

Comparative Properties of Feline Immunodeficiency Virus (FIV) and Human Immunodeficiency Virus Type 1 (HIV-1) Proteinases Prepared by Total Chemical Synthesis

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The aspartyl proteinase (PR) encoded by the feline immunodeficiency virus (FIV) was prepared by total chemical synthesis. The 116-amino-acid polypeptide chain was assembled in a stepwise fashion using a Boc chemistry solid-phase peptide synthesis approach and subsequently folded into the biologically active dimeric proteinase. The synthetic enzyme showed proteolytic activity against a variety of different peptide substrates corresponding to putative cleavage sites of the Gag and Gag–Pol polyproteins of FIV. A comparative study with the proteinase of human immunodeficiency virus type 1 (HIV-1) showed that the FIV and HIV-1 enzymes have related but distinct substrate specificities. In particular, HIV-1 PR and FIV PR each show a strong preference for their own MA/CA substrates, despite identical amino acid residues at four of seven positions from P3–P4' of the substrate including an identical MA/CA cleavage site (between Tyr ~ Pro residues). FIV PR also showed a requirement for a longer peptide substrate than HIV-1 PR. Defining the similarities and the differences in the properties of these two retroviral enzymes will have a significant impact on structure-based drug design. © 1996 Academic Press, Inc.

INTRODUCTION

Retroviruses are important disease-causing agents in man and animals. Most notable is the human immunodeficiency virus (HIV-1), which causes acquired immunodeficiency syndrome (AIDS) in humans (Ratner *et al.*, 1985). Feline immunodeficiency virus (FIV) infection is widespread in domestic and wild cat populations and has been shown to induce an AIDS-like syndrome, with progression and severity similar to AIDS in humans (Ackley *et al.*, 1990; Barlough *et al.*, 1991; Dow *et al.*, 1990; Ishida *et al.*, 1989; Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988, 1989, 1991). Molecular studies of FIV have defined the sequence and organization of the viral genome and determined that FIV was a lentivirus, similar in genetic organization to HIV-1 (Elder *et al.*, 1993; Olmstead *et al.*, 1989; Phillips *et al.*, 1990; Talbott *et al.*, 1989). Because of the similarity of the two viruses and associated diseases at the molecular level, the study of FIV is potentially important for the understanding of HIV and for therapeutic intervention in AIDS.

Retroviruses encode a protease (PR) involved in viral maturation (Crawford *et al.*, 1985; Katoh *et al.*, 1985; Ashorn *et al.*, 1990) that is essential for viral infectivity (Katoh *et al.*, 1985; Kohl *et al.*, 1988; Baboonian *et al.*, 1991).

Thus, disruption of PR function is an excellent target for the design of protease-specific antiviral agents. Indeed, the HIV-1-encoded protease (HIV-1 PR) (Kramer *et al.*, 1986) is one of most important current targets for structure-based drug design (Huff, 1991), with the aim of developing potential therapeutics for AIDS. We have previously defined the sites of the FIV PR-catalyzed cleavages that lead to processing of the FIV Gag and Gag–Pol polyproteins to give the structural proteins and enzymes, including the protease which is involved in virus maturation and infection (Elder *et al.*, 1993).

In the present study, we describe the total chemical synthesis of FIV PR, and the determination of its enzymatic activities. The results of this initial investigation of the comparative enzymatic properties of the FIV PR and HIV-1 PR indicate differences in specificity for the two PRs and in substrate length requirements. Such comparisons facilitate the refining of our understanding of how the viral PRs function and may suggest avenues to the development of more effective PR inhibitors.

MATERIALS AND METHODS

Peptide synthesis

Machine-assisted stepwise solid phase syntheses were carried out on a 0.2 mmol scale using Boc chemistry *in situ* neutralization protocols as previously de-

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scribed (Schnölzer *et al.*, 1992a). Synthesis of the 116-residue FIV PR polypeptide chain was carried out on a Boc-Met-OCH₂-Pam resin (Applied Biosystems, Foster City, CA). Peptide substrates were synthesized manually on 4-MeBHA resin (Peninsula Laboratories Inc., Belmont, CA) and were acetylated at their N-terminus with a solution of 20% acetic anhydride/5% DIEA in DMF for 10 min. Product peptide resins were deprotected and the free peptides were released from the resin by cleavage with HF as previously described (Schnölzer *et al.*, 1992a). The crude peptides were precipitated and washed with ice-cold diethyl ether, dissolved in 20–70% aqueous acetic acid, diluted with H₂O, and lyophilized.

