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# Affinity Purification of HIV-1 and HIV-2 Proteases from Recombinant *E. coli* Strains using Pepstatin-Agarose

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Summary: A procedure is described which employs pepstatin-agarose for the affinity purification of either HIV-1 or HIV-2 protease from two similar recombinant *E. coli* constructs that were developed for the expression of these enzymes. HIV-2 protease was routinely expressed at much higher levels than the HIV-1 enzyme and pepstatin-agarose was the only chromatography step required to isolate pure HIV-2 protease from crude bacterial lysates. A Mono S ionic exchange step following pepstatin-agarose chromatography was sufficient to bring the HIV-1 protease to homogeneity. Purification of either enzyme can be completed in several days yielding homogeneous preparations suitable for crystallization and other physical characterization. 

HIV-1 protease has received considerable attention because of its potential as a therapeutic target in the treatment of AIDS. The basic structural features of this enzyme have been elucidated by X-ray crystallography (1,2) and several classes of inhibitors have been developed which interfere with the proper processing of retroviral polyproteins and assembly of infectious virus (3,4,5).

A number of procedures have been reported for the isolation of HIV-1 protease expressed in soluble and insoluble forms[2] from various recombinant constructs(6-14). HIV-1 protease has also been chemically synthesized and refolded (2, 15-17). These methods involve a variety of chromatographic approaches with yields that are influenced by the level of expression and the number of purification steps required. A recently reported affinity procedure for the HIV-1 enzyme (18) provides a rapid alternative to these methods; however, synthesis of the affinity ligand and its attachment to a preactivated resin are required. Less information is presently available about HIV-2 protease, although both recombinant and synthetic preparations of this enzyme have now been described (19,20).

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[2] "Soluble" refers to active protease found in the supernatant of a cleared bacterial lysate; "insoluble" protease is found typically in inclusion bodies and requires refolding.

The abbreviations used are : dabcyl, 4-(4-dimethylaminophenylazo)benzoic acid; edans, 5-[(2-aminoethyl)amino] naphthalene-1-sulfonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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We report here the development of an affinity purification method that employs commercially-available pepstatin-agarose for the isolation of both HIV-1 and HIV-2 proteases that are expressed in soluble form in different recombinant *E. coli* strains. This procedure allows the rapid purification of either enzyme from crude bacterial extracts to a high degree of purity. HIV-1 protease purified in this manner was recently used for crystallization and X-ray analysis of an enzyme-inhibitor complex (21).

## Methods

Protease Expression- HIV-1 protease was expressed in *E. coli* SCS-1/pBS7-cI which carries the protease gene fused to HIV-1 gag p17 under the control of the temperature-inducible lambda $P_L$ promoter[3] . *E. coli* SCS-1/pKA5 was constructed in the same manner using the HIV-2 protease gene[3]. Conditions for the growth of both strains were identical. Cells were grown at 32°C in 10 liter batches in 20 g/l tryptone, 10 g/l yeast extract, and 10 g/l NaCl to an approximate $A_{600nm}$ of 5. Protease expression was induced for 1 h at 42 °C and the cells were harvested by centrifugation. A substantial proportion of the HIV-1 protease expressed by the pBS7-cI construct was found in a soluble form in cleared bacterial lysates; however, significant amounts of the protease were also detectable by Western blotting in the cellular debris after lysis[3]. HIV-2 is expressed in SCS-1/pKA5 in a soluble form at much higher levels (see Table 1). The reasons for the difference in expression levels are unclear, however, it has been previously noted that HIV-1 protease expression is toxic to *E. coli* cells (7,9).

Preparation of crude extract- All steps are carried out at 0-4°C. Packed cells were resuspended in a 5 to 10 volumes of lysis buffer (50mM Tris-Cl, pH 7.5, 10% glycerol, 5 mM EDTA, 2 mM PMSF, and 5 mM dithiothreitol) containing 10 µg/ml DNase, lysed using a french pressure cell, and centrifuged for 28,000 x g for 40 min. Protease activity was precipitated from the supernatant by the addition of solid ammonium sulfate to 42% saturation for HIV-1 protease and 50% saturation for HIV-2 protease. The precipitate was collected by centrifugation, resuspended at 1/5 the original crude extract volume with lysis buffer at pH 8.5, and treated with 0.31 volumes (of the newly resuspended extract) of 20 mg/ml protamine sulfate for 1 h, and centrifuged for 1 h at 100,000 x g.

