Guidelines for small-scale production and purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris*

Maria Zahid ^{1,2} and Ursula Rinas ^{1,3}

¹ *Leibniz University of Hannover, Technical Chemistry – Life Science, Hannover, Germany*

² *Lahore University of Management Sciences, Department of Biology, Lahore, Pakistan*

³ *Helmholtz Centre for Infection Research, Braunschweig, Germany*

Running head title: Guidelines for small-scale production of HBsAg VLPs

Abstract

Virus-like particle (VLP)-based vaccines have been in the market since decades for preventing viral infection and have proven their usefulness also in other areas of biotechnology. Here, we describe in detail simple small-scale production and purification procedures for the generation of hepatitis B surface antigen (HBsAg) VLPs using *Pichia pastoris* as expression host. This protocol may also be applicable with variations to other HBsAg based VLPs additionally carrying antigens of other pathogens.

Key words

HBsAg, *Pichia pastoris*, Vaccine, Virus, Virus-like particle

1. Introduction

Ever since the development of medical sciences, the approach of prevention being better than cure has been a driving motive to develop vaccines. Vaccines against viral diseases have been quite challenging because of the daunting nature of the viruses.

The first virus-like particle (VLP) product to be launched in the market was against Hepatitis B virus, e.g., HBsAg VLPs [1]. Since then, VLP technology has become more popular, and currently, there are two commercially available VLP-based vaccines already in the market; vaccines against Hepatitis B and human Papilloma virus infections [2]. Moreover, vaccine candidates against many emerging infections such as caused by Zika virus are based on VLP technology [3].

A universal vaccination program has led to a remarkably decreased rate of HBV infections in many countries in Asia, Africa and Europe [4]. There have been approved vaccines against HBV since 1980's and nowadays all of them are produced using recombinant DNA technology [5, 6]. Chimeric HBsAg-based VLPs are also used as scaffold to display epitopes of proteins from other infective agents such as Dengue virus [7] and the Malaria parasite [8].

VLPs offer a compact and stable protein structure which is one of the major requirements for a vaccine product. Many viral surface proteins have an in-built tendency to form VLPs under appropriate conditions [9] and then display immunogenic properties of the actual virions and hence trigger a protective immune response.

An efficient approach to produce HBsAg VLPs is based on subunit expression using yeast based expression systems such as *Saccharomyces cerevisiae* or *Pichia pastoris* [10]. The HBsAg is a very hydrophobic protein and not efficiently secreted [11]. Thus, yeast-based production leads to an intracellular maintained product [12-14]. For a long time, it was

thought that the HBsAg VLPs are formed inside the expressing host cells. Recently, it has been shown that HBsAg VLPs do not assemble inside the cell but are formed during downstream processing [15, 16]

Production and purification of HBsAg VLPs using *P. pastoris* has been reported many times, e.g. [15-19]. However, most published processes are only realizable in specialized laboratories. Here, we would like to offer a protocol which is practicable in non-specialized laboratories and also applicable for those not yet familiar with VLP production and purification procedures.

2. Materials

2.1. Production

- i. *P. pastoris* harboring gene encoding protein of interest under control the the *AOXI* promoter, e.g. HBsAg [17]
- ii. Yeast media components (yeast extract, peptone, yeast nitrogen base, biotin)
Prepare all solutions with (double)-deionized water, ddH₂O (see note 1)
- iii. Shake-flasks with baffles (250 mL, 1 L, if present also larger flasks)
- iv. Glycerol and methanol (biological grade) as non-inducing and inducing carbon sources, respectively
- v. 1 M potassium phosphate buffer, pH 6.0 (stock solution)
- vi. Buffered medium glycerol (BMG): 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base (YNB), 4×10^{-5} % (w/v) biotin, 1% (v/v) glycerol
- vii. Buffered medium methanol (BMM): 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base (YNB), 4×10^{-5} % (w/v) biotin, 0.5% methanol
- viii. Shaking incubator preset to 30°C

