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Expression in and purification of Hepatitis B surface antigen (S-protein) from methylotrophic yeast *Pichia pastoris*

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Abstract

The study describes expression and purification of recombinant hepatitis B small surface antigen (rHBsAg hereafter) in methylotrophic yeast *Pichia pastoris* strain GS115. For expression of the rHBsAg, a single copy of 678 bp cDNA was inserted at the unique *EcoR*I site downstream of the alcohol oxidase (*AOX* 1) promoter of the 8.2 kb pHIL-D2 vector. The cDNA–pHIL-D2 construct was used to transform the strain GS115, resulting in a Mut^S (**M**ethanol **U**tilizing **S**low) phenotype in which the 226 amino acids containing active and full-length rHBsAg protein could be expressed intra-cellularly during slow growth and induction with methanol.

The recombinant protein from the Mut^S expressor was harvested by cell disruption, and purified first by adsorption–desorption on aerosil followed by two-step chromatographic separation i.e. anion exchange on DEAE resin followed by gel permeation on Superdex 75. Reversed passive hem-agglutination assay (RPHA) was used to test the antigenicity while SDS-PAGE was performed to check the purity of the 27 kDa rHBsAg and its aggregates. The results showed that disruption at 12 Kpsi (three cycles), or 30 Kpsi (1 cycle), desorption with 10 mM carbonate buffer (pH 9–10), and storage at 4 °C without detergent did not adversely affect the antigenicity of the rHbsAg. However, the presence of detergents such as TritonX100 and deoxycholate in the disruption and desorption buffers, respectively resulted in reduced antigenicity during storage both at 4 and −20 °C in spite of higher initial yields.

Keywords: *P. pastoris*; Alcohol oxidase; Hepatitis B surface antigen; S-protein; Reversed passive hem-agglutination assay

1. Introduction

Hepatitis B virus (HBV), which belongs to the family Hepadnaviridae, is responsible for causing acute and chronic hepatitis, liver failure, and hepatocellular carcinoma. Although vaccines have been available for over 20 years, the disease remains a significant global problem. Approximately, one third [1] to 50% of the world population is expected to have had encounter with HBV and about 5% of the total infected world population had developed persistent infection [2]. There are more than 350 million HBV carriers world wide [1,3] with an estimated 20–25% risk for death [1]. The geographic distribution of the HBV carriers vary significantly ranging from 10% to 20% in Asia and Sub-Saharan Africa to less than 1% in Northern Europe and America [1]. In the United States, approximately 1 million people are suspected to carry HBV and 5000 die annually while globally approximately 1 million die annually due to HBV [1,3,4].

Structurally, HBV contains a DNA genome of about 3.2 kb which remains surrounded by an inner nucleocapsid core (HBcAg and an outer lipoproteinaceous envelope HBsAg). The envelope possesses antigenic properties and is composed of three regions. These are called major or small (S), medium (M, pre-S2 + S), and large (L, pre-S1 + pre-S2 + S) surface antigens. The three surface antigens are related yet distinct in the sense that they are encoded by a single open reading frame (ORF) having three different in-frame start codons but a common stop codon [5]. These viral surface antigens are suitable candidates for vaccine development because of their role in viral clearance [6]. The pre-S1 region has 108 or 119 amino acids and directly interacts with hepatocytes. The pre-S2 region has 55 amino

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acid residues and is implicated in the polymerized albumin-mediated interaction. The S protein has 226 amino acid residues and is antigenically most significant from vaccine development point of view. It contains complete information for self-assembly and mobilization of cellular lipids to form 42 nm Dane particles. These viral particles are known to be the most potent antigenic form of HBV and are made of 27 nm HBcAg surrounded by the lipoproteinaceous HBsAg [7].

Historically prior to the advent of recombinant anti-HBV vaccines, the prophylaxis against hepatitis B largely relied on the availability of first-generation vaccines which were derived from the sera of infected patients. These vaccines were complex particles (20 nm in diameter) made up of protein, carbohydrate, and lipid. The protein portion carried the antigenic determinants and was composed of two types of sub-units that shared a common polypeptide sequence but occurred either as a non-glycosylated, Mr 25,000 molecule or as a glycosylated, Mr 28,000 form. In the antigenically active HBsAg particle, these polypeptides were joined by disulfide linkage into dimers and higher multimers [8]. These sera-derived HBsAg particles were highly antigenic. However, the high cost, limited availability, poor acceptance, and risks of infections associated with the use of these vaccines led to successful expression of recombinant HBsAg peptide vaccines in a variety of cell lines such as *E. coli*. [9–11], *S. cerevisiae* [12], *P. pastoris* [13–16], Chinese hamster ovary [17,18], mouse [19–21], and *Drosophila* Schneider-2 cells [22]. The commercial anti-HBV vaccine is produced in the yeast and purified on a large scale by chromatographic separation by using the anti-HBsAg CB-Hep.1 monoclonal antibodies produced in Balb/c mouse ascites fluid. The method is fast and efficient, but there is a need for alternate purification techniques due to the ethical and regulatory issues. In recent years, several techniques such as a combination of ion-exchange chromatography and size exclusion chromatography, column chromatography and ultracentrifugation [20], ultracentrifugation [22], ammonium sulfate precipitation and gel permeation chromatography [10], affinity chromatography of 6 × His-tagged [16,23], or GST-tagged recombinant proteins with [11] or without cation exchange chromatography [24], or a single-step purification with monoclonal antibodies [21,25,26] have been tried. However, these studies suggest that purification of recombinant the product vaccine is difficult, highly cost-intensive, and gives low-to-moderate yields due to sub-optimal expression of the antigenic peptides, limited self-assembling, incorrect aggregation, and quick degradation of HBsAg during purification. Nevertheless, the product a proprietary process based on a series of physical and chemical processes for the extraction and purification of intracellularly expressed recombinant HBsAg vaccine (Recombivax HB) from *S. cerevisiae* has been successfully commercialized by Merck & Co. Inc., USA. In addition, interest is also growing for the optimization of the production process of recombinant HBsAg in the methylotrophic yeast *P. pastoris* [13–16]. The cell line offers several advantages due to the ease in genetic manipulation, simple nutritional requirements, and possibility of high-density culturing compared to the mammalian expression system. For the efficient extraction of recombinant HBsAg from *P. pastoris*, a bead mill disintegrator has been found to be useful [27]. In addition, chaotropic salts such as potassium thiocyanate can improve the recovery. Similarly, the effects of pH on the adsorption/desorption of HBsAg from diatomaceous earth matrix [28] and use of potassium thiocyanate and urea for the elution of HBsAg from a murine monoclonal antibody immobilized chromatographic support have been described [25].

