Photoaffinity Labeling of Pepsin

Hiroo YONEZAWA* and Tetsuya WADA
Department of Chemistry, Faculty of Science, Kagoshima University,
1-Korimoto, Kagoshima 890
(Received May 13, 1989)

Synopsis. Photoaffinity reagents for pepsin [Ala-Gly-Phe(N_3), Gly-Gly-Phe(N_3)-Phe-Gly-OEt, Gly-Gly-Phe-Phe(N_3)-Gly-OEt] were synthesized by a solution method. The pentapeptides were cleaved rapidly at the peptide bond between two aromatic amino acid residues by pepsin. This shows that Phe(N_3) residues of the photoaffinity reagents bind with the S_1 or S_1' site of pepsin. Pepsin was irradiated with photoaffinity reagents and the remaining activity was measured. The pepsin activity was decreased more rapidly in the presence of photoaffinity reagents, compared with that in the absence of photoaffinity reagents. Photoaffinity labeling occured at pH 2.0; however, did not occur at pH 4.0. Photoaffinity labeling of pepsin with ^{3}H-labeled Gly-Gly-Phe(N_3)-Phe-Gly-OEt showed that about 9% of the pepsin bound covalently with the photoaffinity reagent.

The specificity of a protease toward substrates was explained by an interaction between subsites of the protease and substrate side chains. Studies of pepsin specificity have shown that the enzyme has at least five subsites: three toward the NH_2-terminate from the sensitive bond (S_3, S_2, S_1) and two toward the COOH-terminate (S_1', S_2'). The S_1 and S_1' sites of pepsin are obviously the primary determinant of specificity.[1] The S_1 and S_1' sites gave a strong preference for aromatic L-amino acid residures. For example, Z-His-Phe-Phe-OMe is hydrolyzed much more rapidly at the Phe-Phe bond than both Z-His-Leu-Phe-OMe and Z-His-Phe-Leu-OMe.[2] Andreeva et al. suggested that the phenolic rings of substrates occupied two hydrophobic pockets which were formed by hydrophobic amino acid residues.[3] These residues are inert in the usual modification reaction. However, it is possible to modify these residues by the use of photoaffinity labeling.[4] The carbenes and nitrenes which were produced by irradiation are very reactive and nonselective in terms of amino acid residues. A peptide containing *p*-azido-L-phenylalanine [Phe(N_3)] at the position of P_1 or P_1' can bind strongly with pepsin, and it may be a good photoaffinity reagent.

Hollands et al. reported that Gly-Gly-Phe(NO_2)-Phe-Gly-OMe was a good substrate of pepsin.[5] Consequently, a peptide which contains an azido group instead of a nitro group of the substrate [Gly-Gly-Phe(N_3)-Phe-Gly-OEt] may covalently bind with the S_1 site of pepsin by irradiation. Similarly, Gly-Gly-Phe-Phe(N_3)-Gly-OEt may covalently bind with the S_1' site. Furthermore, photoaffinity labeling of chymotrypsin with a reagent of product type was carried out.[6] Therefore, Ala-Gly-Phe(N_3) may act as a photoaffinity reagent. In this paper, we report on the synthesis of these three peptides and the photoaffinity labeling of pepsin with these peptides.

Results and Discussion

The substrate and photoaffinity reagents were synthesized by a solution method. A protected tripeptide, Boc-Ala-Gly-Phe(N_3), was prepared by the coupling of Boc-Ala-Gly and Phe(N_3) via an active ester method.[7] The obtained protected tripeptide was treated with hydrogen chloride in ethyl acetate to cleave the protecting group, and free tripeptide was precipitated from aqueous solution.

On the other hand, *p*-azido-L-phenylalanine containing pentapeptides were synthesized by another process. Z-Gly-Gly and Phe(NO_2)-Phe-Gly-OEt or Phe-Phe(NO_2)-Gly-OEt were coupled and the resulting protected pentapeptide esters were hydrogenated. Then, the obtained pentapeptide esters were converted to the corresponding azido compounds by treatment with sodium nitrite and sodium azide. The ^{3}H-labeled Gly-Gly-Phe(N_3)-Phe-Gly-OEt was synthesized by the same procedure. The crude pentapeptides were applied to droplet countercurrent chromatography to give pure peptides. The obtained photoaffinity reagents were employed for enzymatic experiments.

