Molecular Cloning and Characterization of a Unique β-Glucosidase from Vibrio cholerae*

Received for publication, March 27, 2002, and in revised form, May 30, 2002
Published, JBC Papers in Press, May 31, 2002, DOI 10.1074/jbc.M202978200

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We have recently reported the molecular cloning of a gene, gspK, in Vibrio cholerae that encodes a specific glucosamine kinase. We describe here the identification of bglA, a gene contiguous to gspK in a presumptive large chitin catabolic operon. BglA was molecularly cloned into Escherichia coli, and the protein BglA was overexpressed and purified to apparent homogeneity. BglA is 65 kDa (574 amino acids) with an N-terminal amino acid sequence predicted by the gene sequence, suggesting that the enzyme is cytoplasmic. The purified amino acid sequence predicted by the gene sequence, BglA is 65 kDa (574 amino acids) with an N-terminal amino acid sequence predicted by the gene sequence, suggesting that the enzyme is cytoplasmic. The purified enzyme exhibited optimal activity with p-nitrophenyl β-glucoside, cellobiose, and higher oligosaccharides of cellulose. No other glucosides or glycosides tested were hydrolyzed, including Glc-Glc disaccharides where the linkage is β-1-2, β-1-3, and β-1-6, respectively. The predicted BglA sequence bears little similarity to other proteins in the data banks. The Henrissat algorithm places BglA sequence in Family 9 of the glycosidases, suggesting it is an endoglucanase. However, the results summarized above suggested that BglA is an exoenzyme yielding Glc at each cleavage step. To resolve this apparent discrepancy, detailed kinetic studies were conducted with cellotetraose. Only exoglucanase activity was detected. The function of this enzyme in V. cholerae remains to be determined, especially because our strain of this organism does not utilize cellobiose.

Chitin and cellulose are the two most abundant polysaccharides in nature, very similar in structure but differing primarily in the respective monomers. Chitin is composed of β1,4-N-acetylglucosamine residues in place of the Glc residues in cellulose. Chitinases and cellulases are widespread in nature, and many have been isolated from a variety of organisms.

We have previously reported that the chitin catabolic cascade in the marine bacterium Vibrio furnissii comprises several signal transduction pathways and many proteins (summarized in Ref. 1). The complete genomic sequence of Vibrio cholerae has been published recently (2), and virtually all of the genes that we have cloned and sequenced from V. furnissii encode proteins that show great identity/similarity to the comparable proteins from V. cholerae. Therefore, V. cholerae will be the organism of choice for our future studies on chitin catabolism. Among the family Vibrionaceae, V. cholerae is probably the most significant with respect to human health and disease. It is a Gram-negative bacterium that resides in brackish waters and seawater where it is closely associated with copepods, ubiquitous marine microcrustacea that provide a major source of chitin for these bacteria.

Among the unique genes and proteins that we have characterized from V. furnissii, we identified three enzymes, (Glc-NAc)3 phosphorylase (3), Exo I, and β-N-acetylglucosaminidase (4), and a specific glucosamine kinase (5). The three genes are part of what we believe is a (GlcNAc)3 catabolic operon comprising 10 genes in V. cholerae, and our goal is to fully characterize the other seven genes and proteins.

We report here the molecular cloning of a gene, bglA (1.7 kilobases) from the putative operon. In the data banks, it is annotated as an endoglucanase-like glycohydrolase. As shown below, the protein BglA (65 kDa) is a β-1,4-n-glucosidase, more specifically, a cellobiase.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals, reagents, and materials were purchased from the indicated sources. Cellulose, chitin, N-acetylglucosamine (GlcNAc), and p-nitrophenyl (pNP)1 glycosides were from Sigma. Chitin oligosaccharides and (GlcNAc), (n = 2–5) were from Seikagaku America, Inc. Reagents for bacterial media were from Difco and J. T. Baker. pNP-β-glucoside-6-phosphate was kindly provided by Dr. John Thompson (National Institutes of Health, Bethesda, MD). Reagents for molecular biology were obtained from New England Biolabs (Beverly, MA), Stratagene, and U. S. Biochemical Corporation. A glucose assay kit was purchased from Sigma. Other buffers and reagents were of the highest purity commercially available.

