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An avian influenza H5N1 virus vaccine candidate based on the extracellular domain produced in yeast system as subviral particles protects chickens from lethal challenge☆

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ABSTRACT

Highly pathogenic avian influenza is an on-going problem in poultry and a potential human pandemic threat. Pandemics occur suddenly and vaccine production must be fast and effective to be of value in controlling the spread of the virus. In this study we evaluated the potential of a recombinant protein from the extracellular domain of an H5 hemagglutinin protein produced in a yeast expression system to act as an effective vaccine. Protein production was efficient, with up to 200 mg purified from 1 L of culture medium. We showed that the deletion of the multibasic cleavage site from the protein improves oligomerization and, consequentially, its immunogenicity. We also showed that immunization with this deleted protein protected chickens from challenge with a highly pathogenic avian influenza H5N1 virus. Our results suggest that this recombinant protein produced in yeast may be an effective vaccine against H5N1 virus in poultry.

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1. Introduction

Avian influenza (AI) is a highly infectious and economically relevant disease affecting the poultry industry. It also remains a potential public health threat. The recent avian influenza outbreak in the United States led to the destruction of more than 40 million birds, which caused huge economic losses (http://www.usda/avian_influenza.html). All control programs for AI share three broad approaches: prevention, good management and eradication (Swayne, 2012). Vaccines are one tool that may be used in an outbreak to support the eradication of the virus because they boost immunity and decrease virus shedding into the environment. Current licensed vaccines are mainly based on inactivated whole AI viruses. However their application is limited by the difficulty in differentiating infected from vaccinated animals (DIVA) since both have the same antibodies. Recently new generation vaccines have been developed that induce antibodies solely to hemagglutinin (HA),thus facilitating the application of DIVA strategy by using a serological test that detects antibodies to antigens other than HA, so providing evidence for field infection (Swayne and Kapczynski, 2008; Lee et al., 2014). Among these vaccines the recombinant fowlpox-AI-H5 and recombinant herpesvirus-turkey-AI-H5

Abbreviations: HA, hemagglutinin; AOX, alcohol oxidase; Endo H, endoglycosidase H; HPAI, highly pathogenic avian influenza; MBCS, multibasic cleavage site; IMAC, immobilized metal ion affinity chromatography; PAGE, polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; DLS, Dynamic Light Scattering; Rhyd, hydrodynamic radius; TEM, transmission electron microscopy; SPF, specific pathogen-free; EID_{50}, 50% egg infective dose; HI, hemagglutination inhibition; DIVA, differentiating infected from vaccinated animals; HexNAc, *N*-acetylhexoseamine.

☆ To the memory of Professor Krystyna Grzelak and Professor Włodzimierz Zagórski-Ostoja.

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licensed vaccines serve as an alternative. However, recombinant fowlpox-AI-H5 vaccine can only be used in chickens which must be naïve to the fowl pox vector (Swayne et al., 2000). Since the antibodies neutralizing the influenza virus are mainly directed against the HA protein, a dynamic development in subunit vaccine research is currently being observed.

It is a great challenge, using recombinant technology, to produce a properly folded HA protein required for optimal immunogenicity. Hemagglutinin is synthesized as a precursor polypeptide (HA0). This protein is cleaved by proteases to create two subunits, HA1 and HA2, linked by a disulfide bond. HA is post-translationally modified by glycosylation that was shown to play an important role in a proper folding and trimer stabilization (Copeland et al., 1986; Roberts et al., 1993; Sun et al., 2013). On the surface of virions, the trimeric HA is stabilized mainly by residues from the HA2 region (Eisenberg and McLachlan, 1986; Wilson and Cox, 1990). Structurally, the hemagglutinin ectodomain is composed of two distinct regions: (1) a long fibrous stem region, containing residues from both HA1 and HA2 (2) a globular head region containing residues entirely from HA1. Although most of the antibodies neutralizing the influenza virus are directed mainly against the variable globular head of hemagglutinin (Wiley et al., 1981), the HA2 region was also reported to induce neutralizing antibodies (Russ et al., 1987; Gocnik et al., 2008; Pica et al., 2012). These findings suggest that a vaccine based on the extracellular domain of HA would be effective against influenza virus infections.

Another important part of the HA protein is the cleavage site between the HA1 and HA2 domain. The sequence of amino acids in the HA0 cleavage site differs among the virus strains and this is a determining factor of viral pathogenicity (Steinhauer, 1999; Senne et al., 1996). The highly pathogenic avian influenza (HPAI) viruses of the H5 and H7 subtypes contain a multibasic cleavage site (MBCS) motif (Munster et al., 2010). HPAI viruses can be transmitted to humans as well as other mammals (Kuiken and Taubenberger, 2008; Reperant et al., 2009). It has been reported that the modification of MBCS of the H5 HA to a single basic amino acid attenuates viral pathogenicity but preserves the antigenicity of the hemagglutinin protein (Subbarao et al., 2003).

