A Study on the Active site of Glucoamylase from *Aspergillus shirousamii*

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Glucoamylase was inactivated with 1-ethyl-2-(dimethylaminopropyl)carbodiimide (EDC) at pH 5.0. Time course of inactivation of glucoamylase was at least biphasic. From the results of the titration of SH groups with Ellman's reagent and hydroxylamine treatment at pH 7.0, it was concluded that the crucial sites of modification were carboxyl groups of glucoamylase. The CD spectrum of EDC-modified glucoamylase suggested that the gross conformation of the native enzyme was retained. The inactivation of glucoamylase was reduced remarkably in the presence of maltose. The logarithm of the half-life of the inactivation of glucoamylase by EDC was a linear function of log[EDC] in each stage indicating that one carboxyl group among the modified ones was crucial for inactivation of glucoamylase. The change in the binding affinity due to modification was determined by using an affinity column. It indicates that the carboxyl group of glucoamylase seems to play a role in both, the catalysis and substrate binding in the first stage, but in the second stage the binding affinity is recovered almost up to that of native enzyme.

Introduction

A detailed study of the active site of enzymes is a necessary step in learning about their mechanisms of action. Glucoamylase hydrolyzes starch and is an enzyme having potential importance for the food industry. Glucoamylase is among the enzymes that have been little studied in the structural respect. Participation of carboxyl groups in the active site of glucoamylase has been suggested by Hiromi *et al.*[1] They suggested the presence of two functional groups having pKa's of around 2.9 and 5.9 based on the pH-profile of kinetic parameters of glucoamylase from *Rhizopus delemar* with various disaccharides as substrate. Gray and Jolly[2] reported that modification of carboxyl groups of glucoamylase from *A. niger* with water-soluble carbodiimide and the glycine methyl ester resulted in inactivation of the enzyme with the incorporation of about 83 glycine residues. Photooxidation of glucoamylase from *A. niger* in the presence of Methylene Blue or Rose Bengal resulted in a pH-dependent loss of enzymatic activity, which was analogous to the destruction of free L-tryptophan during photo-oxidation.[3] The substrate, maltose in both cases protected the enzyme against inactivation. The tryptophanyl residues of the *A. niger* glucoamylase were oxidized by N-bromosuccinimide in both the presence and the absence of substrates and inhibitors of the enzyme. The results of this experiment indicated that two tryptophan residues were essential in the mechanism of glucoamylase action[4]. The role of histidine and tyrosine residues was studied for the glucoamylase from *A. niger*[5,6]. Ethoxycarbonylation of the histidine by diethylpyrocarbonate did not change the catalytic activity, but affected the binding affinity to some degree. Modification of the tyrosine by N-acetylimidazole also impaired the binding to the substrate without changing the activity. Sulfhydryl groups apparently were not directly involved in either in catalysis or in the binding of substrates of glucoamylase. It has been shown for the glucoamylase from *A. saitoi* that free SH groups are absent both under the physiological conditions and in 8M urea[7].

In the present work, in order to elucidate the role of carboxyl groups in the action of glucoamylase from *A. shirousamii*, the influence of chemical modification of carboxyl groups with water-soluble carbodiimide (EDC) was stuided.

Experiments

Chemicals. Soluble starch was purchased from Junsei Chemical Co., Ltd. Glucose oxidase, peroxidase, glycine methyl ester, 1-ethyl-2-(dimethylaminopropyl) carbodiimide (EDC), 5,5'-dithiobis(2-nitrobenzoic acid), and cyanogen bromide were purchased from Sigma Chemical Co. Hydroxylamine hydrochloride was obtained from Hayashi Pure Chemical Industries, Ltd. Sepharose CL-4B was from Pharmacia Fine Chemicals. Triethylamine (TEA) and hexamethylenediamine were purchased from Junsei Chemical Co., Ltd. Amylopectin was obtained from Sigma. Maltose was purchased from Wako Pure Chemicals Industries, Ltd.

Enzyme. Glucoamylase from *A. shirousamii* was purified as described by Byung Gook Na[8]. Wheat bron koji (a commercial preparation of glucoamylase from *A. shirousamii*) was kindly supplied by Jinro Research Institute.

