

# Expression of the active human and duck hepatitis B virus polymerases in heterologous system of *Pichia methanolica*

Juhyun Choi <sup>a,b</sup>, Eunice E. Kim <sup>c</sup>, Young In Park <sup>b</sup>, Ye Sun Han <sup>a,\*</sup>

<sup>a</sup> Structural Biology Research Center, Korea Institute of Science and Technology, 39-1 Hawallkok-dong Sungbuk-ku, Seoul, South Korea

<sup>b</sup> Graduate School of Biotechnology, Korea University, Seoul, South Korea

<sup>c</sup> LG Chemical Ltd., Research Park, Taejon, South Korea

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## Abstract

We expressed the Hepatitis B virus polymerase (HBV P protein) using a recently introduced yeast system, *Pichia methanolica*. HBV (1–680 amino acids) and Duck Hepatitis B virus (DHBV, 1–780 amino acids) polymerase were expressed and showed DNA dependent DNA polymerase (DDDP). The DHBV polymerase had RNA dependent DNA polymerase (RDDP) and RNase H activities. We present a new simplified way of obtaining active viral P protein using the yeast expression system. The viral P proteins proved to be stable and were not aggregated in the yeast system. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Hepatitis B virus polymerase; *Pichia methanolic*; DNA dependent DNA polymerase; RNA dependent DNA polymerase; RNase H

## 1. Introduction

Hepatitis B virus is one of the causative agents of acute/chronic hepatitis in humans, and is a major health problem worldwide. Over 500 million individuals are hepatitis B carriers worldwide and about 1 million deaths are attributed annually to the effects of HBV (Beasley et al., 1981). There is a strong correlation between chronic hepatitis B infection and the incidence of hepatocellular carcinoma (Zoulim, 1999). Immunization

against HBV has been effective at reducing the number of new infections and interferon and nucleoside analog treatments help some HBV patients. Since therapy cannot eradicate the disease entirely, there is a need to develop an effective drug to cure HBV patients.

Tavis et al. have attempted to express the HBV polymerase and performed its multifunctional enzyme activity. DHBV polymerase was expressed by in vitro translation with rabbit reticulocyte (Wang and Seeger, 1992) and yeast retrotransposon Ty1 (Tavis and Ganem, 1993). The HBV polymerase was expressed by *Baculovirus* (Lanford et al., 1997) and the *E. coli* system (Jeong et al., 1996). However, the biochemical study of

\* Corresponding author. Tel.: +82-2-958-5933; fax: +82-2-958-5939

E-mail address: yshan2@kist.re.kr (Y.S. Han).

HBV polymerase has been limited by difficulties of expressing and purifying the proteins in a heterologous system. The viral polymerases present testing difficulties, because the viral genome binds tightly to the polymerase as an endogenous template in vitro (Summers and Mason, 1982).

We used *Pichia methanolica* as an expression system (Invitrogen Inc., CH Groningen, the Netherlands) to obtain the active HBV polymerase to study HBV replication and inhibition. This system is designed for the high-level production of recombinant proteins in the methylotrophic yeast *P. methanolica*.

Using the *P. methanolica* expression system, we obtained active and soluble viral polymerases (P protein) for both HBV and DHBV. Also, this system offered simplicity and economy compared with bacterial or any other recombinant protein expression system. The P proteins were purified through Ni-NTA resin (Qiagen, Germany) by His-6X fusion tag and had DNA-dependent DNA polymerase activity compared with Klenow polymerase. A recent study revealed that the viral P protein interacts with some cellular chaperones in the host during the viral life cycle (Beck and Nassal, 2001; Park and Jung, 2001). The present study offers a method measuring possible new chaperone effects on P protein activity.

## 2. Methods

### 2.1. Construction of plasmid

HBV polymerase gene (HBV P gene) was amplified using two primers, HBVFwSalI (5'-ACGCGTTCGACGAAATGCCCTATCCTATCAACACTTC-3') and HBVRvSalI (5'-ACGCGTCGACGCAACGGCCCGTGCCCCATTTCC-3'). The PCR product encoded amino acids from the start codon to the 680th amino acid of the HBV P gene, including TP, spacer, RT. The DHBV polymerase gene (DHBV P gene) was amplified with DHFwEcoRI (5'-CGTCCCGGAATTCATGCCCCGACCATTGAAGC A-3') and DHRvSpeI (5'-CCTGATCATGTAAAGTTCACATAGCCTATG-3'). This product encoded

amino acids from the start codon to the 787th amino acid of the DHBV P gene, including the TP, spacer, the RT domains, and the RNase H domains. These genes were cloned into pMet $\alpha$ A and pMetA yeast cloning vectors (Invitrogen Inc., CH Groningen, the Netherlands), and digested with *Sal*I for the HBV P gene and with *Eco*RI and *Spe*I for the DHBV P gene.

### 2.2. Yeast transformation and the selection of transformants

The electrocompetent cell, PMAD16 (*P. methanolica* strain which contains deletion of protease A and B. Invitrogen Inc.), was streaked onto a YAPD (Yeast Extract Adenine Peptone Dextrose Medium) plate to isolate single colonies and incubated at 30 °C for 2 days. After 50 ml YAPD liquid culture of the single colony at 30 °C, 200 ml of YPAD was inoculated until the culture reach an OD<sub>600</sub> of 0.3 overnight. The cells were harvested and resuspended in 40 ml in sterile KD buffer (50 mM potassium phosphate, pH 7.5 and 25 mM dithiothreitol (DTT)). The cell suspension was then incubated for 15 min at 30 °C and collected by centrifugation at 4 °C. The cell pellet was resuspended in 50 ml of ice-cold and sterile STM buffer (270 mM sucrose, 10 mM Tris, pH 7.6 and 1 mM MgCl<sub>2</sub>) at 4 °C. After washing the cell pellet twice with STM buffer, the cells were collected. The cell pellet was then resuspended in 1 ml of ice-cold STM buffer, dispensed in 100  $\mu$ l aliquots and frozen at –80 °C.

