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THE β-D-GLUCOSIDASE SYSTEM OF AN *Actinoplanes* sp.*

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ABSTRACT

Actinoplanes sp. No. 1700, a sporangium-forming, filamentous, soil bacterium possesses a β-D-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21). The enzyme was induced to higher concentrations by addition of methyl or phenyl β-D-glucopyranoside, gentiobiose, or salicin to growing cultures. Addition of D-glucose, lactate, or acetate repressed enzyme induction back to the constitutive level, but never below it. The properties of this inducible system place it in the semi-constitutive category.

Both the constitutive and the inducible enzyme were purified 60-fold; their properties were compared and found to be identical. Their pH optima lay between 5.8 and 6.0; the enzymes were stable for 2 h at 30° at pH 5.5 to 7.3. Rapid inactivation occurred at temperatures above 50°. The enzymes were inactivated by 100 μM Cu^{2+}, Hg^{2+}, Pb^{2+}, and Ag^{+}.

Each of these β-D-glucosidases was inhibited by *p*-chloromercuribenzoate (100 μM); this effect was overcome by cysteine or 2-mercaptoethanol, indicating that the β-D-glucosidase is a sulfhydryl enzyme. Kinetic determinations with chromogenic *p*-nitrophenyl β-D-glucopyranoside established a K_m of 2.5×10^{-4} and an Arrhenius activation-energy of 8.5 kcal.mole^{-1}. The molecular weight of the induced enzyme was 165,000 as determined by elution from Sephadex G-200. Chromatographic studies showed the enzyme to be a hydrolase, not a transferase.

INTRODUCTION

Although β-D-glucosidases (β-D-glucoside glucohydrolases, E.C. 3.2.1.21) are widely distributed among animals, fungi, plants, and yeasts[1–5], they have remained relatively unexplored in bacterial species. Studies on the production, isolation, and characterization of microbial β-D-glucosidases have been almost exclusively limited

*Numbering system according to the *Actinoplanaceae* collection of the University of N. C. Botany Department.

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to the yeasts and certain filamentous fungi. An inducible β-D-glucosidase was purified by Duerksen and Halvorson[4] from *Saccharomyces cerevisiae* grown on methyl β-D-glucopyranoside. The β-D-glucosidase system of *Neurospora crassa* was determined[6] to consist of two enzymes, a thermolabile cellobiase and a thermostable aryl β-D-glucosidase. Coston and Loomis[7] demonstrated the production of isozymes of β-D-glucosidase during the development of the cellular slime-mold *Dictyostelium discoideum*. The enzyme has also been isolated from *Aspergillus niger*[8], *Aspergillus oryzae*[9], and *Stachybotrys atra*[10]. In other studies, certain bacteria have been found to possess β-D-glucosidase activity. Schaefler[11] studied an inducible system for the utilization of β-D-glucopyranosides in *Escherichia coli*, and Han and Srinivasan[12], using *Alcaligenes faecalis* as a source, became the first investigators to isolate and characterize a bacterial β-D-glucosidase.

Metabolic control of β-D-glucosidase in yeasts has been extensively investigated. In contrast to the bacteria, *Saccharomyces cerevisiae* was found by Duerksen and Halvorson[4] to possess an inducible β-D-glucosidase. MacQuillan *et al.*[13] noted that the semi-constitutive state of synthesis of β-D-glucosidase in a yeast hybrid was subject to catabolite repression. Recent studies have also demonstrated the regulatory processes of synthesis of β-D-glucosidase in certain bacteria. An inducible system consisting of β-D-glucopyranoside permease and an aryl β-D-glucopyranoside-splitting enzyme in *E. coli* was reported by Schaefler[11], and *Alcaligenes faecalis* was found by Han and Srinivasan[12] to possess an inducible β-D-glucosidase.

Actinoplanes sp. No. 1700, a member of the *Actinomycetales* has now been found to possess an inducible β-D-glucosidase. No member of this group has hitherto been studied relative to its glycosidase potential. In this article, we report the properties of an inducible β-D-glucosidase system, as well as the properties of the purified enzyme.

EXPERIMENTAL

Organisms, media, and growth. — The organisms used in this study were obtained from the *Actinoplanaceae* culture collection of Prof. J. N. Couch[14] of The University of North Carolina at Chapel Hill. Unless otherwise stated, all strains were grown in 250-ml Erlenmeyer flasks containing 100 ml of D-glucose–peptone–Czapek medium without sucrose (5.0 g of D-glucose, 5.0 g of peptone, 3.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.01 g of $FeSO_4$, and distilled water to 1 liter). The cultures were grown on rotary shakers for 5–7 days at 25°. *Actinoplanes* sp. No. 1700 was maintained on 1.5% agar slants containing 0.5% of Czapek medium without sucrose, 0.5% of peptone, and 3.0% of D-glucose. Liquid-culture stocks of this strain were obtained by inoculating one loopful of mycelia from the agar slants into 250-ml Erlenmeyer flasks containing 100 ml of 0.5% Czapek medium without sucrose, 0.5% of peptone, and 0.5% of D-glucose.

Mycelia from liquid-stock cultures were washed twice with sterile, distilled water, then made to 50.0 ml with sterile, distilled water, and blended for 30 sec in a

sterile, Waring-Blendor cup. Aliquots (1.0 ml) of the cell suspension were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of the liquid-stock medium. Cells were collected from flasks during growth periods by filtration on Whatman No. 1 filter paper, dried for 24 h at 90°, cooled to room temperature in a desiccator, and weighed on an analytical balance. Changes (ΔM) in dry weight were based on the dry-weight determinations made on 10.0-ml portions of cell suspensions from control, induced, or repressed (and chloramphenicol-treated) cell-samples at various intervals of time.

