

In vitro assay for HCV serine proteinase expressed in insect cells

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Abstract

AIM: To produce the recombinant NS3 protease of hepatitis C virus with enzymatic activity in insect cells.

METHODS: The gene of HCV serine proteinase domain which encodes 181 amino acids was inserted into pFastBacHTc and the recombinant plasmid pFBCNS3N was transformed into DH10Bac competent cells for transposition. After the recombinant bacmids had been determined to be correct by both blue-white colonies and PCR analysis, the isolated bacmid DNAs were transfected into Sf9 insect cells. The bacmids DNA was verified to replicate in insect cells and packaged into baculovirus particles via PCR and electronic microscopic analysis. The insect cells infected with recombinant baculovirus were determined by SDS-PAGE and Western-blot assays. The recombinant protein was soluted in N-lauryl sarcosine sodium (NLS) and purified by metal-chelated-affinity chromatography, then the antigenicity of recombinant protease was determined by enzyme-linked immunoabsorbant assay and its enzymatic activity was detected.

RESULTS: The HCV NS3 protease domain was expressed in insect cells at high level and it was partially solved in NLS. Totally 0.2 mg recombinant serine proteinase domain with high purity was obtained by metal-chelated-affinity chromatography from 5×10^7 cells, and both antigenicity and specificity of the protein were evaluated to be high when used as antigen to detect hepatitis C patients' sera in indirect ELISA format. *In vitro* cleavage assay corroborated its enzymatic activity.

CONCLUSION: The recombinant HCV NS3 proteinase expressed by insect cells is a membrane-binding protein with good antigenicity and enzymatic activity.

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INTRODUCTION

Hepatitis C virus, the major etiological agent of post-transfusion non-A, non-B hepatitis, is an enveloped virus obtaining a single-stranded RNA genome of approximately 9.5kb^[1-3]. A single polypeptide of 3 010-3 030 amino acids is translated from

this genome in the order of NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Proteolytic procession of the viral precursor protein is required for replication and is mediated by either viral or host cell proteinases^[4-7]. HCV uses a serine proteinase located at the N-terminus of NS3, to process the cleavage of four sites (3/4A, 4A/4B, 4B/5A and 5A/5B). Since the NS3 protein is very important for releasing functional proteins from the polyprotein, it has been currently targeted in the development of drugs and diagnostics^[8-11].

In the absence of satisfactory cell culture, protein expression is the primary technological methods for HCV^[12-14]. In this report, we described the construction of the recombinant baculovirus, designed to express the NS3 proteinase in insects. The activity of the expressed enzyme has been demonstrated by *in vitro* assay.

MATERIALS AND METHODS

Cells, vectors and strains

Spodoptera frugiperda Sf9 cells were a gift from Professor Gu Shu-Yan, Institute of Virology, CDC, China. The vector pGEX-3X-NS3N containing the gene serine proteinase located at N-terminus of HCV NS3, was constructed in our lab before^[15]. The expression vector pFastBacHTc and *E.coli* DH10Bac were purchased from Invitrogen Inc.

Primers

The primers flanking the vector transfer pFastBacHTc MCS were WB538P (5' -TATTC CCGAT TATTC ATACC-3') and WHT62P (5' -TGGTA TGGCT GATTA TGAT-3'). The primers for bacmid were pUC/M13-R (5' -CAGGA AACAG CTATG AC) and pUC/M13-F (5' -GTTTT CCCAG TCACG AC-3').

Construction of recombinant transfer vector

The gene HCV NS3N serine proteinase was excised from pGEX-3X-NS3N with restriction enzyme *Bam*HI and *Eco*RI, and ligated with vector pFastBacHTc. The recombinant plasmids were transformed into *E.coli* DH5 α . The positive recombinants were identified with PCR and restriction enzyme digestion, then termed as pFBCNS3N.

