Expression and characterization of human foamy virus proteinase

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Abstract The human foamy virus proteinase was expressed in fusion with maltose binding protein in *Escherichia coli* and purified. The specific activity of the fusion protein was similar to that of the processed enzyme. The kinetic constants on foamy virus cleavage site substrates were very low but comparable to those obtained with the *gag*-encoded avian proteinase on its own substrates. The proteinase showed preference for high ionic strength and a pH optimum of 6.6. None of the tested retroviral cleavage site peptides were substrates, however, some peptides representing cleavage sites in retrotransposons were properly processed by the enzyme.

Key words: Human foamy virus; Proteinase; Enzyme kinetics; Substrate specificity

1. Introduction

The retroviral proteinase (PR) plays a crucial role in the maturation of virus by processing the Gag and Gag-Pol polyproteins [1]. The PR of HIV is a very good target for chemotherapy of AIDS and several PR inhibitors are now in clinical use [2]. Much less is known about the structure and specificity of the proteinase of another human retrovirus, that of the human foamy virus (HFV). Retroviruses of the foamy virus subgroup have several unusual features: the Pol protein is synthesized independently from Gag, viral particles contain almost full length reverse-transcribed linear cDNA, and the nucleocapsid domain does not have the consensus zinc finger motif [3,4]. In addition, Gag does not appear to be efficiently processed, except at one site close to the C-terminus [4]. Mutation of the putative HFV PR active site resulted in non-infectious virions, suggesting that the PR is essential for viral infectivity [5]. In a vaccinia virus expression system HFV PR was able to cleave HFV Gag, however, it did not cleave other retroviral Gag precursors [6]. A 100 residue long HFV PR was cloned and found to be active as part of a thioredoxin fusion protein, but the activity was lost after elimination of the heterologous protein part fused with the enzyme coding sequence [7]. Later the HFV PR was cloned with C-terminal His-tag and this enzyme was used to determine the cleavage sites in the Pol polyprotein [8]. However, no kinetic data have thus far been reported on the enzyme. Here we report the expression of a 141 residue long part of the HFV as a fusion protein with maltose binding protein. The effect of salt on the enzyme activity as well as the pH profile of the enzyme have been determined, and the specificity of the proteinase was characterized using oligopeptides representing cleavage sites of various retroviruses and retrotransposons.

2. Materials and methods

2.1. Cloning of the HFV PR

A 451 bp DNA containing the putative HFV PR coding region of the plasmid p13HFV (kindly provided by Dr. R. Flügel, Germany), derived by PCR amplification was cloned into the *Xmn*I and *Bam*HI sites of pMAL-c2 vector (New England Biolabs) after the maltose binding protein (MBP) gene, to produce pMBP-HFVPR. DNA sequencing reaction of the cloned proteinase was performed with ABI-Prism dye terminator cycle sequencing kit (Perkin-Elmer) and analyzed on an Applied Biosystem Model 373A Sequencer. The protein sequence coded by pMBP-HFVPR, as compared to the sequence of HIV-1 and HTLV-1 PRs, is shown in Fig. 1. For the purification of the fusion protein 500 ml freshly prepared *Escherichia coli* cultures bearing the plasmid construct were grown at 37°C up to an absorbance at 600 nm of 0.2–0.5, in Luria-Bertani medium containing 0.1% (w/v) ampicillin. Then, induction with IPTG (1.0 mM) was done for 2 h and cells were harvested by centrifugation at $2000 \times g$ for 20 min at 4°C. After removal of the supernatant, 25 ml of lysis buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 200 mM NaCl, 1 mM DTT) was added. Cells were disrupted by freezing-thawing followed by sonication on ice. Then, samples were centrifuged at $9000 \times g$ for 30 min at 4°C, the supernatant was diluted fivefold with lysis buffer, loaded on a column containing amylose resin (3 ml), and extensively washed with the lysis buffer. The fusion protein was eluted with lysis buffer containing 10 mM maltose. Fractions containing high absorbance at 280 nm were pooled. Typically 12–17 mg fusion protein was obtained per gram wet weight of *E. coli* cells. Cleavage of the isolated fusion protein was performed with factor Xa by 2 h incubation at 37°C. The mixture of the cleaved protease and MBP was purified by gel filtration chromatography, in a Superdex G-75 HR 10/30 column (Pharmacia), equilibrated in 50 mM Tris, pH 8.3, containing 4 M guanidine-HCl. Collected fractions containing the protease were dialyzed against buffer without guanidine-HCl, to regain activity. Purity of the proteinase was assessed by SDS-PAGE, using 10–20% gradient gels. The amino-terminal sequence of the processed protease was determined after SDS-PAGE and electrotransfer to a polyvinylidene difluoride-type membrane using a Knauer Model 910 protein sequencer. Protein con-