We recently demonstrated that recombinant influenza H5 protein produced in *Pichia pastoris* induced a strong immunological response in mice, even though it was cleaved into two subunits (Kopera et al., 2014). The *P. pastoris* system was chosen because of its many advantages. It has been extensively utilized as an industrial platform to produce various proteins of interest, including vaccine antigens (Shanvac™, Elovac™, Gavac™). *Pichia* cells offer the possibility of producing a high level of the desired recombinant protein, are able to carry out post-translational modifications (such as glycosylation and the disulfide bond formation) and in addition efficiently secrete heterologous protein into the culture medium which greatly facilitates its subsequent purification. In the current study, we show that deletion of MBCS in the extracellular region of the H5 antigen improves protein oligomerization. Further, we demonstrate that such a modified antigen is more immunogenic than HA with MBCS, and protects from a lethal challenge with the H5 subtype of HPAI.

2. Materials and methods

2.1. Expression of H5N1 hemagglutinin in P. pastoris

The H5N1 strain A/swan/Poland/305-135V08/2006 clade 2.2.2 (EpiFluDatabase Accession No. EPI15789) was the source of HA gene GenBank accession number: KC172926. The DNA fragment encoding the extracellular domain of HA (Fig. 1) was amplified using forward and reverse primers containing *Cla* and *Sac*II restriction site, respectively. The cleavage site (MBCS, aminoacids RRRKKR) in the hemagglutinin sequence of the influenza H5N1 virus was deleted in a two-step site-directed mutagenesis reaction. The extracellular domain (H5DH and H5DHΔ) was cloned into pPICZαC in frame with α-factor. The His-Tag sequence was added at the C-terminus using the reverse primer. Obtained constructs pPICZαC/H5DH and pPICZαC/H5DHΔ were introduced by electroporation into the *P. pastoris* KM 71 strain (Invitrogen, USA). The yeast transformants were screened for insertion by PCR using 5′ AOX I and 3′ AOX I primers. Yeast clones with verified inserts were grown as previously described (Kopera et al., 2014). Briefly, the positive transformants were inoculated in 100 ml or 400 ml of BMY medium with 1% glycerol and were grown at 30 °C on a plate agitator at 200 rpm agitation. After 48 or 72 h cells were harvested by centrifugation and resuspended in one/fourth of the original volume of BMY with 5% methanol. Subsequent incubation at 26 °C with vigorous shaking was continued for 7 days.

2.2. Purification of H5DH and H5DHΔ proteins

Recombinant hemagglutinins were purified from the culture medium. Protein binding to Ni-NTA Agarose (Qiagen, Germany) was carried out in PBS pH 7.8 with additional 400 mM NaCl. The HA protein was eluted from the column with 250 mM imidazole in PBS pH 7.4. Protein amount was determined using the Bradford method (Bio-Rad, USA). The HA proteins were dialyzed against PBS pH 7.4 followed by lyophilization and stored at −20 °C. The H5DH and H5DHΔ proteins were analyzed using 4–12% SDS-PAGE (Bio-Rad, USA) and by 12% Native-PAGE. H5DH and H5DHΔ proteins (50 μg) were analyzed by High Performance Liquid Chromatography (HPLC, Breeze, Waters, UK) on a C4 column (250 × 4.6 mm, ACE, UK). The eluting solvent A was 0.1% trifluoroacetic acid/water, and solvent B was 0.1% trifluoroacetic acid/90% acetonitrile/water. A linear gradient from 0% B to 100% B in 100 min at a flow rate of 1 ml/min was applied, with dual detection at 220 and 280 nm.

2.3. MS/MS analysis of H5DHΔ protein

H5DHΔHis_6 protein was denatured with denaturing buffer at 95 °C for 10 min. The reaction mix containing denatured HA protein and 0.125 U of endoglycosidase H (Endo H, New England Biolabs, USA) was incubated at 37 °C for 1 h. The non treated protein sample was used as a control. The protein was analyzed using SDS-PAGE. The gel band containing deglycosylated polypeptides was excised. The protein was reduced with 100 mM 1,4 Dithiothreitol (30 min, 56 °C), alkylated with 0.5 M iodoacetamide (45 min in the dark, room temperature) and digested overnight with trypsin (sequencing Grade Modified Trypsin – Promega, USA) by adding the enzyme directly to the reaction mixture. Peptides were analyzed by LC-MS-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using the Nano-Acquity (Waters, UK) LC system and the Orbitrap Velos mass spectrometer (Thermo Electron Corp., USA) as previously described (Kopera et al., 2014). Fragmentation spectra of peptides indicated by Mascot as *N*-glycosylated were manually investigated.

2.4. Characterization of H5DHΔ antigen

H5DHΔ at a concentration of 0.5 mg/ml was loaded on a Superdex 200 10/300 GL column (GE Healthcare, UK), pre-equilibrated with 10 mM Tris pH 7.8 with 200 mM NaCl and the protein elution was monitored at 280 nm. Molecular weight standards (Bio-Rad, USA) were used to calibrate the column and to identify the molecular weights of proteins present in the samples. Dynamic Light Scattering measurements of the purified H5DHΔ

