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Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and *in vivo* analysis of their immunogenic properties

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Abstract
Following earlier studies on high-level intracellular production of hepatitis B surface antigen (HBsAg) using recombinant *Pichia pastoris*, we present here in detail an enhanced method for the purification of recombinant HBsAg virus-like particles (VLPs). We have screened various detergents for their ability to promote the solubilization of recombinant intracellular HBsAg. In addition, we have analyzed the effect of cell disruption and extraction regarding their impact on the release of HBsAg. Our results show that introduction of the mild nonionic detergent Tween 20 in the initial process of cell lysis at ~600 bars by high pressure homogenization lead to the best results. The subsequent purification steps involved polyethylene glycol precipitation of host cell contaminants, hydrophobic adsorption of HBsAg to colloidal silica followed by ion-exchange chromatography and either isopycnic density ultracentrifugation or size exclusion chromatography for the recovery of the VLPs. After final KSCN treatment and dialysis, a total yield of ~3% with a purity of >99% was reached. The pure protein was characterized by electron microscopy, showing the presence of uniform VLPs which are the pre-requisite for immunogenicity. The intramuscular co-administration of HBsAg VLPs, with either alum or a PEGylated-derivative of the toll-like receptor 2/6 agonist MALP-2, to mice resulted in the elicitation of significantly higher HBsAg-specific IgG titers as well as a stronger cellular immune response compared to mice vaccinated with a gold standard vaccine (Engerix™). These results show that *P. pastoris* derived HBsAg VLPs exhibit a high potential as a superior biosimilar vaccine against hepatitis B.

Keywords
Hepatitis B surface antigen virus-like particles; Aerosil-380; Ion-exchange chromatography; Ultracentrifugation; Size-exclusion chromatography; Electron microscopy; Adjuvant; Vaccine
1. Introduction

The development of a safe recombinant hepatitis B vaccine has led to the inclusion of hepatitis B vaccination in the national infant immunization schedules of approximately 160 countries [1]. Recombinant DNA technology was used to produce hepatitis B surface antigen (HBsAg) in form of virus-like particles (VLPs) using the yeast *Saccharomyces cerevisiae* leading to the development of a so-called “second” generation hepatitis B vaccine and the first recombinant subunit vaccine available [2]. This formulation of the hepatitis B vaccine has been on the market since 1986. Initially, HBsAg VLPs of ~22 nm were purified from the plasma of asymptomatic HBV carriers, but due to safety issues and restricted supply, the “first” generation plasma-derived vaccines are no longer in use [2]. Nowadays, as patents have expired, “third” generation “biosimilar” recombinant HBsAg VLP-based vaccines are being introduced into the market by a variety of new manufacturers which try to make the vaccine also more affordable to developing countries [2].

As HBsAg is a very hydrophobic protein, secretion is inefficient in yeast and high-level production has been only achieved as intracellular product. The purification of recombinant HBsAg from yeast cultures is well documented [3-25], [see Tables 1 and 2] and several studies have shown that purified yeast-derived HBsAg can assemble into characteristic ~22 nm VLPs [26-29]. These particles are highly immunogenic and capable of eliciting potent neutralizing antibodies as they mimic the conformation of native viruses but lack the viral genome and can be used as safe and cheap vaccine [26, 30-32].

Previously, we have reported a simple fed-batch technique which leads to the production of ~6-7 g/l HBsAg, with 30% in a “soluble” form competent for assembly into VLPs [29]. Although, the purification of HBsAg VLPs was reported before in
the “Materials and Methods section” [24], optimization studies of the extraction conditions, details of the purification of HBsAg VLPs and the final characterization of their immunogenic properties were not reported. Here, a simple strategy is outlined for the purification of HBsAg leading to VLPs with satisfactory yields, high purity and excellent quality. Finally, we provide evidence in mice about the superior immunogenic properties of these HBsAg VLPs as a parenteral subunit vaccine in combination with either alum or a novel adjuvant, the TLR2/6 agonist MALP-2.
2. Materials and methods

2.1 Strain and culture conditions

The *P. pastoris* strain GS115 carrying 8 copies of the *HBsAg* gene under the control of the *AOX1* promoter has been described previously [33]. The cells were grown on defined medium in a fed-batch procedure as described before [29]. Briefly, the cells were first grown in a batch procedure on glycerol (initial concentration 95 g/l). After depletion of glycerol, production of *HBsAg* was initiated through the addition of methanol to a final concentration of 6 g/l and the methanol concentration was kept constant at 6 g/l by continuous methanol feeding throughout the entire production phase.

2.2 Purification of recombinant *HBsAg*

After harvesting, cells were pelleted by centrifugation at 4,225 g for 15 min at room temperature (1 liter culture broth OD600 ~240 corresponding to ~80 g dry cell mass and ~200 g wet cell mass). The cell pellet was resuspended in the same volume of ice cold buffer [20 mM sodium phosphate buffer, pH 8.0, 5 mM EDTA] for removal of media components and other contaminants and recentrifuged.

