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CHROMOPHORIC PEPTIDE SUBSTRATES FOR THE SPECTROPHOTOMETRIC ASSAY OF HIV-1 PROTEASE

Thaddeus A. Tomaszek, Jr.,* Victoria W. Magaard,** Heidi G. Bryan,**
Michael L. Moore,** and Thomas D. Meek*

Departments of *Medicinal Chemistry and **Peptide Chemistry,
SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

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Purified HIV-1 protease hydrolyzes H-Ser-Gln-Asn-Leu-Phe(NO_2)-Leu-Asp-Gly-NH_2 (Peptide 1) and acetyl-Arg-Lys-Ile-Leu-Phe(NO_2)-Leu-Asp-Gly-NH_2 (Peptide 2) between the (*p*-nitro)phenylalanyl and leucyl residues. The cleavage of Peptides 1 and 2 resulted in a decrease in uv absorbance at 310 nm. The HIV-1 protease-catalyzed peptidolysis of Peptides 1 and 2 was characterized by a linear time course at substrate turnover of $\leq$20%. The solubilities of these substrates at pH 5.0 were sufficient to provide initial rate measurements over a concentration range of 0.05-0.5 mM. Steady-state kinetic data and inhibition constants using both spectrophotometric and high performance liquid chromatography (HPLC) analysis of the peptidolysis of these substrates resulted in comparable values.

The processing of the polyprotein products of the *gag* and *pol* genes of retroviruses is accomplished by a virally-encoded aspartic protease (1). This processing has been shown to be crucial to the replication of retroviruses (2,3), including the human immunodeficiency virus type-1 (HIV-1) (4-6). As a result the protease from HIV-1 has been the subject of intense recent interest. To date, typical kinetic assays for HIV-1 protease involve the separation by HPLC and quantification of the peptidolytic products of oligopeptide substrates (7-11). While the enzyme utilizes peptide substrates which correspond to seven of the known cleavage sequences found within its viral polyprotein substrates (9,10), the most commonly used oligopeptide substrates are based on the cleavage site (designated throughout by asterisk) at the p17-p24 junction of $Pr55^{gag}$, Ser-Gln-Asn-Tyr*Pro-Ile-Val (12). These chromatographic assays have the typical disadvantages of a stopped-time measurement of enzymatic initial rates in that they yield non-continuous measurements of initial rate and are labor-intensive. In a recent report, Nashed *et al.* (13) have reported a chromophoric peptide substrate, Ac-Lys-Ala-Ser-Gln-Asn-Phe(NO_2)-Pro-Val-Val-NH_2, based on the p17-p24 cleavage site (7,8), which exhibits an increase in absorbance at 326 nm upon its hydrolysis between the *p*-nitrophenylalanyl (Phe(NO_2)) and prolyl residues by HIV-1 protease. Substrates of this type have been widely used for spectrophotometric assays of other aspartic proteases (14-16).

Hofmann and Hodges (14) have reported that by situating the chromophoric Phe(NO_2) residue in the $P1'$ position of a peptide substrate, a larger change in absorbance at 310 nm can be achieved upon cleavage than that of a substrate which contains the Phe(NO_2) residue at the P1 position. An oligopeptide based on the reverse transcriptase-endonuclease cleavage site in $Pr160^{gag\text{-}pol}$ of HIV-1, Ac-Arg-Lys-Ile-Leu*Phe-Leu-Asp-Gly-NH_2 is a known substrate for HIV-1 protease (Moore, M. L.,

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et al. unpublished results). This substrate provides not only a Phe residue at its P1' position but also a number of charged residues to enhance its solubility in aqueous media. Accordingly, we have synthesized H-Ser-Gln-Asn-Leu-Phe(NO_2)-Val-Arg-NH_2 (Peptide 1) and Ac-Arg-Lys-Ile-Leu-Phe(NO_2)-Leu-Asp-Gly-NH_2 (Peptide 2), and have characterized their activities as substrates for HIV-1 protease both by spectrophotometry and HPLC. A continuous decrease of absorbance at 310 nm attends HIV-1 protease-catalyzed cleavage between the Leu and Phe(NO_2) of these peptides. In this paper we describe the characterization of a spectrophotometric assay for HIV-1 protease utilizing Peptides 1 and 2.

