

Cloning and expression of NS3 helicase fragment of hepatitis C virus and the study of its immunoreactivity in HCV infected patients

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ABSTRACT

Objective(s): Hepatitis C is a major cause of liver failure worldwide. Current therapies applied for this disease are not fully effective and produce side effects in most cases. Non-structural protein 3 helicase (NS3) of HCV is one of the key enzymes in viral replication and infection. Therefore, this region is a promising target to design new drugs and therapies against HCV infection. The aim of this study was cloning and expression of HCV NS3 helicase fragment in *Escherichia coli* BL21 (DE3) using pET102/D-TOPO expression vector and studying immunoreactivity of the expressed antigen in Iranian infected with hepatitis C.

Materials and Methods: The viral RNA was extracted from the serum of HCV infected patient. The NS3 helicase region was amplified by RT-PCR. The PCR product was directionally cloned into the expression vector pET102/D-TOPO and transformed into the BL21 strain of *E. coli* (DE3). The transformed bacteria were then induced by adding 1mM isopropyl- β -D-thiogalactopyranoside (IPTG) into the culture medium to enhance the protein expression. SDS-PAGE and western blotting were carried out to identify the protein under investigation, and finally purified recombinant fusion protein was used as the antigen for ELISA method.

Results: The insertion of the DNA fragment of the NS3 region into the expression vector was further confirmed by PCR and sequencing. SDS-PAGE analysis showed the successful expression of the recombinant protein of interest. Furthermore, immunoreactivity of fusion NS3 helicase was confirmed by ELISA and western blotting.

Conclusion: It seems that this recombinant protein could be a useful source of antigen for future studies on HCV diagnosis and therapy.

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Introduction

Hepatitis C virus (HCV), the major agent of NANBH (non-A, non-B hepatitis), is a cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV is an enveloped virus which contains a single-strand, positive-sense RNA genome with 9.6 Kb lengths. More than 170 million people are infected with this virus, globally (1-8). Current antiviral therapy for this infection is a combination therapy of pegylated interferon alpha (IFN- α) with ribavirin, which is expensive, frequently ineffective, and associated with many side effects, too. In addition, currently there is no protective vaccine for hepatitis C (6).

One of the key enzymes of HCV that plays an important role in infectivity of the virus is non-structural 3 (NS3) helicase. This enzyme is a necessary factor for unwinding the HCV genome during the process of replication and translation of the viral

genome (7, 9-12). Additionally, NS3 helicase is involved in the replication and virus assembly through interaction with NS5B (RNA dependent RNA polymerase) and core proteins of virus, respectively. On the other hand, because of the strong antigenicity of NS3 helicase, this protein is critical in anti-HCV diagnostic reagents (11, 13, 14). As a result, NS3 helicase could be a promising target for diagnosis and therapy of hepatitis C (6, 7, 9, 14-17).

To expand our knowledge about the NS3 helicase protein, including its immunoreactivity with monoclonal antibodies and potential use in enzyme-linked immunosorbent assay (ELISA) reagent for immunodiagnostic HCV detection in human sera, the goal of this work was focused on cloning and expression of the conserved fragment of this protein for using in new generations of ELISA kits.

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Materials and Methods

Reagents

The expression vector (pET102/D-TOPO), Ni-NTA purification system, anti-His Tag antibody, and HRP-conjugated anti human against IgG were purchased (Invitrogen, Frankfurt, Germany). Primers were synthesized by Metabion (Frankfurt, Germany). Micro bicinchoninic acid (BCA) protein assay Kit (Pierce, Rockford, United States) and Pfu DNA polymerase (Stratagene, La Jolla, United States) were provided. Viral Nucleic acid extraction Kit, PCR product purification Kit, and Plasmid extraction Kit all were obtained from Roche (Frankfurt, Germany). The *Escherichia coli* BL21 (DE3) strain was provided by virology laboratory of Iranian Blood Transfusion Organization. Polyvinylidene difluoride (PVDF) membrane and horseradish peroxidase (HRP) substrate for western blotting were obtained from Amersham (London, United Kingdom).

RNA extraction and cDNA synthesis

Viral RNA was extracted from the serum of a patient who was infected with HCV subtype 1a. RNA extraction was performed according to the manufacturer's instructions of the viral nucleic acid extraction Kit. Reverse transcription (RT) was performed on 5 µl of template RNA with 5.5 µl mixture of RT buffer, RNase Inhibitor, dNTP Mixture, random hexamer primer, and Moloney Murine Leukemia Virus enzyme (MMLV). Reaction was performed at 42°C for 1 hr and 72°C for 10 min in final step for inactivation of reverse transcriptase.