Electroporation was performed as described by Raymond et al. (1998). Transformed cells were selected from the minimal dextrose (MD) plate [1.34% yeast nitrogen base (YNB, Invitrogen Inc.), 4  $\times$  10<sup>–5</sup>% biotin (Sigma), and 2% dextrose (GIBCO)] because the PMAD16 strain lacked 5-aminoimidazole ribonucleotide-carboxylase (the product of the *ADE2* gene from the vector).

### 2.3. Small scale expression tests

Candidate colonies were chosen and re-streaked on a MD plate at 30 °C for 2 days. A single colony was inoculated in 10 ml of Buffered dextrose-complex medium (BMDY, 1% yeast extract,

2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB,  $4 \times 10^{-5}\%$  biotin, and 2% dextrose). The overnight culture was collected and the medium changed to buffered methanol-complex medium (BMMY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB,  $4 \times 10^{-5}\%$  biotin, and 0.5% methanol). After taking 500  $\mu$ l samples from the culture medium, they were centrifuged at 14 000 rpm for 2 min at room temperature. Cell pellets were then treated with yeast protein preparation buffer (0.06 M Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.0025% (w/v) bromophenol blue) and analyzed by 12% SDS acrylamide gel electrophoresis (Gottschling and Berg, 1998). The medium samples were concentrated with trichloroacetic acid (TCA) and also analyzed by 12% SDS-polyacrylamide gel electrophoresis.

#### 2.4. Scale-up and optimization of the protein expression

A single colony was inoculated with 10 ml BMDY in 50 ml conical tube and incubated at 30 °C for 15 h at 300 rpm ( $OD_{600}$ , 2–5). A 1 ml sample from the overnight culture was re-inoculated with 500 ml of BYDY medium in a 2 l baffled flask (Wheaton Inc., USA) and incubated at 30 °C for 16–18 h at 300 rpm. Cells were grown in BMDY to an  $OD_{600}$  value of 1.6–9.0, which has a cell density of approximately  $1.6\text{--}9.0 \times 10^7$  cell per ml. The cells were collected by centrifugation at 4000 rpm at room temperature. The medium was discarded and 200 ml of sterile BMMY medium added. The cell pellet was re-suspended, transferred to a sterile 1 l baffled flask and incubated at 30 °C at 300 rpm. Methanol (Sigma, USA) (1 ml) was added every 24 h and the cells were collected after 72 h of incubation.

#### 2.5. Cell lysis and column purification

A general procedure for cell lysis using French pressure cell (SLM Instrument Inc.) is provided in Current protocols in Molecular Biology (Moore et al., 1987). The  $Ni^{2+}$ -NTA column (Qiagen, Germany) protein purification protein was followed

using a slight modification of the manufacturer's instructions. Briefly, about 3 g (wet cell weight) of cell pellet was suspended in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Triton X-100 (Sigma, USA)). The suspension was treated with lyticase (Sigma, USA) to a final concentration of 20 U/ml and then incubated at 30 °C for 30 min. Lysis was performed using a French pressure cell at 12 000 psi. DNaseI and RNase were added to the cell lysate to final concentrations of 5 and 20  $\mu$ l/ml, respectively. The lysate was incubated for 30 min in an ice bath and then centrifuged for 40 min at 15 000 rpm. The soluble extract was pooled from 10 ml (bed volume) of an  $Ni^{2+}$ -NTA resin packaged column. The column was equilibrated with 50 ml of buffer A, extract was applied and washed with 10 ml of Tris buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Triton X-100, and 10 mM imidazole) three times. The viral P protein fractions were collected using elution buffer (buffer A + imidazole at a final concentration from 25 to 200 mM) and concentrated using an Amicon concentrator. Subsequently, the concentrated protein was loaded on a Superdex-S200 gel filtration (Pharmacia) column equilibrated with buffer A. The viral P protein fractions were pooled and the purity of the protein sample was checked by 12% SDS-polyacrylamide gel electrophoresis. The concentration of the P protein was determined using Bio-Rad dye reagent with BSA as a standard. The zinc ion concentration of the enzyme was determined by atomic absorption spectrophotometry (Varian SpectrAA800).

#### 2.6. Western blot analysis

The western blot analysis using anti- $V_5$  antibody (Invitrogen Inc.) was performed to confirm the expressed viral P proteins (Southern et al., 1991), as recommended by the manufacturer. Transformed yeast cells were grown and induced with methanol (as described in Section 2). The cells (1.5 ml) were centrifuged in a microfuge tube for 1 min at  $14\,000 \times g$ , and washed with  $1 \times$  of water. The cell pellet was resuspended in 100 ml of sample buffer (0.06 M Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.0025% (w/v) bromophenol blue)

(6). The sample mixture was then heated at 95 °C for 5 min and centrifuged at  $14\,000 \times g$  for 5 min. The soluble phase 25  $\mu$ l was loaded per lane for 12% SDS polyacrylamide gel electrophoresis.

