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CHEMICAL MODIFICATION OF PEPSIN WITH 1-ETHOXYCARBONYL-2-ETHOXY-1,2-DIHYDROQUINOLINE

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Abstract

Pepsin was treated with coupling reagents [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC), dicyclohexylcarbodiimide (DCC) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)] in the presence of L-phenylalanine methyl ester hydrochloride (L-Phe-OMe·HCl) and remaining activity was measured. EEDQ inactivated the enzyme activity completely and DCC inactivated about 40% of the activity at the concentration of the coupling reagents of 7.4 mM (160 eq) and the reaction time of 60 min, but WSC did not inactivated at the same condition. Modification reaction of EEDQ was acieved in the presence of glycine methyl ester hydrochloride (Gly-OMe·HCl). The degree of inhibition was falled in the presence of Gly-OMe·HCl compared with in the presence of L-Phe-OMe·HCl. When pepsin was treated with the coupling reagents alone, the extent of inhibition was not greatly altered compared with in the presence of L-Phe-OMe·HCl.

Pepsin is a digestive protease which was secreted from gastric mucosa. Pepsin acts at acidic solution (pH 1.5–5), and early studies on kinetics of the action of pepsin on synthetic peptide substrates suggested the presence in the enzyme of two catalytically important prototropic groups with pK_a values near 1 and 4 (*1, 2*).

It was shown that diazoacetyl-DL-norleucine methyl ester caused the specific and irreversible inactivation of pepsin and complete inactivation was achieved upon the introduction of one such group per molecule of pepsin (*3*). Moreover, Bayliss et al. reported that diazoacetyl-L-phenylalanine methyl ester reacted with Asp-215 (*4*).

Similarly, it was reported that 1,2-epoxy-3-(4-nitrophenoxy) propane inactivated the enzyme with the apparent introduction of two molecules of substituent per molecule of pepsin (*5*). One of the site of reaction was shown to be Asp-32 (*6*).

However, diazo compounds and epoxids react with carboxyl group to give esters. The ester bond is labile, accordingly, it is liable to be hydrolyzed during the procedure of identification of modified aspartyl groups.

On the other hand, Hoare et al. reported a chemical modification of carboxyl groups in

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enzyme with amines and water soluble carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC) (*7*). In this method, carboxyl group changes to amide and amide bond is more stable than ester bond. However, these carbodiimides react nonspecifically with many carboxyl groups containing in the enzyme.

Pepsin cleaves the peptide bond between two hydrophobic amino acid residues (*8*). Thus the binding site of pepsin seems to prefer the hydrophobic side chain of amino acid residue containing a substrate. In addition, Andreeva et al. suggested that substrate binding site of pepsin was formed by hydrophobic amino acids (*9*). Consequently, the coupling reagents which have hydrophobic groups may bind with substrate binding site of pepsin and react with catalytically-active carboxyl groups easily.

Then, modification reactions of pepsin with dicyclohexylcarbodiimide (DCC) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) which have hydrophobic groups were achieved.

Results and Discussion

Pepsin was incubated with the coupling reagents in the presence of L-Phe-OMe·HCl and remaining activities were measured.

The course of inactivation is shown in Fig. 1. After 60 min. EEDQ had inactivated completely and DCC inactivated about 40% of the enzyme activity at the concentration of the coupling reagents of 7.7 mM (160 eq), on the other hand, WSC had not inactivated at the same concentration.

The effect of reaction time for inhibition of pepsin activity was measured. As shown in

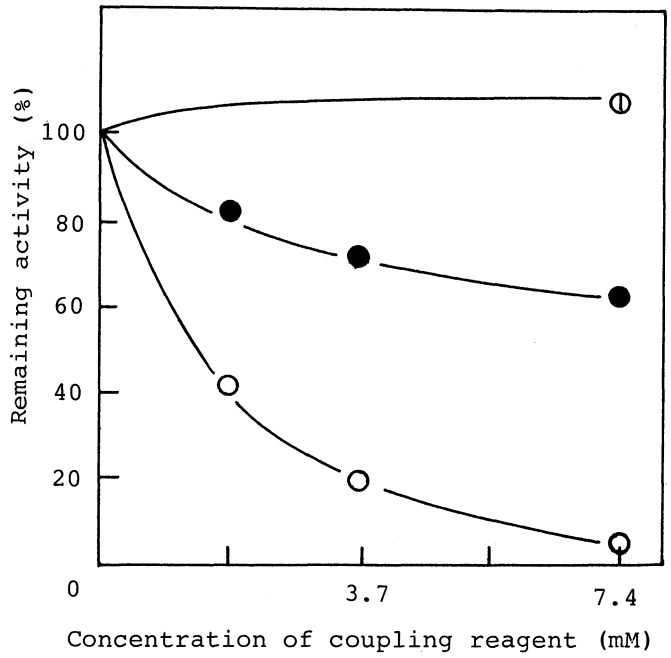

Fig. 1 Inhibition of pepsin activity by coupling reagents in the presence of Phe-OMe·HCl (Effect of concentration of coupling reagents). (Φ) WSC; (●) DCC; (○) EEDQ.

