Solid phase synthesis of the proteinase of bovine leukemia virus

Comparison of its specificity to that of HIV-2 proteinase

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The 126-residue proteinase (PR) of bovine leukemia virus (BLV) was synthesized by solid-phase peptide synthesis and its activity was shown using various oligopeptide substrates representing cleavage sites in BLV, human T-cell leukemia virus type 1 (HTLV-1), murine leukemia virus (MuLV) and human immunodeficiency virus type 1 (HIV-1). The specificity of the BLV PR was also compared to that of chemically synthesized human immunodeficiency virus type 2 (HIV-2) PR. Many of the peptides were cleaved at the expected site, however, 6 out of 15 were hydrolyzed only by one of the PRs. Furthermore, one BLV peptide was processed differently by the two enzymes. These results, together with the relative activities and the lack of inhibition of BLV PR by two HIV-1 PR inhibitors, suggest that the BLV PR specificity is substantially different from that of HIV PRs.

Solid phase peptide synthesis; BLV proteinase; Substrate specificity

1. INTRODUCTION

Human immunodeficiency viruses (HIV) and human T-cell leukemia viruses (HTLV) are intensively studied, since they are the causative agents of acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia (ATL), respectively [1]. The retroviral proteinase (PR), especially that of HIV has also received a great deal of attention as a potential target for chemotherapy [2], since the PR activity is required for the maturation and infectivity of the viruses [3–5]. The lentiviral PR is also suggested to be involved in the early phase of viral life-cycle [6–8]. However, far less is known about the HTLV PR, the proteolytic enzyme of the first bona fide human retrovirus, which has only been recently cloned [9].

Bovine leukemia virus (BLV) is the etiologic agent for enzootic bovine leukosis [10], which is an economically important disease of cattle. BLV shows the closest relationship to HTLV [11–13], therefore, as the virus itself, BLV PR can also be considered as a model of HTLV-1 PR.

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We have used solid phase peptide synthesis to produce the proteolytic enzyme of BLV. Previously, BLV PR has been obtained from purified virus, and partially characterized [14]. It is composed of 126 residues, and it is the largest known retroviral proteinase. In this study the substrate specificity of the BLV PR was compared to that of also chemically synthesized HIV-2 proteinase.

2. MATERIALS AND METHODS

2.1. *Synthesis of peptidyl-resin, hydrogen fluoride cleavage and refolding*

Solid phase peptide synthesis was performed using a Vega Coupler 250C peptide synthesizer (DuPont Co.), starting with 330 mg (0.25 mmol) Boc-Gly-phenylacetamidomethyl resin (Applied Biosystems, Inc.), with a program which has been described earlier [15]. Eight equivalents of protected amino acids (Bachem, Inc.) were used in each cycle. The side chain-protecting groups were benzyl for Asp, Glu, Ser, Thr; 2-chlorobenzyloxycarbonyl for Lys; 2-bromo-benzyloxycarbonyl for Tyr; formyl for Trp and tosyl for Arg. At the end of the 125-cycle synthesis, the peptidyl-resin weighed 2.06 g. The peptide was cleaved from the resin by the following different ways (using 270–270 mg aliquots): (i) stirring for 1 h at 0°C in the mixture of liquid HF (8 ml) and p-cresole (0.3 ml); (ii) stirring for 1 h at 0°C in the mixture of liquid HF (12 ml), anisole (1 ml), and ethanedithiole (1 ml); (iii) by low-high cleavage according to Tam et al. [16]. The crude peptides (25–25 mg) were dissolved in 2 ml 8 M guanidine-HCl, purified by RP-HPLC, and refolded with 20 mM PIPES-HCl, pH 7.0, containing 1 mM EDTA, 100 mM NaCl, 10% glycerol, 5% ethylene glycol and 0.5% Nonidet P-40, as described for HIV-2 PR [17]. Amino acid composition of the obtained protein was determined after hydrolysis in 6 M HCl (110°C, 20 h) on a Durrum D-500 analyzer. Peptide obtained with HF cleavage protocol (ii) was further purified by gel filtration on a Superdex G-75 HR-16/60 column (Pharmacia LKB Biotechnology) and purified

again on RP-HPLC as described for HIV-2 PR [17]. The refolded proteinase solution was concentrated using Amicon-3 concentrator. SDS-PAGE of the purified PR was performed according to Laemmli [18].

