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

ALI RAZA AWAN1*, MUHAMMAD YASIR ZAHOOR2, MUHAMMAD MOHSIN JAVED3, MASROOR ELLAHI BABAR1 AND ZAFAR SALEEM2

1Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Outfall Road, Lahore Pakistan
2Center of Excellence in Molecular Biology, University of the Punjab, 87 West Canal Bank Road, Lahore
3Institute of Industrial Biotechnology, GC University, Lahore
*Corresponding author: arawan77@gmail.com

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 (pPICZαA) for secretary 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; Miyahohara 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 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-cellarly (pPICZαA), 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’ TATTTCT GGGAACCAAGG 3’ and antisense 5’ GCAGCAGAA GCCAAAGG 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’ GGTAACATGCAGTGGAACTCCAC 3’ and antisense 5’ JGGGGGCAGG AATGTACATCCAAA 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 NotI) 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 (ScaI) 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 TTGACAAGGC-3’ and antisense 5’- GCAAATGGCAT TCGACATCC-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 30°C at 250 rpm shaking rate for 48 hours until A600=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 5500g for 25min at 4°C and supernatant concentrated and de-salted.

Purification: De-salted 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 equilibrated 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/Cl, 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, 0.05% 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 Zeocin 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.

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.
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 immunoassy 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.

Acknowledgment

We are thankful to Dr. Sheikh Riazuddin and Dr. Shaheen N. Khan (Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan) for their kind guidance. We are also grateful to Dr. James Cregg and Dr. Knut Madden (KECK Graduate Research Institute, Claremont, CA USA) for his frequent and valuable advice. Higher Education Commission of Pakistan is highly acknowledged for providing financial assistance for the research.

References


(Received for publication 12 February 2011)