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Inhibition of activity of the protease from bovine leukemia virus

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Abstract

In view of the close similarity between bovine leukemia virus (BLV) and human T-cell leukemia virus type I (HTLV-I) we investigated the possibility of developing specific inhibitors of the proteases of these retroviruses using the purified enzyme from BLV. We tested the ability of this protease to specifically cleave various short oligopeptide substrates containing cleavage sites of BLV and HTLV-I proteases, as well as a recombinant BLV Gag precursor. The best substrate, a synthetic decapeptide bearing the natural cleavage site between the matrix and the capsid proteins of BLV Gag precursor polyprotein, was used to develop an inhibition assay. We determined the relative inhibitory effect of synthetic Gag precursor-like peptides in which the cleavable site was replaced by a non-hydrolyzable moiety. The encouraging inhibitory effect of these compounds indicates that potent non-peptidic inhibitors for retroviral proteases are not unattainable.

Key words: Bovine leukemia virus; Protease; Activity and inhibition

1. Introduction

Human T-cell leukemia virus type I (HTLV-I) and bovine leukemia virus (BLV) are closely related retroviruses [1,2] that are the etiological agents of adult T-cell leukemia and enzootic bovine lymphosarcoma, respectively [3–7]. In common with the virus itself, the protease (PR) of BLV strongly resembles the corresponding HTLV-I enzyme [8,9]. Both PRs are responsible for processing both the initial translation products (precursors Gag-Pro-Pol, Gag-Pro and Gag) into functional enzymes (protease, reverse transcriptase and integrase) and the mature structural proteins of the matrix, capsid and nucleocapsid (MA, CA, NC) [10–13], which are required for infectivity [14]. PR is thus a prime target for potential therapeutic agents in the fight against the diseases induced by these retroviruses. Because native BLV PR is easier to obtain to a high degree of purity than HTLV-I PR, the former was employed in the experiments reported here. In order to develop suitable inhibitors, we checked that BLV PR cleaved peptides at the same sites as those cleaved by the HTLV-I enzyme. We therefore developed an in vitro inhibition assay using the substrate with the highest sensitivity that was also the most readily analyzed. Using this test, we examined the potential inhibitory activity of a variety of compounds. BLV and HTLV-I PRs have been isolated by several groups [13,15–19], although the efficiency of specific inhibitors has yet to be reported.

2. Materials and methods

2.1. Source of viral BLV PR

BLV PR was purified from virion particles under non-denaturating conditions. In brief, pure and active PR was obtained by cation-exchange chromatography followed by hydrophobic chromatography. PR in this preparation was found to be most active at pH 6, 40°C and high salt concentration (1–2 M NaCl or ammonium sulfate) using a synthetic peptide as substrate [20].

2.2. Assay for protease activity with synthetic peptides

All the peptides used in the experiments were synthesized by the Merrifield solid-phase method [21]. Proteolytic reactions were carried out in 100 μl of 0.1 M sodium citrate, pH 6, 1 M NaCl containing 10^{-9} mol of peptide. The reaction was initiated by adding 10^{-11} mol of PR, and the reaction mixture was incubated at 37°C for 30 min. Reactions were stopped by immersion in a boiling water bath for 5 min, and the reaction products were analyzed by RP-HPLC.

2.3. Assay for protease activity with Gag precursor

The BLV recombinant Gag precursor was expressed in a vaccinia virus production system (IFFA Laboratories, Rhône Mérieux, Lyon, France) and purified by affinity chromatography using a rabbit anti-p24 polyclonal antibody. The reactions were carried out in 100 μl of 0.1 M sodium citrate, pH 6, 0.1 M NaCl at 40°C for 30 min with 10^{-9} mol of Gag precursor and 10^{-11} mol of PR. Immunoblot analysis of Gag cleavage reaction involved transfer to nitrocellulose, development with murine anti-p24 monoclonal antibody as the primary probe and alkaline phosphatase-conjugated Ig (H and L) mouse antibodies as the detection probe.

