant plasmids kindly provided by Dr. Erich Hoffmann, St. Jude Children's Research Hospital. The H5 gene was modified by removing a polybasic amino acid coding region, known to be associated with high

virulence (Webby et al., 2004). Reverse-genetic viruses rgH1N1, rgH5N1, and rgH1N3 were generated by the 8 plasmid reverse genetics system (Hoffmann et al., 2002). All reverse-genetic viral genes except H5, N1, and N3 were derived from A/PR8/34(H1N1). Oseltamivir carboxylate ([3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-carboxylic acid) was a kind gift from Dr. Tirayut Vilaivan, Department of Chemistry, Faculty of Science, Chulalongkorn University, Thailand.

### 2.2. Construction of pPICZ $\alpha$ A-NA

A gene encoding the NA head domain (residues 63–449) of avian influenza virus A/Viet Nam/DT-036/2005(H5N1) was PCR amplified using forward primer NA.F (5' GCCTCGAGAAAAGAGTAAAATTAGC-GGG 3') and reverse primer NA.R (5' GCTCTAGACCCCTTGCAATGGTGAATGG 3') containing XhoI and XbaI restriction sites (underlined), respectively. PCR was conducted in a total volume of 50  $\mu$ l, containing 2.0 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5 U of DynaZyme EXT DNA polymerase (Finnzymes, Finland), 20 ng of pVn36:N1 (H5N1), and 200 nM primers. Thermal cycling in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) was performed as follows: 94 °C for 5 min, followed by 24 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C, 1 min, with a final step of 72 °C for 10 min.

PCR amplicons were separated by 1% agarose gel-electrophoresis and purified using a QIAquick Gel Extraction kit (QIAGEN, Valencia, CA, USA). Amplicons were then digested with XhoI and XbaI (Promega, Madison, WI, USA) and sub-cloned into pPICZ $\alpha$ A expression vector (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid pPICZ $\alpha$ A-NA was transformed into *Escherichia coli* DH5 $\alpha$  using a standard heat shock method (Sambrook et al., 1989). Transformants harboring pPICZ $\alpha$ A-NA were selected from low salt LB agar containing 25  $\mu$ g/ml Zeocin<sup>TM</sup>, followed by plasmid extraction using QIAprep Miniprep kit (QIAGEN, Valencia, CA, USA). NA gene presence was verified by restriction enzyme digestion and DNA sequencing (BioService Unit, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand).

### 2.3. Transformation of pPICZ $\alpha$ A-NA into *P. pastoris*

Recombinant plasmid pPICZ $\alpha$ A-NA with the validated NA gene sequence was linearized by PmeI digestion and transformed into *P. pastoris* KM71 (Invitrogen Carlsbad, CA, USA) by electroporation (Bio-Rad Gene Pulser; Bio-Rad, Hercules, CA, USA) with setting of 1.5 kV, 25  $\mu$ F and 200  $\Omega$ . Transformants were grown on yeast extract peptone dextrose (YEPD) agar plates containing 100  $\mu$ g/ml Zeocin<sup>TM</sup>. Colonies were selected and screened for NA-integration by colony PCR screening using forward and reverse AOX primers (Invitrogen Carlsbad, CA, USA). PCR was conducted in a total volume of 25  $\mu$ l, containing 2.0 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5 U of DynaZyme II DNA polymerase (Finnzymes, Finland), 2.5  $\mu$ l of cell suspension and 200 nM primers. Thermal cycling was performed as follows: 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, then a final step at 72 °C for 10 min. Amplicons were analyzed by agarose gel-electrophoresis as described above.

