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Fluorescence-based continuous assay for the aspartyl protease of human immunodeficiency virus-1

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5-Dimethylaminonaphthalene-1-sulfonyl-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Trp (Dns-SQNYPIVW) is a fluorescent substrate for the aspartyl protease of human immunodeficiency virus-1. In intact substrate, fluorescence of Trp (λ_{ex} 290 nm, λ_{em} 360 nm) was 60% quenched by energy transfer to the dansyl group. Protease-catalyzed cleavage at the Tyr-Pro bond abolished the energy transfer, and the consequent increase in Trp fluorescence was used to follow the enzymatic reaction. At substrate concentrations $<60\ \mu M$, initial reaction velocity increased as a linear function of substrate concentration, with $k_{cat}/K_M = 9700\ M^{-1}\ s^{-1}$. Limited solubility and internal fluorescence quenching precluded a determination of K_M for Dns-SQNYPIVW, but this was clearly $>100\ \mu M$.

Human immunodeficiency virus; Proteinase; Fluorometric assay; Energy transfer

1. INTRODUCTION

Specific inhibitors of the aspartyl protease of HIV-1[1] may provide the basis of a new therapy for AIDS [1]. The enzyme requires at least a heptapeptide substrate [2], and has generally been assayed by HPLC-based methods in which the specific hydrolysis of peptide substrates is monitored in discontinuous fashion [2]. Recently, an absorbance-based spectrophotometric method was described which allows the enzyme's activity to be monitored continuously [3].

To provide an alternative continuous assay with certain advantages, an established strategy for the assay of hydrolases [4] was applied to the case of HIV-1 protease. A peptide substrate of the protease was modified by the addition of an energy-transfer pair, giving a substrate which allows the enzymatic activity to be monitored directly and continuously. In Dns-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Trp (Dns-SQNYPIVW), the Dns and Trp groups are the N- and C-terminal extensions, respectively, of the heptapeptide sequence at the p17–p24 cleavage site in the viral *gag* polyprotein [5]. HIV-1 protease cleaves this sequence at the Tyr-Pro bond. As Trp and dansyl form an excellent donor–acceptor pair for energy-transfer applications [6], the Dns-octapeptide can be used for direct fluorescence-based assay of the enzyme.

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Abbreviations: HIV-1, human immunodeficiency virus-1; AIDS, acquired immune deficiency syndrome; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Brz, 2-bromobenzyloxycarbonyl; CHO, formyl; PAM, 2-pyridinealdoximine methiodide; DMSO, dimethylsulfoxide; Dns, (dansyl), 5-(dimethylamino)naphthalene-1-sulfonyl; DCM, dichloromethane; DMF, dimethylformamide; TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry

2. MATERIALS AND METHODS

2.1. *HIV-1 protease*

Protease expressed in *E. coli* MM294 was purified as described [7]. Its concentration was determined by amino acid analysis.

2.2. *Peptide synthesis*

Reagents were obtained from Applied Biosystems. Protected amino acids used were Boc-Ser(Bzl), Boc-Tyr(BrZ) and Boc-Trp(CHO). Ethanolamine and Dns chloride were from Aldrich. HPLC was performed on a Waters Delta Prep 3000 with a Vydac C4 column and a H_2O-CH_3CN gradient in 0.1% TFA. A Finnigan 4510 quadrupole FAB-MS instrument was employed.

Ser-Gln-Asn-Tyr-Pro-Ile-Val-Trp (0.5 mmol) was assembled on an Applied Biosystems Model 430A peptide synthesizer using PAM resin and the *N*-methylpyrrolidone/DMSO program (Ver. 1.40). Final acidic deprotection afforded 1.28 g of peptide-resin with a free NH_2-terminus, of which 280 mg swollen with minimum DCM was treated with 5 ml of dry DMF, 0.25 ml of dry triethylamine and 0.4 g of Dns chloride. The mixture was vortexed overnight in the dark (20°C). The peptide-resin was isolated by filtration, washed successively with DMF; DCM; 5% TFA in DCM; DCM; 5% *N,N*-diisopropylethylamine in DCM; and DCM; and dried to afford 290 mg of peptide-resin in which the peptide was shown by quantitative ninhydrin assay [8] to be >99% dansylated. This was vortexed in 10 ml of 10:7:3:80 H_2O:ethanolamine:methanol:DMF for 30 min to deformylate the Trp, isolated by filtration and washed in turn with DMF, methanol and DCM. Next, the resin-linked peptide was further deprotected using HF containing 10% *p*-cresol for 1 h (0°C). Following removal of the HF, the resin-peptide mixture was taken up in minimum TFA, the resin was removed by filtration and the pro-