In the present study, intra-cellular expression of rHBsAg was achieved in a Mut^S strain of *P. pastoris* GS115 during slow growth on methanol under the control of strong *AOX* promoter. The purification of rHBsAg was performed by adsorption/desorption on aerosil followed by ion exchange and gel permeation chromatography (GPC). The study also reports on the effects of detergent, disruption pressure, desorption conditions, and storage temperature on the yield and antigenicity of the rHBsAg.

2. Materials and methods

The summary of materials and methods is presented in Table 1.

2.1. Cloning and transformation

The cDNA encoding the full-length rHBsAg was a generous gift from Pasteur bioMerieux, France. The cDNA was maintained *in vivo* by insertion into the multiple cloning site (MCS) of the pGEM-T Easy vector system I (Promega Corporation, USA) by restriction digestion of both cDNA and the vector with *EcoR*I, ligation, and transformation of the chemically competent *E. coli* strain DH5α (Invitrogen Corporation, USA).

P. pastoris strain GS115 (Invitrogen Corporation, USA) was maintained on Yeast Extract Peptone Dextrose (YEPD) medium (Table 1). The preparation of competent cells, transformation, and screening of the Mut^S phenotype were performed as per the instructions provided in the 'Original *Pichia* Expression Kit' (Invitrogen Corporation, USA). In brief, the cDNA was ligated at the *EcoR*I site downstream of the *AOX*1 promoter of the 8.2 kb pHIL-D2 expression vector (Invitrogen Corporation, USA). The resulting cDNA-pHIL-D2 construct was used to transform the *Pichia* strain GS115. The vector carried the *HIS4* gene for easy selection of transformants on histidine-deficient medium. The Mut^S strain was selected by growing the positive transformants at 30–32 °C on BMMY plates containing a methanol-soaked sterile filter paper inside the lid. The strain that formed very small colony due to slow growth on methanol was designated as Mut^S in which the expression of antigenically active rHBsAg was confirmed by reversed passive hem-agglutination assay

Table 1
Summary of the materials and methods

cDNA sequence of the S-protein (rHBsAg) 678 bp and start codon (underlined)	ATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCGGGGTTTTTCTTG TTGACAAGAATCCTCACAATACCGCAG AGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAA AATTCGCAGTCCCCAACCTCCAATCAC TCACCAACCTCCTGTCCTCAACTTGTCCTCGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCA TCTTCCTCTTCATCCTGCTGCTATGC CTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAG GATCCTCAACCACCAGCACGGGACCA TGCCGAACCTGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAAA CCTTCGGACGGAAATTGCACCTGTATT CCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCC TGGCTCAGTTTACTAGTGCCATTTGTT CAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGG GGGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTT GGGTATACATTTAA (start codon has been underlined)
Maintenance medium for GS115	YEPD: 1% yeast extract, 2% peptone, 2% dextrose
BMGY and BMMY	1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% Yeast nitrogen base (YNB) with ammonium sulfate (without amino acids), 4×10^{-5}% biotin, 1% glycerol (BMGY) or methanol (BMMY)
Cell disruption buffer (PBS/PBST)	PBS (per 1000 mL): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 (pH 7.2–7.5); PBST: PBS with 0.5% (by vol) of Triton X100
Adsorption mixture	Crude extract supernatant and 0.15 M NaCl (1:1 by volume) + aerosil (2%, w/v); gentle stirring on a magnetic stirrer for 16 h at 4 °C
Desorption buffer	10 mM carbonate buffer (Na_2CO_3 and $NaHCO_3$, pH 9–10) with or without deoxycholate (0.25%, w/v)
Ion exchange chromatography	• FPLC system: EKTA Prime (Amersham Pharmacia Biotech) • Resin: DEAE Toyopearl 650 M (Tosoh Corporation, Japan) • Column: C 16/40 (Pharmacia Biotech) • Equilibration buffer: 20 mM Tris (pH 8.0–8.2) • Elution buffer: 0.5 M NaCl in 20 mM Tris (pH 8.0–8.2)
Gel permeation chromatography (GPC)	• FPLC system: EKTA Prime (Amersham Pharmacia Biotech) • Resin: Hiload Superdex 75 Prep grade • Column: XK 16 (Pharmacia Biotech) • Equilibration and elution buffer: PBS (pH 7.2–7.5)

(RPHA) before large-scale cultivation in the fermentor was initiated.

2.2. Production of rHBsAg

Pre-induction growth of the rHBsAg expressor Mut^S strain: The GS115 Mut^S strain was grown in a 3-L BMGY medium (pH 5–6) contained in a 5-L fermentor (model KF-5L, KoBio Tech, S. Korea). Incubation was carried out for 48 h at 30 °C with constant shaking at 250–500 rpm while maintaining the dissolved oxygen (DO) at >30% level.

Induction of rHBsAg expression: After sufficient growth and complete exhaustion of carbon source (glycerol in this case) as indicated by no change in DO levels (100% saturation), induction of rHBsAg expression was started between 48 and 52 h with three doses of 1% (by volume) methanol added once in 24 h under batch conditions. Incubation was continued for additional 72 h at 30 °C under the operational conditions similar to the pre-induction phase.

2.3. Recovery of post-induction expressor cell biomass

The 72 h post-induction *Pichia* culture was harvested by centrifugation at 5000 rpm for 10 min at 4 °C (Sorvall SLA-3000 rotor and Sorvall RC 5C Plus centrifuge, USA). The cell pellet was re-dissolved and washed twice by centrifugation in an ice-chilled PBS (pH 7.2–7.5). The washed cell pellet was finally recovered by centrifugation at 5000 rpm for 10 min at 4 °C.