Induction, repression, and derepression studies. — Equal volumes of cells from liquid-stock cultures of *Actinoplanes* sp. No. 1700 were transferred to sterile, distilled water, and grown for 24 h in order to lessen the endogenous carbon reserves. Cells were allowed to settle, the water was decanted, and they were blended for 30 sec in sterile, metal, Waring-Blendor cups. The suspension (5.0 ml) was inoculated into 250-ml Erlenmeyer flasks containing 100 ml of 0.5% Czapek medium without sucrose. The indicated inducers or repressors, or both, were added *via* millipore filters (0.22 μm), and enzymic activity was determined after growth for 24 h. In order to follow induction, repression, or derepression (or any combination thereof) kinetically, blended mycelia (50.0 ml) were transferred directly into 2.5-liter low-form flasks (Fisher Scientific Co.) containing 1,000.0 ml of 0.5% Czapek medium without sucrose, and allowed to grow overnight; then, the inducers or repressors (or both) were added as indicated. Samples (50.0 ml) were harvested, disrupted, and assayed for enzymic activity at various intervals of time.

Preparation of cell-free extracts. — Cells were harvested in a clinical centrifuge, washed twice with de-ionized, distilled water, and made to double the volume with 0.05M acetate buffer (pH 5.8). The buffered mycelia were disrupted in a Carver Laboratory Press at, or above, a pressure of 8,000 lb.in.$^{-2}$ at 4°. Complete breakage was obtained, as observed by microscopic examination. Cellular debris was removed by centrifugation for 10 min at 0° at 20,000 *g*.

Enzyme and protein assays. — Reaction mixtures contained 0.3 μmole of chromogenic substrate (1.5 ml), 1.4 ml of appropriate buffer (0.05M), and 0.1 ml of enzyme solution. Following incubation for 30 min at 30°, 1.0 ml of 0.5M sodium hydroxide was added, and the nitrophenolate ion liberated was determined spectrophotometrically at 420 nm with a Hitachi Perkin–Elmer 139 spectrophotometer. During purification of the β-D-glucosidase, 10mM 2-mercaptoethanol (BME) was added to the mixture, as the enzyme was found to be susceptible to air denaturation. The specific activity is defined as the number of enzyme units per mg of protein in solution; one unit of the enzyme is the amount of enzyme that will liberate 100 nanomoles of *p*-nitrophenol during 30 min under the conditions stipulated. Protein was assayed by the method of Lowry *et al.*[15], with crystalline, bovine albumin as the standard.

Enzyme purification. — Cell-free extracts were brought to 70 volume percent with cold acetone, and the resulting precipitate was collected by centrifugation at 3,000 *g* for 5 min at 0°. The precipitate was resuspended in 0.05M acetate buffer

(pH 5.8), dialyzed against the same buffer overnight, assayed for enzymic activity, and concentrated to half volume by means of an Amicon PM 30 diaflo ultrafilter membrane. Portions (30.0 ml) of the filtered suspension were passed through a column (2.5 × 50 cm) of Sephadex G-200, and eluted with 0.02M phosphate buffer (pH 7.0). Fractions (5.0 ml) were collected, and assayed for enzymic activity. Active fractions were combined, concentrated to one-third the volume by diaflo ultrafiltration, and subjected to ion-exchange chromatography. *O*-(2-Diethylaminoethyl)-cellulose (DEAE-cellulose) was washed with 0.5M sodium hydroxide, 0.5M hydrochloric acid, and 0.02M phosphate buffer (pH 7.0). Large volumes of the latter buffer were percolated through the column (1.5 × 20 cm), in order to equilibrate the DEAE-cellulose to buffer conditions. After application of the enzyme (30 mg of total protein), elution was effected by a linear gradient of 0 to 5% sodium chloride. Fractions (5.0 ml) were again collected. The active fractions were combined, and concentrated to one-quarter the volume by ultrafiltration. This enzyme solution was finally passed through a column (1.5 × 20 cm) of hydroxylapatite (Bio-Rad HTP), and eluted by a gradient (0–1.0M) of phosphate buffer. Active fractions (5.0 ml) were pooled, and concentrated to one-third the volume. All of the above procedures were performed at 4°; enzyme preparations could be stored at −10° without appreciable loss of activity.

Compounds used. — *p*-Nitrophenyl β-D-glucopyranoside (PNPG), *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-D-mannopyranoside, methyl β-D-glucopyranoside (BMG), methyl α-D-glucopyranoside, phenyl β-D-glucopyranoside, gentiobiose, salicin, methyl β-D-xylopyranoside, methyl α-D-xylopyranoside, cellobiose, methyl α-D-mannopyranoside, trehalose, melibiose, esculin, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Missouri. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside were purchased from Mann Research Laboratories, New York, N.Y.

Thin-layer chromatography. — Aliquots of the assay mixture were chromatographed on glass plates coated with Silica Gel G, and developed with 5:4:3:1 (v/v) butyl alcohol–ethanol–acetone–water. Another solvent, 8:6:3:3 *tert*-butyl alcohol–2-butanone–formic acid–water, was used for the u.v. detection of *p*-nitrophenol and its derivatives on precoated plates (Silufol UV 257, Applied Science Labs., Inc.). Sugars were detected by spraying with an anisaldehyde–sulfuric acid reagent (50 ml of glacial acetic acid, 1 ml of conc. sulfuric acid, and 0.5 ml of anisaldehyde), followed by heating the plates for 10 min at 100°.

Determination of molecular weight. — Molecular weights were determined with a column of Sephadex G-200 (Pharmacia K 25/100) calibrated with Pharmacia standards according to Granath and Kvist[16].

Disc-gel electrophoresis. — Electrophoresis on poly(acrylamide) disc-gel (7.5%) was performed in duplicate in a Tris–glycine buffer system. One tube was stained with amido-Schwarz solution, and then washed with 7% acetic acid to remove the excess of stain (Davis[17]). To locate the active fraction, the second tube was sliced

in segments of 1 mm, and these were incubated with materials for the assay of β-D-glucosidase.

RESULTS

Table I summarizes the results obtained from the enzymic assays of cell-free extracts from selected members of the *Actinoplanaceae*. All strains grown on liquid-stock media were found to possess a variety of glycosidases, with the exception of *Pilimelia terevasa*, which was unable to hydrolyze any of the chromogenic substrates used. Individual isolates were also found to exhibit definite patterns of glycosidase activity. Because No. 1700 had the highest β-D-glucosidase activity, it was selected for further study.

