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# New intramolecularly quenched fluorogenic peptide substrates for the study of the kinetic specificity of papain

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A series of new substrates for determining the catalytic activity of cysteine proteinases is described. The rate of hydrolysis by papain was monitored by a fluorescence continuous assay based on internal resonance energy transfer using 5-[(2-aminoethyl)amino]naphtalene-1-sulfonic acid (EDANS) and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) as fluorescent donor and quenching acceptor, respectively, in peptides with the general structure: DABCYL-Lys-Phe-Gly-Xxx-Ala-Ala-EDANS. The substrates were used to evaluate the effect of amino acid structure in the S1' position on the kinetic parameters for papain catalyzed hydrolysis.

Cysteine proteinase; Papain; Fluorescence; Resonance energy transfer

## 1. INTRODUCTION

Resonance energy transfer [1–3], a phenomenon in which excitation energy is transferred from the excited fluorescent donor chromophore to a quenching chromophore acceptor, has been used to develop spectrophotometric assays for a variety of proteases [4–8]. Cleavage of the fluorogenic substrate produces an increase in fluorescence as the donor and acceptor separate, which can then be used to follow the kinetics of the reaction. In the present paper, we describe the utilization of this methodology to produce a new series of papain substrates with the structure: DABCYL-Lys-Phe-Gly-Xxx-Ala-Ala-EDANS (where Xxx = Phe, Ile, Val, Gly, Asn, Gln; **1a–f**). These new substrates have been utilized to explore the effect of substrate sequence in the S1' position (notation of Schechter and Berger [9]) on the kinetics specificity of papain.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Papain (EC 3.4.22.3) and E-64 were purchased from Sigma and used without further purification. The substrate N$^{\alpha}$-Z-Phe-Arg-MCA (**2** in Table I), and all Boc-amino acids were purchased from Bachem Inc.

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*Abbreviations:* Boc, *tert*-butyloxycarbonyl; DABCYL, 4-(4-dimethylaminophenylazo)benzoic acid; DMSO, dimethylsulfoxide; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; EDANS, 5-[(2-aminoethyl)amino]naphtalene-1-sulfonic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; MCA, methylcoumarylamide; $t_R$, retention time; Z, benzyloxycarbonyl.

Syntheses of the fluorogenic peptidyl substrates **1a–f** were carried out by classical solution methods [10]. N$^{\alpha}$-*tert*-butyloxycarbonyl-protected (Boc) amino acids were used in all coupling steps. The base labile-protecting group, 9-fluorenylmethyloxycarbonyl (Fmoc), was used for the protection of the side chain of lysine, and the benzyl group was employed for the protection of the C-terminal carboxyl group of each peptide. The Boc protecting group was removed before coupling by using 4 N HCl in dioxane. Peptide couplings were achieved by overnight reaction with 1,3-dicyclohexylcarbodiimide or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole in *N,N*-dimethylformamide. After completion of the synthesis, the benzyl group was removed by hydrogenolysis in methanol. The commercially available DABCYL and EDANS groups [11] were attached to the hexapeptides with conventional condensation reactions, and the Fmoc group was removed from the lysine ε-amine group by treatment with piperidine-*N,N*-dimethylformamide (1:1, v/v) to give **1a–f**. Purification of the crude products was accomplished by reverse-phase high pressure liquid chromatography. Final products were evaluated by analytical HPLC on a Vydac C-18 column (4.6 × 250 mm), linear gradient over 20 min of $CH_3CN$-0.045% TFA and $H_2O$-0.036% TFA from 1:19 to 4:1 and 5 min isocratic at 4:1, flow rate 1.2 ml/min, detection at 254 nm; $t_R$= 17.9 min (**1a** in Table I); 17.7 min (**1b**); 17.1 min (**1c**); 16.1 min (**1d**); 16.1 min (**1e**); 16.3 min (**1f**). The integrity of each purified peptide was determined by FAB mass spectrometry, and the observed molecular mass was found to agree with the calculated values.

