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Expression of hepatitis B surface antigen S domain in recombinant *Saccharomyces cerevisiae* using *GAL1* promoter

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ABSTRACT

Hepatitis B virus, a member of the hepadnavirus family, causes the acute and chronic diseases of the human liver. The S domain of hepatitis B virus surface antigen (sHBsAg) was expressed under the control of the galactose-inducible *GAL1* promoter in recombinant *Saccharomyces cerevisiae*. Batch fermentation of *S. cerevisiae* 2805/pδMFα-sHBsAg resulted in 4.92 g l^{-1} dry cell mass and 2.21 mg l^{-1} sHBsAg concentration. To improve the expression level of sHBsAg, the *pdi1* gene encoding protein disulfide isomerase was coexpressed in recombinant *S. cerevisiae*. Batch fermentation of *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI using 21 g l^{-1} glucose and 33 g l^{-1} galactose resulted in 9.75 g l^{-1} dry cell mass and 13.3 mg l^{-1} sHBsAg concentration, which were 2 and 6 times higher than those for *S. cerevisiae* 2805/pδMFα-sHBsAg, respectively. Appearance of three sHBsAgs with different molecular sizes of 24 kDa, 34 kDa and 40 kDa in immunoblot assay and their endoglycosidase treatment indicated that sHBsAg might be expressed in three types of the authentic, MFα signal sequence-containing and N-glycosylated MFα signal sequence-containing forms. Fed-batch fermentation of recombinant *S. cerevisiae* 2805 coexpressing the sHBsAg and *pdi1* genes was carried out by feeding 600 g l^{-1} glucose continuously and controlling galactose concentration at around 20 g l^{-1}. As a result, 20.2 g l^{-1} dry cell mass and 74.4 mg l^{-1} maximum sHBsAg concentration were obtained, which were 4.1 and 33.7 times higher than those for the batch fermentation of *S. cerevisiae* 2805/pδMFα-sHBsAg, respectively.

1. Introduction

Hepatitis B virus (HBV) is a member of the hepadnavirus family causing the acute and chronic diseases of the liver. HBV is an enveloped DNA virus composed of 3.2 kb DNA, core proteins and envelope proteins (Valenzuela et al., 1982). The envelope proteins referring to HBV surface antigen (HBsAg) consist of three structurally related large (L), middle (M) and small (S) proteins. These proteins share a common carboxyl terminus; the L protein contains pre-S1, pre-S2 and S domains, and the M protein possesses pre-S2 and S domains. The S protein equal to the S domain, called small HBV surface antigen (sHBsAg), consists of 226 amino acids and is antigenically most significant from vaccine development point of view (Bruss and Ganem, 1991; Shiosaki et al., 1991). There are three hydrophobic domains in sHBsAg, which are separated by two hydrophilic stretches. The proper orientation of the N-terminus of sHBsAg appears to be important to allow lateral interactions between S proteins in their assembly pathway (Bruss and Ganem, 1991). Thus sHBsAg expressed in heterologous systems should be assembled into 20–22 nm particles for high immunogenicity (Valenzuela et al., 1982). Currently, such a ~22 nm particle form of HBsAg is produced in various eukaryotic systems including CHO cell (Lee et al., 2008). Production of sHBsAg as vaccine in *Escherichia coli* has not succeeded, because sHBsAg expressed in *E. coli* is unstable and deleterious to the host (Miyanohara et al., 1983). Plant-expressed sHBsAg was retained intracellularly (Sojikul et al., 2003). Transgenic tobacco and soybean plant cells cultured in a 6-well plate format produced sHBsAg at its productivities of 0.16 mg l^{-1} d^{-1} and 1.0 mg l^{-1} d^{-1}, respectively. Suspension cultures of soybean resulted in 20–22 mg l^{-1} of maximum sHBsAg concentration (Smith et al., 2002). The production level of sHBsAg in a recombinant *Aspergillus* strain was determined to be about 0.4 mg l^{-1} (Pluddemann and van Zyl, 2003). *P. pastoris* could express and secrete sHBsAg in a particulate form and the specific content of sHBsAg was obtained under 6 μg g^{-1} in a fed-batch fermentation using glycerol and methanol (Vassileva et al., 2001). Constitutive expression of sHBsAg in recombinant *Saccharomyces cerevisiae*

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resulted in about 20 mg l^{-1} of its concentration by a fed-batch fermentation (Gu et al., 1991) and 20–22 nm particles of purified sHBsAg were observed by TEM (Vellanki et al., 2007).

