

## A Sensitive Fluorometric Assay for Activity of Pepsin<sup>#</sup>

Hiroo YONEZAWA,\* Kazuhiko YAMADA, Tetsuya UCHIKOBA, and Makoto KANEDA  
Department of Chemistry, Faculty of Science, Kagoshima University, 1-Korimoto, Kagoshima 890

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Fluorogenic substrates of pepsin; Dns-Ala-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py (**I**), Ala-Ala-Trp-Phe-Leu-NHNHDns (**II**), and Ala-Ala-Trp-Phe-NHNHDns (**III**) were synthesized by a solution method and their properties were compared with Dns-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py (**IV**). The synthetic substrates **I** and **II** were cleaved specifically between Phe and Trp residues by pepsin and increased the fluorescence, although, the substrate **III** was not hydrolyzed by pepsin. The hydrolysis rates of the substrates by pepsin were measured by the increase in fluorescence.

The substrate **I** was hydrolyzed with pepsin by hundreds times faster than the substrates **II** and **IV**. The minimal detectable pepsin concentration of the substrate **I** was 0.5 nM and those of the substrates **II** and **IV** were 20 nM and 50 nM ( $M = \text{mol dm}^{-3}$ ). The substrates were sufficiently soluble in acidic buffers at their optimum pH. The inhibition rates of pepstatin and diazoacetyl-phenylalanine methyl ester for the activity of pepsin were measured by the use of the substrates **IV** and **II**. The activity of pepsin was inhibited by pepstatin within 15 s after mixing and by diazoacetyl-phenylalanine methyl ester within 120 s.

The resultant assay for pepsin is very sensitive and convenient and it is possible to determine rate of hydrolysis continuously.

Recently, it has become apparent that all retroviruses have carboxyl proteases which are essential for the multiplication of viruses.<sup>1,2)</sup> It is necessary to determine the activities of the enzymes rapidly and easily for an investigation of the nature of these enzymes. However, many carboxyl proteases cleave only between two amino acid residues and it is difficult to determine enzymic activities rapidly and continuously.

For example, to determine the activity of pepsin which is a typical carboxyl protease, hemoglobin is used as a protein substrate.<sup>3)</sup> In order to investigate the kinetics and mechanism of pepsin action, many synthetic substrates have been used more favorably than protein substrates. Fruton and Bergmann reported the synthesis of benzoxycarbonyl-dipeptides and the hydrolysis followed by the determination of the amino nitrogen liberated in the Van Slyke microvolumetric apparatus.<sup>4)</sup> Then, Baker synthesized acetyl-dipeptides and determined the rate of hydrolysis by pepsin by ninhydrin method.<sup>5)</sup> Moreover, Terada et al. determined the rate of hydrolysis for peptide substrates by pepsin employing amino acid analyzer.<sup>6)</sup> As these methods required much intension and are time-consuming, it was impossible to determine the change of the pepsin activity during activation of pepsinogen or modification of pepsin. By the use of sulfite esters, May and Kaiser determined the rate of hydrolysis by a spectrophotometric method.<sup>7)</sup>

It was reported that the task of assaying enzymic activity was simplified considerably by the use of chromogenic substrates.<sup>8)</sup> By this method, the change of activity could be determined continuously. A good chromogenic substrate for pepsin, however, has not been obtained. Silver et al. measured the rate of hydrolysis

of acetyl-dipeptide by the adsorption at 237 nm, but in this method, the sensitivity was very low.<sup>9)</sup> Inoue and Fruton synthesized chromogenic substrates containing *p*-nitrophenylalanine at the P<sub>1</sub> position, but the sensitivity of the substrates for the pepsin action was low.<sup>10)</sup>

On the other hand, fluorogenic substrates have been used as highly sensitive assay methods for many protease activities.<sup>11–13)</sup> Meldal et al. reported the synthesis of fluorogenic substrates for subtilisin and pepsin,<sup>14)</sup> although, the minimal detectable concentration of pepsin was not clear. In the previous paper, we reported the synthesis of intramolecularly quenching substrate for pepsin and the measurement of pepsin activity.<sup>15)</sup> In our method, it is possible to determine the pepsin activity rapidly, simply and continuously. However, the rate of hydrolysis of the substrate was relatively slow, and then, the sensitivity was not satisfactory (minimum detectable concentration of pepsin was 50 nM).

