Expression of highly active recombinant NS3 protease domain of hepatitis C virus in *E. coli*

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Abstract The serine protease domain of HCV comprising amino acids 1027–1218 (ΔNS3) was expressed in *E. coli* with a His tag at its N-terminal end. The protease was purified to apparent homogeneity by a single step affinity chromatography resulting in high yields (~3 mg/l of cultured cells). The ΔNS3 efficiently cleaves a 17-mer peptide corresponding to the NS5A-NS5B junction with $k_{cat}/K_m = 160 \times 10^{-3}$ min^{-1} μM^{-1} in the presence of NS4A peptide. Our ΔNS3 represents the minimal domain possessing highly active protease of NS3 constructed so far. The ΔNS3 protein also efficiently processed a longer substrate corresponding to NS5A/5B junction (2203–2506 amino acids) that was synthesized by in vitro transcription and translation system.

Key words: Hepatitis C virus; Serine protease; Non-structural protein 3

1. Introduction

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion non-A, non-B hepatitis, is an enveloped virus containing a single-stranded RNA genome of approximately 9.5 kb nucleotides [1,2]. A single polyprotein of 3010–3030 amino acids is translated from this genome [3] in the order of NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Fig. 1A) [4,5]. This polyprotein is subsequently processed by a combination of host and viral proteases to produce at least 10 viral proteins. The core protein (C) and envelope proteins (E1 and E2) are structural proteins, and NSs are non-structural proteins [6,7].

Previous studies indicate that a host signal peptidase localized in endoplasmic reticulum (ER) catalyzes polyprotein cleavages in the structural region (C/E1, E1/E2, E2/p7 and p7/NS2) [8,5], whereas a HCV encoded serine protease located in the N-terminal one-third of the NS3 protein is responsible for cleavages at four sites (3/4A, 4A/4B, 4B/5A and 5A/5B) (Fig. 1A) [9–14] in the NS region. Using a transient coexpression system it has been shown that proteolytic cleavages in the non-structural protein region (NS3 to NS5B) of HCV polyprotein are effected by two viral proteins, NS3 and NS4A [15–19]. The N-terminal 180 amino acid region of NS3 includes sequences showing homology with the active sites of serine proteases [20–22]. Histidine 1083, aspartate 1107 and serine 1165 (numbers are according to their locations in the polyprotein of HCV subtype J (HCV-Ib) [23,24]) found in this domain have been proposed to constitute the catalytic triad of the NS3 protease similar to other serine proteases belonging to the chymotrypsin family.

NS4A is shown to be the NS3 protease cofactor or effector enhancing cleavage efficiency at various sites NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B [16,19,25–27]. NS4A is an amphipathic protein of 54 amino acids and it has a very hydrophobic N-terminal domain followed by a hydrophilic C-terminal domain [16]. Using an in vitro reconstituted assay system it has been shown that the residues 22–31 of NS4A constitute the putative core sequence for NS4A's effector activity [27]. The mechanism by which NS4A facilitates cleavage remains obscure. Truncation experiments have mapped the N-terminus of NS3 as the domain responsible for interaction with NS4A [25,27].

Since the NS3 protein is very important for releasing functional proteins from the polyprotein, it is currently being targeted in the development of drugs and diagnostics. As a matter of fact, several known serine protease inhibitors have been tested for their action on NS3 activity in vitro and it was found that millimolar concentrations of these compounds were required to show moderate inhibitory effect on NS3 protease activity [3,28]. One major obstacle for these well known inhibitors may be the presence of helicase domain also or alternatively NS3 protease may be structurally different in comparison with other serine protease domains.

In order to carry out detailed characterization of this enzyme in terms of its substrate specificity, kinetics, sensitivity to inhibitors and for structural studies, a reproducible and convenient large-scale purification of the enzymatically active protease domain of NS3 is essential. In this report we show that the region encompassing amino acids 1027–1218 tagged with the His tail (6 His) has greater activity than other expression constructs made so far and the purification procedure is much simpler. We also show that the purified protein possesses the ability to interact with NS4A resulting in increased proteolytic activity.

