Expression and purification of the complete PreS region of hepatitis B Virus

Qiang Deng, Yu-Ying Kong, You-Hua Xie, Yuan Wang

Qiang Deng, Yu-Ying Kong, You-Hua Xie, Yuan Wang, State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes of Life Science, Chinese Academy of Sciences, Shanghai 200031, China
Qiang Deng, You-Hua Xie, Yuan Wang, Sino-France Center for Life Science and Genome Research, Shanghai 200031, China
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Co-correspondents: Yuan Wang and You-Hua Xie
Correspondence to: Yuan Wang, 320 Yue-Yang Road, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China. wangy@sibs.ac.cn
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Abstract

AIM: To express the complete PreS region of HBV in E.coli with good solubility and stability, and to establish an effective method for purification of the recombinant PreS protein.

METHODS: The complete PreS region (PreS1 and PreS2) was fused into a series of tags including glutathione S-transferase (GST), dihydrofolate reductase (DHFR), maltose binding protein (MBP), 6× histidine, chitin binding domain (CBD), and thioredoxin, respectively. Expression of recombinant PreS fusion proteins was examined by SDS-PAGE analysis and confirmed by Western blot. Two fusion proteins, thio-PreS, and PreS-CBD, with desirable solubility and stability, were subjected to affinity purification and further characterization.

RESULTS: Recombinant PreS fusion proteins could be synthesized with good yields in E.coli. However, most of these proteins except for thio-PreS and PreS-CBD were vulnerable to degradation or insoluble as revealed by SDS-PAGE and Western blot. Thio-PreS could be purified by affinity chromatography with nickel-chelating sepharose as the matrix. However, some impurities were also co-purified. A simple freeze-thaw treatment yielded most of the thio-PreS proteins in solution while the impurities were in the precipitate. Purified thio-PreS protein was capable of inhibiting the binding of HBV virion to a specific monoclonal antibody against an epitope within the PreS1 domain.

CONCLUSION: Increased solubility and stability of the complete PreS region synthesized in E.coli can be achieved by fusion with the thioredoxin or the CBD tag. A simple yet highly effective method has been established for the purification of the thio-PreS protein. Purified thio-PreS protein likely assumes a native conformation, which makes it an ideal candidate for studying the structure of the PreS region as well as for screening antivirals.

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Key words: Hepatitis B virus; PreS; Expression; Purification

INTRODUCTION

Virally encoded small (HBs), middle (MHBs), and large (LHBs) surface proteins together with cellular phospholipids form the envelope of HBV virion. These proteins are translated from distinct initiation codons, but share a common reading frame and stop codon. The HBs protein contains 226 amino acids and is the major component of the viral envelope. The MHBs protein has 55 extra amino acids (PreS2) located to the N-terminal of HBs and the LHBs protein carries an additional 119 amino acid (or 109 amino acids depending on the viral subtype, PreS1) N-terminal extension with respect to the MHBs protein[4]. All envelope proteins are co-translationally inserted into the endoplasmic reticulum (ER) membrane directed by the topogenic elements in HBs[2,3].

LHBs protein plays pivotal roles in infection and budding processes during the HBV life cycle. The N-terminal PreS region of the LHBs protein can adopt one of two topological conformations depending on whether it undergoes a posttranslational translocation. Thus, LHBs proteins in virions exhibit a mixed population with their PreS region (PreS1 and PreS2) located either inside or outside of viral envelopes[4,5]. Encapsulation of viral nucleocapsids and secretion of mature viral particles require LHBs proteins with cytoplasmic PreS regions[6-8]. A stretch of amino acids across the PreS1 and the PreS2 is thought to be involved in the interaction with the cytosolic nucleocapsid before the budding event[7]. During the infection process, externally exposed PreS region may mediate the binding of virion to a putative cellular receptor[9]. Amino acids 21-47 of the PreS1 domain likely bear the major epitope for cell attachment[9].

