Recombinant HIV2 Protease Processes HIV1 Pr53\textsuperscript{gag} and Analogous Junction Peptides \textit{in Vitro}\

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A synthetic DNA fragment encoding a protease precursor of the human immunodeficiency virus type 2 (HIV2) was cloned and expressed in bacteria and yeast. A recombinant plasmid encoding a hybrid polypeptide consisting of human superoxide dismutase and an HIV2 protease precursor of 113 amino acids was constructed for regulated intracellular expression in bacteria. Induction of this plasmid produced an autoprocessed form of the retroviral enzyme possessing the correct molecular weight. Overexpression and secretion of the protease from yeast was achieved with an expression vector encoding the yeast phenyl a-factor signal/leader sequence fused to a protease precursor of 115 amino acids. Amino-terminal sequence analysis confirmed that the viral enzyme exported from yeast was correctly processed from its precursor by cleavage of the predicted Ala-Pro peptide bond located at the NH\textsubscript{2} terminus of the protease in the pol open reading frame. No additional amino acid residues were required at the COOH terminus of the protease for this autoproteolytic event. The HIV2 protease expressed in bacteria and yeast was active in an \textit{in vitro} assay when tested on the HIV1 polypetide precursor, myristylated Pr53\textsuperscript{gag}. Two synthetic peptides representing junction sequences in the HIV1 gag-pol precursor were used to purify HIV2 protease. The enzyme exhibited a $K_{M}$ of 23.2 min$^{-1}$ mm$^{-1}$ on the HIV1 matrix-capsid junction peptide and a $K_{M}$ of 71.4 min$^{-1}$ mm$^{-1}$ on the protease-reverse transcriptase junction peptide. These rates show that the HIV2 enzyme is efficient at hydrolyzing the HIV1 peptide junctions, revealing the analogous nature of the substrate specificities of the two enzymes.

The human immunodeficiency virus (HIV)\textsuperscript{1} is the etiological agent of the acquired immunodeficiency syndrome (AIDS), a disease that has evolved into a worldwide public health problem. In addition to HIV type 1 (HIV1) (2–4) which has been implicated in epidemic AIDS in North America, Europe, and Central Africa (5), another retroviral group designated HIV2 (6) has been identified primarily in AIDS patients from West Africa (5, 7). Nucleotide sequence comparisons along with immunological studies carried out on several isolates of HIV2 (6–10) have revealed that this retrovirus is more closely related to the simian immunodeficiency virus (11, 12) than to HIV1. The gag and pol proteins of HIV2 can be precipitated by antibodies in sera from patients infected with HIV1 revealing a conservation of antigenic determinants between the HIV1 and HIV2 proteins. However, the gag and pol products of HIV2 have less than 60% identity in their amino acid sequences (6) when compared with the equivalent HIV1 proteins. Moreover, variation in the size, amino acid composition, and proteolytic cleavage junctions is also evident when the HIV2 polyproteins are compared with their HIV1 counterparts.

The rapid spread of HIV2 (5) underscores the need to understand the molecular and structural biology of this retrovirus for development of strategies to treat and prevent AIDS infections. One attractive target in the effort to arrest HIV replication is the viral protease encoded at the 5' end of the pol gene (13). This enzyme plays an essential role in the viral life cycle by processing the gag and gag/pol polyproteins to the mature structural proteins and enzymes required for virus formation. Recent studies of HIV1 protease have resulted in the elucidation of the three-dimensional structure of the enzyme (14–16). These studies confirm that this retroviral enzyme is a homo-dimeric member of the aspartyl protease family as anticipated by others (17) and permit structure-based design of protease inhibitors that may eventually serve as antiviral agents (18–20). Although the HIV1 and HIV2 proteases presumably play a similar role in viral replication, significant differences exist between the two enzymes. The HIV1 and HIV2 proteases are both 99 amino acids in length but share only 47.5% sequence identity (3, 6) and display unique substrate specificities. Moreover, the HIV2 protease lacks the 2 cysteine amino acid residues found in the HIV1 counterpart, does not react with polyclonal antibodies raised against the HIV1 protease, and is predicted to exhibit a much lower isoelectric point. These differences require that an independent analysis be carried out on the HIV2 protease.

The development of rapid and efficient systems to overproduce a soluble and authentic form of this viral enzyme will facilitate further biochemical and biophysical studies. Furthermore, the availability of \textit{in vitro} assays for the HIV2 protease is essential to address specific questions relating to the mode of action and specificity of the enzyme and its engineered variants. Two recent studies have reported on the chemical synthesis (21) and bacterial expression of the HIV2 protease (22). We have previously reported the expression in
bacteria (23) and yeast (24), purification, and initial characterization of the HIV-1 protease. Here we demonstrate that a precursor form of the HIV-2 protease will autoprocess in bacteria and yeast to yield a mature and active form of the protease. This protease accurately cleaves in vitro the heterologous substrate, HIV-1 myristylated Pr55 as well as synthetic peptides of two junctional sequences of the gag-pol precursor.

MATERIALS AND METHODS

Strains—Escherichia coli D1210 (25) was used for bacterial expression of the recombinant Saccharomyces cerevisiae AB110 (24) was used for yeast expression and secretion of the HIV-2 protease.