2.4. Cell disruption

The washed cell pellet was re-suspended in either phosphate buffer saline (PBS) or phosphate buffer saline with Triton X 100 (PBST) (Table 1) to an optical density (OD_{600} nm) of ~100. The crude cell lysate was prepared by disruption of the washed cell pellet at 12 Kpsi (three cycles) or 30 Kpsi (one cycle) using either a constant cell disruption system (Constant Systems Ltd., UK) or glass beads. During the cell disruption process, the culture and the lysate were constantly maintained in ice. The lysate was

then clarified by removing the cellular debris through centrifugation at 5000 rpm for 10 min at 4 °C.

2.5. Adsorption of rHBsAg on aerosil

The clarified debris-free lysate was mixed (Table 1) with aerosil (Aerosil 380, Degussa AG, Germany) and adsorption was carried out by gentle stirring on a magnetic stirrer for 16 h at 4 °C. Upon completion of the adsorption process, rHBsAg-adsorbed aerosil was recovered by centrifugation at 7000 rpm for 15 min at 4 °C followed by two washings in PBS or PBST (pH 7.2–7.5).

2.6. Desorption of rHBsAg from aerosil

The washed rHBsAg-adsorbed aerosil was re-suspended in one volume of desorption buffer (Table 1) and desorption of rHBsAg from aerosil was carried out by gentle stirring of the mixture on a magnetic stirrer for 6 h at room temperature. The desorbed rHBsAg from aerosil was recovered in the supernatant after centrifugation of the mixture at 24 000 rpm for 30 min at 4 °C. The rHBsAg containing desorbed supernatant was stored for 24 h at 4 °C to provide stabilization and aging prior to purification by chromatography.

2.7. Purification by anion exchange chromatography

For purification by anion exchange chromatography, the DEAE resin was equilibrated with equilibration buffer (Table 1) at a flow rate of 1 mL/min. The desorbed supernatant (250 mL) was loaded onto the column at the same flow rate and elution was carried out with 0.5 M NaCl in equilibration buffer. Five milliliter fractions were collected and the rHBsAg-containing fractions were identified by SDS-PAGE and RPHA.

2.8. Purification by gel permeation chromatography

Superdex 75 resin was equilibrated with three volumes of PBS (pH 7.2–7.5) prior to sample loading. An aliquot (2 mL) of the rHBsAg active fraction obtained from the anion exchange step was loaded onto the resin and elution was carried out at 1 mL/min flow rate. One milliliter fractions were collected and assayed by RPHA and SDS-PAGE.

2.9. Analytical methods

The OD was measured in a 1 cm quartz cell using Agilent 8453 UV-visible spectrophotometer. An appropriately diluted culture was used to produce absorbance between 0.1 and 1.0 at 600 nm against the growth medium as the blank. The total protein was estimated with the BioRad protein assay kit (BioRad Laboratories Inc., USA). For the qualitative and quantitative detection of rHBsAg antigenicity, RPHA was performed with a highly sensitive and specific Hepa-S-ag kit (Greencross Life Sciences Corporation, S. Korea). The RPHA test was based on the agglutination of anti-HBs sensitized sheep erythrocytes in the presence of the antigen. The kit used HBsAg positive and negative human sera as controls. For a qualitative (screening) test, agglutination of anti-HBs sensitized erythrocytes was considered as positive for the presence of the rHBsAg in the test sample. For quantitative test, 25 μL of phosphate buffer saline supplied with the kit was placed in each well of the 96-well micro-plate. A 25 μL of the test material was then mixed into the designated as well 1A (Fig. 3). The solution was mixed gently to avoid formation of air bubbles and transferred to the next file down to H(A → B → C → D → E → F → H) making twofold serial dilution through each well (refer Fig. 3). The dilution in 1H, therefore, corresponded to 256 times and concentration of rHBsAg in well 1H was equal to 25 μg/mL of the test sample. The positive and negative controls were diluted in the same manner. A 25 μL of the erythrocyte suspension was added into each well and mixed gently on a micro-plate mixer for 10 s. The plates were incubated for 1 h at 37 °C. The precipitation of erythrocytes as small mass in the bottom of the wells indicated absence of antigen in the test sample. On the other hand, rHBsAg titer in the sample was indicated by the maximum dilution times at which agglutination pattern was obvious. The HBsAg positive control showed clear agglutination up to the well 1H corresponding to a dilution of 1:256.

SDS-PAGE was performed with 12% polyacrylamide gel under reducing conditions in the presence of 2-mercaptoethanol. A 15 μL sample aliquot was mixed with 5 μL of loading buffer and boiled for 5 min before loading on the gel. Electrophoresis was performed at 150 V (15 min) and 200 V (40 min) for stacking and resolving gels, respectively. The molecular size of the rHBsAg was correlated with Mark 12 MW protein markers (Invitrogen life Technologies, USA) after staining the post-electrophoresis gel with Coommassie stain.

3. Results and discussions

P. pastoris is a methylotrophic yeast that converts methanol into formaldehyde and hydrogen peroxide by alcohol oxidase (AOX), which is tightly regulated by the *AOX* promoter at transcription level. *Pichia* has two *AOX* genes, namely *AOX*1 and *AOX*2, each one being regulated by its own promoter. Growth of *Pichia* on methanol using just *AOX*2 is much slower than with *AOX*1. The present investigation exploited this ability of *Pichia* to produce rHBsAg in a Mut^S strain resulting from insertion of cDNA into the genome and disruption of the endogenous *aox1* locus.

