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Expression of Hepatitis B Virus S Gene in *Pichia pastoris* and Application of the Product for Detection of Anti-HBs Antibody

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Antibody to hepatitis B surface antigen (HBsAb) is the important serological marker of the hepatitis B virus (HBV) infection. Conventionally, the hepatitis B surface antigen (HBsAg) obtained from the plasma of HBV carriers is used as the diagnostic antigen for detection of HBsAb. This blood-origin antigen has some disadvantages involved in high cost, over-elaborate preparation, risk of infection, *et al.* In an attempt to explore the suitable recombinant HBsAg for the diagnostic purpose, the HBV S gene was expressed in *Pichia pastoris* and the product was applied for detection of HBsAb. Hepatitis B virus S gene was inserted into the yeast vector and the expressed product was analyzed by sodium dodecyl sulphate polyacrolamide gel electrophoresis (SDS-PAGE), immunoblot, electronic microscope and enzyme linked immunosorbent assay (ELISA). The preparations of synthesized S protein were applied to detect HBsAb by sandwich ELISA. The S gene encoding the 226 amino acid of HBsAg carrying a hexa-histidine tag at C terminus was successfully expressed in *Pichia pastoris*. The His-Tagged S protein in this strain was expressed at a level of about 14.5% of total cell protein. Immunoblot showed the recombinant HBsAg recognized by monoclonal HBsAb and there was no cross reaction between all proteins from the host and normal sera. HBsAb detection indicated that the sensitivity reached 10 mIu (micro international unit)/ml and the specificity was 100% with HBsAb standard of National Center for Clinical Laboratories. A total of 293 random sera were assayed using recombinant S protein and a commercial HBsAb ELISA kit (produced by blood-origin HBsAg), 35 HBsAb positive sera and 258 HBsAb negative sera were examined. The same results were obtained with two different reagents and there was no significant difference in the value of S/CO between the two reagents. The recombinant HBV S protein with good immunoreactivity and specificity was successfully expressed in *Pichia pastoris*. The reagent for HBsAb detection prepared by *Pichia pastoris*-derived S protein showed high sensitivity and specificity for detection of HBsAb standard. And a good correlation was obtained between the reagent produced by recombinant S protein and commercial kit produced by blood-origin HBsAg in random samples.

Keywords: ELISA, HbsAg, Hepatitis B virus S protein, *Pichia pastoris*

Hepatitis B caused by hepatitis B virus (HBV) contributes significantly to the worldwide incidence of liver diseases. Human bodies immunized by HBV surface antigen (HBsAg) can generate a specific protective antibody--hepatitis B virus surface antibody (HBsAb). Immunoassays of HBsAb have been used to monitor the successful rate of hepatitis B vaccination, and the convalescence and recovery of hepatitis B infected individuals. HBsAb can be detected by a variety of serological tests, such as enzyme immunoassay (EIA), radio immunoassay (RIA), chemiluminescence immunoassay (CLIA), and time-resolved fluoroimmunoassay (TRFIA). The conventional antigen of diagnostic reagent for immunoassay of HBsAb is obtained from the plasma of high titer HBV carriers. Although the plasma-derived antigen is efficacious, its production depends on the availability of suitable human HBV carriers. With the popularization of HBV vaccination, it becomes more and more difficult to find highly viremic individuals to obtain the sera which contain high titre HBsAg. In addition, the nature of blood-origin HBsAg requires each antigen to be

