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Studies on Dextranase. V. Activation of Dextranase from *Penicillium funiculosum* IAM 7013 by Co^{2+} [1a,b]

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The functional role of Co^{2+} in the activation of dextranase (EC 3.2.1.11) from *Penicillium funiculosum* was investigated. (1) Free cobalt ion activated the dextranase, but chelated cobalt compounds did not. (2) Co^{2+} bound to the enzyme and increased its activity, although the Michaelis-constant was not affected. (3) When the dextranase activity was increased by Co^{2+}, the activation energy was decreased and the maximum velocity increased. (4) On the basis of the modification of amino acid residues in the enzyme, it was concluded that the cystein residue was situated out of the active center and was related to the activation. (5) The experimental data obtained suggest a possible function of Co^{2+} for the dextranase activation as follows. Co^{2+} combines immediately with the cysteine residue and the combination leads to the suitable conformational changes of the enzyme for dextran hydrolysis and results in the acceleration of decomposition of the enzyme-substrate complex into products and the enzyme.

The authors have reported purification and general properties of dextranase (EC 3.2.1.11) from the mold[3–5] and the bacterium.[6] In the previous paper,[3] we have also described that dextranases I and II from *Penicillium funiculosum* were activated by Co^{2+}, Mn^{2+} and Cu^{2+}. In particular, the activation was markedly induced by Co^{2+}.

The fact that matallo-enzyme loses its activity when dialyzed against ethylene diamine tetraacetic acid (EDTA) solution is well known. Dextranases I and II, however, were not inactivated by dialysis against EDTA and these results suggested that they were not metallo-enzyme. In order to study the activation mechanisms, the authors examined the effects of Co^{2+} on the substrate and the enzymes, *i.e.* Michaelis constant (K_m), the maximum velocity (V_{max}) and the activation energy of the enzyme. By means of modifications of amino groups in the enzyme, the amino group which combined with Co^{2+} was also investigated.

Materials and Methods

Enzymes and Assay of Dextranase Activity——The dextranase used in this series of experiments was dextranase II from *P. funiculosum* which was purified by the procedures as described in the previous paper.[3] Dextranase I was also used in some experiments together with dextranase II. Assay of dextranase activity was done according to the methods described previously[3] and as follows; reaction was carried out at pH 6.0 and the reducing sugars liberated were determined by Sumner's reagent.[7]

Preparation of Bis(8-quinolinolate)Co^{2+} and Bis(salicyl aldimine)Co^{2+}——To the ethanol solution of 8-oxiquinoline, cobaltic acetate solution was added and then the mixture was kept in a boiling water bath and filtered. Bis(8-quinolinolate)Co^{2+} was recrystallized from ethanol. Bis(salicyl aldimine)Co^{2+} was also prepared as follows. To the salicyl aldehyde in hot-ethanol, ammonium hydroxide and cobaltic acetate

1) *a*) Part IV: M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **22**, 2941 (1974); *b*) This form is part XCVIII of "Studies on Enzymes" by M. Sugiura.
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3) M. Sugiura, A. Ito, T. Ogiso, H. Asano, and K. Kato, *Biochim. Biophys. Acta*, **307**, 357 (1973).
4) M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **22**, 1593 (1974).
5) M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), **22**, 2941 (1974).
6) M. Sugiura, A. Ito, and T. Yamaguchi, *Biochim. Biophys. Acta*, **350**, 61 (1974).
7) J.B. Sumner, *J. Biol. Chem.*, **65**, 393 (1925).

solution were added. On heating the mixture, bis(salicyl aldimine)Co^{2+} was precipitated as dark green compound.

Acetylation of Lysyl and Free Amino Residues in the Enzyme——Acetylation was performed by the method of Fraenkel-Conrat.[8] Five mg of the enzyme was dissolved in 5 ml of cold sodium acetate solution (0.5 saturation) and to the solution, 0.25 ml of acetic anhydride was added for 1 hr. The reaction mixture was dialyzed against distilled water for 5 days.