2. Materials and methods

2.1. Construction of expression plasmid containing the HCV serine protease domain

To construct the expression plasmid pHisBΔNS3, a cDNA fragment encoding amino acid residues 1027–1218 in the HCV polyprotein was obtained by PCR using appropriate oligonucleotides (1: d(CCGCTGCAGCCATGGCGCCTATCACGGCCTAT), 2: d(CCGAAGCTTTCAGGCCGGAGGGGATGAGTT)), which insert a *Pst*I site at the 5′-end and a TAG (stop) codon and *Hin*dIII site at the 3′-end of the sequence. We used plasmid pMANS34NSH [29] as a template to amplify amino acid residues 1027–1218 in the HCV polyprotein. The PCR product amplified using Ex. Taq (Takara) was first cloned into pCR II vector (Invitrogen) according to the manufactur-

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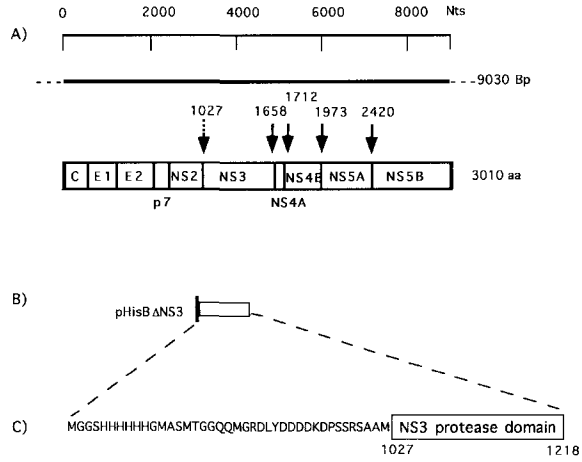

Fig. 1. Schematic representation of HCV serine proteinase domain expressed in *E. coli*. A: The HCV genome and a translated product with names of the processed proteins. The filled arrow shows the cleavage site of HCV serine protease, whereas the dotted arrow indicates the cleavage site of HCV metaloprotease. B: ΔNS3. Closed and open boxes indicate the 6 histidine tag and the protease domain, respectively. C: N-terminal extra amino acid sequence and NS3 protease domain.

er's instructions and digested with *Pst*I and *Hin*dIII to release the fragment encompassing amino acids 1027–1218 of HCV polyprotein. It was subsequently cloned into expression plasmid pTrcHisB (Invitrogen) which was also digested with *Pst*I and *Hin*dIII. The resulting plasmid pHisBΔNS3 (Fig. 1B,C) encodes the protease domain of NS3 (192 amino acids) with a N-terminal 40 non-virus encoded amino acids possessing a consecutive stretch of 6 His residues that allows fusion protein to be purified in a single step by metal chelating affinity chromatography. The cloned DNA fragment was sequenced in order to exclude the introduction of mutations by PCR and also to confirm the in-frameness of the insert.

2.2. Expression and purification of HCV NS3 protease domain

Following transformation of *E. coli* HB101, cells harboring the vector pHisBΔNS3 were grown in LB medium containing 100 μg/ml ampicillin. When the absorbance reached a value of 0.5–0.6 OD_{600} isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to give a final concentration of 1 mM and the incubation was continued for an additional 2 h at 37°C. Under this induction condition a high level expression of ΔNS3 was observed and there was only a small amount present in the insoluble fraction. The cells were harvested by centrifugation and washed extensively with PBS (20 mM sodium phosphate; pH 7.4, 140 mM NaCl). The cell pellet was resuspended in lysis buffer (20 mM sodium phosphate; pH 6.3, 500 mM NaCl) and disrupted by sonication on ice using a Branson 200 sonifier (30 s×6 strokes at 18 W output with 30 s intervals). The homogenate was centrifuged at 30 000×*g* for 30 min to remove cell debris and was chromatographed on a nickel-agarose column (Qiagen). We applied a stepwise gradient of pH to elute the protease in order to overcome the precipitation of the enzyme. The column was washed extensively with several column volumes of lysis buffer and subsequently washed using the same buffer of pH 6.0 and finally with the same buffer of pH 5.0. Resin bound protein was eluted with sodium phosphate buffer (pH 4.0) containing 500 mM NaCl. Eluted fractions were subjected to SDS-PAGE [30], protein containing fractions were pooled and concentrated by using Millipore Ultrafree Biomax 10K concentrator (Millipore). The enzyme was stored in aliquots at −20°C in the same buffer containing 40% glycerol.