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duct peptide isolated by precipitation from diethyl ether (yield; 115 mg of crude dansylated peptide, 8.5% overall, 78% pure by HPLC analysis). The crude peptide (38 mg) was fractionated by HPLC to afford 7.4 mg of pure Dns-SQNYPIVW after lyophilization in the dark. The product gave mass spectral parent ions at masses of 1239 and 1240 Da, with the ratio of isotopic peak heights equal to 0.7. This result identified the product as Dns-SQNYPIVW.

2.3. *HPLC*

HPLC-based assays were performed using a Vydac C-4 column (4.6 mm × 15 cm) and a H_2O-CH_3CN gradient in 0.1% TFA.

2.4. *Fluorimetric enzyme assay*

Dns-SQNYPIVW was dissolved (1–2 mM) in 0.5 M Mes, pH 5.5, and its concentration was estimated using ϵ_{330} for Dns of 4500 M^{-1} cm^{-1}. 'Reaction buffer' was 0.05 M sodium acetate pH 5.5, 13% glycerol, 0.01 M DTT. Fluorescence data were collected using a Perkin-Elmer LS-5B luminescence spectrometer interfaced with a Macintosh computer.

3. RESULTS

3.1. *Synthesis of Dns-SQNYPIVW*

Ser-Gln-Asn-Tyr-Pro-Ile-Val-Trp was α-*N*-dansylated while still coupled to the solid-phase synthesis resin, selectively deprotected at the formyl-Trp using ethanolamine and finally deprotected by HF treatment using 10% *p*-cresol as the sole scavenger (in the dark). This strategy emerged from consideration of (i) the lability of the glutamine residue [9], (ii) a difficulty with migration of the formyl-Trp protecting group observed during development of the HF deprotection, and (iii) the presence of multiple potential dansylation sites in the deprotected peptide. The final preparation of Dns-SQNYPIVW contained an impurity eluted just after the desired product in HPLC (fig.1a) and with mass 1 Da greater than that of Dns-SQNYPIVW; this was identified as a deamidated form of the substrate.

3.2. *Hydrolysis of Dns-SQNYPIVW by HIV-1 protease*

The sequence -Ser-Gln-Asn-Tyr-Pro-Ile-Val- at the p17–p24 junction in the HIV-1 *gag* polyprotein is cleaved at the Tyr-Pro bond by HIV-1 protease [5]. To test the enzyme's ability to accept this sequence flanked by Dns and Trp, the cleavage of Dns-SQNYPIVW was

