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Cleavage Activity of Hepatitis C Virus Serine Proteinase

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To study the character of the hepatitis C virus (HCV) encoding serine proteinase and to search for inhibitors, a practical *in vitro* assay system using the purified enzyme and synthetic peptide substrates was established. The enzyme used was expressed in *Escherichia coli* as a fusion form with protein tags and purified to apparent homogeneity by single-step affinity chromatography. The purified enzyme exhibited proteolytic activity with pH optima of around eight, and the addition of NS4A fragments increased the activity as well as the thermal stability of the enzyme. The activity was inhibited by EDTA and some divalent ions, *i.e.*, copper and zinc, though calcium, magnesium, and manganese were stimulative both in the presence and absence of the NS4A fragment. None of the common protease inhibitors, including serine protease inhibitors, effectively inhibited the activity. Based on the kinetic parameters of the cleavage reaction of the synthetic 20 mer peptides corresponding to the three cleavage sites, NS4A/4B, NS4B/5A, and NS5A/5B, the peptide with the NS5A/5B junction was found to be the most efficient substrate. Analysis of the minimal peptide substrate of NS5A/5B indicated that 5 to 7 amino acids on both sides of the junction were required for efficient cleavage. These findings are expected to contribute to the search for a proteinase inhibitor.

Key words: hepatitis C virus, protease inhibitor, serine proteinase, substrate specificity, synthetic peptide.

Hepatitis C virus (HCV) has been demonstrated to be the causative agent of non-A non-B hepatitis. The cDNA of the entire virus genome has been cloned in recent years and sequenced (*1-4*). The sequence analysis revealed that the virus is closely related to the viruses belonging to the family Flaviviridae, such as yellow fever virus (YFV) and bovine viral diarrhea virus (BVDV), members of the flavi- and pestivirus, respectively. The genome of these viruses is a single-stranded RNA with positive polarity, and it encodes a large open reading frame of approximately 10 kilobases. The polyprotein translated from the RNA is processed into structural and non-structural proteins. These viruses possess a conserved trypsin-like serine proteinase domain within the non-structural region with a conserved active center consisting of an amino acid triad of histidine, aspartic acid, and serine. The virus-encoded proteinase is essential for the cleavage of downstream polyprotein in a *cis*- and *trans*-acting manner (*5-7*). Through the separation of structural and non-structural proteins, and the generation of virus-specific enzymes, the virus-encoded proteinase is considered to be involved in a variety of functions such as coordinated assembly of the virion and maturation (*8*). Thus, inhibition of the virus-specific processing results in a critical reduction of virus propagation. In fact, the infectious virus particle of YFV was not recovered from mammalian cells transfected with RNAs transcribed from a full-length YFV cDNA template containing mutations at serine-138, which is one member of the active triad of viral serine proteinase (*6*). As in these viruses of Flaviviridae, the structural and non-structural proteins of HCV are presumed to be translated from the genome as a single polyprotein, and the genomic organization is predicted as 5′-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ (*9-11*). The activities of two virally encoded HCV proteinases have been identified (*12, 13*). One is a trypsin-like serine proteinase which is encoded at the N-terminal one-third of NS3. This proteinase has been found to be responsible for cleavages at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions (*9, 11, 13*). The second proteinase domain extends over the C-terminus of NS2 and the N-terminus of NS3, and the enzyme is responsible for cleavage at the NS2/NS3 site (*10*). This proteinase is considered to be a metalloproteinase, since a zinc ion is indispensable for the cleavage. We reproduced NS3 serine proteinase activity in COS-1 as well as in *Escherichia coli* cells (*12, 14, 15*). We also reported an *in vitro* assay for the cleavage activity of HCV proteinase fused with protein tags which facilitated the isolation of the proteinase (*16*). The substrates used were either recombinant or synthetic peptides bearing the amino acid sequence of the NS5A/NS5B cleavage site. We showed that the enzyme expressed in *E. coli* had activity compa-

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Abbreviations: APMSF, (4-amidinophenyl)methanesulfonyl fluoride; BVDV, bovine viral diarrhea virus; DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; HCV, hepatitis C virus; MBP, maltose binding protein; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone (*N*-tosyl-L-lysine chloromethyl ketone); YFV, yellow fever virus.

