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Novel membrane extraction procedure for the purification of hepatitis B surface antigen from *Pichia pastoris*

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ABSTRACT

The recombinant hepatitis B surface antigen (HBsAg) vaccine provides excellent protection against hepatitis B virus (HBV). However, high costs of its production prevents many underdeveloped and developing nations from implementing HBsAg vaccination. This in turn increases the risk of contracting HBV related diseases. Majority of the commercial HBV vaccines are derived from purified HBsAg expressed in recombinant yeasts. Most of the cost in production of the vaccine is incurred during the downstream processing. The costs associated with HBsAg purification can be decreased by optimizing the pre-chromatography steps and by reducing the impurity burden on chromatography operations. Here in this work we present a novel strategy for the enriched extraction of recombinant HBsAg from *Pichia pastoris* membranes. We have also developed a simple, easy to operate process for the purification of HBsAg VLPs from the membranes of *P. pastoris*. This novel strategy, while utilizing a single column chromatographic step in the purification scheme results in the highest recovery of HBsAg VLPs reported in the literature. The yield of HBsAg at the end of purification was nearly 5% (85 μg/g of induced wet cell biomass). The HBsAg purified from this process has shown the presence of VLPs. The immunization of these VLPs in BALB/c mice with alhydrogel adjuvant has shown good titers of neutralizing antibodies.

1. Introduction

Hepatitis B virus (HBV) is an enveloped DNA virus of the family hepadnaviridae. The virus transmission occurs through mucocutaneous routes, through infected blood samples, and vertically from mother to child during pregnancy. The virus infection causes inflammation of the liver. Majority of these infected subjects recover quickly, but some progress to fulminant liver failure and death. But, a fraction of other cases progress to become chronic carriers who in the long term might develop conditions like liver cirrhosis and hepatocellular carcinoma (HCC) leading to liver failure. HBV is by far the most important etiological factor for HCC [1] and accounts for nearly 80% of global burden of HCC. Worldwide about 2 billion people are infected with HBV, of which 350 million are chronic carriers, causing around 600,000 deaths every year [2].

Recombinant subunit vaccines for HBV are available from various commercial manufacturers. Majority of the HBV vaccines available in the market are based on HBsAg portion of viral envelope protein. HBsAg is incorporated into the vaccines in the form of virus like particles (VLPs) of 20–25 nm size. Majority of these HBV vaccines are based on purified HBsAg from yeast expression systems like *Saccharomyces cerevisiae* [3], *Pichia pastoris* [4], *Hansenula polymorpha* [5], etc. HBsAg vaccination has also been incorporated as a pediatric vaccine in the Universal Immunization Programme.

Recently, there is a re-emergence in the interest in HBsAg VLPs as these VLPs are being studied as carrier for the presentation of foreign epitopes in the development of novel vaccines for several infectious diseases like *Plasmodium falciparum* [6], dengue [7,8], HIV [9,10], hepatitis C virus [11] and polio virus [12,13]. The advantages of HBsAg VLP vaccines over traditional ones are many, including better safety profile, higher efficacy and lower dosage requirements. HBsAg VLPs are nanomeric in size and structure, and the production of such VLP based vaccines is complex and encounters various constraints [3]. In spite of the obvious merits of these vaccines, their potential is yet to be fully realized [14]. Some of the reasons for this are the difficulties involved in the downstream processing for the purification and isolation of such VLPs. Most of the cost in production of these VLPs is incurred during downstream processing which utilize various chromatography resins which are expensive and, at times, have low throughputs [15]. Although it may not be possible to avoid the use of chromatography resins, a decrease in the number of purification steps would go a long way in decreasing the production costs. One way to achieve this is by optimizing the pre-chromatography steps and by reducing the impurity burden on chromatography operations.

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HBsAg is a hydrophobic protein of molecular weight 24 kDa. The p24s monomers of HBsAg are co-translationally secreted into the endoplasmic reticulum [16,17]. As the protein is localized within the ER, cell lysis followed by a detergent step is necessary for the release these VLPs from the membranes [18]. Detergents facilitate HBsAg recovery by weakening the interactions between HBsAg and the membranes. A study of the extraction of HBsAg from recombinant plant systems has shown that the detergent may also promote VLP vesicle formation by inward budding of the ER membrane [19,20]. An alternative methodology for purification has been investigated in this paper by exploiting the membrane association property of HBsAg. Since lysis of *Pichia* cells in the absence of detergents localizes HBsAg to the membranes, a centrifugation step was introduced after the lysis stage [3]. This would ensure that majority of the host cell proteins and other impurities are removed in the supernatant with hardly any loss of HBsAg. The membranes containing enriched HBsAg were processed for extraction of HBsAg. Based on the properties of HBsAg, a novel purification process was designed from membrane fractions. The purified HBsAg was evaluated for its ability to assemble into VLPs and for their immunogenicity and protective efficacy.

The use of yeast expression systems for the production of recombinant HBsAg is well documented. An examination of the available literature [4,5,17,21–25] shows wide variation in the different conventional processes developed for HBsAg purification (Table 1). Most of these studies have used detergents for the dissociation of HBsAg from the membranes. Majority of these processes use several different purification steps and several different resins for purification which makes the process cumbersome to operate and also time consuming. In this work, we present a simple, easy to operate, single column purification process for HBsAg with high yields. The recovery of HBsAg at the end of purification was ~5%. Later the process which was designed for the purification of HBsAg, was successfully employed in the purification of some of the HBsAg fusion constructs expressed in *P. pastoris* (Section 3.3).

2. Material and methods

2.1. Source of HBsAg P. pastoris clone

The HBsAg clone used in this study was an in house clone of GS115/Mut^S transformant having 8 copies of HBsAg expression cassettes under the control of methanol inducible AOX1 promoter and has been previously described [26].

