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Expression, Purification, and Characterization of Hepatitis B Virus Surface Antigens (HBsAg) in Yeast *Pichia Pastoris*

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Abstract Prevention of the prevalence of HB depends upon the development of efficient diagnostic reagent and preventive vaccine. *Pichia pastoris* offers many advantages over the other expression systems in the production of recombinant HBsAg. In this study, we reported that the recombinant *P. pastoris* strains were cultured in shake flasks and then scaled up in a 5.0-l bioreactor: approximately 27 mg/l of the protein and the maximal cell OD at 600 nm of 310 were achieved in the bioreactor. The recombinant HBsAg was purified by three steps of purification procedures. SDS-PAGE showed that the purified recombinant HBsAg constituted only one homogeneous band of ~24 kDa. CsCl density gradient ultracentrifugation assay indicated that the density of the HBsAg was 1.2 mg/ml, which was in agreement with the natural HBsAg, the HBsAg expressed in *Saccharomyces cerevisiae* and in mammalian cells. Electron microscope observation revealed that the purified recombinant HBsAg was homogeneous 22-nm particles, suggesting the HBsAg expressed in *P. pastoris* was self-assembled to virus-like structures. Competitive ELISA indicated that *P. pastoris*-derived HBsAg possessed the excellent immunoreaction with anti-HBsAg. Animal immunization showed that the immunogenicity of *P. pastoris*-derived HBsAg was superior to that of *S. cerevisiae*-derived HBsAg. Together, our results

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demonstrated that the recombinant HBsAg expressed in *P. pastoris* could provide promising, inexpensive, and large-scale materials for the diagnostic reagent and vaccine to prevent HBV infection.

Keywords Hepatitis B surface antigen · *P. pastoris* · Recombinant HBsAg · Expression · Characterization

Abbreviations

HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
AOX1	*alcohol oxidase* 1
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
ELISA	enzyme-linked immunosorbent assay
PAGE	polyacrylamide gel electrophoresis

Introduction

Human hepatitis B virus (HBV) can cause chronic infection and the infection frequently leads to the development of cirrhosis and hepatoma. Since nearly 350 million people around the world are chronic HBV carriers, the infection has become a severe threat to the health of the people worldwide [1, 2]. HBV nucleocapsid is surrounded by an outer envelope, which consists of three polypeptides. The three overlapping envelope proteins, called major S (S, 226 aa), middle S (M, 281 aa), and large S (L, 400 aa), are virus-coded and share the common C terminus. The major S is retained on the surface of HBV or virus-like particles, i.e. it covers the Dane particle, as well as a baculiform particle and/or a spherical particle with a diameter of 22 nm. Common to all the three particles, the antigen `a` determinant, a major target of both humoral and cellular immune response against HBV, possesses a highly conformational structure projecting from the virus. Antibody against HBsAg is protective against the hepatitis B virus [3, 4]. Human bodies immunized by HBsAg (major S) can generate a specific protective HBsAb. Immunoassays of HBsAb have been used to monitor the rate of hepatitis B vaccination and the convalescence and recovery of HBV-infected patients. Therefore, the 22-nm surface antigen of HBV is the primary component of diagnostic reagent and vaccines against HBV infection.

The epidemic of HB emphasized the needs to combine efficient diagnostic reagent and treatment for the infected population to expand prevention with active prophylaxis by vaccination against HBV [5]. The conventional antigen of diagnostic reagent for immunoassay of HBsAb is mainly obtained from the plasma of high titer HBV carriers. With the popularization of HBV vaccination, it is difficult to find such infected individuals to obtain the sera containing high titer HBsAg. The variety of cell systems were able to express recombinant HBsAg, such as mammalian, insect, and *Saccharomyces cerevisiae* cells, which have been successfully used as HBV vaccines for immunization purposes [6, 7], but the glycosylation of the recombinant HBsAg, occurring in 146 Asn within the sequence of ‘a’ common epitope, affects the immunoreactivity of the product, resulting in the hiding of the epitope. The recombinant HBsAg has also been expressed in *Pichia pastoris*, but the expression level was so low that the band of HBsAg protein could not be detected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [8–10]. Up to now, the recombinant HBsAg has been rarely used for immunoassay to

substitute blood-origin HBsAg. It is well known that the immunoreactivity of antigen is crucial for immunoassay reagents, but current commercial recombinant HBsAg for immunoassay of HBsAb was not satisfactory, especially in their sensitivity [11]. *S. cerevisiae*-derived HBsAg particle vaccine, bound to the cell surface of monocytes through the interaction with the lipopolysaccharide-binding protein and the receptor (CD14), interfered with the lipopolysaccharide and interleukin-2-induced activation of monocytes and reduced the capacity to bind monocytes [12]. Furthermore, plasmid used in *S. cerevisiae* was not stable enough, leading to the increase of the cost and difficulty in the high cell density culture of the recombinant *S. cerevisiae* strain for commercial production [13, 14].

