Kinetic Studies on Substrate Specificity and Active Site of β -D-Glucosidase F₁ from *Streptomyces* sp.

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The substrate specificity and the active site of β -D-glucosidase F₁ (M_r , 50,000; optimum pH, 5.5) purified from a *Streptomyces* sp. were kinetically investigated. The β -D-glucosidase showed a broad substrate specificity for synthetic glycosides and disaccharides having β -glycosidic linkage, but the former was more favorable substrate than the latter. The enzyme was characterized by the ability to hydrolyze rapidly not only *p*-nitrophenyl β -glucoside (K_m , 0.72 mM; k_0 , 63 s⁻¹) but also *p*-nitrophenyl β -fucoside (K_m , 0.19 mM; k_0 , 44 s⁻¹) and laminaribiose (K_m , 1.6 mM; k_0 , 70 s⁻¹). The kinetic study was made as to whether the hydrolyses of these synthetic glycosides were catalyzed at a single active site or at dual active sites in the β -D-glucosidase. In the experiments with the mixed substrates of *p*-nitrophenyl β -glucoside (PNPG) and *p*-nitrophenyl β -fucoside (PNPF) or *p*-nitrophenyl β -galactoside (PNPGal), the kinetic features, the linearity of Lineweaver-Burk plots and the dependence of the apparent maximal velocities and K_m value on the mole fraction (f) of PNPG in the mixed substrate, $f = \frac{[\text{PNPG}]}{[\text{PNPG}] + [\text{PNPF or PNPGal}]}$ agreed very closely with those theoretically predicted for a single catalytic site mechanism. The findings strongly support the notion that the β -D-glucosidase attacks the synthetic glycosides at a common active site.

β -Glucosidase (EC 3.2.1.21, β -D-glucoside glucohydrolase) is defined to be a β -anomeric configuration-retaining enzyme which catalyzes the liberation of β -D-glucose from the non-reducing terminal side of glucosides and gluco-oligosaccharides having β -D-glucosidic linkage. Various types of β -glucosidases are widely distributed in microorganisms,¹⁻⁶⁾ plants,⁷⁻¹²⁾ and animals.¹²⁻¹⁵⁾ Many β -glucosidases have been known to possess a broad substrate specificity showing β -D-fucosidase (EC 3.2.1.38), β -D-galactosidase (EC 3.2.1.23), and even α -L-arabinosidase^{8,14)} activities, although a β -glucosidase non-specific for β -glucoside and lactose has been found in *Aspergillus niger*.⁴⁾ On the contrary, β -fucosidase¹³⁾ has also been noted to show β -glucosidase and β -galactosidase activities, and β -galactosidase,¹⁴⁾ to

show β -glucosidase and β -fucosidase activities.

It is of interest to examine whether such a diversity of the substrate specificity for β -glucosidase is caused by a common catalytic site. The hydrolytic activities of some β -glucosidases from microorganisms,^{1,5)} plants^{7-9,11)} and animals^{12,14,15)} have been reported to be catalyzed at a single active site. In the β -fucosidase from a snail (*Helicella ericetorum*),¹³⁾ however, the existence of two active sites has been suggested, that is, β -fucosidase and β -glucosidase activities are catalyzed at a common active site, and β -galactosidase activity, at another active site.

Ozaki and Yamada²⁾ purified three β -D-glucosidases F₁, F₂ and F₃ from cell free extract of a *Streptomyces* sp. The β -D-glucosidases F₁ and F₂ seem to be enzymes characteristically similar to each other in substrate specificity, but β -D-glucosidase F₃ is different from the other two en-

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zymes in the kinetic feature of hydrolytic reaction. However, the kinetic analysis for the substrate specificity of these β -D-glucosidases has still not been thoroughly made.

