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Development of [Ile^{40}]HTLV-I protease inhibition assay using novel fluorogenic and chromogenic substrate

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HTLV-I is a debilitating and/or lethal retrovirus that causes HTLV-I-associated myelopathy/tropical spastic paraparesis, adult T-cell leukemia and several inflammatory diseases. HTLV-I protease is an aspartic retropepsin involved in HTLV-I replication and its inhibition could treatHTLV-I infection. A recombinant L40I mutant HTLV-I protease was designed and obtained from *Escherichia coli*, self-processingand purification by ion-exchange chromatography. The protease was refolded by a one-step dialysis and recovered activity. The cleavage efficiency of the [Ile^{40}]HTLV-I protease was at least 300 times higher for a fluorescent substratethan that of our previously reported recombinant His-tagged non-mutated HTLV-I protease. In addition, we designed and synthesized a substrate containing a highly fluorescent Mca moiety in the fragment before the scissile bond, and a chromogenic *p*-nitrophenylalanine moiety after the scissile bond that greatly amplified spectrometry detection and improved the HTLV-I protease inhibition potency assay. The HTLV-I protease inhibition assay with the [Ile^{40}]HTLV-I protease and fluorogenic substrate requires distinctively less protease, substrate, inhibitor and assay time than our previous methods. This means our new assay is more cost-effective and more time-efficient while being reproducible and less labor-intensive.

Keywords: human T-cell lymphotropic virus; HTLV-I-associated myelopathy/tropical spastic paraparesis; adult T-cell leukemia; aspartic protease; inhibitor; substrate

Introduction

In 1977, adult T-cell leukemia was defined as distinct clinical entity [1], and in 1980, the oncogenic agent, HTLV-I, was isolated from cultured T cells in a patient with cutaneous T-cell lymphoma [2]. HTLV-I causes a variety of debilitating and/or lethal immunological and neurological disorders including HTLV-I-associated myelopathy/tropical spastic paraparesis [3,4], adult T-cell leukemia (ATL) [5], and a number of chronic inflammatory diseases and opportunistic infections [6]. Globally, 20–30 million patients are infected with HTLV-I with endemic areas found in South Japan, the Caribbean region, central Africa, South America and the Melanesia area [7]. In the United States of America, HTLV-I infects ∼1.2 million patients [8]. ATL develops in 5% of infected patients, which is around 800 patients per year in Japan [9]. The three main routes of transmission are blood, venereal fluids and breast milk.

The genome of HTLV-I is about 9 kb, sandwiched at both ends by long terminal repeats [10]. As a result of reading frame shift, there are three open reading frames encoding the Gag, Gag-Pro and Gag-Pro-Pol precursor proteins [11]. Processing of the Gag precursor protein by HTLV-I protease produces a viral matrix (MA), capsid (CA) and nucleocapsid (NC). Cleavages of the Gag-Pro precursor protein by the protease afford MA, CA, TF1 (transframe 1, a *C*-terminal truncated NC), another HTLV-I protease (PR) and two short 1 and 2 kDa peptides of unidentified functions (p1 and p2). HTLV-I protease hydrolysis of the Gag-Pro-Pol precursor protein results in MA, CA, TF1, PR, p1, reverse transcriptase, ribonuclease H and integrase. Considering HTLV-I protease is responsible for several precursor protein cleavages, the inhibition of HTLV-I protease could disrupt viral replication. From a simplistic viewpoint, our study on HTLV-I protease inhibitors requires an HTLV-I protease, a substrate and an inhibitor.

Much of the literature reported only low- to mid-potency classes of HTLV-I protease inhibitors, because the protease has narrow substrate and inhibitor specificity, and thus the protease is less tolerant to changes in substrate and inhibitor residues [12,13]. Through rational drug design, we discovered the most potent group of small-size inhibitors ever reported in the literature [14,15]. Additionally, HTLV-I protease is difficult to handle because of its propensity for aggregation and self-degradation by cleaving between residues 40 and 41. Consequently, the three-dimensional structure of HTLV-I protease in complex with an inhibitor was unsolved for years, until we designed a mutated and truncated

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Abbreviations used: *HTLV-I, human T-cell leukemia/lymphotropic virus type 1; Gag, group antigen gene; Mca, (7-methoxycoumarin-4-yl)acetyl; Nph, p-nitro-L-phenylalanine; Pol, polymerase gene; Pro, protease gene; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); IPTG, isopropyl β-D-1-thiogalactopyranoside; DTT, dithiothreitol.*