It was reported that elongation of peptide chain of N-terminal portion of the P<sub>1</sub> position resulted in a remarkable increase in the hydrolysis rate by pepsin.<sup>16,17)</sup> Then, the substrates which have longer chain length at the N-terminal portion of the substrates were synthesized and the hydrolysis rate by pepsin and minimal detectable pepsin concentrations were determined. Furthermore, the inhibiting rates of pepsin activity with some inhibitors were determined.

### Results and Discussion

In the previous paper, we reported the synthesis of fluorogenic substrate; Dns-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py (substrate **IV**) and the measurement of pepsin activity.<sup>15)</sup> The rate of hydrolysis of this substrate was relatively slow, and the sensitivity was not satisfactory. In order to increase the rate of hydrolysis, the substrate having Ala residue at the P<sub>3</sub> position (Dns-Ala-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py, substrate **I**) was synthe-

<sup>#</sup>Abbreviations used: Dns, dansyl; Boc, *t*-butoxycarbonyl; Py, 4-pyridyl; EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; AcOH, acetic acid; DMF, dimethylformamide.

sized. However, elongation of the distance between dansyl group and Trp residue resulted in proportionately smaller increase of fluorescence. Then, the substrates (HCl·Ala-Ala-Trp-Phe-Leu-NHNHDns, substrate **II** and HCl·Ala-Ala-Trp-Phe-NHNHDns, substrate **III**) which contain dansyl group at the C-terminal of the peptides and Trp residue at the P<sub>1</sub> position and have free amino group at the N-terminal of the peptides were also synthesized.

The substrates and the peptides which arise from cleavage of the substrates were synthesized by a solution method. The chromatographic pattern for the mixture of substrates **I**, **II**, and estimated reaction products of hydrolysis (Dns-Ala-Ala-Phe and Trp-Val-Leu-OCH<sub>2</sub>Py for substrate **I**, Ala-Ala-Trp and Phe-Leu-NHNHDns for substrate **II**) are shown in Fig. 1A and C.

The substrates **I** and **II** were incubated with pepsin for 24 h and the reaction mixtures were applied on HPLC. As shown in Fig. 1B, the substrate **I** disappeared completely by concomitant with two products (Dns-Ala-Ala-Phe and Trp-Val-Leu-OCH<sub>2</sub>Py), and substrate **I** was completely cleaved at the Phe-Trp bond. Similarly, the substrate **II** was cleaved at Trp-Phe bond (Fig. 1D). On the other hand, the substrate **III** was not hydrolyzed under the same conditions. Presumably, the length of peptide chain of the C-terminal portion was too short. It was reported that Phe-Gly-His-Phe(NO<sub>2</sub>)-Phe-OMe was less susceptible than Phe-Gly-His-Phe(NO<sub>2</sub>)-Phe-Ala-OMe.<sup>18)</sup>

As shown in Fig. 2, hydrolysis of the substrates **I** and **II** increased tryptophan emission (345 nm for substrate **I** and 360 nm for substrate **II**). The increase in emission accompanied by the hydrolysis of the substrate **I** was constant in the pH range of 1.0–5.0, but, on the other hand, that of the substrate **II** was increased linearly with the increase of pH corresponding to a partial deprotonation of carboxyl group of tryptophan residue; at pH 5.0 that was increased to twice the value at pH 1.0.

Comparative measurements of the effect of pH on the pepsin action were made with the substrates **I** and **II**. As shown in Fig. 3, the optimum pH for the substrate **I** was 2.0 and that for the substrate **II** was 4.5. Then, the pH 2.0 buffer and pH 4.5 buffer were used as solvents for the substrates **I** and **II** in the following experiments. It was reported that the substrates containing a positive charge (e. g. Z-His-Phe-Phe-OMe, Gly-Gly-Phe-Phe-OMe) had the optimum pH near 4 and neutral or acidic substrates had the optimum pH near 2.<sup>5,10,19)</sup> The positive charge of the substrates may interact with carboxylate group which is located near the active site of pepsin, and this interaction may be responsible for the decrease of the rate of hydrolysis at low pH. At relatively low pH, the carboxyl group does not dissociate and the interaction with positive charge of the substrates may be lost. The peptide pyridyl esters con-