The paper describes the kinetic analyses of the substrate specificity and the catalytic site of β -D-glucosidase F₁.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl β -D-glucosidase (PNPG), *p*-nitrophenyl β -D-galactoside (PNPGal), *p*-nitrophenyl β -D-fucoside (PNPF), *p*-nitrophenyl β -L-fucoside, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Chemical Co. (St. Louis, USA). Lactose monohydrate and cellobiose for substrate were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and cellobiose for culture medium from Sigma Chemical Co. Laminaribiose was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), gentiobiose, from Fluka Fine Chemical (Tokyo, Japan), and sophorose monohydrate, from Senn Chemicals USA Co. (San Diego, USA). Glucose AR-II Kit was a product of Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Yeast extract, malt extract, yeast malt extract agar, and yeast nitrogen base were obtained from Difco Laboratories (Detroit, USA). DEAE-Toyopearl 650 M was obtained from Tosoh Co. (Tokyo, Japan); Sephacryl S-200 HR and Mono Q HR 5/5, from Amersham Pharmacia Biotech (Uppsala, Sweden).

Bacterial strain and growth conditions. *Streptomyces* sp. No. 35 was isolated from soil as described previously.²⁾ Inoculated slants were incubated at 45°C for 48 h, and then stored at 4°C.

Spores scraped from one slant of *Streptomyces* sp. No. 35 were inoculated to 100 mL of pre-cultivate medium (yeast extract 4 g/L, malt extract 10 g/L and glucose 4 g/L; pH 7.0; autoclaved at 121°C for 20 min) in a flask and incubated at 45°C on a rotatory shaker (120 rpm) for 12 h. Then 3 mL of the pre-cultivate medium was inoculated to 100 mL of cultivate medium (yeast nitrogen base 6.7 g/L, cellobiose 2 g/L and CaCO₃ 20 g/L; pH 7.0; autoclaved at 121°C for 20 min) in a flask and incubated at 45°C on a rotatory shaker (120 rpm) for 5 h. Mycelia harvested were washed

with 0.2 % (w/v) KCl and frozen with liquid nitrogen. Frozen mycelia were stored at -180°C.

Enzyme assay. The standard reaction mixture for measuring β -glucosidase activity consisted of 200 μ L of substrate (12.5 mM glucoside or 125 mM disaccharide), 200 μ L of 0.2 M MES-NaOH buffer (pH 5.5), and 100 μ L of enzyme solution containing 0.1 % bovine serum albumin (BSA). The reaction mixture was incubated at 30°C for 10 min. The reaction for *p*-nitrophenyl β -glycoside was stopped by adding 1 mL of 1 M Na₂CO₃ and the absorbance of liberated *p*-nitrophenol was measured at 400 nm. One unit of the β -glucosidase activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol per 1 min from *p*-nitrophenyl β -glucoside in the standard reaction mixture. The amount of glucose liberated from glucobiose was measured by the glucose oxidase-peroxidase method^{16,17)} with our modification using¹⁸⁾ "Glucose AR-II" Wako Pure Chemical Ind., Ltd. The reaction was stopped by adding 1 mL of 2 M Tris-HCl buffer (pH 7.0) and boiling for 3 min.

Protein determination. The concentration of β -D-glucosidase F₁ was determined spectrophotometrically on the basis of the value for $E_{1\text{cm}}^{1\%}$ at 280 nm was 13.8, which was obtained from the relationship between the dry weight and optical density for the purified enzyme preparation.¹⁹⁾

Purification. The β -D-glucosidase F₁ was newly purified according to a modification of the procedure described by Ozaki and Yamada.²⁾ In the present purification procedures, the column chromatographies using Toyopearl TSK-HW55 and using Sephacryl S-200HR and Bio-Gel HT were as stated below. Frozen mycelium (20 g) in a blue top falcon tube (50 mL) was suspended in 10 mL of 50 mM sodium phosphate buffer (pH 6.5), and then sonicated with Branson Sonifier 250 (Branson Co.) under the conditions of 40 W, duty cycle 50 %, for 4 min in ice-water. The sonicated suspension was centrifuged twice at 28,000 $\times g$ for 30 min, and the supernatant was used as crude extract. All purification procedures were carried out at 4°C unless otherwise noted. The crude extract was chromatographed twice on a DEAE-Toyopearl 650M column (2.0 $\times$ 30 cm) that had been equili-

