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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

Chandrasekhar Gurramkonda [1,2,3*], Maria Zahid [4], Satish Kumar Nemani [4], Ahmad Adnan [1,5], Satheesh Kumar Gudi [2], Navin Khanna [2], Thomas Ebensen [6], Heinrich Lünsdorf [6], Carlos A. Guzmán [6], and Ursula Rinas [1,4*]

[1] *Department of Structural Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany*
[2] *International Centre for Genetic Engineering and Biotechnology, New Delhi, India*
[3] *Current address: Technology Research Centre, Department of Chemical, Biochemical and Environmental Engineering, University of Maryland Baltimore County, Baltimore, USA*
[4] *Institute of Technical Chemistry – Life Sciences, University of Hannover, Hannover, Germany*
[5] *Current address: Department of Chemistry, GC University Lahore, Pakistan*
[6] *Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany*

***Corresponding authors**
chskrg@umbc.edu; chskrg@gmail.com (CG)
ursula.rinas@helmholtz-hzi.de (UR)

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^{TM}). 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

84 the “Materials and Methods section” [24], optimization studies of the extraction
85 conditions, details of the purification of HBsAg VLPs and the final characterization
86 of their immunogenic properties were not reported. Here, a simple strategy is
87 outlined for the purification of HBsAg leading to VLPs with satisfactory yields, high
88 purity and excellent quality. Finally, we provide evidence in mice about the superior
89 immunogenic properties of these HBsAg VLPs as a parenteral subunit vaccine in
90 combination with either alum or a novel adjuvant, the TLR2/6 agonist MALP-2.
91

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 OD 100 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^{o}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-Chem, 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

192 detects all conformational versions of HBsAg. Using the described conditions for
193 sample preparation and chromatography [29], the standard HBsAg (NIBSC code
194 number – 00/588) as well as the purified HBsAg eluted at a retention time of 10.9
195 min. The standard HBsAg was used for calibration. The proteins eluting at 10.9 min
196 (standard and purified HBsAg) were collected, dried to remove traces of organic
197 solvents, and subjected to SDS-PAGE analysis and immunoblotting using a linear
198 epitope-specific anti-HBsAg in-house monoclonal antibody to confirm the presence
199 of HBsAg.
200
201 **2.4 Other protein analytical methods**
202 The protein concentration in total cell extracts and other samples was determined
203 using the bicinchoninic acid (BCA) method [35]. SDS-PAGE analysis was
204 performed as reported [29]. Electron microscopy of HBsAg VLPs was carried out as
205 described previously [24, 29].
206
207 **2.5 *In vivo* immunization studies**
208 *2.5.1 Mice*
209 Female BALB/c (H-2d) mice 6-8 weeks old were purchased from Harlan (Germany).
210 All animal experiments in this study were performed in agreement with the local
211 government of Lower Saxony (Germany) with the permission No. 33.11.42502-04-
212 017/08.
213
214 *2.5.2 Immunization protocols*
215 The mice were immunized by the i.m. route on days 0, 14 and 28 with Engerix™ (2
216 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

242 10^5 per well) were incubated for 24 h (IFNγ) up to 48 h (IL-2, IL-4 and IL-17) in the
243 absence or in the presence of the HBsAg VLPs with a concentration of 2 µg/ml.
244 Then, cells were removed and the plates were processed. Colored spots were counted
245 with an ELISpot reader (C.T.L.) and analyzed using the ImmunoSpot image analyzer
246 software v3.2 [40].
247
248 *2.5.6 Statistical analysis*
249 The statistical significance of the differences observed between the different
250 experimental groups was analyzed using the Student's unpaired *t*-test and the non-
251 parametric Mann-Whitney test of SigmaStat 3.10 (Build 3.10.0) or alternatively with
252 Graph Pad Prism 5 for Windows (Version 5.04) using the two-way ANOVA test.
253 Differences were considered significant at $p < 0.05$.
254

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

280 experiments we observed a decline of “bioactive” HBsAg during prolonged
281 incubation (data not shown). For pilot scale purification, cell breakage is best
282 performed by high pressure homogenization in the presence of detergent (0.5 – 1%
283 Tween 20) and the crude lysate obtained after homogenization can be immediately
284 treated with NaCl and PEG 6000 (4°C, 2 h stirring followed by 12-16 h w/o stirring).
285 This process combines the solubilization of HBsAg and precipitation of host cell
286 contaminants. Previous studies using different molecular weight forms of PEG (Mr
287 300 – 100,000) indicated best results using PEG 6000 [41-43] and a sequential
288 precipitation of host cell contaminants and HBsAg by step-wise increasing
289 concentrations of PEG 6000 [12]. We also tested different molecular weight forms of
290 PEG (Mr 1,000 – 20,000) at concentrations of 1-8% and found best results, e.g.
291 highest amount of soluble HBsAg with a minimum of soluble host cell contaminants
292 by using a single precipitation step with 4-6% PEG 6000 (data not shown).
293
294 ***3.5 Aerosil-380 extraction and ion-exchange chromatography***
295 Equilibrated colloidal silica (Aerosil-380) was used to bind HBsAg in
296 clarified PEG extracts at neutral pH through hydrophobic adsorption. Elution of
297 bound HBsAg from silica using 50 mM sodium carbonate-bicarbonate buffer, pH
298 10.5, resulted in an unsatisfactory recovery. However, the recovery increased ~10-
299 fold by supplementing the elution buffer with 1.2 M urea leading to an HBsAg eluate
300 with a purity of 60-70% (Table 3). The following ion exchange chromatography step
301 (Figure 2A) further increased the purity of HBsAg to 90-95% (Table 3, Figure 2B).
302 SDS-PAGE analysis of the ion exchange eluate fractions under strong reducing
303 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