HTLV-I protease that formed complexes with several of our highly potent inhibitors [16,17]. However, our previous accounts heavily focused on inhibitor design and structural data with minimal discussion on the protease and its substrate. The inclusion of our HTLV-I protease assay development would render the aforementioned manuscripts too long. Herein, we detail our development of HTLV-I protease and substrate for HTLV-I protease inhibition potency determination. We describe the construction, expression, self-processing, purification and refolding of an L40I mutant HTLV-I protease. The L40I mutation was necessary to prevent undesired autolysis [18]. As a point of interest, we prepared the recombinant immature His-tagged [Ile^{40}]HTLV-I protease, and took advantage of the protease's self-processing to efficiently remove the His-tag and afford the desired [Ile^{40}]HTLV-I protease. Other groups reported C90A and C109A mutations or *C*-terminal truncation of the last nine residues were necessary to reduce protease self-aggregation [16,18]. These modifications are not necessary in our case because our [Ile^{40}]HTLV-I protease is stable against aggregation. In addition, we evaluate a novel fluorogenic and chromogenic substrate that greatly enhances both UV and fluorescence detection of the substrate and its two cleaved fragments. Our current improved HTLV-I protease potency assay system requires much less protease, substrate, inhibitor and time than our previous method.

Materials and Methods

Construction of [Ile^{40}]HTLV-I Protease Fusion Protein Expression Plasmid

An L40I mutant HTLV-I protease gene was synthesized with the following features: (i) an *Nde*I restriction site was added to the 5′ end, corresponding to translation-enhancing element (TEE) sequence (MNHKV), His-tag sequence, factor Xa cleavage site sequence (IEGR) and the mature protein of HTLV-I protease sequence; (ii) the Leu^{40} codon was mutated to Ile to block undesired autolysis; and (iii) a stop codon and a *Pst*I restriction site were introduced to the 3′ position of the Leu^{125} codon (Figure 1(D)). The mutated HTLV-I protease gene was then cloned into pColdI (TaKaRa-Bio Inc., Otsu, Japan) using the *Nde*I and *Pst*I restriction sites to give pColdI-protease, and the construct was sequenced to confirm the structure.

Expression of [Ile^{40}]HTLV-I Protease Fusion Protein

The pColdI-[Ile^{40}]HTLV-I protease was transformed into *Escherichia coli* BL21 cells (Novagen, Merck KGaA, Darmstadt, Germany). The bacterial cells were grown in lysogeny broth (4 ml) containing 100 μg ml^{-1} ampicillin to an optical density of ∼0.5 at 600 nm. The expression of the [Ile^{40}]HTLV-I protease fusion protein was induced with incubation at 15 °C ('cold-shock') and the addition of IPTG to a final concentration of 1 mM. The induced culture was incubated at 15 °C overnight. The cultured cells were collected by a centrifugation at 6000× g at 4 °C for 30 min. The collected cells were resuspended and the inclusion body was dissolved in buffer A (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, 10 mM 2-mercaptoethanol, pH 8.0). The mixture was incubated at 25 °C for 60 min with shaking, and then centrifuged at 9000× g at 4 °C for 30 min. The solution was incubated with cell debris remover (CDR; Whatman-GE Healthcare, Tokyo, Japan, 5 ml) equilibrated in buffer A at 25 °C for 60 min with shaking. CDR was removed through a Büchner funnel to afford a clear supernatant solution.

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A          - ---------- ---------- ---------- ----------
B          - ---------- ---------- ------MGHH HHHHHHHHSS
C          - ---------- ---------- ---MGHHHHH HHHHHSSGHI
D          M NHKVHHHHHH IEGRHMGSHP TPKKLHRGGG LTSPPTLQQV
E          - ---------- ---------- ---------- ----------

                    1         10         20         30         40
A ---------- PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVL
B GHIDDDDKHM PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVL
C DDDDKHMLED PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVL
D FLNQDPASIL PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVI
E ---------- PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVI

                   50         60         70         80         90
A PIALFSSNTP LKNTSVLGAG GQTQDHFKLT SLPVLIRLPF RTTPIVLTSC
B PIALFSSNTP LKNTSVLGAG GQTQDHFKLT SLPVLIRLPF RTTPIVLTSC
C PIALFSSNTP LKNTSVLGAG GQTQDHFKLT SLPVLIRLPF RTTPIVLTSC
D PIALFSSNTP LKNTSVLGAG GQTQDHFKLT SLPVLIRLPF RTTPIVLTSC
E PIALFSSNTP LKNTSVLGAG GQTQDHFKLT SLPVLIRLPF RTTPIVLTSC