S. cerevisiae is a popular eukaryotic microorganism recognized as GRAS and used as a host cell for the production of recombinant proteins. Among several constitutive and inducible expression systems, the galactose-inducible promoter systems are known to provide the tight expression control and overproduction of the target proteins (Cha et al., 2004; Park et al., 2005). In this study, recombinant *S. cerevisiae* strains were constructed for the expression of sHBsAg under the control of the *GAL1* promoter. The effects of protein disulfide isomerase (PDI) coexpression on the production and expression patterns of sHBsAg were investigated by batch and galactose-controlled fed-batch fermentations of recombinant *S. cerevisiae* strains.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae 2805 (*MATα pep4::HIS3 prb-Δ1.6 his3 ura3-52 gal2 can1*) was used as a host strain for the sHBsAg gene expression (Park et al., 2007). Plasmid pδMFα-sHBsAg containing the sHBsAg gene fused with the mating factor alpha (MFα) signal sequence was constructed on the basis of plasmid pδ-neo (Cha et al., 2004) and given by Mogam Biotechnology Institute (Yongin, Korea). The KEX2 protease recognition site (-Glu-Lys-Arg-) was added behind the MFα signal sequence for its efficient elimination. Plasmid pPDI containing the *S. cerevisiae pdi1* gene was constructed previously (Cha et al., 2006). Expression of both sHBsAg and PDI was controlled by the galactose-inducible *GAL1* promoter.

2.2. Culture conditions

YNB (0.67% yeast nitrogen base without amino acid and 2% glucose) with 2% filter-sterilized G418 sulfate known as geneticin (Q-BIO gene Inc., Carlsbad, CA, USA) was used for the maintenance of yeast transformants. Batch culture was carried out in a 2.5 l-scale jar fermentor (Bioengineering AG, Wald, Switzerland) with 1 l of YP medium (2% peptone and 1% yeast extract) containing appropriate amounts of glucose and galactose as carbon sources. An agitation speed of 500 rpm, aeration of 1 vvm and 30 °C of culture temperature were maintained throughout the culture. Medium acidity was controlled at pH 5.5 by the addition of 2N HCl or ammonia water. The fed-batch mode of operation was started after the depletion of initially added glucose. To maintain glucose concentration at a basal level, 600 g l^{-1} glucose was fed into the vessel at 1.8 g h^{-1} of feed rate. Galactose concentration during the fed-batch mode was controlled between 15 g l^{-1} and 25 g l^{-1} by intermittent feeding of 500 g l^{-1} galactose.

2.3. Quantification of sHBsAg

Enhanced chemiluminescence (ECL) western blot was carried out for the determination of sHBsAg content. After centrifugation of 1 ml culture broth, the cell pellets and supernatant were collected separately. After resuspending the pellets with 100 μl of 0.154 M PBS buffer (pH 7.0), and adding 100 μl of 2× SDS sample buffer and 0.2 g glass bead (I.D. 0.5 mm, Biospec, Bartlesville, OK, USA), the mixture was subjected to three times boiling for 4 min and vortexing for 1 min, and then centrifugation for 3 min at 4 °C. The supernatant was loaded into 12%T sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Kim et al., 2007). Proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Pall Co., East Hills, NY, USA). The membrane was treated with 5% skim milk for 1 h and washed with TBST (6 g l^{-1} Tris, 29.22 g l^{-1} NaCl and 0.1% Tween 20) for 30 min. After 2 h of incubation with a goat anti-sHBsAg antibody (Greencross Co., Yongin, Korea) diluted with 5% skim milk at 1:3000, the membrane was washed with TBST. As the second antibody, a horseradish peroxidase conjugated rabbit anti-goat IgG (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) was diluted with 5% skim milk at 1:5000. The membrane was soaked in the second antibody solution for 1 h. After washing with TBST for 30 min, sHBsAg on the membrane was visualized with the Supersignal kit (Pierce Biotech. Inc., Rockford, IL, USA) and a medical X-ray film (Kodak, Rochester, NY, USA). Protein bands were analyzed with a densitometry ImageMaster VDS system (Pharmacia Biotechnology, Sweden). Standard sHBsAg was given by Mogam Biotechnology Institute (Korea) and ECL standard was purchased from ELPIS Biotechnology Co. (Seoul, Korea). The specific content of sHBsAg was estimated by dividing sHBsAg concentration by dry cell mass.