## 2.7. Polymerase activity assay

DNA-dependent DNA polymerase (DDDP) and RNA-dependent DNA polymerase (RDDP) activities of the viral P proteins were tested using a synthesized DNA substrate (polA; 5'-CTCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCC-3' and polB; 5'-GGATCCTCTAGAGTC-3') and RNA-DNA hybrid substrate (RpolA; 5'-GCAUGCCUGCAGGUCGACUCUAGAGGAUCC-3, and polB), respectively. PolA and polB oligomers were annealed to the complementary oligonucleotide in the following buffer (20 mM Tris, pH 7.4, 80 mM NaCl, 1 mM DTT, 1 mM EDTA, and 3% glycerol) at 75 °C for 5 min and then the incubation temperature was gradually reduced in a water bath over 6 h. The RNA-DNA hybrid substrate was annealed with annealing buffer plus 1 U/ $\mu$ l of RNase inhibitor (Ambion). Annealed DNA and RNA-DNA hybrid substrates were concentrated and washed with sodium acetate and ethanol in RNase-free water. The final concentrations of the DNA and the RNA-DNA hybrid substrates were 10 and 100  $\mu$ M, respectively. DDDP activity was determined using the protein from the Ni<sup>+</sup>-NTA (Quiagen, Germany) purification. The assay to determine the DNA polymerization activity of viral P proteins was performed with the annealed DNA substrate (10 pmol), which was incubated in a 50  $\mu$ l reaction mixture (50 mM Tris, pH 7.4, 100 mM MgCl<sub>2</sub>, 1 mM DTT, 100 mM NaCl, 10  $\mu$ M dNTP (– dCTP), 0.1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, NEN-Du Pont Co., Chadds Ford, PA), and 10 pmol of the viral P proteins) for 2 h at 37 °C. The reverse transcriptase activity assays of the P proteins were performed with RNA-DNA hybrid substrate (100 pmol), which was incubated in a 50  $\mu$ l reaction mixture (50 mM Tris, pH 7.4, 100 mM MgCl<sub>2</sub>, 1 mM DTT, 100 mM NaCl, 10  $\mu$ M dNTP (– dCTP), 0.1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, NEN-Du Pont Co., Chadds Ford, PA), 0.1% Nonidet P-40, and 10 pmol of the viral P

proteins) for 2 h at 37 °C. In the RDDP activity test reaction, 1 U/ $\mu$ l of RNase inhibitor was added. After incubation, the reaction mixtures were stopped by adding 50 mM of EDTA, treated with protease K (0.2 mg/ml) at 37 °C for 30 min and extracted with phenol. The radioactivities of the reaction products were measured on DEAE-cellulose (DE-81) paper disks (Whatman Co.) as described previously (Grandgenette and Rho, 1975). The stop buffer (95% formamide, 1  $\times$  TBE, 0.05% bromophenol blue, 0.05% Xylene cyanol, and 0.2% SDS) was added to the reaction mixture, which was then heated at 95 °C for 5 min. The samples were then chilled on ice, subjected to Sephadex G-50 chromatography and analyzed by electrophoresis in 15% polyacrylamide gel containing 7 M urea in TBE buffer (90 mM Tris-borate and 2.5 mM EDTA) at 300 V. Gels were dried under the vacuum and the extension products were visualized by autoradiography. The dried gel or DEAE-cellulose (DE-81) paper disks (Whatman Co.) was quantified by scanning using a FUJIX BAS 2000 phosphorimager.

RNase H activity of the DHBV P protein was determined using RNA-DNA hybrid substrate (RpolA and polB). RpolA was radiolabeled by incubating 200 pmol of the synthesized 30-mer RNA, 100  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol, NEN-Du Pont Co., Chadds Ford, PA) and 20 U of T4 polynucleotide kinase (Promega, Madison, WI, USA) for 60 min at 37 °C. After incubating the enzyme for 10 min at 70 °C, the un-incorporated radiolabeled ATP was removed by Sephadex G-50 chromatography. The substrate was obtained by annealing the two oligonucleotides (labeled RpolA and polB) in annealing buffer (described above), and the mixture was slowly cooled to room temperature after heating at 80 °C for 3 min. The molar ratio of the components, labeled RpolA and polB in annealing mixture was 1:3. The labeled RNA-DNA hybrid substrate was concentrated and washed with sodium acetate and ethanol in RNase-free water. The final concentration of the RNA-DNA hybrid substrate was 200  $\mu$ M. The RNase H activity assay of the DHBV P proteins was performed with the RNA-DNA hybrid substrate (200 pmol), which was incubated in a 20  $\mu$ l reaction mixture (50 mM Tris, pH 7.4, 4

mM  $\text{MgCl}_2$ , 5 mM DTT, 100 mM NaCl, 0.1% Nonidet P-40, and 10 pmol of DHBV P protein) for 1 h at 37 °C. All Reactions containing the RNA-DNA hybrid substrate were treated with 1 U/ $\mu\text{l}$  of RNase inhibitor (ambion) 1 U/ $\mu\text{l}$ . After incubation, the reaction mixtures were stopped by adding 50 mM of EDTA and then treated with protease K (0.2 mg/ml) at 37 °C for 30 min and phenol-extracted. Radioactivities of the reaction products were measured by 15% polyacrylamide gel electrophoresis containing 7 M urea in TBE buffer (90 mM Tris–borate and 2.5 mM EDTA) at a constant 300 V. The gel was dried, and the product bands were analyzed by autoradiography on Fuji RX X-ray film.

### 3. Results

#### 3.1. Cloning and expression of the viral P proteins

Expressions of the HBV (1–680 amino acids) and DHBV (1–780 amino acids) P proteins were accomplished using the recently developed yeast expression system, *P. metanolica*, for a structurally dependent functional study. The cloning and expression were performed using pMet $\alpha$ A vector (secreted form of the *Saccharomyces cerevisiae*  $\alpha$  factor prepro signal peptide) initially, but the P proteins were not detected by western blot with anti V<sub>5</sub>-HRP antibody. Therefore, we cloned the HBV and DHBV P genes into pMetA vector (endogenous expression form) (Fig. 1). The cloned HBV P protein contained three domains, TP, Spacer, and polymerase (1–680 amino acids) and the DHBV P protein was cloned with all domains (1–787 amino acids).