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stringently inactivated, highly purified, and subject to extensive safety testing for the presence of human infectious agents. The disadvantages associated with its over-elaborate preparation as well as high cost lead to the development of an alternative to the blood-origin HBsAg based on recombinant DNA technology. Today a variety of cell systems are able to synthesize HBsAg. The recombinant HBsAg produced by mammalian and yeast cells has been used as HBV vaccines for immunization purposes successfully (Valenzuela *et al*.,1982; Shouval *et al*., 1994; Raz *et al*., 1996). However, there have been few reports on the recombinant HBsAg that is used for immunoassay instead of blood-origin HBsAg. The requirement for immunoassay reagent differs from that for vaccines. The immunogenicity is the most important for vaccines because of the purpose for initiation and enhancement of specific immune response. But the quality of immunoassay, such as accuracy, sensitivity, specificity as well as linearity, depends on the efficiency of specific binding between antigen and antibody so that the immunoreactivity of antigen is crucial for immunoassay reagent. We have used some commercial HBV recombinant vaccines as the diagnostic antigen for immunoassay of HBsAb but the results are not satisfactory, especially in their sensitivity.

HBsAg proteins are encoded by two adjacent regions of the HBV S open reading frame (S ORF), the pre-S and the S region. S protein of 226 aa, which is the major protein constituent of the HBV envelope, is encoded by the S gene, a 'middle' gene carrying 55 aa at the N-terminus encoded by the pre-S2 portion of the pre-S region and a 'large' protein encoded by the whole ORF (pre-S1, pre-S2 and S, 389aa) which are the minor envelope constituents (Stibbe *et al*., 1983; Heermann *et al*., 1984; Neurath *et al*., 1986) (Fig. 1).

S protein has the highest density of epitopes against HBsAb in the three HBsAg molecules. The affinity between polyclonal antibody and multivalence antigen depends on the density of epitopes of antigen and the higher the density, the higher the affinity. High affinity between antibody and antigen is crucial for increasing the sensitivity of immunoassay. The epitopes of the Pre-S1 and Pre-S2 proteins which can be recognized by B cell and T cell receptor, can enhance the immunogenicity of HBsAg (Neurath *et al*., 1986; Milich *et al*., 1985; Budkowska *et al*., 1985). As the diagnostic antigen, S protein possibly has an advantage because of its higher reactivity to polyclonal HBsAb. Therefore, we focused our studies on S protein. There are two kinds of epitopes in protein antigen, continuous epitopes and discontinuous epitopes. Continuous epitopes consist of a number of consecutive residues in the protein sequence and may be part of a large so-called discontinuous epitope composed of residues distant in the sequence that are brought together by the folding of the peptide chain (Berzofsky, 1985). It is well known that the choice of expression systems (host, plasmid) is the key to successful production of a correctly folded protein. In this paper, we described the characteristics of S protein with the fusion tails of C-terminal myc and hexa-histidine tag expressed by *Pichia pastoris* and the application of the recombinant antigen in immunoassay of HBsAb.

Materials and Methods

Specimens The HBsAb standard panel was from the National Center for Clinical Laboratories (NCCL, China). The random samples were collected from the clinical department of The Third Affiliated Hospital of Sun Yat-sen University.

Template, vector and strains A plasmid containing hepatitis B virus genome (PHBV1-adw) was kept by our laboratory. The vector containing 3' terminal thymidine at both ends (pGEM-T) was purchased from Promega (Madison, USA). The yeast vector expressing the protein intracellularly (pPICZB) and yeast strain KM71H (mut^s, Arg^+) were purchased from Invitrogen and used following the instructions of the manufacture. E.coli JM109 (Invitrogen Co., San Diego, USA) was used for all plasmid manipulation. Zeocin™ (a formulation of phleomycin D1, a basic, water-soluble, copper-chelated glycopeptide isolated from Streptomyces verticillus) (Invitrogen Co.) was employed for positive selection in E. coli and Pichia. Pastoris.

Enzymes and chemicals All restriction enzymes and T_4 DNA ligase were purchased from New England Biolabs. Peptone and YNB were purchased from Difco.

