

Expression, purification and characterization of glycosylated influenza H5N1 hemagglutinin produced in *Pichia pastoris*

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The A/swan/Poland/305-135V08/2006 (H5N1-subtype) hemagglutinin (HA) gene was cloned and expressed in yeast *Pichia pastoris* (*P. pastoris*). The HA cDNA lacking the C-terminal transmembrane anchor-coding sequence was fused to an α -factor leader peptide and placed under control of the methanol-inducible *P. pastoris* alcohol oxidase 1 (*AOX1*) promoter. Two *P. pastoris* strains: SMD 1168 and KM 71 were used for protein expression. Recombinant HA protein was secreted into the culture medium reaching an approximately 15 mg/L (KM 71 strain). Fusion protein with a His₆ tag was purified to homogeneity in one step affinity chromatography. SDS-PAGE and MS/MS analysis indicated that the protein is cleaved into HA1 and HA2 domains linked by a disulfide bond. Analysis of the N-linked glycans revealed that the overexpressed HA is fully glycosylated at the same sites as the native HA in the vaccine strain. Immunological activity of the hemagglutinin protein was tested in mice, where rHA elicited a high immune response.

Key words: Avian influenza, H5N1, recombinant hemagglutinin, *Pichia pastoris*

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INTRODUCTION

Methylotrophic yeast *Pichia pastoris* efficiently expresses recombinant genes. *Pichia* has the following main advantages: (i) it is able to express heterologous genes in high yield (Romanos, 1995; Rodriguez *et al.*, 1998), (ii) the proteins produced in this system may be secreted into the culture medium preserving correctly formed disulfide bonds (White *et al.*, 1994). Additionally, *P. pastoris* secretes only a few other proteins to the medium which facilitates protein purification. (iii) The carbohydrate cores incorporated into the N-glycosylation sites (Asn-X-Ser/Thr) of recombinant protein molecules are frequently at similar positions to those found in natural glycoproteins. Outer sugar of *P. pastoris* secreted proteins is Man8-10 GlcNAc2 (Grinna & Tschopp, 1989). Efficiency of the *P. pastoris* expression system is based on an unusually strong promoter from the alcohol oxidase 1 gene (*AOX1*) driving the expression of the foreign gene. The *AOX1* promoter is repressed in cells grown on glucose and most other carbon sources, but is induced over

1000-fold when cells are shifted to a medium containing methanol as a sole carbon source (Careghino *et al.*, 2002). Nowadays, *Pichia* is widely accepted as an important biotechnological host organism. Various pharmacologically important proteins were produced in this expression system e.g. malaria vaccine antigen (Brady *et al.*, 2001), human serum albumin (Bushell *et al.*, 2003), IGF-1 (Brealey, 1998), insulin (Kjeldsen *et al.*, 1999) and hepatitis B antigen (Cregg *et al.*, 1987). This study is aimed at expression of the hemagglutinin (HA) gene of H5N1 avian influenza virus (AIV) in *Pichia* cells. The HA protein plays a crucial role in the virus infection. HA glycoprotein is synthesized as an inactive HA0 molecule assembled as noncovalently bound homotrimers on the viral surface. This precursor protein is cleaved (RRKKR/G multi-basic cleavage site for H5 subtype) by trypsin-like proteases to create two subunits, HA1 and HA2 which are linked by a single disulfide bond (Skehel & Wiley, 2000). After cleavage, at the specific site, these two disulfide-bonded protein domains produce the mature form of the HA protein. Cleavage is a prerequisite for the infection. The HA0 molecule is post-translationally modified by glycosylation. Glycans are attached *via* N-glycosylic linkages to the asparagine residue of the conserved glycosylation site motif Asn-X-Ser/Thr (X-any amino acid) (Grinna & Tschopp, 1989).

There are several strategies to develop a new generation of vaccines. One of the simplest is using the surface proteins of the virus. It was demonstrated that HA is the major target for the development of influenza vaccines (Crawford *et al.*, 1999; Athmaram *et al.*, 2011). In this report, we describe expression of the HA gene of H5N1 avian influenza virus in *Pichia pastoris* system. rHA was produced in a soluble form with high yield and was shown to be highly immunogenic in mice.