Gene and Plasmid Constructions—A 360-base pair synthetic DNA fragment was constructed that encoded an 112-amino acid precursor of the HIV-2 protease from isolate ROD-6. The precursor contained 13 additional amino acids at the NH2 terminus of the 99-amino acid protease and was constructed from 17 overlapping oligonucleotides. Viral codons were chosen to construct the coding sequence except at Val-10 (GTA→GTG), Thr-12 (ACA→ACC), and Val-71 (GTA→GTC). Codons 10 and 12 were chosen to incorporate a unique BstEII site and codon 71 was chosen to incorporate a unique NplII site. The endonuclease restriction sites were included in the Sall site and was included at the 3′ end of the synthetic gene. Fifteen of the oligonucleotides used in the construction were 45 bases in length and had 22- or 23-base pair overlaps. The 5′ and 3′ terminal oligonucleotides were 27 and 18 bases in length, respectively (Fig. 1A). The DNA construct included a stop codon at the COOH terminus of the protease that provided the first G of the Sall site at the 3′ end of the gene. The endonuclease restriction sites located at the 5′ and 3′ ends of the DNA segment facilitated the subsequent cloning of the gene into appropriate plasmids and expression vectors. The BstEII and NplII sites were introduced to permit dissection of the gene. Oligonucleotides were synthesized using solid-phase phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer and were purified as described elsewhere (24). Oligonucleotides were phosphorylated, annealed, and ligated following standard protocols (26). Equimolar concentrations (5 pmol) of each phosphorylated oligonucleotide were mixed together in 2 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 10 mM dithiothreitol and heated to 90°C for 2 min in a 1.5-ml microcentrifuge tube in a covered water bath. The temperature control of the bath was then shut off and the oligonucleotides allowed to anneal for the 4 h needed for the bath temperature to reach 20°C. The mixture was then ligated at 25°C for 20 min using T4 DNA ligase. The DNA was precipitated with ethanol, dried, and suspended in water. After digestion with the enzymes XbaI and Sall, the 360-base pair DNA fragment was purified from a 7% polyacrylamide gel and cloned into the M13 vector, mpl9. Nine independent clones were isolated and purified, single-stranded DNA was harvested, and the insert of each cloned gene was sequenced using the dideoxynucleotide chain-termination method (27). Each isolate contained discrepancies when compared with the expected sequence.

One isolate contained three transitions Val-67 (GTA→ATA), Asp-79 (GAC→AAC), and Asn-83 (AAT→AAC), and one deletion Ala-92 (GCG→GC) in the coding region of the mature protease. This isolate also contained an insertion/substitution at Arg-5 (AGA→AAGC).

Four isolates were identical to the previous isolate except that the C deletion at codon 92 was not present. Three isolates contained four substitutions in the mature protease, Ala-34 (GCA→GTA), Lys-45 (AAA→AAC), Val-71 (GTC→GTA), and Ala-92 (GCG→GCT). One isolate contained two transitions Asp-79 (GAC→AAG) and Asn-83 (AAT→AAC) in the mature protease and one insertion/substitution at Arg-5 (AGA→AAGC). Although the G to A and C to T transitions can be accounted for by guanine modification during chemical DNA synthesis (28), no normal pattern can be discerned to account for the discrepancies which may have resulted from machine failure or random inaccuracies. The isolate with one insertion and two single-base substitutions was chosen for repair. The substitution at position 83 was a silent mutation and was left uncorrected. Two oligonucleotides 5′-GTTGCGCCCAAGTCTCGTTGGTCGTCGCC′ and 5′-TGGTTGCGCCCAAGTCTCGTTGGTCGTCGCC′ were used to repair the insertion/full substitution at Arg-5 and the substitution at Asp-79, respectively. One round of site-directed mutagenesis using both oligonucleotides and methods previously described (29) was used to obtain the desired gene sequence. The repaired DNA was sequenced completely and used for all subsequent DNA constructions.

For secretion of the HIV-2 protease from yeast, the plasmid pHIVi2PR11 was constructed. The 339-base BglII-SalI DNA fragment encoding the HIV-2 protease was cloned into the BglII-SalI digest vector pSUODCF2 (30). This vector provided the β-lactamase gene for selection, the CoEI origin of replication for autonomous replication in E. coli and the inducible tac promoter for transcriptional control. Plasmid pSUODCF2 encodes a β-lactamase consisting of human superoxide dismutase (SOD) fused to a 113-amino acid precursor of the HIV-2 protease (Fig. 1B). The cloning strategy involved replacing the COOH-terminal alanine of human SOD with methionine and glycine 72 and threonine 74 of the pol polyprotein. For secretion, plasmid pHIVi2PR11 encoding the recombinant protease precursor was linearized with BglII and ligated to a Sall-digested vector pSUODCF2. This vector provided the β-lactamase gene for selection. For secretion, the β-lactamase gene and the CoEI origin of replication were excised using the Sall restriction enzyme and the pol polyprotein was spliced into the remaining vector. The Sall-digested plasmid pHIVi2PR11 was ligated to a Sall-digested vector pSUODCF2. The Sall digested vector pSUODCF2 was then transformed into E. coli to form the recombinant plasmid pSUODCF2R11. The recombinant plasmid was screened for the presence of the antibiotic resistance gene and the expression of the HIV-2 protease.