Transposition

The recombinant transfer vector pFBCNS3N was transformed into the competent DH10Bac cells. After growth in the shaking incubator at 37 °C for 6 h, the cells were placed on the plates (containing 50 mg/L kanamycin, 7 mg/L gentamicin, 10 mg/L tetracycline, 40 mg/L IPTG and 100 mg/L X-gal) with serial dilution, then incubated at 37 °C for 48 h. The white colonies containing the recombinant bacmid, therefore, were selected for the isolation of recombinant bacmid DNA. The bacmid, termed as bacmid-NS3N, was analyzed by 0.5 % agarose gel electrophoresis to confirm the presence of high molecular weight DNA.

Production and analysis of recombinant baculovirus

9×10^5 of Sf9 cells were seeded to each well of a 6-well plate in TNM-FH medium and incubated at 27 °C. Bacmid-NS3N was transfected into Sf9 cells using Lipofectamine reagent (Gibco

BRL) after cells attached to the wells. The supernatant was collected as virus stock when cytopathic effect appeared. At the same time, virus particles in the supernatant were precipitated using PEG8000 to extract viral genome for PCR amplification with the primers WB538P, WHT62P and pUC/M13-R, F to confirm the recombinant virus containing the interested gene. The morpha of recombinant baculovirus was observed under transmission electron microscope.

Expression and purification of serine proteinase in insect cells

Monolayer insect cells were transfected by the recombinant baculovirus at a multiplicity of infection (MOI) of 10. The cells were harvested after incubation for 72 h at 27 °C, resuspended in lysis buffer (0.5 % NP-40, 20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 100 mg/L PMSF), and disrupted by ultrasonic. The supernatant and precipitation of the mixture were collected for SDS-PAGE and Western blotting. 5×10^7 of Sf9 cells were collected by centrifugation after transfection for 72 h to purify the recombinant serine proteinase. The cell pellet was resuspended in TNG buffer (50 mmol/L Tris-HCl pH 7.4, 0.2 % NLS (N-Lauryl Sarcosine Sodium), 10 % glycerol, 150 mmol/L NaCl, 100 mg/L PMSF), and disrupted by ultrasonic. The interested protein was purified from the supernatant by means of affinity chromatography on Ni-NTA columns (Qiagen).

In vitro assay of HCV NS3 proteinase activity

Fifteen μ g recombinant serine proteinase was mixed with 15 μ g substrate HCV NS5ab in 100 μ L of 25 mmol/L Tris-HCl pH 7.4, 10 % glycerol, 0.5 mol/L NaCl, 10 mmol/L DTT, 0.5 % NP-40. The incubation was carried out at room temperature for different time periods and the result was read through SDS-PAGE.

Antigenicity of recombinant HCV serine proteinase

The identity of recombinant proteins was confirmed by 50 HCV-positive and 10 HCV-negative sera. Microtitre cells were coated by overnight incubation at 4 °C with 100 μ L recombinant protein solution (10 mg/L proteins in 0.1 mol/L sodium carbonate buffer, pH 9.6). Serum samples diluted at 1:20 in PBSTM (PBS+0.3 % Tween-20+3 % fat-free milk) were added, and incubated at 37 °C for 1 hour after blocking. The specific binding was revealed using HRP anti-human IgG detection system (Sigma, USA).

RESULTS

Construction of recombinant transfer vector

A 540 bp fragment was excised from the plasmid pFBCNS3N with restriction endonuclease enzymes *HindIII* and *EcoRI* (Figure 1). The result showed that the construction of recombinant transfer vector was successful.

Generation of recombinant baculovirus

The recombinant baculoviruses were generated after the site-specific transposition of transfer plasmids in *E. coli*, transfection and packaging in insect cells. Large amounts of baculoviruses were observed in the nuclei of cells under transmission electron microscope (Figure 2). In PCR detection, genome DNA of the recombinant baculoviruses as template, 800 bp and 3 000 bp fragments appeared respectively with primers WB538P, WHT62P and pUC/M13-R, F. The result conformed to our previous expectation and revealed HCV serine proteinase gene existed in the genome of recombinant baculoviruses.

Efficient expression of HCV serine proteinase by recombinant baculovirus

Sf9 cells were transfected with the recombinant baculovirus

at a MOI of 10, and serious CPE appeared after 72 h. The total cell extracts were separated by 15 % SDS-PAGE and stained with Coomassie blue. A predominant band at 23 kDa (approximately 20 % of total proteins) was detected in the cell extracts from bacmic-NS3N infected cells but not in the uninfected cell extracts (Figure 3A). Immunoblot analysis with the HCV-positive sera (Figure 3B) revealed that the 23 kDa protein was clearly detected in cells infected with the recombinant baculovirus.