Characterization of synthetic peptides

Crude peptide products were analyzed by reverse-phase HPLC using a Vydac C₁₈ column (0.46 × 15 cm) at a flow of 1 ml/min with a linear gradient from 0.1% aqueous TFA to 65% acetonitrile/35% water containing 0.1% TFA. Sample absorbance was recorded at 214 nm. Semipreparative HPLC was carried out on a Vydac C₄ column (1.0 × 25 cm) using linear solvent gradients on a Waters Delta Prep 4000 HPLC system. Solvents A and B were as described above. The concentration of buffer B in A was linearly increased at a rate of 1% per minute at a flow rate of 3 ml/min. Absorbance was recorded at 220 nm.

Peptide products, both crude and purified, were also characterized by electrospray mass spectrometry (Model API III, Sciex, Thornhill, Ontario, Canada). Peptides were dissolved in either 50% acetic acid or 50% water/acetonitrile/0.1% TFA to a concentration of 1 mg/ml. Samples were introduced into the atmospheric pressure ionization (API) source of the mass spectrometer by direct infusion using a syringe pump (Harvard Instruments, Boston, MA) at 5 μl/min as previously described (Schnölzer *et al.*, 1992b). Data were acquired onto an Apple Macintosh IIx computer and were processed using the data analysis program MacSpec 3.11b25 (Sciex). Molecular masses were calculated from the multiply charged ions using the HyperMass program (MacSpec 3.11b25).

Folding of FIV PR

Crude lyophilized FIV PR monomer (8 mg) was dissolved in 8 M urea, 0.1 M Na phosphate, pH 5.5, 1 mM DTT to a concentration of 2 mg/ml and dialyzed at 4° against 500-ml batches of buffer containing 0.1 M sodium acetate, pH 5.5, 0.1 M NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, and varying amounts of urea. The concentration of urea in the dialysis buffer was reduced in each batch from 4, to 2, 1, 0.5, 0.25 M, and then no urea. The resulting turbid solution (~16 ml) was centrifuged and the supernatant concentrated in P3 Centricon tubes to ~2 ml. The final enzyme solution was adjusted to 30% glycerol and stored at -20°C.

Assay of enzymatic activity

Assays were carried out at 37° in 0.05 M sodium citrate/0.1 M sodium phosphate buffer, pH 5.25, containing 0.1 mM EDTA, 1 mM DTT, and 1 M NaCl. The peptide substrates were dissolved in DMSO at a concentration of 20 mg/ml, diluted 20-fold into assay buffer, and then 1:1 into the reaction mixture. The final concentration of substrates in the reaction mixture was 0.5 μg/μl. For a 20-μl assay, 2 μl of the above enzyme solution was used. The progress of the cleavage reaction was followed by reverse-phase HPLC on a Vydac C₁₈ column (0.46 × 15 cm) at a flow rate of 1 ml/min using a linear gradient from 0 to 40% acetonitrile in 0.1% aqueous TFA over 15 min. Cleavage products were collected from analytical HPLC runs based on their absorbance at 214 nm and directly analyzed by electrospray mass spectrometry without further manipulation.

Effect of salt concentration. Standard cleavage assays with the 21-residue MA/CA peptide substrate were carried out at NaCl concentrations from 0 to 5 M. Quantitation of cleavage products after an incubation time of 1 hr at 37° was based on integration of peak areas obtained by analytical HPLC.

pH dependence. Assays (10 μl) were carried out at different pH using 5 μg of the 21-residue MA/CA peptide substrate and 1 μl PR solution. The pH was varied in steps of 0.5 pH units by mixing different amounts of 0.1 M sodium citrate/0.2 M Na₂HPO₄ buffers containing 0.1 mM EDTA and 1 mM DTT. Quantitation of product formation after a 20-min incubation time was done by HPLC as described above.

