Purification of recombinant HBsAg expressed in methylotrophic yeast *Pichia pastoris*

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Introduction

Hepatitis B virus (HBV) responsible for chronic hapatitis, liver failure, and hepatocellular carcinoma affects over 50% of the world population with ~ 100 million deaths annually.

The virus contains a DNA genome (3.2 kb), nucleocapsid core, and an envelope. The three envelope surface antigens called short (S), medium (M), and large (L), are currently used as vaccines for effective prophylaxis (Brechot et al., 2000). For commercial production of the recombinant vaccine, eukaryotic cell lines mainly *S. cerevisiae*, *P. pastoris*, and Chinese hamster ovary (CHO) are employed. However, the main technological bottlenecks in large-scale production of the vaccine-grade product include; sub-optimal expression, difficult, cost-intensive downstream processing, limited self-assembling, incorrect aggregation, and degradation during purification and storage.

The present study reports on purification of recombinant HBsAg (S peptide) in *P. pastoris,* expressed intra-cellularly under the control of the strong alcohol oxidase promoter (*AOX*1) during slow growth on methanol (Mut^{S}) as carbon source.

Materials and Methods

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

Strain	*P. pastoris* GS115 (Invitrogen Corporation, USA)
cDNA sequence encoding the S protein of HBsAg	ATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAG AGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCAC TCACCAACCTCCTGTCCTCAACTTGTCCTCGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGC CTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCAACCACCAGCACGGGACCA TGCCGAACCTGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATT CCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTT CAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCTTGAGT CCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAA
Vector	8.2 kb pHIL-D2 (Invitrogen Corporation, USA)
Cloning	*P. pastoris* GS115 Mut^{S}: *Pichia* expression kit (Invitrogen Corporation USA)
Media	
a) GS115 strain	YPD
b) GS115 Mut^{S}	BMGY (glycerol) and BMM (methanol)
Fermentation	
a) strain	GS115 Mut^{S}
b) growth	5 liter fermentor, 3 liter BMGY, 30^{o}C, 250-500 rpm, >30% DO, 48 h

c) induction	3 doses of 1% (v/v) methanol added after every 24 h under batch conditions
d) harvesting	72 h post-induction culture centrifuged at 5000 rpm for 10 min at 4°C.
e) cell disruption	PBS washed (twice) pellet re-suspended in PBS (pH 7.4) with or without 0.5% Triton x 100, cell disruption at 12 Kpsi (3 cycle) or 30 Kpsi (1 cycle), again centrifuge.
f) adsorption on aerosil	crude extract supernatant and 0.15 M NaCl (1:1) + aerosil (2% w/v); gentle stirring on a magnetic stirrer at 4°C, 16 h
g) desorption	▪ *Recovery of adsorbed HBsAg:* centrifuge (7000 rpm, 15 min, 4°C) to collect pellet ▪ *Washing (2):* PBS (pH 7.4) ▪ *Desorption buffer:* pellet resuspended in 10 mM carbonate buffer (Na_2CO_3 and $NaHCO_3$, pH 10.0) containing 0.25% deoxycholate ▪ *HBsAg desorption:* gently stir at RT, for 6 h on a magnetic stirrer ▪ *Recovery of HBsAg:* centrifuge (4000 rpm, 4°C) and collect the supernatant ▪ *Aging:* store at 4°C, 24 h
Ion Exchange chromatography	▪ *FPLC system:*EKTA Prime (Amersham Pharmacia Biotech) ▪ *Resin:* DEAE Toyopearl 650 M, packed vol 100 ml ▪ *Column:*C 16/40 Pharmacia Biotech ▪ *Flow rate:* 1ml/min ▪ *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) ▪ *Fraction volume:* 5 ml
Gel permeation chromatography (GPC)	▪ *FPLC system:*EKTA Prime (Amersham Pharmacia Biotech) ▪ *Resin:* Hiload Superdex 75 Prep grade, packed vol 120 ml ▪ *Column:* XK 16 Pharmacia Biotech ▪ *Flow rate:* 1ml/min ▪ *Equilibration buffer:* PBS (pH 7.4) ▪ *Elution buffer:* PBS (pH 7.4) ▪ *Fraction volume:* 1 ml
Analytical methods	Cell density, Total protein, RPHA, SDS-PAGE, Western blot (rabbit anti-HBsAg primary antibody, goat anti rabbit IgG secondary antibody labeled with Horse-peroxidase (Kirkegaard and Perry Laboratories, Maryland, USA)