Construction of recombinant yeast Using a pair of primers (sense: 5'-GGAATTCATGGAGAACATCACATCAGG-3'; antisense: 5'-CG GCGGCCGCAATGTATACCCAAAGAC-3':), HBV S gene encoding the 'major' 226-aa envelope protein was amplified by polymerase chain reaction (PCR) from plasmid PHBV1 and inserted into PGEM-T vector by T/A cloning strategy, leading to subcloning

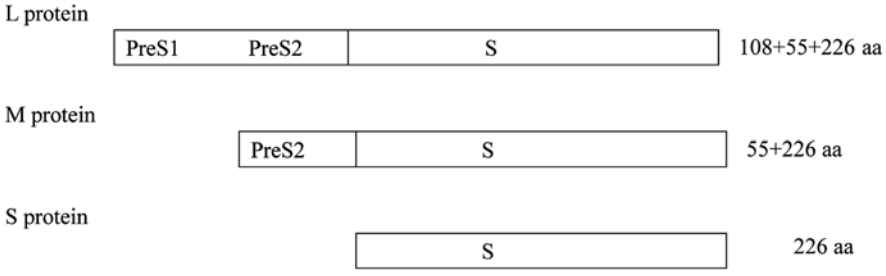

Fig. 1. HBsAg encoded by S, PreS1, PreS2 region of HBV ORF S ("L", "M", "S" protein).

vector PGEM/Sag (containing S gene). By restriction enzyme analysis and sequencing, the PGEM/SAg was confirmed to contain S gene which was then inserted in the correct orientation between the *Eco*RI and *Not*I sites of pPICZB leading pPICZB-SAg. The pPICZB-SAg was transformed into *E. coli*. After transformation, plate transformation was mixed onto low salt LB plates(1% Tryptone (OXOID), 0.5% Yeast Extract(OXOID), 0.5% NaCl) with 25 mg/L Zeocin™ and Zeocin™ resistant colonies were selected. Zeocin™ resistant transformants were inoculated into 2 ml low salt LB medium with 25 mg/L Zeocin™, grown overnight at 37°C with shaking. Plasmid DNA was isolated by miniprep for restriction analysis and sequencing to confirm the gene of interest in frame with the C-terminal peptide. The construct pPICZB-SAg, linearized by cleaving with *Sac*I, was transformed into the KM71H yeast by electroporation to generate Mut^s transformants. The transformants were plated on yeast extract peptone dextrose medium with sorbitol (YPDS) (1% yeast extract, 2% peptone, 2% dextrose, 1 M Sorbitol, 2% agar) containing Zeocin™ (100 mg/L) for positive selection. The transformants grown on plates were confirmed by PCR to contain the pPICZB-SAg.

Expression of S protein The recombinant yeasts confirmed to contain the S protein coding sequence was cultured in 100 ml buffered glycerol-complex medium (BMGY) (1% yeast extract, 2% peptone, 100 mM potassium phosphate, PH 6.0, 1.34% yeast nitrogen base, 1% glycerol, 0.0005 biotin) and grown in flask at 30°C with shaking for 2 days until A_{600} = 6. Then the cells were harvested, subsequently resuspended in 20 ml buffered methanol-complex medium (BMMY) (1% yeast extract, 2% peptone, 100 mM potassium phosphate, PH 6.0, 1.34% yeast nitrogen base, 0.5% methanol, 0.0005 biotin) and induced by adding 100% methanol to a final concentration of 0.5% for 4 days.

Characterization of the S protein of HBV Cells were harvested from the flask, washed and resuspended, and lysed by digestion of lyticase and agitated with glass beads. After centrifugation of the lysate, the recombinant HBsAg that was recovered in the supernatant was processed on Coomassie-stain sodium dodecyl sulphate polyacrolamide gel electrophoresis (SDS-PAGE) and thin-layer protein scanning of SDS-PAGE gel for quantitative measurement. The proteins in the identical supernatant were transferred from 12% SDS gel to polyinylidene difluoride (PVDF) membrane (Milipore, Massachasett,USA) to be used for the monoclonal HBsAb against HBsAg. Thirty serum samples which were confirmed to be negative for serological markers were indicative of hepatitis B infection by microparticle enzyme immunoassay (Axsym System, Abbot Diagnostic Division, USA). The alkaline phosphatase-conjugated rabbit anti-mouse IgG and anti-human IgG were used respectively for immunoblot. The titre of recombinant HBsAg in clarified lysate was assayed with sandwich ELISA. The lysate containing S protein was layered on the top of a sucrose gradient formed in polycarbonate tube [10 ml of 60% (w/v) sucrose, 5 ml 50% (w/v) sucrose, 5 ml 40% (w/v) sucrose, 5 ml 30% (w/v) sucrose and 5 ml 20% (w/v) sucrose in Tris-HCl 50 mM (pH 7.8)] and spun at 75,600 g for 21 h at 4°C. The fractions were detected by ELISA respectively and the HBsAg positive fraction was analyzed under an electronmicroscope.