                  100        110        120   125
A LVDTKNNWAI IGRDALQQCQ GVLYLPEAKG PPVIL
B LVDTKNNWAI IGRDALQQCQ GVLYLPEAKG PPVIL
C LVDTKNNWAI IGRDALQQCQ GVLYLPEAKG PPVIL
D LVDTKNNWAI IGRDALQQCQ GVLYLPEAKG PPVIL
E LVDTKNNWAI IGRDALQQCQ GVLYLPEAKG PPVIL
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Figure 1. The amino acid sequence of HTLV-I proteolytic proteins.(A) Mature HTLV-I protease, M.W. = 13 459; (B) mature recombinant His-tagged HTLV-I protease, M.W. = 16 357; (C) mature recombinant His-LED-tagged HTLV-I protease, M.W. = 16714; (D) immature recombinant His-tagged [Ile^{40}]HTLV-I protease, M.W. = 19 190; (E) mature recombinant [Ile^{40}]HTLV-I protease, M.W. = 13 459. Blue 'H' represents the His-tag sequence. Underlined 'MNHKV' represents TEE. Green 'IEGR' represents the factor Xa cleavage site. Red characters represent the prematuration sequence. Boxed 'I' represents the L40I mutation. Residue numbering is relative to the mature HTLV-I protease sequence.

This solution was incubated with Ni-NTA agarose (Qiagen, Tokyo, Japan, 10 ml) at 25 °C for 60 min with shaking, packed into an empty column and the largest fraction was eluted from the His-Bind affinity column with buffer A. The partially purified protease was loaded on a Sephadex G-25 column (GE Healthcare, Tokyo, Japan) equilibrated with buffer B (20 mM sodium acetate, 6 M urea, 5 mM EDTA, 5 mM 2-mercaptoethanol, pH 3.0), and the eluted fraction of the protease was concentrated using a centrifugal filter (Centricon Plus 20, Billerica, MA, USA), as needed. The protease was loaded on a Hitrap HP SP column (GE-Healthcare) equilibrated with buffer B and eluted with 0.12–0.15 M NaCl in buffer B.

[Ile^{40}]HTLV-I Protease Refolding

The [Ile^{40}]HTLV-I protease solution (*ca* 0.2 mg ml^{-1}, Figure 1(E)) was dialyzed against a mixed solution of 20 mM PIPES, 2 mM DTT, 1 mM EDTA, 150 mM NaCl and 10% glycerol at pH 7.0. The protease was stored in the mixed solution (240 μM) at −20 °C until use.

Fluorescent Substrate

The fluorogenic and chromogenic HTLV-I substrate, Lys(Mca)-Ala-Pro-Gln-Val-Leu*Nph-Val-Met-His-Pro-Leu (Figure 2), was synthesized by standard solid phase peptide synthesis by which sequential elongation from an Fmoc-Leu-(2-chrolotrityl chloride) resin. Trityl was used to protect the side-chains of Gln and His. Each coupling consisted of the removal of the N^{α}-Fmoc protection by piperidine (20% v/v in DMF), and a single 120 min coupling of the activated hydroxybenzotriazole ester form of the N^{α}-Fmoc-amino acid (2.5 equiv.) in DMF. From the initial Fmoc-Leu-(2-chrolotrityl chloride) resin (0.433 mmol g^{-1}, 0.296 g, 0.128 mmol), the side-chain-protected Fmoc-Ala-Pro-Gln-Val-Leu*Nph-Val-Met-His-Pro-Leu resin (0.421 g) was synthesized. Some of the resin