2.2.1 Step 1: Cell lysis and detergent mediated solubilization of *HBsAg*

For the initial detergent optimization studies, a cell pellet (corresponding to 1 ml OD100 culture broth) was resuspended with glass beads [0.5 g of ~ 0.5 mm size] in 1 ml of a basic lysis buffer [10 mM sodium phosphate buffer, pH 8.0, 5 mM EDTA, 500 mM NaCl, 8% glycerol]. This basic lysis buffer was additionally supplemented with 0-2% detergents [Tween 20 or Triton X-100 or CHAPS or NP-40 or sodium deoxycholate] for detergent testing and the whole mixture incubated at 4°C using a
thermomixer. In pilot scale studies, cell lysis was essentially carried out as described previously [24]. The washed cell pellet from 1 liter culture broth was resuspended in 1 liter ice-cold lysis buffer [25 mM phosphate buffer, pH 8.0, 5 mM EDTA, 0.6% (v/v) Tween-20] and the pre-cooled cell suspension disrupted by high pressure homogenization (Gaulin Lab 60, APV Gaulin, Germany) using four cycles at 600 bar and ~4°C. Cell lysis was confirmed by microscopy.

2.2.2 Step 2: Polyethylene glycol (PEG) precipitation
To the lysate collected after high pressure homogenization, a 5 M NaCl solution was slowly added within 30 min to a final concentration of 500 mM followed by the addition of polyethylene glycol 6000 (S. D. Fine-Ch em, India, 50% w/v) to a final concentration of 5% (w/v). This suspension was stirred for 2 h at 4°C and precipitation was then allowed to occur for 12-16 h at 4°C without stirring. The suspension was then clarified by centrifugation at 4°C and 4,225 g for 15 min.

2.2.3 Step 3: Aerosil-380 adsorption
Prior to use, Aerosil-380 (Evonik, Hanau, Germany) was pre-equilibrated, e.g. washed twice, with 25 mM sodium phosphate buffer, pH 7.2, 500 mM NaCl (centrifuged at 4,225 g for 15 min and 4°C). The clarified supernatant obtained after PEG precipitation (removal of host cell proteins and other host contaminants) was mixed with Aerosil-380 (0.13 g of dry Aerosil-380 pre-equilibrated per g initial wet cell mass). This suspension was stirred for 4 h at 4°C and centrifuged at 4°C and 4,225 g for 15 min. The pellet (corresponding to 1 liter of initial culture broth) was washed twice with 25 mM phosphate buffer (pH 7.2), centrifuged as above, finally resuspended in 800 ml of 50 mM sodium carbonate-bi-carbonate buffer, pH 10.8, 1.2
M urea and kept at 37°C for 12 h with stirring. This suspension was then centrifuged at 25°C and 15,180 g for 60 min and the supernatant pH adjusted to pH 8.5 for better removal of silica particles (Aerosil-380) and the solution clarified by vacuum-filtration (0.45 µm) before proceeding to the next step.

2.2.4 Step 4: Ion-exchange chromatography

The clarified Aerosil-380 eluate was further processed by anion exchange chromatography. An XK column (Amersham Pharmacia Biotech, Sweden) packed with 200 ml of DEAE Sepharose FF (GE Healthcare) and pre-equilibrated with 50 mM Tris-HCl, pH 8.5 (conductivity ~3.2 mS/cm) was employed and the column loaded with the Aerosil-380 eluate (~800 ml) using a flow rate of 4 ml/min. After loading, the column was washed with washing buffer [50 mM Tris-HCl, pH 8.5, conductivity ~ 3.2 mS/cm] until the absorbance at 280 nm in the eluate returned to baseline. The bound HBsAg was eluted using a salt step [50 mM Tris-HCl, pH 8.5, 500 mM NaCl, conductivity ~50 mS/cm]. The protein containing fractions (absorbance at 280 nm) were analyzed by SDS-PAGE.

2.2.5 Step 5: Isopycnic density ultracentrifugation and size-exclusion chromatography

To the pooled HBsAg-containing fractions obtained after ion-exchange chromatography, CsCl was added to a final density of 1.2 d/ml. This solution was ultra-centrifuged (Sorval rotor: TV865B) at 236,525 g for 12 h at ~ 23°C without break. Alternatively, size-exclusion chromatography was used for further purification. The DEAE Sepharose FF eluate was concentrated by ultrafiltration (Vivaspin membrane 10,000 MWCO, Sartorius Stedium Biotech GmbH, Germany)
and loaded onto a pre-equilibrated Sephacryl S-300 (Hiprep 26/60) pre-packed column. Elution was carried out with PBS (pH 7.2) and monitored at 280 nm.