2.4. Analysis

Dry cell mass was measured with a spectrophotometer (Ultrospec 2000, Amersham Phamacia Biotech, Uppsala, Sweden) at 600 nm. Concentrations of glucose, galactose, and ethanol were determined by an HPLC (1100LC, Agilent, Santa Clara, CA, USA) equipped with an RI detector. Samples were separated by the Carbohydrate Analysis column (Phenomenex, Torrance, CA, USA) heated at 60 °C. The mobile phase composed of 5 mM H_2SO_4 was flowed at 0.6 ml min^{-1}.

3. Results

3.1. Batch production of sHBsAg

To investigate the culture behavior of recombinant *S. cerevisiae* 2805/pδMFα-sHBsAg, batch fermentation was carried out in YP medium containing 20 g l^{-1} glucose and 33 g l^{-1} galactose (Fig. 1). After the complete depletion of glucose initially added, the cells consumed ethanol as a carbon source and were grown gradually. Galactose induced the expression of the sHBsAg gene under the *GAL1* promoter after 20 h of cultivation. Finally, 4.92 g l^{-1} dry cell mass and 2.21 mg l^{-1} sHBsAg concentration were obtained in 70 h of batch fermentation. Immunoblot analysis provided that the specific content of sHBsAg was maintained at 0.45 mg g^{-1} in the period of galactose-induced sHBsAg production.

3.2. Effects of PDI coexpression on sHBsAg expression

PDI is known to be an essential yeast protein that catalyzes the oxidation, reduction, and isomerization of disulfide bonds (Laboissiere et al., 1995). The *pdi1* gene coding for PDI was episomally coexpressed to help the disulfide bond formation between inter or intra-molecules of sHBsAg and its expression was also induced by galactose. Recombinant *S. cerevisiae* 2805 containing plasmids pδMFα-sHBsAg and pPDI was cultivated batch-wise in YP medium with 21 g l^{-1} glucose and 33 g l^{-1} galactose (Fig. 2). Glucose was consumed rapidly in 11 h of culture. Galactose added initially and ethanol produced from glucose were used simultaneously by the cells, of which dry mass reached 9.75 g l^{-1} in 82 h of culture. Different from the case without PDI coexpression, about 17.9 g l^{-1} galactose was consumed and strongly induced the sHBsAg expression. As illustrated in the immunoblotting of sHBsAg (Fig. 2), the density analysis of sHBsAg present at the same location of the standard showed an average sHBsAg content of 0.58 mg g^{-1} which was 1.3 times higher than the case without PDI coexpression. Interestingly, two new protein bands were observed between the monomer