TABLE I

SPECIFIC ACTIVITIES OF VARIOUS GLYCOSIDASES[a] OF SELECTED GENERA OF THE *Actinoplanaceae*

Genus and species	*Strain*	*α-Glc*	*β-Glc*	*α-Gal*	*β-Gal*	*GlcNAc*	*α-Man*
Actinoplanes sp.	1700	10.67	12.22	Tr[b]	3.46	Tr[b]	—
	2a97	7.16	4 83	Tr	2 38	Tr	—
utahensis	258	8.60	7.49	Tr	2.60	Tr	—
philippinensis	2	8.70	3.40	—	2.77	—	—
Spirillospora sp.	1496	2.67	Tr	—	Tr	—	—
albida	1030	3.78	—	Tr	Tr	—	—
sp.	1309b	3.93	Tr	—	Tr	—	—
Pilimelia terevasa	1778	—	—	—	—	—	—
Ampullariella digitata	33	5.35	1.65	—	Tr	1.34	1.30
campanulata	65	5.50	1.85	—	Tr	3.26	1.72
regularis	168	—	—	—	—	3.78	Tr
Streptosporangium roseum	499	2.43	—	—	—	3.76	—
sp.	72	1.88	—	—	—	1.68	—
roseum	27	—	—	—	—	1.50	—
Amorphosporangium auranti-	253	5.15	4.69	—	1.36	2.38	Tr
color	262	5.00	4.00	—	Tr	2.71	1.75

[a]Abbreviations: α-Glc, α-D-glucosidase; β-Glc, β-D-glucosidase; α-Gal, α-D-galactosidase; β-Gal, β-D-galactosidase; GlcNAc, 2-acetamido-2-deoxy-β-D-glucosidase; α-Man, α-D-mannosidase. α-Glc, β-Gal, and GlcNAc were buffered with phosphate buffer at pH 7.0; α-Gal and α-Man were buffered with acetate buffer, pH 5.2; and β-Glc was buffered with acetate buffer, pH 5.8. [b]Tr = trace, or a specific activity of <1 00.

In order to determine when enzyme production was at its peak, cells grown in liquid culture were assayed for α- and β-D-glucosidase activity at various periods during growth. Fig. 1 reveals that both enzyme production and cell growth reached a maximum after 5 days. All experiments were conducted with actively growing cells.

Induction. — The response of *Actinoplanes* sp. No. 1700 to a variety of glycosides is summarized in Tables II and III. No significant increase in α-D-glucosidase

synthesis could be detected in cells grown on such likely inducers as methyl α-D-glucopyranoside and maltose, suggesting that this enzyme is constitutive. However, β-D-glucosidase was induced to levels 2 to 2.5 times the basal level in cells grown on gentiobiose, salicin, phenyl β-D-glucopyranoside, and methyl β-D-glucopyranoside. Methyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside, methyl β-D-xylopyranoside, esculin, and cellobiose were less effective as inducers of β-D-glucosidase activity. Superficially, it would appear that *Actinoplanes* sp. No. 1700 produces a semi-

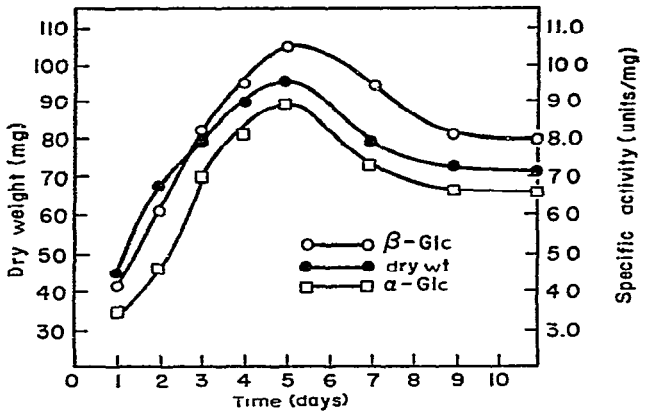

Fig. 1. Enzyme production during growth of cultures of *Actinoplanes* sp. No. 1700.

TABLE II

EFFECT OF VARIOUS CARBOHYDRATE SOURCES ON THE β-D-GLUCOSIDASE ACTIVITY OF *Actinoplanes* sp. NO. 1700[a]

Carbohydrate source (5 mM)	*Specific activity (units/mg of protein)*
Control	10.22
Methyl β-D-glucopyranoside	26.70
Phenyl β-D-glucopyranoside	25.92
Salicin	23.40
Gentiobiose	20.62
Methyl 1-thio-β-D-glucopyranoside	14.46
Methyl β-D-xylopyranoside	14.02
Esculin	13.32
Cellobiose	13.20
Sucrose	10.24
D-Glucose	10.20
Methyl α-D-glucopyranoside	10.04
Maltose	9.89
α,α-Trehalose	9.42
Methyl α-D-mannopyranoside	9.28

[a]The organism was grown for 24 h in 100 ml of 0.5% Czapek medium without sucrose, plus the indicated glycoside (5 mM) as the sole source of carbon.

TABLE III

EFFECT OF VARIOUS CARBOHYDRATE SOURCES ON THE α-D-GLUCOSIDASE ACTIVITY OF *Actinoplanes* sp. NO. 1700[a]

Carbohydrate source (5 mM)	*Specific activity (units/mg of protein)*
Control	9.45
Methyl α-D-glucopyranoside	10.12
Methyl α-D-xylopyranoside	9.76
Methyl β-D-glucopyranoside	9.62
Maltose	9.46
Melibiose	9.30
α,α-Trehalose	8.65
Sucrose	8.52

[a]The organism was grown for 24 h in 100 ml of 0.5% Czapek medium without sucrose, plus the indicated glycoside (5 mM) as the sole source of carbon.

constitutive β-D-glucosidase whose high, basal, enzyme level can be increased by induction. From Fig. 2, it may be seen that induced synthesis of β-D-glucosidase can be detected almost immediately, without any of the apparent lag observed for induction of yeast β-D-glucosidase and for *E. coli* β-D-galactosidase. An increase in total protein was also observed during induction (see Fig. 3). In the presence of chloramphenicol, an inhibitor of protein synthesis, the amount of total protein, as well as the specific activity of the induced β-D-glucosidase, rapidly decreased, suggesting that increased protein synthesis occurs during induction.

