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Establishment of an in vitro assay system for screening hepatitis C virus protease inhibitors using high performance liquid chromatography

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Abstract

The hepatitis C virus (HCV) genome contains the code for a conserved, serine-type protease, called NS3, for the processing of the non-structural protein region of the viral polyproteins. Furthermore, a related protein, NS4A, is an effector or cofactor of NS3 protease activity in the cleavage of NS3-4A, NS4A-4B, NS4B-5A and NS5A-5B junctions. To establish an in vitro assay system for the screening of those enzyme inhibitors that inhibit the protease NS3-4A, we prepared a maltose-binding protein-NS3-NS4A fusion protein and a synthetic peptide substrate that mimics the NS5A-5B junction. Cleavage of the synthetic peptide was analyzed by reversed-phase high performance liquid chromatography (HPLC). We showed that the enzymatic activity of the NS3-NS4A fusion protein was enhanced in comparison to the NS3 protein alone. The assay conditions for optimum NS3-4A protease activity were determined to be pH 7.6 and 37°C. In addition, we evaluated several protease inhibitors using the same HPLC assay system. The activity of HCV protease NS3-4A was inhibited by 2714.4 μM diisopropyl fluorophosphate, 270.8 μM *N*-tosyl-L-lysyl chloromethyl ketone, and 825.5 μM chymostatin. The results of the present study indicated that the synthetic peptide substrate and HPLC assay system are suitable for studying HCV protease activity and may facilitate the development of anti-HCV therapeutic reagents.

Keywords: Hepatitis C virus; NS3-NS4A fusion protein; Synthetic peptide substrate; Protease inhibitors; High performance liquid chromatography

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1. Introduction

Hepatitis C virus (HCV) is the major etiologic agent of post transfusion non-A, non-B hepatitis (Alter et al., 1989; Choo et al., 1989; Kuo et al., 1989). HCV infection is estimated to affect approximately 50 million people worldwide. Nearly 50% of the afflicted individuals become chronically infected, and liver cirrhosis develops in approximately 20% (Mast and Alter, 1993). In addition, an increased incidence of hepatocellular carcinoma in patients with non-A, non-B hepatitis suggests that HCV may be involved in the pathogenesis of this malignancy (Chien et al., 1992; Shimotohno, 1993). The lack of an efficient HCV replication system contributes to the absence of a screening methodology for anti-HCV agents, although two recent reports describe the in vitro replication of HCV (Shimizu et al., 1993; Lanford et al., 1994).

HCV contains a single-stranded, positive-sense RNA genome that encodes a single, long polyprotein with the gene order: 5′-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′, where E = envelope protein and NS = non-structural protein. The NS3 protein displays serine protease-like enzymatic activity in the N-terminal one-third (Bazan and Fletterick, 1989; Miller and Purcell, 1990), and its amino acid sequence is homologous with some helicases in the C-terminal protein (Miller and Purcell, 1990; Gobalenya et al., 1989; Suzich et al., 1993). Cleavage of the NS3-4A, NS4A-4B, NS4B-5A, and NS5A-5B sites depends on the activity of the serine protease NS3, observed in the expression of different parts of the NS proteins in eukaryotic cell expression systems and in in vitro translation systems (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993a,b; Hijikata et al., 1993; Tomei et al., 1993). NS4A is an amphipathic protein of 54 amino acids length; it has a hydrophobic N-terminal domain and a hydrophilic C-terminal domain (Failla et al., 1994). NS4A acts as an effector or cofactor of the protease activity of NS3 in the cleavage of the NS3-4A, NS4A-4B, NS4B-5A, and NS5A-5B junctions. In addition, it can act in trans to affect NS3 protease activity (Hahm et al., 1995; Lin et al., 1994; Bartenschlager et al., 1994; Failla et al., 1994; Lin and Rice, 1995a). Lin and Rice (1995a) suggested that the central region of NS4A is involved in the NS4A-dependent cleavage of the NS4B-5A site. Furthermore, Shimizu et al. (1996) demonstrated that residues 22–31 of NS4A are required for its effector activity in the processing of a synthetic peptide as substrate that mimics the NS5A-5B junction.

