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Use of a Fluorescence Plate Reader for Measuring Kinetic Parameters with Inner Filter Effect Correction

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A general method is presented here for the determination of the K_m, k_{cat}, and k_{cat}/K_m of fluorescence resonance energy transfer (FRET) substrates using a fluorescence plate reader. A simple empirical method for correcting for the inner filter effect is shown to enable accurate and undistorted measurements of these very important kinetic parameters. Inner filter effect corrected rates of hydrolysis of a FRET peptide substrate by hepatitis C virus (HCV) NS3 protease at various substrate concentrations enabled measurement of a K_m value of 4.4 ± 0.3 μM and k_{cat}/K_m value of 96,500 ± 5800 M^{-1} s^{-1}. These values are very close to the HPLC-determined K_m value of 4.6 ± 0.7 μM and k_{cat}/K_m value of 92,600 ± 14,000 M^{-1} s^{-1}. We demonstrate that the inner filter effect correction of microtiter plate reader velocities enables rapid measurement of K_i and K_i' values and kinetic inhibition mechanisms for HCV NS3 protease inhibitors.

FRET[2] (fluorescence resonance energy transfer) molecules (1) have been extensively used as substrates for cellulase (2), hydrolases (3, 4), and especially proteases (5–9). The intramolecular FRET protease substrates consist of a peptide with a fluorescence donor at one side of the enzymatic cleavage bond and a quenching acceptor at the other side. The intrinsic fluorescence of the donor is dramatically reduced in these substrates because of intramolecular resonance energy transfer to the quenching group. Since this energy transfer becomes insignificant beyond distances of about 100 Å, the full fluorescence quantum yield of the donor group is restored after enzymatic cleavage.

Fluorogenic substrates employing the EDANS (5-[(2′-aminoethyl)amino]naphthalene sulfonic acid) and DABCYL (4-[[4′-(dimethylamino)phenyl]azo]benzoic acid as donor–quencher pair were first introduced in 1990 by Matayoshi *et al.* for HIV protease (5). The EDANS/DABCYL pair is still considered as the "first-choice couple" because of its excellent energy overlap and the relatively long lifetime of the excited state of the EDANS fluorophore. The EDANS/DABCYL pair has been successfully used in renin (6), cathepsin D (7), cytomegalovirus (8), and HCV NS3 protease (9) substrates. Cleavage of a FRET substrate by an enzyme separates the fluorophore from the quenching group which results in the generation of a fluorescence signal. Under some conditions the fluoresced light is absorbed by quenching groups on neighboring substrates or cleaved product molecules so that only a fraction of the fluoresced light impinges upon the detector system of the fluorometer. This phenomenon is known as the inner filter effect. The magnitude of the inner filter effect depends on the wavelength range, pathlength, and concentration of quenching components and must be measured for each instrumental configuration if accurate fluorescence measurements are to be obtained. The inner filter effect associated with this kind of substrate has prevented some researchers from measuring kinetic parameters such as K_m, k_{cat}, and k_{cat}/K_m from the fluorescence signal and so these values were often measured using a time-consuming HPLC assay (8, 9). However, empirical procedures for correcting inner filter effects have been used quite extensively in fluorescence spectroscopy (10, 11). We now introduce an empirical inner filter effect correction procedure for FRET substrates using a 96-well fluorescence plate reader.

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[2] Abreviations used: FRET, fluorescence resonance energy transfer; EDANS, 5-[(2′-aminoethyl)amino]naphthalene sulfonic acid; DABCYL, 4-[[4′-(dimethylamino) phenyl]azo]benzoic acid; HCV, hepatitis C virus; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide.

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MATERIALS AND METHODS

HCV NS3 protease substrate Ac-DED (EDANS) EEAbu-ψ [COO]ASK (DABCYL)-NH_2 and its C-terminal product HO-(ASK) DABCYL-NH_2 were obtained from AnaSpec and their purities were greater than 95% by HPLC analysis. 5-[(2′-Aminoethyl)amino]naphthalene sulfonic acid was obtained from Sigma Chemical Co. (A-4535). The N-terminal EDANS product and Fmoc-Glu–EDANS were synthesized by Abbott chemists and have been purified by HPLC. The HCV NS3 serine protease was expressed as glutathione *S*-transferase fusion protein in *Escherichia coli* and purified by affinity column chromatography. Detailed experimental procedures for protease expression and purification will be published elsewhere.

