Intracellular Distribution and Some Properties of β -Glucosidases of a Cellulolytic *Pseudomonad*

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Received May 17, 1976

Two distinct forms of β -glucosidase, A and B, were found to occur in the cells of *Pseudomonas fluorescens* var. *cellulosa*: A was membrane-bound, while B cytosolic. They differed also from each other in some properties, such as molecular size, kinetic parameters, and susceptibility to various compounds. β -Glucosidase B was partially purified and studied especially of its substrate specificity. The results indicated that it may be an atypical β -glucosidase which possesses a certain character of exo-cellulase.

Our previous papers have shown that the cellulolytic system of *Pseudomonas fluorescens* var. *cellulosa* consists of a cell-bound and two extracellular endo- β -1, 4-glucanases (cellulases C, A and B) and intracellular β -glucosidases.¹⁻⁴⁾ Another extracellular cellulase has been indicated to occur, which was much more active than the above enzymes in saccharification of a microcrystalline cellulose (Avicel) and consequently seemed to be a β -1, 4-glucanase of exo-type.⁵⁾ However, information concerning the character and intracellular localization of these enzymes is still too little to understand the whole process of cellulolysis by this pseudomonad.

The present paper reports the intracellular distribution, partial purification and some properties of two β -glucosidases of this bacterium.

MATERIALS AND METHODS

Organism and its culture conditions. The same strain of *Pseudomonas fluorescens* var. *cellulosa* as used in the previous papers¹⁻⁵⁾ was grown at 37°C on a rotary shaker (210~230 rpm) in appropriate Erlenmeyer flasks containing 1% (w/v) CMC** in the basal

medium of a volume of around 80% of the flask volume.

Crude intracellular enzyme preparation (S7). The bacterial cells were harvested after 9-hr of incubation by centrifugation at 7000 $\times g$ for 20 min, suspended in 0.02 M phosphate buffer, pH 6.0, and disintegrated by sonication at 5 kc for 15 min. Supernatant (S7) obtained from the sonicate by centrifugation at 7000 $\times g$ for 30 min was used as a starting material for enzyme fractionation.

Enzyme assays. The reaction mixture (1 ml) was composed of a substrate solution, 0.02 M phosphate buffer of pH 6.0 and an enzyme solution in 1:2:1 by volume and incubated at 30°C for appropriate period. The substrate solution contained, unless otherwise stated, 0.5% NPG, 0.032% cellobiose or other cellooligosaccharides, 1% CMC, and 1% Avicel (suspension).

NPGase activity was assayed as before⁴⁾ by measuring NP liberated, and expressed in terms of extinction at 420 nm (E_{420}) or as NPGase activity unit; one unit corresponds to an enzyme amount which releases 1 μ mole of NP per ml per hr.

Cellobiase activity was assayed by estimating the increase in reducing power in terms of E_{800} according to the method of Somogyi-Nelson.⁶⁾ In some cases, liberated glucose was determined by the glucose oxidase method.⁷⁾ Hydrolysis of other cellooligosaccharides was pursued in the same way as for cellobiase activity.

CMCase and Avicel-saccharifying activities were assayed similarly by measuring the increase in reducing power (E_{800}).

Gel-filtration on Bio-Gel P-150 column. The sample was applied on a column (4 $\times$ 60 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.0. Elution was performed with the same buffer at 5°C at a flow

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** Abbreviations: CMC, sodium carboxymethyl cellulose; CMCase, carboxymethyl cellulase (CMC-saccharification); DOC, sodium deoxycholate; DP, degree of polymerization; DS, degree of substitution; NP, *p*-nitrophenol; NPC, *p*-nitrophenyl β -cellobioside; NPG, *p*-nitrophenyl β -glucoside; NPGase, *p*-nitrophenyl β -glucosidase.

rate of 20 ml/hr.

Column chromatography with DEAE- and CM-cellulose. Each of the ion-exchange cellulose columns (2×20 cm) was equilibrated with 0.02 M phosphate buffer, pH 6.0. Elution was carried out with the same buffer at 5°C at a flow rate of 50 ml/hr.