Determination of Glucoamylase Activity. The enzymatic activity with soluble starch as a substrate was measured at pH 4.5 and 37 °C. The glucose released was measured by the D-glucose oxidase method of Dahlqvist with slight modification[9].

Modification of Glucoamylase with EDC. Glucoamylase (5.65 μM) in 0.02 M cacodylate buffer (pH 5.0) was modified at 25 °C. Final EDC and glycine methyl ester concentrations are 20-30 mM and 0.133 M, respectively. To stop the reaction, aliquots were withdrawn at appropriate times and added to 0.2 M acetate buffer (pH 4.5). Then the enzymatic activity of each aliquot was measured.

Treatment of EDC-modified Glucoamylase with Hydroxylamine. EDC-modified enzyme with up to 27.7% residual activity was dialyzed against phosphate buffer (pH 7.0), then incubated with 1N hydroxylamine adjusted to pH 7.0 for 5-6 hr. at room temperature. The final protein concentration was 0.79 μM. To measure the enzymatic activity, en-

zyme samples treated with hydroxylamine were dialyzed against 0.02 M acetate buffer (pH 4.5).

Circular Dichroism Spectra. CD spectra were measured with a JASCO J-20 spectropolarimeter at room temp. in cells of 1 cm light path. The enzyme concentration used were 15.1 μM and 2.97 μM for wavelength of 310-250 nm and 250-200 nm, respectively.

Affinity Column Preparation. Affinity column preparation was accomplished by activation of Sepharose CL-4B with cyanogen bromide and linking hexamethylenediamine as a spacer to the activated Sepharose CL-4B. This is called as AH-Sepharose CL-4B. Amylopectin as ligand was coupled to the AH-Sepharose CL-4B. The activation of Sepharose CL-4B was performed by the method of Kohn and Wilchek[10]. Ten grams of wet Sepharose CL-4B were washed with 30% acetone, followed by 60% acetone, and resuspended in 10 m*l* 60% acetone. The mixture was cooled to -15 °C. A quarter grams of CNBr was added to the Sepharose suspension. With cooling and vigorous stirring, the corresponding volume of TEA solution (1.5 M in 60% acetone) was added dropwise over a period of 1-3 min. (final molar ratio of CNBr:TEA of about 1:1.5). Then the entire reaction mixture was quickly poured into 100 m*l* of ice cold washing medium (Acetone: 0.1 N HCl = 1:1). The resin can be stored in cold washing medium for about 1 hr. without significant loss of activation. The resin was washed with cold 60% acetone, followed by quick washings with cold 30% acetone and cold water. Then the resin was resuspended in coupling medium (hexamethylenediamine 400 mg in 5 m*l* distilled water adjusted to pH 9.5) at room temperature. After gentle stirring for 1 hr., ethanolamine was added to the reaction mixture to eliminate unreacted cyanate esters. Above AH-Sepharose CL-4B was washed with distilled water, followed with carbonate buffer (pH 9.16). Amylopectin was also activated similarly with CNBr at 0 °C. After adding the triethylamine solution, activated amylopectin was coupled to the AH-Sepharose CL-4B under the same conditions as did for coupling of hexamethylenediamine to the activated Sepharose CL-4B.

Determination of the Change in Binding Affinity. The change in binding affinity was determined according to the method of Hoschke[5]. The affinity gel (200-300 mg, Y) was added to an 1 m*l* of enzyme solution in 0.005 M acetate buffer (pH 4.5) of known absorbance (E_o), and the mixture was stirred at 4 °C until equilibrium was attained (about 40-50 min). The mixture was stored for 15 min. and the absorbance (E_m) was measured at 280 nm after sedimentation of the gel grains. The amount of enzyme bound to the gel could be obtained by using the expression $100 \times (E_o-E_m)/E_o \cdot Y$. By knowing the affinity of the original enzyme, the change of binding due to modification can be determined.