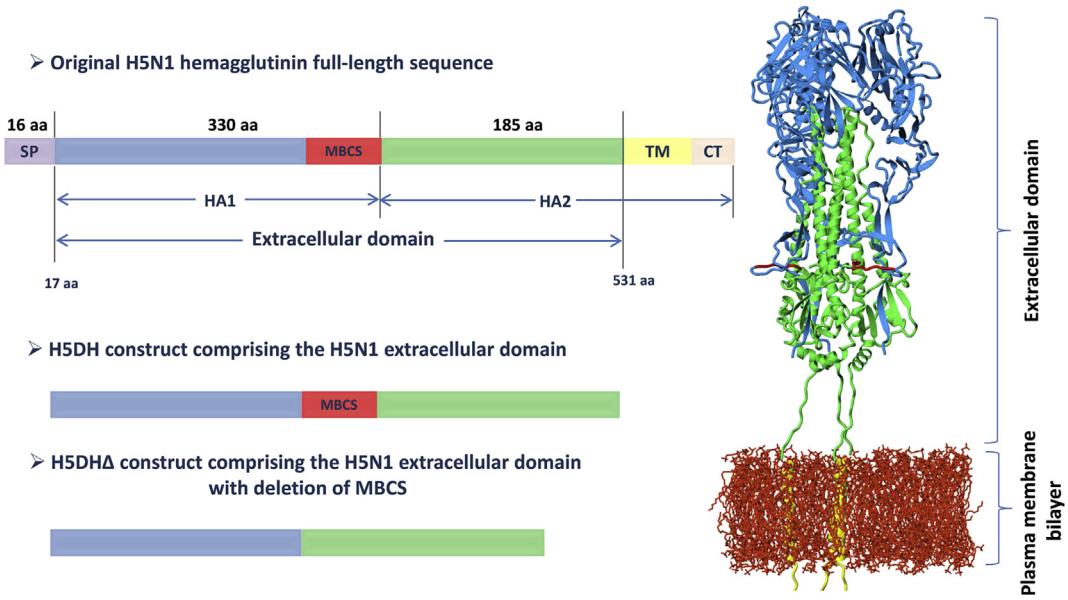

Fig. 1. Schematic diagram of the HA protein and two constructs – H5DH and H5DHΔ. SP – signal peptide, TM – transmembrane domain, CT – cytoplasmic domain, MBCS – multibasic cleavage site. The schematic trimer structure was modelled from the PDB ID code 5E2Y in the YASARA program.

protein were performed on the DynaPro NanoStar DLS instrument (Wyatt Technology Corp., USA). Selected fractions containing high molecular weight forms (HMW) and low molecular weight forms (LMW) of H5 protein from the SEC analysis were diluted with buffer (10 mM Tris pH 7.8, 200 mM NaCl) to 0.1–1.0 mg/ml, and spun down at 20,000×g for 5 min. All measurements were taken at 25 °C in 50 μl disposable cuvettes (UVette, Eppendorf, USA) with a 5 s acquisition time and 10 acquisitions per measurement. The collected data was analyzed on the DYNAMICS v7 software (Wyatt Technology Corp., USA). The H5DHΔ protein sample was applied to the clean side of carbon on mica and negatively stained with 2% (w/v) sodium silico tungstate. A grid was then placed on top of the carbon film which was subsequently air-dried.

Images were taken under low dose conditions (less than 20 e-/A2) with a T12 FEI electron microscope at 120 kV using an ORIUS SC1000 camera (Gatan, Inc., Pleasanton, CA).

2.5. Ethic statements

The experiments were approved by the Second Local Ethical Committee for Animal Experiments at the University of Life Sciences in Lublin, Permit No. 70/2013. All procedures were according to Regulation of the Minister of Agriculture and Rural Development on the Specific Conditions of Keeping Animals in Laboratory Animal Farms and in Entities Carrying out Experiments and Tests and the performance of experiments on animals were governed by the Act on Experiments on Animals (Polish Law, 2005). All the chickens were raised and handled humanely. All efforts were made to minimize suffering. The chickens were monitored two times a day, including weekends. At the end of experiment the chickens were humanely euthanized (cervical dislocation).

2.6. Chicken immunization

Layer chickens (ISA brown) were housed in poultry-house in cages, in standard commercial conditions. Groups of ten chicken were immunized subcutaneously in the neck skin fold twice at the 21st and 49th day of life with 25 μg of H5DH or H5DHΔ supplemented with 0.3% Alhydrogel (aluminium hydroxide, Gentaur, Germany). The control group received only adjuvant according to the same schedule. Four weeks after booster blood was collected from the wing veins, allowed to coagulate, and centrifuged. The collected sera were kept at −80 °C. For the wild-type H5N1 challenge study, ten specific pathogen free (SPF) White Leghorn chickens (VALO BioMedia GmbH, Germany), housed in a biosafety level 3 containment, were immunized subcutaneously into the neck skin fold with 25 μg of H5DHΔ protein. The recombinant antigen suspended in PBS was supplemented with 0.3% Alhydrogel. Control group (5 chickens) was only administered specific adjuvant according to the same schedule. Re-vaccination was carried out 4 weeks later as described above. Prior to challenge, the chickens were placed in HEPA-filtered isolators (Montair Andersen B.V., Netherlands).

