Fluorogenic Substrate of Pepsin

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Synopsis. Fluorogenic substrates of pepsin; Dns-Ala-Phe-Trp-Val-Leu-OCH_2Py and Dns-Phe-Trp-Val-Leu-OCH_2Py were synthesized by a solution method. Incubation of pepsin with Dns-Ala-Phe-Trp-Val-Leu-OCH_2Py resulted in specific cleavage at the Phe-Trp bond and the time-dependent increase in fluorescence intensity at 345 nm paralleling the extent of substrate hydrolysis. The optimum pH of the substrate was 3.0 and the K_m and k_{cat} values were 8.0×10^{-5} M and 0.10 s^{-1}, respectively. The linearity of the plot of fluorescence intensity vs. enzyme concentration was satisfactory in the range of 50 nM to 500 nM of pepsin at a substrate concentration of 50 μM. Because of its simplicity and rapidity, this method is useful for the measurement of pepsin action. In contrast, Dns-Phe-Trp-Val-Leu-OCH_2Py was not cleaved by pepsin.

Pepsin [EC. 3.4.4.1.] is a gastric proteinase active in acidic solutions (pH 1—5). To determine the pepsin activity, hemoglobin is generally used as a protein substrate.[2] In addition, many synthetic substrates have been used for the measurement of pepsin activity.[3,4] The widely used analytical procedures to estimate the hydrolysis rate are based on the determination of amine products by observing their reaction with ninhydrin. These procedures usually require much intension and are time-consuming. The task of enzymic activity assay is simplified by use of chromogenic substrates. However, a good chromogenic substrate for pepsin has not been obtained. For example, pepsin does not cleave *p*-nitroanilide substrates.

Inouye et al. synthesized chromogenic substrates containing *p*-nitrophenylalanine at the P_1 site such as Z-His-Phe (NO_2)-Phe-OMe and determined the rate of hydrolysis in terms of the increase in absorbance at 310 nm.[5] However, the sensitivity of these substrates for the pepsin action was low.

Fluorometric methods are employed for the detection of peptidase activity as convenient and very sensitive methods. Most of the fluorogenic substrates for peptidases are acyl derivatives of highly fluorescent amines.[6,7] These substrates would not be cleaved by pepsin in analogy with *p*-nitroanilide substrates.[8,9] Latt et al. reported the synthesis of intramolecularly quenching substrates for carboxypeptidase, where the resonance energy transfer phenomenon was utilized with dansylated tryptophan-peptides.[10] Recently, Sato et al. reported applications of 3,4,6,7-tetramethyl-9,10-dioxa-*syn*-bimane to prepare intramolecularly quenching substrates for assay of some peptidases.[11,12]

Since pepsin cleaves the peptide bond between two aromatic amino acids, the peptides containing a dansyl group at the NH_2-terminal, phenylalanine at the P_1 site and tryptophan at the P_1' site must be cleaved between two aromatic amino acid residues by pepsin, with a concomitant increase in the tryptophan fluorescence. However, N-protected peptides are not soluble in acidic buffers. Then, 4-pyridylmethyl ester of N-protected substrates for pepsin was employed, since it confers moderate solubility in acidic buffers.[13] Z-Ala-Ala-Phe(NO_2)-Phe-$O(CH_2)_3Py$ and Phe-Gly-His-Phe(NO_2)-Phe-Val-Leu-OMe are reported by good substrates for pepsin.[9] In view of this, Dns-Phe-Trp-Val-Leu-OCH_2Py (**1**) and Dns-Ala-Phe-Trp-Val-Leu-OCH_2Py (**2**) have been synthesized in the present work as fluorogenic substrates for pepsin.