### 2.4. Expression of secreted NA

A single integrant-containing colony was inoculated into YEPD media containing 100  $\mu$ g/ml Zeocin<sup>TM</sup> and incubated at 30 °C for 48 h with shaking at 250 rpm. The cell culture was then transferred to fresh BMGY medium (100 mM phosphate buffer (pH 6.0), 1.34% (w/v) yeast nitrogen base, 1.0% (w/v) yeast extract, 2.0% (w/v) pep-

tone, 0.00004% (w/v) biotin, 1.0% (v/v) glycerol) and grown until an  $OD_{600}$  of 5–6 was reached. Cells were harvested and resuspended in BMMY medium (100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 1.0% (w/v) yeast extract, 2.0% (w/v) peptone, 0.00004% (w/v) biotin). Absolute methanol was added at 24, 48, 72, and 96 h of growth to a final concentration of 0.5, 1.0, 2.0, 3.0, and 4.0% (v/v), respectively. At each time point, cell culture supernatant was collected for determination of NA expression and enzyme activity. Protein expression of pPICZ $\alpha$ A-transformed *P. pastoris* KM71 was conducted as a negative control.

## 2.5. Characterization of yeast-expressed NA

### 2.5.1. SDS-PAGE analysis

To monitor the expression of secreted NA, proteins of the cell culture supernatant were separated by 12.5% SDS-PAGE (Laemmli, 1970). Protein bands were visualized by staining with Coomassie<sup>®</sup> brilliant blue G-250, ultra pure (USB Corporation, Cleveland, OH, USA). To identify glycoproteins, *P. pastoris* culture supernatant was digested with *N*-glycosidase F (PNGase F) (New England BioLabs, MA, USA) according to manufacturer's protocol, separated by 12.5% SDS-PAGE and gels stained using a SilverSNAP<sup>®</sup> Stain Kit II and a GelCode Glycoprotein Staining Kit (PIERCE, Rockford, IL, USA).

### 2.5.2. Western blot analysis

Immunoblotting was conducted using mouse anti-His-probe monoclonal IgG<sub>1</sub> antibody (H-3) (Santa Cruz Biotechnology, CA, USA) as a primary antibody and goat anti-mouse IgG<sub>1</sub> conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, CA, USA) as a secondary antibody. In addition, mouse antiserum against recombinant N1 NA (kindly provided by Dr. Potjanee Srimanote, Graduate Program of Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Thailand) or rabbit polyclonal anti-NA antibody (United States Biological, MA, USA) was also used as a primary antibody and subsequently probed with goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA), respectively. Proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Bioscience, Little Chalfont, Bucks, UK). The membranes were blocked with 5% non-fat dried milk in TBS buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) before incubation for 2 h at room temperature with primary antibodies, diluted 1:2000 with TBST buffer (TBS buffer containing 0.05% Tween 20) and 5% non-fat dried milk, followed by washing twice with TBST buffer and then incubating for 1 h at room temperature with secondary antibodies, diluted 1:5000 with TBST buffer. Proteins were detected using 2,6-dichloroindophenol (Sigma, St. Louis, MO, USA) or Super Signal West Pico Chemiluminescent Substrate Detection reagents (PIERCE), according to manufacturer's instructions.

## 2.6. NA activity assay

NA enzymatic activity was determined using an endpoint fluorescence-based assay employing a fluorogenic substrate, 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO, USA), as described previously (Potier et al., 1979). In brief, 10  $\mu$ l of sample was mixed with an equal volume of assay buffer (32.5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.5, containing 4 mM CaCl<sub>2</sub>) and the enzymatic reaction initiated by addition of 30  $\mu$ l of 833  $\mu$ M MUNANA, followed by a 30 min incubation at 37 °C. The reaction was terminated by the addition of 150  $\mu$ l of stop solution (100  $\mu$ M glycine, pH 10.7, in 25% ethanol). The amount of fluorescent product, 4-methylumbelliferone (4-MU) released was measured in a Victor<sup>3</sup> V spectrofluorometer (Perkin Elmer, MA, USA) with excitation

and emission wavelengths of 355 and 460 nm respectively. Blank control reactions contained substrate alone. All reactions were conducted in triplicate in 96-well flat-bottom polystyrene plates (Corning Costar, Corning, NY, USA). A standard curve was generated by plotting relative fluorescence intensity against the amount of free 4-MU. One unit of NA was defined as one micromolar of 4-MU produced per min at 37 °C. Protein concentration was determined using Bradford's method with bovine serum albumin as standard (Bradford, 1976).