Materials and Methods

HIV-1 Protease. Recombinant HIV-1 protease was obtained from the PRO4 expression vector in *E. coli* strain AR58 as described (17). The protease was purified to >90% homogeneity by the method of Strickler *et al.* (18). The purified protease was stored at -20° C in 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 200 mM NaCl, 40% glycerol at protein concentrations of 10-30 µg/ml.

Peptide synthesis. Peptide substrates were prepared by solid phase peptide synthesis on benzhydrylamine resin and cleaved with anhydrous liquid HF at 0° C. Peptides which contained a reduced peptide bond isostere replacement for the scissile dipeptide were prepared as described (8). All peptides were purified to homogeneity by thin layer chromatography and HPLC, and their structures were confirmed by amino acid analysis and fast atom bombardment mass spectrometry.

Enzyme assays. Initial characterization of all peptide substrates was performed using HPLC separation and quantification of the peptidolysis products (7,8). Assay mixtures (100 µL, 37° C) contained variable concentrations of peptide substrate in 80 mM sodium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 0.8 M NaCl (pH 4.7) (Buffer A). Reactions were initiated by the addition of 20 ng of protease and quenched after 10 min with an equal volume of 1% trifluoroacetic acid. Reaction products were analyzed by reverse phase HPLC (Beckman Ultrasphere ODS (4.5 mm x 25 cm), mobile phase: 5-40% acetonitrile gradient (20 min), 40% acetonitrile (5 min) in 0.05% trifluoroacetic acid at 1.5 ml/min). Percent of reaction was calculated by integration of the substrate and product peaks detected at 220 nm.

The spectrophotometric assay of Peptides 1 and 2 was performed with a Perkin Elmer Lambda 4C spectrophotometer equipped with a constant temperature bath (37° C) which circulated water through jacketted 1-cm (0.5-mL) cuvettes.

Assay mixtures (400 µL) contained variable concentrations of substrates in either Buffer A (pH 4.7) or for variable pH studies, in a mixed buffer (Buffer B; consisting of Buffer A + 80 mM each of glycine, 2-(N-morpholino)ethanesulfonic acid (Mes), and Tris) adjusted to each pH and held at constant ionic strength by the addition of small aliquots of 3 M NaCl. Reactions were initiated by the addition of 5-40 ng of protease and the absorbance at 310 nm was recorded. Initial rates were determined from the slope of the observed linear decrease in absorbance. Extinction coefficients were determined from the total absorbance change at 310 nm resulting from the complete consumption of the peptide substrate in reaction mixtures that were initiated by the addition of a small concentration of peptide.

Product analysis. Samples of the peptidolytic products of Ac-Arg-Lys-Ile-Leu-Phe(NO_2)-Leu-Asp-Gly-NH_2 and H-Ser-Gln-Asn-Leu-Phe(NO_2)-Val-Arg-NH_2 from assay mixtures containing 400 µM of either substrate and 20 ng protease (60 min, 37° C) were prepared by HPLC under conditions as described above. The product peaks were collected, dried under argon at room temperature, redissolved in 500 µL of H_2O, and the lyophilized products were characterized by fast atom bombardment mass spectrometry.

Kinetic analysis. Kinetic constants were determined from either the HPLC or spectrophotometric assays by fitting of initial rate data obtained at 10-20% substrate turnover to the Michealis-Menten equation ($v = V_{max}[S]/(K_m + [S])$) using the FORTRAN program of Cleland (19). Inhibition constants were measured by the method of Dixon (20) at a fixed substrate concentration of 200 μM Ac-Arg-Lys-Ile-Phe(NO_2)-Leu-Asp-Gly-NH_2 (spectrophotometric assay) or 1 mM Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 (HPLC assay (7,8)) assuming competitive inhibition.

