Comparison of the \(\alpha\)-Amylase of *Bacillus subtilis* and *Bacillus amyloliquefaciens*

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The \(\alpha\)-amylase \((\text{\(\alpha\)-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1})\) of *Bacillus subtilis* strain W23 is less negatively-charged than the \(\alpha\)-amylase of *B. amyloliquefaciens* strain F, as determined by electrophoretic mobility in polyacrylamide gel at pH 8.6. The \(\alpha\)-amylase of strain W23 is immunologically unrelated to the \(\alpha\)-amylase of strain F, as judged by lack of cross-reaction in Ouchterlony immunodiffusion studies. The pH range of maximal activity for the enzyme of strain W23 was 5.7 to 6.7, with a maximum at 6.3. The pH range of activity for the \(\alpha\)-amylase of strain F was 5.5 to 6.5, with a maximum at 5.9. No significant difference was found in the effect of temperature on the activity of the \(\alpha\)-amylase of strain W23 and strain F. \(\alpha\)-Amylase production by strain W23 occurs throughout the 7-hr growth period, whereas enzyme production by strain F does not begin until the culture enters the stationary phase of growth. The total amounts of enzyme produced by strains W23 and F after 7 hr of growth were 0.3 and 25.5 units/ml, respectively.

We have shown that the highly amylolytic organisms isolated from commercial, \(\alpha\)-amylase concentrates (reportedly prepared from strains of *Bacillus subtilis*) are not biochemically or genetically related to *B. subtilis* Marburg but are strains of *B. amyloliquefaciens* Fukumoto (16). Owing to the fact that extensive studies have been conducted on the characterization of the \(\alpha\)-amylase obtained from these commercial enzyme concentrates [see review by Fischer and Stein (3)], it was of interest to compare the \(\alpha\)-amylase of an authentic strain of *B. subtilis* with that of *B. amyloliquefaciens*. In this paper, we describe some of the differences between the \(\alpha\)-amylases produced by these two groups of organisms.

Materials and Methods

Organisms. The organisms used in this investigation were *B. subtilis* strain W23 and *B. amyloliquefaciens* strain F. The media, conditions of growth, and maintenance of stock cultures were described by Welker and Campbell (16).

Assay of \(\alpha\)-amylase. The saccharogenic assay of Fischer and Stein (4) as modified by Welker and Campbell (15) was used to measure enzyme activity. The absorbancy was read at 400 nm in a Bausch & Lomb Spectronic-20 colorimeter. Protein was determined by the method of Lowry et al. (10) with crystalline bovine serum albumin as a standard. One unit of \(\alpha\)-amylase activity was defined as that amount of protein which will release 1 mg of reducing sugars, calculated as maltose, in 3 min at 25 C. *B. subtilis* \(\alpha\)-amylase was assayed at pH 6.3; *B. amyloliquefaciens* \(\alpha\)-amylase was assayed at pH 5.9 (17).

Enzyme production. The medium and conditions used for \(\alpha\)-amylase production by *B. amyloliquefaciens* strain F (17) were also employed for enzyme production by *B. subtilis* W23.

Enzyme purification. The \(\alpha\)-amylase of *B. amyloliquefaciens* was purified and crystallized as described by Welker and Campbell (17). The \(\alpha\)-amylase of *B. subtilis* W23 was purified by column chromatography on diethylaminoethyl (DEAE) cellulose. The DEAE cellulose (standard capacity, 0.9 ± 0.1 meq; Mann Research Laboratories, Inc., New York, N.Y.) was washed exhaustively with 0.005 m sodium phosphate buffer, pH 7.2 (equilibration buffer), and packed to a height of 20 cm in a chromatographic column (4 cm in diameter) fitted with a sintered-glass filter. The DEAE cellulose was washed exhaustively with equilibration buffer, and the \(\alpha\)-amylase solution (dialyzed thoroughly against equilibration buffer) was applied to the column. The \(\alpha\)-amylase was eluted by step-wise elution with increasing molarities of sodium phosphate buffer (0.005, 0.01, 0.02, 0.05, and 0.1 M), pH 7.2. The fractionation was carried out at 4 C and the eluate was collected in 30-ml fractions. Each fraction was assayed for enzyme activity. The \(\alpha\)-amylase was quantitatively eluted with 0.1 M sodium phosphate. The frac-

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tions, containing α-amylase activity, were combined, placed in dialysis tubing, and concentrated (4 C) by immersion in a glass cylinder containing Carbowax 4000 (Union Carbide Chemical Co., Charleston, W.Va.). The concentrated enzyme was dialyzed (4 C) against 0.02 M sodium-glycophosphate buffer, pH 7.0 (GP buffer). The dialyzed enzyme solution was lyophilized and dissolved in 1 ml of distilled water.

