Simple One-step Purification of Hepatitis B Core Antigen in *Escherichia coli*

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**Abstract**

Hepatitis B core antigen (HBcAg) is one of the most important diagnostic reagents in hepatitis B virus (HBV) infection. The purification of HBcAg using conventional methods, such as sucrose density gradient ultracentrifugation, is time and cost consuming. To overcome this difficulty, we aimed at developing an efficient method for producing a soluble form of HBcAg for diagnostic application. The HBcAg construct was secreted into periplasm space of *E. coli*, which facilitates purification by selective disruption of the outer membrane. The His-tagged HBcAg was purified from prepared periplasmic fraction using Ni-NTA affinity chromatography with an average yield of 20 mg/L culture. The results of competitive ELISA indicated that the antigenicity of periplasmic HBcAg is comparable with commercial *E. coli*-derived antigen. These results demonstrate the periplasmic system is suitable for the rapid and simple purification of bioactive and soluble HBcAg.

**Keywords:** Hepatitis B Core Antigen, Periplasmic Expression, Purification

1. **Background**

HBcAg is a 183 amino acid protein, which can be divided into an assembly (1 - 149) and a protamine-like domain (150 - 183). These domains are responsible for polymerization and RNA packing, respectively (1, 2). HBcAg is not only used in acute Hepatitis B virus infection detection but also used in development of hybrid vaccines such as malaria, influenza A virus and simian immunodeficiency virus DNA vaccine. HBcAg protein is highly immune and elicits potent B cells, cytotoxic T cells, and T-helper cells. Therefore, such fused epitopes elicit a strong immune response in animal models, particularly if inserted in the immunodominant region (3).

Different expression systems, such as insect cells, yeast, and *Escherichia coli* were used for HBcAg preparation (4-6). Although HBcAg prepared in insect cells has proper antigenicity, it is unsuitable for large-scale expression due to high cost and complicated manipulation. *Pichia pastoris* is able to secrete HBcAg to culture media, thus facilitating long and complicated HBcAg purifications. Long incubation time and low yield is main disadvantages of *Pichia pastoris* expression system. *E. coli* is a proper host for foreign protein expression due to a high growth rate, low cost, simple culture condition and it is easy to manipulate (7).

HBcAg was cloned, expressed, and purified in *E. coli* in previous studies. Various purification strategies suggested for HBcAg from *E. coli* such as affinity chromatography using His-tag in combination with non-affinity methods such as ion exchange, size exclusion, and selective participation. Protein was purified from the cytosol as a soluble form or by refolding after the inclusion of insoluble bodies (8-10).

*E. coli* has high ability to transport expressed proteins to periplasm. Expression of protein periplasm system can be considered as first purification step, since periplasm contain less complex mixture than cytoplasm. The outer membrane of *E. coli* can be selectively degraded by osmotic shock and spheroplast containing proteins can be easily isolated (11-13).

In this study, we modify the HBcAg coding sequence according to *E. coli* codon usage without amino acid alteration. The synthetic DNA was sub-cloned into pET22b+ expression vector and was successfully expressed in *E. coli* periplasmic space. Finally, we compared antigenicity of HBcAg with commercial *E. coli*-derived HBcAg.

2. **Methods**

2.1. **Serum Samples**

Serum samples were collected from patients with HBV infection (patients who were positive for HBcAg antibodies) who were referred to the Blood Transfusion Center,
Mashhad, Iran. All the serum samples yielded positive results when tested using commercial anti-HBcAg ELISA kits. Blood samples from healthy volunteers were used as controls and tested using ELISA.

2.2. Computational Analysis of the Construct

Amino acid sequence coding HBcAg (access number: P03146) was obtained from UniProt, which is available at (http://www.uniprot.org/). The amino acid sequence was reverse translated into nucleotide sequencing using a reverse translator available at (http://www.bioinformatics.org/sms2/rev_trans.html). DNA sequence was optimized according to E. coli codon usage using OPTIMIZER, which was available at (http://genomes.urv.es/OPTIMIZER/). The effectiveness of codon optimization was evaluated by codon adaptation index (CAI) and guanine-cytosine (GC) content calculation using rare codon analysis (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis.html). Protein molecular weight was predicted using ExPasy available at (http://web.expasy.org/protparam/).

2.3. Construction of Recombinant Plasmid

DNA encoding HBcAg was synthesized by Biomatik and sub-cloned into BamHI and EcoRI restriction sites of pET22b+ expression vector using standard protocols to construct the pET22b-HbcAg construct. The ligation products were transformed into chemically competent E. coli BL21 (DE3), and grown on LB plate supplemented with 100 µg/mL ampicillin. Some of the survival colonies were selected and sub-cloning was verified using DNA sequencing.

2.4. Expression of the HbcAg Protein

The method used for expression of recombinant HBcAg described in our previous studies (6-8). One colony of E. coli BL21 (DE3) harboring pET22b-HBcAg was inoculated into 5 mL LB containing 100 µg/mL ampicillin and incubated overnight at 37°C under constant shaking 250 rpm. One mL of overnight culture was inoculated in 100 mL fresh LB containing 100 µg/mL ampicillin and incubated at 37°C under OD600 nm reached at 0.6. In this time, expression of HBcAg was induced by addition of IPTG with a final concentration of 0.4 mM and further incubated at 18°C for 12 hours under shaking 250 rpm.

2.5. Expression Analysis by SDS-PAGE

To analyze expression of recombinant HbcAg, 750 µL of culture containing induced bacteria was transferred into a 1.5 mL micro-tube and cells were harvested by centrifugation at 5000rpm for 5min. The cells were suspended in 30 µL 2 x loading buffer and heated at 100°C for 10 minutes, and analyzed by 12% SDS-PAGE in a discontinuous buffer system. Non-induced cells were analyzed as negative controls in parallel.