rable to that expressed in animal cells. However, the efficiency of cleaving the synthetic peptide substrates declined at temperatures higher than 30°C. It was recently found that a part of the NS4A protein is indispensable for cleavage at the NS3/NS4A and NS4B/NS5A sites, and that it accelerates the rate of cleavage at the NS5A/NS5B junction (*17, 18*). The protein was found to form a stable complex with NS3 protein (*13*). Based on these findings as well as the similarity in hydropathy, the NS4A protein is considered to correspond to the NS2B protein of flaviviruses. The NS2B protein of flaviviruses and possibly p10 of BVDV and its homologues in the plant viruses act as chaperonins, assisting the folding of the proteinase to bind with substrates (*17*). Crystal structure analyses of the complex of the enzyme and an NS4A fragment peptide have just been reported and have shown that the NS4A peptide intercalates within a β sheet of the enzyme core and provides a more rigid and precise framework to form the prime-side of the substrate-binding channel (*19*). The minimum functional domain of NS4A was revealed 10 amino acids from 1678-1687 in a transient protein expression system in COS-1 cells (*18*). An attempt was made to reconstruct the HCV serine proteinase activity *in vitro* using the fused proteinase and parts of the NS4A fragments (*20*). The result showed that the addition of 4A18-40, the peptide consisting of the 18-40 amino acid sequence of NS4A, accelerated the cleavage activity of NS3. In the present article, we report the establishment of an *in vitro* assay system with or without an NS4A peptide and we present information about the enzyme including pH preference, effect of ions and thermal stability. We also compare kinetic data of the cleavage reaction of each cleavage site. The amino acid sequences of the cleavage sites have been revealed in recent years by using bacterial and animal cell expression methods and *in vitro* transcription-translation assays (*9, 14, 15*). It was found that all four cleavage sites which were cleaved by NS3 serine proteinase had conserved amino acid residues at P6, P1, and P1′. Our kinetic study has established the differences in cleavage rate among these sites, which may reflect the order of appearance of the viral proteins in infected cells. The present information about the character of the enzyme should facilitate the search for an HCV proteinase-specific inhibitor.

MATERIALS AND METHODS

The basic method for the production and preparation of the fused form of HCV serine proteinase, MBP-NS3, has been reported (*16*). The peptide substrates were obtained from the Peptide Institute (Osaka) and were dissolved in dimethyl sulfoxide (DMSO). The NS4A fragment peptides were purchased from Sawady Technology (Tokyo). The proteinase inhibitors were the products of Boehringer Mannheim (Germany) and were dissolved in DMSO.

Recombinant Substrates—The structures and constructions of the expression plasmids for the recombinant substrates with the amino acid sequences of the NS3/4A, NS4A/4B, and NS4B/5A, and NS5A/5B junctions in between maltose binding protein (MBP) and dihydrofolate reductase (DHFR), pMD34, pMD4AB, pMD45, and pMD-5AB have been reported (*15*). The recombinant substrates were produced in *E. coli* and were purified as described previously (*16*). In short, the expression plasmids were transformed in an *E. coli* strain, JM109, by the standard method. The transformed bacteria were cultured in Luria broth containing 50 mM ampicillin at 37°C, and the expression of the substrate proteins was induced by an addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacteria were harvested and homogenized in buffer containing 10 mM sodium phosphate (pH 7.2), 30 mM NaCl, and 10 mM DTT. The homogenate was loaded on a methotrexate-bound resin column. The column was rinsed with the elution buffer, and then proteins were eluted with the elution buffer containing sodium dihydrofolate.