The assembly of HBsAg particle appears not to occur in *Escherichia coli*-based expression system [4, 15, 16]. Due to the above-mentioned limitations of the *S. cerevisiae* and the low expression of the heterogenous protein in mammalian cells, both of them were not suitable for the expression of HBsAg. *P. pastoris* provides an efficient and inexpensive heterologous system for the production of high-level functionally active recombinant proteins [17, 18] and becomes the trend to substitute *S. cerevisiae* in biopharmaceutical production [19]. Since all serotypes of HBsAg contain only `a` common epitope, the S protein meet the requirements for detecting all serotypes of HBsAb. In addition, as the diagnostic antigen, the S protein has an advantage for its higher reactivity to polyclonal HBsAb and is also the major component of the HB vaccine. In this paper, we report that high-level expression of the recombinant HBsAg 'S' gene in *P. pastoris* was achieved in the bioreactor. SDS-PAGE showed the purified recombinant HBsAg constituted only one homogeneous band of ~24 kDa. Electron microscope observation revealed that homogeneous 22-nm particles were formed in the recombinant HBsAg. Competitive enzyme-linked immunosorbent assay (ELISA) and animal immunization showed that the recombinant HBsAg possesses the excellent immunoreaction and immunogenicity against HBsAb. Our results demonstrated that the recombinant HBsAg expressed in *P. pastoris* could provide promising, inexpensive, and large-scale materials for the diagnostic reagent and vaccine to prevent the HBV infection.

Materials and Methods

Cloning of HBsAg 'S' Gene and Construction of Recombinant *Pichia* Strains

HBV genomic DNA was directly extracted from the serum of HBV-infected patient in the Second Xiangya Affiliated Hospital of Central South University (China). The S gene (155~835) primer was designed according to the standard sequence of HBV in China (HBsAgFP: 5`-CGC*GGATCC*ATGGAGAACATCGCATCAG-3`, with a *Bam*H I site; HBsAgRP: 5`-AAGGAAAAAA*GCGGCCGC*TTAAATGTATACCCAAAGACAAA AG-3`, with a *Not* I site). The sequence of S gene (the full-length DNA sequence of HBsAg "S" gene is listed in supplementary information) was subtype *adr* compared with the standard sequence of HBV in China). The S gene was amplified by PCR using genome DNA as template and the PCR production was digested, inserted at *Bam*H I and *Not* I sites in pPIC3.5 K vector (Invitrogen), downstream of *alcohol oxidase* 1 promoter. The constructed expression plasmid was confirmed by enzyme digestion and sequencing. Linearized pPIC3.5 K-HBsAg using *Sal* I (for *his4* integration) was transformed into GS115 cells. Briefly, 80 μl of electrocompetent GS115 cells were mixed with 5 μg of linearized HBsAg expression plasmid in a 0.2-cm cuvette and pushed on the GenePulser (Biorad). The push parameters were 7.5 KV/cm, 50 μF, and 400 Ω.

Analysis of *Pichia* Integrants and Biomass

The recombinant transformants were cultured on MM and MD plates to differentiate Mut^+ from Mut^s. The genomic DNA was isolated from the transformants as described in multi-copy *Pichia* expression kit (Invitrogen) and analyzed for the presence of the HBsAg gene using the 5`*AOX1* and 3`*AOX1* primers. The isolated genomic DNAs from recombinant *P. pastoris*, which were transformed with a parent plasmid, or *P. pastoris* GS115, were used as control for PCR. Positive clones were screened for HBsAg expression using HBsAg quantitative ELISA system (Beijing Wantai Pharmacy Enterprise). The biomass was determined by measuring the absorbance of the broth at 600 nm (OD_{600}) in a spectrophotometer (Eppendorf). The samples were diluted if the absorbance was above 0.6 at 600 nm.

Expression of Recombinant HBsAg 'S' Gene

The recombinant strains in *P. pastoris* were grown in 15-ml baffled flasks containing BMGY medium at 29 °C with constant shaking (300 rpm). At the absorbance of 6 (OD_{600}), cells were harvested, re-suspended to the cells of 1.5 OD at 600 nm in shake flasks containing BMMY medium, and induced in shaking incubator (300 rpm) at 29 °C. The cells were induced with 1% (*v*/*v*) methanol. The transformed *P. pastoris* with the parent vector was used as the control [19]. At 72 h after induction, the cells were collected and disrupted using glass beads. The cell lysis was centrifuged at the maximal speed for 10 min at 4 °C, and the clear supernatant was transferred to a clean microcentrifuge tube. HBsAg concentration in the supernatant was detected by HBsAg quantitative ELISA.