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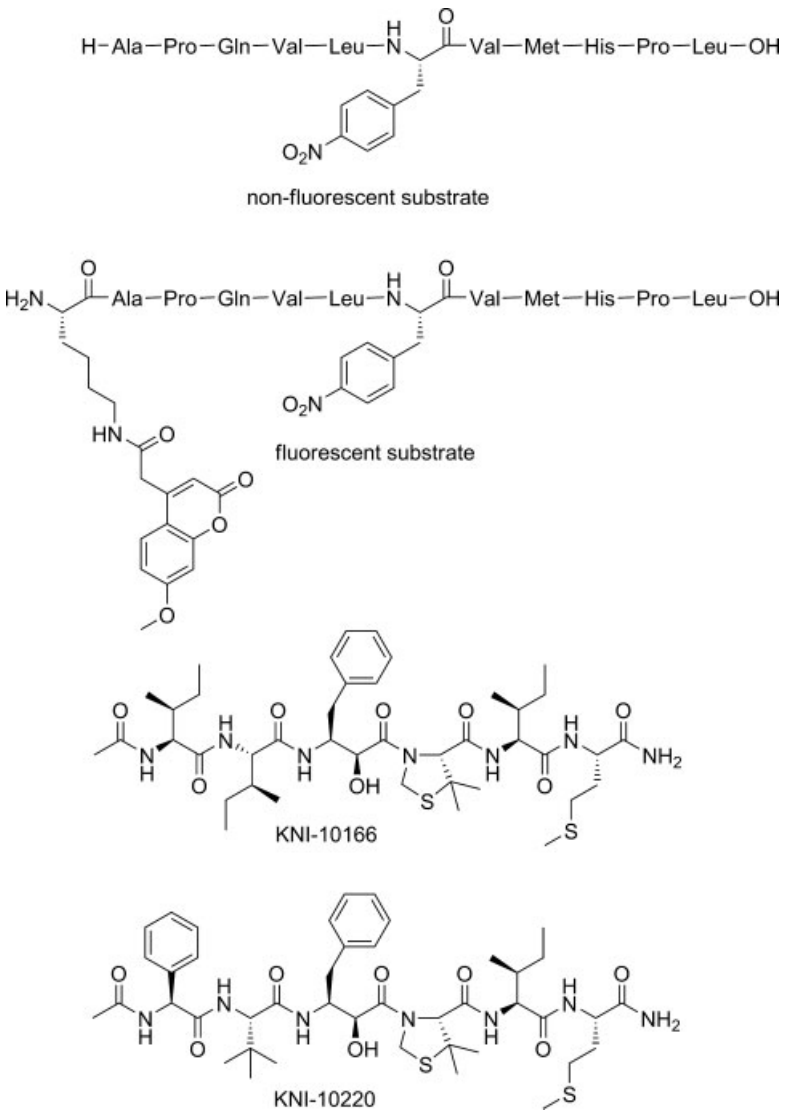

Figure 2. The non-fluorescent and fluorescent substrates used in the study are both chromogenic. The two HTLV-I protease inhibitors, KNI-10166 and KNI-10220, were originally designed from the MA/CA cleavage site sequence.

(84 mg) was further elongated to obtain a Boc-Lys(Fmoc)-Ala-Pro-Gln-Val-Leu*Nph-Val-Met-His-Pro-Leu resin. After the removal of the Fmoc group, (7-methoxylcoumarin-4-yl)acetic acid (15.0 mg, 0.064 mmol) was coupled in the presence of 1-hydroxybenzotriazole (8.7 mg, 0.064 mmol) and *N*,*N*′-diisopropylcarbodiimide (10.1 µl, 0.064 mmol) in DMF. The peptides were cleaved from the resin by TFA in the presence of *m*-cresol, thioanisole and distilled water (93 : 2.3 : 2.3:2.3 v/v/v/v, 22 µl per 1-mg resin) for 90 min at room temperature, concentrated *in vacuo*, and precipitated with diethyl ether at 0 °C followed by centrifugation at 6000×g for 5 min (3×). The resultant peptide was suspended in water and lyophilized for at least 12 h. The crude substrate (41.9 mg) was purified by preparative reverse-phase HPLC with a 0.1% aqueous TFA-CH_3CN system, immediately cooled to −78 °C and lyophilized for at least 12 h to afford the pure peptide (21.8 mg, 13.3 µmol, 52% yield; MALDI-MS(TOF): $[M+H]_{calcd}$: 1641, $[M+H]_{found}$: 1641; HPLC analysis at 230 nm: purity was >95%). The substrate was stored in DMSO (4 mM) at −20 °C until use.

[Ile^{40}]HTLV-I Protease Activity Assay

The activity of the [Ile^{40}]HTLV-I protease was determined from the rate of hydrolysis of the fluorescent substrate. The substrate was dissolved in DMSO at desired concentrations. The protease activity was evaluated over time at 37 °C in a 50 µl mixed solution of an assay buffer (24 µl of 417 mM citric acid, 2.08 mM DTT, 2.08 M NaCl, 10.42 mM EDTA, 12.5% glycerol, pH 5.3) with protease (20 µl, final conc. 18.6 nM) and different substrate concentrations (6 µl: 1 µl DMSO, 5 µl buffer). The reaction was terminated by the addition of 20% trichloroacetic acid (15 µl). Cleavage of the substrate was detected by UV and fluorescence during reverse-phase HPLC with a 0.1% aqueous TFA-CH_3CN system (column: YMC-Pack Pro C18, Kyoto, Japan, gradient: 19–34% (15 min), flow rate: 1.0 ml min^{-1}, 328–393 nm, 40 °C). The concentrations of the intact substrate and the two hydrolyzed substrate fragments were calculated from their respective area-under-curve and standard concentration curve. Errors were expressed as standard errors.