2.4. Inhibition assay

The proteolytic reactions were carried out in 10 μl of 0.1 M sodium citrate, pH 6, 1 M NaCl containing 10^{-10} mol of the ^{125}I-labeled 9003 substrate, including various concentrations of inhibitor. The reaction

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Abbreviations: BLV, bovine leukemia virus; HTLV-I, human T-cell leukemia virus Type I; PR, protease; MA, matrix; CA, capsid; NC, nucleocapsid; RP-HPLC, reverse-phase high performance liquid chromatography; R_T, retention time; STA, (3R, 4S) 4-amino 3-hydroxy 6-methylheptanoic acid.

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was initiated by adding 10^{-12} mol of PR, and the reaction mixture was incubated at 37°C for 30 min. Reactions were stopped by immersion in a boiling water bath for 5 min, and the reactions products were analyzed by thin layer electrophoresis on cellulose plates. The plates were dried and autoradiographed. Radioactivity was counted from spots removed from the autoradiograms and the reaction efficiency determined [20].

3. Results and discussion

Under the optimum activity conditions of BLV PR (see section 2), we tested peptidic substrates harboring the recognition cleavage sites of the BLV and HTLV-I PRs.

Peptide 9003 (Table 1) contains the cleavage site between the MA and CA domains of the BLV Gag polyprotein. As previously described, this peptide was cleaved at the expected site by PR [20], and was used as a positive control.

Peptide 9214 harbored the cleavage site of the N-terminal end of BLV PR. It contained aminobutyric acid instead of the cystein residue to prevent possible disulfide bonding between identical peptides. One of the expected hydrolyzed products was peptide 9215. This latter peptide had a retention time (R_T) of 12.4 min on a RP-HPLC column (data not shown). Under the same conditions, native peptide 9214 had a R_T of 21.2 min, while the same peptide incubated with PR gave, in addition to 9214 itself (R_T = 21.2 min), an additional peak (R_T = 12.4 min) corresponding to the hydrolyzed byproduct (Fig. 1A).

We also checked whether BLV PR processed its natural Gag precursor substrate correctly. The proteolytic reaction was carried as detailed in section 2. After 30 min incubation, three major bands (39, 36 and 24 kDa) were detected on SDS-PAGE, corresponding to the predicted molecular weights of the CA linked to the NC, the MA linked to the CA, and the CA, respectively (Fig. 2). These results suggested that BLV PR cleaves at the predicted site of the BLV Gag precursor.

Peptides 9306 and 8812 harbor the cleavage site between the MA and CA domains of the HTLV-I Gag precursor. The reaction was carried out and analyzed as described in section 2. Peptide 9306 was cleaved at the expected site by PR, generating a peptide corresponding to the hydrolyzed by-product 9307 (Fig. 1B). We also checked whether the BLV PR could cleave a shorter peptide than peptide 9306. We tested activity on peptide 8812 using peptide 8814 as the control. We found that 8812 was uncleaved even with longer incubation times (3, 6, 12 and 24 h) and/or higher concentrations of PR (data not shown). It is noteworthy that peptide 9306 was cleaved whereas 8812 was not. This is indicative of an influence on site recognition by PR of the length of the peptide and the surrounding three-dimensional structure.