PCR amplification and construction of recombinant plasmid

To amplify the NS3 helicase of HCV, PCR was carried out on the synthesized cDNA. The conserved region was selected for designing the primers. Nested PCR was performed with outer (F: 5' GTG GAG TGG CTA AGG CGG TGG A 3' and R: 5' GTT GAG TGC GGG AGA CAG CAT CCT 3') and inner (F: 5' CAC CAT GAG CTT CCA GGT GGC CC 3' and R: 5' GAC ACA CGT GTT GCA GTC TAT CA 3') primers. To enable directional cloning with pET102/D-TOPO, the "CACC" sequence was added at the 5' end of inner forward primer. Total volume of PCR reaction was 100 µl, containing 2 µl Pfu DNA polymerase, 0.6 µl (100 pmol/µl) of each of the forward and reverse primers, 5 µl of 10X reaction buffer, 4 µl dNTPs (10 mM), 8 µl template, and sterile water which was added into the mixture to 100 µl. PCR products of the first round of PCR were used as the template for the second round of PCR. PCR was performed according to the following program: initial denaturation at 94°C for 2 min, 40 cycles containing denaturation at 94°C for 45 sec, annealing at 58°C in first round of PCR and 59°C in second round of PCR for 10 sec, extension at 72°C for 2 min, and the last final extension at 72°C for

10 min. After electrophoresis using 1.5% agarose gel containing ethidium bromide, final PCR product was visualized under a UV transilluminator. PCR product was purified with PCR product purification Kit, and ligated with pET102/D-TOPO expression vector according to the standard protocol of the Kit. N-terminal His-patch thioredoxin and His-Tag fragment of pET102/D-TOPO vector were also expressed with NS3 fragment to increase the solubility and purify of it, respectively (18).

The ligation product was transformed into *E. coli* BL21 (DE3) strain containing T7 polymerase gene for expression of the recombinant plasmid and subsequently these bacteria were cultured in Luria Bertani (LB) agar plate containing 100 µg/ml of ampicillin. For confirmation of the cloning of recombinant plasmid, PCR and DNA sequencing techniques were performed.

Expression and purification of recombinant 6His-NS3 protein

E. coli BL21 (DE3) cells carrying recombinant plasmid were inoculated into LB-broth containing 100 µg/ml ampicillin and allowed to grow in a shaker incubator at 37°C overnight. Once an optical density (OD) of the culture at 600 nm reached to 0.6, induction was performed by addition of 1 mM IPTG (isopropyl-L-D-thiogalactoside) and allowed to grow for 5 more hr at 37°C in a shaker incubator. After this time, bacterial culture was centrifuged at 8,000 rpm for 10 min. Then, the pellet was resuspended in lysis buffer (50 mM Tris HCL, 30 mM NaCl, pH 7.6) containing cocktail of protease inhibitor and subsequently sonicated for 20 cycles of 30 sec with 30 sec of incubation on ice between pulses. In the next step, sonicated bacteria were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was purified using Ni-NTA purification system under denaturing conditions. The collected elution was analyzed by performing BCA assay to determine the expression yield of the recombinant protein and was applied for western blot and ELISA tests.

Western blotting

Protein samples, including recombinant 6His-NS3 helicase, were prepared, loaded in 12% SDS-PAGE gel, and run to separate. The protein bands were transferred onto PVDF membrane. Subsequently, the membrane was incubated at room temperature (RT) for 1.5 hr with blocking buffer (5% non-fat dry milk in TBS-Tween); then, it was incubated with the HCV positive serum (with a pool sera from antigens of 1a, 1b, and 3a genotypes) as the primary antibody diluted 1:15000 in the blocking buffer (3% non-fat dry milk in TBS plus 0.1% Tween-20) at 4°C overnight. The membrane was washed with TBST (TBS plus 0.1% Tween 20) once for 15 min and three times for five min. After washing, the membrane

