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Cloning, expression and purification of a recombinant poly-histidine-linked HIV-1 protease

Andreas Leuthardt and Johannes L. Roesel

Ciba-Geigy Ltd., Oncology and Virology Research Department, Pharmaceutical Division, K125.308, CH-4002 Basel, Switzerland

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The gene coding for the HIV-1 protease was cloned in an *Escherichia coli* expression vector adding three-histidine codons to the amino and carboxy terminus of the protease sequence. Expression of the protease from this construct led to the accumulation of high amounts of insoluble histidine-linked protease entrapped in inclusion bodies. The histidine-linked protease could be efficiently released from purified inclusion bodies with 6 M guanidine hydrochloride and further purified by metal chelate affinity chromatography. The refolded protease cleaved synthetic peptide substrates and the viral polyprotein p55 with the same specificity as the wild type protease. It displays a specific activity of 4.4 μmol/min/mg.

HIV-1 protease; Metal affinity chromatography; Acetyl-pepstatin

1. INTRODUCTION

The protease of human immunodeficiency virus type 1 (HIV-1) is essential for the viral life cycle [1,2]. It processes the viral *gag* and *gag-pol* polyproteins to the mature structural and enzymatically active proteins [3]. Point mutations that inactivated the viral protease gave rise to the production of noninfectious progeny virus [1]. Due to this crucial role in the viral replication the HIV-1 protease is regarded as one of the most promising targets for antiviral intervention [4]. Therefore, production and purification of large quantities of this enzyme are prerequisites for the development of assays which allow the identification of potent and selective inhibitors. Several laboratories have described the production of HIV-1 protease either by expression in *Escherichia coli* or *Saccharomyces cerevisiae* [5–15] or by the chemical synthesis of the 99 amino acids-long monomer [14,16,17].

Here we report a method for expression and easy purification of large quantities of a recombinant histidine-linked HIV-1 protease in *E. coli* and a rapid and easy purification procedure for this enzyme.

Correspondence address: J.L. Roesel, Ciba-Geigy Ltd., Oncology and Virology Research Department, Pharmaceutical Division, K125.308, CH-4002 Basel, Switzerland. Fax: (41) (61) 696-3835.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; MES, morpholinoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Nph, *p*-nitrophenylalanine; Nle, norleucine.

2. MATERIALS AND METHODS

2.1. *Cloning and expression of the histidine-linked protease*

A *Bam*HI–*Bgl*II fragment coding for the HIV-1 protease was synthesized by amplifying the protease open reading frame of the HIV-1 provirus harboring plasmid pNL 4.3 using the two primers shown in Fig. 1A. This fragment was cloned in-frame in the *Bam*HI–*Bgl*II site of the IPTG-inducible *E. coli* expression vector pDS 56/3H-3H [18,19]. This expression vector provides a ATG start codon and three His codons upstream of the *Bam*HI–*Bgl*II site and another three terminal His codons downstream of the cloning site. The resulting plasmid pDS 56/3H-Hprt-3H was transformed into the *E. coli* strain M15 containing the *lacI*-producing repressor plasmid PDM1.I [20]. Transformants were selected on LB-agar plates supplemented with 25 μg/ml kanamycin and 100 μg/ml ampicillin. Recombinant plasmids were identified by restriction endonuclease analysis and clones containing the correct sized insert were sequenced [21].

For induction of gene expression *E. coli* M15 PDM1.I containing plasmid pDS 56/3H-Hprt-3H were grown at 37°C in 1 litre 2 × YT medium containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. At an OD_{600} = 0.7 the culture was divided in 4 × 250 ml and IPTG added to a final concentration of 400 μg/ml. The cultures were induced for a period of up to 5 h.

2.2. *Purification and solubilization of histidine-linked protease containing inclusion bodies*

The purification of inclusion bodies and the solubilization of the histidine-linked protease was performed using a modified protocol of a published procedure [22].