2.7. ELISA

The 96-well polystyrene plates (Nunc, Denmark) were coated overnight at 4 °C with recombinant H5 HA (A/Bar-headed Goose/Qinghai/12/05 H5N1, clade 2.2) produced in a mammalian cell system (Immune Technology, USA) diluted in PBS to 6 μg/ml. Bound IgY were detected using goat anti-chicken IgY (Fc-specific)-HRP (Thermo Scientific, USA) antibodies. After incubation with TMB chromogen substrate solution (Sigma-Aldrich) absorbance was measured at 450 nm (A_{450}) with a microplate reader (Synergy 2, BioTek Instruments). The *cut off* for the ELISA was set as the mean A_{450} of the control group plus two standard deviations.

2.8. Challenge studies

The immunized chickens as well as control birds were inoculated oculonasally with 10^6 50% egg infectious dose (EID_{50}) of the H5N1 A/turkey/Poland/35/2007 (clade 2.2.3, isolated in 2007 from a meat turkey flock in Poland) virus in the volume of 100 μl intraocularly (50 μl) and intranasally (50 μl). Clinical observation was performed twice daily. Oropharyngeal and cloacal swabs were collected from all birds at 4, 7, 10 and 14 days post inoculation and in the case of control birds only at 3 days post inoculation. RNA was extracted from 0.2 ml of the transport medium using a NucleoSpin

8 Virus Core kit (Macherey-Nagel, Germany) in a JANUS® automated workstation (PerkinElmer). RT-PCR/M with primers developed by Spackman et al (Spackman et al., 2008). and Quantitect Probe PCR reagents (Qiagen, USA) was performed. Ten-fold dilutions of the RNA extracted from allantoic fluids collected from SPF eggs inoculated with a titrated homologous H5N1 virus were also tested. The results of the qRT-PCR were converted from cycle threshold values and expressed as equivalent EID_{50} per milliliter of swab medium. At the end of the experiment, the chickens that survived infection with virulent virus were euthanized by cervical dislocation.

2.9. Hemagglutation inhibition test

Two and four weeks post prime vaccination, two and three weeks post booster and two week after challenge, samples of sera were collected from birds and tested in a hemagglutination-inhibition (HI) test. The HI test was performed according to a standard procedure (OIE, 2015). Briefly, 10 μl of sera in serial twofold dilutions were incubated for 25–30 min with four HA units of the inactivated antigen A/turkey/Poland/35/2007 H5N1. Next, a 1% suspension of chicken erythrocytes was added and incubated for 30 min. The HI titer was assessed as the reciprocal of the highest dilution in which hemagglutination was inhibited.

3. Results

3.1. Characterization of glycosylated hemagglutinin variants

The extracellular domain of H5N1 hemagglutinin (residues 17-531) was selected because it was previously shown to induce a strong immunological response in mice (Kopera et al., 2014). Therefore we presumed that the recombinant protein encompassing these residues may adopt the correct three-dimensional structure required for trimer formation and/or higher oligomerization. The transmembrane region and cytoplasmic tail of HA2 were excluded in order to favour secretion of the protein into the culture medium. In order to obtain uncleaved protein product we excluded the 18 nucleotides coding for the MBCS site. Finally we obtained two variants of the H5 antigen: H5DH (residues 17-531) and H5DHΔ (residues 17-531, Δ341-346, amino acids RRRKKR excluded) (Fig. 1). Coomassie blue staining after the SDS-PAGE separation of the purified H5DH and H5DHΔ protein showed a high level of purity after one-step purification (Fig. 2A and B). The purity of the proteins was further confirmed by HPLC (Fig. 3). Both SDS-PAGE and HPLC analysis indicated that the H5DHΔ protein was produced as an uncleaved polypeptide, while H5DH was proteolytically processed into separate domains (HA1 and HA2). Analysis of the HA (A/swan/Poland/305-135V08/2006) sequence determined six potential *N*-glycosylation sites (5 sites in HA1 and 1 site in HA2) in the extracellular domain of HA protein using NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/). In order to examine *N*-glycosylation sites of recombinant HA antigens we used a standard proteomic procedure for Endo H treated proteins. Mass spectrometry analysis confirmed that all predicted *N*-glycosylation sites in H5DH (Kopera et al., 2014) and three sites in H5DHΔ (Table 1) are glycosylated.

Yields as high as 20 mg and 200 mg of final purified recombinant antigen per 1 L were obtained after the IMAC procedure for H5DH and H5DHΔ, respectively. Repeated production batches indicated excellent reproducibility.

3.2. Immunogenicity of H5DH and H5DHΔ antigens

To identify potential differences of the responses in the two groups (H5DH and H5DHΔ) sera of the individual birds were tested.

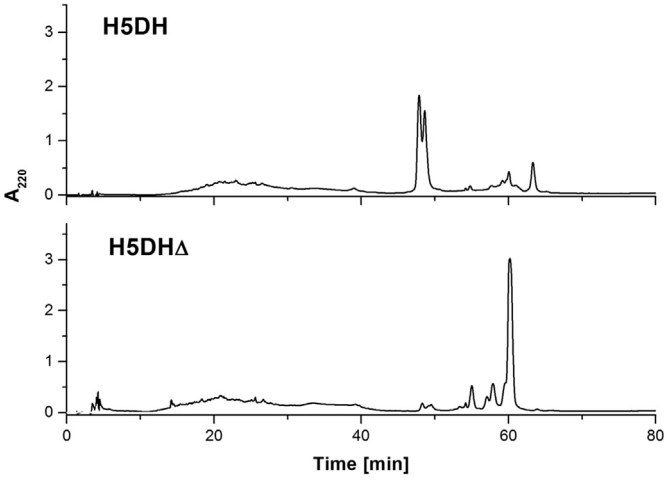

Fig. 3. Analytical HPLC chromatograms of H5DH and H5DHΔ protein. Absorbance at 220 nm is shown. A volume corresponding to 50 μg of protein was injected onto ACE C4 column as described in Materials and methods.