2.2. Reagents, instruments and apparatus

Phenyl-600M Toyopearl chromatography resin was purchased from Tosoh Bioscience (Stuttgart, Germany). Sephacryl-300 HR resin was purchased from GE Healthcare (Uppsala, Sweden). Goat anti-mouse IgG (H&L chain-specific)-horseradish peroxidase (HRP) conjugate was purchased from Calbiochem (La Jolla, CA, USA). Substrate 3,3′,5,5′-tetramethylbenzidine (TMB) insoluble for membrane and TMB soluble for ELISA were obtained from Sigma–Aldrich (St. Louis, MO, USA). 5S-mAb (which detects HBsAg) was an in-house preparation. Costar 96-well EIA/RIA polystyrene plates were purchased from Corning Incorporated, Corning, NY, USA. BCA protein assay kit was purchased from Thermo Scientific (Rockford, USA). Non-fat dry milk and Bradford reagent were procured from Bio-Rad Laboratories (Hercules, CA). PVDF membranes for Western blot analysis were purchased from Advanced Microdevices Pt. Ltd. (Ambala Cantt, India). Hepanostika HBsAg Ultra Microelisa system kit was purchased from Biomerieux (Marcy L'Etoile, France). Monolisa Anti HBs plus kit was purchased from Bio-Rad, (Marnes-la-Coquette, France). Acid-washed glass beads (~450 μm) were from Sigma–Aldrich Inc. (St. Louis, USA). Alhydrogel adjuvant for vaccine formulation was purchased from BrennTag Biosector (Frederikssund, Denmark). Yeast extract, bacto-peptone and yeast nitrogen base (without amino acids and ammonium sulphate) were purchased from Becton, Dickinson and Company, (Sparks MD, USA).

The AKTA-FPLC system, AKTA purifier system was from GE Healthcare (Uppsala, Sweden). Chromatography columns were procured from Bio-Rad (Marnes-la-Coquette, France). *Pichia* culture shakers were from Kunher Shaker (Birsfelden, Switzerland). Thermomixer was from Eppendorf AG (Hamburg, Germany). Dynomill was from WAB (Muttenz, Switzerland). Tangential flow filtration (TFF) apparatus was from Sartorius Stedim Biotech (Goettingen, Germany). Peristaltic pump was from Watson Marlow (Wilmington, USA). The Pellicon-2 polyethersulphone membrane of 300 kDa for TFF was procured from Millipore (Billerica, USA). XK 26/70 chromatography column was purchased from GE Healthcare (Uppsala, Sweden). Sorvall RC6+ centrifuge was from Thermo Scientific (Germany).

2.3. Shake flask cultures

YPD starter culture was set up with HBsAg *Pichia* clone and grown for 18 h at 30 °C with shaking at 250 rpm. The starter culture was diluted into 4 L of BMGY growth medium, into eight 2 L baffled flasks at an initial OD_{600} (Optical density at 600 nm) ~0.06. The cultures were incubated in the shaker at 30 °C at 250 rpm until the OD_{600} of the culture reached ~25–30. At this point, the cells were pelleted down by centrifugation at room temperature, washed with sterile PBS (20 mM Phosphate buffer pH 6.0 + 150 mM NaCl) and resuspended in 2 L of BMMY induction medium into eight 2 L baffled flasks. Induction was carried out at 30 °C with shaking at 250 rpm and by addition of 0.5% (v/v) methanol twice daily. The induction was carried up to 96–100 h [27]. At the end of induction, the cells were pelleted by centrifugation at 4 °C, washed twice with sterile PBS and stored at −70 °C.

2.4. Compartmentalization of HBsAg

Induced *P. pastoris* (100 OD cells) were lysed with 0.5 ml of 450 μm size glass beads, in a thermomixer with 0.5 ml of lysis buffer containing 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 8% glycerol. The total lysate (around 0.5 ml) along with the membranes was collected. The glass beads were washed with 0.5 ml of lysis buffer, to remove residual lysate, and were pooled with the lysate collected earlier. The lysate was then spun down, and the supernatant was separated from the membranes. The membranes were then resuspended into 1 ml of lysis buffer. The lysate, the supernatant after lysis and the resuspended membrane fractions were analyzed by Western blot with 5S-mAb for the amount of HBsAg present in supernatant and membrane fractions.

2.5. Optimization of membrane extraction

Induced *P. pastoris* (100 OD cells) were lysed as mentioned in Section 2.4. The total lysate (around 0.5 ml) along with the membranes were collected. The glass beads were washed with 0.5 ml of lysis buffer, to remove residual lysate, and were pooled with the lysate collected earlier. The lysate was then spun down, and the supernatant was separated from the membranes. The membranes were then extracted at 25 °C for 2 h with different extraction buffers containing; 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and various extraction agents like 1% Tween-20, 2% Tween-20, 1% Triton X-100, 1 M urea, 4 M urea, 8 M urea, 0.5% NP-40, 0.5% sarcosine, 0.1% SDS and 0.5% CHAPS. The membrane extracts were estimated for HBsAg recovery by using Hepanostika HBsAg microelisa kit. Based on the results of Hepanostika ELISA, the

Table 1
Estimated recoveries of recombinant HBsAg from yeast expression systems [4,5,17,21–25]. The values mentioned in this table are either the values given in the article or have been calculated based on the data provided in the article.

Author	Expression	Purification steps and resins	No. of columns/resins	Estimated recoveries	Method of HBsAg estimation
Present study	*P. pastoris*, GS115, Mut ^{s}	PEG-6000 precipitation, tangential flow filtration, phenyl-600 M toyopearl	1	20%	Hepanostika ELISA[a]
Liu et al. [21]	*P. pastoris*, GS115, Mut ^{s}	Ammonium sulphate precipitation, phenyl-5PW, CsCl density gradient	1	37%	Sandwich ELISA
Hardy et al. [4]	*P. pastoris*	Acid precipitation, diatomaceous earth matrix, immunoaffinity, anion exchange and size exclusion chromatographies	4	6–13.6%	Sandwich ELISA
Huang et al. [5]	*H. polymorpha*	DEAE sepharose FF, butyl-S-QZT, ultrafilration-500 kDa, sepharose 4 FF	4	21%	Sandwich ELISA
Bardiya et al. [22]	*P. pastoris*, GS115, Mut ^{s}	Aerosil-380, DEAE toyopearl 650 M, superdex 75	3	nd	RPHA
Bo et al. [23]	*P. pastoris*, KM71H, Mut ^{s}	Ammonium sulphate precipitation, Ni-NTA IMAC	1	nd	Sandwich ELISA
Kee et al. [24]	*S. cerevisiae*	XAD-4 beads, Ammonium sulphate, butyl sepharose	2	nd	Sandwich ELISA
Wampler et al. [25]	*S. cerevisiae*	Tangential flow filtration, XAD-2 beads, aerosil 380, butyl agarose or immunoaffinity chromatography	3	nd	nd
Lunsdorf et al. [17]	*P. pastoris*, GS115, Mut ^{s}	PEG-6000 precipitation, Aerosil 380, DEAE-sepharose FF, CsCl density gradient	2	nd	nd

nd is not determined. Recovery of HBsAg per gram of induced wet cell biomass (wcb) was approximately 85 μg/g of wcb in the present study, and it was approximately 27 μg/g of wcb in the study by Liu et al. [21].