Enzyme Assay—Principally, 80 μg/ml of MBP-NS3 in a buffer consisting of 50 mM Tris HCl (pH 7.8), 5 mM $CaCl_2$, 10 mM DTT, and 30 mM NaCl was pre-incubated with inhibitors dissolved in DMSO or DMSO itself at a concentration of 2% at 25°C for 15 min. The concentration of MBP-NS3 in the stock solution was 2-6 mg/ ml in 50% glycerol, and the final glycerol concentration in the reaction mixture was less than 5%. The reaction was started by addition of the substrate (the synthetic peptide or the recombinant protein) solution, and was continued for 60 min. The NS4A cofactors were added before preincubation. The reaction with the cofactor was carried out at 37°C for 10 min. A peptide substrate, Pep 5 (Dns-GEAGDDIVPC SMSYTWT), at a final concentration of 86 μM was used as the standard substrate for the assays. The reaction using the peptide substrates was quenched by addition of 5 N NaOH at a final concentration of 0.25 N and the products were analyzed by HPLC. The progress of the reactions using the recombinant protein substrates was evaluated with a *Personal Densitometer*™ from Molecular Dynamics after SDS-PAGE and Coomassie Brilliant Blue staining.

High-Performance Liquid Chromatography (HPLC) Procedure—The HPLC analysis was made on a Shimadzu LC-9A liquid chromatography system equipped with a TSK gel ODS-80 TM column (Tosoh, Tokyo). The column was eluted with acetonitrile gradients in 50 mM ammonium acetate (pH 6.5). The fluorescence signal of the dansyl moiety of the N-terminal portion of the cleaved product and the uncleaved substrate was detected with a fluorescence monitor, Shimadzu RF5000 (excitation at 340 nm and emission at 510 nm).

RESULTS

Effect of pH and Divalent Ions—The enzyme used, MBP-NS3, was expressed in *E. coli* as a fusion form with protein tags and purified to apparent homogeneity by single-step affinity chromatography (*16*). The cleavage activity of MBP-NS3 towards the synthetic substrate, Pep 5, was compared at different pHs, at 25°C (Fig. 1). MBP-NS3 cleaved Pep 5 most efficiently in buffers at about pH 8, although the activity was slightly different among buffers. The effect of divalent ions at a concentration of 2 mM was examined in Tris HCl buffer at pH 7.8 (Table I). The stimulative and inhibitory effects of the divalent ions were similar with and without the NS4A peptide, P41. Calcium, magnesium, and manganese ions accelerated peptide cleavage. In contrast, copper and zinc ions reduced the reaction rate to less than 15% of the control. EDTA at a concentration of 1 mM reduced the cleavage reaction to one-fourth of the control. The addition of calcium or magnesium ion at

twice the amount of EDTA restored, at least partially, the activity of the enzyme preincubated with 1 mM EDTA. Sodium and potassium ions were stimulative at 0.1 M, but not at 0.5 M (Table II). The stimulative effect of these ions as well as the additive effect of sodium and calcium ions reached a maximum, at which the rate was about 70% higher than that of the control.

Effect of the NS4A Fragments—Studies of the effect of NS4A (amino acid number 1658-1711) on HCV serine proteinase using cell cultures have shown that a C-terminal 33-amino acid sequence (*17*) or 10-amino acid sequence from 1678 to 1687 (*18*) was required. To reconstitute the activity of the NS3-NS4A complex *in vitro*, a peptide fragment of NS4A, P41, with the amino acid sequence 1673 to 1697, was added to the reaction mixture. In preliminary experiments, when the ratio of P41/enzyme was higher than ten, the reaction rate reached the maximum. Thus, the fragment was used at a tenfold excess amount. Figure 2 illustrates the cleavage rate of MBP-NS3 in the presence or absence of P41 as a function of temperature. The highest cleavage reaction by MBP-NS3 alone was found at 25°C, and the reaction rate declined at temperatures higher than 30°C. In the presence of P41, the maximum reaction rate was found at 37°C, and was 4-6 times higher than that of MBP-NS3 alone at 25°C. Several NS4A fragment peptides having 6 to 30 amino acids were added to the enzyme reaction fluid. Two of the seven fragments, P41 and P46,