354 the HBsAg VLPs co-administered with alum or the MALP-2 derivative as compared
355 to the control groups, but the HBsAg-specific IL-17, IFNγ and IL-2 secreting cells
356 were also increased (Figure 5C). To further characterize the capacity of HBsAg
357 VLPs to induce the cellular immune responses, spleen cells isolated from vaccinated
358 mice on day 42 were re-stimulated *in vitro* with HBsAg VLPs and their proliferation
359 capacity was then assessed. A strong dose-dependent proliferative response was only
360 observed in mice vaccinated with HBsAg VLPs co-administered with alum (SI >4)
361 or the PEGylated MALP-2 derivative (SI >4), as shown in Figure 5D. In contrast, no
362 or only marginal responses were observed with cells derived from mice vaccinated
363 with either HBsAg VLPs alone (SI >2), Engerix™ or PBS (SI <2). The differences
364 observed between the results obtained with adjuvanted HBsAg VLPs compared with
365 those obtained from either the control, non-adjuvanted HBsAg VLPs or Engerix™
366 vaccinated mice were significantly higher ($p < 0.05$).
367

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 straight forward 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

393 protein formulations has proven to be effective in preventing shear induced
394 aggregation of antibodies [50] and also aggregation of murine polyomavirus VLPs
395 during storage [51]. Thus, replacement of Triton X-100 by Tween 20 presumably
396 helps to reduce shear stress induced denaturation and “irreversible” aggregation of
397 HBsAg during mechanical cell breakage. The other important objective for the first
398 downstream purification steps relates to the removal of host cell contaminants. As we
399 aimed for releasing “bioactive” HBsAg into the soluble fraction of the cell
400 homogenate host cell contaminants should be transferred preferably to the insoluble
401 fraction of the lysate. Substitution of Triton X-100 by Tween 20 presumably also
402 helps to achieve this objective as it is less effective in (host cell) membrane
403 solubilization. The intended transfer of host cell contaminants into the insoluble
404 fraction is further accomplished by addition of 5% PEG 6000, a hydrophilic nonionic
405 polymer, which is known to precipitate the majority of host cell proteins,
406 polysaccharides, and nucleic acids but not the HBsAg when employed at this
407 concentration [8]. After precipitate removal, the following purification steps e.g.
408 hydrophobic adsorption to colloidal silica, desorption from silica and subsequent
409 chromatography and final maturation are with minor modifications in accordance
410 with previously published procedures. The final yields are certainly in need of
411 improvement but we would expect high robust yields under standardized industrial
412 GMP production and purification conditions. The quality of the final product,
413 however, is outstanding as it outperforms, in particular when adjuvanted with the
414 novel adjuvant MALP-2, the gold standard HBsAg vaccine Engerix™ in stimulating
415 humoral and cellular immune responses.
416

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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.*

562 **Figure 5.** Induction of efficient humoral and cellular immune responses in BALB/c
563 mice following vaccination with HBsAg VLPs: (A) Humoral immune responses
564 stimulated in vaccinated mice. Analysis of HBsAg VLPs specific IgG responses in
565 sera from mice (n=5) immunized on days 0, 14 and 28 with PBS (control), Engerix™
566 (2 µg/dose) co-administered with alum (1:1) or HBsAg VLPs (2 µg/dose) alone or
567 co-administered with either alum (1:1) or the PEGylated derivative of MALP-2 (5
568 µg/dose) by the i.m. route. (B) Determination of HBsAg VLPs specific IgG1 and 2a
569 titers present in sera. Results are expressed as mean end point titers. (C) Analysis of
570 the T helper responses stimulated in mice vaccinated with HBsAg VLPs. Detection
571 of IFNγ, IL-2, IL-4 and IL-17-secreting spleen cells by ELISpot. Splenocytes
572 recovered from vaccinated mice were incubated for 24 or 48 h in the presence of
573 HBsAg VLPs. Results are presented as spot forming units per 10^6 cells, which were
574 subtracted from the values obtained from non-stimulated cells. SEM is indicated by
575 vertical lines. (D) Cellular immune responses stimulated in vaccinated animals.
576 Cellular proliferation was assessed by determination of the [^{3}H] thymidine
577 incorporated into the DNA of replicating cells. Results are averages of triplicates and
578 they are expressed as stimulation index (SI).
579 The results obtained in animals vaccinated with HBsAg VLPs co-administered with
580 different adjuvants were statistically significant with respect to those observed in
581 mice receiving HBsAg VLPs alone or the gold vaccine standard (Engerix^{TM}) at
582 $p<0.05$ (*).