Results and Discussion

The substrates and the peptides which will arise from cleavage of the substrates were synthesized by a solution method. Each protected amino acid was coupled with amino acid 4-pyridylmethyl ester or peptide 4-pyridylmethyl esters from C-terminus. The obtained Boc-peptide-OCH_2Pys were treated with HCl/AcOH and the resulting deblocked peptide 4-pyridylmethyl esters were dansylated by the usual manner. The dansyl peptides obtained gave one spot by thin layer chromatography and a single peak by HPLC. Compounds **1** and **2** were sufficiently soluble in an acidic buffer containing 10% DMF for use in kinetic studies. The chromatographic patterns for the mixtures of substrates (**2** and **1**) and products (Dns-Ala-Phe, Dns-Phe, and Trp-Val-Leu-OCH_2Py) are shown in Fig. 1-A and 1-C. The elution times of synthetic peptides were as follows: **2**, 27.2 min; **1**, 27.2 min; Dns-Ala-Phe, 20.0 min; Dns-Phe, 18.9 min; Trp-Val-Leu-OCH_2Py, 18.9 min.

The substrates **2** and **1** were incubated, respectively, with pepsin for 3 h and the reaction mixtures were applied on HPLC. Fig. 1-B indicates disappearance of the substrate (**2**) and concomitant appearance of two products (Dns-Ala-Phe and Trp-Val-Leu-OCH_2Py). This result suggests that the substrate was cleaved quantitatively at a single bond between phenylalanine and tryptophan. On the other hand, compound **1** was not cleaved by pepsin (Fig. 1-D).

As seen in Fig. 2, hydrolysis of the substrate (**2**) decreased dansyl emission (500 nm) and increased tryptophan fluorescence (345 nm). When pepsin was added to the substrate solution, the fluorescence at 345 nm increased momentarily, then decreased gradually up to about 2 min. Subsequently, the fluorescence at 345 nm increased linearly in proportion to the concentration of added pepsin. Consequently, measurement of the increase in the fluorescence was started at the incubation time of 2 min. Similarly, addition of pepsin to the substrate produced an increase in the fluorescence intensity at 500 nm. This indicates that the dansyl group of the substrate was bound at a hydrophobic region of the enzyme.[14] Then, the fluo-

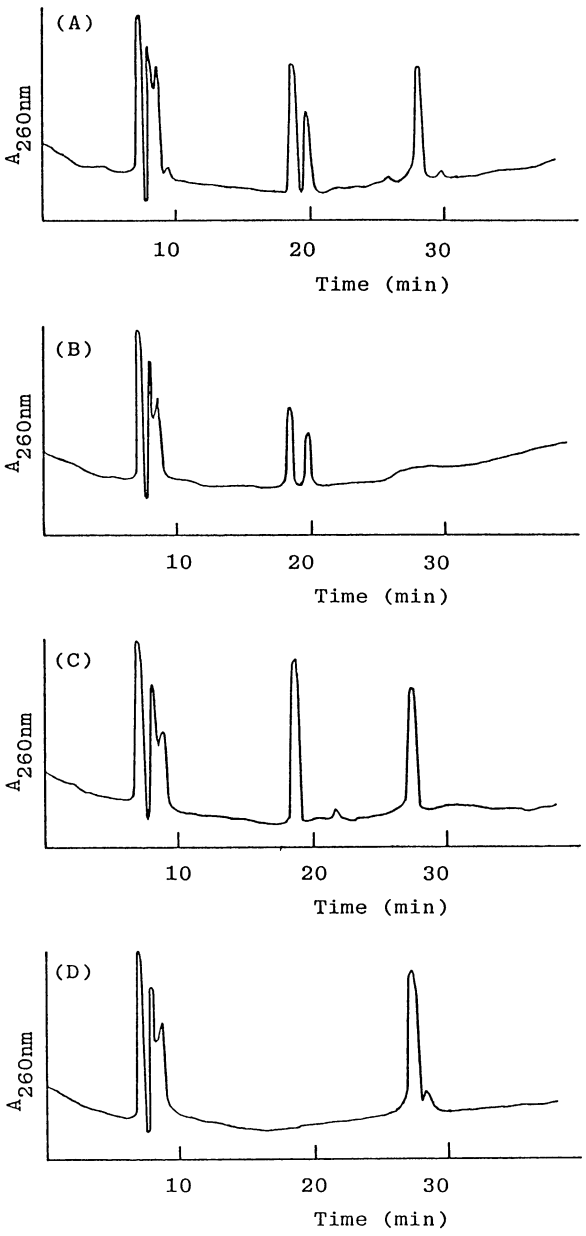

Fig. 1. HPLC traces of fluorogenic substrates and reaction products. (A) Mixture of **2**, Dns-Ala-Phe, and Trp-Val-Leu-OCH_2Py; (B) Reaction mixture of **2** and pepsin; (C) Mixture of **1**, Dns-Phe, and Trp-Val-Leu-OCH_2Py; (D) Reaction mixture of **1** and pepsin.