Fig. 2, modification reaction terminated after 40～60 min. These coupling reagents are presumably inactivated by water up to this time. Since EEDQ inhibited pepsin activity completely, the subsequent experiments were carried out with EEDQ.

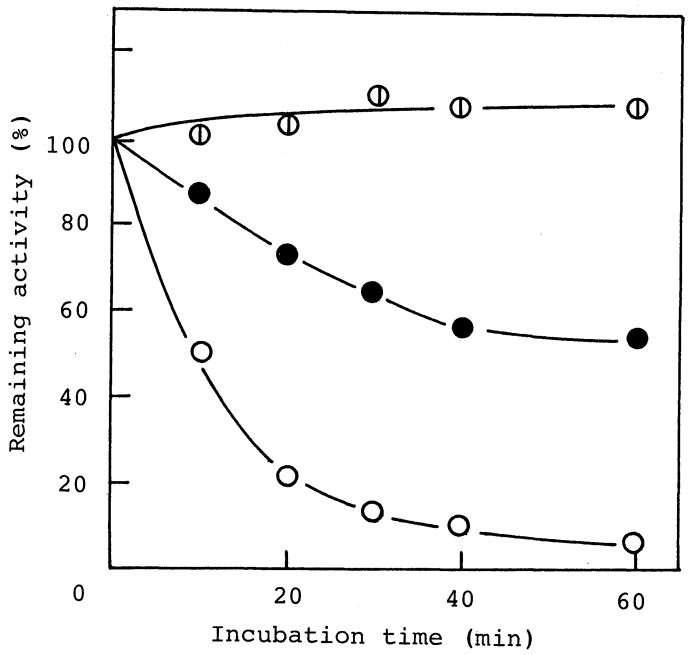

Fig. 2 Inhibition of pepsin activity by coupling reagents in the presence of Phe-OMe·HCl (Effect of reaction time). (Φ) WSC; (●) DCC; (○) EEDQ.

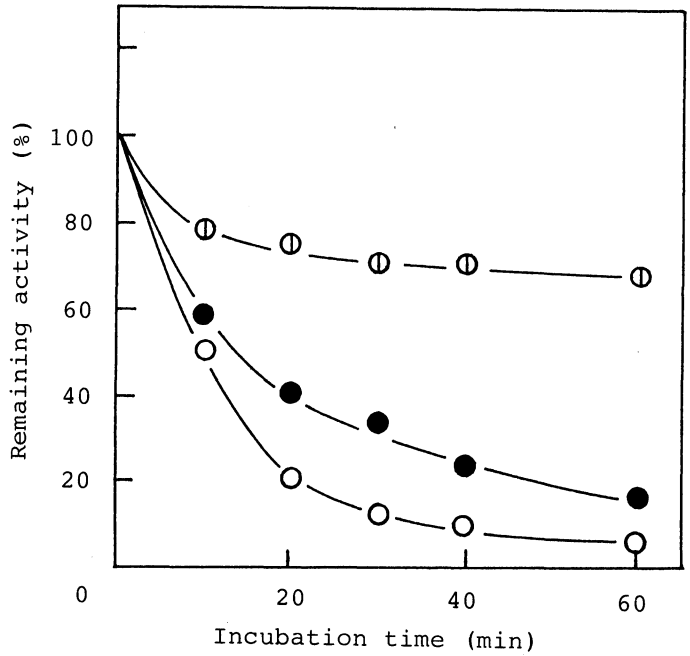

Fig. 3 Inhibition of pepsin activity by EEDQ. (○) Phe-OMe·HCl; (Φ) Gly-OMe·HCl; (●) Phe-Gly-OMe·HCl.

The effect of intensity of binding between amines and binding site of pepsin was examined. L-Phe-Gly-OMe·HCl may bind with the binding site of pepsin stronger than L-Phe-OMe·HCl, and Gly-OMe·HCl may bind weaker. When pepsin was treated with EEDQ and L-Phe-Gly-OMe·HCl, the extent of inhibition was not greatly altered in the presence of L-Phe-OMe·HCl (Fig. 3). On the other hand, when pepsin was treated with EEDQ and Gly-OMe·HCl, the extent of inhibition was weakened compared with in the presence of L-Phe-OMe·HCl (Fig. 3).

When pepsin was treated with the coupling reagents alone, the extent of inhibition was not greatly altered compared with in the presence of L-Phe-OMe·HCl (Fig. 4).