Pepstatin-agarose chromatography- The pH of the extract is adjusted to 7.5 with HEPES (free acid) and applied at a rate of 2 ml/min to the bottom(designated forward flow in Figure 1) of a 25 ml column ( 3.5 cm diameter) of pepstatin-agarose (Sigma, lot #48F-9550) equilibrated with 50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM PMSF and 1mM dithiothrietol (equilibration buffer) in the cold room. The column was washed in the same direction with 400 ml of the same buffer containing 0.4 M ammonium sulfate (wash buffer). The column was connected to an FPLC system (Pharmacia) such that flow occurred in the reverse direction and it was washed with chilled buffers as follows: 25 ml equilibration buffer, 25 ml wash buffer, 25 ml equilibration buffer, 325 ml wash buffer, and 75 ml equilibration buffer. Protease was then eluted at 4 ml/min using: 250 mM Na-ε-aminocaproate, pH 10.5; 5% glycerol; 5% ethylene glycol; 1 mM EDTA; 1 mM PMSF; and 1 mM dithiothreitol(elution buffer). 12 ml fractions are collected, neutralized immediately with 0.6 ml 2M HEPES, and stored at -20°C. The column was cleaned immediately after use with 5 ml of 6M guanidine-HCl followed by buffer containing 20% ethanol.

Mono-S chromatography (HIV-1 Protease only)- 10 mg/ml solid MES was added to pooled samples, the pH was adjusted to 6.0 with 2 M acetic acid, and the sample was applied to a 1 ml Mono-S column (Pharmacia HR 5/5) equilibrated with 50 mM MES, pH 6.5, containing 1 mM EDTA, and 1 mM dithiothreitol. The column was washed with 15 ml of equilibration buffer and the protease was eluted with a 0 to 1M NaCl gradient (12 ml total volume).

To prevent loss of activity when storing these enzymes beyond several days, glycerol and ethylene glycol are added to a final concentration of 5% each. The samples are then quick-frozen in a dry ice-ethanol bath and stored at -80°C.

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[3] Simmer, R. et al., in preparation.

Analytical methods- Both HIV-1 and HIV-2 proteases were assayed at 30°C using the fluorogenic substrate, dabcyl-SQNYPIVQ-edans , at 3.0 μM under the conditions described previously (22). Substrate levels far below the $K_m$ (22) were used because of limited solubility and to conserve substrate. Because it was not feasible to assay either enzyme at $V_{max}$, the enzyme units reported in Table 1 are based arbitrarily on the rate of fluorescence increase per minute under a standardized set of conditions.

The Bradford assay (23) was used for the determination of protein in crude extracts. Different estimates of the protein in highly purified fractions were obtained when the Bradford, modified Lowry(24), and $A_{280nm}$ were used. Consequently, the levels of purified proteases were determined from the $A_{280\,nm}$ of samples denatured in 6M guanidine-HCL using extinction coefficients calculated from the predicted sequences (25).

SDS-PAGE was carried out using the PhastSystem (Pharmacia) followed by silver staining according to the manufacturers directions. Amino acid composition (Beckman 6300 amino acid analyser) and N-terminal sequence analyses (Applied Biosystems 477 and 471A protein sequencers) were done on samples purified by reversed-phase HPLC on a Vydac $C_4$ column using an acetonitrile gradient with 0.1% trifluroacetic acid.

## Results and Discussion

Immobilized pepstatin has been used for the affinity purification of a number of aspartyl proteases such as renin (26) and cathepsin D (27,28) (see reference 29 for discussion and additional examples). The recent findings that pepstatin is a potent inhibitor of both HIV-1 and HIV-2 proteases (30,31) suggested that pepstatin-agarose might provide an efficient affinity method that could be used to purify both of these enzymes. The development of the procedure described here was aided by the availability of the fluorogenic HIV-1 protease substrate, dabcyl-SQNYPIVQ-edans (22), and the observation made during these studies that this synthetic substrate is also readily cleaved by HIV-2 protease. Comparison of the specific activities of purified HIV protease preparations (Table 1) suggests that HIV-1 protease is significantly more efficient at cleaving this substrate, however, the conditions for optimal HIV-2 protease activity have not been defined. A thorough comparison of the kinetic characteristics of these two enzymes is currently underway.

