Enzymatic characterization of purified NS3 serine proteinase of hepatitis C virus expressed in *Escherichia coli*

Akiko Mori[a,*], Kazunori Yamada[a], Junko Kimura[a], Tomoko Koide[b], Satoshi Yuasa[b], Ei Yamada[a], Tatsuo Miyamura[c]

[a]*Laboratory of Molecular Medicine, Yokohama Research Center, Mitsubishi Chemical Corp., 1000 Kamoshida-cho, Aoba-ku, Yokohama 227, Japan*
[b]*Laboratory of Pharmaceuticals II, Yokohama Research Center, Mitsubishi Chemical Corp., 1000 Kamoshida-cho, Aoba-ku, Yokohama 227, Japan*
[c]*Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan*

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Abstract Non-structural protein 3 (NS3) of the hepatitis C virus (HCV) has been shown to be a serine proteinase which cleaves the HCV polyprotein thus activating its replicative machinery. To characterize enzymatic activities of NS3 serine proteinase, the proteinase region was expressed in *Escherichia coli* and purified. The purified proteinase specifically cleaved a purified fusion protein sandwiching the NS5A/5B cleavage sequence. In addition to serine proteinase inhibitors, some chelators also inhibited the cleavage activity. Metal ions were not required for its activity, suggesting that the proteinase may be a novel serine proteinase having a unique binding site for chelators.

Key words: Hepatitis C virus; Non-structural protein; Serine proteinase; Inhibitor; Chelator

1. Introduction

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion non-A, non-B hepatitis worldwide [1,2]. HCV infection results in mild and acute liver disease, but chronic infections are common and may eventually develop into cirrhosis or hepatocellular carcinoma [3,4]. Although interferons are currently used for the treatment of chronic hepatitis, their efficacy is limited to a small portion of the patients owing to insufficient suppression of HCV replication [5]. Therefore another reliable anti-HCV agent is necessary to control HCV hepatitis.

Experiments using recombinant DNA techniques have revealed that HCV has positive-strand RNA approximately 9400 nucleotides long which codes a single polyprotein of about 3010 amino acids [1,2,6–9]. Since its genomic organization is similar to that of the flaviviruses and pestiviruses, HCV is classified as a member of the family flaviviridae [10]. Following the 5′-untranslated region, the viral structural proteins are located at the amino- (N-) terminal region of the polyprotein in the order of core, E1 and E2. The replicative machinery is located in the carboxyl (C)-terminal region as non-structural proteins and the gene order is NS2-NS3-NS4A-NS4B-NS5A-NS5B [11]. After being translated as a precursor polyprotein, the structural proteins were shown to be processed by a host cell signal peptidase with no apparent involvement of virus-coded proteinases.

In contrast to the cleavage of the structural proteins by the host enzyme, the non-structural proteins are released by the action of virus-coded proteinase. Amino acid sequence analysis of a number of different isolates of HCV showed that the NS3 protein contained three highly conserved amino acid residues, His-1083, Asp-1107, and Ser-1165, which are well known as a catalytic triad of the serine proteinase family [12,13]. In fact, it was confirmed that the Ser-1165 in the NS3 protein was essential to cleave the downstream portion of the polyprotein by using in vitro transcription–translation systems and some mammalian cell culture systems [14–18]. From these studies, the N-terminal region of the NS3 protein was shown to cleave the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions but not the NS2/3 junction [19]. Comparison of the sequences around each cleavage site revealed the unique substrate specificity of the proteinase: Cys or Thr at the P1 position, Ser or Ala at the P1′ position, and Asp or Glu at the P6 position [16]. Further analysis revealed that cleavage at NS2-NS3 was mediated by a second proteinase encoded within a region composed of the C-terminal portion of the NS2 gene and the N-terminal portion of the NS3 gene. This enzyme was likely a zinc-dependent metallo-proteinase whose His-952 and Cys-993 were involved in catalysis [20,21]. This putative metallo-proteinase overlaps the NS3 serine proteinase and presumably works in a self-inactivating manner.

