Production, Purification, and Characterization of α-Amylase from *Thermomonospora curvata*

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*Thermomonospora curvata* produces an extracellular α-amylase. Maximal amylase production by cultures in a starch-mineral salts medium occurred at pH 7.5 and 53°C. The crude enzyme was unstable to heating (65°C) at pH 4 to 6, and was activated when heated at pH 8. The enzyme was purified 66-fold with a 9% yield and appeared homogeneous on discontinuous gel electrophoresis. The pH and temperature optima for activity of the purified enzyme were 5.5 to 6.0 and 65°C. The molecular weight was calculated to be 62,000. The *Kₘ* for starch was 0.39 mg/ml. The amylolytic pattern consisted of a mixture of maltotetraose and maltopentaose.

A U.S. Public Health Service study (19) has demonstrated the feasibility of open-windrow composting for the conversion of biodegradable municipal solid waste to a usable product. The finished compost is useful as a soil supplement in strip mine reclamation and as an agricultural mulch (7). In the evaluation of finished compost quality, starch utilization has been advocated as a rate indicator in the composting process and as one criterion in determining the biological stability of the product (13, 26). Although many studies (10, 20–23) have been published on some of the thermophilic cellulolytic flora isolated from the composting process, no report has characterized the amylase produced by any of the isolates. *Thermomonospora curvata*, a cellulolytic, thermophilic actinomycete frequently isolated from composting material, produces an extracellular amylase. The purification and characterization of this amylase was undertaken for two reasons: (i) the literature contains no reports on the characterization of highly purified amylases from thermophilic actinomycetes, and (ii) the study of the amylase would help to evaluate the role of *T. curvata* in the starch degradation which must occur before the compost can be considered a finished, marketable product.

**MATERIALS AND METHODS**

Culture maintenance. Cultures of *T. curvata* were maintained at 4°C on a 1% agar medium containing the constituents listed in the next section.

Minimal medium for amylase production. The minimal medium for amylase production contained: 1% soluble starch (Fisher Scientific Co.) or 1% maltose, 0.2% (NH₄)₂SO₄, 0.02% MgCl₂·6H₂O, 0.1-μg/ml concentrations of biotin and thiamine, 10⁻⁴ M CaCl₂, and 10⁻¹ M potassium phosphate (final pH, 7.8). Appropriate amounts of dry carbohydrate, mineral salts, and vitamins were autoclaved separately and combined aseptically after cooling.

**Amylase production.** Erlenmeyer flasks (500 ml) containing 200 ml of medium were inoculated with approximately 8 mg (dry weight) of washed inoculum. Cultures were routinely incubated at 50 to 53°C in a New Brunswick G-76 rotary water bath shaker at 160 rpm for 3 days.

**Amylase assay.** Amylase activity was measured in magnetically stirred reaction mixtures containing 2 ml of 4% soluble starch (Fisher), 1 ml of 1.0 M sodium acetate buffer (pH 6.0), and 0.6 ml of water. After equilibration in a water chamber maintained at 65°C by a Haake FK2 constant-temperature circulator, the reaction was initiated by addition of 0.4 ml of appropriately diluted enzyme. One-milliliter samples were drawn at zero time and after 10 min of incubation. Accumulation of reducing sugar was estimated by the dinitrosalicylic acid method of Bernfeld (2). An amylase activity unit was defined as the release of 1 μmol of reducing sugar (as glucose) per min.

