Milk-clotting Enzyme from Microorganisms

Part IX. Reaction of Diazo-H-tetrazole on Mucor-rennin (Crystalline Milk-clotting Enzyme)

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Received March 8, 1971

The milk-clotting activity of Mucor-rennin (Milk clotting enzyme of *Mucor pusillus* Lindt) was inhibited by reaction of diazo-1-H-tetrazole accompanied with increase of the value of the absorbance of biazohistidine at 480 nm. The activity did not remain when the absorbance reached 50% of maximum value. It is concluded from these results that one mole of histidine in 2 moles of histidine contained in the enzyme has a relation to active center.

In the previous papers,^{1,2)} it was reported that the crude milk-clotting enzyme was produced from *Mucor pusillus* Lindt cultivated on wheat bran. The enzyme was purified from the crude sample and crystallized. And, it was named Mucor-rennin.^{1,2)} The general properties and the amino acid composition were studied,¹⁻⁵⁾ and it was revealed that one or two moles of histidine in Mucor-rennin were related to active center by the chemical modification.^{6,7)} This paper describes the inhibition of the activity of the Mucor-rennin by the

reaction of diazo-1-H-tetrazole (DHT).

EXPERIMENTAL METHOD AND MATERIALS

Mucor-rennin crystal. The crystalline milk-clotting enzyme, Mucor-rennin, was obtained from *Mucor pusillus* Lindt by the purification procedures using Amberlite CG-50, DEAE Sephadex A-50 and Sephadex G-100 columns as reported in the previous paper.¹⁾ One gram of the crystalline enzyme was dissolved in 10 ml of distilled water and was dialysed twice against 200 volumes of distilled water for 24 hr to remove ammonium sulfate. The enzyme was lyophilized for the experiment.

Determination of milk-clotting activity. To 0.5 ml of the enzyme solution in a tube of 25 ml volume, 5 ml of 10% (w/w) skim milk powder solution (Snow Milk Products Co., Ltd., Japan) in 0.01 M CaCl₂ was added. The tube was incubated at 35°C. The milk-clotting activity was determined by the method described previously.¹⁾ The amount of enzyme which clotted the substrate within 1 min under the above condition was defined as 400 units.

Reaction of diazo-1-H-tetrazole. The reaction of diazo-1-H-tetrazole was performed with the modified Horinishi method.⁸⁾ One gram of 5-amino-1-H-tetra-

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zole was dissolved in an ice bath by slow addition of 0.7 g of sodium nitrite in 10 ml of water. The pH value of this acidic solution of DHT was adjusted to pH 5.0 with KOH before mixing with the enzyme solution. The final volume of the DHT solution was filled up to 100 ml. This solution was used as initial DHT solution. DHT solution was mixed with 2 ml of 0.5 M KH_2PO_4 -NaOH buffer (pH 6.0) and enzyme solution (1 ml). The solution was reacted at room temperature. After completion of the reaction, the amount of the diazotized histidine residue (biazo-histidine) in the enzyme was determined by the measurement of value of the absorbance at 480 nm with Hitachi spectrophotometer.

RESULTS

Effect of DHT concentration on the milk-clotting activity

Diazo-1-H-tetrazole solution was diluted to double the original volume. One ml of the diluted DHT solution was mixed with 0.015% Mucor-rennin (10 ml) and KH_2PO_4 -NaOH buffer (pH 6.0, 2 ml). The mixture was reacted

FIG. 1. Effect of Concentration of Diazo-1-H-tetrazole on Inhibition of Milk-clotting Activity of Mucor-rennin.

Residual milk-clotting activity was determined after Mucor-rennin-diazo-1-H-tetrazole solution was allowed to stand at 35°C for 90 min (○) and 180 min (●).

at room temperature for 90 to 180 min. Milk-clotting activity was inhibited by the addition of DHT. The residual milk-clotting activity was gradually decreased as the concentration of added DHT increased. The activity was completely inactivated with the addition of 0.6 ml of the diluted DHT solution (Fig. 1).

Effect of DHT on absorbancy at 480 nm milk-clotting activity of mucor-rennin

The initial DHT solution or the DHT solution which was diluted to double the original volume was added to the enzyme solution. The residual activity was inactivated gradually with the reaction-time accompanied with the increase of the absorbance at 480 nm. The absorbance value curves were observed as two step type as shown in Fig. 2. When the absorbance at 480 nm reached around 0.19, the

FIG. 2. Relation between Absorption at 480 nm and Milk-clotting Activity through Reaction Time of Diazo-1-H-tetrazole.

Milk-clotting activity (1/2 DHT): ●, absorption at 480 nm: (1/2 DHT) ○, DHT ▽.