Since the viral serine proteinase was thought to be an attractive target for antiviral therapy, several assay systems of NS3 proteinase using in vitro transcription–translation systems and some mammalian cell culture systems have already been developed. It is important to develop an enzymatic assay system which displays clear activity of the enzyme to facilitate inhibitor screening. Aiming at establishing such an assay system, we have cloned original HCV proteinase genes from the HCV-positive serum of chronic hepatitis patients into a plasmid of *E. coli*, and have examined their ability to cleave a substrate fusion protein containing the NS5A/5B cleavage site between MBP and protein A, expressed in identical cells. In the course of these studies, we found some highly active NS3 clones which cleaved the protein substrate in *E. coli* (Yamada, K. et al., unpublished results). Using one such clone, D51, and the recombinant substrate described above, we succeeded in detecting sufficient NS3 proteinase activity for application to inhibitor screening in vitro. In this paper, we describe the establishment of the

*Corresponding author. Fax: (81) (45) 963-3992.

Abbreviations: HCV, hepatitis C virus; NS, non-structural protein, APMSF, 4-(amidinophenyl)-methanesulfonyl fluoride; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-bu tane; PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyllysylchloromethane; TPCK, tosylphenylalanylchloromethane; Pefabloc/SC, 4-(2-aminoethyl)-benzenesulfonylfluoride; CyDTA, *trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid.

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enzymatic NS3 proteinase assay system and the characteristics of the NS3 proteinase which was investigated by this system.

2. Experimental

2.1. Construction of expression plasmids

The cDNA fragment encoding amino acid residues 1050–1214 in the HCV polyprotein was isolated by PCR, using our newly obtained HCV cDNA clone, pWNHD51, as a template and was inserted into the expression plasmid pNH71 which contains 13 N-terminal amino acid leaders derived from a nitrile hydratase gene (Yamada, K., unpublished data). This leader sequence possesses a consecutive stretch of histidine residues that allows a fusion protein to be purified in a single step by metal chelating affinity chromatography. The resulting plasmid, pNHJ13-9, encodes a fusion protein of 178 amino acid residues (Fig. 1A). In the same way, the expression plasmid pNHJ13-9A, which encodes a mutant type proteinase in which Ser-1165 is substituted to Ala, was constructed by using pWNHD51A as a template for PCR (Fig. 1A). The details of the plasmids pWNHD51 and pWNHD51A will be reported elsewhere.

Expression plasmid pMCP1, which encodes the *E. coli* MBP and the *Staphylococcus aureus* protein A in tandem, is a derivative of pMAL-c2 (New England BioLabs Inc., USA) and pRIT2. Plasmid pMCP1 also contains multiple cloning sites of pUC19 between the MBP gene and protein A gene. Substrate expression plasmids, pMCP-34, pMCP-44, pMCP-45 and pMCP-C2, which encode fusion proteins containing appropriate amino acid sequences for the NS3 proteinase cleavage site between the MBP and protein A junctions (Fig. 1B), were constructed by inserting corresponding sequences into pMCP1.

2.2. Expression and purification of HCV NS3 proteinase

E. coli DH5 harboring plasmid pNHJ13-9 or pNHJ13-9A was cultured in LB broth and induction was performed by cultivation under isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.5 mM final concentration) at 25°C for 18 h [22]. Cells were suspended in Tris/NaCl (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 1 mM EDTA, 1 mM dithiothreitol and 5% glycerol and treated with lysozyme (120 mg/ml) for 30 min. After sonication on ice with a Sonifier 450 (Branson Sonic Power Co., USA), the supernatant was obtained by ultracentrifugation (200,000 × *g* for 30 min) and underwent buffer exchange with Buffer A (50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 10 mM imidazol, 5% glycerol and 0.05% Tween 20) using a P6-DG desalting column (Bio-Rad Laboratories Inc., USA). Then it was applied to His Bind Resin (Novagen) pre-treated with Ni^{2+} solution. The His-tag-conjugated HCV NS3 proteinase was purified according to the protocol of the supplier. The buffer of the final proteinase fraction was exchanged with Tris/NaCl containing 0.05% Tween 20 and 20% glycerol.