As shown in Fig. 1, these two photoaffinity reagents were cleaved at the same rate as that of the substrate. It is well-known that pepsin cleaves the peptide bond between two aromatic amino acid residues. It is clear that Phe(N_3) residues of the photoaffinity reagents bind with the S_1 or S_1' site of pepsin.

When pepsin was irradiated in the presence of the photoaffinity reagents, the pepsin activity decreased more rapidly than in the absence of the reagents (Fig. 2). The difference in the remaining activities of both cases was small, within 5 minutes. Then, the pepsin activity was decreased enormously after irradiation for 15 minutes, even in the absence of photoaffinity reagents. It was concluded that the appropriate

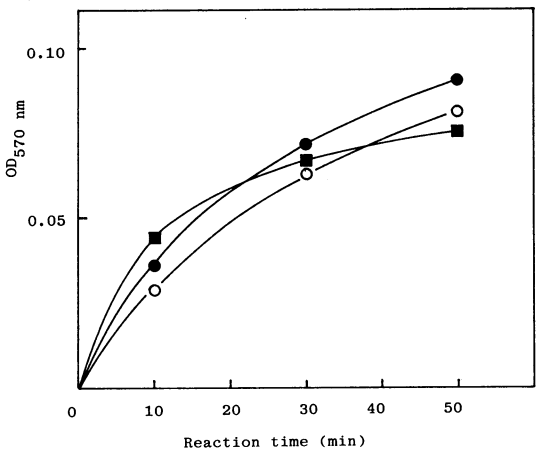

Fig. 1. Hydrolysis of photoaffinity reagents. (○) Gly-Gly-Phe(NO_2)-Phe-Gly-OEt; (●) Gly-Gly-Phe(N_3)-Phe-Gly-OEt; (■) Gly-Gly-Phe-Phe(N_3)-Gly-OEt.

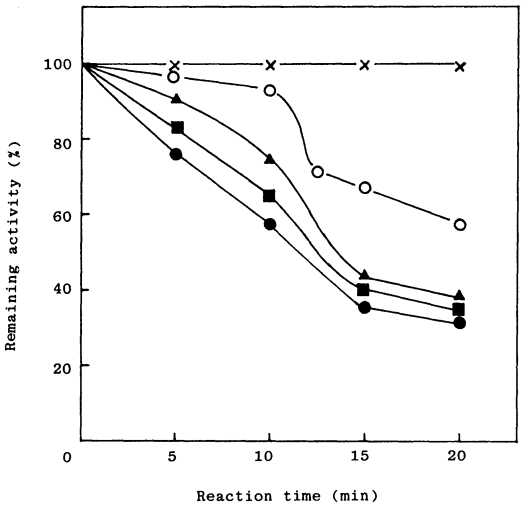

Fig. 2. Effect of irradiation time on photoaffinity labeling. (×) in dark; (○) absence of photoaffinity reagent; (▲) Ala-Gly-Phe(N_3); (■) Gly-Gly-Phe-Phe(N_3)-Gly-OEt; (●) Gly-Gly-Phe(N_3)-Phe-Gly-OEt.

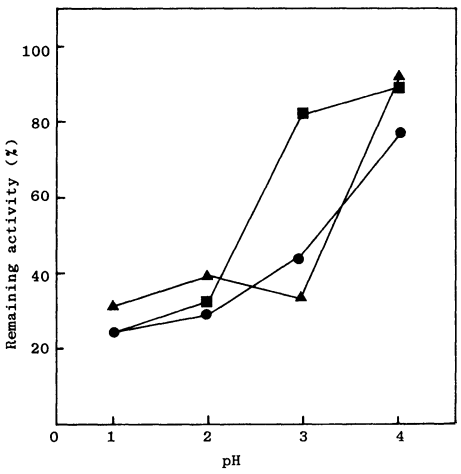

Fig. 3. Effect of pH of solution on photoaffinity labeling. (▲) Ala-Gly-Phe(N_3); (■) Gly-Gly-Phe-Phe(N_3)-Gly-OEt; (●) Gly-Gly-Phe(N_3)-Phe-Gly-OEt.

irradiation time should be 10 minutes. At this time, the remaining pepsin activities were as follows: 93% (absence of the photoaffinity reagent), 68% [Ala-Gly-Phe(N_3)], 65% [Gly-Gly-Phe-Phe(N_3)-Gly-OEt], 60% [Gly-Gly-Phe(N_3)-Phe-Gly-OEt].

