Volume 280, number 2, 347–350 FEBS 09527 March 1991
ADONIS 0014579391002160

Purification of HIV-1 wild-type protease and characterization of proteolytically inactive HIV-1 protease mutants by pepstatin A affinity chromatography

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Received 28 November 1990; revised version received 22 January 1991

Recombinant wild-type protease of human immunodeficiency virus, type 1 (HIV-1) expressed in *E. coli* was purified by pepstatin A affinity chromatography. An 88-fold purification was achieved giving a protease preparation with a specific enzymatic activity of approximately 3700 pmol/min/μg. Two proteolytically inactive HIV-1 mutant proteases (Arg-87→Lys; Asn-88→Glu) were found to bind to pepstatin A agarose, and they were purified as the wild-type protease. A third mutant protease (Arg-87→Glu) was apparently unable to bind to pepstatin A under similar conditions. Binding to pepstatin A indicates the binding ability of the substrate binding site and the ability to form dimers. These features may be used to purify and to characterize other mutated HIV-1 proteases.

HIV-1 protease; Mutant protease; Pepstatin A affinity chromatography

1. INTRODUCTION

The virus encoded proteases have an essential function in the life cycle of retroviruses [1–3]. Studies have been focussed on the protease of the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome [4]. As a potential target for chemotherapy, its enzymatic properties and inhibitors (for review see [5–9]) as well as its structure have been studied [10].

Also, mutants of the protease have been generated to identify the active site and other functionally important regions within the enzyme ([11–13] and references therein). The second conserved region, Gly-Arg-Asn at position 86–88, which is unique for retroviral proteases (PR), and is not present in pepsins, was found to be essential for proteolytic activity [12]. Based on the 3-dimensional structure [10,14], an interaction by ion pairing of Arg-87 with Asp-29 has been assumed to be required for enzymatic activity.

Retroviral PRs represent a subclass of pepsin-like aspartic proteases and the general inhibitor pepstatin A was used to purify the PR of bovine leukaemia virus (BLV) by inhibitor affinity column chromatography [15]. For the purification of HIV-1 PR, inhibitor affinity chromatography was established by using a peptide substrate analogue coupled to agarose resin [16]. HIV-1 PR is also inhibited by pepstatin A [17–20]. In this report, we describe pepstatin A affinity chromatography purification of HIV-1 wild-type PR and proteolytically inactive mutant proteases.

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2. MATERIAL AND METHODS

2.1. *Purification of recombinant HIV-1 protease*

Lysates of *E. coli*, expressing the HIV-1 PR [21] were clarified by centrifugation for 30 min at 200 000 × *g* at 4°C. An equal volume of ice-cold saturated ammonium sulfate solution containing 1 mM dithiothreitol (DTT) was added, stirred overnight at 4°C and centrifuged for 15 min at 15 000 × *g*. The pellet was resuspended in 10 ml of buffer A (50 mM Pipes, pH 6.8, 1 M NaCl, 1 M ammonium sulfate, 1 mM DTT, 1 mM EDTA, 1 mM Na/K-tartrate, 10% glycerol) and centrifuged for 30 min at 100 000 × *g*. The supernatant was loaded on a pepstatin A agarose (Pierce) column. After washing the column with 5 vols of buffer A, the PR was eluted with buffer B (100 mM Tris-HCl, pH 8.2, 1 mM DTT, 1 mM EDTA, 1 mM Na/K tartrate, 10% glycerol, 5% ethylene glycol) at a flow rate of 1 cm/min.

2.2. *Protease assays*

Unless noted otherwise, 1 μl of the fractions were assayed for PR activity using the synthetic nonapeptide substrate, Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-NH_2 [22], according to the procedure described earlier [20,21]. Assays were carried out in a total volume of 10 μl containing 3000 pmoles of substrate, 5 μl of 2× reaction buffer (0.5 M potassium phosphate, pH 6.5, 5% (v/v) glycerol, 1 mM DTT, 1 mM EDTA and 1.5 M ammonium sulphate), and were incubated for 30 min at 37°C.