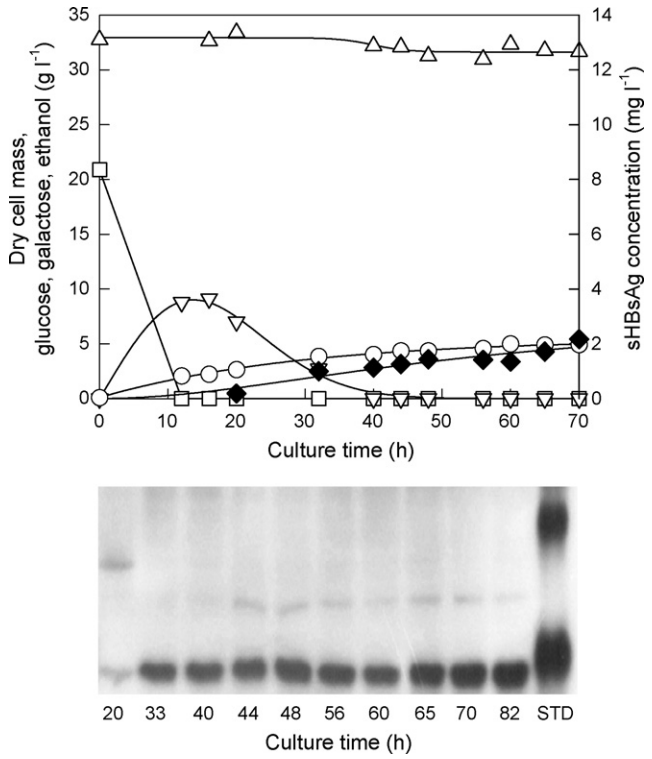

Fig. 1. Batch fermentation of recombinant *S. cerevisiae* 2805/pδMFα-sHBsAg in YP medium containing 20 g l^{-1} glucose and 33 g l^{-1} galactose at 30 °C, 500 rpm and 1 vvm. Symbols denote as follows; dry cell mass (○), glucose concentration (□), galactose concentration (△), ethanol concentration (▽), and sHBsAg concentration (♦). The cell suspension was diluted appropriately to have the identical optical density of 10 and used for immunoblot assay. Concentration of the standard sHBsAg was 5 mg l^{-1}.

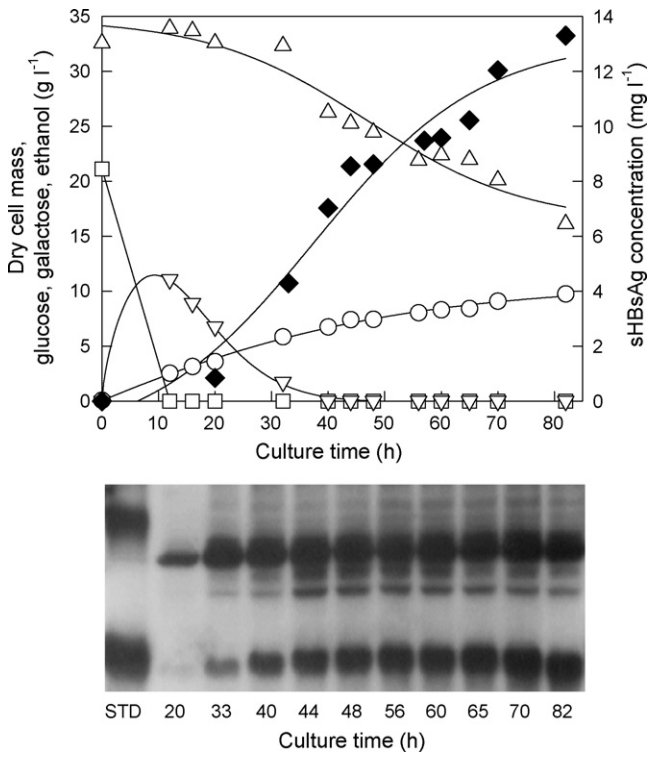

Fig. 2. Batch fermentation of recombinant *S. cerevisiae* 2805 transformed with pδMFα-sHBsAg and pPDI vectors in YP medium containing 21 g l^{-1} glucose and 33 g l^{-1} galactose at 30 °C, 500 rpm and 1 vvm. Symbols denote as follows; dry cell mass (○), glucose concentration (□), galactose concentration (△), ethanol concentration (▽), sHBsAg concentration (♦). The cell suspension was diluted appropriately to have the identical optical density of 10 and used for immunoblot assay. Concentration of the standard sHBsAg was 5 mg l^{-1}.

(lower case) and dimmer (upper case) of the sHBsAg standard, suggesting that the two proteins seemed to be unprocessed forms of sHBsAg. At the end of fermentation, these unprocessed sHBsAgs occupied about 50% of total sHBsAg content. Finally, 13.3 mg l^{-1} sHBsAg was obtained in the batch cultivation.