To confirm the expression of DHBV P proteins, we used anti V<sub>5</sub>-HRP antibody for the recombinant proteins, which has a V<sub>5</sub> epitope in its C-terminal region. In the western blot analysis using the antibody, 7 clones revealed a single positive band at about 90 kDa (Fig. 2). The N-terminal sequence of the band confirmed that it was P protein. In case of HBV P protein expression, western blot detected a positive band about 74 kDa (Fig. 3B). Blank pMet A vector was trans-

formed in the PMAD16 and induced by methanol as a negative expression control. The induced band of blank pMet A vector was not detected by western blot analysis (data not shown).

#### 3.2. Purification of the viral P proteins

$\text{Ni}^{2+}$ -NTA resin was used to purify the proteins because the P proteins have a six-histidine tag at C-terminal region. DHBV and HBV P proteins were eluted in buffer A containing 75 and 100 mM imidazole, respectively. The asterisks of Fig. 3 indicate expressed HBV and DHBV P proteins (Fig. 3A and B). These proteins were confirmed by western blot analysis. DHBV P protein was concentrated using an Amicon concentrator, applied through gel-filtration chromatography, Su-

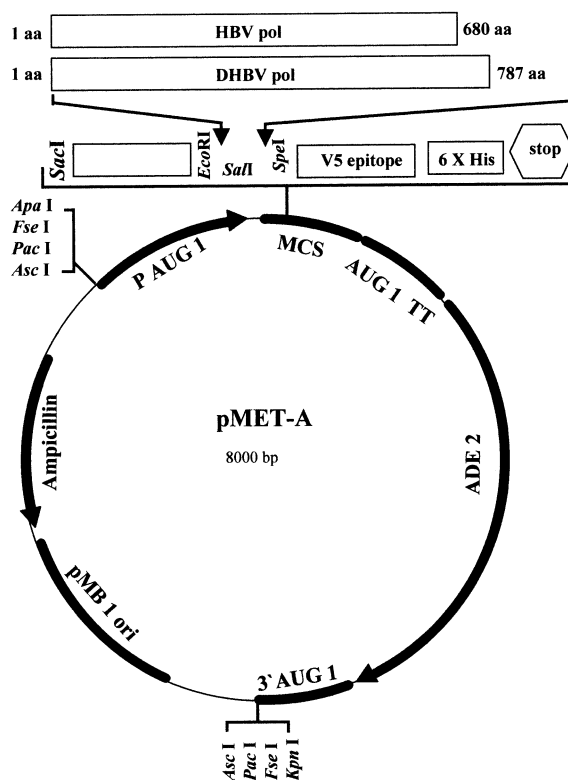


Fig. 1. Schematic representation of recombinant HBV and DHBV P genes in pMet A, yeast expression vector. These genes were cloned into pMet A yeast cloning vector using the restriction endonuclease site of the vector (HBV P: *Sal*I, DHBV P: *Eco*RI and *Spe*I).

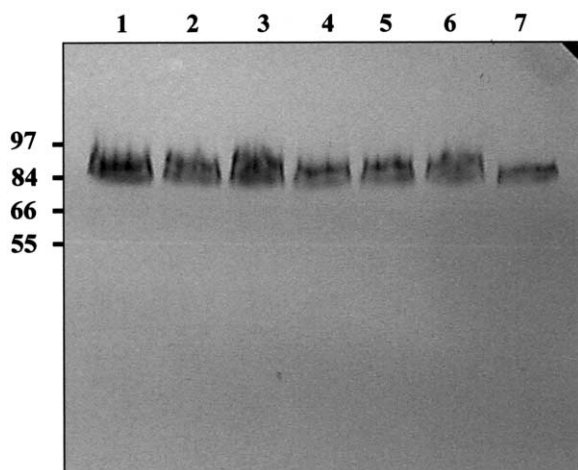


Fig. 2. Western blot analysis of DHBV P protein with anti- $V_5$  antibody. Western blot analysis was performed using anti- $V_5$  antibody. The lanes 1–7 represent the selected DHBV P gene colony numbers and seven clones revealed a single positive band at about 90 kDa. Left hand side of the gel contained the protein size marker.

perdex-S200 (Pharmacia), and purified as a fine single band (Fig. 4). The protein concentration of the enzyme was determined using the Bio-Rad dye reagent with BSA as a standard. DHBV P protein was purified up to 0.7  $\mu$ g in 100 ml methanol induced cell culture. In the case of HBV P protein we could not purify the protein further that achieved by  $Ni^{2+}$ -NTA column purification, because this protein was unstable after 2–3 h at 4 °C. Dextrose, glucose, and glycerol were added at various concentrations to the partially purified HBV P protein for stability, but complete stability of the P protein was not possible (data not shown).

### 3.3. DNA polymerase activity assay using DNA template

The P proteins have multiple functions on pathogenesis. Among these functions, we checked DDDP activity using the DEAE-cellulose (DE-81) paper disk method. The DEAE-cellulose (DE-81) paper disk method is more rapid and accurate than the other assay methods for confirming the viral protein activity. Un-labeled free isotopes were fully eliminated by Sephadex G-50 chro-