2.2. *Oligopeptides*

Oligopeptides were synthesized by solid-phase peptide synthesis on a Model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or on the Vega Coupler 250C using Boc chemistry, and were purified by RP-HPLC. Stock solutions and dilutions were made in distilled water (or in 5 mM dithiothreitol for the Cys-containing peptide), and the proper peptide concentration was determined by amino acid analysis on either a Durrum D-500 or a Waters Pico-Tag analyzer.

2.3. *Proteinase assay*

Proteinase assays were performed at 37°C using chemically synthesized BLV and HIV-2 PRs [17,19–21] in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 5 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40 and 2 M NaCl, followed by the separation of the substrates and cleavage products by RP-HPLC as it was described [15,17,20,21]. Amino acid analysis of the collected peaks was used to confirm the cleavage site in the substrates.

3. RESULTS AND DISCUSSION

3.1. *Chemical synthesis of the BLV proteinase*

While earlier techniques limited chemical peptide synthesis to produce only relatively small biologically active oligopeptides and enzymes like ribonucleases [22,23], recent developments and automatization of the solid-phase peptide synthesis have made it possible to produce larger biologically active polypeptide molecules [24–26], including the 99-residue HIV-1 and HIV-2 PRs [19,27,28]. However, the routine synthesis of large peptides (over 50 residues) has not yet been completely resolved. Although BLV PR has already been expressed in a bacterial expression vector [29], chemical synthesis may have some advantages: it is less labourious, faster, and contamination/degradation by cellular proteinases is not a concern.

Using the solid phase peptide synthesis, we were able to prepare the BLV PR, a 126 residue polypeptide, so far the largest proteinase which has been chemically synthesized. Peptides obtained with any of the HF cleavage method showed activity after refolding, however, the peptide subjected to HF cleavage protocol (ii) had the highest specific activity (data not shown). The amino acid composition of the peptide obtained with cleavage method (ii) and RP-HPLC purification was determined to be as follows (the numbers in parentheses represent the expected values): Asx 9.63 (10); Thr 5.08 (5); Ser 7.39 (9); Glx 10.60 (11); Pro 13.88 (14); Gly 9.61 (9); Ala 8.96 (9); Val 10.85 (11); Met 2.01 (2); Ile 7.36 (8); Leu 16.91 (17); Tyr 3.11 (3); Phe 2.41 (2); Lys 3.74 (3); Arg 8.45 (9); Trp was not determined. N-terminal sequence analysis performed up to 22 residues with a model 470A gas-phase sequencer (Applied Biosystems, Inc.) gave the proper sequence. The peptide was further purified by gel filtration and RP-HPLC. The last purification step is shown in Fig. 1. The peak fraction of the HPLC separation was active after refolding and it was found to be homogenous using SDS-PAGE (Fig. 1) having the expected molecular weight (15 kDa). The active fraction eluted on the RP-HPLC at 50% acetonitrile which is in good agreement to that found with the viral enzyme (52–55%) [30]. This preparation was able to cleave peptides in a time-dependent manner. The cleavage of the peptide KTKVL*VVQPK followed the Michaelis–Menten kinetics with an apparent K_m of 0.05 mM and apparent K_{cat} of 0.4 nmol·s^{-1}·mg^{-1} (using 1 h incubation time).