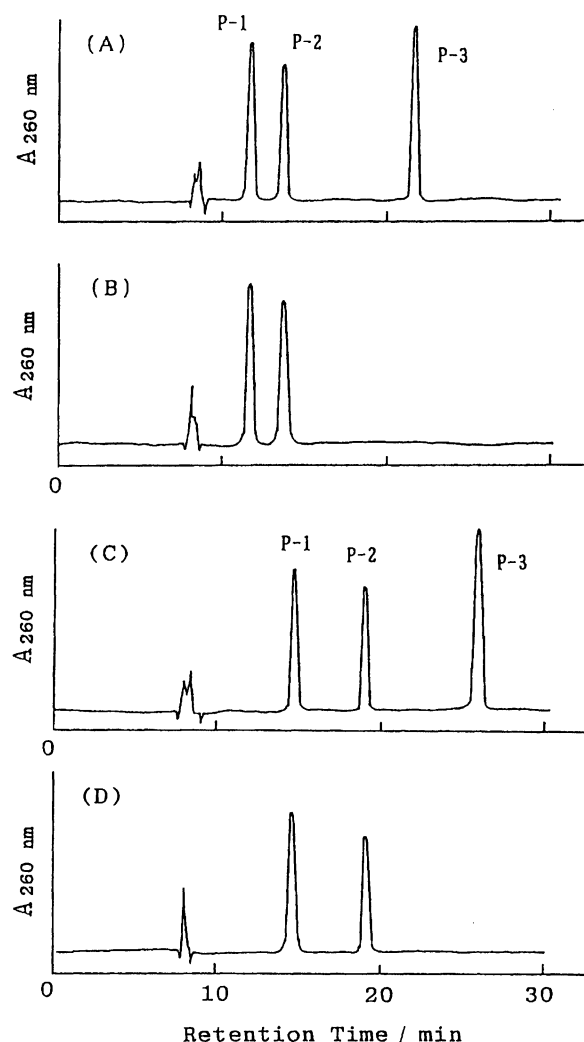


Fig. 1. HPLC trace of substrate **I**, **II**, and reaction mixtures. (A) Mixture of substrate **I**, Dns-Ala-Ala-Phe and Trp-Val-Leu-OCH<sub>2</sub>Py. P-1, Trp-Val-Leu-OCH<sub>2</sub>Py; P-2, Dns-Ala-Ala-Phe; P-3, substrate **I**. (B) Reaction mixture of substrate **I** and pepsin. (C) Mixture of substrate **II**, Ala-Ala-Trp and Phe-Leu-NHNHDns. P-1, Ala-Ala-Trp; P-2, Phe-Leu-NHNHDns; P-3, substrate **II**. (D) Reaction mixture of substrate **II** and pepsin. The reaction mixtures (100  $\mu$ l) were applied to a C<sub>18</sub> reversed-phase column (DYNAMAX-60A, 4.6 $\times$ 250 mm, Rainin Instrument) and eluted with a linear gradient of 20–80% (substrate **I**) and 10–60% (substrate **II**) 2-propanol/acetonitrile (7/3, v/v) containing 0.1% trifluoroacetic acid in 0.1% trifluoroacetic acid for 30 min at a flow rate of 0.5 ml min<sup>-1</sup>.

tain a positive charge at C-terminal, and the optimum pH of Z-Gly-Phe-Phe-OCH<sub>2</sub>Py is 3.5, while the optimum pH of Z-Gly-Phe-Phe-Gly-OCH<sub>2</sub>Py is pH 2.<sup>20)</sup> In this substrate, the positive charge is relatively remoted from sensitive peptide bond, and therefore, the interaction with the carboxylate group may not occur. The substrate **I** has longer peptide chain at the C-terminal

