Expression of Truncated Sequences of Influenza A Virus Subtype H5 in *Pichia Pastoris*

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Abstract: Influenza A virus haemagglutinin (HA) is the major target for protective immune responses in the natural host. Vaccines based on conserved antigenic determinants could provide efficient protection from disease and infection. Therefore, four non-overlapping sequences (designated P1, P2, P5 and rHA1) of different functional domains of influenza A virus subtype H5 were cloned and expressed in *Pichia pastoris (P.pastoris)*. P1, P2 and rHA1 polypeptides were purified using nickel affinity chromatography, whereas, P5 was purified using lectin affinity chromatography. Correct expression was analysed by SDS-PAGE, Western blot, glycosylation analysis and MALDI-TOF. These results form the basis for generation of monoclonal antibodies, development of recombinant Elisa and subunit vaccines.

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1. Introduction

Avian influenza (AI) affects the respiratory, digestive and/or nervous system of many bird species. AI viruses are influenza A viruses belonging to the Orthomyxovirus family, and they are classified according to their pathogenicity and the antigenicity of the surface proteins haemagglutinin (HA) and neuraminidase (NA) of which 16 and 9 variants, respectively, are known to date [1]. Viruses containing subtypes H5 and H7 are highly pathogenic in poultry and cause outbreaks of highly pathogenic AI (HPAI), with mortality rates reaching 100% [2]. HA-specific antibodies are protective as a result of their ability to prevent virus attachment and penetration of the host cell. HA is a homotrimer, each monomer is synthesized as a single polypeptide (HA0) that is cleaved by host proteases into HA1 and HA2. HA1 contains receptor-binding pocket surrounded by antigenic binding sites. H5 and H7 subtypes emerge at irregular intervals and cause severe economic losses in poultry, furthermore, it was demonstrated that H5N1 subtype could directly cross the species barrier to replicate in humans and cause severe disease [4]. Vaccination can be a powerful tool to support eradication programs if used in conjunction with other control methods**.** Adjuvanted killed vaccines can provide a strong humoral immune response and they provide an effective protection against homologous low pathogenic AI (LPAI) and HPAI challenges. One of the concerns in the use of the commercially available vaccines (consisting of inactivated AI virus) to control HPAI in poultry farms is the possibility that while these vaccines may protect from disease, they do not hinder infection. Thus asymptomatic virus circulation may continue, resulting in spread of infection to non-immunised birds, e.g. in other (neighbouring) farms [3]. Inactivated influenza vaccines will develop antibodies not only to the protective epitopes on the HA and NA , but also to the internal proteins which make the differentiation between infected and vaccinated animals (DIVA) difficult [5]**.** On the other hand, inactivated hetrologous vaccines are manufactured in a similar way to inactivated homologous ones. The use of hetrologous neuraminidase DIVA strategy is an acceptable approach but availability of diagnostics is an issue. Several recombinant fowl pox (FP) viruses expressing the H5 antigen have been developed and one has been licensed in Mexico [6]. Other vectors have been used to successfully deliver the H5 or H7 antigens, such as constructs using infectious laryngotracheitis virus (ILT). Recombinant vectored vaccines also enable the differentiation between infected and vaccinated birds. However, their use is restricted to countries in which they are legally available. In addition, the use of these vaccines is also restricted to species in which the vector virus will replicate [7]. Peptide vaccination could be an alternative to commercially available vaccines. Subunit vaccines based on conserved antigens provide broader protection.

Moreover, HA protein derived recombinant peptides would not elicit an immune response against internal viral proteins which facilitate DIVA. The HA1 antigenic domain of HA has been shown to induce an immune response equal to that of the full-size protein [8]. *P. pastoris* has the potential of rapid growth to very high cell densities in inexpensive media as strong promoters are available [9]. It can produce high-level of foreign proteins either intracellular or extracellular. In addition, it has the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing [10;11]. Accordingly conserved sequences of influenza A subtype H5N1 were expressed in *P. pastoris* and the possibilities to be used for diagnostic and vaccination purposes was explored.

2. Materials and Methods:

2.1 Avian influenza virus

A highly pathogenic influenza virus A/Thailand/1 (Kan-1)/2004 (H5N1), accession number: AY555150, isolated from Thailand [12] was obtained from Dr Puthavathana, Department of Microbiology, Bangkok as a supernatant of infected MDCK-cells. All operations with H5N1 virus were performed in bio-safety level 3 facilities.