Table 1

**HIV Protease Purification Summary[1]**

| Step | HIV-1 Protease | | HIV-2 Protease | |
|---|---|---|---|---|
| | total protein (mg) | total activity (units) | total protein (mg) | total activity (units) |
| supernatant | 6271 | -[2] | 15121 | 298[2] |
| ammonium sulfate | 624 | -[2] | 4504 | 370 |
| protamine sulfate | 610 | 251 | 4337 | 358 |
| pepstatin-agarose | 8.4 | 141 | 18 | 196 |
| Mono-S | 0.6 | 115 | - | - |

[1]Material from 10 liter fermentations of E. coli SCS-1/pBS7-cI and SCS-1/pKA-5 for HIV-1 and HIV-2 protease purifications respectively.

[2]not possible to obtain reliable estimates of total activity.

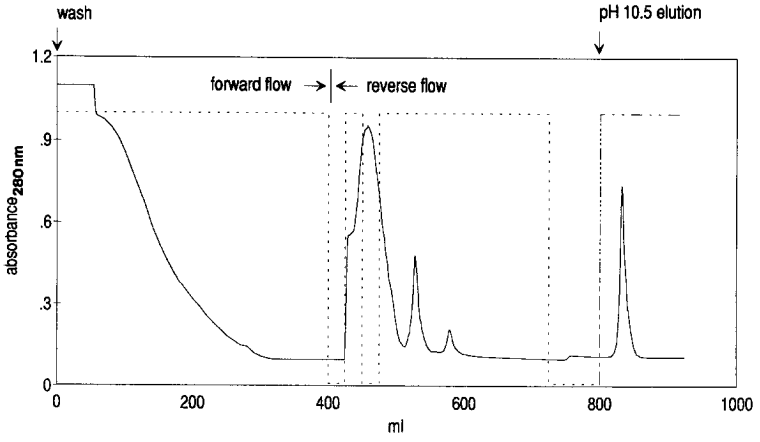


Figure 1. Elution profile of a pepstatin-agarose affinity column as described in Methods after loading a partially purified extract of *E. coli* SCS-1/pKA5 that contains HIV-2 protease. The broken lines indicate the changes in the wash buffer that occur during the column washing steps (- - - -) and when the pH 10.5 elution begins(- · · · · · -).

Crude bacterial lysates from both SCS-1/pBS7-cI and SCS-1/pKA5 required treatment with ammonium sulfate and protamine sulfate for efficient binding of HIV protease to pepstatin-agarose. These steps removed a considerable amount of protein (Table 1), as well as other components in the crude extract that interfered with protease binding (data not shown). During control experiments, using extracts containing HIV-2 protease, it was observed that protease binds efficiently to pepstatin agarose in buffers containing up to 1 M ammonium sulfate; however, the retention of other proteins varied considerably depending of the level of salt used (data not shown). Optimal purity of HIV protease preparations were achieved when extracts were diluted at the volumes described in Methods. The conductivity of the sample applied to the pepstatin agarose column then approximates that of the wash buffer.

Figure 1 shows a representative elution profile of HIV-2 protease from pepstatin agarose and it outlines the wash procedure that was used for both HIV-1 and HIV-2 protease purification. A considerable amount of bacterial protein bound to the resin, which necessitated an extensive (15 column volumes) buffer wash. We discovered that low-salt/high-salt wash cycles (beginning at 400 ml in Figure 1) removed additional protein and this step has proven to be essential for achieving high purity of either HIV-1 or HIV-2 protease. The adsorbed protein removed by these wash cycles appeared to be nonspecifically bound to the resin through hydrophobic interactions since ω-aminohexyl-agarose, which is structurally similar to the support and linker arm of pepstatin-agarose, displayed similar behavior when extracts containing HIV-2 protease were applied. Protease was not effectively retained by the ω-aminohexyl-agarose; however, extensive washing, followed by low- and high-salt wash cyles, were necessary to completely elute nonspecifically bound protein (data not shown).

Buffers containing pepstatin were inefficient at eluting bound protease from pepstatin-agarose (data not shown). Several other elution methods were tried and ε-aminocaproate at pH

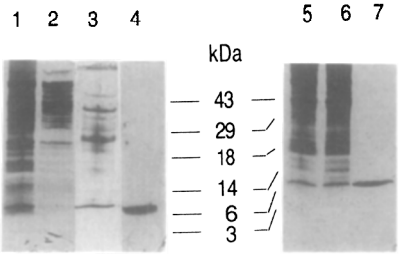


Figure 2. Silver-stained SDS-PAGE PhastGel (Pharmacia) of fractions obtained during purification of HIV-1 (lanes 1-4) and HIV-2 proteases (lanes 5-7). The fractions shown are ammonium sulfate (lanes 1 and 5), protamine sulfate (lanes 2 and 6), pepstatin-agarose (lanes 3 and 7) and Mono S (lane 4 only ). The amounts of protein in the highly purified HIV-1 (lane 4) and HIV-2 (lane7) samples were 0.5 and 0.3 µg respectively. The mobilities of low molecular weight, prestained markers (Bethesda Research Labs) are also indicated.

