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High-level expression of enzymatically active bovine leukemia virus proteinase in *E. coli*

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An *E. coli* plasmid expressing efficiently an artificial precursor of bovine leukemia virus (BLV) proteinase under transcriptional control of the phage T7 promoter was constructed. The expression product accumulates in the induced *E. coli* cells in the form of insoluble cytoplasmic inclusions. Solubilization of the inclusions and a refolding step yield almost pure and completely self-processed proteinase. Purification to homogeneity was achieved by ion-exchange chromatography and reverse-phase HPLC. On a preparative scale, a high yield of enzymatically active proteinase was obtained. An initial study using a series of synthetic peptide substrates shows a distinct substrate specificity of BLV proteinase.

Retroviral proteinase; Polyprotein precursor processing; Recombinant product accumulation; Synthetic peptide substrate; Bovine leukemia virus; *E. coli*

1. INTRODUCTION

Bovine leukemia virus (BLV), the causative agent of chronic leukemia in cattle, sheep and goats, requires in its life cycle, like other retroviruses, specific activity of a virally coded processing proteinase [1].

DNA sequence homology analysis reveals that BLV is closely related to human T-cell leukemia viruses HTLV1 and HTLV2 (see [2]). The nucleotide sequence of proviral BLV DNA was determined independently in two laboratories [3,4]. Comparison of the deduced amino acid sequence with the sequence of proteinase isolated from virions [5] indicates that the proteinase is coded for by its own reading frame, different from both *gag* and *pol* genes. The synthesis of the polyprotein precursor Gag-Pro-Pol thus requires two frameshift events (one upstream and one downstream of the proteinase ORF) at sites whose exact positions, however, have not been determined conclusively [2]. In contrast to HIV1 proteinase which is, for obvious reasons, probably the best characterized retroviral processing proteinase (see e.g. [6,7]), data on the BLV enzyme remain rather scanty.

We report here the construction of an *E. coli* expression plasmid (pB603T7Q) that encodes an artificial 22 kDa proteinase precursor. This precursor undergoes accurate selfprocessing in vitro, yielding an active 14 kDa enzyme. Preliminary characterization of substrate specificity of the mature, chromatographically purified recombinant enzyme is also described.

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2. EXPERIMENTAL

The source of a coding sequence of BLV proteinase was the 823 bp *Sau*3A fragment (nt 1756 to 2578, [4]) from pBLV (a plasmid comprising full-length BLV proviral DNA, provided generously by Dr. N. Sagata). This fragment was placed under transcriptional and translational control of bacteriophage T7 gene 10 in a vector pT7Q9 (M.F. and M.A., unpublished results), a derivative of pRK172 [8], yielding the expression plasmid pB603T7Q. A host strain *E. coli* BL21(DE3) containing one copy of T7 RNA polymerase gene in its genome (as detailed in [9]), was used to overexpress the proteinase precursor from pB603T7Q. Bacteria were grown in a rich medium containing 100 μg/ml ampicillin and 25% glycerol at 37°C in flasks on a rotary shaker. The expression of the BLV proteinase precursor was induced at $A_{550} = 1.2$–1.5 by adding IPTG to a final concentration of 1 mM and the cells were harvested 90 min after induction. Cell lysates were obtained by a freeze–thaw cycle, addition of lysozyme and sonication. Cytoplasmic inclusions were pelleted and washed with 0.1% Triton X-100 [10]. The pellets of inclusion bodies were suspended in 0.5 ml of 0.2 M Tris-HCl (pH 7.0), 0.1 M sodium phosphate, 0.2 M NaCl, 0.01 M EDTA, 0.5% 2-mercaptoethanol, and dissolved in 10 ml of warm saturated solution of urea, and dialyzed overnight at 4°C against 3 litres of 0.01 M sodium acetate (pH 5), 0.002 M EDTA, 20% glycerol, 5% ethyleneglycol. The dialysate was centrifuged and the mature proteinase contained mainly in the supernatant was purified by SE-Sephadex chromatography and HPLC on C4 Vydac column (see section 3 for detailed procedures).