2.3. Expression and purification of substrates

The recombinant cells harboring a substrate expression plasmid were cultured in LB broth and induction was performed by IPTG (0.8 mM) for 3 h at 37°C. The cells were collected and sonicated in buffer containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.05% Tween 20, 1 mM PMSF, and 1 mM EDTA. The supernatant obtained by centrifugation (15,000 × *g* for 30 min) was loaded onto an IgG-Sepharose column (2 ml) and the substrate was purified according to the instruction manual. The eluted substrates were dialyzed against Tris/NaCl.

2.4. Detection of the proteinase activity

Purified enzyme (60 ng) was mixed with purified substrate (3.2 μg) in a reaction mixture (20 μl) composed of Tris/NaCl and 10 mM dithiothreitol, and incubated at 37°C for 4 h. After incubation, the reaction was stopped by adding SDS-PAGE sample buffer and heating at 100°C for 5 min. The digested pattern was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 (CBB R-250). The absorbance of the protein A band (29 kDa) and the MBP band (43 kDa), produced by the cleavage reaction, and that of the remaining substrate band (67 kDa) were scanned by He/Ne laser at 632.8 nm with a densitometric scanner FAST SCAN (Molecular Dynamics, USA). On the basis of the data obtained, proteinase activity was calculated by determining the percentage of cleaved substrate. When effects of proteinase inhibitors were evaluated, the enzyme was pre-incubated with each inhibitor at an appropriate concentration in 18 μl of Tris/NaCl at 30°C for 30 min and 2 μl of the substrate containing 0.1 M dithiothreitol were added. The inhibitory effects were calculated by defining the density at 0 time as 0% and that at 4 h without an inhibitor as 100%. The 50% inhibitory concentration (IC_{50}) for each inhibitor was calculated from these data.

3. Results

3.1. Expression and affinity purification of recombinant HCV NS3 proteinases and substrates

To detect NS3-specific proteolitic activity in vitro, we prepared a wild type and a mutant type of the enzyme (D51ΔNS3S and D51ΔNS3A, respectively) and a substrate (MCP-C2) (Fig. 1).

D51ΔNS3S and D51ΔNS3A were purified to nearly homogeneity by a single step of metal chelating affinity chromatography (Fig. 2A, lanes 1 and 2). The 21-kDa bands were confirmed to be D51ΔNS3S or D51ΔNS3A by Western blotting using anti-NS3 antibody (data not shown). As substrates, fusion proteins composed of MBP and protein A sandwiching the NS3/

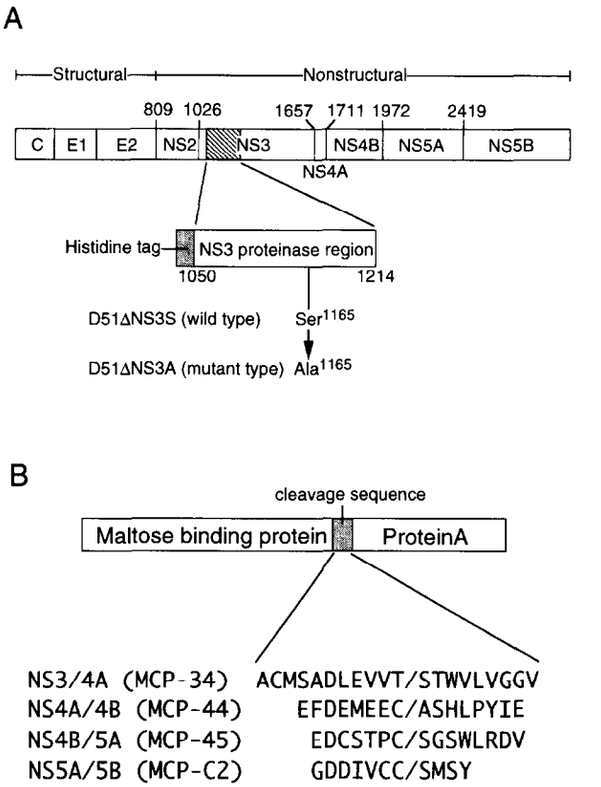

Fig. 1. Structural organization of the constructs used to express HCV NS3 proteinases and their substrates in *E. coli*. (A) The entire HCV polyprotein, made up of structural proteins (Core to E2) and nonstructual proteins (NS2 to NS5B), is shown at the top. Constructs for the expression and purification of the NS3 proteinase are indicated with their coded region below the HCV polyprotein. A serine residue of the wild type enzyme (D51ΔNS3S) was replaced by alanine in a mutant enzyme (D51ΔNS3A). (B) Schematic representation of various recombinant substrates containing a cleavage site of the NS3 proteinase. The indicated cleavage sequences, corresponding to the NS3/4A, NS4A/4B, NS4B/5A, NS5A/5B cleavage sites, were inserted between MBP and protein A.