EEDQ and DCC have hydrophobic groups (quinonoid or cyclohexyl) but WSC had not

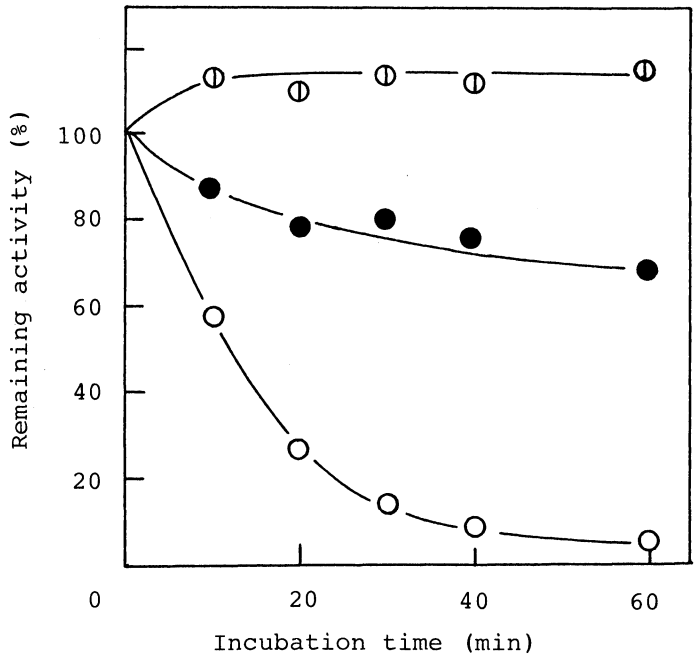

Fig. 4 Inhibition of pepsin activity by coupling reagents alone. (Φ) WSC; (●) DCC; (○) EEDQ.

hydrophobic group, since, this result shows that hydrophobic interaction between the coupling reagents and active site of pepsin is important for binding of the coupling reagents. It was reported that the S_1 and S_1' sites of pepsin gave a strong preference for aromatic L-amino acid residues which has flat phenyl groups. In practice, pepsin cleaves L-phenylalanyl-L-phenylalanine bond 6 times faster than L-β-cyclohexylalanyl-L-phenylalanine bond (*10*). This may shows that the binding site of pepsin prefer a flat phenyl group. EEDQ has a flat quinonoid group and DCC has a bent cyclohexyl group. Consequently, EEDQ binds with the binding site of pepsin faster than DCC and reacts with the catalytically-active carboxyl group rapidly.

Experiments

Pepsin was purchased from SIGMA Chemical Co. Pepsin and amino acid esters were dissolved in 10% glycerol solution (pH was adjusted to 3.2 by hydrochloric acid). Coupling reagents were dissolved in acetonitril.

Determination of the Proteolytic Activity of Pepsin Solution. ..... The proteolytic activity of pepsin solution was estimated by a modified form of the method of Kunitz. Casein (100 mg) was dissolved in 100 ml of a sodium citrate buffer (pH 1.8) under heating, and undissolved casein was filtered off. A 3 ml of the casein solution was pipetted into a test tube, which was placed in a water bath at 37°C. After 10 min. 20 μl portion of the pepsin solution to be assayed was added to the casein solution, and incubated for 5 min. Then, 0.3 ml of trichloroacetic acid solution (3 M) was added rapidly. The test tube was kept for a further 10 min. in the water bath and the resulting precipitate was then filtered off. To the filtrate (2 ml), were added 2 ml of NaOH solution (1 M) and 0.5 ml of Phenol reagent. After 30 min. the absorbance at 660 nm of the solution was measured.

Modification of Pepsin (Effect of Concentration of Coupling Reagents) ..... A 17.5 mg portion of pepsin (0.5 μmole) was dissolved in 10 ml of L-Phe-OMe·HCl solution (12.5 mM in 10% glycerol solution). Forty μl of the coupling reagent solution of each concentration was added to 0.5 ml of the pepsin solution and incubated at 30°C, and after 60 min. 20 μl of the solution was taken and remaining activity was measured.

Modification of Pepsin (Effect of Reaction Time) ..... A 17.5 mg portion of pepsin was dissolved in 10 ml of amino acid ester solution (12.5 mM in 10% glycerol solution). Forty μl of the coupling reagent solution (100 mM) was added to 0.5 ml of the pepsin solution and incubated at 30°C. At selected intervals, 20 μl of the solution was taken and remaining activity was measured.

References

1) A. J. Cornish-Bowden and J. R. Knowles, *Biochem. J.*, **113**, 353 (1969)
2) J. S. Fruton, *Adv. Enzymol.*, **33**, 401 (1970)
3) T. G. Rajagopalan, W. H. Stein and S. Moore, *J. Biol. Chem.*, **241**, 4295 (1966)
4) R. S. Bayliss, J. R. Knowles and G. B. Wybrandt, *Biochem. J.*, **113**, 377 (1969)
5) J. Tang, *J. Biol. Chem.*, **246**, 4510 (1971)
6) J. A. Hartsuck and J. Tang, *J. Biol. Chem.*, **247**, 2575 (1972)
7) D. G. Hoare and D. E. Koshland, *J. Am. Chem. Soc.*, 88, 2057 (1966)
8) J. S. Fruton. "Hydrolytic Enzymes". ed. by A. Neuberger and K. Brocklehurst. (1987), p. 1, Elsevier, New York.
9) N. S. Andreeva, A. S. Zdanov, A. E. Gustchina and A. A. Fedorov, *J. Biol. Chem.*, **259**, 11353 (1984)