3.1. Growth and expression of rHBsAg in GS115 Mut^S strain

Fig. 1. shows the strategy adopted for the production and purification of rHBsAg. In brief, sufficient growth of

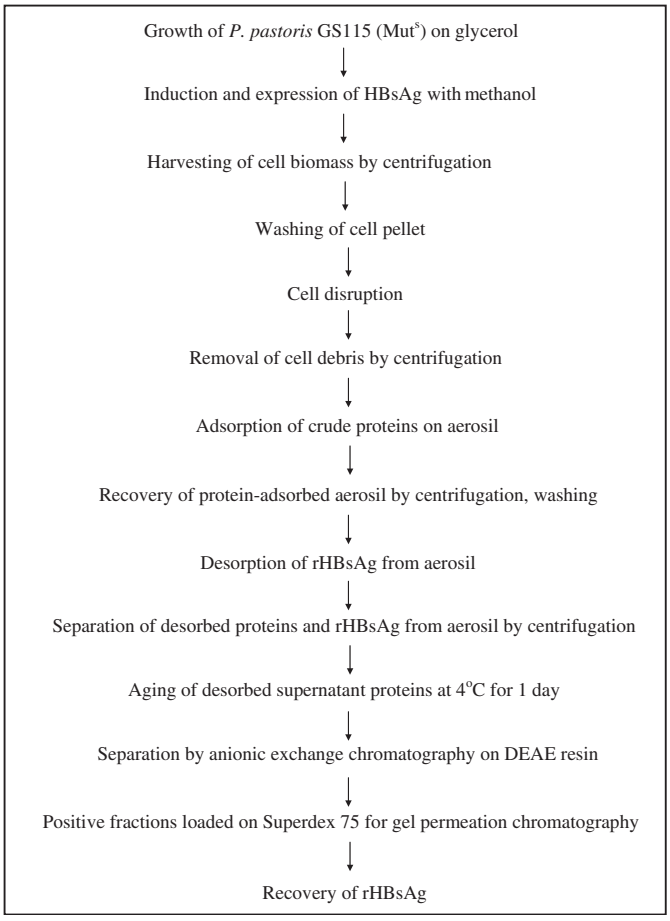

Fig. 1. Schematic illustration of rHBsAg production and purification process.

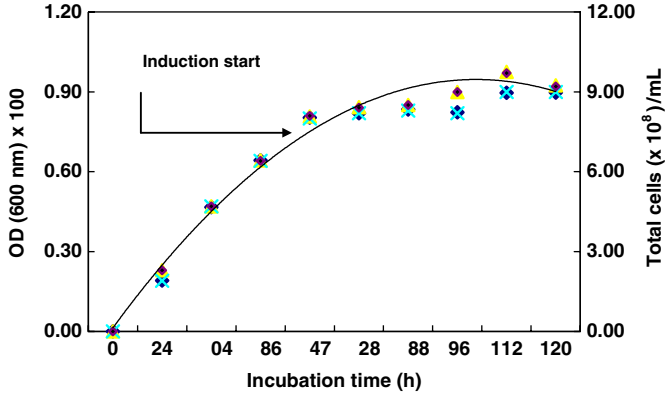

Fig. 2. Exponential growth phase of *P. pastoris* glycerol followed by slow growth and induction of rHBsAg with methanol. Different symbols represent separate replicates.

the yeast was obtained first on glycerol for 48 h. After 100% DO level was achieved due to the exhaustion of glycerol which occurred between 48 and 52 h, slow growth, induction of *AOX* promoter, and expression of rHBsAg were initiated with methanol (Fig. 2). The total protein in the 72 h post-induction culture medium prior to cell disruption was low (40–60 mg/L) which is in agreement with the fact that *Pichia* secretes very few native proteins. Inability of the erythrocytes to agglutinate with the medium aliquot further indicated that rHBsAg was not released in the growth medium (Fig. 3; panel 1, lane 1). The total protein in the 72 h post-induction culture after cell disruption in PBST was approximately 2.4 ± 0.4 g/L cell lysate which could be due to batch fermentation compared to the reported yields of 5–10 g total protein/L cell lysate of *Pichia* under continuous fermentation conditions.

3.1.1. *Effect of induction doses*

During the various stages of rHBsAg expression and purification, the activity was assayed by RPHA (Fig. 3, Panels 1–3). There was an increase in the rHBsAg activity with increase in induction doses at 24, 48, and 72 h post-induction, corresponding to 1, 2, and 3 methanol doses, respectively (panel 1, lanes 2–4). The rHBsAg concentration at 24 h post-induction was about 0.20 μg/mL that increased to over 6.25 and 12.5 μg/mL in cell lysate after 48 and 72 h, respectively.

3.2. *Extraction of rHBsAg*

3.2.1. *Effect of detergents and disruption methods*

Paez et al. [27] earlier reported on the usefulness of a bead mill disintegrator (3 passes of 4 min each) for efficient cell disruption and extraction of the rHBsAg from *P. pastoris*. During the present study, performance of two cell disruption methods viz., glass bead and cell disruptor were evaluated. During disruption of high cell density culture (88–120 h total incubation) with glass beads, the total protein recovery in PBS was only 23–28% (0.6–0.8 g/L) of that obtained (2.6–2.8 g/L) with PBST (PBS with Triton X 100) under the same conditions. However, the initial rHBsAg activity was higher both in PBST and PBS compared to the corresponding crude extracts prepared by a cell disruptor (lane 4 versus lanes 7 and 9; lane 5 versus 6 and 8, respectively). The total protein recovery in PBS at 12 Kpsi (3 cycles) was 76.52% (1.76 g/L) of that obtained with PBST (2.3 g/L). However, at 30 Kpsi (1 cycle), 93.91% (2.16 g/L) of the total protein could be recovered in PBS compared to that obtained in PBST (2.3 g/L). In other words, at 12 Kpsi, 81.48% of the total protein could be recovered in PBS compared to that obtained at 30 Kpsi. However, there was no difference in the total protein yields at the two pressure conditions when PBST was used.

3.2.2. *rHBsAg antigenicity during storage*

The aliquots of above-mentioned crude extracts were stored at 4 and −20 °C for 4 weeks and analysed by RPHA (panel 2; lanes 10–15 and 18–21, respectively). At 4 °C after storage, the crude extracts prepared in PBST by cell disruptor at both the pressures showed over 50% loss in activity (lanes 12 and 14) compared to when analysed immediately (lanes 7 and 9). However, a reverse trend was observed for crude extracts prepared in PBS (lanes 13 and

15 versus lanes 6 and 8). Similar results were also noticed after storage at −20 °C (lanes 18–21). However, the loss of activity by PBST could be reduced by glass bead disruption (lanes 10 and 11 versus lanes 4 and 5, respectively), suggesting that pressure employed for cell disruption might play accessory role in causing loss in antigenicity of rHBsAg in the presence of detergent.

