The Myanmar Health Science Research Journal Vol. 21, No. 1, 2009

Cloning of the hepatitis B surface antigen gene (1): extraction and transformation of the recombinant HBsAg gene containing plasmid into the *E. coli* DH5 alpha cells

Win Aung,Yeon-Hee Kim,**Hyunah Kim,** Suk-Hoon Ha & *Khin Pyone Kyi*

*Diagnostics and Vaccine Research Centre
Department of Medical Research (Lower Myanmar)
**Institute of Science and Technology, CJ Corporation, Republic of Korea.

The majority of currently available recombinant hepatitis B (HB) vaccines in the market have been produced by using appropriate expression plasmids in *Saccharomyces cerevisiae* and *Hansenula polymorpha* yeast cells as host systems. Recently, attempts were made to use *Pichia pastoris* as an alternative and more productive host for cloning of yeast expression plasmid carrying the hepatitis B surface antigen (HBsAg) structural gene for production of recombinant HB vaccines in the future. DNA cloning involves separating a specific gene or DNA segment from a larger chromosome, attaching it to a small molecule of carrier DNA, and then replicating this modified DNA thousands or millions of times through both the increase in cell number and the creation of multiple copies of the cloned DNA in each cell, followed by expression of desired protein. In this study, the HBsAg gene was first extracted and purified from chromosomal DNA of *H. polymorpha* transformant cells. It was then ligated with pGEM-T vector followed by transformation into the competent *E. coli* DH5 alpha cells which had already been prepared in our laboratory. The results from each and every steps were confirmed by direct PCR identification, restriction enzyme analysis followed by agarose gel electrophoresis determination and DNA sequencing analysis. The nucleotide sequences of the HBsAg gene, extracted and purified from the final transformant *E. coli* were found to be totally identical to that of the HBsAg gene which had been initially extracted and purified from *H. polymorpha* cell. Research works on amplification and ligation of the HBsAg gene and yeast plasmid in *E. coli* DH5 alpha cells for further transformation of the recombinant HBsAg gene containing plasmid into the *P. pastoris* yeast cells are in progress at the Research and Development Centre of Pharmaceuticals, Institute of Science and Technology, CJ Corporation, Ichon City, Republic of Korea.

INTRODUCTION

'Genes' are the fundamental units of genetic information in living system. 'DNA' is the chemical basis of hereditary and is organized into genes. Biochemically, a 'gene' is defined as a segment of DNA (or, in few cases, RNA) that encodes the information required to produce a functional biological product. The final product is usually a protein. Therefore, genes control the synthesis of various types of protein [1]. Advances in molecular genetics and nucleic acid chemistry have made possible to identify genes coding for biologically active substances, to analyze them in detail, to transfer them within and between organisms, and to obtain gene expression under controlled conditions with efficient synthesis of the encoded product [2]. To clone means to make identical copies. Cloning of DNA from any organism entails five general procedures; cutting of DNA at precise location by restriction endonuclease (RE), joining two DNA fragments covalently by DNA ligase to construct the recombinant DNA, selecting a

small molecule of DNA capable of self-replication, moving recombinant DNA from the test tube to a host cell, and selecting or identifying host cells that contain recombinant DNA. A gene that codes for a specific product can be isolated and propagated by insertion into a suitable vector with the aid of highly specific RE enzymes which cleave the vector DNA at predetermined sites, and ligases which join the gene insert to the vector. The vector can then be introduced into host organisms and individual clones that carry the desired gene can be selected and propagated in mass culture [3].