Bacterial Strains—V. cholerae E1 Tor N16961 is the strain used for obtaining the DNA sequences of the two chromosomes (2) and was used in this work along with V. furnissii 1519, similar to 1514, which was the strain used in our earlier work. These and Escherichia coli strains BL21 (DE3) (Novagen) and XL1-Blue (Stratagene) harboring designated plasmid constructs were stored as frozen cultures in Luria Broth (LB) medium with 5% glycerol. Typically, E. coli strains were grown overnight in LB medium plus appropriate antibiotics with vigorous shaking. Fresh medium was inoculated with cells from the overnight culture at a 1:20 dilution, and this culture was grown to the desired density at either a midexponential or stationary phase as specified.

Molecular Analysis, Construction of pET-bglA (β1,4-N-Glucosidase Overexpression Vector)—DNA preparations, restriction enzyme digests, ligation, and transformations were performed using standard techniques (6).

The β-1,4-n-glucosidase gene, bglA, was amplified by PCR using synthetic primers based on the complete sequence of gene VC0615 in the chromosomal DNA of V. cholerae. The 5′-PCR primer was designed containing a NdeI restriction site to facilitate cloning into the start site following a T7 promoter in the overexpression vector pET21a (Novagen, Madison, WI). The primers used to construct the overexpression plas-

* This work was supported by Grant GM51215 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: pNP, p-nitrophenyl; BglA, β-glucosidase; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
Routine assays were conducted at 37 °C.

**Enzyme Assays—**Protein concentration was measured by using a Bio-Rad protein assay using bovine serum albumin as the standard. Enzyme assays were conducted at 37 °C, and in all assays, product formation was proportional to the time of incubation and the quantity of enzyme. Control incubation mixtures lacked enzyme, contained heat-denatured enzyme, or lacked substrate. No activity was observed with these negative controls. In kinetic studies with the purified enzyme, the mixtures contained 0.1-0.5 μg of BglA unless specified otherwise.

Cell extracts or protein fractions to be assayed were adjusted to 100-μl volumes with 50 mM sodium phosphate buffer, pH 6.5, and mixed with 100 μl of the same buffer containing 5 μl pNP-β-glucoside. Routine assays were conducted at 37 °C taking 3-4 time points from 0-0.5 h. The reaction was quenched with 900 μl of 1 M Tris base, pH 11, and absorbance was measured at 405 nm. The quantity of p-nitrophenol produced per minute was calculated using an extinction coefficient of 18,300 M cm⁻¹⁻¹.

A discontinuous assay was also used for determining the kinetics of hydrolysis of the cellobioseglycosidases Glc (a = 2-5) and to test other α- or β-glucosides. The product Glc was quantitated with a glucose oxidase kit (Sigma). The reaction mixture (200 μl) contained 50 mM sodium phosphate buffer, pH 6.5, and 5 mM sugar substrates, or other concentrations as specified. The reactions were initiated by adding BglA (0.5-4.0 μg), incubated at 37 °C over a time course (typically 0-1 h), and heated at 100 °C for 3 min. The glucose assay reagent (400 μl) was added to the reaction mixture and incubated at 37 °C for exactly 30 min, and 400 μl of 12 N H₂SO₄ were added. The absorbance of the mixture versus the reagent blank was measured at 540 nm. A standard curve was obtained with each of the analyses containing Glc in an incubation mixture lacking BglA. In the mixtures containing glycosides or oligoglycosidases of GlcNac, product formation was determined by the Morgan-Elson method (7) for free GlcNac.

**Kinetic Hydrolysis of Cellotetraose and Analysis of Oligosaccharide Mixtures—**To 0.15 ml of 3 mct cellotetraose in 50 mM phosphate buffer, pH 6.6, were added 15 μl of homogenous enzyme (45 μg), and the mixture incubated at 37 °C. Aliquots were removed at 0.5, 1, 2, 5, 10, 20, 60 min, and after 12 h. The aliquots were heated at 100 °C to stop the reaction and diluted to 150 μl with water. Generally, 20 of 150 μl of aliquots were injected into the ion-exchange column.

The mixture was analyzed by HPAEC (8) using a Dionex DX600 chromatography system (Dionex Corp., Sunnyvale, California) equipped with an electrochemical detector (ED). The column was CarboPac PA (4 × 250 mm), and the eluants were (a) 0.1 M NaOH and (b) 1 M sodium acetate in 0.1 M NaOH. The flow rate of the column was set at 1 ml/min, and the gradients were as follows: 5% B for 0-4 min; 5-20% B from 4-8 min; and 20% B from 8-15 min or longer.