10.5 proved to be an efficient buffer for eluting both HIV-1 and HIV-2 proteases. As shown in Figure 1, HIV-2 protease was routinely recovered as a sharp peak under these conditions. This enzyme was routinely isolated to homogeneity from protamine sulfate-treated extracts of SCS-1/pKA5 in a single step using pepstatin-agarose (Fig 2, lane 7). Identical elution characteristics were observed with HIV-1 protease expressed in SCS-1/pBS7-cI. However, the purity of the HIV-1 enzyme was more variable at this step, possibly reflecting the lower level of expression. More highly purified HIV-1 protease fractions than shown in lane 3 of Figure 2 are frequently seen after pepstain-agarose. Subsequent chromatography on a Mono S column (Pharmacia) was the only additional step required to obtain homogeneous preparations as judged by SDS-PAGE (Fig. 2, lane 4). It should be noted that factors other than pH appear to influence HIV protease elution, since glycine at pH 10 was not an effective eluent. Similar conditions were reported previously for the desorption of HIV-1 protease from an affinity column prepared with a peptide-based inhibitor of HIV-1 protease (18).

Yields from the pepstatin-agarose step were approximately 50%. The greatest losses of both HIV-1 and HIV-2 proteases occur during the long wash step. Significant losses of HIV-1 protease were also observed during the low-salt/ high-salt wash cycles; whereas, loss of the HIV-2 enzyme was minimal during this step. It was also noted that higher levels of the HIV-2 enzyme could be retained per ml of resin. Although these results may reflect structural differences between the two enzymes, interpretation is difficult in view of the higher relative abundance of the HIV-2 enzyme in the bacterial lysates.

The estimated molecular weights (determined by SDS-PAGE and FAB-mass spectrometry), the predicted amino acid compositions, and N-terminal sequences of both proteases correlated well with those predicted by the gene sequence(data not shown). Positive western blots were seen with HIV-1 protease preparations using rabbit antisera raised against synthetic HIV-1 peptides. A weak cross reactivity was seen with HIV-2 protease preparations (data not shown). The complete three-dimensional atomic structure of the HIV-1 protease isolated using pepstatin-agarose affinity chromatography was verified recently by the X-ray crystal structure determination of an enzyme-inhibitor complex (21).

## Conclusions

Pepstatin-agarose affinity chromatography provides a means of rapid purification of both HIV-1 and HIV-2 proteases using a commercially-available affinity resin. Under the conditions described here, HIV-2 protease can be purified from crude extracts to homogeneity in a single step. HIV-1 protease, which was expressed at lower levels, required only one additional chromatography step to obtain homogeneous material suitable for crystallization. Such methods, combined with continuous, fluorometric protease assays (22), should facilitate the rapid assessment of potential HIV-protease inhibitors, allow comparisons between these two closely related enzymes, and provide the reagents necessary for large scale screening or bioassay.