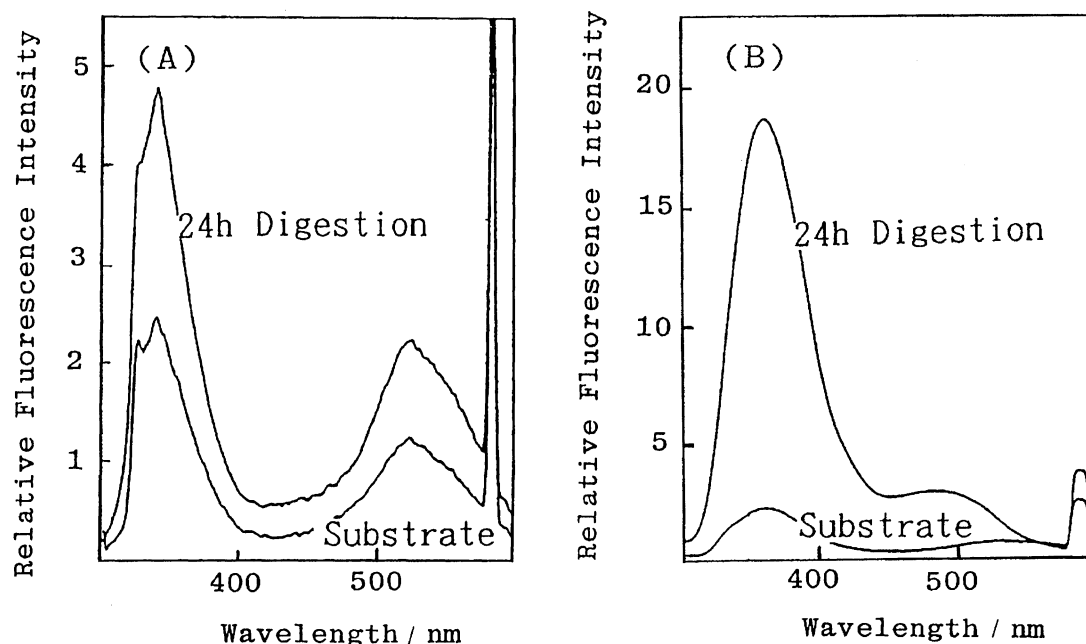


Fig. 2. Fluorescence emission spectra of substrates **I** and **II** before and after hydrolysis. (A) The substrate **I** and pepsin solution were mixed and after 10 s and 24 h, fluorescence emission spectra was measured. (B) The fluorescence emission spectra of the substrate **II** was measured in the same manner.

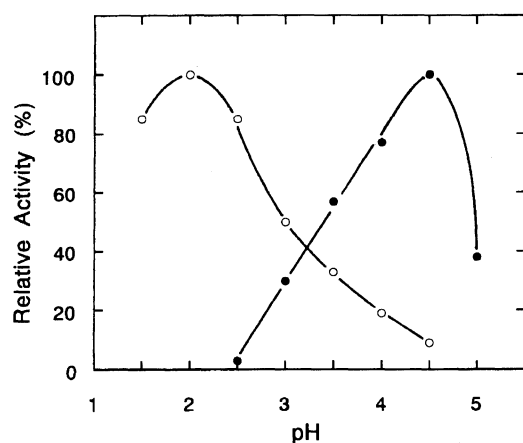


Fig. 3. pH Dependence of hydrolysis of substrates. The substrates were dissolved in the buffer of various pH and pepsin solution was added. After incubation for 1 min, the increase in fluorescence was measured during the following 6 min. (○) substrate **I**; (●) substrate **II**.

portion, accordingly the optimum pH is 2.0. The substrates **I** and **II** were sufficiently soluble in acidic buffers at their optimum pH (up to 50  $\mu$ M for the substrate **I** and up to 100  $\mu$ M for the substrate **II**).

When pepsin solution was added to the substrates solution, the fluorescence at 345 nm or 360 nm increased linearly in proportion to the concentration of added pepsin solution (Figs. 4A and 4B). The minimal detectable pepsin concentration for the substrate **I** was 0.5 nM and that for the substrate **II** was 20 nM. The minimal detectable pepsin concentration of the substrate

**IV** was 50 nM, and therefore, the substrate **I** was more sensitive than the substrate **IV** by 100 times.

As shown in Table 1, the substrate **I** was hydrolyzed 200 times faster than the substrate **IV**. Similarly, Sachdev et al. reported that Z-Gly-Gly-Phe-Phe-O-(CH<sub>2</sub>)<sub>3</sub>Py was hydrolyzed 20 times faster than Z-Gly-Phe-Phe-O-(CH<sub>2</sub>)<sub>3</sub>Py.<sup>20</sup> The substrate **II** and the substrate **IV** were hydrolyzed at nearly the same rate. The P<sub>1</sub> position of the substrate **I** is Phe and that of the substrate **II** is Trp, then, the substrate **I** was hydrolyzed much faster than the substrate **II**. Sachdev et al. and Yonezawa et al. reported that pepsin cleaved the substrates containing Phe residue at the P<sub>1</sub> position 20 times faster than the substrates which contained Trp or Tyr residues.<sup>19,20</sup> On the other hand, the amino acid residues at the P'<sub>1</sub> position did not give significant influence on the hydrolysis rate.<sup>20,21</sup>

The fluorogenic substrates were used to determine the rate of inhibition of pepstatin on pepsin activity. The substrate **IV** solution and pepsin solution were mixed and then pepstatin solution was added to the mixture in the course of measurement of the increase in the fluorescence. The increase in fluorescence was stopped within 15 s (Fig. 5), and, as the result, pepsin was shown to be inhibited by pepstatin within 15 s.