The α-amylase was then purified by disc electrophoresis in the Canalco model 12 disc electrophoresis apparatus by the procedure outlined in the model 12 instruction manual. Electrophoresis (3 ma per column) was carried out for 40 min at ambient room temperature; the gels were immediately removed, and the upper 10 mm from each gel was combined. The combined gels were mashed and suspended in 3 ml of GP buffer and placed at 4 C for 12 hr. The gel was removed by suction filtration through a coarse sintered-glass funnel and washed with 5 ml of GP buffer. The purified α-amylase was found to be free from contaminating protein, as judged by disc electrophoresis (Fig. 1A). Table 1 summarizes a typical purification procedure.

**Immunological procedures.** Antiserum against the crystalline α-amylase of *B. amyloliquefaciens* strain F was prepared as described by Welker and Campbell (17). Antibody was not prepared against the α-amylase of *B. subtilis* W23 owing to the limited supply of the enzyme. Ouchterlony plates were prepared according to Postgate and Campbell (13).

**RESULTS**

**Effect of pH and temperature on enzyme activity.** The procedures used for the determination of pH and temperature optima were described by Welker and Campbell (17). The α-amylase of *B. subtilis* W23 used in these studies was purified through step 3 of Table 1 and had an activity of 31 units/ml. The pH range of maximal enzyme activity for the α-amylase of *B. subtilis* W23 was found to be 5.7 to 6.7, with a maximum at 6.3. The pH range for the α-amylase of *B. amyloliquefaciens* is 5.5 to 6.5, with a single maximum at 5.9 (17). At pH 3.5, the α-amylase of *B. subtilis* W23 and *B. amyloliquefaciens* had 50 and 15%, respectively, of their maximal activity. The stability of the α-amylase of *B. subtilis* W23 with respect to pH was not examined owing to the small quantity of purified enzyme available, but the α-amylase of *B. amyloliquefaciens* is rapidly inactivated below pH 5.0 (17).

Activity measurements of the two α-amylases were carried out at their respective pH optima over the temperature range of 0 to 70 C at 5 C increments. No significant difference was noted in the temperature optimum; both enzymes exhibited a temperature optimum of 65 C.

**Electrophoretic mobility.** The α-amylase of *B. subtilis* W23 and *B. amyloliquefaciens* F were compared with respect to their electrophoretic mobility in polyacrylamide gel. The results show that the α-amylase of *B. subtilis* W23 (Fig. 1A) is less negatively charged than the α-amylase of *B. amyloliquefaciens* F (Fig. 1C). A control gel (Fig. 1B) containing both enzymes shows that there is no interaction between the two types of α-amylase. Duplicate gels were cut into 3-mm sections, and each section was placed in 0.5 ml of GP buffer. The total α-amylase activity per 3-mm section is shown in Fig. 2. These data show that the two enzymes retain activity during electrophoresis, and that the position of each enzyme

**TABLE 1. Purification of the α-amylase of Bacillus subtilis W23**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>Total units</th>
<th>Specific activity (units/mg of protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>8,500</td>
<td>12,750</td>
<td>0.47</td>
<td>100</td>
</tr>
<tr>
<td>Step 2</td>
<td>560</td>
<td>3,136</td>
<td>0.59</td>
<td>24.6</td>
</tr>
<tr>
<td>Step 3</td>
<td>36</td>
<td>1,098</td>
<td>1.85</td>
<td>8.7</td>
</tr>
<tr>
<td>DEAE cellulose column</td>
<td>33</td>
<td>1,040</td>
<td>2.95</td>
<td>8.2</td>
</tr>
<tr>
<td>Concentration by lyophilization</td>
<td>3.7</td>
<td>721</td>
<td>3.20</td>
<td>5.7</td>
</tr>
<tr>
<td>Disc electrophoresis</td>
<td>8.3</td>
<td>683</td>
<td>10.52</td>
<td>5.3</td>
</tr>
</tbody>
</table>

a One unit of α-amylase activity is equal to 1 mg of reducing sugar, measured as maltose, released in 3 min at 25 C, pH 6.3.