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## References

1. Navia, M.A., Fitzgerald, P.M.D., McKeever, B.M., Leu, C.-T., Heimbach, J.C., Herber, W.K., Sigal, I.S., Darke, P.L. and Springer, J.P. (1989) Nature 337, 615-620.
2. Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J. and Kent, S.B.H. (1989) Science 245, 616-621.
3. Meek, T.D., Lambert, D.M., Dreyer, G.B, Carr, T.J., Tomaszek, T.A., Moore, M.L., Strickler, J.E., Debouck, C., Hyland, L.J., Matthews, T.J., Metcalf, B.W. and Petteway, S.R. (1990) Nature 343, 90-92.
4. Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Krohn, A., Lambert, R.W., Merrett, J.H., Mills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L. Thomas, G.J., and Machin, P.J. (1990) Science 248, 358-361.
5. McQuade,T.J., Tomasselli, A.G., Liu,L., Karacostas, V., Moss, B., Sawyer, T.K., Heinrikson, R.L., Tarpley, W.G. (1990) Science 247, 454-456.
6. McKeever, B.M., Navia, M.A., Fitzgerald, P.M.D., Springer, J.P., Leu, C.-T., Heimbach, J.C., Herber, W.K., Sigal, I.S., and Darke, P.L. (1989) J. Biol. Chem. 264, 1919-1921.
7. Darke, P.L., Leu, C.T., Davis, L.J., Heimbach, J.C., Diehl, R.F., Hill, W.S., Dixon, R.A.F., Sigal, I. (1989) J. Biol. Chem. 264, 2307-2312.
8. Meek, T.D., Dayton, B.D., Metcalf, B.W., Dreyer, G.B., Strickler, J.E., Gorniak, J.G., Rosenberg, M., Moore, M.L., Magaard, V.W. and Debouck, C. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 1841-1845.
9. Danley, D.E., Geoghegan, K.F., Scheld, K.G., Lee, S.E., Merson, J.R., Hawrylick, S.J., Rickett, G.A., Ammirati, M.J. and Hobart, P.M. (1989) Biochem. Biophys. Res. Comm. 165, 1043-1050.
10. Billich, A., Hammerschmid, F. and Winkler, G. (1990) Biol. Chem. Hoppe-Seyler 371, 265-272.
11. Pichuantes, S., Babe, L.M., Barr, P.J., Craik, C.S. (1989) Proteins: Structure, Function, and Genetics 6, 324-337.
12. Giam, C.-Z. and Boros, I. (1988) J. Biol. Chem. 263, 14617-14620.
13. Tomaselli, A.G., Olsen, M.K., Hui, J.O., Staples, D.J., Sawyer, T.K., Heinrikson, R.L. and Tomich, C.-S. C. (1990) Biochemistry 29, 264-269.
14. Cheng, Y.-S.E., McGowan, M.H., Kettner, C.A., Schloss, J.V., Erickson-Viitanen, S. and Yin, F.H. (1990) Gene 87, 243-248.

15. Copeland, T.D. and Oroszlan, S. (1988) Gene Anal. Tech. 5, 109-115.
16. Nutt, R.F., Brady, S.F., Darke, P.L., Ciccarone, T.M., Colton, C.D., Nutt, E.M., Rodkey, J.A., Bennett, C.D., Waxman, L.H., Sigal, I.S., Anderson, P.S. and Verber, D.F. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7129-7133.
17. Schneider, J. and Kent, S.B.H. (1988) Cell 54, 363-368.
18. Heimbach, J.C., Garsky, V.M., Michelson, S.R., Dixon, R.A.F., Sigal, I.S., Darke, P.L. (1989) Biochem. Biophys. Res. Comm. 164, 955-960.
19. Le Grice, S.F.J., Ette, R., Mills, J. and Mous, J. (1989) J. Biol. Chem. 264, 14902-14908.
20. Wu, J.C., Carr, S.F., Jarnagin, K., Kirsher, S., Barnett, J., Chow, J., Chan, H.W., Chen, M.S., Medzihradszky, D., Yamashiro, D. and Santi, D.V. (1990) Arch. Biochem. Biophys. 277, 306-311
21. Erickson, J., Neidhart, D.J., VanDrie, J., Kempf, D.J., Wang, X.C., Norbeck, D., Plattner, J.J., Rittenhouse, J., Turon, M., Wideburg, N., Kohlbrenner, W.E., Simmer, R., Helfrich, R., Paul, D. and Knigge, M. (1990) Science, in press
22. Matayoshi, E.D., Wang, G.T., Krafft, G.A., and Erickson, J. (1990) Science 247, 954-958
23. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
24. Hartree, E.F. (1972) Anal. Biochem. 48, 422-427.
25. Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319-326.
26. Dzau, V.J., Slater, E.E. and Haber, E. (1979) Biochemistry 18, 5224-5228
27. Afting, E.G. and Becker, M.L. (1981) Biochem. J. 197, 519-522
28. Helseth, D.L. Jr. and Veis, A. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3302-3306.
29. Rich, D. (1986) in Proteinase Inhibitors (Barrett, A.J. and Salvesen, G., eds.) pp. 179-217. Elsevier Science Publishers BV, New York
30. Richards, A.D., Roberts, R., Dunn, B.M., Graves, M.C., Kay, J. (1989) FEBS Letters 247, 113-117.
31. Richards, A.D., Broadhurst,A.V., Ritchie, A.J., Dunn, B.M., and Kay, J. (1989) FEBS Letters 253, 214-216