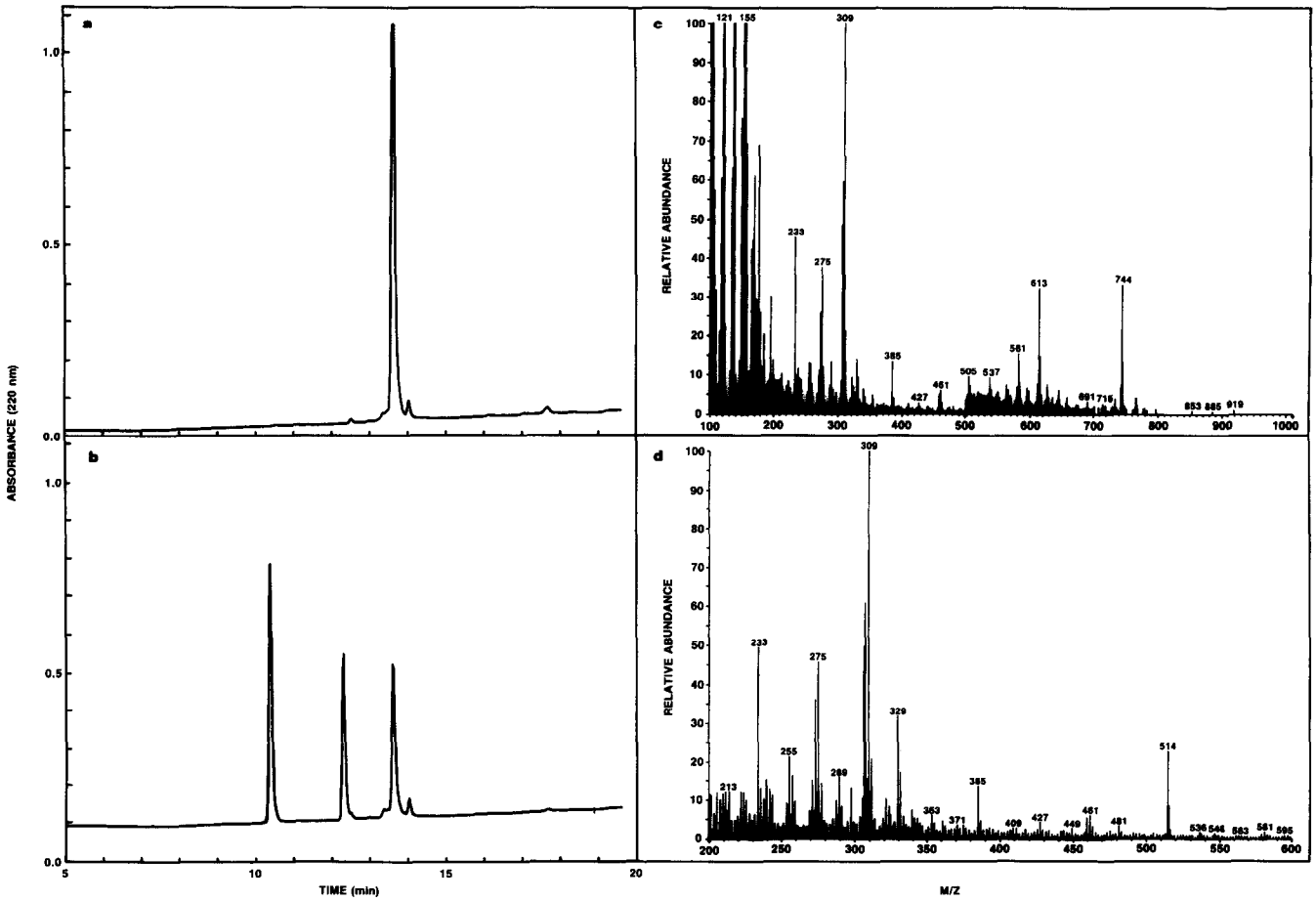

Fig.1. Action of HIV-1 protease on Dns-SQNYPIVW. (a) HPLC of Dns-SQNYPIVW before addition of HIV-1 protease (see section 2). (b) HPLC analysis of 20 μl sample withdrawn from a reaction mixture containing Dns-SQNYPIVW (1.3×10^{-4} M) with HIV-1 protease (7×10^{-7} M); the sample was diluted by addition to 220 μl 10% acetic acid and 200 μl was injected. (c) FAB-MS of first-eluted product peak fraction from panel b; the predicted M_r for Dns-SQNY is 743.7 (note peak at $M/Z = 744$). (d) FAB-MS of second eluted product peak fraction from (b); the predicted M_r for PIVW is 513.7 (note peak at $M/Z = 514$).

analyzed by HPLC (fig.1a,b). The two products were identified by mass spectrometry (fig.1c,d). The earlier eluted product (molecular ion of mass 744) was Dns-SQNY (calculated M_r of 743.7) (fig.1c), and the later eluted product (molecular ion of mass 514) was PIVW (calculated M_r of 513.7) (fig.1d). Thus, HIV-1 protease cleaved Dns-SQNYPIVW at the Tyr-Pro bond.