Measurement of kinetic parameters. V_{max} and K_m for the cleavage of the 21-residue MA/CA peptide substrate were calculated from plots of 1/velocity versus 1/[substrate]. Standard assay conditions were used with substrate concentrations between 5 and 150 μM. Initial velocities were determined from cleavage product formation analyzed by reverse-phase HPLC. Assays were set up so that less than 20% of the substrate was cleaved.

Inhibition by pepstatin A. Cleavage assays were performed in the absence of NaCl using otherwise standard conditions. Pepstatin A concentrations were 1, 5, 10, 50, and 100 μM, respectively. Quantitation of the cleavage reaction was as described above.

RESULTS

FIV PR amino acid sequence

The FIV PR is released from the region of the gag-pol translation product corresponding to the N-terminal domain of the Pol polyprotein (Talbot *et al.*, 1989). We have previously defined the proteolytic processing sites at the N-terminus of the PR and RT regions of the pol open reading frame (Elder *et al.*, 1993). In conjunction with the translated cDNA sequence, these data define

1	YNKVGTITTL	EKRPEILIFV	NGYPIKFLLD	30
31	TGADITILNR	RDFQVKNSIE	NGRQNMIGVG	60
61	GGKRGTYIN	VHLEIRDENY	KTQCIFGNVC	90
91	VLEDNSLIQP	LLGRDNMIKF	NIRLVM	116

FIG. 1. The amino acid sequence of the FIV protease (FIV PR). The N-terminus was established by Edman degradation of the PR purified from virus. The C-terminus was inferred from N-terminal sequence analysis of FIV reverse transcriptase, adjacent to the PR toward the 3' end of the Pol open reading frame. The predicted mass from the 116-amino-acid residue sequence shown is in agreement with the observed mass of the PR present in viral preparations [Observed: $13,235 \pm 2$ Da; calculated: 13,234 Da (average isotope composition)] (Elder *et al.*, 1993).

the 116-residue PR monomer amino acid sequence (Fig. 1). Direct HPLC–mass spectrometry of the proteins from disrupted FIV virions identified a component in the expected amount (i.e., in an amount comparable to other similarly sized components of the virus pol-encoded enzyme cassette, and in a much smaller amount than the gag-derived structural proteins), with a mass in exact agreement with the sequence shown in Fig. 1 (Elder *et al.*, 1993).

FIV PR chemical synthesis

The 116-amino-acid-residue PR was prepared by total chemical synthesis using highly optimized stepwise solid-phase methods (Schnölzer *et al.*, 1992a). Synthesis started with the C-terminal Met residue, linked to a cross-linked polystyrene resin support. Chain assembly proceeded in stepwise fashion from the C-terminal to the amino-terminal residue, as described under Materials and Methods. The machine-assisted synthesis proceeded at a rate of 75 residues per day, for a total of just over 1.5 days. Colorimetric assay for residual free amine (Sarin *et al.*, 1981) showed that all amino acid additions had proceeded with >99.7% yield. After completion of the assembly of the protected peptide chain, the DNP protecting group was removed from the His side chain of the resin-bound peptide, the N^α-Boc was removed, and then the remainder of the protecting groups were removed and the peptide was simultaneously cleaved from the resin support, by treatment with HF plus scavenger.

The crude peptide product was examined by analytical reverse-phase HPLC and by electrospray mass spectrometry. Material of the target MW was observed (Found: $13,235 \pm 2$ Da; calculated: 13,234.4 Da (average isotope composition)). Purification was performed by semipreparative reverse-phase HPLC, using electrospray mass spectrometry to identify fractions containing the desired product in large proportion. The combined fractions were lyophilized, and the partially purified product was folded by dialysis from 8 M urea into native buffer conditions to give partially purified, folded FIV PR.

Enzymatic activities

Specificity. Aliquots of the solution containing the folded synthetic FIV PR were assayed for the ability to cleave a synthetic peptide substrate corresponding to the the matrix and capsid proteins cleavage site (MA/CA) of the gag translation product (Fig. 2). The sequence of the substrate was (R)KEEGPPQAY ~ PIQTVNGPOY(R). The N- and C-terminal Arg residues were added to improve the solubility of the peptide in the assay buffers. Specific cleavage products were observed. Mass spectrometry on the collected fractions corresponding to the two products showed that cleavage occurred between the Tyr and Pro residues. This corresponds to the MA/CA cleavage site observed during viral maturation *in vivo* (Elder *et al.*, 1993).