Given its important functional role in the HBV life cycle and being a potential target for the development of novel antivirals, efforts have been made to express the PreS region...
for structural analysis, however, with little success. To date, the PreS region synthesized in E. coli is either insoluble or quickly degraded\cite{15,16}. Nunez et al\cite{13}, produced the PreS region fused with a 6x histidine (6x His) tag that is largely insoluble. Purification of the His-tagged PreS protein under non-denaturing condition failed owing to a severe proteolysis. Structure analysis is difficult due to the fragile nature of the PreS region synthesized in E. coli after denature-renaturing cycles. Study with UV-CD spectra indicates that almost half of the proteins display non-ordered conformations\cite{13}, which may reflect an inherent instability of the PreS region or the improper folding of the recombinant PreS region. Alternative ways have been adopted to synthesize partial regions of PreS$^{[15,16]}$. However, it is not clear whether these partial regions retain their original structures.

In this study, we fused the PreS region with different tags respectively and studied the expression of these PreS fusion proteins in E. coli, in the hope of stabilizing the PreS region by a structurally linked tag. We observed increased solubility and stability of the PreS fused with the thioredoxin or the CBD tag. We have further established a simple yet highly effective method for purification of thio-PreS. The thio-PreS protein purified with this method is capable of inhibiting the binding of viral particle to a PreS1-specific monoclonal antibody.

**MATERIALS AND METHODS**

**Plasmid construction**

Primers (PreS-forward: 5'-ATGGGAGGTTGGTCTTC-CAAC-3'; PreS-reverse: 5'-GGTTCGCTGACAGGCT-CCCAGTC-3') with adaptors harboring appropriate restriction endonuclease sites were used to amplify the fragment encoding the PreS region of HBV was amplified. The DNA product was inserted into several prokaryotic expression vectors, respectively. Expression vectors used in this study were pET28α (Novagen), pQE40 (Qiagen), pMalC2x (NEB), pGEX2T (Pharmacia), pThioHisA (Invitrogen), and pTXB1 (NEB) (Figure 1). Clones containing correct inserts were verified by sequencing (Bioasia, Shanghai).

**Expression of fusion proteins**

E. coli Top10 (F') strain was used for the expression of DHFR-PreS and thio-PreS while the BL21 (DE3) strain was used for the expression of other fusion proteins. Expression was induced by IPTG for 4 h at room temperature. Optimized IPTG concentrations were 0.1 mmol/L for DHFR-PreS, 1 mmol/L for thio-PreS, and 0.3 mmol/L for other fusion proteins. Bacteria were harvested and resuspended in PBS with 1 mmol/L EDTA and 100 mmol/L PMSF. Lysates were prepared by sonication on ice and centrifuged before further SDS-PAGE analysis.

**Purification of fusion proteins**

Purification of PreS-CBD was performed with chitin resin (NEB) according to the manufacturer’s instructions. PreS-CBD coupled on the resin was subjected to an intermediate self-cleavage, induced by incubation overnight with 50 mmol/L DTT in PBS at 4 °C. Purification of thio-PreS was performed with ProBond\textsuperscript{TM}-nicel-chelating sepharose resin (Invitrogen), taking advantage of a conformational His-Patch motif within the thioredoxin tag. Supernatant of the bacteria lysate was mixed with the resin and rocked gently. The resin was washed thoroughly with PBS, followed by a stringent wash with five column volumes of wash buffer (500 mmol/L NaCl, 5 mmol/L imidazole, 20 mmol/L phosphate, pH 6.0) at a flow rate of 1 mL/min. Recombinant proteins were collected in 2.5 column volumes of elution buffer (500 mmol/L NaCl, 250 mmol/L imidazole, 20 mmol/L phosphate, pH 6.0). Impurities were removed by centrifugation of the thawed sample having undergone an overnight freeze at -70 °C. Purified proteins were subjected to desalting with Sephadex G25 (Pharmacia). Protein concentration was determined by the Bradford assay.

**Virus capture assay**

125E11\textsuperscript{[19,20]} is a monoclonal antibody against PreS1. For virus capturing, the antibody was immobilized on a microplate (Nunc) in carbonate buffer. PBS diluted sera of HBV patients (provided by Ruijin Hospital, Shanghai) were added at 10\textsuperscript{4} infectious virions per well and incubated for 1 h at room temperature. HBV virions were then detected with HRP conjugated anti-HBs antibody (Sino-America Biotech) and subjected to TMB developed color reaction. The optical density values were measured at 450 nm ($A_{450}$) with an automatic photometer (Bio-rad). For competitive binding assays, different amount of purified thio-PreS, or thioredoxin as the control, was added to the diluted sera prior to virus capturing.

