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Purification and Characterization of a *Bacillus polymyxa* β-Glucosidase Expressed in *Escherichia coli*

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The β-glucosidase encoded by the *bglA* gene from *Bacillus polymyxa* was overproduced in *Escherichia coli* by using a plasmid in which *bglA* is under control of the *lacI* promoter. Induction with isopropyl-β-D-thiogalactopyranoside allowed an increase in the specific activity of the enzyme of about 100 times the basal level of gene expression. The enzyme was purified by a two-step procedure involving salting out with ammonium sulfate and ion-exchange chromatography with DEAE-cellulose. Fractions of β-glucosidase activity recovered by this procedure, after electrophoresis in an acrylamide gel and staining with silver nitrate, yielded a single band of protein. This band was shown by a zymogram to correspond to β-glucosidase activity. The purified protein showed an apparent molecular mass of 50 kDa and an isoelectric point of 4.6, values in agreement with those expected from the nucleotide sequence of the gene. K_m values of the enzyme, with either cellobiose or *p*-nitrophenyl-β-D-glucoside as the substrate, were determined. It was shown that the enzyme is competitively inhibited by glucose. The effects of different metallic ions and other agents were studied. Hg^{2+} was strongly inhibitory, while none of the other cations tested had any significant effect. Ethanol did not show the stimulating effect observed with other β-glucosidases. The mechanism of enzyme action was investigated. High-pressure liquid chromatography analysis with cellobiose as the substrate confirmed previous data revealing the formation of two products, glucose and another, unidentified, compound. Results presented here indicate that this compound is cellotriose formed by transglycosylation.

β-Glucosidases are a heterogeneous group of enzymes with a broad substrate specificity over different aryl- and alkyl-β-D-glucosides. These enzymes have aroused considerable interest primarily because of their involvement in the biological saccharification of cellulosic material (6, 29, 34).

Genes that code for β-glucosidases have been cloned from different species of bacteria and fungi. We have classified the products of these genes into two groups of homology, types I and II, which include enzymes with polypeptide chains of about 450 and 800 amino acids, respectively. All type I β-glucosidases described are bacterial, whereas type II includes enzymes from both bacteria and fungi. β-Glucosidases belonging to type I are homologous to a group of bacterial phospho-β-galactosidases (13).

Bacillus polymyxa β-glucosidase-encoding genes that have been expressed in *Escherichia coli* and *Saccharomyces cerevisiae* provide a useful system to study the relationship between primary structure and enzyme function. These genes are efficiently expressed in *E. coli*, either from their own promoters or under control of inducible promoters in expression vectors, and their activity is easily detected in colonies by using commercial chromogenic substrates (1, 12; this work). This greatly facilitates modification of the properties of the enzymes by random or site-directed mutagenesis.

In addition to their function in cellulolysis, β-glucosidases play other important biological roles. Plant β-glucosidases are involved in activation of phytohormones (10, 11) and in formation of fruit aromas (16, 22, 32, 33), and they have been associated with mechanisms of resistance to phytopathogens (34). β-Glucosidases are added to cellulolytic enzyme complexes produced by fungi to increase the efficiency of saccharification in processes which require digestion of plant material (9, 21, 30). Overproduction, modification, and heterologous expression of these enzymes offer new possibilities. Improved saccharification of cellulosic substrates by *Trichoderma reesei* has been achieved by amplification of a β-glucosidase-encoding gene from this species (5). A strain of *S. cerevisiae* able to ferment cellobiose has been constructed by expression in this yeast of the β-glucosidase-encoding gene *bglA* from *B. polymyxa* (1), which represents a step toward the development of new yeast strains able to produce ethanol from cellulosic substrates.

To expand the basic knowledge of β-glucosidases, we undertook the purification and characterization of the enzyme encoded by the *bglA* gene from *B. polymyxa* expressed in *E. coli*.

Production of *B. polymyxa bglA*-encoded β-glucosidase activity by *E. coli*. Cultures of *E. coli* W3110 (*lacI*^{q} *tnaA2 trpR*) harboring plasmid pLGBGA (1) were used for production of the enzyme. Plasmid pLGBGA consists of a 1,677-bp DNA fragment containing the β-glucosidase-encoding gene *bglA* from *B. polymyxa* cloned at the polylinker site of vector pUC18. The initial ATG codon of *bglA* is separated by 107 bp from *lacI*, which makes *bglA* inducible by isopropyl-β-D-thiogalactopyranoside. This plasmid was generated after sequential deletion of a DNA fragment from *B. polymyxa* which contained the *bglA* gene.

