PURIFICATION AND PROPERTIES OF AMYLASE FROM *NOCARDIA ASTEROIDES*

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The enzyme alpha amylase was purified from cells of *Nocardia asteroides* by gel filtration on Sephadex G-150 and by DEAE-Sepharose column chromatography. The purified enzyme revealed a single band on a polyacrylamide gel electrophoresis under native conditions, indicating its homogeneity. The native molecular weight of the purified enzyme on a Sephadex G-200 column was estimated to be 150,000. The enzyme showed two protein bands of molecular weights 65,000 and 56,000 on a 10% SDS-PAGE under denatured conditions, indicating that the native enzyme has two or more subunits of different molecular weights. The purified enzyme had an optimal pH of 6.9 and an optimal temperature of 50°C. The enzyme activity was enhanced by MgCl₂ and inhibited by EDTA. Starch or maltose in the culture medium significantly enhanced the enzyme production compared with the culture medium containing glucose.

Complex polysaccharides, such as starch and glycogen, function as reserve nutrients for the growth and development of all living organisms. The α- and β-amylases are of primary importance to the degradation of these compounds. Microbes like the thermophilic bacteria *Halobacterium halobium*, the moderate halophile *Micrococcus halobius* (3, 18), and *Bacillus subtilis* (20) produce hydrolytic enzymes such as amylase (α-1-4-glucan glucanohydrolase) [EC 3.2.1.1.], which attacks at random any of the α-1-4-glycosidic linkages on a starch molecule. Similarly, halophilic bacteria, such as *M. halobius*, *H. halobium*, and *Acinetobacter* sp., produce large amounts of amylase if cultivated aerobically in media containing 1 to 3 M NaCl (17).

Amylase has been purified to homogeneity from *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Streptomyces hygroscopius*, *B. subtilis*, *Bacillus stearothermophilus*, *Acinetobacter* sp., *Pseudomonas saccharophila*, and *M. halobius* (15, 23). The

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Amylases of *B. licheniformis* are thermostable and show high activity at 90°C and over a wide pH range (pH 5–10) (8). Amylase from *M. halobius* is sensitive to an optimal temperature of 50 to 55°C and a pH range of 6.5 to 7.5 (18). Earlier studies demonstrated that the amylase of *Vigna mungo* cotyledons requires calcium for its activity and heat stability (7).

*Nocardia asteroides* a facultative, intracellular opportunistic, aerobic actinomycete infects the immunosuppressed host and causes systemic nocardiosis. This organism is known to produce different hydrolytic enzymes (6, 16) to catabolize the complex store materials of the host for energy production. Because the properties of amylases are different in different organisms and no reports were available on the role of amylase in *Nocardia*, we initiated a study, to describe the optimal conditions for amylase synthesis and the characteristics of the purified amylase from *N. asteroides*.

**MATERIALS AND METHODS**

Stock cultures of different *Nocardia* species, such as *N. asteroides*, *N. brasilensis*, and *N. caviae*, were maintained in Sabouraud dextrose agar (SDA, Difco, Detroit, MI, U.S.A.) at 37°C. *N. asteroodes* obtained from ATCC (27938) was used.

Soluble starch, D-glucose, maltose, and other chemicals were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A., dinitrosalicylic acid was obtained from Kodak Eastman Co., Rochester, NY, U.S.A. All other chemicals were reagent grade.

*N. asteroides* was grown in Sabouraud dextrose broth (SDB) medium and in synthetic medium (SM) containing 4.0% glucose, 0.2% (NH₄)₂SO₄, 0.1% K₂HPO₄, 0.1% NaCl, and 0.1 ml of trace element solution per 100 ml. The trace element solution contained 0.1% FeSO₄·7H₂O, 0.1% MnCl₂, and 0.1% ZnSO₄·7H₂O (pH 7.0) (1). To study the influence of different nitrogen sources on amylase production, SDB media were supplemented with 0.1% peptone, peptone and yeast extract, respectively. In addition, starch was substituted for glucose in the SDB medium to study its influence on amylase activity.

Each inoculum (2.0 ml), prepared from the 3-day-old culture grown on the SDA plates, was homogenized gently to break the clumps, and then transferred to Erlenmeyer flasks (250 ml) containing 50 ml of SDB medium. The inoculated flasks were incubated at 37°C for 3 days on a shaker operating at 120 rpm. Cells were harvested from the culture media by centrifugation, homogenized in 100 mM Tris-HCl buffer (pH 7.4) containing 5 mM CaCl₂ and 10 mM β-mercaptoethanol. The supernatant was used to measure the intracellular enzyme activity, and culture filtrates were used to measure the extracellular enzyme activity. To study the effects of inducers on amylase synthesis in *N. asteroides*, the SDB media were supplemented with 1% starch, glucose, and maltose, respectively.