Protein concentrations were estimated from UV absorbance at 280 nm: an extinction coefficient of ε = 20 800 M^{-1} cm^{-1} was calculated on the basis of primary sequence data according to published procedures [31] and concentration of ΔNS3 was determined according to the Lambert Beer law. Alternatively, ΔNS3 concentration was determined by Bradford (BioRad) assay using lysozyme as standard.

2.3. HCV NS3 protease assays

To characterize the enzymatic activity of the purified protease (ΔNS3) we investigated its ability to cleave a synthetic peptide, S-1 (Dns-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Δ-Ser-Met-Ser-Tyr-Thr-Trp-Thr-COOH, Δ; cleavage site) corresponding to the cleavage site of the NS5A/5B. Protease activity of ΔNS3 and maltose binding NS3 fusion protein, MBP-NS3 (amino acids 985–1647) [29] was analyzed either in the presence or absence of NS4A peptide, P41 (amino acids 1673–1692 of HCV polyprotein).

Kinetic constants were determined from enzyme assays with the synthetic substrate concentration ranging from 25 to 960 μM. The K_m and k_{cat} values were determined by Lineweaver-Burk plots. Kinetic reactions were analyzed either in the presence or absence of P41. For evaluating the rate of reaction in the absence of P41, the ΔNS3 or MBP-NS3 (0.72 mM) was incubated with various concentrations of substrate in a buffer (Tris-HCl (pH 7.8), 30 mM $NaCl_2$, 5 mM $CaCl_2$, 10 mM DTT) at 25°C for 60 min, whereas reactions carried out in the presence of P41 (10 mM) were initially equilibrated with ΔNS3 or MBP-NS3 for 15 min at 37°C in the above buffer. The reaction was initiated by addition of substrate at 37°C and data points were collected for 10 min.

In order to test the ability of the ΔNS3 to cleave longer substrate representing NS5A/B junction protein in the amino acid sequence 2203–2506 of HCV polyprotein which closely resembles the situation in vivo, radiolabelled NS5A/5B substrate was produced using a coupled transcription-translation system (TNT Promega) according to the manufacturer's protocol. The DNA encoding amino acids 2203–2506 of polyprotein representing the NS5A/B site was amplified by PCR and was added to a 25 μl TNT reaction in the presence of [^{35}S]methionine (Amersham) at 300 μCi/ml and incubated at 30°C for 1–2 h. For experiments to assess proteolytic cleavage of pre-formed substrate the TNT reaction mixture was diluted by adding an equal volume of buffer (100 mM HEPES (pH 7.6), 300 mM NaCl, 6 mM $MgCl_2$, 20 mM DTT). To 6 μl (≈300 cpm of translated product) of diluted TNT reaction mixture, ΔNS3 (750 nM) was added and incubated for 30 min at 30°C. In experiments to study the effect of NS4A peptide, the protease was pre-incubated with P41 for 5 min at 30°C before the addition of substrate. Samples were withdrawn at different time intervals and the reaction was stopped by adding SDS-PAGE sample buffer followed by denaturing at 95°C for 3 min. All samples were loaded on to SDS-PAGE and the radioactivity was quantitated by image analyzer (BAS 2000, Fuji Film).

3. Results and discussion

The purified ΔNS3 protease migrated as a single band with a molecular mass consistent with that calculated from the primary sequence (molecular mass of 25 kDa as judged from SDS-PAGE) (Fig. 2). The final yield was ~3 mg of purified protein per liter of cultured cells and the purity of the preparate was estimated to be over 95% as judged by SDS gel. Though several constructs have been reported for the expression of the NS3, considering the yield and the simplicity of the purification procedure which we described here offers clear advantage for structural studies, wherein large amounts of enzyme are required. Table 1 shows that in the absence of P41 the K_m of ΔNS3 was 250 μM and k_{cat} was 2.0 min^{-1}. In

Table 1
Effect of P41 on the cleavage kinetics of peptide NS5A/5B by ΔNS3 and MBP-NS3[a]

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
ΔNS3	250	2.0	8×10^{-3}
ΔNS3+P41	99	15.8	160×10^{-3}
MBP-NS3	360	1.3	3.6×10^{-3}
MBP-NS3+P41	196	5.1	26×10^{-3}

[a]Data are the mean values from three independent experiments.