[a] Recovery of ELISA activity as per Hepanostika ELISA, RPHA (Reverse passive hemagglutination assay).

membrane extraction experiment was repeated again to further evaluate the optimal condition for extraction of HBsAg. The membranes were extracted with extraction buffers containing; 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and different concentrations of Tween-20 and urea like; 0% Tween-20 + 0 M urea, 2% Tween-20 + 0 M urea, 2% Tween-20 + 1 M urea, 2% Tween-20 + 4 M urea, 2% Tween-20 + 8 M urea and 0% Tween-20 + 8 M urea. The membrane extracts were then analyzed by SDS-PAGE gel silver stain and by Western blot with 5S-mAb to determine the optimal condition for recovery of HBsAg from membranes. The total protein recovered by each of these extraction conditions was calculated by bicinchoninic acid (BCA) method of protein estimation. The percentage of HBsAg present in each of the extractions was estimated by Image J densitometry scan of the silver stained gel. Based on these values the specific activity of HBsAg in different extractions was estimated as μg of HBsAg per mg of total protein.

2.6. Purification of HBsAg

Induced *P. pastoris* biomass (100 g) (Section 2.3) was lysed with glass beads in dyno-mill in 400 ml of lysis buffer, containing 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 8% glycerol at 10 °C. The lysate was spun down at 4 °C, and the membranes were washed twice with the lysis buffer. HBsAg was extracted from the membranes with 200 ml of membrane extraction buffer, containing 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 2% Tween-20 at 30 °C for 2–2.5 h. The membrane extract was separated from the cell debris by centrifugation. The supernatant after the centrifugation was turbid and hence it was subjected to 5% PEG-6000 precipitation by adding 20-25 ml of 50% PEG-6000 over 30 min, and left overnight at 4 °C for complete precipitation. Next day, the precipitate was removed by centrifugation and the supernatant of PEG precipitation was collected. The PEG supernatant was then subjected to tangential flow filtration through 300 kDa polyethersulphone membrane. The TFF was carried out at 4 °C. The buffer used for TFF contained 20 mM phosphate buffer pH 7.2, 5 mM EDTA and 150 mM NaCl. 10 L of this buffer was used to filter 220 ml of PEG supernatant. Small molecular impurities and smaller oligomeric forms of HBsAg were removed in the filtrate. As the TFF buffer did not contain any Tween-20, the percentage of Tween-20, which remained in the retentate, also got reduced. The unfiltered material/retentate, which contained higher order structures, and VLPs of HBsAg were collected. The NaCl concentration of the retentate was increased to 1 M at pH 7.2 by adding 5 M NaCl. The binding of the retentate to 35 ml of pre-equilibrated phenyl-600M Toyopearl resin (equilibration buffer contained 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 1 M NaCl) was carried out as a batch process for 2.5 h at 30 °C. The bound resin was then packed into a 50 ml Bio-Rad column and further chromatographic steps were performed with an AKTA-FPLC apparatus. The resin was then washed and eluted on AKTA system by programmed buffer conditions as outlined here; 0 ml to 100 ml – 20 mM phosphate buffer pH 7.2 + 5 mM EDTA + 1 M NaCl; 100 ml to 200 ml – 20 mM phosphate buffer pH 7.2 + 5 mM EDTA + 1 M NaCl to 20 mM phosphate buffer pH 7.2 over 100 ml base run; 200 to 300 ml – 20 mM Phosphate buffer pH 7.2; 300 to 380 ml – 20 mM Bicarbonate buffer pH 9.6; 380 to 460 ml – 20 mM bicarbonate buffer pH 9.6 + 1 M urea; 460 to 540 ml – 20 mM bicarbonate buffer pH 9.6 + 4 M urea.

The elution fractions of 4 M urea elution peak, were pooled and dialyzed against 20 mM phosphate buffer pH 7.2 and 150 mM NaCl, to remove urea, and the protein was stored in this buffer after sterile filtration. This protein was later used for all other biochemical and immunological studies.

The purified Phenyl eluate of HBsAg was further characterized by gel filtration. The gel filtration was carried out to evaluate the proportion of VLPs in the phenyl eluate of HBsAg. The HBsAg VLPs have an average molecular weight of 2 MDa [28], and since the Sephacryl-300 HR resin has an exclusion limit of 1 MDa, it was chosen for gel filtration. XK 26/70 column was packed manually with 320 ml of Sephacryl-300 HR resin. This would give a void volume of ∼130 ml (determined by calibration markers). The column was equilibrated with 5 column volumes of 20 mM Phosphate buffer pH 7.2 + 150 mM NaCl. The purified HBsAg protein from the phenyl elution was dialyzed against 20 mM Bicarbonate buffer pH 9.6 + 1.2 M urea. 10 ml of this protein, at a concentration of 0.6 mg/ml, was loaded onto gel filtration column. The gel filtration

was performed with the equilibration buffer (20 mM Phosphate buffer pH 7.2 + 150 mM NaCl) at a flow rate of 0.5 ml/min (11 cm/h). The peak fractions were collected and analyzed by SDS-PAGE gel silver stain and Western blot with 5S-mAb.

2.7. SDS-PAGE gel, sample buffer and silver staining of SDS-PAGE gels

SDS-PAGE was carried out using standard protocols. Gels were prepared and run in the presence of 0.1% SDS (denaturing) [29,30]. The 2× sample buffer contained 0.5 M DTT, 50% β-mercaptoethanol, 10% SDS and 3% glycerol [27].

Protein bands on SDS-PAGE gels were visualized by silver staining. The gel was first soaked in water for 10 min to remove traces of SDS and fixed with 10% methanol and 10% acetic acid for 2 h. The fixative was removed and the gel was thoroughly washed with water overnight with 3–4 changes to remove the traces of fixative embedded in the gel. It was then treated with 5 μl of 1 M DTT in 100 ml water for 30 min and incubated with silver nitrate (1 mg/ml) in water for exactly 30 min at room temperature. The gel was then washed quickly 2–3 times with the developer solution (3 g sodium carbonate + 50 μl 37–41% formaldehyde in 100 ml water) and developed with the same. The reaction was stopped by adding stopping solution (3 ml of 2.3 M citric acid in 100 ml water).