MATERIALS AND METHODS

Strain and plasmid vector. Two *P. pastoris* strains: KM 71 (*his4*, *aox1::ARG4*) and the protease deficient SMD 1168 (*his4* and *pep4*), and the yeast expression pPICZ α C vector were obtained from Invitrogen.

Construction of pPICZ α C/HAHis₆ vector and transformation of *Pichia pastoris*. The IV strain H5N1

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Abbreviations: HA, hemagglutinin; AOX, alcohol oxidase; Endo H, endoglycosidase H, AIV, influenza virus.

A/swan/Poland/305-135V08-2008 was the source of HA gene. The DNA fragment encoding HA protein lacking 16 N-terminal aminoacids and C-terminal transmembrane anchor and cytoplasmic domain was amplified using forward and reverse primers containing *Clal* and *NcoI* restriction sites, respectively, and pGEM/HA as the template. The modified fragment codes for HA truncated peptide. Appropriate PCR fragment was inserted into pPICZ α C plasmid and introduced into *P. pastoris* strain KM73 or SMD 1168 by electroporation as described in the Invitrogen manual. Yeast transformants were also screened for the construct insertion at the alcohol oxidase promoter (AOX I) site by PCR, using 5' AOX I and 3' AOX I primers (Invitrogen).

Cell growth. Insert-positive yeast clones were grown initially in 100 or 400 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 1% glycerol) at 30°C on a plate agitator at 160 rpm agitation. When the culture reached OD₆₀₀ of 2, the cells were centrifuged at $3000 \times g$ for 5 min and resuspended in one-fourth of the original volume of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.8, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 5% methanol) for induction of the AOX I promoter. Subsequent incubation at 26°C with vigorous shaking was continued for 5 or 7 days. The culture medium was cleared of yeast cells by centrifugation at $3000 \times g$ at 4°C for 6 min and the supernatant was collected for further HA purification.

Purification of His₆-rHA. Recombinant protein containing the hexahistidine tag was purified by affinity chromatography on Ni-NTA resin (Qiagen). 100 ml of post culture medium aliquot was diluted to 200 ml with a PBS buffer pH 7.4 containing 500 mM NaCl and gently mixed with 3 ml Ni-NTA resin at 4°C for 2.5 h. The resin was packed into a column and washed with one volume of PBS buffer, then the bound protein was eluted with 3 ml portions of the elution buffer (250 mM imidazole in PBS). Fractions containing protein were analyzed by SDS/PAGE and Western blotting. All fractions containing purified protein were pooled and dialyzed against PBS buffer pH 7.4. Subsequently the protein was lyophilized and stored at -80°C.

Western blotting. Proteins separated by 12% SDS/PAGE were electrotransferred onto an Immobilon-P transfer membrane (Millipore). The membrane was incubated for 2 h with 5% non-fat dried milk in a TBS-T buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). His₆-tagged HA protein was detected using a mouse monoclonal anti-His-HRP antibody (1:5000, Abcam). The membrane was analyzed using SuperSignal West Femto Trial Kit (Thermo Scientific). His₆-tagged HA protein was also detected with monoclonal anti-HA1 antibody (a gift from the Institute of Biotechnology and Antibiotics, Warsaw). Alkaline phosphatase-conjugated anti-mouse antibody was used as a secondary antibody diluted at 1:30000 (Sigma-Aldrich). The blot was visualized with Western Blue Stabilized Substrate for alkaline phosphatase (Promega).

Determination of protein concentration. Protein concentration was determined according to the Bradford protocol. Bovine serum albumin was used as the standard.

Enzymatic deglycosylation. 5 μ g of rHA protein was denatured with 1x Glycoprotein Denaturing Buffer at 95°C for 10 min. 20 μ l of reaction mix containing denatured HA protein and 0.125 U of endoglycosidase H (Endo H, NEB) in $1 \times G5$ reaction buffer (50 mM so-

dium citrate, pH 5.5) was incubated at 37°C for 1 h. An untreated protein sample was incubated under the same conditions without the enzyme.