Modification of Arginine Residues and Photooxidation of Histidine Residues in the Enzyme——Modification of arginine residues in the enzyme was carried out according to the method of Liu, *et al.*[9] To 10 mg of the enzyme in 4.5 ml of 100 mM triethanolamine buffer (pH 8.0), 1,2-cyclohexanedione (10 mg) in 0.5 ml of water was added. As the control, 0.5 ml of water without 1,2-cyclohexanedione was used. The reaction was allowed to proceed at room temperature in a dark place for 12 hr. The mixture was dialyzed against water for 48 hr at 4°.

Photooxidation of histidine residues in the enzyme was also done by the methods of Horae[10] and Weil.[11] To 1 ml of the enzyme solution containing 5 mg, 1 ml of 200 mM phosphate buffer (pH 7.0) and 0.5 ml of methylene blue solution (0.04%) were added and the irradiation was done at 20° for 2 hr with a 100 V-200 W tungsten lamp. During all the procedures, air without CO_2 was flowed into the reaction vessel.

Modification of Carboxyl Groups in the Enzyme[12]——One ml of N-ethyl-N(3-dimethylaminopropyl) carbodiimide solution (in acetone), 1 ml of 20 mM phosphate buffer (pH 7.0), 1 ml of glycine ethyl ester solution and 3 ml of 30 mM KCl were mixed in a test tube and allowed to stand at $25\pm2°$ for 2 hr. The reaction mixture was dialyzed against water for 6 days to remove excess reagents.

Determination of Free Amino Groups and SH-groups in the Enzyme——Amino groups were determined according to the ninhydrin reaction.[13] Determination of SH-groups was carried out by the procedure of Negi, *et al.*[14] To 1 ml of the enzyme solution, 2 ml of 30 mM phosphate buffer (pH 8.0) and 0.2 ml of 5,5′-dithiobis(2-nitrobenzoic acid) solution (0.396% in 40 mM phosphate buffer, pH 7.0) were added and the mixture was allowed to stand for 10 min, The absorbance was determined at 412 nm in a 10 mm cell.

Substrate and Chemicals Used——Dextran was produced by *Leuconostoc mesenteroides* N-4, and was partially hydrolyzed and fractionated. One fraction (mol. wt. 5×10^4) was used as the substrate in all the experiments. Other chemicals used were of reagent grade.

Results and Discussion

Effect of Co^{2+} on the Substrate

In order to determine whether the increase of the enzyme activity was derived from the activation of the substrate by Co^{2+} or not, the following experiments were performed. The mixture of $CoCl_2$ and dextran was treated with cation ion-exchanger, Amberlite XE-100, and the dextran freed of Co^{2+} was used as the substrate in the experiments. Results are presented in Table I. In the dextran solution containing Co^{2+}, 134.6% of relative activity

Table I. Effect of the Treatment of Amberlite XE-100 on the Co^{2+} Activation of Dextranase Reaction

Substrate system	Amberlite XE-100 treatment	Relative activity (%)
Substrate alone	−	100
Substrate and Co^{2+}	−	134.6
Substrate alone	+	100
Substrate and Co^{2+}	+	97.9

Dextran solution in the presence (3.0×10^{-4}M) or absence of $CoCl_2$ was applied to the column of Amberlite XE-100. The treated substrate solution was used in determination of the dextranase activity.

8) H. Fraenkel-Conrat, *J. Biol. Chem.*, **177**, 385 (1949).
9) W.-H. Liu, G. Feinstein, D.T. Osuga, R. Haynes, and R.E. Feeny, *Biochemistry*, **7**, 2886 (1968).
10) D.G. Hoare and D.E. Koshland, Jr., *J. Am. Chem. Soc.*, **88**, 2057 (1966).
11) L. Weil, A.R. Buchert, and J. Maher, *Arch. Biochem. Biophys.*, **40**, 245 (1952).
12) H. Horinishi, K. Nakaya, A. Tani, and K. Shibata, *J. Biochem.* (Tokyo), **63**, 41 (1968).
13) E.W. Yemm and E.C. Cocking, *Analyst*, **80**, 209 (1955).
14) T. Negi, T. Samejima, and M. Irie, *J. Biochem.* (Tokyo), **70**, 359 (1971).

was observed, whereas, in the dextran treated with Amberlite XE-100, dextranase was not activated. These results suggested that Co^{2+} did not activate the substrate but acted directly on the enzyme.