2.8. BCA protein estimation

Protein concentrations were estimated by bicinchoninic acid (BCA) method [31] using BSA as reference protein. The procedure was carried out as per the manufacturer's instructions.

2.9. Electron microscopy

The membrane purified phenyl eluate of HBsAg was diluted in 20 mM bicarbonate buffer pH 9.6 to a concentration of 3 μg/ml. The protein sample was then adsorbed onto 400 mesh carbon coated copper grids. The grids were contrasted with 2% aqueous uranyl acetate, dried under an infrared lamp and visualized with FEI, Tecnai, 120 kV transmission electron microscope.

2.10. Immunization of mice

Animal experiments were reviewed and approved by the International Centre for Genetic Engineering and Biotechnology's Institutional Animal Ethics Committee and the Guidelines of the Government of India were followed. The HBsAg protein was adsorbed onto alhydrogel adjuvant as per the manufacturer's instructions. 25 μg of alum was used for 1 μg of protein formulation. A group of 5 female Balb/c mice aged 4–6 weeks were immunized intraperitoneally on 0, 28 and 56 days. Another group of 5 female Balb/c mice were mock immunized with equal amount of adjuvant in PBS. For collection of sera for analysis, mice were bled 3 days prior to primary immunization and 1 week after both the boosters.

2.11. Anti-HBsAg protective antibody response

The mice sera samples were evaluated for the levels of protective antibodies generated in response to HBsAg immunization. This was done by using Monolisa Anti-HBs Plus kit, which is a direct antibody sandwich ELISA system for quantitative and qualitative estimation of protective antibody titers against HBsAg. The determination of anti-HBsAg levels has been standardized by the use of WHO Anti-HBsAg reference preparation expressed in milli-International Units per milliliter (mIU/ml). A level greater than or equal to 10 mIU/mL is generally considered as the standard for demonstrating post-vaccination protection against HBV.

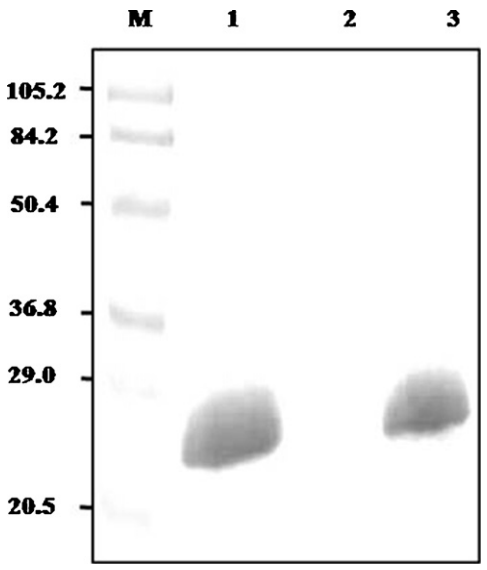

Fig. 1. Western blot showing the compartmentalization of HBsAg. Induced *P. pastoris* (100 OD) were lysed with glass beads on thermomixer in lysis buffer containing 20 mM phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 8% glycerol. The total cell lysate (around 0.5 ml) along with the membranes was collected. The beads were washed with 0.5 ml of lysis buffer and mixed with the lysate collected earlier. The lysate was spun down and the supernatant and the membrane portions were separated. The total cell lyaste (lane 1), the lysate supernatant (lane 2) and the membrane portions (lane 3) were analyzed by Western blot with 5S-mAb for the amount of HBsAg present. Lane M shows pre-stained low molecular weight markers. The HBsAg is seen at 25 kDa position.

3. Results and discussion

3.1. HBsAg is associated with membrane

HBsAg is a lipid envelope VLP and is a hydrophobic protein. HBsAg when expressed to high levels in *Pichia*, is seen localized with the endoplasmic reticulum [16–18,24]. This necessitates the need for the use of detergents for the release of HBsAg VLPs from the membranes [3]. The investigation of the membrane association property of HBsAg showed (Fig. 1) that of the total amount of HBsAg present in the *Pichia* cells (Fig. 1, lane 1), most of it remained associated with the membranes (Fig. 1, lane 3). The lysate supernatant did not contain any detectable amount of HBsAg (Fig. 1, lane 2). This indicated that when *Pichia* cells are lysed with buffer without any detergents, most of the recombinant HBsAg remains associated with the membranes. This was of advantage in the purification. The *Pichia* cells were lysed with buffer without detergent followed by a centrifugation step to remove proteins present in the supernatant. The membrane fraction, which then contained HBsAg was extracted with suitable membrane extraction buffer. This ensured that majority of the host cell impurities were removed in the supernatant, while the enriched HBsAg protein could be extracted from the membranes [24]. This reduced the burden of host cell impurities in the downstream processing.

3.2. Optimal recovery with 2% Tween-20

Since majority of HBsAg, on lysis of *Pichia* cells, remained associated with the membranes, experiments were carried out to identify the optimal condition for the recovery of HBsAg from the lysed cell membranes. *Pichia* cells were lysed and the membranes were extracted with different detergents and urea as mentioned in

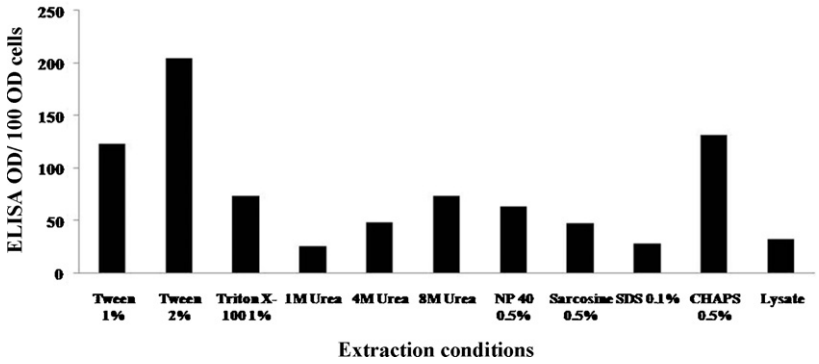

Fig. 2. Membrane extraction of HBsAg by different extraction conditions. Graph showing the amount of HBsAg extracted by different extraction conditions. Induced *P. pastoris* (100 OD) were lysed with glass beads on thermomixer in lysis buffer containing 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 8% glycerol. The total cell lysate (around 0.5 ml) along with the membranes was collected. The beads were washed with 0.5 ml of the lysis buffer and mixed with the lysate collected earlier. The lysate was spun down and the supernatant and the membrane portions were separated. The membranes were then extracted with different extraction conditions like 1% Tween-20, 2% Tween-20, 1% Triton X-100, 1 M urea, 4 M urea, 8 M urea, 0.5% NP-40, 0.5% Sarcosine, 0.1% SDS, 0.5% CHAPS. The amount of HBsAg extracted was calculated by Hepanostika HBsAg Microelisa system. The graph shows ELISA readings per 100 OD of cells.