Amylase activity was assayed, using soluble starch as substrate, by the method
Amylase from *N. asteroides* of Miller (14). The reaction mixture contained 0.5 ml of enzyme, 0.5 ml of 0.05% soluble starch, and 0.5 ml of 25 mM Tris-HCl buffer (pH 7.0). After incubation at 37°C for 30 min, 3 ml of dinitrosalicly acid (DNS) was added, and the mixture was boiled for 15 min. After cooling, the absorbance was measured at 640 nm. The specific activity of the enzyme was expressed as micromoles of glucose formed per minute per milligram of protein.

In step one, 3-day-old cultures were centrifuged at 12,000 × g for 20 min, and the cells were washed twice with 100 mM Tris-HCl buffer (pH 7.4) containing 5 mM CaCl₂ and 10 mM β-mercaptoethanol. The washed cells were sonicated and centrifuged at 2,500 × g for 20 min at 4°C and the resulting supernatant served as the crude enzyme. All the steps of purification were carried out at 4°C.

In the step two, the glycogen-amylase complex was prepared according to the method described by Loyter and Schramm (10). Cold ethanol was added to the crude cell-free extract to bring it to 40% of the final concentration. The mixture was allowed to stand for 30 min and was centrifuged at 12,000 × g for 30 min. The pellet was discarded. To the supernatant, 2% glycogen was added to one-eighth of the final volume to form a complex with amylase. The mixture was allowed to stand at 4°C for 30 min and was centrifuged at 40,000 × g at 4°C for 30 min. The resulting pellet was dissolved in 50 mM Tris-HCl buffer (pH 7.0) containing 50 mM CaCl₂ and was dialyzed against the same buffer to dissociate and remove the glycogen from the amylase.

In step three, the enzyme obtained in step 2 was loaded on to a Sephadex G-150 column (1.5 × 40 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM CaCl₂, 10 mM β-mercaptoethanol, and 0.1 M NaCl. The flow rate of the column was maintained at 3.5 ml/h. Amylase was eluted from the column by using the same buffer, and 1.0-ml fractions were collected.

In step four, the fractions containing the amylase activity from the gel filtration column were pooled and loaded on a DEAE-Sepharose column (1.5 × 20 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 50 mM CaCl₂. The flow rate of the column was adjusted to 45 ml/h. Unbound protein was removed by washing the column with the same buffer. The enzyme was eluted from the column by using the buffer containing 0-0.5 M sodium chloride. Then 3-ml fractions were collected, and the fractions containing the amylase activity were pooled, lyophilized, and used to study the properties of the enzyme.

The protein concentration during different stages of enzyme purification was determined by the method of Spector (22), using Coomassie Brilliant Blue and bovine serum albumin as the standard. The protein concentration of each fraction during column chromatography was monitored by the absorbance at 280 nm.

The homogeneity of the purified enzyme under native conditions was determined on 5% polyacrylamide gel electrophoresis (PAGE) according to the method of Davis (2). PAGE under denaturing conditions was carried out by the procedure described by Laemmli (9) to determine the subunits of the enzyme. Standard proteins were used for calibration: myosin (205,000), β-galactosidase (116,000),...
phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The gels were stained with Coomassie Brilliant Blue R-250.

The native molecular mass of the α-amylase was determined by gel filtration, using Sephadex G-200 column (1.5 X 35 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM CaCl₂, 10 mM β-mercaptoethanol and 0.1 M NaCl. The flow rate of the column was maintained at 4 ml/h. The standard marker proteins used for calibration were thyroglobulin 669,000, apoferritin 443,000, β-amylase 200,000 and alcohol dehydrogenase 150,000.

RESULTS

The maximal growth of Nocardia asteroides occurred in SDB medium supplemented with 1% soluble starch, compared with SM supplemented and yeast extract, with peptone and yeast extract, or with peptone alone. Maximal growth occurred at 72 h after inoculation and was significant after the SDB media were supplemented with inducers, such as starch, glucose, and maltose (Table 1). The extracellular enzyme showed a very low specific activity, and therefore further studies were made only with intracellular enzyme. The binding of α-amylase with amylopectin and glycogen was studied. Because the enzyme did not bind efficiently with amylopectin, glycogen was used to form the glycogen-amylase complex, which is one of the steps in the purification of amylase.