In short, cells were centrifuged and the pellet (9–10 g/l) resuspended in 5 ml/g buffer A containing 50 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, pH 8.0. Lysozyme was added (1 mg/ml f.c.) and the cells were kept on ice for 30 min and then frozen to −70°C. After thawing, NP-40 was added (0.25% (v/v) f.c.) followed by 20 min incubation on ice. DNase I (0.1 mg/ml f.c.) with $MgCl_2$ (10 mM f.c.) were added. This suspension was centrifuged at 17,000 × *g* for 15 min in a Sorvall centrifuge using an SS34 rotor. The insoluble fraction was gently resuspended with a Pasteur pipette at 5 ml/g in buffer A containing 0.5% (v/v) Triton X-100, put on a table shaker for 30 min at room temperature and again collected by centrifugation (17,000 × *g*; 15 min). The Triton-extraction step was repeated twice followed by a wash with an equal volume of deionized water. The pellet was resus-

pended at 5 mg/ml in buffer B (50 mM Tris-HCl, 50 mM NaCl, 6 M guanidine hydrochloride, pH 8.0).

The inclusion bodies were solubilized overnight by gentle shaking. Samples were taken from all purification steps and analyzed by SDS-PAGE on 17% tricine-gels [23]. The separated protein bands were visualized either by Coomassie blue staining or by immunoblot techniques using a mouse monoclonal antibody raised against recombinant HIV-1 protease.

2.3. *Nickel iminodiacetic acid affinity chromatography and renaturation of the histidine-linked protease*

Iminodiacetic acid (IDA) adsorbent (Chelating Sepharose, Pharmacia) charged with Ni^{2+} as described by Hochueli et al. [24] was packed into a column (7.5 cm × 1.6 cm) and equilibrated with buffer B. All chromatographic steps were performed at a flow rate of 20 ml/h. The solubilized inclusion bodies were loaded onto the Ni^{2+}-IDA column and after extensive washing with buffer B, the bound material was eluted by an imidazole gradient ranging from 0–100 mM in buffer B. The chromatogram was monitored by UV detection at 280 nm and the collected fractions were analyzed by 17% tricine SDS-PAGE and Western blot analysis. Fractions containing pure histidine-linked protease were diluted to a final protein concentration of 10–30 μg/ml, adjusted to 10 mM DTT and dialyzed for 24 h against 100 × volume of MTEG buffer (20 mM MES pH 6.0, 0.01% Triton X-100, 5 mM EDTA, 29% glycerol) supplemented with 10 mM DTT. Aliquots were stored at −70°C.

2.4. *Protease activity assay*

The specific activity of the histidine-linked protease was determined with the chromophoric substrate H-K-A-R-V-L-Nph-E-A-Nle-S. Proteolytic cleavage of this substrate by the protease leads to the formation of an amino-terminal Nph fragment. The reaction mixture contained 8.4 nmol substrate, 20 mM MES pH 6.0 and 40 ng purified protease in a final volume of 100 μl. After incubation at 37°C for 30 min, proteolysis was stopped by the addition of 100 μl 0.3 M $HClO_4$. For the determination of the K_m, substrate concentrations ranging from 1 to 50 μM in 0.1 M Na-acetate, 0.9 M NaCl, pH 4.7 were used. Reactions were started by the addition of 47.6 ng enzyme and the final assay volume was 200 μl. After 45 min incubation at 37°C proteolysis was stopped by addition of 20 μl 0.3 M $HClO_4$. The assay mixture was analyzed by RP-HPLC using a Vydac C 18 column, equilibrated with 22% acetonitrile, 78% H_2O and 0.08% TFA at a flow rate of 1 ml/min. The formation of cleavage products was monitored at 280 nm. Kinetic constants were determined with less than 20% substrate turnover.