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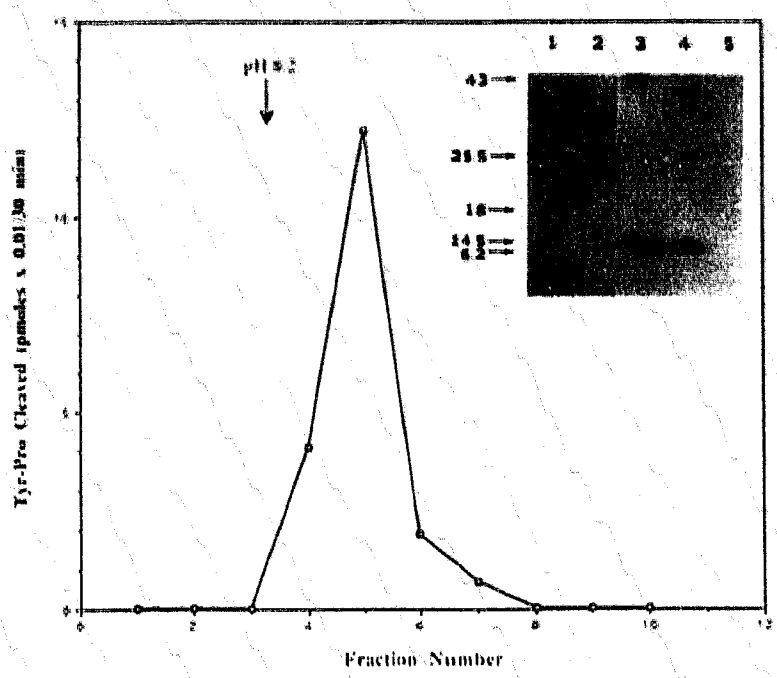

Fig. 1. Purification of recombinant HIV-1 wild-type and mutant protease by pepstatin A chromatography. Fractions of wild-type protease were assayed for proteolytic activity, and mutant protease (Mut 3-11; Arg-87→Lys) was detected by Western blotting (insert). Lanes 1 and 2 show effluent fractions at the maximum of the protein peak monitored at 280 nm, and lanes 3-5 demonstrate fractions of the eluate, corresponding to fractions 5-7.

2.3. *Immunoblotting*

Equal volumes of the effluent and eluate fractions after pepstatin A chromatography were electrophoresed on 10-20% gradient tricine-polyacrylamide gels (Novex Experimental Technology, CA) or dot blotted. The proteins were transferred to 0.22 μm nitrocellulose paper (Schleicher & Schüll) and immunoblotted using antiserum specific to HIV-1 protease [22] preadsorbed using 1:10 volumes of a 2 mg/ml *E. coli* lysate for 1 h at room temperature.

3. RESULTS AND DISCUSSION

Immobilized pepstatin A has been used in the purification of cathepsin D, pepsinogenes, renin ([23] and references therein), and BLV PR [15]. We defined conditions for purifying HIV-1 PR by pepstatin A affinity chromatography, which provides a fast procedure for isolating recombinant HIV-1 PR. The protease was bound to pepstatin A agarose at a high salt concentration, pH 6.8, and was eluted from the resin at pH 8.2 without salt as shown in Fig. 1. This result is in accordance with inhibitor studies, which indicated that the binding of pepstatin A is pH-dependent. It occurs readily at low pHs, but is markedly reduced above pH 7 [17,18]. In addition, high ionic strength is of importance for pepstatin binding [17]. After pepstatin A affinity chromatography, 19% of PR activity was recovered, and the specific enzyme activity increased by a factor of 88, from 42 pmol/min/μg in the crude *E. coli* lysate to 3700 pmol/min/μg after the column procedure. Under similar conditions, the HIV-1 PR did not bind to agarose beads (Bio-Gel A-0.5m, 200-400 mesh), demonstrating a specific binding to pepstatin A. Additional procedures involving hydrophobic interaction

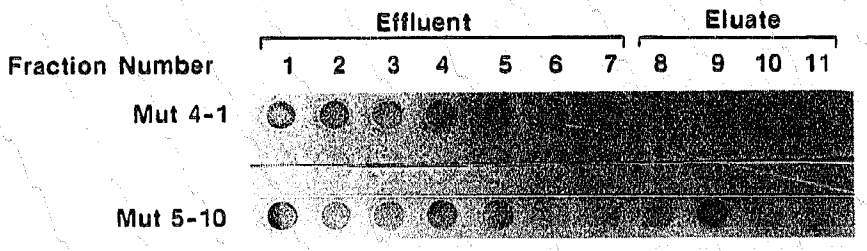

Fig. 2. Characterization of HIV-1 mutants proteases by pepstatin A chromatography. Mut 4-1 (Arg-87→Glu) and Mut 5-10 (Asn-88→Glu) were applied to pepstatin A affinity chromatography. 100 μl of effluent and eluate fractions were tested for mutant protease by dot blotting using a preadsorbed HIV-1 protease specific antiserum and horseradish peroxidase staining.