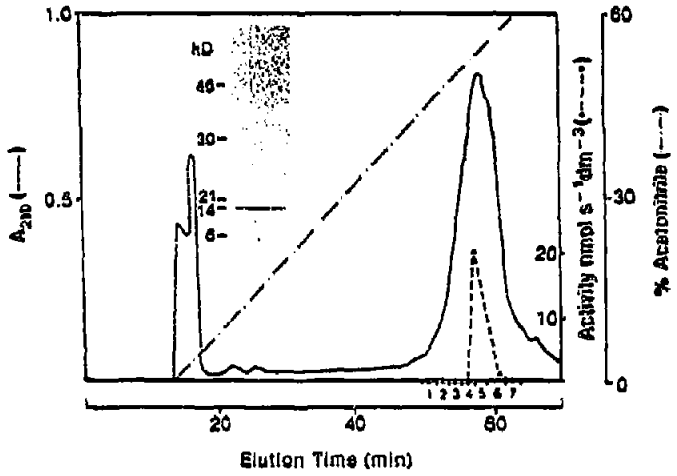

Fig. 1. Reversed-phase HPLC purification of chemically synthesized BLV proteinase. The polypeptide was cleaved from the resin by HF in the presence of anisole and ethanedithiole, purified by reversed-phase HPLC, then purified by gel filtration in 6 M Guanidine-HCl, 100 mM Tris-HCl. The peak fraction of the gel filtration was acidified to pH 2 by the addition of TFA and applied to a μBondapak C18 column (19 × 150 mm). The proteinase was eluted with an acetonitrile gradient into water in the presence of 0.05% TFA. Fractions were collected manually, lyophilized and refolded as described in section 2. Activity was measured using the oligopeptide KTKVL*VVQPK (representing the CA/NC cleavage site in HTLV-1). Insert shows the purity of the peak fraction (fraction 5) of RP-HPLC on SDS-polyacrylamide gel. The protein was stained with Coomassie brillant blue R-250.

3.2. *Specificity of BLV proteinase, comparison to that of chemically synthesized HIV-2 proteinase*

Oligopeptides representing determined cleavage sites in the polyproteins of BLV, HTLV-1, MuLV and HIV-1 were investigated as substrates of BLV PR. To compare the substrate specificity of BLV PR with HIV-2 PR, the peptides were also investigated as substrates of chemically synthesized HIV-2 PR.

Oligopeptides representing known Gag and Gag-Pro cleavage sites in BLV were all hydrolyzed by the BLV PR (Table I). Similar results were found with BLV PR expressed in *E. coli* [29]. This suggests, that the retroviral PR may be able to process the respective polyproteins, as it was suggested for HIV-1 and HIV-2 PR, using the same approach [20,31]. However, one BLV cleavage site peptide (peptide 2) was not hydrolyzed by

Table I

Comparison of the substrate specificity of chemically synthesized BLV and HIV-2 proteinases

Peptide	Represented cleavage site			Relative activity[a]	
				BLV PR	HIV-2 PR
1.	PPAIL•PIISE	BLV	MA/CA[b]	1.00	1.00
2.	KQPAIL•VHTPG	BLV	CA/NC	0.80	not hydrolyzed[c]
3.	ELECL•LSIPL	BLV	TF/PR	0.32	0.15[d]
4.	PPMVG•VLDAP	BLV	PR/p3	0.20	0.23
5.	APQVL•PVMHP	HTLV-1	MA/CA	1.2	1.5
6.	KTKVL•VVQPK	HTLV-1	CA/NC	1.9	3.1
7.	DPASIL•PVIP	HTLV-1	TF/PR	0.52	not hydrolyzed[c]
8.	RSSLY•PALTP[e]	MuLV	MA/p12	0.02	12.4
9.	TSQAF•PLRAG[e]	MuLV	p12/CA	0.21	11.8
10.	MSKLL•ATVVS[e]	MuLV	CA/NC	0.07	not hydrolyzed[c]
11.	PQTSLL•TLDDQ[e]	MuLV	NC/PR	0.14	not hydrolized[c]
12.	VSQNY•PIVQ	HIV-1	MA/CA	not hydrolyzed[c]	28.8
13.	KARVL•AEAMS	HIV-1	CA/X	0.13	6.1
14.	TATIM•MQRGN	HIV-1	X/NC	0.16	26.9
15.	VSFNF•PQITL	HIV-1	TF/PR	not hydrolyzed[c]	0.25