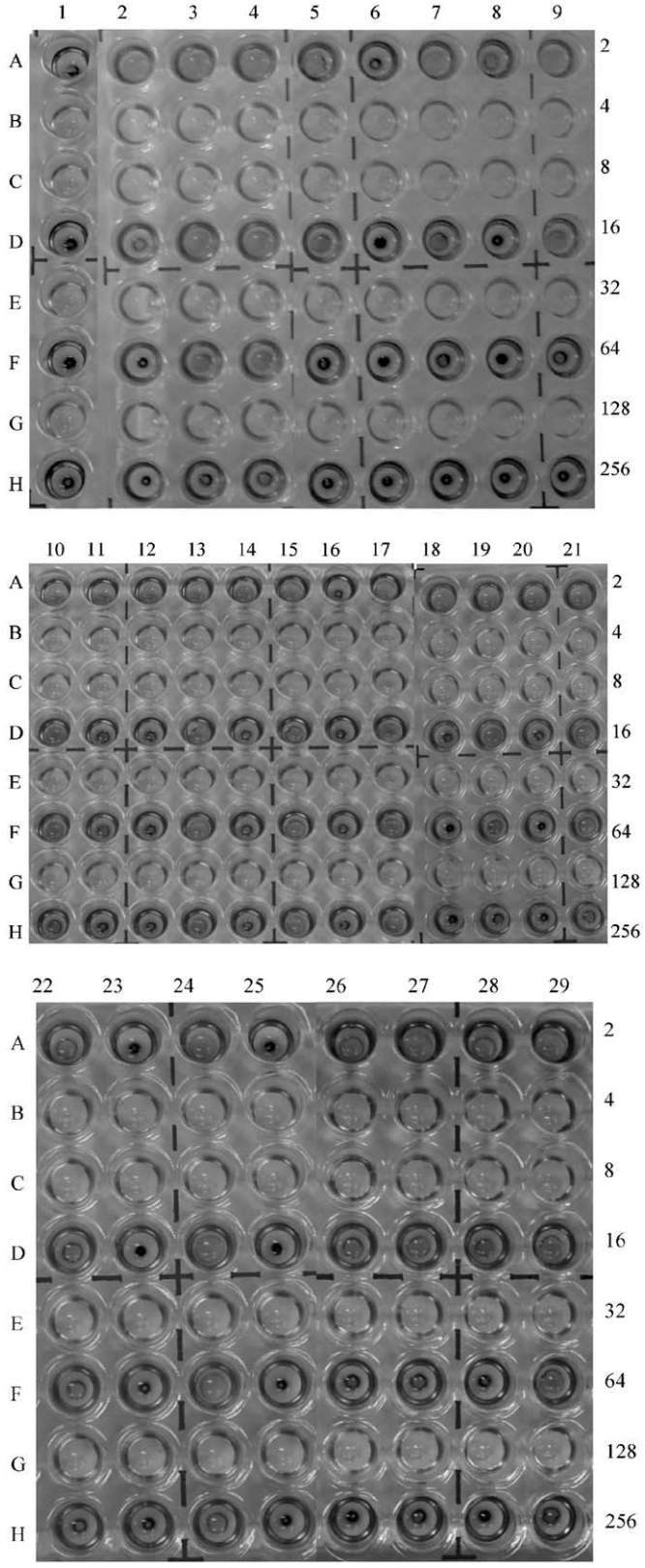

Fig. 3. rHBsAg activities in cell extracts during adsoption–desorption. Panel 1: effect of cell disruption conditions on initial rHBsAg activity (values on the right indicate dilution times). Lane 1: culture supernatant after harvesting of yeast cell biomass; (a) Cell disruption by glass bead: (lane 2: 24 h post-induction, disruption in PBST; lane 3: 48 h post-induction, disruption in PBST; lane 4: 72 h post-induction, disruption in PBST; lane 5: same as lane 4, but disruption in PBS). (b) Cell disruption by cell disruptor: (lane 6: disruption in PBS at 12 Kpsi × 3 cycles; lane 7: same as lane 6 but disruption in PBST; lane 8: disruption in PBS at 30 Kpsi × 1 cycle; lane 9: same as lane 8, but disruption in PBST). Panel 2: Effect of cell disruption conditions on rHBsAg activity during storage (values on the right indicate dilution times). (a) Cell disruption by glass bead and storage of lysates for 4 weeks at 4 °C: (Lane 10: disruption in PBST; lane 11: disruption in PBS). (b) Cell disruption by cell disruptor and storage of lysates for 4 weeks at 4 °C: (lanes 12: disruption in PBST at 12 Kpsi × 3 cycles; lane 13: same as lane 12, but disruption in PBS; lane 14: disruption in PBST at 30 Kpsi × 1 cycle; lane 15: same as lane 14, but disruption in PBS) lane 16: negative control; lane 17: positive control. (c) Cell disruption by cell disruptor and storage of lysates for 4 weeks at −20 °C (lane 18: disruption in PBST at 12 Kpsi × 3 cycle; lane 19: same as lane 18, but disruption in PBS; lane 20: disruption in PBST at 30 Kpsi × 1 cycle; lane 21: same as lane 20, but disruption in PBS). Panel 3: Effect of desorption conditions on rHBsAg activity during storage (values on the right indicate dilution times). (a) Aging of desorbed supernatants for 24 h at 4 °C: (Lane 22: disruption in PBST at 12 Kpsi × 3 cycles, desorption in 10 mM carbonate buffer with deoxycholate; lane 23: disruption in PBS at 12 Kpsi × 3 cycles, desorption in 10 mM carbonate buffer without deoxycholate; lane 24: disruption in PBST at 30 Kpsi × 1 cycle, desorption in 10 mM carbonate buffer with deoxycholate; lane 25: disruption in PBS at 30 Kpsi × 1 cycle, desorption in 10 mM carbonate buffer without deoxycholate). (b) Storage of desorbed supernatants for 4 weeks at 4 °C: (lane 26: same as lane 22; lane 27: same as lane 23; lane 28: same as lane 24; lane 29: same as lane 25).