brated with 10 mM phosphate buffer (pH 8.3), followed by elution with 0 to 0.5 M linear gradient of NaCl. β -Glucosidase active fractions were collected, concentrated, and dialyzed to 10 mM phosphate buffer (pH 8.3) containing 0.1 M NaCl overnight, and loaded on a Sephacryl S-200 HR column (3.0 $\times$ 73 cm) that had been equilibrated with 10 mM phosphate buffer (pH 8.3) and 0.1 M NaCl, and eluted with the same buffer. The active fractions were collected, concentrated, and dialyzed to 10 mM phosphate buffer (pH 6.3) overnight, and chromatographed at room temperature in a fast performance liquid chromatography system using a Mono Q HR 5/5 column that had been equilibrated with 10 mM phosphate buffer (pH 6.3), to elute with 0 to 0.3 M linear gradient of NaCl. The active fractions were collected and dialyzed to 10 mM phosphate buffer (pH 6.2) overnight, and chromatographed on a Bio-Gel HT column (0.7 $\times$ 4 cm) that had been equilibrated with 10 mM phosphate buffer (pH 6.2). β -Glucosidase was eluted in the non-adsorbed fraction from the column. The non-adsorbed fraction was concentrated, and dialyzed to 10 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl overnight, and was chromatographed again by a Sephacryl S-200 HR column (3.0 $\times$ 73 cm), that had been equilibrated with 10 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl and eluted with the same buffer. The active fractions were combined and used as the purified β -D-glucosidase F₁. The enzyme preparation migrated as a single protein band by disc polyacrylamide gel electrophoresis, and its sugar content was found to be less than 0.5% by the phenol-sulfuric acid method.²⁰⁾

Determination of molecular weight. Molecular weight of β -D-glucosidase F₁ was estimated by SDS-polyacrylamide gel electrophoresis (PAGE).²¹⁾ In SDS-PAGE, myosin (M_r , 200,000), phosphorylase *b* (M_r , 97,000), BSA (M_r , 68,000), ovalbumin (M_r , 43,000) and carbonic anhydrase (M_r , 29,000) were used as standards. The detection of protein was done with Coomassie Brilliant Blue G-250 (CBB).

RESULTS AND DISCUSSION

Properties of β -D-glucosidase F₁.

Figure 1 shows the final elution pattern of β -D-glucosidase F₁ from Sephacryl S-100 HR column. SDS-PAGE indicated that the molecular weight was estimated to be 50,000 by a comparison with the marker proteins, and to be 45,000 by gel chromatography with a Sephacryl S-200 HR column (3.0 $\times$ 73 cm, eluted with 10 mM phosphate buffer at pH 6.5 containing 0.1 M NaCl; data not shown), implying that the enzyme was a monomeric protein. Though Ozaki and Yamada²⁾ reported that β -D-glucosidase F₁ was dimer, we could not obtain evidence in support of the dimerization. The reason for such a difference in the estimation of the molecular weight remains equivocal.

Optimum pH of β -D-glucosidase F₁ has been described to be in the range of pH 6.0 to 6.5.²⁾ As shown in Fig. 2 (a), however, the optimum pH of the newly purified β -D-glucosidase F₁ was ob-

Fig. 1. Chromatography of *Streptomyces* sp. β -D-glucosidase F₁ on Sephacryl S-100 HR column and SDS-PAGE of the purified enzyme.

Details of procedure are described in MATERIALS AND METHODS. Fraction nos. 35, 36 and 37 were pooled as purified β -glucosidase F₁. $\circ$, activity (U/mL); $\bullet$, absorbance at 280 nm. The purified enzyme (1.9 μ g) was subjected to a 10% SDS polyacrylamide gel electrophoresis, and the detection was done with CBB G-250. Lane 1, molecular size markers; myosin (M_r , 200,000), phosphorylase *b* (M_r , 97,000), BSA (M_r , 68,000), ovalbumin (M_r , 43,000) and carbonic anhydrase (M_r , 29,000). Lane 2, purified β -glucosidase F₁ from a *Streptomyces* sp. The enzyme is indicated by an arrowhead.

Fig. 2. Effects of pH and temperature on β -D-glucosidase F_1 from a *Streptomyces* sp.