rescence at 500 nm decreased with the hydrolysis of the substrates. The increase in the fluorescence at 345 nm subsequent to complete hydrolysis of the substrate by pepsin was not influenced by the amount of added pepsin. The molar fluorescence intensity of the complete hydrolysis of compound **2** was 50000.

Comparative measurements of the effect of pH on the pepsin action were made with compound **2**. The optimum pH was 3.0. A pH 3.0 buffer was thus used as solvent for the substrate in the following experiments. K_m and k_{cat} values of 8.0×10^{-5} M and 0.10 s^{-1} were obtained from the Lineweaver-Burk plot of compound **2**. The linearity of the plot of fluorescence

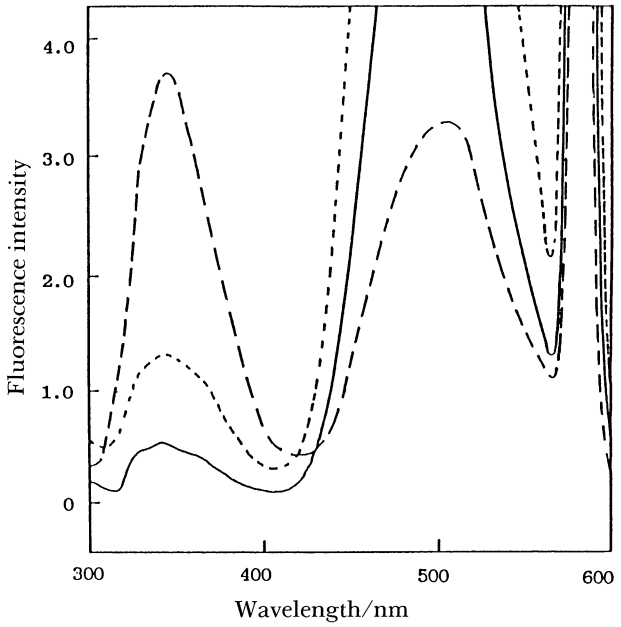

Fig. 2. Fluorescence emission spectra of **2** and the hydrolysate of **2**. (——) **2**; (------) **2** was incubated with pepsin for 10 s; (— —) **2** was incubated with pepsin for 3 h.

intensity vs. enzyme concentration was satisfactory in the range of 50 nM to 500 nM of pepsin.

Compound **2** is the first fluorogenic substrate of pepsin. The resultant assay for pepsin was sensitive and convenient. The product formed can be detected directly as hydrolysis occurs. This contrasts with the case of the nynhydrin methods, where at least several minutes elapse between the formation and detection of the products. The fluorogenic substrate described here may be useful for the assay of pepsin activity.

Experimental

All melting points are uncorrected. Optical rotations were determined with a JASCO spectropolarimeter J-20A. Fluorescence measurements were performed with a Shimadzu spectrofluorophotometer RF-5000.

Synthesis of Substrates. Boc-Leu-OCH_2Py (3). To a solution of Boc-Leu (2.31 g, 10 mmol) and 4-pyridylmethanol (1.09 g, 10 mmol) in CH_2Cl_2 (20 ml) was added DCC (2.06 g, 10 mmol) at 0 °C. The mixture was stirred for 2 h at 0 °C and overnight at room temperature, then evaporated in vacuo, and ethyl acetate was added to the residue. After dicyclohexylurea was filtered off, the filtrate was washed successively with 4% sodium bicarbonate, 10% citric acid and water. The organic layer was dried over anhydrous sodium sulfate and was evaporated in vacuo to yield Boc-Leu-OCH_2Py as an oil (3.07 g). ^1H NMR ($CDCl_3$) δ=0.94 (6H, d, J=5 Hz), 1.40 (9H, s) 1.40—1.92 (3H, m), 4.48 (1H, q, J=6 Hz), 4.90 (1H, d, J= 6 Hz), 5.15 (2H, s), 7.22 (2H, dd, J=1 and 5 Hz), 8.58 (2H, dd, J=1 and 5 Hz).