3.3. *Fluorescence properties of Dns SQNYPIVW*

Addition of HIV-1 protease led to time-dependent intensity changes in the fluorescence emission spectrum of Dns-SQNYPIVW (λ_{ex} 290 nm). The 360 nm Trp emission peak progressively increased to ~2.5 times its initial intensity while the Dns group's emission band declined in intensity (fig.2a). The data could also be viewed as a series of difference spectra (fig.2b). The 487 nm isosbestic point showed that the proces underlying the spectral changes was a conversion involving only two states (i.e. substrate + H_2O to products).

3.4. *Correlation of fluorescence changes with hydrolysis of Dns-SQNYPIVW*

To verify the presumed link between changes in the fluorescence of Dns-SQNYPIVW and its hydrolysis, the reaction (initially 2.4 ml) shown in fig. 2 was sampled between scans (20 μl aliquots) for HPLC analysis.

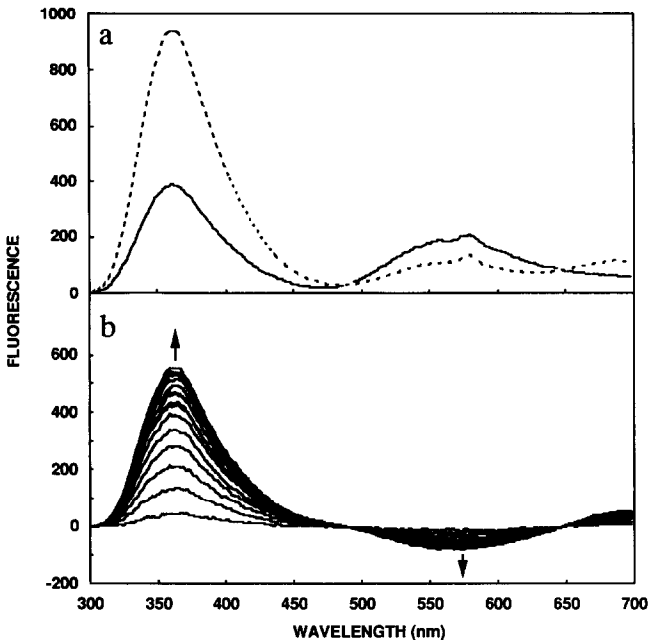

Fig.2. Fluorescence emission (λ_{ex} 290 nm) of Dns-SQNYPIVW and its hydrolysis products. See section 2 for conditions. (a) Solid line: fluorescence of Dns-SQNYPIVW (3.3×10^{-5} M). Dashed line: the same sample following 90 min reaction at 20°C in the presence of HIV-1 protease (2×10^{-8} M). (b) Difference spectra from the same experiment; the initial spectrum was subtracted from a series of spectra recorded at time intervals during the 90 min reaction period. The first spectrum was recorded 2 min after the addition of enzyme, and subsequent scans were initiated at 6 min intervals thereafter. Arrows show the directions of spectral changes.

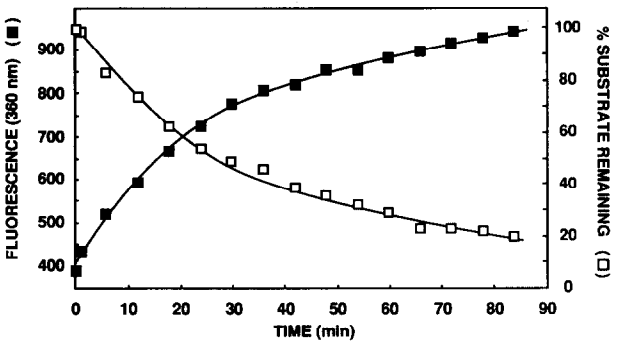

Fig.3. Progress of fluorescence change at 360 nm (closed symbols) and hydrolysis of substrate as measured by HPLC (open symbols). The experiment is the same as that shown in fig. 2. 20 μl aliquots of the reaction (initial volume 2.4 ml) were quenched by addition to 220 μl of ice-cold 10% acetic acid.