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Abbreviations: BLV, bovine leukemia virus; HIV, human immunodeficiency virus; HFV, human foamy virus; HTLV, human T-cell leukemia virus; MMTV, mouse mammary tumor virus; RSV, Rous sarcoma virus; Nomenclature of retroviral proteins: MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, proteinase; IN, integrase; RT, reverse transcriptase

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centrations were determined by the Bradford spectrophotometric method [9]. Rabbit antiserum against the conserved active site region of the HFV protease [10] was used for immunoblotting.

2.2. Proteolytic assay

Oligopeptides were synthesized as described previously [11]. The PR assays were initiated by the mixing of 5 μl purified fusion proteinase or processed proteinase (15–130 μM) with 10 μl 2× incubation buffer (0.1 M MES, 0.2 M Tris, 0.1 M acetate and 2 M NaCl, pH 6.6) and 5 μl 0.01–0.6 mM substrate at 37°C for 1 or 2 h. The reaction was stopped by the addition of 180 μl 1% trifluoroacetic acid, and an aliquot was analyzed by reversed-phase HPLC as described previously [11]. Oligopeptides not hydrolyzed during the 2 h period were assayed at 0.2 mM final substrate concentration by incubating them with 50 μM enzyme overnight. Based on the detection limit of the assay, the estimated k_{cat} threshold value for these substrates is smaller than 1×10^{-5} s^{-1}. Cleavage products of PR-catalyzed hydrolysis were identified by amino acid analysis and/or by N-terminal sequencing. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis–Menten equation (or to linear equation if $K_m \ll [S]$) by using the Fig. P program (Fig. P Software Corp.).

2.3. Determination of the ionic strength effect on the activity, pH optimum and urea denaturation curve of the enzyme

The pH optimum of the enzyme was determined in 100 mM MES, 200 mM Tris, 100 mM acetate buffer, in the pH range of 5–8, in the presence of 0.15 mM substrate. The effect of ionic strength was determined at pH 7.0 in the same buffer by the addition of NaCl to the indicated final concentration. Urea dissociation curve was determined in the presence of 1 M NaCl in the same buffer by addition of urea to the indicated final concentration

3. Results

Using a template plasmid containing an infectious HFV clone [12], the 5′ region of HFV containing the PR coding sequence (Fig. 1) was amplified by PCR, the obtained DNA was cloned into pMAL-c2, and expressed in fusion with MBP in *E. coli*. The fusion protein was purified on amylose resin, followed by gel filtration under denaturing conditions, as shown in Fig. 2. The purified chimeric protein cleaved an oligopeptide substrate (SRAVN*TVTQS, asterisk indicates the site of cleavage) having the cleavage site sequence in the nucleocapsid domain of the Gag [7] at the expected site as determined by Edman degradation and amino acid analysis of the fragments (Fig. 3). Oligopeptides representing the Pol cleavage sites of HFV were also hydrolyzed at the sites previously determined [8], and kinetic parameters were determined for some of these peptides (Table 1). While the K_m value was within the expected range, the obtained catalytic constants were much lower than those we previously determined for various mammalian retroviral proteinases coded on *pol* genes [13–15] but similar to those obtained with *gag*-encoded avian PR [11]. The avian PR is produced in an equimolar amount to the structural Gag proteins, while *pol*-encoded PRs are present in only 10–20-fold lower concentration in the virion. It is important to note that the HFV PR contains a Ser in the active site triplet, similar to the avian PR as well as yeast retrotransposons, as compared to Thr in the PR of HIV and most other retroviruses (see Fig. 1). Furthermore, substitution of Ser to Thr in the avian enzyme substantially increased its activity [16] while the reverse mutation in HIV-1 PR decreased the catalytic efficiency [17]. However, it should be noted that substitution of the active site Ser of HFV PR to Thr did not cause a change in virion infectivity [5].

