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EXPRESSION OF PRES2/S ANTIGEN OF HEPATITIS B VIRUS ISOLATED FROM PAKISTAN IN YEAST CELLS

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Abstract

Recombinant vaccines are an indispensable component of disease management of Hepatitis B infection. Genetic evolution of Hepatitis B Virus (HBV) necessitates the production of new types of antigens for next generation vaccine. This study aimed the expression of HBV middle surface (PreS2/S) protein in *Pichia pastoris* for production of an antigen for improved HBV vaccine formulation. A PreS2/S antigen-gene was cloned into a vector (pPICZAα) for secretory expression in *P. pastoris*. Expression of recombinant *P. pastoris* was optimized under control of AOX1 promoter to produce PreS2/S recombinant protein, which was purified through column chromatography. Specificity of the protein was confirmed through SDS-PAGE, western blot and ELISA. For immunization study, recombinant PreS2/S antigen and commercially available HBV vaccine comprising HBsAg alone were injected to immunize four groups of rabbits. The results of analysis of immunization showed that recombinant PreS2/S protein produced double antibody-titer than that of commercially available HBsAg indicating that PreS2/S antigen could be an ideal candidate for new generation HBV vaccines. This is the first report of secretory expression of biologically active PreS2/S antigen in *P. pastoris* from Pakistan and it will help to produce more effective vaccine to immunize 180 million people of Pakistan against HBV.

Introduction

Hepatitis B virus infection is a major human health hazard worldwide and is estimated to affect more than 350 million people in Asia, North America, Europe, and Africa (McMahon, 2005, Lu and Hu, 2005)**.** Its causative agent is HBV, which is an enveloped virus containing partially double stranded, circular DNA genome, and classified within the family hepadnaviridae (Stuyver *et al.,* 2000)**.** The HBV envelope consists of three major immunogenic proteins called small (HBsAg), medium (*PreS2/S*) and large (*PreS1/PreS2/S*) (Zhao *et al.,* 2006). HBsAg consisting of 226 amino acids (aa) is encoded by *S* gene whereas medium (*PreS2/S*) antigen coded by the pre-S2 gene (55AA) and the S gene, whilst the large (*PreS1/PreS2/S*) antigen is encoded by pre-S1 (119 or 108 AA, depending on subtypes), pre-S2 and S genes. World Health Organization recommends making HBV vaccination as a part of all national immunization programs (McMahon, 2005). To date, the majority of licensed recombinant vaccines containing HBsAg (S-protein) have been expressed in the yeast (Borchani-Chabchoub *et al.,* 2003, Cregg *et al.,* 1987, Miyanohara *et al.,* 1983, Stephenne, 1990, Vassileva *et al.,* 2001) or mammalian cells (Diminsky *et al.,* 1999) and demonstrated effective immunization against the virus. Interferon and nucleoside analog treatments are also reported to help some HBV patients (Karayiannis, 2003). However, 10% of adults still show no response to these vaccines (McMahon, 2005) and therapy cannot eradicate the disease entirely which necessitates to produce new candidate antigens that include the *PreS* proportion of the polypeptide, which could lead to the production of a more effective therapeutic vaccine. Inclusion of *PreS2/S* antigen in vaccine formulation could also mitigate the chances of non-responsiveness of vaccines against escape variants (Yamada *et al.,* 2001). The *PreS2/S* gene is comprises of *S* gene with an extra 165 nucleotides at 5' end (Tai *et al.,* 1997) and antigen is involved in nucleocapsid interaction (Poisson *et al.,* 1997) and extra cellular secretion of HBV particles (Le Seyec *et al.,* 1998) playing an important role in full stimulation of humoral and cellular immune responses. In addition, the *PreS2/S* protein is also important for immunological responses at T-cell level and has T-cell and B-cell recognition sites, spanning amino acids 1-25 of the protein (Neurath *et al.,* 1986) making it most suitable candidate antigen for HBV vaccine development. This study describes the heterologous expression of a characterized *PreS2/S* gene from genotype D of HBV in *P. pastoris*. The antigen will help to increase the efficacy of HBV recombinant vaccines and diagnostics in Pakistan.

