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Modelling, synthesis and biological activity of a BLV proteinase, made of (only) 116 amino acids

G. Précigoux[a], S. Geoffre[a], R. Léonard[a], S. Llido[a], A. Dautant[a], B. Langlois d'Estaintot[a], P. Picard[a], A. Ménard[b], B. Guillemain[b] and M. Hospital[a]

[a]*Laboratoire de Cristallographie, URA 144 CNRS, Université de Bordeaux I, 351 cours de la Libération, 33405 - Talence Cedex, France* and [b]*Fondation Bergonié, INSERM 328, Université de Bordeaux, 229 cours de l'Argonne, 33076 - Bordeaux Cedex, France*

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Bovine leukaemia virus (BLV) is the aetiological agent of *Leukosis enzootica bovis* [Viral Oncology (1980), G. Klein (Ed.) Raven Press, New York, pp. 231–238], a widely spread disease in cattle. BLV is reported as the animal model of human T-cell leukaemia virus (HLTV) which is the causative agent of adult T-cell leukaemia and tropical spastic paraparesis. Like the viruses themselves, the two retroviral proteinases (PR) are very closely related [Virology 142 (1985) 357–377]. BLV and HTLV-I PR are reported as putative proteins made of 126 [J. Virol. 57 (1986) 826–832] and 125 [FEBS Lett. 293 (1991) 106–110] amino acids, respectively (*long sequences*), belonging to the aspartyl proteinase family [Nature 329 (1987) 351–354], with the aid of molecular modelling, we show that BLV and HLTV-I proteinases made of only 116 and 115 amino acids, respectively (*short sequences*), display three-dimensional structures similar to that observed for other retroviral aspartyl proteinases. The models are based on three-dimensional structures of the Rous sarcoma virus (RSV PR) and the human immunodeficiency virus (HIV-1 PR). We used solid phase peptide synthesis to produce the putative proteolytic enzyme of BLV (116 amino acids). In this study, we show that the folded synthetic protease accurately hydrolyzes a decapeptide corresponding to the sequence of the Matrice–Capside (MA/CA) cleavage site of the *gag* polyprotein. In addition, the proteolytic activity is inhibited by a statine ((4*S*,3*S*)-4-amino-3-hydroxyl-6-methylheptanoic acid) containing an analogous sequence.

BLV protease; Modelling; Solid phase synthesis; Protease activity and inhibition

1. INTRODUCTION

Since all known aspartic proteinases contain a conserved active site and core structure, it is reasonable to attempt to use them to model the unknown structure of another aspartic proteinase. The crystal structure knowledge of homologous proteins is useful for sequence alignment and modelling of related protein. The crystal structures of RSV [6] and HIV-1 PR [7] were used to align the sequences of BLV and HTLV-I PR and to construct structural models of the latter. The amino acid sequences are shown in a structural alignment for RSV, BLV, HTLV-I and HIV-1 proteinases in Table I. As described previously [5,8,9], the structure alignment positioned all the deletions at surface turns between antiparallel β strands. The carboxyl extremities of RSV and HIV PR are aligned with hydrophobic residues Ile and Leu, followed by Pro, in the *long sequences* of BLV and HTLV-I PR, respectively. Such dipeptides are known to belong to the privileged cleavage sites of the BLV and HTLV-I PR. From Pro to the carboxyl extremity, ten amino acids of the BLV and HTLV-I *long sequences* do not have any equivalent residue in the alignment of the active RSV and HIV-1 PR, and subsequently were not used in the modelling studies.

Correspondence address: Dr. G. Precigoux, Université de Bordeaux, CNRS URA 144, Lab. de Cristallographie 351 cours de la Liberation, 33405 Talence Cedex, France. Fax: (33) (56) 846686.