matography. The positive control of the activity test using klenow DNA polymerase was shown as a clear single extended product band (data not shown). To confirm the artifact signals on the paper disks as remaining free isotopes, we performed the negative reaction, which was the same as the positive reaction without the enzyme. This test was unable to detect any other signal on the film. The result showed that Sephadex G-50 chromatography could fully remove un-labeled free isotopes. We tested the DDDP activity using the synthesized DNA substrates, polA and polB (sequences of these DNA substrates are described in Section 2), and these oligomers were annealed to the complementary oligonucleotide in annealing buffer. The eluant of the  $Ni^{2+}$ -NTA (Quiagen, Germany) column were collected to determine the DDDP activity, which for the DHBV P proteins was found to range from 10 to 100 mM of imidazole buffer (Fig. 5A). The DDDP activity of the DHBV P protein was approximately one third of the 0.5 U klenow DNA polymerase (0.5 U, Promega) activity (data not shown). Despite of weak activity of the first fractions eluted, the 75 mM fraction had strong activity (Fig. 5B and C). Compared with the DDDP activity of the DHBV P protein, HBV P protein had DDDP activity in the broad range of the imidazole buffer concentration. Maximum activity of the HBV P protein was detected in 100 mM of imidazole buffer (Fig. 6A and B). For the negative control assay, only the pMet A vector was transformed into PMAD16 and processed using the same method as the HBV and DHBV P proteins. We could not detect the positive signals on the gel for the pMet A vector only. Unexpected DDDP activity was shown by the fraction before giving the imidazole gradient, which might have been due to yeast endogenous DNA polymerase activity or that of unbound P protein. However, this signal was weak compared with the DHBV and HBV P protein DDDP activities. Fig. 6C was acquired by over-exposing the film (Fig. 6C).

### 3.4. DNA polymerase activity assay using RNA template

The P protein binds in cis to its own mRNA

and this complex is packaged into immature viral nucleocapsids, then synthesize minus strand DNA using RNA template. RNA-dependent DNA polymerase activity test on DEAE-cellulose (DE-81) paper disks was similar to the RDDP activity test. The positive control used for the activity test was AMV reverse transcriptase (Avian Myeloblastosis virus RT, Promega) and this showed a clear single 30-mer DNA product band. To confirm the artifact signals on the paper disks for the remaining free isotopes, we performed the negative reaction, which was the same as the positive reaction condition without the enzyme. In this test we were unable to detect any other signal on the film (data not shown). We then tested RDDP activity using the synthesized RNA–DNA hybrid substrates, RpolA and polB (sequences of these RNA and DNA substrate were described in Section 2), and these oligomers

were annealed to the complementary oligonucleotide in annealing buffer in RNase-free water. Compared with the RDDP activity of the DHBV P protein, RDDP activity of HBV P protein had not shown significant positive signal (Fig. 7). The activity of AMV reverse transcriptase (9 U) was shown on the paper disk. We could not detect the positive signal for the pMet A vector only. Fig. 7 A and B indicate that only DHBV P protein has RDDP activity.

### 3.5. RNase H activity assay

DHBV P protein has an RNase H domain, and RNase H activity was determined using labeled RNA and DNA hybrid substrate (as described in Section 2). The P protein that was used in the RDDP activity test was used in this assay. The

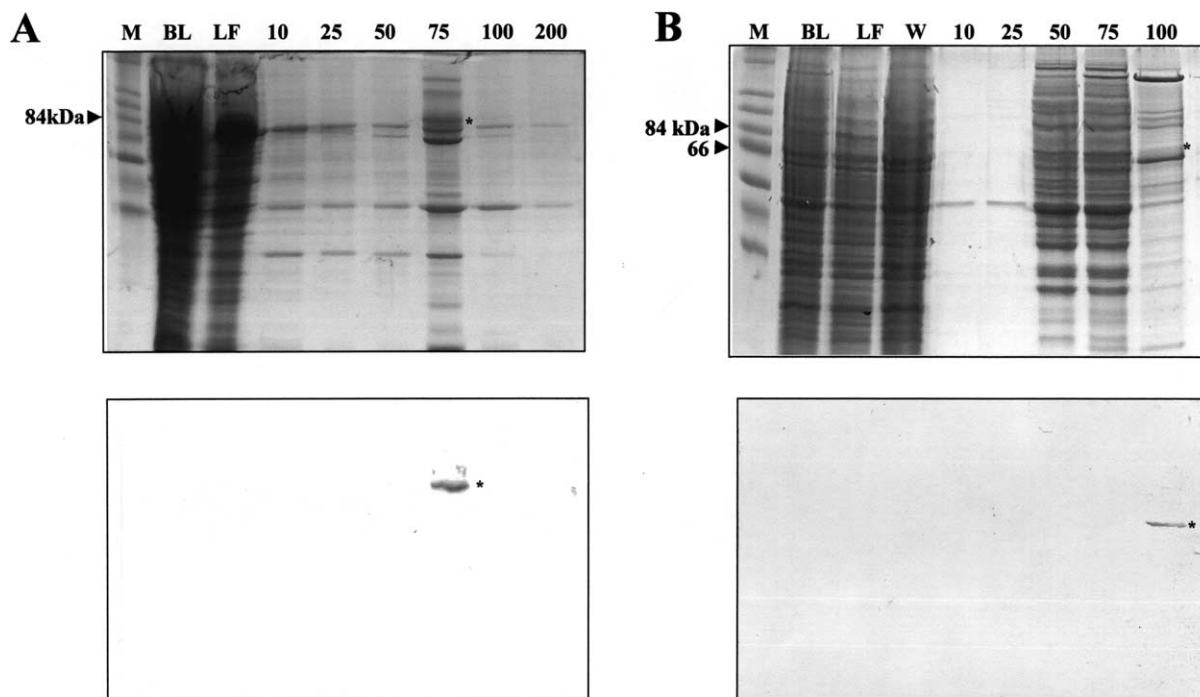


Fig. 3. Purification and Western blot analysis of the expressed P proteins. The cell were lysed and treated using the procedure described in Section 2. The extracted sample was introduced to  $\text{Ni}^{2+}$ -NTA resin packed column. (A) The DHBV P protein was eluted with 75 mM of imidazole buffer (upper panel) and this protein was confirmed by western blot analysis using anti- $\text{V}_s$  antibody (lower panel). The 90 kDa protein band was the expressed DHBV P protein. (B) The HBV P protein was collected in 100 mM of imidazole buffer (upper panel) and confirmed by western blot analysis (lower panel). The asterisks indicate the expressed DHBV and HBV P proteins (BL and LF indicate crude cell and the fraction that flows through before loading the sample, respectively).