### 2.2. *Methods*

Fluorescence was monitored by using a Perkin-Elmer fluorescence spectrometer model MPF-4 equipped with a Xenon Power Supply 156 and a recorder model 56. Papain activity was assayed with N$^{\alpha}$-Z-Phe-Arg-MCA at pH 6.45 according to the method of Barrett et al. [12]. The fluorescence of 7-amino-4-methylcoumarin liberated from the substrate was monitored at 458 nm with excitation at 370 nm. The active concentration of papain was determined by active site titration with E-64 for 30 min at 30°C as described [12].

Hydrolyses of fluorogenic substrates **1a–f** by papain were monitored at 30°C in 1 cm cuvettes. The poorly soluble substrates were dissolved in DMSO and diluted with buffer; final concentration of DMSO was ≈2%. All reactions were conducted in 0.4 M phosphate buffer (pH 6.45); dithiothreitol (8 mM) and EDTA (4 mM) were also present. The excitation wavelength was 336 nm and the emission

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wavelength was 472 nm with a slit of 10 nm. All peptides were cleaved at one site, which was identified by analytical HPLC of the products.

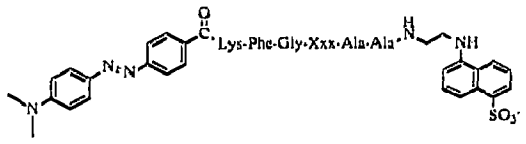
The rate of fluorescent substrate hydrolysis was computed from a non-linear least squares fit of enzyme kinetic data [13] using a home-made program. For each substrate in Table I, the initial velocities' $v_0$ were determined in triplicate and these values were used to compute the Michaelis–Menten constant ($K_m$) and the turnover constant ($k_{cat}$) as described.

## 3. RESULTS

Papain digestion of substrates **1a–f** was followed by monitoring the increase in the fluorescence of the peptide moiety H-Xxx-Ala-Ala-EDANS (where Xxx = Phe, Ile, Val, Gly, Asn, Gln) at 472 nm upon excitation at 336 nm. The difference between the fluorescence intensity at 472 nm obtained from the exhaustive enzymic digestion of the substrate and the fluorescence intensity of the unhydrolyzed substrate was used as the reference. The rate of increase in fluorescence was directly proportional to the amount of substrate concentration. The fluorescence enhancements of substrates after hydrolysis ranged from 19 to 49. The increase in fluorescence intensity observed for Ile (49), Asn (46), Gly (34) and Glu (28) suggested that these substrates adopt folded conformations in solution since fluorescent quenching is related to distance in accordance with the equation formulated by Förster [14].

Table I shows the kinetic parameters for the hydrolysis of the new fluorogenic substrates **1a–f**. Partition coefficients (log*P*) for each amino acid side chain in the P1' position were calculated from atomic increments by using the values reported by Ghose and Crippen [16].

Table 1. Kinetic parameters for papain[a] catalyzed hydrolysis of intramolecularly quenched fluorogenic substrates 1a-f.




| Substrate | P1' | $k_{cat}$ ($s^{-1}$) | $K_M$ (M) | $k_{cat}/K_M$ ($M^{-1} \times s^{-1}$) | log*P*[b] |
|---|---|---|---|---|---|
| 1a | Phe | 4.9 ± 1.9 | 1.9 ± 1.1 x $10^{-5}$ | 2.63 x $10^5$ | 2.383 |
| b | Ile | 1.5 ± 0.2 | 3.0 ± 1.2 x $10^{-5}$ | 5.06 x $10^4$ | 1.932 |
| c | Val | 2.4 ± 0.6 | 1.1 ± 0.6 x $10^{-5}$ | 2.22 x $10^5$ | 1.470 |
| d | Gly | 3.3 ± 0.3 | 7.6 ± 2.1 x $10^{-6}$ | 4.32 x $10^5$ | 0.242 |
| e | Gln | 3.8 ± 0.3 | 8.8 ± 1.8 x $10^{-6}$ | 4.32 x $10^5$ | - 0.396 |
| f | Asn | 4.6 ± 0.6 | 9.9 ± 2.8 x $10^{-6}$ | 4.64 x $10^5$ | - 0.858 |
| 2[c] | MCA | 52 ± 6 | 0.4 ± 0.1 x $10^{-3}$ | 1.20 x $10^5$ | |

[a] Kinetic parameters were determined as described in section 2 at pH 6.45 and 30°C.
[b] The correlation equation obtained between the turnover constants ($k_{cat}$) from papain and hydrophobicity (log*P*) of amino acid Xxx side chain in the fluorescent substrates **1b-f** was $k_{cat}$ = 3.6log*P* - 1.0 ($r^2$ = 0.972).
[c] Values taken from reference [15].