In all three panels, open circle, open triangle, and open square indicate PNPG, PNPGal and PNPF, respectively. (a) pH activity curves: 500 μ L of each reaction mixture containing 0.18 μ g of the purified enzyme, 0.02% BSA, 5 mM substrate and Britton-Robinson buffer was incubated at 30°C for 5 min; (b) pH stability curves: 100 μ L of a mixture containing 1.8 μ g of the purified enzyme, 0.04% BSA and Britton-Robinson buffer had been kept at 4°C for 24 h, and 10 μ L of an aliquot was incubated with 90 μ L of 0.1% BSA/0.1 M MES-NaOH (pH 5.5), 200 μ L of 0.2 M MES-NaOH (pH 5.5), and 200 μ L of 12.5 mM substrate at 30°C for 5 min; (c) thermal stability curves: 100 μ L of a mixture containing 0.18 μ g of the purified enzyme, 0.1% BSA, and 0.1 M MES-NaOH (pH 5.5) had been kept at various temperatures for 15 min, then 200 μ L of 0.2 M MES-NaOH (pH 5.5) and 200 μ L of 12.5 mM substrate were added, and the mixture was incubated at 30°C for 5 min.

served to be about 5.5 by using three kinds of *p*-nitrophenyl β -glycosides.

Activities on *p*-nitrophenyl β -glycosides agreed closely with one another in the pH-stability and thermal-stability curves in Fig. 2 (b) and (c), suggesting that the substrates were hydrolyzed at a single catalytic site.

Substrate specificity.

The substrate specificity of β -D-glucosidase F_1 was examined with various kinds of glycosides and disaccharides having α - or β -glucosidic linkage. Table 1 lists the relative rates for hydrolyses of various glycosides at 5 mM and disaccharides at 50 mM. The β -D-glucosidase was characterized by an ability to hydrolyze rapidly not only *p*-nitrophenyl β -D-glucoside (PNPG) but also *p*-nitrophenyl β -fucoside (PNPF) as glycosides, and laminaribiose as disaccharide. The measurement of hydrolytic activity for PNPG of a favorable substrate has not been done by Ozaki and Yamada.²⁾ Among β -glucobioses, laminaribiose is the most preferable to the enzyme, but the relative rate in hydrolysis of laminaribiose to cellobiose differs too greatly between the data of the present paper and their paper.²⁾ The comparison for the substrate

Table 1. Relative rates for the hydrolyses of glycosides and disaccharides by β -glucosidase F_1 from a *Streptomyces* sp.

Substrate		Relative rate (%)
Glycoside (5 mM)		
<i>p</i> -Nitrophenyl β -D-glucoside		100
<i>p</i> -Nitrophenyl α -D-glucoside		0
<i>p</i> -Nitrophenyl β -D-galactoside		26
<i>p</i> -Nitrophenyl α -D-galactoside		0
<i>p</i> -Nitrophenyl β -D-fucoside		92
<i>p</i> -Nitrophenyl β -L-fucoside		0
<i>p</i> -Nitrophenyl α -L-arabinopyranoside		0
<i>p</i> -Nitrophenyl α -L-arabinofuranoside		0
<i>p</i> -Nitrophenyl α -D-mannoside		0
Disaccharide (50 mM)		
(linkage)		
Cellobiose	(Glc β -1, 4 Glc)	100
Sophorose	(Glc β -1, 2 Glc)	110
Laminaribiose	(Glc β -1, 3 Glc)	220
Gentiobiose	(Glc β -1, 6 Glc)	15
Lactose	(Gal β -1, 4 Glc)	23
Maltose	(Glc α -1, 4 Glc)	0
Melibiose	(Gal α -1, 6 Glc)	0

specificity at a definite substrate concentration is qualitative and not always reasonable. Therefore, it is the most appropriate to quantitatively compare the substrate specificity on the basis of the rate parameters kinetically measured.

The Lineweaver-Burk double reciprocal plots

Fig. 3. Lineweaver-Burk plots for the hydrolyses of several disaccharides and glycosides having β -glycosidic linkage.