For secretion of the HIV-2 protease from yeast, the plasmid pHIVi2PR11 was constructed. The 339-base BglII-SalI DNA fragment encoding the HIV-2 protease was cloned into the pBS241-derived plasmid pPR11 (24). This yeast expression vector contains 2 copurifying sequences for autonomous replication in yeast, the glucose-regulated hybrid promoter ADH2/GAPDH, the α-factor terminator to ensure transcription termination, and the yeast genes, leuc-3 and ura3 for selection. The β-lactamase gene and the CoEI origin of replication were excised using the Sall restriction enzyme and the pol polyprotein was spliced into the remaining vector. The Sall-digested plasmid pHIVi2PR11 was ligated to a Sall-digested vector pBS241. The recombinant plasmid was screened for the presence of the antibiotic resistance gene and the expression of the HIV-2 protease.

Expression of HIV-2 Protease in Bacteria and Yeast—E. coli D1210 cells harboring plasmid pSUODCF2R11 were grown for 15-18 hr at 37°C in 3 ml of Luria broth (26) containing ampicillin at 40 μg/ml. The overnight culture was used to inoculate 60 ml of M9 minimal medium containing ampicillin at 40 μg/ml (26) and cells were grown for 2 hr at 37°C with vigorous shaking. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 200 μM final concentration, and cultures were incubated for another 18 hr. Aliquots of the induced culture containing 0.3 OD600 were collected by centrifugation at 10,000 × g for 10 min, and pellets were lysed by repeated boiling and freezing in 40 μl of 63 mM Tris-HCl, pH 6.8, 80 mM dithiothreitol, 10% glycerol, 3% sodium dodecyl sulfate (SDS), 0.02% bromphenol blue. Proteins were separated by SDSPAGE in 15% polyacrylamide gels, transferred to nitrocellulose filters for 1 h at 75 volts using a Bio-Rad Trans-Blot apparatus. The HIV-2 protease was identified on immunoblots using rabbit polyclonal antibodies raised against a synthetic HIV-2 protease as the primary antibody. A goat anti-rabbit antibody conjugated to horseradish peroxidase (Tago) was used as the secondary antibody for immunodetection.

Cultures of S. cerevisiae AB110 cells harboring pSUODCF2R11 were grown in 50 ml of leucine- and uracil-deficient media for 24-48 hr at 30°C, and cells were collected by centrifugation at 7000 × g for 20 min. Proteins in 5 ml of supernatants were precipitated with 2.5 ml of 50% trichloroacetic acid containing 2 mg/ml deoxycholate. After incubation at 4°C for 30 min, pellets were collected, washed with acetone, dried, and suspended in 20 μl of sample buffer (33). Proteins fractionated by SDS-PAGE were visualized by Coomassie Blue staining or transferred to nitrocellulose filters for immunodetection.

For large scale yeast fermentation and expression of the HIV-2 protease, a 115-liter fermentor was prepared with 88 liters of leucine- and uracil-deficient media (32) containing 0.05% (w/v) deoxycholate. The fermentor was then inoculated with a 6-liter seed culture of S. cerevisiae AB110 harboring the recombinant plasmid pHIVi2PR11. The seed culture had been grown for 24 h in leucine-deficient media to ensure a high copy number of the expression plasmid. Culture conditions were 30°C, 5.0 psi and 300 rpm. Cell density, pH, CO2 concentration, and glucose concentration measurements were made every 2 h throughout the duration of the fermentation run, which lasted 72 h. Cell density measurements were made using an inline spectrophotometer set at 650 nm (OD650). The pH was determined by pH electrode and the concentration of CO2 was determined by infrared spectroscopy using an IR-703 gas analyzer (In-
Recombinant HIV Protease

The HIV protease was assayed against the decapeptide, Ala-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-Trp and the octapeptide, Ser-Gln-Tyr-Pro-Ile-Val-Gln. The decapeptide corresponds to the HIV carboxy-terminal autoprocessing site, and the octapeptide corresponds to the HIV matrix-capsid cleavage site (2). The peptides were synthesized using conventional solid-phase methods. Concentrations of the enzyme stock solutions were determined by titration with the substrate-based inhibitor Val-Ser-Gln-Asn-Leu-Val (CH(OH)CH)Val-Ile-Val (34). Reactions were carried out in 0.1 M sodium acetate buffer, pH 4.7, containing 5 mM EDTA, 1 mM NaCl, and 0.05-0.6 mM peptide substrate. The effect of salt and pH on enzyme activity was tested by using 0.1 M sodium acetate buffer at either pH 4.7 or 5.5 and containing either 0.25 or 1 mM NaCl. Typically, 1-3 x 10^4 ng of HIV protease was added to initiate the reaction, which was stopped after 2 h at 37 °C by addition of 50 μL of cold 0.1% trifluoroacetic acid on ice. Conditions were adjusted so that <25% of the substrate was hydrolyzed during the incubation. Reaction products were separated by reverse-phase HPLC using a C18 Porec column. Products of the decapeptide (Ala-Thr-Leu-Asn-Phe and Pro-Ile-Ser-Pro-Trp) were resolved with a gradient of 10-50% acetonitrile in 0.1% trifluoroacetic acid, while the octapeptide fragments (Ser-Gln-Tyr-Pro and Ile-Val-Gln) were observed with a gradient of 0-55% acetonitrile. Absorbance was monitored at 280 nm, and hydrolysis of the peptides was quantitated by integration of the peaks and comparison with standard curves. Each data point was measured in triplicate. The program Enzfitter was used to fit data to Michaelis-Menten kinetics (37).