## 4. DISCUSSION

Many cysteine proteinases hydrolyze protein substrates in vivo and therefore recognize and cleave extended peptide sequences found in the natural substrates. However, most systematic kinetic studies of the effect of substrate sequence on kinetic parameters of cysteine proteinases have employed chromophoric peptides (e.g. substrate **2**), which have been designed for liberating the chromophoric aromatic group. Although these substrates have proven to be extremely valuable for characterizing many cysteine proteinases, by their design they cannot provide information about the effects of peptide sequence in the *prime* region of substrate (P1', P2', ...) that could interact with the corresponding *prime* region of the enzyme (S1', S2', ...) [9]. We were interested to evaluate if variations of the P1'–P3' positions of substrates for cysteine proteinases might affect the kinetic parameters for substrate hydrolysis, and thereby gain information relating to substrate specificity for members of this class of enzyme.

Fluorescence resonance energy transfer is a useful method for continuous assay of protease activity that has been employed to develop substrates of other proteases [4–8]. Cleavage of the substrate results in a relief of fluorescence quenching, which is directly proportional to the concentration of the fluorophore. A major advantage of this approach is that it permits systematic modification of both portions of the peptide substrates that interact with the enzyme, and therefore provides information not attainable with substrates used previously to characterize these proteases. For these reasons, we developed a new series of substrates for cysteine proteinases by attaching the recently described fluorescent donor (EDANS) and quenching acceptor (DABCYL) [7] to the C- and N-terminus of the peptide substrates.

The choice of the oligopeptide sequence, H-Lys-Phe-Gly-Xxx-Ala-Ala-OH (where Xxx = Phe, Ile, Val, Gly, Asn, Gln) was guided by the available data on the specificity of papain [17–19] and the requirement that only a single peptide bond (Gly-Xxx) be split by the enzyme. The subsite S2 of papain is known to preferentially bind hydrophobic amino acids; phenylalanine is one of the best amino acids for inserting into the P2 cleft [20]. Glycine was placed at P1 since the S1 binding site S1(P1) is relatively non-selective in papain [17–20] compared to other proteases and glycine fits quite well at this site. Analysis by HPLC of the products obtained upon hydrolysis established that cleavage occurred between the Gly-Xxx bond as expected. The data obtained (Table I) indicate that hexapeptides **1a–f** have $k_{cat}/K_m$ values similar to the $k_{cat}/K_m$ value reported for the methylcoumarylamide substrate **2** [15]; however, **2** is hydrolyzed faster and has a higher Michaelis–Menten constant.

Attempts to correlate physical-chemical parameters of substrates with the enzymatic activity of papain have

shown that this is a multivariate problem [21]. In 1974, Alecio et al. [22] using a series of *N*-benzyloxycarbonyl-glycyl-L-amino acid amides suggested that papain preferred hydrophobic residues in the S1' subsite. Following on this suggestion, we evaluated the relationship between the hydrophobicity of the amino acid side chains in P1' and the kinetic parameters of the corresponding substrate. As expected from previously reported data [21] no correlation equation was found for $K_m$ or $k_{cat}/K_m$ using a single parameter. However, for substrates **1b–f** the turnover constant linearly correlates with the logarithm of the water-octanol partition coefficient (log*P*). This relationship between $k_{cat}$ and log*P* shows that the reactivity of the enzyme increased by decreasing the hydrophobic nature of the amino acid in the S1' position for the non-aromatic series of substrates; in fact, the $k_{cat}$ of Asn substrate **1f** is equivalent to the $k_{cat}$ of Phe substrate **1a** for this enzyme. These results suggest a complex relationship between structure and kinetic parameters and confirm that multiple factors beyond hydrophobic interactions affect the kinetic parameters for papain-catalyzed hydrolysis of substrates.

In summary, we have shown that the resonance energy transfer method can be used to develop new substrates for cysteine proteinases. This method also permits systematic evaluation of the effect of structural changes in the prime region of protease substrates on kinetic parameters. The application of these substrates to other cysteine proteases is currently under investigation.

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