The recombinant strains were then cultured in the bioreactor, a BIOSTAT-B 10.0 l fermentor of 5.0-l working volume with control modules for pH, temperature, agitation, dissolved oxygen, and air flow. Seed culture for the fermentation was started from the fresh glycerol stock and inoculated directly into baffled flask (50-ml working volume) containing the BMGY medium. After 24-h growth, seed culture was inoculated with 1% inoculation, grown for 16~18 h and then used to inoculate for the fermentation. Five percent of inoculation was used for a 10-1 bioreactor containing 5 l of basal salt medium (85% o-phosphoric acid (26.7 ml/l), 0.93 g/l calcium sulfate·$2H_2O$, 18.2 g/l potassium sulfate, 14.9 g/l magnesium sulfate·$7H_2O$, 4.13 g/l potassium hydroxide, 40 g/l glycerol, and 8.0 ml trace metal salt PTM1 (contained 6.0 g/l cupric sulfate·$5H_2O$, 0.08 g/l sodium iodide, 3.0 g/l manganese sulfate H_2O, 0.2 g/l sodium molybdate·$2H_2O$, 0.02 g/l boric acid, 0.5 g/l cobalt chloride, 20.0 g/l zinc chloride, 65.0 g/l ferrous sulfate·$7H_2O$, 5.0 ml sulfuric acid, and 0.2 g/l biotin). To guarantee the optimal growth, the bioreactor was set to operate at 29 °C and pH value was controlled at 6.0 by automatic feeding of 30% (*w*/*w*) ammonium hydroxide. The impeller speed was set from 400 to 800 rpm. The oxygen of medical grade was fed to the bioreactor to maintain the dissolved oxygen concentration above 30% of air saturation. If the glycerol in the bioreactor was depleted, methanol was continuously added at 1.8~9.6 g/l/h to induce the synthesis of HBsAg in the cells.

Lysis of Cells and Detection of Recombinant HBsAg

Aliquots of induced cells were harvested by centrifugation at 10,000×*g* at 4 °C and washed with sterile water. The cells were then re-suspended in breaking buffer (25 mmol/l sodium phosphate, 150 mmol/l sodium chloride, 1 mmol/l EDTA, 1 mmol/l DTT; 6.0 ml breaking buffer/g wet cells) for 18 h at 4 °C and disrupted by four passes through a sanitized APV

Gaulin 30CD homogenizer at chamber pressures of 13,000 psi. The recombinant HBsAg in the partial supernatant was detected by quantitative ELISA and SDS-PAGE.

Purifications of Recombinant HBsAg

The crude HBsAg solution was precipitated in 20–40% (*w*/*v*) saturation range of ammonium sulfate for 30 min and centrifuged at 12,500×*g* for 15 min. The pellet was dissolved in a minimal volume of 100 mmol/l PBS (pH 7.2), containing 5 mmol/l EDTA. The protein solution was dialyzed twice against 2 l of 100 mmol/l PBS containing 8.5% saturation of ammonium sulfate (buffer A) overnight. The dialyzed protein was further purified by hydrophobic interaction chromatography HPLC using Phenyl-5PW 0.75× 7.5 cm column (TOSOH, Japan) equilibrated with buffer A (approximately ten times of bed volume). The column was washed with ten times of bed volumes of buffer A and then the column was washed in 100 mmol/l PBS with linear descendent gradient of ammonium sulfate from 8.5% to 0% (*w*/*v*). The absorbed HBsAg was eluted with 100 mmol/l PBS containing 10% (*v*/*v*) ethanol. The column flow rate was maintained at 1 ml/min and the fractions were collected (1.0 ml/tube). Recombinant HBsAg, 1.0 ml, was further layered on 15 ml of 22% CsCl in 20 mmol/l PBS (pH 7.2) and centrifuged in a Beckman T70i rotor at 60,000 rpm for 10 h at 20 °C. The centrifuge tube was punctured at the bottom and 1.0 ml of fractions of the gradient was collected and analyzed for the presence of HBsAg particles by electron microscope.

Electron Microscope

A drop of purified sample was placed on a 400-mesh copper grid with formvar-carbon film for 2 min and the excess sample was blotted off. The grids were then stained with 2% aqueous solution of uranyl acetate and examined in an F30 transmission electron microscope (Philips, Netherlands) operating at 200 kV [20].

Concentration and Immunoreactivity Assays of Recombinant HBsAg

The concentration of soluble HBsAg in cell extracts was determined with HBsAg quantitative ELISA system (Beijing Wantai Pharmacy Enterprise). Briefly, the clarified extracts were diluted into a series of fivefold with 20 mmol/l PBS (pH 7.2). One hundred microliters of the diluted extracts was added to each well of a 96-well plate coated with anti-HBsAg polyclonal antibody. The plate was incubated for 1 h at 37 °C, emptied and taped dry, and then washed with PBS containing 0.02% Tween-20 for five times. One hundred microliters of horseradish peroxidase (HRP) conjugated mouse anti-HBsAg was added to each well. The plate was incubated for 1 h at 37 °C, emptied, and tapped dry. After five washes as described above, 100 μl of substrates was added. The plate was allowed to develop for 30 min at 37 °C and the reaction was stopped by adding 50 μl of 2 mol/l H_2SO_4. Each well of the plate was read at a double-wavelength of $A_{450/620}$ nm (Bio-Rad 680). Each sample was done in triplicate. The assay cutoff was calculated on the basis of the means of four negative controls, a series of standard dilution panel containing 0~4 ng of HBsAg was done to make a reference curve.