Results

Time Course and pH-Profile of Inactivation of glucoamylase by EDC. Inactivation of glucoamylase by EDC was performed in cacodylate buffer (pH 5.0). During the course of the reaction, the pH of the reaction mixture was not changed. The time course of the inactivation of glucoamylase by EDC was at least biphasic (Figure 1, 2). The reaction up to 50% inactivation followed pseudo-first order kinetics, then slow inactivation followed up to 80% inactivation. The pH-dependence of the reaction is shown in Figure

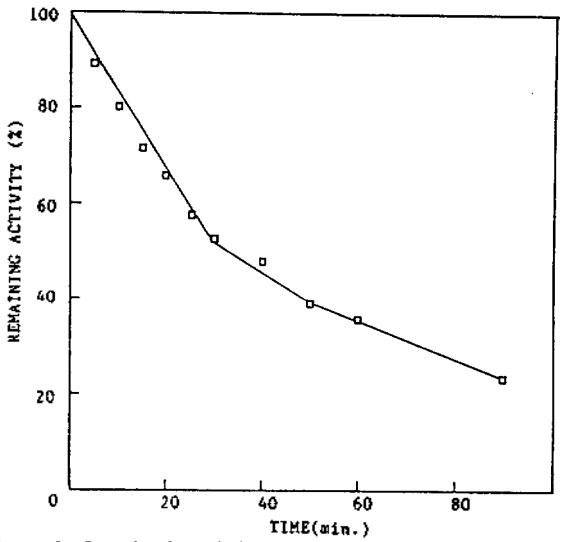

Figure 1. Inactivation of glucoamylase with EDC Treatment at pH 5.0 and 25 °C; concentrations of reaction were 5.65 μM enzyme and 20 mM EDC. The enzymatic activity was measured as described in the methods, with soluble starch as a substrate.

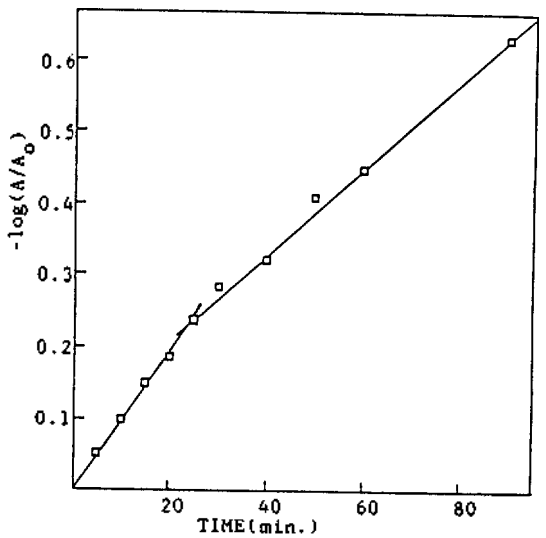

Figure 2. Semilog plot of the data from Figure 1.

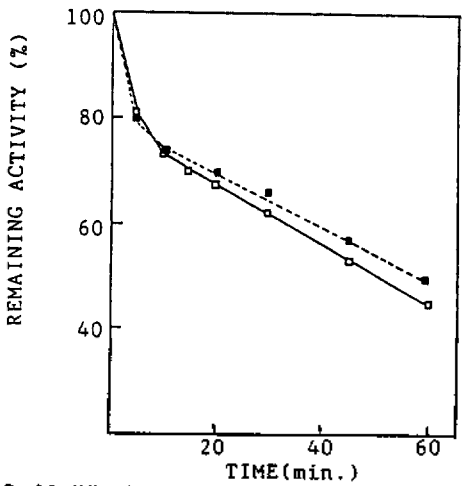

Figure 3. Modification of glucoamylase by EDC with and without preincubation; with preincubation of EDC (□), without preincubation of EDC (■).