Synthetic peptide substrates corresponding to all the proteolytic processing sites of the FIV Gag and Gag–Pol polyprotein translation products were similarly examined (Table 1). Specific cleavage was observed in each case, and for those substrates where the processing sites were known from studies of the viral proteins (Elder *et al.*, 1993), there was precise correlation between the studies with synthetic enzyme on synthetic substrate and the processing events *in vivo*. These results demonstrate that the synthetic FIV PR enzyme has specificity consistent with that required for viral maturation.

Kinetics. The rate of FIV PR activity was measured using an HPLC assay, with the synthetic peptide analog of the MA/CA cleavage site as substrate (Fig. 2). Enzymatic activity was enhanced at increasing concentrations of NaCl; the maximum rate was obtained at ~1.5 M NaCl. This effect of salt concentration is similar to that observed for the HIV-1 PR (Wondrak *et al.*, 1991). The effect of pH on activity was also measured, at constant

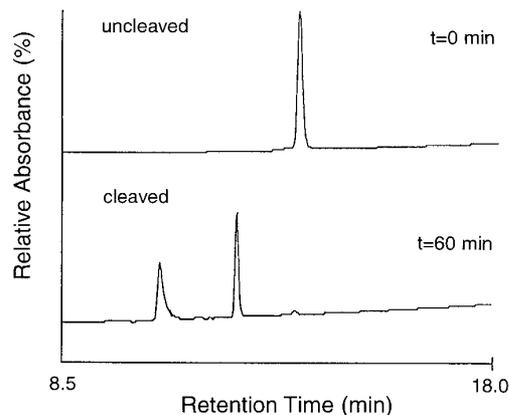


FIG. 2. Specific cleavage of a synthetic peptide corresponding to the matrix–capsid junction in the FIV Gag open reading frame. The MA/CA substrate had sequence (R)KEEGPPQAY ~ EPIQTVNGPOY(R). The N- and C-terminal Arg residues were added to improve solubility of the peptide in the assay buffers. Mass spectrometry on the two products showed that cleavage occurred between the Tyr and Pro residues. This corresponds to the same site of processing observed in viral maturation *in vivo*. (Elder *et al.*, 1993).

TABLE 1

FIV PR Cleavage of Synthetic Peptides Corresponding to *in Vivo* Processing Sites of the FIV Gag and Gag-Pol Polyproteins

Cleavage site	Sequence	Proteolytic activity
MA/CA	$P_4 P_3 P_2 P_1 \quad P'_1 P'_2 P'_3 P'_4$ R K E E G P P Q A Y ~ P I Q T V N G V P Q Y R	+
	R P Q A Y ~ P I Q T R	+
	P Q A Y ~ P I Q T	+
	R Q A Y ~ P I Q R	±
	Q A Y ~ P I Q	-
CA/NC 1	Y K M Q L ~ L A E A L T K	+
	M Q L ~ L A E	-
CA/NC 2	T K V Q ~ V V Q S K G S	
NC/PR	R R G E T I G F V N ~ Y N K V G R R	+
PR/RT	R I R L V M ~ A Q I S D K R	+
RT/RNase H	R R G A E T W ~ Y I D G G R K	+
RNase H/DU	R R C Q T M ~ M I I E R R	
DU/IN	Y G S T G V F ~ S S W V D R I E E A	+

ionic strength. Under the conditions used, the enzyme was saturated with substrate. Maximum enzymatic activity was observed in the range of pH 5–6. Again, this is similar to the pH of maximum activity observed for the HIV-1 PR, under comparable conditions (Hyland *et al.*, 1991a).