## 2.7. Steady-state kinetics and NA inhibition assay

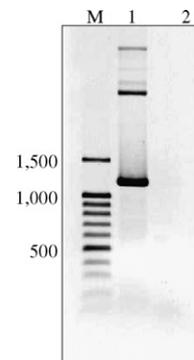
The amount of NA used in the determination of Michaelis constant ( $K_m$ ) and the inhibition constant ( $K_i$ ) was 0.005–0.02 units. To obtain  $K_m$  values, the assay was performed in the presence of 0.05–125  $\mu$ M substrate and calculated by fitting the data to the Michaelis-Menten equation using the KaleidaGraph software package (Synergy Software).

To determine  $K_i$  values, NA activity was measured in the presence of 0.00005–50 nM oseltamivir carboxylate. The sample was pre-incubated with the inhibitor at 37 °C for 30 min, followed by addition of 125  $\mu$ M MUNANA. The reaction was then incubated at 37 °C for 30 min and terminated by the stop solution. The fluorescent intensity was measured as described above. The fluorescent intensity of the reaction without the inhibitor was taken as the maximum NA activity ( $V_{max}$ ). The  $V_{max}$  and calculated  $K_m$  values (see above) were used in the determination of  $K_i$  values. The data were fitted to a single-site competitive inhibition function (Segal, 1975):  $v_i = V_{max}(1 - ([I]/([I] + (K_i(1 + ([S]/K_m))))))$  using KaleidaGraph software package (Synergy Software), where  $v_i$  is measured activity,  $V_{max}$  is maximum activity,  $[I]$  is inhibitor concentration,  $K_i$  is the inhibition constant,  $[S]$  is substrate concentration, and  $K_m$  is the Michaelis constant. From the calculated  $K_m$  and  $K_i$  values, half maximal inhibitory concentrations ( $IC_{50}$ ) of oseltamivir carboxylate were inferred using the equation  $IC_{50} = K_i(1 + ([S]/K_m))$  (Segal, 1975).

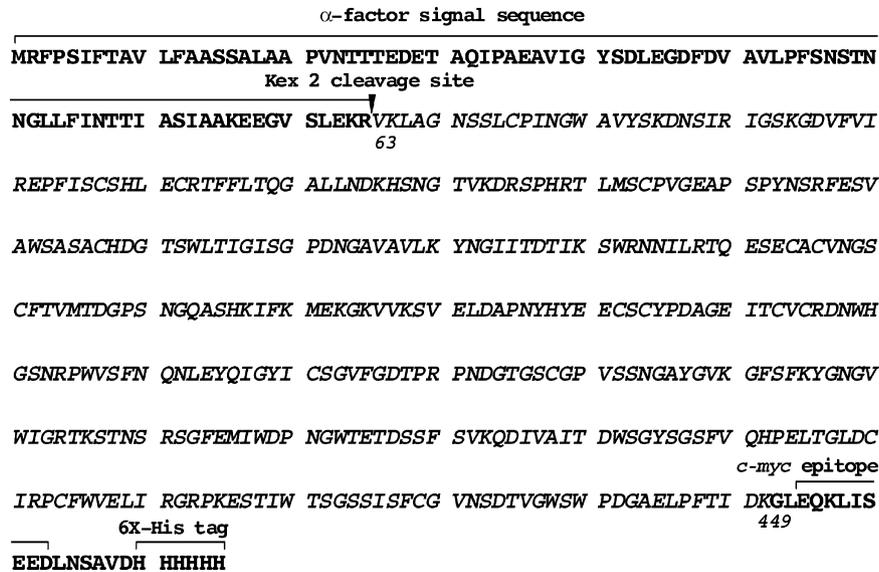
## 3. Results and discussion

### 3.1. Construction of N1 NA-integrated *P. pastoris* clones

The 1185 bp gene encoding the NA head domain (residues 63–449) of A/Viet Nam/DT-036/2005(H5N1) virus was cloned into the expression plasmid pPICZ $\alpha$ A to obtain pPICZ $\alpha$ A-NA (Fig. 1). DNA sequence analysis showed an in-frame direct fusion of the NA gene (387 encoded amino acids) with the secretion signal of