2.2 Pichia pastoris strains

Two *P. pastoris* strains, supplied by Invitrogen Life Technologies (Invitrogen, Karlsruhe Germany) were used. GS115 (*his4*) strain is an auxotrophic mutant deficient in histidine dehydrogenase, while SMD1168 H *(his4, pep4*) is additionally defective in the vacuole peptidase *A (pep4)*.

2.3 Expression vectors

For recombinant protein expression, pGAPZαC vector (Invitrogen GmbH, Karlsruhe, Germany) was modified and designated pAOX. Briefly, the AOX promoter was PCR amplified from SMD1168H genomic DNA with appropriate primers and used to replace the GAP promoter. 5′ AOX1 promoter region induces expression in the presence of methanol. The plasmid contains on α- factor signal sequence (responsible for secretion of target protein), multiple cloning sites (to insert gene of interest) and polyhistidine (6xHis-tag to facilitate purification and protein detection). Moreover, Zeocin *She ble* resistance gene is incorporated into the cloning vector and used as a selectable marker for transformation (Figure 1).

2.4 Molecular cloning

To identify regions within H5 protein that are highly conserved, protein alignments were performed using MacVector^{TM}7.0. Four coding DNA fragments of epitope based truncated sequences of HA were chosen and designated P1, P2, P5 and rHA1 as shown in figure (2) and table (1). RNA was extracted from influenza A subtype H5N1 (A/Thailand/1(Kan-1)/2004) and cDNA was synthesized followed by amplification of these truncated sequences. Two primers for each coding sequence were used for a series of synthetic reactions. The primers were designed with specific restriction enzymes sites to create compatible ends (vector-PCR products). In the reverse primers, stop codons were not induced in order to fuse the coding sequence with 6xHis-tag. Both DNA-product and pAOX plasmid were digested by Xho1 and Not1 restriction enzymes (Fermentas, Leon-Rot, Germany), analysed using gel electrophoresis and purified using Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany). After determination of the concentration of purified DNA using NanoDrop (peQLAb Biotechnology GmbH), ligation was done for digested DNA and plasmid using T4 –DNA ligase (Fermentas, Leon-Rot, Germany). The ligation mixture was transformed into XL10-Gold ultracompetent *E-coli* cells (Stratagene, La Jolla, CA 92037 USA) and selected on Low salt LB agar (1 % peptone, 0.5 % NaCl, 0.5 % yeast extract, 1.5% Agar Agar, pH, 7.5) with 25 µg / ml Zeocin (Invitrogen GmbH, Karlsruhe, Germany). Selected colonies were picked up and inoculated in to 5 ml Low salt LB liquid medium (1% peptone, 0.5% NaCl, 0.5% yeast extract, pH, 7.5) and incubated overnight at 37 °C. The recombinant plasmids (designated pAOX H5-P1, pAOX H5-P2, pAOX H5-P5 and pAOX H5-HA1) were isolated using miniprep (Zyppy^{TM} Plasmid Miniprep Kits, Qiagen, Hilden, Germany) for restriction analysis and sequencing. pAOX H5-P1, pAOX H5-P2 and pAOX H5-HA1 plasmids were subjected to double digestion with Bgl II (Fermentas, Leo-Rot, Germany), whereas Bg1 II and Xho1 were used for double digestion of pAOX H5-P5. Sequencing of the gene of interest was performed using cycle sequencing kit (Big Dye Terminator v1.1; Applied Biosystem, Lincoln Centre Drive Foster City, USA) followed by analysis in an ABIPRISM^{TM} 310 Genetic analyzer (Applied Biosystems).