Effect of Co^{2+}-chelate Compounds on Dextranase Activity

To investigate the necessity of free Co^{2+} ion in the activation system, Co^{2+}-chelate compounds were used as the activator instead of free cobalt ion. Results are presented in Table II indicating that these chelate compounds did not activate dextranases I and II. These results also suggested that free cobalt ion was necessary in the dextranase activation.

Table II. Effect of Metal-chelates on Dextranases I and II

Metal-chelate	Relative activity (%)	
	Dextranase I	Dextranase II
None	100	100
Bis(8-quinolinolate)Co^{2+}	91.4	91.2
Bis(salicylaldimine)Co^{2+}	91.0	91.6

Dextranase was incubated with metal-chelate (1.0×10^{-3}M) at 37° for 30 min, and then the dextranase activity was determined under the standard procedures.

Effect of Co^{2+} Concentration on Dextranase Activity

For an investigation of the effect of Co^{2+} concentration on the enzyme activity, the enzyme was preincubated with various concentrations of Co^{2+}, then the solution was diluted 500-times with deionized water. The dextranase activity was determined according to the previous methods.[3] As shown in Fig. 1, after the dilution the effect of Co^{2+} on the activation was observed at 1×10^{-2}—1×10^{-4}M, and at these concentrations, the activation rate was dependent on Co^{2+} concentration.

Effect of Adding Order of the Components to the Reaction Mixtures on the Reaction Rate

When metal ion activates the enzyme reaction, there are two possible cases, *i.e.* metal ion can bind to the enzyme and/or substrate and form active enzyme and/or active substrate.[15]

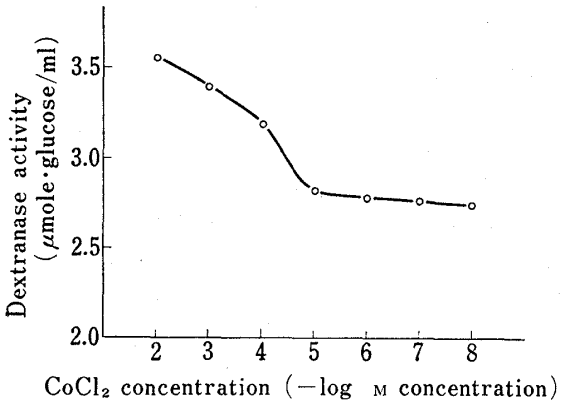

Fig. 1. Effect of Co^{2+} Concentration on Dextranase Activity

The enzyme was incubated with indicated concentrations of $CoCl_2$ at 30° for 60 min and diluted with cold deionized H_2O. The enzyme activity was determined under the standard conditions.

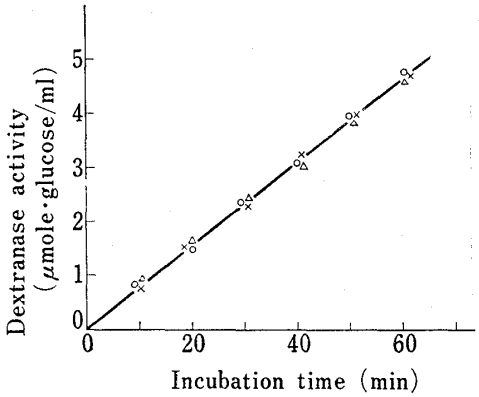

Fig. 2. Effect of the Adding Order of the Components in the Reaction Mixture on the Reaction Rate

$CoCl_2$ concentration was 3.0×10^{-4}M.
○: Mixture ot the enzyme and metal ion was incubated at 20° for 30 min and then the substrate was added. △: Mixture of substrate and metal ion was incubated at 20° for 30 min and then the enzyme was added. ×: The enzyme, metal ion and substrate were simultaneously mixed and dextranase activity was determined under the standard conditions.

