CHEMISTRY LETTERS, pp. 77-80, 1992. 

# 2-Aminobenzoyl-peptide-2,4-dinitroanilinoethylamides. Facile Fluorescent Detection System for Sequence Specific Proteases

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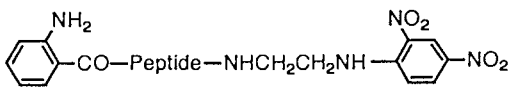
New fluorogenic peptides with efficient quenching group were synthesized and subjected to the action of thermolysin and trypsin. The hydrolysis of the peptide bond resulted in the increase in fluorescence intensity by 50-200-fold of the quenched peptides.

For the detection of protease activity, substrates consisting of the appropriate peptides with chromophore or fluorophore at the *C*-termini are often employed. Typically, 4-nitroanilide (NA) and 4-methylcoumaryl-7-amide (MCA) substrates are widely used and commercially available in varieties. Generally, proteases exhibit the primary specificity to the particular amino acid residue ($P_1$)[1] and hydrolyze the following peptide bond (in this case C-type protease). Secondarily they recognize the peptide sequences (Pn) preceding the amino acid of the primary specificity. Since the releasing chromophore has to be attached to the end of peptide, however, it is totally impossible to investigate the specific recognition of the other side of scissile bond. In addition, there are a group of proteases (N-type proteases) which hydrolyze the preceding peptide bond of the primarily recognizing amino acid residue ($P_1'$). The substrates mentioned above cannot be applied for them.

For the convenient detection system for proteases covering the specificities to the both sides of the scissile bond, several intramolecularly quenched fluorescent substrates (IQFS) have been proposed.[2] The combination of 2-aminobenzoyl (Abz) and 4-nitrobenzylamide (Nba) (Abz-peptide-Nba system) was hopeful in general use but the quenching efficiency was somewhat poor.[3] The energy transfer from Trp to 5-dimethylaminonaphthalene-1-sulfonyl group accomplished some improvement.[4] Very recently, more efficient IQFS systems appeared for detection of activities of HIV-1 proteases[5] and matrix metalloproteinases.[6] They include different pairs of fluorogenic and quenching moieties, namely, 5-sulfo-1-naphthylaminoethylamide and 4-(4'-dimethylaminophenylazo)benzoyl, and Trp and 2,4-dinitrophenyl. Considering the increasing needs for the sensitive and specific assays of various types of proteases involving disease-related ones and physiologically important processing enzymes, we attempted to reinforce the quenching ability by the use of dinitrophenyl group instead of Nba. Thus, for the new IQFS system, Abz-peptide-Dna (Dna, 2,4-dinitroanilino-




| Peptide | |
|---|---|
| Ala-Gly-Leu-Ala | 1 |
| Gly-Phe-Arg-Leu | 2 |
| Gly-Phe-Arg-Leu-Leu | 3 |

Fig. 1. Intramolecularly quenched fluorogenic substrates.

ethylamide) was designed (Fig. 1).[7] The wide usefulness for the aimed search for the sequence specific proteases also requires facile synthesis of various peptide substrates and high quenching efficiency.

We synthesized **1** by conventional method according to the procedure reported earlier,[3] and **2** and **3** much more conveniently by Kaiser's oxime resin solid-phase-method.[8, 9] Especially, the efficient amidation cleavage with Dna and Leu-Dna of the peptide assembled on the resin (Boc-Abz-Gly-Phe-Arg(Tos)-Leu-resin) produced Boc-Abz-Gly-Phe-Arg(Tos)-Leu-Dna and Boc-Abz-Gly-Phe-Arg(Tos)-Leu-Leu-Dna, respectively. This procedure not only saved the time for synthesis but also allowed the easy isolation and purification of the protected peptides. They were finally treated with anhydrous HF and purified by RP-HPLC to obtain the desired fluorogenic substrates.[10]