**Amylase purification.** After clarification by centrifugation (17,500 × g at 4°C for 15 min), culture fluids were concentrated on an Amicon model TCF10 ultrafiltration unit by using a membrane filter (PM-10; molecular weight exclusion of >10⁶) under an atmosphere of N₂ with a pressure of 25 lb/in² at 6°C. Approximately 3 liters of culture fluid could be concentrated to 50 ml in 25 h. The concentrated culture filtrate was adjusted to pH 8 and stirred slowly at 2°C as precooled ethanol (95%, -10°C) was added dropwise to a final concentration of 75%. The precipitate from this step was redissolved in 4 ml of 0.01 M phosphate buffer, pH 8, and applied to a Sephadex G-150 column (1.5 by 60 cm) equilibrated at 6°C with a 0.01 M phosphate-0.1 mM calcium acetate buffer, pH 8, containing 0.02% sodium azide. Fractions (3 ml) were collected at a flow rate of 15
ml/h; those containing amylase activity were combined and applied to diethylaminoethyl (DEAE)-cellulose (Sigma, medium mesh) equilibrated with the same buffer in a column (1.5 by 30 cm) at 6°C. Fractions (2 ml) were collected during elution with a linear NaCl gradient (0 to 0.6 M) in buffer at a flow rate of 12 ml/h. Fractions with the highest specific activity were combined and concentrated on an ultrafiltration unit (model 12, Amicon) with a membrane filter (UM-10) under the same conditions as previously described.

Electrophoresis. Polyacrylamide gels, prepared according to the procedure of Davis (4), were used in discontinuous electrophoresis equipment (model 12, Canalco). Standard 7% gels were prepared in tubes (0.5 by 7.5 cm) and run at pH 8.3. Samples (0.2 ml) containing about 100 μg of protein were mixed with a 40% sucrose solution and pipetted directly onto the stacking gels. A 1-mA current was applied to each tube until the tracking dye entered the separating gel. The current was then raised to 3 mA per tube for the remainder of the run (approximately 3 h). Gels were stained with either amido black or Coomassie brilliant blue R250 and de-stained electrophoretically (amido black), with 7% acetic acid as the electrolyte, or by diffusion (Coomassie brilliant blue) at 25°C in the same solvent. Unstained gels were sliced into sections corresponding to the electrophoretic pattern of stained gels, macerated in tubes containing 2 ml of buffer, stored overnight at 2°C, and assayed for enzyme activity. The eluate from active slices was pooled and concentrated. Gels were stained for carbohydrate by the procedure of Zacharius et al. (30).

Chromatography of hydrolysis products. The enzymatic action on starch was followed by a method similar to the procedure of Welker and Campbell (29). Samples (0.5 ml) from standard amylase assay mixtures were removed at 5-min intervals up to 30 min and placed in precooled tubes in an ethanol bath at −20°C. Samples were thawed and heated in a boiling water bath for 7 min, deionized with 2 g of a mixture (1:1) of Amberlite 1R-4B and IRC-50 chemically pure resin, and placed at 2°C overnight. Resin was removed by centrifugation at 14,500 × g at 4°C, and unknown samples (10 μl), along with the reference sugars (glucose, maltose, and maltotriose at 1% concentrations) were spotted 4 cm apart on Whatman no. 3 chromatography paper (24 by 30 cm). Chromatograms were run for 4 h with a solvent consisting of n-butanol-pyridine-water (6:4:3) and air dried. Chromatograms were redeveloped for a total of five ascents. Reducing sugars were detected by the silver-dip method of Mayer and Larner (16), modified as follows. Solution A was prepared by diluting 2 ml of saturated AgNO₃ to 12 ml with distilled water and then to 400 ml with acetone. Solution B contained 100 ml of 10% aqueous solution of 0.05 M sodium thiourea. Solutions were poured into separate pans, and the paper was dipped rapidly in solution A and allowed to dry. The paper was next dipped into solution B until the characteristic black spots appeared (about five dips). After washing in tap water for 1 h with continuous flow, the chromatogram was dipped into solution C until the background coloration disappeared (about 5 min). A 60-min wash in tap water with continuous flow yielded a stable chromatogram.

Determination of molecular weight. The sodium dodecyl sulfate-polyacrylamide gel method of Weber and Osborn (27) was used. Gels (10%) were prepared in tubes (0.75 by 10.2 cm). Protein samples were prepared according to method 1 in the presence of sodium dodecyl sulfate at 100°C for 3 min. A 1-mA current was applied per tube until the tracking dye entered the sample gel (1.5 h), at which time the current was raised to 3 mA per tube. The average time for all runs was 4.5 h. Gels were stained with Coomassie brilliant blue R250 (Bio-Rad) and de-stained by diffusion. Proteins of known molecular weight, bovine serum albumin (0.5 mg/ml), ovalbumin (1 mg/ml), pepsin (1 mg/ml), and chymotrypsin (1 mg/ml), served as reference markers.

