Cloning and Characterization of the Beta-Amylase Gene from

Bacillus polymyxa

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The gene for beta-amyrase was isolated from Bacillus polymyxa by molecular cloning in B. subtilis. B. subtilis cells containing this gene express and secrete an amylase which resembles the B. polymyxa beta-amyrase and barley beta-amylase in terms of the products it generates during carbohydrate hydrolysis. Starch hydrolysis with this beta-amyrase produces maltose, not glucose, whereas maltotriose and cyclodextrins are resistant to the action of this beta-amyrase. The enzyme has a molecular weight of approximately 68,000. Restriction endonuclease mapping demonstrated that the DNA inserted in pBD64 and containing the gene is approximately 3 kilobases in length.

Bacilli are known to secrete a number of enzymes into the extracellular medium. One of the major classes of these extracellular enzymes are amylases. Both alpha-amylases and beta-amylases hydrolyze alpha C1-O-C4 bonds. However, alpha-amylases and beta-amylases are distinguished by different enzymatic action patterns observed in the hydrolysis of starch.

During the early stages of starch digestion, alpha-amylases split starch essentially at random (13). The products of this hydrolysis are alpha-maltose, alpha-glucose, and alpha-limit dextrins. The alpha-amylases are endoenzymes that can bypass the alpha-1,6 branch points of amylopectin. Their action results in a rapid decrease in viscosity (liquefaction) and a loss of iodine-binding color (dextrinogenic capacity).

In contrast, beta-amylases are exoenzymes that remove maltose stepwise from the nonreducing end of starch (13). The products of this hydrolysis are beta-maltose, beta-limit dextrins, and very small amounts of beta-glucose (formed by the degradation of amylase molecules containing an odd number of sugar residues). Beta-amylases do not hydrolyze amylpectin internally to the alpha-1,6 branch points. During the early stages of starch hydrolysis, beta-amylases generate relatively large amounts of maltose, as compared with the amounts of dextrin fragments liberated. That is, beta-amylases are saccharifying enzymes.

It should, however, be realized that alpha-amylases are "partially" saccharifying and that beta-amylases are "partially" liquefying and dextrinogenic. Indeed, Bacillus subtilis was once thought to produce two types of alpha-amylases, one liquefying and the other saccharifying. Based upon DNA hybridization results, the organism secreting the liquefying enzyme has been renamed B. amylooliquefaciens (18).

Until recently, it was believed that beta-amylase existed only in higher plants. Observations that beta-amylases are found in B. polymyxa (12), B. megaterium (7), and a Bacillus species that secretes an unusual amylase in an alkaline environment (1) established that beta-amylases are produced by several Bacillus species as extracellular enzymes.

Although there have been reports of the cloning of secreted alpha-amylases from Bacillus species, including the amylases from B. amylooliquefaciens (17), B. subtilis (19), and B. licheniformis (16), comparable attempts with beta-amylases have not appeared in the literature. Here we describe the isolation of the gene coding for beta-amyrase (EC 3.2.1.2.) from B. polymyxa, the characterization of the crude enzyme, and the restriction endonuclease mapping of the DNA fragments containing the gene.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. B. polymyxa ATCC 8523 provided the chromosomal DNA. B. subtilis BR151 (trpC2 metB10 lys-3) was used as the host for the amylase gene. BR151 secretes only traces of alpha-amyrase. These bacterial strains were grown in L broth (10) in a rotary shaker at 37°C. To screen for amyrase activity, we streaked the bacteria onto plates containing L broth and 0.8% soluble potato starch (LS plates). The LS plates were kept for at least 4 weeks under refrigeration before use to allow precipitation of the starch. When the LS plates were stained to detect amyrase activity, a solution containing 0.1% iodine and 0.2% potassium iodide was used. The plasmids used were pUB110 (2) and pBD64 (6). When the bacteria were carrying plasmid pUB110, the growth medium was supplemented with 5 μg of kanamycin per ml; when they were carrying plasmid pBD64, it was supplemented with 10 μg of chloramphenicol per ml.