The effect of pH on photoaffinity labeling was determined. As shown in Fig. 3, all photoaffinity reagents did not act at pH 4.0. Photoaffinity reagets which contained the azido group at the P_1 acted at pH 3.0; however, the reagent which contained an azido group at the P_1' did not act at this pH.

Then, pepsin was irradiated with a ^{3}H-labeled photoaffinity reagent and the incorporated radioactivity was measured. In this experiment, the measurement was made at concentrations of 0.05 mM of pepsin and 1 mM of the photoaffinity reagent (1 M=1 mol dm^{-3}). The amount of radioactivity incorporated into 4.75×10^{-8} mol of pepsin was 5820 dpm, which corresponded to 4.2×10^{-9} mol of the photoaffinity reagent. Consequently, about 9% of the pepsin was labeled upon irradiation. At this time, the remaining pepsin activities were 89 and 68%, respectively, in the absence and presence of the photoaffinity reagent. The decreased pepsin activity was larger than the amount of incorporated photoaffinity reagent.

Gennari et al. reported the photooxidation of chymotrypsin by *N*-acetyl-3-nitrotyrosine.[8] The possibility of a photooxidation reaction of pepsin with Phe(N_3) residues is not ruled out in these procedures. However, when pepsin was irradiated with 2 mM of Phe(N_3), the remaining activity was equal in the absence of Phe(N_3), indicating that a nonspecific photooxidation reaction did not occur. It may be possible to identify the amino acid residues which form S_1 and S_1' sites of pepsin by photoaffinity labeling.

Experimental

Boc-Ala-Gly-Phe(N_3) (I). This was prepared from Phe(N_3) (410 mg, 2 mmol) and Boc-Ala-Gly-OSu (690 mg, 2 mmol) using the usual procedure. The product was recrystallized from methanol-ether-petroleum ether; yield, 710 mg, (82%); mp 127—128 °C; $[\alpha]_D^{25}$ +18.0° (*c* 1, MeOH); Found: C, 52.35; H, 6.29; N, 19.48%. Calcd for $C_{19}H_{26}O_6N_6$: C, 52.52; H, 6.03; N, 19.35%.

Ala-Gly-Phe(N_3) (II). The **I** (434 mg, 1 mmol) was dissolved in 1.5 M HCl in ethyl acetate (5 ml) at 0 °C. The solution was stirred for 30 min at 0 °C and the resulting crystals were collected and washed with a mixed solvent of ethyl acetate and ether (1 : 1 v/v). The crystals were dissolved in cold water (5 ml) and the free tripeptide was separated by the addition of pyridine to pH 7. The resulting crystals were filtered and washed with a small amount of cold water; yield, 170 mg, (51%); mp 215 °C; $[\alpha]_D^{25}$ +38.9° (*c* 1, 0.1 M HCl); Found: C, 50.24; H, 5.46; N, 25.19%. Calcd for $C_{14}H_{18}O_4N_6$: C, 50.29; H, 5.43; N, 25.14%.

Boc-Phe-Gly-OEt (III). This was prepared from Boc-Phe (5.30 g, 20 mmol), and Gly-OEt·HCl (2.80 g, 20 mmol) by the 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) method. The product was recrystallized from ethyl acetate-petroleum ether; yield, 6.31 g, (90%); mp 88—89 °C; $[\alpha]_D^{25}$ −6.0° (*c* 1, MeOH).

Boc-Phe(NO_2)-Phe-Gly-OEt (IV). The **III** (3.50 g, 10 mmol) was dissolved in 1M HCl in acetic acid (50 ml). After 1 h at room temperature, the solution was evaporated and treated with ether. The obtained crystals were coupled with Boc-Phe(NO_2) (3.10 g, 10 mmol), as described above. Recrystallization from DMF-ether gave 4.20 g, (77%); mp 207—209 °C; $[\alpha]_D^{25}$ −14.0° (*c* 1, DMF); Found: C, 60.01; H, 6.17; N, 10.41%. Calcd for $C_{27}H_{34}O_8N_4$: C, 59.77; H, 6.32; N, 10.33%.