Enzymatic activity of the histidine-linked protease was also tested, using HIV-1 p55 *gag* precursor protein expressed in *E. coli*. 300 ng partially purified HIV-1 *gag* polyprotein from bacterial lysates was incubated for 2 h in 20 mM MES buffer pH 6.0 containing 20 ng histidine-linked protease in a final volume of 20 μl. The reaction was stopped by addition of an equal volume of 2 × sample buffer and the reaction mixture was applied to 12% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to immobilon and the blot was developed using an anti p24 mouse monoclonal antibody, recognizing also p55 and processing intermediates.

3. RESULTS AND DISCUSSION

3.1. *Construction and expression of the histidine-linked protease in* E. coli

Fig. 1A shows the cloning strategy for the histidine-linked protease as described in section 2. It resulted in the construction of an IPTG-inducible gene coding for the 99 amino acids of the HIV-1 protease sequence preceded by an initiator methionine and eight additional amino acids including three histidines (MRGSHHHGS-) and extended at the C terminus by arginine-serine followed by three histidines (-RSHHH) (Fig. 1B). The histidine-linked protease has a calculated molecular weight of 12.46 kDa. Extracts from induced and non-induced bacterial cultures containing the expression vector pDS 56/3H-Hprt-3H were analyzed by SDS-PAGE. Induction by IPTG yielded a product which migrated at the expected position of 12.5 kDa (Fig. 2A, lane 2). The 12.5 kDa protein band was absent in either noninduced cells (lane 3) or non-transformed bacteria (lane 1). Western blot analysis (Fig. 2B) showed that the 12.5 kDa protein identified by Coomassie blue staining reacted specifically with a HIV-1 protease specific antibody. Therefore, the 12.5 kDa protein represents the histidine-linked HIV-1 protease. The histidine-linked protease could be detected 15 min after induction and the amount increased until approximately 60 min. Prolonged incubation until 5 h did not alter the yield significantly. But 5 h after induction a minor contamination probably corresponding to a truncated form of the protease could be detected.

3.2. *Purification and refolding of the histidine-linked protease*

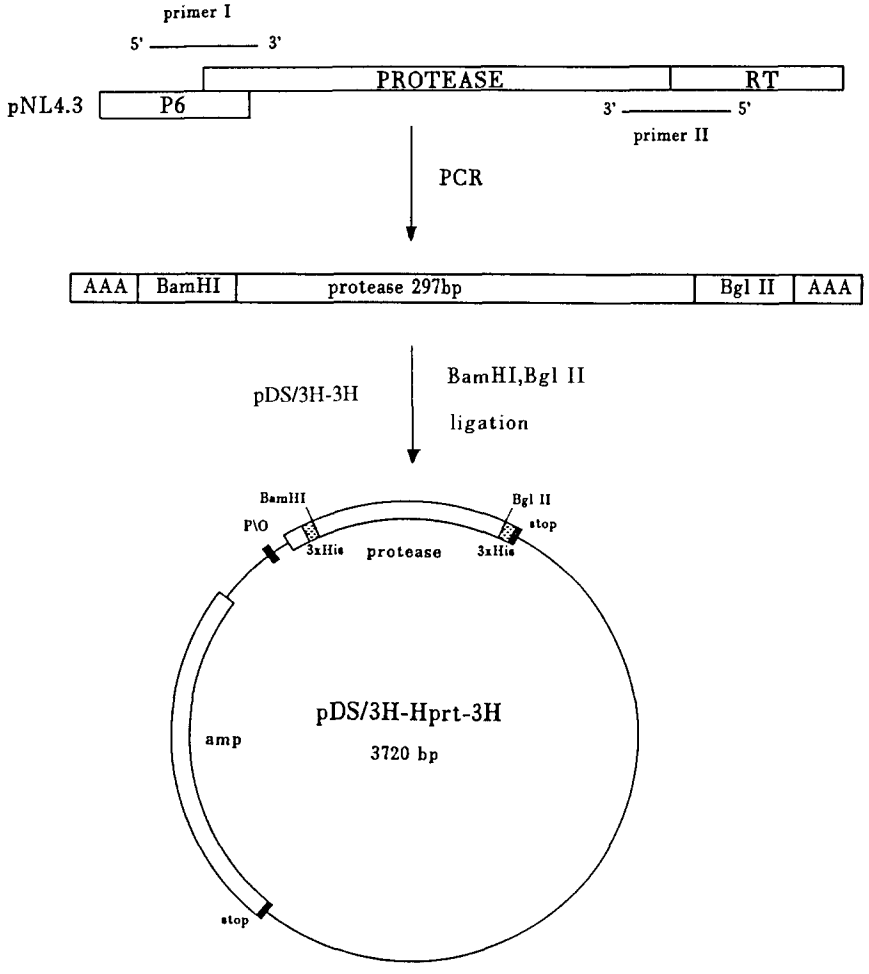
Bacterial lysate from cells collected 5 h after induction was used for the purification of the protease. First, the distribution of the histidine-linked protease between the pellet fraction and the supernatant fraction was analyzed after centrifugation of the bacterial lysate at 17,000 × *g* (see section 2). Western blot analysis revealed that approximately 90% of the expressed histidine-linked protease was in the insoluble fraction (Fig. 3B, lane 1–3). Therefore, the insoluble fraction was used for further purification.