Activity assays. In the course of purification the BLV proteinase activity was assayed against the peptides PPAILPIIS and QPAVLNphVAP in 0.05 M sodium acetate (pH 5.3), 0.3 M NaCl at 37°C. Cleavage products were detected by HPLC (Vydac C18 column, linear methanol gradient). The specificity studies were performed under the same conditions (salt concentration, pH, temperature) using 0.4–0.8 mM concentrations of substrates incubated for 1 h in a final volume of 60 μl with 1 μg of proteinase.

Amino-terminal amino acid sequences were determined with an Applied Biosystems 470A protein sequencer.

3. RESULTS AND DISCUSSION

Heterologous expression in *E. coli* has proved a useful source of avian (e.g. [10]) as well as mammalian (e.g. [11]) retroviral proteinases in preparative amounts (for review see [6,7]).

In order to overexpress BLV proteinase in *E. coli* we constructed plasmid pB603T7Q based on the phage T7 RNA polymerase/promoter system [9] (see section 2). Similar expression vectors have been used for expression of HIV-1 proteinase (e.g. [11], see also [7]). The expected primary translation product of pB603T7Q is a fusion protein containing 4 amino acids derived from phage T7 gene 10 and linker, followed by BLV proteinase ORF sequence which comprises 126 amino acids of mature proteinase flanked on both N and C termini by 42 and 26 amino acids, respectively (Fig. 1). The exact sites of ribosomal frameshifts occurring during translation of viral messenger in infected eukaryotic cells are not known. Thus, it is not possible to surmise how far upstream and downstream of mature proteinase, the reading frame of our artificial precursor corresponds to that of the Gag-Pro and/or Gag-Pro-Pol viral translates. Regardless of this, both cleavage sites are preserved, making the artificial 22 kDa precursor synthesized in *E. coli* a potential substrate for selfprocessing leading to a mature 14 kDa proteinase.

All *E. coli* BL21(DE3)/pB603T7Q cells in induced culture formed cytoplasmic inclusion bodies. The inclusions formed within 90 min following induction contained unprocessed 22 kDa precursor as a major component (Fig. 2, lane 3). Some minor in vivo processing (of various extent in individual cultivations) was regularly observed (analyses not shown). Prolonged, overnight cultivations yielded a processed 14 kDa protein which, however, lacked proteolytic activity in in vitro assays. The 90 min induction period was therefore chosen for all experiments. The 22 kDa artificial precursor thus obtained was found to undergo readily selfprocessing in vitro, i.e. in the course of solubilization of inclusion bodies and subsequent dialysis. The resultant 14 kDa soluble protein (Fig. 2, compare lanes 3 and 4) displayed proteolytic activity. Due to the low solubility of BLV proteinase, some of the processed enzyme, as well as varying amounts of the unprocessed precursor were trapped in the precipitate (Fig. 2, lane 5) that had formed during dialysis. Yields of the active mature enzyme were further improved by subjecting the insoluble precipitate to additional rounds of solubilization/dialysis procedure.

The soluble fraction obtained after dialysis of dissolved inclusions is substantially enriched for the mature proteinase and shows a high degree of purity on SDS-PAGE (Fig. 2, lane 4). Further purification is achieved by SE-Sephadex column chromatography (Fig. 3a), which removes unprocessed precursor, peptide contaminants and most of lysozyme that was added when lysing the cells. BLV proteinase elutes at 0.23 M NaCl in the NaCl gradient (0–1 M, in sodium acetate buffer pH 5.0) (Fig. 3a). Similar elution profiles were observed with MAV and HIV1 proteinases ([12] and I. Pichová, personal communication). The final purification by HPLC (Fig. 3b) removes the last traces of contaminants and results in a homogeneous preparation of the enzyme (Fig. 2, lane 6). In all, 7 g of wet cell paste yielded approx. 1 g of washed inclusion bodies and these in turn yielded approx. 25 mg of homogeneous enzyme.