DNA isolation and plasmid construction. B. polymyxa cellular DNA was isolated (11) and partially cleaved with restriction endonuclease MboI to yield DNA fragments of 2 to 14 kilobases (kb) in size (14). DNA fragments were recovered from the reaction mixture by phenol extraction and ethanol precipitation. Plasmid pBD64 was isolated from B. subtilis 1E22 (pBD64) (5). After dialysis and ethanol precipitation, the plasmid was linearized by BamHI digestion. For ligation, approximately equal amounts (weight-to-weight ratio) of linearized pBD64 and partially digested B. polymyxa chromosome were incubated in the presence of T4 ligase at 16°C overnight. The ligated DNA was used to

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transform *B. subtilis* BR151 cells containing pUB110 (6). Transformations were done on LS plates in overlays containing 2.5 ml of LB broth, 0.7% agar, and 150 μg of chloramphenicol. DNA from clones on these plates was used to transform BR151 cells not containing pUB110. To prepare sufficient plasmid DNA for restriction map construction, we inoculated L broth containing chloramphenicol and kanamycin with frozen cells from a beta-amylase-producing transformant. *B. subtilis* cells (1 liter) were grown for 5 to 6 h. The cells were suspended in 40 ml of 10 mM Tris hydrochloride (pH 8.0)—50 mM EDTA—100 mM NaCl, and 1.5 ml of a 10-mg/ml lysozyme solution was added. The suspension was kept at 37°C until lysis occurred (approximately 30 min) and was then rapidly boiled. After centrifugation, 1 ml of a 10-mg/ml proteinase K solution (Boehringer-Mannheim Biochemicals) was added, and the mixture was kept at 70°C for 40 min. The DNA was then precipitated with an equal volume of isopropanol, and the plasmid DNA was purified by cesium chloride equilibrium density centrifugation in the presence of ethidium bromide. The removal of ethidium bromide by n-butanol extraction was followed by dialysis against 10 mM Tris hydrochloride—1 mM EDTA.

**Enzyme studies and extracellular protein separation.** The medium in which the cells had been grown was saturated to 100% with (NH₄)₂SO₄ in the presence of 1 mM phenylmethanesulfonyl fluoride. The precipitate was dissolved in and dialyzed against 0.01 M calcium acetate and lyophilized. Crystalline enzyme (1 mg) or 5 mg of ammonium sulfate-precipitated culture medium (dialyzed and lyophilized) and 4 mg of substrate were dissolved in 0.3 ml of 0.01 M calcium acetate and incubated at 37°C for 3 h. Prior to incubation with the substrate, the enzyme secreted by *B. polymyxa* was partially purified by fractionation on a DEAE column (4) to avoid possible contamination of the beta-amylase by a debranching enzyme. Paper chromatography and subsequent staining with AgNO₃ were performed as described by Roby and French (15).

Crystalline *B. amyloliqufaciens* alpha-amylase (A 6380) and crude *Aspergillus oryzae* alpha-amylase (takadiastase) (A 0273) were purchased from Sigma Chemical Co. Crystalline porcine alpha-amylase (171535) and barley beta-amylase (17157) were obtained from Calbiochem-Behring.

Protein samples for gel electrophoresis were dissolved in Laemmli sample buffer (9), boiled for 5 min, and subjected to electrophoresis on a 10% polyacrylamide—sodium dodecyl sulfate gel. After electrophoresis, one-half of the gel was

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**FIG. 1.** Plate assay. LS plates contained the following bacteria (not stained with iodine): A, *B. subtilis* BR151(pGX2509) (BR151 amy [Bam]); B, *B. subtilis* BR151 (pGX2566) (BR151 amy [Bpo]); C, *B. polymyxa* ATCC 8523; and D, *B. subtilis* BR151.
stained for 45 min with 0.1% Coomassie brilliant blue-R–50% methanol–7.5% acetic acid and then destained overnight with 10% acetic acid–5% methanol. The other half of the gel was dialyzed against H2O for 2 h (to remove the sodium dodecyl sulfate) and was then incubated for 4 h on an agarose gel containing soluble potato starch. After incubation, the agarose gel was stained with an iodine solution containing 0.1% iodine and 0.2% potassium iodine. Staining occurred in approximately 5 min. The iodine was then rinsed off with H2O. The agarose gel was observed as the iodine evaporated to obtain maximum contrast for photography.

RESULTS

Isolation of the gene coding for the beta-amylase (amy [Bpo]) from B. polymyxa. B. subtilis BR151(pUB110) was transformed with B. polymyxa DNA which had been partially digested with MboI and ligated to BamHI-digested pBD64. After 24 h of incubation at 37°C, a total of 10⁶ transformants were obtained on LS plates. Zones of precipitated starch

FIG. 2. Paper-chromatographic analysis of the products from the reaction of pure crystalline porcine pancreatic alpha-amylase with starch (lane 1), cyclomaltoheptaose (lane 2), and maltotriose (lane 3) and from the reaction of the partially purified enzyme secreted by A. oryzae with the same three substrates in the same order (lanes 4, 5, and 6). Lane 7 is a glucose marker.