2.6. Preparation of Periplasmic Fraction

Harvested cells were suspended in 25 mL of hypertonic solution and incubated at 4°C for 30 minutes. Then, cells were harvested by centrifugation and supernatant was collected. These were re-suspended in 25 mL of MgSO4 5 mM, incubated at 4°C for 30 minutes and additionally centrifuged. Supernatant resulted from hypertonic and hypotonic treatment was centrifuged to remove any debris and dialyzed against PBS overnight at 4°C. The total yield of protein was determined by Bradford assay and percent of target protein was calculated by gel-scanning method. The final yield of the target protein was calculated by multiplication of protein percent in total protein concentration. This analysis was performed in triplicate and results were shown as an average of the results.

2.7. Protein Purification

Supernatants collected in previous steps were centrifuged at 12000 rpm at 4°C for 20 minutes to remove any debris. Then, supernatant was transferred into a 1 x 5 cm column, which was packed with 1.5 mL of Ni-NTA resin. Resin was washed by three columns of binding buffer and five volumes of washing buffer. The absorbed HbcAg was eluted by imidazole gradient. The flow rate of 0.5 mL/min was used in all chromatographic steps.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

The wells of the ELISA plate were coated with 200 µL of HBcAg (1 µg/mL) and incubated overnight at 4°C. After this period, wells were washed twice with washing buffer and blocked by blocking buffer for 2 hours at room temperature. Then, 50 µL of serum samples containing anti-HbcAg antibodies and 50 µL of monoclonal anti-HbcAg HRP-conjugated antibody were added to each well and incubated for 1 hour at 37°C. After washing six times with washing buffer, 100 µL of TMB solution was added to each well and incubated at room temperature for 30 minutes. The absorbance was read at 450 nm by ELISA reader. The anti-HbcAg antibody negative serum was analyzed as a negative control in the same condition.

3. Results

3.1. Construction of pET22b-HbcAg-His Tag

The DNA sequencing result indicated the gene of HBcAg was successfully transferred into pET22b+...
BamHI/EcoRI expression vector. This vector incorporated PelB signal to N-terminal of protein, which resulted in secretion of protein to periplasmic space. Expression cassette in this vector is under control of the T7 promoter and high-level expression could be achieved with IPTG induction. The theoretical molecular mass of HBCAg expressed with pET22b in E. coli cells was 14.5 kDa.

3.2. Small-Scale Expression

Small-scale expression was achieved by IPTG induction (final concentration 0.4 mM), and incubation at 18°C for 12 hours. The SDS-PAGE results indicated protein was successfully expressed in induced cells. No similar band was observed in non-induced cells harboring pET22b-HBcAg.

3.3. Purification

All purification steps were performed at room temperature. The HBcAg-His tagged was immobilized on Ni-NTA resin and non-bound proteins washed out. The absorbed antigen recovered using elution buffer and dialyzed against PBS overnight. Total yield of purified protein was approximately 20 mg/L. The purity of protein on 12% SDS-PAGE was more than 90%.

3.4. ELISA

ELISA was performed to evaluate antigenicity of purified antigen. The competitive ELISA results indicated periplasmic HBcAg reacts with HRP-conjugated anti-HBcAg monoclonal antibody. The results of 10 infected and 10 healthy sera indicated this protein could discriminate infected and healthy sera.

4. Discussion

Codon usage of E.coli is different with viruses. The viral protein gene sequence contains codons, which are rarely used in E.coli cells. tRNA of such codons is less frequent, and rapidly depleted during high-level expression, which is also difficult to such proteins. It is suggested that codon optimization increases the expression levels of recombinant proteins. We optimized the interest gene with substituting rare codons with preferred codons (14).

Expression of the optimized protein sequence under control of T7 promoter is an effective method for high-level expression of proteins. However, overproduction of foreign proteins in E. coli generally leads to formation of inclusion bodies (15). Although purification of inclusion bodies is easy, denaturation and renaturation of purified proteins are prerequisites and thus complicate purification.
It is suggested low temperature induction could improve protein solubility expressed in cytoplasm (16).

Expression of protein as periplasmic not only results in soluble expression, it also targets proteins easily separated from most of the host contaminant proteins. Periplasm provides a good compartment for protein structure due to low protease activity.

PelB signal peptides were removed during secretion of protein to periplasm, resulting in correct N-terminal peptides (17).

It is suggested low temperature induction in periplasmic expression increases transportation of protein into periplasm. It is likely that high-temperature induction increases hydrophobic interaction, which results in the aggregation of proteins. Low-temperature induction allows more time for protein chains to pass through the membrane, resulting in lower protein aggregation in cytosol (18).

Mechanical and physical cell disruption methods are available to release periplasmic proteins. Mechanical proteins release less selectively proteins, which have difficult downstream purification. With this knowledge, we used osmonic shock for protein release from periplasm. Selective releasing and low cost are the most important advantages of this method (18).

Overall yield of recombinant protein in this system was 20 mg/dL, which is lower than some previous strategies that used cytosolic expression systems. However, low yield with regard to ease of purification and low cost can be considered a way for HbcAg preparation.

The strong signal observed in ELISA is most likely due to the interaction of monoclonal antibodies and core protein.

4.1. Conclusion

His-tagged HbcAg expressed by periplasmic systems can be purified easily without denaturation and renaturation steps or using complicated method. Moreover, the incorporation of His-tag does not affect the antigenicity of HbcAg with serum antibodies.

References


Table 1. Results of HbcAg Antibodies Detection in HBV Infected and Healthy Volunteer Sera

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