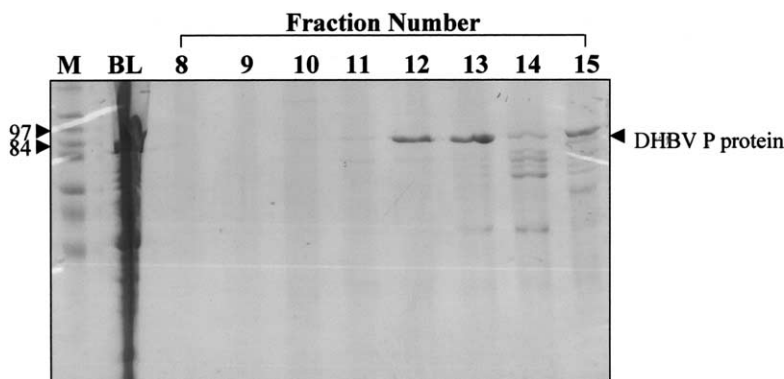


Fig. 4. Purification of the DHBV P protein by gel filtration chromatography. The DHBV P protein that was collected in the 75 mM imidazole buffer was concentrated using an Amicon concentrator and subjected to gel-filtration chromatography, i.e. Superdex-S200 equilibrated with buffer A. The viral P protein fractions were pooled and the purity of the protein sample was determined by electrophoresis using 12% SDS-polyacrylamide gel electrophoresis. Lane numbers indicate fraction numbers, M; molecular size marker, and BL; Supernatant of the cell lysate (5  $\mu$ l). The purified DHBV P protein is shown in Lane 12.

DHBV P protein had RNase H activity. To compare the RNA substrate digestion pattern of DHBV P protein with RNase H and RNase A, we performed an RNase A reaction. In the RNase A containing reaction, a 5'-labeled 30-mer RNA template was digested into small nucleotides. The RpoA nucleotide complementary to polB was digested by RNase H activity of DHBV P protein and a 15-mer RNA product was shown on 15% denaturing polyacrylamide gel electrophoresis (Fig. 8, lanes 1 and 2). For the negative control, pMetA vector only transformant was used, and no other significant RNase H activity was detected.

#### 4. Discussion

The P gene consisted of four domains, terminal protein, spacer, reverse transcriptase and RNase H. Of these functional proteins, P protein was been interested because of its multiple functions in the viral life cycle and pathogenesis. Much effort has gone into isolating the P protein and performing its multi-functional enzymatic activities, but to date insufficient P protein is available for biochemical and structural studies. In many cases, the expressed recombinant P proteins aggregated during the purification step, and also the viral genome bound tightly to the expressed poly-

merase as an endogenous template in-vitro (Summers and Mason, 1982).

In our study, we tried to express of the viral P gene using the recently developed yeast expression system, *P. metanolica*, for a structurally dependent functional study. We present a new method for producing the protein in reasonable amounts. In addition, this system is economic and reliable with a potential for the large scale screening of cofactors and inhibitors of the P protein functions.

In a small-scale expression test of HBV P proteins, we were able get a single P protein band by western blotting, but large-scale expression tests failed to produce P proteins in abundance. We varied the expression conditions with respect to temperature (20–30  $^{\circ}$ C), and nutrients [dextrose, adenine, or YNB (yeast nitrogen base, Invitrogen, Inc.)], and methanol concentrations (from 0.5 up to 10%). More P protein was expressed on a small-scale than on a large-scale. In the large-scale expression, aeration of the culture is very important. If the culture is not shaken properly, below 300 rpm or by using a simple un baffled culture flask, the cells form spores.

Study of viral P protein activity is important to the understanding of the viral replication mechanism. We developed a new method to check the viral P protein activity. The paper disk methods, described in the Section 2, allowed the measure-



ment of viral DDDP and RDDP activities. Many inhibitors of endogenous polymerase were tested to rule out cellular yeast polymerase activity on HBV and DHBV polymerase activity. We used 20  $\mu$ M of Aphidicolin for inhibition of yeast DNA polymerase ( $\alpha$  and  $\beta$  forms) and 80  $\mu$ M of Actinomycin D for inhibition of DNA directed DNA

synthesis. We could observe that aphidicolin inhibition was the same as the pMet vector only as a negative control. Therefore, we chose pMet vector as a negative control in DNA dependent DNA polymerase activity test. Also, actinomycin D reduced HBV and DHBV DDDP activities (Offensperger, et al., 1988; Howe, et al., 1992;