The fluorescence changes and hydrolysis of the substrate occurred at the same rate (fig. 3), each following a first-order time course with $t_{1/2}$ of 30 min.

3.5. *Kinetic characterization of proteasecatalyzed hydrolysis of Dns-SQNYPIVW*

As the maximum fluorescent change during hydrolysis was at 360 nm (fig.2), this wavelength was selected for single-wavelength kinetic studies of the reaction. Dns-SQNYPIVW was prepared as a stock solution of 1–2 mM (the limit of its solubility) in 0.5 M Mes, pH 5.5, and diluted into reaction buffer for each assay; dilution always exceeded 1:20, so that Mes was reduced to below 0.025 M. At substrate concentrations of 1–60 μM, the initial reaction velocity had linear dependence on substrate concentration (fig.4). Thus, a lower limit of 100 μM could be firmly set upon the K_M of Dns-SQNYPIVW. With substrate concentrations >100 μM, fluorescence quenching was evident from a nonlinear dependence of initial fluorescence on substrate concentration. This, and the limited solubility of the substrate, precluded exploration of its kinetic

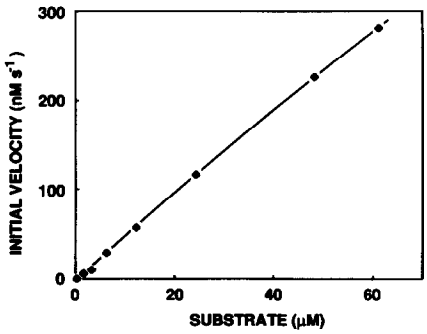

Fig.4. Dependence of initial reaction velocity on substrate concentration. Assays were performed under the conditions described in section 2, using an enzyme concentration of 4.8×10^{-7} M. Reaction volumes were 0.24 ml. λ_{ex} was 290 nm, and λ_{em} was 360 nm.

behavior at these concentrations. The same difficulties precluded an estimate of k_{cat}. From the slope of the line in fig.4, it was estimated that $k_{cat}/K_M = 9700\ M^{-1}\ s^{-1}$ for Dns-SQNYPIVW hydrolysis, consistent with previously reported values for peptides containing the Tyr-Pro cleavage site [2].

4. DISCUSSION

The seven-residue sequence between the energy donor and energy acceptor groups in Dns-SQNYPIVW is unusually long, but may have to be so to satisfy the specificity requirements of HIV-1 protease [2]. A computer-generated model indicates that the dansyl and Trp groups are separated by about 2.5 nm if the substrate exists in a maximally extended conformation, but the 60% fluorescence quenching observed with Dns-SQNYPIVW suggests an average separation of rather less than 2.0 nm [10,11]. Ng and Auld [11] reported near-total quenching in a substrate in which Trp and Dns were separated by five residues, noting the possible benefit in this respect of including a proline in the peptide sequence. Quenching in Dns-SQNYPIVW may also be assisted by a proline-dependent conformational effect.

The method can be refined. Improvements might follow from selection of other substrate sequences for placement between the energy donor and acceptor groups (to give better kinetic properties), addition of extra hydrophilic groups to enhance substrate solubility, and the exploitation of other fluorophore pairs. Placing a range of potential substrate sequences between the energy donor and energy acceptor groups will allow a fuller and more precise exploration of the enzyme's specificity.

Fluorescent substrates designed along these lines will complement the existing chromophoric substrate for HIV-1 protease [3]. The simplicity of an absorbance-based assay is valuable, but the requirement for a specific chromophoric residue always to be present at the cleavage site restricts the potential for exploration of the enzyme's specificity. Current interest in this problem [3,12,13] suggests that future studies of HIV-1 protease and other retroviral proteases wil benefit from the availability of several optical methods of assay.

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