**Amino-terminal Segment Sequence Analysis**—The retroviral protease in 20 ml of yeast supernatant was obtained from yeast AB110 cultures expressing the recombinant plasmid pHSV2PI115. Proteins were precipitated with trichloroacetic acid, fractionated in a 15% polyacrylamide gel containing SDS, transferred to nitrocellulose paper, treated with trypsin, and eluted in 50 mM ammonium bicarbonate buffer. The column was washed with a linear gradient of 10-100 mM ammonium bicarbonate in buffer followed by two column volumes of water. The eluates, which eluted in the later fractions of the water wash in approximately 250 ml, was further purified by preparative isoelectric focusing on a Rotofor unit (Bio-Rad). A mixture of 0.5 ml, pH 3-10, Ampholytes and 0.25 ml, pH 5-8, Ampholytes was added to 65 ml of protease sample to precipitate five Rotorfor fractions. The fractions in the range of pH 5.0-6.5 were pooled. These samples were further purified by reversed-phase HPLC using a preparative C8 column (Pro 10/300 Protein Plus, Du Pont, 4.6 x 21.2 cm) and eluted with a linear gradient of 25-85% acetonitrile/water in 0.1% trifluoroacetic acid over a period of 60 min at a flow rate of 10 ml/min. The protein eluted at approximately 45% acetonitrile was lyophilized and stored at -20 °C.

The resulting enzyme is 95% pure as judged by NH2-terminal sequence analysis as well as silver staining of SDS-PAGE gels. For enzymatic analysis, the lyophilized enzyme was denatured in 0.05 mM sodium acetate, pH 5.5, containing 8 M urea, and refolded with a 10-fold dilution into 0.2 M sodium acetate, pH 5.5, containing 1 mM EDTA, 5 mM dithiobitol, 10% glycerol, and 5% ethylene glycol. This procedure has been shown to restore maximal activity to preparations of HIV-1 enzyme that have become inactive due to denaturation (34).

**In Vitro Assay for HIV Protease on Pr53R**—A recombinant form of the natural substrate Pr53R has previously been expressed in yeast (35). The myristylated polypeptide was purified and shown to be a substrate for the HIV protease (24). In a similar fashion, we monitored the activity of the HIV protease expressed in bacteria and yeast on myristylated Pr53R. The heterologous substrate (1 μg) was incubated in 10 mM Tris-HCl, pH 7.0, containing 180 mM NaCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride with the concentrated yeast media or partially purified bacterial lysates. After 8 h of incubation at 25 °C, the reaction products were analyzed by SDS-PAGE in 15% polyacrylamide gels on an immunoblot containing AIDS patient sera or the primary antibody. The serum was inactivated in a Biosafety Level 3 facility by heating at 86 °C for 35 min, treating with psoralen at 25 μg/ml final concentration, and UV irradiation on ice. Visualization of the specific bands on the immunoblot was achieved with goat anti-human antibodies conjugated to horseradish peroxidase (Tago) as the secondary antibody. Lysates of different yeast cells (36) were included as markers to visualize HIV viral proteins. The lysates were inactivated in a Biosafety Level 3 facility by treating with 0.5% Triton X-100 final concentration. Reaction products were used at various concentrations in some assays to inhibit the activity of the retroviral protease.

**Bacterial expression of the HIV protease was achieved by cloning a synthetic DNA fragment (Fig. 1A) encoding the retroviral enzyme into a bacterial expression vector derived from pSOD/PRI79 (23). The resulting recombinant plasmid pSOD/HIV2PR183 (Fig. 1B) encodes a polypeptide containing a 180-amino acid human superoxide dismutase and an HIV2 protease precursor of 113 amino acids. The hSOD sequences are essential for expression of the 113-amino acid protease precursor since a similar construction lacking the hSOD sequences resulted in no detectable HIV2 protease. The viral protease precursor contains an NH2-terminal extension of 14 amino acids to the 99-amino acid protease. There is no COOH-terminal extension since a stop codon was placed immediately downstream of the COOH-terminal leucine of the protease. The expression of the fusion protein was controlled by IPTG induction of the tac promoter located 5' to the hSOD gene. Protein expression was monitored in total cell lysates by SDS-PAGE and immunoblotting. An immunoreactive band of approximately 10 kDa (PR) can be observed within 0.25 to 0.5 h of induction using rabbit polyclonal antibodies raised against a synthetic HIV2 protease (Fig. 2, lanes 2 and 3). The 10-kDa band was not detected using rabbit polyclonal antibodies raised against the HIV1 protease. The 10-kDa species is in agreement with the 10,721-dalton pre-
A. HIV2 protease synthetic gene