3.3. Expression patterns of sHBsAg

As shown in Fig. 2, sHBsAgs with three different molecular sizes were observed by the immunoblot assay of the intracellular proteins. The authentic sHBsAg was mainly expressed in *S. cerevisiae* 2805/pδMFα-sHBsAg. On the other hand, the authentic and unprocessed sHBsAgs with different molecular weights were equally produced in *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI. To explore the expression patterns of three different sHBsAgs, the sHBsAg sample obtained in the batch fermentation of *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI was treated with endoglycosidase H (endo H) (Fig. 3). Endo H is able to cleave off high mannose- and some hybrid-oligosaccharides in N-glycoproteins (Baldari et al., 1987; Lee et al., 2003). Immunoblot analysis of sHBsAg before the endoH treatment (Fig. 3, lane 1) showed that the actual molecular weights of the three sHBsAgs were estimated to be about 24 kDa, 34 kDa and 40 kDa. The theoretical molecular weights of the authentic sHBsAg and MFα signal sequence-containing sHBsAg are calculated to be 25.4 kDa and 34.3 kDa by using the ExPASy Proteomic Server, respectively. By the endoH reaction, the density of a protein band at 40 kDa decreased considerably and the other protein band at 34 kDa enlarged concomitantly. Meanwhile, the band density of the authentic sHBsAg located at 24 kDa did not increase but decreased by a dilution effect mediated by the endoH reaction. These results by the endoH treatment suggested that sHBsAgs with

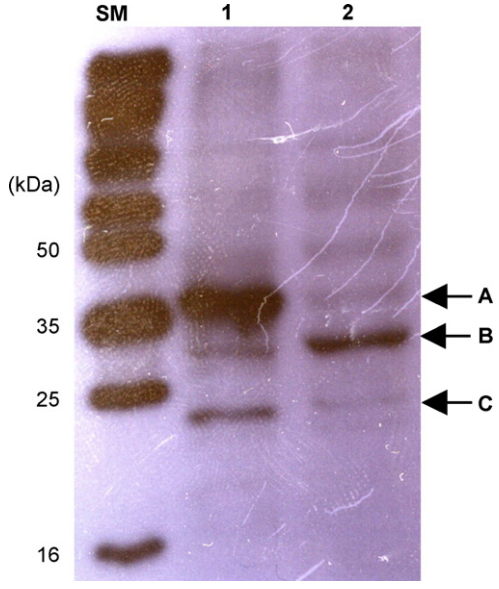

Fig. 3. EndoH treatment of sHBsAg expressed in recombinant *S. cerevisiae* transformed with plasmid pδMFα-sHBsAg and pPDI. Lane 1 and 2 indicate the samples before and after the endoH treatment, respectively. SM shows the protein size marker. Alphabetic letters of A, B and C beside the arrows indicate the protein bands of the N-glycosylated MFα signal sequence-containing sHBsAg, the MFα signal sequence-combining sHBsAg and authentic sHBsAg, respectively.

Table 1
Summarized results of sHBsAg production in batch and fed-batch fermentations of recombinant *S. cerevisiae* 2805 strains harboring the sHBsAg gene.

Plasmid	Culture type	Dry cell mass ($g\,l^{-1}$)	Max. sHBsAg concentration ($mg\,l^{-1}$)	Max. specific sHBsAg content ($mg\,g^{-1}$)
pδMFα-sHBsAg	Batch	4.92	2.21	0.45
pδMFα-sHBsAg + pPDI	Batch	9.75	13.3	1.36
pδMFα-sHBsAg + pPDI	Fed-batch	20.2	74.4	5.63

34 kDa and 40 kDa of molecular weight seemed to be fused with the MFα signal sequence and the N-glycosylated MFα signal sequence, respectively.