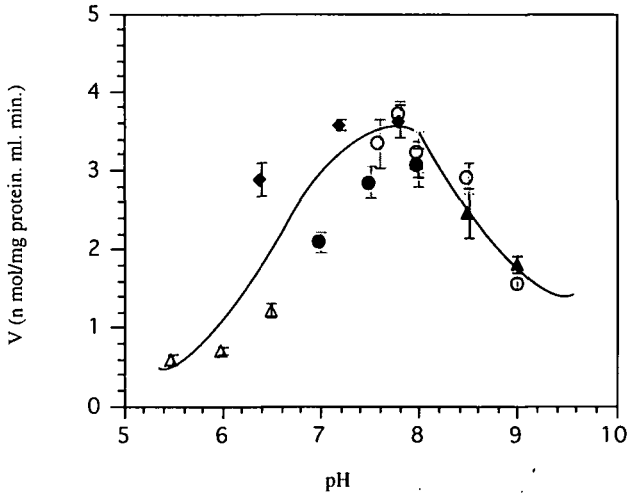

Fig. 1. **Effect of buffers and pH on cleavage activity of MBP-NS3.** The cleavage activity was measured by the hydrolysis assay of P5. The reactions were performed in reaction mixtures consisting of Na MES (△), Na HEPES (●), Na TAPS (▲), Na phosphate (◆), or Tris HCl (○), 30 mM NaCl and 10 mM DTT at 25°C for 60 min. The values are the means±standard deviations of triplicate assays.

TABLE I. **Effect of divalent cations and EDTA on cleavage activity of MBP-NS3.** The reactions were performed in reaction mixtures consisting of 50 mM Tris HCl buffer (pH 7.8), 30 mM NaCl, and 10 mM DTT at 25°C for 60 min (in the absence of P41) or at 37°C for 10 min (in the presence of P41). The values are the means± standard deviations of triplicate assays.

Divalent ion or EDTA	*V* (nmol/mg protein·ml·min) (−) P41	(+) P41
Control	3.97±0.35	25.5±2.99
$BaCl_2$ (2 mM)	4.06±0.55	31.3±1.82
$CaCl_2$ (2 mM)	4.47±0.52	34.0±1.55
$CuCl_2$ (2 mM)	<0.5	0.55±0.94
$MgCl_2$ (2 mM)	4.50±0.19	33.1±1.35
$MnCl_2$ (2 mM)	4.88±0.07	35.1±1.17
$ZnCl_2$ (2 mM)	<0.5	<0.5
EDTA (1 mM)	1.21±0.07	7.01±0.78
EDTA (1 mM)+$CaCl_2$ (2 mM)	3.28±0.06	9.35±1.03
EDTA (1 mM)+$MnCl_2$ (2 mM)	4.16±0.07	18.6±1.51
EDTA (1 mM)+$ZnCl_2$ (2 mM)	<0.5	1.62±1.51

TABLE II. **Effect of cations on cleavage activity of MBP-NS3.** The reactions were performed in reaction mixtures consisting of 50 mM Tris HCl buffer (pH 7.8), and 10 mM DTT at 25°C for 60 min. The values are the means±standard deviations of triplicate assays.

	V (nmol/mg protein·ml·min)
Control	2.41±0.19
+30 mM NaCl	3.53±0.06
+0.1 M NaCl	4.37±0.10
+0.5 M NaCl	2.81±0.15
+0.1 M KCl	3.93±0.14
+0.5 M KCl	2.52±0.04
+30 mM NaCl+5 mM $CaCl_2$	4.14±0.49
+0.1 M NaCl+5 mM $CaCl_2$	4.19±0.35

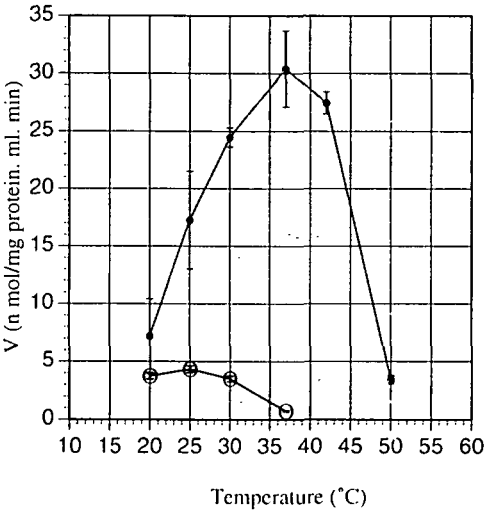

Fig. 2. **Effect of temperatures on cleavage activity of MBP-NS3 with or without P41.** The cleavage activity was measured by the hydrolysis assay of P5 with (●) or without P41 (○) in 10-fold excess over the enzyme in the standard buffer. The values are expressed as the means±standard deviations of triplicate assays.