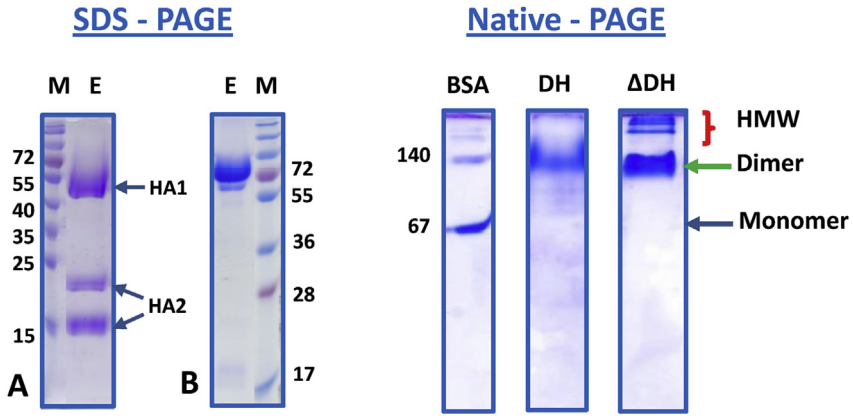

Fig. 2. Electrophoretic analysis of H5DH and H5DHΔ after the one step IMAC purification. Fractions of H5DH (A) and H5DHΔ (B) protein were analyzed by SDS-PAGE. Samples containing molecular weight marker (M), 15 μl of eluted protein (E) were electrophoresed in 12% SDS-PAGE gel and stained with Coomassie Blue. Reducing conditions during SDS-PAGE analysis disrupted all disulphide bonds (e.g. between cysteine 4 and cysteine 461) and visualized bands of both HA1 and HA2 domains. Two forms of HA2 domain suggest their different glycosylation states. Fractions of H5DH and H5DHΔ protein and bovine serum albumin (BSA) were also analyzed under native condition.

Table 1
N-glycosylated peptides from the H5DHΔ protein confirmed by LC/MS/MS analysis. N-linked glycosylation sites are underlined. Molecular mass of modified peptides (one HexNAc attached to the asparagine residue) was determined by spectrometer.

Residue	Region	Amino acid sequence	Mass
22–35	HA1	**NVT**VTHAQDILEK + HexNAc	1669.85
163–189	HA1	SY**NNT**NQEDLLVLWGIHHPNDAAEQTR + HexNAc	3337.56
500–513	HA2	**NGT**YDYPQYSEEAR + HexNAc	1894.78

HexNAc (*N*-acetylhexoseamine).
N-linked glycosylation sites are bolded and modified asparagine residues are underlined.

Immunization efficacy was measured at the humoral response level using an indirect ELISA and the hemagglutination inhibition (HI) test. Both variants of H5 antigen elicited specific anti-HA-IgY antibodies and only minor differences between these two antigens were observed (Fig. 4A). However, the significant differences in the immunological properties between these two antigens were disclosed in the HI test. Only 50% of the group immunized with H5DH were HI positive and no titers higher than 64 were observed, whereas 100% of chicken immunized with H5DHΔ were positive with higher HI titers (Fig. 4B). These results prompted us to investigate the oligomeric status of H5DH and H5DHΔ antigens.

3.3. H5DHΔ but not H5DH forms of higher oligomers

Electrophoresis under native conditions showed the high molecular weight forms in the sample of H5DHΔ (Fig. 2). Although under native condition the H5DH protein migrated as a whole molecule (under non-reducing conditions disulphide bonds are preserved), this variant of antigen formed only dimers. The H5DHΔ antigen was further analyzed by size exclusion chromatography (SEC) revealing two major peaks (Fig. 5A). The first peak eluting prior to a thyroglobulin (670 kDa) demonstrates that the recombinant antigen forms high molecular weight complexes. The second peak eluting between γ-globulin (158 kDa) and ovalbumin (44 kDa) indicates the presence of monomers or dimers. To assess the oligomeric profile of the HMW and LMW fraction after a lyophilization/resuspension cycle, we re-analyzed them separately. Both SEC-purified fractions remain stable (Fig. 5B and C). SEC-purified fractions were also analyzed using Dynamic Light Scattering (DLS). DLS data showed that for the HMW fraction the measured size corresponded to multimeric protein particles with an average hydrodynamic radius (Rhyd) of 30 nm. The average size of the particles eluting in the LMW fraction was 10 nm. In order to establish whether H5DHΔ protein forms biological nanostructures in a form of subviral particles electron microscopy analysis was performed. Transmission electron microscopy (TEM) showed that HMW oligomers formed regular, spherical-shaped nanostructures with an average size of 30 nm in diameter (Fig. 6). The size of the particles is consistent with the data obtained using DLS (Rhyd 30 nm). However, the morphology of H5DHΔ nanostructures visualized by TEM is variable.