By optimized washing procedures (i.e. 3 × Triton X-100, 1 × H_2O) the majority of contaminating bacterial proteins were removed from the insoluble material without loss of protease (lane 4–10). However, two major contaminants migrating at about 35 kDa and 15 kDa, respectively, remained in the insoluble fraction (lane 11). Harsher mechanical treatment of pellets (i.e. vortexing) partially disrupted the inclusion bodies and led to loss of material (data not shown). Finally, the histidine-linked protease together with the two major contaminants could be completely solubilized from the purified inclusion bodies by 6 M guanidine hydrochloride. No protease was left in the pellet of this fraction (Fig. 3B, lane 13).

The fraction containing the solubilized histidine-linked protease was then loaded on the nickel affinity column equilibrated with 6 M GuHCl. Fig. 4B shows that the majority of contaminants could be found in the flow through (I), whereas the histidine-linked protease was efficiently bound to the column. Applying an imidazole gradient a split peak could be detected in the chromatogram (Fig. 4A). The first maximum, corresponding to fraction 24, contained mainly protein contaminants, whereas the second maximum represented the highest concentration of the protease. Pure protease

1A) primer I 5' AAA GGA TCC CCT CAG ATC ACT CTT TGG C 3'

primer II 5' AAA AGA TCT CAA CAC TTG CAG GGG CTG C 3'



1B)

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                                  Bam HI
ATGAGAGGATCGCATCACCATGGATCCCCTCAGATCACTCTTTGGCAGCGACCCCTCGTC    60
 M  R  G  S  H  H  H  G  S  P  Q  I  T  L  W  Q  R  P  L  V

ACAATAAAGATAGGGGGGCAATTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACA   120
 T  I  K  I  G  G  Q  L  K  E  A  L  L  D  T  G  A  D  D  T

GTATTAGAAGAAATGAATTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGA   180
 V  L  E  E  M  N  L  P  G  R  W  K  P  K  M  I  G  G  I  G

GGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGCGGACATAAAGCT   240
 G  F  I  K  V  R  Q  Y  D  Q  I  L  I  E  I  C  G  H  K  A

ATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACT   300
 I  G  T  V  L  V  G  P  T  P  V  N  I  I  G  R  N  L  L  T
                        Bgl II
CAGATTGGCTGCACTTTAAATTTTAGATCTCACCATCACTAA                     342
 Q  A  G  C  T  L  N  F  R  S  H  H  H  -
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Fig. 1. Cloning strategy for the histidine-linked HIV-1 protease. (A) The HIV-1 protease open reading frame in pNL4.3 was amplified by PCR using primer I and II. The resulting fragment was cloned into the *Bam*HI–*Bgl*II site of the expression vector pDS/3H-3H. The amino acid sequence of the recombinant protein is depicted in B.