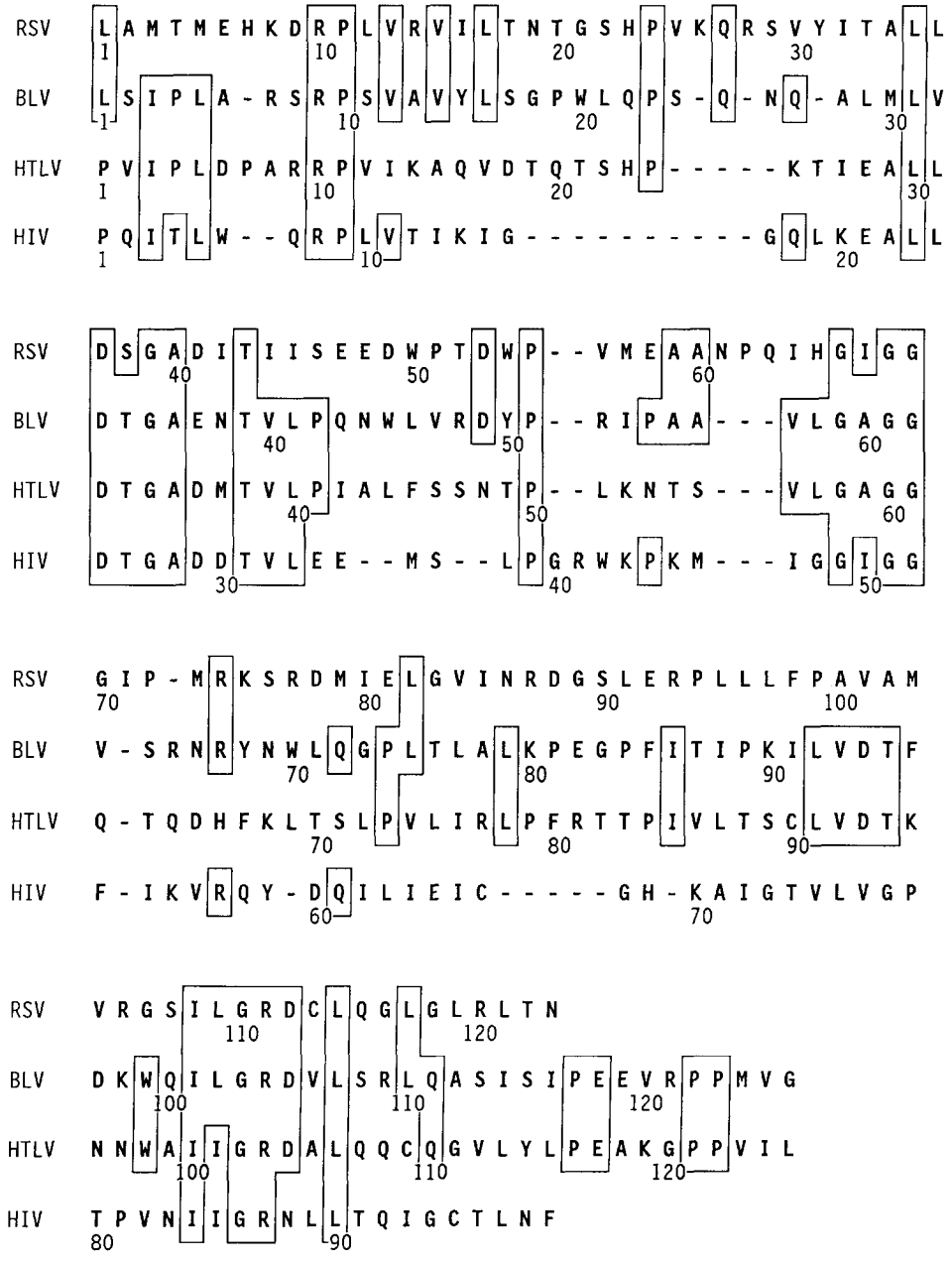
2. EXPERIMENTAL

Fig. 1 shows a stereo-view of the superimposed three-dimensional structures of BLV, HTLV-I and HIV PR. The model of RSV PR crystal structure [6], which was refined to 2.0 Å resolution with an agreement factor of 0.16 was used to place the main chain atoms. The amino acid side chains were placed either in conformation identical to those in RSV PR or in sterically acceptable conformations. The structural alignment of HTLV-I and BLV PR with RSV PR positioned one or two short deletions at surface turns located in the regions 25 or 7 and 25, respectively (RSV numbering). The conformations for the shortened loops were built as tight turns. A difficult part of the model building occurred at the surface loop called flap which is not completely visible in the electron density map of RSV PR. The model of the crystal structure of HIV-1 PR complexed with an inhibitor [7] was used to place the main and side chains of a few missing amino acids at the end of the flap. In such models, the ten supplementary residues, at the carboxyl extremity of the BLV and HTLV-I PR, would be located on the opposite side of the active site and would not act in the catalysis process.

We used solid phase peptide synthesis to produce the *short sequence*, a 116 amino acid polypeptide of the proteolytic enzyme of BLV. The synthesis was performed on an Applied Biosystems 431A peptide synthesizer using a minimum of two coupling steps followed by a capping step at each addition. The protein was cleaved from the resin by the hydrogen fluoride (HF) procedure in presence of anisole and ethanethiol. The crude extract was lyophilized and purified on a Sephadex G-50 column and reverse phase HPLC. The purified protein was analysed for composition. Furthermore, the NH_2-microsequence analysis of fifteen amino acids was achieved by the Applied Biosystem

Table I

Amino acid sequence alignment for the RSV, BLV, HTLV-I and HIV-1 PR. Boxes enclose regions where the BLV PR amino acid sequence is found conserved in other three proteinases.



manufacturer. Both experiments confirm the identity of the BLV proteinase.

