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HTLV-I Protease Cleavage of P19/24 Substrates Is Not Dependent on NaCl Concentration

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Understanding the factors that affect the activity of Human T-cell Leukemia Virus type I (HTLV-I) protease is essential for the discovery of inhibitors to be used for the treatment of HTLV-I infection, but little has been reported on the protease to date. Here we report the production of HTLV-I protease in purified yields greater than 150 mg/L, determination of its extinction coefficient, and determination of the optimum conditions for cleavage of the p19/24 substrates (DABCYL)-(GABA)-PQVL-Nph-VMH-(EDANS), (DABSYL)-(GABA)-PQVL-Nph-VMH-(EDANS), and (DABSYL)-(GABA)-PQVLPVMH-(EDANS). The highest activity was found at pH 5.2–5.3 and 37°C. There was no effect on activity upon change in sodium chloride concentration from 0 to 1500 mM. The values of K_m and k_{cat} for cleavage of these substrates by the protease with and without the histidine tag were determined.

Key Words: HTLV; aspartic acid; protease; salt; kinetics.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I), isolated in the early 1980s, was the first oncogenic human retrovirus to be discovered, and it has recently been classified as a dangerous emerging pathogen by the Centers for Disease Control (*1*). HTLV-I is an oncovirus in the *Retroviridae* family (*2*) and infection with this virus has been linked to adult T-cell leukemia/lymphoma (ATL) and tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM) (*3*). It is estimated that at least 2.5 million United States residents and an additional 15 to 20 million individuals worldwide are infected with HTLV-I, and currently approximately 10% of these infected individuals will develop the fatal disease ATL (*4*). The Caribbean strains of HTLV-I are becoming increasingly infectious as indicated by patients with this strain developing the above

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diseases at earlier ages than those infected with Japanese strains (*5*), which suggests that HTLV-I might be evolving into a more dangerous virus.

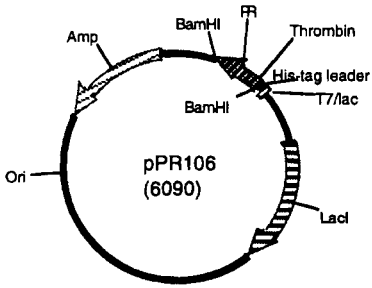
HTLV-I exhibits homology to other leukemia viruses such as bovine leukemia virus, avian sarcoma/leukemia virus, simian T-cell leukemia virus, mouse mammary tumor virus and Rous sarcoma virus, and, to a lesser extent, human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) (*6*). The protease from HTLV-I, like the proteases from these other viruses, is an aspartic acid protease that is involved in the proteolytic processing of the transcribed viral polyproteins. It processes the Gag and Gag-Pro-Pol precursors to produce the mature structural proteins and enzymes essential for viral replication (*6–8*). The protease is therefore an attractive target for inhibitor design in the treatment of HTLV-I infection, especially given the low mutation rates of HTLV-I (*9*). HIV-1 and HTLV-I proteases are only 30% homologous and exhibit distinct differences in response to pepstatin based inhibitors (*10*), rendering HIV-1 protease inhibitors ineffective in the treatment of HTLV-I infection. To date, relatively little has been reported on HTLV-I protease, and only a few reports on the activity of the protease have appeared, none of which describe systematic studies of the factors influencing activity. It is critical to understand the factors that affect the activity of HTLV-I protease for the design of inhibitors to treat HTLV-I infection. Here, we report the efficient expression and purification of HTLV-I protease, the determination of the molar extinction coefficient, and the dependence of protease activity on temperature, pH, and NaCl concentration.

MATERIALS AND METHODS

Chemicals and instrumentation. Sodium chloride was purchased from J. T. Baker, imidazole from Sigma, and Tris, urea, sodium acetate, and Luria–Bertani (LB) broth Miller from Fisher. All chemicals were reagent grade and used without further purification. The water used in all experiments was deionized and filtered using a NANOpure Infinity base unit from Barnstead/Thermolyne Corporation. Dynamic light scattering measurements were carried out using a DynaPro from Protein Solutions, Inc.