Tables

Table 1: Snapshot view on purification processes for hepatitis B surface antigen from yeast cultures

Host	Purification steps	Ref.
S. cerevisiae	Lysis → Centrifugation → Amicon concentration → XAD-2 → Centrifugation → Aerosil 380	[3]
S. cerevisiae	Lysis → Centrifugation → Aerosil 380 → Ammoniumsulfate precipitation → Sepharose 4B	[4]
S. cerevisiae	Lysis → Centrifugation → Aerosil 380 → ECTHAM-cellulose → Sepharose 6B → Ammonium thiocynate treatment → Dialysis	[5]
S. cerevisiae	Lysis → Centrifugation → Urea treatment → Aerosil 380 → Amicon concentration → Sepharose CL-4B → Dextran sulfate → CsCl ultracentrifugation	[6]
S. cerevisiae	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)	[7]
S. cerevisiae	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)	[8]
S. cerevisiae	Lysis → Centrifugation → Solubilization using Triton X-100 → Concentration → Diafiltration → Urea treatment → Diafiltration → KSCN → Dialysis	[9]
S. cerevisiae	Lysis → Acidification → Centrifugation → Ammonium sulfate precipitation at pH 6.5 → Centrifugation → Suspension of precipitate → Dialysis → Hydroxyapatite (repeat: 2 times) → Dialysis followed by ultrafiltration	[10]
S. cerevisiae [a]	Precipitation → Immunoaffinity chromatography → Size-exclusion chromatography	[11]
S. cerevisiae	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 → Diafiltration → Ultrafiltration → TSK HW 65 → CsCl ultracentrifugation → Dialysis and ultrafiltration	[12]
S. cerevisiae	Lysis → Centrifugation → Detergent treatment → Centrifugation → XAD-4 → Hydrophobic interaction chromatography	[13]
H. polymorpha	Lysis → Precipitation of cell debris with PEG → Separation of PEG supernatant → Adsorbtion 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	[14]
H. polymorpha	Lysis → Centrifugation → Anion exchange chromatography → Butyl-S QZT → Ultrafiltration → Size-exclusion chromatography	[15]
P. pastoris	Lysis → Acid precipitation → Hyflo Super Cel	[16]
P. pastoris	Lysis → Centrifugation → Amberlyte XAD-2 column → Macroprep High Q chromatography → Cellufine sulfate chromatography → Ultrafiltration → Formulation	[17]
P. pastoris	Lysis → Centrifugation → Treatment with colloidal silica → Macroprep High Q chromatography → Butyl Sepharose-4 fast flow → Ultrafiltration → Sepharose CL-4B → Ultrafiltration → Formulation	[18]
P. pastoris	Lysis → Centrifugation → Acid precipitation → Aerosil 380 → Immunoaffinity chromatography → Ion-exchange chromatography → Size-exclusion chromatography	[19]
P. pastoris	Lysis → Centrifugation → Aerosil 380 → DEAE Toyopearl 650M → HiLoad Superdex 75	[20]
P. pastoris	Lysis → Centrifugation → Ultrafiltration of supernatant → Immunoaffinity purification → Ultrafiltration	[21]
P. pastoris	Lysis → Centrifugation → Membrane extraction with detergent → Centrifugation → "HIMAX" technology → Centrifugation → DEAE → Diafiltration	[22]
P. pastoris	Lysis → Precipitation → Centrifugation → Phenyl-5PW HIC → Ultracentrifugation	[23]
P. pastoris	Lysis → PEG Precipitation → Centrifugation → Aerosil 380 → DEAE Sepharose FF → Ultracentrifugation → KSCN treatment and dialysis → Formulation	[24]
P. pastoris	Lysis → Centrifugation → Membrane extraction → Centrifugation → PEG Precipitation → Centrifugation → Diafiltration → Phenyl 600M → Size exclusion chromatography → Dialysis	[25]

[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) [a]

Yeast	Purification steps[b]	Final recovery[c] (mg/l culture broth)	Purity [c] (%)	Reference
S. cerevisiae	6 UC	~0.3	90	[6]
S. cerevisiae	13 UC	nd	nd	[12]
P. pastoris	3 UC	10	nd	[23]
P. pastoris	4 UC	nd	nd	[24]
S. cerevisiae [d]	3 SEC	~0.06	nd	[11]
H. polymorpha	5 SEC	nd	95	[14]
H. polymorpha	4 SEC	nd	99	[15]
P. pastoris	4 SEC	nd	95	[17]
P. pastoris	5 SEC	nd	95	[19]
P. pastoris	4 UC or SEC	~50	>99	this study

