Osong Public Health Res Perspect 2012 3(2), 79–84
doi:10.1016/j.phrp.2012.04.003
pISSN 2210-9099 eISSN 2233-6052

ORIGINAL ARTICLE

Non-chromatographic Method for the Hepatitis B Virus X Protein Using Elastin-Like Polypeptide Fusion Protein

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Received: December 16, 2011
Revised: January 15, 2012
Accepted: January 20, 2012

KEYWORDS:
elastin-like polypeptide,
HBx,
hepatitis B virus,
inverse transition cycling,
recombinant protein purification

Abstract
Objectives: Hepatitis B virus (HBV) is a member of the hepadnavirus family. The HBV genome contains four genes designated as S, C, P, and X. The HBV X (HBx) gene encodes for a 16.5-kDa regulatory protein that enhances HBV replication and exerts multifunctional activities. The aim of this study is to describe the rapid and easy purification of HBx using ELP (elastin-like polypeptide) fusion protein.
Methods: The ELP–HBx fusion protein was overexpressed in *Escherichia coli*. Environmental sensitivity was demonstrated via turbidity and dynamic light scattering as a function of temperature. HBx was purified as an ELP fusion protein. ELPs are biopolymers of the pentapeptide repeat Val-Pro-Gly-Xaa-Gly that undergo an inverse temperature phase transition. ELP follows in temperature and salt consistency, precipitation, and solution repetition (inverse transition cycling) with polypeptide, where it purifies the protein in a simple manner.
Results: Fusion proteins underwent supramolecular aggregation at 40 °C in 1 M NaCl and slowly resolubilized at subphysiologic temperatures. ELP domain proteolysis liberated a peptide of comparable size and immunoreactivity to the commercial HBx.
Conclusion: This study suggests that HBx can be purified rapidly and easily using inverse transition cycling, and that this method can be applied in determination of HBx 3D structures and HBx stability study.

1. Introduction

In alcohol or virus infection, persistent hepatic inflammation precedes chronic liver injury. A series of recent reports strongly indicated that chronic inflammation is closely linked to the development of liver cancer, implicating a theory of inflammation–fibrosis–cancer axis [1,2]. In Western countries, the most common cause of liver diseases is alcohol; by contrast, more than 90% of liver disease cases in Korea

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are due to viral infections and progressive hepatocellular carcinoma, which is often initiated by chronic hepatitis B virus (HBV) or hepatitis C virus infection and is considered a major worldwide health problem [3,4]. Approximately 350 million people worldwide are chronically infected by HBV. In Korea, in particular, 70% of liver cancer cases are caused by HBV infection, representing a serious national health issue.

HBV contains four genes designated as S, C, P, and X. As with the rest, the X protein (HBx) encodes a 16.5-kDa regulatory protein including 154 amino acids. HBx enhances HBV replication and exerts multifunctional activities [5]. It acts as a transcriptional coactivator in the nucleus and stimulates various signal transduction pathways in the cytoplasm. Recently, HBx is known to have a kunitz domain, where it performs a special function as a serine protease inhibitor and transcriptional activatior [6].

HBx does not bind directly to the DNA but regulates the activity of cellular proteins such as p53, BubR1, Pin1, UV-DDB, and HSP40/DanJ to bind directly or indirectly and contributes to liver cancer progression [7−10].

The objective of this study was to develop an HBx purification process with elastin-like polypeptide (ELP) domains. ELPs are genetically engineered polypentapeptide biopolymers like mammalian elastin. They are composed of pentapeptide repeats of Val-Pro-Gly-Xaa-Gly, where the guest residue is any amino acid residue other than proline [11−14]. These polypeptides are soluble below their characteristic temperature transition and undergo an abrupt inverse temperature phase transition to aggregates upon heating and addition of salt.

The hypothesis of this study was that a fusion protein constructed from ELP and HBx would result in HBx purification by ELP inverse phase-transitioning behavior. The ELP−HBx gene was designed, and the protein product was expressed in *Escherichia coli*. Results are presented for HBx purification. This study suggests that HBx can be purified in a rapid and simple manner using inverse transition cycling (ITC), and this method can be applied in determination of HBx 3D structures and HBx stability study.

2. Materials and Methods

2.1. Cloning of full-length HBx gene

To establish the HBx expression construct, polymerase chain reaction was performed with the inserting forward primer (5′-AAG CTT CTG GTT CCG CGT GGA TCC ATG GCT GCT AGG CTG TGC-3′) and reverse primer (5′-AAG CTC AGC TAG GCA GAG GTG AAA AAG TTG CAT GG-3′). These resulting polymerase chain reaction fragments were digested with *Hin*dIII and *Blp*I, for the purpose of directional and in-frame ligation to the pQE-His-ELP vector, and inserted to pQE30-His-ELP (kindly provided by Dr. Yoon, Kyonggi University). Expression vectors containing the fusion gene were transformed into *E coli* BL21 (DE3) (Novagen, Madison, WI, USA) for protein overexpression.

2.2. Overexpression of recombinant fusion protein

Fifty milliliters of LB and M9 minimum media with 100 μg/ml of ampicillin was inoculated with the expression strain and induced at a cell density of 0.6 ($A_{600\ nm}$) by the addition of 0.3 mM isopropyl-thiogalactoside (IPTG) at 3−4 hours.

2.3. Inclusion body purification

Overexpressed cells were harvested by centrifugation (3200 × *g* for 15 minutes) and resuspended in 5 ml phosphate-buffered saline (PBS) with lysozme. Cells were lysed via sonication on ice and centrifuged (13,000 rpm for 15 minutes, 4 °C) to harvest the inclusion body. The inclusion body pellet was washed (50 mM PBS, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA) and centrifuged (15 minutes at 13,000 rpm at 4 °C). The supernatant was decanted, and the precipitate was washed in 2 M urea for 20 minutes. Inclusion bodies were separated by centrifugation at 13,000 rpm for 30 minutes and washed in 0.5% Triton X-100 (v/v) and 10 mM EDTA (20 minutes incubation at room temperature). After washing, the inclusion bodies were recovered by centrifugation at 13,000 rpm for 15 minutes.

2.4. Inclusion body solubilisation and purification of recombinant ELP−HBx

Washed inclusion bodies were dissolved in 0.1 M Tris−HCl, pH 8.6, containing 8 M urea for 24 hours at room temperature. Dissolved ELP−HBx fusion protein was separated by centrifugation at 13,000 rpm for 30 minutes, and the supernatant was collected. Then, denaturated fusion protein was dialyzed in 1 M urea solution. The recombinant ELP−HBx was purified by three rounds of ITC as described previously [13].

2.5. In vitro degradation of fusion protein

Thrombin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS to a concentration of 300 units/ml. Next, 3 μl of the enzyme solution was added to 100 μl of a purified ELP−HBx solution in PBS for a final activity of 0.3 units. The mixture was incubated overnight with gentle agitation at 37 °C, after which sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described above.

2.6. Protein analysis

Purified ELP−HBx fusion proteins were separated on a 12% SDS-PAGE gel and stained with silver. For Western blotting, ELP−HBx fusion proteins were separated and transferred onto a polyvinylidene