MS/MS analysis of rHA. rHA protein after reaction with Endo H was analyzed by SDS/PAGE. A gel band containing deglycosylated polypeptides was excised. Protein was reduced with 100 mM DTT (for 30 minutes at 56°C), alkylated with 0.5 M iodoacetamide (45 min in a dark at room temperature) and digested overnight with 12.5 ng/ μ l of trypsin (sequencing Grade Modified Trypsin — Promega V5111) by adding the enzyme directly to the reaction mixture. Peptides were analyzed by LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using Nano-Acquity (Waters) LC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp.), by applying peptides to RP-18 precolumn (nanoACQUITY Symmetry[®] C18 — Waters 186003514) using water containing 0.1% TFA as a mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18 — Waters 186003545) using an acetonitrile gradient (0–35 % AcN in 180 minutes) in the presence of 0.05% formic acid with the flow rate of 250 nl/min. Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis. Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, on-site license) against users' defined database. Search parameters for precursor and product ions mass tolerance were 40 ppm and 0.8 Da, respectively, enzyme specificity: trypsin, missed cleavage sites allowed: 1, fixed modification of cysteine by carbamidomethylation and variable modification of methionine oxidation and HexNAc at asparagine. Peptides with Mascot Score exceeding the threshold value corresponding to <5% False Positive Rate, calculated by Mascot procedure were considered to be positively identified. Fragmentation spectra of peptides indicated by Mascot as N-glycosylated were manually investigated.

Immunization experiments. Seven-week old, pathogen-free, female Balb/c mice were used for vaccination. The animals were housed in a temperature controlled environment at 24°C with 12 h day-night cycles and received food and water *ad libitum*. Immunization experiments were done with groups consisting of 5 or 7 mice, in the animal house of Institute of Experimental Medicine PAS (Warsaw) under control of the Bioethics Committee (permission no 11/2012). In the first experiment mice were immunized three times intradermally in the hind paw at 3-week intervals with 100 μ l containing 1, 5 or 25 μ g of HA. An adequate portion of the protein was dissolved in saline plus adjuvant. For immunization, 25 μ g of Sigma Adjuvant System containing 0.5 mg Monophosphoryl Lipid A (detoxified endotoxin) from *Salmonella minnesota* and 0.5 mg synthetic Trehalose Dico-rynomycolate in 2% oil (squalene)-Tween[®] 80-water was used. Two booster injections were adjuvanted with 25 μ g monophosphoryl lipid A and 25 μ g muramyl dipeptide (Sigma-Aldrich). Control mice received adjuvant only. One week prior to the first immunization and 2 weeks after each immunization, blood samples were collected, allowed to coagulate and centrifuged. The collected sera were stored at -20°C before measuring the antibody level. In the second experiment, two different methods of administration were applied. Mice were immunized intradermally in the hind paw and subcutaneously in the neck with 100 μ l of 25 μ g rHA plus adjuvant. The immunization scheme was identical as in the first experiment.

ELISA test. Sera from experimental animals were analyzed by indirect ELISA for detection of anti-HA IgG antibodies. The 96-well polystyrene plates (Nunc) were coated overnight at 4°C with recombinant H5 HA (17-530, Δ341-346, 6xHis-tag at C-terminus) antigens: HA (A/Bar-headed Goose/Qinghai/12/05(H5N1)) produced in a mammalian cell system (Immune Technology) and HA (A/swan/Poland/305-135V08/2006(H5N1)) produced in a baculovirus system (Oxford Expression Technologies), diluted in PBS to 1 μg/ml for coating MaxiSorp wells (mouse trial 1) and 6 μg/ml for coating MediSorp wells (mouse trial 2), respectively. On the next day, plates were washed with PBST, blocked for 1.5 h with 2% BSA/PBS at 37°C. Bound IgG were detected using goat anti-mouse IgG (γ-chain specific)-HRP antibodies (Sigma-Aldrich). After incubation with TMB chromogen substrate solution (Sigma-Aldrich), absorbance was measured at 450 nm with a microplate reader (Synergy 2; BioTek Instruments).