**Fig. 1.** PCR amplicon of the head domain of H5N1 NA (residues 63–449). PCR amplification of head domain of N1 NA gene yielded a product approximately 1185 bp product. Lane M, 100 bp ladder DNA marker; lane 1, PCR amplicon; lane 2, negative control (PCR minus template).



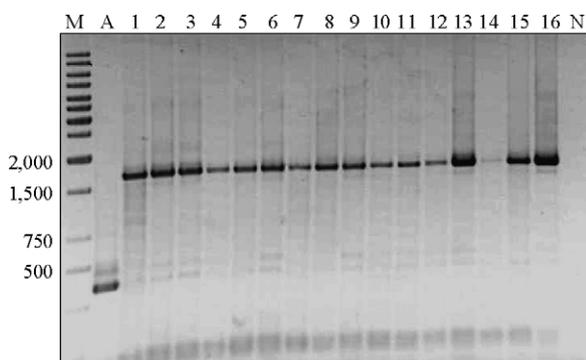
**Fig. 2.** Sequence of recombinant N1 NA open reading frame. Head domain (residue 63–449) of H5N1 NA was fused in-frame with  $\alpha$ -factor signal sequence and Kex 2 cleavage site at the N-terminus, and c-myc epitope and 6-His tag at the C-terminus.

*Saccharomyces cerevisiae*  $\alpha$ -factor of pPICZ $\alpha$ A vector (Fig. 2). The  $\alpha$ -factor secretion signal and Kex2 cleavage site allow the expressed NA to be efficiently released from the yeast cell as a native N-terminus protein fragment. In addition, the c-myc epitope and 6-His tag C-terminal to NA allow for detection and purification of the expressed NA.

Following transformation of PmeI-linearized pPICZ $\alpha$ A-NA plasmid into *P. pastoris* KM71, 16 colony clones were randomly selected for screening of NA integration. All tested clones yielded the expected PCR amplicon size (1669 bases), indicating that the NA gene had been integrated into the *P. pastoris* genome (Fig. 3).

### 3.2. Expression and detection of secreted recombinant N1 NA

Small-scale expressions of the 16 NA-integrated clones and negative control pPICZ $\alpha$ A-transformed *P. pastoris* KM71 were conducted using 3% (v/v) absolute methanol for induction. After 72 h of incubation, the culture supernatants were collected for SDS-PAGE analysis and NA activity determination. As shown in Fig. 4, SDS-PAGE revealed that heterogeneous proteins 65–95 kDa were produced in the integrant clones (hereafter referred to as smeared



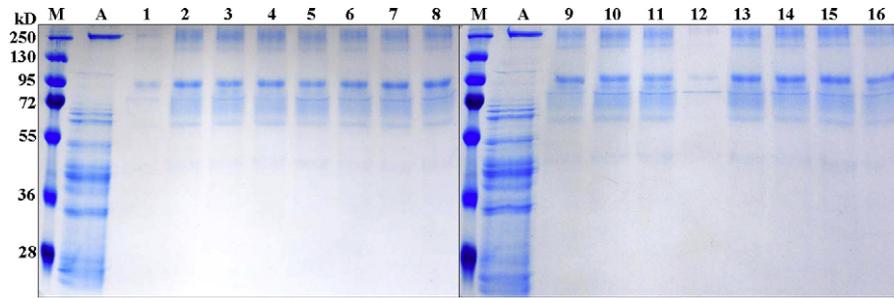
**Fig. 3.** PCR analysis of *P. pastoris* integrants. Integration of N1 NA gene into the *P. pastoris* genome was tested by colony PCR screening using 5' AOX and 3' AOX primers. 1669 bp amplicons are indicative of N1 NA gene integration. Lane M, 100 bp ladder DNA marker; lanes 1–16, amplicons of pPICZ $\alpha$ A-NA transformant clones no. 1–16; lane A, amplicon of *P. pastoris* integrant containing pPICZ $\alpha$ A; lane N, negative control (PCR minus template).

