Kinetic Properties of Galactose Oxidase from *Gibberella fujikuroi*

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The kinetic properties of galactose oxidase of *Gibberella fujikuroi* were investigated. The enzyme oxidized D-galactose, 2-substituted D-galactoses, 2-epimer D-talose, and D-galactose-containing oligosaccharides, but not other saccharides. The enzyme was highly specific for molecular oxygen as an electron acceptor. The enzyme gave a nonlinear Lineweaver-Burk plot when the oxidation rate at a variety of galactose concentrations was investigated. The data were well explained by the assumption that the enzyme has two binding sites for galactose. K_1 and K_2 , which are the dissociation constants for the first and second bindings of galactose to the enzyme, were calculated to be 1.3 mM and 42 mM, respectively. The enzyme was mixedly inhibited by D-galacturonic acid.

Galactose oxidase (EC 1.1.3.9) is an enzyme which catalyzes the oxidation of D-galactose to D-galactohexodialdose with the reduction of molecular oxygen to hydrogen peroxide.¹⁾ In our previous studies, we reported that *Gibberella fujikuroi* excretes significant amounts of galactose oxidase into the culture medium,²⁾ and also reported the purification and some properties of the enzyme.³⁾ The present paper describes the substrate specificity and kinetic properties of *Gibberella* galactose oxidase.

MATERIALS AND METHODS

Enzyme preparation. Galactose oxidase was purified from a culture filtrate of *Gibberella fujikuroi* according to the methods described previously.^{2,3)} The purified enzyme preparation had a specific activity of 3.3 units/mg protein, and showed one major protein band of the enzyme and 2 or 3 other minor faint bands by silver staining after polyacrylamide gel electrophoresis.

Enzyme assay. Galactose oxidase activity was assayed by determining the hydrogen peroxide produced during oxidation of galactose with a 4-aminoantipyrine-phenol-peroxidase system, according to the method described previously.²⁾

Chemicals. D-Galactose was purchased from the

Nakarai Kagaku Co., Kyoto. Peroxidase from horseradish (Grade III, 100 units/mg solid) was a product of the Toyobo Co., Osaka. Other chemicals were the best commercial products available.

RESULTS

Substrate specificity

The substrate specificity was examined with various sugar substrates. In addition to D-galactose, a number of D-galactose analogues were oxidized, as shown in Table I. 2-Substituted galactoses such as 2-deoxy-D-galactose, D-galactosamine, and N-acetyl-D-galactosamine, a 2-epimer such as D-talose, and galactosides were effectively oxidized. However, L-galactose, D-galactose-1-phosphate, and other isomeric D-aldohexoses were not oxidized at all. D-Galactose-containing di- and oligosaccharides such as melibiose, raffinose, and stachyose were oxidized at about the same rate as was D-galactose. Dihydroxyacetone was also a good substrate.

The effect of various acceptors on the activity was also examined. Molecular oxygen was the only effective acceptor for electrons, and other acceptors tested were completely ineffective (Table II).

TABLE I. SUBSTRATE SPECIFICITY OF GALACTOSE OXIDASE IN THE OXIDATION OF VARIOUS SUGARS

The rates of oxidation were determined with 20 munits of purified enzyme under the standard assay conditions, and were expressed as a percentage of that of D-galactose.

Substrate	Relative activity (%)
D-Galactose	100
L-Galactose	0
D-Galactono- γ -lactone	0
D-Galacturonic acid	0
Galactitol	0
2-Deoxy-D-galactose	43
D-Galactosamine	11
N-Acetyl-D-galactosamine	60
D-Galactose-1-phosphate	0
D-Galactose-6-phosphate	0
Methyl- α -D-galactopyranoside	84
Methyl- β -D-galactopyranoside	123
UDP-galactose	6
D-Allose	0
D-Altose	0
D-Glucose	0
D-Mannose	0
D-Gulose	0
D-Idose	0
D-Talose	52
D-Fructose	1
Inositol	0
Maltose	0
Sucrose	0
Lactose	1
Melibiose	68
Raffinose	98
Stachyose	79
Glycerol	1
DL-Glyceraldehyde	0
Dihydroxyacetone	135
Dihydroxyacetone phosphate	0