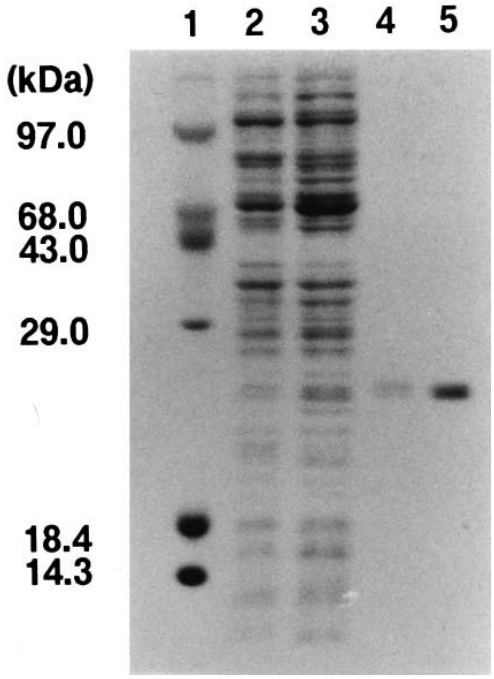

Fig. 2. Purification of the NS3 protease domain. Samples deriving from single steps of the purification were loaded on an SDS-15% polyacrylamide gel, and bands were visualized by Coomassie staining. Lane 1: molecular mass markers; lane 2: homogenate from *E. coli* without construct; lane 3: homogenate from pTrcHisBΔNS3; lane 4: 750 ng of purified ΔNS3 after affinity chromatography; lane 5: 1.5 μg of purified ΔNS3 after affinity chromatography.

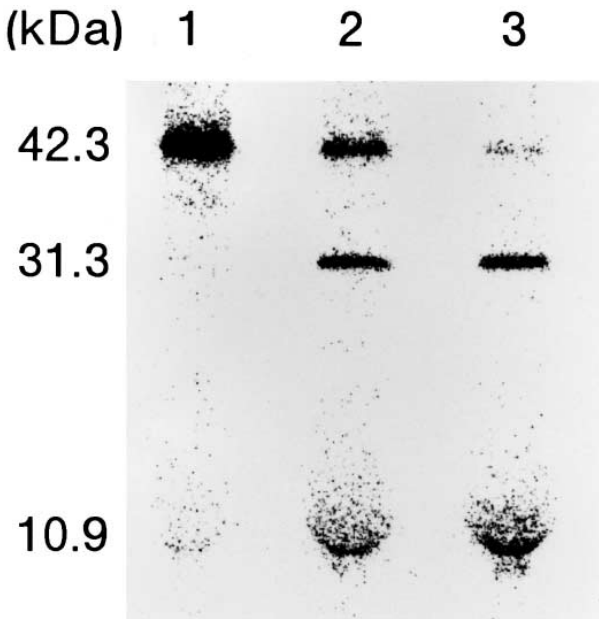

Fig. 3. TNT analysis of cleavage of NS5A/5B substrate by ΔNS3. Substrate representing NS5A/5B incubated, lane 1: without ΔNS3; lane 2: with ΔNS3; lane 3: with both ΔNS3 and P41. All samples were incubated at 30°C for 30 min; proteins were labelled with [^{35}S]methionine and analyzed by SDS-PAGE followed by fluorography.

contrast, in the presence of a 10-fold molar excess of P41 the K_m (99 μM) was about 2.5 times lower than that in the case of ΔNS3 alone, and the k_{cat} (15.8 min^{-1}) was about 8 times higher than that for ΔNS3 alone. As a result the k_{cat}/K_m value increased 20 times in the presence of P41. When we compared the k_{cat}/K_m value of MBP-NS3, ΔNS3 protease activity was increased to 2-fold in the absence and 6-fold in the presence of P41. Our ΔNS3 protease activity was found to be the highest reported so far. The data presented above suggest that region 1027–1218 represents the minimal domain required for the protease activity, since the region 1027–1218 exemplifies all the cleavage kinetics of the MBP-NS3 (985–1647) reported earlier [27].