72 kDa proteins) which were relatively more intense than the negative control, and could represent different forms of recombinant N1 NA.

To verify the presence of recombinant N1 NA with NA activity, fluorescence-based NA activity assay was conducted on NA of all 16 clones. To correct for background fluorescence due to the media, heat-inactivated culture supernatant of each sample was used as a control blank in NA activity assay. As shown in Fig. 5, 5 out of 16 clones (clone nos. 2, 8, 9, 10, and 15) produced NA activity  $\geq$  0.35 units, and the lowest NA activity (0.05 and 0.06 units) was observed in clone nos. 1 and 12 respectively. No NA activity was observed in the negative control. Variation of NA activity between the different clones may reflect differences in the copy number of the NA gene integrated into the yeast genome. Taken together, the SDS-PAGE and activity data indicate that the head domain (residues 63–449) of H5N1 NA could be expressed in *P. pastoris* as a secreted soluble protein with measurable NA activity, albeit at a low level of expression compared with typical recombinant protein expression in *P. pastoris*.

To optimize NA expression, integrant clone no. 9 was subjected to different induction conditions, varying the methanol concentration (0.5–4% (v/v)) and incubation time (24–96 h). Induction with 2–3% (v/v) of absolute methanol for a period of 96 h yielded the highest NA activity,  $\sim$ 0.50 units, with a culture supernatant protein concentration of  $\sim$ 0.10 mg/ml (data not shown). These conditions were then used in all further experiments.

NA expressed in yeast is hyperglycosylated, resulting in multiple species with reduced mobility relative to unmodified NA in SDS-PAGE (Martinet et al., 1997). The expected size of unmodified N1 NA secreted head domain is 45 kDa, and thus the observed smeared 72 kDa proteins in the integrant clones (Fig. 4) could represent NA protein with varying levels of glycosylation. SDS-PAGE with silver staining showed heterogeneous protein-band patterns similar to Coomassie staining, in which it was difficult to distinguish negative control from *P. pastoris* NA gene integrant (Fig. 6A, lane 1 and lane 3). However, N-glycosidase F treatment of the NA-expressing *P. pastoris* culture supernatant resulted in the appearance of two proteins 45 and 60 kDa, with a concomitant reduction in the intensity of the smeared 72 kDa proteins (Fig. 6A, lane 4). Moreover, glycoprotein staining was positive for the smeared 72 kDa proteins, but not for 45 and 60 kDa proteins (Fig. 6B, lane 4). Thus,

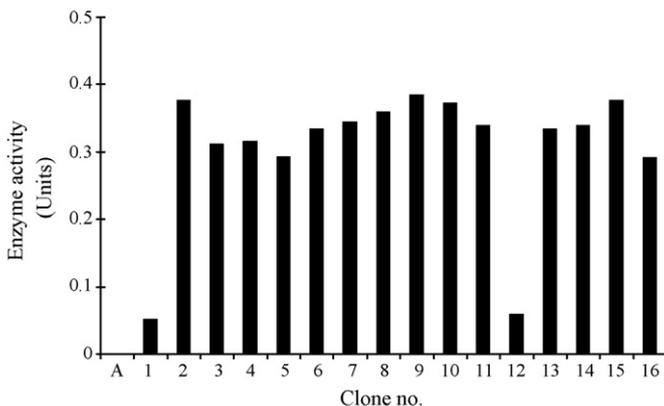


**Fig. 4.** SDS-PAGE analysis of *P. pastoris* integrants. Integrant clones no. 1–16 were grown in BMGY media and induced in BMMY supplemented with 3% (v/v) methanol. Culture supernatants were collected after induction for 3 days for SDS-PAGE analysis. Twenty-microliter aliquots of the culture supernatant were separated by 12.5% SDS-PAGE. Lane M, molecular weight markers; lane A, *P. pastoris* containing only pPICZ $\alpha$ A vector; lanes 1–16, *P. pastoris* integrant clones no. 1–16. The heterogeneous proteins (65–95 kDa) observed in the integrant clone samples are marked on the right.