Then, the rate of inhibition of diazoacetyl-phenylalanine methyl ester was measured. As shown in Fig. 6, the activity of pepsin was also lost in 120 s in the presence of copper(II) ion. In the previous paper, Rajagopalan et al. reported that the pepsin activity was completely lost in 10 min.<sup>22</sup> These pepsin activities were measured by the hemoglobin method.<sup>3</sup> In this

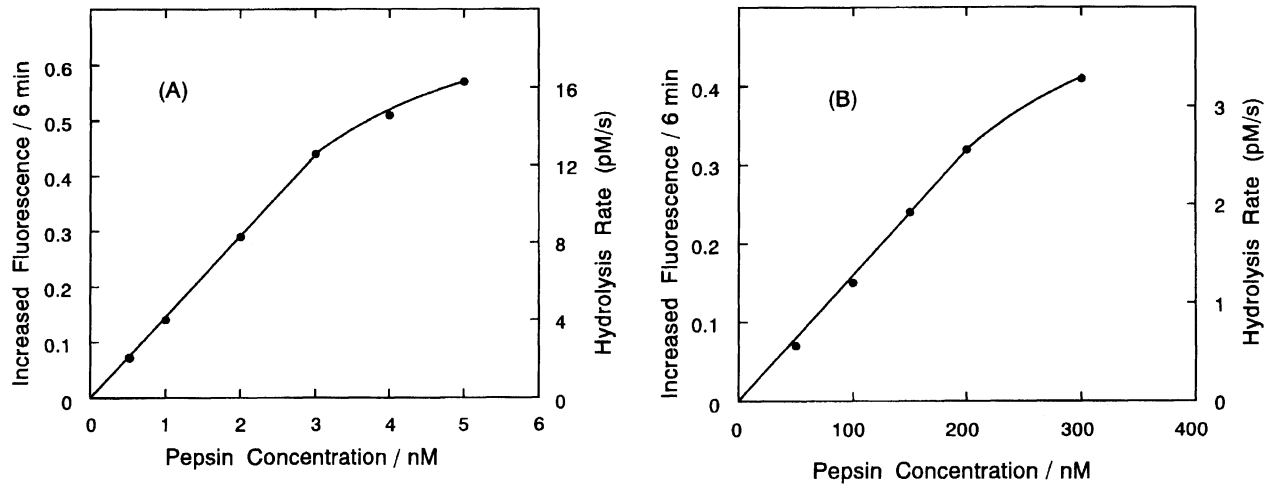


Fig. 4. Increase in fluorescence vs. pepsin concentration. Various amounts of pepsin solutions were added to the substrate solutions and the increase in fluorescence was measured. (A) substrate I; (B) substrate II.

Table 1. Kinetic Constants of Substrates

| P <sub>3</sub>                                                 | P <sub>2</sub> | P <sub>1</sub> | P <sub>1</sub> ' | P <sub>2</sub> ' | P <sub>3</sub> ' | $K_m$<br>μM | $k_{cat}$<br>s <sup>-1</sup> | $k_{cat}/K_m$<br>μM s <sup>-1</sup> |
|----------------------------------------------------------------|----------------|----------------|------------------|------------------|------------------|-------------|------------------------------|-------------------------------------|
| Dns-Phe-Trp-Val-Leu-OCH <sub>2</sub> Py <sup>a,c)</sup>        |                |                |                  |                  |                  | —           | —                            | —                                   |
| Dns-Ala-Phe-Trp-Val-Leu-OCH <sub>2</sub> Py (IV) <sup>c)</sup> |                |                |                  |                  |                  | 80          | 0.1                          | 0.00125                             |
| Dns-Ala-Ala-Phe-Trp-Val-Leu-OCH <sub>2</sub> Py (I)            |                |                |                  |                  |                  | 35.8        | 9.23                         | 0.258                               |
| Ala-Ala-Trp-Phe-NHNHDns (III) <sup>b)</sup>                    |                |                |                  |                  |                  | —           | —                            | —                                   |
| Ala-Ala-Trp-Phe-Leu-NHNHDns (II)                               |                |                |                  |                  |                  | 80.7        | 0.054                        | 0.00067                             |

a) Not hydrolyzed with pepsin at a concentration of 300 nM. b) Not hydrolyzed with pepsin at a concentration of 2 μM. c) Previously reported data.<sup>15)</sup>