Material and Methods

Vector and strains: The vector containing 3' terminal thymidine at both ends (PCR2.1 vector), the yeast vector expressing the proteins extra-cellularly (pPICZα), *P. pastoris* strain GS115 (His4), *E.coli* Top10F' strain for plasmid manipulation and ZeocinTM selection drug were obtained from Invitrogen Co., USA.

Blood sampling and DNA extraction: 10 patients having positive surface antigen markers were identified randomly from different hospitals of Punjab province of Pakistan. From each patient 5 ml blood was taken in sterile tubes having 60 ul of 0.5M EDTA. Viral DNA was isolated through Proteinase K digestion method (Persing *et al.,* 1993).

PCR amplification and gene cloning: PCR optimization was carried out using 50ng of template, 10 picomole of each primer, 2 units of Taq polymerase, 200 µM of dNTPs and different thermo cycling programs for

amplification of complete surface region from Pakistani HBV isolates using primers (sense 5' TATTCTT GGGAACAAGAG 3' and antisense 5' GCAGCAAA GCCCAAAAG 3). These DNA fragments were cloned into a T-A cloning vector and confirmed through PCR and restriction digestion. Using Big Dye Terminator Cycle Sequencing Ready Reaction Kit on ABI-3100 DNA analyzer, cloned DNA fragments were sequenced and the sequences of the HBV surface genes were submitted to NCBI gene data bank under accession numbers from FJ670505 to FJ67014. Genetic variability analysis was performed using NCBI Blast search and ClustalW freeware, which revealed the genotype of the isolates as D (unpublished data). A selected isolate PKHBV5 (accession no. FJ670505) was used for amplification of *PreS2/S* gene through PCR with gene specific primers (sense: 5' GGTACCATGCAG TGGAACTCCAC 3' and antisense 5' JGCGGCCGC AATGTATACCCAAA GACAAAAG 3'). PCR product of *PreS2/S* gene of selected HBV isolate was ligated into PCR2.1 T-A cloning vector. Cloning of gene was confirmed through PCR and restriction digestion. The cloned *PreS2/S* gene was digested from T-A vector with the help of restriction endonuclease (*KpN1* and *Not1*) and was subcloned into the *P.pastoris* expression vector pPICZα-A.

Recombination of yeast cells: The expression vector pPICZα-A-*PreS2/S* was linearized with restriction endonuclease (*SacI*) and introduced chemically into *P. pastoris* strain GS115. The transformants were plated on yeast extract peptone dextrose medium with sorbitol (YPDS) (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar) containing Zeocin™ (100 mg/L) for positive selection. The integration of expression cassette into yeast genome was confirmed by PCR using AOX1 locus specific primers (sense 5´- GACTGGTTCCAA TTGACAAGC-3´ and antisense 5´- GCAAATGGCATT CTGACATCC-3´) (Invitrogen Co., USA).

Determination of methanol utilizing phenotype: The mutant phenotype of transformed *P.pastoris* strain was determined by the procedure outlined in Easy Select™ *P.pastoris* expression manual (Invitrogen Co., USA). Cell growth on minimal media with dextrose (MDH) was compared to growth on minimal media using methanol (MMH) replacing dextrose. Growth rate of recombinant cells was then compared to control strains *P.pastoris* GS115/ HSA/Muts and GS115/LacZ/ Mut+.

Gene expression: The recombinant yeast cells were cultured in 100 ml buffered glycerol-complexmedium (BMGY) (1% yeast extract, 2% peptone, 100 mM potassium phosphate, PH 6.0,1.34% yeast nitrogen base, 1% glycerol, 0.0005% biotin). The expression of recombinant protein was allowed to grow in flask at 30ºC at 250 rpm shaking rate for 48 hours until A_{600}=6. The cells were then harvested, subsequently resuspended in 100 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 96 hours. The strain with the highest expression level was selected by SDS-PAGE analysis of the concentrated supernatant and fermented in 1L culture medium. The fermentation broth was centrifuged at 5500*g* for 25min at 4°C and supernatant concentrated and de-salted.