it is inferred that the smeared 72 kDa proteins possess variable glycosylations, which can be deglycosylated to generate two smaller proteins.

To demonstrate that the 45, 60 and 72 kDa proteins were indeed recombinant N1 NA, immunoblotting with anti-His (Fig. 6C) and anti-NA antibodies (Fig. 6D) was performed. The efficiency of mouse antisera against recombinant N1 NA and commercial rabbit polyclonal anti-NA antibodies in immunoblotting experiments was similar (data not shown), and therefore, mouse antisera were used in all subsequent experiments. The 45, 60 and 72 kDa proteins were recognized by anti-His (Fig. 6C, lanes 3 and 4) and anti-NA (Fig. 6D, lanes 3 and 4) antibodies. Prolonged *N*-glycosidase F treatment reduced the 60 kDa protein intensity (Fig. 6D, lane 4), suggesting that the 60 kDa protein may be intermediate in glycosylation status between the 45 and 72 kDa forms. The Western blot results thus show that the predominant glycoform of recombinant N1 NA has a molecular weight approximately 72 kDa.

Analysis of detergent-disrupted whole viral particles using non-reducing SDS-PAGE, has shown the presence of NA protein of ~200 kDa (Johansson et al., 1989; Johansson and Kilbourne, 1996), and virus NA head domain of 50 kDa (Blok et al., 1982). Moreover, the 70 kDa monomeric and 250 kDa tetrameric forms of NA have been observed from purified NA fractions (Tanimoto et al., 2005). Taken together, it could be suggested that monomeric NA has a range of molecular size from ~50 to 70 kDa. We have also observed viral N1 NA protein of ~55 kDa (Fig. 6D, lane 5), and under non-reducing SDS-PAGE condition that of the ~250 kDa form (data not shown).



**Fig. 5.** NA activity of *P. pastoris* integrants. Culture supernatants of *P. pastoris* integrant clones no. 1–16 were assayed for NA activity. The experiment was done in triplicate. The unit of NA activity is the average value and standard deviation of the measurement is typically  $\pm 0.0002$ . Clone A is negative control *P. pastoris* containing pPICZ $\alpha$ A vector.

Although the core peptide of viral N1 NA is 49 kDa in size, glycosylation can increase the apparent molecular weight to 55 kDa. The recombinant N1 NA lacking both the transmembrane and stalk regions has an expected core peptide size of 45 kDa. Quantitative analysis of *N*-linked glycosylated NA revealed an average ratio of *N*-acetyl-*D*-glucosamine:mannose:glucose of 2:30:0.5–1 residue per glycosylation site (Martinet et al., 1997), leading to a postulated increase in molecular mass of 6 kDa per glycosylation site. The N1 NA contains eight potential glycosylation sites (Bragstad et al., 2008) of which four sites, Asn68, Asn126, Asn215, and Ser 414 are in the head domain. Although the presence of *O*-linked glycosylation of viral NA has not been demonstrated to date, *P. pastoris* is capable of introducing not only *N*-linked but also *O*-linked glycans to heterologous proteins (Bretthauer and Castellino, 1999; Gemmill and Trimble, 1999). Hence, the recombinant N1 NA may also contain some additional long chain *N*-linked glycans at other sites or *O*-linked glycans.