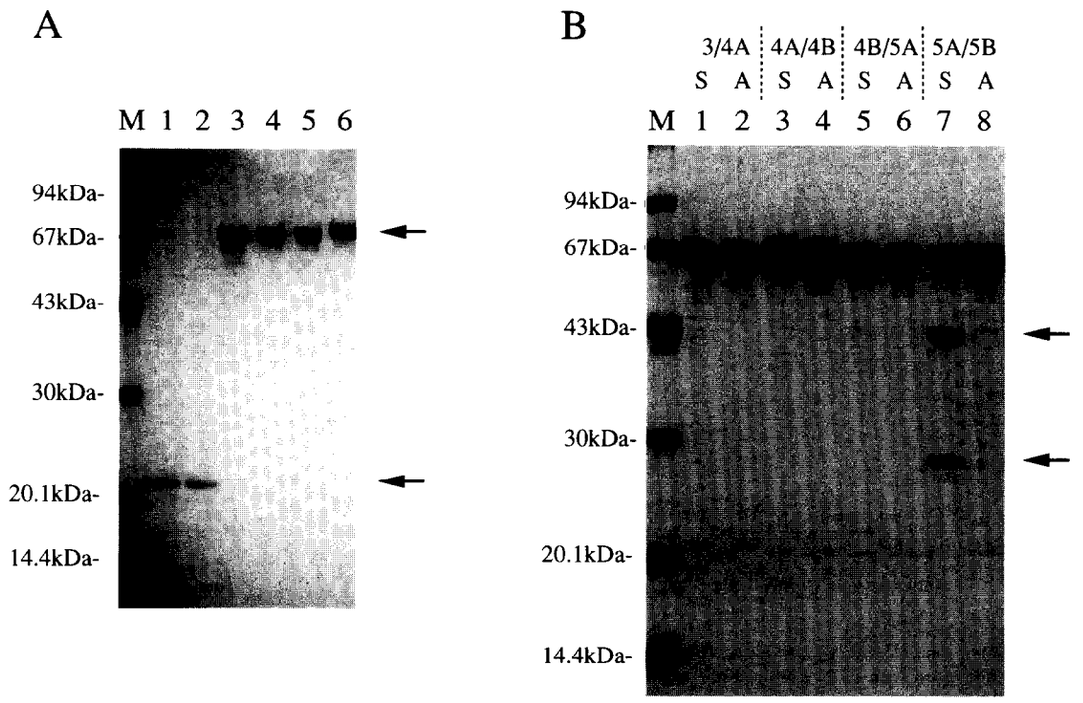

Fig. 2. Detection of the HCV NS3 proteinase activity using purified enzymes and substrates. (A) Purified NS3 proteinases and their substrates expressed in *E. coli*. D51ΔNS3S and D51ΔNS3A were purified by metal chelating affinity chromatography (lanes 1 and 2, respectively). The recombinant substrates MCP-C2, MCP-34, MCP-44, and MCP-45 (shown in Fig. 1B) were purified by using IgG Sepharose resin (lanes 3–6, respectively). The bands of purified enzymes and substrates are indicated by arrows. (B) Detection of NS3 serine proteinase activity. D51ΔNS3S (lanes 1, 3, 5, and 7) or D51ΔNS3A (lanes 2, 4, 6, and 8) was mixed with MCP-34 (lanes 1 and 2), MCP-44 (lanes 3 and 4), MCP-45 (lanes 5 and 6) or MCP-C2 (lanes 7 and 8), and the mixtures were incubated under the standard assay condition. Each sample was analyzed by SDS-PAGE followed by staining with CBB R-250. The MBP band (43 kDa) and the protein A band (29 kDa) produced by the cleavage are indicated by arrows.

4A, NS4A/4B, NS4B/5A or NS5A/5B cleavage site were purified with IgG Sepharose resin (Fig. 2A, lanes 3–6).