3.3. Purification of rHBsAg

3.3.1. Adsorption of rHBsAg on aerosil

The behavior of adsorption/desorption of rHBsAg on aerosil was monitored by SDS-PAGE (Fig. 4). The adsorption of rHBsAg on aerosil was non-specific and most of the cellular proteins were also adsorbed (lane 2). Similar non-specific adsorption of cellular proteins during purification of rHBsAg from *P. pastoris* also occurs on a diatomaceous earth matrix [28].

The adsorption media left obtained after the recovery and washing of rHBsAg-adsorbed aerosil by centrifugation (refer section 2.5) were analysed for the presence of unbound cellular proteins (Fig. 5). The results indicated that the adsorption of total cellular proteins on aerosil was more efficient when cell lysates were prepared in PBS (T-2 and T-4) instead of PBST (T-1 and T-3). As a result, non-bound total proteins at 150–170 mg/L were found in the supernatant compared to 400–660 mg/L when PBST was used. Similarly, when PBS was used, the pressure used for cell disruption had no effect on adsorption efficiency as supernatants contained same amount of proteins suggesting

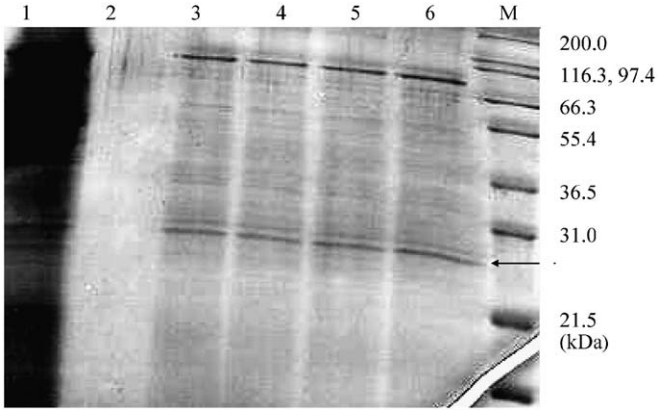

Fig. 4. SDS-PAGE analysis of rHBsAg during adsorption–desorption from aerosil. Lane 1: crude extract prepared by disruption in PBST at 30 Kpsi × 1 cycle; lane 2: supernatant after adsorption of proteins on aerosil; lane 3: desorption with 10 mM carbonate buffer with deoxycholate for 2 h; lane 4: same as lane 3, but desorption for 4 h; lane 5: same as lane 3, but desorption for 6 h; lane 6: after aging of desorbed supernatant at 4 °C for 1 day; M-Mark 12 MW protein markers (Invitrogen life Technologies, USA).

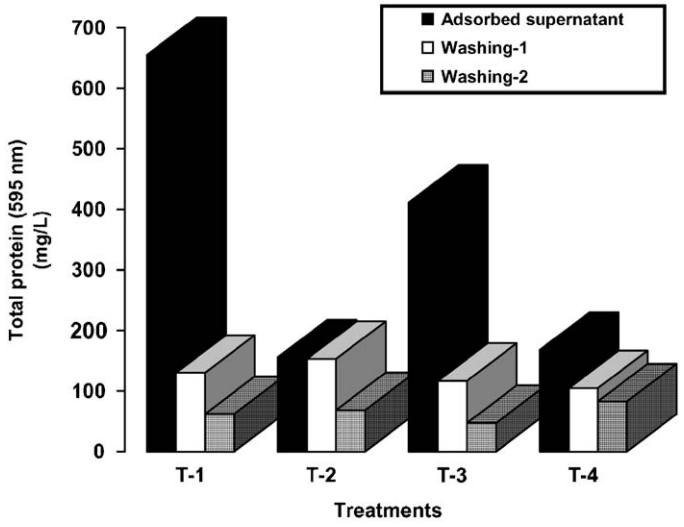

Fig. 5. Non-bound proteins in the supernatant after adsorption of rHBsAg on aerosil. T-1: cell disruption in PBST at 12 Kpsi × 3 cycles; T-2: cell disruption in PBS at 12 Kpsi × 3 cycles; T-3: cell disruption in PBST at 30 Kpsi × 1 cycle; T-4: cell disruption in PBS at 30 Kpsi × 1 cycle.

that TritonX100 interfered and reduced the efficiency or capacity of aerosil to bind proteins. However, the differences in the loss of proteins from rHBsAg- adsorbed aerosil during subsequent washings (W-1, W-2) with PBS or PBST became negligible among the treatments, indicating that TritonX100 in the disruption buffer interfered in initial uptake of proteins by aerosil, but did not cause dislodging once the proteins were adsorbed onto the aerosil. Earlier studies on the adsorption/desorption behavior of recombinant HBsAg suggest that capacity of the diatomaceous earth matrix for adsorption of HBsAg could also be influenced by changes in the pH [28].

3.3.2. Desorption of rHBsAg from aerosil

During the present study, for the recovery of rHBsAg from the aerosil, a desorption buffer with a basic pH (Table 1) was used and a concentrated rHBsAg monomer was recovered within first 2 h of desorption process as indicated by the SDS-PAGE analysis (Fig. 4, lanes 3–5). No qualitative or quantitative differences between lanes 3–5 corresponding to 2, 4, and 6 h desorption time suggested that desorption of most of the rHBsAg from the aerosil took place within the initial 2 h and the extended desorption time beyond 2 h did not cause much desorption of contaminating proteins, suggesting that the rHBsAg was less tightly bound to the aerosil compared to the contaminating proteins. These results are in agreement with earlier reports on more specific desorption of recombinant HBsAg than contaminant proteins by use of a basic (pH 8.2) pH buffer [28].