### 3.3. Kinetic properties and inhibition of NAs

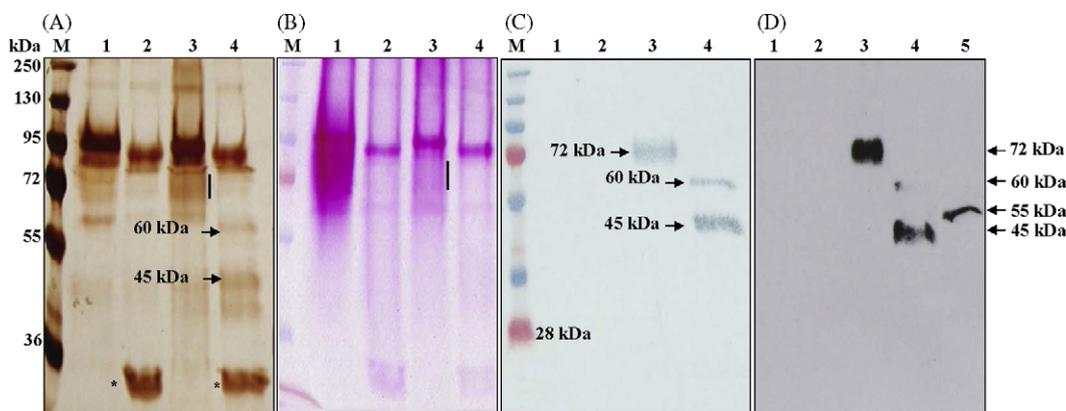
To verify whether the enzymatic properties of recombinant N1 NA were similar to those of viral N1 NA, the specific NA activity, Michaelis-Menten constants ( $K_m$ ) for MUNANA, and the inhibition constants ( $K_i$ ) for oseltamivir carboxylate of the recombinant N1 NA and viral NAs were determined. The rgH1N1, rgH5N1, and rgH1N3 reverse-genetic viruses were used as sources of viral NAs and *P. pastoris* integrant clone no. 9 for recombinant N1 NA. The kinetic studies were performed using suspensions of allantoic fluid containing reverse-genetic viruses and *P. pastoris* culture supernatant.

The results showed that the specific activity of recombinant N1 NA (314 units/mg) is similar to that of viral NAs (rgH1N1 = 294 units/mg, rgH5N1 = 378 units/mg, and rgH1N3 = 356 units/mg), indicating that the recombinant N1 NA has activity comparable to viral NA. The  $K_m$  values for MUNANA substrate of recombinant N1 NA (17  $\mu$ M) and viral NAs (15–18  $\mu$ M) are similar (Table 1). The  $K_m$  values obtained from this study are similar to those reported from native viruses (Rameix-Welti et al., 2006).

**Table 1**  
Steady kinetic parameters of the recombinant N1 NA and viral NAs.

	$K_m$ ( $\mu$ M)	$K_i$ (nM)	IC <sub>50</sub> (nM)
rgH1N1 NA	18 $\pm$ 2	0.0020 $\pm$ 0.0004	0.0159
rgH5N1 NA	15 $\pm$ 3	0.0013 $\pm$ 0.0004	0.0121
rgH1N3 NA	18 $\pm$ 2	0.0017 $\pm$ 0.0002	0.0132
Recombinant N1 NA	17 $\pm$ 4	0.0024 $\pm$ 0.0003	0.0182

$K_m$  and  $K_i$  values shown are the average from three independent experiments, in which the values were obtained from fitting to dose–response curves, as described in Section 2. Half maximal inhibitory concentrations (IC<sub>50</sub>) of oseltamivir carboxylate were inferred from calculated  $K_m$  and  $K_i$  values.