Cultures of *E. coli* W3110/pLGBGA were grown in complete (Luria-Bertani) medium supplemented with 250 mg of ampicillin per liter at 37°C to an A_{600} of 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to 2 mM, and the cultures were further incubated until the β-glucosidase activity encoded by *bglA* reached a maximum. The cells were harvested by centrifugation, washed with cold 50 mM citrate-phosphate buffer, pH 7.0, and resuspended in the same buffer. The cells were disrupted by sonication, and the cell

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TABLE 1. Purification of β-glucosidase

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude extract	93	4,930	53	100	1
Ammonium sulfate precipitation	23	3,295	143	67	2.7
Ion-exchange chromatography					
Pooled fractions 34–47	4.5	2,434	541	49	10.2
Fraction 43	0.024	58	2,417	1.2	46

debris was eliminated by centrifugation. β-Glucosidase activity was assayed as described previously, by using *p*-nitrophenyl-β-D-glucopyranoside (PNPG) or cellobiose as the substrate (12). Protein concentration was determined by the method of Bradford (7), with bovine serum albumin as the standard.

Specific activity in crude extracts of the culture reached a maximum of about 100 times the basal level of gene expression at 8 h after addition of isopropyl-β-D-thiogalactopyranoside. This level was maintained in the induced culture for more than 12 h.

Purification of β-glucosidase activity. All purification steps were carried out at 0 to 4°C. Enzyme activity was monitored with PNPG as the substrate.

Salting out of proteins in the crude extract was the first step of purification. The lower and upper ammonium sulfate concentration limits which allowed maintenance of the enzyme in solution were determined. Increasing amounts of the salt were added to crude extract samples, and the ratio of precipitated activity versus that kept in solution was calculated for each sample. Concentrations of 35 and 53% were found to be useful limits for fractionation of the activity. Therefore, the salt was added to the crude extract to a concentration of 35% and the precipitated protein (about 25% of the total) was eliminated by centrifugation, while 95% of the activity was maintained in solution. The salt concentration was then increased up to 53%: the precipitated protein (70% of that saved in the previous step), which contained most of the activity (96%), was kept, while the protein still in solution was discarded. The pellet obtained after precipitation with 53% salt was redissolved in a small volume of citrate-phosphate buffer and desalted by using Sephadex G-25 PD-10 columns (Pharmacia). Values of activity and protein recovered after this first purification step are summarized in Table 1.

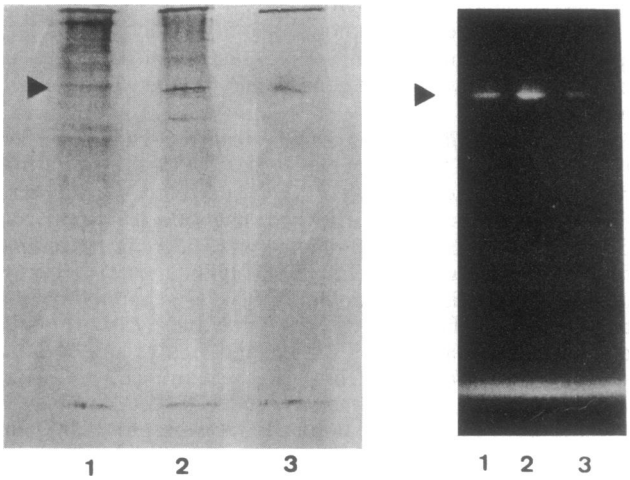

FIG. 1. Nondenaturing PAGE (left) and zymogram (right) of samples taken at different stages of β-glucosidase purification. Lanes: 1, crude extract of an isopropyl-β-D-thiogalactopyranoside-induced culture; 2, fraction recovered after salting out with ammonium sulfate; 3, fraction 38 recovered after DEAE chromatography. The arrowheads indicate the overproduced β-glucosidase and the corresponding activity.

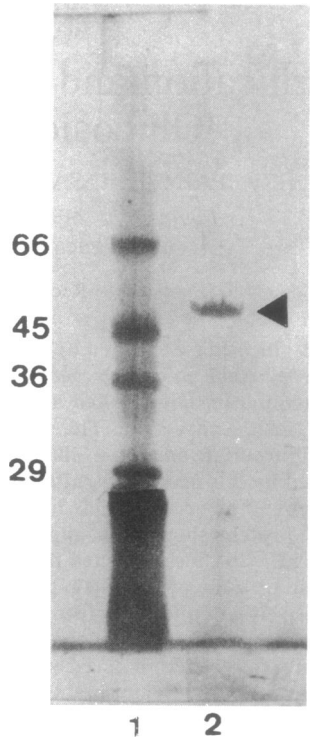

FIG. 2. Denaturing SDS-PAGE of purified β-glucosidase. Lanes: 1, molecular weight standards; 2, fraction 38 recovered after DEAE chromatography. The arrowhead indicates the purified β-glucosidase. The numbers on the left are molecular weights in thousands.