**Western blot**

Western blot analyses of PreS fusion proteins were performed according to a standard method\cite{21}. Supernatants of the expression lysates were separated on SDS-PAGE, and transferred to nitrocellulose filters. 125E11 (1:1 000) was used as the primary antibody for PreS detection. Blots were developed using the ECL method (PerfectBio) with HRP-labeled rabbit anti-mouse Ig (1:2 000, Dako).

**RESULTS**

**Construction and expression of PreS fusion proteins**

A series of prokaryotic expression vectors (Figure 1) were employed to construct PreS expression plasmids. The DNA fragment encoding the PreS region of HBV was amplified.
by PCR and inserted into these vectors respectively. 6× His tag was fused to either the N-terminal or the C-terminal of PreS. The CBD tag was joined to the C-terminal of PreS by an intein (Figure 1). Other tags (GST, DHFR, MBP, and thioredoxin) were located at N-terminal respectively.

SDS-PAGE was performed to examine the synthesis of fusion proteins. His-tagged PreS fusion proteins were insoluble, though fairly high yields were achieved (Figure 2A). GST-PreS was found in soluble fraction of the bacterial lysate. However, severe degradation was obvious. Even worse degradation was observed with DHFR-PreS (data not shown). Previous studies by Cho et al. [14], suggested a more stable recombinant product of MBP fused PreS. In our study, however, moderate proteolysis still occurred. Besides the full-length 60 ku protein, other bands with a smaller molecular weight were observed as indicated on SDS-PAGE, probably represented degraded proteins (Figure 2B). On the contrary, thio-PreS showed an excellent stability in solution (Figure 2C) with a high yield reaching about 0.2-0.5 mg per ml of the bacteria culture. Similarly, PreS-CBD was found predominantly in the supernatant of the bacteria lysate, and importantly, without any obvious degradation by SDS-PAGE analysis (Figure 2D).

**Purification of PreS fusion proteins**

Given their desirable solubility and stability, PreS-CBD and thio-PreS were subjected to further purification. The chitin matrix was used to purify the PreS-CBD protein. As shown in Figure 2D, PreS-CBD could be successfully separated from the lysate mixture and coupled to the chitin resin. Binding of PreS-CBD to the chitin resin was highly specific, and few contaminants were detected by SDS-PAGE analysis (lane 3, Figure 2D). However, the purification process was handicapped in the elution step. The intact fusion product was difficult to be eluted off the chitin resin, and inter-based cleavage was unsuccessful due to the fragile nature of the free PreS region (data not shown).

Thioredoxin tag as in thio-PreS harbored a His-Patch motif that allowed purification of recombinant proteins on nickel chelating sepharose. The His-Patch motif differed from other linear His-tags in conformation. Thus, recombinant proteins could be purified under a non-denaturing condition. Figure 3A shows the affinity purification of thio-PreS with nickel chelating sepharose. Proteins were eluted with a buffer containing imidazole at different concentrations. An elution buffer containing 250 mmol/L imidazole, 500 mmol/L NaCl and 20 mmol/L sodium phosphate, pH 6.0, gave an optimized elution of thio-PreS. However, some impurities were apparently present even under a highly stringent wash condition. We also tried the resin of thio-bond (Invitrogen) that is specific for the thioredoxin tag rather than the His-Patch motif, but with little success (data not shown).

Since thio-PreS showed a great solubility, freezing the affinity purified sample followed by a quick thaw might improve the purity of thio-PreS by eliminating the impurities that were slow in redissolving. As shown in Figure 3B, with
this approach, most impurities were completely fractioned into the precipitate while the majority of thio-PreS remained in the supernatant without any proteolysis. Densitometry scan (Bio-Rad) of the sample on SDS-PAGE suggested a high purity of above 95%. The purified thio-PreS protein was very stable. A procedure of desalting chromatography with Sephadex G25 did little harm to the integrity of this fusion protein even at room temperature (data not shown).