Purification and horseradish peroxidase (HRP)-conjugation of recombinant S protein The S protein in the lysate was salted out by 40% $(NH_4)_2SO_4$ and purified with hexahistidine tag at C terminus of the recombinant fusion protein on the metal-chelating resin (ProBond, Invitrogen, Ni-NTA), following the manufacturer's recommendation. Recombinant S protein was conjugated with HRP according to the reference (Ivan *et al*., 1981).

Coating and detection of HBsAb The purified S protein was dissolved in 0.045 mol/L phosphate-buffered saline (PBS, pH 7.4). The polystyrene microplate was coated with the S protein at 2 μg per well. The unoccupied protein binding sites of wells were blocked with 10% normal goat serum in PBS containing 0.3% Tween-20.

The coated recombinant S protein served as the solid-phase antigen. The HRP-labeled recombinant S protein (zhongshan Bioengineering co.) was utilized as a probe to detect HBsAb (HBsAb standard and random clinical sera) by one-step sandwich ELISA. Tetra-methyl-benzidine (TMB) (sigma) was used as the substrate for colorimetric detection, and OD (optical density) value of absorbance was measured at 645 nm. Optimal working concentrations of both solid-phase antigen and the HRP-labeled recombinant S protein were determined based on the HBsAb standard of the National Center for Clinical Laboratories.

Statistical analyses Randomized paired t-test was used to analyze the results. Analyses were performed using the SPSS software package.

Results

Molecular cloning and expression of S protein The restriction analysis and DNA sequencing confirmed that the HBV S gene was amplified from plasmid PHBV1 by PCR and inserted to yeast expression vector pPICZB in the correct orientation (Fig. 2).

Coomassie-stained SDS-PAGE revealed a band of apparent molecular weight 26,000 in lysate of KM71H yeast carrying pPICZB-SAg induced by methanol, corresponding in size to the unglycosylated form of S protein with fusion tails of C-terminal myc and polyhistidine (6 × His) (myc and polyhistidine tag contribute molecular weight 2,100 to recombinant protein). The expression level in this strain was about 14.5% of the total cell protein (Fig. 3). No such bands could be shown in non-induced transformants or in culture medium.

Characterization of recombinant S protein Immunoblot showed a single band (molecular weight 26,000) identified by monoclonal HBsAb, corresponding in size to the unglycosylated fusion S protein. No higher molecular bands could be detected (Fig. 4). There was no cross reaction between all proteins derived from *Pichia pastoris* and normal sera. The titre of the recombinant HBsAg in the cell lysate was 1 : 25,600 using ELISA kit.

The recombinant S protein in the HBsAg positive fractions

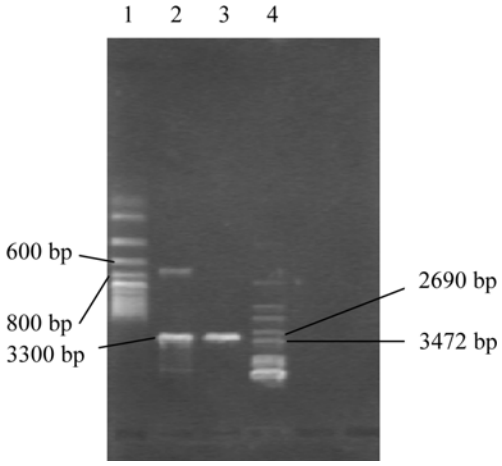

Fig. 2. Restriction endonuclease map of recombinant plasmid pPICZB-sAg. lane 1: 200 bp DNA Ladder; lane 2: recombinant plasmid pPICZB-sAg/EcoRI and NotI; lane 3: pPICZB plasmid/ EcoRI and NotI; lane 4: λ-ECOT14I digest Marker.