2.5 P. pastoris transformation and expression

After analysis of the insert, an amount of recombinant plasmid DNA was prepared. 5-10 µg (per transformation) were linearized by Bstx1 (Fermentas, Leon-Rot, Germany) ,checked by agarose gel electrophoresis and transformed into SMD1168H *P. pastoris* cells using an optimized protocol according to EasyComp kits (Invitrogen, Karlsruhe, Germany) after some modification. Cells were spread on yeast peptone agar (1 % yeast extract,

2% peptone, 2% Agar, 2% D.Glucose) containing 100 µg Zeocin. The selected zeocin transformants were analysed for the presence of the insert by colony PCR using gene and promptor specific primers. Briefly, to perform colony PCR, primer seq2 (5´GCAGCTCGCTCATTCCAATTCC 3´) was used as promoter specific primer, however, specific sense primers for P1, P2, P5 and HA1 (table 1) were used as antisense primers. Swabs from selected clones were suspended in dH_2O and heated 95 °C for 10 min. followed by centrifugation for 5 min. at 4000 rpm. 10 µl of the supernatant was used to perform PCR. Small- scale expression was done to identify and confirm a recombinant *pichia* clones that express the correct protein and also to optimize the condition of expression. Primary culture was done from GS115 or SMD1168H *Pichia* cells in YP glycerol (1% yeast extract, 2% peptone, 2% glycerol) using microtiter plate 24 wells and incubated at 28 °C for 24 hrs at 250 rpm. An ensuing preparatory culture was initiated when OD_{600} = 0.8- 1.0 and incubated at 28 °C for 24 at 250 rpm. Induction of expression was done in YP methanol (1% yeast extract, 2% peptone, 2% methanol) either pH 6 or 8 at OD_{600} = 30-70. To inhibit peptide degradation 2 µg/ml Pepstatin (Sigma Aldrich, Munich, Germany) was used during protein expression. Protein expression was analyzed by Sodium Dodecyle-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [13] followed by Western blot [14] using mouse anti-his-tag antibody (Dianova, Hamburg, Germany).

2.6 Expression of recombinant polypeptides in large scale using fermenter

rHA1 and rP1 polypeptides were expressed in *P. pastoris* (SMD1168H) using a 10 Litre fomenter to establish a high-density cell fermentation method BIOSTAT Cplus-C15-3 fermenter (Institute for Biochemistry, Faculty of Medicine, Leipzig University). The culture temperature in pre-induced stage was optimized at 28 °C to adapt cell growth and recombinant protein expression in YP 2% glycerine, pH 6. Induction was done in the same media (at OD_{600} = 40) using 2% methanol after adjusting pH to be 8. For purification, protein was firstly precipitated using ammonium sulfate (Sigma, Germany). Briefly, ammonium sulfate was added slowly to a final concentration of 80% (470 g / litre of solution) and stirred at 4 °C for 15 min. The sample was centrifuged by ultracentrifugation (Surespin Rotor, 11,000 xg/4 °C/30 min). The pellet was resuspended in an appropriate volume of equilibration buffer that used in purification.

2.7 Purification of recombinant polypeptides

All purification steps were performed at room temperature. The purification protocol was optimised for each polypeptide using nickel-nitrilotriacetic acid (Ni-NTA, Qiagen, Hilden, Germany) or lectin affinity chromatography (Lectin Peroxidase Concanavalin, Sigma, Munich, Germany). Briefly, for nickel affinity chromatography, column was prepared according to the manufactures' protocol (Qiagen, Hilden, Germany) using 4 ml Ni-NTA agarose. The column was equilibrated by passing 10 ml equilibration buffer (10 mM imidazole, 300 mM NaCl and 50 mM Tris-Cl). Culture supernatant containing peptides of interest were passed through the column and washed with 10 ml equilibration buffer. Elution was done by increasing the concentration of imidazole (100-200 mM imidazole). For lectin affinity chromatography, equilibration was done by 150 mM NaCl in 10 mM PBS pH, 7.4 The unbound fraction was collected and the column was washed with 10 ml equilibration buffer. The bound fraction was eluted from the column with 0.4 M methyl α- D- manopyranoside (Sigma Aldrich, Munich, Germany) in equilibration buffer. The fractions were dialyzed and concentrated by using vivaspin ultrafilter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with a molecular weight cutoff of 5,000 – 50.000 Da according to the size of polypeptide. Alternatively, purification was done under native or denaturing conditions using 4 M Urea and 1 mM Phenylmethylsalfonylfluorid (PMSF, Roth, Karlsruhe, Germany). Fractions of high protein concentration were pooled and total protein amount was quantified using Bradford kits (Biorad, Hercules, CA, USA).