RESULTS

Conditions for maximal amylase production. The pH and temperature requirements for amylase production in maltose-mineral salts-vitamin medium are illustrated in Fig. 1 and 2, respectively. During variation of both parameters, the accumulation of amylase activity in culture fluids was proportional to growth, the optima being pH 7.5 and 53°C.

Heat stability of crude amylase. During testing of the heat stability of the crude amylase composting temperatures (60 to 70°C), the enzyme was rapidly inactivated at pH 4 to 5, relatively stable at pH 6 to 7, and activated at pH 7 to 8 (Fig. 3). When incubated at 65°C and

![FIG. 1. Effect of pH on amylase production, accumulation of extracellular protein, and dry cell weight after 3 days of incubation in shaken culture, 50°C, with 1% starch as carbohydrate source.](http://aem.asm.org/)
pH 8 for 5 to 10 min before mixing with substrate, an increase in activity of about 60% was observed when later measured in the standard reaction mixture.

Purification of the amylase. A summary of results from the purification procedure is given in Table 1. A 66-fold purification was achieved, with a yield of 9% of the original activity. The purified preparation appeared to be homogeneous on discontinuous gel electrophoresis (Fig. 4), and all detectable amylase activity in the gel coincided with a single protein band. The amylase from this section of the gel could be quantitatively eluted (>85%) by maceration in hydroxymethylaminomethane-glycine buffer, pH 8.3, and allowing the elution to proceed overnight at 2°C.

Temperature and pH optima. Under the conditions of the routine assay system, the optimal temperature for the purified amylase was 65°C (Fig. 5). The Q₁₀ values (calculated from triplicate experiments) for the range 45 to 65°C varied from 1.75 to 1.82. The enzyme exhibited a rather broad pH optimum in the range of pH 5.5 to 6.5, with half-maxima at pH 4.8 and 7.5 (Fig. 6). The purified enzyme was rapidly inactivated at pH values below 7.0 when held at 65°C. At pH 7 to 8, the enzyme was stable, but did not show the heat activation observed with the crude preparations.

In stability studies on microbial thermostable amylases (1, 3), the presence of bovine serum albumin (BSA) afforded substantial protection against heat denaturation. However, the presence of BSA at 65°C caused no change in the heat stability of the purified T. curvata amylase, and at 90°C it caused more rapid inactivation. This deleterious effect is shown in Fig. 7. The enzyme in the presence of BSA was completely inactivated within 5 min at 90°C, whereas enzyme alone or in the presence of substrate lost only about 40% of its activity. The presence of the substrate had no significant protective effect.

### Table 1. Purification summary of amylase from T. curvata

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Sp act</th>
<th>Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Crude culture filtrate</td>
<td>2,733</td>
<td>12,300</td>
<td>752</td>
<td>16.35</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(2) Retentate from PM-10 filter</td>
<td>50</td>
<td>12,500</td>
<td>60</td>
<td>208</td>
<td>12.7</td>
<td>100</td>
</tr>
<tr>
<td>(3) Ethanol precipitate at 75% saturation</td>
<td>4</td>
<td>6,480</td>
<td>13</td>
<td>498</td>
<td>30.5</td>
<td>53</td>
</tr>
<tr>
<td>(4) Eluted from Sephadex G-150</td>
<td>9</td>
<td>2,313</td>
<td>3.8</td>
<td>608</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>(5) Eluted from DEAE-cellulose</td>
<td>14</td>
<td>1,086</td>
<td>1.0</td>
<td>1,086</td>
<td>66</td>
<td>9</td>
</tr>
</tbody>
</table>
yielded a $K_m$ of 0.39 mg/ml. Reaction rates for the purified enzyme with either starch or amyllose were initially linear under standard assay conditions (Fig. 9). The enzyme caused no detectable hydrolysis of maltose or maltotriose.