- ix. Pipette set and tips, tubes for centrifugation and storage
- x. Centrifuges with temperature control for harvesting the culture and separating soluble and insoluble fractions

2.2. Downstream processing

- i. Acid-washed glass beads (0.45 mm diameter)
- ii. Tween 20 (biological grade)
- iii. Polyethylenglycol (PEG) 6000
- iv. Aerosil 380 (Evonik, Hanau, Germany)
- v. FPLC system with ion exchange (DEAE Sepharose FF, 20 – 200 mL, GE healthcare) and gel filtration (Sephacryl-S300, 26/60, 320 mL, GE healthcare) columns
- vi. FPLC buffers (for detailed recipes, see Table 1)
- vii. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , pH 7.2
- viii. Concentrators, 10 kDa molecular weight cut-off (MWCO)
- ix. Cellulose acetate dialysis membrane (14 kDa MWCO)

2.3. Analytical methods

- i. UV-VIS spectrophotometer for measuring the optical density at 600 nm (OD_{600}) and cuvettes (1mm path length)
- ii. Protein quantification reagent (Bradford, BCA etc)
- iii. SDS-PAGE sample buffer: 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 2.5% (w/v) SDS, 0.2% Bromophenol blue, 45% (v/v) β -mercaptoethanol, 5% (v/v) glycerol
- iv. SDS-PAGE gels (12%), molecular weight marker, and electrophoresis apparatus

- v. Fixing and Colloidal Coomassie Blue staining solution [20]
- vi. Transfer apparatus for Western blotting
- vii. Polyvinylidene difluoride (PVDF) membrane
- viii. Buffers for immunoblotting
 - i. Tris buffer saline (TBS): 25 mM Tris-HCl, pH 7.5, 150 mM NaCl
 - ii. TBS-Tween 20 (TBS-T): TBS, pH 7.5, 0.5% Tween 20
 - iii. Blocking buffer: TBS-T, pH 7.5, 2% bovine serum albumin (or 2% skim milk powder)
- ix. Primary and horse radish peroxidase (HRP)-labeled secondary antibodies for HBsAg
- x. Tetramethylbenzidine (TMB) detection substrate (“insoluble” for immunoblotting, generating insoluble product and “soluble” for ELISA, generating soluble product), plate reader ELISA plates and 96-well ELISA plates
- xi. CFormvar coated Cu-grids (300 mesh)
- xii. 2% (w/v) uranylacetate, pH 4.5

3. Methods

3.1. Shake-flask cultivation for HBsAg production

To test for HBsAg production, shake-flask cultures of *P. pastoris* harboring the HBsAg gene are grown at 30°C and 250 rpm, according to manufacturer’s instructions (*Pichia* expression Kit, Catalog no. K1710-01, Invitrogen USA). As inoculum, 100 µL of glycerol stock culture is introduced to 50 mL BMG medium and incubated for 12-16 h or until the OD₆₀₀ reaches 15. Subsequently, 10 mL of this inoculum is introduced into 1 L BMG medium to start the main culture(s) (see note 2), which is further incubated until OD₆₀₀ = 8. After approx. 14 h,

the cells are harvested and resuspended into BMM to start production. Induction is continued with regular addition of methanol i.e., 0.5% volume after every 24 hours and HBsAg accumulation is analyzed by SDS-PAGE analysis (Fig. 1, see note 3).

3.2. High cell density cultivation for HBsAg production using *P. pastoris* GS115

If the laboratory is equipped to run controlled bioreactor cultivations (e.g. equipped for controlled continuous methanol feeding to keep the methanol concentration constant) detailed information on fed-batch procedures to produce HBsAg can be found elsewhere [13] (see note 4).

3.3. Culture harvesting

In shake flask cultures, production continues up to 120 h after induction. Best timing needs to be tested empirically. For harvesting the culture, use continuous-flow centrifuges (for controlled bioreactor fed-batch cultivations) or buckets of 250 mL to pellet the biomass at 4°C and 4,000 rpm ($3,345 \times g$) for 20 min. Time course samples and biomass for HBsAg purification can be stored at -20°C for about one week and for longer periods at -80°C.