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B. pSOD/HIV2PR113

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NcoI  BglII  NcoI
|      |      |      |
| Stop | TAG   | ATTCAGCT |
|      |      |         |
| CATGGCA CGTCTAG |
|      |      |         |
| Met Ala |
| tac Promoter | Human SOD | 152 aa |
| HIV2 pol sequences | 13 aa |
| HIV2 Protease Sequences | 99 aa |
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C. pHIV2PR115

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XbaI  BglII  NcoI  NcoI
|      |      |      |      |
| Stop | TAG   | ATTCAGCT |
|      |      |         |
| LeuAspLysArgPheArgGlu |
| CTAGATAAAGATTTAGAGAA |
| TATTTTCTAAATCTCTTCTTAG |
|      |      |         |
| ADH2/GAPDH Promoter | α-factor leader | HIV2 pol sequences | 13 aa |
| HIV2 Protease Sequences | 99 aa |
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**Fig. 1.** Gene construction and recombinant plasmids for the expression of the HIV2 protease. A. synthetic HIV2 protease gene. Schematic representation of the HIV2 protease synthetic gene. A 360-bp XbaI-Sall DNA fragment was constructed using 17 overlapping synthetic oligonucleotides of 18, 27, and 45 nucleotides in length. The XbaI, BglII, and SalI restriction sites were included to facilitate subcloning of the final construction. The BstEII and HpaII restriction sites present in the coding region of the 99-amino acid protease (hatched) were introduced to permit dissection of the gene. B, bacterial expression plasmid pSOD/HIV2 PR113. A 339-bp synthetic BglII-Sall DNA fragment encoding the 99-amino acids HIV2 protease and containing 13 additional residues at its NH2-terminus was cloned into the plasmid pSOD/PR179 (23). The resulting plasmid encodes a hSOD/HIV2 protease hybrid precursor whose expression is under the control of the tac promoter. C, yeast expression plasmid pHIV2PR115. The BglII-Sall restriction fragment described above was cloned into the plasmid pPR179 (24) that provided the yeast α-factor signal/leader sequence containing the KEX2 processing site to promote secretion of the retroviral enzyme from the cell. The HIV2 recombinant plasmid also contains the ADH2/GAPDH promoter, the α-factor terminator, 2μ yeast sequences and the genetic markers leu2-de, ura3, and β-lactamase.

dicted mass of the mature protease. This indicates that the HIV2 protease is capable of autoprocessing within the bacterial host as previously reported for the HIV1 protease (23, 39-41). Another major band of approximately 30 kDa (SOD/PR) was also detected by the antibodies to the HIV2 protease. This band is also detected by a mouse monoclonal antibody raised against hSOD (results not shown), and probably represents the 27,865 kDa SOD/HIV2 protease fusion containing the unprocessed viral enzyme. As expected, the protease and the hybrid precursor were not observed in the uninduced cultures.

Expression levels of the hybrid polypeptide and the mature protease reach their maximum levels at approximately 3-4- and 5-6 h post-induction, respectively (Fig. 2, lanes s-11). Expression levels of both proteins are greatly reduced after 18 h suggesting that these proteins are susceptible to degradation by E. coli proteases. Increasing levels of the 30-kDa precursor band do not result in a concomitant increase of the 10-kDa protease band suggesting that release of the protease from the hybrid polypeptide is slow relative to the proteolytic turnover of the viral protease. The HIV2 protease is completely soluble in this expression system and at maximum expression levels is approximately 0.1% of the total cellular proteins.
**Recombinant HIV-2 Protease**

**Fig. 2. Immunodetection of HIV-2 protease expressed in E. coli D12110.** Cells harboring plasmid pSOD/HIV2PR113 were grown in 60 ml of M9 minimal media (26) for 2 h, and cultures were induced by the addition of IPTG at a final concentration of 0.2 mM. Samples were removed at different times post-induction and 0.3 OD₆₀₀ of cells were lysed as described under “Materials and Methods.” Proteins fractionated by electrophoresis in a 15% polyacrylamide gel containing SDS were transferred to nitrocellulose filters. Immunoblot analysis was performed using rabbit polyclonal sera raised against a synthetic HIV-2 protease. The column numbers indicate hours after induction with IPTG. PR is the protease; SOD/PR is the hybrid precursor. Prestained proteins were loaded as molecular weight standards (column M).

**Fig. 3. Detection of HIV-2 protease in yeast supernatants of S. cerevisiae AB110.** A, Coomassie Blue-stained proteins. B, immunoreactivity with rabbit sera containing antibodies to a synthetic HIV-2 protease. Cells harboring pHIV2PR115 were grown in 50 ml of leucine and uracil deficient media at 30 °C. After 24 (lane 2) or 48 h (lane 3) of incubation, 10-ml aliquots of the cultures were pelleted by centrifugation and proteins in 5 ml of yeast supernatants were precipitated with trichloroacetic acid and fractionated by SDS-PAGE in a 18% polyacrylamide gel containing SDS. Immunoblot analysis was performed as described in Fig. 2. Trichloroacetic acid-precipitated proteins of yeast supernatants of AB110 cells harboring the parental plasmid pBS24.1 (24) are shown in lane 4. PR is the mature HIV-2 protease.