**Fig. 6.** SDS-PAGE and immunoblot analysis of recombinant N1 NA. Proteins in culture supernatant were separated by 12.5% SDS-PAGE and visualized by silver (A) and glycoprotein staining (B). In immunoblotting, proteins were probed with anti-His (C) and anti-NA (D) antibodies. Lane M, molecular weight markers; lane 1 and 2, culture supernatant of *P. pastoris* containing only pPICZ $\alpha$  vector treated without and with *N*-glycosidase F respectively; lanes 3 and 4, culture supernatant of integrant clone no. 9 without and with *N*-glycosidase F respectively; lane 5, purified rgH1N1 viral particles. Arrow and asterisk indicate the expected migrations of recombinant N1 NA and *N*-glycosidase F, respectively. The smeared 72 kDa proteins are indicated by a vertical line.

Taken together, it could be suggested that the recombinant N1 NA likely possesses similar kinetic properties to that of native N1 NA. It is thought that reporting  $K_i$  values is more useful for comparison of inhibitors than  $IC_{50}$ , which varies according to the substrate concentration used. Nonetheless, both  $K_i$  and  $IC_{50}$  values were determined for oseltamivir carboxylate to permit comparison with other studies. As shown in Table 1,  $K_i$  and  $IC_{50}$  values for oseltamivir carboxylate from both recombinant and viral N1 NA were not appreciably different. The inferred  $IC_{50}$  values for oseltamivir carboxylate obtained from this study are lower than reported by others (Collins et al., 2008; Rameix-Welti et al., 2006; Yen et al., 2007), which could be due to the differences in the source of oseltamivir carboxylate or variation in the assay conditions.

Although heterologous expression of N1 NA in *P. pastoris* may result in differences in glycosylation from that of viral N1 NA, the recombinant and viral N1 NAs were shown to exhibit similar enzymatic properties. Consequently, regardless of the difference in glycosylation, both recombinant and viral N1 NAs possess similar kinetic properties. Based on known NA structures (Russell et al., 2006; Varghese et al., 1998; White et al., 1995), the glycosylation sites are known to be localized on the surface of NA head domain and away from the substrate binding site. Presumably, the glycosylations of recombinant N1 NA are on the surface of head domain and do not perturb the accessibility of small substrate (i.e. MUNANA) to the catalytic site of the enzyme. Nonetheless, the *in vivo* role of glycosylation and its effects on the molecular properties of NA are unknown.

Antibodies directed against HA appear to have greater effect on viral infectivity than those against NA; hence, current viral vaccines are more focused on HA. On the other hand, there is ample evidence to support the usefulness of vaccines against NA to help build protective immunity against influenza virus, by either interfering with viral replication or its spread (Aymard et al., 1998; Jahiel and Kilbourne, 1966; Johansson et al., 1989, 1998; Kilbourne et al., 1968; Schulman et al., 1968; Webster et al., 1988). Purified viral NA has been used to supplement conventional influenza vaccine and shown to enhance immune responses against viruses (Johansson et al., 1998; Kilbourne et al., 1968; Schulman et al., 1968). Moreover, recombinant NA derived from a baculovirus-expression system induces NA-specific antibodies, resulting in the reduction of viral replication in mice (Kilbourne et al., 2004). Yeast-produced N2 NA has been tested for protective efficacy in influenza-challenged mice, and shown to give 50% protection (Martinet et al., 1997). Thus the immunogenic potential of NA and the molecular mechanism of NA

as a protective antigen are still interesting issues worthy of further investigation. The recombinant N1 NA derived from *P. pastoris* described in this report is active, and thus has a structural conformation of biological and immunological relevance. Hence, this protein could be used as an influenza vaccine, with the caveat that the extraneous c-myc epitope and His tag peptide may interfere with the desired immune response.

In conclusion, heterologous expression of avian influenza virus A/Viet Nam/DT-036/2005(H5N1) NA in *P. pastoris* provided functionally active N1 NA with similar kinetic properties to the viral N1 NA. This expression system provides a simple alternative means to produce biologically active N1 NA suitable for high-throughput screening of novel inhibitors, biochemical studies of the N1 NA-inhibitor complex, and characterization of the immunogenic properties of avian influenza virus N1 NA. These studies using recombinant N1 NA will require large-scale expression in a fermentation system, followed by protein purification.

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