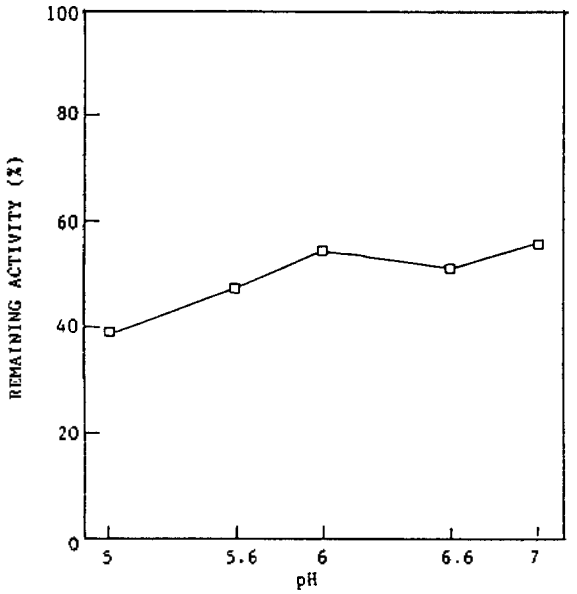

Figure 4. pH-Profile of Inactivation of glucoamylase by EDC. The reaction conditions were essentially the same as those described in Figure 1 except for pH.

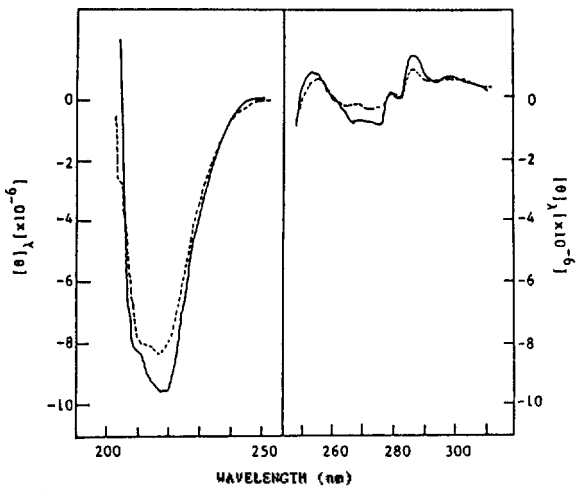

Figure 5. CD spectra of Native and EDC-modified glucoamylase at pH 5.0. Native glucoamylase, —; EDC-modified glucoamylase (20% residual activity), ----.

4. The inactivation was optimum at pH 5.0. Thus, the modification of glucoamylase was performed at pH 5.0 in the following experiments.

Test of the Possibility of Side Reactions. When the EDC-modified glucoamylase was treated with 1 N hydroxylamine at pH 7.0 for 5 hr. at room temperature, regeneration of enzyme activity was not observed. This result indicated that the tyrosine residues were not the site of EDC modification. In the native glucoamylase, 0.53 mol. equivalent of free SH group was titrated by Ellman's reagent only in the presence of 6.4 M guanidine-HCl. In the EDC-modified one, 0.54 mol. equivalent of free SH group was also titrated by Ellman's reagent in the presence of the denaturant. Thus, the free SH group was not the site of EDC-modification.

CD Spectrum of EDC-Modified Glucoamylase. The conformation of EDC-modified glucoamylase having about 20% residual activity was examined by CD spectroscopy.

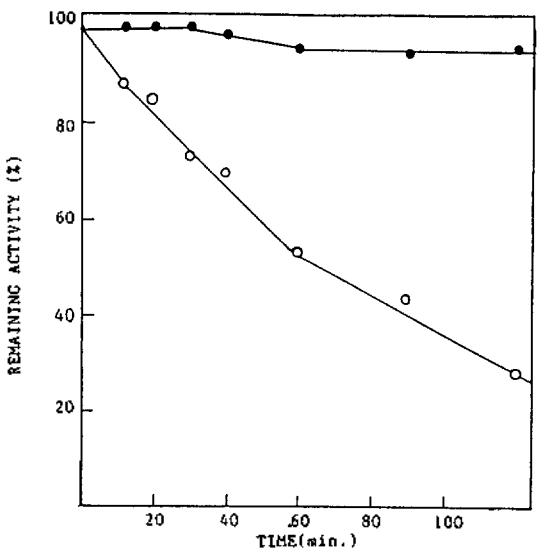

Figure 6. Modifications of glucoamylase by EDC in the Presence and the Absence of Maltose. The reaction conditions were essentially the same as those described in Figure 1. The concentration of maltose is 0.6 M.