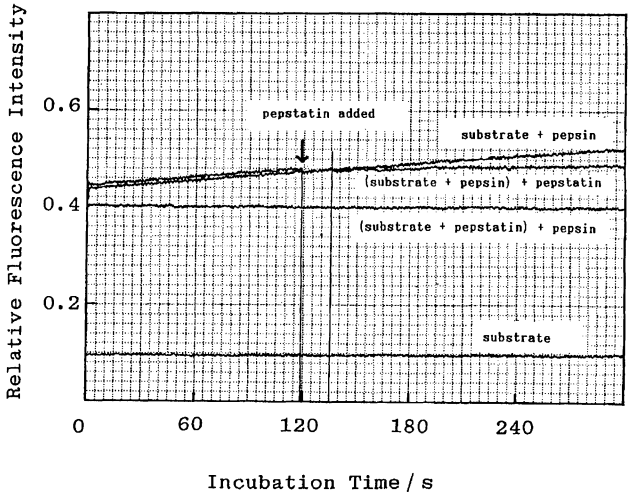


Fig. 5. Inhibition of pepsin activity with pepstatin. The pepsin solution was added to the substrate IV solution, and after 120 s, pepstatin solution was added and the increase in fluorescence was measured.

method, the pepsin activity was changed continuously during the digestion of hemoglobin (10 min). On the other hand, by the use of our synthetic fluorogenic substrates, pepsin activity can be determined continuously. It may be possible to determine the rate of activation

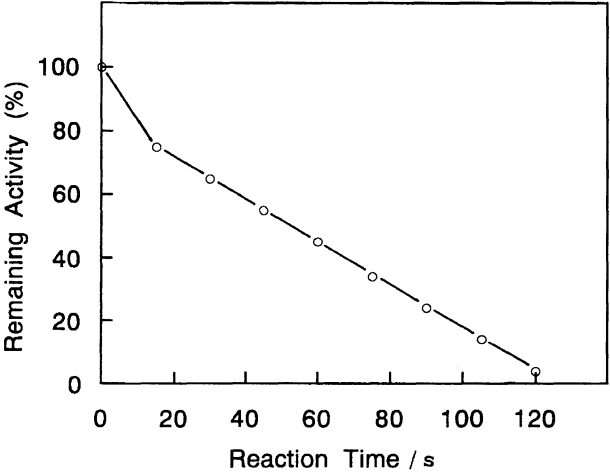


Fig. 6. Inhibition of pepsin activity with diazoacetyl-phenylalanine methyl ester. The pepsin solution was added to the substrate II solution containing cupric ion, and after 60 s, diazoacetyl-phenylalanine methyl ester solution was added and the increase in fluorescence was measured.

or inactivation of pepsin in more detail. As the resultant assay for pepsin is very sensitive and convenient, this method is useful for research of pepsin

action.

## Experimental

**Synthesis of Substrates. Dns-Ala-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py (1), (substrate I):** Boc-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py<sup>15)</sup> (150 mg, 0.15 mmol) was dissolved in 10 ml of 1 M HCl/AcOH containing 2% 2-mercaptoethanol and allowed to stand for 2 h at room temperature. The solution was evaporated in vacuo to dryness, and the resulting solid was collected with the aid of ether. The product was dissolved in water (10 ml) and cooled in an ice bath. To this solution was added 50% saturated K<sub>2</sub>CO<sub>3</sub> (10 ml) and resulting precipitate was collected by filtration, washed with water and dried. The resulting Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py and Dns-Ala (50 mg, 0.15 mmol) were dissolved in 1 ml of DMF and 5 ml of CH<sub>2</sub>Cl<sub>2</sub>, and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (37 mg, 0.15 mmol) was added at 0 °C. The mixture was stirred for 2 h at 0 °C and overnight at room temperature, then evaporated in vacuo, and 2% HCl was added to the residue. The resulting precipitate was collected by filtration, and washed with 4% sodium hydrogencarbonate solution and water and dried. The product was recrystallized from DMF-ethanol; yield, 51 mg, (33%); mp 255–258 °C;  $[\alpha]_D^{25}$  –19° (c 0.5, DMF).

Found: C, 63.16; H, 6.56; N, 11.87%. Calcd for C<sub>55</sub>H<sub>67</sub>O<sub>9</sub>N<sub>9</sub>S<sub>1</sub>·H<sub>2</sub>O: C, 63.02; H, 6.63; N, 12.03%.