The proteolytic processing steps in the HCV life cycle are required to produce infectious virus particles; this makes viral proteases primary target for anti-viral reagents. Lin and Rice (1995b) described an in vitro trans-cleavage assay for the HCV serine protease NS3, using cell-free translation. The cleavage products, however, were analyzed by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) using ^{35}S-labeled substrate, rendering them useless for inhibition studies. Therefore, we produced the NS3-NS4A fusion protein in *Escherichia coli*, and used high performance liquid chromatography (HPLC) to analyze the effectiveness of a synthetic peptide substrate that mimics the NS5A-5B junction. Furthermore, several common protease inhibitors were tested.

2. Materials and methods

2.1. Construction of expression vectors containing NS3-NS4A

An HCV cDNA clone containing nucleotides 3421–5475 (NS3-NS4A) was amplified by polymerase chain reaction (PCR). Oligonucleotides 5′-CCGCTGCAG-CCATGGCGCCTATCACG-GCCTAT-3′ and 5′-CCGAAGCTTTCAG-CACTCTTC-CATTTCATC-3′ were used as plus- and minus-strand primers, respectively. PCR products were digested with *Pst*I and *Hin*dIII, and then ligated into pMAL-c2 (New England Biolabs Inc., Berarly, MA, USA). A plasmid containing the HCV genome nucleotides 3421–5313 (NS3) was constructed by the same method as described above. Oligonucleotides 5′-CCGCT-GCAGCCATGGCGCCTATCACGG-CCTAT-3′ and 5′-CCGAAGCTTTCAAGTGACGACCTC-CAGGTC-3′ were used as plus- and minus-

strand primers, respectively. The *E. coli* strain JM109 used to transform the plasmids described above, was grown in Luria broth (LB) containing ampicillin (50 μg/ml) at 37°C. Enzyme expression was induced by adding 0.5 mM isopropyl-ß-D-(−)-thiogalactopyranoside (IPTG) and by incubating the culture at 20°C for 3 h. Cells were harvested by centrifugation and stored at −80°C.

2.2. Preparation of maltose-binding protein (MBP)-NS3-NS4A fusion protein.

The pellet from a 3-l culture was resuspended in 300 ml buffer A (10 mM sodium phosphate buffer (pH 7.2), containing 30 mM sodium chloride (NaCl) and 2 mM ß-mercaptoethanol (ME)), and then sonicated. The cell lysate was centrifuged at 12 000 rpm for 15 min. The clear supernatant was fractionated with 70% saturated ammonium sulfate. The precipitate was dissolved in 30 ml buffer B (10 mM sodium phosphate buffer (pH 7.2), containing 30 mM NaCl, 2 mM ME and 0.25% Tween 20) and applied onto an amylose resin column (1.6 × 10 cm). The column was rinsed with buffer B, then with buffer C (10 mM sodium phosphate buffer (pH 7.8), containing 0.5 M NaCl and 2 mM ME), and eluted with buffer D (10 mM sodium phosphate buffer (pH 7.8), containing 0.5 M NaCl, 2 mM ME and 10 mM maltose). The enzyme was stored at −20°C in 50% glycerol for biochemical characterization. The concentration of the purified MBP-NS3-NS4A fusion protein was determined by amino acid composition analysis using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The purity of the fusion protein was estimated by 12.5% SDS-PAGE, stained with Coomassie Brilliant Blue using the Rapid Stain CCB Kit (Nakalai Tesque, Kyoto, Japan). Next, proteins were transferred to a PVDF membrane (IPVH, 0.45 μm, Millipore Corporation, Beaford, MA, USA) and incubated in Towbin buffer (25 mM Tris Base, containing 192 mM glycin and 20% methanol) for 1 h at 4°C, and at a constant current of 150 mA. The membranes were briefly incubated in TBST buffer (10 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and 0.05% Tween 20) containing 1% nonfat dried milk. Rabbit polyclonal antibodies specific for either the NS3 or the NS4A fusion protein were diluted 1 : 1000 in TBST containing 1% nonfat dried milk. The primary antibody solutions were incubated with the membranes for 3 h at room temperature. The membranes were washed three times with TBST (5 min each), then incubated for 1 h with secondary antibody diluted 1 : 10 000 in TBST containing 1% nonfat dried milk. The membranes were again washed three times (5 min each) with TBST. The secondary antibody used for both proteases was a goat anti-rabbit antibody conjugated to biotin. The immunoblot signal was visualized with the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Peptide synthesis