Results and discussion

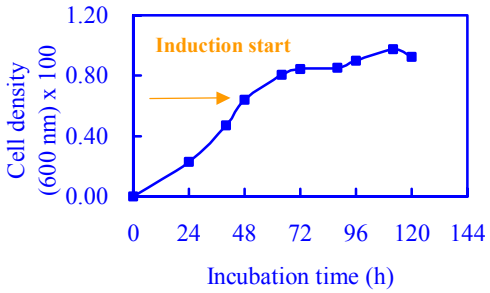
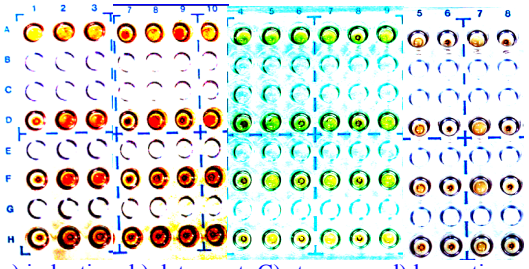


Fig. 1: Growth curve of *P. pastoris* on glycerol followed by methanol

For the production of HBsAg, first sufficient growth of the yeast is obtained on glycerol (48 h), followed by slow growth, induction of *AOX 1* promoter with methanol and expression of the recombinant HBsAg protein. For initiation of the expression of the HBsAg, complete exhaustion of glycerol is essential.



a) induction; b) detergent; C) storage; d)desorption

Fig. 2: RPHA analysis of crude extracts

Fig. 2; Lanes (From top towards bottom);
a) Post-induction: 1) 24 h; 2) 48 h; 3) 72 h
b) Disruption: (1) 12 Kpsi x 3, only PBS; (2) 12 Kpsi x 3, PBS+ Triton x 100; (3) 30 Kpsi x 1, only PBS; (4) 30 Kpsi x 1, PBS+ Triton x 100
c) Storage at 4°C for 4 weeks: (1) 12 Kpsi x 3, PBS+ Triton x 100; (2) 12 Kpsi x 3, only PBS; (3) 30 Kpsi x 1, PBS+ Triton x 100; (4) 30 Kpsi x 1, only PBS; 5) negative control; 6) positive control
d) Desorption: (1) 12 Kpsi x 3, PBS+ Triton x 100, with deoxycholate; (2) 12 Kpsi x 3, only PBS, without deoxycholate; (3) 30 Kpsi x 1, PBS+ Triton x 100, with deoxycholate; (4) 30 Kpsi x 1, only PBS, without deoxycholate

- With increase in induction doses, there was increased expression of HBsAg (Fig.2 a)
- Both at 12 Kpsi (3 cycles) and 30 Kpsi (1 cycle), the initial HBsAg activity was higher when Triton x 100 was used in PBS for cell disruption compared to PBS only case (Fig. 2 b)
- There was significant loss of HBsAg activity in presence of Triton x 100 when stored at 4^{o}C for 4 weeks irrespective of the cell disruption pressure applied. This indicated adverse effects of detergents on HBsAg activity during long-term storage. On the other hand, when only PBS was used, HBsAg activity increased tremendously during the corresponding time (Fig. 2 c).
- Although, addition of deoxycholate in 10 mM carbonate buffer for desorption gave higher initial HBsAg activity (Fig. 2 d), significant loss of activity was observed subsequently during storage at 4^{o}C. Contrarily, an increase in activity was recorded when desorption was carried out without deoxyclolate followed by storage at 4^{o}C.
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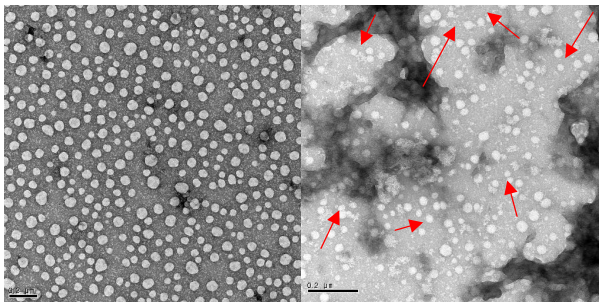