The N-terminal sequence LSIPLARSRP determined for SE-Sephadex and HPLC purified mature 14 kDa proteinase corresponds with that of the enzyme isolated from BLV virions [5]. The identical N-terminal sequences of the natural and recombinant enzymes suggest that the processing of the recombinant precursor at the amino terminus is accurate.

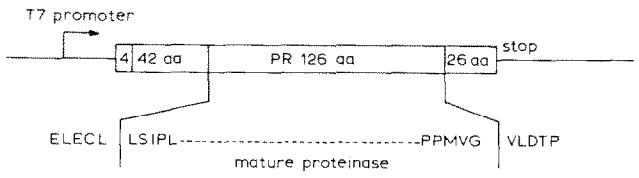

Fig. 1. Construction of BLV proteinase expression plasmid. Phage T7 promoter directs synthesis of a recombinant precursor comprising 4 vector-derived amino acids and 194 amino acids of BLV proteinase reading frame. The cleavage sites and the surrounding sequences at both the N- and C-termini of the mature proteinase are indicated. PR, proteinase; aa, amino acid.

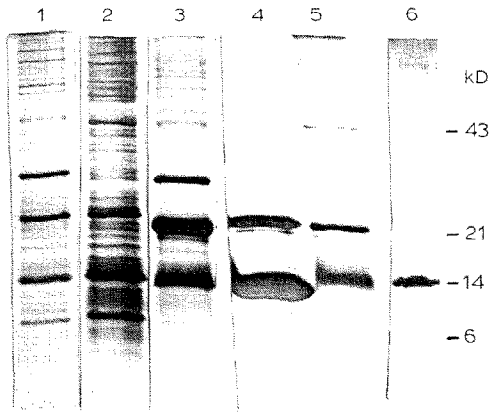

Fig. 2. BLV proteinase expression in IPTG-induced *E. coli* BL21(DE3)/pB603T7Q. SDS-PAGE shows the protein composition of the cell lysate (lane 1); soluble cytoplasmic fraction (lane 2); dissolved inclusions (lane 3); supernatant (lane 4) and sediment (lane 5) after dialysis of the dissolved inclusions; homogeneous SE-Sephadex and HPLC purified BLV proteinase (lane 6). Silver staining, 18% SDS-PAGE. The molecular weight markers are indicated on the right. For details see text.

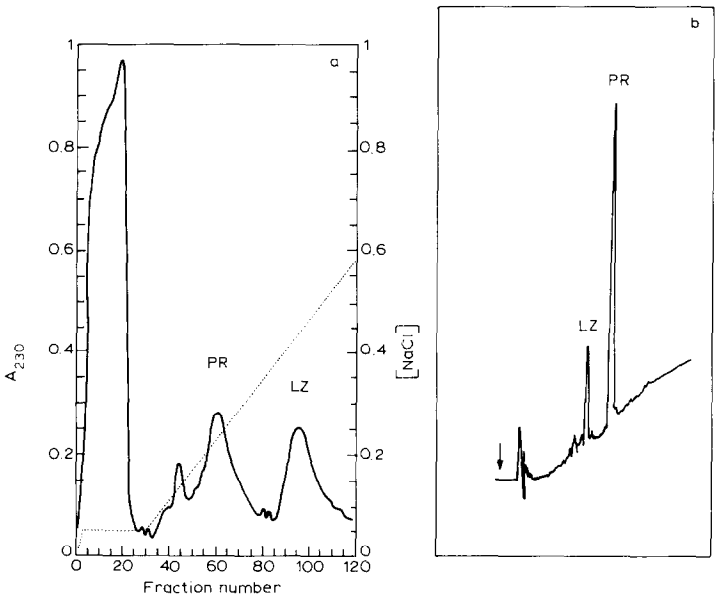

Fig. 3. Purification of BLV proteinase by chromatography on (a) SE-Sephadex and (b) reverse-phase HPLC. (a) The SE-Sephadex column was equilibrated in 0.05 M sodium acetate, pH 5. An elution gradient of NaCl was applied as indicated by the concentration curve. Solid line, elution profile; dotted line, NaCl gradient. (b) The material obtained after the cation-exchange chromatography was applied to a C4 Vydac column in water with 0.05% trifluoroacetic acid (TFA) and eluted with a gradient of acetonitrile containing 0.05% TFA. PR, proteinase; LZ, lysozyme.