With respect to the C-terminal end of the HTLV-I PR, the dipeptide L-147/P-148 has been proposed as a cleavage site by Oroszlan and Luftig [22]. A fusion protein with an HTLV-I PR containing only 115 amino acids (P-33 to L-147) has also been shown to be active on peptide 9003 (Llido et al., manuscript in preparation). We thus tested the ability of the BLV PR to cleave the dipeptide L-147/P-148. For this, we assayed peptides 8911 and 9101 using the expected hydrolysis product

Table 1
Activity of BLV protease towards the different substrates

Peptide number	Amino acid sequence	Represented cleavage site	Relative activity
BLV			
9003	Y-D-P-P-A-I-L↓P-I-I	MA/CA	1.0
9002	Y-D-P-P-A-I-L		–
9214	A-E-L-E-aBu-L↓L-S-I-P-L-A	NTermPR	0.7
9215	L-S-I-P-L-A		–
HTLV-I			
9306	A-P-Q-V-L↓P-V-M-H-P	MA/CA	2
9307	P-V-M-H-P		–
8812	Ac P-Q-V-L↓P-V-M	MA/CA	0
8814	Ac P-Q-V-L		–
8911	G-V-L-Y-L↓P-E-A-K-R	CTermPR(1)	0
9101	G-V-L-Y-L↓P-E-A	CTermPR(1)	0
9000	G-V-L-Y-L		–
9312	K-G-P-P-V-I-L↓P-I-Q-D-P	CTermPR(2)	0.9
9313	P-I-Q-D-P		–

↓ represents the cleavage site. aBu represents aminobutyric acid. The 9002, 9215, 9307, 8814, 9000 and 9313 peptides (non-cleavable controls) represent the expected products on proteolysis of peptides 9003, 9214, 9306, 8812, 8911, 9101 and 9313, respectively.
CTermPR(1) and CTermPR(2) represent the cleavage site of the C-terminal end of the HTLV-I PR which has been proposed by Oroszlan and Luftig [22] and by Kobayashi *et al.* [17], respectively.