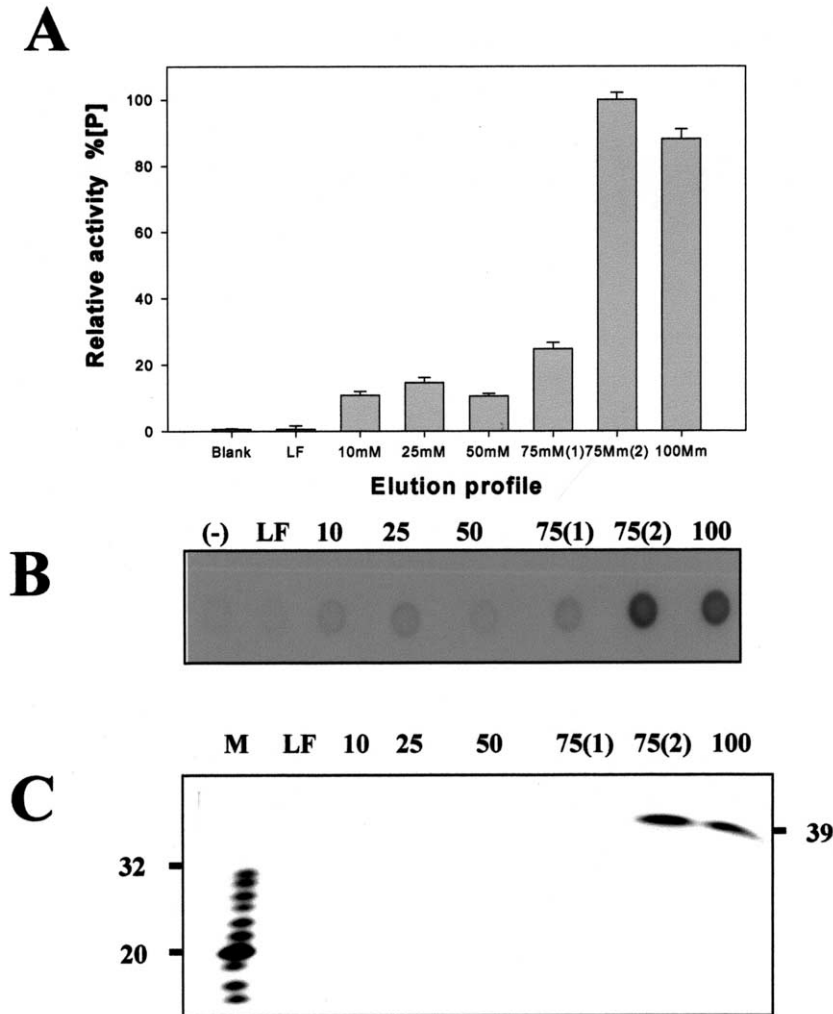


Fig. 5. The DNA-dependent DNA polymerase activity of the DHBV P protein. (A) The relative DDDP activities of the P protein collected from the  $\text{Ni}^{2+}$ -NTA purification were compared. The protein from the 75 mM imidazole buffer revealed the strongest activity. 'Blank' indicates the vector transformant's activity only and LF represents the loading buffer passed through the  $\text{Ni}^{2+}$ -NTA purification. (B) Radio activities of the reaction products were measured on DEAE-cellulose (DE-81) paper disks (Whatman Co.). As was found by western blot analysis, the fraction in the 75 mM imidazole buffer had the strongest activity. (C) The reaction products were analyzed by 15% polyacrylamide gel electrophoresis containing 7 M urea. Gels were dried under vacuum and the products were visualized by autoradiography. The 39-mer labeled polymerized DNA is shown in 75 and 100 mM imidazole buffer collection. M is the size marker.

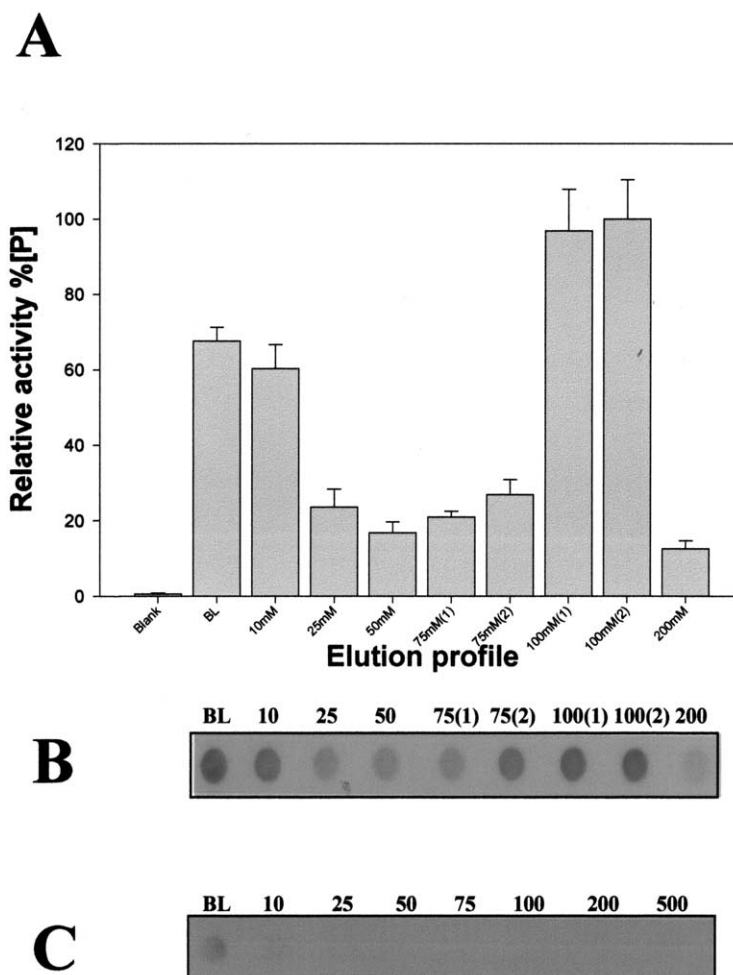


Fig. 6. DNA-dependent DNA polymerase activity of HBV P protein. (A) The DDDP activity of the HBV P protein was compared with other elution collections. Western blot band of the HBV P protein was positive in 100 mM imidazole buffer (as shown in Fig. 3B). The 100 mM elution contained the majority of the protein, and had the strongest DDDP activity. Blank indicates the negative control, and represents the activity of the transformant pMet A vector only. (B) Radio activities of the reaction products were measured on DEAE-cellulose (DE-81) paper disks (Whatman Co.) as described previously (5). In agreement with the western blot analysis, 100 mM elution collection revealed the strongest activity. (C) For an assay negative control, pMet A vector was transformed into PMAD16 and processed as described for the HBV and DHBV P proteins. We did not detect positive signals on the gel except for a faint spot before applying the imidazole gradient, but this signal was very weak compared with the DHBV and HBV P protein positive signals. BL represents the fraction before applying the imidazole gradient.