Z-Gly-Gly-Phe(NO_2)-Phe-Gly-OEt (V). This was prepared from Z-Gly-Gly (1.06 g, 4 mmol) and Phe(NO_2)-Phe-Gly-OEt·HCl (1.92 g, 4 mmol) by EEDQ method. The product was recrystallized from DMF-ether; yield, 2.38 g, (86%); mp 187—190 °C; $[\alpha]_D^{25}$ −15.1° (*c* 1, DMF); Found: C, 59.34; H, 5.38; N, 12.01%. Calcd for $C_{34}H_{38}O_{10}N_6$: C, 59.12; H, 5.55; N, 12.17%.

Gly-Gly-Phe(NH_2)-Phe-Gly-OEt·2HCl (VI). The **V** (2.76 g, 4 mmol) was suspended in a mixed solvent of H_2O-acetic acid-methanol (30 ml), and concd HCl (0.7 ml) was added. The **V** was hydrogenated in the presence of palladium black at room temperature. After 24 h, the

catalyst was filtered off, and the filtrate was evaporated in vacuo. The resulting crystals were collected with the aid of acetone; yield, 1.97 g, (82%); mp 219—221 °C; $[\alpha]_D^{25}$ −7.1° (*c* 1, 0.1 M HCl); Found: C, 50.33; H, 6.42; N, 13.51%. Calcd for $C_{26}H_{34}O_6N_6 \cdot 2HCl \cdot H_2O$: C, 50.57; H, 6.20; N, 13.61%.

Gly-Gly-Phe(N_3)-Phe-Gly-OEt·HCl (VII). To a solution of **VI** (1.07 g, 1.78 mmol) in 2 M HCl (1.78 ml), was added a solution of 2 M $NaNO_2$ (0.89 ml, 1.78 mmol) at 0 °C and stirred for 1 h at 0 °C. To the solution was added a solution of 2 M NaN_3 (0.89 ml, 1.78 mmol); the reaction solution was then stirred for 1 h at 0 °C. To the solution was added NaCl until saturated; resulting precipitate was then filtered and dried. The resulting crude **VII** (250 mg) was purified by droplet countercurrent chromatography,[9] using the mixed solvent of cyclohexane–1-butanol–acetic acid–water (1:4:2:5 v/v); the upper phase of the mixture was used as a moving phase. Fractions containing **VII** were collected and evaporated. The precipitate was collected with the aid of ether; yield, 170 mg, (16%); mp 204—205 °C; $[\alpha]_D^{25}$ −31.0° (*c* 1, DMF); Found: C, 51.41; H, 5.85; N, 18.50%. Calcd for $C_{26}H_{32}O_6N_8 \cdot HCl \cdot H_2O$: C, 51.44; H, 5.81; N, 18.46%.

Gly-Gly-Phe-Phe(NH_2)-Gly-OEt·2HCl (VIII). This was prepared from Z-Gly-Gly-Phe-Phe(NO_2)-Gly-OEt (4.14 g, 6 mmol), as described for the preparation of **VI**; yield, 3.18 g, (88%); mp 241—242 °C; $[\alpha]_D^{25}$ −11.2° (*c* 1, 0.1 M HCl); Found: C, 50.71; H, 6.34; N, 13.48%. Calcd for $C_{26}H_{34}O_6N_6 \cdot 2HCl \cdot H_2O$: C, 50.57; H, 6.20; N, 13.61%.

Gly-Gly-Phe-Phe(N_3)-Gly-OEt·HCl (IX). The **VIII** (1.00 g, 1.67 mmol) was converted to the corresponding azido compound according to the same manner described above and recrystallized from a mixed solvent of 1-butanol–acetic acid–pyridine–ethanol (4:2:2:1 v/v)–acetone; yield, 760 mg, (77%); mp 208—209 °C; $[\alpha]_D^{25}$ −46.6° (*c* 1, DMF); Found: C, 48.61; H, 6.09; N, 17.44%. Calcd for $C_{26}H_{32}O_6N_8 \cdot HCl \cdot 3H_2O$: C, 48.56; H, 6.11; N, 17.42%.