Hemagglutinin Inhibition (HI) test. Sera samples were heat inactivated at 56°C for 30 min. and then were pretreated by kaolin and chicken erythrocytes to avoid a false positive reaction in the HI test (Bizanov & Tamosiunas, 2005). The pretreated sera (25 μl of sera in serial twofold dilutions) were incubated for 25 min in a titration plate with 4 HA units of the inactivated antigen (Bizanov and Tamosiunas, 2005). Next, the suspension of 1% hen's erythrocytes was added and incubated for 30 min. The HI titer was determined as the reciprocal of the highest dilution in which hemagglutination is inhibited. Samples were assigned as positive when their titer was ≥ 16. Sera from the immunization experiments (Trials 1 and 2) were tested using a heterologous strain H5N2 A/chicken/Belgium/150/1999 virus (GD Deventer).

Accession numbers. Sequences used in this study are available in public databases: H5N1 A/swan/Poland/305-135V08/2006 (EpiFluDatabase [http://platform.gisaid.org], GenBank Accession No. EP1156789).

RESULTS

Protein expression and purification

In order to evaluate the potential of *P. pastoris* as an expression host for production of a recombinant influenza H5 hemagglutinin, the pPICZαC/HAHis₆ vector was constructed. Template for amplifying the HA sequence was derived from H5N1 influenza virus (A/swan/Poland/305-135V08/2006). Primers were designed to produce an anchor-free HA cDNA with extended ends that facilitated cloning and yielded a product containing the affinity tag. The amplified his- HA sequence fused to the heterologous α-mating factor prepro-secretion signal from *S. cerevisiae* that directs protein export into the medium was ligated into appropriate restriction sites of the pPICZαC plasmid. DNA sequencing confirmed correct orientation of the insert. Following amplification in the *E. coli* DH5α strain, the pPICZαC/HAHis₆ plasmid was linearized with *PmeI* and used for transformation of *P. pastoris* KM71 and SMD 1168 strains. Expression of the HA gene was induced by the activation of *AOX1* promoter with methanol. *P. pastoris* transformants grown in the methanol-containing BMMY medium started to secrete the HA protein. Induction of HA was already visible after 24 h, but was potentiated with time, an optimum being attained after 5–7 days. Figure 1 shows the rHA overexpression in a mean time of induction. SDS/

PAGE followed by Coomassie Brilliant Blue staining indicated that almost 90% of the overexpressed protein was secreted into the medium (not shown). Two *P. pastoris* strains were tested for protein expression and KM 71 strain yielded a higher protein level in comparison to that of SMD 1168 (15 mg and 9 mg of total purified HA protein per liter, respectively). Therefore, this strain was selected for further optimization experiments. We observed a significant effect of the pH of the extracellular medium on the protein expression and the pH 7.8 was selected for efficient protein production. In preliminary experiments, 1% methanol was added every 24 h until the optimal time of induction was reached. Further experiments have shown that a single induction with 5% methanol and 7 day cultivation after induction yielded the highest rHA level. For purification purposes *P. pastoris* shake-flask culture was scaled up to 400 ml BMGY medium and 100 ml of BMMY medium. The recombinant protein secreted into the culture medium was purified using affinity chromatography under native conditions. After one-step purification on the Ni-NTA resin the rHA protein was pure when analyzed by SDS/PAGE (Fig. 2). Typically, 1.3–1.5 mg of recombinant antigen was obtained from 100 ml of induced yeast-culture supernatant.

Characterization of purified rHA

The theoretical molecular weight of His-tagged HA lacking C-terminal transmembrane anchor and cytoplasmic domain is 59.5 kDa. The molecular weight of HA1 and HA2 domains is 39.5 and 20 kDa, respectively. SDS/PAGE analysis has shown that the rHA protein expressed in *P. pastoris* displayed three bands with molecular weight about 65, 47 and 22 kDa (Fig. 2). Two of the higher molecular weight bands gave a positive signal in Western blot analysis using anti-His and anti-HA1 antibodies (Fig. 1A and B). Mass spectrometry analysis of protein bands confirmed the presence of the whole rHA and two cleaved subunits. In order to determine whether the