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

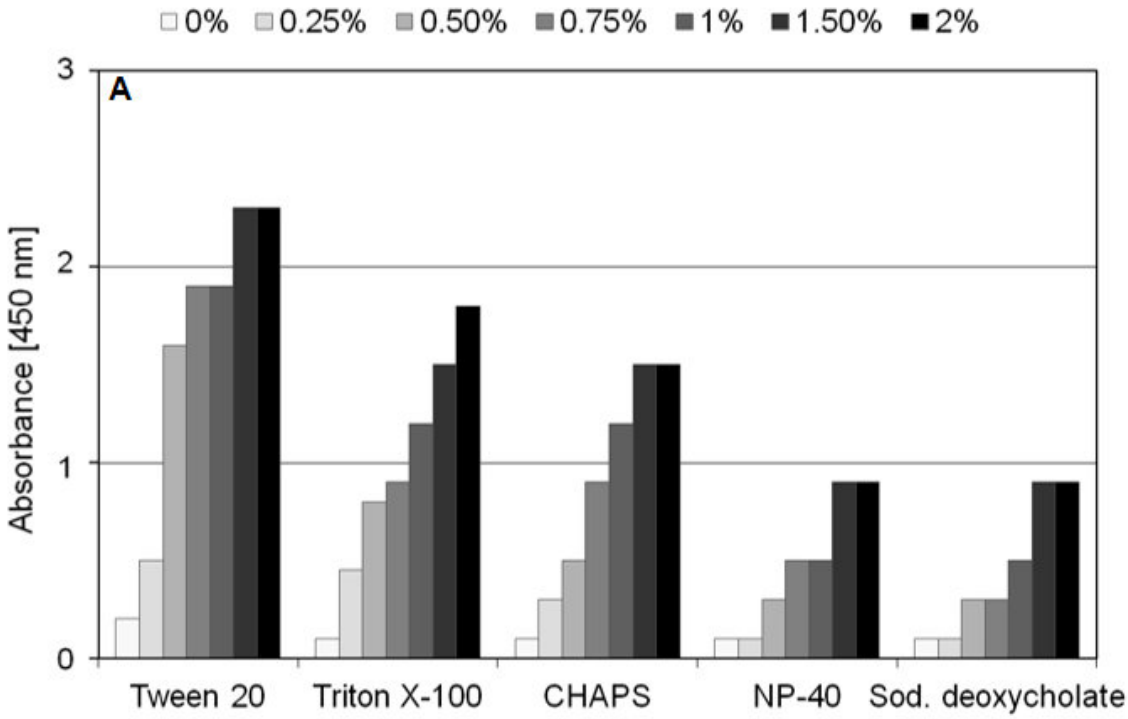
UC (ultracentrifugation), SEC (size exclusion chromatography), nd (not determined)

Table 3: Summary of purification process for recombinant HBsAg VLPs

Steps	Recovery (%) [a]	Purity (%) [b]
Cell lysate	100	nd
PEG precipitation supernatant	~75	nd
Eluate from colloidal silica (Aerosil 380)	~30	60-70
DEAE Sepharose FF eluate	~7	90-95
Ultracentrifugation → KSCN treatment → dialysis or Size-exclusion chromatography → KSCN treatment → dialysis	~3	>99

[a] Based on Sandwich-ELISA
[b] Based on BCA test and RP-HPLC
nd (not determined)

Figure 1


0%
0.25%
0.50%
0.75%
1%
1.50%
2%
A
Absorbance [450 nm]
3
2
1
0
Tween 20
Triton X-100
CHAPS
NP-40
Sod. deoxycholate