To avoid the problems associated with intracellular proteolysis in bacteria as well as to facilitate its purification, the protease was expressed and secreted in yeast. Yeast expression of the HIV-2 protease was accomplished by fusing the synthetic DNA fragment encoding the HIV-2 protease to DNA encoding the α-factor signal/leader sequences that contain the KEX2 recognition cleavage site (Fig. 1C). The glucose-regulatable hybrid promoter ADH2/GAPDH (42) is used to control expression of the cloned gene. The α-factor terminator (43) is included to ensure transcription termination. The plasmid pHIV2PR115 encodes an HIV-2 pol precursor of 115 amino acids with a predicted molecular weight of 12,435. A protein band of approximately 10 kDa (PR) is observed by Coomassie Blue staining (Fig. 3A, lane 3) and immunoblotting (Fig. 3B, lane 2) in yeast supernatants of cells transformed with pHIV2PR115, indicating autoprocessing of the precursor. The viral protease is not observed in yeast supernatants of cells grown for 24 h (A and B, lane 2) since glucose consumption and the resultant induction of the ADH2/GAPDH promoter takes place after 28–30 h of growth under the culture conditions used (see below). Maximum levels of expression (0.8–2.0 mg/liter of media) are observed after 48–72 h. The HIV-2 protease proved to be stable at 30 °C in the yeast media for up to 6 days as judged by immunoblotting and enzymatic analyses (results not shown) and represents a predominant yeast-secreted protein. This protein is not seen in yeast supernatants of cells harboring the parental plasmid pBS24.1 (A and B, lane 4).

The NH₂ terminus of the yeast-secreted HIV-2 protease was sequenced to unequivocally demonstrate that the 10-kDa product represents the correctly processed, mature, retroviral protease. The first 13-amino acid residues displayed the sequence Pro-Gln-Pro-Ser-Leu-Trp-Lys-Arg-Pro-Val-Val-Thr-Ala which is identical to that predicted for the HIV-2 protease (6). This confirms that the viral enzyme secreted from yeast was correctly released from the precursor by cleavage at the Ala-Pro junction.

To determine whether the recombinant HIV-2 protease expressed in bacteria and yeast represents an active form of the viral enzyme, its ability to correctly cleave the HIV-1 polyprotein substrate, myristylated Pr53Kₑₑₑ, was established. Both the bacterial- (Fig. 4, lane 2) and yeast- (lane 6) expressed protease can accurately process the HIV-1 protein precursor as judged by the generation of capsid protein (p24) and matrix protein (p17) along the course of the incubation. Another protein band of approximately 6 kDa (p6 or p7) that probably represents a processing product of nucleocapsid protein (p15) (44) was also detected by the human AIDS serum. This protein had remained undetected when similar experiments were performed with the HIV-1 protease (24) due probably to a low titer of antibodies against this protein. These processed species are indistinguishable from those generated by a purified HIV-1 protease expressed in yeast (lane 3) (24) and comigrate with their counterparts expressed in vivo (lane 4). Bacterial extracts and yeast supernatants that do not contain the HIV-2 protease do not generate specific viral proteins when incu-
bated with Pr53<sup>946</sup>. Nonspecific proteolysis of the HIV1 polyprotein precursor (lanes 1 and 5) is presumably caused by endogenous bacterial and yeast proteases. Processing of Pr53<sup>946</sup> by the HIV2 protease was completely abolished when 10 mM pepstatin was included in the assay (results not shown). This observation provides evidence that the HIV2 protease is an aspartyl protease as already established for the HIV1 enzyme (15).

The HIV2 protease was purified from yeast supernatants using a three-step purification procedure. Fig. 5 shows representative samples from the various stages of the protocol. We estimate that the initial concentration of the protease is approximately 0.8 mg/liter of yeast supernatant (lane 2). The subsequent steps involving phenyl-Sepharose chromatography, preparative isoelectric focusing fractionation, and reversed-phase HPLC result in homogeneous enzyme (lanes 3–5). The phenyl-Sepharose column, which mimics the hydrophobic peptide substrate, is used to concentrate the enzyme from the large volume of yeast media. The protease-containing fractions eluted from the column are subjected to preparative isoelectric focusing on a Rotofor unit to remove high molecular weight proteins and melanin polymers of heterogeneous size. Migration of the HIV2 protease to its expected isoelectric point of approximately 5.5 separates the enzyme from the negatively charged polymeric contaminants which migrate as a dark brown band in the pH range of 1–3. Following removal of the Ampholytes by dialysis, the pH 5.0–6.5 Rotophor fractions are loaded onto a preparative C<sub>5</sub> column and the protease is eluted as a single peak at approximately 60% acetonitrile; this is in agreement with its expected hydrophobic nature. The single band shown in Fig. 5, lane 5, is 95% pure as judged by amino acid analysis and silver staining (data not shown). The overall yield of the protein is between 10 and 20%. The purified protein is stable to rapid freeze/thaw cycles and storage at -20 °C in 10% glycerol, 5% ethylene glycol buffer. However, concentrated enzyme solutions (0.25 mg/ml) kept at 0 °C can lose activity, presumably due to autodegradation. For cases where enzyme activity is lost due to denaturation, activity can be restored by refolding the enzyme using a procedure similar to that used for HIV1 protease (34). The purified enzyme was assayed against the HIV1 matrix-capsid octapeptide Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln to yield a specific activity of 3.0 μmol/min/mg. This value is comparable to that obtained for hydrolysis of the same substrate by purified HIV1 protease (45).