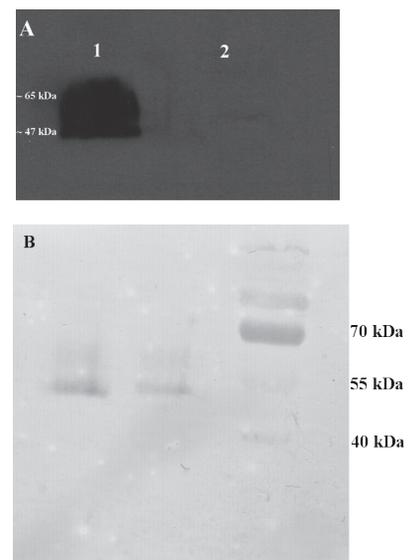


Figure 1. Western blot analysis of rHA overexpression. Protein samples were electrophoresed in 12% SDS/PAGE and transferred onto membrane and identified by anti-His (A) and anti-HA1 (B) antibody. A — culture after 70 h of methanol induction. Line 1 — 1 ml of culture medium precipitated with TCA. Line 2 — cell pellet from 1 ml of culture. B — Ni-NTA column purified rHA, line 1 and 2 — about 2 μg of protein eluted consecutively from column by 250 mM imidazole. M — protein marker.

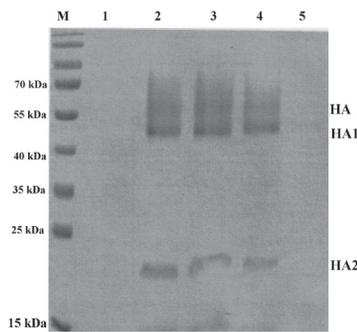


Figure 2. Purification of rHA by Ni-NTA chromatography.

M — protein marker, Lane 1 — wash with PBS, Lane 2 to 5 — protein eluted with 250 mM imidazole.

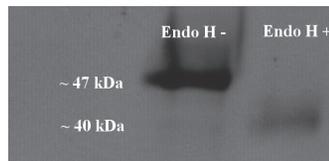


Figure 4. Western blot analysis of rHA1 treated with Endo H. rHA purified from *P. pastoris* KM 71 strain was deglycosylated with Endo H.

Protein samples were electrophoresed in 12% SDS/PAGE and transferred onto membrane and identified by anti-His antibody.

rHA protein is partially cleaved into HA1 and HA2 subunits, an excess of a reducing agent was added to the elution fractions. Under reducing conditions only two bands: about 47 and 22 kDa were seen, indicating that the whole pool of rHA expressed in *P. pastoris* is cleaved into HA1 and HA2 subunits (Fig. 3).

MS/MS analysis of protein

The difference between the calculated molecular weight of rHA and its subunits and the apparent molecular weight of SDS/PAGE bands indicated protein modifications. After a reaction with Endo H, the molecular weight of about 47 kDa band decreased to about 40 kDa (Fig. 4). This shift in molecular weight suggests glycosylation of the rHA protein produced in *P. pastoris*. Mass spectrometry analysis confirmed that all of the predicted glycosylation sites in HA1 subunit are glycosylated (see Table 1).

Glycosylated and truncated rHA elicits a high antibody titer

Purified, His-tagged hemagglutinin was tested for immunogenicity in a mice model. To determine an optimal dose of the antigen, mice were immunized intradermally with three doses of rHA given at 3-week intervals. A control group received only adjuvant at the same intervals and it did not induce an HA-spe-

Table 1. Table represents N-glycosylated peptides from the HA of H5N1 influenza strain (A/swan/Poland/305-135V08/2006) confirmed by LC/MS analysis.

N-linked glycosylation sites are underlined and in bold. In case of 1-22 peptide, two neighboring asparagine residues are modified.