HTLV-I protease expression vector. Plasmid pPR101, whose preparation was previously reported (*11*), was digested with *Bam*HI and isolated on a 2% NuSieve low melting gel. The *Escherichia coli* expression vector pET-15b (Novagen) was digested with *Bam*HI, dephosphorylated with calf intestinal alkaline phosphatase, and isolated on 1% SeaPlaque gel. The DNA fragment from pPR101 (382 bp) was ligated into the *Bam*HI site of pET-15b to construct the expression vector pPR106. This new construct has a His-Tag leader followed by a thrombin cleavage site and the HTLV-I protease gene (Fig. 1, the fusion protein will be referred to as HisTPr).

Expression and purification of HisTPr. The previously reported purification (*11*) was slightly modified as described below to isolate the fusion protein HisTPr. *E. coli* BL21(DE3) cells containing the expression plasmid pPR106 were grown in 500 ml of LB Miller medium containing ampicillin at 37°C to an optical density at 600 nm (OD_{600}) of 0.6–0.8 and induced by addition of isopropyl-β-D-thiogalactoside (IPTG, 0.1 mM final concentration). After 3 h, the cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris, pH 7.9, 5 mM imidazole, and 500 mM NaCl), and sonicated. The insoluble fraction was collected by centrifugation then resuspended in buffer B (buffer A with 8 M urea). After incubating at 0°C for 1 h, the mixture

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                10         20         30         40
MGSSHHHHHH SSGLVPRGSH MLEDPVIPLD PARRPVIKAQ
                50         60         70         80
VDTQTSHPKT IEALLDTGAD MTVLPIALFS SNTPLKNTSV
                90        100        110        120
LGAGGQTQDH FKLTSLPVLI RLPFRTTPIV LTSCLVDTKN
               130        140
NWAIIGRDAL QQCQGVLYLP EAKGPPVIL
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FIG. 1. Sequence of pPR106. The boxed region highlights the thrombin cleavage site and the arrow indicates the beginning of the protease sequence.

was separated by centrifugation and the supernatant was loaded onto an 8 ml His-Bind (Novagen) column. The resin with bound protease was washed first with buffer B, then with buffer C (20 mM Tris, pH 7.9, 20 mM imidazole, 500 mM NaCl, and 8 M urea), and eluted with buffer D (20 mM Tris, pH 7.9, 1 M imidazole, 500 mM NaCl, and 8 M urea) to provide a 16-kDa protein. This molecular weight agrees with the expected molecular weight of the HTLV-1 protease monomer with the attached leader containing the His-Tag and thrombin cleavage site.

The isolated fusion protein was refolded by dialysis against buffer E (10 mM sodium acetate), at pH 3.5, then water. Aliquots of 1 ml were dried under vacuum using Labconco centrivap concentrator operating at 35°C. Stock solutions for concentration determination were prepared by dissolving the protein in water.

Concentration determination. Solutions of HisTPr in water at pH 5.1 (ranging from 0.14–2.5 mg/ml) were prepared and the OD_{280} for each solution was recorded. Three sets of data were collected, and experimental data were compared to the theoretical predictions based on the following extinction coefficients: W, 5.559; Y, 1.197; F, 0.0007 L/mol · cm.

Solutions of HisTPr at pH 5.1 in water (ranging in concentration from 0.112–8.4 μg/ml) were prepared and an 800-μl aliquot from each was mixed with 200 μl of Bradford reagent dye to yield 1 ml total volume. After incubation at room temperature for 10 min, the OD_{595} was recorded using a plastic disposable cuvette with a pathlength of 1 cm. Water (800 μl) and 200 μl of dye was used as a blank. The cuvette was rinsed three times with water in between samples. The HisTPr was compared to the commercially available protease pepsin. Solutions of pepsin were prepared with concentrations ranging from 0.025–0.348 mg/ml, the OD_{595} of these solutions were recorded.

Intrinsic fluorescence measurement. The HisTPr cleavage of the fluorogenic substrate (4-(4-dimethylaminophenylazo)benzoyl)-(γ-aminobutyric acid)-PQVL-*p*-nitrophenylalanine-VMH-(5-(2-aminoethylamino)-1-napthalenesulfonic acid) (abbreviated (DABCYL)-(GABA)-PQVL-Nph-VMH-(EDANS)) (SynPep Corp.), a modified p19/24 site, was monitored using a RF5301PC Spectrofluorimeter (Shimadzu) (*12*). The reactions were carried out in reaction buffer E at a substrate concentration of 100 μM and a HisTPr concentration of 50 nM in a volume of 1 ml. The pH dependence was determined by monitoring solutions at various pHs at 37°C and 0 mM NaCl.