3.4. H5DHΔ antigen protects from lethal H5N1 challenge

Our results strongly suggest that deletion of the MBCS motif in the H5 protein improves its oligomerization and immunogenicity. Therefore we selected the H5DHΔ protein to evaluate the protective efficacy of a recombinant vaccine. Serological examination using

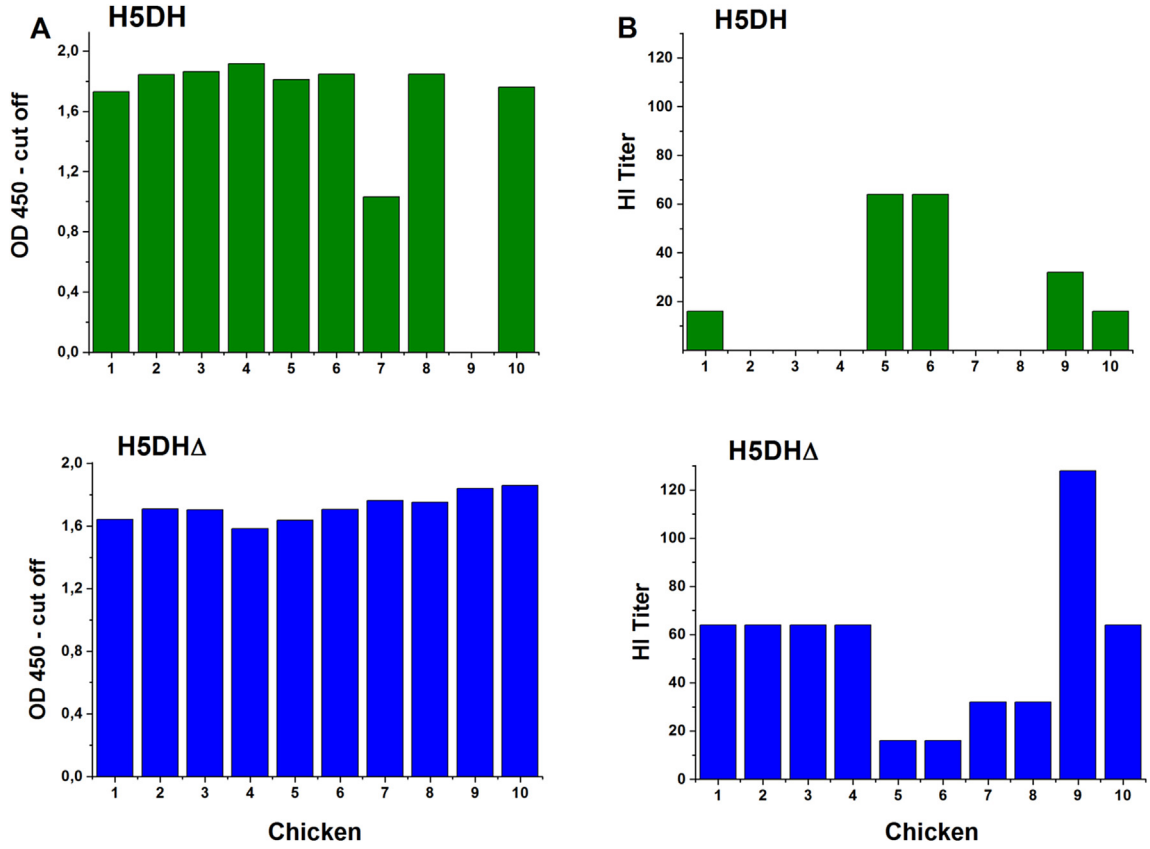

Fig. 4. Chickens response after immunization with H5DH and H5DHΔ antigen. Immune response of individual chicken immunized with 25 μg of H5DH or H5DHΔ were measured 4 weeks after booster by indirect ELISA (A) and HI test (B).