Figure 2 shows the absorption spectra of Abz-Ala-Gly, Leu-Ala-Dna, and **1**. The Abz group showed λmax at 311 nm, where Dna has a window in the spectrum. The excitation of Abz-group at 320 nm emitted fluorescence at 425 nm which overlapped the shoulder of the absorption spectrum of Dna. In fact efficient quenching by energy migration was achieved by the attachment of them on both sides of peptides as shown in Fig. 2. When **1** was subjected to the hydrolysis with thermolysin, the fluorescence intensity increased up to 54-fold of the intrinsic one at 50 μM (1 M=1 mol·dm$^{-3}$). The progress curve was shown in Fig. 3 which may allow the continuosly recording fluorescent assays of thermolysin. Both thermolysin and trypsin also hydrolyzed **2** and **3**, and afforded 190- and 52-folds fluorescence increase at 50 μM, respectively. In the longer peptide the quenching was less effective due to the distance to the fluorophore, but still sufficient for the routine assays of proteases. Furthermore the high contrast in fluorescence intensities before and after hydrolysis suggests that this IQFS system may be useful in the 96-well micro plate reader analysis. Figure 4 shows the fluorescence intensities of the equimolar mixture of Abz-Ala-Gly and Leu-Ala-Dna in buffer solution in various concentrations. The intermolecular quenching was observed at higher than 70 μM. Therefore, it should be recommended that this IQFS system is effectively utilized at lower than 50 μM of the substrate

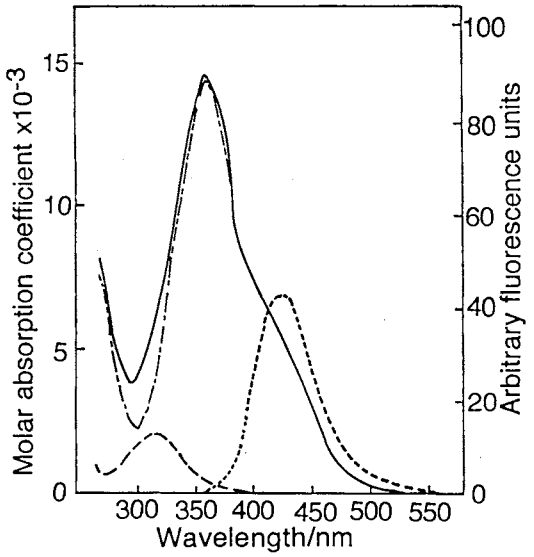



Fig. 2. Absorption spectra of Abz-Ala-Gly (– –), Leu-Ala-Dna (— -), and **1** (——), and emission spectrum of Abz-Ala-Gly (- - -). The compounds (20 mM) were dissolved in 50 mM Tris·HCl, 2.5 mM $CaCl_2$, pH 7.2, containing 2.5% DMF; excited at 320 nm.

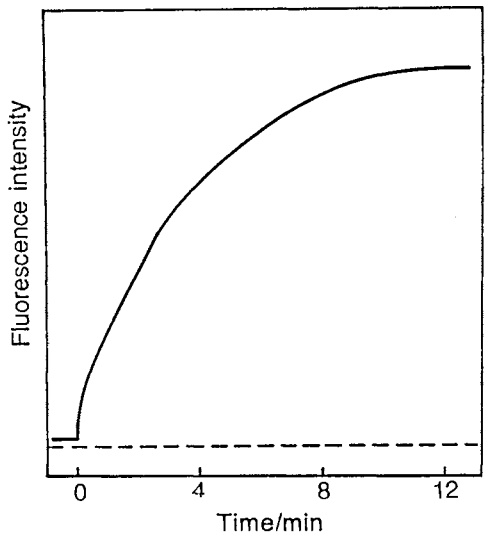



Fig. 3. Progress curve for the complete hydrolysis of **1** (50 μM) under the condition in Table 1.

concentration.

The second advantage of this IQFS system is the possible application for screening of the sequence specific proteases from various origins. Some proteases depend on not only single amino acid but the peptide sequence in specificities. One of the examples was found as shown in Table 1. Thermolysin preferencially hydrolyzes the preceding peptide bond of Leu. Therefore, it hydrolyzed all of three peptides at the expected points. The cleavage points were determined by RP-HPLC analysis of the hydrolysates.[11] The kcat/Km value, determined by the Lineweaver-Burk plot, of **1** suggests that it is a susceptible substrate for thermolysin. However, another tetrapeptide **2** was 290-fold less susceptible. In addition, the pentapeptide **3** appeared 260-fold more susceptible than **2** with similar sequence, which lacks P2' residue. This fact emphasizes that the presence of P2'-S2' interaction is so

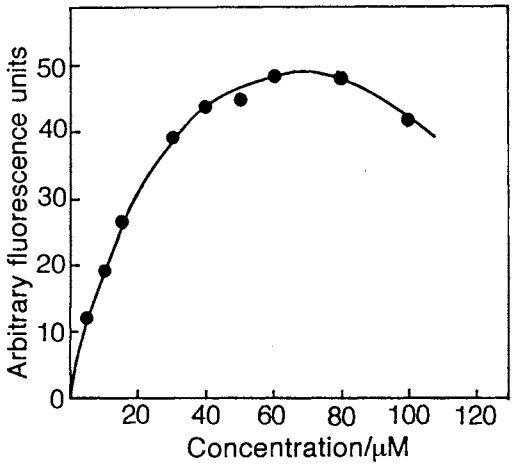



Fig. 4. Fluorescence intensities of equimolar mixture solution of Abz-Ala-Gly and Leu-Ala-Dna in 50 mM Tris·HCl, 2.5 mM $CaCl_2$, pH 7.2, containing 2.5% DMF at various concentrations. λex=320 nm, λem=425 nm.