For development of currently available recombinant hepatitis B (HB) vaccines, hepatitis B virus (HBV) recovered from plasma of a hepatitis B carrier has been used to prepare viral DNA: That DNA has been cloned in *Escherichia coli* and the gene coding for hepatitis B surface antigen (HBsAg) has been isolated. This gene has been inserted into yeasts by means of appropriate expression vectors. Purified HBsAg proteins expressed from transfected yeast cells have been formulated into HB vaccines and shown to induce protective antibody response in human [2]. The majority of currently available recombinant hepatitis B (HB) vaccines in the market have been produced by using appropriate expression plasmids in *Saccharomyces cerevisiae* and *Hansenula polymorpha* yeast cells as host systems. In this study, an attempt was made to extract the HBsAg gene from the transformed *Hansenula polymorpha* yeast cells containing it which was then transformed into the competent *E. coli* cells for further transformation into final host, *Pichia pastoris* yeast cells with an aim to produce recombinant HB vaccine in the near future by using as an alternative and a more productive strain.

MATERIALS AND METHODS

HBsAg structural gene

The HBsAg gene was extracted and purified from the Master Cell Bank (MCB) containing the structural gene coding for the HBsAg protein, integrated in the genomic DNA of the *H. polymorpha* transformant yeast cells for production of recombinant HB vaccine. It was provided by the CJ Pharmaceutical Corporation, Republic of Korea. The HBsAg gene consists of 678 base pairs, coding the hepatitis B surface antigen (S) protein with 226 amino acids [4].

Host strain

E. coli DH5 alpha strain, grown in Luria-Bertani (LB) media supplemented with ampicillin and stored at -20^{o}C was used in cloning process for plasmid amplification. This media was prepared under the clean bench by addition of 10 gm tryptone, 5 gm yeast extract, 10 gm sodium chloride, 15 gm agar and 1 ml ampicillin solution (stock concentration of 100 mg/ml) into deionized water to make a final volume of 1 liter, followed by plating [5].

Expression vectors

The pGEM-T vector (3003bp) with terminal thymidine to both ends containing ampicillin resistance gene was commercially available from the Promega Co, USA [6].

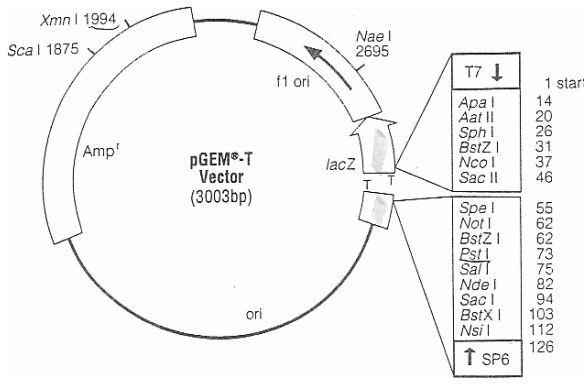


Fig. 1. Physical map of plasmid pGEM- T(3003 bp)

Extraction and purification of the HBsAg gene from H. polymorpha cell

First, the HBsAg coding DNA segment, integrated in the chromosomal DNA of *H. polymorpha* transformant cell from Master Cell Bank (MCB) was extracted by using the QIAGEN test kit, USA [7]. The extracted DNA segment containing the gene

of interest was amplified by Thermocycler using; PCR reaction buffer, dNTPs, DNA template, polymerase enzyme, double distilled water, and specific primers; AY21 F (5'-ATG GAG AAC ATC ACA TCA GGA-3' and CTR (5'-CTC TTT GTT TTG TTA GGG T-3'), with an annealing temperature of 45^{o}C and repeated cycles of 30. The DNA segments were purified by using PCR purification test kit from QIAGEN. The purified HBsAg gene of 678 base pairs was confirmed by agarose gel electrophoresis and DNA sequencing analysis by using the ABI Prism 3100 Genetic Analyzer [8].

Ligation of the HBsAg gene and plasmid pGEM-T

To obtain the plasmid-insert DNA construct, the above purified HBsAg gene was ligated to the commercially available plasmid pGEM-T vector which had already been linearized with *EcoRV* at base 51, by using T4 ligase enzyme at 16^{o}C for 3 hours followed by 4^{o}C for overnight [9].

Preparation of competent E. coli DH5 alpha cells

Just before transformation, the competent *E. coli* DH5 alpha cells were prepared by calcium chloride method [10]. For efficient transformation, it is essential that number of viable cells should not exceed 10^{8} cells per milliliter for which *E. coli* is equivalent to a OD of 0.35–0.45 at which these cells are competent for an efficient transformation.