Competitive ELISA was used to detect the immunoreactivity of recombinant HBsAg with HBsAg quantitative ELISA system (Beijing Wantai Pharmacy Enterprise). Briefly, 50 μl of appropriate dilution HBsAb positive sera and recombinant HBsAg (~1.0 mg/ml) were added to each well of a 96-well plate and simultaneously 50 μl of 20 mmol/l PBS and

50 μl of recombinant HBsAg were added to another plate of 96 wells as the negative control. The following steps were done as described above in the concentration assays of recombinant HBsAg. The following formula was used to calculate the inhabit efficiency of competition: inhabit efficiency of competition %=(No HBsAg competition $OD_{450/620}$− HBsAg competition $OD_{450/620}$)/No HBsAg competition $OD_{450/620}\times 100$.

Immunogenicity Assays of Recombinant HBsAg

BALB/c female mice (eight per group) were immunized by intraperitoneal injections. Briefly, on day 0, 7, and 14, the mice were injected with the purified HBsAg derived from blood-origin, *P. pastoris*, *S. cerevisiae*, or PBS (2.0 μg per animal per injection) in complete Freund's adjuvant, incomplete Freund's adjuvant, and saline, respectively, and followed by two injections of the same dose of antigens in incomplete Freund's adjuvant at intervals of 3–4 weeks. Mice were bled before the start of immunization and at weekly intervals for a period of 10 weeks. The levels of anti-HBsAg-specific antibody were monitored with HBsAb ELISA system (Beijing Wantai Pharmacy Enterprise). Briefly, the sera were appropriately diluted and each of 50 μl diluted sera was added to each well of a 96-well plate, including appropriate negative and positive controls. The plates were incubated for 45 min at 37 °C, emptied and taped dry, and washed with PBS containing 0.02% Tween-20 for three times. Fifty microliters of HRP-conjugated goat anti-mouse IgG antibody was added to each well to detect bound serum antibody.

Results

Screening of Positive Transformant Strains

Twenty G418-resistant colonies were selected on MD and MM plates. Their growth rates were almost the same as the parent *P. pastoris* GS115 strain, demonstrating that they were all $His^{+}Mut^{+}$. The wild-type *alcohol oxidase* 1 gene (2.2 kb) and the S gene (~700 bp) were amplified from the recombinant strains by PCR (Fig. 1), confirming that the S gene was integrated into the chromosome of the recombinant *P. pastoris*. The Screening of Mut+ transformants on MD/MM plates were consistent with PCR analysis of the *P. pastoris*

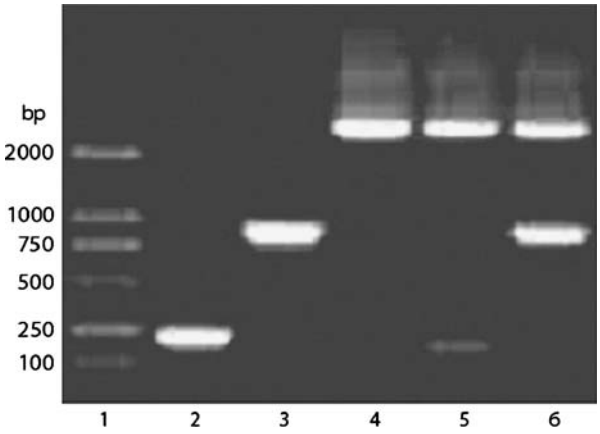

Fig. 1 Integration of HBsAg 'S' gene into the *P. pastoris* GS115 was confirmed by PCR in various DNA templates using 5`AOX1 primer and 3`AOX1 primer .*1* DNA ladder, *2* pPIC3.5K, *3* pPIC3.5K-HBsAg, *4 P. pastoris* GS115 without transformation, *5* control strains, *6* pPIC3.5K-HBsAg recombinants

transformants. The S gene was integrated into the *P. pastoris* genome at the *his4* locus arising from a single crossover event between the *his4* locus in the chromosome and *HIS4* gene on the HBsAg expression vector, therefore, the recombinant *P. pastoris* possessed much better genetic stability than the recombinant *S. cerevisiae*.