The kinetic properties of the purified HIV2 protease were evaluated using the two synthetic peptide substrates Ala-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-Trp and Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln. To ensure accuracy in all enzymatic analyses, active site titrations using the substrate-based inhibitor Val-Ser-Gln-Asn-Leu-[CH(OH)]<sub>3</sub>Val-Ile-Val (34) were used to determine the concentration of enzyme stock solutions. Evaluation of two buffer conditions commonly used for HIV1 protease assays showed that an 80% increase in enzyme activity on either peptide substrate resulted by increasing the salt concentration from 25 mM to 1.0 M. A further increase in enzyme activity of approximately 10% resulted by lowering the pH from 5.5 to 4.7. The optimal conditions of high salt and low pH were used for determining the kinetic constants of the octapeptide and decapptide substrates. The enzyme exhibited a k<sub>cat</sub> of 65 min<sup>-1</sup> and a K<sub>M</sub> of 2.8 ± 0.5 mM on the octapeptide substrate and a k<sub>cat</sub> of 10 min<sup>-1</sup> and a K<sub>M</sub> of 0.140 ± 0.045 mM on the decapptide substrate (Fig. 6).

The behavior of yeast expressing the HIV2 protease in large scale fermentation conditions was evaluated to establish baseline growth and production profiles. Sixty-eight liters of pHIV2PR115 transformed yeast were grown in a 115-liter vessel. Results shown in Fig. 7A reveal that the yeast culture remains in lag phase for approximately 15 h until an increase in the cell density and a simultaneous decrease in the pH is observed. The stationary phase is reached after approximately 65 h. Glucose utilization by the yeast resulted in a sharp evolution of CO<sub>2</sub> production from 20 to 28 h post-inoculation (B). A concomitant shift in pH was also noted during this time interval (A). As expected, the HIV2 protease was expressed upon depletion of available glucose and was detected immunologically after 30 h (C, lane 4). Maximal expression

Fig. 5. Purification of secreted HIV2 protease from yeast. Samples from various steps of the purification procedure were run on a 17.5% polyacrylamide gel containing SDS and stained with Coomassie Blue. Samples were precipitated with trichloroacetic acid/ deoxycholate as described under "Materials and Methods" to reduce the volume of the sample. Lane 1, molecular weight markers; lane 2, yeast culture supernatant (5 μl, 0.1% of total); lane 3, pooled, protease-containing fractions from the phenyl-Sepharose column (0.2% of total); lane 4, preparative isoelectric focusing fractions migrating in the pH 5.0–5.5 range (0.6% of total); lane 5, purified protease (3.5 μg, 0.8% of total) isolated by reversed-phase chromatography on a preparative C<sub>5</sub> HPLC column.

Fig. 6. Double-reciprocal plot of HIV-2 protease activity versus concentration of peptide substrates ATLNFPISWP (■) and SQNYPIVQ (○). Either 2.5 × 10<sup>-4</sup> mg (■) or 8.9 × 10<sup>-5</sup> mg (○) HIV-2 protease was incubated with varying amounts of substrate for 2 h at 37 °C in 0.1 M sodium acetate buffer, pH 4.7, containing 1 M NaCl and 2 mM EDTA. Enzyme activity was measured as described under "Materials and Methods." Data were fit to the Michaelis-Menten equation and kinetic constants were calculated using a nonlinear regression program ("Enzfitter" from Biosoft).
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![Graphs and images related to HIV2 protease](image)

**Fig. 7.** Fermentation profiles of yeast expressing and secreting HIV2 protease. A, cell density (OD600) and pH profiles. B, percent glucose concentration and percent CO2 concentration. C, expression levels of secreted HIV2 protease from yeast. The HIV2 protease in 4 mL of yeast supernatant was precipitated with trichloroacetic acid/deoxycholate, fractionated by electrophoresis in a 15% polyacrylamide gel containing SDS and transferred to nitrocellulose filters for immunoblot analysis as described under "Materials and Methods." Protein molecular weight markers, lane 1; media from pBS24.1 transformed yeast, 72 h, lane 2; media from pHIV2PR115 transformed yeast, 24 h, lane 3; 30 h, lane 4; 48 h, lane 5; 52 h, lane 6; 72 h, lane 7.

Levels were obtained at 72 h (C, lane 7) and the growth was terminated due to the increasing pH of the media.

**DISCUSSION**

The emergence and rapid spread of HIV2 as another causative agent of AIDS (5, 6) raises the challenge to better understand the molecular biology of this retrovirus. Since the protease is essential for viral replication and represents an attractive target for anti-AIDS therapeutics we have cloned and expressed the HIV2 protease in bacteria and yeast. This should facilitate the purification of large quantities of protein for further biochemical and biophysical studies. In addition, the expression systems allow us to study the autoprocessing event as well as the proteolytic activity of the mature protease on the heterologous substrate HIV1 Pr53(35).

