EFFECT OF SOLVENT COMPOSITION ON SOLUBILITY AND ACTIVITY OF BOVINE LEUKEMIA VIRUS PROTEINASE

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The solubility and activity of bovine leukemia virus proteinase (BLV PR) has been examined as a function of solvent composition in order to find a buffer system which would enable to prepare samples concentrated sufficiently for crystallization attempts and other studies. Low solubility and frequent precipitation was one of the major difficulties in the course of experiments carried out with BLV PR in several laboratories. We found that buffers composed of polyvalent ions, and containing glycerol, ethylene glycol or dimethyl sulfoxide, enabled to concentrate the enzyme up to 2 mg/ml. Presence of 4–20% of DMSO, isopropyl alcohol or ethylene glycol results in activation of BLV PR, while glycerol does not significantly affect the BLV PR activity. Zinc or magnesium ions substituting for sodium ions in commonly used buffers caused decrease both in solubility and activity of BLV PR.
Key words: BLV proteinase; Specific activity; Solubility; Solvent composition.

Bovine leukemia virus (BLV) is a B-cell lymphotropic retrovirus closely related to human T-cell leukemia viruses. BLV can naturally infect cattle and be experimentally transmitted to sheep, in which it induces a disease complex termed enzootic bovine leukosis[1]. Experimental infection of rabbits, however, causes severe depression in immune function[2,3]. Therefore, BLV is considered one of the models for chemotherapy of diseases caused by human retroviruses.

Retroviral proteinases (PR's) belong to the aspartic proteinase family. Proteinase of bovine leukemia virus (BLV PR) is very likely to function as a homodimeric enzyme, similarly to the HIV-1, HIV-2 and RSV proteinases, whose structures have been solved by X-ray crystallography (for review see refs[4,5] and references therein)**. However, BLV PR differs from the retroviral PR's studied so far in several important features: it

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has a different substrate specificity[6,7], it exhibits unusual thermostability[8] and it creates at least two active forms by stepwise selfprocessing[9]. These facts make the BLV PR an attractive subject for structural studies.

We have cloned, purified and characterized BLV PR (ref.[6]) and similar results were reported also by others[7,10]. However, both the kinetic studies and ongoing crystallization trials were complicated by the limited solubility of the BLV PR. Therefore it was necessary to examine the behavior of the enzyme under a set of conditions, in order to find more a favorable solvent system to handle the proteinase samples. In this report, we present an analysis of some of the components often used in storing and assay buffers for the retroviral proteinases, to determine their effects on the activity, solubility and stability of the BLV proteinase.

EXPERIMENTAL

Protein Expression and Purification

BLV proteinase was expressed as described previously[6] using *E. coli* strain BL21(DE3) and an expression vector pB603T7Q, coding for the 22 kDa proteinase precursor. Bacteria were grown in a rich medium containing 100 μg/ml ampicilin at 37 °C in flasks on a rotary shaker. The expression of BLV proteinase precursor was induced at A_{550} = 1.2–1.5 by adding IPTG to final concentration of 0.5 mmol/l. Cells were harvested 90 min after induction and disintegrated by a French press and sonication. Cytoplasmic inclusion bodies harboring the proteinase precursor, were pelleted, solubilized in warm saturated solution of urea and dialyzed. Proteinase cleaves itself out of the precursor in course of the renaturation. The dialysate was centrifuged and the mature proteinase contained mainly in the supernatant was purified using affinity chromatography on affigel-Boc-F[CH_2NH]FAFGKOMe. The support was equilibrated in 20 mM sodium citrate, 2 mM EDTA, pH 5.5 and incubated 30 min with the proteinase sample. The proteinase was eluted with 0.03 M TRIS-HCl, pH 9, 15% glycerol. The pH of the eluted material was immediately adjusted to 5.5. The material obtained was screened by activity assay (see below) and electrophoresis in 18% or 15% polyacrylamide gel in presence of sodium dodecylsulfate. The samples of BLV PR were stored at 4 °C.

The active enzyme fractions were concentrated by placing the samples in dialyzation tubing (Spectrapore 3500) on solid PEG 6000 at a room temperature.

Activity Assays

In the course of isolation, purification and storing, the activity of proteinase was monitored using a synthetic peptide containing the chromogenic nitrophenylalanine residue[6], Nph-Pro-Pro-Ala-Ile-Leu*Pro-Ile-Ile-Ser (derived from a cleavage site on the BLV structural polyprotein. The scissile

**Abbreviations used: MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TRIS, tris-(hydroxymethyl)aminomethane; DMSO, dimethylsulfoxide; HIV-1, HIV-2, human immunodeficiency virus type 1 and 2; RSV, Rous sarcoma virus; IPTG, isopropylthiogalactoside; PEG, polyethylene glycol; Nph nitrophenylalanine.

bond is indicated by asterisk). Cleavage was performed in 0.05 M sodium acetate, 0.3 M NaCl buffer pH 5.3 and followed by HPLC (Vydac C-18 column, linear methanol gradient). Typically, 50 pmol of a concentrated BLV PR sample was added to 100 μl of the substrate solution in the assay buffer and incubated at 37 °C for 15 min. The final concentration of substrate was 0.4–0.8 mmol/l. The reaction was stopped by the addition of 20% trifluoroacetic acid to a final concentration of 2% (v/v).