**Molecular weight determination.** Electrophoretic mobility profiles of the known molecular weight proteins, together with the purified amylase in SDS-gel electrophoresis, are shown in Fig. 10. From these results, the molecular weight of the amylase was calculated to be 62,000 at pH 8.0.

**Determination of the amylolytic pattern.** Chromatography of products of the amylase acting on starch for 10, 20, and 30 min under the routine assay conditions yielded only two spots with identical $R_f$ values (0.29 and 0.39) for all three reaction times. In various studies (8, 24, 28) on the correlation of carbohydrate

**Kinetic parameters.** Although the Lineweaver-Burk linear transformation of the Michaelis-Menten equation is most commonly used for the calculation of $K_m$ and $V_m$, the Hofstee transformation (9) has been shown to be the most reliable and discerning method for the calculation of the kinetic parameters (6). When reaction rates for the purified amylase were measured over a 100-fold range of starch concentrations, under standard assay conditions, a Hofstee plot of $V$ versus $V/S$ (Fig. 8)
AMYLASE OF T. CURVATA

The requirements for amylase production by T. curvata are markedly different from those of the closely related Thermomonospora viridis (25) and Thermoactinomyces vulgaris (11). Both T. viridis and T. vulgaris required a complex nitrogen source such as peptone for amylase production, whereas T. curvata produced high levels of the enzyme in a chemically defined medium. The crude amylase of T. curvata is unusual in its heat stability as related to pH. It was inactivated by heating at 65°C at pH 4 to 6 and significantly activated in the pH range of 7 to 8. The amylase of T. vulgaris has opposite requirements for heat stability, being more stable at pH 5 to 6.5 and rapidly inacti-

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vated at pH 7 to 8. The temperature optimum for amylase production by *T. curvata* is 53°C; during the first 7 to 10 days of the composting process, the average temperatures of municipal solid waste reach 50 to 60°C to provide that optimum (20). By that time, the average pH of the refuse has reached the range of 7 to 8 (19), which also encompasses the optimum for amylase production by *T. curvata*. Therefore, it would appear that in these respects composting conditions during the early stages of the process would be the most favorable for starch degradation by *T. curvata*. During analysis of carbohydrate content in composting (19), the soluble sugar content (initially about 0.8%) was rapidly depleted, followed by starch (from 4% initially down to less than 1.0% by 28 days), and finally by cellulose (from 50% initially down to 30% at the end of the 49-day process). *T. curvata*, which can utilize all three classes of carbohydrates and which could probably establish sizable population densities early in the process, has the potential of playing a major role in the rate of conversion to a finished product.

The purified amylase described here has a pH optimum of 5.5 to 6.0 and a temperature optimum of 65°C. In this regard, it appears similar to the amylases from other thermophiles (1, 18, 12). However, the *Kₘ* for starch, 0.39 mg/ml, is considerably lower than that reported for thermostable amylases from *Thermomonospora vulgaris*, 1.4 mg/ml (1), *Bacillus macerans*, 3.3 mg/ml (5), or *Bacillus stearothermophilus*, 1.0 mg/ml (15). A molecular weight of 62,000 was calculated for the amylase by SDS-gel electrophoresis. We could find no report of a molecular weight for a purified amylase of another therophilic actinomycete. However, the amylase of *Aspergillus niger* (which is also unstable at acidic pH, like the *T. curvata* amylase) has a molecular weight of 61,000 (17), and most bacterial amylases have molecular weights in the range of 50,000 to 60,000.

One unusual feature of the amylase from *T. curvata* is its amylolytic pattern, consisting of oligosaccharides that appear to be malto-tetraose and maltopentaose. In contrast, the partially purified amylase from *T. vulgaris* (1) produced only glucose and maltose from soluble starch. This apparent difference between amylases from closely related species may be due to the presence of an associated α-glucosidase that hydrolyzes the larger oligosaccharides to glucose and maltose in the *T. vulgaris* amylase preparations. A comparison of the action patterns of pure amylase preparations from the two species on a variety of malto-oligosaccharides is needed to resolve this question.

LITERATURE CITED

23. Stutzenberger, F. J. 1972. Cellulolytic activity of *Thermomonospora curvata*: optimal assay conditions, par-