was eluted starting from fraction 30. A single band with the expected molecular weight of 12.5 kDa could be identified in the silver stained gel (Fig. 4C). We estimated a purity of greater than 95% for the histidine-linked protease. For renaturation samples of pure protease were diluted with buffer B to give a final protein

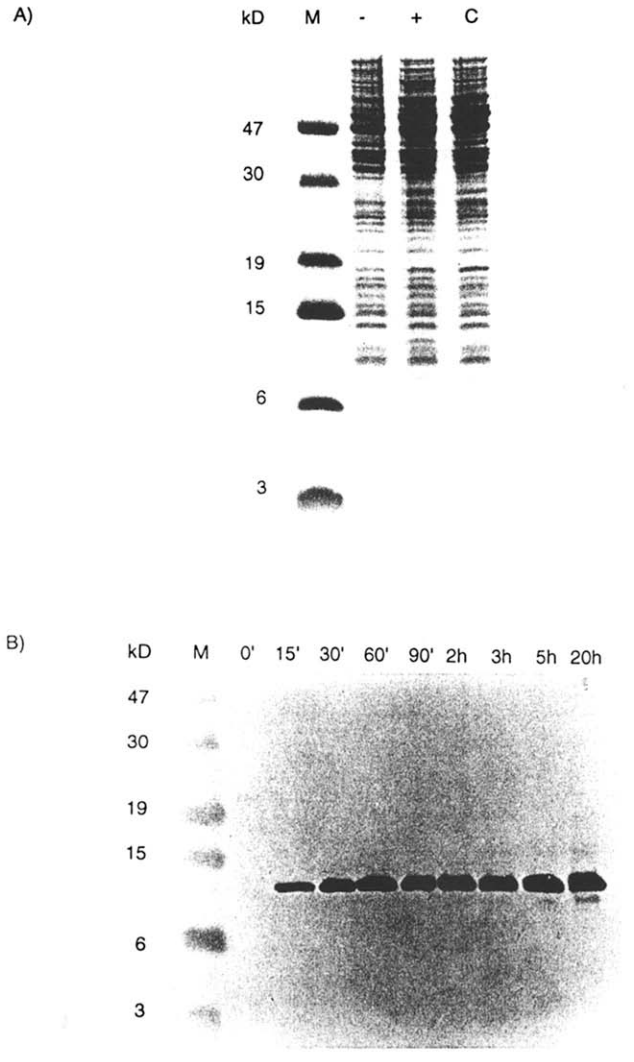

Fig. 2. Expression of the histidine-linked protease. (A) Extracts from bacterial cultures containing the expression vector pDS56/3H-Hprt-3H before (−) or after (+) induction or from cultures without the expression vector (c) were analysed by PAGE and Coomassie blue staining. (B) Time course of expression for the histidine-linked protease. Western blot analysis of bacterial extracts drawn at various time points after IPTG induction. Lane M contains molecular weight size markers.

concentration of 10–30 μg/ml. This protein concentration proved to be optimal for dialysis. At higher protein concentrations precipitates formed during dialysis and the specific activity decreased (data not shown). The refolded histidine-linked protease could be stored at −70°C for several weeks without considerable loss of activity.