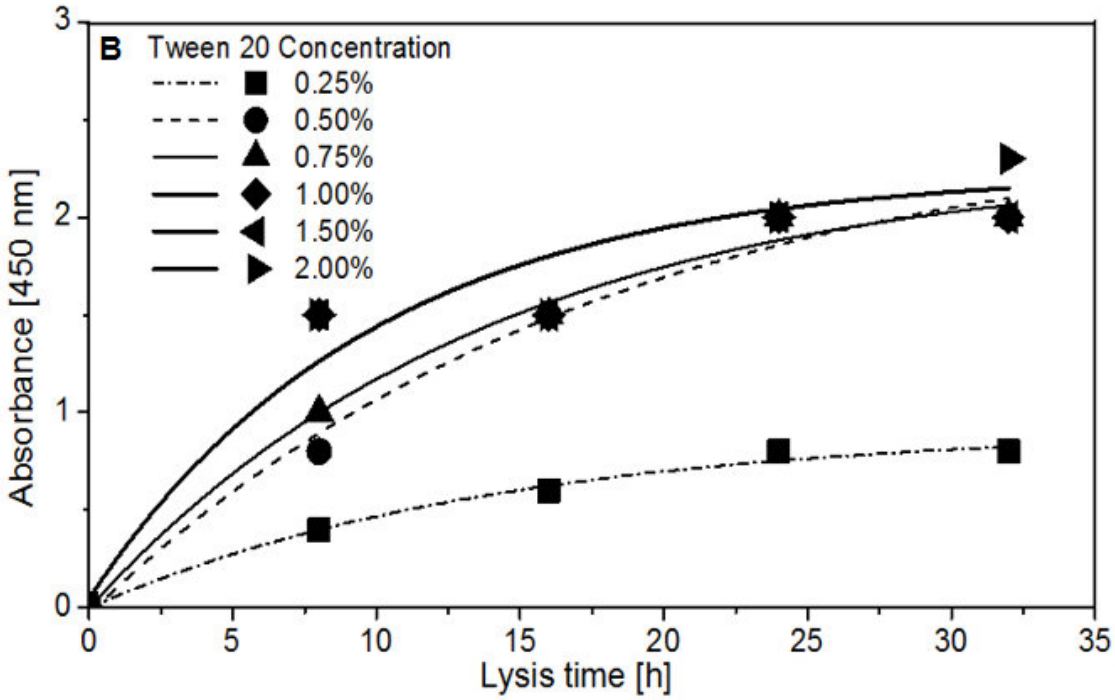
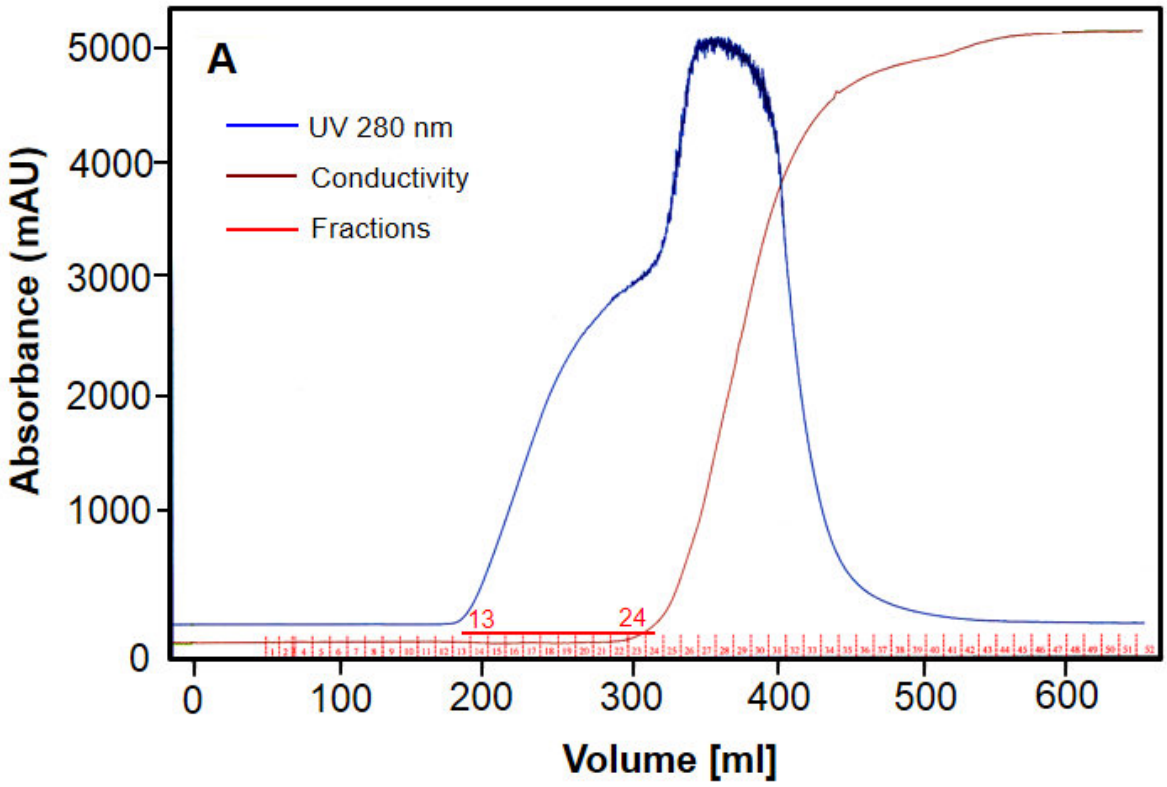

B
Tween 20 Concentration
0.25%
0.50%
0.75%
1.00%
1.50%
2.00%
Absorbance [450 nm]
3
2
1
0
0
5
10
15
20
25
30
35
Lysis time [h]

Figure 2


A
UV 280 nm
Conductivity
Fractions
Absorbance (mAU)
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4000
3000
2000
1000
0
13
24
0
100
200
300
400
500
600
Volume [ml]