3.4. Fed-batch production of sHBsAg

To obtain a large amount of sHBsAg, fed-batch fermentation of recombinant *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI was carried out in YP medium initially containing $19\,g\,l^{-1}$ glucose and $32\,g\,l^{-1}$ galactose (Fig. 4). After the depletion of glucose, the concentrated glucose solution was fed continuously to maintain a basal level. To control galactose concentrations between $15\,g\,l^{-1}$ and $25\,g\,l^{-1}$, the galactose solution was supplied intermittently after 32 h. The recombinant cells utilized glucose, galactose and ethanol as carbon sources and a final dry cell mass of $20.2\,g\,l^{-1}$ was obtained. For sHBsAg production, sHBsAg expression was induced by galactose consumption in 39 h of culture to reach its maximum concentration of $74.4\,mg\,l^{-1}$. But sHBsAg content was reduced gradually after 39 h and $24.7\,mg\,l^{-1}$ sHBsAg concentration was obtained at the end of fermentation. Immunoblot analysis of intracellular sHBsAg exhibited that the amount of sHBsAg at 24 kDa increased during the galactose-induced condition but the protein band of sHBsAg at 40 kDa smeared after 32 of cultivation. A decrease in sHBsAg concentration might be ascribed to the degradation of sHBsAg containing the N-glycosylated MFα signal sequence after the middle of fed-batch fermentation.

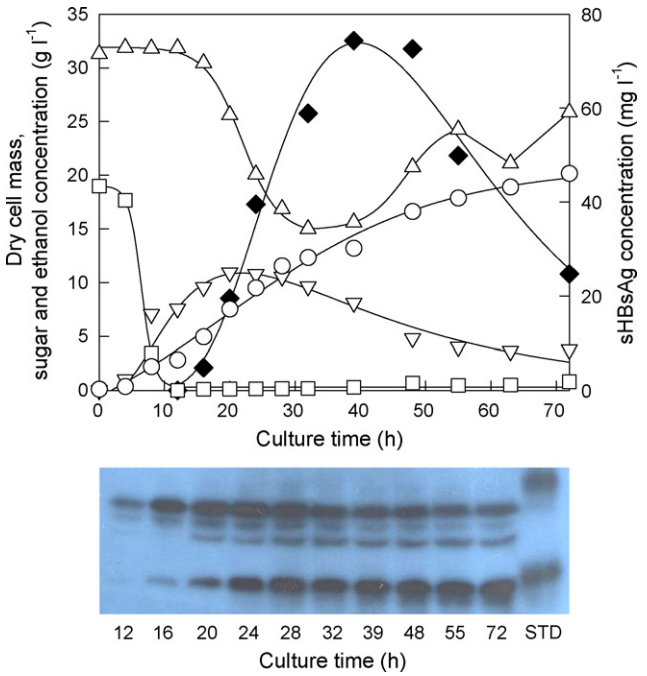

Fig. 4. Fed-batch fermentation of recombinant *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI in YP medium containing $19\,g\,l^{-1}$ glucose and $32\,g\,l^{-1}$ galactose at 30 °C, 500 rpm and 1 vvm. After complete deletion of glucose initially added, a glucose solution of $600\,g\,l^{-1}$ was fed constantly at $1.8\,g\,h^{-1}$ of feed rate. A galactose solution of $500\,g\,l^{-1}$ was fed intermittently in order to control the galactose level between $15\,g\,l^{-1}$ and $25\,g\,l^{-1}$ in culture broth. Symbols denote as follows; dry cell mass (○), glucose concentration (□), galactose concentration (△), ethanol concentration (▽), sHBsAg concentration (♦). One milliliter of culture broth was collected and its pellets were used for immunoblot assay. Concentration of the standard sHBsAg was $5\,mg\,l^{-1}$.

4. Discussion

The surface antigen of hepatitis B virus causing the chronic liver diseases is composed of three related proteins. Among them, the S protein (sHBsAg) is shared by the two proteins and known to be most important for vaccine development. By batch and fed-batch fermentations of recombinant *S. cerevisiae* coexpressing the sHBsAg and *pdi1* genes under the control of the *GAL1* promoter, mass-production of sHBsAg was successfully carried out in this study.