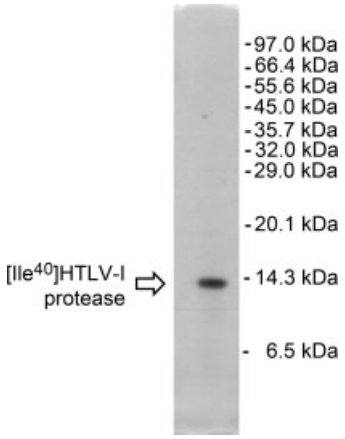

Figure 3. The recombinant [Ile^{40}]HTLV-I protease on SDS-PAGE. The purified protein (0.4 μg) was applied to electrophoresis on SDS-PAGE (16.3% polyacrylamide) at a constant current of 50 mA for 3.5 h and stained with a silver stain. On the right side, phosphorylase b (97.0 kDa), bovine serum albumin (66.4 kDa), glutamic dehydrogenase (55.6 kDa), ovalbumin (45 kDa), glycelaldehyde-3-phosphate dehydrogenase (35.7 kDa), carbonic anhydrase II (32.0 and 29.0 kDa), soybean trypsin inhibitor A (20 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa) were loaded and migrated as protein markers.

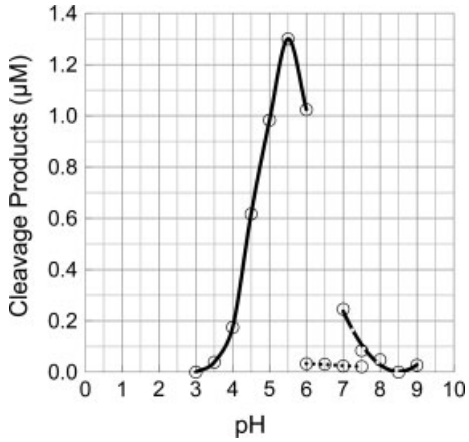

Figure 4. Optimal pH of [Ile^{40}]HTLV-I protease. Cleavage of the fluorescent substrate occurs optimally at pH 5.5 as evaluated in citrate (solid), PIPES (dotted) and Tris buffer (dashed) solutions to cover pH range 3.0–9.0. The plot depicts product detection by fluorescence.

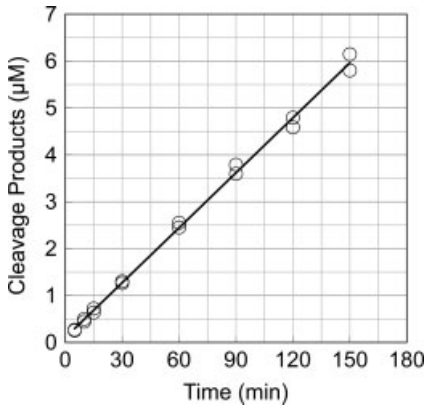

Figure 5. Time course of [Ile^{40}]HTLV-I protease cleavage of the fluorescent substrate. Data were obtained from two independent experiments. The plot depicts product detection by fluorescence.

[Ile^{40}]HTLV-I Protease Inhibition Potency Assay

Percent inhibition was expressed as the percent of intact substrate. The inhibition potency assay was similar to the [Ile^{40}]HTLV-I protease activity assay with the following differences: (i) the inhibitor was dissolved in DMSO at desired concentrations; and (ii) the protease activity was evaluated after 30 min at 37 °C in a 50 μl mixed solution of the assay buffer with protease (20 μl, final conc. 18.6 nM), substrate (5 μl, final conc. 100 μM) and different inhibitor concentrations (1 μl). The IC_{50} value was calculated from the sigmoid plot derived by percent inhibition potency at 1, 5, 10, 20, 50 and 100 nM of the test inhibitor, as a single determination at each concentration, using KaleidaGraph (Synergy Software, Reading, PA, USA). The error range for the IC_{50} value was calculated from the root-mean-square deviation (RMSD) of the plot, i.e. 50% ± RMSD inhibition.