The lyophilized protein was refolded with a procedure similar to the one described by Tamburini et al. [10], starting with an 8 M urea concentration. The three last dialysis steps versus 50-volumes of a solution containing 100 mM sodium citrate, pH 6.2, 0.5 M sodium chloride, 1 mM dithio-1,4-threitol (DTT) and 0.05% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), caused precipitation of the unfolded proteinase. Removal of precipitated protein by centrifugation (20,000 × *g* for 30 min) yielded a soluble proteinase with a concentration ca. 1 mg/ml as determined by Bradford assay [11]. As shown in Fig. 2, the proteinase was eluted at 60% acetonitrile by HPLC and was found to be homogenous using SDS-PAGE.

Proteolytic reactions were carried out with 10 μl of the supernatant and 10 μl of a 1 mM solution of the synthetic MA/CA analog decapeptide containing the cleavage site Leu-Pro, Tyr-Asp-Pro-Pro-Ala-Ile-Leu-Pro-Ile-Ile, as substrate at 37°C. Fig. 2 shows that the BLV PR can cleave the decapeptide. Two peptide products, distinct from the starting material were resolved by HPLC. The amino acid composition analysis of the two peptides verified that peak 1 contains a mixture of Pro and Ile (ratio 1:2) and peak two contains a solution of Tyr, Asp, Ala, Ile, Leu and Pro, ratio (1,1,1,1,1:2). The same experiment was done with the octapeptide Ser-Ile-Ser-Ile-Pro-Glu-Glu-Val, corre-

Fig. 1. Stereo view of the C_α chains of the superimposed BLV and HTLV-I models together with the HIV-1 complexed crystal structure. For clarity, only three proteinases were represented: thick line, HIV-1 PR; medium-thickness line, BLV PR and thin line, HTLV-I PR. The computer graphics program TURBO-FRODO [16] was run on the Silicon Graphics computer system. The minimization process was performed with X-PLOR [17] program, and required about 1,500 iterations to bring the systems to a stable state defined by a maximum derivative of the potential function < 0.01.

sponding to our putative conformational C-terminal end of the BLV PR. The peptide remained uncleaved, even with increased amounts of BLV PR and incubation time, confirming the effective presence of ten more residues in the native proteinase.

One putative statine containing inhibitor, Tyr-Asp-Pro-Pro-Ala-Ile-Sta-Ile-Ile, mimicking the MA/CA decapeptide substrate sequence, was synthesized and assayed as inhibitor for BLV PR. As shown on Fig. 3, as the concentration of this molecule increases, the enzymatic activity is inhibited. Similar experiments will be done using the recombinant HTLV-I PR *short sequence*.

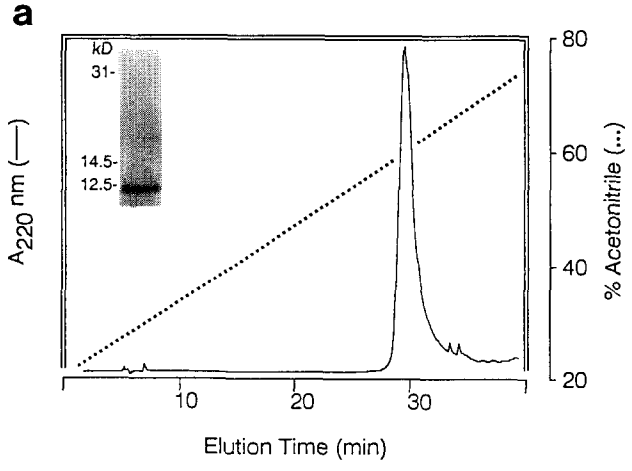
3. RESULTS AND DISCUSSION

The real value of our BLV PR model (*short sequence*) is underlined by the in vitro activity and inhibition of the synthetic proteinase.

Of the many questions that can be raised by the model, the biological role of the ten more amino acid residues, located at the C-terminus of the native proteinase, is of particular interest.

A recent work from Hayakawa et al. [12] on HTLV-I PR described amino acids 116–120 (HTLV-I numbering) as essential to process the *gag* p53 precursor polyprotein.