Paper chromatography of sugars. Development was performed by the descending method on Tōyō No. 51 filter paper with *n*-butanol: pyridine: water (6: 4: 3) at room temperature. Spots were detected by the dipping technique in silver nitrate-sodium hydroxide reagents.⁸⁾

Chemicals. NPG and NPC were synthesized by the method of Glaser and Wulvek.⁹⁾ Cellooligosaccharides were prepared from Avicel by the method of Miller *et al.*¹⁰⁻¹¹⁾ Nojirimycin bisulfite was a gift of Dr. T. Niwa, Meiji Seika Kaisha, Ltd. CMC of DS 0.62 (Daiichi Kogyo Seiyaku Co.), Avicel (American Viscose Co.), and all other chemicals were commercial products.

RESULTS

Occurrence of two forms of intracellular β -glucosidase

As shown in Fig. 1-A, two peaks of NPGase activity (PI and PII) were detected in a crude intracellular enzyme preparation (S7) upon its gel-filtration on Bio-Gel P-150 column. S7 was then fractionated as shown in Fig. 2 and the fractions (S26, S105 and P105) were each analyzed for PI and PII by gel-filtration (Fig. 1-B, C and D). As the centrifugal force increased, PI disappeared from the supernatant fractions and appeared in the pellet fractions. Thus, PI seemed to be a particulate or membrane-bound enzyme. On the other hand, PII was always recovered in the supernatant, indicating that PII is a cytosolic enzyme. Furthermore, when PI was solubilized by treating P 26 with DOC (S 26'), the enzyme activity was still eluted from the column at the position near that of PI (Fig. 1-E). This suggested that the enzyme proteins in PI and PII fractions may differ from each other in their molecular sizes.

Partial purification of β -glucosidase components

According to the procedure outlined in Fig.

FIG. 1. Gel-filtration Patterns of β -Glucosidase on Bio-gel P-150 Column of the Fractions Prepared as Shown in Fig. 2.

NPGase activities (E_{420}): after 3-hr reaction with eluates from A (S7, supernatant fraction at $7000 \times g$ from cell-sonicates), B (S26, supernatant fraction at $26,000 \times g$ from S7) and C (S105, supernatant fraction at $105,000 \times g$ from S26); after 24-hr reaction with eluates from D (P105, precipitate fraction at $105,000 \times g$ from S26) and E (S26', supernatant fraction at $26,000 \times g$ from DOC-treated P26).

2, S26 was prepared in a large scale (10 liters of culture) and subjected to gel-filtration. Figure 3 shows one of the patterns obtained. PI (fractions 16~20) and PII (fractions 21~30) were concentrated separately by ultrafiltration through collodion bag at 5°C.

It was found from preliminary tests that almost all NPGase activities of PI and PII were recovered in the unadsorbed fraction on ion-exchange cellulose column chromatography, while CMCase activities were desorbed only after elution with the buffer containing 0.1 M or more NaCl. PI and PII were therefore chromatographed on DEAE- and CM-cellulose columns, respectively, to remove contaminating cellulase. Fractions with NPGase-activity from PI (8~14) and those from PII (9~11) were concentrated by ultrafiltration, which yielded β -glucosidase A and B, respectively. Purification of both enzymes is summarized in Table I.

β -Glucosidase A showed no detectable activi-

FIG. 2. Fractionation of Two Forms of β -Glucosidase in a Crude Enzyme Extract (S7).

activities toward these three substrates were eluted to form similar profiles to one another, as in the first chromatography. Furthermore, the ratios of these enzyme activities for three substrates were different between PII and β -glucosidase B, but nearly identical before and after rechromatography of β -glucosidase B (Table II). These results seem to suggest that these three activities of β -glucosidase B may be due to the same enzyme.

TABLE I. PARTIAL PURIFICATION OF β -GLUCOSIDASE A AND B

Fractionation step	Total protein ^{a)} (mg)	Total NPGase activity (units)	Specific activity (unit/mg)
Supernatant of cell-sonicates at 26,000 $\times g$: S26	135	6.98	0.052
Bio-Gel P-150 gel-filtration of S26: PI	48	1.03	0.022
PII	24	3.18	0.132
DEAE-cellulose column chromato- graphy of PI: β -glucosidase A	5.6	0.57	0.102
CM-cellulose column chromato- graphy of PII: β -glucosidase B	4.1	2.49	0.609

^{a)} Measured from E_{290} using bovine serum albumin as a standard.