Purification: De-slated supernatant was centrifuged in ultracentrifuge at 60,000g for 90 minutes and the sedimentation was dissolved in TNE buffer (10mM /L EDTA, 50mM/L Tris-Cl (pH 7.6) and 100mM/L NaCl). The suspended recombinant protein was purified by sucrose density gradient (10-80% sucrose concentrated) at 40,000g for 60 minutes. Recombinant HBV *PreS2/S* antigen was then subjected to ion-exchange chromatography at 25°C followed by pH adjustment of the concentrated and dialyzed solution to 8.0 with 20mM Tris-Cl buffer. A DEAE-Sepharose Fast Flow (DEAE-SFF) was equilibriumized with buffer (20mM Tris-Cl,, pH 8.0, 50mM NaCl). The protein solution was applied to column and adsorbed protein was eluted using a linear gradient 0-1M NaCl in 20mM Tris-Cl buffer pH 8.0.

Characterization of recombinant protein: The analysis of recombinant *PreS2/S* protein secreted by cells into cell culture was performed using SDS-PAGE. The concentration of purified recombinant protein was estimated using Bio-Rad assay. Each sample was prepared by mixing 5µl of Bmercaptoethanol, 10µl of 25% SDS solution, 75 µl supernatant, 8 µl TE buffer (10 mM TRIS/HCl, 1 mM EDTA, pH 8.0) and 2µl sample loading dye containing bromophenol blue and the gel was stained with comassie blue stain. Western blot analysis was carried out with monoclonal antibody, (Santa Cruz Biotechnology, Inc., USA) specifically produced against amino acid 12-17 of *PreS2/S* protein, having no reactivity with the main antigenic epitope 'a' determinant of the protein. ELISA procedure was applied with HBs ELISA kit (DRG International Inc., USA) for testing bio- activity of recombinant HBV *PreS2/S* protein. Antibodies in ELISA had an affinity only for 'a' determinant region of HBsAg.

Immunization study: Purified recombinant *PreS2/S* protein was emulsified with alum adjuvant for injecting. 12 Rabbits were grouped in four groups (3 in each group) for immunization study. Groups 1 & 2 were immunized with 2 & 10 µg of recombinant HBV *PreS2/S* protein respectively whereas groups 3 and 4 were immunized with 2and 10 µg of commercially available hepatitis B vaccine (HBsAg) respectively. A booster dose was also given at day 21. Serum samples were collected for 119 days with 7 days interval and titer of antibodies against HBsAg were analyzed using ELISA kit (DRG International Inc., USA). Antibodies in ELISA had an affinity only for 'a' determinant region of HBsAg. Serum samples were then prepared through serial dilution in [0.1% (v/v) Tween 20, 140mM NaCl, .005% thimerosal, 25% Feta Bovine Serum in Phosphate Buffer Saline (PBS)] diluents.

Results

PCR amplification of *PreS2/S* antigen-gene was carried out from the cloned complete surface gene of HBV isolate PKHBV5 under investigation and then cloned into

the *pPICZα-A* plasmid. PCR and restriction analysis confirmed the insertion of an 860 bp DNA fragment of *PreS2/S* gene. Subsequent DNA sequencing confirmed their identity and further determined the right orientation of the gene. The plasmid was transformed into *P. pastoris* cells by chemical method (Ausubel *et al.,* 1994, Sambrook *et al.,* 1989) and the positive Zecocin resistant colonies were analyzed by colony PCR. For this analysis PCR products of 588 bp and 1448 bp (860bp of *PreS2/S* gene + 588bp of vector) confirmed the integration of *pPICZα-A-PreS2/S* into *P. pastoris* genome. Mutants were recognized through screening of culture media Mut+ and selected in comparison to controls (data not shown). The recombinant *P. pastoris* strains were selected on the basis of resistance for increased drug concentration. Five highly resistant strains were selected from 2.0-3.0 mg/ml concentration of ZeocinTM. Based on SDS-PAGE analysis, the strain #5 showed highest level of expression (data not shown). The culture was fed for four days with 5 ml/L, methanol to 0.5% (final concentration), each day. Culture supernatant was collected on each time point and subjected to SDS-PAGE, indicating the expression of heterologous *PreS2/S* protein in *P. pastoris.* The *PreS2/S* was purified by DEAE-SFF chromatography. Eluted samples with major peaks separated by chromatography were analyzed through SDS-PAGE and western blot. A 34kD protein was detected by western blot using a mouse monoclonal *pre-S2* antibody (better go to some appropriate place in M&M). The anti-*preS2* specifically detected the first sub-dominant epitope of *PreS2/S* protein that spanned from 12-17 amino acid positions and had no affinity for the 'a' determinant. The western blot showed a single positive reacting band at 34kD on the nitrocellulose membrane Fig 1.