3.4. Cell lysis

To analyze for intracellular HBsAg production, glass beads (0.45 mm diameter) are an efficient and cheap option for lysis and useful for analytical and small scale purification procedures. Additionally, a homogenizer can be used for cell lysis of suspension volumes equal or larger than 10 mL (see note 5).

3.4.1. Cell lysis using glass beads

1. Wash cell pellet equivalent to a cell pellet from 1 ml culture with an $OD_{600} = 100$ with 25 mM phosphate buffer (PB), pH 8.0 and resuspend in lysis buffer.

2. Add glass beads (0.45 mm diameter) of roughly equal weight of wet cell pellet.
3. Vortex for 40 sec at maximum speed and keep on ice for next 40 sec. Repeat the cycle of vortexing and chilling 8-10 times.
4. Centrifuge for 10 min at 13,000 rpm ($17,000 \times g$) to remove cell debris and insoluble fraction of HBsAg.
5. Collect the supernatant in a fresh tube and store at -20°C (-80°C for longer periods).

3.4.2. Cell lysis via homogenizer

1. To perform lysis via homogenizer (e.g. Microfluidizer, Microfluidics, Newton MA, USA), resuspend 100 g wet biomass (obtained from 1-2 L bioreactor culture or 5 L shake-flask culture broth) in lysis buffer (25 mM sodium phosphate buffer, pH 8, 5 mM EDTA) to a final volume of 1 L ($\text{OD}_{600} \approx 200$).
2. Add Tween 20 to a final concentration of 0.6 % (v/v) and readjust the pH to pH 8.0.
3. Prepare the homogenizer by chilling to 4°C , turn on the air supply and create pressure (5-8 bar).
4. Wash the homogenizer chamber with ddH₂O and lysis buffer.
5. Pass cells at 12,000 psi (38 psi at inlet) through the homogenizer for 12-14 times.
6. Proceed for the further steps of downstream processing (as shown in Fig. 2).

3.5. Purification of HBsAg

Lysed cells are subjected to further downstream processing mainly based on different chromatographic methods (summarized in Fig. 2).

3.5.1. Clarification of cell lysate

1. Perform lysis of 100 g wet biomass using homogenizer as described in the previous section.

2. Slowly add solid polyethylene glycol 6000 (PEG 6000) to the cell lysate to a final concentration of 5% (w/v) followed by the addition of 5 M NaCl to obtain a final concentration of 500 mM NaCl in about half an hour keeping everything at 4°C.
3. Stir the mixture for 2 h and then let it incubate for further 12-16 h at 4°C to facilitate precipitation.
4. This suspension is clarified by centrifugation at 4°C and 4,000 rpm ($3,345 \times g$) for 25 min.

3.5.2. Aerosil 380 adsorption

1. For preparation of pre-equilibrated Aerosil, gently mix the Aerosil in 500 mL binding buffer (0.13 g Aerosil per 1 g wet biomass), centrifuge at 4,000 rpm ($3,345 \times g$) and discard the supernatant (for recipe, see Table 1).
2. Add the PEG supernatant to the pre-equilibrated slurry of Aerosil 380 in the Aerosil binding buffer.
3. Stir the suspension obtained by mixing the PEG supernatant and the Aerosil pellet at 4°C and 300 rpm.
4. After 4 hours, centrifuge the Aerosil suspension at 4°C and 4000 rpm ($3,345 \times g$) for 25 min.
5. Wash the pellet twice using the 100 mL Aerosil washing buffer (Table 1), resuspend in 800 mL elution buffer (Table 1) and incubate for 12 h at 37°C and 120 rpm.
6. Separate the Aerosil eluate (supernatant fraction) from the pellet after centrifugation for 150 min at 25°C and 10,000 rpm ($8664.5 \times g$) and further clarify by vacuum-filtration (0.2 μm pore size).
7. Store the filtered eluate at 4°C for 24 h to improve stability of HBsAg, a process called aging [18].