[a] Activities were measured at 0.2 mM substrate concentration, and a relative activity was expressed as the activity on a given peptide divided by the activity obtained for each enzyme with peptide 1.
[b] MA, matrix protein; CA, capsid protein; X, peptide between MA and CA in HIV-1 Gag; NC, nucleocapsid protein; TF, transframe protein; PR, retroviral proteinase (retroviral protein nomenclature from Leis et al. [37]). Cleavage sites are indicated by asterisks, and taken from [9,36].
[c] Not hydrolyzed during 24 h incubation.
[d] This peptide was hydrolyzed by the HIV-2 PR at the -Cys•Leu- bond, instead of the -Leu•Leu- bond found by the BLV PR.
[e] The peptides representing MuLV cleavage sites were also hydrolyzed by disrupted MuLV, at the predicted site.

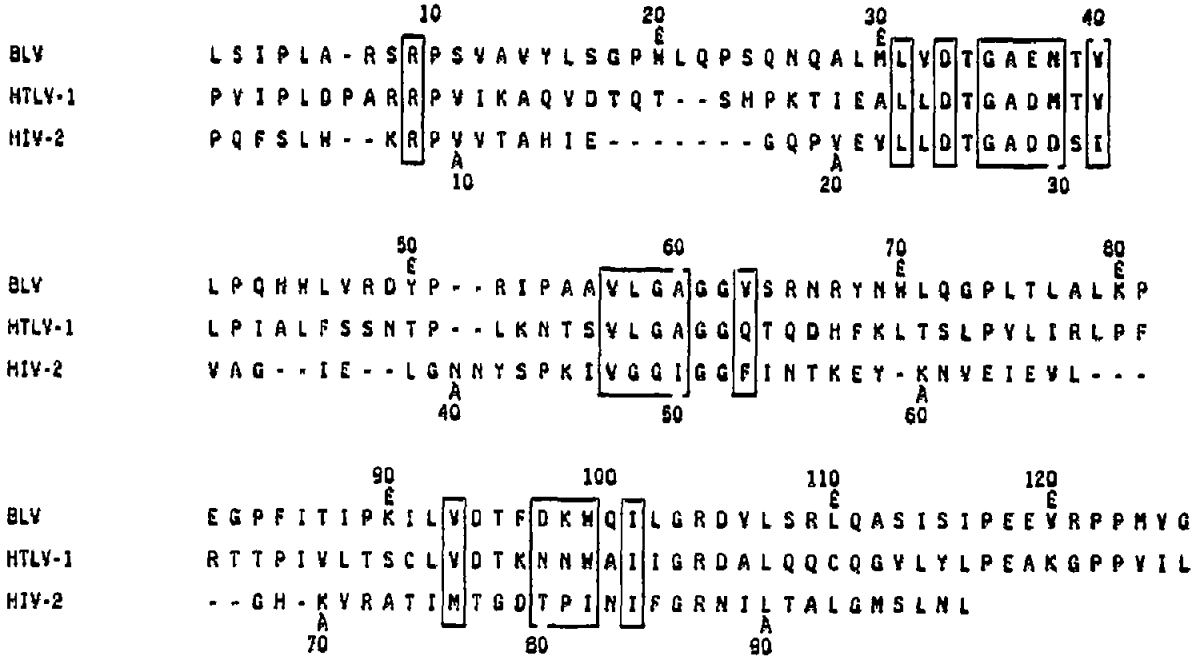

Fig. 2. Amino acid alignment of BLV, HTLV-1 and HIV-2 proteinases. The amino acid sequences are taken from the following literatures: BLV PR [12,14]; HTLV-1 PR [9]; HIV-2 PR [19]. Comparison is based on multiple alignment [38]. Those amino acids which are predicted to be involved in the enzyme-substrate interactions (S_4-S_3' region) based on the structures of HIV-1 PR-inhibitor complexes [39–41] and molecular modeling of HIV-PR substrate and inhibitor interactions [17,42] are boxed.

the HIV-2 PR, while another one (peptide 3) was hydrolyzed differently, at the -Cys*Leu- bond instead of the -Leu*Leu- bond.