Transformation of the HBsAg gene containing plasmid into E. coli cells

The recombinant product, plasmid-insert DNA construct, was then transformed into competent *E. coli* DH5 alpha cells by using the heat-shock procedure. i.e 42^{o}C for 90 seconds without shaking [9]. It is well known that only transformed cells containing the ampicillin resistance gene (*AMP*) on the plasmid vector can survive on an indicator plate containing LB media with ampicillin. In our study, transformants containing recombinant genes were identified by the appearance of white colonies whereas non-transformed colonies showed blue colour with a relatively smaller in size on the indicator plates. The closed circular recombinant plasmid of about 3700 base pairs was then extracted by using AccuPrep plasmid extraction kit (Bioneer) [11] and confirmed by RE analysis with *EcoRI* followed by agarose gel electrophoresis. The presence of the HBsAg structural gene in this plasmid-insert DNA construct was also detected by direct colony PCR identification (using primers AY21F and CTR) followed by purification with PCR purification QIAGEN test kit [7]. The purified HBsAg gene of 678 base pairs was confirmed by agarose gel electrophoresis determination and DNA sequencing analysis by using the ABI Prism 3100 Genetic Analyzer [8].

RESULTS

Electrophoretic identification of the HBsAg gene, extracted from the chromosomal DNA of *H. polymorpha* transformant (MCB of CJ Corporation) followed by PCR purification, is shown in Fig. 2.

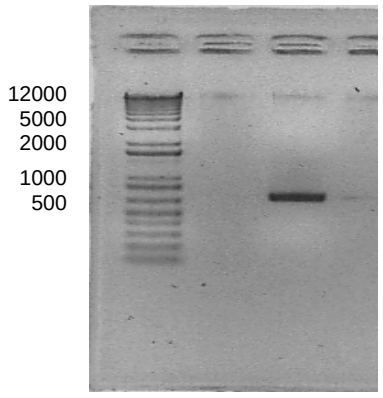


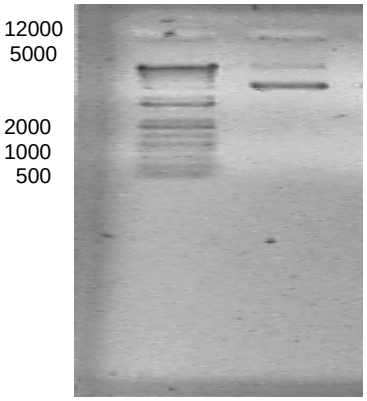
Fig 2. Electrophoretic identification of the HBsAg gene, extracted from the genomic DNA of *H. polymorpha* transformant

The distinct DNA band was observed at 700 base pairs level and consistent with the number of base pairs identified in the HBsAg gene. Fig. 3 illustrates the nucleotide sequences of the DNA segment, extracted from the chromosomal DNA of *H. polymorpha* transformant, analyzed by using the ABI

ATGGAGAACA TCACATCAGG ATTCCTAGGA CCCCTGCTCG TGTTACAGGC
GGGGTTTTTC TTGTTGACAA GAATCCTCAC AATACCGCAG AGTCTAGACT
CGTGGTGGAC TTCTCTCAAT TTTCTAGGGG GAACTACCGT GTGTCTTGGC
CAAAATTCGC AGTCCCCAAC CTCCAATCAC TCACCAACCT CCTGTCCTCC
AACTTGTCCT GGTTATCGCT GGATGTGTCT GCGGCGTTTT ATCATCTTCC
TCTTCATCCT GCTGCTATGC CTCATCTTCT TGTTGGTTCT TCTGGACTAT
CAAGGTATGT TGCCCGTTTG TCCTCTAATT CCAGGATCTT CAACTACCAG
CACGGGACCA TGCAGAACCT GCACGACTCC TGCTCAAGGA ACCTCTATGT
ATCCCTCCTG TTGCTGTACC AAACCTTCGG ACGGAAATTG CACCTGTATT
CCCATCCCAT CATCCTGGGC TTTCGGAAAA TTCCTATGGG AGTGGGCCTC
AGCCCGTTTC TCCTGGCTCA GTTTACTAGT GCCATTTGTT CAGTGGTTCG
TAGGGCTTTC CCCCACTGTT TGGCTTTCAG TTATATGGAT GATGTGGTAT
TGGGGGCCAA GTCTGTACAG CATCTTGAGT CCCTTTTTAC CGCTGTTACC
AATTTTCTTC TGTCTTTGGG TATACATT