Results and Discussion

Cleavage of Peptide 1 by the protease resulted in an HPLC chromatogram consisting of two major peaks at 7.9 min and 13.6 min, corresponding to H-Phe(NO_2)-Val-Arg-NH_2 and H-Ser-Gln-Asn-Leu-Phe(NO_2)-Val-Arg-NH_2, respectively. The other expected peptidolytic fragment, Ac-Ser-Gln-Asn-Leu-OH, could not be detected. Likewise, limited peptidolysis of Ac-Arg-Lys-Ile-Leu-Phe(NO_2)-Leu-Asp-Gly-NH_2 (Peptide 2) with the protease resulted in the appearance of two product peaks at 11.2 and 12.7 min which were identified by fast atom mass spectrometric analysis as H-Phe(NO_2)-Leu-Asp-Gly-NH_2 (m/e = 495.5) and Ac-Arg-Lys-Ile-Leu-OH (m/e = 570.7), respectively.

The protease cleaved Peptides 1 and 2 between their leucyl and *p*-nitrophenylalanyl residues. Initial rate data obtained from the HPLC assay at 0.05-0.5 mM Peptides 1 and 2 were fitted to the Michaelis-Menten equation to yield the kinetic constants shown in Table 1.

Formation of the carboxyl-terminal cleavage products of Peptides 1 and 2 is expected to manifest a decrease in UV absorption at wavelengths above 280 nm. Temporal changes in the UV absorption spectra of Peptide 2 upon treatment with HIV-1 protease for 120 min are shown in Figure 1. Cleavage of the Leu-Phe(NO_2) bond of Peptide 2 results in a hypsochromic shift of the absorption maximum (λ_{max} shifts from 277 nm to 272 nm). Spectral changes in the absence of the protease are negligible. Similar to other chromophoric peptide substrates of this type (14,15), this shift in λ_{max} allows spectrophotometric monitoring of the peptidolytic reaction by recording the decrease in

Table 1. Comparison of Kinetic Parameters Using HPLC and Spectrophotometric Assays [a]

	HPLC Assay			Spectrophotometric Assay		
Substrate/Inhibitor	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Peptide 1	0.16	1.9	12	0.12	2.0	17
Peptide 2	0.46	13	28	0.28	7.7	28
		K_i (μM)			K_i (μM)	
Pepstatin A		0.20			0.14	
Ac-Ser-Gln-Asn-Tyrψ[CH_2NH]Pro-Val-Val-NH_2		7.1			7.8	

[a] Initial rate data were obtained in Buffer A (pH 4.7) at 0.05-0.5 mM substrate. Reactions were initiated with 20 ng purified HIV-1 protease (37° C) as described in Materials and Methods. Inhibition constants were obtained by Dixon analysis (20) under identical reaction conditions using a fixed 200 μM concentration of Peptide 2 (spectrophotometric assay) or for the hplc assay, at pH 6.0 using a fixed concentration of the substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 as described (7,8). Apparent inhibition constants (K_i) were determined assuming competitive inhibition (8,22).