The standards, Glc, and cellobioseglycosidases eluted as sharp well resolved peaks at the following times (in min): Glc, 3.15; (Glc), 5.7; (Glc), 9.1; (Glc), 10.1; and (Glc), 10.6.

The quantity of each sugar was determined from the area under the corresponding peak where the PED response, i.e., the area, was calibrated by injecting standard samples of the corresponding sugar. The cellotetraose substrate was ~5% contaminated with cellobiose, and a suitable correction was made for this contaminant in the value for G₄ detected in the hydrolysate.

The kinetic data points for Fig. 4 were fitted by a program available on the Internet, KYPlot. The modeling of the reaction sequence and simulations of the progress curves for Fig. 5 were conducted likewise with a computer program, KinTekSim.

| Step | Total protein | Total activity | Specific activity | Purification |
|------|---------------|----------------|------------------|--------------|
| Crude extract | mg | units | units/mg | /fold |
| Streptomycin | 221 | 12,000 | 33.5 | 1.0 |
| Ammonium sulfate | 146 | 11,200 | 74.3 | 1.6 |
| DEAE-Sepharose | 54 | 10,100 | 187 | 5.6 |
| IMAC-Zn²⁺ column | 26 | 8390 | 323 | 9.6 |

**Note:** Activity measured with pNP-β-glucoside in 50 mM phosphate buffer, pH 6.5, at 37 °C. One unit is 1 nmol of pNP was released per min.

In some cases, the course of the hydrolysis was also followed by subjecting aliquots of the incubation mixture to thin layer chromatography on HPTLC Silica Gel 60 plates (EM Scientific) using the following solvent mixture: n-butanol, methanol, ammonium hydroxide, and water, 5:4:2:1 (v/v). This procedure separated cellulose oligosaccharides Glc through Glc₅. The oligosaccharides Glc₅ were detected by spraying the plate with a 3% cupric acetate, 8% phosphoric acid followed by heating on a hot plate at 100 °C for 20 min followed by 200 °C until the compounds charred and the spots were visible.

**Overexpression and Purification of BglA**

**Step 1: Crude Extracts—**A single colony of E. coli strain BL21 (DE3) harboring pET·βgIA was inoculated in 200 ml of LB medium supplemented with 50 μg/ml ampicillin and grown overnight at 37 °C with aeration. Two liters each of LB medium supplemented with 1 mM isopropyl-1-thio-β-D-galactoside (final concentration) in two 6-liter flasks were inoculated with 50 ml of the overnight culture and allowed to grow at 37 °C with aeration until the absorbance at 600 nm = 3.0. The cells were harvested by centrifugation at 4000 rpm for 30 min at 4 °C. Subsequent steps were performed at 4 °C unless otherwise specified.

The cell pellet (8.5 g) was washed twice with 800 ml of 20 mM Tris-HCl buffer containing 0.1 mM NaCl and 1 mM EDTA, pH 7.5, and finally resuspended in 32 ml of 20 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.5. The cells were disrupted by two passages through a Wabash French pressure. Non-lysed cells were removed by centrifugation at 10,000 × g for 1 h.

**Step 2: Streptomycin Sulfate Precipitation—**Nucleic acids were precipitated using streptomycin sulfate (160 μl of 10% stock solution/ml crude extract) added dropwise with stirring. The mixture was stirred for an additional 45 min and centrifuged at 100,000 × g for 30 min.

**Step 3: Ammonium Sulfate Fractionation—**The supernatant of Step 2 was treated with saturated ammonium sulfate solution added dropwise with stirring to a final concentration of 55%. After stirring overnight, the suspension was centrifuged at 150,000 × g for 1 h. The ammonium sulfate pellet was resuspended in 30 ml of 20 mM potassium phosphate buffer containing 0.15 M NaCl, pH 7.0, and dialyzed against the same buffer.

**Step 4: DEAE Column Chromatography—**The 55% ammonium sulfate fraction was transferred to a 200-ml DEAE-Sepharose fast-flow column equilibrated with the phosphate, NaCl buffer of Step 3. After transferring the sample to the column, it was washed with 500 ml of the same buffer, and a gradient (700 ml) from 0.15 M NaCl to 1.2 M NaCl in the phosphate buffer was applied to elute the column. The activity eluted between 0.4 and 0.5 M NaCl. The active fractions were pooled and dialyzed against 20 mM potassium phosphate buffer, pH 6.8, containing 0.2 M NaCl.