Kinetics

The effect of galactose concentration in the range of 0.5 to 200 mM on the oxidation rate was examined. The substrate saturation curve showed a sigmoidal character at very low galactose concentrations, and the double reciprocal plot gave a downward convex curve, as shown in Fig. 1. From Hill's plot,⁴⁾ a value of 1.3 for n (Hill coefficient) was obtained, though data not shown. This type of behavior has been termed substrate activation, and may be explained by a mechanism in which the

substrate itself acts as a modifier.⁵⁾ Therefore, in order to account for the experimental data obtained here, the following assumptions were made:

1) *Gibberella* galactose oxidase has two binding sites for galactose, and the oxidation of galactose normally proceeds only when both of the two binding sites are occupied by galactose.

2) The two binding sites are qualitatively identical, and consequently which of the two sites is first occupied by galactose is decided by chance.

3) However, the first binding of galactose to the enzyme affects the second binding of galactose to the enzyme.

4) The other substrate, molecular oxygen, does not affect the bindings of galactose to the enzyme, and is not rate-limiting under the assay conditions used here.

The following scheme can be written based on these assumptions^{6,7)}:

where, K_1 stands for the dissociation constant in the first binding, and K_2 for the dissociation constant in the second binding ($K_1 \neq K_2$), as defined by the following equations:

$$K_1 = \frac{[E][S]}{[ES]} \quad (2)$$

$$K_2 = \frac{[ES][S]}{[ESS]} \quad (3)$$

Rapid-equilibrium treatment leads to the following rate equation:

$$v = k_{+5}[ESS] = \frac{V[S]^2}{K_1 K_2 + K_2[S] + [S]^2} \quad (4)$$

Where v is the oxidation rate of D-galactose. The following two cases were considered.

i) At high galactose concentrations where $[S] \gg K_1$ holds, Eq. (4) becomes the Michaelis-Menten type of equation as shown below:

$$v = \frac{V[S]}{K_2 + [S]} \quad (5)$$

TABLE II. SUBSTRATE SPECIFICITY OF GALACTOSE OXIDASE IN THE ACCEPTORS

The enzyme reaction was carried out in an evacuated Thunberg tube containing a mixture composed of 250 μ mol of galactose, 50 μ mol of TES-NH₄OH buffer, pH 7.0, 50 munits of purified enzyme, and each electron acceptor at the concentration specified in a total volume of 3 ml.

The reduction rate of each acceptor was determined by following the absorbance changes at the wavelengths indicated, and was expressed as a percentage of that of molecular oxygen, which was determined under the standard assay conditions.

Electron acceptor	Concentration (mM)	Wavelength observed (nm)	Relative activity (%)
O ₂	Dissolved ^a		100
Cytochrome <i>c</i> ^b	0.1	550	0
2,6-Dichlorophenolindophenol	0.1	600	0
2,6-Dichlorophenolindophenol plus phenazine methosulfate	0.1, 1	600	0
Ferricyanide	1	420	0
Methylene blue	0.01	650	0
Nitro blue tetrazolium	1	535	0
NAD	1	340	0
NADP	1	340	0

^a In distilled water.

^b Horse heart.

FIG. 1. Effect of D-Galactose Concentration on the *Gibberella* Galactose Oxidase Reaction.

A: The initial velocities (v) were determined under the standard assay conditions except that varying concentrations of D-galactose were used. Reaction time was 5 min in the range of 15–200 mM galactose, and 60 min in the range of 1.0–10 mM. The rate is expressed in mM per min. The insert shows the initial velocities in the range of 1.0–20 mM.

B: The double reciprocal plot was obtained from the data of A.

This equation is transformed into:

$$\frac{[S]}{v} = \frac{1}{V}[S] + \frac{K_2}{V} \quad (6)$$

The plot of $[S]/v$ vs. $[S]$ gave a straight line at high galactose concentrations above 30 mM, as shown in Fig. 2. The value of K_2 , which is the

intercept of the plot on the $[S]$ axis, was 42 mM.

ii) At low galactose concentrations where $[S] \ll K_2$ holds, Eq. (4) becomes:

$$v = \frac{V[S]^2}{K_1K_2 + K_2[S]} \quad (7)$$

This equation is transformed into:

FIG. 2. $[S]/v$ vs. $[S]$ Plot in the Range of High Galactose Concentrations.