The enriched fraction recovered from ammonium sulfate fractionation was applied to a DEAE MemSep 1010 ion-exchange chromatography cartridge (Millipore) equilibrated with citrate-phosphate buffer. The proteins were eluted with a linear NaCl gradient (0 to 1.0 M) in the same buffer. Values of activity and protein in the recovered fractions are given in Table 1. About 50% of the total activity was recovered in a pool of fractions (34 to 47), achieving a purification factor of 10. Several fractions (38 to 45) showed a high degree of specific activity, over 40 times higher than that of the crude extract.

Samples taken at different stages of purification were analyzed by electrophoresis in nondenaturing polyacrylamide gels (Fig. 1) by using the discontinuous buffer system of Ornstein-Davis (17). Two 10% acrylamide gels were run in parallel. One of them was prepared as a zymogram by adding 4-methylumbelliferyl-β-D-glucopyranoside, an artificial substrate for β-glucosidase (6, 31), at a concentration of 2 mM. Hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside releases methylumbelliferone, a fluorescent compound which

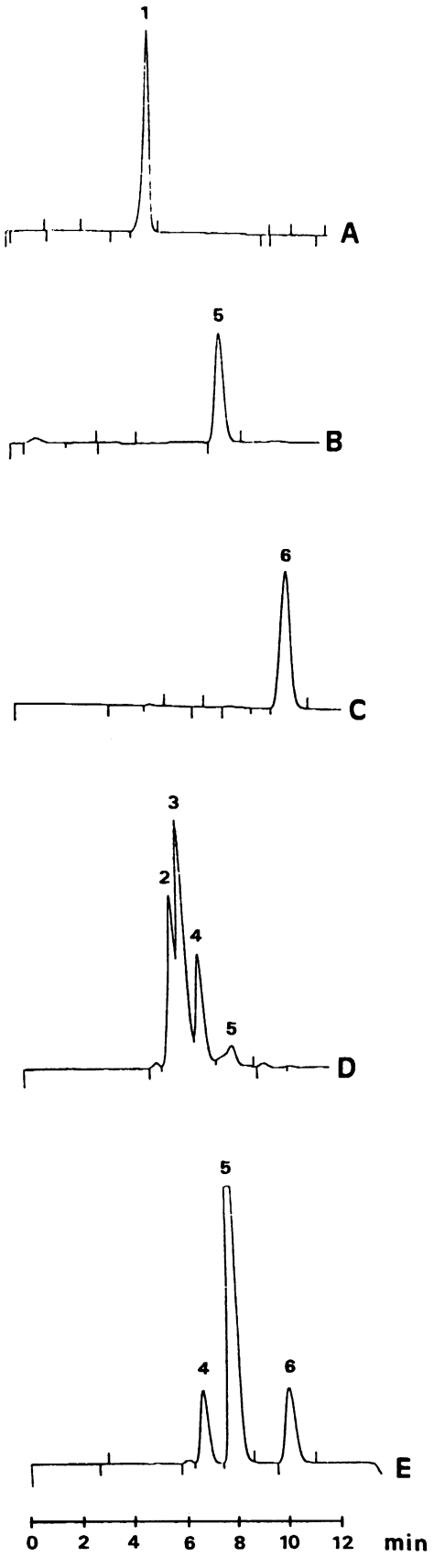

FIG. 3. HPLC analysis of the products of β-glucosidase activity with cellobiose as the substrate. Peaks are numbered on the basis of increasing retention time. Peaks having the same retention time are marked by the same number. Panels: A, mixture of glucose 1-phosphate and glucose 6-phosphate; B, cellobiose; C, glucose; D, mixture of cellodextrins (2 to 5 glucose units); E, reaction products of β-glucosidase with cellobiose as the substrate.

can be visualized under UV light. The two gels were run overnight at 5 mA. The gel without 4-methylumbelliferyl-β-D-glucopyranoside was stained with Coomassie brilliant blue R-250 and photographed under visible light, while the one containing 4-methylumbelliferyl-β-D-glucopyranoside was photographed under UV light (Fig. 1). The zymogram showed that the single band of protein observable in the highly purified fractions, visible as a prominent band in the tracks of crude extract and the ammonium sulfate fractionation product, had β-glucosidase activity.

It can be estimated that about 5% of the soluble protein present in crude extracts of induced cultures was β-glucosidase. The simple two-step purification procedure described can be scaled up and represents an important asset for structural studies which require high amounts of purified protein.

