PHOTOAFFINITY LABELING OF PEPSIN WITH Phe(N3)-Gly-OEt

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PHOTOAFFINITY LABELING OF PEPSIN WITH Phe(N_3)-Gly-OEt

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Abstract

Photoaffinity reagent for pepsin, Phe(N_3)-Gly-OEt was synthesized by a solution method. The photoaffinity reagent was a competitive inhibitor of pepsin. This showes that the photoaffinity reagent binds to the binding site of pepsin. Pepsin was irradiated with photoaffinity reagent and remaining activity was measured. Photoaffinity labeling occured at pH 1. 0; however, did not occure at pH 4. 16. The photoaffinity labeling was inhibited in the presence of Gly-Gly-Phe and Phe-Gly-OEt.

Pepsin is a gastric proteinase active at acid pH (pH 1-5). Studies of pepsin specificity have shown that the S_1 site gave a strong preference for aromatic amino acid residues (*1, 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*). In the previous paper, we reported the photoaffinity labeling of pepsin (*5*). The photoaffinity reagents used were Gly-Gly-Phe(N_3)-Phe-Gly-OEt, Gly-Gly-Phe-Phe(N_3)-Gly-OEt, which were the substrates of pepsin. Moreover, Ala-Gly-Phe(N_3), which was a competitive inhibitor of pepsin, acted as a photoaffinity reagent. In this paper, we report the synthesis of a competitive inhibitor of pepsin which contained the azido group at the P_1' and photoaffinity labeling of pepsin.

Results and Discussion

The photoaffinity reagent was synthesized by a solution method. A protected dipeptide, Z-Phe(NO_2)-Gly-OEt was prepared by the coupling of Z-Phe(NO_2) and Gly-OEt · HCl. The obtained protected dipeptide ester was hydrogenated and the obtained dipeptide ester was converted to the azido compound by treatment with sodium nitrite and sodium azide. The obtained photoaffinity reagent was employed for enzymic experiments.

As shown in Fig. 1, the photoaffinity reagent showed competitive inhibition for

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pepsin. The values of K_I for the photoaffinity reagent was 6.4×10^{-4} M. This showed that the photoaffinity reagent binded with the S_1' site of pepsin. The effect of pH on photoaffinity labeling was determined. As shown in Fig. 2, Phe (N_3) -Gly-OEt acted at pH 4.16, however, did not act at pH 1.0. On the other hand, Ala-Gly-Phe (N_3) acted at pH 1.0 and did not act at pH 4.16.

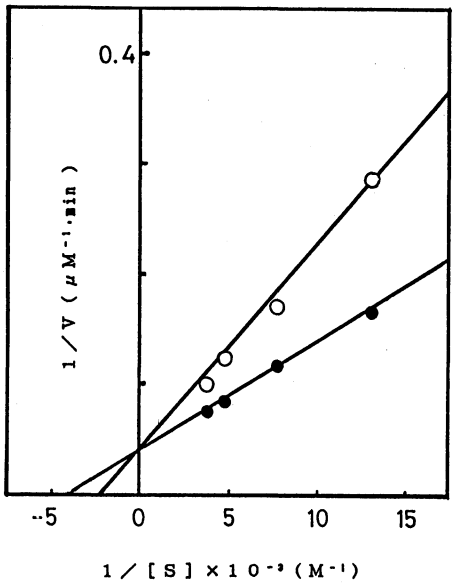

Fig. 1. Inhibition of pepsin action by photoaffinity reagent.
(●) absence of photoaffinity reagent; (○) presence of photoaffinity reagent.

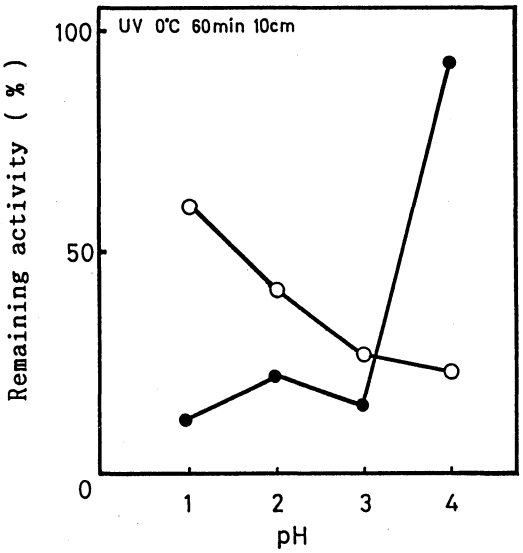

Fig. 2. Effect of pH of solution on photoaffinity labeling.
(○) Phe (N_3)-Gly-OEt; (●) Ala-Gly-Phe (N_3).