The data of Fig. 1 in the range of 30~200 mM galactose were plotted in the form of $[S]/v$ vs. $[S]$.

FIG. 3. $[S]^2/v$ vs. $[S]$ Plot in the Range of Low Galactose Concentrations.

The data of Fig. 1 in the range of 0.5~30 mM galactose were plotted in the form of $[S]^2/v$ vs. $[S]$.

$$\frac{[S]^2}{v} = \frac{K_2}{V} [S] + \frac{K_1 K_2}{V} \quad (8)$$

The plot of $[S]^2/v$ vs. $[S]$ gave a straight line, as shown in Fig. 3. The value of K_1 , which is the intercept of the plot on the $[S]$ axis, was 1.3 mM.

The K_m value, which is $[S]$ at which $V/2$ is obtained, was calculated to be 43 mM from the relationship of

$$K_m = (K_2 + \sqrt{K_2^2 + 4K_1 K_2})/2.$$

Thus, the results obtained indicate that Eq. (4), derived from the two binding sites model, is consistent with the kinetic data obtained experimentally with *Gibberella* galactose oxidase.

TABLE III. EFFECT OF VARIOUS SUGARS ON THE ACTIVITY OF GALACTOSE OXIDASE

The enzyme activities were determined with 20 munits of purified enzyme under the standard assay conditions containing the substrate, galactose (250 μ mol), and the various other sugar effectors (250 μ mol) indicated, and were expressed as a percentage of the activity with no addition.

Sugar added	Relative activity (%)
No addition	100
D-Galactono- γ -lactone	76.3
D-Galacturonic acid	33.2
Galactitol	103
D-Glucose	99.4
D-Mannose	97.2
D-Fructose	87.5
D-Fucose	97.6
Inositol	101
Lactose	97.1
Maltose	104
Sucrose	105
Glycerol	102

FIG. 4. Double Reciprocal Plots of the Inhibitory Effect of D-Galacturonic Acid on Galactose Oxidase.

The initial velocities were determined under the standard assay conditions except that varying concentrations of D-galactose and D-galacturonic acid were used. D-Galacturonic acid concentration (mM): $\bullet$, 0; $\circ$, 20; $\blacktriangle$, 50.

Inhibition studies

The effects of various sugars on the enzyme activity were examined. Galactose analogues such as D-galacturonic acid and D-galactono- γ -lactone inhibited the enzyme activity, but sugars having no galactose configuration did

FIG. 5. Secondary Plots of the Slopes and Intercepts against D-Galacturonic Acid Concentration.

A: The slopes of the primary plots in Fig. 4 were replotted against the inhibitor concentration.

B: The intercepts of the primary plots in Fig. 4 were replotted against the inhibitor concentration.

not affect the enzyme activity (Table III). When the mode of inhibition was tested with

D-galacturonic acid, it mixedly inhibited the enzyme activity towards D-galactose, as shown in Fig. 4.

From this result, the following scheme was written⁸;

where, Ki , Ki' , and Ki'' are the inhibition constants defined by the following equations:

$$Ki = \frac{[E][I]}{[EI]} \quad (10)$$

$$Ki' = \frac{[ES][I]}{[ESI]} \quad (11)$$

$$Ki'' = \frac{[ESS][I]}{[ESSI]} \quad (12)$$

Rapid-equilibrium treatment leads to the following rate equation:

$$v = \frac{V[S]^2}{\left(1 + \frac{[I]}{Ki}\right) K_1 K_2 + \left(1 + \frac{[I]}{Ki'}\right) K_2 [S] + \left(1 + \frac{[I]}{Ki''}\right) [S]^2} \quad (13)$$

At high galactose concentrations where $[S] \gg K_1$ holds, Eq. (13) becomes:

$$v = \frac{V[S]}{\left(1 + \frac{[I]}{Ki'}\right) K_2 + \left(1 + \frac{[I]}{Ki''}\right) [S]} \quad (14)$$

From the secondary plots (Fig. 5), the inhibition constants, Ki' and Ki'' , were calculated to be 36 and 110 mM, respectively. From the relationship of $K_2/K_2' = Ki'/Ki''$, K_2' was calculated to be 127 mM. However, the inhibitory effect (Ki value) of D-galacturonic acid in the presence of low galactose concentrations could not be determined exactly because of experimental error.