b DEAE cellulose column chromatography followed by concentration of enzyme with Carbowax 4000

![Fig. 1. Electrophoretic separation in polyacrylamide gels of the α-amylase of Bacillus subtilis W23 and B. amyloliquefaciens F. Electrophoresis in 7.5% polyacrylamide gel was performed as described in the Canalco model 12 instruction manual. The samples were run (3 ma per column) in tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.6, for 40 min at ambient room temperature in 0.5 × 6.0 cm columns. The protein bands were visualized by staining with aniline blue-black (0.5%) in 7.5% acetic acid. (A) B. subtilis W23 α-amylase (10 µg). (B) Mixture of B. subtilis W23 α-amylase (10 µg) and B. amyloliquefaciens F α-amylase (25 µg). (C) B. amyloliquefaciens F α-amylase (50 µg).](http://jb.asm.org/Downloadedfrom/September30232017byguest)
activity corresponds to the appropriate position of the stained protein bands in Fig. 1.

Immunodiffusion studies. An immunological comparison was made between the purified \( \alpha \)-amylase of \( B. \ subtilis \) W23 (194 units/ml) and the \( \alpha \)-amylase of \( B. \ amyloliquefaciens \) by use of antiserum against the crystalline \( \alpha \)-amylase of \( B. \ amyloliquefaciens \) F. Figure 3 shows that there is no reaction between the \( \alpha \)-amylase of \( B. \ subtilis \) W23 and the heterologous antiserum, whereas the \( \alpha \)-amylase of \( B. \ amyloliquefaciens \) shows a homologous reaction. Identical results were obtained when the \( \alpha \)-amylase of \( B. \ subtilis \) W23 was tested with antiserum against the \( \alpha \)-amylase of \( B. \ amyloliquefaciens \), strains SB, T, P, and N. These data clearly demonstrate that the two enzymes do not share a common precipitating antigenic site.

\( \alpha \)-Amylase production by growing cultures.

\[ A \]

\[ B \]

\[ C \]

**FIG. 2.** Enzymatic activity of the \( \alpha \)-amylase of \( B. \ subtilis \) W23 and \( B. \ amyloliquefaciens \) F separated by electrophoresis in polyacrylamide gels. The experimental conditions were the same as described in Fig. 1. After electrophoresis, each gel was immediately cut into 3-mm sections. The 3-mm sections were placed in 13 \( \times \) 100 mm tubes containing 0.5 ml of GP buffer and placed at 4 C for 12 hr. Enzymatic activity in each tube was measured by the assay procedures described in the text. (A) \( B. \ subtilis \) W23 \( \alpha \)-amylase (10 \( \mu \)g). (B) Mixture of \( B. \ subtilis \) W23 \( \alpha \)-amylase (10 \( \mu \)g) and \( B. \ amyloliquefaciens \) F \( \alpha \)-amylase (25 \( \mu \)g). (C) \( B. \ amyloliquefaciens \) F \( \alpha \)-amylase (50 \( \mu \)g).

\[ \text{Fig. 3. Immunological comparison of the } \alpha \text{-amylase of } B. \ subtilis \text{ W23 and } B. \ amyloliquefaciens \text{ F. Center well: antiserum against } \alpha \text{-amylase of } B. \ amyloliquefaciens \text{ F, 0.4 ml; well 1: } \alpha \text{-amylase of } B. \ subtilis \text{ W23 (8.8 units/ml), 0.2 ml; wells 2 and 3: } \alpha \text{-amylase of } B. \ amyloliquefaciens \text{ F (1.6 units/ml), 0.4 ml; well 4: } \alpha \text{-amylase of } B. \ subtilis \text{ W23 (8.8 units/ml), 0.4 ml; wells 5 and 6: } \alpha \text{-amylase of } B. \ amyloliquefaciens \text{ F (1.6 units/ml), 1 } \mu \text{g, 0.2 ml. The plate was held at 16 C for 24 hr. The Ouchterlony plate was held at 16 C for photography at 24, 48, and 72 hr.}\]