3.2. Detection of NS3 proteinase activity in vitro

When the substrate MCP-C2 was mixed with wild type enzyme D51ΔNS3S in the presence of 10 mM dithiothreitol, the enzyme specifically cleaved MCP-C2 to produce an MBP fragment and a protein A fragment (Fig. 2B, lane 7). However, when the mutant enzyme D51ΔNS3A was mixed with MCP-C2, cleavage was not detected (Fig. 2B, lane 8). The purified NS3 proteinase cleaved the substrate only by the wild type enzyme, suggesting that the cleavage did not result from contaminating bacterial proteinases. To confirm the NS3 proteinase activity, we determined the N-terminal amino acid sequence of the protein A fragment produced in the reaction. The sequence was SMSY-, which was identical to the predicted one (Fig. 1B) [16], assuring the cleavage by HCV NS3 proteinase.

To study the basic characteristics of the NS3 proteinase, we performed the reaction at various pHs. It was found that the enzyme was most reactive at pH 7.5. Its activity was mostly lost below pH 6.0 and above pH 9.0 as previously shown in baculovirus expression system [23]. We also measured the enzyme activity at various temperatures. The activity was highest at 37°C and considerably lost at 4°C (data not shown).

When D51ΔNS3S or D51ΔNS3A was incubated with MCP-34, MCP-44, or MCP-45, which contain other cleavage sequences (Fig. 1B, Fig. 2A lanes 4–6), the cleavage were not observed (Fig. 2B, lanes 1–6). Although slight cleavage of the

Table 1
Effects of different types of proteinase inhibitors on the ability of NS3 proteinase to cleave the substrate containing NS5A/5B cleavage site

Type of target proteinase	Inhibitor	IC_{50}[a] (mM)
Ser	PMSF	0.60
	APMSF	1.89
	Pefabloc/SC	0.45
	3,4-dichloroisocoumarin	0.03
	TLCK	0.15
	TPCK	0.03
	Chymostatin	0.71
	Antipain	>1
	Leupeptin	>1
	Soy bean trypsin inhibitor	>2 mg/ml
Cys	E-64	>1
	Iodoacetamide	0.26
Asp	Pepstatin	>1
Exo(amino)	Bestatin	>1
Metallo	EDTA	0.007
	EGTA	1.6
	CyDTA	0.007
	1,10-Phenanthroline	0.26
	1,7-Phenanthroline	>2

[a]The IC_{50} values were calculated from dose-response curves involving at least three different concentrations.