0.086% Mucor-rennin was reacted with DHT at pH 6.0 and room temperature (21°C).

curve manifested a shoulder, which indicated a two step reaction. The reaction of DHT coupling was faster under the initial DHT concentration than that under the diluted DHT solution. The absorbance at 480 nm was 0.19 after the diluted DHT solution was mixed with the enzyme and the residual milk-clotting

activity was completely lost. The maximum value at 480 nm was 0.38, which was assumed to be 100%. Relation between residual milk-clotting activity and rate of the absorbance at 480 nm is illustrated in Fig. 3. The milk-clotting activity became zero unit when the rate of the absorbance reached 50%.

FIG. 3. Relation between Residual Milk-clotting Activity and Absorbancy at O.D. 480 nm by Diazo-1-H-tetrazole-reaction at pH 6.0.

DISCUSSION

DHT was found to possess the following characteristics superior to those of common diazonium compounds for the quantitative determination of histidine in amino acid mixtures or proteins.⁸⁾ Histidine biazotyl-H-tetrazole has a strong absorption band at 480 nm, while tyrosine biazotyl-H-tetrazole has a weaker band at a considerably longer wave length of 550 nm, so that the effect of overlapping of the biazotyrosine band on the absorption at 480 nm is much smaller than those observed with common diazonium compounds. Moreover, the bis coupling to histidine residues in proteins is completed before the bis coupling to tyrosine residues. The only exception was the coupling to the strong bond residue in

chymotrypsinogen. One can, therefore, observe the bis coupling to histidine residues separately from that to tyrosine residues. All of the histidine residues in lysozyme, insulin, albumin, and trypsin were found to react uniformly with DHT to give biazotyl-histidine residues. On the other hand, the reaction curves obtained for trypsinogen, chymotrypsinogen and α -chymotrypsin showed two steps of absorbancy change and revealed the presence of bound histidine residues.

Mucor pusillus is a thermophilic mold⁹⁾ which produces a crude milk-clotting enzyme under aerated condition.^{10,11)} The crude enzyme was purified and crystallized.¹⁾ The general physicochemical properties, reaction of the Mucor rennin on casein in agar gel of the Mucor rennin were studied.¹⁻⁵⁾ The crystalline milk-clotting enzyme has an acid protease activity. The optimum pH for the digestion of κ -casein is 4.5, while that for hemoglobin digestion is 4.0. The physical properties of the crystalline enzyme are as follows; partial specific volume is 0.74; sedimentation coefficient is 2.39×10^{-13} (cm.g.sec⁻¹. dyne⁻¹); diffusion coefficient (7.9×10^{-7} cm² sec⁻¹); frictional coefficient (f/f_0), 1.33. Molecular weight was given as 29,000 by Svedberg's method and 30,600 by Yphantis's method. The number of amino acid residues contained in 1 mole of protein of the crystalline enzyme is 277~281 ((1/2 Cys)₂, Met₃, Asp₄₄, Thr₂₁, Ser₂₂, Glu₂₀, Pro₁₄, Gly₃₄, Ala₁₆₋₁₇, Val₂₄, Ile₁₂, Leu₁₅, Tyr₁₃, Phe₉, His₁₋₂, Lys₁₁₋₁₂, Arg₄, Trp₂₋₃). It is found that the content of histidine in the enzyme is 2 mole by Goodwin's corrected method. In the studies of active site of Mucor-rennin by chemical modification,^{6,7)} the activity was inhibited by the reaction of iodine and photooxidation of methylene blue.

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The results suggested that tyrosine, tryptophane and histidine residues in the enzyme has a relation to active site. Among these amino acids, the histidine residue was more rapidly reacted than other amino acids. A parallel relation was observed between the decreased amount of histidine residue and inactivation of the enzyme. From the results, it is concluded that one or two mole of the histidine residue contained in one mole of Mucor-rennin has a relation to active center of this enzyme. Under alkaline condition, the enzyme was inactivated spontaneously. Therefore, the experiment of DHT was not performed. At pH 6.0, the activity was inhibited and the absorbance at 480 nm

increased. The absorbance curve showed two step-reaction at 0.19 of O.D. 480 nm. The activity was completely lost at this point. At the first step below 0.19, active histidine residue is diazonated earlier than other residue, and the absorbance value of more than 0.19 is due to coupling of inactive histidine residue. On the other hand, when the absorbance reach 50% of the maximum value, the activity was completely lost. It is evident from these results that histidine is involved in the active site of Mucor-rennin as illustrated in previous papers,^{6,7)} and that one of the two molecules of histidine contained in the enzyme has a relation to active center.