Results

The crude His-tagged [Ile^{40}]HTLV-I protease concentration was 42.4 mg l^{-1} in the lysogeny broth. After removal of the tag and purification, the [Ile^{40}]HTLV-I protease was obtained as a single band at about 13 kDa from silver-stained SDS-PAGE (Figure 3). The *m/z* ratio of the [Ile^{40}]HTLV-I protease was determined by MALDI-TOF-MS Voyager-DE RP (Applied Biosystems Inc., Foster City, CA, USA): $[M+H]_{calcd}$: 13460, $[M+H]_{found}$: 13454. The *N*-terminal amino acid sequence was determined with a gas-phase protein sequencer model 470A and a model 120A analyzer (Applied Biosystems Inc.), and the sequence, Pro-Val-Ile-Pro-Leu-Asp, matched the sequence of the mature HTLV-I protease (Figure 1(A)). The protease concentration was determined using Bradford assay kit (Bio-Rad, Tokyo, Japan) and an ~0.050 optical density of the dimer at 595 nm was observed. The optimal pH for the [Ile^{40}]HTLV-I protease to cleave the fluorescent substrate was elucidated as 5.5 with 1 M salt (Figure 4). The concentration of the hydrolyzed substrate fragments from cleavage by the protease followed a linear relationship over time (Figure 5). The K_m, V_{max}, k_{cat} and k_{cat}/K_m values were determined (Table 1). The [Ile^{40}]HTLV-I protease exhibited higher relative activity than the His-tagged HTLV-I protease used in our previous reports [13,19]. A protease inhibition study with the two proteases and two substrates was

Table 1. Fluorescent substrate-enzyme kinetic parameters

HTLV-I protease	K_m (mM)	V_{max} (mmol s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
His-tagged	$54.7 \pm 13.1 \times 10^{-3}$	$24.6 \pm 3.0 \times 10^{-6}$	0.641 ± 0.078	11.7 ± 3.1
L40I mutant	$7.31 \pm 2.11 \times 10^{-3}$	$1.65 \pm 0.04 \times 10^{-3}$	42.9 ± 1.0	$5.86 \pm 1.70 \times 10^{3}$

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performed on two potent HTLV-I protease inhibitors, KNI-10166 and KNI-10220 (Figure 2, Table 2).

Discussion

HTLV-I protease is a comparatively small enzyme consisting of 125 amino acid residues with a characteristic aspartic protease's Asp-Thr-Gly arrangement. Our X-ray crystallography study suggests that HTLV-I protease is active as a C2 symmetry homodimer fold [16,17]. Recently, we constructed an expression system for recombinant HTLV-I protease fused to an *N*-terminal His-tagged peptide [19] to develop HTLV-I protease inhibitors (Figure 1(B)) [13]. However, this recombinant His-tagged HTLV-I protease was prone to autolysis. The literature reported facile autoproteolysis in approximately half of the HTLV-I protease expression protein during isolation and purification, resulting in a truncated protein cleavage between Leu^{40}*Pro^{41} of the full-length mature protease (Figure 1(A)) [18]. This undesired self-hydrolysis occurs because Leu^{40}*Pro^{41}'s regional sequence is similar to those found at several common HTLV-I protease cleavage sites. Fortunately, autolysis can be prevented by a Leu to Ile mutation at position 40 without greatly affecting enzyme kinetic parameters [18]. From this information, we developed a recombinant [Ile^{40}]HTLV-I protease (Figure 1(E)). Although a recombinant His-tagged [Ile^{40}]HTLV-I protease fusion protein was prepared (Figure 1(D)), the His-tag peptide was attached to the protease sequence by a Ser-Ile-Leu*Pro-Val-Ile-Pro sequence that matched the TF1/PR cleavage site sequence, and consequently, the His-tag was removed by a [Ile^{40}]HTLV-I protease self-processing between Leu*Pro. Although the factor Xa cleavage sequence, Ile-Glu-Gly-Arg, was engineered in the His-tagged [Ile^{40}]HTLV-I protease in case the protease could not efficiently remove its own His-tag, cleavage by factor Xa protease was deemed unnecessary. Considering the His-tagged non-mutated protease has a band at 16 kDa from SDS-PAGE [19], the removal of the His-tag in the protease mutant was evidenced by a single band at 13 kDa (Figure 3), matching MALDI-TOF-MS *m/z* ratio and matching six residues of the *N*-terminal sequence by gas-phase protein sequencer-analyzer.

