Isolation of Recombinant HIV1 Protease Expressed in *E. coli* and *S. cerevisiae*

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Abstract

Studies of the life cycle and composition of the human immunodeficiency virus (HIV) have identified a virally encoded aspartyl protease. This enzyme is responsible for the processing of the initially translated *gag* and *gag/pol* polyprotein precursors to yield the mature capsid proteins (p17, p24, p9, and p6) and the three viral enzymes (protease, reverse transcriptase, and integrase). Mutations within the protease coding regions of the retroviruses, including HIV1, have led to the formation of non-infectious virions composed of non-processed polyproteins. Efficient inhibition of HIV1 proteolytic activity would interfere with the replication and assembly of the virus and hence reduce viral proliferation.[1]

Rational design of a specific viral protease inhibitor requires knowledge of the structure and function of the protease. Such information can be generated only if reagent levels of purified protein are available. For this reason, two expression systems were developed to express the HIV1 protease in *E. coli* and *S. cerevisiae*. Purification schemes have been established for the recombinant enzyme from these systems.[2,3]

Bacterial and yeast expression systems produce the viral protease as an active soluble protein. Expression levels of the biosynthetic product are limited by the toxicity of the protease to its host. Low yields, of between 0.1 and 1.0 mg of viral protease per liter of saturated cell culture, together with the strong hydrophobic nature of the 22 kD dimeric protease, provide an exceptionally challenging protein purification task.

A unique property of the viral protease is its extremely high isoelectric point of 9.95. This pI value is markedly higher than the pI values of the majority of bacterial and yeast proteins, and provides the basis for 200–500 fold purification in one step using preparative isoelectric focusing in the Rotofor® cell.

Methods

EXPRESSION OF HIV PROTEASE IN *E. COLI*

DNA sequences encoding the viral protease were subcloned downstream (3') from the human superoxide dismutase (hSOD) gene and their expression controlled by the *tac* promoter. Induction with IPTG[4] resulted in the expression of a hSOD/protease fusion protein that self-processed to yield the mature protease. The protease monomer migrates with an apparent molecular weight of 11,000 in SDS-PAGE and can be readily distinguished from the high molecular weight precursor. Purification of the protease expressed in *E. coli* is described in detail elsewhere.[2]

TREATMENT OF BACTERIAL LYSATE FOR ISOELECTRIC FOCUSING

Three hundred grams of bacterial cells were collected by centrifugation, resuspended in 800 ml of 50 mM Tris/HCl buffer (pH 8.0) containing 5 mM EDTA, 1 mM DTT, 1 mM PMSF (phenylmethylsulfonyl fluoride), 100 mM KCl and 0.5% Triton® X-100 detergent, and sonicated. Bacterial DNA was removed by protamine sulfate precipitation, and the clarified supernatant was then adjusted to 40% ammonium sulfate. The salt-precipitated fraction, containing the protease, was then applied to a CM-Sepharose® column equilibrated with 20 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.1% Triton X-100 detergent. Application of a linear NaCl gradient in the same buffer resulted in the elution of the viral protease in several hundred ml at about 80 mM salt. This sample was then dialyzed against water and concentrated several fold by dialysis in a bed of polyethylene glycol (PEG 6000) crystals. A Bio-Lyte® ampholyte blend resulting in final concentrations of 1% pH 3–10 and 0.25% pH 8–10 was added to ~55 ml of dialyzed, concentrated material and the mixture was loaded into the Rotofor cell without further treatment.

RUNNING CONDITIONS

Focusing was carried out at 12 W constant power for 3 to 4 hr at 4 °C. The initial conditions were generally 600 V and 20 mA. At equilibrium, the conditions were approximately 1,200 V and 10 mA. Twenty fractions of 2.5 ml each were collected and their pH and A_{280} measured. See Figure 1, panel A.

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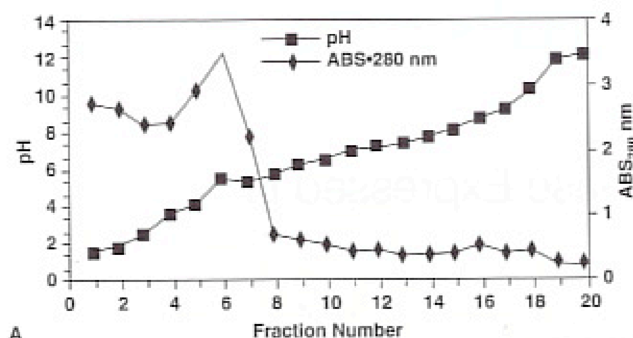