TABLE III. **Effect of NS4A fragment peptides on cleavage activity of MBP-NS3.** The reactions were performed using the NS4A fragment peptide in 10-fold excess over the enzyme in the standard buffer at 37°C for 10 min. The values are the means± standard deviations of triplicate assays.

NS4A Fragment	Amino acid number		K_m (mM)
P41	1673-1692	32.3±1.92	0.020
P42	1678-1687	<0.1	–
P43	1673-1682	<0.1	–
P44	1683-1692	<0.1	–
P45	1680-1685	<0.1	–
P46	1668-1697	28.75±1.54	0.019
P47	1683-1702	<0.1	–

TABLE IV. **Comparison of recombinant and synthetic substrates.** The kinetic parameters of the hydrolysis of the substrates were determined in the standard buffer with P41 in 10-fold excess over the enzyme at 37°C. The values are the means±standard deviations of triplicate assays.

Cleavage site	k_{cat} (1/min)			
	Recombinant substrate		Synthetic peptide substrate	
NS3-4A	SADLEVVT ↓ STWVL	0.015±0.014	Dns-DLEVVT ↓ STWVLV	<0.01
NS4A-4B	EFDEMEEC ↓ ASHL	0.019±0.005	Dns-YQEFDEMEEC ↓ ASHLPYIEQG	0.81±0.09
NS4B-5A	INEDCSTPC ↓ SGSWL	0.054±0.013	Dns-WINEDCSTPC ↓ SGSWLKDVWD	1.0±0.2
NS5A-5B	GDDIVCC ↓ SMSYTWTG	0.70±0.18	Dns-GEAGDDIVPC ↓ SMSYTWTGAL	3.3±0.3

TABLE V. **Comparison of peptide substrates.** The kinetic parameters of the hydrolysis of the substrates were determined in the standard buffer. The values are the means±standard deviations of triplicate assays. a: The reactions were performed using P41 in 10-fold excess over the enzyme at 37°C for 10 min. b: The reactions were performed at 25°C for 60 min. c: The substrate was hardly dissolved in the reaction mixture. d: The small letter represents the (D)-amino acid. e: An unknown product was formed immediately after the addition of the peptide.

	Substrate	MBP-NS3+P41[b]		MBP-NS3[c]	
		k_{cat} (1/min)[a]	K_m (mM)[a]	k_{cat} (1/min)[a]	K_m (mM)[a]
Pep1	Dns GEAGDDIVPC ↓ SMSYTWTGAL	3.3±0.3	0.015±0.002	1.0+0.1	0.15+0.02
Pep2	Dns-GEAGDDIVPN SMSYTWTGAL	<0.1	–	<0.1	–
Pep3	Dns-GEAGDDIVAC SMSYTWTGAL	<0.1[d]	–	<0.1	–
Pep4	Dns-GEAGDDIVPc SMSYTWTGAL[e]	<0.1	–	<0.1	–
Pep5	Dns GEAGDDIVPC SMSYTWT	5.1±0.6	0.026±0.007	1.3+0.2	0.19+0.04
Pep6	Dns GEAGDDIVPC SMS	2.0±0.1	0.29±0.01	<0.1	–
Pep7	Dns GEAGDDIVAC SMS	<0.1	–	<0.1	–
Pep8	Dns GEAGDDIVCC SMS	<0.1	–	<0.1	–
Pep9	Dns DDIVPC SMSYTWT	4.3±0.9	0.12±0.06	0.87+0.23	0.27+0.11
Pep10	Dns DDIVPC SMSYT	3.7±0.08	0.20±0.001	0.34+0.09	0.69+0.11
Pep11	Dns DDIVPC SMS	3.2±1.3	1.9±1.0	<0.1	–

accelerated the reaction (Table III). Both of these fragments have the HCV amino acid sequence number 1673 to 1692. However, P42, P43, and P44 which contain the HCV amino acid sequence numbers 1678 to 1687, 1673 to 1682, and 1683 to 1692, respectively, were not effective accelerators.