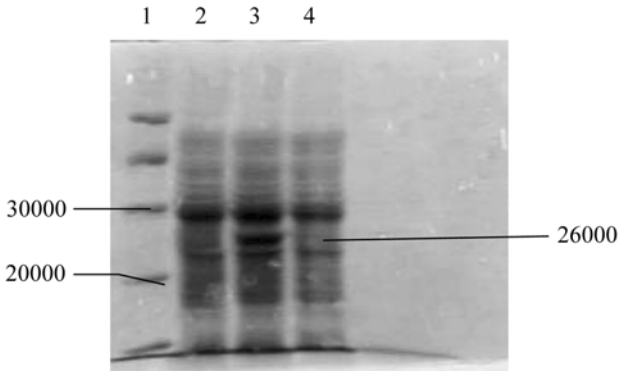

Fig. 3. SDS-PAGE analysis of recombinant protein. Lane 1: protein marker; lanes 2: Cell lysate of non-induced KM71H/ pPICZB-sAg; lanes 3: Cell lysate of induced KM71H/pPICZB-sAg strain, S protein expressed in 26,000 Da; lane 4: Cell lysate of induced KM71H/pPICZB.

on 20%-30% (w/v) sucrose gradient by ultracentrifugation formed spherical particles with a diameter of 28-30 nm (Fig. 5), which appear somewhat larger than blood-origin HBsAg spheres of 20-22 nm.

Purification of recombinant S protein Coomassie-stained SDS-PAGE revealed a band (molecular weight 26,000) of recombinant yeast product purified by the metal-chelating resin (Ni-NTA), corresponding in size to the recombinant fusion S protein and the purity was over 87% (Fig. 6).

Results of HbsAb detection By using S protein synthesized preparations as the coated antigen and the conjugate to detect HBsAb standard, the results indicated that the sensitivity reached 10 mIu (microinternational unit)/ml and the specificity was 100%. The 293 random sera were assayed using

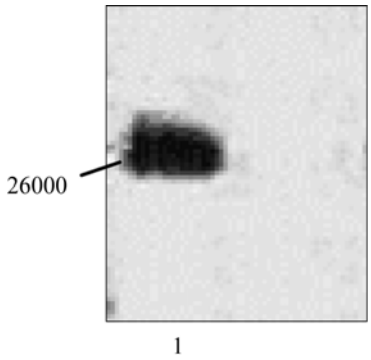

Fig. 4. Immunoblot analysis of recombinant protein. Lane 1: recombinant S protein identified by monoclonal HBsAb.

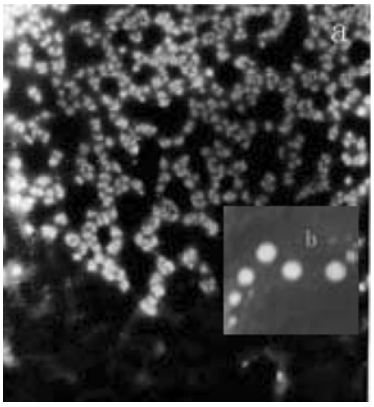

Fig. 5. Electron micrograph of recombinant protein. (a) Magnification, 52,000. (b) Magnification,73000. Sample was negatively stained by uranyl acetate.