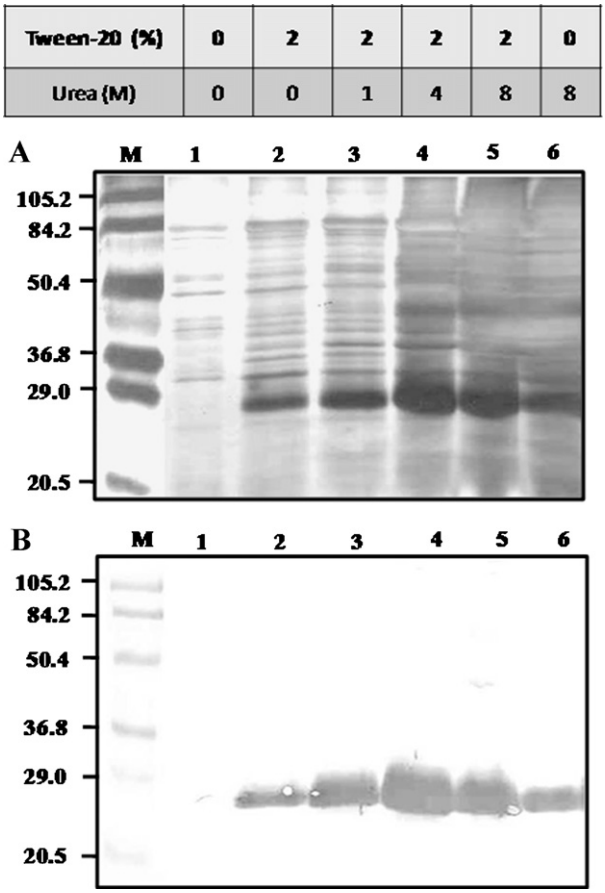

Fig. 3. Optimization of membrane extraction conditions for HBsAg. (A) Silver stained SDS-PAGE gel. (B) Western blot of the gel on panel A with 5S-mAb showing the amount of HBsAg extracted. Induced *P. pastoris* (100 OD) were lysed with glass beads on thermomixer in lysis buffer containing 20 mM Phosphate buffer pH 7.2, 5 mM EDTA, 150 mM NaCl and 8% glycerol. The total cell lysate (around 0.5 ml) along with the membranes was collected. The beads were washed with 0.5 ml of the lysis buffer and mixed with the lysate collected earlier. The lysate was spun down and the supernatant and the membrane portions were separated. The membranes were then extracted with different combinations of Tween-20 and urea. All the membrane extraction buffers also contained 20 mM Phosphate buffer pH 7.2, 5 mM EDTA and 150 mM NaCl. The table on the top of panel A shows the percentage of Tween-20 and Molarity of urea present in the extraction conditions corresponding to respective lanes. Lane M shows pre-stained low molecular weight markers. Lane 1–6 show extractions with different concentrations of Tween-20 and urea. lane 1: 0% Tween-20 + 0 M urea; lane 2: 2% Tween-20 + 0 M urea; lane 3: 2% Tween-20 + 1 M urea; lane 4: 2% Tween-20 + 4 M urea; lane 5: 2% Tween-20 + 8 M urea; lane 6: 0% Tween-20 + 8 M urea.

Section 2.5. The HBsAg activity extracted was measured by Hepanostika microelisa system. Of the different detergents used in extraction, 2% Tween-20 gave the highest HBsAg recovery (Fig. 2). Of the different concentrations of urea, 8 M urea gave the highest recovery (Fig. 2). Further extraction experiments were carried out using a combination of different concentrations of Tween-20 and urea (Section 2.5). Analysis of these membrane extracts by SDS-PAGE gel silver stain and by Western blot with 5S-mAb (Fig. 3A and B) showed that the extraction of membranes with PBS (Fig. 3A and B, lane 1) did not recover any HBsAg. However, with the incorporation of 2% Tween-20 (Fig. 3A and B, lane 2) in the membrane extraction buffer, it was possible to recover significant amount of HBsAg. The addition of 1 M urea (Fig. 3A and B, lane 3) and 4 M urea (Fig. 3A and B, lane 4) into 2% Tween-20 membrane extraction buffer increased the yield of HBsAg in a concentration dependent manner. The addition of 8 M urea (Fig. 3A and B, lane 5) into 2% Tween-20 membrane extraction buffer, although did increase the recovery of HBsAg as compared to that of 2% Tween-20 (Fig. 3A and B, lane 2), however, the HBsAg yield was less as compared with that of 2% Tween-20 + 4 M urea (Fig. 3A and B, lane 4). Extraction with 8 M urea (Fig. 3A and B, lane 6) without any Tween-20 did recover significant amounts of HBsAg, however the HBsAg recovery was less as compared with that of 1 M, 4 M and 8 M urea with 2% Tween-20 (Fig. 3A and B, lanes 3–5). It was also observed that addition of 2% Tween-20 (Fig. 3A, lane 2) besides recovering HBsAg, also extracted more impurities as compared to PBS (Fig. 3A, lane 1). With increasing urea concentration in presence of 2% Tween-20, there was also increased extraction of impurities (Fig. 3A, lanes 3–5). Extraction with 8 M urea alone (Fig. 3A, lane 6) also yielded almost equivalent amounts of impurities as compared to 2% Tween-20 + 4 M and 8 M urea (Fig. 3A, lanes 4 and 5) even though the amount of HBsAg recovered was less as compared to Fig. 3A and B (lanes 3–5).