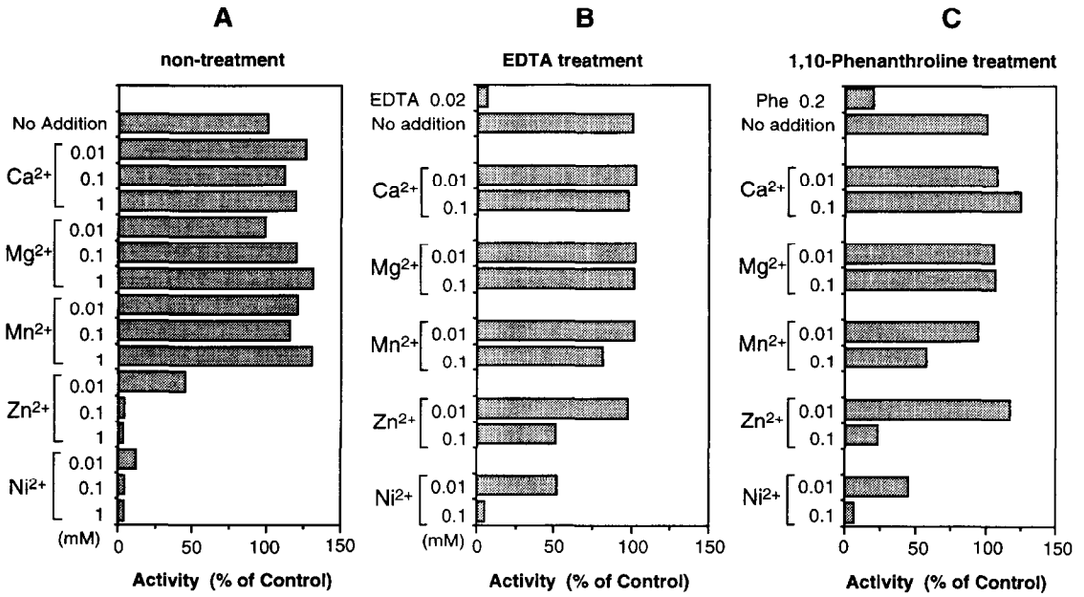

Fig. 3. Study of the involvement of metal ions to the enzyme activity. (A) Effect of metal ions on the enzyme activity. Several metal ions of indicated concentrations were added to the assay mixture and the enzyme activity was measured under the standard assay condition. (B,C) Effect of metal ions on the enzyme pre-treated with EDTA or 1,10-phenanthroline, respectively. The proteinase fraction treated with 0.2 mM EDTA (B) or 1 mM 1,10-phenanthroline (C) for 30 min were dialyzed against Tris/NaCl buffer containing 0.1% Tween 20. The enzyme activity was measured under the standard assay condition with or without metal ions. The cleavage activity is shown as a percentage of that without addition of metal ions.

NS4A/4B site was shown, no digestion of the NS3/4A and NS4B/5A sites was detected by Western blotting analysis with the anti-MBP antibody (data not shown).

3.3. Susceptibility to various proteinase inhibitors

Up to now, there was no report in which effect of commercial available inhibitors was studierd in the purified enzymatic assay system. To characterize the enzymatic activity of D51ΔNS3S, the effect of various proteinase inhibitors was studied. The NS3 proteinase has been demonstrated to be a serine proteinase by mutation analysis at Ser-1165 (Fig. 2) and its sequence homology with other serine proteinases, which was biochemically ascertained by the effectiveness of some serine proteinase inhibitors (Table 1). In addition to PMSF, Pefabloc/SC, and 3,4-dichloroisocoumarin, which inhibit a wide range of serine proteinases, TLCK and TPCK effectively inhibited NS3 proteinase activity. Chymostatin, known as a competitive inhibitor of chymotrypsin, weakly inhibited the NS3 proteinase, while antipain and leupeptin, which are known to be reversible competitive inhibitors of trypsin-like proteinases, did not decrease the activity.

Interestingly, in addition to some serine proteinase inhibitors, chelators such as EDTA, CyDTA and 1,10-phenanthroline, also inhibited the proteinase activity (Table 1). 1,7-Phenanthroline, a non-chelating analog of 1,10-phenanthroline, had no effect on the proteinase activity, suggesting importance of the chelating portion of 1,10-phenanthroline. However, among tetraacetic acid derivatives, EGTA showed only weak inhibition of the reaction while EDTA and CyDTA very efficiently inhibited it.

3.4. Examination of the inhibitory effect of chelators

The inhibition by some chelators raised two possibilities that a metal ion might be participating in the NS3 proteinase activity or that binding of chelators to the proteinase resulting in inhibition of the activity. In the former case, the NS3 proteinase is classified as a metal (Ca^{2+})-activated serine proteinase, or Zn^{2+} is involved in the proteinase activity since the NS3 serine proteinase region is located within the zinc-dependent metalloproteinase region responsible for processing at the NS2/3 site [20,21].

To study the contribution of metal ions to the proteinase activity, some metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, and Ni^{2+}) were added to the reaction mixture. The enzyme was not activated by any metal ions, and was inhibited by Zn^{2+} and Ni^{2+} even at the low concentration of 0.01 mM (Fig. 3A). To further examine the effect of metal ions on the enzyme activity, the enzyme activity was measured after removal of possibly pre-existing metal ions from the enzyme fraction. The enzyme was treated with 0.2 mM EDTA or 1 mM 1,10-phenanthroline and dialyzed against Tris/NaCl containing 0.1% Tween 20. It was found that the proteinase activity was restored after dialysis (approximately 80% or 50% of the original enzyme activity, when treated with EDTA or 1,10-phenanthroline, respectively). Even when the metal ions were added to these enzymes, no activation was observed (Mg^{2+}, Ca^{2+} and Mn^{2+}) and similar inhibition to Fig. 3A was shown (Zn^{2+} and Ni^{2+}) in Fig. 3B and C. Furthermore, when the enzyme inactivated by treatment with EDTA or 1,10-phenanthroline was diluted ten times with metal-free water, the enzyme activity was recovered to the control level without supplement of metal ions (data not shown).