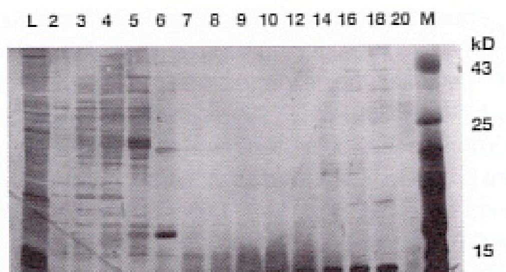

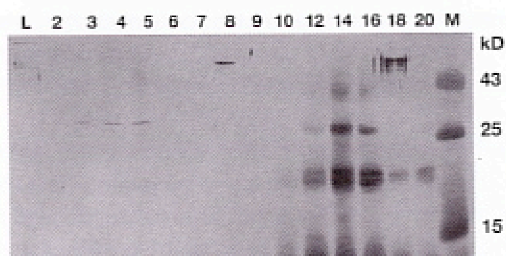

Fig. 1. Analysis of Rotofor fractionation of HIV1 protease expressed in E. coli. *Panel A. A plot of A_{280} and pH for Rotofor fraction numbers 2–20. Panel B. SDS-PAGE of Rotofor fraction numbers 2–20. 12.5% polyacrylamide gels were Coomassie® blue/silver stained. Lane L contains an aliquot of the crude material loaded on the Rotofor cell and lane M contains prestained molecular weight standards. Panel C. A polyacrylamide gel similar to the one in panel B was immunoblotted with rabbit polyclonal antibodies against the protease and developed with a peroxidase conjugated second antibody.*

VISUALIZATION OF PROTEINS USING SDS-PAGE

Aliquots from each Rotofor fraction were separated by SDS-PAGE and the polyacrylamide gels were stained with Coomassie blue/silver (Figure 1, panel B). To identify the protease-containing fractions, separated fractions were immunoblotted with antibodies to the protease (Figure 1, panel C). The fractions in the pH 9 to 11 range contained the majority of the HIV1 protease in agreement with the predicted pI of the enzyme. These fractions were pooled, concentrated, and then subjected to reversed phase HPLC on a C_3 column. The protease eluted as a homogeneous fraction as determined by SDS-PAGE. The amino acid composition was confirmed by amino acid analysis and both amino- and carboxyl-terminal sequencing.[2] In *in vitro* assays, the purified enzyme specifically cleaved the natural polyprotein substrates pr53[gag 2-3] and reverse transcriptase (p66),[5] as well as a series of synthetic polypeptides containing the target scissile bonds.[2] Furthermore, the proteolytic activity could be inhibited by pepstatin A as expected for an aspartyl protease.

YEAST EXPRESSION OF VIRAL HIV PROTEASE

A yeast expression vector encoding the signal sequence of the yeast pheromone alpha factor was used to express and secrete the HIV1 protease from *Saccharomyces cerevisiae*.[3] The correctly processed protease is easily detected in the crude culture media of yeast cells grown for 72 to 96 hours. The cells were removed by centrifugation, and the clarified media was concentrated about 50-fold by ultrafiltration. Following extensive dialysis against water, the medium was adjusted to 0.1% Triton X-100 detergent and 1 mM PMSF and applied to the Rotofor cell in the presence of 1% Bio-Lyte ampholytes, pH 3–10. The viral protease was detected in the Rotofor fractions above pH 9. These fractions were further purified using HPLC under the conditions described for the bacterially expressed protease.

Results

Purification of the HIV1 protease was greatly facilitated by the use of the Rotofor cell. This procedure confirmed the predicted isoelectric point of 9.95 for this protease and yielded substantial purification. The Rotofor cell proved useful for purification of the protease from either bacterial cell extracts or yeast culture supernatants. In the case of the *E. coli* expressed protein, the large quantities of contaminating bacterial proteins in the crude sample required initial purification steps prior to the separation on the Rotofor cell in order to avoid precipitation due to overloading of the apparatus. Rotofor fractionation of the bacterial lysate resulted in a near 200-fold purification of the protease. In the case of the yeast expression system, the viral protein is secreted into the culture while the majority of the contaminating proteins and nucleic acids are contained within the yeast cells. The yeast cell culture supernatant fluid, which is not rich in proteins can be concentrated extensively before loading the sample into the Rotofor cell. Fractionation of the concentrate by the Rotofor cell resulted in a purification of the protease of nearly 500-fold. The protease-containing fractions required further purification by HPLC in order to obtain single band homogeneity as evidenced by silver staining of polyacrylamide gels.[2,3] The yields of purified material ranged from 5 to 10 mg from 600 grams of wet bacterial pellet to approximately 1 mg from 12 liters of saturated yeast culture media.

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