2.2.6 Step 6: Potassium thiocyanate (KSCN) treatment and dialysis of the final bulk

The HBsAg positive fractions were pooled and treated with KSCN to a final concentration of 1.2 M. The mixture was stirred at 37°C for ~ 4 h. The KSCN treated HBsAg was extensively dialyzed against PBS (pH 7.2) and the final pure protein (the so called bulk protein) filter sterilized and used for immunization studies.

2.3 Analytical methods for HBsAg determination

2.3.1 Quantitative analysis of HBsAg by ELISA

The concentration of HBsAg in cell extracts and other samples was determined using a quantitative Sandwich ELISA (Hepanostika HBsAg Ultra, Biomerieux, The Netherlands) following the manufacturer’s instructions. This ELISA was originally developed for analyzing HBsAg in human sera and most likely detects preferentially the immunogenic (“bioactive”) versions of HBsAg (e.g. VLPs and rod-shaped structures). The clarified samples were diluted appropriately with a buffer containing 0.1% BSA in PBS (pH 7.2) and analyzed in triplicates. For calibration, a dilution series containing 0 to 1 ng/ml of HBsAg standard (NIBSC code number – 00/588) and 0 to 100 ng/ml of in-house prepared pure HBsAg was employed. All samples were analyzed in triplicates.

2.3.2 Quantitative analysis of HBsAg by RP-HPLC

The amount of HBsAg was also analyzed by reversed phase-high performance liquid chromatography (RP-HPLC), essentially as reported previously [34]. This assay
detects all conformational versions of HBsAg. Using the described conditions for sample preparation and chromatography [29], the standard HBsAg (NIBSC code number – 00/588) as well as the purified HBsAg eluted at a retention time of 10.9 min. The standard HBsAg was used for calibration. The proteins eluting at 10.9 min (standard and purified HBsAg) were collected, dried to remove traces of organic solvents, and subjected to SDS-PAGE analysis and immunoblotting using a linear epitope-specific anti-HBsAg in-house monoclonal antibody to confirm the presence of HBsAg.

### 2.4 Other protein analytical methods

The protein concentration in total cell extracts and other samples was determined using the bicinchoninic acid (BCA) method [35]. SDS-PAGE analysis was performed as reported [29]. Electron microscopy of HBsAg VLPs was carried out as described previously [24, 29].

### 2.5 In vivo immunization studies

#### 2.5.1 Mice

Female BALB/c (H-2d) mice 6-8 weeks old were purchased from Harlan (Germany). All animal experiments in this study were performed in agreement with the local government of Lower Saxony (Germany) with the permission No. 33.11.42502-04-017/08.

#### 2.5.2 Immunization protocols

The mice were immunized by the i.m. route on days 0, 14 and 28 with Engerix™ (2 µg; GSK, England) or 2 µg of HBsAg VLPs alone or co-administered with alum
(1:1) or PEGylated MALP-2 (10 µg/dose) in a total volume of 50 µl of PBS [36]. Mixing of antigen and adjuvant in PBS was performed 30 min before the i.m. injection into the right hind leg. The optimal dose of the adjuvant was determined in preliminary studies (data not shown). Animals in the negative control group received only PBS.

2.5.3 Detection of antigen-specific IgG in the sera

The HBsAg VLP-specific antibodies were determined in the serum samples by ELISA using microtitre plates coated with 100 µl/well of the respective antigen (2 µg/ml in 0.05 M carbonate buffer, pH 9.6, as previously described [37].

2.5.4 Measurement of cellular proliferation

The spleens of vaccinated mice were aseptically removed, single-cell suspensions were prepared and the erythrocytes lysed by 2 min incubation in ACK buffer. The cells were washed twice and adjusted to 2 x 10^6 cells/ml in complete RPMI medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The splenocytes were seeded at 100 µl/well (1 x 10^5) in a U-bottomed 96-well microtitre plate (Sarstedt, Germany) and cultured in quadruplicate for 4 days in the presence of different concentrations of HBsAg VLPs, 5 µg/ml concanavalin A or medium alone [38, 39].

2.5.5 ELISPOT assay

For the determination of the amount of cytokine secreting T helper cells in the spleen, the murine IFN-γ, IL-2, IL-4 and IL-17 ELISpot kits (BD Pharmingen, USA) were used according to the manufacturer’s instructions. Spleen cells (1 x 10^6 or 5 x
$10^5$ per well) were incubated for 24 h (IFN$\gamma$) up to 48 h (IL-2, IL-4 and IL-17) in the absence or in the presence of the HBsAg VLPs with a concentration of 2 $\mu$g/ml. Then, cells were removed and the plates were processed. Colored spots were counted with an ELISpot reader (C.T.L.) and analyzed using the ImmunoSpot image analyzer software v3.2 [40].