HPLC assay of cleaved EDANS and DABCYL peptide product. Cleavage product formation was monitored by both fluorescence signal of the EDANS peptide and UV signal of the DABCYL peptide and quantitated by HPLC using authentic EDANS and DABCYL products. Enzymatic reactions (144 μl) were stopped at different time intervals (from 100 to 1200 s) by the addition of 3 μl of 50% TFA which was followed by 3 μl of 0.05 mM free EDANS as an HPLC internal reference. The components in the reaction mixtures were separated by a Lichrospher C-18 reverse-phase column (4 $\times$ 125 mm, 5 μm, Merck) using a 10–50% acetonitrile gradient in H_2O/0.1% TFA at 1 ml/min. Enzyme-generated DABCYL peptide was monitored at 512 nm and quantitated using a standard curve of authentic DABCYL peptide run under identical conditions. Initial rates of EDANS and DABCYL peptide product formation were measured using five time points. Kinetic evaluation of the cleavage reactions was performed by fitting the initial rates to the Michaelis–Menten equation using nonlinear least-squares regression analysis performed with the help of KaleidaGraph software.

Plate reader fluorogenic assay of HCV NS3 protease activity. Continuous assays were performed on an ICN Titertek Fluoroskan II 96-well microtiter fluorescence plate reader at ambient temperature using white microfluor U-bottom plates from Dynex, Inc. Excitation and emission wavelength filters of 355 and 485 nm, respectively, were used. Typically, 150 μl assay buffer (50 mM triethanolamine, 50 mM NaCl, and 3 mM $MgCl_2$ at pH 7.4) with 10 μl DMSO or inhibitor solution in DMSO was mixed with 20 μl of substrate. The reaction was then initiated by addition of 20 μl HCV NS3 protease in 50 mM Tricine, 1% Triton X-100 at pH 8.0. The time-dependent increase of fluorescence intensity was monitored every 40 s for 30 min. Initial reaction velocities, expressed as fluorescence units per minute, were obtained by least-squares analysis of the initial phase of the reaction using Deltasoft data-collecting and -analyzing software (BioMetallics, Inc.).

Inner filter effect correction on the microtiter plate reader. Fluorescence end-point readings were taken for different substrate concentrations in 198 μl assay buffer. Afterward, 2 μl of 50 μM free EDANS was added to each substrate concentration and mixed thoroughly and then a second round of fluorescence end-point readings were taken.

Micromolar fluorescence units for EDANS product. Micromolar fluorescence units for the EDANS product were obtained by diluting the EDANS product into assay buffer at concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 2.0 μM, and then a fluorescence end-point reading was taken. The linear relationship between fluorescence units and concentrations yields the micromolar fluorescence units for this specific EDANS peptide. The EDANS product stock solution concentration was calculated using an extinction coefficient of 7380 M^{-1} cm^{-1} at wavelength 344 nm obtained from Fmoc-Glu–EDANS.

K_i and K_i' determination for inhibitor. Initial rates of substrate hydrolysis were obtained by linear least-squares data fitting of actual experimental data. Apparent K_m and V_{max} values at each inhibitor concentration were obtained from nonlinear regression fitting using the Michaelis–Menten equation and the inner filter effect corrected initial rates of substrate hydrolysis. K_i and K_i' were obtained from the X-axis intercepts of plots of measured K_m/V_{max} and $1/V_{max}$ versus inhibitor concentration, respectively.