Fig. 3. Nucleotide sequence of the HBsAg gene extracted from *H. polymorpha* transformant (678 base pairs)

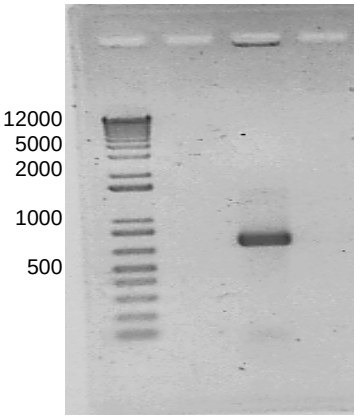
Prism 3100 Genetic Analyzer. The nucleotide sequences obtained were identical to that of the HBsAg gene. On determination of the optical density of cultured *E. coli*, it was found to be 0.38, indicating the competency of *E. coli* cells for an efficient transformation. Electrophoretic identification of the extracted plasmid-insert DNA construct, transformed into *E. coli* cell, is demonstrated in Fig. 4.



Lane 1 = Nucleic acid markers
Lane 2 = Gene of interest

Fig. 4. Electrophoretic determination of the extracted plasmid-insert DNA construct in *E. coli* cell

The DNA band was observed at the level of about 3700 base pairs, indicating the formation of cloned DNA in the transformant. Direct colony PCR identification of the HBsAg DNA segment, transformed into *E. coli* cell by using the plasmid pGEM–T vector in agarose gel electrophoresis, is depicted in Fig. 5**.**



Lane 1 = Nucleic acid markers
Lane 2 = Gene of interest

Fig. 5. Direct colony PCR identification of the HBsAg coding DNA segment in the *E. coli* transformant

The distinct DNA band was detected at the expected level of 700 base pairs, confirming the presence of the DNA of interest in the transformant. The nucleotide sequence of the purified HBsAg segment, extracted and purified from transformant *E. coli* cell, was analyzed by using the ABI Prism 3100 Genetic Analyzer**.** The nucleotide sequences obtained were found to be identical to that of the HBsAg gene initially extracted and purified from chromosomal DNA of *H. polymorpha* cell as illustrated in Fig. 2.

DISCUSSION

E. coli are prokaryotic unicellular, gram-negative bacilli, and usually grow readily on ordinary culture media. They are non-pathogenic in their habitat. The length of a typical *E. coli* cell is 2 µm and its chromosome is a single double-stranded circular DNA molecule. The *E. coli is* the first organism used for recombinant DNA work and still the most common host cell. *E. coli* has many advantage: its DNA metabolism and many other biochemical processes are well understood, many naturally occurring cloning vectors and plasmids associated with *E. coli* are well characterized and effective

techniques are available for moving DNA from one bacterial cell to another [1]. Yeasts are eukaryotic, unicellular, microscopic and usually spherical in shape with diameter of 3-15 µm. Plasmids are circular DNA molecules that replicate separately from the host chromosome. They can be introduced into bacterial cells by a process called transformation [10].

Pichia pastoris is one of the methylotrophic yeast cells, capable of metabolizing methanol as its sole energy and carbon source. It is also well documented that *P. pastoris* has many of the advantage of higher eukaryotic expression system such as protein procession, protein folding and post translational modification. The heterologous expression in *Pichia* can be either intracellular or secreted. The major advantage of expressing heterologous protein as secreted protein is that it secrets very low level of native proteins. Therefore, the secreted heterologous protein comprises the vast majority of the total protein in the medium and serves as the first step in purification of protein [12, 13, 14, 15]. However, the yeast plasmid amplification can never be achieved in yeast cells, an initial plasmid construction was performed in *E. coli* cells before transfer of plasmid-insert DNA construct (recombinant HBsAg gene) into *P. pastoris* [1].