2.5.6 Statistical analysis

The statistical significance of the differences observed between the different experimental groups was analyzed using the Student’s unpaired $t$-test and the non-parametric Mann-Whitney test of SigmaStat 3.10 (Build 3.10.0) or alternatively with Graph Pad Prism 5 for Windows (Version 5.04) using the two-way ANOVA test. Differences were considered significant at $p < 0.05$. 

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3. Results

The generation and purification of HBsAg VLPs from *Pichia* cells include several steps which are outlined below. First, cell breakage is required and the target protein needs to be released from the endoplasmic reticulum where it is found assembled into defined multi-layered lamellar structures [24]. This first step is of crucial importance as it combines the mechanical destruction of cells and cell compartments with the detergent-assisted solubilization of membranes and membranous structures. The steps following release and solubilization of HBsAg encompass removal of the majority of host cell contaminants by precipitation, hydrophobic adsorption of HBsAg to colloidal silica and final purification and maturation of HBsAg using chromatography and KSCN treatment.

3.1 Cell lysis, detergent-assisted solubilization of HBsAg from crude cell lysates and precipitation of host cell contaminants

For small scale purification, cell lysis is best performed using glass beads. For larger scale purification, high pressure homogenization is preferred as it simplifies the following downstream steps of purification. At first, different detergents were analyzed regarding their effect on the solubilization of HBsAg from crude cell lysates. Best results regarding the solubilization of “bioactive” HBsAg were obtained using the nonionic detergent Tween 20 as compared with the other tested detergents such as Triton X-100 (nonionic), CHAPS (zwitterionic), NP-40 (nonionic), or sodium deoxycholate (anionic, bile salt) (Figure 1A). The results also revealed that the concentration of Tween 20 should be at least or above 0.5% and that lysis with glass beads in the thermomixer should last for at least 12 to 16 h at 4°C for maximum solubilization (Figure 1B). Longer lysis is not recommended as in some
experiments we observed a decline of “bioactive” HBsAg during prolonged incubation (data not shown). For pilot scale purification, cell breakage is best performed by high pressure homogenization in the presence of detergent (0.5 – 1% Tween 20) and the crude lysate obtained after homogenization can be immediately treated with NaCl and PEG 6000 (4°C, 2 h stirring followed by 12-16 h w/o stirring). This process combines the solubilization of HBsAg and precipitation of host cell contaminants. Previous studies using different molecular weight forms of PEG (Mr 300 – 100,000) indicated best results using PEG 6000 [41-43] and a sequential precipitation of host cell contaminants and HBsAg by step-wise increasing concentrations of PEG 6000 [12]. We also tested different molecular weight forms of PEG (Mr 1,000 – 20,000) at concentrations of 1-8% and found best results, e.g. highest amount of soluble HBsAg with a minimum of soluble host cell contaminants by using a single precipitation step with 4-6% PEG 6000 (data not shown).

3.5 Aerosil-380 extraction and ion-exchange chromatography

Equilibrated colloidal silica (Aerosil-380) was used to bind HBsAg in clarified PEG extracts at neutral pH through hydrophobic adsorption. Elution of bound HBsAg from silica using 50 mM sodium carbonate-bicarbonate buffer, pH 10.5, resulted in an unsatisfactory recovery. However, the recovery increased ~10-fold by supplementing the elution buffer with 1.2 M urea leading to an HBsAg eluate with a purity of 60-70% (Table 3). The following ion exchange chromatography step (Figure 2A) further increased the purity of HBsAg to 90-95% (Table 3, Figure 2B). SDS-PAGE analysis of the ion exchange eluate fractions under strong reducing conditions already revealed the expected properties of HBsAg appearing at positions
corresponding to monomeric (~25 kDa) and dimeric versions of the antigen (~50 kDa) (Figure 2B, [44]).

3.6 Isopycnic density ultracentrifugation versus size-exclusion chromatography and preparation of final bulk

The ion-exchange chromatography eluate fractions containing HBsAg were pooled and subjected to ultracentrifugation. The different sections of the ultracentrifugation tubes were analyzed by SDS-PAGE and revealed the presence of HBsAg in the upper parts of the tube with high purity (>99%) and the expected SDS-PAGE running profile (Figure 3A). Alternatively, the pooled and concentrated HBsAg containing ion-exchange chromatography eluate fractions were subjected to size exclusion chromatography where oligomeric components eluted at the void volume (Figure 3B). Both techniques, the ultracentrifugation and size exclusion chromatography appear to be equally effective for the final generation of HBsAg VLPs (Figure 3). HBsAg positive fractions after either isopycnic density ultracentrifugation or size-exclusion chromatography were pooled and treated with KSCN. This mixture was then extensively dialyzed against PBS for removal of CsCl and KSCN. In total, approx. 50 mg HBsAg VLPs with a purity of >99% can be recovered from one liter culture broth with a final yield of around 3% (Tables 2 and 3). The entire HBsAg production and purification process is outlined in Figure 4.