RESULTS

An inner filter effect correction may be necessary whenever the experimentally measured fluorescence intensities are not proportional to the concentration of the fluorescent compound. This phenomenon may result when the fluorescent compound self-absorbs a fraction of the emitted light which might occur under conditions of high optical densities (12). We have examined this possibility and the results in Fig. 1 show a linear relationship between fluorescence intensity and concentration of the N-terminal EDANS product. We find no evidence for a significant inner filter effect due to self-absorption by the EDANS fluorophore. However, the EDANS fluorophore is linked to the quenching DABCYL group in our HCV protease substrate. The excellent spectral overlap between the DABCYL absorbance and the EDANS fluorescence spectra results in a high degree of absorbance of the EDANS emission spectrum by the DABCYL group (5). Thus, our substrate exhibits internal fluorescence quenching when intact, but cleavage of the substrate by HCV protease separates the EDANS fluorophore from the DABCYL quenching group which results in an increased fluorescence signal. However, at high optical densities of substrate, some of the fluoresced light may

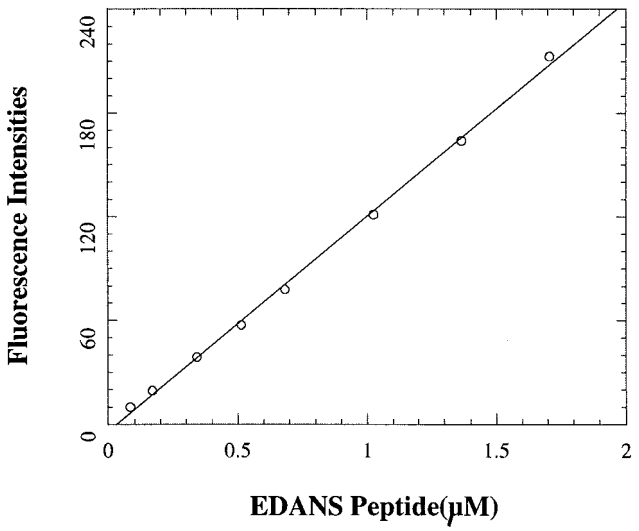

FIG. 1. Fluorescence intensities of various concentrations of N-terminal EDANS product.

be absorbed by DABCYL groups on neighboring substrates or cleaved product molecules so that only a fraction of the fluoresced light impinges on the detector system of the fluorometer. This type of inner filter effect is the main source of error in our HCV protease FRET substrate assay and the mathematical correction for this effect is described below.

Inner filter effect correction on the microtiter plate reader. The method which we introduce here involves measurement of the free EDANS fluorescence signal at various concentrations of quenching DABCYL groups from the FRET substrate. The reduction in the observed fluorescence signal of the free EDANS in the presence of various substrate concentrations reflects the inner filter effect at those specific substrate concentrations. Analysis of Table 1 shows that 0.5 μM EDANS generated 62.69 fluorescence units in our plate reader after subtracting the background fluorescence of 4.38 fluorescence units. However, in the presence of 0.5 μM FRET substrate, the 0.5 μM free EDANS only generated 60.46 fluorescence units or 96.4% of the intrinsic free EDANS fluorescence. The inner filter effect can become quite large, as shown by the data at 20 μM FRET substrate which indicate that only 32.4% of the intrinsic free EDANS fluorescence is able to be detected by the instrument.

The inner filter effect becomes important when conducting enzyme kinetic experiments using multiple concentrations of FRET substrate. The EDANS peptide product generated by enzymatic cleavage will fluoresce, but only a fraction of the signal will reach the detector due to the inner filter effect. Therefore, the measured enzymatic reaction velocities must be corrected for the inner filter effect by dividing the velocity at each substrate concentration by the corresponding Corr value in the table.

$$\text{Corr\%} = f_{\text{EDANS}} \text{ (a teach substrate concentration)/}$$
$$f_{\text{EDANS}} \text{ (in the absence of substrate)}$$

Kinetic characterization. The initial rates of substrate cleavage by HCV NS3 protease (0.3–0.6 nM) at different substrate concentrations were measured using the microtiter plate reader. At high substrate concentrations, initial rates appear to decrease due to the inner filter effect. However, when the inner filter effect correction for each substrate concentration was applied, the corrected rates could be fit to the Michaelis–Menten equation which yielded a K_m of 4.4 $\pm$ 0.3 μM. This value is in excellent agreement with K_m values of 4.6 $\pm$ 0.7 and 4.7 μM obtained by ourselves and Taliani *et al.* (9), respectively, using HPLC methods. The V_{max} value from the plate reader experiment was converted to micromolar EDANS product per minute using the experimentally measured factor of 120 fluorescence units per micromolar EDANS product. The k_{cat} and k_{cat}/K_m values are 0.425 $\pm$ 0.010 s^{-1} and 96,500 $\pm$ 5800 M^{-1} s^{-1}, respectively, from plate reader measurement and 0.426 $\pm$ 0.02 s^{-1} and 92,600 $\pm$ 14,000 M^{-1} s^{-1}, respectively, from our HPLC measurement (Fig. 2).