A 20-mer peptide Dansyl-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Ala-Leu-OH, which mimics the NS5A-5B junction, was synthesized on an automated multiple peptide synthesizer (Model PSSM-8, Shimazu, Kyoto, Japan). The peptide was purified by preparative reversed-phase HPLC (ODS-80Tm, 2.15 × 30 cm, Tosoh, Tokyo, Japan), using 0.1% aqueous trifluoroacetic acid (TFA)/acetonitrile-based mobile phases. The synthetic peptide was characterized by amino acid analysis, reversed-phase HPLC, and electron-spray bombardment mass spectrometry. Its purity was 92.7%. The peptide was stored frozen at −20°C.

2.4. HCV NS3-NS4A assay

Protease activity was determined in a total volume of 200 μl of assay buffer [50 mM Tris-HCl (pH 7.6), containing 30 mM NaCl, 1 mM CaCl2 and 2 mM (±)-dithiothreitol (DTT)] containing 1.4 μg of MBP-NS3-NS4A (a final concentration of 0.0603 μM) and 12 μg of the synthetic peptide (a final concentration of 26 μM). The reaction temperature was 37°C. After an adequate incubation period, the reaction was stopped by adding 4 μl of 2.5-M acetic acid, and then the reaction mixture was transferred into a vial for HPLC

analysis. Analytical HPLC was carried out using a Tosoh SC-8020 liquid chromatography system (Tokyo, Japan), complete with a binary solvent delivery system, a heated column compartment, and an auto-injection system. The HPLC conditions were as follows: ODS-80Ts column, 0.46 × 15 cm, Tosoh, Tokyo; Buffer D, 0.1% TFA in water; Buffer E, 0.09% TFA in 90% acetonitrile; 0–50% Buffer D gradient over a 20-min period at a flow rate of 1.0 ml/min. Peptides were detected by UV absorbance at 214 nm. The cleavage products, product A (Dansyl-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys) and product B (Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Ala-Leu-OH), were quantified by calculating the peak height.

2.5. Inhibition of NS3-NS4A protease activity

Diisopropyl fluorophosphate (DFP) and 4-amidinophenylmethanesulfonyl fluoride hydrochloride (pAPMSF) were purchased from Fluka Chemie AG, Buchs, Switzerland. Aprotinin, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), *N*-tosyl-L-phenylalanyl ketone (TPCK), Leupeptine, and Pepstatin were purchased from Nacalai tesque, Kyoto, Japan. 4-(2-aminoethyl)-benzolsulfonylfluoride hydrochloride (Pefabloc SC) was purchased from E. Merck, Darmastadt, Germany. *N*-Tosyl-L-lysyl chloromethyl ketone (TLCK) was purchased from Funakoshi Co., Tokyo, Japan. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. Antipain, E-64, and Elastinal were purchased from Peptide Institute, Inc., Osaka, Japan. Chymostatin was purchased from Genosys Bio Technologies, Inc., Cambridge, UK. Cystatin and 3,4-dichloroisocoumarin were purchased from Sigma Chemical Company, St. Louis, MO, USA. MBP-NS3-NS4A was pretreated with inhibitors (a final concentration of 100 μg/ml) for 15 min at 37°C. Then, the proteolytic reactions were initiated by the addition of the synthetic substrate described above, and incubated for 1 h at 37°C. The percentage of NS3-NS4A inhibition activity was calculated using the peak height of product A.