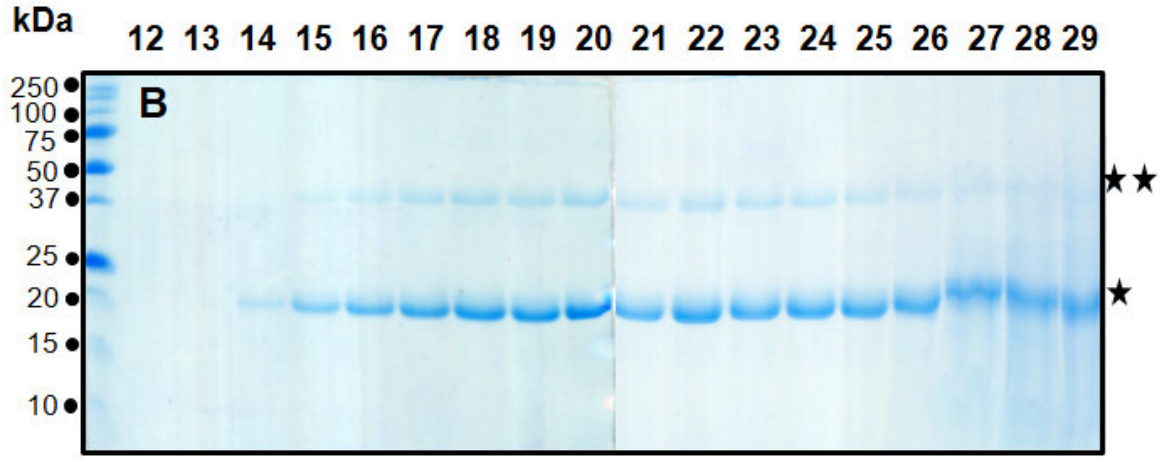
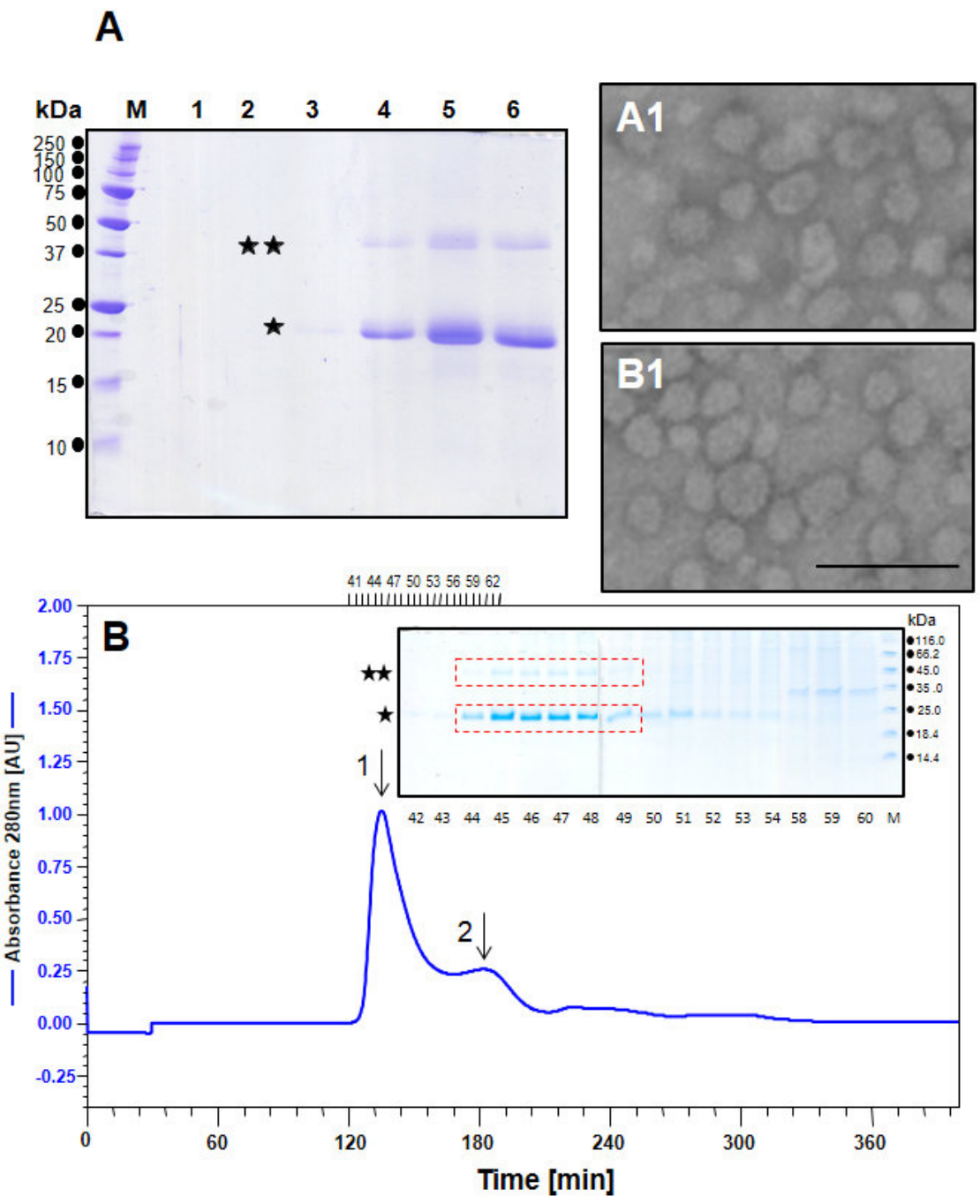

kDa
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
B
250
100
75
50
37
25
20
15
10
★★
★