\( \alpha \)-Amylase production in cultures of \( B. \ amyloliquefaciens \) F and \( B. \ subtilis \) W 23 is shown in Fig. 4. The production of \( \alpha \)-amylase by \( B. \ subtilis \) W23 occurs at a low rate throughout the 7-hr growth period, whereas \( \alpha \)-amylase production by \( B. \ amyloliquefaciens \) F does not occur until the culture enters the stationary phase of growth. The amounts of \( \alpha \)-amylase produced by \( B. \ subtilis \) W23 and \( B. \ amyloliquefaciens \) F after 7 hr of growth were 0.3 and 25.5 units/ml, respectively. Growth periods of 12 to 16 hr did not increase the \( \alpha \)-amylase content (units per milliliter) of either culture. Identical results have been obtained with the other strains of \( B. \ amyloliquefaciens \) and \( B. \ subtilis \) used in our previous study (16).

**DISCUSSION**

The difference in the electrophoretic mobility of the \( \alpha \)-amylase of \( B. \ subtilis \) W23 and \( B. \ amyloliquefaciens \) F is reminiscent of the studies of Ottesen and co-workers (7, 8, 12) and Smith et al. (14) on the proteinases from various strains of "\( B. \ subtilis.\)" Ottesen and Spector (12) demonstrated that subtilisin and the Novo bacterial proteinase differed in their electrophoretic
mobility in the alkaline range. Hunt and Ottesen (8) subsequently showed that the Nagarse proteinase derived from \textit{B. subtilis} BPN' was similar to the Novo proteinase and differed from subtilisin in its substrate specificity and its trypsin peptide fingerprint. Later studies by Smith et al. (14) revealed that the amino acid sequence of subtilisin differs in a total of 83 residues from the sequence of the BPN' proteinase. These findings are not too surprising in view of our study (16), which revealed that \textit{B. subtilis} BPN' is a strain of \textit{B. amyloliquefaciens} (designated as strain N).

Green and Colarusso (6) have reported differences in the electrophoretic mobility of the \(\alpha\)-amylase of \textit{B. subtilis} SB-1 (a histidine- and tryptophan-requiring derivative of \textit{B. subtilis} 168) and a prototrophic strain of \textit{B. subtilis} strain 1088. We have not examined \textit{B. subtilis} strain 1088, but the fact that it makes substantially more enzyme than the SB-1 strain and will not grow at pH 2.2 suggests that strain 1088 may be improperly classified. The fact that the deoxyribonucleic acid (DNA) of strain 1088 will transform strain SB-1, however, suggests that it is not a strain of \textit{B. amyloliquefaciens} since the DNA of \textit{B. amyloliquefaciens} will not transform auxotrophic strains of \textit{B. subtilis} (16).

The lack of cross-reaction of the \(\alpha\)-amylase of \textit{B. subtilis} W23 with antisera against the \(\alpha\)-amylase of \textit{B. amyloliquefaciens} strains F, SB, N, P, and T clearly demonstrates that these two types of \(\alpha\)-amylase do not share a common precipitating determinant. Inoue et al. (9) reported that the \(\alpha\)-amylases of \textit{B. subtilis} strains H, N, and K show cross-reactions with the antiserum prepared against the \(\alpha\)-amylase of strain H; they also reported that the \(\alpha\)-amylase from \textit{B. subtilis} var. \textit{globigii} does not cross-react with the antiserum against the \(\alpha\)-amylase of strain H. Our demonstration (16) that the H, N, and K strains of \textit{B. subtilis} are actually strains of \textit{B. amyloliquefaciens} provides an explanation for their results.

The synthesis of \(\alpha\)-amylase by growing cultures of \textit{"B. subtilis"} has been reported by several groups of workers (1, 2, 5, 11). In all of these studies, the maximal \(\alpha\)-amylase activity was shown to be produced in the postlogarithmic phase of cell growth. It should be noted, however, that these investigators worked with strains of \textit{B. amyloliquefaciens} rather than \textit{B. subtilis}: Fukumoto et al. (5) with strain F, Nomura et al. (11) with strain H, and Coleman and Elliott (1) and Coleman and Grant (2) with strain T [all strains as identified by Welker and Campbell (16)]. Thus, our results on the production of \(\alpha\)-amylase by \textit{B. amyloliquefaciens} F confirm those of the above investigators. They also establish that the production of \(\alpha\)-amylase by an authentic strain of \textit{B. subtilis} differs markedly from that of \textit{B. amyloliquefaciens}.

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\section*{Literature Cited}