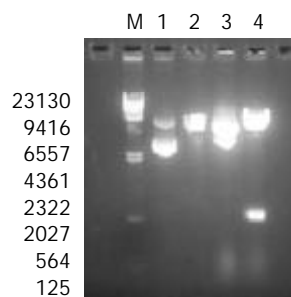


Figure 1 Restriction endonuclease enzyme analysis for recombinant bacmid. Lane M. λ /*HindIII* standard DNA molecular weight marker, Lane 1. pFastBacHTc plasmid, Lane 2. pFastBacHTc plasmid digested with *HindIII* and *EcoRI*, Lane 3. pFBCNS3N plasmid, Lane 4. pFBCNS3N plasmid digested with *HindIII* and *EcoRI*.

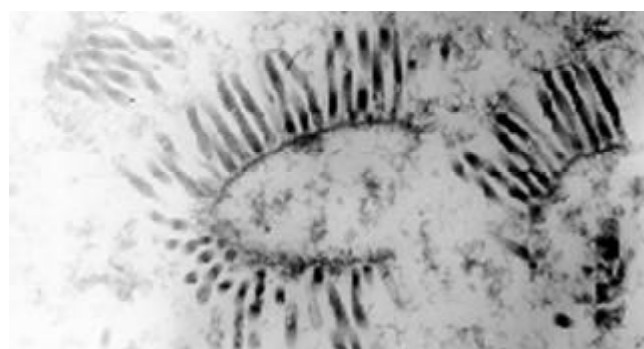


Figure 2 Recombinant baculovirus in insect cells under transmission electron microscope ($\times 36\ 000$).

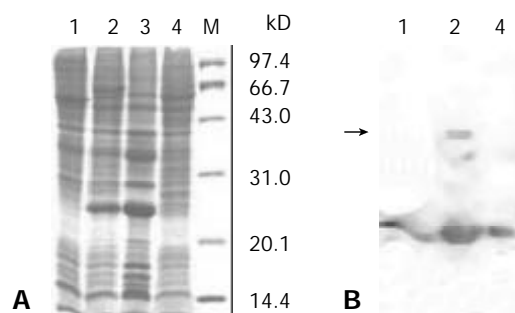


Figure 3 SDS-PAGE analysis (3A) and Western blotting analysis (3B) for the recombinant HCV serine proteinase expressed in insect cells. 3A. lane 1. untransfected Sf9 cells, lane 2. Sf9 cells transfected with rvBacNS3N, lane 3. the pellet of the cell lysate transfected with rvBacNS3N, lane 4. the supernatant of the cell lysate transfected with rvBacNS3N, Lane M. low molecular weight markers. 3B lanes 1,2,4 corresponded to Figure 3A.

Analysis of solubility of recombinant HCV serine proteinase

Though HCV serine proteinase was expressed efficiently in insect cells, a little was laid in the supernatant of the cell lysate.

Only by immunoblot analysis was the protein in the supernatant detected. Did the protein's misfolding or binding with endomembrane system in cells lead to insoluble protein? This question was analyzed by changing the composition of the lysis buffer. When the cell lysates were dissolved in TNG buffer containing N-lauryl sarcosine sodium, more interested protein was soluble because TNG as a detergent could dissolve proteins binding with membrane. Therefore, it was presumed that the recombinant HCV serine proteinase binding with membrane resulted in its insolubility. The HCV serine proteinase was extensively purified from 5×10^7 Sf9 cells and demonstrated a single protein band of 23kDa (more than 90 % of the total proteins by densitometric analysis) by using Ni-NTA columns. The total yield of the purified protein was about 0.2 mg.