Specific activity of HBsAg in each of the extractions was calculated to find out an optimal extraction condition. A summary of specific activity of HBsAg membrane extractions is shown in Table 2. The PBS extraction (without the addition of Tween-20) recovered least amounts of protein and also it did not recover any HBsAg (Table 2, lane 1). This was in congruence with the gel picture and Western blot (Fig. 3A and B, lane 1). While extraction with 8 M urea without Tween-20 (Fig. 3A and B, lane 6 and Table 2) yielded sufficient quantity of protein, the recovery of HBsAg was lower as compared with that of 2% Tween-20 + 1 M, 4 M and 8 M urea (Fig. 3A and B, lane 3–5 and Table 2) and hence the specific activity of HBsAg was also low. 2% Tween-20 along with extracting proteins from the membranes also recovered significant amounts of HBsAg, and hence the specific activity of HBsAg was highest (Fig. 3A and B, lane

Table 2
Table showing estimation of total protein and specific activity of HBsAg extracted in different extraction buffers. The silver stained gel picture in Fig. 3A was used to estimate the percentage of HBsAg protein present among total proteins in the respective lanes. The calculations were made by the use of Image J software densitometry scan of the gel. Please note that all the extraction conditions mentioned in the above table apart from their respective components also contained 20 mM phosphate buffer pH 7.2, 5 mM EDTA and 150 mM NaCl.

Lane no.	Extraction	Total protein[a] (mg)	% of HBsAg[b]	Total HBsAg[c] (μg)	Specific activity[d]
Without Tween-20					
1.	PBS	0.2	0	0	0
6.	8 M urea	2.2	9	199	90
With 2% Tween-20					
2.	0 M urea	0.8	19	155	190
3.	1 M urea	1	18	190	180
4.	4 M urea	2.3	13	292	130
5.	8 M urea	3.1	9	279	90

[a] Total protein was estimated by BCA protein estimation kit using BSA as standard.
[b] % of HBsAg in total protein was estimated by Image J software analysis silver stained gel.
[c] Total HBsAg was estimated by values of total protein and % of HBsAg.
[d] Specific activity of HBsAg (in μg of HBsAg per mg of total protein) was estimated by values of total protein in mg and total HBsAg in μg.

2 and Table 2). Addition of 1 M and 4 M urea with 2% Tween-20 (Fig. 3A and B, lanes 3 and 4; Table 2) increased the yield of total HBsAg, but extracted far more impurities than HBsAg and hence their specific activity was low as compared with that of 2% Tween-20 alone (Table 2, lane 2). 2% Tween-20 + 8 M urea (Fig. 3A and B, lane 5; Table 2) extracted the highest amount of total protein, while the amount of HBsAg recovered was less as compared to 2% Tween-20 + 4 M urea (Fig. 3A and B, lane 4; Table 2) and hence the specific activity of HBsAg was lower. It can be seen from Table 2 lanes 2–5 that as the amount of urea increased in 2% Tween-20 membrane extraction buffer, the amount of total protein extracted also increased, while the amount of total HBsAg recovered increased in 1 M and 4 M urea, but decreased with 8 M urea. The specific activity of HBsAg also decreased with increasing urea.

3.3. *Purification and characterization of HBsAg*

The purification of a protein would be easier if the specific activity of the starting material is high. The results indicated 2% Tween-20 as ideal condition for membrane extraction of HBsAg, since the amount of impurities extracted by this condition was less as compared to the recovery of HBsAg. Hence during purification of HBsAg the condition of 2% Tween-20 along with other buffer components was selected for the extraction of HBsAg from the lysed *Pichia* cell membranes. A novel downstream process was also designed (Section 2.6) which involved steps like membrane extraction, PEG precipitation, tangential flow filtration and Phenyl hydrophobic interaction chromatography (Fig. 4). The lysate supernatant contained majority of proteins (~4 g) (Table 3). As the lysis was carried out in the absence of detergents, majority of HBsAg remained associated with the membranes. The membrane extraction recovered significant amount of HBsAg (Fig. 5A and B, lane 1). The amount of total protein extracted was also low (~50%) as compared to lysate supernatant (Table 3). In the case of membrane extraction of HBsAg with 2% Tween-20, the yield of HBsAg was ~20% on small-scale lysis with thermomixer (Table 2), whereas it was ~9% on large-scale lysis with dynomill (Table 3). This difference in the yield of HBsAg is attributable to the vigorous lysis in dynomill, which in turn extracts more host cell proteins from membranes. But the amount of total HBsAg recovered per 100 OD of cells was similar in lysis with both thermomixer (155 μg/100 OD cells) and dyno-mill (186 μg/100 OD cells). The membrane extract was turbid and not suitable for TFF and hence it was subjected to PEG-6000 precipitation. The PEG precipitation removed nearly 50% of the total proteins with over 17% loss of HBsAg (Fig. 5A and B, lane 2; Table 3), and also removed the turbidity making it suitable for TFF. Majority of the purification was achieved by TFF, which removed small molecular weight impurities and also the lower oligomers of HBsAg (Fig. 5A and B, lane 3; Table 3). Thus the TFF procedure functioned as purification and a pre-chromatography step for isolation of VLPs.

Since HBsAg is a hydrophobic protein it binds very strongly on the Phenyl hydrophobic column, leaving behind some of the impurities in flow through (Fig. 5A and B, lane 4). The strong interaction of HBsAg and Phenyl column ensures that the column can be washed with stringent conditions to remove the remaining impurities. The Phenyl chromatography profile (Fig. 4) showed two minor peaks (Peak-1 and 2) and a major peak (Peak-3). There was a small hump in the chromatogram between peak-2 and 3. During chromatography, some minor impurities were removed in the wash with 20 mM Phosphate buffer pH 7.2 + 1 M NaCl (0–100 ml, Fig. 4) and 20 mM Phosphate buffer pH 7.2 + 1 M to 0 M NaCl (100–200 ml, Fig. 4). During wash with 20 mM phosphate buffer pH 7.2 (200–300 ml, Fig. 4), a small peak (Fig. 4, peak-1) was seen. The analysis of this peak by SDS-PAGE silver staining and by Western blot (Fig. 5A and B, lane 5) showed the loss of some amount of HBsAg along with few impurities. Another minor peak (Fig. 4, peak-2) was seen when the column was washed with 20 mM Bicarbonate buffer pH 9.6 (300–380 ml, Fig. 4). The analysis of this peak by SDS-PAGE silver staining and by Western blot (Fig. 5A and B, lane 6) showed the loss of some amount of HBsAg along with few