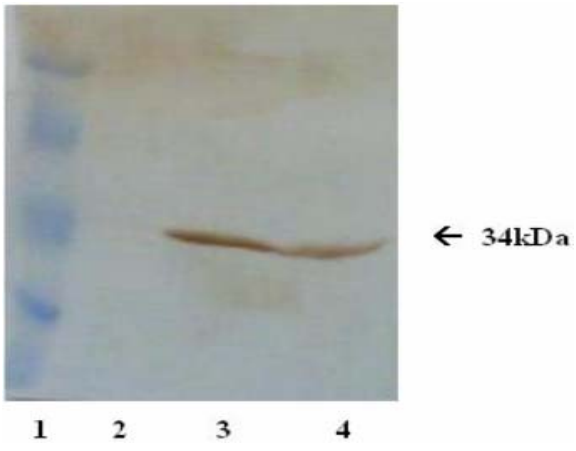

Fig. 1. Western blot: (Lane 1: Prestained protein marker; Lane 2: negative control; Lane 2-3: recombinant *PreS2/S* protein).

The reactivity and antigenicity of recombinant *PreS2/S* protein were confirmed through ELISA (Fig. 2). Antibodies used in ELISA had an affinity only for 'a' determinant of *S* domain of *PreS2/S*. The reactivity of recombinant *PreS2/S* protein was 0.233 against a cutoff standard value of 0.008 for ELISA test. The ELISA showed a high level of reactivity confirming that recombinant *PreS2/S* protein was biochemically active. For immunization studies, four groups of rabbits were immunized with 2ug and 10ug of purified recombinant *PreS2/S* protein (group 1 & 2) and commercially available HbsAg vaccine (group 3 & 4). The analysis of antibodies-titer showed that each group of rabbits developed different titers of antibodies at different times from day one. Results of immunization study demonstrated that group 1 developed detectable antibodies by day 14 while group 3 & 4 developed antibodies on/after day 28. The antibody titer produced in each group is shown in Fig. 3. The level of antibody-titer abruptly increased following booster dose on day 21 in all groups attaining highest level in week 7. Data analysis showed that group attaining highest level of antibodies in their serum was 1: 3000 compared to 1: 2000 for group 4 in week 7. Group 3 on the other hand attained antibody titer 1:2500 that is higher than group 4. The analysis of results identified recombinant *PreS2/S* protein with significant immunization ability as 2 ug of the protein produced higher antibody-titer compared to 10ug of HBsAg.

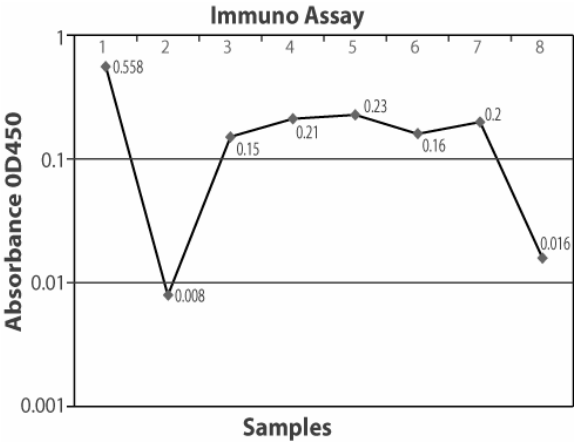

Fig. 2. ELISA: yellow chrome confirmed the reactivity and bioactivity of recombinant *Pre-S2* protein (Lane 1: negative control; Lane 2-5: recombinant Pre-S2 protein; Lane: 6: ELISA kit positive control; Lane 7: ELISA kit negative control).