In vitro characterization of purified HBsAg

The final bulk protein was also analyzed by RP-HPLC and compared with the NIBSC standard (code number – 00/588). A retention time of 10.9 min was observed as was found for the standard (data not shown). The HBsAg did not show any
binding to lectins, thus proving absence of glycosylation (data not shown). Finally, electron microscopy of pure HBsAg, obtained using either ultracentrifugation or size-exclusion chromatography, revealed in both cases the presence of the characteristic icosahedral symmetrical structures with a diameter of ~22 nm, the so-called HBsAg “VLPs” (included in Figure 3).

3.7 In vivo immunogenic properties

To analyze the antigenic properties of HBsAg VLPs in vivo, BALB/c mice were immunized with a gold standard vaccine (Engerix™ which contains alum as adjuvant), HBsAg VLPs alone (2 µg/dose), or HBsAg VLPs co-administered with either alum (1:1) or a PEGylated derivative of MALP-2 (5 µg/dose) by the i.m. route. The obtained results demonstrated that the co-administration of HBsAg VLPs with adjuvants resulted in enhanced stimulation of the antigen-specific IgG-titers in comparison to the results observed in animals which received HBsAg VLPs alone or Engerix™ (Figure 5A). Significantly higher IgG titers (p < 0.05) were only observed in mice receiving the PEGylated MALP-2 derivative (5 µg/dose; Figure 5A). To evaluate the effect of HBsAg VLPs on the stimulated T helper response, the subclass distribution of the HBsAg-specific IgG (IgG1 and 2a) was analyzed. Although the levels of anti-HBsAg IgG1 were significantly higher, the levels of HBsAg-specific IgG2a antibodies were also increased in mice vaccinated with HBsAg VLPs co-administered with either alum or the MALP-2 derivative (Figure 5B). This suggested that the parenteral immunization by the i.m. route using HBsAg VLPs as a vaccine resulted in the stimulation of a more Th2 dominated T helper response. The analysis of the cytokines secretion by HBsAg-restimulated splenocytes by ELIspot showed that not only IL-4 secreting cells were increased in number in mice which received
the HBsAg VLPs co-administered with alum or the MALP-2 derivative as compared
to the control groups, but the HBsAg-specific IL-17, IFNγ and IL-2 secreting cells
were also increased (Figure 5C). To further characterize the capacity of HBsAg
VLPs to induce the cellular immune responses, spleen cells isolated from vaccinated
mice on day 42 were re-stimulated *in vitro* with HBsAg VLPs and their proliferation
capacity was then assessed. A strong dose-dependent proliferative response was only
observed in mice vaccinated with HBsAg VLPs co-administered with alum (SI >4)
or the PEGylated MALP-2 derivative (SI >4), as shown in Figure 5D. In contrast, no
or only marginal responses were observed with cells derived from mice vaccinated
with either HBsAg VLPs alone (SI >2), Engerix™ or PBS (SI <2). The differences
observed between the results obtained with adjuvanted HBsAg VLPs compared with
those obtained from either the control, non-adjuvanted HBsAg VLPs or Engerix™
vaccinated mice were significantly higher (p < 0.05).
4. Discussion

HBsAg is a very hydrophobic protein with long stretches of connected hydrophobic amino acids. Only recently it was shown that HBsAg – when produced in yeast, e.g. *P. pastoris* – does not assemble into VLPs within the cell as was assumed previously nor does it insert in significant amounts into ER membranes as was also proposed. Evidence has been presented that the HBsAg remains in the endoplasmic reticulum (ER) where it does not form VLPs but where a major fraction assembles into well-ordered multi-layered lamellar structures [24]. The layering order of HBsAg in these lamellar structures strongly suggests the presence of well-ordered HBsAg subunits [24], which should be solubilizable without getting structurally disordered to reassemble into VLPs under appropriate conditions. The remainder of HBsAg forms non-structured aggregates in the ER, which are only solubilizable using extremely harsh protein-structure breaking conditions [29].