**Step 5: IMAC Chromatography—**The pooled sample from Step 4 was transferred to an immobilized Zn²⁺ affinity column (20-ml bed volume, Amersham Biosciences) that had first been equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 0.2 M NaCl. The column was then eluted with a gradient of 0-0.5 M imidazole in the same buffer. The active fractions were pooled and dialyzed against 20 mM potassium phosphate buffer, pH 6.8, containing 10 mM NaCl and 1 mM EDTA followed by the same buffer without EDTA. Purity was monitored throughout the fractionation by SDS-PAGE and enzyme activity (Table 1). The purified enzyme was stored in small aliquots with dithiothreitol (0.5 mM, final concentration) at −20 or −80 °C. The activity was stable under these conditions.

**N-terminal Amino Acid Determination**

The N-terminal amino acid residues of purified recombinant BglA were determined by Dr. Robert Cole using an Applied Biosystems 475A protein sequencer (Amino Acid Sequencing Facility, Dept. of Biological Chemistry, Johns Hopkins School of Medicine, Baltimore, MD).
Effects of pH, Ionic Strength, and Temperature on Enzyme Activity

The effects of these parameters on enzyme activity were studied with the purified enzyme at 37 °C to determine the optimal conditions for kinetic characterization.

Effects of pH—The substrate concentrations of 2.5 mM pNP-Glc or celllobiose were used. The following buffer systems were employed: citrate (pH 3.0–6.5), imidazole (pH 6.5–7.7), TAPS (pH 7.7–9.0), and glycine-NaOH (pH 8.5–10.0). Where possible, overlapping pH ranges were used with different buffers.

Effect of Ionic Strength—The effect of ionic strength on enzyme activity was determined using 50 mM MES buffer, pH 6.5, supplemented with 0–2.0 M NaCl or KCl. Activity was determined as described above.

Effect of Temperature—The optimum temperature and thermal stability of the enzyme were investigated using pNP-glucoside or celllobiose as the substrate. The optimum temperature was determined by incubating reaction mixtures as described above over a temperature range of 0–70 °C. Thermal stability of the enzyme (0.5–3.0 μg) was measured in 100 μL of buffer by incubating at the desired temperature for 30 min, cooling to room temperature, and then initiating the reactions by the addition of the substrate. The formation of p-nitrophenol or free glucose was determined as described above.

RESULTS

Molecular Cloning, Expression and Purification of BglA

The genomic sequence of V. cholerae was employed to design primers for subcloning the bglA gene into the pET21a overexpression vector, yielding pET-bglA. The vector was used to transform E. coli BL21 (DE3). Fig. 1 shows that the protein BglA was expressed at a high level in these cells.

The enzyme was purified 10-fold from the crude extract in approximately a 25% yield, giving an apparently homogeneous protein (Fig. 1). The N-terminal amino acid sequence of the purified recombinant protein was MLLLTNHHIGTET. This sequence is identical to the N terminus predicted by the nucleotide sequence of the gene. The gene sequence also predicts that BglA contains 574 amino acids with a calculated molecular mass of 65 kDa (Fig. 1). A search of various data banks with the BglA sequence has not shown significant similarities with other proteins but indicates that BglA is a member of glycosyl hydrolase Family 9, which consists of a variety of endoglucanases and explains why it has been annotated a hydrolase Family 9, which consists of a variety of endoglucanases but indicates that BglA is a member of glycosyl hydrolase Family 9.

Kinetic Studies

Two types of substrates were used to define the optimal conditions for measuring the activity of the enzyme, pNP-β-Glc and celllobiose. p-Nitrophenol was measured spectrophotometrically, and Glc liberated from celllobiose was assayed with glucose oxidase.