The high propensity for HTLV-I protease to self-aggregate is a major reported problem. The Cys^{90} and Cys^{109} residues are not involved in the formation of structurally important disulfide bonds [18]. The [L40I,C90A,C109A]-mutant protease was reported to exhibit little change in kinetic properties relative to the wild-type HTLV-I protease and are considered more stable because of lower risks for undesired intramolecular disulfide bond formations [18,20]. In addition, removal of the last nine or ten *C*-terminal residues (116–125 or 117–125) does not drastically negatively impact the proteases' kinetic profiles [16,21,22], despite a conflicting report [23]. Due to the intense self-aggregation, the three-dimensional structural data of several protein–inhibitor complexes could only be solved when the nine *C*-terminal residue was removed from the [Ile^{40}]HTLV-I protease [16,17]. In our case, these modifications were not necessary. Indeed, in contrast to our His-tagged non-mutated protease, our [Ile^{40}]HTLV-I protease was less prone to aggregation, most likely because of its shorter sequence than that of the His-tagged protease. Our [Ile^{40}]HTLV-I protease did not form aggregates and was stable as a 240 μM pH 7.0 stock solution for at least 2 months at 4 °C, even with freeze–thawing. Hence, our [Ile^{40}]HTLV-I protease is easy to handle in enzyme assays.

The literature reported the [Ile^{40}]HTLV-I protease (Figure 1(E)) was indistinguishable from the wild-type protease (Figure 1(A)) in terms of catalytically efficient (k_{cat}/K_m) with the MA/CA, CA/NC, TF1/PR and PR/p3 substrates [18]. Although we did not study the wild-type HTLV-I protease, our [Ile^{40}]HTLV-I protease is sequence-wise closer to the wild-type protease than our His-tagged non-mutated protease (Figure 1(B)), yet our His-tagged HTLV-I protease was found enzymatically similar to a literature wild-type protease [19]. The literature also reported a His-LED-tagged HTLV-I protease (Figure 1(C)) that differs sequence-wise from our His-tagged HTLV-I protease (Figure 1(B)) by only three residues, Leu-Glu-Asp (LED). The wild-type and His-LED-tagged HTLV-I proteases are kinetically equivalent, and thus suggesting the two proteases are enzymatically similar because the His-LED-tag does not radically alter the enzymatic properties of the protease [10]. These reports suggest our L40I mutant protease should have similar catalytic efficiency as our His-tagged non-mutated protease. However, there are also huge discrepancies in the literature: the catalytic efficiencies of the wild-type HTLV-I protease and CA/NC substrate have been reported as 158.7 mM^{-1} s^{-1} in one research group [23] and 0.019 mM^{-1} s^{-1} in another [10]. Another research group showed the inconsistency was a result of different refolding conditions [22]. We believe the discrepancy is likely a consequence of autolysis in the wild-type HTLV-I protease that leads to different proportions of intact protease. Experimentally, we observed significant dissimilarities in the kinetic profiles of our [Ile^{40}]HTLV-I protease and His-tagged HTLV-I protease (Table 1). The catalytic efficiency of the protease mutant is at least 300 times higher for a fluorescent substrate than that of the His-tagged non-mutated protease. Because of this higher activity, the incubation time used in the inhibition potency assay could be shortened from 6 h to 30 min [24].

Concurrent with our HTLV-I protease study, we developed a more chromogenic substrate based on the MA/CA cleavage site sequence. In a recent work, Ala-Pro-Gln-Val-Leu*Pro-Val-Met-His-Pro was used as substrate and was subsequently improved by incorporating a chromogenic reporter group, Nph as the P_1' residue [19]. The substrate, Ala-Pro-Gln-Val-Leu*Nph-Val-Met-His-Pro-Leu, ameliorated UV detection [25] and cleavage efficiency [10] (Figure 2). However, once hydrolyzed, the Ala-Pro-Gln-Val-Leu fragment does not have the Nph chromophore. In this study, we appended a fluorescent chromogenic reporter group Mca in substrate Lys(Mca)-Ala-Pro-Gln-Val-Leu*Nph-Val-Met-His-Pro-Leu to the distant P_6 residue to minimize interference with protease activity (Figure 2). Hence, the intact substrate and its two hydrolyzed fragments are all chromogenic, and thereby facilitating UV detection. Additionally, the fluorescence of the intact substrate and the fluorogenic fragment was also detected. The pH optimum for cleavage of the fluorescent substrate by the [Ile^{40}]HTLV-I protease was determined as 5.5 with 1 M salt (Figure 4). This observation is within range of literature reports on other substrate and HTLV-I protease types: pH 5.0–5.5 and 1–3 M salt [26]. On a related note, because the [Ile^{40}]HTLV-I protease was less active against the fluorescent substrate in PIPES buffer solution from pH 6.0–7.5, the stock protease was stored under similar conditions. PIPES does not interfere with fluorescence determination, because we can detect the intact fluorescent substrate in the pH study. We are currently studying the effect of PIPES on the L40I protease.