**Boc-Leu-NHNHDns (2):** To a chilled solution of Boc-Leu (230 mg, 1 mmol) and Dns-NHNH<sub>2</sub> (270 mg, 1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 ml), was added 250 mg (1 mmol) of EEDQ. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature, then evaporated in vacuo, and ethyl acetate was added to the residue. The reaction mixture was washed successively with 10% citric acid, 4% sodium hydrogencarbonate, and water. The organic layer was dried over anhydrous sodium sulfate. It was evaporated in vacuo and the crystals were collected by filtration with the aid of petroleum ether. The product was recrystallized from ethyl acetate-petroleum ether; yield, 420 mg, (87%); mp 165–167 °C;  $[\alpha]_D^{25}$  –37° (c 0.5, methanol).

Found: C, 57.51; H, 7.23; N, 11.64%. Calcd for C<sub>23</sub>H<sub>34</sub>O<sub>5</sub>N<sub>4</sub>S<sub>1</sub>: C, 57.72; H, 7.16; N, 11.71%.

**Boc-Phe-Leu-NHNHDns (3):** Compound 2 (410 mg, 0.86 mmol) was treated with 10 ml of 1 M HCl/AcOH in the same manner as described above. The resulting HCl-Leu-NHNHDns, Boc-Phe (255 mg, 0.96 mmol), and triethylamine (0.12 ml, 0.85 mmol) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and coupled in the same manner using EEDQ as described above. The product was recrystallized from ethyl acetate-petroleum ether; yield, 390 mg, (72%); mp 122–124 °C;  $[\alpha]_D^{25}$  –42° (c 0.5, methanol).

Found: C, 61.25; H, 7.02; N, 10.96%. Calcd for C<sub>32</sub>H<sub>43</sub>O<sub>6</sub>N<sub>5</sub>S<sub>1</sub>: C, 61.42; H, 6.93; N, 11.19%.

**Boc-Trp-Phe-Leu-NHNHDns (4):** Compound 3 (320 mg, 0.51 mmol) was treated with 1 M HCl/AcOH in the same manner as described above. The resulting HCl-Phe-Leu-NHNHDns and Boc-Trp (200 mg, 0.66 mmol) were coupled in the same manner using EEDQ. The product was recrystallized from ethyl acetate-petroleum ether; yield, 370 mg, (89%); mp 134–136 °C;  $[\alpha]_D^{25}$  –46° (c 0.5, methanol).

Found: C, 62.89; H, 6.63; N, 11.88%. Calcd for C<sub>43</sub>H<sub>53</sub>O<sub>7</sub>N<sub>7</sub>S<sub>1</sub>·1/2H<sub>2</sub>O: C, 62.91; H, 6.63; N, 11.94%.

**Boc-Ala-Trp-Phe-Leu-NHNHDns (5):** Compound 4 (220 mg, 0.27 mmol) was treated with HCl/AcOH containing 2% 2-mercaptoethanol in the same manner as described above. The resulting HCl-Trp-Phe-Leu-NHNHDns and Boc-Ala (60 mg, 0.32 mmol) were coupled in the same manner using EEDQ. The product was recrystallized from ethyl acetate-petroleum ether; yield, 171 mg, (71%); mp 229–231 °C;  $[\alpha]_D^{25}$  –31° (c 0.5, DMF).

Found: C, 61.12; H, 6.58; N, 12.14%. Calcd for C<sub>46</sub>H<sub>58</sub>O<sub>8</sub>N<sub>8</sub>S<sub>1</sub>·H<sub>2</sub>O: C, 61.32; H, 6.71; N, 12.43%.

**Boc-Ala-Ala-Trp-Phe-Leu-NHNHDns (6):** Compound 5 (132 mg, 0.15 mmol) was treated with HCl/AcOH containing 2% 2-mercaptoethanol in the same manner as described above. The resulting HCl-Ala-Trp-Phe-Leu-NHNHDns and Boc-Ala (30 mg, 0.16 mmol) were coupled in the same manner using EEDQ. The product was recrystallized from ethyl acetate-petroleum ether; yield, 106 mg, (74%); mp 210–212 °C;  $[\alpha]_D^{25}$  –53° (c 0.4, methanol).

Found: C, 60.85; H, 6.72; N, 12.78%. Calcd for C<sub>49</sub>H<sub>63</sub>O<sub>9</sub>N<sub>9</sub>S<sub>1</sub>·H<sub>2</sub>O: C, 60.54; H, 6.74; N, 12.97%.