The native glucoamylase gave a through at 217 nm and a shoulder at 210 nm in the wavelength region responsible for the backbone conformation of protein (200-250 nm), and 2 peaks at 255 and 287 nm and 4 troughs at 262, 268, 277, and 283 nm in the wavelength region which is attributed to the aromatic side chains of amino acid residues in glucoamylase (Figure 5). The CD spectrum of EDC-modified glucoamylase in the short wavelength region was almost superimposable on that of native glucoamylase, indicating that the peptide backbone conformation of the enzyme was maintained during the reaction with EDC. The CD bands in the wavelength region between 250-310 nm of EDC-modified glucoamylase were slightly different from those of native glucoamylase, though the peak and trough positions were similar.

Modification of Glucoamylase by EDC in the Presence of Maltose. Maltose is a poor substrate of glucoamylase. As shown in Figure 6, maltose protected the enzyme from inactivation by EDC. Until the enzyme modified in the absence of maltose inactivated up to about 70% of original activity, the enzyme modified in the presence of maltose had almost original activity. Even when the enzyme modified in the absence of maltose had 30% of original activity, the enzyme modified in the presence of maltose inactivated only up to 5%. Thus the maltose inhibited the inactivation of glucoamylase by EDC very effectively.

Inactivation of Glucoamylase by Various Concentrations of EDC. As shown in Figure 1, inactivation of glucoamylase by EDC consisted of 2 or 3 stages. The reaction up to about 50% approximates first-order kinetics with respect to time at any fixed concentration of EDC. However, as shown in Figure 7, the enzymatic activity is not completely lost; instead, it is reduced to a low residual level. Moreover, the rate of reduction to this residual activity varies with EDC concentration. Thus, the rate constants of the first state of inactivation were measured at various concentrations of EDC and the half-lives of the enzyme ($T_{1/2}$) were determined. According to Levy *et al.*, the $\log(T_{1/2})$-log[EDC] relation was a useful tool to estimate the amount of the functional groups

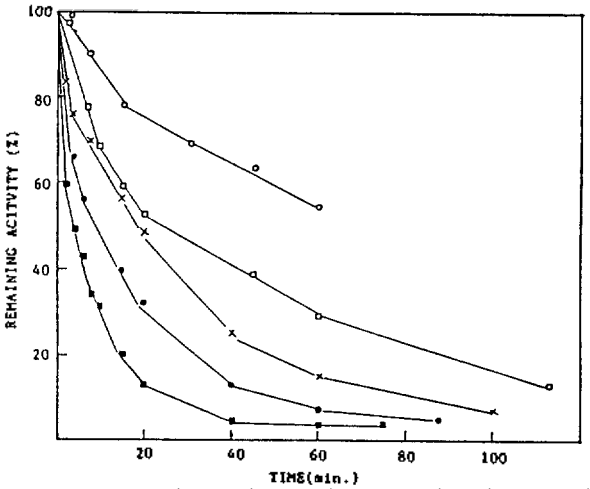

Figure 7. Inactivation of glucoamylase by Various Concentrations of EDC. Glucoamylase (5.65 μM) was treated as described in Figure 1 with various concentrations of EDC (10-100 mM); 10.7 mM(○), 20.8 mM(□), 31.7 mM(×), 49.0 mM(●), 100.7 mM(■).

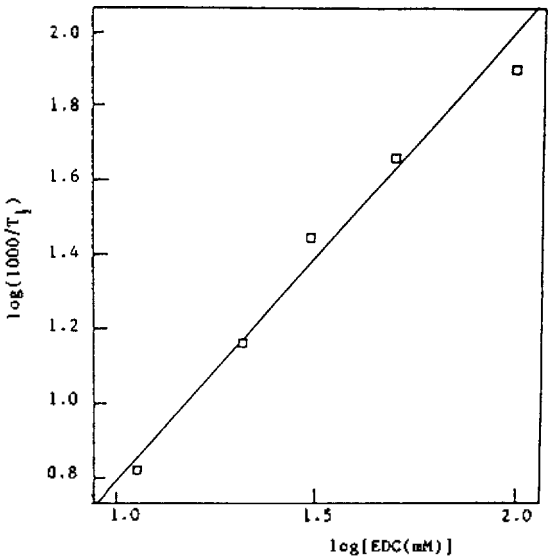

Figure 8. Plot of log($T_{½}$)-log[EDC] Relation. The reaction conditions were the same as those described in Figure 7.

responsible for the enzymatic action[11]. In the case of glucoamylase, the slop of log($T_{½}$)-log[EDC] was almost equal to one (Figure 8).