The enzyme reactions were done at 30 °C and at pH 5.5. When synthetic glycosides were used as substrates, kinetic parameters were calculated by measuring the amount of liberated *p*-nitrophenol. When disaccharides were used as substrates, kinetic parameters were calculated by measuring the amount of liberated glucose from the non-reducing terminal of substrate. The amount of enzyme used was as follows: for 0.25–0.5 mM and 1.0–4.0 mM PNP, 0.38 μ g and 0.19 μ g, respectively, for PNPGal and PNP, 0.057 μ g; for sophorose, 0.57 μ g; for laminaribiose, 0.28 μ g; for cellobiose, 0.51 μ g; for gentiobiose, 2.3 μ g; for lactose, 1.0 μ g. ○, PNP; ●, PNPGal; △, PNP; ▲, sophorose; □, laminaribiose; ■, cellobiose; ◇, gentiobiose; ◆, lactose.

Table 2. Kinetic parameters for the hydrolyses of various substrates by β -glucosidase F₁ from a *Streptomyces* sp.

Substrate	K_m (mM)	V (μ mol/min/mg protein)	k_0 (s ⁻¹)	k_0/K_m (s ⁻¹ · mM ⁻¹)
<i>p</i> -Nitrophenyl β -D-glucoside	0.72	55	63	88
<i>p</i> -Nitrophenyl β -D-fucoside	0.19	39	44	230
<i>p</i> -Nitrophenyl β -D-galactoside	31	64	73	2.4
Cellobiose	6.4	31	35	5.5
Sophorose	7.0	50	57	8.1
Laminaribiose	1.6	59	70	44
Gentiobiose	2.6	5.0	5.7	2.2
Lactose	88	23	26	0.30

for some glycosides and disaccharides are shown in Fig. 3. The kinetic parameters for the hydrolyses of the substrates are summarized in Table 2. The data demonstrate that glycosides seem to be more favorable substrate than disaccharides; in particular, the catalytic efficiency (k_0/K_m) for PNP is significantly higher than those for other substrates. Therefore, the β -glucosidase from a *Streptomyces* sp. may be designated as a β -D-fucosidase as well as other β -glycosidases.^{1,7,10,11,14)}

The β -D-glucosidase showed no activity toward α -L-arabinoside.

Evidence for a single active site: kinetic behavior on mixed substrates.

When a certain enzyme acts on a mixture of two types of substrates (A and B) to form a common product, the kinetic features are different, depending on whether the two substrate are hydrolyzed at a single active site, or at double active

sites specific for each substrate. Conclusive evidence for a single active site mechanism or dual sites mechanism is provided by analyzing the kinetic features^{22,23)} in the mixed substrate system: 1) the possibility of competition between the two substrates, 2) the linearity of Lineweaver-Burk plots, and 3) the dependence of the apparent maximum velocity (V) on the mole fraction (f) of A, $f = [A]/([A] + [B])$. In this case, A is PNPG and B is PNPGal or PNPF.

Figure 4 shows the Lineweaver-Burk plots for hydrolyses of the mixed substrates, PNPG and

Fig. 4. Profiles of $1/v$ versus $1/s$ for the hydrolyses of mixed substrates.

Each reaction mixture containing $0.08 \mu\text{g}$ of the purified enzyme, 0.02% BSA, 0.1 M MES-NaOH (pH 5.5), and substrates in a total volume of $500 \mu\text{L}$ was incubated at 30°C . In each panel, the theoretical curves and the experimental values are represented by solid lines and open circles, respectively. (a) The mixed substrates of PNPG and PNPGal; $f_{g\text{-gal}} = [\text{PNPG}]/([\text{PNPG}] + [\text{PNPGal}])$. (b) The mixed substrates of PNPG and PNPF; $f_{g\text{-f}} = [\text{PNPG}]/([\text{PNPG}] + [\text{PNPF}])$.