3.3. *Enzymatic activity of the purified histidine-linked protease*

First, we tested the histidine-linked protease in its ability to cleave an endogenous substrate. Therefore, partially purified p55 *gag* precursor protein was incubated with purified protease and the reaction monitored by Western blot analysis using a monoclonal antibody directed against the p24 capsid protein. As shown in Fig. 5, incubation of p55 *gag* without protease (lane C) left the p55 *gag* polyprotein intact. In contrast, incubation of p55 *gag* with purified histidine-linked protease processed the precursor protein to the p24 capsid protein and intermediates with a molecular weight of 31 kDa and 41 kDa, respectively (lane 0). Addition of > 10 μM acetyl-pepstatin, a specific inhibitor of aspartic proteases, abolished the proteolytic activity of the histidine-linked protease. Concentrations of 1 μM or less resulted in only partial inhibition.

The kinetic parameters of the histidine-linked protease were determined using the chromophoric substrate H-K-A-R-V-L-Nph-F-Nle-S. This peptide mimics the cleavage site between the p24 capsid protein and the p6 nuclear capsid protein. The cleavage process was monitored by RP HPLC.

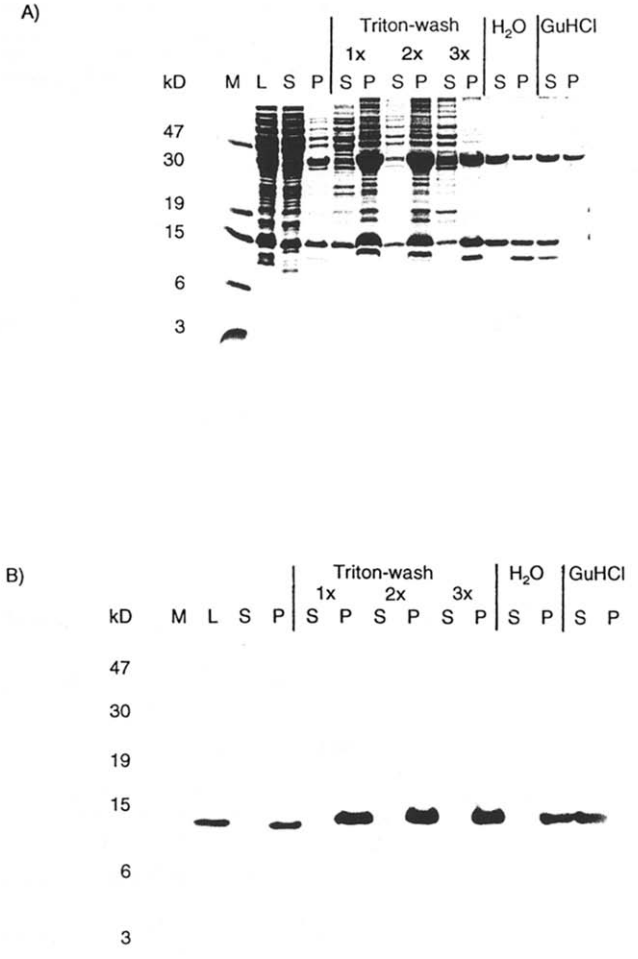

Fig. 3. Purification of inclusion bodies containing the histidine-linked protease. (A) Western blot analysis of aliquots from the purification procedure outlined in section 2. From left to right: the whole bacterial lysate (L), or the supernatant (S) and the pellet fraction (P) after the 17,000 × *g* centrifugation; the supernatant (S) and the pellet (P) fractions after each of three repetitive Triton X-100 washes, one water wash and the final solubilisation in GuHCl. (B) Coomassie blue-stained gel of the identical samples after PAGE. Lane M contains molecular weight size markers.