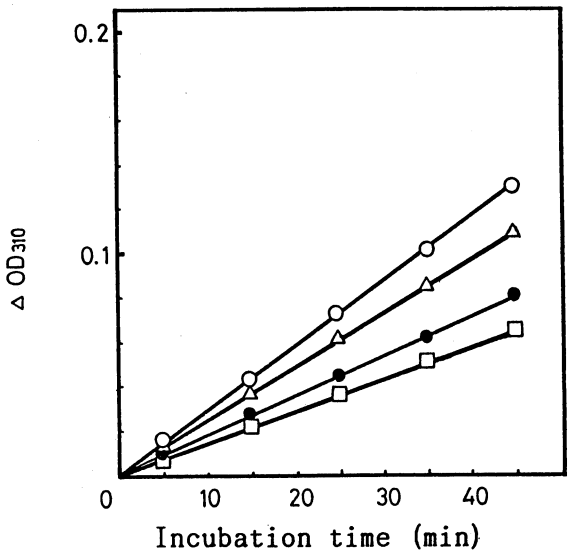

Fig. 3. Inhibition of photoaffinity labeling by Phe-Gly-OEt. (○) 0.5 ml of pepsin solution + 0.5 ml of buffer + 0.5 ml of photoaffinity reagent solution, not irradiated; (△) 0.5 ml of pepsin solution + 1.0 ml of buffer, irradiated; (●) 0.5 ml of pepsin solution + 0.5 ml of Phe-Gly-OEt solution + 0.5 ml of photoaffinity reagent solution, irradiated; (□) 0.5 ml of pepsin solution + 0.5 ml of buffer + 0.5 ml of photoaffinity reagent solution, irradiated.

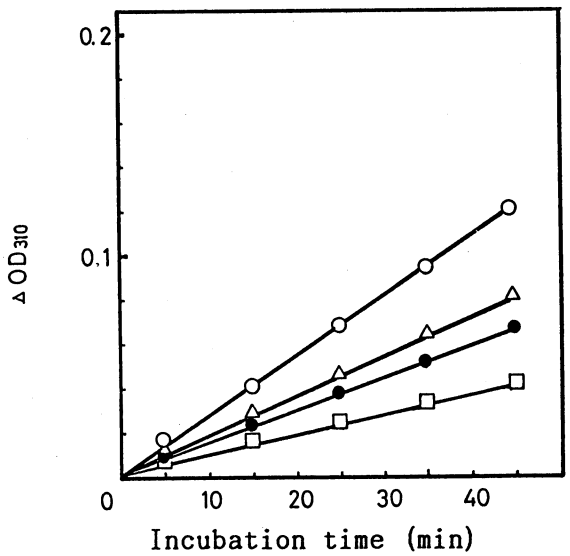

Fig. 4. Inhibition of photoaffinity labeling by Gly-Gly-Phe. (○) 0.5 ml of pepsin solution + 0.5 ml of buffer + 0.5 ml of photoaffinity reagent solution, not irradiated; (△) 0.5 ml of pepsin solution + 1.0 ml of buffer, irradiated; (●) 0.5 ml of pepsin solution + 0.5 ml of Gly-Gly-Phe solution + 0.5 ml of photoaffinity reagent solution, irradiated; (□) 0.5 ml of pepsin solution + 0.5 ml of buffer + 0.5 ml of photoaffinity reagent solution, irradiated.

The photoaffinity labeling was inhibited by the presence of Phe-Gly-OEt and Gly-Gly-Phe (Fig. 3, 4). Phenylalanine residue of Phe-Gly-OEt may bind to S_1' site of pepsin in competition with the photoaffinity reagent. On the other hand, phenylalanine residue of Gly-Gly-Phe may bind to S_1 site of pepsin. Consequently, S_1' site of pepsin is probably empty. This may show that when S_1 site of pepsin is filled with the hydrophobic side chain of amino acid residue, the conformation of S_1' site changes and affiinity of S_1' site and phenolic ring of P_1' becomes to be weakened.