FIG. 3. Paper-chromatographic analysis of the products from the reaction of crystalline B. amyloliquefaciens enzyme with starch (lane 1), cyclomaltoheptaose (lane 2), and maltotriose (lane 3) and from the reaction of crystalline barley beta-amylase with the same three substrates in the same order (lanes 4, 5, and 6) and of the partially purified protein from BR151(pGX2566) cultures with the same three substrates in the same order (lanes 7, 8, and 9). Lane 10 is a glucose marker.
clearing were observed in two areas of confluent transformants. Colonies from these areas were diluted and replated onto LS plates containing chloramphenicol to isolate single positive colonies. Plasmid DNA was prepared from each of the two isolates. This DNA was used to transform BR151 (plasmid free), and chloramphenicol-resistant transformants that cleared starch were again isolated. One of the plasmids from a starch-clearing isolate was designated pGX2566.

**Characterization of the secreted amylase by a plate assay.** B. subtilis BR151, B. polymyxa, B. subtilis BR151(pGX2566), and B. subtilis BR151 containing the cloned alpha-amylase gene from B. amyloliquefaciens (amy [Bam]) pGX2509 (C. Rhodes and C. Banner, unpublished data) were grown in L broth overnight and plated onto LS plates containing chloramphenicol. A comparison of these strains showed that BR151 made no detectable amylase (Fig. 1D), B. polymyxa developed first a cloudy halo (data not shown) and then a clear halo (Fig. 1C), BR151(pGX2509) developed a clear halo (Fig. 1A), and BR151(pGX2566) developed a cloudy halo (Fig. 1B).

**Characterization of the secreted amylase from cells carrying pGX2566.** The medium in which the cells had been grown was saturated to 100% with (NH₄)₂SO₄. The precipitate was dissolved in and dialyzed against 0.01 M calcium acetate and lyophilized. The products resulting from the action of B. polymyxa-derived beta-amylase were compared with the products obtained by the action of other amylases. Paper-chromatographic analysis revealed the following. When starch, cyclomaltotriose, and maltotriose were used as the substrates, alpha-amylases such as crystalline porcine pancreatic enzyme (Fig. 2, lanes 1, 2, and 3, respectively), crystalline B. amyloliquefaciens enzyme (Fig. 3, lanes 1, 2, and 3, respectively), and nonpurified enzyme secreted by B. subtilis IS311, an amylase-positive strain (Fig. 4, lanes 5, 6, and 7, respectively) resulted in the production of glucose and

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**FIG. 4.** Paper-chromatographic analysis of the products from the reaction of the partially purified B. polymyxa enzyme with starch (lane 2), cyclomaltotriose (lane 3), and maltotriose (lane 4) and of the nonpurified enzyme secreted by B. subtilis IS311 with the same three substrates in the same order (lanes 5, 6, and 7). Lane 1 is a glucose marker.

**FIG. 5.** (A) Separation of the secreted protein on a 10% polyacrylamide–sodium dodecyl sulfate gel. Lanes: 1, Medium from a B. subtilis(pGX2566) culture; 2, the standards myosin (heavy chain) (200,000), phosphorylase b (92,500), bovine serum albumin (68,000), and ovalbumin (43,000); and 3, medium from a B. subtilis (pGX2509) culture. (B) Gel from panel A after overnight incubation on 1% agarose containing 0.1% starch and staining with iodine. Lanes 1, 2, and 3 are the same as in panel A. MW, Molecular weight.
maltose from the starch and the maltotriose. However, none of the bacterial alpha-amylases catalyzed the hydrolysis of the cyclic compound. Partially purified takadiastase from A. oryzae (Fig. 2, lanes 4, 5, and 6) acted on all three substrates (starch, cyclomaltoheptaose, and maltotriose, respectively) to produce maltose and glucose.

When beta-amylases were incubated with the same substrates (starch, cyclomaltoheptaose, and maltotriose), starch was the only substrate digested effectively; data are shown for crystalline barley beta-amylase (Fig. 3, lanes 4, 5, and 6, respectively) and partially purified B. polymyxa enzyme (Fig. 4, lanes 2, 3, and 4, respectively). These enzymes did not act on cyclomaltoheptaose or maltotriose and yielded maltose and little appreciable glucose from starch. The extracellular enzymes from cultures of BR151(pGX2566) (Fig. 3, lanes 7, 8, and 9) acted in the same manner as the B. polymyxa enzyme when exposed to starch, cyclomaltoheptaose, and maltotriose, respectively.

**Determination of the molecular weight of the B. polymyxa beta-amylase.** To identify and obtain a molecular weight for the beta-amylase protein, we made crude extracellular enzyme preparations from BR151(pGX2566), BR151(pBD64), and BR151(pGX2509). The crude extracellular preparation from BR151(pGX2566), molecular weights standards, and the crude extracellular preparation from BR151(pGX2509) were run in duplicate on a polyacrylamide-sodium dodecyl sulfate gel. The gel was cut in half. One-half was stained with Coomassie blue, and the other half was allowed to diffuse into an agarose gel containing starch. Figure 5 shows the position of the beta-amylase protein in comparison with the position of the alpha-amylase protein. The alpha-amylase protein has a molecular weight of 54,778. The beta-amylase from BR151(pGX2566) appeared to have a molecular weight of approximately 68,000. The BR151(pBD64) crude extracellular protein preparation did not have a major band at the location for either the alpha-amylase or the beta-amylase (data not shown).