3. Results

3.1. Enzyme purification and analysis of NS3-NS4A and NS3 protease activity

We chose the region of the HCV genome encoding NS3-NS4A for expression in *E. coli*. NS3-NS4A spans the HCV genome nucleotides 3421–5475 (amino acids 1027–1711). We expressed the NS3-4A fusion protein as a complex with MBP, which has an affinity for specific ligands that assist enzyme purification. The resulting 116-kDa protease preparation contained one major band on SDS-PAGE (Fig. 1A). The partially purified MBP-NS3-NS4A fusion protein was greater than 80% pure. The immunological identity of the fusion protein was comfirmed by immunoblot analysis, using NS3- and NS4A-specific polyclonal antibodies (Fig. 1B).

We also constructed a plasmid encoding only the NS3 region, spanning the HCV genome nucleotides 3421–5313 (amino acids 1027–1657). The expressed MBP-NS3 fusion protein (112-kDa) was also $>80\%$ pure. As shown in Fig. 2, we compared the time course of the proteolytic cleavage of the synthetic substrate by NS3-NS4A and NS3 (0.0603 μM). NS3 alone hydrolyzed the substrate very slowly (15% substrate hydrolysis in 4 h). On the other hand, the rate of the substrate cleavage by NS3-NS4A was significantly faster (80% substrate cleavage in 90 min).

3.2. Cleavage of a synthetic peptide and enzymatic characterization of protease activity

The synthetic peptide was incubated with purified NS3-NS4A as described under Section 2, and the cleavage products were analyzed by reversed-phase HPLC. The HPLC cleavage profiles of the peptide are illustrated in Fig. 3. HPLC analysis of the reaction mixture after incubation shows that the elution signal of the substrate at 17–18 min is significantly reduced, compared to the elution profile produced with untreated substrate. Two cleavage products are visible at 14.9 min (product A) and 14.3 min (product B). Furthermore, we performed a kinetic analysis and quantified the cleavage products by HPLC. A

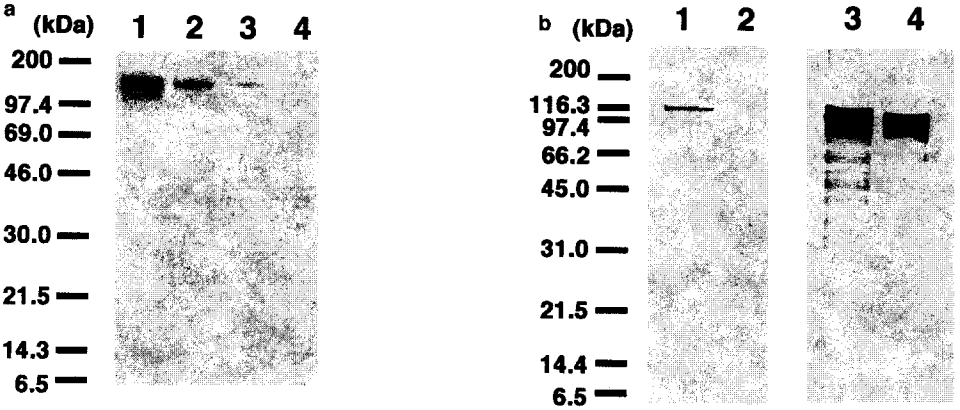

Fig. 1. (A) SDS-PAGE of partially purified NS3-NS4A protease. Two-fold dilutions of the protease were made: lane 1, 0.875 μg; lane 2, 0.438 μg; lane 3, 0.219 μg; lane 4, 0.110 μg. (B) Immunoblot of NS3-NS4A (lanes 1 and 3) and NS3 (lanes 2 and 4). The separated proteases were then transferred to an IPVH membrane and subjected to Western blot analysis as follows: lanes 1 and 2 were probed with the NS4A antibody, and lanes 3 and 4 with the NS3 antibody.