Boc-Val-Leu-OCH_2Py (4). Compound **3** (3.07 g) was treated with 1M (1 M=1 mol dm^{-3}) HCl/AcOH for 1 h at room temperature and the resulting HCl · Leu-OCH_2Py was converted into free base as described previously.[13] The obtained oily Leu-OCH_2Py was coupled with Boc-Val (2.17 g, 10 mmol) in the same manner using DCC (1.65 g, 8 mmol) in CH_2Cl_2 (30 ml) to yield Boc-Val-Leu-OCH_2Py as an oil (3.16 g). ^1H NMR ($CDCl_3$) δ=0.95 (12H, d, J=6 Hz), 1.40

(9H, s), 1.37—1.87 (2H, m), 2.03 (2H, t, J=6 Hz), 3.88 (1H, dd, J=6 and 8 Hz), 4.73 (1H, q, J=7 Hz), 5.06 (1H, brd, J=7 Hz), 5.13 (2H, s), 6.47 (1H, brd, J=8 Hz), 7.23 (2H, brd, J=4 Hz), 8.62 (2H, dd, J=2 and 4 Hz).

Boc-Trp-Val-Leu-OCH$_2$Py (5). Boc-Trp (3.0 g, 10 mmol) and Val-Leu-OCH$_2$Py (derived from 3.16 g of **4** in the manner descrived for Leu-OCH$_2$Py) were coupled in the same manner using DCC (1.56 g, 7.6 mmol) in CH_2Cl_2 (30 ml). The product was recrystallized from ethyl acetate-petroleum ether; yield, 3.75 g, (81%); mp 89—91 °C; $[\alpha]_D^{25}$ −22.5° (c 1, DMF).

Found: C, 64.59; H, 7.72; N, 11.04%. Calcd for $C_{33}H_{45}O_6N_5 \cdot 1/2H_2O$: C, 64.26; H, 7.35; N, 11.36%.

Boc-Phe-Trp-Val-Leu-OCH$_2$Py (6). Compound **5** (3.1 g, 5.1 mmol) was treated with 1 M HCl/AcOH for 1 h at room temperature and the solvent evaporated in vacuo. The resulting crystals were collected with the aid of ether; yield, 3.5 g. The product (3.5 g) was dissolved in water (8 ml) and cooled in an ice bath. To this solution was added 50% saturated aqueous K_2CO_3 (13 ml) and the resulting precipitate was collected by filtration, washed with water and dried. The resulting Trp-Val-Leu-OCH$_2$Py and Boc-Phe (1.33 g, 5 mmol) were coupled in the same manner using DCC (0.9 g, 4.5 mmol) in DMF (10 ml) and CH_2Cl_2 (20 ml). The product was recrystallized from ethyl acetate-petroleum ether; yield, 1.94 g, (57%); mp 121—123 °C; $[\alpha]_D^{25}$ −18.0° (c 1, DMF).

Found: C, 66.15; H, 7.05; N, 11.00%. Calcd for $C_{42}H_{54}O_7N_6 \cdot 1/2H_2O$: C, 66.03; H, 7.26; N, 11.00%.

Boc-Ala-Phe-Trp-Val-Leu-OCH$_2$Py (7). Boc-Ala (80 mg, 0.42 mmol) and Phe-Trp-Val-Leu-OCH$_2$Py (derived from 0.3 mmol of **6** in the manner described for Leu-OCH$_2$Py) were coupled in the same manner using DCC (65 mg, 0.32 mmol) in DMF (1 ml) and CH_2Cl_2 (4 ml). The product was recrystallized from ethyl acetate-petroleum ether; yield, 200 mg, (81%); mp 161—163 °C; $[\alpha]_D^{25}$ −19.5° (c 1, DMF).