Glycosite	Residue	Region	Amino acid sequence
1	1-22	HA1	HANN ST EQVDTIMEK
2	22-35	HA1	NVT VTTHAQDILEK
3	163-189	HA1	SY NNT NQEDLLVLWGIIHPNDAAEQTR
4	278-304	HA1	CQTPIGAI NS SMPF

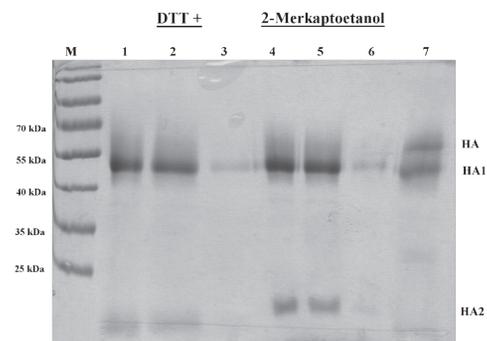


Figure 3. Electrophoretic analysis of rHA protein under reducing and non-reducing conditions.

cific immune response. After the second, and more clearly, after the third antigen injection, a significant immune response was detected in mice immunized with 1, 5 and 25 μ g of rHA (Fig. 5A). Sera samples of mice immunized with the lowest antigen dose (1 μ g) show the highest IgG titer reached ca. 1 mln after the second booster, however, with a significant scatter. Not surprisingly, the individual response seems to normalize with higher antigen doses, i.e. 25 μ g. Therefore, the next immunization series were done with an elevated antigen concentration. Titer in the HI assay was considered as a preliminary and indirect measure of neutralizing antibody response against the rHA antigen. Group immunized with 1 μ g of rHA showed a maximum of 512 HI titer but only 3 mice out of 5 were positive. To explore whether the route of immunization affected the efficacy of humoral response in mice, the animals were immunized intradermally and subcutaneously with 25 μ g of rHA. After third immunization, mice immunized subcutaneously showed a higher IgG titer (Fig. 5B).

DISCUSSION

Influenza virus is a pathogen inducing respiratory disease with symptoms varying from mild to severe. Certain strains of the virus could be even lethal to the host. The virus recurring epidemics, quite often evolving into pandemics, are a constant menace to public health. Besides humans, the virus spreads among swine, horses, and dogs, but mainly in birds, often pointed out as the primary host of the pathogen. Until now, there are no safe medications against influenza. Effectiveness of a quite recently introduced drug — tami-flu — is under discussion, and the strains resistant to the drug already circulate in the population. A realistic barrier against the disease remains therefore by vaccination against influenza. It is well established that the virus' hemagglutinin is the main antigen, inducing neutralizing antibodies. However, the HA gene mutates fast and the resulting "antigenic drift" necessitates constant creation of novel vaccine versions acting against seasonal versions of the pathogen. This is a rather compelling task as the standard vaccines are prepared from the viruses grown on a large scale in chicken embryos. Production of the recombinant HA in appropriate expression systems is an alternative approach. Some of such systems were already successfully adapted to production of a recombinant