The substrate specificity of the recombinant BLV proteinase was analyzed using a series of synthetic peptides representing putative cleavage sites of BLV, HTLV1, HIV1 and MAV polyproteins (Table I). Peptides 1, 2 and 3 are based on natural cleavage sites within BLV polyproteins [5], and peptide 5 is designed according to the consensus substrate sequence of proteinases of C-type retrovirus family [13]; they all were hydrolyzed readily. For peptide 1, K_m value 47 μM and V_{max}[2] μmol·min^{-1}·mg^{-1} were determined. In contrast, peptide 4 based on the BLV CA/NC processing site [5] was cleaved poorly. Interestingly, peptides 4 and 5 differ strikingly in their rate of cleavage, despite having almost identical non-prime residues. Much slower cleavage of peptide 4 is probably caused by the presence of a His residue in the P2′ position; in most retroviral proteinases studied so far, small hydrophobic side chains are preferred in P2 or P2′. The His residue in P2′ is unfavourable and synthetic peptide is cleaved poorly. In native polyprotein this might be compensated by other effects, like aggregation. With MAV proteinase we have also observed that some sequences which are efficiently processed in the virus were poorly cleaved in synthetic peptides [12,13]. The substrate 6 corresponding to HTLV1 MA/CA junction [14] was also cleaved efficiently.

Table I

Cleavage of synthetic substrates by BLV proteinase

Peptide substrate	Origin	Relative cleavage
1 ELECL*LSIPL	BLV n/PR	1.00
2 PPMVG*VLDAP	BLV PR/p13	0.60
3 PPAIL*PIIS	BLV MA/CA	0.90
4 QPALL*VHTP	BLV CA/NC	<0.01
5 QPAVL*NphVAP	Consensus C-type	0.65
6 APQVL*PVMHP	HTLV1 MA/CA	0.55
7 KARVY*NphEANle	HIV1 CA/X (modified)	0
8 KARVL*NphEAM	HIV1 CA/X (modified)	0
9 ATFQA*NphPLREA	MAV RT/IN (modified)	0
10 ATHQVY*NphVRKA	MAV RT/IN (modified)	0

Peptides 1–4 and 6 were designed according to processing sites of BLV [5] and HTLV1 [14] polyproteins, respectively; 5 is a chromogenic peptide, representing a consensus processing site based on target sites of polyproteins of C-type retroviruses [13]; 7–10 are prominent chromogenic peptide substrates based on HIV1 CA/X target site (7,8) [15] and on MAV RT/IN target site (9,10) [13]. The nomenclature of retroviral proteins is according to [16].

The asterisk indicates the expected cleavage site. Nph, 4-nitrophenylalanine; Nle, norleucine. The hydrolysis was followed by HPLC and amino acid analysis of cleavage products. The relative cleavage rates were calculated from the integrated areas of substrate and product peaks. For conditions see section 2.

Peptides 7 to 10 were found almost resistant to the BLV proteinase under the conditions used. Peptides 7 and 8 are prominent substrates of HIV1 proteinase (with K_m values lower than 15 μM and k_{cat} higher than 10 s^{-1} under similar conditions [15], and peptides 9 and 10 are good chromogenic substrates of MAV proteinase [13]. Nitrophenylalanine residue (Nph) introduced into P1′ position of peptides 5, 7, 8, 9 and 10 [13,15] increases extinction coefficients and improves detection of cleavage products on HPLC.

The substrate specificity of the BLV proteinase as characterized up to now is substantially distinct from

that of HIV1 and MAV proteinases. Possible similarity of BLV and HTLV proteinases should be further studied.

In conclusion, we have constructed an efficient system for bacterial expression of enzymatically active BLV proteinase, as witnessed by accurate selfprocessing of its precursor, and by specific cleavage of synthetic peptide substrates by the mature enzyme.

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