Thus, cell breakage and release of HBsAg form the ER in a “bioactive” form competent for VLP assembly is the major objective for the first step of the purification procedure. Usually, the nonionic detergent Triton X-100 is employed for solubilization of HBsAg form yeast homogenates, e.g. [9, 13, 45, 46]. However, there have been early indications that the nonionic detergent Tween 20 might be less harsh to “intact” HBsAg compared to Triton X-100 [6]. In our hands Tween 20 released more “bioactive” HBsAg from the yeast homogenate compared to Triton X-100 as measured by an HBsAg Sandwich ELISA developed for the determination of HBsAg particles in human plasma. We do not have a straightforward explanation why Tween 20 performs better compared to Triton X-100 in releasing “bioactive” HBsAg. Tween 20 is considered a milder detergent being less effective in membrane solubilization compared to Triton X-100 [47-49]. Moreover, addition of Tween 20 to
protein formulations has proven to be effective in preventing shear induced aggregation of antibodies [50] and also aggregation of murine polyomavirus VLPs during storage [51]. Thus, replacement of Triton X-100 by Tween 20 presumably helps to reduce shear stress induced denaturation and “irreversible” aggregation of HBsAg during mechanical cell breakage. The other important objective for the first downstream purification steps relates to the removal of host cell contaminants. As we aimed for releasing “bioactive” HBsAg into the soluble fraction of the cell homogenate host cell contaminants should be transferred preferably to the insoluble fraction of the lysate. Substitution of Triton X-100 by Tween 20 presumably also helps to achieve this objective as it is less effective in (host cell) membrane solubilization. The intended transfer of host cell contaminants into the insoluble fraction is further accomplished by addition of 5% PEG 6000, a hydrophilic nonionic polymer, which is known to precipitate the majority of host cell proteins, polysaccharides, and nucleic acids but not the HBsAg when employed at this concentration [8]. After precipitate removal, the following purification steps e.g. hydrophobic adsorption to colloidal silica, desorption from silica and subsequent chromatography and final maturation are with minor modifications in accordance with previously published procedures. The final yields are certainly in need of improvement but we would expect high robust yields under standardized industrial GMP production and purification conditions. The quality of the final product, however, is outstanding as it outperforms, in particular when adjuvanted with the novel adjuvant MALP-2, the gold standard HBsAg vaccine Engerix™ in stimulating humoral and cellular immune responses.
Acknowledgements

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Reference List


Figure legends

Figure 1. Detergent solubilization of HBsAg from cell lysates: (A) Cells were lysed with glass beads in basic lysis buffer additionally containing detergents at the indicated concentrations and incubated at 4°C in a thermomixer for 48 h. The final amount of “bioactive” HBsAg released into the soluble lysate fraction is given in relative units of the Sandwich ELISA readout. (B) The time-dependent release of “bioactive” HBsAg into the soluble fraction of the lysate as followed by the Sandwich-ELISA.

Figure 2. Anion exchange chromatography (DEAE Sepharose FF): (A) Elution of bound proteins during ion exchange chromatography. The eluate fractions 13 to 24 were pooled (each fraction 12 ml), filtered and subjected to the next purification step. (B) Analysis of eluate fractions 12 to 29 by SDS-PAGE (10 µl of each sample loaded). The single and double asterisks refer to the monomer and dimer of HBsAg, respectively.
Figure 3. Ultracentrifugation versus size exclusion chromatography: (A) The pooled and filtered ion-exchange chromatography eluate fractions (fractions 13 to 24) were subjected to isopycnic density ultracentrifugation. After centrifugation tubes were punctured to collect the fractions 1 (bottom of the ultra-tube) to 6 (top of the ultra-tube) which were analyzed by SDS-PAGE (10 µl of each sample loaded). Marker ‘M’, fractions 1 to 6 (lanes 1 to 6). The single and double asterisks refer to the monomer and dimer of HBsAg, respectively. (A1) Electron microscopy of HBsAg VLPs obtained after isopycnic density ultracentrifugation, KSCN treatment and dialysis. These HBsAg VLPs were used after sterile filtration for the mice immunization studies (data shown in Fig. 5). (B) Pooled and concentrated HBsAg containing ion-exchange chromatography eluate fractions were subjected to size-exclusion chromatography. Protein elution was followed by UV (280 nm). The first arrow (1) points to the HBsAg VLPs (void volume) and the second arrow (2) to host cell impurities. Insert: The eluate fractions (42 to 53 and 59 to 60) corresponding to the peaks 1 and 2, respectively, were analyzed by SDS-PAGE. The single and double asterisks refer to the monomer and dimer of HBsAg, respectively. (B1) Electron microscopy of HBsAg VLPs obtained after size exclusion chromatography (eluate fractions 44 to 49), KSCN treatment and dialysis. The bar corresponds to 100 nm.

Figure 4. Schematic process flow chart for production and purification of HBsAg VLPs using recombinant P. pastoris.
Figure 5. Induction of efficient humoral and cellular immune responses in BALB/c mice following vaccination with HBsAg VLPs: (A) Humoral immune responses stimulated in vaccinated mice. Analysis of HBsAg VLPs specific IgG responses in sera from mice (n=5) immunized on days 0, 14 and 28 with PBS (control), Engerix™ (2 µg/dose) co-administered with alum (1:1) or HBsAg VLPs (2 µg/dose) alone or co-administered with either alum (1:1) or the PEGylated derivative of MALP-2 (5 µg/dose) by the i.m. route. (B) Determination of HBsAg VLPs specific IgG1 and 2a titers present in sera. Results are expressed as mean end point titers. (C) Analysis of the T helper responses stimulated in mice vaccinated with HBsAg VLPs. Detection of IFNγ, IL-2, IL-4 and IL-17-secreting spleen cells by ELISpot. Splenocytes recovered from vaccinated mice were incubated for 24 or 48 h in the presence of HBsAg VLPs. Results are presented as spot forming units per 10^6 cells, which were subtracted from the values obtained from non-stimulated cells. SEM is indicated by vertical lines. (D) Cellular immune responses stimulated in vaccinated animals. Cellular proliferation was assessed by determination of the [³H] thymidine incorporated into the DNA of replicating cells. Results are averages of triplicates and they are expressed as stimulation index (SI).