Induced and constitutive β-D-glucosidase appear to be located in the cytoplasm of *Actinoplanes* sp. No. 1700 (see Table IV).

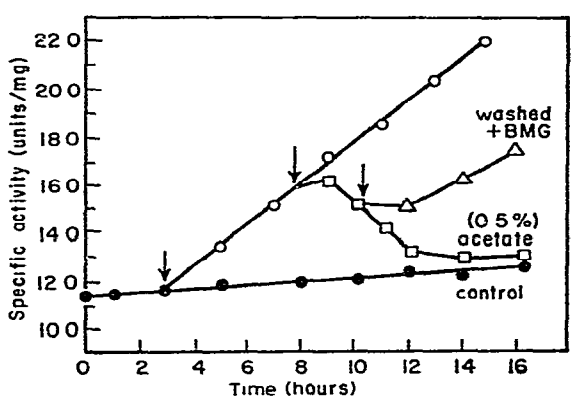

Fig. 2. Induction and repression of β-D-glucosidase in cultures of *Actinoplanes* sp. No. 1700. The arrow at 3 h shows when inducer, methyl β-D-glucopyranoside (5mM), was added. The arrow at 8 h indicates addition of acetate repressor. The arrow at 10 h shows the alleviation of repression when the cells were washed and more inducer was added.

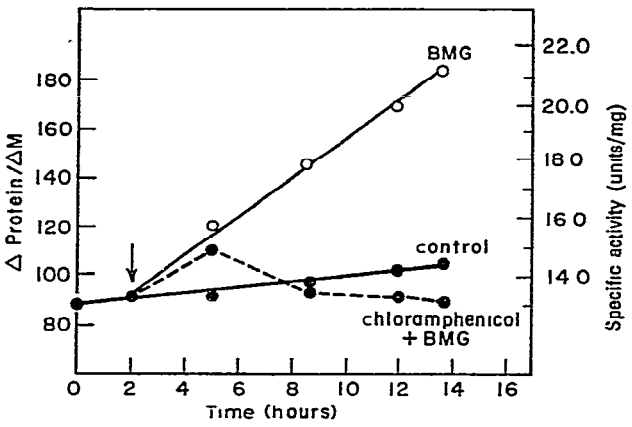

Fig. 3. The effect of chloramphenicol on the production of β-D-glucosidase. The arrow at 2 h indicates the addition of inducer and chloramphenicol (100 μg/ml).

TABLE IV

LOCATION OF THE β-D-GLUCOSIDASE ACTIVITY IN *Actinoplanes* sp. NO. 1700

Material	*Specific activity (units/mg of protein)*	
	Constitutive enzyme	*Induced enzyme*
Whole cells	3.07	3.62
Cell fragments	3 20	4.04
Extract	12.28	27.65

Repression and derepression. — In the presence of certain carbon sources, induced β-D-glucosidase synthesis was noticeably affected. Although D-glucose, acetate, and D-lactate were found to be the most effective repressors, oxalacetate, glycerol, maleate, L-lactate, succinate, and pyruvate were also able to influence induced enzyme-synthesis (see Table V). However, synthesis of β-D-glucosidase was never repressed below the basal level, as may be seen in Figs. 2 and 4.

Repression of catabolite by D-glucose or acetate was effectively reversed when the treated cells were washed and then grown[2,4] in the presence of BMG. Derepression of synthesis of β-D-glucosidase was observed within 2 to 4 h, as shown by the increase in specific activity. Cyclic AMP did not relieve the repression by D-glucose in this system.

Purification of β-D-glucosidase. — Table VI summarizes the procedures followed in the purification of methyl β-D-glucopyranoside-induced β-D-glucosidase. The enzyme was considerably purified by passing the redissolved, acetone precipitate through Sephadex G-200. A substantial amount of nonspecific protein was removed before the β-D-glucosidase was eluted from the column (see Fig. 5). Column chromatography of the β-D-glucosidase on DEAE-cellulose resulted in further purification of

TABLE V

CATABOLITE REPRESSION OF INDUCED β-D-GLUCOSIDASE[a]

Carbon source (0.5%)	*Repression of induced β-D-glucosidase (%)*
Control	0
D-Glucose	95
Acetate	94
D-Lactate	91
Oxalacetate	84
Glycerol	79
Maleate	74
L-Lactate	70
Succinate	60
Pyruvate	56

[a]The organism was grown for 24 h in 100 ml of 0.5% Czapek medium without sucrose, 5 mM BMG, and 0.5% of the indicated source of carbon.

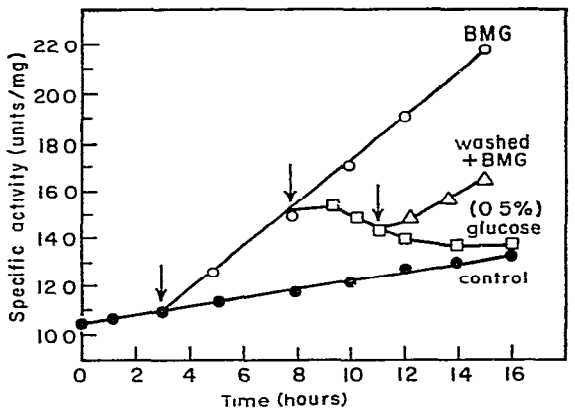

Fig. 4. Repression of β-D-glucosidase synthesis by D-glucose on cultures induced to produce the enzyme by methyl β-D-glucopyranoside. Arrow at 3 h indicates addition of inducer; arrow at 8 h indicates addition of D-glucose (0.50%); and arrow at 11 h indicates when the cells were washed free of repressor and re-induced.

TABLE VI

PURIFICATION OF β-D-GLUCOSIDASE[a]

Fraction	*Total volume (ml)*	*Total enzyme (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Enzyme yield (%)*
Crude extract	600	40,490	1,397	28.98	100
70% Acetone	335	36,960	616	60.04	89
Sephadex (G-200)	200	25,580	152	151.62	57
DEAE-cellulose (0–5% NaCl)	105	10,895	22.7	482.06	28
Hydroxylapatite (0–0.1 M phosphate buffer)	67	3,452	2.0	1,726	8.5

[a]Described in the text.