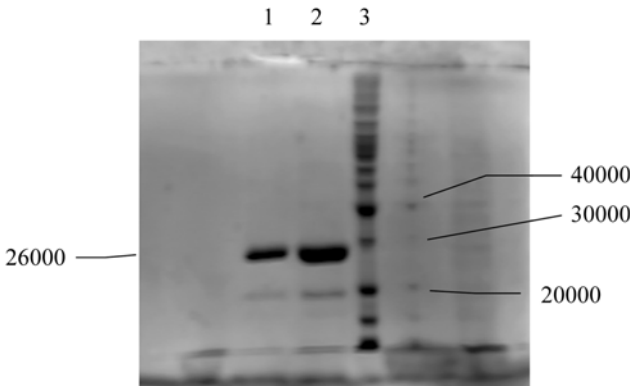

Fig. 6. Purification of recombinant S protein. Lane 1,2: recombinant fusion S protein purified by Ni-NTA; Lane 3: protein marker.

recombinant S protein and a commercial HBsAb ELISA kit (produced by blood-origin HBsAg, Zhongshan Bio-Tech Limited Company) and 35 HBsAb positive sera and 258 HBsAb negative sera were examined. The same results were obtained with two different reagents and there was no significant difference in the value HBsAb S/CO ($t = 0.652$, $p > 0.05$) between the two reagents (Table 1).

Table 1. Comparison of the result of the HBsAb were assayed using recombinant S protein with a commercial HBsAb ELISA kit in 293 random sera*

	No. Samples Tested	No. Positive	No. Negative	S/CO
Recombinant S protein	293	35	258	1.10 ± 1.75
Commercial HBsAb ELISA kit	293	35	258	1.08 ± 1.82
T				0.652
P				>0.05

*S/CO values are expressed as mean ± standard deviation.

Discussion

The necessary nucleotide sequence of the HBsAg coding region was chosen according to requirements for immunoassay reagents. All serotypes of HBsAg contain only 'a' common epitope which consists of 124-137aa and 139-147aa of the S protein sequence so that S protein as the diagnostic antigen has to be required for the detection of all serotypes of HBsAb. In ELISA reactive system, the antigen or antibody molecules have previously been coated on the solid phase resulting in much higher concentration of detective target in solid phase than that in liquid phase because of the specific binding between antibody and antigen. Once the detective targets were captured to solid phase by the first step of immune response, the secondary binding between conjugate probe and detective target took place easily because the working concentration of conjugate probe had been previously optimized. As indicated above, it is the rule of heterogeneous EIA. In this test, the sensitivity of detection was determined by whether HBsAg on the solid surface could efficiently capture HBsAb in the sample. S protein is the major and the smallest protein of HBsAg which has higher density of epitope against HBsAb than that of the 'middle' and 'large' proteins. As compared with the use of 'middle' and 'large' proteins , using only S protein as coated antigen can enable the solid surface of microplate to possess more antigenic sites per area. The high density of epitope enables the solid phase antigen to possess stronger ability to capture HBsAb. There are two forms of S proteins, non-glycosylated form protein 24(P24) and its glycosylated derivative glycoprotein 27(GP27) (molecular weight of 24,000 and 27,000, respectively) (Shil *et al*., 1977; Peterson, 1981). The S proteins synthesized by various cell expression systems, such as *Pichia pastoris*, Saccharomyces cerevisiaea, insect expression system, etc, appear in various glycosylated forms which affect the immunoreactivity of the product. The glycosylation of S protein always occurs in 146 Asn within the sequence of 'a' common epitope resulting in the hiding of the epitope. The epitope mapping using different HBsAb monoclonal antibodies indicated that the epitopes on P24 were quantitatively different from those on GP27. The epitopes of high affinity against HBsAb had a dominant position in P24 (Zuckerman *et al*., 2003).