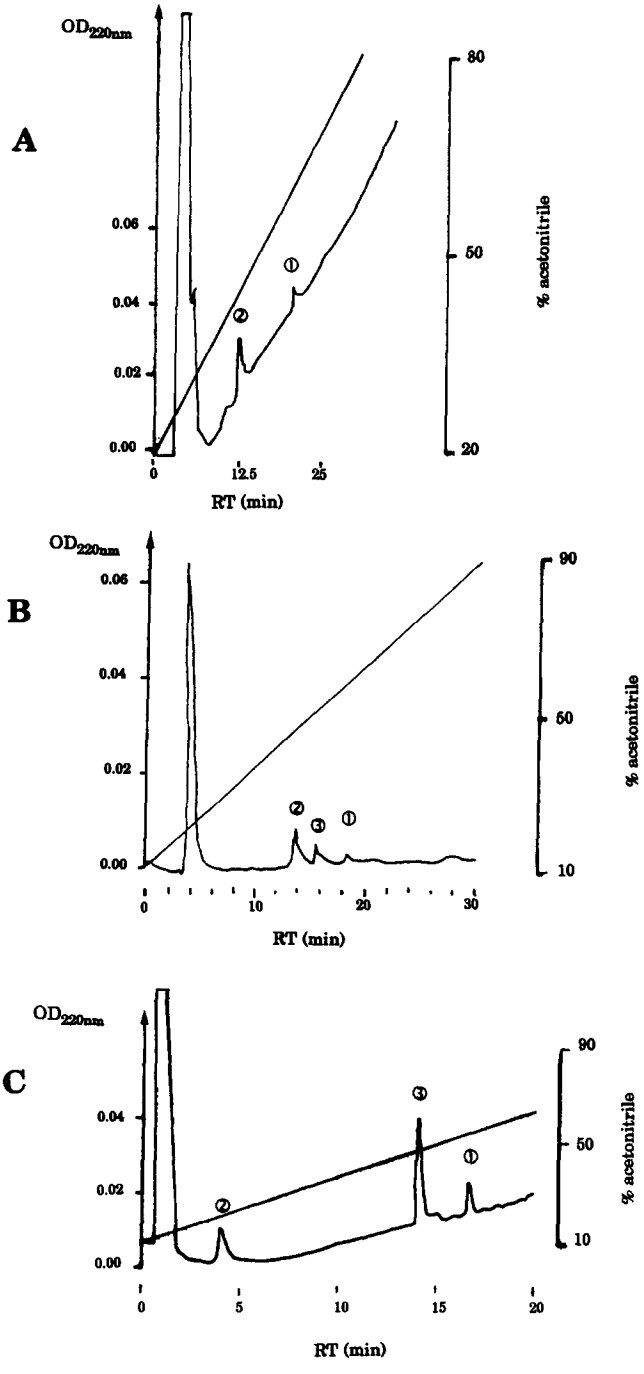

Fig. 1. Activity of BLV PR against peptides 9214 (A), 9306 (B) and 9312 (C). The peptidic reaction products from the activity test were loaded onto a RP-HPLC V5C4–25FC4 column (5 μm, 250 × 4.6 mm; Interchim) and eluted in 0.1% TFA at a constant flow rate of 1 ml/min with a 30 min linear gradient of acetonitrile at 20°C. The diagonal line represents progress of the gradient. (A) Peptide 9214 (1) is cleaved by BLV PR generating the peptide (2) corresponding to the hydrolyzed byproduct 9215. The peptide corresponding to the sequence A-E-L-E-aBu-L was eluted at the beginning of the gradient. (B) Peptide 9306 (1) is cleaved by BLV PR, generating two peptides corresponding to the hydrolyzed by-product 9307 (2) and to the peptide A-P-Q-V-L (3). (C) Peptide 9312 (1) is cleaved by BLV PR, generating two peptides corresponding to the hydrolyzed by-product 9313 (2) and to the peptide K-G-P-P-V-I-L (3).

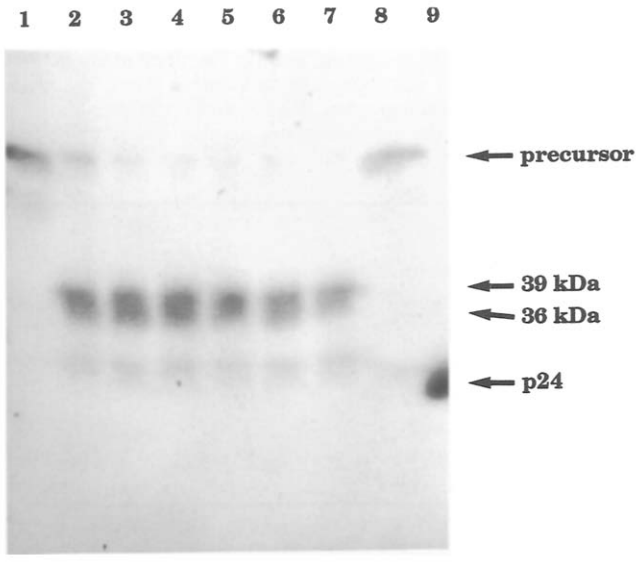

Fig. 2. Immunoblot analysis of cleavage products of BLV PR on BLV recombinant Gag precursor. Lanes 1–7 correspond to incubation times of 0, 30 min, 1, 2, 3, 6 and 24 h, respectively. Lanes 8 and 9 correspond to the Gag precursor incubated for 24 h without PR and to 2 μg of BLV proteins, respectively.

9000 as a control (see section 2). The 8911 and 9101 peptides remained uncleaved even with longer incubation times (3, 6, 12 and 24 h) and/or higher PR concentrations (data not shown). In contrast, peptide 9312, containing the dipeptide L-157/P-158, proposed by Kobayashi et al. [17] as the cleavage site of the HTLV-I PR C-terminal end, gave, in addition to 9312 itself, two additional peaks corresponding to the hydrolyzed byproduct 9313 and to the sequence K-G-P-P-V-I-L, respectively (Fig. 1C). These results indicated that this site is recognized, and that the dipeptide L-157/P-158 is probably the cleavage site of the HTLV-I PR C-terminal end.