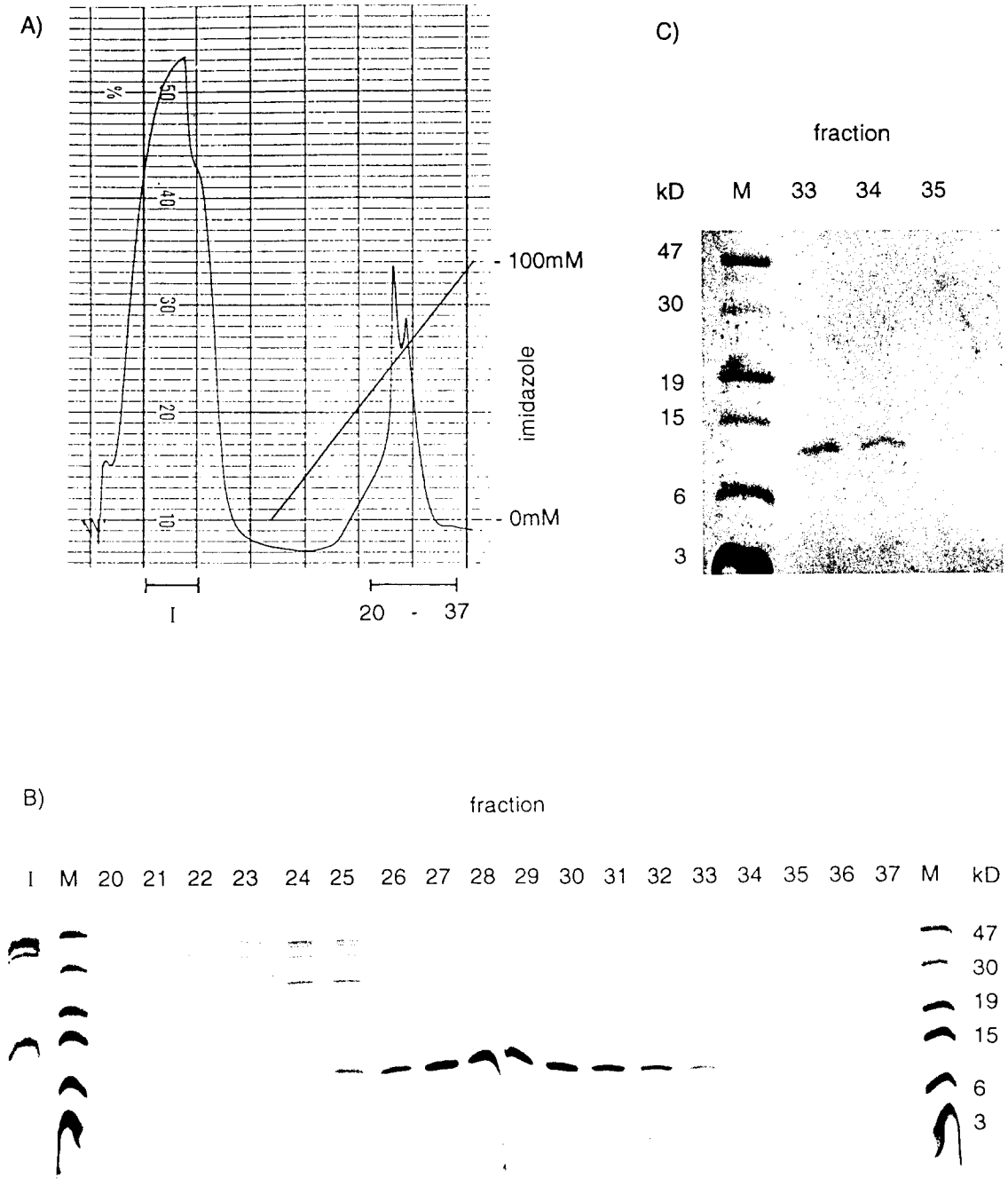

Fig. 4. Affinity chromatography on nickel-loaded chelating Sepharose column. (A) UV absorbance profile of the guanidinium-HCl solubilized material after chromatography on the affinity column. Fraction I indicates the flowthrough. Fractions 20 to 37 contained material eluted in the presence of increasing amounts of imidazole and was analysed by PAGE. (B) Coomassie blue-stained gel analysing fraction 20 to 37. (C) Silver stained gel analysing fraction 33 to 35. Lane M contains molecular weight size markers.