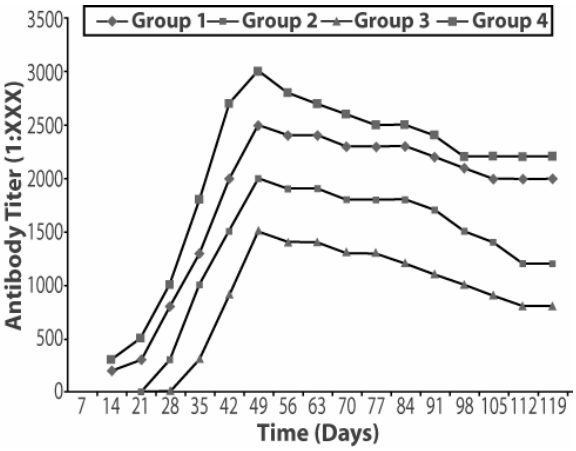

Fig. 3. Y-axis: days of serum sample collection; X-axis: antibody titer; group-1 (♦) immunized with 2 ug of recombinant *PreS2/S* protein; group 2 (■) immunized with 10 ug of recombinant *PreS2/S* protein; group 3 (▲) immunized with 2 ug of HBV vaccine containing HBsAg alone; group 4 (■) immunized with 10 ug HB vaccine containing.

Discussion

Recombinant DNA vaccines are the most effective disease management tools of the present therapeutic era. It has been suggested that antigen comprising of *PreS2/S* protein enhances the immune response to HBV infection and effectively controls escape mutants (Yamada *et al.,* 2001, Sambrook *et al.,* 1989, Chengalvala *et al.,* 1999, Ijaz *et al.,* 2003). In present study a biologically active *PreS2/S* antigen in *P. pastoris* was expressed efficiently. *P. pastoris* is a powerful and inexpensive heterologous expression system for the production of functionally active recombinant proteins. This system combines the advantage of high expression levels, easy scale-up, inexpensive growth media, and the capacity to perform many post-translational modifications, characteristic of higher eukaryotes (Cereghino and Cregg, 2000, Gellissen, 2000). The proteins produced by *P. pastoris* are correctly folded and form structures similar to epitopes in native HBV protein. Molecular weight of *PreS2/S* recombinant protein expressed in *P. pastoris* is 34 kDa corresponding to *PreS2/S* protein in size (Borchani-Chabchoub *et al.,* 2003). Expressed protein band on SDS-PAGE and western blot confirmed efficient secretion of recombinant *PreS2/S* protein. This recombinant protein was immunoreactive against anti-HBs as shown by ELISA. Previously *PreS2/S* protein was expressed intracellularly in *Saccharomyces cerevisiae* (Borchani-Chabchoub *et al.,* 2003), human hepatocellular carcinoma cells (Ge *et al.,* 2004) and Silkworm Larvae (Likhoradova *et al.,* 2004). Similarly, Ottone and co workers (Ottone *et al.,* 2007) demonstrated intracellular expression in *P. pastoris* of HBsAg(s) corresponding to S surface antigen gene of the four major subtypes: adr, adw2, ayr and ayw3, and to PreS2-S (*PreS2/S* gene) region of the two subtypes adr and adw2. It was inferred that *P. pastoris* Mut+ strain could express *PreS2/S* protein intracellularly (Ottone *et al.,* 2007). It was described previously that *PreS2/S* protein was not secreted into the culture medium and was recovered from the cytoplasm (Ge *et al.,* 2004) however this study demonstrated that antigenically active recombinant *PreS2/S* protein could also be secreted in culture medium. The immunization results of this study in rabbits showed that *PreS2/S* protein has greater immunogenic potency compared to HBsAg that may be due to additional epitopes present in *PreS2* region of PreS2/S protein. It is expected that HBV recombinant *PreS2/S* antigen will perk up the development of recombinant vaccines and immunoassay reagents. Many efforts have been employed to produce antigens with larger surface polypeptide (Han *et al.,* 2006). This leads us to suggest that the *PreS2/S* protein is an ideal candidate for a new generation of Hepatitis B vaccine and a better substitute for the current HBsAg antigen. Further studies on detailed assessment of the immunogenicity of humeral immunity and cellular immunity may provide conclusive evidence.

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