Expression of Recombinant HBsAg

After sufficient growth and induction, the expression of recombinant HBsAg was detected by ELISA. The results indicated that recombinant HBsAg was successfully expressed in *P. pastoris* in shake flasks: the biomass of the strains and expression level of HBsAg were 58 OD_{600} and 5.8 mg/l, respectively. In the bioreactor, at the beginning of the glycerol batch phase, the dissolve oxygen concentration was high, and then decreased shortly as the cells grew rapidly. At 28 h after inoculation, the fermentation was transferred to fed-batch phase and glycerol was added at a growth-limiting rate to derepress the methanol metabolic machinery and allow the cells to transit smoothly from glycerol to methanol feeding. The dissolved oxygen concentration ranged from 15% to 54%. Since the Mut^+ recombinants were sensitive to high concentration of residual methanol, the methanol level in the bioreactor was carefully judged by the dissolved oxygen concentration to prevent the accumulation of methanol in the medium during the induction phase from being oxidized to being formaldehyded. During this phase, the maximal expression yield of HBsAg appeared after 78 h induction. The expression level reached up to the maximum, 27 mg/l (Fig. 2). Simultaneously, the biomass also reached the maximum, 310 OD_{600}. Subsequently, the expression of HBsAg decreased, suggesting that the cells were autolyzed and the recombinant protein was digested by proteases. The biomass of the strains and expression level of HBsAg in the bioreactor were 5.3-fold and 4.7-fold more than in shake flask respectively. Since the conditions in the bioreactor were better for the growth of the recombinant *P. pastoris*, and methanol metabolism was faster during fermentation, the HBsAg yield from density fermentation were higher than in shake-flasks.

Purification of Recombinant HBsAg

The extracted supernatant proteins from crude cell culture were purified by ammonium sulfate precipitation, hydrophobic chromatography and CsCl density gradient ultracentri-

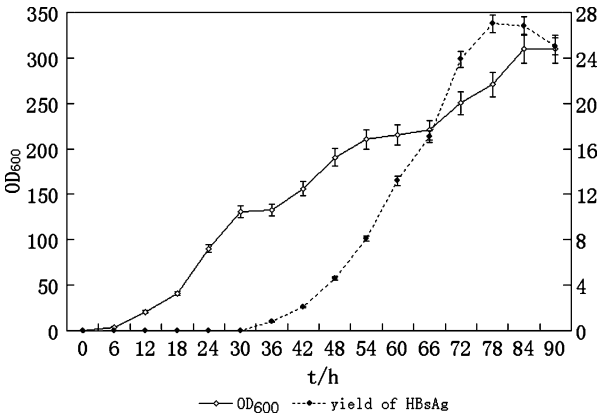

Fig. 2 The curve of cell density and expression of recombinant HBsAg during the high cell density culture ($p<0.005$ against OD_{600}, $p<0.001$ against yield of HBsAg)

fugation. The purification efficiency was detected with silver-stained SDS-PAGE and HBsAg quantitative ELISA. The recombinant HBsAg was precipitated in the 20–40% (*w*/*v*) saturation range of ammonium sulfate (data not shown). The contaminated proteins were clearly eliminated and the HBsAg was concentrated obviously and the yield of recovery of purified HBsAg was 62.4%. The dialyzed protein was further purified by hydrophobic chromatography. HBsAg quantitative ELISA indicated that the HBsAg was eluted in 100 mmol/l PBS containing 10% (*v*/*v*) ethanol (Fig. 3, the last peak) and almost no HBsAg protein was measured in other effluent solution, suggesting that the HBsAg was a strong hydrophobic protein and similar to that expressed in mammalian cells. SDS-PAGE showed that almost 90% contaminating proteins were removed and the yield of recovery of purified HBsAg was 81.3%. The HBsAg effluent solution was further purified by 22% CsCl density gradient centrifugation and individual fraction was assayed for the presence of HBsAg by quantitative ELISA. The results showed that the major peak fractions containing the HBsAg migrated to a position of about 1.2 mg/ml. In addition, lower and higher density of HBsAg fraction was also observed, but there was very little HBsAg in these fractions, suggesting that the HBsAg expressed in *P. pastoris* could be self-assembled into particles. Purified protein constituted only one homogeneous band of ~24 kDa (Fig. 4a), which was similar to the values obtained from native HBsAg, HBsAg expressed in mammalian cells, and *S. cerevisiae* [21, 22], and correspondent to the band identified in crude lysis. The yield of recovery of purified HBsAg was 72.5%. The purity of recombinant HBsAg through three steps of purification was confirmed by silver-stained SDS-PAGE and the total yield of recovery was 36.8%. It means that a fed-batch culture in 10 l bioreactor could produce 100 mg purified HBsAg. The purified protein constituted only one homogeneous band of ~24 kDa, implying that the glycosylation was not undergone in recombinant HBsAg expressed in *P. pastoris*. This advantage was good for the exposure of the epitope and increased the immunoreactivity and immunogenicity of recombinant HBsAg. The electron microscope observation revealed that large quantities of global structures with an average diameter of 22 nm were formed in the purified recombinant HBsAg (Fig. 4b). It was consistent with those expressed in *S. cerevisiae* and in mammalian cells, and further indicated that the recombinant HBsAg expressed in *P. pastoris* could be self-assembled into the virus-like structures in the absence of other viral proteins, and the morphology of the particles was similar to that observed in the sera of HBV-infected patients.