Table 1. Kinetic Constants for the Hydrolysis of the Fluorogenic Peptides

| Peptide $P_3$ $P_2$ $P_1$ $P_1'$ $P_2'$ | | Enzyme | [S] μM | kcat $s^{-1}$ | Km μM | kcat/Km $s^{-1}M^{-1}$ | Ft/Fi[c] |
|---|---|---|---|---|---|---|---|
| Ala-Gly-Leu-Ala | **1** | thermolysin[a] | 17-99 | 29 | 26 | $1.1\times10^6$ | 51 |
| Gly-Phe-Arg-Leu | **2** | thermolysin[a] | 16-98 | 0.10 | 26 | $3.8\times10^3$ | 190 |
| | | trypsin[b] | 5-98 | 23 | 8.3 | $2.7\times10^6$ | 190 |
| Gly-Phe-Arg-Leu-Leu | **3** | thermolysin[a] | 16-78 | 31 | 30 | $1.0\times10^6$ | 52 |
| | | trypsin[b] | 5-78 | 22 | 14 | $1.6\times10^6$ | 52 |

a) 50 mM Tris·HCl, 2.5 mM $CaCl_2$, pH 7.2, containing 2.5% DMF, 25 °C. Thermolysin, 1.5 nM for **1**, 150 nM for **2**. b) 50 mM Tris.HCl, 100 mM NaCl, 10 mM CaCl2, pH 7.8, containing 2.5% DMF, 25 °C. Trypsin, 3.8 nM. c) Increase in fluorescence intensity at 100% hydrolysis.

important in fast processing of substrate peptides by thermolysin. It should be noteworthy that thermolysin hydrolyzed definitely Arg-Leu bond, though there are three potentially labile bonds in **3**, Gly-Phe, Arg-Leu, and Leu-Leu. On the other hand, tryptic hydrolysis was not effected so much by P2' residue as shown in Table 1. These observations clearly demonstrated that thermolysin has more sequence specificity than trypsin and only the IQFS system could conveniently discover this fact.

Not only the digestive proteases, there are a number of processing enzymes in nature. They recognize the specific sequences in the natural substrates. For the correct detection of particular protease, the appropriate sequence should be adopted. By the facility in synthesis, the high contrast in fluorescence intensities, and the broad covering for the extended binding site, the improved IQFS system (Abz-peptide-Dna) is superior to the

other systems. For the processing of the biologically active peptides produced by bacteria, the sequence specific proteases are awaited. In the screening of such proteases, guiding fluorogenic substrates may be useful especially on the 96-wells micro plate reader.

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9) Boc-Leu (4 mmol) was reacted with the oxime resin (4.0 g) by DCC. The incorporated amount was determined by picrate assay to be 0.48 mol/g resin. The Boc-Leu-resin (4.36 g) was treated with 25% trifluoroacetic acid (TFA) in $CH_2Cl_2$ (60 ml). Boc-Arg(Tos) (3 equiv.) was coupled with DCC/1-hydroxybenzotriazole (HOBt). The third amino acid Boc-Phe was coupled by symmetrical anhydride method to avoid the spontaneous cleavage of dipeptide, the further elongation of peptide with Boc-Gly and Boc-Abz was perfomed by DCC/HOBt. Finally, the peptide resin, Boc-Abz-Gly-Phe-Arg(Tos)-Leu-resin was treated with Dna or Leu-Dna (4 equiv.) in the presence of acetic acid (4 equiv.) in DMF (30 ml). The yields of precursors of **2** and **3** were 85 and 71%, respectively.
10) MS-GEL C18 column (10 mm x 250 mm) with 43% $CH_3CN$/0.1% TFA. Two peptides **2** and **3** were obtained as fluffy powder after lyophylization in 72% and 67%, respectively. FAB-MS: **2**, 819 $(M+H)^+$; **3**, 932 $(M+H)^+$.
11) MS-GEL C18 column (4.6 mm x 150 mm) with a linear gradient of 10-100% $CH_3CN$/0.1% TFA.

(Received October 11, 1991)