Cleavage of Synthetic Peptides and Recombinant Proteins Having the Amino Acid Sequence of NS3/4A, NS4A/4B, NS4B/5A, or NS5A/5B—The recombinant protein and synthetic peptide substrates covering four cleavage sites, NS3/4A, NS4A/4B, NS4B/NS5A, and NS5A/5B, were cleaved by MBP-NS3 in the presence of P41. The recombinant protein substrates consisted of 12-15 amino acids from the vicinity of the cleavage sites sandwiched between MBP and *E. coli* DHFR domains. The synthetic peptides (except NS3/4A, which has a 15 amino acid length) have 20 amino acid sequences harboring each cleavage site. The synthetic peptides were more effectively cleaved than were the recombinant protein counterparts. The substrates with the amino acid sequence of the NS5A/5B junction, both recombinant and synthetic, were the most efficiently cleaved. The N-terminal amino acid sequences of the C-terminal portion of the cleaved products derived from the synthetic peptides were analyzed. It was confirmed that the cleavage occurred at the expected sites.

Cleavage of Synthetic Peptides Having the NS5A/5B Cleavage Site—For elucidating the minimum length required for effective cleavage, the peptide substrate, Pep 1, which has a 20 amino acid length surrounding the NS5A/5B junction, was subjected to N- and C-terminal deletions. The cleavage rates were compared in the presence or absence of P41. In both cases, Pep 5 was cleaved most efficiently. Comparing Pep 1, 5, and 6, the shortening of the three C-terminal residues favored cleavage, but that of seven residues resulted in a large reduction of the cleavage rate.

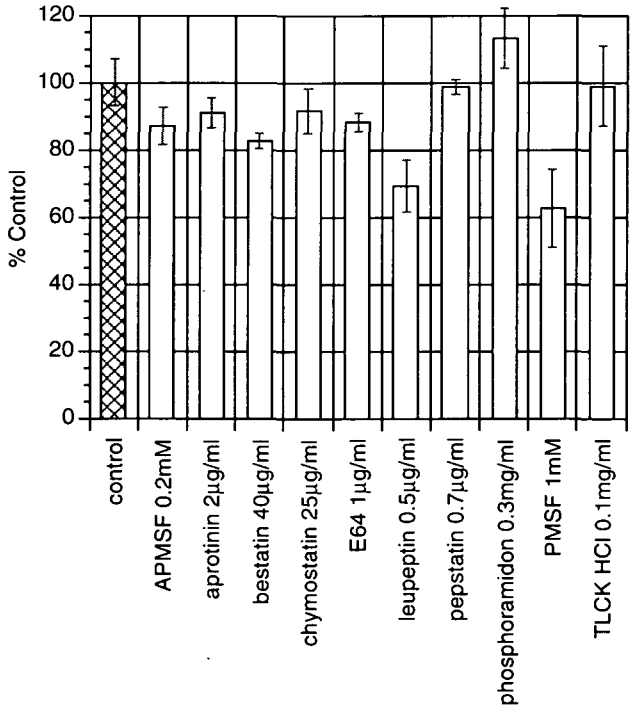

Fig. 3. **Effect of proteinase inhibitors on cleavage activity of MBP-NS3.** The cleavage activity was measured by the hydrolysis assay of P5. The reactions were performed in the standard buffer at 25°C for 60 min. The cleavage activity in the control reaction without any proteinase inhibitors was designated as 100%. The values are expressed as the means±standard deviations of triplicate assays.

In parallel with the reduction of the cleavage rate, the affinity of the substrate for the enzyme, which is represent-

ed by K_m, was reduced. A similar tendency was found in the comparison of Pep 9, 10, and 11. The substrates which had seven C-terminal residues were the most efficiently cleaved. In addition, the deletion of the four N-terminal residues reduced the cleavage efficiency based on the comparison of Pep 5 and 9, but not Pep 6 and 11. Pep 2 and 4, whose cysteine residues at the P1 position were replaced with asparagine and (D)-cysteine, respectively, were not cleaved by the enzyme.