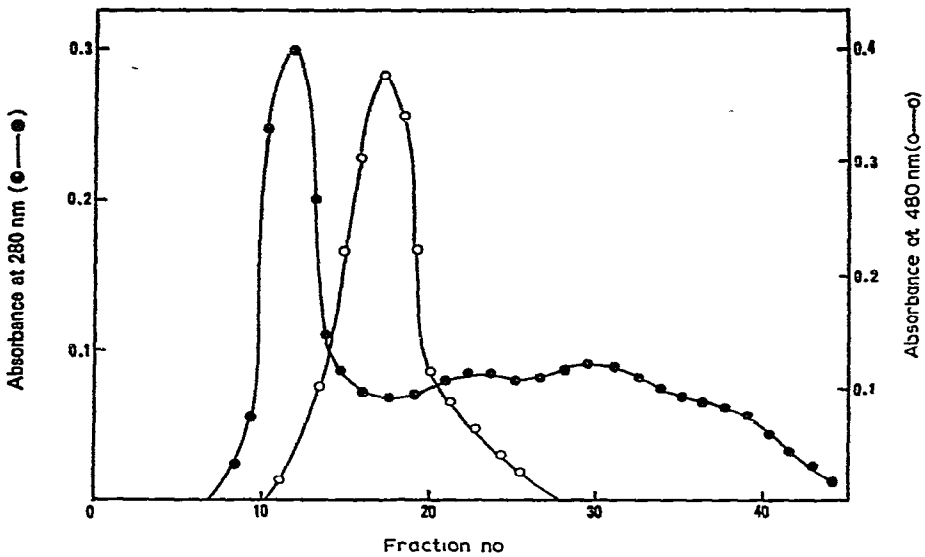

Fig. 5. Elution pattern of β-D-glucosidase through Sephadex G-200 (●——●, protein; ○——○, β-D-glucosidase).

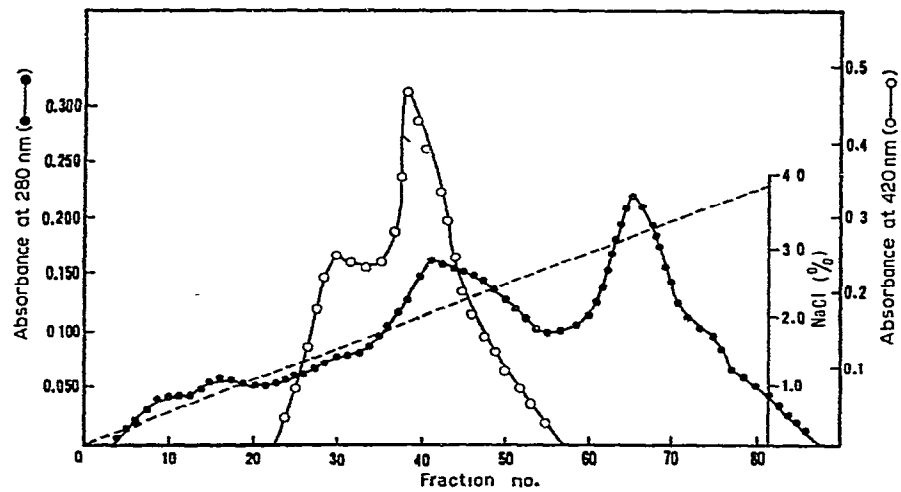

Fig. 6. DEAE-cellulose fractionation of β-D-glucosidase (●——●, protein; ○——○, β-D-glucosidase).

the enzyme. The gradient pattern of the enzyme, as shown in Fig. 6, shows that β-D-glucosidase was eluted from the column with ~2% sodium chloride. Passage of β-D-glucosidase through a column of hydroxylapatite resulted in a 60-fold purification, with a yield of 8.5%; this step not only resulted in higher purification, but also served to remove the sodium chloride from the enzyme solution. Elution of the

enzyme was achieved with ~0.04M phosphate buffer, pH 7.0 (see Fig. 7). Enzyme preparations obtained by this procedure were retained for characterization and comparative studies. Constitutive enzyme obtained from cells grown on control media was purified in the same way. Data only for the induced enzyme are shown, as the constitutive enzyme was identical in its properties.

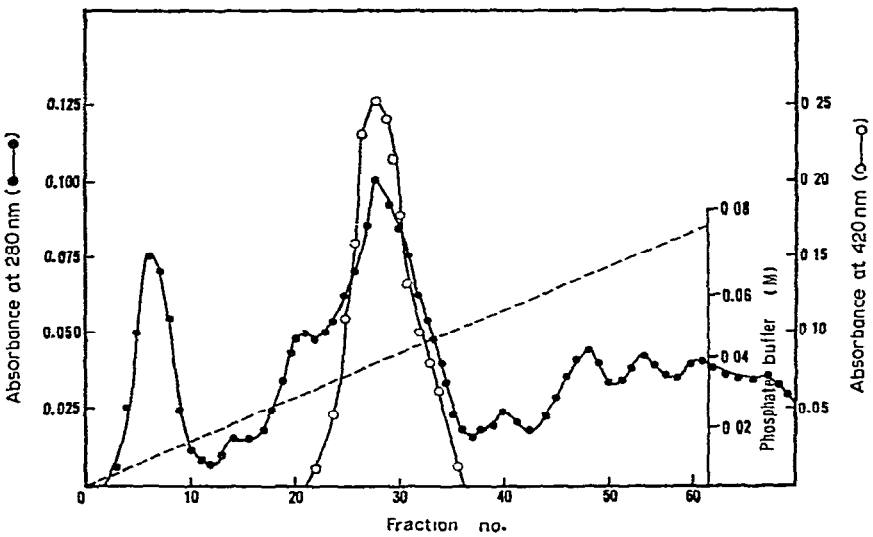

Fig. 7. Elution of β-D-glucosidase through a column of hydroxylapatite (●——●, protein; ○——○, β-D-glucosidase).