Figure 3


A
kDa
M
1
2
3
4
5
6
250
150
100
75
50
37
25
20
15
10
A1
B1
B
41 44 47 50 53 56 59 62
kDa
116.0
66.2
45.0
35 .0
25.0
18.4
14.4
42 43 44 45 46 47 48 49 50 51 52 53 54 58 59 60 M
1
2
Absorbance 280nm [AU]
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
0
60
120
180
240
300
360
Time [min]

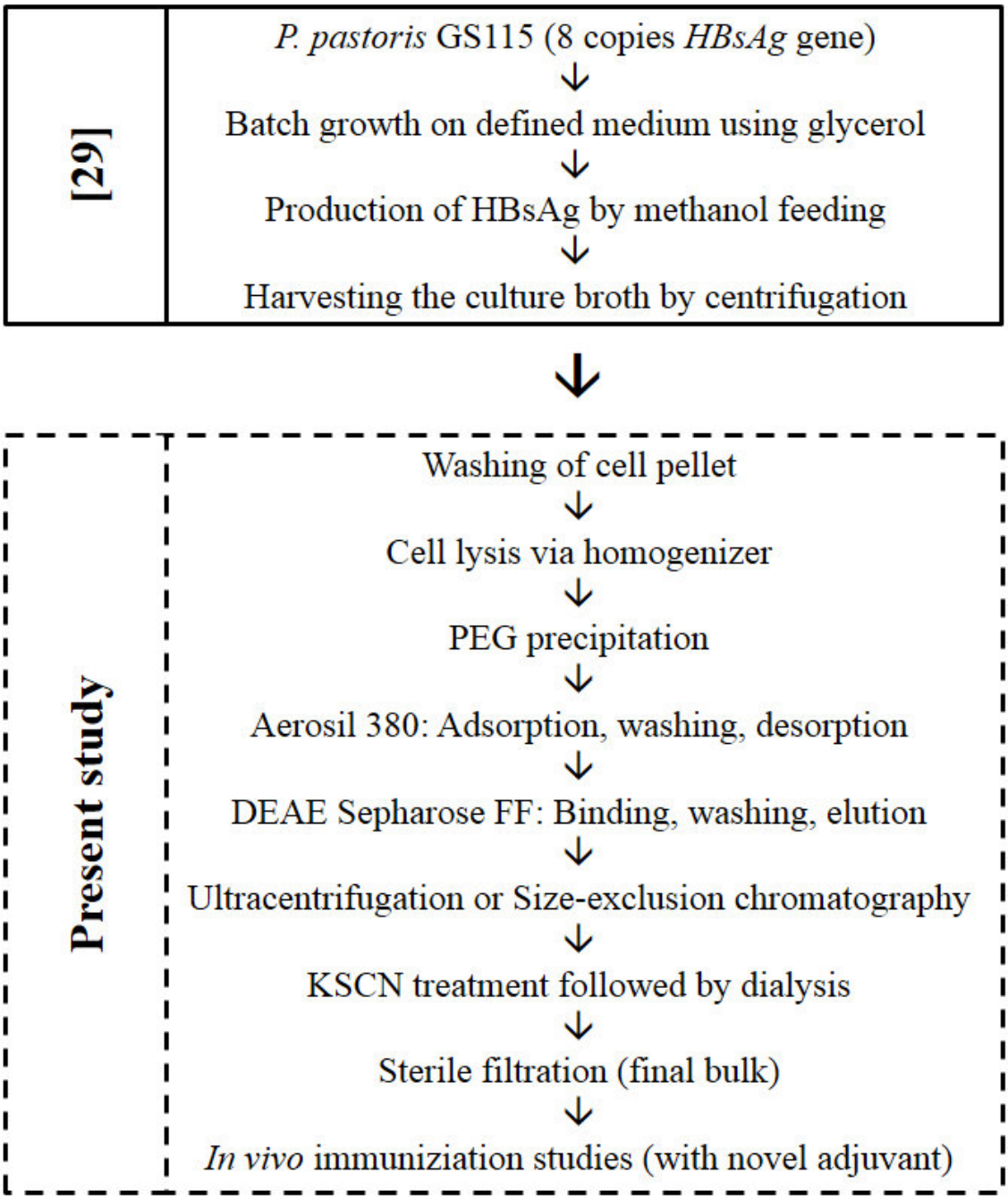
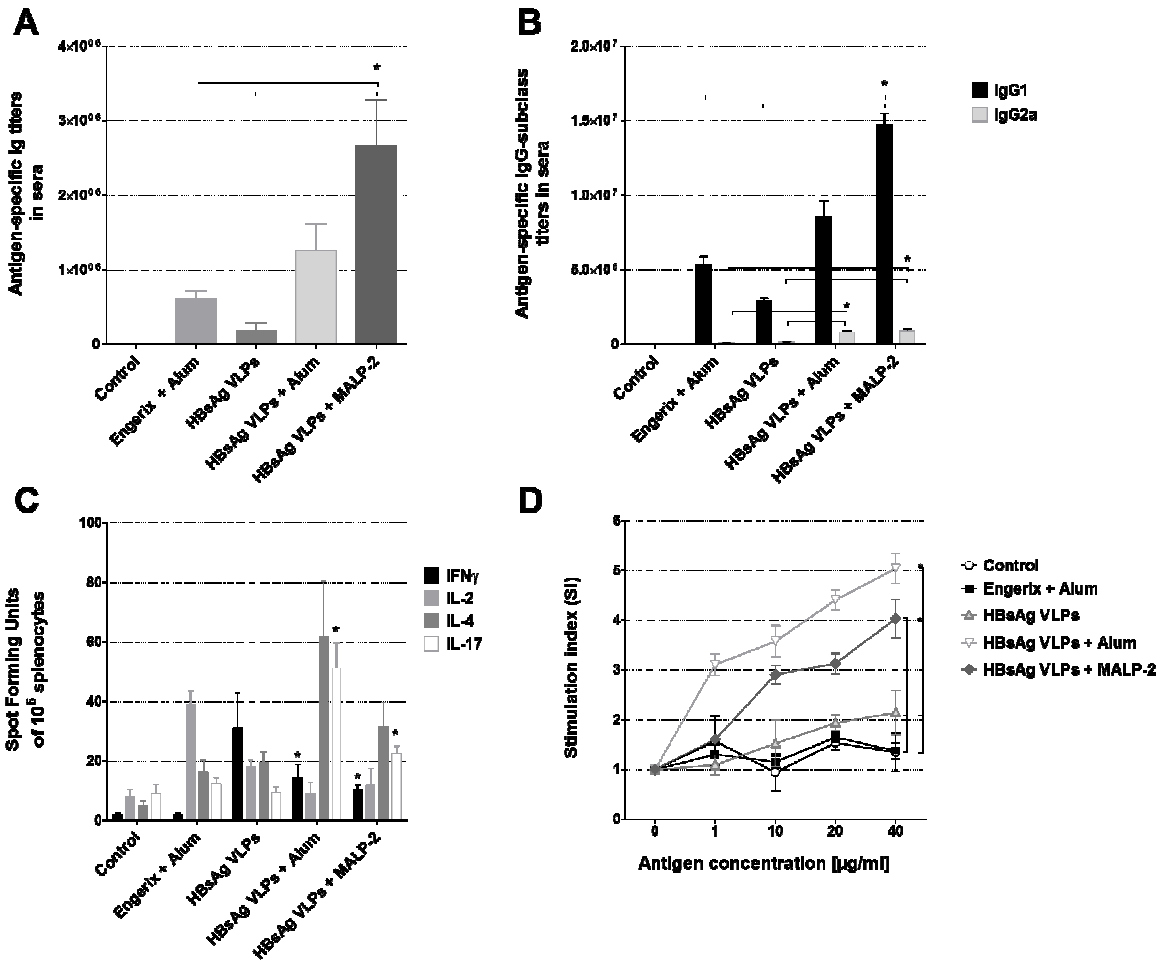

[29]
P. pastoris GS115 (8 copies HBsAg gene)
Batch growth on defined medium using glycerol
Production of HBsAg by methanol feeding
Harvesting the culture broth by centrifugation
Present study
Washing of cell pellet
Cell lysis via homogenizer
PEG precipitation
Aerosil 380: Adsorption, washing, desorption
DEAE Sepharose FF: Binding, washing, elution
Ultracentrifugation or Size-exclusion chromatography
KSCN treatment followed by dialysis
Sterile filtration (final bulk)
In vivo immuniziation studies (with novel adjuvant)

Figure 4

Figure 5


A
Antigen-specific Ig titers in sera
Control
Engerix + Alum
HBsAg VLPs
HBsAg VLPs + Alum
HBsAg VLPs + MALP-2
B
Antigen-specific IgG-subclass titers in sera
IgG1
IgG2a
C
Spot Forming Units of 10^5 splenocytes
IFNγ
IL-2
IL-4
IL-17
D
Stimulation Index (SI)
Antigen concentration [µg/ml]
Control
Engerix + Alum
HBsAg VLPs
HBsAg VLPs + Alum
HBsAg VLPs + MALP-2