Effect of Protease Inhibitors—The effect of some proteinase inhibitors on MBP-NS3 without P41 was examined at 25°C. (4-Amidinophenyl)methanesulfonyl fluoride (APMSF) and aprotinin are serine proteinase inhibitors, whereas bestatin, E-64, and pepstatin are aminopeptidase, metalloproteinase and acidic proteinase inhibitors, respectively. Leupeptin and phenymethylsulfonyl fluoride (PMSF) inhibited both serine and cysteine proteases. None of these inhibitors reduced the reaction rate to less than 50% of the control. PMSF and leupeptin reduced the reaction rate by approximately 40%.

DISCUSSION

Studies of the processing of HCV polyproteins have been successfully performed using the transient expression of HCV genome in human, animal, and bacterial cell cultures (*9, 11, 14, 15, 17, 18, 21*) or cell-free transcription-translation assays (*12, 13, 22*). In these studies, the viral proteinase activities were detected, and the cleavage sites were identified. Both NS3 and NS4A were also found to be necessary for the serine proteinase activity. However, a kinetic study using a transient expression system obtained complex results (*17*). Therefore, we and other investigators have proposed that *in vitro* assays using the purified enzyme and peptide substrates harboring NS5A/5B (*16, 20*), NS4A/4B (*23*), or NS4B/5A (*24*) site would be more suitable for examining the kinetic parameters and for characterization of HCV proteinase activity.

The enzyme we used in the present study, MBP-NS3, has features in common with the enzyme activity observed in transient expression in animal cells or *in vitro* transcription-translation (*16*). This fusion enzyme had several advantages: the majority of the enzyme was recovered as soluble form, so that solubilizing agents such as guanidine sodium hydrochloride and urea, or detergents were unnecessary. The soluble enzyme was easily purified by one affinity chromatography, and the yield was 4-6 mg per liter of bacteria culture. After storage for one year at −20°C in 50% glycerol, the change of cleavage activity was within 20% (Kakiuchi, N., unpublished observations).

In this study, we obtained more precise information about the characteristics of the enzyme and the kinetic parameters of each cleavage site. The results revealed a new aspect of the enzyme: the enzyme prefers neutral to weak alkaline pHs for cleavage reaction. Divalent ions showed ambivalent effects, *i.e.*, calcium, magnesium, and manganese were stimulative, whereas copper and zinc were suppressive to the enzyme reaction. EDTA had an inhibitory effect on the enzyme, and the inhibition was reversed by addition of excess calcium or manganese. The inhibitory effect of EDTA was also observed in an assay using a cell-free system (*22*). The results with divalent cations, taken together with the EDTA inhibition, suggested that the stimulative divalent ions may not only neutralize the negative charge of the enzyme and substrates to help interaction of these molecules, but may also assist the correct folding of the enzyme, and the inhibitory ions may compete with them. Copper ion was reported to compete with Ag^+ in inhibiting chymotrypsin (*25*), and silver ion binds specifically between asparagine-102 and histidine-57 in DIP-trypsin (*26*). The crystal structure of HCV NS3 proteinase was recently reported (*19, 27*). It was shown that the binding site of zinc (*27*) or tetrahedral coordinated metal ion (*19*) was in the region distal to the active center. The binding site consisted of cysteine-1123, cysteine-1125, cysteine-1171, and histidine-1175. The mutation of these residues showed that they may be important, but not essential, for the serine proteinase activity (*13*). Though the role of these ions in serine proteinase activity is not clear, these ions may have a significant effect on the proteinase structure, and consequently, on its cleavage activity. It is also possible that the binding of the tetrahedral coordinated metals is necessary for metalloproteinase, whose functional region overlaps with the serine proteinase.