linear reaction was observed for approximately 1 h, indicating that the enzyme was highly stable in the incubation buffer (Fig. 4A). The optimum temperature for NS3-NS4A activity was determined in the same manner as the peptide cleavage assay (Fig. 4B); incubations were carried out during the linear range of the enzyme reaction. The optimum temperature for NS3-NS4A activity was determined to be 37°C. We observed a decrease in activity of > 20% below 30°C and at 60°C. Below 20°C the activity was markedly decreased. All further experiments were carried out at 37°C, the optimum temperature. The optimum pH was determined to be pH 7.6 (Fig. 4C).

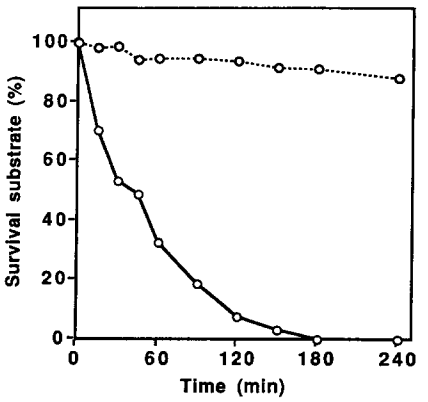

Fig. 2. Comparison of the reactivity of HCV proteases NS3-NS4A (——) and NS3 (· · ·). Protease activity, expressed as percent survival of substrate peptides, was measured at several different incubation times at 37°C. Substrate (26 μM) was incubated with either NS3 or NS3-NS4A (0.0603 μM) in a total volume of 200 μl.

3.3. Inhibition of NS3-NS4A protease activity by several protease inhibitors

Several potent protease inhibitors were evaluated by HPLC for their inhibition of NS3-NS4A protease activity. NS3-NS4A was pretreated with the inhibitors for 15 min at 37°C, before the addition of the synthetic substrate. DMSO solution (0.2–1%) was used as solvent, having no effect on the cleavage of the synthetic peptide by NS3-NS4A protease. The percent inhibition produced by serine protease inhibitors (DFP, Aprotinin, Pefabloc SC, TLCK, TPCK, PMSF, Elastinal, pAPMSF, AEBSF, 3,4-dichloroisocoumarin), cysteine protease inhibitors (cystatin, E-64), serine-cysteine protease inhibitors (leupeptine, chymostatin), an aspartic protease inhibitor (pepstatin), and a selenium-cysteine protease inhibitor (antipain) was determined (Fig. 5). Three serine protease inhibitors produced > 50% inhibition: DFP (500 μg/ml, 2714.4 μM), TLCK (100 μg/ml, 270.8 μM), and chymostatin (500 μg/ml, 825.5 μM). Two other serine protease inhibitors, aprot-

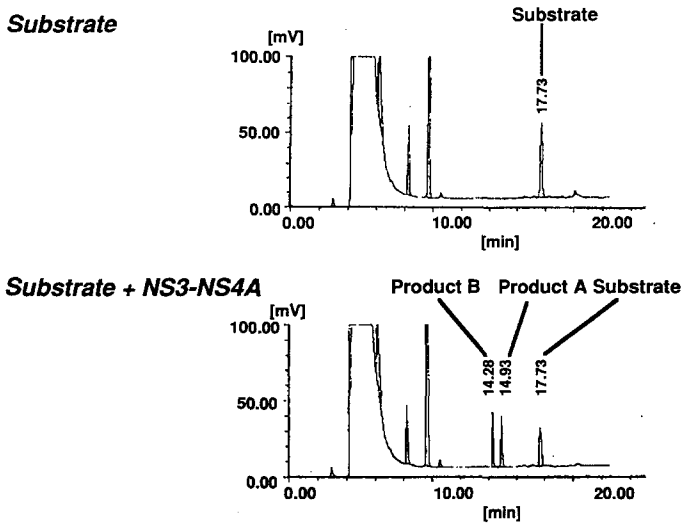

Fig. 3. HPLC profiles of the peptide cleavage products produced by NS3-NS4A. The cleavage products, A and B, and the cleaved and uncleaved substrates are indicated. The HPLC profiles of the reaction mixture are displayed both before (top) and after (bottom) cleavage.