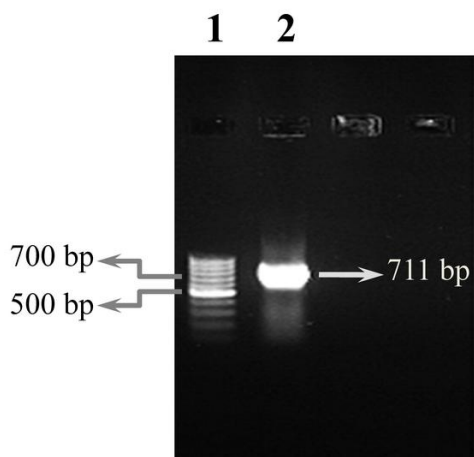


Figure 1. PCR amplification of NS3 helicase
Lane 1:100 bp DNA ladder, lane 2: PCR product of NS3 helicase fragment

was incubated with HRP-conjugated rabbit anti-human IgG, diluted 1:80,000 in TBST containing 3% non-fat dry milk for 1 hr at RT. Again, washing of the membrane was performed as mentioned before; then, it was incubated with chemiluminescent HRP substrate for five min at RT. Finally, the produced light from the luminescent chemical reactions was recorded on a film.

In a parallel experiment, western blot analysis was performed on the recombinant protein with anti-His tag monoclonal antibody (diluted 1:2000). This antibody specifically binds to His-tag fusion of recombinant protein and confirms the presence of the protein of interest.

ELISA

The purified recombinant protein was used as the antigen coating the ELISA strips in order to test sera in an ELISA format. The wells of microplates were coated with 100 μ l of the antigen (5 μ g/ml) in 100 mM sodium carbonate buffer pH 9.6, at 4 $^{\circ}$ C overnight. Next day, the wells were washed three times with TBST and blocked with 200 μ l of 5% BSA in TBS for 1 hr at 37 $^{\circ}$ C. After three times washing with wash buffer, 100 μ l of diluted serum (1:1000) was added in duplicate to the wells and was incubated for 30 min at 37 $^{\circ}$ C. The wells were washed three times and subsequently incubated with 100 μ l of HRP-conjugated goat anti-human IgG, diluted 1:1000 for 30 min at 37 $^{\circ}$ C. Washing the wells were performed four times, then 100 μ l of the TMB substrate was added to each well and incubated for 20 min at 37 $^{\circ}$ C, while microplates were coverslipped with foil. The reaction was stopped by adding 50 μ l of 0.2 M H₂SO₄ and absorbance of the resulting yellow color was measured at 450 nm with the Biochrom Asys Expert 96 microplate reader.

50 HCV positive sera, from different HCV subtypes (1a, 1b, and 3a), and 50 HCV negative sera were used in ELISA test

Results

PCR amplification and construction of recombinant plasmid

The PCR products were analyzed on agarose gel in parallel with 100 bp DNA size marker. The expected 711 bp PCR product fragment was observed (Figure 1). The PCR product was ligated to pET102/D-TOPO expression vector and transformed into *E. coli* BL21 (DE3) strain. The recombinant plasmid was confirmed by PCR and sequencing analysis. The results of BLAST search in NCBI showed that the determined sequence has 95% similarity with a HCV subtype 1a with the accession number EU687194.

Expression and identification of recombinant protein

Bacterial culture containing the recombinant plasmid was induced by 1 mM IPTG once the OD (600 nm) reached 0.6. After five hr shaking the medium at 37 $^{\circ}$ C, SDS-PAGE and western blotting were done. Western blot assay was performed using both HCV positive serum and anti His-tag antibody as a primary antibody. The results clearly have shown the expression of recombinant NS3 helicase protein in comparison with the control well (Figure 2). Additionally, the results of the western blotting using HCV positive serum as the primary antibody confirmed that the recombinant NS3 helicase strongly reacted with anti-HCV antibodies in the serum of HCV infected patient.

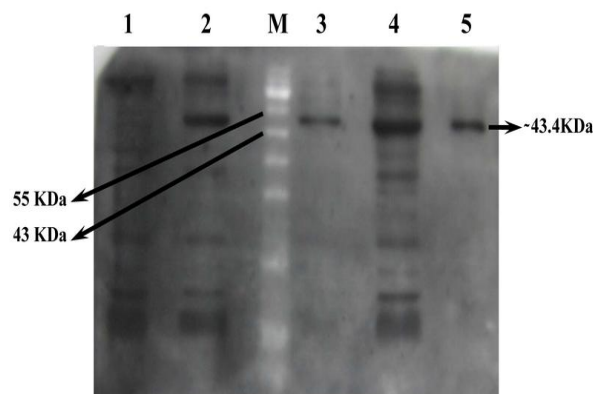


Figure 2. Western blotting of NS3 helicase protein was performed with HCV positive serum as primary antibody. lane1: Total protein of *Escherichia coli* BL21 as negative control; lane2: Total protein of *E. coli* BL21 carrying pET102-NS3 without IPTG induction; lane 3, 5: The purified NS3 from *E. coli* BL21 transforming with pET102-NS3 using infected serum and His-tag antibody respectively; lane 4: Total protein of *E. coli* BL21 Carrying pET102-NS3 after IPTG induction for 5 hr at 37 $^{\circ}$ C; lane M: molecular weight markers