NS4A or parts of it was reported to be indispensable for the cleavage of NS4B/5A and to accelerate the cleavage of NS4A/4B and NS5A/5B using transient expression systems in animal cell culture (*17, 18*). In the present study, it was found that the addition of two NS4A fragments, P41 and P46, covering 1673-1692 and 1668-1697, respectively, accelerated the cleavage of Pep 5. Their effects on the enzyme activity were almost the same. In contrast, the smaller fragments, P42 and P46, covering 1678-1687 and 1680-1685, respectively, did not alter the enzyme activity. In our earlier study, utilizing transient expression of the HCV protein in COS-1 cells, a 10-amino acid sequence of NS4A, 1678-1687, was sufficient for the enzyme activation (*18*), whereas the longer region of 1680-1692 was sufficient for the *in vitro* cleavage of NS5A/5B (*20*) and NS4B/5A (*23*). The extra sequence which was required for the enhancement of enzyme activity, 1688-1692, may contribute to the interaction with the enzyme in the reaction fluid. Analysis of the crystal structure showed that the amino acids at 1680-1689 interacted with NS3 protein (*19*). Thus, we used P41 to reconstitute the enzyme activity *in vitro* in this study. The addition of P41 to the reaction mixture resulted in not only acceleration of the cleavage of Pep 5, but also the thermal stabilization of the enzyme activity. As a result of the addition of P41 in tenfold excess over the enzyme, the optimum temperature of the cleavage reaction was shifted from 25 to 37°C, and the reaction rate increased about 4-6 times.

In the comparison of the kinetic parameters of cleavage at the four junctions in the presence of P41, we found that the order of cleavage rates in both recombinant and peptide substrates was NS5A/5B>NS4B/5A≥NS4A/NS4B>NS3/4A. In earlier studies using a vaccinia virus transfection system or the transformation of *E. coli* by an expression plasmid, the NS3/4A junction was cleaved in *cis* mode, and the cleavage reaction seemed to occur co-translationally. In this study, the cleavage of the NS3/4A junction was not observed, since only intermolecular reactions can be detected by *in vitro* enzyme assay. We found that the cleavage rate of NS4B/5A was comparable to that of NS4A/NS4B, though the data reported earlier showed that

the NS4B/5A site was cleaved with the lowest efficiency (*20*). This discrepancy may be due to the size and the sequence of the substrate used. Another possibility is the difference in reaction conditions: our assays were performed in fluid containing less glycerol, and thus having lower viscosity, and without detergent at 37°C. Among the cleavage sites of NS4-NS5, the NS5A/5B junction was cleaved at the highest rate. NS5B protein may be needed most in the early stage of virus propagation. From the comparison of the substrates bearing the NS5A/5B junction, five to seven C-terminal residues and six N-terminal residues were required for a sufficient cleavage rate of the peptide substrate. The consensus sequences seemed insufficient for an efficient substrate, and the flanking sequences, especially the C-terminal hydrophobic residues, favored enzyme binding. This sequence preference for the substrate of the enzyme indicates that not only the peptide substrates, but also the substrate-mimicking inhibitors of the enzyme need a sequence longer than that of common serine proteinases. However, the peptide whose cysteine at the P1 position was replaced with asparagine or with (D)-cysteine was not an efficient substrate, and the co-existence of these non-cleaved substrates did not affect the cleavage efficiency of the substrates. This result suggest that the non-cleaved substrate did not compete with the substrates in binding with the enzyme.

Though the HCV NS3 proteinase has the catalytic triad domain of serine proteinase, the other parts of the proteinase domains, including the predicted substrate binding pockets, have poor sequence homology with the serine proteinases encoded in flaviviruses and the well-studied enzymes such as trypsin, chymotrypsin, and elastase (*11*). Studies using cell cultures and *in vitro* coupled transcription-translation assay revealed that the enzyme has different cleavage site specificity from these serine proteases (*9, 11, 15*). Indeed, common proteinase inhibitors including serine proteinase inhibitors, such as aprotinin, APMSF, leupeptin, PMSF, and TLCK, did not effectively inhibit the enzyme activity. Only leupeptin and PMSF reduced the cleavage reaction moderately. This result, together with the different cleavage specificity, suggests that the substrate and the inhibitor specificity of HCV serine proteinase are dissimilar to those of the common proteinases and the virus-encoded proteinases. Thus, we are optimistic that HCV serine proteinase-specific inhibitors, which could be candidate anti-HCV agents, will be found. However, a large number of compounds may have to be screened. The properties of the enzyme we used would facilitate large-scale screening. The use of the peptide substrate bearing NS5A/5B, P5, and its derivatives would also save time. We have begun screening studies with this system (*28*).

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