TABLE II. NPGASE, CMCase AND CELLOBIASE ACTIVITIES OF PII AND β -GLUCOSIDASE B BEFORE AND AFTER RECHROMATOGRAPHY ON CM-CELLULOSE COLUMN

Enzyme preparation	Protein (mg/ml)	NPGase activity (μ mole NP/hr)	CMCase activity ^{a)} (μ mole glucose/hr)	Cellobiase activity ^{a)} (μ mole glucose/hr)
PII	22	0.243 (1.00) ^{b)}	0.514 (2.12) ^{b)}	1.48 (6.09) ^{b)}
β -Glucosidase B	6.3	0.202 (1.00)	0.079 (0.48)	0.93 (4.60)
Rechromatographed β -glucosidase B	4.2	0.168 (1.00)	0.081 (0.48)	0.89 (5.29)

^{a)} Assayed by the glucose oxidase method.

^{b)} Relative values to NPGase activity.

Some properties of β -glucosidase A and B

pH-activity relation: The pH optima of NPGase activities of both enzymes were at about 7 (Fig. 5).

Thermostability: As shown in Fig. 6, β -glucosidase B was more thermostable than A.

FIG. 5. Effect of pH on the Activities of β -Glucosidase A and B.

NPGase activities at various pH values (McIlvaine buffer, 0.05 M) were expressed as percentage of the maximum.

●—●, β -glucosidase A; ○—○, β -glucosidase B.

Effect of various metal salts, EDTA and Nojirimycin bisulfite: As summarized in Table III, both enzymes were inhibited by Hg^{2+} , Zn^{2+} , Cu^{2+} , IO_3^- and N_3^- , but not by K^+ , Na^+ and HSO_4^{2-} . β -Glucosidase A was inhibited by Mn^{2+} , CN^- , NO_2^- and EDTA, but B was not inhibited by them. Nojirimycin,

FIG. 6. Thermostabilities of β -Glucosidase A and B.

Each enzyme solution was heated for 5 min at pH 6.0 at temperature indicated, and cooled in ice bath. Residual NPGase activity was expressed as percentage of the maximum.

●—●, β -glucosidase A; ○—○, β -glucosidase B.

TABLE III. EFFECTS OF VARIOUS COMPOUNDS ON NPGASE ACTIVITIES OF β -GLUCOSIDASE A AND B

Compound (1 mM)	Relative activity ^{a)}	
	A	B
None (control)	100	100
KCl	96	100
NaCl	98	115
MgCl ₂	100	95
CaCl ₂	74	110
MnCl ₂	57	90
CuSO ₄	13	20
ZnCl ₂	51	20
HgCl ₂	0	0
KCN	65	190
NaIO ₃	65	30
NaNO ₂	24	100
NaN ₃	30	16
Na ₂ HAsO ₄	96	100
EDTA	22	120
Nojirimycin bisulfite ^{b)}	104	58

^{a)} Reaction mixtures with β -glucosidase A and B were incubated for 24 hr and 4 hr, respectively.

^{b)} 2.3×10^{-5} M.

FIG. 7. Double Reciprocal Plots of Reaction Velocity (V , $E_{420} \cdot \text{hr}^{-1}$) versus NPG Concentration (S , mM).

●—●, β -glucosidase A; ○—○, β -glucosidase B.

known as a potent inhibitor of both α - and β -glucosidases,¹²⁾ inhibited β -glucosidase B but not A.

Substrate concentration-activity relation: Apparent K_m -values of both enzymes for NPG were determined as 1.9×10^{-3} and 6.1×10^{-3} M, respectively (Fig. 7).

Substrate specificity of β -glucosidase B

Activities of β -glucosidase B for various

TABLE IV. HYDROLYSIS OF VARIOUS β -GLUCOSIDES BY β -GLUCOSIDASE B^{a)}

Substrate	Concentration (mM)	m μ mole glucose liberated/min
Cellobiose	0.175	15.4
Gentiobiose	"	0.0
Laminaribiose	"	11.3
Sophorose	"	10.3
NPC	1.08	36.0 (0.633) ^{b)}

^{a)} The reaction mixture contained 0.06 NPGase unit of enzyme.

^{b)} m μ mole NP liberated/min.

β -glucosides are shown in Table IV. Under the reaction condition employed, this enzyme split all the β -glucosides tested except gentiobiose. Evidently, it split NPC preferentially at the holosidic bond.

Hydrolysis products from celooligosaccharides and CMC were studied by paper chromatography (Fig. 8). The main product was glucose in all cases.