Experiments

Z-Phe (NO_2) -Gly-OEt (I). To a chilled solution of Z-Phe (NO_2) (1.93 g, 5.6 mmol), Gly-OEt · HCl (0.79 g, 5.6 mmol) and triethylamine (0.78 ml, 5.6 mmol) in DMF (5 ml) and CH_2Cl_2 (15 ml), was added 1-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (1.36 g, 5.6 mmol). The reaction mixture was stirred for 1 hr at 0° C and allowed to stand overnight at room temperature. The reaction mixture was evaporated *in vacuo* and was added 2% HCl. The resulting precipitate was collected by filtration, washed with 4% sodium bicarbonate solution and water. The product was recrystallized from hot ethanol-ether; yield, 1.36 g, (56%); mp 158-159° C.

Phe (NH_2) -Gly-OEt · 2HCl (II). Compound *I* (1.20 g, 2.8 mmol) was suspended in a mixture of water-acetic acid-methanol (30 ml) and was added conc. HCl (0.5 ml, 6 mmol). The *I* was hydrogenated in the presence of palladium black at room temperature. After 24 hr, the catalyst was filtered off, and the filtrate was evaporated *in vacuo*; yield of oil (1.05 g).

Phe (N_3) -Gly-OEt · HCl (III). To a solution of the *II* (1.05 g, 2.8 mmol) in 1 M HCl (5 ml), was added a solution of 1.05 M $NaNO_2$ (2.7 ml, 2.8 mmol) at 0° C and stirred for 10 min at 0°C. To the solution, was added a solution of 1.05 M NaN_3 (2.7 ml, 2.8 mmol) and the reaction mixture was then stirred for 1 hr at 0° C and evaporated *in vacuo*. The resulting precipitate was filtered and dried. The product was recrystallized from ethanol-ethyl acetate; yield, 0.54 g, (59%); mp 135-139° C.

Enzyme and Methods. The pepsin used was a salt free crystalline sample from Worthington Biochemical Co. U. S. A. The substrate used was Gly-Gly-Phe (NO_2) -Phe-Gly-OEt. The kinetic constants of the substrate were K_m=0.83 mM, K_{cat}=5.5 sec^{-1}.

Inhibition of Pepsin Action with Photoaffinity Reagent. The photoaffinity reagent, substrate and pepsin were dissolved in citrate buffer (pH 4.16). To a mixture of 3 ml of the photoaffinity reagent solution (1 mmol) and 3 ml of the substrate solutions (0.075 mM, 0.125 mM, 0.20 mM, 0.25 mM), was added 50 μl of pepsin solution (0.06 mM) at 37° C. At selected intervals, the increase in absorbance at 310 nm was measured (*5*).

Effect of pH of Solution on Photoaffinity Labeling. Mixtures of 0.5 ml of pepsin solution (0.06 mM) and 0.5 ml of the photoaffinity reagent solutions (2 mM) of various pH were incubated for 10 min at room temperature and cooled in an ice-bath for 5 min. The solution was irradiated for 15 min with a Toshiba mercury lamp H400-P at a distance of 60 cm at 0° C. The irradiated enzyme solution (0.1 ml) was taken and added

to the substrate solution (0.25 mM, 10 ml) at 37° C and the increase in absorbance at 310 nm was measured.

Inhibition of Photoaffinity Labeling with Peptides. The photoaffinity reagent, peptides (Gly-Gly-Phe and HCl · Phe-Gly-OEt) and pepsin were dissolved in citrate buffer (pH 4.16). A mixture of 0.5 ml of pepsin solution (0.06mM) and 0.5 ml of peptide solution (20 mM) was incubated for 10 min at room temperature. To the mixture, was added 0.5 ml of photoaffinity reagent solution (2 mM) and irradiated as described above. The irradiated enzyme solution (0.1 ml) was taken and added to the substrate solution (0.25mM, 10 ml) at 37° C. At selected intervals, the increase in absorbance at 310 nm was measured.

Refernces

1) J. S. Fruton, "Acid Proteases", ed. by J. Tang, (1977), p. 131, Prenam Press, New York.
2) J. S. Fruton. "Hydrolytic Enzymes", ed. by A. Neuberger and K. Brocklehurst, (1987), p. 1, Elsevier, New York.
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 Enzymology", (1977), Vol. XLVI, p. 69, Academic Press, New York.
5) H. Yonezawa and T. Wada, *Bull. Chem. Soc. Jpn.* **62**, 3730 (1989).