Kinetic parameters were determined on the same MA/CA peptide substrate (Fig. 2). Measurements of initial rate as a function of substrate concentration were performed by HPLC assay. Only the expected cleavage at the Tyr ~ Pro site was observed. Typical saturation kinetics were observed (Fig. 3a). A double reciprocal plot (Fig. 3b) of the data gave $K_m \sim 10$ mM for the synthetic peptide analogue of the FIV MA/CA cleavage site. This K_m is considerably higher than the high micromolar to millimolar K_m values reported for the HIV-1 PR on unmodified peptide substrate analogues of the HIV-1 MA/CA processing site (Hyland *et al.*, 1991b; Ido *et al.*, 1991; Griffiths *et al.*, 1992). The observed V_{max} was similar to that of HIV-1 PR acting on the analogous HIV-1 substrate. This V_{max} measurement is only approximate, because protein concentration measurements for FIV PR have not yet been calibrated. However, K_m values are unaffected by concentration of active enzyme. Further comparative studies, now under way, will clarify this point.

Substrate specificity—comparison with HIV-1 PR

In addition to the determination of processing sites in the viral Gag-Pol polyprotein described above, a variety of studies of the specificity of FIV PR were carried out on synthetic peptide substrates. Assays were analyzed by HPLC and identities of the cleavage

products were determined in all cases by electrospray mass spectrometry. Comparative measurements on HIV-1 PR with HIV-1 synthetic peptide substrates were also performed. Some of the results are shown in Figs. 4 and 5 and Table 2.

In the comparative activity studies of the two retroviral enzymes, two clear-cut results stand out: (i) FIV PR will not cut an acetyl-(6-residue peptide)amide, whereas HIV-1 PR will cut the corresponding HIV 6-residue substrate (Kent, unpublished results; Wlodawer *et al.*, 1989); FIV PR does, however, specifically cut an acetyl-(8-residue peptide)amide (Table 2). Thus there are significant differences in the substrate length requirement for the two enzymes. (ii) Although both enzymes cut their corresponding Gag MA/CA cleavage sites between Tyr and Pro residues, the FIV PR and HIV-1 PR show a strong preference for their own MA/CA Tyr ~ Pro substrates, despite four identical amino acids in the P3–P3' positions (see Fig. 4). HIV-1 PR cuts the FIV MA/CA Tyr ~ Pro site approximately 50 times slower than the FIV PR does (Fig. 4a). FIV PR cuts the HIV-1 MA/CA Tyr ~ Pro site approximately 200 times slower than HIV-1 PR cuts the same substrate (Fig. 4b). The small difference in reciprocal rates may be due to uncertainties in determining the concentration of active enzyme present in each case.

It is interesting to note that FIV PR cleaves a synthetic peptide spanning the NC/PR processing site [sequence Ac-Arg¹-Arg-Ile-Gly⁴-Phe⁵-Val-Asn⁷ ~ Tyr⁸-Asn-Lys-Val-Gly-Arg-Arg· amide; italicized residues added for solubility] at the expected Asn⁷ ~ Tyr⁸, and also between the Gly⁴ ~ Phe⁵ residues. An additional cleavage site has been fortuitously created by the use