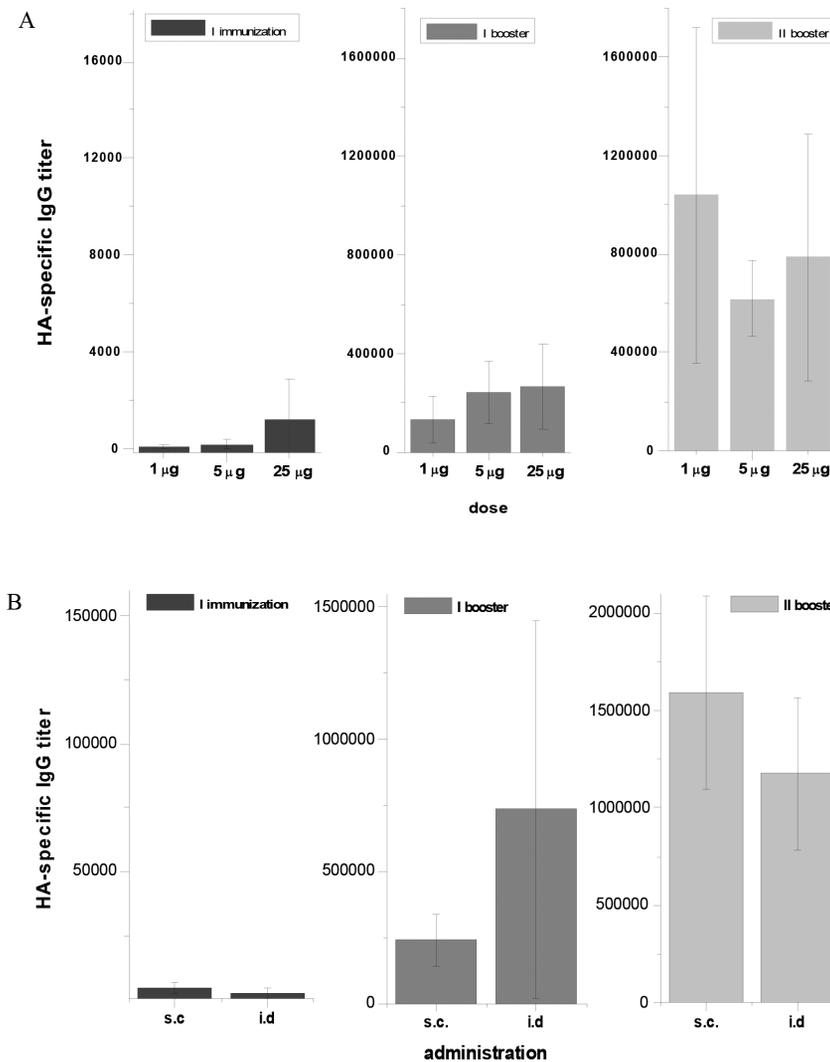


Figure 5. A. HA specific serum IG endpoint titers after mice immunization with *P. pastoris* rHA.

Bars represent the average of the titers in sera collected separately from 5 individuals. For each bar, the range of scattering is indicated. The endpoint titer is defined as the highest dilution of serum yielding in ELISA test absorbance 4 times over background. **B.** Evaluation of the route of antigen administration. Mice were injected with 25 µg doses of rHA antigen either subcutaneously (s.c) or intradermally (i.d.).

antigen, useful as a so called “subunit” or “new generation” vaccine, but have some disadvantages. Production in mammalian or insect cell lines (Wu *et al.*, 2010; Crawford *et al.*, 1999) is costly, requires high-tech mass cell production that is under constant threat of foreign pathogen introduction. HA may be overexpressed in *E. coli*, but the product is insoluble and the purification is tedious. Searching for an optimal antigen production method we opted for a simple and inexpensive *Pichia pastoris* system. Here, secretion of the overexpressed polypeptide considerably facilitates the purification of the product. In the literature, there are reported several attempts at the production of HA in *P. pastoris*. Full length HA protein of H1 type (Athmaram *et al.*, 2011; 2012) and H5 type virus (Wang *et al.*, 2007) were expressed in *P. pastoris* as partially secreted, soluble proteins. The level of expressions appeared to be low. Expression of the H5 type polypeptide was also reported by the Subathra group (Subathra *et al.*, 2014) but the protein was not exported out of the cells. In the system described here, the overproduced protein is secreted up

to at least 90%, considerably facilitating further purification of the antigen. We anticipated that such efficient secretion results from the truncation of the sequence coding for 16 N-terminal amino acids (signal peptide) and the C-terminal transmembrane domain. This HA fragment is responsible for anchoring in the cell membrane and its deletion may be beneficial for secretion. Our selection of an appropriate producer strain is also advantageous. Contrary to the common observations, it is not the standard SMD 1168 secreting strain, but KM 71 which efficiently secretes the HA antigen. This is surprising as in our previous experiments with expression of several polypeptides, SMD 1168 appeared to be an efficient secretion strain (Grzelak *et al.*, 2003; Kludkiewicz *et al.*, 2005). An important factor for activity of HA is its specific cleavage into H1 and H2 subunits (Klenk *et al.*, 1975; Lazarowitz & Choppin, 1975; Steinhauer, 1999). In contrast to other reports (Wang *et al.*, 2007) the rHA produced in our system is fully processed into subunits, without traces of further proteolysis (see Fig. 2). The data presented here demon-

strate that the influenza antigen produced in *P. pastoris* with good efficiency is highly immunogenic and might be considered as a candidate for a subunit vaccine.

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