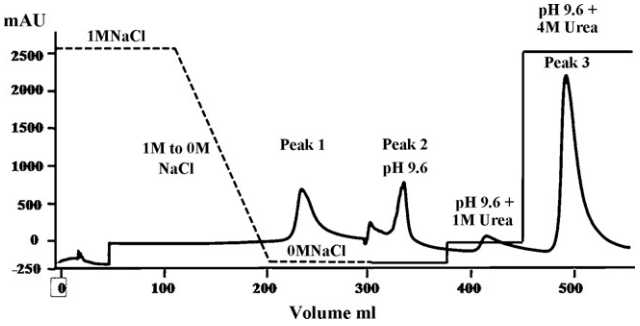

Fig. 4. Chromatogram showing the elution profile of HBsAg from phenyl-600 M Toyopearl resin. The NaCl concentration of the retentate was increased to 1 M by adding 5 M NaCl. It was then bound for 2.5 h at 30 °C, on to pre-equilibrated toyopearl phenyl-600 M resin. The resin was then washed and eluted. The dark colored curve is the elution profile of the protein from phenyl-600 M toyopearl column. The *Y*-axis shows absorbance of protein at 280 nm in mAU. The *X*-axis shows volume in ml. The dotted line shows wash with a salt gradient of 20 mM Phosphate buffer pH 7.2 + 1 M NaCl (0 to 100 ml); 20 mM Phosphate buffer pH 7.2 + 1–0 M NaCl (100–200 ml); 20 mM phosphate buffer pH 7.2 + 0 M NaCl (200–300 ml). The protein was eluted as shown in the figure with a step gradient (in dark colored straight line curve) of 20 mM Bicarbonate buffer pH 9.6 (300–380 ml), 20 mM bicarbonate buffer pH 9.6 + 1 M urea (380–460 ml) and 20 mM bicarbonate buffer pH 9.6 + 4 M urea (460–540 ml). Majority of HBsAg was eluted at 20 mM Bicarbonate buffer pH 9.6 with 4 M urea.

Table 3
Purification table summarizing different steps of purification of HBsAg. The silver stained gel picture in Fig. 5A was used to estimate the percentage of HBsAg protein present among total proteins in the respective lanes. The calculations were made by the use of Image J software densitometry scan of the gel. NA refers to not applicable.

Step	Total protein[a] (mg)	% of HBsAg[b]	Total HBsAg[c] (mg)	Estimated recovery[d] (%)	Purification fold[e]
Lysate supernatant	3880	NA	NA	NA	NA
Membrane extract	2170.2	8.6	186.6	100	1
PEG precipitated supernatant	1095.7	14.1	154.5	82.8	2
Retentate of TFF	85.1	36.3	30.9	16.6	13
Phenyl eluate	8.5	99.6	8.5	4.6	10

[a] Total protein was estimated by BCA protein estimation kit using BSA as standard.
[b] % of HBsAg in total protein was estimated by Image J software analysis silver stained gel.
[c] Total HBsAg was estimated by values of total protein (mg) and % of HBsAg.
[d] Recovery % was estimated by values of total HBsAg (μg).
[e] Purification fold was estimated by values of total protein (mg).

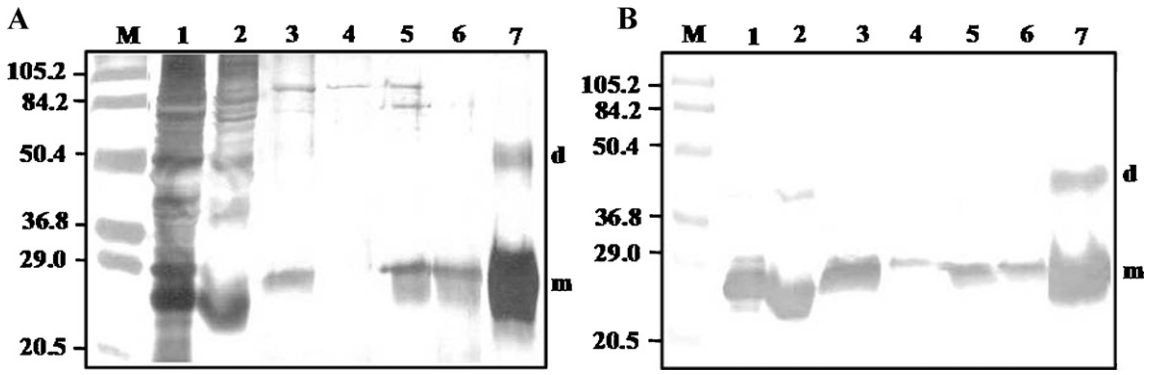

Fig. 5. Different steps of purification of HBsAg. Silver stained SDS-PAGE gel (A) with corresponding Western blot (B) developed with 5S-mAb. Lane M shows pre-stained low molecular weight markers. Lane 1: Membrane extract; lane 2: PEG precipitated supernatant. Lane 3: Retentate after TFF; lane 4: Phenyl-600 M toyopearl flow through; lane 5: 20 mM phosphate buffer pH 7.2 elution peak (Fig. 4, peak-1); lane 6: 20 mM bicarbonate buffer pH 9.6 elution peak (Fig. 4, peak-2) and lane 7: Purified HBsAg. HBsAg is seen as 25 kDa monomer (m) and 50 kDa dimer (d).

impurities. However these washes were required to ensure that the column was thoroughly washed before elution. To ensure complete removal of impurities from the column, one more wash was given with 20 mM Bicarbonate buffer pH 9.6 + 1 M urea (380–460 ml, Fig. 4). During this wash there was only a minor hump seen with very little protein loss. Following this wash, the purified HBsAg was eluted from the column with 20 mM Bicarbonate buffer pH 9.6 + 4 M urea (460–540 ml, Fig. 4). This elution step showed up as a major peak in the chromatogram (460–540 ml, Fig. 4, peak-3). The analysis of this peak by SDS-PAGE silver staining and by Western blot showed the presence of purified HBsAg, majority of which was seen as a 25 kDa monomer (Fig. 5A and B, lane 7, m) while a small amount was also seen as 50 kDa dimer (Fig. 5A and B, lane 7, d). The elution fractions were pooled and dialyzed against 20 mM phosphate buffer pH 7.2 + 150 mM NaCl. Even after elution of Phenyl column with 20 mM bicarbonate buffer pH 9.6 + 4 M urea, some amount of HBsAg was still bound to the resin (data not shown). A further elution with 8 M urea was not preferred, as it would result in loss of biological activity of VLPs. The total HBsAg recovered at this stage was nearly 8.5 mg with recovery % of 4.6 (Table 3).

To be an effective immunogen, the viral envelope protein should have the ability to assemble into VLPs. HBsAg is known to assemble into VLPs. To address this issue, the phenyl eluate of HBsAg was visualized under transmission electron microscope (TEM), which showed the presence of VLPs of 20–40 nm size (Fig. 6).