The same method of obtaining accurate K_m and V_{max} values using inner filter effect correction was also applied to an inhibitor mechanism study. An inhibitor identified from our screening group was used at 0, 0.8, 1.6, 4, and 8 μM to measure K_m and V_{max}. Inner filter effect corrected initial velocities were transformed into a double-reciprocal plot of $1/V_{corr}$ vs $1/[S]$ which indicated that this compound is a competitive inhibitor of HCV NS3 protease with a K_i value of 1.5 μM from K_m/V_{max} versus $[I]$ plot and a K_i' value of 21 μM from a $1/V_{max}$ versus $[I]$ plot (Figs. 3 and 4).

DISCUSSION

We have demonstrated that this inner filter effect correction can be used to accurately determine K_m,

TABLE 1

Inner Filter Effect Correction on Microtiter Plate Reader at Different HCV NS3 Protease Substrate Concentrations

[S] μM	$f(S)$	f(EDANS + S)	f(EDANS)	Corr%
0	4.38	67.07	62.69	1
0.5	6.15	66.61	60.46	0.964
1	8.22	67.95	59.73	0.953
2	12.35	65.92	53.57	0.854
4	16.02	62.42	46.40	0.740
8	21.27	59.26	37.99	0.606
12	25.22	56.20	30.98	0.494
16	27.12	52.96	25.84	0.412
20	29.02	49.33	20.31	0.324

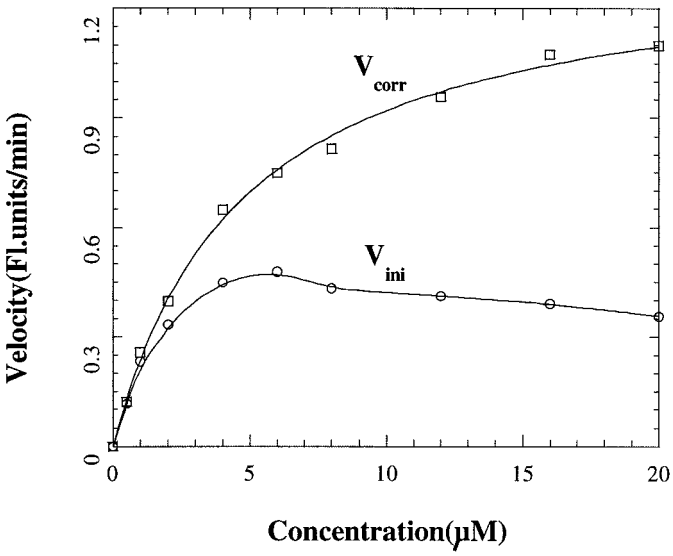

FIG. 2. HCV NS3 protease catalyzed substrate hydrolysis rates at different substrate concentrations: uncorrected rates (circles), inner filter effect corrected rates (squares).

V_{max}, and k_{cat}/K_m for this EDANS–DABCYL HCV NS3 protease substrate using a microtiter plate reader. Inner filter effect corrected plate reader velocities yielded values for K_m, k_{cat}, and k_{cat}/K_m of 4.4 ± 0.3 μM, 0.425 ± 0.010 s^{-1}, and 95,600 ± 5,800 M^{-1} s^{-1}, respectively, which are in excellent agreement with HPLC measured values of 4.6 ± 0.7 μM, 0.426 ± 0.02 s^{-1}, and 92,600 ± 14,000 M^{-1} s^{-1}, respectively. We have also demonstrated that inner filter effect corrected rates can be used to determine the kinetic inhibition mechanism by double-reciprocal plot analysis, enabling accurate measurement of K_i and K_i'.