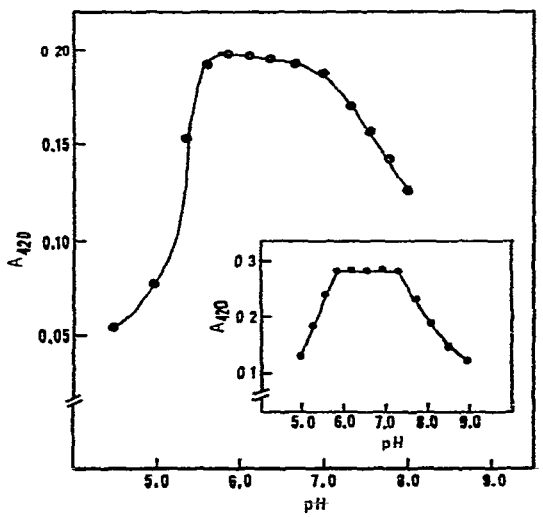

Fig. 8. pH-optimal stability. (Inset: pH of optimal activity.)

Properties of the enzyme. — The effect of pH on the hydrolysis of PNPG by β-D-glucosidase was studied by using sodium acetate and sodium phosphate buffers. Although the enzyme functions optimally at pH 5.8–7.0, it was stable in solution from pH 5.5 to 7.3 when kept for 2 h at 30° (see Fig. 8). Rapid inactivation occurred at pH values <5.0 and >8.0.

Hydrolysis of PNPG was maximal at 40°, and a conventional, Arrhenius plot was obtained in the range of 10 to 40° (see Fig. 9). Deviation from linearity was noted above 40°. An activation energy of 8.5 kcal.mole^{-1} was calculated from the linear portion of the line. Heat-inactivation studies showed that the enzyme was very

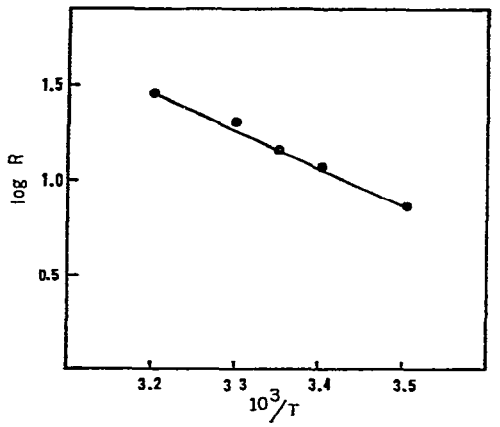

Fig. 9. Arrhenius activation-energy plot for β-D-glucosidase.

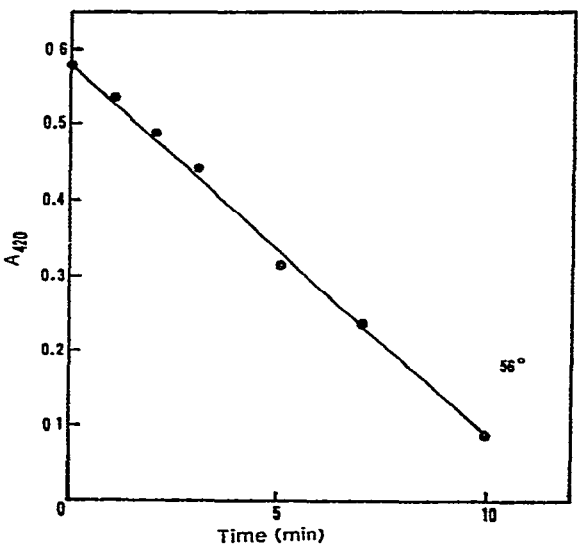

Fig. 10. Thermal inactivation of β-D-glucosidase at 56°.

sensitive to temperatures above 50°. At 56°, enzymic activity was almost completely destroyed within 10 min (see Fig. 10).

Enzymic hydrolysis of PNPG also proceeded linearly for at least 30 min (see Fig. 11). The initial velocity of the reaction was directly proportional to the concentration of the enzyme (see Fig. 12). A typical Michaelis–Menten relationship between the initial velocity and the concentration of the substrate was obtained. The Lineweaver–Burk plot yielded a straight line, from which an apparent K_m of 2.5×10^{-4}

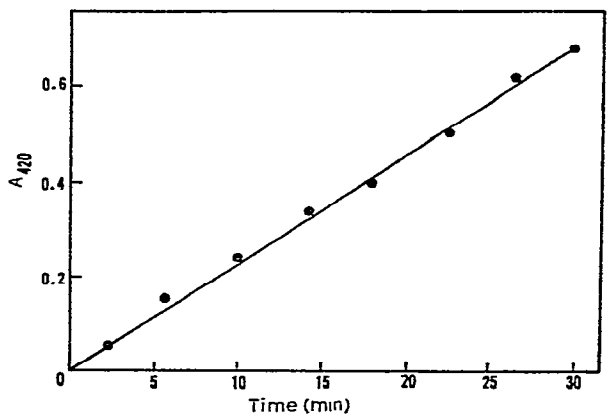

Fig. 11. Enzymic hydrolysis of *p*-nitrophenyl β-D-glucopyranoside by β-D-glucosidase, showing linearity for at least 30 min.

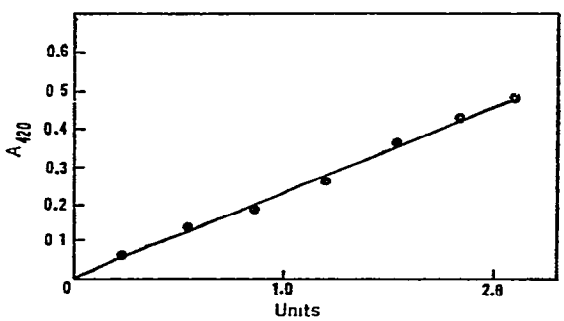

Fig. 12. Proportionality of initial velocities to enzyme concentration.

was determined (see Fig. 13). As illustrated in Fig. 14, competitive inhibition of hydrolysis of PNPG by 1mM cellobiose was observed. At a concentration as high as 10mM, BMG only slightly inhibited hydrolysis.