15) G. Danno, *Agr. Biol. Chem.* (Tokyo), 35, 997 (1971).

Therefore, the effect of preincubation on the enzyme or substrate with Co^{2+} was investigated. Results are presented in Fig. 2. At the same incubation time, the initial reaction rates were the same in all experimental systems. Under each condition, the rate increases with incubation time. The results of Table I and these experiments suggested that Co^{2+} immediately bound to the enzyme and increased the dextranase activity.

Table III. Effect of EDTA on the Dextranase Activity in the Presence or Absence of Co^{2+}

Reaction system	Relative activity (%)
Enzyme+$CoCl_2$ (1×10^{-5}M)+EDTA (1×10^{-5}M)	99.8
Enzyme+$CoCl_2$ (2×10^{-5}M)+EDTA (2×10^{-5}M)	100
Enzyme+H_2O+EDTA (1×10^{-5}M)	98.1
Enzyme+H_2O+EDTA (2×10^{-5}M)	98.1
Enzyme+$CoCl_2$ (1×10^{-5}M)+H_2O	124.9
Enzyme+$CoCl_2$ (2×10^{-5}M)+H_2O	141.1

The enzyme was incubated with $CoCl_2$ or water at 37° for 20 min. Then the EDTA solution was added to the enzyme solution and the dextranase activity was assayed under the standard conditions.

Effect of EDTA on Co^{2+} Activation of Dextranase

In the presence of EDTA, the effect of Co^{2+} on dextranase was investigated, *i.e.* the effect of EDTA on the enzyme activated by Co^{2+} was observed. Results are presented in Table III. The enzyme activated by Co^{2+} showed 141% of relative activity at 1×10^{-4}M and 125% of it at 1×10^{-5}M, respectively, whereas these activations were not observed with the addition of EDTA in the dextranase reaction system. These results suggested that the activation of dextranase by Co^{2+} requires the simultaneous presence of free Co^{2+} and the enzyme in the dextranase reaction system.

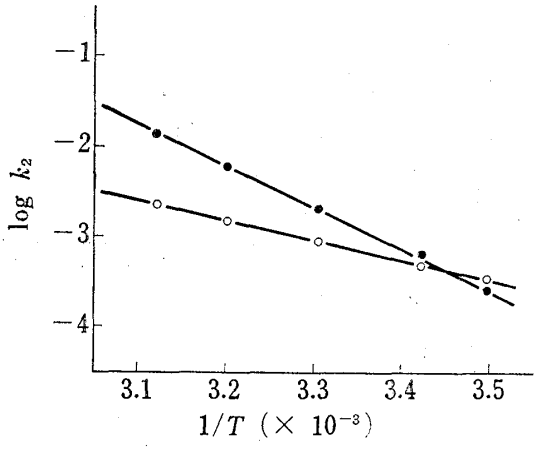

Fig. 3. Arrhenius Plots for Dextranase II Reaction in the Presence and Absence of $CoCl_2$

Reaction was done at indicated temperatures with or without $CoCl_2$ and other experimental conditions were the same as described in the text.
○: with $CoCl_2$ (3×10^{-4}M) ●: without $CoCl_2$

Effect of Co^{2+} on the Activation Energy, Michaelis Constant (K_m) and Maximum Reaction Rate (V_{max})

In the presence and absence of Co^{2+}, the activation energy was calculated from Arrhenius plots. As shown in Fig. 3, in the presence of Co^{2+}, the activation energy decreased from 9.0×10^3 cal/mole to 5.53×10^3 cal/mole. With dextranase reaction, K_m and V_{max} were also investigated at various Co^{2+} concentrations from Lineweaver-Burk plots. Results are presented in Table IV. K_m values were constant at various Co^{2+} concentrations, on the other hand, V_{max} value increased along with the increase of Co^{2+} concentration. Assuming the dextranase reaction mechanisms as shown in Table V, these results suggested that, in the dextranase reaction, Co^{2+} accelerates the decomposition of the enzyme-substrate complex into the enzyme and products, *i.e.* it causes the increase of the rate constant k, and that simultaneously the rate constant k_2 is larger than k_1.