Further quantitative analyses of the desorbed supernatant both in the presence or absence of deoxycholate in desorption buffer did not show major differences (620–750 mg/L) in the total protein content. But, the initial rHBsAg activity was higher in the presence of deoxycholate (Fig. 3; panel 3, lanes 22 and 24 versus lanes 23 and 25) possibly due to the reduction in the lipopolysaccharide contamination [25]. However, when the desorbed supernatant aliquots were analysed after 24 h aging at 4 °C (Fig. 3, panel 3, lanes 22–25) and storage for 4 weeks at 4 °C (lanes 26–29), minor loss of activity in the presence of deoxycholate occurred (lanes 22 and 24 versus lanes 26 and 28, respectively) while a reverse trend was noticed when deoxycholate was not included in the desorption buffer (lanes 27 and 29 versus lanes 23 and 25). Similarly, when 30 mM carbonate buffer was used with or without deoxycholate instead of 10 mM carbonate buffer activity by RPHA was low. A similar phenomenon of reduction in antigenicity of HBsAg was observed but at elevated pH by Wampler et al. [8].

3.3.3. Purification by ion exchange chromatography

The desorbed supernatant (250 mL) after 24 h aging at 4 °C was fractionated on a DEAE resin (Fig. 6, panel 1). The fraction-24 showed a strong rHBsAg monomer band in SDS-PAGE (Fig. 7; panel 1, lane 1), high activity by RPHA (upto 6 well), and a total protein concentration of 725 mg/L. During the anion exchange chromatography, the binding of rHBsAg on the DEAE resin was very efficient even when fivefold concentrated desorbed supernatant was loaded (Fig. 7; panel 2). As a result, no loss of rHBsAg was observed either in the flow through (lane 2) or during the initial stages of elution when NaCl concentration in the elution buffer was building up (lane 4). However, an rHBsAg fraction similar to fraction 32 obtained from the GPC (Fig. 7; panel 1, lane 2) was seen during the pre-elution washing of the DEAE resin with 20 mM Tris buffer (Fig. 7; panel 2, lane 3) suggesting that some loss of rHBsAg occurred possibly due to the saturation of the resin. Otherwise,

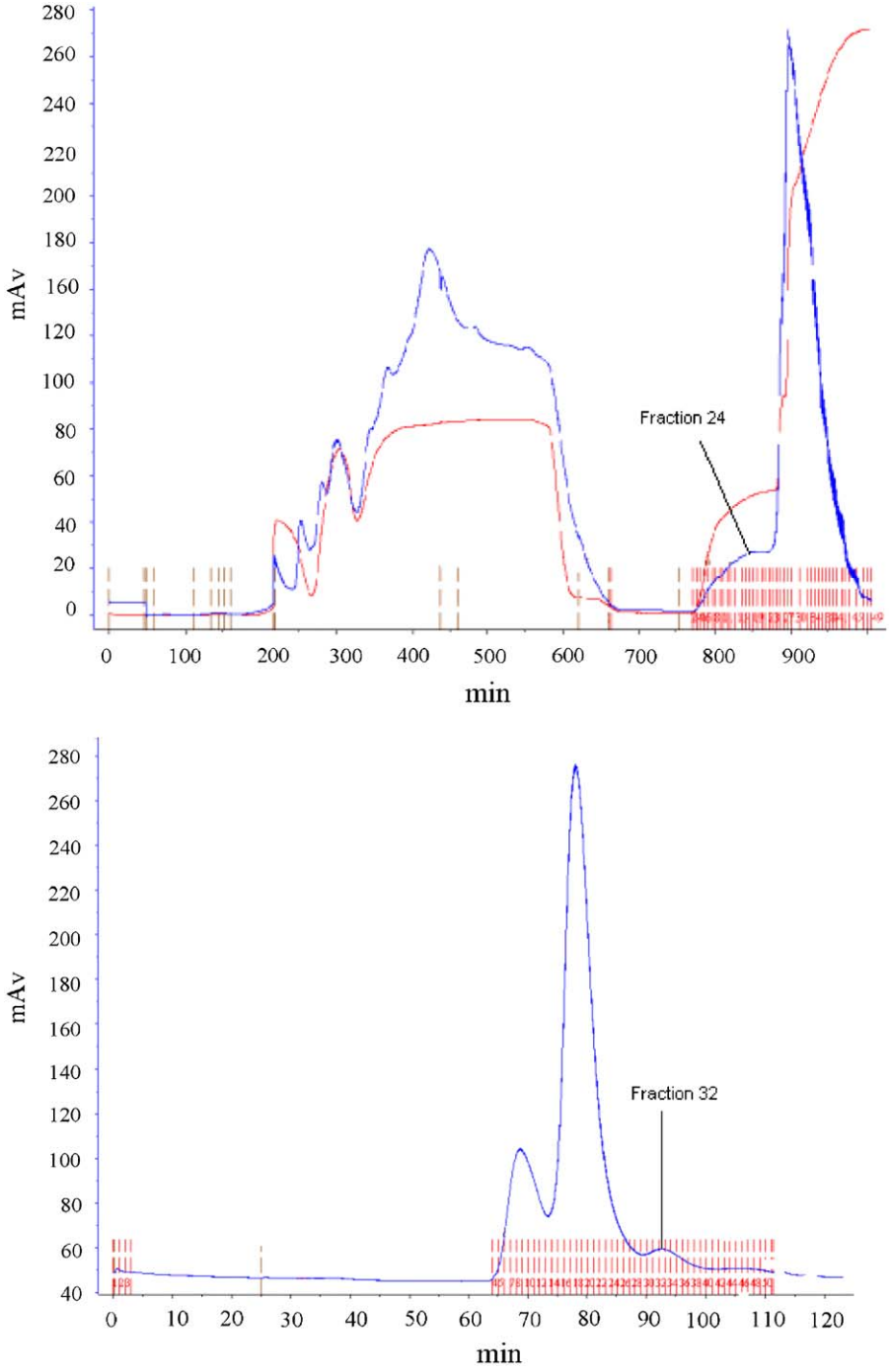

Fig. 6. Elution profile of rHBsAg during fractionation by ion exchange and GPC. Panel 1: Purification of rHBsAg by ion exchange chromatography. Panel 2: Purification of rHBsAg by gel permeation chromatography.

elution of most of the rHBsAg showing high RPHA activity (similar to fraction 24 as described above) occurred with 0.5 M NaCl in 20 mM Tris buffer (Fig. 7; panel 2, lane 5).