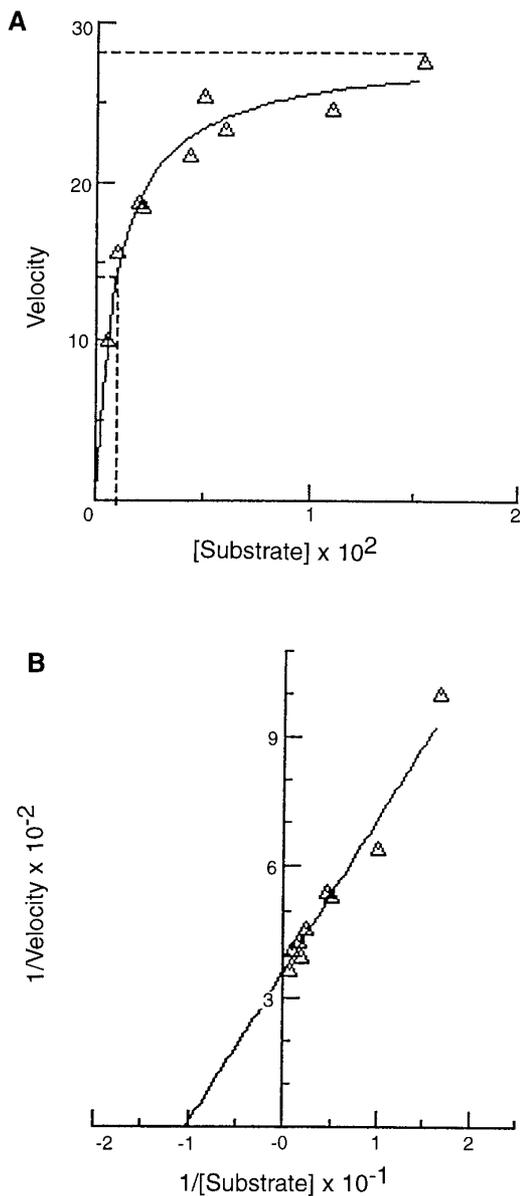


FIG. 3. Initial velocity kinetic measurements at 37°C, pH 5.25, [NaCl] = 1 M, of the cleavage by FIV PR of the synthetic MA/CA substrate described in the legend to Fig. 2. (a) Velocity versus (substrate concentration) showed saturation as is typical of enzyme-catalyzed reactions; (b) a double reciprocal plot of 1/velocity versus 1/[substrate]. Observed $K_m \sim 10$ mM.

of the N-terminal Arg residues to enhance solubility. A longer natural substrate sequence [Ac-Arg-Arg-Gly-Glu-Thr-Ile-Gly-Phe-Val-Asn ~ Tyr-Asn-Lys-Val-Gly-Arg-Arg·amide] did not display this phenomenon and was specifically processed only at the expected Asn-Tyr cleavage site (Elder *et al.*, 1993).

In addition, with the CA/NC No. 1 cleavage site, the enzymes exhibit somewhat different specificities: the FIV PR cuts the CA/NC No. 1 peptide between Leu ~ Leu residues, while the HIV-1 PR cuts the corresponding HIV-1 substrate between Leu ~ Ala residues. However, the

HIV-1 PR cleaved the FIV CA/NC No. 1 peptide between the Leu ~ Ala and Leu ~ Leu peptide bonds (Fig. 5 and Table 2). This vividly demonstrates the similarities and differences in the specificity of the two enzymes on peptide substrates.

CONCLUSIONS

The FIV PR represents an important addition to the family of well-characterized retroviral proteases (Miller *et al.*, 1989a,b). As our understanding of the FIV PR increases, it will be important to relate that knowledge to the existing database for HIV-1 PR. The two enzymes have related activities, but at the same time we have defined critical differences, the most important being the observation that HIV-1 PR and FIV PR each show a strong preference for their own MA/CA substrates, despite an identical -Tyr ~ Pro- cleavage site in each case and substantially similar neighboring sequences. This type of subtle distinction in enzymatic properties makes the comparative study of the properties of FIV PR in the context of the HIV-1 PR an important model system for structure-based drug design. Further study will be facilitated by the recent completion of the crystal structure of FIV PR (Dunn *et al.*, 1995).

Currently, HIV-1 PR inhibitors show promise as effective AIDS therapeutics (Huff, 1991; Mitsuya *et al.*, 1990; Vacca *et al.*, 1994), although the development of PR inhibitor-resistant strains of HIV-1 has been reported from clinical trials (Otto *et al.*, 1993; El-Farrash *et al.*, 1994). This is not unexpected, since the HIV-1 reverse transcriptase has a high error rate that leads to rapid mutation of viral proteins subject to selective pressure. However, it does point to the utility of designing PR inhibitors which demonstrate inhibition against closely related PRs (i.e., HIV-1 and FIV) in that they may be better able to inhibit mutant forms of the PRs.

The observation that FIV PR requires a substrate 8 amino acids in length is important in that many PR inhibitors for HIV-1 PR are based on peptides less than 7 amino acids in length. In requiring a longer substrate, the FIV PR is in effect being more specific in needing the S4 through S4' substrate binding pockets to be filled. Inhibitors based on an 8-amino-acid peptide may be more effective against FIV PR, as well as HIV-1 PR and mutant PRs. Initial results with pyrrolidine-containing α -keto amide-based inhibitors (Slee *et al.*, 1995) support this conclusion for FIV PR since addition of FIV-specific amino acid residues to the primary core structure (Slee *et al.*, 1995) increases the specificity for FIV PR dramatically (Wong, personal communication).