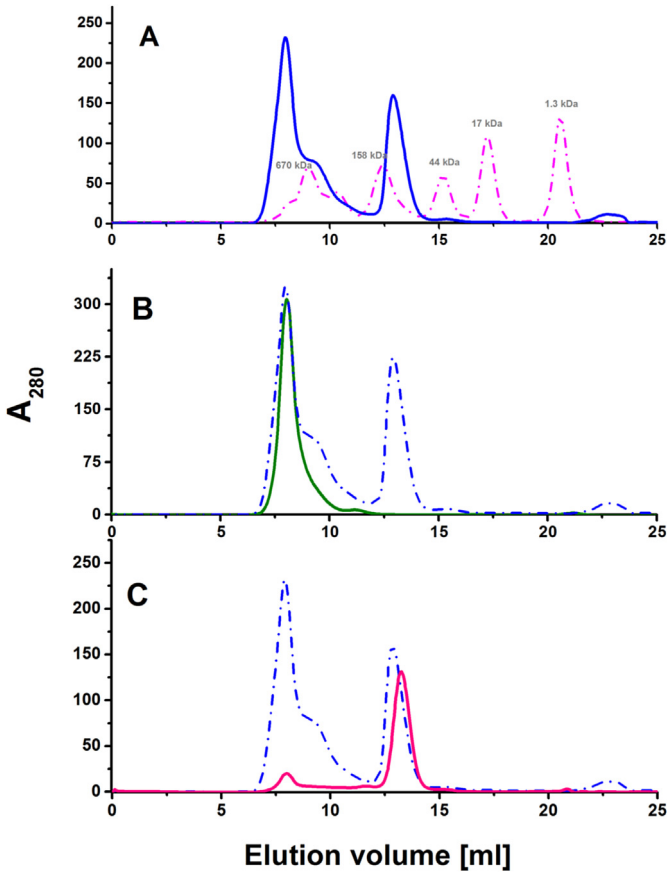

Fig. 5. Characterization of H5DHΔ oligomers and monomers by Size Exclusion Chromatography on Superdex 200 10/300 GL column. (A) Chromatogram of the IMAC - elution fraction (blue line). Molecular weight standard indicated by the magenta dotted line. Fractions of the H5DHΔ protein were lyophilized separately and dissolved in water followed by re-injection onto Superdex 10/300 GL column. The panels present superimposed elution profiles of purified H5DHΔ protein (dotted line) overlaid with SEC profile of stable oligomers (B) and monomers (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

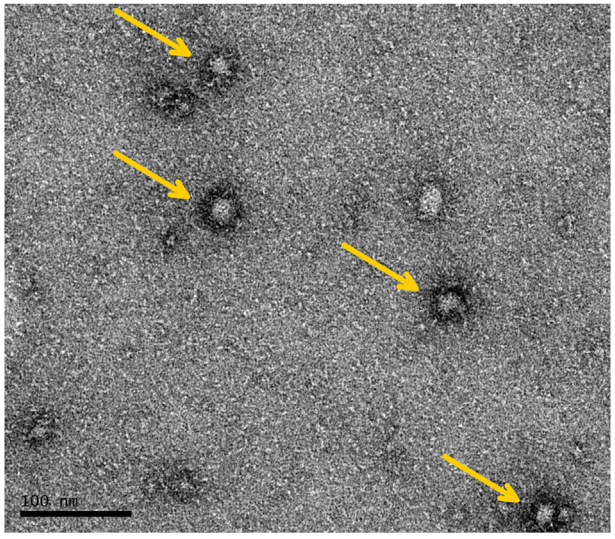

Fig. 6. Transmission electron microscopy of the purified H5DHΔ protein. Images were obtained at nominal 30 000 magnification. The black scale bar represents 100 nm.

the HI test revealed the steady increase of the humoral response after SPF chicken immunization with H5DHΔ antigen. Kinetics of the HI titer is presented in Fig. 7. Two weeks after the first vaccination the HI response was rather weak and only 50% of the group were HI positive. Two weeks later the immunological response had increased and 9/10 chickens were HI positive with antibody titer no higher than 64 (Mean $\log_2$ 4.4 ± 0.97). The second vaccination boosted the antibody levels (Mean $\log_2$ 6.1 ± 0.99) and all chickens had seroconverted. HI titers as high as 4096 were observed after challenge ($\log_2$ 9.6 ± 1.35). All vaccinated chickens survived challenge and no clinical signs were observed whereas control chickens died by 4 days post inoculation. High amounts of viral RNA (usually $>10^6$ eqEID$_{50}$ per ml swab fluid) were detected in samples collected from control birds. Only in three of ten immunized birds were small amounts of the virus detected (10^3–10^5 eqEID$_{50}$ per ml of swab fluid) in oropharyngeal and cloacal swabs (4 and 10 days post inoculation).

4. Discussion

For effective poultry vaccination a low-cost vaccine with a specific match to the antigen(s) of the responsible virus is strongly required. Recombinant hemagglutinin-based vaccines are a potential alternative for influenza vaccine manufacture. Here, we investigated the use of the *P. pastoris* expression system to produce a soluble HA antigen, which is the primary target of the neutralizing antibody. We demonstrated the feasibility of producing H5N1 HA antigen in yeast, that would allow rapid scale-up to high-volume production. Using simple fed-batch growth procedures we obtained high-level protein expression reaching 200 mg of the purified H5DHΔ antigen from 1 L of yeast medium. A difference in the expression levels between the H5DH and H5DHΔ proteins was observed. The reason is unknown but it might be caused by the differences in protein processing involving folding and glycosylation. However, further studies are needed to address this question.

One crucial issue for a veterinary sub-unit vaccine is the development of a simple and efficient process for purification of the desired antigen. The vaccine should ideally contain only desired compound and must be consistent in its composition. The *P. pastoris*

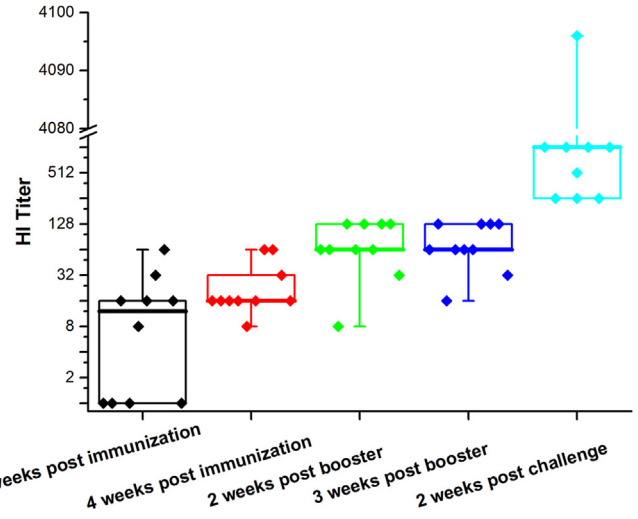

Fig. 7. A box-and-whisker plot showing kinetics of serum hemagglutination inhibition (HI) titers in chicken following prime and booster immunization and after challenge with homologous H5N1 strain (clade 2.2.3). The median (thick line) is shown with the interquartile range (25% and 75%, box) and the maximum and minimum (whiskers), n = 10.