Determination of the Change in Binding Affinity. The relation between the changes in activity and binding affinity was given in Figure 9. As shown in Figure 9, the binding affinity reduced rapidly up to 50%, in which the enzyme had about 70% activity and then increased slowly. When the enzyme modified having 20% enzymatic activity, the binding affinity was recovered up to 95% of the native enzyme.

Discussion

Water-soluble carbodiimides in combination with various kinds of nucleophiles have been used as tools for the analysis of essential carboxyl groups for various enzymes. Though carbodiimides selectively modify the carboxyl groups, they also modify the tyrosine and free SH groups. Thus, we tested

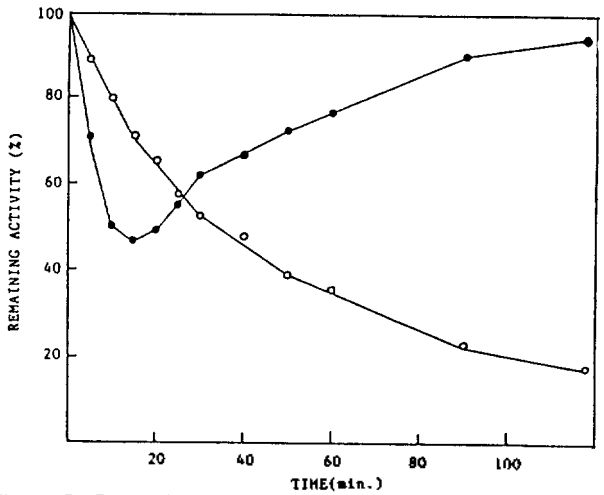

Figure 9. Determination of the Change in Binding Affinity. Reaction conditions: enzyme concentrations, 2.0 μM; 0.005 M acetate buffer (pH 4.5); temperature, 4 °C.; enzymatic activity (○); binding activity (●).

the side reactions of carbodiimide. The evidence described in the result confirmed that the inactivation of glucoamylase of the enzyme. And from the result of Figure 6, it is evident that the carboxyl groups modified by EDC are in the active site.

As shown in Figure 1, the inactivation of glucoamylase by EDC did not follow simple pseudo-first order kinetics, and probably consisted of more than two inactivation processes. To eliminate the ambiguity due to the decrease in effective EDC concentration during incubation, EDC was incubated for 30 min. prior to the addition of glucoamylase. The results were practically identical with those without preincubation. Both cases showed same patterns of inactivation (Figure 3). Thus it was not likely that degradation of the reagent complicated the reaction kinetics. The results described in this report suggest that more than two carboxyl groups are contained in the active site of glucoamylase. As shown in Figure 6, the logarithms of the half-lives of the first stage were linear functions of log[EDC] with a slope of one. Those of the second stage were also linear function with a slope of one. Therefore, at least one carboxyl group was involved in each stage of inactivation. There are some reports suggesting that two carboxyl groups are involved in active site[1,12,13]. Another possible explanation for the complex time course of inactivation is that the inactivation progressed with the modification of one carboxyl group, but due to the modification of other carboxyl group not involved in the catalytic site very small changes of conformation (not apparent in the CD spectrum) were induced, resulting in decrease of the rate of inactivation. Thus, the inactivation kinetics would become more complex. In the binding test, as shown in Figure 9, decrease in the binding affinity correlates with the first stage of inactivation and increase up to 95% of the binding affinity of the native enzyme correlates with the second stage of inactivation. These results can be described that one carboxyl group involved in the first state of inactivation plays a role in catalysis and in the same time induces a conformational change resulting in decrease of binding affinity. Another carboxyl group involved in the second stage of inactivation induces an another conformational change and this compensates for the first conformational change to restore the binding affinity up

to that of native enzyme. Thus the rate of second atage inactivation decreases.

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