These results show that metal ions are not involved in the proteinase activity and that the chelators themselves inhibit the NS3 serine proteinase by binding to the enzyme as a reversible inhibitor, not by removing metal ions from the enzyme.

4. Discussion

To establish an enzymatic assay system, we selected the D51ΔNS3S clone, which covers the region 1050–1214 of the NS3 protein, from the results of deletion analyses of our original HCV NS3 proteinase clones. This clone expressed the highest ability to cleave a recombinant substrate containing the NS5A/5B cleavage site in an *E. coli* co-expression system (Yamada, K. et al., unpublished results). By using this enzyme, we succeeded in detecting enzymatic cleavage activity in vitro at the level of CBB R-250 staining.

Our in vitro assay system revealed that the peptide sequence of 11 amino acids around the NS5A/5B site was a favorable substrate for *trans* cleavage by the NS3 proteinase, while those of the other sites were hardly cleaved at all (Fig. 2B). These results confirmed previous reports suggesting that in vivo cleavages by the NS3 proteinase could be separated into two types: cleavages which require NS4A as a cofactor (NS3/4A, NS4A/4B, and NS4B/5A sites) and one which does not (NS5A/5B site) [24–27]. It was also presented that NS4A might cause the enzyme to change substrate specificity. The effect of NS4A on the NS3 proteinase activity remains to be studied in our enzymatic assay system.

We examined the effect of well-known proteinase inhibitors by the established in vitro assay system. The NS3 proteinase has been predicted to be a chymotrypsin-like serine proteinase based on the sequence homology around the active center [13]. Supporting data were reported using an in vitro transcription and translation system, in which only the chymotrypsin-specific inhibitors TPCK, chymostatin and Pefablok/SC inhibited the proteinase activity [28]. Although it was reported that 300 μM of TLCK (effective against trypsin-like serine proteinases) did not inhibit the activity [28], we found that the same concentration of TLCK inhibited 67% of the activity in our assay system. As shown in this study, some chelators also inhibited the enzyme activity (Table 1, Fig. 3) in spite of the observation by Hahm et al. that 2 mM of EDTA did not show any inhibitory effect. The contradiction to the data of Hahm et al. seems to be derived from the differences of the purity and the conditions of the assay systems. These inhibition profile might reflect the existence of a unique active site pocket, which would define the unique substrate specificity (requirement of a cysteine residue at the P1 position, especially in the NS5A/5B cleavage site [29,30]) of the enzyme.

Although the chelating portion of 1,10-phenanthroline seemed to be involved in the inhibition, it was shown that the chelator itself inhibited the enzyme activity in a manner independent of metal ions, such as by direct binding to the enzyme (Fig. 3). Among EDTA, CyDTA and EGTA, which contain tetraacetic acid in the molecules, only the former two chelators efficiently inhibited the enzyme activity (Table 1). Considering that the whole structure of EDTA is identical to the part of CyDTA, it is suggested that the structure has a suitable size and shape for binding to the enzyme. It is reported that the model of the specificity pocket of the enzyme includes Lys-1162 and Arg-1181 [31]. It could be speculated that negative charges of EDTA/CyDTA or electron pairs of 1,10-phenanthroine might interact with the side chains of such amino acids and thus inhibit the enzyme activity. Alternatively, these inhibitors might attach to a distinct site away from the active center, change the tertiary structure of the enzyme, and allosterically render it inactive. Since the purified recombinant NS3 proteinase has a relatively small molecular size compared to other proteinases, X-ray crystallographical analysis would also be useful to elucidate the tertiary structure of this enzyme which possesses unique substrate specificity and sensitivity to chelators.

Our enzymatic system, established for assay of the HCV NS3 proteinase which is an attractive target for anti-HCV agents, provides a simple and clear strategy for the first screening of anti-HCV reagents. Considering the unique characteristics of HCV NS3 proteinase described in this report, specific inhibitors against the proteinase could be identified in this system.

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