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A simple, continuous fluorometric assay for HIV protease

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Novel fluorogenic substrates for human immunodeficiency viral protease have been developed based on the principle of fluorescence energy transfer. Starting from a p24/p15 cleavage site-derived hexapeptide substrate, Ac-Thr-Ile-Nle-Nle-Gln-Arg-NH_2, incorporation of 2-aminobenzoic acid in place of the acetyl group as the donor and *p*-NO_2-Phe at the P1′ position as acceptor gave the intramolecularly quenched fluorogenic substrate. Cleavage of the substrate by HIV protease released the fluorescent *N*-terminal tripeptide from its close apposition to the quenching nitrobenzyl group, resulting in enhanced fluorescence. An automated assay based on 96-well microtiter plates and a fluorometric plate reader have been developed, which allow high throughput of compounds in the search for HIV protease inhibitors.

Key words: fluorogenic substrate; fluorometric assay; HIV protease inhibitors

Human immunodeficiency viral (HIV) protease is a logical therapeutic target in the search for AIDS drugs. In order to allow the exploration of potential inhibitors of HIV protease and to optimize lead compounds, a convenient assay to kinetically characterize the inhibitor which allowed for automation and high throughput was necessary. Initial assays (1–3) used in the characterization of HIV protease were based on HPLC separation of products and substrate at fixed time intervals. This HPLC assay quickly became the rate-limiting step in the development of HIV protease inhibitors with desirable therapeutic properties. A continuous assay which allowed for quantitative kinetic characterization of the interaction of the inhibitors with HIV protease was needed. The strategy of developing either a chromogenic or a fluorogenic substrate was chosen based on work characterizing short peptide substrates of known HIV protease cleavage sites (4).

Our initial attempts focused on developing a chromogenic substrate. Because of the known accommodation of Phe and Tyr at position P1 in HIV protease substrates, we prepared a series of potential substrate analogs containing *p*-NO_2-Phe at either P1 or P1′. Of the compounds we examined which were octapeptides or smaller, only the P1′ analogs were substrates and the changes in absorbance upon hydrolysis were insufficient for a sensitive, continuous assay. Recently, Nashed *et al.* (5) and Tomaszek *et al.* (6) have reported a spectrophotometric assay based on cleavage of chromogenic substrates containing *p*-NO_2-Phe at position P1. In these cases, the substrates were an octapeptide, a nonapeptide, and a decapeptide. Richards *et al.* (7) have recently reported a nonapeptide substrate with *p*-NO_2-Phe at the P1′ site. A high-throughput, radiometric assay for screening for HIV protease inhibitors, which is not continuous, has been reported by Hyland *et al.* (8). Tamburini *et al.* (9) have developed a sensitive HPLC assay based on a dansylated heptapeptide substrate and fluorescence detection.

Fluorogenic substrates for hydrolytic enzymes have been widely used in biochemistry because of their high sensitivity. In many cases, the fluorophore, e.g. *C*-terminal peptide anilides, is linked directly to the bond undergoing cleavage, resulting in a highly fluorescent group upon hydrolysis. As the minimum length for a substrate of HIV protease from our studies was a hexapeptide, which is cleaved into two tripeptides, such a strategy was not applicable. The idea of using intramolecularly quenched fluorogenic substrates containing a donor and an acceptor chromophore in

Abbreviations: HIV, human immunodeficiency virus; DMF, dimethylformamide; CH_2Cl_2, dichloromethane; HPLC, high performance liquid chromatography; Abz, 2-aminobenzoic acid, or anthranilic acid; DMSO, dimethylsulfoxide; TFA, trifluoroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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the same molecule which would become separated upon hydrolysis has been widely used (10, 11). Recent examples of applications include a bacterial collagenase (12), vertebrate collagenase and gelatinase (13), enkephalinase (14), and atrial dipeptidyl carboxyhydrolase (15). As we had demonstrated that a substrate for HIV protease could contain *p*-NO_2-Phe, a known acceptor, at position P1′, incorporation of a fluorescent donor moiety in the *N*-terminal segment was attempted. The peptide, Ac-Thr-Ile-Nle-Phe(*p*-NO_2)-Gln-Arg-NH_2, was modified by replacement of the *N*-terminal acetyl group with various fluorescent groups based on anthranilic acid, 2-aminobenzoic acid (Abz). A suitable fluorogenic substrate resulted, and an automated assay utilizing microtiter plates and a fluorescence plate reader has been developed. During the course of preparing this paper, we became aware of similar efforts to develop such an assay by Weidner *et al.* (16), Matayoshi *et al.* (17), and Geoghegan *et al.* (18) utilizing different fluorogenic substrates.