Taken together the results reported above indicated that 9003 and the BLV recombinant Gag precursor were the best BLV substrates for the development of an inhibition assay. However, as the proteolysis of substrate 9003 could be more readily analyzed than that of the BLV recombinant Gag precursor substrate, the former was employed to examine the inhibitory effect of test compounds. Pepstatin A (Sigma) was used as the reference agent for qualitative and quantitative inhibition. Under our experimental conditions, the concentration for 50% inhibition (IC_{50}) of BLV PR by pepstatin A was in the range of $3–5 \times 10^{-4}$ M.

It has been demonstrated that the site located at the junction of the MA and CA fragments is efficiently cleaved by PR [15,18]. We therefore decided to synthesize a primary inhibitor containing the MA and CA environment for use as a standard. Since peptide 9003 was found to be the best substrate for BLV PR, we synthesized a peptide (9004) in which the two amino acids of the cleavable site were replaced by the non-hydrolyzable

Table 2
Inhibitory activity of peptides against BLV protease

Peptide number	Sequence		Cleavage site	IC_{50}(M)
	Pepstatin A			$3–5 \times 10^{-4}$
9004	Y-D-P-P-A-I-**STA**-I-I	BLV	MA/CA	5.0×10^{-7}
8901	Ac P-Q-V-L-**ʳ**-P-V-M	HTLV-I	MA/CA	1.0×10^{-5}
8903	Ac P-Q-V-L-**PIP**-V-M	HTLV-I	MA/CA	1.5×10^{-5}
8904	Ac P-Q-V-L-**AZE**-V-M	HTLV-I	MA/CA	2.5×10^{-4}
8813	P-Q-V-**STA**-A-L	HTLV-I	MA/CA	1.0×10^{-4}

STA, **ʳ**, **PIP**, and **AZE** represent the statin residue, the reduced bond, the pipecolic acid and the azetidine residues, respectively.

statin residue (Table 2). The IC_{50} of BLV PR by 9004 was around 5.0×10^{-7} M.

As BLV PR has been found to correctly process an HTLV-I recombinant Gag precursor [20] and peptide 9306, a HTLV-I peptide substrate, we also tested the activity of potential inhibitors of HTLV-I PR based on models of the enzyme structure. Although the 8812 peptide was not cleaved by BLV PR, the models indicated that molecules derived from its 3D structure should inhibit BLV and HTLV-I PRs (unpublished results). Compounds 8901, 8903, 8904 and 8813 were thus synthesized as potential inhibitors [24]. These molecules were constructed from the 111–117 sequence in which the cleavage site was replaced by a non-hydrolyzable moiety (Table 2). Best inhibition was obtained with compounds 8903, which contained the pipecolic acid residue (IC_{50} 15 μM), and 8901 containing the reduced bond (IC_{50} 10 μM), whereas compounds bearing the azetidine (8904) or the statin residues (8813) displayed almost no inhibitory effect against BLV PR (IC_{50} of around 2.5×10^{-4} M and 1.0×10^{-4} M, respectively).

Leupeptin, soybean trypsin inhibitor, chymostatin, antipapain and pepstatin A have all been tested on BLV and HTLV-I PRs [25], but only pepstatin A was found to produce a significant inhibition. Our results with pepstatin A are in good agreement with these findings. Since this compound cannot be employed in the treatment of leukemia, more specific inhibitors are required. On the basis of the results on the various substrates, we tested several modified substrates as inhibitors. The greatest inhibition of the BLV PR was observed with peptide 9004 (100-fold more active than pepstatin A), which is derived from the BLV PR cleavage site. Most of the inhibitors bearing a modified HTLV-I PR cleavage site were less efficient than compound 9004, but were all significantly more efficient than pepstatin A.

As peptidic inhibitors are susceptible to proteolytic degradation in vivo, they are likely to be ineffective therapeutically. Nevertheless the results reported here indicate that more specific, non-peptidic inhibitors of retroviral proteases could be developed.

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