ELISA

The purified NS3 protein was used as a potential antigen to set up the ELISA method for studying the immunoreactivity of Iranian HCV patient sera. ELISA test was performed on 50 positive samples from different HCV subtypes (1a, 1b, and 3a). The obtained results from these experiments showed that 80% of positive samples were above the cutoff value calculated from the data which were obtained from negative samples. Among these 80% positive samples, 1a genotype with more than 75% frequency has the highest reactivity compared to other genotypes (1b and 3a).

Discussion

Hepatitis C referred to a "silent disease" is often asymptomatic infection. Therefore, detection and treatment of this disease in the early stage is critical. Because of the important role of the NS3 helicase in viral replication and also its antigenicity due to highly conserved epitopes of this region, this protein fragment could be a promising target for developing new therapeutic agents (7, 14, 17, 19). Additionally, NS3 helicase protein is a key antigen in anti-HCV diagnostic kits (5, 9). In one study, a recombinant fusion protein 6-His NS3 helicase was used as an antigen to produce monoclonal antibodies MABs specific for HCV NS3 helicase. Their results showed that NS3 helicase activity could be inhibited by a concentration of these MABs in a nanomolar range (8). The helicase NS3 belongs to superfamily 2 (SF2) of the Non-ring helicase group (9). This portion of HCV forms three domains and contains a number of highly conserved helicase motifs. These conserved regions line the cleft between the domain 1 and 2 (20). These domains (1 and 2) are tandem repeats on the translated polypeptide and form a molecular motor. Structural and biochemical studies have shown that movement of these domains during the NS3 activity process, initiates the moving of the helicase along nucleic acids and eventually unwinding the strand (9). Also, studies suggest that ATP binding site is likely located in the cleft between domain 1 and 2 (which is referred as RecA-like motor domains) (9).

In this research, a fragment of NS3 helicase, contained domain 1 and domain 2, was cloned into pET102/D-TOPO expression vector and induced by IPTG to express the recombinant protein in BL21 (DE3) strain of *E. coli*. This vector contains a tagged polyhistidine region at C-termini of the recombinant protein and used for purification by Ni-NTA affinity column; In addition, this region is useful for detection of the fusion protein with anti His-tag monoclonal antibodies in western blotting. Using pET102/D-TOPO expression vector helps us to get a considerable amount of expressed protein for appraising and studying in other steps of the project. In another

experiment that infected Iranian patients sera (subtype 1a) was applied as the primary antibody, a high antigenicity of this NS3 helicase region was seen.

Although, the predicted molecular mass of the expressed NS3 fragment was around 26.1 kDa, because of the molecular weight of His-patch sequence (has about 16 kDa) and His-tag which added around 1 kDa to the fused protein, the total molecular mass of the expressed recombinant protein raised to around 43.4 kDa. Considering the obtained results from subtype 1a, we tested 50 positive sera samples from different HCV subtypes (1a, 1b, 3a, etc) by means of ELISA method. The results showed that 80% of the samples were immunoreactive.

In new generations of HCV diagnostic kits more than three antigens (from nonstructural 3, nonstructural 4, nonstructural 5, and core genes) are used for increasing the sensitivity of the test to reach about 100% (21, 22). Because these antigens represent in the early stages of infection with HCV; thus, having 80% sensitivity in just one of these antigens used in diagnostic kits could be promising to develop new generations of HCV detection kits (21).

Conclusion

The results of the present study showed that NS3 helicase fragment of HCV was successfully cloned and expressed in *E. coli* BL21 (DE3) using pET102/D-TOPO expression vector. Noticing to the past reports which tried different approaches such as production of human single-chain antibody (sFV) fragment as intracellular antibodies and intrabodies to inhibit the HCV NS3 helicase or use of HCV NS3, core, and E1 epitopes to produce a novel combined vaccine, it can be suggested that using the expressed protein from domains (1 and 2) of HCV NS3 helicase would be a promising target for future. This recombinant protein was proposed to be used as an efficient protein fragment to design new therapeutic antibodies; while, it can also be considered as an important antigen in HCV immunodiagnosis assays.

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