Protein folding in the lumen of ER, where the efficiencies of disulfide bond formation and folding are interrelated, has been known to be a rate-limiting step in protein production and secretion (Son et al., 2007; Tu et al., 2000). Several reports for improving the capacity of protein production and secretion in recombinant *S. cerevisiae* suggested that ER-resident molecular chaperones including protein disulfide isomerase (PDI) facilitated disulfide bond formation (Tu et al., 2000; Xiao et al., 2004). PDI has both disulfide isomerase and oxidoreductase activities, and also rearranges pre-existing disulfide linkages. It was reported that coexpression of PDI increased the production of human apolipoprotein(a) KV kringle fragment and beta-glucosidase in *S. cerevisiae* (Cha et al., 2006; Powers and Robinson, 2007), and an antibody Fab fragment against human immunodeficiency virus type 1 in *Pichia pastoris* (Gasser et al., 2007). This positive effect of PDI coexpression was realized in sHBsAg production in recombinant *S. cerevisiae*. PDI coexpression not only increased the amount of the authentic sHBsAg with 24 kDa of molecular weight but also allowed the cells to newly produce unprocessed or modified sHBsAgs with 34 kDa and 40 kDa. Endo H treatment with the crude extract containing the three types of sHBsAgs revealed that sHBsAgs with 34 kDa and 40 kDa were probably fused with the MFα signal sequence and N-glycosylated MFα signal sequence, respectively. In previous studies, the authentic form of sHBsAg was visualized at 23–25 kDa of molecular weight in an immunoblotting analysis (Huovila et al., 1992; Smith et al., 2002). Considering no increase in the 24 kDa protein band as shown in Fig. 3, the authentic sHBsAg expressed in recombinant *S. cerevisiae* was not N-glycosylated in spite of the presence of two N-glycosylated sites (Lee et al., 2003; Park et al., 2005). Most of HBsAg without its presumptive precursor similar to a leader sequence was not glycosylated when expressed in recombinant yeast (XV610-8C). Even though sHBsAg was N-glycosylated or not, it could form the typical structure of the 22-nm particle with strong immunogenicity (Valenzuela et al., 1982). Meanwhile, the HBV large surface antigen containing the S domain is not O-glycosylated when expressed in recombinant *S. cerevisiae* (Park et al., 2005). The reason for the accumulation of the recombinant sHBsAg combined with the N-glycosylated MFα signal sequence is not clear. However, it is likely that PDI coexpression may cause a rate-imbalance between sHBsAg overexpression and MFα signal sequence cleavage mediated by endogenous KEX2 protease. The KEX2 protease cleavage site was designed to be located between the MFα signal sequence and sHBsAg polypeptide when constructing plasmid pδMFα-sHBsAg. For more production of the authentic sHBsAg, a methanol-induced production of sHBsAg in *P. pastoris* suggests that a slower growth

rate is favorable for its efficient processing and particle assembly (Vassileva et al., 2001).

The results of sHBsAg production in batch and fed-batch fermentations were summarized in Table 1. In batch fermentation, PDI coexpression gave higher values of cell growth and sHBsAg production; 2.0 and 6.0-fold increases in dry cell mass and sHBsAg concentration compared with no expression of PDI. The strategies of feeding glucose continuously and controlling galactose concentration between 15 g l^{-1} and 25 g l^{-1} in the fed-batch fermentation enhanced the culture performance considerably. The fed-batch fermentation of *S. cerevisiae* 2805/pδMFα-sHBsAg + pPDI resulted in 4.1 and 33.7 times higher dry cell mass and sHBsAg concentration than the batch fermentation without PDI coexpression (Fig. 1), respectively. SDS-PAGE analysis of intracellular proteins collected in the fed-batch fermentation showed the density change of sHBsAg bands according to their forms (Fig. 4). More accumulation of the authentic sHBsAg and reduction of sHBsAg with 40 kDa suggested that the authentic sHBsAg was expressed stably in the recombinant *S. cerevisiae* strain whereas unprocessed sHBsAg containing the N-glycosylated MFα signal sequence seemed to be degraded by non-specific serine and aspartyl proteases (Gimenez et al., 2000). Similar proteolytic degradation was observed when HBV pre-S antigen fused with the killer toxin signal sequence was expressed in recombinant *S. cerevisiae* (Lee et al., 2003).

More research is in progress for the further improvement of sHBsAg production in recombinant *S. cerevisiae* such as the coexpression of KEX2 protease and other chaperones, which are expected to overexpress the authentic form of sHBsAg by minimizing the production of unprocessed sHBsAg.

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