MATERIALS AND METHODS

Solid phase synthesis of HIV protease substrates

Acetyl hexapeptide amides were prepared by solid phase peptide synthesis using the *p*-methylbenzhydrylamine resin (Sigma, St. Louis, MO). For each synthesis, 0.5 g of polymer (1 mmol free amine/g of resin) was used. The following BOC-amino acids were used (Bachem, Torrance, CA): Boc-Arg(Tos), Boc-Gln, Boc-Thr(Bzl), Boc-Asp(OBzl), Boc-Lys(Cl-Z), Ser(Bzl), Boc-Leu, Boc-Ile, Boc-Val, Boc-Nle, and Boc-Phe(*p*-NO_2). The Phe(*m*-NO_2) used was kindly provided by Drs. John Talley and Dan Getman of Monsanto (St. Louis) and protected by reaction with di-*t*-butylpyrocarbonate. The following synthetic protocol was used for incorporation of the Boc-amino acids:

Deprotection. 50% trifluoroacetic acid/CH_2Cl_2 5 min and 25 min

CH_2Cl_2	2 × 1 min
Isopropanol	2 × 1 min
CH_2Cl_2	2 × 1 min

Neutralization. 10% diisopropylethylamine/CH_2Cl_2 3 min and 5 min

CH_2Cl_2	2 × 1 min
DMF	2 × 1 min

Coupling. 4 equiv. of Boc-amino acid and 4 equiv. of diisopropylcarbodiimide in the presence of 4 equiv. of hydroxybenzotriazole in DMF for 2 h. Coupling in DMF was repeated if the Kaiser test (19) was positive. Acetylation was performed with acetic anhydride in DMF in the presence of an equivalent amount of DIPEA for 30 min. Completed peptides were cleaved with HF/anisole, 9:1. Crude peptides were dissolved in 20–50% acetic acid and lyophilized. They were purified by reversed-phase HPLC on a C_{18} semipreparative column using a 0.05% TFA/H_2O and 0.05% TFA/acetonitrile gradient. Their identity was confirmed by high-resolution mass spectrometry, NMR, and amino acid analyses.

Synthesis of 2-aminobenzoyl-Thr-Ile-Nle-Phe (p-NO_2)-Gln-Arg-NH_2, Abz-NF-6*

The protocol was essentially that used for the other substrates with the following modifications. Abz was coupled using an equivalent amount of BOP reagent (20) and 3 equiv. of DIPEA in DMF for 2 h. The cleaved peptide was dissolved in glacial acetic acid, diluted with water, and lyophilized. Small portions of the crude peptide were dissolved in glacial acetic acid, and purified by a Vydac C_{18} semipreparative HPLC column (1 × 25 cm) using a 25–45% acetonitrile gradient (0.05% TFA) for 40 min. The main peak at 35% acetonitrile was collected and lyophilized. The structure of the peptide was confirmed by FABMS (MH^+ = 940), by amino acid analysis (Beckman System Gold), and by UV and fluorescence spectroscopy.

HPLC assay

The HPLC HIV protease assay was conducted using either synthetic HIV protease (1) in which the two Cys residues (Cys^{67}, Cys^{95}) had been replaced by the isosteric α-aminobutyric acid to eliminate the complications of free sulfhydryl groups (21), or cloned material expressed in *E. coli* supplied by Dr. George Glover of Monsanto (St. Louis). In all cases examined, the cleavage patterns and inhibition results were identical. Synthetic HPLC-purified protease was dissolved in a buffer of 6 M guanidine·HCl, 100 mM Tris, pH 7.5, 40% glycerol at a concentration of 50 μg/mL. It was dialyzed versus a buffer of 2 M guanidine·HCl, 100 mM Tris, pH 7.5, 40% glycerol (100 × volume, 2 × 1 h, room temp.), followed by dialysis against 20 mM phosphate buffer, pH 7.5, 40% glycerol, 0.1% CHAPS (100 × volume, 2 × 1 h, room temp.), and finally a buffer (1 mM phosphate buffer, pH 7.5, 40% glycerol, 0.1% CHAPS) according to J. Schneider and S.B.H. Kent (personal communication). The precipitated solid material was removed by centrifugation, and the refolded HIV protease was stored at −70°. Twenty microliters of 0.1 mM substrate (dissolved in DMSO and diluted with assay buffer) was mixed with 20 μL assay buffer (20 mM phosphate buffer, pH 6.4, 20% glycerol, 0.1% CHAPS). Ten microliters of HIV protease stock was added, and the mixture was incubated at 25° for the desired time. The reaction was stopped by the addition of 60 μL of 10% TFA and the sample was applied to an HPLC column (Vydac C18,

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0.46 × 25 cm), developed with 0.05% TFA for 5 min, followed by a gradient of 0–40% acetonitrile in 40 min. For inhibitor studies, 10 μL of the protease solution was preincubated at 25° for 10 min with 20 μL of 0.1 mM inhibitor (dissolved in DMSO and diluted to 0.1 mM with assay buffer). Then 20 μL of test substrate, Ac-Thr-Ile-Met-Met-Gln-Arg-NH_2, or Abz-Thr-Ile-Nle-Phe-(p-NO_2)-Gln-Arg-NH_2 (Abz-NF*-6), was added in order to determine inhibition of cleavage. Reactions were stopped and cleavage rates were monitored by HPLC as above. In order to confirm the cleavage pattern of Abz-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2, cleavage was allowed to finish as judged by HPLC and the two product peaks were isolated by HPLC. Incubation with either synthetic HIV protease or enzyme expressed and purified from *E. coli* (kindly supplied by Dr. George Glover of Monsanto) gave the same HPLC pattern (Fig. 1). Retention time of the substrate was 42.1 min, while Abz-Thr-Ile-Nle-OH was 37.4 min and H-Phe(p-NO_2)-Gln-Arg-NH_2 was 17.7 min. Identity of the product peptides was confirmed by FABMS and amino acid analysis.