inine (500 μg/ml) and elastinal (500 μg/ml, 975.4 μM) had no effect on NS3-NS4A protease activity. Furthermore, antipain and pepstatin slightly inhibited NS3-NS4A protease activity (approximately 28%), while cystatin and E-64 produced no inhibition. These results indicate that NS3-NS4A protease activity is not inhibited by one specific class of protease inhibitors. The inhibition produced by of 3,4-dichlorocoumarin was not evaluated because of its effect on the synthetic peptide substrate. Likewise, the inhibition produced by Pefabloc SC, TLCK, TPCK, leupeptin, and PMSF could not be determined because of their effects on the substrate at concentrations in excess of those indicated in Fig. 5.

4. Discussion

The HCV serine protease NS3 is a likely target for the development of anti-HCV therapeutic agents, because its activity is essential for viral proliferation. Recently, it was reported that NS4A enhanced the cleavage of the NS3-specific cleavage sites of the HCV genome (Bartenschlager et al., 1994; Failla et al., 1994). Since NS5B was produced efficiently from NS△4A, it has been proposed that NS4A is not essential for cleavage at the NS5A-5B junction (Failla et al., 1994). Similarly, using a synthetic substrate, we demonstrated that the proteolytic cleavage activity of NS3-NS4A was greater than that of NS3 alone. Thus, we established an HCV protease assay system for screening anti-HCV agents by using a bacterially-expressed NS3-NS4A fusion protein and by preparing a synthetic substrate that mimics the NS5A-5B junction.

The partially purified MBP-NS3-NS4A protease was identified as a 116-kDa protein by SDS-PAGE and by Western blot analysis using NS4A- and NS3-specific polyclonal antibodies. The Dansyl-labeled synthetic peptide used in the present study has been previously described (Kakiuchi et al., 1995). The peptide is made up of the 20 amino acids surrounding the NS5A-5B junction, but substitutes proline for cysteine at P2 to prevent the formation of disulfide linkages upon solubilization. Proteolytic inhibition was quantitatively evaluated by HPLC.

Hahm et al. (1995) reported that NS3-NS4A is a chymotrypsin-like protease. The authors developed a trans-cleavage assay, using in vitro transcription and translation. In their study, the HCV protease NS3-NS4A was inhibited by the chy-