DISCUSSION

The sugar substrate specificity of galactose oxidase of *Polyporus circinatus* (and/or *Dactylium dendroides*) is characterized as follows^{9~11}:

- 1) The pyranose ring is important for the enzyme activity.
- 2) The galactose configuration at carbon 4 is essential, but the configuration at carbon 2 is not so critical.
- 3) The carbon 1 position need not be free, and the β -configuration is somewhat favored.
- 4) Oligosaccharides containing a galactose residue at the nonreducing end are oxidized more rapidly than is free galactose. The sugar substrate specificity of *Gibberella* galactose

oxidase was essentially the same as that of galactose oxidase of *Polyporus circinatus* (and/or *Dactylium dendroides*), although oligosaccharides containing galactose residues were not necessarily oxidized more rapidly. These results suggest that galactose oxidases produced by these two microorganisms resemble each other closely in their catalytic properties, in spite of the differences in some of molecular properties described previously.³⁾ The structure of dihydroxyacetone seems to be apparently far from the galactose configuration, but may be in the proper orientation to be enzymatically oxidized. The kinetics of oxidation of dihydroxyacetone ($K_1=1.0$ mM and $K_2=10$ mM) was the same as those of the oxidation of galactose (data not shown), and hence both substrates seemed to be oxidized at the same catalytic sites of *Gibberella* galactose oxidase.

A concave curve of the Lineweaver–Burk plot was obtained when the oxidation rate of galactose by *Gibberella* galactose oxidase was examined by the use of a peroxidase–chromogen assay system. In order to confirm the completeness of the coupling reaction, the reactivity of peroxidase to hydrogen peroxide was examined under the same assay conditions. The quinoneimine dye was immediately formed (within one minute), and the amount was a linear function of the concentration of hydrogen peroxide added in the range of 0.01 to 0.20 mM (data not shown). Therefore, it seemed that this concave curve of the Lineweaver–Burk plot was based on the kinetic properties of *Gibberella* galactose oxidase itself rather than on those of peroxidase. However, it may be necessary to confirm the results obtained here further by other assay methods. Similar curvatures are reported in many enzymes, including the saccharifying α -amylase of *Bacillus subtilis*,¹²⁾ L-threonine deaminase of *Clostridium tetanomorphum*,¹³⁾ and deoxythymidine kinase of *E. coli*.¹⁴⁾ Yoshida *et al.*¹²⁾ accounted for this phenomenon by assuming that two kinds of substrate binding sites, one active and one activating, are present in the saccharifying α -amylase of *Bacillus sub-*

tilis, and that the binding of substrate to the activating site does not affect the binding at the active site, and *vice versa*. On the other hand, we could interpret the kinetic properties of *Gibberella* galactose oxidase by assuming the two identical galactose binding sites. Several different explanations of nonlinear plots may be possible.⁵⁾ These are seldom kinetically distinguishable, and must be studied by other methods. Furthermore, the numbers of galactose binding sites and the binding constants may be estimated more correctly by the binding assay with ¹⁴C-labeled galactose. However, the simple model described here may be chosen as a working hypothesis up to that time.

It has not been reported that the galactose oxidase of *Polyporus circinatus* (and/or *Dactylium dendroides*) shows a nonlinear Lineweaver–Burk plot, although *K_m* values for D-galactose varying from 2.8¹⁵⁾ to 240 mM⁹⁾ have been reported. *Gibberella* galactose oxidase showed a nonlinear Lineweaver–Burk plot when examined at a wide range of galactose concentrations, but showed a normal Lineweaver–Burk plot at high galactose concentrations, above 30 mM. These results suggest that more detailed studies using a wide range of galactose concentrations are necessary for the kinetic elucidation of the galactose oxidase of *Polyporus circinatus* (and/or *Dactylium dendroides*).

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