2.8 Glycosylation analysis

2.8.1 Blot with Concanavalin

Glycosylated polypeptides (P2, P5 and rHA1) were separated by SDS – PAGE and transferred to nitrocellulose. The membrane was blocked by incubation in PBS containing 2% (v/v) Tween 20 for 2 minutes. The blot rinsed twice in PBS followed by incubation with 10 µg/ml of lectin peroxidase (lectin from Concanavalin A, L6397, Sigma, Munich, Germany) in PBS containing 0,05% (v/v) Tween 20, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ for 16 hrs at 20 °C. The blot was rinsed in PBS and developed using 3, 3´diaminobenzidin tetra- hydrochloride (DAB) (Applichem, Darmstadt, Germany).

2.8. 2 Deglycosylation with Endoglycosidase

Deglycosylation was done using Endoglycosidase H kit (Endo H_f, New England Biolabs, U.K.). Briefly, 1 µl of 10X Glycoprotein denaturating buffer was added to 9 µl of purified HA1 then denatured at 100 °C /10 min. 4 µl 10x GS reaction buffer was added. Deglycosylation was done by incubation of the mixture with Endo H_f at a concentration of 1:500. Deglycosylation was analyzed after 0 min, 15 min, 30 min, 1 hr, 2 hrs, and

3 hrs by SDS-PAGE, Western blot and blotting using Concanavalin.

3. Results

3.1 Molecular cloning

The coding sequences of genes of interest were isolated and amplified from influenza A subtype H5N1 (A / Thailand/1(Kan-1)/2004) using primers designed with Xho1 and Not1 restriction sites. The size of the H5-P1, H5-P2, H5-P5 and H5-HA1 coding sequences were 159, 880, 128 and 1007 bp, respectively. PCR products were digested using Xho1 and Not1, purified and ligated with pAOX. After transformation of pAOXH5-P1, pAOXH5-P2, pAOXH5-P5 and pAOXH5-HA1 to *E-coli* cells, many colonies arose on selecting plates. Single colonies were cultured in liquid medium for plasmid isolation. Double digestion of pAOXH5-P1, pAOXH5-P2, pAOXH5-P5 and pAOXH5-HA1 resulted in large segments (2614, 2400, 2454 and 2799 bp, respectively) and small segments (1224, 1206, 1183 and 1716 bp, respectively) (Figure 3). Plasmids containing genes of interest were sequenced and after confirming the presence of desired sequences. Linearised plasmids were transformed into *P. pastoris* strains GS115 and SMD1168H. Inserts were verified by colony PCR using promoter and gene specific primers.

3.2 Expression and analysis of recombinant polypeptides

P1 and P2 polypeptides were expressed in YP 2% methanol, pH 8 for 24 hrs/29 °C/250 rpm. Purification was carried out using Ni-NTA chromatography under natural condition (Figure 3.A). For P1 polypeptide, washing and elution were achieved by increasing imidazole to 100 mM. Washing of P2 polypeptide was done by lowering pH to 6.0 and eluted at pH of 2-3. P5 polypeptide was demonstrated in cell lysate of both GS115 and SMD1168H *Pichia* cells by SDS-PAGE and Western blot after induction in YP 2% methanol, pH 6, the expected band was detected in supernatant of SMD1168H after expression in YP 2 %, pH 8. For purification, P5 polypeptide could not bind to Ni-NTA neither under natural nor under denaturing condition. It was purified by lectin affinity chromatography (Figure 4.A). P1, P2 and P5 polypeptides were analyzed in culture supernatant by Western blot using anti-His-tag antibodies (Figure 4.B). rHA1 polypeptide was demonstrated in cell lysate and supernatant of SMD1168H cells by SDS-PAGE and Western blot 24, 36 and 48 hrs after induction of expression, some protein degradation was observed. Addition of 2 µg pepstatin/ml prevented protein degradation. rHA1 polypeptide could not bind to Ni-NTA under natural condition; however, it binds under denaturing condition using 4 M urea. 1 mM PMSF was added to prevent the degradation during purification. Washing was done using 10 mM imidazole, 300 mM NaCl and 50 mM Tris-Cl. However, the desired rHA1 polypeptide was eluted using 100 mM imidazole, 300 mM NaCl and 50 mM Tris–Cl. Analysis of rHA1 polypeptide by SDS-PAGE and Western blot showed a broad smear above the expected size (above 39.67 kDa) and it reacted with lectin from Con A which reveals the presence of glycosylation. Analysis of rHA1 polypeptide treated with Endo H_f by SDS-PAGE and Western blot showed a band which is in accordance with the expected size (Figures 4.C). On the other hand, other glycosylated polypeptides (P2 and P5) could not react with Con A and could not deglycosylated with Endo H_f. MALDI-TOF analysis of P1 polypeptide showed a molecular weight of 7592.1 Da $[M+ H]^+$ compared to the theoretical mass: 7591,52 Da.