**Characterization of the PreS fusion protein**

To verify the correctness of synthesized fusion proteins, Western blot was performed with the monoclonal antibody 125E11. As shown in Figure 4, MBP-PreS, PreS-CBD, and thio-PreS with a correct molecular weight could be detected, respectively. Proteolysis of MBP-PreS was also apparent, while only tiny traces of degradation of thio-PreS and PreS-CBD were found. 125E11 could recognize a PreS1 epitope exposed on the surface of the virion. As shown in Figure 5, when immobilized on the solid surface of microplate wells, the antibody could specifically capture viral particles from HBV patient sera. Addition of purified thio-PreS protein to the sera could inhibit the capturing process efficiently and in a dose-dependent manner, suggesting that the PreS region in the thio-PreS protein likely adopts a native conformation that mimics that of the HBV virion.

**DISCUSSION**

The PreS region of LHBs has been implicated in the attachment of HBV virion to the putative receptor on host cells, while its inward conformation is thought to be involved in viral morphogenesis. Other important roles have also been assigned to PreS, including regulation of viral replication and transactivation of a variety of promoter elements[22-24]. Thus, multiple virological functions of the PreS region provide a useful target for anti-HBV drug intervention. Inhibition on viral infection by *E. coli* expressed PreS occurs through a direct interference with the binding of HBV to the putative cell surface receptor[25]. Recombinant PreS product could also be developed as a protein vaccine that elicits B and T cell immune responses on a broader range of MHC haplotypes[26-29]. Nevertheless, detailed structural studies of PreS are complicated by the difficulties in isolating large quantities of LHBs from viral particles and in expressing soluble and stable PreS proteins.

The complete PreS on the surface of HBV virions has been shown as an independent domain with a native structure[30,31]. Thus, recombinant PreS proteins expressed in the *E. coli* system may accurately reflect the proper structure. However, according to previous works, the PreS region is susceptible to heavy proteolysis during expression and purification. In this paper, attempts have been made to express the PreS fused with various tags in *E. coli*, in the hope of stabilizing the recombinant products by structurally linked tags[30]. Although most PreS fusion proteins expressed in this study are still vulnerable to degradation, or exist as insoluble inclusion bodies, a stable and soluble PreS fusion protein containing the thioredoxin or the CBD tag has been synthesized with a good yield. Thioredoxin is a highly hydrophilic protein tag, designed for achieving increased solubility of fusion proteins expressed in *E. coli*, and usually accumulates at sites on the cytoplasmic inner membrane known as adhesion zones[31]. We proposed that the environment of adhesion zones as well as the great solubility of thioredoxin could contribute to the better folding of the recombinant PreS protein. The impact of intein-CBD (Figure 1) on a better stability of PreS is also obvious. The underlying mechanism is currently unknown. It is possible that the intein-CBD tag forms a structure that may hinder the access of proteases to the proteolytic sites in the PreS region.

Affinity purification based on the His-Patch motif under a non-denaturing condition is not always efficient. In purification of thio-PreS, the contamination of impurities is obvious. Interestingly, after a freeze-thaw treatment, the affinity purified thio-PreS sample is dramatically purified. The majority of thio-PreS remains in the supernatant while the impurities are largely precipitated. This process may take advantage of the differential solubility between thio-PreS and the impurities or act as a salting-out step. The purified thio-PreS might adopt a native structure, since the soluble expression product in a stable fashion usually suggests a proper folding, and His-Patch based affinity purification would imply a correct conformation of the thioredoxin tag. Importantly, thio-PreS can efficiently inhibit the binding of viral particle to the PreS1 specific monoclonal antibody, suggesting exposed epitopes in the recombinant protein as those on the surface of the native virion. PreS-CBD is also soluble and shows a high stability. Purified PreS-CBD coupled to the chitin resin could be obtained, but the following intein-based cleavage by DTT was unsuccessful which may be due to the fragile nature of the PreS region.
Similarly, thio-PreS was subjected to an enterokinase-cleavage that is engineered in the thio-fusion expression system to separate the desired PreS region from the thio-fusion tag. However, severe proteolysis was observed during this treatment (data not shown), suggesting an important role of the thio-endoxin tag in stabilizing the PreS region.

In conclusion, the complete PreS region fused with the thio-endoxin or CBD tag has been successfully synthesized. A simple yet efficient method has been established for purification of the thio-PreS protein. The thio-PreS protein is highly stable and soluble. The purified thio-PreS protein may be a valuable candidate for studying the structure of the PreS region as well as for screening antivirals.

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