Fig. 6 shows the pH dependence of the proteolytic activity for the histidine-linked protease in the MES buffer system. The proteolytic activity was only slightly affected between pH 4.5 and 6.0 but dropped considerably at higher pH. This pH dependence of the histidine-linked protease is slightly different compared to the wild type HIV-1 protease. The wild-type protease is more sensitive to pH changes between 4.0 and 6.0 with a narrow optimum at pH 6.0 (data not shown). The ionic strength was determined to be optimal without adding salt.

Therefore, 20 mM MES buffer pH 6.0 was selected for the determination of the specific activity of the enzyme. Under these conditions, the specific activity of the refolded enzyme was measured to be 4.4 μmol/min/mg. This compares well to the specific activity of the wild type enzyme, as published by others [12,15,22,25–27].

The K_m for the chromophoric peptide could not be

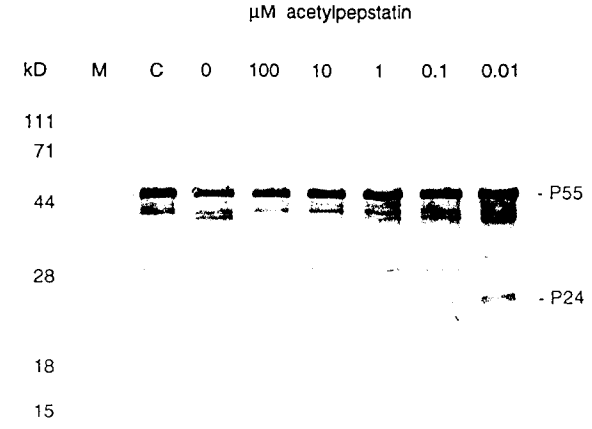

Fig. 5. Processing of $p55^{gag}$ by purified histidine-linked HIV-1 protease in the presence of acetyl-pepstatin. Western blot analysis; from left to right: 300 ng partially purified $p55^{gag}$ poly protein were incubated without (C) enzyme, enzyme without inhibitor (0), or decreasing amounts (100 to 0.01 μM) of acetyl pepstatin. Lane M contains molecular weight size markers. Positions of the p55 poly protein and the cleavage product p24 are indicated.

determined with the histidine-linked protease in the MES buffer system because the enzymatic activity of the enzyme was unaffected over a substrate concentration range of 1–50 μM. The K_m was therefore determined in 0.1 M Na-acetate, 0.9 M NaCl buffer pH 4.7. Using double reciprocal Lineweaver–Burk plot a K_m of 51 μM was calculated for the peptide substrate using the histidine-linked protease. It has to be emphasized that the specific activity is 8-fold lower in the Na-acetate buffer system when compared with the MES buffer system.

Many investigators studied the substrate affinity of the authentic HIV-1 protease purified by various purification procedures. The chromophoric peptide was identified with a high V_{max}/K_m and the K_m was reported to be 21 μM [28]. Therefore, the histidine-linked HIV-1 protease shows enzymatic characteristics similar to the wild type enzyme.

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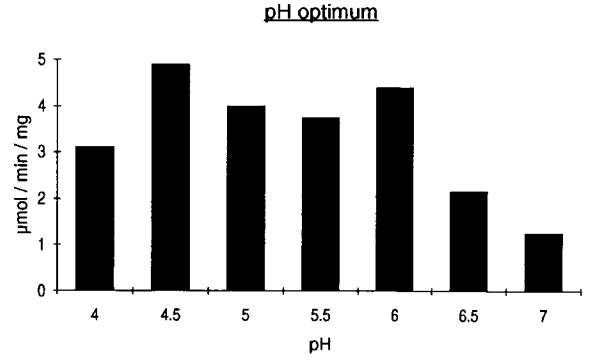

Fig. 6. pH-optimum of the histidine-linked HIV-1 protease. pH dependence of the protease between pH 4 and 7.

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