The fusion protein was processed with factor Xa. The first 11 residues of the processed PR was ISMNPLQLLQP– as

Table 1
Oligopeptide substrates representing naturally occurring cleavage sites in retroviruses and retrotransposons tested as substrates of the HFV PR

No.	Sequence	Source	Site	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1.	SRAVN*TVTQS	HFV	Gag(NC)[a]	0.56 ± 0.10	0.006 ± 0.0005	0.011
2.	WQHWEN*QVGHR	HFV	PR/RT[b]	not determined[c]		
3.	YEGVF*YTDGS	HFV	RT/RH[b]	> 1.0[d]		0.001[d]
4.	GSYVVN*CNTKK	HFV	RH/IN[b]	0.91 ± 0.08	0.003 ± 0.0002	0.003
5.	NGYVH*TVRTR	Ty3	Gag(NC)[e]	not hydrolyzed		
6.	SRAVY*TITPE	Ty3	RT/IN[e]	0.15 ± 0.05	0.001 ± 0.0002	0.007
7.	SNVVS*TIQS	Ty3	RT/IN[e]	not determined[c]		
8.	TARAH*NVSTS	Ty1	Gag(NC)[f]	not hydrolyzed		
9.	VPTIN*NVHTS	Ty1	PR/IN[f]	0.03 ± 0.01	0.0002 ± 0.00003	0.007
10.	IHLIA*AVKAV	Ty1	IN/RT[f]	1.00 ± 0.16	0.002 ± 0.0002	0.002
11.	ELECL*LSIPL	BLV	TF/PR[g]	not hydrolyzed		
12.	PPMVG*VLDAP	BLV	PR/p13[g]	not hydrolyzed		
13.	DSKAF*LATDQ	MMTV	pp21/p3[g]	not hydrolyzed		
14.	DELIL*PVKRK	MMTV	p3/p8[g]	not hydrolyzed		
15.	IQPLIM*AVVNR	RSV	X2/NC[g]	not hydrolyzed		
16.	RATVL*TVALH	RSV	PR′/RT[g]	not hydrolyzed		
17.	TFQAY*PLREA	RSV	RT/IN[g]	not hydrolyzed		
18.	VSQNY*PIVQ	HIV-1	MA/CA[g]	not hydrolyzed		
19.	KARVL*AEAMS	HIV-1	CA/p2[g]	not hydrolyzed		
20.	TATIM*MQRG	HIV-1	p2/NC[g]	not hydrolyzed		
21.	RPQNF*LQSRP	HIV-1	p1/p6[g]	not hydrolyzed		
22.	APQVL*PVMHP	HTLV-1	MA/CA[g]	not hydrolyzed		
23.	KTKVL*VVQPK	HTLV-1	CA/NC[g]	not hydrolyzed		

[a]Cleavage site determined by Pfrepper et al. [7].
[b]Cleavage site determined by Pfrepper et al. [8].
[c]Small degree of hydrolysis was observed at the indicated position during overnight incubation.
[d]For this substrate no saturation of the enzyme was achieved within the studied substrate concentration range. The k_{cat}/K_m value was calculated from measurements performed in first-order kinetic conditions ($[S] \ll K_m$).
[e]Cleavage sites published by Kirchner and Sendmeyer [28].
[f]Cleavage sites published by Merkulov et al. [23].
[g]These cleavage site peptides were substrates of the respective proteinases [11,15,18,22].

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                                                *
HIV1            PQITLW--QRPLVTIRIGGQLK-----EALLDTGADDTVLEEMNLPG---KWKPKMIGGI

HTLV-1          PVIPLDPARRPVIKAQVDTQTSHPKTIEALLDTGADMTVLPIALFSSNTPLKNTSVLGAG

HFV            ISMNPLQ-LLQPLPAEIKGTKLL------AHWDSGATITCIPESFLEDEQPIKKTLIKTIH


HIV1            GGFIKVRQYDQIPVEICG----HKAIGTVLVGPTPVN--IIGRNLLTQIGCTLNF

HTLV1           GQTQDHFKLTSLPVLIRLPFRTTPIVLTSCLVDTKNNWAIIGRDALQQCQGVLYLPEAKGPPVIL

HFV             GEKQQNVYYVTFKVKGRK---VEAEVIASPYEYILLSPTDVPWLTQQPLQLTILVPLQEYQEKIL


HIV1            -
HTLV1           -
HFV             SKTALPEDQKQQLKTLFVKYDNLWQHW
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Fig. 1. Sequence comparison of HIV-1, HTLV-1 and HFV proteinases. Alignment was made with the CLUSTAL W (1.74) program. Identical amino acids are shown in bold. The active site aspartate is indicated by an asterisk. The cloned HFV proteinase contained two extra residues (underlined) at the N-terminus to maintain *Xmn*I and factor Xa cleavage sites.