As HLTV-I and BLV PR are very closely related [2] a few questions still remain unanswered. Do the ten last amino acid residues of the two retroviral proteinases confer any functional advantage on the rate of enzymatic activity, or do they have a major function in the recognition of a privileged sequence of the gag precursor polyprotein?



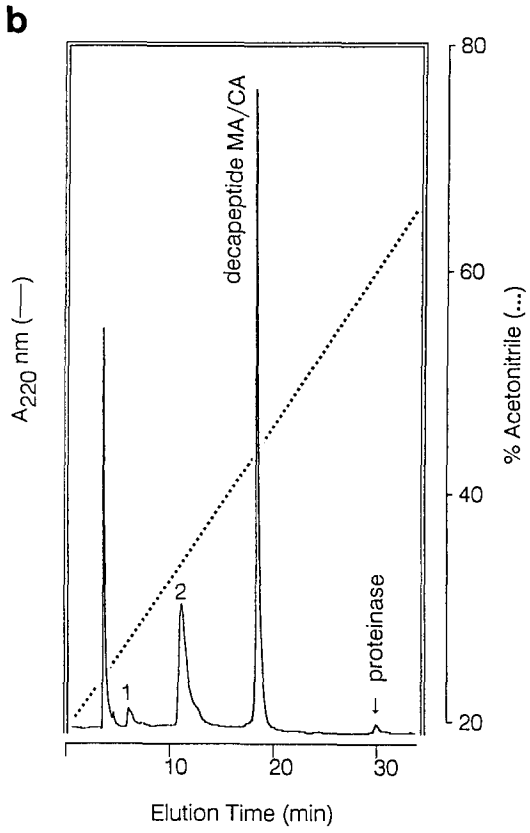


Fig. 2. (a) Reverse-phase HPLC of chemically synthesized BLV PR (116 amino acids). The proteinase applied to a Hyperbond C18, 10 μm (0.39 × 30 cm, SFCC/Shandon Scientific Ltd.) column is eluted in 30 min with a linear gradient, at a constant flow rate of 1 ml/min at (60:40, buffer A/buffer B). Buffer A: 0.08% trifluoroacetic acid (TFA) in acetonitrile. Buffer B: 0.1% TFA in water. The insert shows the purity of the HPLC peak on SDS-PAGE. The protein was silver nitrate stained. (b) Proteinase assays were performed at 37°C using chemically synthesized BLV PR in 100 mM sodium citrate, pH 6.2, 0.5 M NaCl, 1 mM DTT and 0.05% CHAPS, followed by the separation of the substrate and cleavage products by reverse phase HPLC. Amino acid analysis of the collected peaks 1 and 2 confirms the sequences Pro-Ile-Ile and Tyr-Asp-Pro-Pro-Ala-Ile-Leu, respectively.

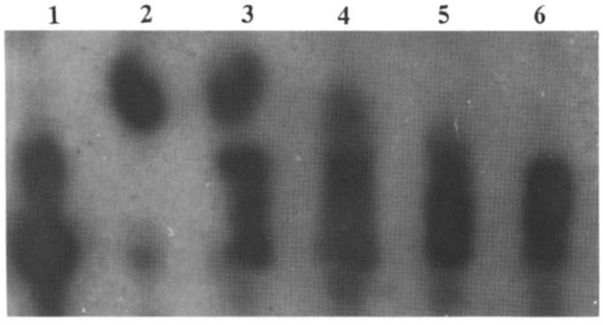


Fig. 3. Autoradiogram of the thin layer electrophoresis analysis. Proteolytic reactions were carried out in 10 μl of a proteinase solution (1 mg/ml), 100 mM sodium citrate, pH 6.2, 0.5 M NaCl, 1 mM DTT and 0.05% CHAPS, containing 10^{-10} mol of the ^{125}I-labeled Tyr-Asp-Pro-Pro-Ala-Ile-Leu-Pro-Ile-Ile peptide substrate. Lane 1, ^{125}I-labeled peptide substrate; lane 2, ^{125}I-labeled Tyr-Asp-Pro-Pro-Ala-Ile-Leu; lane 3, incubation at 37°C for 24 h in the presence of the peptide substrate; lanes 4 to 6, addition of 1 μl of inhibitor solutions, Tyr-Asp-Pro-Pro-Ala-Ile-Sta-Ile-Ile, with concentrations of 0.01, 0.1, 1 mg/ml, respectively.

Different means to obtain the BLV PR, chemical synthesis of the *long sequence* [13], expression in *E. coli* [14], extraction from the virus [3,15], will allow comparative studies of their specific activities. These experiments, together with crystallographic studies would shed a light on the possible role of these intriguing supplementary amino acids.

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