system enables secretion of the overexpressed polypeptide which considerably facilitates purification of the product. Taking advantage of this secretion and applying affinity chromatography to the growth medium from which the yeast cells had been removed we managed to purify the H5DHΔ antigen to homogeneity in a one-step process.

The design of effective vaccines for the prevention of influenza is a challenging task. We selected residues 17 to 531 of H5 protein representing the extracellular domain because it was reported to induce a strong immunological response in mice (Kopera et al., 2014; Shoji et al., 2009). We tested two variants of the H5 antigen. Both H5DH and H5DHΔ were immunogenic in the chicken model. This is consistent with a previous study in which the modification of MBCS of H5HA to a single basic residue failed to alter the antigenicity of HA (Subbarao et al., 2003). It was also reported that influenza VLPs containing mutant HA without the multibasic cleavage site displayed an uncleaved precursor conformation and elicited immune responses comparable to VLPs containing wild type HA (Song et al., 2010). Our study demonstrated that only the H5DHΔ antigen elicited high HI titers. Analysis of the oligomerization status of H5DH and H5DHΔ antigen revealed that deletion of the MBCS improves protein oligomerization. We demonstrated that H5DHΔ formed oligomers with no requirement for the addition of any foreign trimerization sequence. It was reported that the expression of the recombinant HA ectodomain in mammalian cells or the baculovirus system required the addition of multimerization 'foldon' at the C terminus in order to produce a stable oligomeric structure (Wei et al., 2008; Weldon et al., 2010). Wei and colleagues analyzed various forms of rHA protein for their potential efficacy as vaccines (Wei et al., 2008). They reported that different rH5HA proteins vary in their ability to elicit neutralizing antibodies depending on their multimeric nature. High-molecular weight oligomers stimulated the strongest antibody response, followed by the trimeric form of the H5HA molecule, while H5HA monomers were poorly immunogenic. On the other hand, despite being monomeric, the H3HA protein expressed in the *P. pastoris* was protective against a lethal influenza challenge in mice (Saelens et al., 1999).

Transmission electron microscopy visualized H5DHΔ particles with the average size of 30 nm that morphologically resembled influenza virions. Pushko and colleagues showed that purified full-length HA formed oligomeric pleomorphic subviral particles of 20 nm in diameter (Pushko et al., 2015). It was also reported that the oligomeric fraction of rHA1 forms rosette-like structures (Verma et al., 2012). For the first time we showed that the H5DHΔ protein that comprised the extracellular domain of hemagglutinin assembled into spherical structures. However, the mechanism of the H5DHΔ subviral particles formation remains to be elucidated.

The chicken model has been commonly used to evaluate the virulence and the pathogenicity of the avian influenza viruses as well as vaccine effectiveness. The assessment of protection by avian influenza vaccines is best accomplished using a challenge model. Vaccine efficacy can be quantified by measuring the prevention of morbidity and mortality, prevention of egg production drops and quantitative reduction in shedding of the challenge virus (Swayne, 2009). In the challenge experiment the H5DHΔ antigen proved to be strongly immunogenic for SPF chickens, which mounted satisfactory level of antibodies and were fully protected against challenge with homologous H5N1 strain (clade 2.2.3) that is slightly different from the original strain (clade 2.2.2). It should be pointed out that a minimum of 80% protection from mortality is sufficient in efficacy requirements for a vaccine in countries where vaccination against avian influenza is permitted (OIE, 2015). Virus shedding was also significantly reduced compared to sham-vaccinated controls. Only small amounts of virus in three of ten vaccinated chickens were found in oropharyngeal and cloacal swabs. It is well known that vaccines against highly pathogenic avian influenza viruses reduce sickness, clinical signs and death, but they would not completely prevent birds from becoming infected and shedding virus into the environment (USDA, Avian Influenza).

In summary, the H5 antigen based on the extracellular region of hemagglutinin with deletion of the multibasic cleavage site oligomerizes into functional spherical-like structures. H5DHΔ is highly immunogenic and provides clinical protection following a challenge with a homologous strain. The presented data demonstrated that the influenza antigen efficiently produced in *P. pastoris* can be considered as a candidate for a subunit vaccine.

5. Conclusion

The H5DHΔ antigen expressed by yeast contains a high proportion of oligomeric antigen that appears to be an effective vaccine. When it is combined with the appropriate adjuvant it generates high-affinity antibodies with the capacity to neutralize the homologous strain. A high level of production and ease of differentiating infected from vaccinated animals (DIVA) are the major advantages of this vaccine candidate.

Conflicts of interest

None.

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