Tavis and Ganem, 1993; Seifer and Standring, 1993; Shin and Rho, 1995).

We used partially purified viral P-proteins in our activity studies. However, for the negative control used for the activity test no significant DDDP or RDDP signals were detected for the DNA and RNA–DNA hybrid substrates. The DHBV P protein has shown RDDP activity with-

out  $\epsilon$  motif. Recent studies have proposed that some chaperones might be needed for RDDP activity at the start point of DHBV replication (Beck and Nassal, 2001). The RDDP activity of our DHBV P protein was very weak compared with DDDP activity and the HBV P protein did not have RDDP activity. These results showed that HBV replication occurred via a different

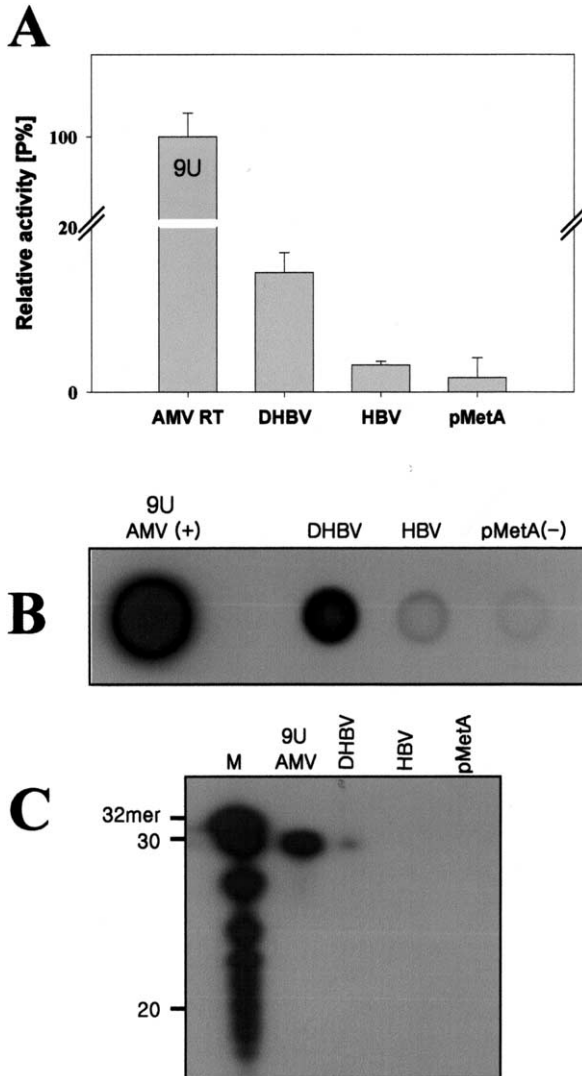


Fig. 7. The RNA-dependent DNA polymerase activity of DHBV and HBV P proteins. (A) The relative RDDP activities of HBV, DHBV P protein and 9 U of AMV RT (Avian Myeloblastosis Virus Reverse Transcriptase, Promega). (B) Radio activities of the products were measured on DEAE-cellulose (DE-81) paper disks (Whatman Co.). 'pMetA (-)' indicates the vector transformant's activity only. (C) The reaction products were analyzed by electrophoresis on 15% polyacrylamide gel containing 7 M urea. Gels were dried under vacuum and the products were visualized by autoradiography. The 30-mer labeled polymerized DNA was shown in DHBV P protein. For the positive control reaction, 9 U of AMV RT was used. M indicates size marker.

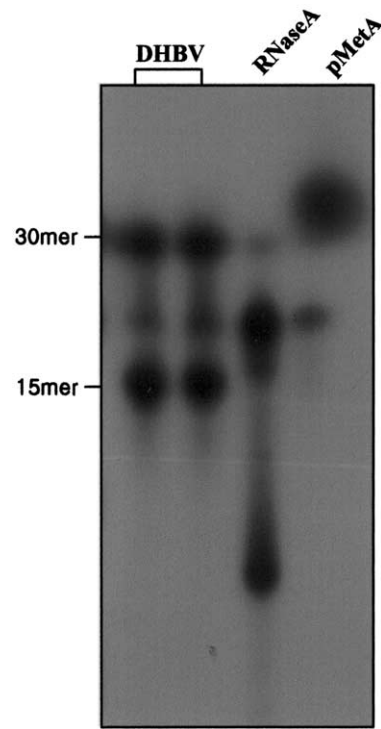


Fig. 8. The RNase H activity of DHBV P protein. The RNase H activity of the DHBV P protein was tested using a synthesized RNA–DNA hybrid substrate (RpolA and polB). Lane 1 and 2 indicate the RNase H activity of the DHBV P protein, lane 3 shows RNase A activity, and lane 4 is a pMetA only transformant used as a negative control.

mechanism from that of the DHBV or the DHBV P proteins used yeast chaperones, which were existed in the protein preparation substrate. It is possible that the HBV P protein may not be folding correctly when expressed in this system. The HBV P protein which did not have RNaseH domain could exhibit no RDDP activity. Furthermore, DHBV P protein had strong RNase H activity for the short synthetic RNA substrate (RpolA). Although these activities might be partial compared with the full activities of the P protein, which needs other components like the  $\epsilon$  motif, the DHBV and HBV P proteins showed several activities in the absence of such component in our system.

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