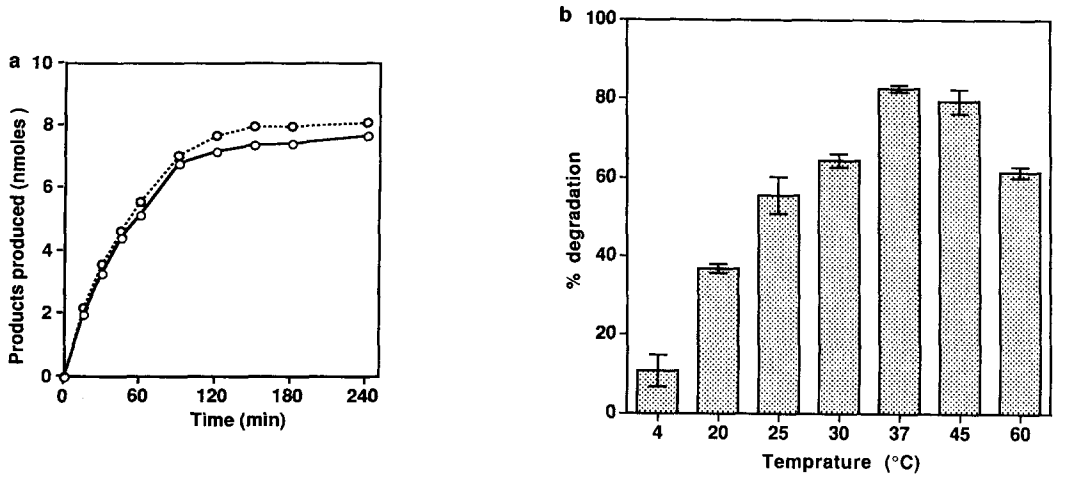

Fig. 4. (A) The kinetic analysis of the NS3-NS4A protease reaction. The cleavage products A (——) and B (· · ·) are indicated. Assay conditions are described in the legend for Fig. 2. (B) The determination of the optimum temperature for NS3-NS4A protease activity. Substrate (26 μM) was incubated as described in Fig. 2, with the following modifications: samples were incubated at the indicated temperature for 90 min in a total volume of 200 μl, then analyzed by HPLC. (C) The determination of the optimum pH for NS3-NS4A protease activity. Substrate (26 μM) was incubated as described in Fig. 2, with the following modifications: 50 mM Tris -HCl buffer was substituted for a buffer adjusted to the pH value indicated. Samples were incubated at 37°C for 2 h, then analyzed by HPLC.

motrypsin-specific inhibitors TPCK (300 μM), chymostatin (1 mM), and Pefabloc SC (8 mM), but not by the trypsin-specific inhibitors antipain (500 μM), leupeptin (400 μM) and TLCK (300 μM). These results conflict with the enzyme inhibition observed by the assay system employed in the present study. TLCK showed the greatest inhibition (50% inhibition at a concentration of 149.9 μM), followed by chymostatin (50% inhibition at a concentration of 596.3 μM) and DFP (50% inhibition at a concentration of 2257.9 μM). TPCK (100 μg/ml, 283.9 μM) and Pefabloc SC (100 μg/ml, 417.2 μM) slightly inhibited protease activity. The differences observed between the present study and Hahm et al. may be due to our use of the 20-mer peptide substrate, rather than the recombinant substrate. In addition, we used intact NS3-NS4A, while Hahm et al. used an NS3$\triangle$C-4A-4B polypeptide containing an internal deletion of the C-terminal two-thirds of NS3. Lin and Rice (1995b) also examined the effects of several protease inhibitors. They found that millimolar concentrations of TLCK and AEBSF inhibited the proteases $NS3_{181}$ (which denoted the N-terminal 181 residues of NS3) and NS3-4A in a trans-cleavage assay, utilizing either the NS4A-4B or NS4B-5A substrate. The authors also found that PMSF (2 mM) and TPCK (0.2 mM) showed slight inhibition. Moreover, aprotinin (2 mg/ml), chymostatin (2 mM), 3,4-dichloroisocoumarin (2 mM), and leupeptin (2 mM) had no detectable effect. In addition, D'Souza et al. (1995) studied the inhibition of the NS3 protease expressed as a polyhistidine fusion protein. Although TPCK (250 μM) and TLCK (600 μM) showed significant inhibition, DFP (1 mM) had no observable effect. Their results, like the data presented here, suggest that serine protease inhibitors do not consistently inhibit the HCV proteases studied.

Furthermore, we evaluated the inhibitory effects against NS3 protease of three protease inhibitors, producing $> 50\%$ inhibition against NS3-NS4A protease activity. The IC_{50} (50% inhibitory concentration) values of DFP, TLCK and chymostatin were 726 μM, 95.0 μM and 428 μM. The IC_{50} values were similar to those obtained in the NS3-NS4A inhibition assay. However, the NS3 inhibition assay was performed by 5-fold protease, compared with the NS3-NS4A protease and had an incubation period of 3 h.