Sensitivity of β-D-glucosidase to heavy metals was observed when the enzyme was pre-incubated in the presence of various cations. The enzyme was completely inhibited by Cu^{2+}, Pb^{2+}, Hg^{2+}, and Ag^{+} at concentrations as low as 100 μM (see Table VII). The instability of the enzyme in the presence of heavy metals led to

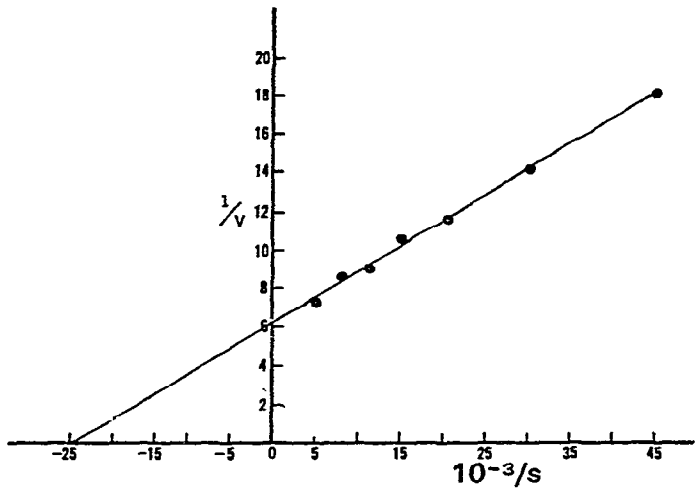

Fig. 13. Enzyme–substrate (*p*-nitrophenyl β-D-glucopyranoside) relationship as a Lineweaver–Burk plot. (K_m of 2.5×10^{-4}M was determined for this system.)

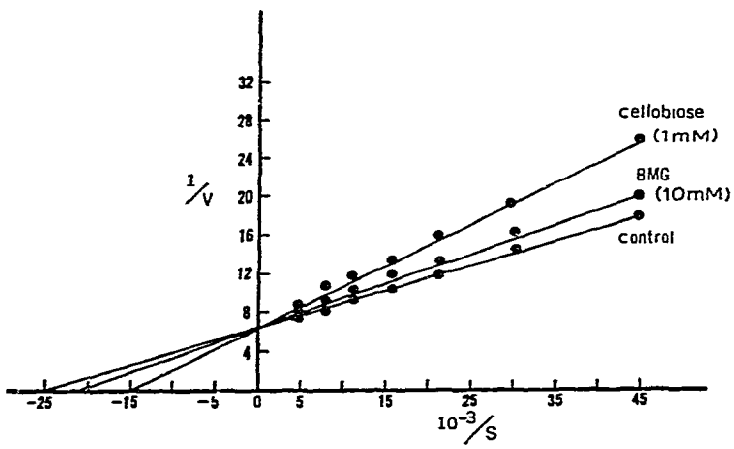

Fig. 14. Competitive inhibition, by cellobiose, of hydrolyses of *p*-nitrophenyl β-D-glucopyranoside with β-D-glucosidase.

the examination of the effects of sulfhydryl inhibitors on the β-D-glucosidase activity. As indicated in Table VIII, *p*-chloromercuribenzoate (CMB) at a concentration of 100 μM almost completely inhibited the enzyme. Iodoacetate was less effective. Cysteine and 2-mercaptoethanol reversed the inhibition.

Table IX summarizes the results obtained when various glycosides were subjected to enzymic hydrolysis in the presence of equal proportions of β-D-glucosidase. Cellobiose was hydrolyzed at about twice the rate for PNPG, whereas salicin, esculin, and phenyl β-D-glucopyranoside were hydrolyzed at much lower rates. Hydrolysis of BMG was scarcely detectable.

The results of thin-layer chromatography showed that the action of the enzyme was strictly hydrolytic, and not transferring. Thus, cellobiose was hydrolyzed to glucose, whereas PNPG showed only glucose and *p*-nitrophenol; this behavior is in marked contrast to that of the β-D-glucosidase[18] from *Aspergillis niger* or from *Lagenidium* sp.*.

The molecular weight of the enzyme was found to be ~165,000, a value quite close to that of that from *Lagenidium* sp. The enzyme was not pure, as 5 discrete bands were detected by disc-gel electrophoresis; however, only one band possessed β-D-glucosidase activity.

TABLE VII

EFFECT OF VARIOUS CATIONS ON THE ACTIVITY OF INDUCED β-D-GLUCOSIDASE[a]

Cation	*Concentration* (M)	*Inhibition* (%)
Na^+	1 m	0
K^+	1 m	0
Mg^{2+}	1 m	0
Ca^{2+}	1 m	0
Co^{2+}	1 m	0
Ni^{2+}	1 m	0
Fe^{3+}	1 m	10
Zn^{2+}	1 m	13
Cu^{2+}	100 μ	100
Hg^{2+}	100 μ	100
Pb^{2+}	100 μ	100
Ag^+	100 μ	100

[a]All the cations were added as chlorides, except Ag^+, which was added as the sulfate, and Ni^{2+} and Pb^{2+}, which were added as the acetates. Reaction mixtures contained 0.5 ml of cation solution, 1.4 ml of acetate buffer (pH 5.8), 0.1 ml of the enzyme solution, and 1.0 ml of 5mM PNPG.

TABLE VIII

EFFECT OF SULFHYDRYL AGENTS ON β-D-GLUCOSIDASE ACTIVITY[a]

Inhibitor	*Concentration* (M)	*Inhibition* (%)
None		0
CMB	100 μ	86
Iodoacetate	5 m	11
Sodium azide	10 m	6
CMB+BME (10mM)	100 μ	1
CMB+cysteine (10mM)	100 μ	4

[a]Reaction mixtures contained 0.5 ml of the inhibitor, 1.5 ml of 0.5M acetate buffer (pH 5.8), and 0.1 ml of purified enzyme. PNPG (1mM), or PNPG plus BME or cysteine, was added after a 20-min pre-incubation period.

*T. M. McInnis and A. Domnas, unpublished results.