To evaluate the efficacy of the [Ile^{40}]HTLV-I protease and fluorescent substrate in inhibitor potency determination, we selected two previously reported potent HTLV-I protease inhibitors, KNI-10166 [13] and KNI-10220 [24] (Figure 2, Table 2). Because the protease mutant and fluorescent substrate offer several advantages over the His-tagged non-mutated protease and non-fluorescent substrate,

Table 2. Potency of two HTLV-I protease inhibitors under different assay methods

Inhibitor	IC_{50} (μM)		
	His-tagged protease (743.0 nM) Non-fluorescent substrate (200 μM)	L40I mutant protease (18.6 nM) Non-fluorescent substrate (200 μM)	L40I mutant protease (18.6 nM) fluorescent Substrate (100 μM)
KNI-10166	2.15 ± 0.27	0.120 ± 0.017	0.0230 ± 0.0035
KNI-10220	0.564 ± 0.032	0.116 ± 0.020	0.00630 ± 0.00166

the concentrations used in inhibition profiling were considerably lower using the protease mutant (18.6 nM) and fluorescent substrate (100 μM) than the His-tagged protease (743.0 nM) and non-fluorescent substrate (200 μM). The IC_{50} values determined with the His-tagged protease and non-fluorescent substrate were higher than those obtained from the protease mutant and non-fluorescent substrate, which in turn were higher than those from the protease mutant and fluorescent substrate assay.

From these differences in IC_{50} values, the ideal assay method comes in debate. Our study indicates the [Ile^{40}]HTLV-I protease offers better stability against aggregation over the His-tagged protease and thereby partially contributing to higher proteolytic activity, which consequently permits shorter incubation assay time (from 6 h to 30 min) [24]. The [Ile^{40}]HTLV-I protease is structurally closer to the wild-type protease and is more practical for inhibition profiling than the His-tagged protease. The two tested inhibitors, KNI-10166 and KNI-10220, were 5 and 18 times more potent, respectively, against the protease mutant than the His-tagged protease.

The literature described incorporation of the chromogenic reporter group Nph at the P_1' position of several substrates allows for greater UV detection sensitivity [25]. In the study, the overall catalytic rate (k_{cat}) of the Nph substrates and HTLV-I protease were similar suggesting the mechanism of hydrolysis of these substrates once bound were the same, and thus, leaving substrate affinity (K_m) as the main determinant for cleavage efficiency (k_{cat}/K_m). The authors concluded the efficiency with which their HTLV-I protease cleaves its substrate was found higher than that previously reported by other research groups. From the same line of reasoning, our substrate containing an Mca fluorogenic and an Nph chromogenic reporter group allows even greater UV and fluorescence detection sensitivity. Both fluorescent and non-fluorescent substrates are structurally identical except for an additional fluorogenic P_6 residue which lies completely solvent exposed and outside of the protease's active site, based on an extrapolation of structural data from our X-ray diffraction crystallography study on inhibitors and des-(117–125)-[Ile^{40}]HTLV-I protease [17]. We observed the IC_{50} values of the two examined inhibitors, KNI-10166 and KNI-10220, were 5 and 18 times lower, respectively, in assays using the [Ile^{40}]HTLV-I protease and the fluorescent substrate than those using the non-fluorescent substrate.

Summarizing our discussion, an HTLV-I protease inhibition potency assay requires a protease, substrate and inhibitor. Because none of these are commercially available, the newly designed assay system must be cost-effective and time-efficient while being reproducible and less labor-intensive. In the past, HTLV-I protease inhibition potency assays have been limited by the activity and stability of the protease, the detection sensitivity of the substrate, the efficiency of the assay method and the quantity of the HTLV-I protease inhibitor. In the current work, we describe the preparation and characterization of a recombinant [Ile^{40}]HTLV-I protease that exhibits higher proteolytic activity (at least 300 times) for a fluorescent substrate and physicochemical stability (2 months at 4 °C, even with freeze–thaw) than our reported recombinant His-tagged HTLV-I protease. Moreover, we discovered a bifunctional fluorogenic and chromogenic substrate that improves UV and fluorescence detection sensitivity over our reported mono-functional chromogenic substrate. With the [Ile^{40}]HTLV-I protease and fluorescent substrate, much smaller amount of protease (19 vs 743 nM), substrate (100 vs 200 μM) and inhibitor (e.g. KNI-10166: IC_{50} = 23 vs 2153 nM) along with a drastically shorter incubation time (30 min vs 6 h) are needed to perform the HTLV-I protease inhibition potency assay than our previous methods.

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