The methylotrophic yeast *Pichia pastoris* has been found to be an efficient system for high-level production of foreign proteins (Ouyang *et al*., 2003; Zhu *et al*., 2003; Murasugi *et al*., 2003; Chen *et al*., 2004; Boue *et al*., 2004; Lin *et al*., 2004; Murthy *et al*., 2004; Lenassi *et al*., 2004). The proteins produced by *Pichia pastoris* have the correct folding that form the similar discontinued epitopes to parent proteins. In comparison with Saccharomyces cerevisiae, Pichia may not hyperglycosylate in proteins synthesized. Both Saccharomyces cerevisiaea and *Pichia pastoris* have a majority of N-linked glycosylation of the high-mannose type, but the length of the oligosaccharide chains added post-translationally to proteins in Pichia (average 8-14 mannose residues per side chain) is much shorter than that in Saccharomyces cerevisiae (50-150 mannose residues) (Kalidas *et al*., 2001; Kim *et al*., 2004). Furthermore, Saccharomyces cerevisiae core oligosaccharides have terminal α1,3 glycan linkages whereas *Pichia pastoris* does not. It is believed that the α1,3 glycan linkages in glycosylated proteins produced from Saccharomyces cerevisiae are primarily responsible for the alteration of the genetically antigenic nature of the primary proteins. The proteins synthesized by *Pichia pastoris* may resemble the glycoprotein structure of higher eukaryotes (Bretthauer *et al*., 1999). Additionally, *Pichia pastoris* has other advantages, such as high level and stability of protein expression and easy manipulation, which are also important for large-scale biological production. The recombinant protein for preparation of probe (conjugate) has to be purified, otherwise HRP conjugated to the recombinant protein will be resolved by the protease of lysate. Fusion tails, such as hexa-histidine and GST, have been widely used for purification of recombinant protein by immobilized metal ion affinity chromatography (IMAC). This approach promotes efficient recovery and purity of recombinant protein from crude cell extracts.

For the reasons presented above, we chose the S gene of HBV as the gene of interest and the *Pichia pastoris* as the host for expression. The S protein of HBV was expressed in Pichia pastorios efficiently. The molecular weight of product was approximately 26,000 corresponding in size to the fusion unglycosylated S protein(Vanlandschoot *et al*., 2003). No higher molecular bands could be detected by immunoblot, indicating that the molecules of S protein were not glycosylated under these conditions. The unglycosylated species of HBsAg avoid reduction of the immunoreactivity caused by glycosylation. Since there is no reaction between all proteins from *Pichia Pastoris* (including recombinant and

native proteins) and the normal human serum, there will be no false positive result using this recombinant protein for the immunoassay with human serum samples. The titre of the recombinant HBsAg in the cell lysate showed the good immunoreactivity with HBsAb by ELISA. The 14.5% expression level of the total proteins satisfies the large-scale biological production. Using recombinant S protein as the ELISA solid phase antigen and conjugate probe, the results of detecting HBsAb standard indicated that the sensitivity reached 10 mIu/ml, the specificity 100% and nice duplication and linearity were achieved simultaneously. A good correlation was found between the reagent produced by recombinant S protein and that produced by blood-origin HBsAg in the randomly selected samples. The reagent produced by recombinant S protein of HBV met with the requirements of quality for immunoassay of HBsAb.

We have described the expression of the S protein (adw serotype) of HBV in *Pichia pastoris* and its application for detection of HBsAb by ELISA. The data shown in this paper indicate that this reagent produced by recombinant S protein can meet the quality control requirements of immunoassay of HBsAb in sensitivity, specificity, accuracy and linearity. Because the S protein synthesized appears to possess high degree of immunoreactivity and specificity, use of S protein synthesized as the diagnostic antigen, instead of blood-origin HBsAg, might be able to detect HBsAb. The yeast-derived S protein offers the possibility of an alternative to the blood-origin HBsAg as the HBsAb ELISA diagnostic reagent for qualitative and quantitative immunoassay. In this way, we can solve the problems caused by plasma-derived HBsAg, such as complicated preparation, risk of infection, difficulty of quality control, high cost, etc. The recombinant HBsAg is safe, stable, simple and inexpensive while it is as effective as the blood-origin reagent. Therefore, it is believed that the recombinant HBsAg will improve the manufacture of HBsAb immunoassay reagents.

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