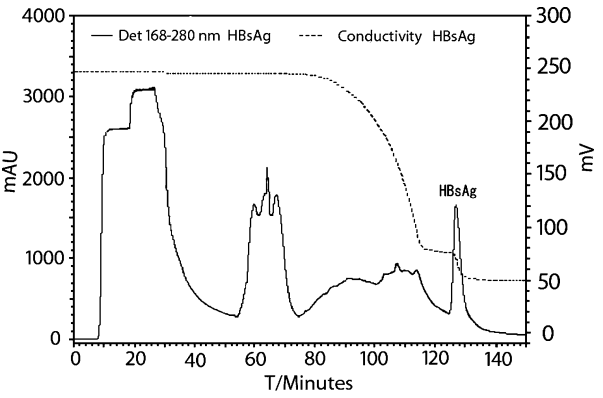

Fig. 3 Hydrophobic interaction chromatogram of recombinant HBsAg. The last small peak showed the elution HBsAg protein (during 120~140 min)

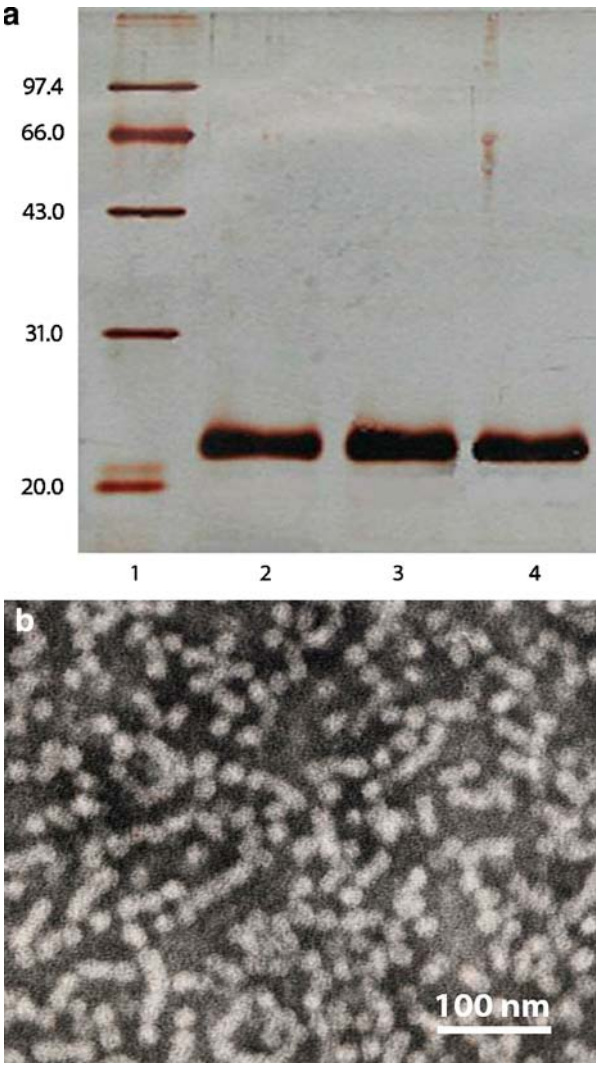

Fig. 4 CsCl density gradient centrifugation and electron microscope observation of the recombinant HBsAg. **a** Purity of recombinant HBsAg analyzed by SDS-PAGE after CsCl (22% (*w*/*v*)) density gradient centrifugation. Lane *1* protein marker, lane *2*, *3*, *4* the fraction of protein in 8, 9, 10 tube after CsCl density gradient centrifugation analysis; **b** electron micrograph of recombinant HBsAg particles, the fractions of 1.2 mg/ml were collected, negatively stained with uranyl acetate and observed by electron microscope

Characterization of Immunoreaction and Immunogenicity of Recombinant HBsAg

The immunoreactivity of the purified recombinant HBsAg was assayed by the competitive ELISA. The results demonstrated that the recombinant HBsAg had obvious effect of competitive inhibition to all six human HBsAb-positive sera (Fig. 5a). The efficiency of competitive inhibition was 73%, 95%, 89%, 72%, 76%, and 83% respectively. The varied competitive inhibition efficiency was probably caused by different concentration or affinity of the anti-HBsAg in different sera. It also indicated that the recombinant HBsAg had an excellent immunoreactivity.