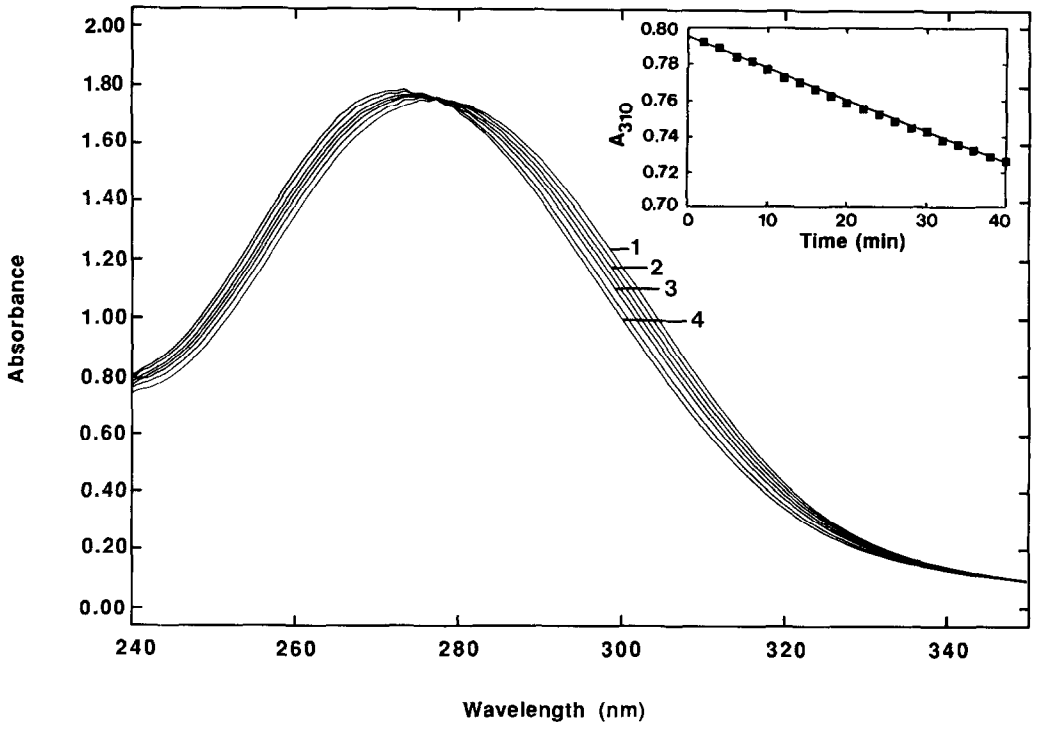

Figure 1. Time course (120 min) of changes in the UV absorption spectra of 250 μM Peptide 2 in Buffer A (pH 5.0) upon the addition of 20 ng HIV-1 protease as described in Materials and Methods. Spectra 1-4 were recorded at 2, 30, 60 and 120 min, respectively, following the addition of purified HIV-1 protease. The inset is a plot of the decrease (0-40 min) in absorbance at 310 nm.

absorbance at 310 nm. The overall change in extinction coefficient at 310 nm for the formation of H-Phe(NO_2)-Leu-Asp-Gly-NH_2, as obtained from the absorbance measurements at 310 nm after complete peptidolysis of Peptide 2 at several concentrations, was found to be $\Delta\varepsilon^{310\ nm} = 1200\pm100$ $M^{-1}cm^{-1}$. The change in spectra observed upon peptidolysis of Peptide 1 (data not shown) was comparable to that shown for Peptide 2 in Figure 1 ($\Delta\varepsilon^{310\ nm} = 700\pm40$ $M^{-1}cm^{-1}$). As expected, the incorporation of the Phe(NO_2) residue at the P_1' position of Peptides 1 and 2 provides chromophoric products of greater sensitivity than that of the previously characterized substrate of HIV-1 protease, Ac-Lys-Ala-Ser-Gln-Asn-Phe(NO_2)-Pro-Val-Val-NH_2 ($\Delta\varepsilon$ = 600 $M^{-1}cm^{-1}$ (13)).

From these findings, linear spectrophotometric time courses of the hydrolysis of Peptides 1 and 2 could be used to generate initial rate data in units of nmol/min. The initial time course of the protease-catalyzed peptidolysis of Peptide 2, shown in the inset in Figure 1, is linear for 40 min, corresponding to approximately 20% substrate turnover. As a result, initial rate data were taken from the slopes of the time courses at $\leq$20% substrate turnover.

Initial rate data of HIV-1 protease-catalyzed peptidolysis were measured at variable concentrations of Peptides 1 and 2 (0.05-0.5 mM). Apparent substrate inhibition was observed at concentrations greater than 0.6 mM, suggesting that this concentration may approach the limit of its solubility. In the buffers used, the solubility of Peptides 1 and 2 was sufficient to allow initial rate measurements at substrate concentrations which were below and above their K_m values. This provides a suitable range of concentrations for kinetic analysis by double-reciprocal plots of 1/v vs. 1/[S] as shown in