Table I

Comparison of HIV-1 wild-type and mutant proteases

Designation	Amino acid replacement	Enzyme activity (pmol/min/μg)	Binding to pepstatin agarose
wild-type	-	3700	100%
Mut 3-11	Arg87→Lys	not active	100%
Mut 4-1	Arg87→Glu	not active	< 5%*
Mut 5-10	Asn88→Glu	not active	35%*

* Percent of recovered protease detected in the eluate after pepstatin A agarose affinity chromatography as assessed by density scanning

chromatography and cation exchange chromatography slightly improved the specific activity of the final enzyme preparation (4200 pmol/min/μg). With both procedures we obtained an enzyme which was 20–30% pure (PR concentration 10–18 nM) as determined by active site titration using compound 3 of Grobelny et al. [25], a potent transition state inhibitor of HIV proteases. These enzyme preparations have been used for developing a solid phase assay [20], a continuous spectrophotometric assay and enzyme kinetics [24] and for inhibitor studies [25,26].

As described earlier [12,13], mutations in the second conserved region of the HIV-1 PR destroy the ability of this enzyme to cleave its substrate. Even a relatively minor change by replacing the Arg-87 with Lys (maintaining a positive charge on the side chain) did not show any specific proteolytic activity. We have shown earlier, that the product expressed in the mutants were identical in size to the wild-type product, and approximately equal amounts were observed after 30 min of induction [12]. As demonstrated in Fig. 1, insert, Mut 3-11 binds to pepstatin A as well as the wild-type PR. The Western blot shows only one single band of 11 kDa, no non-specific signals have been observed with the preadsorbed antibody. Under identical conditions, the mutant protease derived from 4-1 and 5-10 were analyzed for their capacity to bind to pepstatin A agarose by dot blotting analysis. Apparently, Mut 5-10 binds weaker to pepstatin A, approximately 35% of Mut 5-10 PR was bound, and the majority of Mut 4-1 PR was found in the eff[illegible]ated by densitometric scanning of the dot blots shown in Fig. 2. The results are summarized in Table I.

The change of a positive to a negatively charged amino acid at position 87 (Mut 4-1) apparently leads to the loss of inhibitor binding ability, which may be caused by conformational changes altering either the structure of the binding pocket, or the dimerization, or both. However, the change of a neutral to a negatively charged amino acid at position 88 (Mut 5-10) still allowed binding of the inhibitor, assuming that this mutation has only a minor influence on the structure/conformation of the enzyme. A more conservative mutation (Arg-87→Lys) in the second conserved region may lead to minor changes in the micro-environment of the cleavage site preventing the hydrolysis of the substrate without affecting the binding of the inhibitor/substrate, whereas the mutations Asn-88→Glu and Arg-87→Glu may cause conformational changes of the secondary structure decreasing or abolishing the inhibitor/substrate binding, respectively, under the conditions used. Further characterization of these mutants by determining the substrate binding (K_m) and dimerization (K_d) may give a better understanding of the functional role of the Gly-Arg-Asn structural element of HIV-1 proteases and in the design of specific inhibitors capable of recognizing the viral protease but not the related pepsin-like host proteases. Additionally, inhibitor affinity chromatography allows to screen and purify other mutated HIV proteases still capable of inhibitor-binding. While this manuscript was in preparation, another group reported the use of pepstatin A affinity chromatography for the purification of HIV-1 and HIV-2 wild-type PRs [27].

Acknowledgements: We thank T. Unangst and L. Fox for their excellent technical assistance. Research sponsored by the National Cancer Institute, DHHS under contract no. NO1-CO-74101 with ABL.

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