Apparent K_m -values for celooligosaccharides of DP 3–6 were estimated from Lineweaver-Burk plots (Fig. 9) as 0.162, 0.145,

FIG. 8. Paper Chromatograms of Hydrolyzed Products of Celooligosaccharides and CMC by β -Glucosidase B.

The reaction mixture consisted of 5 mg of each substrate indicated and 0.5 ml (0.6 NPGase unit) of enzyme preparation. Incubation was for 15 min for celooligosaccharides, but it was for 4 hr for CMC. M, markers: G, glucose; G₂–G₆, cellobiose-cellohexaose.

FIG. 9. Double Reciprocal Plots of Reaction Velocities (V , E_{420}/hr) of Hydrolysis with β -Glucosidase B versus Cellooligosaccharide Concentrations (S , mg/ml).

Cellobiose, $\bullet$ — $\bullet$; cellotriose, $\circ$ — $\circ$; cellotetraose, $\blacktriangle$ — $\blacktriangle$; cellopentaose, $\triangle$ — $\triangle$; cellohexaose, $\blacksquare$ — $\blacksquare$.

0.140 and 0.143 mM, respectively, but V_{max} -values were similar (about 1.2 E_{420}/hr) in all cases. With cellobiose as a substrate, extremely high K_m and V_{max} values were obtained (5.8 mM and 19.8 E_{420}/hr).

The reaction with CMC was leveled off at a hydrolysis extent of about 4% and Avicel was hardly hydrolyzed (Fig. 10).

FIG. 10. Time Courses of Hydrolysis of CMC ($\bullet$ — $\bullet$) and Avicel ($\circ$ — $\circ$) by β -Glucosidase B.

The mixture with CMC contained 0.06 NPGase unit of enzyme, and the one with Avicel 0.6 unit. Increase in reducing power was followed by the Nelson-Somogyi method.

DISCUSSION

At least two intracellular β -glucosidases were found to occur in *Pseudomonas fluorescens* var. *cellulosa*. β -Glucosidase A seemed to be membrane-bound, while β -glucosidase B

cytosolic.

It has been reported^{3,4} that only a little NPGase activity of this bacterium can be released from the cells by spheroplasting with EDTA-lysozyme-treatment or by treatment according to the Heppel's method of osmotic shock, and that about one half of the total NPGase activity is recovered in the supernatant upon centrifugation at $60,000 \times g$ of the cell sonicates or broken spheroplasts.

The present work revealed that about 90% of NPGase activity of S26 is attributable to PII. Consequently, most of the NPGase activity of this bacterium seems to be due to β -glucosidase B.

Though definite statement should not be made until further purification would be achieved, β -glucosidase A and B appear to be distinctive from each other in their molecular size, thermostability, apparent K_m -values, susceptibility to various inhibitory and stimulatory compounds, and relative substrate specificity.

Properties of β -glucosidase B were investigated in some details. This enzyme was capable of attacking not only NPG, NPC, cellooligosaccharides of DP 2–6 and CMC, but also sophorose and laminaribiose. Following facts show that β -glucosidase B splits the terminal β -glucosyl unit of these substrates: 1) The amount of glucose liberated from NPC was far larger than that of NP. 2) The major initial products detected in the reactions with cellooligosaccharides were generally glucose and an oligomer which was smaller by one glucose unit than the original. 3) Only glucose was detected throughout the enzymolysis of CMC.

Values of V_{max}/K_m , relative activities of β -glucosidase B for cellooligosaccharides of DP 3–6 at lower concentrations than the K_m values, were almost equal to each other and about 2.5 times as large as those for cellobiose. This fact indicates that the three consecutive glucose units may be needed to fulfill the specificity region of this enzyme.

β -Glucosidase B split CMC of DS 0.62 up to a hydrolysis extent of about 4%, but practi-

cally Avicel could not be a substrate of this enzyme. This fact and those mentioned above suggest that the enzyme may be an atypical β -glucosidase which possesses a character of exoglucanase and attacks the amorphous cellulose chains from the non-reducing end, but does not split the chains in a crystalline state. In this respect, this enzyme resembles an exocellulase of *Trichoderma viride*.¹³⁾ For the present, however, it is difficult to explain such properties as well as the intracellular localization of this enzyme in correlation with its possible role in the cellulolysis by this pseudomonad.

Acknowledgement. The authors are grateful to Dr. K. Nisizawa, Professor Emeritus of the university, for his interest in this work.

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