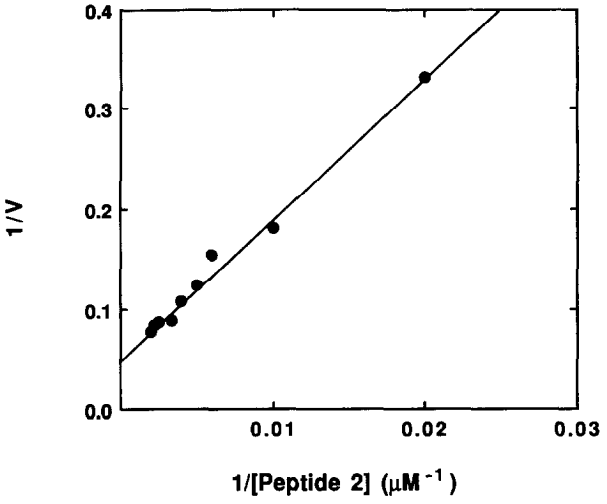

Figure 2. Initial rate data from the spectrophotometric assay of Peptide 2 at variable concentrations plotted in double-reciprocal form (1/v vs. 1/[S]. Reaction mixtures (0.4 mL) containing Buffer B (pH 5.0) and 0.05-0.5 mM Peptides 2 were initiated (at 37° C) by the addition of 20 ng purified HIV-1 protease, and the linear decrease in absorbance at 310 nm was recorded as described in Materials and Methods. Initial rates (v) were calculated from the extinction coefficients and the enzyme concentration in units of nmol product/min-μg, and the plots were obtained by fitting the rate data to the Michaelis-Menten equation as described (19).

Figure 2. Under the conditions developed by Nashed et al. (13), the limited solubility of the substrate, Ac-Lys-Ala-Ser-Gln-Asn-Phe(NO_2)-Val-Val-NH_2, rendered detailed kinetic analysis by double-reciprocal plots difficult.

The kinetic constants of the HIV-1 protease-catalyzed cleavage of Peptides 1 and 2 were obtained by fitting initial rate data to the Michaelis-Menten equation are shown in Table 1. The k_{cat}, K_m, and k_{cat}/K_m values derived from the spectrophotometric assay compare quite favorably with those obtained from the HPLC assay. The K_m values of Peptides 1 and 2, 0.12 mM and 0.28 mM, respectively, are approximately ten-fold lower than values which are typical for peptide substrates of this size (7-9). This may reflect tighter binding of these substrates at the high concentration of NaCl (0.8 M) and at pH 4.7, as has been reported for another substrate of HIV-1 protease (21).

Table 2. pH Variation of Kinetic Constants for Peptide 2 [a]

pH	k_{cat}/K_m ($mM^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)
4.0	13	3.2	0.25
5.0	28	7.7	0.28
6.0	21	7.3	0.35
7.0	6.7	2.1	0.31

[a] In Buffer B (37° C), adjusted to pH cited as described in Materials and Methods.

The variable concentrations used for Peptide 1, 0.05-0.5 mM, are suitable for kinetic analysis in view of the K_m value of this substrate (K_m = 0.12 mM). Based on a molecular weight of 22,000 for the protease, the calculated k_{cat} and k_{cat}/K_m values from either assay were: Peptide 1 (2 s^{-1} and 14 $mM^{-1}s^{-1}$) and Peptide 2 (8 s^{-1} and 28 $mM^{-1}s^{-1}$). Although the K_m and k_{cat} values of these substrates are proportionally lower than those typical for other peptide substrates, such as Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 (k_{cat} = 70 s^{-1}, K_m = 6 mM, k_{cat}/K_m = 13 $mM^{-1}s^{-1}$ (7)), the similarity of the k_{cat}/K_m values suggests comparable rates of association of enzyme and substrate for these peptides. Similarly, apparent inhibition constants obtained using either assay (Table 1) are nearly identical for pepstatin A and a heptapeptide inhibitor containing a reduced amide isosteric replacement (designated as $\Psi[CH_2NH]$) for the scissile Tyr-Pro amide bond, Ac-Ser-Gln-Asn-Tyr$\Psi[CH_2NH]$Pro-Val-Val-NH_2 (8).

The pH variation of the kinetic parameters k_{cat}/K_m and k_{cat} for Peptide 2 is shown in Table 2. At each of these values of pH, the initial rates measured by the spectrophotometric assay in a mixed buffer were linear and the protease activity was found to be completely stable. Both k_{cat}/K_m and k_{cat} decreased at both high and low pH, while little variation in K_m was observed. Clearly, pH 5-6 represents an optimal range for the peptidolysis of Peptide 2. These preliminary results demonstrate the utility of these chromophoric peptide substrates in the kinetic characterization of HIV-1 protease. Further development of this spectrophotometric assay will greatly facilitate the acquisition of steady-state kinetic data of the enzyme and allow detailed characterization of its inhibitors.

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