Found: C, 64.85; H, 7.23; N, 11.75%. Calcd for $C_{45}H_{59}O_8N_7 \cdot 1/2H_2O$: C, 64.73; H, 7.24; N, 11.74%.

Dns-Phe-Trp-Val-Leu-OCH$_2$Py (1). Compound **6** (90 mg, 0.12 mmol) was treated with 1 M HCl/AcOH for 1 h at room temperature, and the resulting deblocked peptide ester was dansylated according to the procedure described by Gray.[15] The product was recrystallized from ethyl acetate-petroleum ether; yield, 65 mg, (61%); mp 125—127 °C; $[\alpha]_D^{25}$ −41.0° (c 1, DMF).

Found: C, 65.48; H, 6.34; N, 10.87%. Calcd for $C_{49}H_{57}O_7N_7S_1 \cdot 1/2H_2O$: C, 65.60; H, 6.52; N, 10.93%.

Dns-Ala-Phe-Trp-Val-Leu-OCH$_2$Py (2). This compound was obtained from **7** (120 mg, 0.15 mmol) by the same procedure as described above, and recrystallized from DMF-ether; yield, 72 mg, (50%); mp 232—234 °C; $[\alpha]_D^{25}$ −31.5° (c 1, DMF).

Found: C, 64.29; H, 6.52; N, 11.68%. Calcd for $C_{52}H_{62}O_8N_8S_1 \cdot 1/2H_2O$: C, 64.51; H, 6.56; N, 11.57%.

Dns-Ala-Phe (8). This compound was obtained from Ala-Phe (200 mg, 0.17 mmol) by the same procedure as described above. The product was recrystallized from ethyl acetate-petroleum ether; yield, 160 mg, (48%); mp 114—116 °C; $[\alpha]_D^{25}$ +7.5° (c 1, DMF).

Enzyme and Methods. Pepsin (salt free crystalline sample from Worthington Biochemical Co. U.S.A.) was dissolved in 0.1 M citrate buffer pH 3.0. The substrates were dissolved in 0.1 M citrate buffer (pH 3.0) containing 10% DMF.

Analysis of Reaction Products. Fifty microliters of the pepsin solution (0.1 mM) was added to 5 ml of the substrate solutions (50 μM) at 37 °C. After incubation for 3 h, the reaction mixtures were subjected HPLC assays. The reaction mixtures (100 μl) were applied to a C_{18} reversed-phase column (DYNAMAX-60A, 4.6×250 mm, Rainin Instrument) and eluted with a linear gradient of 10—70% 2-propanol/acetonitrile (7/3. v/v) in 0.1% trifluoroacetic acid for 30 min at a flow rate of 0.5 ml min^{-1}.

Measurement of Fluorescence Emission Spectrum. The substrate (**2**) was dissolved in a 0.1 M citrate buffer (50 μM). Fifty microliters of the pepsin solution (0.1 mM) was added to the substrate solution (5 ml) at 37 °C. After incubation for 10 s and 3 h, the fluorescence emission spectrum was measured (excitation at 290 nm).

pH-Activity Curve. The substrate (**2**) was dissolved in 0.1 M citrate buffers in the pH range 2.0—4.5 (50 μM). Fifty microliters of the pepsin solution (0.1 mM) was added to the substrate solutions (5 ml) at 37 °C. After incubation for 2 min, the increase in emission at 345 nm was recorded during the following 6 min of the incubation time at 37 °C (excitation at 290 nm).

Linear Relation of the Fluorescence Intensity vs. Enzyme Concentration. Various amounts (5—100 μl) of the pepsin solution (0.1 mM) was added to 5 ml of the substrate solutions (50 μM, pH 3.0) at 37 °C and the increase in emission was measured in the manner described above.

Kinetic Parameter Measurement. Twenty five microliters of the pepsin solution (0.1 mM) was added to various concentrations (5—50 μM) of the substrate solution (5 ml) at 37 °C, and the increase in emission was measured in the manner described above.

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1) Abbreviations used in this work: Dns, dansyl; Py, 4-pyridyl; DCC, dicyclohexylcarbodiimide; Phe(NO_2), p-nitrophenylalanine.

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