The HIV2 protease is capable of autoprocessing in bacteria to form a mature and active form of the enzyme when expressed as part of a human superoxide dismutase fusion protein. The protease in the hSOD/HIV2 polyprotein has only a 14-amino acid NH2-terminal extension and does not contain additional amino acids at its COOH terminus. The mature HIV2 protease was completely soluble, and the onset of degradation could be monitored after 4 h of induction. The autoprocessing event appears to be less efficient than that observed for the hSOD/HIV1 protease precursor (23) since a large percentage of the HIV2 protease remains in the hybrid polyepitide throughout an 18-h time course of expression. Although the lack of a COOH-terminal extension did not prevent autoprocessing of HIV2, it may have contributed to the inefficiency of the process. Analogous amino acid sequences to those found in the hSOD/HIV1 polyepitide (23) may be required in the hSOD/HIV2 polyepitide for efficient autolysis from the polyepitide precursor.

When expressed and secreted in yeast, the HIV2 protease was correctly released from a 115-amino acid pol precursor lacking a COOH-terminal extension. This observation can also be extended to the HIV1 protease as well since a 154-amino acid HIV1 pol precursor that does not contain additional amino acid sequences at the COOH-terminus of the protease efficiently self-processes in yeast. It has been postulated that the HIV protease undergoes autolysis activation when expressed as part of a higher molecular weight precursor and that this activation is initiated at the COOH terminus of the protease (40). The mature HIV1 and HIV2 proteases that we have obtained using yeast expression plasmids encoding HIV precursors with no additional amino acid sequence at their COOH termini, support this idea and shows that the autoprocessing event does not require that the COOH-terminal amino acids be present for processing the NH2-terminal site.

The HIV2 protease monomer is predicted to be a 99-amino acid protein that is released from the gag/pol polyprotein after cleavage of NH2-terminal Ala-Pro and COOH-terminal Leu-Pro peptide bonds. The amino-terminal sequence that we determined for the yeast-expressed HIV2 protease represents the first direct evidence that the potential Ala-Pro cleavage site is recognized and hydrolyzed by the HIV2 enzyme during autolysis. Autoprocessing of the HIV1 protease at the analogous Phe-Pro cleavage sites has been reported (23, 24, 39-41). Mass spectral analysis of purified HIV2 protease provides additional evidence that the mature HIV2 protease is correctly processed to form the 99-amino acid enzyme monomer.4

To determine whether the bacterial and yeast-expressed HIV2 protease represents an active viral product with authentic enzymatic activity, the pattern of proteolysis using purified HIV1 Pr53(35) precursor as a substrate (35) was determined. Processing of murine leukemia virus and feline leukemia virus gag precursors by a heterologous retroviral protease has been reported (46-49). Both the bacterial and yeast-expressed 4S. Richiante, L. M. Babé, P. J. Barr, D. L. DeCamp, and C. S. Craik, unpublished results.

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HIV protease process the HIV gag polyprotein to yield a product pattern that is indistinguishable from that generated by an HIV protease expressed in yeast. The migration of the peptides generated in vitro is also identical to the migration of structural proteins from HIV virions. This shows that the HIV protease expressed in both the bacterial and yeast systems are enzymatically active and capable of authentic processing of the HIV gag precursor in vitro.

The HIV gag polyprotein has a calculated molecular weight of 57,100 and is 20 amino acids larger than its HIV counterpart (3, 6). The HIV and HIV gag precursors share 58% identity in their amino acid sequences. Processing of the HIV Pr55gag by the HIV protease suggests that the HIV and HIV gag polyproteins adopt similar conformations that allow the recognition and hydrolysis of a select number of peptide bonds within the polyprotein precursor. Synthetic peptides with sequences of the HIV matrix/capsid junction and the protease reverse transcriptase junction were shown to be substrates for the HIV protease in quantitative assays using purified protease whose concentration was verified by active site titration. This permits an accurate comparison of the efficiencies of the HIV1 and HIV2 enzymes on the same substrate. The HIV2 protease hydrolyzes the octapeptide substrate with a $k_{cat}$ of 65 min$^{-1}$ and a $K_M$ of 2.8 mm. The similarity of these kinetic parameters to those described for the HIV1 protease on the identical substrate ($k_{cat}$ = 75 min$^{-1}$, $K_M$ = 1.5 mm) (45) shows the analogous substrate specificity of the two enzymes and confirms the observation of efficient HIV2 protease processing of Pr55gag, in vitro. A detailed analysis of the HIV1 and HIV2 substrate specificities on various synthetic peptides is described elsewhere (49). These results suggest that active site-directed inhibitors currently being developed against the HIV1 protease may also serve as effective inhibitors of the HIV2 protease. Preliminary results using the purified HIV2 protease and synthetic inhibitors support this proposal (49).

The expression systems described here are shown to be useful in the production of a mature, active HIV2 protease. These systems may also prove to be useful for the expression of variant forms of the HIV2 protease as well as related aspartyl proteases. The large scale production of active HIV2 protease achieved by using the yeast expression system will facilitate its purification as well as permit the development of sensitive and efficient in vitro assays for this protein. The availability of reagent levels of homogeneous material will also permit biophysical analysis of the retroviral enzyme to provide a better understanding of structure/function relationships and aid in the rational design of inhibitors and eventual antiviral pharmaceuticals.

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