Amino Acid Residues in the Enzyme Concerning Co^{2+} Activation

In order to investigate amino acid residues in dextranase relating to Co^{2+} activation, the effect of Co^{2+} on the enzyme modified with various reagents was examined. Results are presented in Table VI. Lysyl residues, arginyl residues, hystidyl residues and glutamyl residues in the enzyme were found to be not concerned with Co^{2+} activation, whereas in the

Table IV. K_m and V_{max} Values for Dextranases I and II at Various Co^{2+} Concentrations

$CoCl_2$ concentration (M)	V_{max} (μmole as glucose)	K_m ($\times 10^{-1}$%)
Dextranase I		
None	4.92	2.66
2×10^{-4}	6.50	2.87
1×10^{-4}	5.26	2.87
5×10^{-5}	5.21	2.87
2.5×10^{-5}	5.04	2.87
Dextranase II		
None	4.76	2.86
2×10^{-4}	6.42	2.99
1×10^{-4}	5.54	2.99
5×10^{-5}	5.32	2.97
2.5×10^{-5}	4.82	2.99

Dextranase activity was assayed in the presence of $CoCl_2$ under the standard conditions and each value was calculated by Lineweaver-Burk plots.

Table V. Reaction Mechanisms and Equations for the Velocity

In the absence of Co^{2+}

$$K_m:\ E + S \longrightarrow ES \xrightarrow{k_1} E + \text{products}$$

In the presence of Co^{2+}

$$K_m':\ E + S \longrightarrow ES \xrightarrow{k_2} E + \text{products}$$

The equations for the velocity are as follows, respectively.

$$v_1 = \frac{k_1 \cdot e}{\frac{K_m}{s}+1} \qquad v_2 = \frac{k_2 \cdot e}{\frac{K_m'}{s}+1}$$

$$\frac{1}{v_1} = \frac{K_m}{V_1}\cdot\frac{1}{s} + \frac{1}{V_1} \qquad \frac{1}{v_2} = \frac{K_m'}{V_2}\cdot\frac{1}{s} + \frac{1}{V_2}$$

Where, e and s are the concentrations of enzyme and substrate, respectively. K_m and K_m' are the dissociation constants of the enzyme-substrate complex.

Table VI. Effect of the Chemical Modification of Amino Groups in Dextranase II on Co^{2+} Activation

Modified amino group	Ratio of activation	Modified amino group	Ratio of activation
Native enzyme	1.00	Aspartic acid and glutamic acid	1.29
Lysine and free amino group	1.28	Cysteine[a]	1.08
Arginine	1.34	Cysteine[b]	1.01
Histidine	1.42		

The modification procedures were described in the text. Activity of the modified enzyme was determined under standard procedures.
a) modified by *p*-chlormercuribenzoic acid b) modified by iodoacetic acid

case of modification of cysteine residues, dextranase II was not activated by Co^{2+}. When the molecular weight of dextranase II was calculated to be 4.4×10^4(3), from the modification of SH-residues in the enzyme by DTNB,[16] the enzyme was found to contain two SH-residues per mole. By the modification with iodoacetic acid, these two SH-residues were carboxymethylated and then Co^{2+} activation of dextranase was not observed, but the enzyme activity was lost only partially and the residual activity was shown 43% of the native enzyme. These observations suggested that cysteine residues were not directly concerned with the enzyme activity.

On the basis of results described as above, a possible function of Co^{2+} for the dextranase activation is considered that Co^{2+} combines immediately with the cysteine residue which is situated out of the active center of the enzyme. This combination leads to the suitable conformational changes of dextranase for dextran hydrolysis and results in the acceleration of decomposition of the enzyme-substrate complex into products and the enzyme.

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16) 5,5′-Dithiobis(2-nitrobenzoic acid).