3.5.3. Ion exchange (IEX) chromatography

1. Re-adjust the pH of the Aerosil eluate (approx. 800 mL) to pH 8.0 with phosphoric acid before loading on IEX column.
2. Load the Aerosil eluate (approx. 800 mL) on a 200 mL DEAE Sepharose FF IEX column, prewashed with 1 M NaOH, ddH₂O and equilibrated with IEX binding buffer as described previously [15]. Alternatively, 20 mL of DEAE Sepharose FF IEX resin prewashed with 1 M NaOH, ddH₂O and equilibrated with IEX binding buffer (Tables 1 and 2) can be suspended in the Aerosil eluate and subsequently used to pack a 20 mL column. The following purification steps are based on the utilization of a DEAE Sepharose FF self-packed 20 mL column (Tables 1 and 2).
3. The self-packed column is washed with IEX washing buffer (Tables 1 and 2) and the HBsAg is eluted via isocratic flow using IEX elution buffer (Tables 1 and 2).
4. The fractions containing mono- and dimeric forms of HBsAg (25 and 50 kDa, respectively) are pooled (approx. 30-40 mL eluate) (Fig. 3) and concentrated using ultrafiltration concentrator of 10 kDa MWCO. The concentrated fractions (approx. 5 mL) are further purified via gel filtration chromatography.

3.5.4. Gel filtration (GF) chromatography and KSCN treatment

1. For the polishing step, load the HBsAg concentrate (approx. 5 mL) on pre-equilibrated gel filtration (GF) column (column volume 360 mL) and elute with PBS, pH 7.2 at 1 mL/min flow rate (Tables 1 and 3). For a smaller volume of the concentrate (e.g. 1-2 mL), a GF column with a bed volume of 120 mL is recommended.

2. Pool the fractions containing HBsAg (mono- and dimeric versions) (Fig. 4) and treat with KSCN, to a final molarity of 1.2 M, for partial “surface unfolding”.
3. Incubate this mixture at 100 rpm and 37°C for 5 h in an orbital shaker.
4. Dialyze the KSCN treated HBsAg against PBS, pH 7.2 using a cellulose acetate dialysis membrane of 14 kDa MWCO. Filter-sterilize the purified HBsAg VLPs (0.2 µm pore size) and store at 4°C.

3.6. General analytical methods for analysis of cell growth and HBsAg concentration

3.6.1. Monitoring cell growth

Cell growth is monitored by measuring the optical density at 600 nm wavelength via UV-vis spectrophotometer. For each sample, take an average of three readings (see note 6).

3.6.2. SDS-PAGE analysis for (soluble) intracellular HBsAg production

Cell samples collected during cultivation are lysed by glass beads and the soluble fraction of cell lysates corresponding to OD₆₀₀ = 100 are mixed with an equal volume of the SDS-PAGE sample buffer, vortexed for up to 1 min and incubated for 10 min at 95°C. 15 µL of each sample are loaded per well on a 12% SDS-polyacrylamide gel. The gels are stained overnight with the Colloidal Coomassie Blue staining solution.

3.6.3. Protein concentration

Quantify the total amount of protein by bicinchoninic acid (BCA) or Bradford assay. For pure HBsAg samples, the concentration is quantified by measuring the absorbance at 280 nm by using $\epsilon = 3.2 \text{ M}^{-1} \text{ cm}^{-1}$ [17] (<http://web.expasy.org/protparam/>). All protein samples are analyzed in triplicates.

3.6.4. Western blot and immunostaining

The cell lysates are also analyzed via Western blot and immunostaining to verify the presence of HBsAg. The samples are electro-transferred from SDS-PAGE gels to an equilibrated PVDF membrane for 60 minutes. The transfer is verified by co-transfer of a prestained molecular weight marker. After blocking with blocking buffer the membrane is incubated in a solution with the primary antibody against HBsAg (e.g. linear epitope specific mouse monoclonal anti-HBsAg antibody) for 1 h at room temperature. After three washings steps with TBS-T the membrane is incubated for 1 h with horse radish peroxidase (HRP)-labelled goat-anti mouse secondary antibody and later washed twice with TBS. The immune-blot is developed using “insoluble “ TMB substrate. Once the bands are visible, the reaction is stopped by washing the membrane two to four times with ddH₂O. Bands corresponding to mono- and dimeric HBsAg are visible in the soluble fraction of the cell lysate (Fig. 5).