**Mapping the insert.** When treated with restriction endonuclease PvuII, plasmid pGX2566 (approximate size, 7.8 kb) yielded two pieces (i.e., there is one cut in the parent plasmid and a second cut in the insert) 6 and 1.7 kb in size (Fig. 6). When both EcoRI and PvuII were used, two more pieces were split from the 6-kb fragment. Thus, in addition to the 1.7-kb piece, 3.8-, 1.3-, and 1-kb pieces were seen when the mixture was electrophoresed on agar (Fig. 6). When plasmid pGX2566 was digested either with PvuII alone or with EcoRI alone, ligated, and used to transform BR151 cells, both beta-amylase-positive and -negative clones were obtained. Plasmid minipreparations (8) from these transformants showed that deletion of the 1.3-kb EcoRI fragment or the 1.7-kb PvuII fragment resulted in the amylase-negative phenotype.

**Southern analysis.** A blot with a 32P-labeled pGX2566 EcoRI-HindIII digest as a probe showed homology only with B. polymyxa ATCC 8523 chromosomal DNA (Fig. 7, lanes 3 and 4) and not with B. subtilis BR151 chromosomal DNA (Fig. 7, lanes 2 and 5) or B. amyloliquefaciens ATCC 23845 chromosomal DNA (Fig. 7, lane 7).

**DISCUSSION**

Through ligation of B. polymyxa ATCC 8523 chromosomal DNA into plasmid pBD64 and subsequent transformation of BR151(pUB110), we were able to identify clones positive for beta-amylase production. Screening of the transformants was accomplished with LS plates that had been kept in storage (at 4°C) for at least 4 weeks. On these opaque plates...
beta-amylase-producing bacteria formed a partially cleared area (probably owing to a background of precipitated beta-limit dextrin). This is in contrast to results obtained with alpha-amylase-producing bacteria, which generated a completely clear area or halo (Fig. 1). Iodine staining of starch cleared by alpha-amylase and starch partially cleared by beta-amylase resulted in clear and reddish halos, respectively (data not shown).

B. subtilis BR151 (pGX2566) secretes a beta-amylase having an approximate molecular weight of 68,000. Restriction endonuclease mapping suggested that the inserted DNA is approximately 3 kb; thus, at least 70% of the insert should represent the beta-amylase structural gene and its control elements. The fact that the crude enzyme catalyzes the breakdown of starch with the production of maltose but without the liberation of glucose indicates that it is a beta-amylase rather than an alpha-amylase. This is supported by the finding that the enzyme did not effectively digest maltotriose or cyclodextrins. Indeed, it has been reported that the amylolytic enzyme from B. polymyxa ATCC 8523 and sweet potato beta-amylase generate the same products (12).

It has been observed that there are two enzymes in the starch-degrading system of B. polymyxa (4). This bacterium secretes a beta-amylase and a debranching enzyme which is specific for the alpha-1,6 linkage in starch. A cloned gene for a debranching enzyme would generate very little maltose and would also produce maltotriose. Paper-chromatographic analysis of products generated during starch hydrolysis with extracts from BR151 (pGX2566) cultures indicated that only maltose is released; thus, there is no evidence for debranching enzyme activity.

Interest in the eventual sequencing of the B. polymyxa gene coding for beta-amylase exists not only because the establishment of more secretion sequences and other Bacillus regulatory signals (promoters, ribosome binding sites, etc.) should be worthwhile. There may be a family of enzymes (such as the serine proteases) of carbohydrate hydrolyses in which certain amino acid residues compose the active site, and although the sequences may not be homologous, the arrangements of the catalytic sites may be (as is the case for trypsin and subtilisin). This question is intriguing because of the nuances in the specificity of action that exist for the amylases (e.g., alpha and beta, saccharifying and liquefying, etc.). When tested immunologically, the alpha-amylase produced by B. amyloliquefaciens does not cross-react with that produced by B. subtilis or with that elaborated by A. oryzae. Nor is there a cross-reaction with the alpha-amylase isolated from pig pancreas (unpublished results). Although there is indeed little homology in the amino acid sequences in these proteins, three short homologous regions have been found (3). In the region coding for the B. polymyxa beta-amylase sequenced so far, the homologous short sequence present in all the alpha-amylases is absent (unpublished results).

LITERATURE CITED