determined by Edman degradation, verifying that the processing occurred at the expected site. The PR contained two extra amino acids (underlined) required to maintain the integrity of *Xmn*I and Factor Xa cleavage sites, not found in the viral proteinase sequence. The effect of the two extra residues is expected to be negligible on the PR activity [18]. Specific activity of the processed HFV PR on the decapeptide substrate was $110 \pm 2\%$ of the unprocessed enzyme as determined at 0.2 mM substrate concentration in a duplicate measurement. The processed fusion protein was purified from the maltose binding protein by gel filtration chromatography in the presence of 4 M guanidine-HCl as shown in Fig. 2. The PR was estimated to be 90% pure based on Coomassie staining, and reacted with rabbit anti-HFV PR antiserum (data not shown). The purified PR was refolded by dialysis, however, only residual activity was detected as compared to the non-separated HFV PR (data not shown). The loss of activity after processing from another type of fusion protein has also been reported in the literature [7].

The activity of the foamy PR in the fusion with MBP was strongly dependent on the ionic strength of the buffer as shown in Fig. 4A, a common characteristics of retroviral PRs [19]. The pH optimum of the fusion protein was also determined at low and high ionic strength and found to be 6.6–6.8 (Fig. 4B). The processed enzyme gave an identical pH profile (data not shown).

The HFV PR was found to dimerize [8] and the enzyme is expected to be active in the dimeric form, similar to other retroviral proteinases [1]. Previous studies indicated the Ser in place of Thr in the 'fireman's grip' formed by the active site triplet may result in less stable dimers of retroviral proteinases [20]. Activity measurements with the HFV PR required concentrated enzyme, which also suggested that besides the low catalytic activity, the stability of the PR dimer could also be low, as compared to other retroviral PRs. Therefore the urea denaturation curves for the unprocessed fusion protein and the processed HFV PR were determined (Fig. 5). The urea concentration leading to 50% loss in enzymatic activity of both forms of enzyme was 0.75 M as compared to the 1.85 M for the HIV-1 PR [21].

Several oligopeptide substrates representing naturally occurring cleavage sites in retroviruses were tested as substrates of the HFV PR (Table 1). These substrates were previously found to be properly hydrolyzed by their respective proteinases [11,13,15,18,22]. Furthermore, all of these peptides were also substrates of the HIV-1 PR ([11,13,15,22], our unpublished results), however, none of them were hydrolyzed by the HFV enzyme (Table 1). Peptides representing cleavage sites in yeast retrotransposons were also assayed, and four out of the six tested were found to be cleaved by HFV PR (Table 1).

4. Discussion

Unlike other mammalian retroviruses which encode the PR in the Pol part of the Gag-Pol (Gag-Pro-Pol) polyproteins, the HFV PR was found to be coded on a separate Pol gene and transcribed from a spliced mRNA [3,4]. Sequence comparison with the PRs of the other human retroviruses (Fig. 1), as well as molecular modeling of the HFV PR based on the crystal structure of several retroviral PRs (P. Bagossi, Gy. Fenyöfalvi

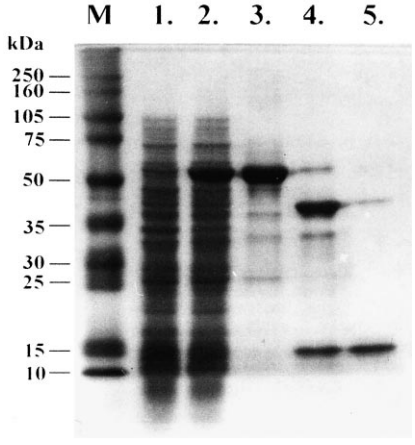

Fig. 2. Expression and purification of HFV proteinase. Analysis of total cell lysate prepared from *E. coli* cells bearing the construct pHFVPR-MBP before (lane 1) and after (lane 2) IPTG induction are shown. The fusion protein was purified on an amylose column (lane 3), processed with factor X (lane 4) then the processed PR was purified by gel filtration (lane 5). Rainbow protein markers (Amersham) were also run for comparison (lane M). The gel was stained with Coomassie blue.