Table 1. Effect of inducers on amylase production.

<table>
<thead>
<tr>
<th>Inducers</th>
<th>Concentration (%)</th>
<th>Specific activity (μmol of glucose formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>1</td>
<td>0.033</td>
</tr>
<tr>
<td>Maltose</td>
<td>1</td>
<td>0.026</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Starch, maltose and glucose were used as inducers and added separately at the concentration of 1% to Sabouraud dextrose broth.

Table 2. Purification scheme of amylase from Nocardia asteroides.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>total activity units a</th>
<th>Specific activity units b (mg/protein)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free homogenate</td>
<td>120.0</td>
<td>156.0</td>
<td>26.22</td>
<td>0.17</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>199.0</td>
<td>44.9</td>
<td>10.03</td>
<td>0.22</td>
<td>38.3</td>
<td>1.39</td>
</tr>
<tr>
<td>Glycogen-amylase complex</td>
<td>20.0</td>
<td>19.6</td>
<td>4.37</td>
<td>0.23</td>
<td>16.6</td>
<td>1.46</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>19.0</td>
<td>0.43</td>
<td>3.19</td>
<td>7.90</td>
<td>12.2</td>
<td>49.3</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>15.0</td>
<td>0.28</td>
<td>2.39</td>
<td>12.0</td>
<td>9.11</td>
<td>74.6</td>
</tr>
</tbody>
</table>

* One unit of enzyme is defined as the amount of enzyme required to liberate 1 μmol of glucose per minute. The specific activity of the enzyme is expressed as units/mg of protein.

* Specific activity is expressed as number of units (mg protein)⁻¹.
Fig. 1. Homogeneity of α-amylase.
Polyacrylamide slab gel electrophoresis was run on a 5% gel in native condition at 5°C. A, purified alpha amylase from *Nocardia asteroides*.

Fig. 2. Elution profile of α-amylase on Sephadex G-150 column (1.5×40 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0).
The flow rate was adjusted to 3.5 ml/h. The enzyme containing pellet from 40,000×g was applied to the column and eluted with the same buffer by collecting 1 ml/fraction. Absorbance at 280 nm (●) and α-amylase activity (◆) are shown.
Purification of amylase

The summary of the purification of amylase is shown in Table 2. The enzyme was purified to homogeneity by glycogen-amylase complex, gel filtration, and ion exchange chromatography on a DEAE-Sepharose column. The purified amylase showed a single band on polyacrylamide gel electrophoresis confirming the homogeneity of the enzyme (Fig. 1). The enzyme was purified 75-fold and had a specific activity of 12 units.

The elution pattern of the amylase on a Sephadex G-150 column is shown in Fig. 2. Two protein peaks were obtained from the column. The first peak appeared between fractions 25 and 45, and the second peak appeared between fractions 65 and 100. Only the protein eluted in the first peak showed amylase activity. The chromatographic pattern of α-amylase on DEAE-Sepharose is presented in Fig. 3. The enzyme was eluted as a single peak.

Enzyme characteristics

Purified amylase from N. asteroides revealed a native molecular weight of 150,000 daltons on Sephadex G-200 column chromatography. The purified amylase revealed two bands on PAGE (denatured conditions), having the molecular weight of 150,000 daltons. The chromatographic pattern of α-amylase on DEAE-Sepharose ion-exchange chromatography (1.5 × 20 cm).

The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 50 mM CaCl₂, and the flow rate of the column was adjusted to 45 ml/h. A concentrated solution of active fractions obtained from the gel filtration was applied on the column. Amylase was eluted with the same buffer containing sodium chloride between 0 and 0.5 M. Absorbance at 280 nm (●) and α-amylase activity (■) are expressed, active fractions were combined and lyophilized.
Fig. 4. SDS-polyacrylamide gel electrophoresis (PAGE) for subunit molecular weight determination.

SDS-PAGE was performed under denaturing conditions in 1.5 mm slab gel containing 10% gel and the proteins were stained with Coomassie Brilliant Blue R-250. Lane 1: standard-molecular-weight proteins. A, carbonic anhydrase, 29,000; B, ovalbumin, 45,000; C, bovine serum albumin, 66,000; D, phosphorylase, 97,000; E, β-galactosidase, 116,000; and F, myosin, 205,000. Lane 2: contained the purified enzyme preparation exhibiting the two subunits, G1 and G2.

Fig. 5. Effect of pH on enzyme activity.