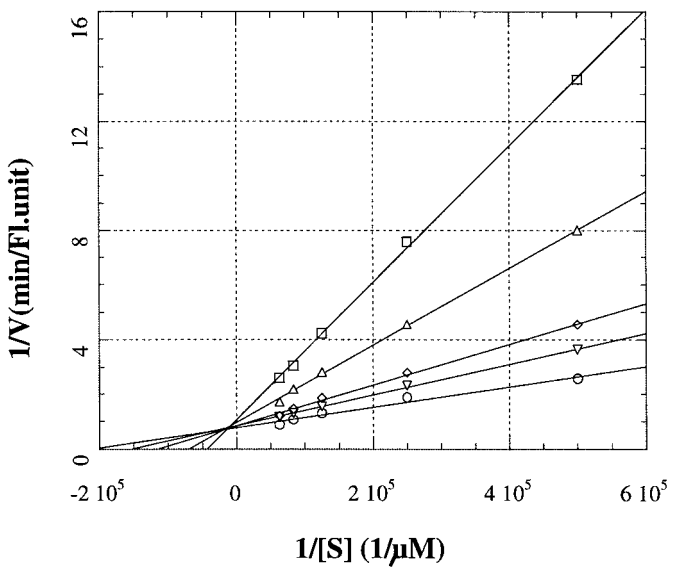

FIG. 3. Double-reciprocal plots of inner filter effect corrected rates versus substrate concentrations with inhibitor concentrations of 0 (circles), 0.8 (inverted triangles), 1.6 (diamonds), 4 (triangles), and 8 μM (squares).

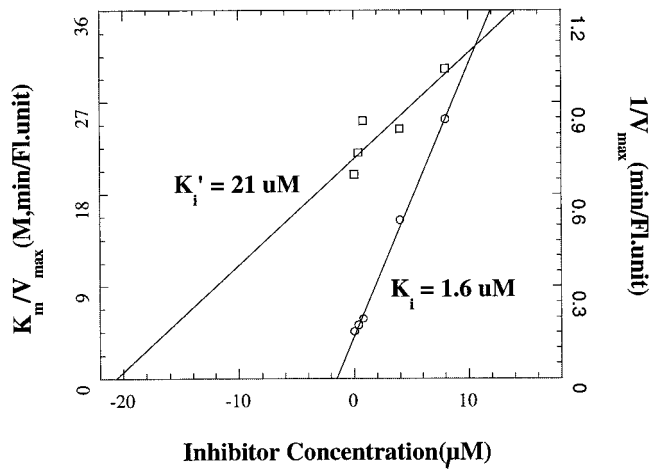

FIG. 4. K_m/V_{max} (circles) and $1/V_{max}$ (squares) versus inhibitor concentrations which yield a K_i of 1.6 μM and a K_i' of 21 μM.

Inner filter effect correction is usually done by a theoretical calculation making assumptions about path length and slit width (11). Matayoshi *et al.* first introduced the empirical free EDANS addition method to correct the inner filter effect. These studies enabled the K_m value measurement for an HIV protease substrate using a Spex Fluoromax fluorometer (5). The Spex instrument has less inner filter effect due to shorter path length when compared with a microtiter plate reader. However, the Spex instrument can only measure one rate at a time and in some case photo bleaching could prevent accurate inner filter correction. We have applied the inner filter effect correction to velocities measured in a microtiter format. It is now possible to run a complete inhibition mechanism study in only a half hour on a microtiter plate reader, while the same set of 36 velocity data points requires 2 days to collect on a cuvette-reading fluorometer or by HPLC. In summary, this method provides a fast and accurate way to determine K_m, k_{cat}, and k_{cat}/K_m for HCV NS3 protease as well as K_i values and the kinetic inhibition mechanism of NS3 protease inhibitors. However, the method should apply to all FRET substrates as long as there are no solubility, high substrate inhibition, or aggregation problems. We believe that inner filter effect correction of enzymatic rates obtained on a microtiter plate reader could be more extensively applied to other fluorescence resonance energy transfer substrates and their corresponding enzymes.

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