Measurements

The purified and precipitated BLV PR samples were solubilized in the saturated urea solution. The solubilized samples were renaturated by dialysis against the buffers of interest. The dialysis was performed for 24 h at least, at 4 °C and the tubing was transferred 3 times to a fresh batch of buffer. Then the tubings containing the BLV PR samples in various buffers were placed on PEG 6000 and concentrated at a room temperature. The absorbance at 550 nm was checked in the individual samples every 30 min, in order to monitor the occurrence of precipitation. Approximately one hour after the occurrence of precipitate, the protein concentration in the soluble fraction was determined. The activity of the soluble fraction was checked as described above and a specific activity was calculated. Routinely, we determined the protein concentration using the Bradford method[11]. The concentration of selected samples was verified by amino acid analysis. Since all the samples used in this study were purified using the affinity column, the results obtained from the Bradford method were in a good agreement with those from amino acid analysis.

For the examination of the effect of cations on the BLV PR solubility, the PIPES-citrate buffer pH 6.8 was used. The activity of BLV PR in presence of various chemical additives was tested using the HEPES-citrate buffer pH 7.8.

RESULTS AND DISCUSSION

Effect of Buffer on the Solubility of BLV PR

Long term observations revealed that even a prolonged storing does not cause a substantial decrease in the specific activity of the BLV PR. The samples examined were stored up to two years in 4 °C, usually in sodium acetate, sodium phosphate or TRIS-HCl buffer and protein concentration was 0.02–0.7 mg/ml. Storing in pH ranging from about 5.5 to 8.8 does not cause any damage of the enzyme and neither does a short term (several hours) storing at a room temperature. However, precipitation occurred usually with time in the stock solutions.

The active proteinase can be recovered from the precipitated material by resolubilization in the saturated urea solution and dialysis against an appropriate buffer. Such a procedure may be followed by a decrease in the specific activity of the enzyme (up to 10%). Therefore, a set of various buffers has been examined in order to find a system convenient for a long term storage, concentrating or resolubilization of the BLV PR.

The calculated isoelectric point of BLV PR (ref.[10]) is above 10.2. The data summarized in the Table I suggest that the pH value of the buffer used, when ranging from 5.5 to 7.8, is of a less importance than the ions present. Though, the data obtained up to now indicate, that the buffers of pH above 6 are more favorable for the solubility of the

BLV proteinase. No significant changes in the specific activity have been observed when BLV PR was concentrated in the buffers listed in Table I.

The results also demonstrate that polyvalent ions (citrate, TRIS) stabilize the BLV PR solutions more efficiently than the acetate buffer that has been used in our initial studies. In 20 mM sodium citrate solution, the maximum enzyme concentration may reach more than a double value than in the acetate buffer of the same pH. Similarly, Mason–Pfizer monkey virus proteinase, another retroviral PR, displays significantly better solubility in MES-chloride than in acetate buffer. For the BLV PR, the HEPES-citrate system was found to be the most favorable of the buffers tested, enabling to concentrate the samples up to 1.3 mg/ml, without any decrease in the specific activity and without precipitation. The concentration of HEPES or PIPES buffers (20 mmol/l) appeared to be optimal for the solubility of the BLV PR. Both in more diluted and more concentrated buffers, the maximum proteinase concentration was slightly lower (data not shown).

Effect of Cations

The solubility of BLV proteinase was examined in presence of various cations substituting for the sodium ions, which are present in the most of commonly used buffers.

TABLE I

Effect of buffer composition and pH on the solubility and specific activity of BLV PR. The specific activity is expressed relatively to that determined in sodium acetate buffer. The protein concentration was determined by Bradford method[11]. Each data point represents an average of three activity and concentration determinations. The experimental errors range from 10 to 15% for RP HPLC activity assays and up to 10% for values of protein concentration

Buffer component 20 mmol	pH	Specific activity relative	Maximum protein concentration mg/ml
Sodium acetate	5.5	1.00	0.30
Sodium citrate	5.5	1.00	0.75
	6.5	1.05	0.92
Sodium succinate	6.0	0.96	0.52
MES chloride	6.0	0.98	0.98
Sodium PIPES	6.8	1.02	1.02
Sodium phosphate	7.0	1.00	1.10
TRIS-HCl	7.5	1.02	0.93
Sodium HEPES	7.8	1.05	1.10
PIPES-citrate	6.8	1.02	1.25
HEPES-citrate	7.8	1.05	1.32