rHA1 and P1 were expressed in large scale using high-density cell fermentation. Analysis of expressed polypeptide at 12, 24, 36 and 48 hrs after induction revealed that the best cultural condition for obtaining better expression level of rHA1 is 28 °C for 36 hrs while and best cultural condition for obtaining better expression level of P1 polypeptide is 28 °C for 36 hrs. The expression level of rHA1 and P1 polypeptides produced with optimized fermentation process reached 80 mg/L and 75 mg/L, which is about ten-fold higher than the one produced in regular shaking flasks.

4. Discussion

The influenza HA glycoprotein is the primary target of neutralizing antibodies [15]. The H3 structure was initially used to characterize the antigenic structure of H5 [16]. Before, Kaverin and coworkers [17] described the fine structure of H5 antigenic sites. The H3 secondary and tertiary structure is similar to H5 [18]. Site 1 is an exposed loop comprising HA1 residues 136 –141 (H5 numbering strain H5N2 A/Mallard /Pennsylvani a/10218 / 84) corresponding to antigenic site A of H3 [16] (in this paper referred as P5). Site 2 comprises two sub-sites, one (HA1 residues 156-157) that corresponds to site B in H3 subtype. H5 124 - 129, which corresponds to 129 - 133 in the H3 sequence, is located outside any site in the H3 HA structure recognized by virus-neutralizing mAbs but partially overlaps a region involved in the antigenic site Sa in H1 HA [19]. Several recent reports demonstrated that HA and HA1 fragment containing the majority of antigenic determinants are responsible for generation of virus-neutralizing antibodies and vaccines based on conserved antigens provide broader protection

[8;20;21]. Recently, reassortant influenza vaccines were developed by reverse genetics [22-25]. These vaccines have similar performances as conventional inactivated vaccines; however, large scale vaccine production still depends on egg-based production. As an alternative, viral proteins can be expressed in an in vitro system and can be used with adjuvant as a vaccine. By this technology, production of viral antigens does not involve culture of live AIVs and avoids biosecurity concerns.

Table (1): Primers and Peptide Description

[a]Peptide	[b]Primer sequence	[c]Peptide description
P1	**Site E-sense:** GTA **CTC GAG** AAG AGA GAG GCT GAA GCA GAT CTA GAT GGA GTG AAG CC **Site E-as:** CAT **GCG GCC GC**C TTC TCC ACT ATG TAG GAC C	corresponds to the neutralizing epitope of site E of H3, conserved in H5, 40 amino acids residues long, molecular mass is 7.5 kDa.
P2	**RBS-sense:** GTA **CTC GAG** AAG AGA GAG GCT GAA GCA AAT AAT ACC AAC CAA GAA GAT C **RBS-as:** CAT **GCG GCC GC**G TCC CCT TTC TTG ACA ATT TTG	consists of the receptor binding site, site D and parts of site B, conserved in H5, 97 amino acid residues long, contains a glycosylation site, molecular mass is 13.9 kDa
P5	**Site A-sense:** ´5 GTA **CTC GAG** AAG AGA GAG GCT GAA GCA TCA TTA GGG GTG AGC TCA GC 3´ **Site A-as**: 5´CAT **GCG GCC GC**G TAT GTA CTG TTC TTT TTG ATA AGC C 3´	conformational epitope in H5, not conserved in H5, 30 amino acid residues long, contains a glycosylation site, molecular mass is 6.5 kDa
rHA1	**HA1-sense:** 5´GTA **CTC GAG** AAG AGA GAG GCT GAA GCA GAT CAG ATT TGC ATT GGT TAC C 3´ **HA1-as:** ´5GAT **GCG GCC GC**T CTT TGA GGG CTA TTT CTG AGC C´3	contains the majority of those antigenic determinants of HA that are responsible for generation of virus-neutralizing antibodies, 320 amino acid residues long, contains 5 glycolsyation sites, molecular mass is 39.6 kDa