TABLE IX

HYDROLYSIS OF VARIOUS D-GLUCOPYRANOSIDES BY β-D-GLUCOSIDASE

Substrate	*Type of linkage*	*D-glucose liberated*[a] *(μg)*
Cellobiose	(1→4)-β-D-glucoside	86 4
PNPG	β-D-glucoside	44.6
Salicin (salicyl β-D-glucopyranoside)		14.1
Esculin (7-hydroxycoumarin-6-yl β-D-glucopyranoside)	(1→4)-α-D-glucoside	9.6
Phenyl β-D-glucopyranoside		9.2
BMG	(1→4)-β-D-galactoside	3.4
Maltose		2.2
Methyl α-D-glucopyranoside		1.9
Lactose		0

[a]Reaction mixtures contained 2.0 ml of 0 02M substrate, 1.9 ml of 0.05M acetate buffer (pH 5.8), and 0.1 ml of purified enzyme. Following 30-min incubation at 30°, the mixture was boiled for 5 min to denature the β-D-glucosidase This solution (1 0 ml) was then added to 1.0 ml of de-ionized, distilled water and 2.0 ml of glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). After a 10-min incubation at 30°, 6 drops of 4M hydrochloric acid were added to stop the reaction, and the colorimetric determination of glucose was made at 420 nm.

DISCUSSION

The discovery that the *Actinoplanaceae*, as a group, contain a variety of glycosidases was not unexpected. As soil inhabitants, these organisms are most probably active in the degradation of organic matter, for which the action of hydrolytic enzymes of this type would be essential. Nutritional studies[19,20] have demonstrated that the *Actinoplanaceae* are capable of utilizing numerous glycosides, including di- and oligo-saccharides, as the sole carbon source, indicating the presence of glycosidases necessary for the hydrolysis of these carbohydrates. The almost complete absence of α- and β-D-galactosidase activity among the strains tested is surprising. The possibilities that (*a*) α- and β-D-galactosidases are inducible enzymes whose synthesis is repressed by the D-glucose in the growth medium, or (*b*) the organisms have to grow in the presence of a suitable substrate in order to produce the enzymes, could account for this observation. Low to zero specific activities for 2-acetamido-2-deoxy-β-D-glucosidase may be explained by the fact that this enzyme is often exocellular, and thus it would not be detected in certain cell-free extracts. However, the results reveal definite patterns of enzymic activity for a given genus, illustrating that biochemical, as well as morphological, differences exist between related members of the family.

Actinoplanes sp. No. 1700 maintained a high, basal level of β-D-glucosidase, which, in response to specific inducers, resulted in increased levels of enzyme. This semi-constitutive state of enzyme synthesis has been observed for yeast β-D-glucosidase[13], *E. coli* β-D-galactosidase[24], and *Proteus mirabilis* F-lac β-D-galactosidase[21]. MacQuillan and Halvorson[22] also observed that, in the yeast system, repression of

D-glucose could not be relieved by inducers of β-D-glucosidase synthesis, and suggested that at least two distinct sites must be involved in the regulation of enzyme synthesis: an induction site and a repressor site. Similar results obtained with constitutive and semi-constitutive mutants of *E. coli* led Loomis and Magasanik[24] to draw similar conclusions. Catabolite repression of β-D-glucosidase synthesis in *Actinoplanes* sp. No. 1700 was, however, overcome by the addition of BMG, implying that induction and repression are most probably linked to a common site. Repressed levels of enzyme never fell below the basal level of unrepressed synthesis of enzyme. A similar observation was made by Colby *et al.*[21] for the regulation of synthesis of β-D-galactosidase in *Proteus mirabilis* F-lac, a recombinant containing the episomal lac operon of *E. coli*. The high basal level of β-D-galactosidase was induced several-fold by isopropyl 1-thio-β-D-glucopyranoside. Colby *et al.*[21] attributed their observations to the presence of a conformationally altered, repressor molecule (altered by the change in the cytoplasmic environment) unable to interact completely with the operator, causing a high basal level of enzyme. An altered conformation could also affect the interaction between the repressor and the inducer molecules, changing the relative effectiveness of various inducers. Catabolite repression by D-glucose did not alter the basal level, but it did effect induced β-D-galactoside permease (rather than the β-D-galactosidase), as uptake of D-galactoside in whole cells was inhibited by D-glucose.

As the genome of *Actinoplanes* No. 1700 had not been altered, it must be concluded that the semi-constitutive mechanism is the way in which the organism regulates its β-D-glucosidase activity.

Purified β-D-glucosidase from *Actinoplanes* sp. No. 1700 exhibits properties common to both the fungal and the bacterial enzymes. In general, fungal (as well as plant) β-D-glucosidases show very little cellobiase activity[23], although that of *Neurospora crassa* has been shown to consist of a cellobiase and an aryl β-D-glucopyranosidase which differ in temperature sensitivity[6]. The bacterial β-D-glucosidase isolated[12] from *Alcaligenes faecalis* was reported to possess high cellobiase activity, a property common to *Actinoplanes* sp. β-D-glucosidase. At this time, we cannot exclude the possibility that the β-D-glucosidase system of *Actinoplanes* sp. No. 1700 consists of two enzymes, and that aryl β-D-glucosidase and cellobiase have been purified concomitantly. No significant differences were noted between the *Actinoplanes* enzyme and the enzymes from other sources with respect to pH and temperature optima, pH stability, heat inactivation, energy of activation, inhibitor studies, and K_m.

Inhibition of β-D-glucosidase activity by *p*-chloromercuribenzoate suggests the existence of sulfhydryl groups in the enzyme molecule. Such reductants as cysteine and 2-mercaptoethanol not only relieved this inhibition, but also allowed for increased activity of uninhibited enzyme, indicating its sensitivity to oxidation. It is not yet known whether the active site is affected.

The enzyme remained quite stable during purification, and its activity was not appreciably affected by repeated freezing and thawing. Purified enzyme stored at $-10°$ for as long as 6 months retained most of its original activity.

It is, therefore, concluded that, although the β-D-glucosidase of *Actinoplanes* sp. No. 1700 is bacterial with respect to its source, the enzyme does not appear to be characteristically different from other β-D-glucosidases.

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