In order to investigate the ratio of VLP and non-VLP species in the purified HBsAg, a gel filtration was carried out on a Sephacryl-300 HR column (Section 2.6). The VLPs are expected to elute near the void volume. The chromatography profile showed a broad peak near the void volume (Fig. 7). The analysis of peak fractions by SDS-PAGE silver staining and by Western blot with 5S-mAb showed the presence of HBsAg in all the void volume peak fractions (Fig. 7, inset A and B). However on SDS-PAGE silver stained gel or on Western blot there was no difference among HBsAg eluted at different portions of the peak. This result was expected as the difference would probably lie in the quality or in the size of the HBsAg VLPs. Previously it has been shown that the purified HBsAg on gel filtration elutes as a broad void volume peak [32]. The TEM analysis of HBsAg from different portions of this void volume peak had shown the presence of VLPs of similar size and shape [32]. The fact that the membrane purified HBsAg assembles into VLPs and that the membrane purified HBsAg elutes as a broad volume peak on gel filtration shows that most of the HBsAg protein eluting from the Phenyl column was assembled into VLPs. Hence the Phenyl eluate of HBsAg probably did not need any further fractionation. We believe that

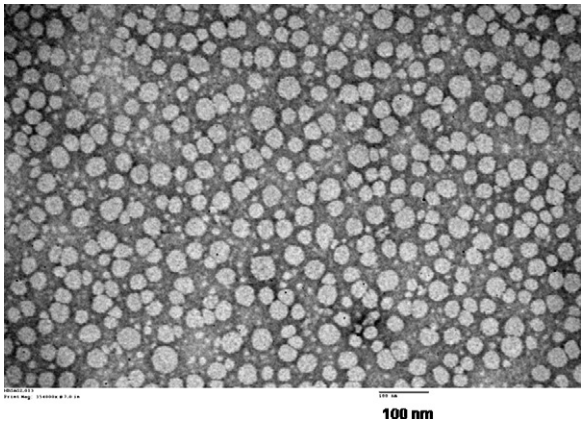

Fig. 6. Transmission electron microscope image of purified HBsAg protein. The purified phenyl eluate of HBsAg in 20 mM Bicarbonate buffer pH 9.6 at 3 μg/ml concentration was coated on to 400 mesh carbon coated copper grid and stained negatively with 2% uranyl acetate and visualized under TEM.

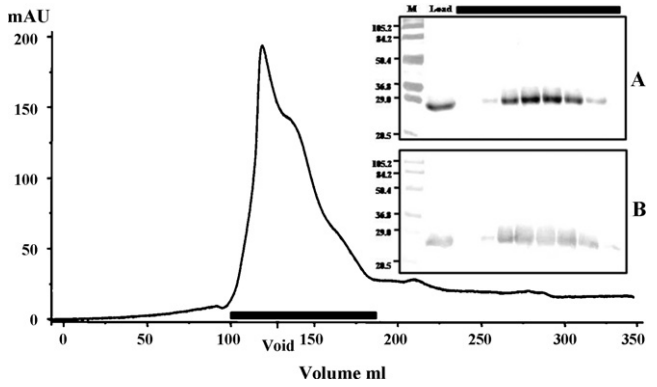

Fig. 7. Gel filtration profile of membrane purified HBsAg protein on sephacryl-300HR. The HBsAg protein eluted from the Phenyl column was pooled and dialyzed against 20 mM Bicarbonate buffer pH 9.6 + 1.2 M urea. The protein was then concentrated to 0.6 mg/ml, and 10 ml (6 mg) of this protein was loaded onto sephacryl-300HR gel filtration column. The thick dark colored curve is the elution profile of HBsAg protein from the gel filtration column. The *Y*-axis shows absorbance of protein at 280 nm in mAU. The *X*-axis shows volume in ml. The inset shows silver stained SDS-PAGE gel (A) and corresponding Western blot (B) developed with 5S-mAb. Lane M shows low molecular weight pre-stained markers. Load refers to the protein load onto the gel filtration column.

the incorporation of tangential flow filtration with 300 kDa membrane in the initial steps of purification scheme must have removed majority of the monomeric and lower oligomeric forms of HBsAg, thus enriching the VLP forms of HBsAg in the purified preparation. The mice immunized with the Phenyl eluate of HBsAg (Section 2.10) elicited high titers of anti-HBsAg antibodies. After two boosters, the levels of protective anti-HBsAg antibodies were raised to an extent of 12,000 mIU/ml, which is far higher compared to the minimum requirement of 10 mIU/ml. This indicates that the membrane purified HBsAg can be used as a vaccine.

The process which was developed for the purification of HBsAg, is based on the unique properties of HBsAg. Hence this process can be expected to work in the purification of some of the HBsAg fusion proteins. To this end, the process has been successfully applied in the purification of at least three other constructs of HBsAg fusion proteins (data pending publication) expressed in *P. pastoris* like; Den2EDIII-HBsAg (Dengue virus-2 envelope protein domain-III (EDIII) as an N-terminal fusion of HBsAg), Den2EDIII-PreS2-S (Dengue virus-2 EDIII as an N-terminal fusion of PreS2-S of HBV) and HEV (ORF2 nAg) fusion with HBsAg. In all these above constructs, the HBsAg purification process was successfully employed in the purification and isolation of these recombinant VLPs. Hence this method can be a useful and generic purification process for the purification of HBsAg and other HBsAg chimeric proteins.

4. Conclusions

HBsAg protein, when over expressed to high levels in *P. patoris* gets associated with cell membrane. This membrane association of HBsAg can be disrupted by 2% Tween-20. The membrane association property of HBsAg was exploited in its purification. The *Pichia* cells were lysed with buffer lacking detergents. A centrifugation step followed by the cell lysis ensured that majority of the host cell impurities are removed in the supernatant. The enriched HBsAg was then extracted from the lysed cell membranes by treatment with 2% Tween-20. Further, a downstream process involving membrane extraction, PEG precipitation, TFF and Phenyl hydrophobic chromatography ensured the complete purification of HBsAg. Majority of the HBsAg purified from the Phenyl column, existed in the form of VLPs and eluted as a broad void volume peak on gel filtration with Sephacryl-300 HR. The immunization of HBsAg purified from the Phenyl column showed that the membrane purified HBsAg VLPs were immunogenic and elicited high titer anti-HBsAg protective antibody responses in Balb/c mice.

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