The coefficient for proteolytic efficiency was comparable to that of two reports published recently [27,32], whereas the K_m and k_{cat} values are comparable with only that of NS3 (encompassing both the protease and helicase domains fused to MBP) [27]. In the case of ΔNS3, the presence of P41 clearly increases the affinity for the substrate (as evident from K_m values) and thereby increases the catalytic rate (Table 1). Shimizu et al. [27] have made a similar observation with NS3 which has both protease and helicase functional domains, whereas Steinkuhler et al. [32] have expressed NS3 protease domain encompassing amino acids 1038–1226 of HCV polyprotein and analyzed its activity on NS4A/NS4B cleavage site both in the presence and in the absence of NS4A protein. Though the kinetic differences were observed using a peptide derived from a NS4A-independent cleavage site (NS4A/4B), the data clearly show the activation role of NS4A in altering the NS3 activity in general. However, the affinity (K_m) was not altered in the presence of NS4A as evident from their published data. This may possibly be due to the absence of essential N-terminal amino acids for interaction with NS4A in their expressed protease domain. The fact that NS4A can interact with ΔNS3 in a similar way as with full NS3 and brings about similar changes confirms that ΔNS3 despite its truncated version and fusion retains the favorable conformation for interaction. Based on the above observations we can conclude that amino acid residues 1027–1218 are sufficient to represent the complete protease function of NS3 even considering its ability to interact with NS4A. Detailed analyses of

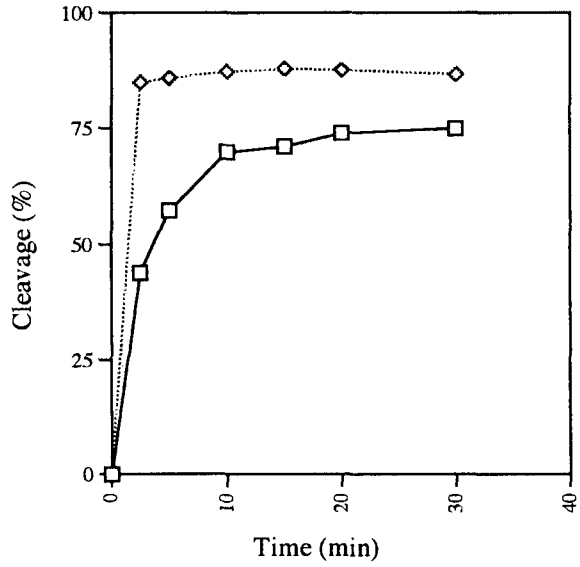

Fig. 4. TNT analysis on effect of P41 on ΔNS3 rate of cleavage. Normalized cleavage % is shown against different time intervals. Without P41 (□) and with P41 (◇).

the regulation and the substrate requirements of the protease are expected to offer further insights into the mechanism of activation by P41.

In addition, when we tested the ability of ΔNS3 to cleave a longer substrate representing the NS5A/B junction, as shown in Fig. 3, ΔNS3 could cleave the NS5A/B junction protein (amino acids 2203–2506) in close agreement with the observation when the small synthetic peptide substrate NS5A/B was used. The addition of P41 again clearly enhanced the cleavage reaction. From the time course of these reactions (Fig. 4) the rate of cleavage was stimulated more than 2-fold by the addition of P41. Since the purified recombinant ΔNS3 protease has a relatively smaller molecular size, representing the complete catalytic function of NS3 protease, and considering the ease and yield of purification, we believe that it is an ideal candidate to generate further studies elucidating the structure-function relationships, mechanism of interaction with NS4A peptide as well as developing protease inhibitors.

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