PNPGal (or PNPF), at several mole fractions, where v is the initial velocity for the total substrate concentration s , the sum of the concentrations of the two substrates, and the mole fraction of each substrate is indicated as $f_{g\text{-gal}} = [\text{PNPG}]/([\text{PNPG}] + [\text{PNPGal}])$ in Fig. 4 (a), or $f_{g\text{-f}} = [\text{PNPG}]/([\text{PNPG}] + [\text{PNPF}])$ in Fig. 4 (b): at $f_{g\text{-gal}}$ and $f_{g\text{-f}} = 1$, only PNPG is present, and at $f_{g\text{-gal}}$ and $f_{g\text{-f}} = 0$, only PNPGal (or PNPF) is present. The v values for each $f_{g\text{-gal}}$ (or $f_{g\text{-f}}$) were observed to be always less than the sum of the initial velocities for the hydrolysis of each substrate, v_1 and v_2 : $v < v_1 + v_2$; that is, the competition between the two substrates was observed. The relationship between $1/v$ versus $1/s$ indicates a clear linearity at each f value. The V values calculated from each straight line changed continuously with f values without a maximum or minimum. Such kinetic features suggest characteristics of a reaction in which the enzyme hydrolyzes the two substrates at a single active site.

If PNPG and PNPGal (or PNPF) are hydrolyzed

Fig. 5. Dependence of K_m and V on f .

The K_m and V values were calculated from profiles of $1/v$ versus $1/s$ plots in Fig. 4. In each panel, the theoretical curves and the experimental values are represented by solid lines and open circles, respectively. (a) the K_m values as functions of the mole fraction $f_{g\text{-gal}}$, (b) the V values as functions of the mole fraction $f_{g\text{-gal}}$, (c) the K_m values as functions of the mole fraction $f_{g\text{-f}}$, (d) the V values as functions of the mole fraction $f_{g\text{-f}}$.

at a common catalytic site, the apparent maximum velocity (V) and the apparent Michaelis constant (K_m) can be expressed as a function of f by the following formula,^[1,22-25]

$$V = \frac{V_1 K_A f + V_2 K_B (1-f)}{K_A f + K_B (1-f)}$$

$$K_m = \frac{1}{K_A f + K_B (1-f)}$$

where K_A and K_B are reciprocals of K_m values for PNPG and PNPGal (or PNPF), and V_1 and V_2 are V values at $f=1$ and $f=0$, respectively. Figure 5 demonstrates the dependence of the experimental and theoretical values of V and K_m on f . The experimental data are in good agreement with the theoretical ones predicted for a single active site mechanism. The findings strongly support the notion that PNPG, PNPGal and PNPF are hydrolyzed at a common catalytic site in *Streptomyces* sp. β -D-glucosidase F_I.

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Streptomyces sp. β -D-グルコシダーゼ F_1 の基質特異性および活性部位に関する反応速度論的解析

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Streptomyces sp. から精製した β -D-グルコシダーゼ

F_1 (分子量, 50,000; 最適 pH, 5.5) の基質特異性と活性部位について反応速度論的な検討を行った. 本酵素は, β -グルコシド結合からなる各種の合成基質や二糖に対し広い基質特異性を示したが, 二糖よりも合成基質に対し高い特異性を示した. 合成基質では p -ニトロフェニル β -グルコシド (K_m , 0.72 mM; k_a , 63 s⁻¹) よりも p -ニトロフェニル β -フコシド (K_m , 0.19 mM; k_a , 44 s⁻¹) が, 二糖ではセロビオースよりもラミナリビオース (K_m , 1.6 mM; k_a , 70 s⁻¹) が加水分解の反応効率からみてより優れた基質と考えられた. p -ニトロフェニル β -グルコシド (PNPG), β -フコシド (PNPF) および β -ガラクトシド (PNPGal) が共通の活性部位で加水分解されるか否かについて, 混合基質を用いた反応系において速度論的解析を行った. PNPG と PNPF (または PNPGal) の混合基質の加水分解におけるいくつかの速度論的特徴, 特に Lineweaver-Burk プロットの直線性, あるいは PNPG の他基質に対するモル分率 (f), $f = [\text{PNPG}] / ([\text{PNPG}] + [\text{PNPF} \text{ または } \text{PNPGal}])$ の最大速度とミカエリス定数への依存性の実験値は単一触媒部位機構において理論的に予測される値によく一致した. これらの実験結果は, 本酵素がこれら三種の合成基質を共通の活性部位で分解していることを支持している.