3.3.4. Purification by gel permeation chromatography

Fraction 24 as shown in Fig. 7 (panel 1, lane 1) was further purified by GPC (Fig. 6, panel 2) and the monomer could be obtained in fraction 32 (Fig. 7; panel 1, lane 2). The total protein concentration in this fraction was 27 mg/L.

3.4. Trypsin treatment of the rHBsAg obtained from GPC

The primary structure of HBsAg contains 14 cysteine residues per 226 amino acid containing monomer, which

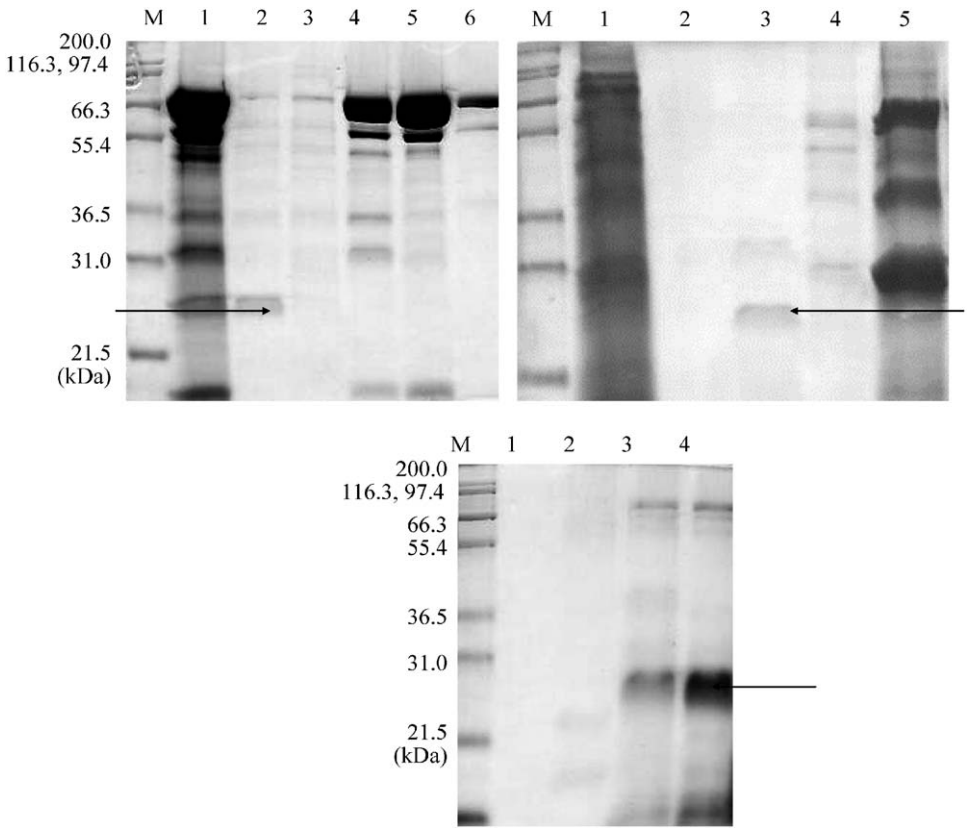

Fig. 7. SDS-PAGE analysis of the rHBsAg-active fractions obtained by ion exchange chromatography and GPC. Panel 1: Ion exchange and GPC. Panel 1: M-Mark 12 MW protein markers; Lane 1: fraction-24 from ion exchange; lane 2: fraction-32 from GPC showing rHBsAg monomer (27 kDa); lane 3: GPC fraction-29; lane 4: GPC fraction-21; lane 5: GPC fraction-18; lane 6: GPC fraction-15. Panel 2: Ion exchange of concentrated sample. Panel 2: M-Mark 12 MW Protein markers; Lane 1: Crude extract in PBST at 30 Kpsi × 1 cycle; lane 2: flow through during loading; lane 3: pre-elution washing showing rHBsAg monomer (27 kDa) similar to fraction-32 from GPC; lane 4: early elution fraction; lane 5: rHBsAg fraction. Panel 3: GPC fractions 18 and 21 after Trypsin treatment. Panel 3: M-Mark 12 MW protein markers; Lane 3: GPC fraction-21 after trypsin treatment; lane 4: GPC fraction-18 after trypsin treatment.

provides opportunity for formation of numerous disulfide bonds. During *in vitro* purification of the yeast antigen, some disulfide bonds form spontaneously between the antigen subunits, converting the antigen into dimers. The *in vitro* formation of disulfide bonds between the dimers creates large aggregate particle giving rise to a mixture of heterogeneous particles [8]. This can lead to difficulties in purification and identification of the purified product. On the other hand, breaks of disulfide bonds in HBsAg particles can cause reduction in antigenicity. During the present study, the GPC fractions 21 and 18 (Fig. 7, panel 1, lanes 4 and 5, respectively) showed very high RPHA activity and therefore, were suspected to carry high-molecular-weight aggregates of rHBsAg. Hence, both the fractions were treated with trypsin (Sigma-Aldrich Co., USA) for 5 h at 37 °C and the results on appearance of rHBsAg monomers are illustrated in SDS-PAGE (Fig. 7, panel 3, lanes 3 and 4). The observations therefore, of the present study are in agreement with earlier reports suggesting that HBsAg travels as a high-molecular-weight aggregate during size-exclusion chromatography [29].

4. Conclusions

1. Higher expression of rHBsAg can be achieved with increase in number of induction doses of methanol.
2. Presence of TritonX100 and deoxycholate adversely affected the rHBsAg activity during storage at 4 and −20 °C.
3. Adsorption of cellular proteins on aerosil was non-specific while desorption produced concentrated rHBsAg.
4. For higher yields and recovery of rHBsAg, trypsin treatment after first GPC could be useful.
5. A polishing step by second GPC may be useful to get uniformly aggregated product.

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