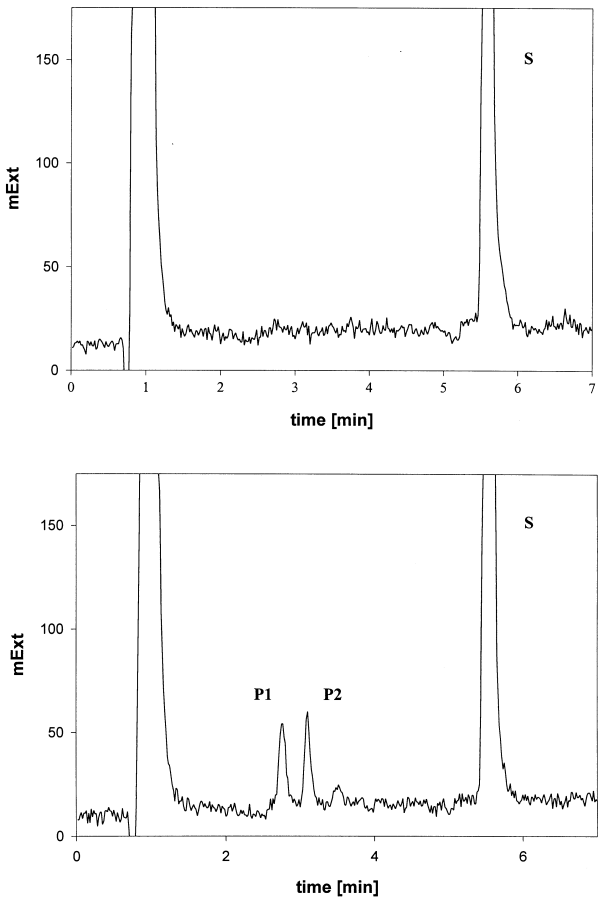

Fig. 3. Cleavage of an oligopeptide substrate with HFV PR. Substrate 1 of Table 1 was incubated in the presence (lower panel) or absence (upper panel) of fusion protein for 1 h as described in Section 2. After incubation the reaction mixture was separated by reversed-phase HPLC. Peaks were identified by amino acid analysis and Edman degradation. P1: SRAVN; P2: TVTQS; S: uncleaved substrate (SRAVNTVTQS).

and J. Tözsér, unpublished results) suggested that HFV PR structure starts with the initiator Met encoded by the spliced mRNA, and should be at least 100–107 residues long. Mutagenesis studies identified the Asp of the Pol as the active site in the Pol fusion protein (see Fig. 1, [5]), however, the C-terminal -Arg-Gly-Asn/Asp- conserved region characteristic of the retroviral PR [1] is missing from the sequence (Fig. 1). Since the enzyme may contain a flanking sequence at the C-terminus, similar to the case of HTLV-1 PR, we cloned a 141 amino acid long region of the Pol. Since the coded proteinase was active on oligopeptide substrate (Fig. 2), and it was ended at the same site as the PR fragment previously found to be active on its polyprotein substrate [6], it appears to contain the entire PR coding sequence.

Foamy viruses have several unusual features, and their replication in some aspects resembles that of hepadnaviruses [3,4]. The higher pH optimum of the HFV PR may be related to the replication cycle distinct from other retroviruses. Unlike in other retroviruses, Gag does not appear to be efficiently cleaved into smaller proteins, rather it is processed at the C-terminal end intracellularly, in preassembled cores [10]. This may require a pH optimum which is close to the intracellular pH. Another feature of the HFV PR is its low dimer stability, determined by urea dissociation, as compared to the more stable HIV-1 PR.