Fluorescence spectra

The excitation and emission spectra were measured on an SLM 8000 C spectrofluorometer. Both the substrate, Abz-NH*-6, and the *N*-terminal product, Abz-Thr-Ile-Nle-OH, show absorption maxima at 337 nm and broad emission maxima between 390 and 440 nm. The comparison between substrate and product at the same concentration shows dramatically the tenfold increase in excitation and sixfold increase in emission upon enzymatic hydrolysis. Initial kinetics were monitored at 25° with magnetic stirring at optimal conditions (excitation = 337 nm and emission = 410 nm).

UV spectra

The absorption spectra of the substrate, Abz-NH*-6, and the *N*-terminal product, Abz-Thr-Ile-Nle-OH, were recorded on a Beckman DU-8 spectrophotometer. The substrate shows maxima at 284 nm and 258 nm, while the cleavage product has maxima at 318 nm and 252 nm.

Fluorescence assay

Fluorescence measurements on 96-well ELISA plates were made with the Titertek Fluoroskan II, version 3.1. An excitation filter of 355 nm (bandwidth 35 ± 4 nm) and an emission filter of 430 nm (bandwidth 25 ± 3 nm) were used. Ten microliters of a stock solution (0.05 mg/mL) of HIV protease were incubated with five different concentrations of Abz-NF*-6 in a final volume of 100 μL of assay buffer at 37° with the increase in fluorescence monitored in each well every 2 min for 20 min. A stock solution of 1 mM Abz-NF*-6 in DMSO was diluted to 0.1 mM with assay buffer and used for the assay. The highest concentration of DMSO tested was 5%, which shows no effects of cleavage. A standard curve (Fig. 2) relating

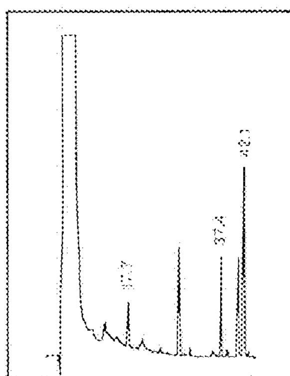

FIGURE 1

HPLC profile of elution pattern of Abz-Thr-Ile-Nle-Phe-(p-NO_2)-Gln-Arg-NH_2 (Abz-NF*-6, elution time = 42.1 min) and products, Abz-Thr-Ile-Nle-OH (elution time = 37.4) and H-Phe(p-NO_2)-Gln-Arg-NH_2 (elution time = 17.7 min). Forty microliters of 50 μM Abz-NF*-6 in assay buffer were incubated with 10 μL of HIV protease solution for 60 min at room temperature. Cleavage was stopped by addition of 60 μL of 10% TFA and the mixture was analyzed by reversed phase HPLC (Vydac C_{18}, 0.46 × 25 cm column). Mobile phase 0.05% TFA/H_2O (5 min), 0–40% acetonitrile (0.05% TFA) at 1 mL/min, detection at 220 nm. Other peaks are due to buffer components. UV absorption (aufs: 0.2) plotted versus time.

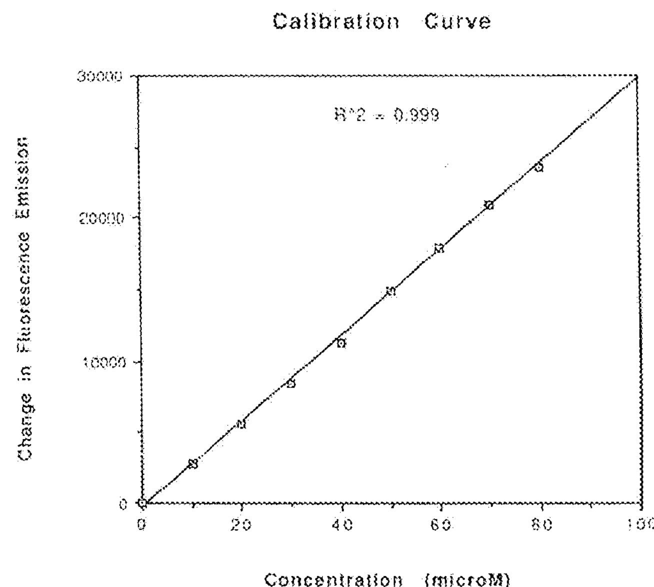

FIGURE 2

Standard calibration curve of fluorescence increase versus concentration. The fluorogenic substrate (Abz-NleF*-6) at eight different concentrations was incubated overnight with 0.5 μg of HIV protease in assay buffer (final volume = 100 μL) on a microtiter plate. The changes in fluorescence emission were measured and are plotted versus the concentration of substrate.

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