Activity of baculovirus-expressing HCV serine proteinase in trans

In order to determine whether the baculovirus-expressing cleavage N-terminus of HCV NS3 has a proteinase activity, an *in vitro* proteolytic assay with HCV NS5ab protein (235aa) as a substrate containing the cleavage site of HCV NS5a and NS5b was designed. Figure 4 shows that the baculovirus-expressing HCV serine proteinase could cleave the substrate HCV NS5ab (about 35kDa) into bands of 24kDa and 11kDa (Figure 4). This means that the baculovirus-expressing HCV serine proteinase has a high proteolytic activity.

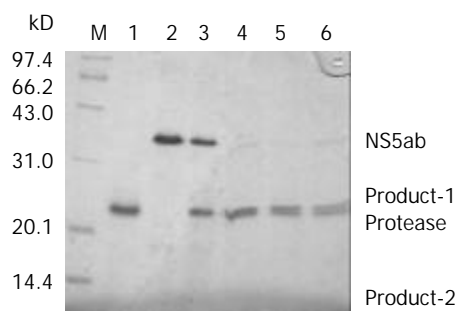


Figure 4 *In vitro* trans-cleavage at the NS5A/5B site of the recombinant HCV serine proteinase. Lane M. low molecular weight markers, lane 1. the purified HCV serine proteinase, lane 2. the substrate HCV NS5ab protetin, lanes 3-6. cleavage reaction after 0, 10, 20 and 30 min.

Antigenicity of recombinant HCV serine proteinase

Fifty HCV positive sera and 10 HCV negative sera were evaluated for their reactivities against the purified HCV serine proteinase. A high degree of immunoreactivity was observed in 30 HCV positive sera, showing good antigenicity of HCV serine proteinase.

DISCUSSION

It has been proved that HCV NS3 protein with 631 amino acids possesses both serine proteinase in its N-terminus and NTP/RNA helicase activity in its C-terminus^[16]. Both *in vitro* translation and transient mammalian cells have shown that NS3 is a serine proteinase and is responsible for proteolytic processing of the non-structural region (NS3, NS4A, NS4B, NS5A and NS5B) of HCV polyprotein. Since the maturation of these non-structural proteins is the prerequisites for replication, and packaging of HCV, NS3 serine proteinase plays an important role in the life cycle of HCV and is an attractive target for antiviral therapy. We expressed only 181 amino acids in HCV NS3 N-terminus because previous experiments had corroborated it with good enzyme activity^[17].

Baculovirus-insect cell expression system has two advantages over others: first, in most cases the recombinant

protein in insect cells is processed, modified, and targeted to its appropriate cellular location, where it is functionally similar to its authentic counterparts. Second, high levels of heterogenous gene expression, up to 20 % of total cellular proteins, are often achieved compared with other eukaryotic expression systems^[18-20]. Here we chose the Bac-to-Bac expression system which is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E.coli*^[21,22]. Colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then is used to transfect insect cells, eliminating the need for multiple rounds of plaque purification.

In our experiment, the interested gene was efficiently expressed in insect cells through the recombinant virus BacNS3N containing HCV NS3 serine proteinase gene, and the identity of the protein was validated by use of immunoblot. But the recombinant protein predominantly lay in the insoluble part, and the solubility could not be improved even by adding 1 % NP-40 into the lysate. Overton *et al* illuminated that HCV NS3 serine proteinase expressed in insect cells was a membrane-associated protein through discontinuous sucrose density gradients and only by adding some detergents did part of the protein gain the enzyme activity^[23]. Suzuki *et al* discovered that HCV serine proteinase from insect cells was soluble and active with 0.5 % NLS^[24]. Therefore, the TNG buffer including NLS and glycerol was used to dissolve the pellet. The results showed that part of the recombinant protein became soluble and more proteins were soluble when enhancing the percentage of NLS and adding EDTA both. The recombinant protein fused with 6 His tag at its N- terminus could be purified through metal-chelating chromatography. Finally, 0.2 mg purified recombinant protein was obtained from 5×10^7 insect cells. An *in vitro* detection system with SDS-PAGE and staining was developed with the recombinant HCV NS5ab as a substrate. The system eliminates the need of expensive instrument and reagent, heavy work, as compared with other cleavage systems^[25-27] and provides a simple and clear strategy for the screening of inhibitors against the HCV proteinase.

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