Fig. 2 shows the rate of hydrolysis of pNP-Glc was constant with a time of incubation for at least 30 min, indicating that neither product, p-nitrophenol nor more importantly Glc, inhibited the enzyme at the low concentrations formed under these conditions. The kinetic modeling results described below suggest that high concentrations of Glc do inhibit the enzyme. End product inhibition has been observed with some glycohydrolases.
The kinetic studies summarized above suggest that VC0165 encodes an exoglucosidase, i.e. specifically a $\beta$-4-glucosidase. One possible explanation for the incongruity between our results and the assignment based on sequence is the statement made in the latest update of CAZy that "some glycoside hydrolases are multifunctional enzymes that contain catalytic domains that belong to different GH families." In other words, perhaps BglA is both an exoglucanase and endoglucanase. To resolve this question, more extensive kinetic studies were conducted with cellobiose.

The expected products are the monosaccharides, disaccharides, and trisaccharides, but the relative rates at which they appear kinetically could answer the question raised above. An ion-exchange method was developed using HPAEC as described under "Experimental Procedures" to determine these potential intermediates quantitatively. The standards were well resolved, and the areas under the peaks were used for quantitation following calibration with known concentrations of the respective standards. It should be noted that the columns were eluted for ~20 min in the event (although unlikely) that higher oligosaccharides were formed by transglycosylation, a reaction that is catalyzed under appropriate conditions by some glycosidases.

Fig. 3. Effect of cellobiose concentration on BglA activity. Activity was measured at 37 °C, pH 6.5, by determining the quantity of Glc liberated from cellobiose as described under "Experimental Procedures." A, initial rate of Glc formed versus cellobiose concentration. B, Woolf-Augustinsson-Hofstee plot of the data in A. The rate $v$ (nmol/min/μg protein) is plotted versus $v/[S]$ yielding an apparent $V_{max} = 1.6$ nmol/min/μg of protein and $K_m = 6.96$ mM for cellobiose.

Activity was determined as a function of pH, ionic strength, and temperature as described under "Experimental Procedures." The pH optimum of the purified recombinant enzyme was between 6.0–6.5. At the pH optimum, NaCl and KCl gave similar results with an optimum at 10 mM, and the enzyme retained 75% of its activity in 0.5 M salt. The purified enzyme retained greater than 90% of its activity in 20 mM EDTA or EGTA.

Purified BglA displayed a temperature optimum from 37 to 42 °C. The enzyme was stable for at least 1 h from 4–37 °C. The incubation at 40 and 70 °C for 3 h in dilute solution and in the absence of potential protective agents such as albumin or dithiothreitol resulted in 40 and 100% loss of enzyme activity, respectively.

Effects of Substrate Concentrations on Enzyme Activity; Specificity Studies—Fig. 2B shows the rate $v$ versus substrate concentration for pNP-glucoside. Kinetic parameters for pNP-glucoside were calculated from Woolf-Augustinsson-Hofstee ($v$ versus $v/[S]$) plots (9). As shown in Fig. 2C, the kinetic constants were found to be $K_m = 0.45$ mM and $V_{max} = 0.34$ nmol/min/μg.

Similar studies with cellobiose showed again that the rate of hydrolysis was constant with time of incubation and proportional to protein concentration. Fig. 3A shows the effect of cellobiose concentration on the rate of glucose formation, and Fig. 3B shows the Woolf-Augustinsson-Hofstee plot. The calculated kinetic parameters for cellobiose were $K_m = 6.96$ mM and $V_{max} = 1.60$ nmol/min/μg.

Normally, the aryl glycosides are hydrolyzed more rapidly by glycosidases than the corresponding alkyl glycosides or oligosaccharides, but in this case, the $V_{max}$ (and the $k_{cat}$) for cellobiose was 4.7-fold greater than that for pNP-glucoside. However, there is a compensating effect because of the differences in $K_m$ for the two substrates. The catalytic efficiency ($k_{cat}/K_m$) of the enzyme for the two substrates were approximately 45 and 13.7 for pNP-Glc and cellobiose, respectively.

The enzyme was also active with the higher $\beta$-1→4Glc-linked oligosaccharides, cellobiose, cellotetraose, and cellopentaose. At 1 mM concentrations, pH 6.5, at 37 °C, the rate of Glc formation from these oligomers was around the same as that observed with cellobiose. Attempts to obtain interpretable kinetic data were hampered by the fact that in addition to Glc, the product of each reaction was also a substrate for the enzyme.

pNP was released from pNP-cellobiose, but this came only after a lag period completely unlike the kinetics of hydrolysis of pNP-Glc, which proceeds rapidly with no lag. An analysis of the pNP-cellobiose/BglA incubation mixtures by TLC indicated that Glc and pNP-Glc were the first products of the reaction. The pNP was apparently released from the first hydrolysis product, PNP-Glc. This result is discussed below.