In our study, we extracted and purified the HBsAg gene from genomic DNA of *H. polymorpha* transformant cell. It was then ligated with pGEM-T vector followed by transformation into the competent *E. coli* DH5 alpha cells which had already been prepared. The results from each and every steps were confirmed by direct PCR identification, RE analysis followed by agarose gel electrophoresis determination and DNA sequencing analysis. It was found that the nucleotide sequences of the HBsAg gene, extracted and purified from final transformant *E. coli* were identical to that of the HBsAg gene which had been initially extracted and purified from chromosomal DNA of *H. polymorpha* cell, indicating the successful transformation of the HBsAg gene into the *E. coli* cells for further cloning process .

This study described the possible use of *E. coli* DH5 alpha cells as an intermediate host for cloning of the HBsAg gene containing plasmid prior to the transformation into an ultimate host, *P. pastoris* for expression of HBsAg protein. Research works on amplification and ligation of the HBsAg gene and yeast plasmid in *E. coli* DH5 alpha cells for further transformation of the recombinant HBsAg gene containing plasmid into the *P. pastoris* yeast cells are in progress at the Research and Development Centre of Pharmaceuticals, Institute of Science and Technology, CJ Corporation, Ichon City, Republic of Korea.

REFERENCES

1. Nelson DL & Cox MM. Lehninger. Recombinant DNA technology. In*: Principles of Biochemistry*. Third edition, 2000; 1119-1148.
2. WHO Requirements for hepatitis B vaccines made by recombinant DNA technology. *WHO Technical Report Series* 1989; No.786: 38-60.
3. Glick BR & Pasternak JJ. Principles and applications of recombinant DNA. Recombinant DNA technology. In: *Molecular Biotechnology* 1994; 17-54.
4. Product Manual. Safety and effectiveness of CJC 50100, Hepatitis B vaccine (Recombinant). CJ Pharmaceutical Company. Republic of Korea.
5. Sambrook J & Russell DW. Liquid media for *E. coli*. In: *Molecular cloning: a laboratory manual*.Vol 1, 3rd edition. 2001; A. 2.2- A 2.6.
6. Manual of the Multicopy *Pichia* Expression Kit, Invitrogen, USA, 2001.
7. Handbook of the QIAGEN Genomic DNA. 1995; 1-50.
8. Sequencing Chemistry Guide for ABI Prism 3100 Genetic Analyzer. HITACHI. 1-21.
9. Technical Manual. Promega. pGEM-T and pGEM-T Easy Vector Systems. Ligations using the pGEM-T and pGEM-T Easy Vectors and the 2X rapid ligation buffer, 1998 ; 11-12.
10. Sambrook J& Russell DW. Preparation and transformation of competent *E.coli* using calcium chloride. In: *Molecular cloning: a laboratory manual*, vol.1, 3rd edition. 2001; 1: 1.116-1.118.

11. User's guide of the Bioneer AccuPrep Plasmid Extraction Kit. 1999; 1-13.
12. Barnett JA, Payne RW & Yarrow D. Yeasts: characteristics and identification. Published by the Press Syndicate of the University of Cambridge USA 1983.
13. Cregg JM, Barringer KJ & Hessler AY. *Pichia pastoris* as a host system for transformation. *Molecular Cell Biology* 1985; 5 : 3376-3385.
14. Cregg JM & Huggins DR. Production of foreign proteins in the yeast *Pichia pastoris. Canadian Journal of Botany* 1995 *Supp;* 73: 5981-5987.
15. Creeg JM, Vedvick TS & Raschke WC. Recent advances in the expression of Foreign Genes in *Pichia pastoris. Biotechnology* 1993; 11: 905-910.