a) cell extract supernatant b) adsoption on aerosil

Fig. 3: Transmission electron micrographs

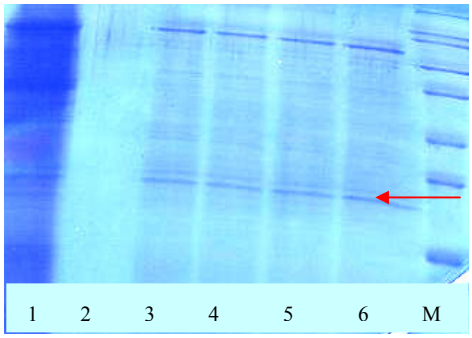
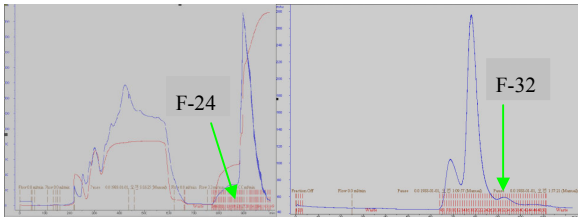


Fig. 4: SDS-PAGE analysis during adsorption-desorption process

Lanes; 1) crude extract 30 Kpsi x 1 cycle with Triton x 100, 2) adsorbed supernatant, 3) 2 h desorption with 10 mM carbonate and deoxycholate, 4) 4 h desorption 5) 6 h desorption, 6) after 1 d aging of desorbed supernatant at 4^{o}C, M- Protein molecular markers



a) Ion exchange b) GPC

Fig. 5a: Elution profile during separation by ion exchange and GPC

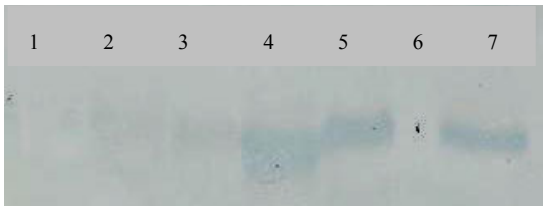
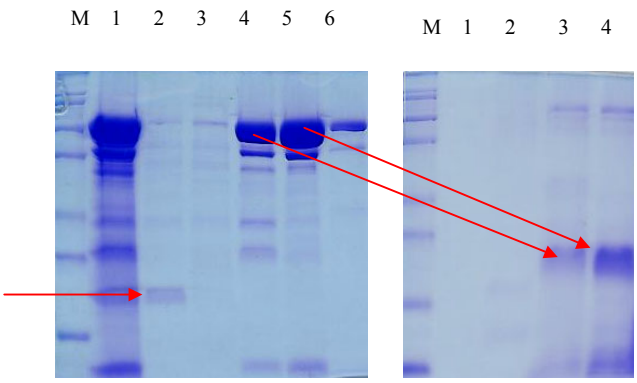


Fig. 6. Western blot analysis

Lanes; 2, 3, and 4 recombinant HBsAg, Lanes 5 and 7 commercial HBsAg standard for comparison



a) Ion exchange and GPC b) After Trypsin treatment

Fig. 5b: SDS-PAGE analysis during separation by ion exchange and GPC

a) M- Protein molecular markers, Lanes; 1) fraction-24 from ion exchange, 2) fraction-32 from GPC showing HBsAg monomer (27 kDa), 3) GPC fraction-29, 4) GPC fraction-21, 5) GPC fraction-18, 6) GPC fraction-15

b) M- Protein molecular markers, Lanes; 3) GPC fraction–21 after trypsin treatment, 4) GPC fraction-18 after trypsin treatment

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Conclusions

- Higher expression of HBsAg with more number of induction doses
- Triton x 100 and deoxycholate adversely affected the HBsAg activity during storage at 4°C
- The adsorption on aerosil is non-specific (Fig. 4, lane 2), while desorption yields HBsAg monomer (Fig. 4, Lane 3-6).
- For higher yields and recovery of HBsAg, trypsin treatment prior to GPC or after first GPC followed by second GPC could be useful.
- A polishing step by second GPC could be essential to get higher purity product

References

Brechot, C. Gozuacik, D. Murakami, Y. and Brechot, P.P., "Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC)", seminars in *CANCER BIOLOGY, **10,*** 211 (2000).