The results obtained in animals vaccinated with HBsAg VLPs co-administered with different adjuvants were statistically significant with respect to those observed in mice receiving HBsAg VLPs alone or the gold vaccine standard (Engerix™) at p<0.05 (*).
Table 1: Snapshot view on purification processes for hepatitis B surface antigen from yeast cultures

<table>
<thead>
<tr>
<th>Host</th>
<th>Purification steps</th>
<th>Ref.</th>
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<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → Centrifugation → Amicon concentration → XAD-2 → Centrifugation → Aerosil 380</td>
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<tr>
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<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → PEG followed by acetic acid treatment → Calcium chloride treatment → Centrifugation → Amicon concentration → Fractogel TSK HW65(F) → Fractogel TSK DEAE 650 (M) → Fractogel TSK HW65(F)</td>
<td>[7]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → PEG followed by acetic acid treatment → Calcium chloride treatment → Centrifugation → Amicon concentration → Fractogel TSK HW65(F) → Fractogel TSK DEAE 650 (M) → Fractogel TSK HW65(F)</td>
<td>[8]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → Centrifugation → Solubilization using Triton X-100 → Concentration → Diafiltration → Urea treatment → Diafiltration → KSCN → Dialysis</td>
<td>[9]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → Acidification → Centrifugation → Ammonium sulfate precipitation at pH 6.5 → Centrifugation → Suspension of precipitate → Dialysis → Hydroxyapatite (repeat: 2 times) → Dialysis followed by ultrafiltration</td>
<td>[10]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Precipitation → Immunoaffinity chromatography → Size-exclusion chromatography</td>
<td>[11]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → Centrifugation → PEG precipitation (8 %) → Centrifugation → Pellet suspension and homogenization → PEG precipitation (3 %) → Centrifugation → PEG precipitation (8 %) → Centrifugation → Pellet suspension and homogenization → Diafiltration → Sucrose density gradient centrifugation → Ultrafiltration → CsCl ultracentrifugation → Dialfiltration → Ultrafiltration → TSK HW 65 → CsCl ultracentrifugation → Dialysis and ultrafiltration</td>
<td>[12]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Lysis → Centrifugation → Detergent treatment → Centrifugation → XAD-4 → Hydrophobic interaction chromatography</td>
<td>[13]</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>Lysis → Precipitation of cell debris with PEG → Separation of PEG supernatant → Adsorption on a silica matrix → Separation of the silica matrix → Desorption of the product from the silica matrix → Separation of the supernatant of the silica matrix → Ion exchange chromatography → Ultrafiltration → Density gradient ultracentrifugation → Size-exclusion chromatography → Sterile filtration</td>
<td>[14]</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>Lysis → Centrifugation → Anion exchange chromatography → Butyl-S QZT → Ultrafiltration → Size-exclusion chromatography</td>
<td>[15]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Acid precipitation → Hyflo Super Cel</td>
<td>[16]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Amberlyte XAD-2 column → Macroprep High Q chromatography → Cellufine sulfate chromatography → Ultrafiltration → Formulation</td>
<td>[17]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Treatment with colloidal silica → Macroprep High Q chromatography → Butyl Sepharose-4 fast flow → Ultrafiltration → Sepharose CL-4B → Ultrafiltration → Formulation</td>
<td>[18]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Acid precipitation → Aerosil 380 → Immunoaffinity chromatography → Ion-exchange chromatography → Size-exclusion chromatography</td>
<td>[19]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Aerosil 380 → DEAE Toyopearl 650M → HiLoad Superdex 75</td>
<td>[20]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Ultrafiltration of supernatant → Immunoaffinity purification → Ultrafiltration</td>
<td>[21]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Membrane extraction with detergent → Centrifugation → “HIMAX” technology → Centrifugation → DEAE → Dialfiltration</td>
<td>[22]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Precipitation → Centrifugation → Pheny1-5PW HIC → Ultracentrifugation</td>
<td>[23]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → PEG Precipitation → Centrifugation → Aerosil 380 → DEAE Sepharose FF → Ultrafiltration → KSCN treatment and dialysis → Formulation</td>
<td>[24]</td>
</tr>
<tr>
<td><em>P. pastoris</em></td>
<td>Lysis → Centrifugation → Membrane extraction → Centrifugation → PEG Precipitation → Centrifugation → Diafiltration → Phenyl 600M → Size exclusion chromatography → Dialysis</td>
<td>[25]</td>
</tr>
</tbody>
</table>