**HCl-Ala-Ala-Trp-Phe-Leu-NHNHDns (7), (substrate II):** Compound 6 (90 mg, 0.093 mmol) was treated with HCl/AcOH containing 2% 2-mercaptoethanol in the same manner as described above. The resulting product was recrystallized from DMF-ethanol-petroleum ether; yield, 74 mg, (80%); mp 192–195 °C;  $[\alpha]_D^{25}$  –30° (c 0.5, DMF).

Found: C, 52.81; H, 6.63; N, 12.78%. Calcd for C<sub>44</sub>H<sub>56</sub>O<sub>7</sub>N<sub>9</sub>S<sub>1</sub>Cl<sub>1</sub>·6H<sub>2</sub>O: C, 52.92; H, 6.86; N, 12.62%.

**Materials and Enzymic Studies.** HCl-Ala-Ala-Trp-Phe-NHNHDns (substrate III) was synthesized in the same manner as described above. Dns-Ala-Phe-Trp-Val-Leu-OCH<sub>2</sub>Py (substrate IV) was prepared in the same manner as described in the previous paper.<sup>15)</sup> 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 containing 10% DMF. Fluorescence measurements were performed with a Shimadzu spectrofluorometer RF-5000.

**Analysis of Reaction Products.** One hundred  $\mu$ l of the pepsin solutions (100  $\mu$ M) were added to 5 ml of the substrate I solution (25  $\mu$ M, pH 2.0) and substrates II and III solutions (50  $\mu$ M, pH 3.0) at 37 °C. After incubation for 24 h, the reaction mixtures were subjected HPLC assays.

**Measurement of Fluorescence Emission Spectrum.** Ten  $\mu$ l of the pepsin solutions (100  $\mu$ M) were added to 5 ml of the substrates I and II solutions under the same conditions described above. After incubation for 10 s and 24 h, the fluorescence emission spectrum was measured (excitation at 290 nm).

**Influence of pH for Increase in Fluorescence.** The substrates I and II were hydrolyzed for 24 h under the same conditions described above. The substrates solutions (pepsin was not added) or reaction mixtures (100  $\mu$ l) were added to 3 ml of the 0.1 M citrate buffers in the pH range 1.0–5.0 and the fluorescence emission spectrum was measured.

**pH-Activity Curve.** The substrates I and II were dissolved in the 0.1 M citrate buffer in the pH range 1.0–5.0 and pepsin solutions (10  $\mu$ l of 10  $\mu$ M solution for the substrate I and 15  $\mu$ l of 50  $\mu$ M solution for the substrate II) were added to the substrate solutions under the same conditions described above. After incubation for 1 min, the

increase in fluorescence (345 nm for the substrate **I** and 360 nm for the substrate **II**) was recorded during the following 6 min of the incubation time.

**Linear Relation of the Increase in Fluorescence vs. Enzyme Concentration.** Various amounts of the pepsin solution was added to the substrate **I** solution (25  $\mu$ M, pH 2.0) and the substrate **II** solution (50  $\mu$ M, pH 4.5) and the increase in fluorescence was measured in the manner described above.

**Measurement of Inhibition Time of Pepstatin.** Fifty  $\mu$ l of the pepsin solution (50  $\mu$ M) was added to 5 ml of the substrate **IV** solution (25  $\mu$ M, pH 2.0), and the increase in fluorescence at 345 nm was measured as described above. And then, after 120 s, 100  $\mu$ l of the pepstatin solution (100  $\mu$ M, in methanol) was added rapidly, and the increase in fluorescence was measured subsequently.

**Measurement of Inhibition Time of Diazoacetyl-phenylalanine Methyl Ester.** The substrate **II** was dissolved in a 0.2 M acetate buffer (pH 4.5) containing 10% of DMF and 2 mM  $\text{CuSO}_4$  (50  $\mu$ M). Fifty  $\mu$ l of the pepsin solution (100  $\mu$ M) was added to 3 ml of the substrate solution (50  $\mu$ M, pH 4.5), and the increase in fluorescence at 360 nm was measured as described above. And then, after 60 s, 60  $\mu$ l of the diazoacetyl-phenylalanine methyl ester solution (100 mM, in methanol) was added rapidly, and the increase in fluorescence was measured subsequently. The activity of pepsin was determined by differentiation of the curve of the increase of fluorescence.

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