The BLV PR was also able to hydrolyze the HTLV-I cleavage site peptides at the expected site (Table I). In fact, the highest relative activity was obtained with the oligopeptide representing the CA/NC cleavage site in HTLV-I (peptide 6), which was also sufficiently hydrolyzed by the HIV-2 PR.

Since it has been noted earlier, that the HTLV/BLV cleavage sites are homologous to the MuLV cleavage sites [32], we also investigated the cleavage of oligopeptides representing four MuLV Gag and Gag-Pol cleavage sites. In accordance with the cleavage site homology, these peptides were also hydrolyzed by the BLV PR at the expected site (Table I). Although two of the peptides representing MuLV cleavage sites were also substrates for HIV-2 PR, two of them were hydrolyzed only by the BLV PR.

Two peptides of HIV-1 cleavage site sequences were not hydrolyzable by the BLV PR, while one of them (peptide 12) was the most efficient substrate of HIV-2 PR from the selected peptides. These results suggest, that the HIV proteinase has a substrate specificity distinct from that of the BLV (and possibly the HTLV) PR.

3.3. *Lack of inhibition of the BLV PR by inhibitors of HIV proteinases*

Two specific inhibitors of HIV proteinases, SP-346 [33] and a phosphinicomethyl isostere (Compound 3 [34]) were assayed as inhibitors of BLV PR. None of these inhibitors were able to inhibit the BLV PR up to the highest concentration assayed (40 μM and 1 μM, respectively), while both were able to inhibit completely HIV-2 PR under identical conditions (IC_{50} values were 8 μM and 0.02 μM, respectively). This result further emphasizes the differences between the HIV and BLV proteinases. The lack of inhibition with SP-346 is not surprising, since it is a pipecolic acid containing analog of SP-211, an HIV PR substrate (peptide 12 in Table I), which is not hydrolyzed by the BLV PR. This peptide contains a typical Class I cleavage site of the immunodeficiency viruses having Asn at P_2, aromatic amino acid at P_1 and Pro at P_1' position [35]. Since another peptide representing Class 1 cleavage site was also not hydrolyzed by BLV PR (peptide 15), the typical Class 1 cleavage sites of HIV are not efficient substrates of BLV PR. In this regard, it is important to point out, that -Tyr/Phe*Pro- cleavage site does not occur in BLV and HTLV Gag polyproteins [36].

Although the HIV-2 PR substantially differs in size and amino acid composition from BLV and HTLV-1 PRs, many of the amino acids expected to be involved in enzyme-substrate interaction are conserved (Fig. 2). However, certain amino acid changes at the crucial parts of the substrate binding pocket like the change of Ile^{50} to Ala at the tip of the flap or the change of Thr^{80}-Pro^{81}-Ile^{82} as part of the S_1-S_1' binding pockets may be responsible for the substantial differences in specificity of the HIV and BLV proteinases.

HIV proteinases are potential targets for chemotherapy [2]. By analogy, it may be possible to treat ATL and enzootic bovine leukosis by administration of inhibitors of HTLV and BLV PRs. However, our initial results indicate, that the BLV PR (and possibly the HTLV PR) has a substantially different substrate specificity from that of HIV PRs, as also suggested by others [29]. Therefore it is likely, that inhibitors designed against the HIV PRs will not be suitable in therapy of enzootic bovine leukosis and ATL.

The protease of HTLV-1 has recently been expressed in *E. coli* [9]. The availability of both BLV and HTLV-1 PRs will allow comparative studies of their substrate specificity.

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