BALB/c female mice were immunized with the purified HBsAg derived from blood-origin, *P. pastoris* and *S. cerevisiae* to investigate the immunogenicity of the purified

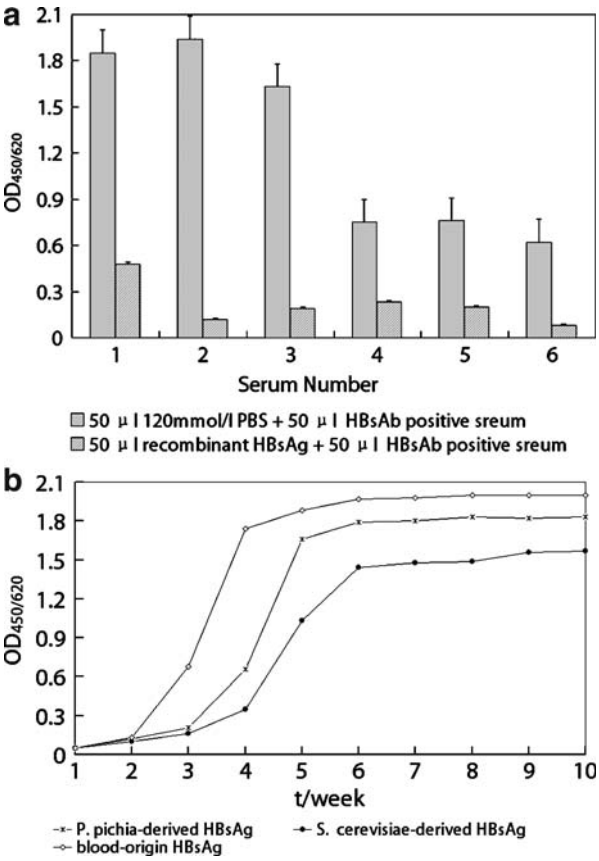

Fig. 5 Immunoreaction and immunogenicity assays of the *P. pastoris*-derived HBsAg. **a** Competitive ELISA assays the immunoreaction of the recombinant HBsAg. Each recombinant HBsAg and 50 μl of HBsAb-positive serum (total 100 μl) was added to the HBsAg ELISA kit to assay the immunoreaction, at the same time, each 50 μl of 20 mmol/l PBS and 50 μl of HBsAb-positive serum (total 100 μl) was added to the HBsAg ELISA kit as controls. *1–6* six different HBsAb positive sera. **b** Antibody response in BALB/c mice elicited by immunization with *P. pastoris*-derived HBsAg, *S. cerevisiae*-derived HBsAg and blood-origin HBsAg respectively

recombinant HBsAg, and the levels of antibody against HBsAg in the sera of the mice for the certain periods of time were measured with ELISA. Figure 5b illustrated that the specific antibody was first detected 1 week after the last injection in all groups of immunized mice, but later their responses varied. In the group of mice immunized with blood-origin HBsAg, the responses increased sharply, from 0.1 to 1.75 $OD_{450/620}$ within 2 days, reached near-maximal values at week 4, and then remained at a high plateau (up to 2.0 $OD_{450/620}$). In comparison, the antibody levels in the groups immunized with *P. pastoris*-derived HBsAg or *S. cerevisiae*-derived HBsAg increased steadily, while the antibody levels in the groups immunized with *P. pastoris*-derived HBsAg reached the near-maximal values (up to 1.70 $OD_{450/620}$) at week 5, the antibody levels in the groups immunized with *S. cerevisiae*-derived HBsAg reached the near-maximal values (up to 1.42 $OD_{450/620}$) at week 6, and then both of them remained at relatively high levels during the entire course studied, indicating the antibody response and levels of immunized mice with *P. pastoris*-derived HBsAg and *S. cerevisiae*-derived HBsAg developed relatively more slowly and lower than immunized with blood-origin HBsAg, but compared to both yeast expression systems, the immunogenicity of *P. pastoris*-derived HBsAg was obviously superior in antibody response and levels to that of the *S. cerevisiae*-derived HBsAg, demonstrating that *P. pastoris*-derived HBsAg possessed better immunogenicity.

Discussion

Recombinant HBsAg S gene has been expressed previously in the systems such as *E. coli*; yeast systems including *S. cerevisiae*, *Hansenula polymorpha*, *P. pastoris*, *Yarrowia lipolytica*; and mammalian cells [6, 8–10, 21–23]. However, the certain limitations restrict the expression of heterologous protein in mammalian cells and in *S. cerevisiae*. The expression level of HBsAg S protein in mammalian cells was very low, while the culture medium was more expensive than any other culture medium; therefore, the production cost of the HBsAg was too high to accept. The *S. cerevisiae*-based expression system for the expression of HBsAg relies on the use of the episomal vectors derived from the endogenous 2-μm circle, an autonomously replicating plasmid, which is unstable and reduces the overall yield of the desired product [13]. Moreover, plasmid maintenance requires the use of the expensive selective medium and the yield of product is usually not high with the exception of some products. The assembly of HBsAg particle appeared not to occur in *E. coli*-based expression system [15, 16].