[a]P1, P2, P5 and rHA1 coding DNA fragments of epitope based truncated sequences of HA influenza A subtype H5N1 (A/Thailand/1(Kan-1)/2004).
[b]Bold sequences indicate the sequence of restriction sites (Xho1 in sense primers and Not1 in antisense primers) , the sequence of alpha factor is underlined.
[c]Molecular mass was calculated for his-tag polypeptide when alpha factor is completely processed (secreted in supernatant).

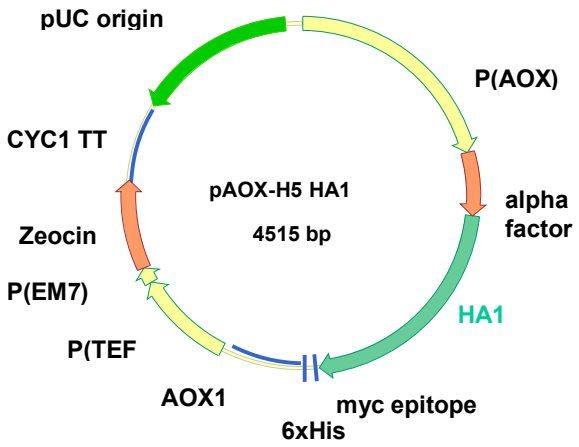

Figure (1): Schematic diagram of *P. pastoris* expression (pAOX) vector containing gene of interest.

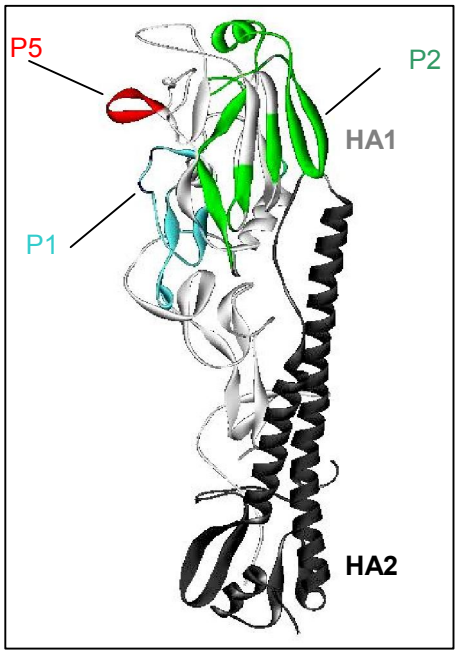

Figure (2): Crystal structure of influenza A subtype H5 monomer. The location of coding sequences used for expression within HA1 was coloured using wlviewerlite35.exe and WebLab ViewerLite (1JSM.pdb) programs.

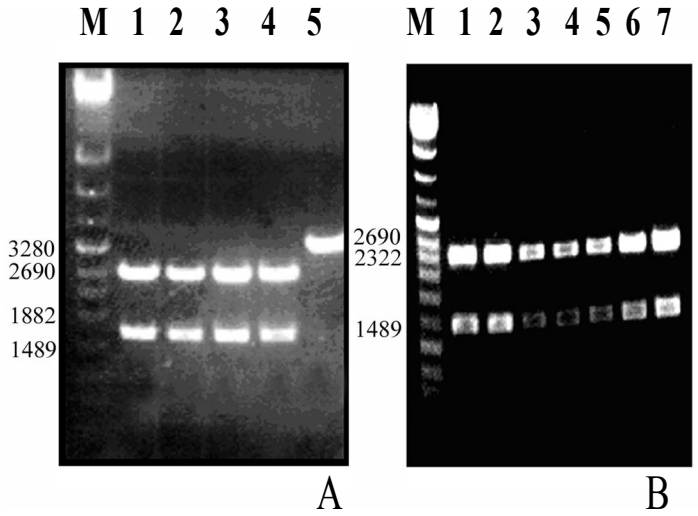

Figure (3): A) Double digest of pAOX-H5HA1 using Bgl II restriction enzyme resulting in a large segment (2799 bp) and a small segment (1716 bp): lanes1-4 different clones subjected to double digest. Lane 5: intact pAOX-HA1 plasmid. B) Double digest of pAOX-H5P5 using Bgl II and Xho1 restriction enzymes resulting large segment (2453 bp) and small segment (1183 bp). Lanes 1-7 are different clones.