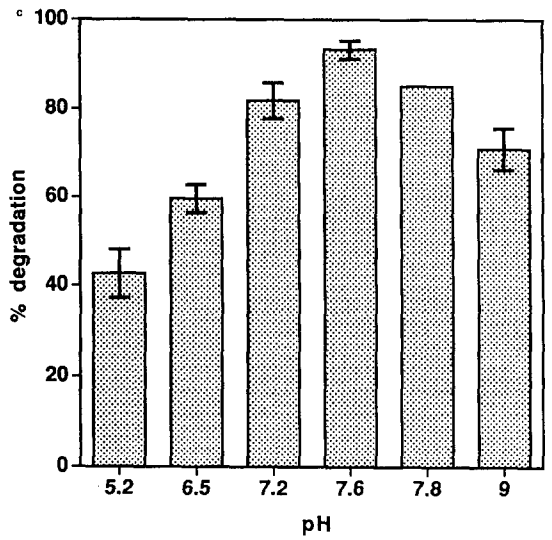

Fig. 4C

The formation of a stable complex between the catalytic domain of NS3 and the central region of NS4A had been previously reported (Lin and Rice, 1995a; Satoh et al., 1995). We confirmed the immunological identify of the MBP-NS3-NS4A fusion protein complex by immunoblotting with NS3- and NS4A-specific polyclonal antibodies. Results from the present study indicate that the proteolytic activity of NS3-NS4A was greater than that of NS3 alone. Using the synthetic peptide substrate, we compared the kinetic constants of the two proteases at 37°C. The K_m for NS3 was 270.0 μM, while that for NS3-NS4A was approximately four times lower, 68.6 μM. In addition, the V_{max} for NS3 was 3.6 nmol/h, while that for NS3-NS4A was approximately 3.6 times higher, 12.8 nmol/h. Kakiuchi et al. (1995) showed that the kinetic parameters of the enzyme reaction were the same using the substrate containing the Cpro-2 substitution as they were with the 20-mer substrate. The enzyme used, however, included the HCV genome nucleotides 3293–5283 (amino acids 985–1647), which contain the genome of NS2. Shimizu et al. (1996) showed that NS3 protease activity was enhanced in a dose-dependent manner by an NS4A fragment, derived from residues 18–40 of NS4A, with optimal activity observed at a 50-fold molar excess of the

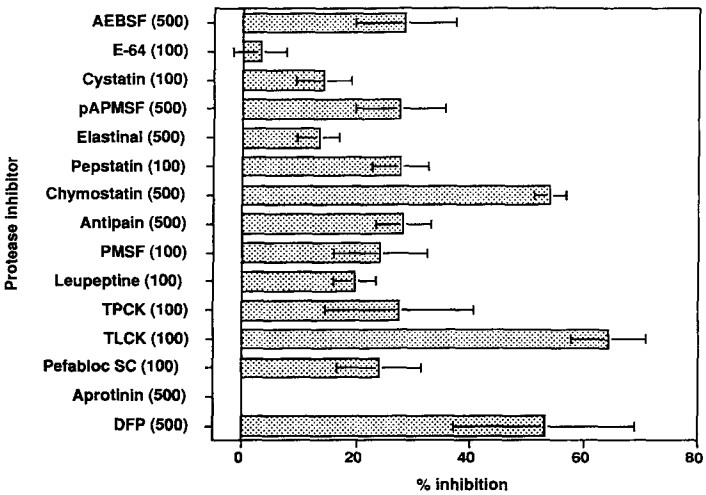

Fig. 5. Effect of various protease inhibitors on the activity of NS3-NS4A. The values in parentheses are concentrations of protease inhibitors and are expressed in μg/ml. Data represent the means $\pm$ standard deviation values from at least three separate experiments.

NS4A fragment. In the present study, however, it was not clear whether NS3 interacted in equimolar proportions with NS4A in the production of the NS3-NS4A complex.

Our results demonstrate the usefulness of synthetic substrates in determining the biochemical properties and cleavage-site specificity, of NS3-NS4A. Use of the synthetic substrate appears to provide a simpler and faster means of obtaining quantitative assay results than did the recombinant substrate. The in vitro assay system and preliminary results with various protease inhibitors, described in the present study, show promise for the future development of specific inhibitors of the HCV serine protease NS3-NS4A.

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