A similarity also exists between the processing profile of foamy viruses and yeast retrotransposons. Similar to HFV, in the yeast retrotransposon Ty1, a p4 protein is C-terminally cleaved from the NC domain, which processing is essential for transposition [23]. Although the peptides representing retrotransposon cleavage sites in the NC domain were not hydrolyzed (Table 1), three other peptides representing Pol cleavage sites in yeast retrotransposons were cleaved by the HFV PR, suggesting that the retrotransposon proteases and HFV PR share similarities in specificity (Table 1). A common characteristics of the cleaved substrates is that, with one exception, both P2 and P2′ residues are β branched. This is also true for the other Gag cleavage sites which were determined very recently [24]. However, this may only be part of the sequence requirement, since other peptides having such residues at P2 and P2′ (like peptides 5, 7, 14–16, 22–23 in Table 1) were not substrates of the enzyme. Similar to other retroviral proteinases the specificity of HFV PR may also be governed by extended interactions between the substrate and the enzyme, could be very complex, and strongly dependent on the context of the substrate sequence.

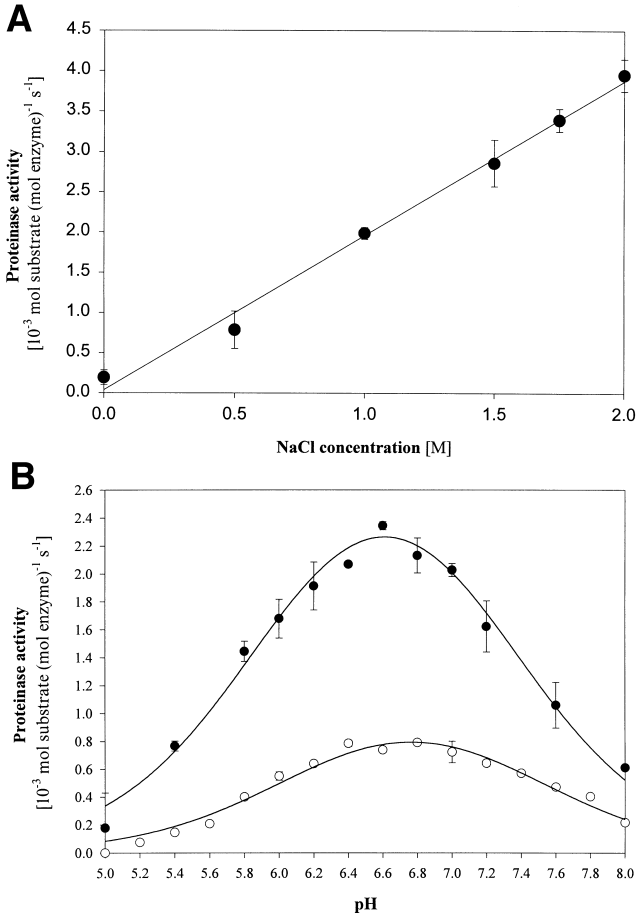

Fig. 4. Effects of ionic strength and pH on the activity of recombinant HFV PR. A: The activity of the MBP-HFV PR fusion protein was assayed in the presence of various NaCl concentrations at pH 6.6. B: The activity of the MBP-HFV PR fusion protein was measured at the indicated pH as described in Section 2, in the presence (●) or absence (○) of 1 M NaCl.

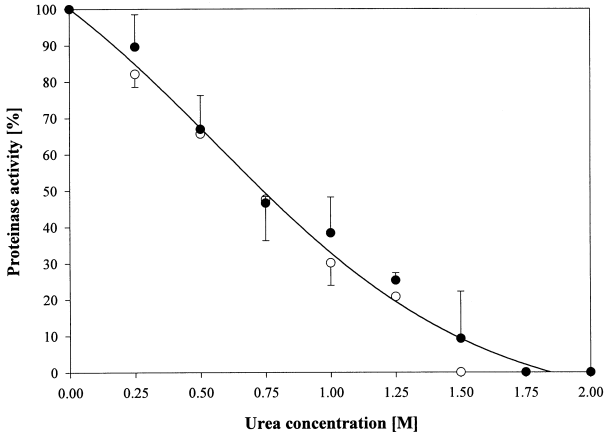

Fig. 5. Urea denaturation curves for recombinant HFV PR. Changes in enzymatic activity were determined with substrate 1 of Table 1 for the purified fusion protein (●) or processed proteinase (○) as described in Section 2.

The low catalytic efficiency of the HFV proteinase is in line with the lower specific activities of the HFV RT and IN as compared to those of other retroviruses [25,26]. As opposed to other retroviruses, in which the Gag-Pol fusion protein is present only in relatively smaller amounts as compared to the Gag proteins, a significant amount of Pol is produced by HFV-infected cells [27], which may be required to have larger total concentrations of the active HFV enzymes.

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