Physicochemical and kinetic properties of the enzyme. The purified protein was used to characterize its physicochemical and kinetic properties. The apparent molecular weight was determined by subjecting a highly pure fraction to denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and staining the gel with silver nitrate. The gel (Fig. 2) indicated that the β-glucosidase has a molecular mass of about 50 kDa. The isoelectric point of the protein, determined by using Ampholine PAG plates in a Multiphor Electrofocusing Unit (LKB-Pharmacia), was 4.6. Both molecular weight and isoelectric point determinations are well in agreement with the values deduced from the nucleotide sequence of the gene (13).

K_m values for PNPG and cellobiose were determined from Lineweaver-Burk plots. PNPG and cellobiose were used as substrates at concentrations of 80 μM to 3 mM and 10 to 100 mM, respectively. The enzyme showed K_m values of 0.6 mM for PNPG and 13 mM for cellobiose. Higher affinity for PNPG is also shown by several other β-glucosidases (34), suggesting that the in vivo role of this enzyme is not cellobiose hydrolysis. Inhibition by glucose was also investigated by measuring enzyme activity with PNPG as the substrate in the presence of different concentrations of the sugar (0, 0.04, and 0.15 M). The Lineweaver-Burk plots obtained showed that glucose caused competitive inhibition with a K_i value of 19 mM. Inhibition by glucose, a common characteristic of β-glucosidases (3, 28, 30, 34) although there are exceptions (2), is an important drawback for industrial use of these enzymes.

The effects of different metallic ions (Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, and Hg^{2+}) and other agents (dithiothreitol, SDS, and ethanol) on the activity of the purified enzyme were tested. Among the different cations, Hg^{2+}, which is known to affect thiol groups, was strongly inhibitory at 1 mM, while none of the others had any significant effect. Dithiothreitol and SDS, both at 5 mM, reduced the activity by 30 and 85%, respectively. Ethanol, which at a certain range of concentrations has been proved to increase the activity of some, but not all, β-glucosidases (14, 15, 27), had no stimulating effect on this enzyme.

Mechanism of enzyme action. Chromatographic analysis of the products of β-glucosidase activity was carried out by using a high-pressure liquid chromatograph (HPLC) equipped with a Waters Sugar-PAK I column (Millipore). Samples of purified β-glucosidase (150 mU of PNPGase activity) were incubated with the substrate (cellobiose at 10 mg/ml in citrate-phosphate buffer). The reactions were allowed to proceed at 37°C for different times. The samples were then deionized with AG 50W-X8 cation-exchange resin and AG 4-X4 anion-exchange resin (Bio-Rad) and injected into the chromatograph. The column was operated at 40°C with water as the mobile phase at a flow rate of 0.5 ml/min, and the reaction products were monitored with a refractive index detector.

Previous analysis by HPLC of the products of reaction of *bglA*-encoded activity present in crude extracts of *E. coli* with cellobiose as the substrate revealed the formation of glucose and another, uncharacterized, compound (12). It is known that β-glucosidases have different mechanisms of action that yield products other than glucose. One type is phosphorylation, which leads to formation of glucose 1-phosphate (4). Formation of glucose 1-phosphate from cellobiose and P_i by P_i glucosyltransferase (EC 2.4.1.20) is characteristic of cellulolytic organisms which grow better on cellobiose than on glucose as the sole carbon source, because the energy of the β-1,4-glucosidically bound fraction is conserved in the phosphorylated glucose (18, 19, 24, 28). ATP-dependent phosphorylation of cellobiose yielding glucose and glucose 6-phosphate has also been described (26). Another mechanism is transglycosylation, which can give rise to cellotriose or other products, depending on the nature of the acceptor molecule (25). The chromatograms presented in Fig. 3 confirm previous data revealing the formation of glucose and another product and show that this compound can be neither glucose 1-phosphate nor glucose 6-phosphate (both phosphorylated sugars appear unresolved in peak 1 in Fig. 3A). According to the described mechanisms of β-glucosidase action, the unidentified compound could only be cellotriose generated by transglycosylation (25). That this is indeed the case was indicated by the control shown in Fig. 3D, in which a mixture of cellodextrins, 2 to 5 U, was injected into the chromatograph. Cellotriose (peak 4 in Fig. 3D) showed exactly the same retention time as the unidentified compound resulting from β-glucosidase action (Fig. 3E, peak 4). Transglycosylation as a mechanism of action of a β-glycosidase has an important precedent in the literature: the *lacZ*-encoded β-galactosidase of *E. coli*, in the presence of lactose, catalyzes the formation of allolactose (23), which acts as the true inducer of the *lac* operon (8, 20).

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