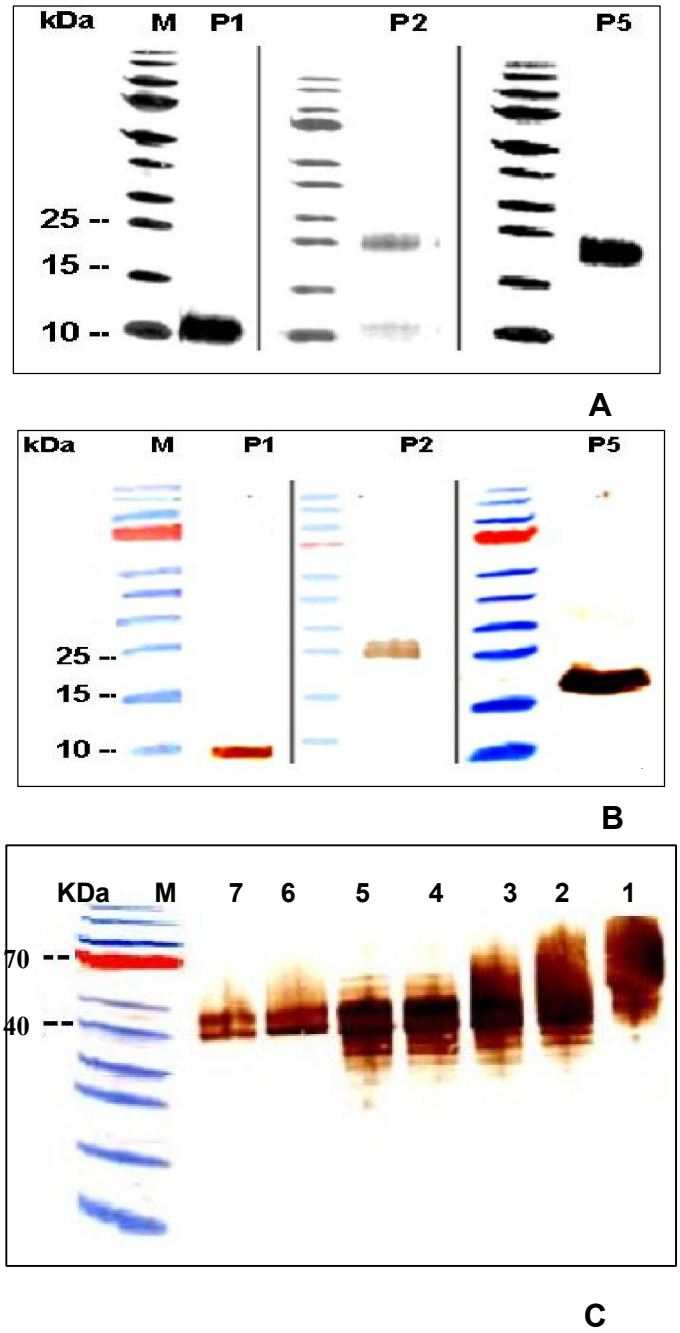

Figure (4): Analysis of recombinant polypeptide. A and B): SDS-PAGE and Western blot analysis of purified P1, P2 and P5 polypeptides expressed in *P. pastoris* cells (strain SMD1168H) and secreted into supernatant, respectively. Lane 1): P1 polypeptide purified by Nickel affinity chromatography, theoretical molecular weight 7.5 kDa. Lane 2): P2 polypeptide purified by Nickel affinity chromatography, theoretical molecular weight 13.9 kDa. Multiple bands are due to different glycosylation as contains P2 contains a glycosylation site. Lane 3) P5 polypeptide purified by Lectin affinity chromatography, theoretical molecular weight 6.5 kDa. The increase in size is attributed to presence of a glycosylation site. Figure (4.C): Western blot analysis of rHA1 polypeptide. Lane 1: purified rHA1, Lanes 2, 3, 4, 5, 6 and 7 are purified rHA1 and treated with Endo H_f for 15 min, 30 min, 1hr, 2 hrs 3 hrs and 4 hrs, respectively.