The enzyme displayed no activity toward any other $\alpha$ or $\beta$ sugar substrate tested including di(N-acetylglucosamine) (GlCNAC)$_2$, chitobiose (GlcN)$_2$, chitin oligosaccharides (GlcNAC)$_n$ ($n = 3–5$), trehalose, lactose, maltose, and sucrose. More importantly, it exhibited no activity with positional isomers of cellobiose: sophorose ($\beta$1→2), laminaribiose ($\beta$1→3), and gentiobiose ($\beta$1→6). No activity was detected with pNP-$\beta$-GalNAc, pNP-$\beta$-glucose-6-phosphate, or with pNP-(GlcNAc)$_n$ ($n = 1–3$).

Endoglucanase or Exoglucanase?

The data bank lists gene VC0615 as encoding an endoglucanase-related protein, and the CAZy (carbohydrate-active enzyme) database algorithm of Henrissat and co-workers (10–12) places this enzyme in Family 9 of the glycohydrolases. This large family comprises endoglucanases primarily of the cellulase type. The characterized enzymes do not hydrolyze cellobiose nor yield Glc as an end product. In fact, many of them produce cellobiose as an end product.

The kinetic studies summarized above suggest that VC0165 encodes an exoglucosidase, i.e. specifically a $\beta$-1,4-glucosidase.
BglA and the cellotetraose were incubated for 12 h at which point only Glc was detected in the incubation mixture. Aliquots were taken periodically starting at 0.5 min. Some of the results are shown in Fig. 4 and are discussed below.

**DISCUSSION**

As indicated in the Introduction, we have identified 10 contiguous genes in the *V. cholerae* genome that may constitute a chitin disaccharide transport and catabolic operon. In this report, one of these genes, VC0615 designated here as *bglA*, was molecularly cloned from *V. cholerae* DNA into a high expression strain of *E. coli*. The protein was purified to apparent homogeneity and was characterized as a \( \beta \)-1,4-glucosidase. The enzyme hydrolyzed cellobiose and aryl \( \beta \)-glucosides such as \( p \)-nitrophenyl \( \beta \)-D-glucopyranoside. It was also active with higher cellulose oligosaccharides, e.g. the trisaccharides, tetrasaccharides, and pentasaccharides, and in each case Glc was formed. However, BglA did not hydrolyze any other glucoside tested including the positional isomers of cellobiose, nor did it act on \((\text{GlcNAc})_2\). The question raised under "Results" is whether BglA is an exoglucosidase as suggested by the biochemical data or an endoglucosidase as suggested by the sequence or both.

In an attempt to answer this question, BglA was incubated with cellotetraose (\( \text{G}_4 \)) and the products were analyzed as a function of time of incubation. In the following, it is assumed that the rate constants for hydrolysis of the glycosidic bonds far exceed the rate constants in the opposite direction. For purposes of this discussion the reactions can be treated as "irreversible." If the enzyme is a pure endoglucanase, the reaction would be as follows.

\[
\text{Glc}_4 \rightarrow 2\text{Glc}_2
\]

**REACTION 1**

If the enzyme is a pure exoglucanase, the products would be as follows.

\[
\text{Glc}_4 \rightarrow \text{Glc} + \text{Glc}_3 \rightarrow 2\text{Glc} + \text{Glc}_2 \rightarrow 4\text{Glc}
\]

**REACTION 2**

The enzyme could function processively as follows.

\[
\text{Glc}_4 + \text{BglA} \rightarrow \text{BglA}\text{-Glc}_4 \rightarrow \text{BglA}\text{-Glc}_3 
\]

\[
\text{BglA}\text{-Glc}_2 \rightarrow \text{BglA}\text{-Glc} \rightarrow \text{BglA} + 4\text{Glc}
\]

**REACTION 3**

That is, once the enzyme binds the tetrasaccharide, the hydrolytic cleavages proceed sequentially until the final products (4Glc) are formed. Because BglA also hydrolyzes the trisaccharides and disaccharides, the on and off rates of dissociation of the intermediates BglA\text{-Glc}_3 and BglA\text{-Glc}_2 would have to be included in the model. If these rates favor dissociation, the enzyme would be partially processive.