*a* HBsAg was secreted into the culture medium.
Table 2: Purification of HBsAg from yeast cultures using ultracentrifugation (UC) or size exclusion chromatography (SEC) as final step (prior to KSCN treatment)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Purification steps\textsuperscript{b}</th>
<th>Final recovery\textsuperscript{c} (mg/l culture broth)</th>
<th>Purity \textsuperscript{c} (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. cerevisiae}</td>
<td>6 UC</td>
<td>~0.3</td>
<td>90</td>
<td>[6]</td>
</tr>
<tr>
<td>\textit{S. cerevisiae}</td>
<td>13 UC</td>
<td>nd</td>
<td>nd</td>
<td>[12]</td>
</tr>
<tr>
<td>\textit{P. pastoris}</td>
<td>3 UC</td>
<td>10</td>
<td>nd</td>
<td>[23]</td>
</tr>
<tr>
<td>\textit{P. pastoris}</td>
<td>4 UC</td>
<td>nd</td>
<td>nd</td>
<td>[24]</td>
</tr>
<tr>
<td>\textit{S. cerevisiae} \textsuperscript{d}</td>
<td>3 SEC</td>
<td>~0.06</td>
<td>nd</td>
<td>[11]</td>
</tr>
<tr>
<td>\textit{H. polymorpha}</td>
<td>5 SEC</td>
<td>nd</td>
<td>95</td>
<td>[14]</td>
</tr>
<tr>
<td>\textit{H. polymorpha}</td>
<td>4 SEC</td>
<td>nd</td>
<td>99</td>
<td>[15]</td>
</tr>
<tr>
<td>\textit{P. pastoris}</td>
<td>4 SEC</td>
<td>nd</td>
<td>95</td>
<td>[17]</td>
</tr>
<tr>
<td>\textit{P. pastoris}</td>
<td>5 SEC</td>
<td>nd</td>
<td>95</td>
<td>[19]</td>
</tr>
<tr>
<td>\textit{P. pastoris}</td>
<td>4 UC or SEC</td>
<td>~50</td>
<td>&gt;99</td>
<td>this study</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Only references on HBsAg purification included containing respective quantitative data
\textsuperscript{b} Number of purification steps before final ultracentrifugation (UC) or size exclusion chromatography (SEC); normal centrifugation step is not considered as purification step
\textsuperscript{c} Recovery and purity relates to the final pure bulk protein
\textsuperscript{d} HBsAg was secreted into the culture medium

UC (ultracentrifugation), SEC (size exclusion chromatography), nd (not determined)

Table 3: Summary of purification process for recombinant HBsAg VLPs

<table>
<thead>
<tr>
<th>Steps</th>
<th>Recovery (%)\textsuperscript{a}</th>
<th>Purity (%)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>100</td>
<td>nd</td>
</tr>
<tr>
<td>PEG precipitation supernatant</td>
<td>~75</td>
<td>nd</td>
</tr>
<tr>
<td>Eluate from colloidal silica (Aerosil 380)</td>
<td>~30</td>
<td>60-70</td>
</tr>
<tr>
<td>DEAE Sepharose FF eluate</td>
<td>~7</td>
<td>90-95</td>
</tr>
<tr>
<td>Ultracentrifugation $\rightarrow$ KSCN treatment $\rightarrow$ dialysis or Size-exclusion chromatography $\rightarrow$ KSCN treatment $\rightarrow$ dialysis</td>
<td>~3</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Based on Sandwich-ELISA
\textsuperscript{b} Based on BCA test and RP-HPLC
nd (not determined)
Figure 1

A

Absorbance [450 nm]

0 1 2 3

Tween 20  Triton X-100  CHAPS  NP-40  Sod. deoxycholate

B

Tween 20 Concentration

- - - 0.25%
- - - 0.50%
- - - 0.75%
- - - 1.00%
- - - 1.50%
- - - 2.00%

Absorbance [450 nm]

0 1 2 3

Lysis time [h] 0 5 10 15 20 25 30 35
Figure 3

A

B

Absorbance 280 nm [AU]

Time [min]

0 60 120 180 240 300 360

0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00

0

2

1

**

*
Figure 4

**Present study**

- Washing of cell pellet
- Cell lysis via homogenizer
- PEG precipitation
- Aerosil 380: Adsorption, washing, desorption
- DEAE Sepharose FF: Binding, washing, elution
- Ultracentrifugation or Size-exclusion chromatography
- KSCN treatment followed by dialysis
- Sterile filtration (final bulk)

*In vivo* immunization studies (with novel adjuvant)
Figure 5