3.6.5. ELISA analysis

For quantification of soluble HBsAg, the samples collected during the cultivation can be analyzed by ELISA (see note 7). Soluble fractions of cell lysate samples corresponding to OD₆₀₀ = 100 are diluted 100 times with PBS, pH 7.2 and 100 µL of each sample and control, positive and negative, are loaded on the ELISA plate. A serial dilution of pure HBsAg VLP standard prepared in PBS is also included for the quantification. The plate is incubated at 37°C for 1 h. The wells are washed with PBS and 50 µL of the HRP labeled anti-HBs conjugate is added to each well and further incubated at 37°C for 1 h. The wells are washed 6 times with PBS, 100 µL of substrate (e.g. urea peroxide + H₂O₂ or “soluble” TMB) is added and the plate is incubated at room temperature for 30 min. 100 µL of 1 M H₂SO₄ is added to stop the reaction, the plate is scanned at 450 nm.

3.6.6. Transmission electron microscopy

The best choice to confirm and characterize the presence of HBsAg VLPs is transmission electron microscopy (TEM) (Fig. 6). Briefly, dilute the purified HBsAg VLPs with PBS, pH 7.2 to an appropriate protein concentration (0.2-0.5 mg/mL), adsorb for 1 minute to a glow-discharged CFormvar coated Cu-grids (300 mesh) and negatively stain with 2% (w/v) uranylacetate, pH 4.5. Further details for TEM analysis of HBsAg VLPs are given elsewhere [15, 16]. In addition to TEM, other useful analytical tools for VLP analysis are atomic force microscopy (AFM) and multiangle light scattering (MALS) techniques [21, 22].

4. Notes

1. Sterilize all media components by autoclaving at 121°C for 20 min. Sensitive components, e.g. biotin, should be filter sterilized.
2. For *Pichia* cultures, the culture volume should not exceed 20% of the total flask volume to ensure proper aeration.
3. The parental non-producing strain *P. pastoris* GS115 should be grown as a negative control to analyze for HBsAg production. Purified HBsAg VLPs can show different migration properties on reducing SDS-PAGE gels compared to non-purified intracellular HBsAg presumably because of the presence of different non-reduced disulfide bonds. Please see also the amount of reducing agent needed for sample preparation for SDS-PAGE analysis (see also Fig. 1).
4. In case there is no access to bioreactor facilities, multiple shake-flask cultures can be used to produce adequate biomass for the purification of VLPs.
5. Complete lysis should be confirmed by probing the cell lysate under a light microscope with 40x magnification.

6. For OD measurement, collect the samples from the cultures regularly and dilute to suitable extent (between OD_{600nm} 0.1 – 0.8) with 0.9% (w/v) NaCl.
7. For ELISA, a commercially available HBsAg ELISA kit (Hepanostika micro ELISA, bioMérieux France) can be used. However, it is also feasible to use “in-house” ELISA strips with the appropriate specific antibodies.

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Tables - Guidelines for small-scale production of HBsAg VLPs

Step	Buffer	pH
Lysis	25 mM PB + 5 mM EDTA + 0.6% Tween 20	8.0
PEG precipitation (overnight)	lysis buffer + 500 mM NaCl + 5% PEG 6000	8.0
Aerosil binding	25 mM PB + 500 mM NaCl	7.2
Aerosil washing	25 mM PB	7.2
Aerosil elution	50 mM CBB + 1.2 M Urea	10.8
Aging	50 mM CBB	10.8
IEX binding	50 mM CBB	8.0
IEX washing	50 mM Tris-HCl	8.0
IEX elution	Tris-HCl + 500 mM NaCl	8.0
Concentration (e.g. via Vivaspin column)	50 mM Tris-HCl + 500 mM NaCl	8.0
Gel filtration	PBS	7.2
KSCN	PBS + 1.2 M KSCN	7.2
Dialysis	PBS	7.2

Table 1: List of buffers used during downstream processing of HBsAg VLP. IEX: ion exchange chromatography; PB: (Sodium) phosphate buffer; CBB: Sodium carbonate-bicarbonate buffer; PBS: Phosphate buffered saline.