This particular experiment was carried out using the PIPES-citrate buffer pH 6.8 and the metal ions of interest were added as chlorides. The BLV PR samples were transferred to the individual buffers by dialysis, as described in Experimental. The results summarized in the Table II demonstrate that lithium, potassium, zinc, calcium and magnesium, when used at 20 mM concentration, do not help to enhance the solubility of BLV PR. On the contrary, presence of these cations caused decrease both in solubility and the specific activity of the enzyme. Particularly, in presence of magnesium or zinc, the specific activity drops significantly. Similar observations with HIV-1 PR were reported by Zhang et al.[12] and Woon et al.[13].

Effect of Chemical Additives

Jordan et al.[14] carried out a study with the HIV-1 and HIV-2 PR's, in order to evaluate an effect of solvent on the enzyme activity and to explain the discordance in the data on dimerization constants of the proteinases published so far. It has been demonstrated that the behavior of HIV-1 PR is affected dramatically by many factors which should be analyzed thoroughly in order to avoid errors in all types of experiments. The activity of HIV-1 PR was found to be particularly sensitive to organic solvents such as glycerol or DMSO. Therefore, we analyzed the effect of these compounds on both activity and solubility of BLV PR.

The changes in the activity which occur with the increasing concentration of the above mentioned compounds are illustrated in Fig. 1. The rate of the substrate hydrolysis by BLV PR increases in presence of DMSO, ethylene glycol or isopropyl alcohol. In presence of glycerol, only minor changes in the specific activity have been observed.

TABLE II

Effect of cations on the solubility and specific activity of BLV PR. Maximum concentration of the BLV PR achieved in presence of various cations. The specific activity of the enzyme is expressed relatively to that determined in the sodium containing buffer. Each result is an average of three determinations. Experimental errors correspond to those mentioned in Table I

Cation, 20 mmol	Specific activity relative	Maximum protein cocncentration mg/ml
Na^+	1.0	1.26
K^+	0.96	1.16
Li^+	0.90	0.90
Ca^{2+}	0.88	0.93
Zn^{2+}	0.30	0.80
Mg^{2+}	0.27	0.84

The increase of the BLV PR activity caused by 20% (v/v) either of DMSO or of ethylene glycol is about 30%. The substrate hydrolysis rate increases even more rapidly at the increasing concentration of isopropyl alcohol: at the highest concentration tested, 20%, the increase of the BLV PR activity is as high as 82%. Such a behavior of BLV PR is surprising, since e.g. the HIV-1 PR activity is inhibited by glycerol or DMSO (ref.[14]). These findings are of a great importance in particular for the kinetic measurements, because a number of substrates and inhibitors of BLV PR are soluble only in DMSO, while glycerol or ethylene glycol are frequently used in storing or assay buffers.

While 4–10% DMSO or isopropyl alcohol do not significantly affect the maximum concentration achieved in the BLV PR solutions (data not shown), ethylene glycol and glycerol help to prevent precipitation in concentrated samples and to reach higher protein concentrations. In presence of 7–10% glycerol (HEPES-citrate buffer, pH 7.8), it was possible to concentrate BLV PR up to 2 mg/ml. Such samples are still not concentrated sufficiently for crystallization attempts which require protein solutions of about 6 mg/ml at least. Therefore, other experiments are needed to further improve the conditions of the BLV PR treatment.

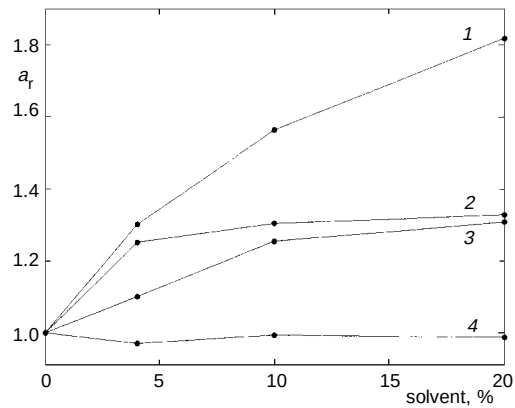

FIG. 1
Effect of isopropyl alcohol (*1*), DMSO (*2*), ethylene glycol (*3*) and glycerol (*4*) on the specific activity of BLV PR. The activity of of BLV PR was monitored using the HPLC assay (see Experimental). The activity of BLV PR is expressed relatively to that determined in absence of the additives tested: $a_r = a/a_0$. a_r Relative activity, a activity in the presence of the solvent addition, a_0 activity without the solvent addition. Each data point represents an average of three activity values determined with the error reflecting the standard deviation of the used method

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