Some of the kinetic results are shown in Fig. 4 (A (0–5 min), B (0–20 min), and C (0–60 min)). The curves are fitted to the experimental data points by a computer program KYPlot. It should be noted that the conditions were deliberately chosen so that if cellobiose and especially cellulbiose were produced, they would accumulate and could be detected before there was significant hydrolysis. That is, the initial concentration of substrate is 3 mM, but the \( K_m \) for hydrolysis of cellulbiose is \( \sim 7 \) mM.

**Fig. 4. Analysis of kinetics of hydrolysis of cellotetraose.** Progress curves are shown for the hydrolysis of cellotetraose at 0–5 min (A), 0–20 min (B), and 0–60 min (C). See "Experimental Procedures" for description of incubation mixture and analysis. There was no detectable \( \text{G}_2 \) until 5 min. The data points for \( \text{G}_4 \) were calculated by subtracting the sum of \( \text{G}_1 \), \( \text{G}_2 \), and \( \text{G}_3 \) (as total Glc) from 3 mM \( \text{G}_4 \) (total Glc = 12 mM). The lines are best fit curves obtained by the KYPlot program.

**Fig. 5. Simulation of the progress curve from 0–20 min incubation.** The results shown in Fig. 4 were modeled according to the simple stepwise hydrolysis scheme (see "Discussion"). The 0–20 min incubation (Fig. 4B) is shown as an example. The lines give the results of the modeling program. The fit to the data points suggests that the model is correct, and that the enzyme is a simple exoglucosidase that hydrolyzes each of the substrates \((\text{G}_4, \text{G}_3, \text{G}_2)\) at about the same rate.
and the values for cellobiose and cellotetraose appear to be around the same.

At the early time points, the products are Glc and Glc$_3$. As the concentration of substrate falls, so do the rates of formation of the two major products Glc and Glc$_3$. The concentration of Glc$_3$ reaches a steady state as it is formed from Glc$_4$ and hydrolyzed to Glc$_2$.

According to the equations written above, the key to the question of exoglucanase versus endoglucanase is the rate of formation of the substrate, Glc$_4$, Glc$_3$, and Glc$_2$. The concentration of Glc$_4$ falls below its $K_m$; whereas the concentration of Glc$_3$ rises to 5 mM, and Glc$_2$ is hydrolyzed to Glc$_2$.

Thus, all of our results are consistent with the conclusion that BglA is an exoglucosidase, and that its assignment as an endoglucanase (based on sequence) must be re-evaluated.

There is a hint that the enzyme may be partially processive. At the earliest time points, the only detectable products are Glc and cellobiose, and Glc exceeds Glc$_3$ by $\sim$60%. But these should be equimolar if the enzyme is not processive. If it is partially processive and the excess Glc is corrected for the fact that 1 mM Glc$_3$ yields 4Glc, the results suggest that the enzyme may be $\sim$15% processive under these conditions.

The specificity of BglA raises an important question. What is the physiological function of this enzyme, and why is it in the cluster of genes we presume to be a (GlcNAc)$_2$ transport/catabolic operon? We can only speculate. To begin with, the same gene is present in the genome of V. furnissii. However, neither V. furnissii nor V. cholerae catabolizes cellobiose or cellulose in contrast to their rapid utilization of chinin oligosaccharides and, more slowly, of chinin. There is at least one possibility that we can suggest. In plants, lignin is a polymerization product of the phenols formed from phenolic $\beta$-glucosidases such as coniferyl and syringin. The glucosides are cleaved by essential plant aryl-$\beta$-glucosidases (13). However, the phenols can also serve other functions. The plant pathogen Agrobacterium tumefaciens provokes tumor growth in infected plants, and the major pre-inducer of the bacterial virulence genes is the plant $\beta$-glucoside, coniferin. But the glucoside is first processed by the bacterial $\beta$-glucosidase to yield the phenol, coniferyl alcohol, the true inducer of the virulence genes (14). In other words, the $\beta$-glucosidase serves as part of an important signaling sequence. Because V. cholerae spends part of its life cycle associated with marine plants, it is conceivable that BglA has a similar although unknown function, namely to hydrolyze certain aryl-$\beta$-glucosides.

Studies are in progress to define the functions of BglA by constructing an in-frame deletion of the gene in the V. cholerae genome, which may result in a significant change in the phenotype of the cells.

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