Method step	Volume	Flow-rate
Washing	5 CV	1 mL/min
Elution	5 CV	0.5 mL/min
Washing	5 CV	1 mL/min

Table 2: Protocol for the ion exchange chromatography (IEX) of HBsAg. CV (column volume) of self-packed column is 20 mL. The eluate is collected in 5 mL fractions.

Step	Volume	Flow rate
Equilibrium	0.1 CV	1 mL/min
Sample application	5 mL	1 mL/min
Elution	1.5 CV	1 mL/min

Table 3: Protocol for the gel filtration chromatography of HBsAg VLPs using Sephacryl-S300, 26/60 (GE Healthcare). The total column volume (CV) is 320 mL. The eluate is collected in 5 mL fractions.

Figure and Figure captions - Guidelines for small-scale production of HBsAg VLPs

Figure 1: Production of HBsAg in shake-flask cultures. Cells were grown first in BMG and induction was started by resuspending cells in BMM containing 0.5% methanol. Cell samples were collected 0, 24, 48, 72, 96 and 120 h post-induction, concentrated/diluted to $OD_{600} = 100$, and lysed via glass beads and the soluble cell fraction analyzed by SDS-PAGE. The arrow on the left gel points towards HBsAg. The gel on the right side shows samples from the non-producing parental strain GS115 grown under the same conditions as a control culture. Std, purified HBsAg VLPs at a concentration of 0.2 mg/mL. BI: before induction. M: molecular weight marker.

Production of HBsAg in *Pichia pastoris* GS115

Figure 2: Flow-sheet illustration of HBsAg purification. The three blocks represent three stages of purification i.e. lysis and lysate treatment, capturing of HBsAg and semi-purification and, ultimately, polishing. After salt mediated PEG precipitation, the soluble part of the cell lysate is subjected to HBsAg capturing (adsorption of HBsAg to fumed silica, Aerosil 380, through hydrophobic binding). The Aerosil eluate is processed via ion exchange (IEX) chromatography to remove further contaminants. The semi-purified product is further purified via gel filtration (GF) chromatography and treated with potassium thiocyanate (KSCN). Finally, the VLP containing solution is filtered and stored at 4°C until further use.

Figure 3: Ion exchange chromatography of HBsAg. (A) Chromatogram, elution step with 500 mM NaCl. (B) SDS-PAGE analysis of peak fractions (numbers given on top of chromatogram and gel). The mono- and dimeric HBsAg containing fractions (marked with red rectangle) are processed for GF chromatography.

Figure 4: Polishing of HBsAg VLPs is done via gel chromatography. (A) GF chromatogram of IEX-purified, pooled and concentrated HBsAg. The two peaks of the chromatogram correspond to HBsAg VLPs (*) and HBsAg not assembled into VLPs (**). (B) SDS-PAGE analysis of GF load and peak fractions (numbers given on top of chromatogram and gel). HBsAg VLP peak fractions showing both mono- and dimeric HBsAg on reducing SDS-PAGE gels (fractions marked by red rectangles are pooled and used for further processing). M: protein molecular weight marker.

Figure 5: Immunoblot of (A) the soluble fraction of the GS115 lysate and (B) the soluble fraction of the cell lysate of HBsAg producing cells. Note the bands corresponding to mono- and dimeric HBsAg.

Figure 6: TEM image of purified HBsAg VLPs (HBsAg VLPs of 22-25 nm size, scale bar = 50 nm, energy-filtered TEM Libra 120, Zeiss, Oberkochen, Germany; Photo courtesy: H. Lünsdorf).