The results from the FIV and HIV-1 MA/CA cleavage site are interesting in that they indicate the importance of the P2 and P3' positions in the recognition and cleavage of the peptide by the FIV and HIV-1 PRs. Incorporation of amino acid side chains at these positions, re-

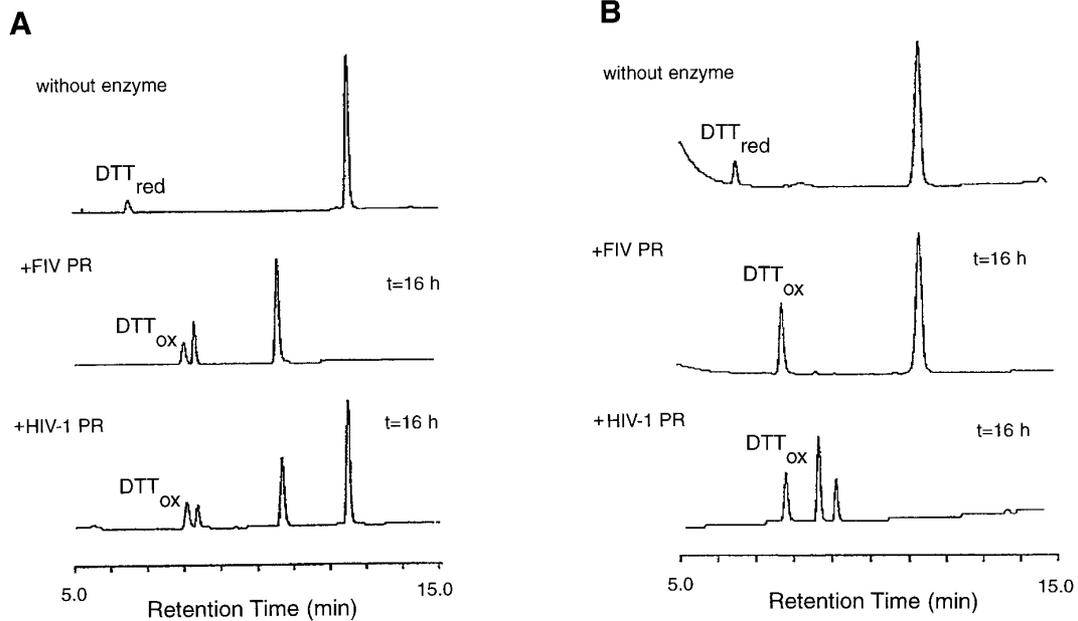


FIG. 4. Action of FIV PR and HIV-1 PR on synthetic peptide analogues of the MA/CA Tyr ~ Pro processing site in the FIV and HIV-1 Gag translation products. Note the strong preference of each retroviral enzyme for the substrate from the same virus. (a) FIV substrate Ac-RPOAY-PIQTR-NH₂ (top) treated for 16 hr with FIV PR (middle) or HIV-1 PR (bottom). (b) HIV-1 substrate Ac-RSONY-PIVQR-NH₂ (top) treated for 16 hr with FIV PR (middle) or HIV-1 PR (bottom). The digestions were analyzed by HPLC and the hydrolysis products identified by electrospray mass spectrometry. Reduced and oxidized forms of DTT are marked.

sulting in a peptide that is cleaved equally by both PRs, may facilitate the development of peptide-based inhibitors that are equally effective for FIV PR and HIV-1 PR.

By exploiting the similarities and differences in substrate specificity between the FIV and HIV-1 PRs, in conjunction with the three-dimensional structures, it may be