The methylotrophic yeast *P. pastoris* has raised an increasing interest for the production of recombinant proteins offering fast growth rates in low cost media, ease of genetic manipulation and the capability of post-translational modifications as glycosylation, disulfide bond formation and protein folding. In addition, *P. pastoris* system has many advantages as it secrets only low levels of endogenous proteins and a secreted heterologous protein comprises the vast majority of the total protein in the medium, secretion facilitates purification significantly [10;11]. Four regions were chosen for production of recombinant polypeptides and designated P1, P2, P5 and rHA1 (Table 1 and Figure 2). Coding DNA fragments of full length or epitope-based truncated sequences of influenza A subtype H5N1 (A/Thailand/1(Kan-1)/2004) were cloned in to pAOX vector for recombinant production using gene specific primers. Appropriate expression cassettes were used for transformation of *P. pastoris* cells (strains GS115, SMD1168H). Selected clones were used for secretory expression of polypeptides fused to his-tag facilitating detection in culture supernatants using Western blot. The four developed polypeptides were identified by SDS-PAGE and Western blot in both cell lysate and culture supernatant. Secretion requires the presence of a signal sequence on the foreign protein to target it to the secretory pathway. While several different secretion signal sequences have been used including the native secretion signal present on some hetrologous proteins, success has been variable. However the secretion sequences from *S. Servisae* factor PrePro peptide has been used with the most success. *S. Cervisae* factor prepro peptide consists of a 19aa signal pre sequence followed by a 66- residue pro sequence [26]. *P. pastoris* has the potential of performing post-translational modifications including N-glycosylation. It begins in the endoplasmic reticulum (ER) with the transfer of a lipid-linked oligosaccharide unit Glc3Man9GlcNAc2 (Glc: Glucose GlcNAc: N. acetylglucosamine) to Asparagine Asn-Xser/Thr. rHA1 has a theoretical molecular mass of 39.67 kDa but when the protein was expressed in *P. pastoris* it gave a broad smear above the expected size as analysed by SDS-PAGE and western blot. Treatment of purified HA1 with Endo H_f revealed that its aberrant migration resulted from post-translational glycosylation hence HA1 of influenza A H5N1 subtype isolated from Thailand [12] contains 5 glycosylation sites. Although the exact structure of the oligosaccharides were not analyzed, the most commonly observed N-linked glycans in *P. Pastoris* secreted recombinant protein are short Man8Glc NAC and Man9 GLCNAc. Endo H_f cleaves within chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoprotein [27]. Analysis of P2 polypeptide by Western blot showed multiple bands due to different glycosylation pattern as it contains a glycosylation site. Analysis of culture supernatant of P5 by Western blot revealed that P5 polypeptide is secreted in a very low amount (detected only after concentration by ultraconcentration). Analysis of P5 polypeptide in cell lysate by SDS-PAGE and Western blot showed a size of about 21 Kda. This attributed to the glycosylation of alpha factor. The pro sequence of alpha factor contains three N-linked- glycosylation sites and a dibasic- kex2 -endopeptidase. P5 polypeptide could not bind with Ni-NTA affinity chromatography either under natural or denaturing condition. As optimal purification using Ni-NTA is dependent on the amount of 6x his-tagged protein, possibly, P5 polypeptide hist-tag in the N-terminal might be removed by proteolysis. Eshaghi and others [28] mentioned that His-tag in N terminal of expressed proteins was suspected to proteolytic removal in sf-9 cells. P2 polypeptide contains one glycosylation site and its theoretical mass is 14 Kda, however, analysis by SDS-PAGE and western blot resulted 2 bands (about 10 and 22 Kda). This attributed to not all the protein glycosylated. Endo H_f could not remove the glycosylation residues of P2 and P5 polypeptide.

In the present study, different polypeptides were expressed successfully in yeast system. This will be helpful in the future study of antigen detection and antibody detection kits. Moreover, together with the safety, reliability and economic potential of *P. pastoris*, as well as the flexibility and fast adaptation of the expression system may allow development of an effective recombinant influenza vaccine based on truncated sequences of HA that might provide broader protection against H5 HPAI viruses.

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