P. pastoris offers an excellent eukaryotic expression system for the inexpensive, large-scale production of functional recombinant protein because of the strong, tightly regulated promoter elements. *P. pastoris* expression system involving *alcohol oxidase* 1 gene regulatory elements has been characterized as one of the most efficient systems among all varieties of yeast, and the gene of interest is integrated into the genome of *P. pastoris*, therefore the genome of *P. pastoris* is more genetically stable than the genome of *S. cerevisiae*. The proteins expressed in *P. pastoris* are non-hyperglycosylated and lack a terminal 1,3-linked mannose[24]. More importantly, *P.pastoris*, unlike *S. cerevisiae*, is particularly suitable for the fermentative growth. *Pichia* has the ability to reach very high cell densities during fermentation, which may improve overall protein yields when the parameters throughout the fermentation process, such as temperature, dissolved oxygen, pH, agitation, aeration, and carbon source are accurately monitored and controlled [19]. High-level expression and efficient assembly of HBsAg particles has been reported in *P. pastoris* Mut^s host; however, the time required to achieve peak product concentration is very long, for instance, fermentation of *P. pastoris* takes about 240 h for optimal production because of the low metabolic activity of the Mut^s strain during the 150–200-h methanol induction period [25]. There were some reports on the expression of recombinant HBsAg in *P. pastoris* Mut^+ strain, but the expression level was so low that only very weak band of proteins could be detected in SDS-PAGE, thus, most of these results were merely descriptive [9, 26].

To fully exploit the potential of the *Pichia* expression system for the production of recombinant proteins, we constructed the recombinant Mut^+ strains in *P. pastoris* and cultured the strains in shake flasks on trial. ELISA and SDS-PAGE showed that the recombinant HBsAg was authentically expressed in *P. pastoris* and the production of HBsAg reached 4.2 mg/l in the shake-flasks. We scaled up the production of recombinant HBsAg from shake-flasks of 15 ml to the fermentor of 5.0 l. At 78 h after induction, the biomass reached the maximum 310 OD_{600}, and the recombinant HBsAg reached up to the maximum 27 mg/l. Our expression yield of the recombinant HBsAg was higher than the previous reports [9, 26]. The recombinant strain screened in the study was multiple-copy integration of recombinant genes in *P. pastoris* and resulted in the increasing expression of the HBsAg [27, 28]. The cultures in bioreactor have been set up for 90 h for Mut^+ strains, which is shorter than the previously described fermentation protocols for the Mut^s strains to achieve the comparable, higher HBsAg production efficiency and lower production cost.

The purity and identity of the purified recombinant HBsAg were confirmed by SDS-PAGE. Its molecular weight was approximately 24 kDa corresponding to the unglycosylated S protein in size, indicating that the glycosylation had not undergone in the molecules of S protein expressed in *P. pastoris*, therefore the reduction in the immunogenicity and immunoreactivity of the recombinant HBsAg caused by the glycosylation could be avoided. CsCl sedimentation analysis indicated the recombinant HBsAg banded at a density of 1.2 g/ml, was a typical 22 nm subviral particle, and the electron microscope examination further confirmed the presence of particular structures with a diameter of approximately 22 nm. In addition, the buoyant density, shape, and size of the structures, resembled the correctly folded HBsAg, indicating that the recombinant HBsAg expressed in *P. pastoris* was the same as that expressed in *S. cerevisiae* and in mammalian cells. The competitive ELISA showed that the efficiency of competitive inhibition of the purified recombinant HBsAg was from 72% to 95%, implying that the purified HBsAg possessed the excellent immunoreactivity with HBsAb in human serum and could be substituted for blood-origin HBsAg as the diagnostic antigen for detecting HBsAb, which could eliminate the risks caused by the using of plasma-derived HBsAg, such as high cost, complicated preparation, infection, difficulty in quality control, etc.

Animal immunization showed that the immunogenicity of *P. pastoris*-derived HBsAg ranged between blood-origin HBsAg and *S. cerevisiae*-derived HBsAg, but was better than that of *S. cerevisiae*-derived HBsAg, which might be related to the relationship with the attachment to monocytes [12]. The S protein synthesized in various expression systems, such as *S. cerevisiaea*, *P. pastoris*, and insect, appeared in various glycosyglated forms. The glycosylation of S protein could result in the reduction in the immunoreaction and immunogenicity by the hiding of the epitope. The epitope mapping using different HBsAg monoclonal antibody indicated that the epitopes on P24 were quantitatively different from those on P27. The epitopes of high affinity against HBsAb had a dominant position on P24 [29].

In summary, the recombinant HBsAg expressed in *P. pastoris* we described here possessed good physical and immunological characteristics, resembled 22-nm particles present in the serum of HBV carriers, and was significantly better in the immunoreactivity and immunogenicity than *S. cerevisiae*-derived HBsAg. The recombinant S protein was safe, simple, and inexpensive, but was as efficient as the blood-origin HBsAg; therefore, it was a good substitute material for blood-origin HBsAg. The recombinant HBsAg expressed in *P. pastoris* could provide promising, inexpensive, and large-scale materials of the diagnostic reagent and vaccine for the prevention of HBV infection.

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