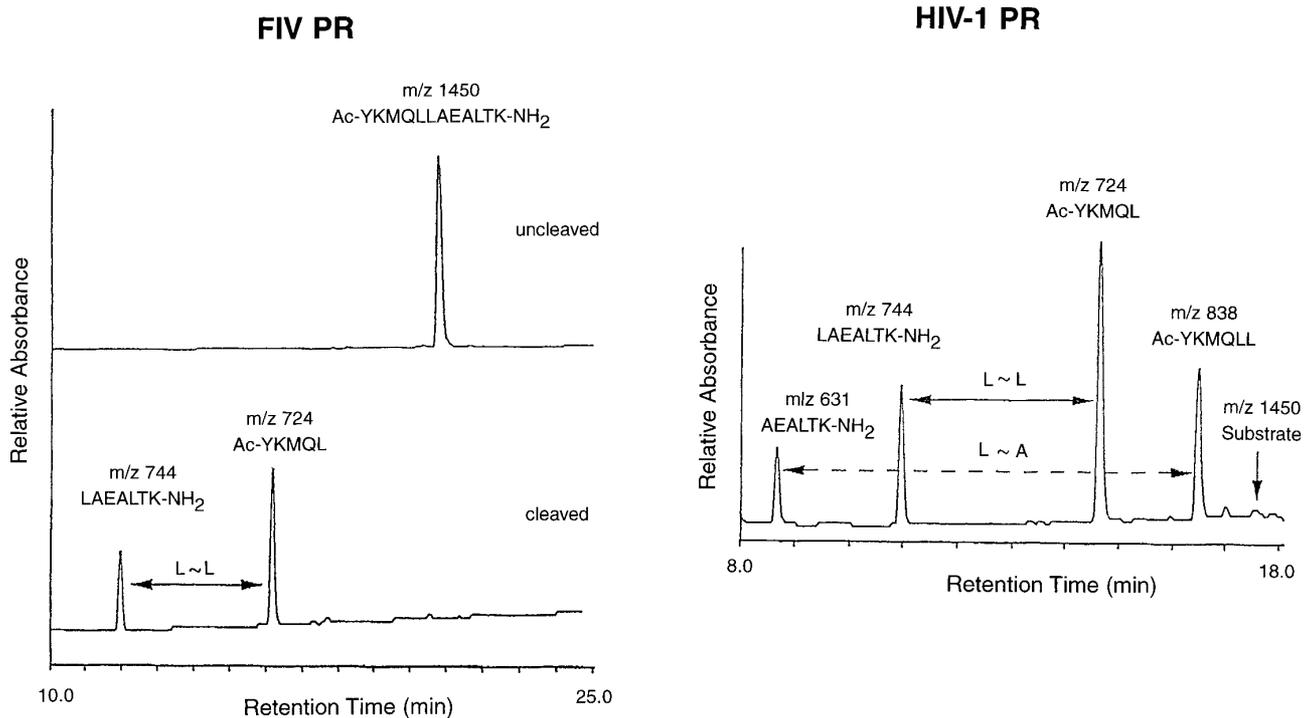


FIG. 5. Action of FIV PR and HIV-1 PR on a synthetic peptide analogue of the FIV first (5'-most) capsid/nucleocapsid processing site. The FIV PR cuts the substrate at only one site, corresponding to the -Leu ~ Leu- processing site observed *in vivo*. The HIV-1 PR cuts the same -Leu ~ Leu- site and also the neighboring -Leu ~ Ala- peptide bond, at similar rates. (In HIV-1, the CA/NC#1 processing event occurs at a -Leu ~ Ala- site in the analogous HIV-1 Gag sequence). (Left) FIV PR; (Right) HIV-1 PR.

TABLE 2
Substrate Length Requirements for FIV PR and HIV-1 PR

Cleavage site	Peptide sequence ^a	Activity detected	
		FIV PR	HIV PR
MA/CA	R K K K G P P Q A Y ~ P I Q T V N G V P Q Y	Yes	Yes
8-mer	P Q A Y ~ P I Q T	Yes	Yes
6-mer	Q A Y ~ P I Q	No	Yes
CA/NC 1	Y K M Q L ~ L A E A L T K	Yes	Yes ^b
6-mer	M Q L ~ L A E	No	Yes

^a All substrates were Acetyl-Peptide-amides. Activities were observed by reverse phase HPLC.

^b HIV-1 PR cuts equally at Leu⁵ ~ Leu⁶ and at Leu⁶ ~ Ala⁷.

possible to design PR inhibitors which are highly specific for retroviral PRs and resistant to mutations in the PR primary amino acid sequence, which to date have led to PR escape from inhibition.

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