Gly-Gly-^{3}H-Phe(N_3)-Phe-Gly-OEt·HCl (X). This compound was prepared by the mentioned reaction sequence, starting with 230 mg (1.1 mmol, 0.6 mCi) of ^{3}H-labeled *p*-nitro-L-phenylalanine. The obtained crude product was purified by droplet countercurrent chromatography; yield, 210 mg. The specific radioactivity was 2298 dpm μg^{-1}.

Enzymatic Study. The substrate used was Gly-Gly-Phe(NO_2)-Phe-Gly-OEt·HCl. The cleavage of the substrate was measured at 37 °C, and 0.1 M sodium citrate buffer (pH 4.16) was used to control the pH. Fifty μl of pepsin solution (0.06 mM) was added to 10 ml of the substrate solution (0.25 mM) and the increase in absorbance at 310 nm was measured.[10] The kinetic constant of the substrate was K_m=0.83 mM, k_{cat}=5.5 s^{-1}.

Hydrolysis of VII and IX with Pepsin. Fifty μl of pepsin solution (0.06 mM, pH 4.16) was added to the substrate (**VII** or **IX**) solution (0.5 mM, 10 ml) and incubated at 37 °C. At selected intervals, 0.1 ml of the reaction mixture was taken out and the rate of the cleavage was determined by the ninhydrin method.[11]

Effect of Irradiation Time on Photoaffinity Labeling. Pepsin and photoaffinity reagents were dissolved in a 0.1 M sodium citrate buffer (pH 2.0). A mixture of 0.5 ml of a pepsin solution (0.06 mM) and 0.5 ml of the photoaffinity reagent solution (2 mM) was incubated for 10 min at room temperature and cooled in an ice-bath for 5 min. The solution was irradiated with a Toshiba mercury lamp H400-P at a distance of 60 cm at 0 °C. At selected intervals, 0.1 ml of the solution was taken out and the remaining activity was measured.

Effect of pH of Solution on Photoaffinity Labeling. Mixtures of 0.5 ml of a pepsin solution (0.06 mM) and 0.5 ml of the photoaffinity reagents solution (2 mM) of various pH were irradiated for 15 min at 0 °C under the same conditions described above and the remaining activity was measured.

Photoaffinity Labeling with X. A mixture of 0.5 ml of a pepsin solution (0.1 mM, pH 2.0) and 0.5 ml of a **X** solution (2 mM, pH 2.0) was irradiated for 10 min at 0 °C under the same conditions described above. To the solution, 2 M TCA (1 ml) was added and allowed to stand for 20 min. The reaction mixture (1.9 ml) was filtered with a glass fiber filter, Whatman GF/B and the filter was washed twice with 1 M TCA (1 ml) and ethanol (1 ml). The filter was dried and the radioactivity measured.

References

1) J. S. Fruton, "Acid Proteases," ed. by J. Tang, (1977), p. 131.
2) G. E. Trout and J. S. Fruton, *Biochem.*, **8**, 4183 (1969).
3) N. S. Andreeva, A. S. Zdanov, A. E. Gustchina, and A. A. Fedorov, *J. Biol. Chem.*, **259**, 11353 (1984).
4) H. Bayley and J. R. Knowles, "Methods in Enzymolgy," (1977). Vol. XLVI, p. 69.
5) K. Medzihradszky, I. M. Voynick, H. Medzihradszky-Schweiger, and J. S. Fruton, *Biochem.*, **9**, 1154 (1970).
6) E. Escher and R. Schwyzer, *FEBS Lett.* **46**, 347 (1974).
7) E. Escher and R. Schwyzer, *Helv. Chim. Acta*, **58**, 1465 (1975).
8) G. Gennari, G. Jori, G. Galiazyo, and E. Scoffon, *J. Am. Chem. Soc.*, **92**, 4140 (1970).
9) H. Yonezawa, M. Kaneda, N. Tominaga, S. Higashi, and N. Izumiya, *J. Biochem.*, **90**, 1081 (1981).
10) K. Inoue and J. S. Fruton, *Biochem.*, **6**, 1765 (1967).
11) S. Moor and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

Abbreviations: Phe(N_3), *p*-azido-L-phenylalanine; -OSu, *N*-hydroxysuccinimide ester; EEDQ, 1-ethoxycarbonyl-2-ethoxyl-1,2-dihydroquinoline.