Succinate (■) (pH 5.0–5.8), phosphate (●) (pH 6.0–7.5), and Tris-HCl (■) (pH 7.2–9.0) buffer were used to study the effect of pH on α-amylase activity.
weights of 65,000 and 56,000 indicating that the native enzyme has at least two subunits of different molecular weights (Fig. 4).

The effect of pH on the enzyme activity was studied using three types of buffers (succinate, phosphate and Tris-HCl) with pH values ranging from 4.0 to 9.0. The

![Graph](image)

**Fig. 6.** Effect of temperature on the activity of α-amylase.

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Final concentration (mM)</th>
<th>Activity % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>1.0</td>
<td>59.6</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>89.4</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1.0</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>119.2</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1.0</td>
<td>149.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>316.3</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>1.0</td>
<td>103.6</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>85.7</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>1.0</td>
<td>29.7</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>52.1</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>1.0</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mo$^{2+}$</td>
<td>1.0</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>14.8</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>1.0</td>
<td>22.3</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Values given are percentage of activation and reduction of amylase activity by metal ions to control values. The control values is taken as 100% activity.
optimal pH for the enzyme activity was 6.9 (Fig. 5). The enzyme activity was retained over the pH range of 6.5 to 7.2, beyond which the activity decreased considerably.

The effect of temperature on the activity of amylase is presented in Fig. 6. The highest activity of amylase occurred at 50°C after which the activity decreased, regardless of the presence of ions. Lineweaver-Burk plots of the data yielded an apparent $K_m$ value of the enzyme of 0.35 mg/ml for starch and 0.34 mg/ml for maltose.

The enzyme activity in the presence of different metal ions at concentrations of 1 and 10 mM was measured (Table 3), and at each concentration the enzyme activity was enhanced. Incubation of the enzyme with 0.1 M EDTA for 30 min at 37°C, caused a 90% loss of activity. However, the enzyme activity was not inhibited by trypsin at 1 or 10 mM.

**DISCUSSION**

*Nocardia asteroides* is a Gram-positive actinomycete commonly found in the soil. It affects immunosuppressed hosts and causes nocardiosis, an acute or chronic infection which increasingly affects the population of the U.S. (4, 21). We found that starch and maltose were inducers for the amylase in *N. asteroides*. Similar results were observed in the case of ruminant bacteria, *Bacteroides ruminicola*, *Butyrivibrio fibrisolvens*, and *Streptococcus bovis JBI* (13). Higher levels of amylase were produced by a culture grown in a maltose-containing medium than in a medium with glucose. The highest activity was obtained when starch was added after 24 h of inoculation.

The amylase activity of a *Clostridium* isolate responded to lactose, glucose, fructose, and ribose as inducers, but the response was considerably lower than to maltose, starch, or pullan. Enzyme production can be induced by glucans containing 1, 4 or 1, 6 linkages. This mechanism is established for several amylolytic enzymes of bacterial or fungal origin (5, 11). The amylase from *N. asteroides* was purified to homogeneity by the glycogen-amylose complex preparation, gel filtration of Sephadex G-150, and by DEAE-Sepharose column chromatography. The purified enzyme showed a single band on polyacrylamide gel electrophoresis under native conditions, indicating its homogeneity. The enzyme was purified 75-fold and had a specific activity of 12 units.

The *Nocardia* amylase was stable in buffer containing β-mercaptoethanol and CaCl$_2$. Ions like manganese and magnesium had pronounced effects, on the enzyme activity, but *Nocardia* amylase activity without the divalent cations was not lost completely. This is in contrast to the amylase of *Acinetobacter* that requires the presence of 10 mM CaCl$_2$ (18).

The optimal temperature for amylase from *N. asteroides* was 50°C, beyond which the enzyme activity decreased. Similar findings have been reported for the amylase of *Clostridium*, which has maximal activity between 40 and 60°C. Further
increased in temperature reduced the activity drastically (12). The optimal temperature for the amylase from *Bacillus licheniformis* is 91°C (8).

The optimal pH of amylase from *N. asteroides* is 6.9 which is similar to that of the moderate halophile *M. halobius* but different from *Clostridium*, in which the enzyme is active at pH 5.2. Most bacterial amylases have molecular weights of about 50,000. The molecular weight of halophilic *Acinetobacter* and *Bacillus subtilis* were 65,000 and 68,000, respectively. The native molecular weight of the purified amylase from *N. asteroides* was estimated to be 150,000 daltons. This is very similar to the molecular weight of amylase from *Asellus aquaticus* (19). The subunit molecular weight of the amylase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme showed two protein bands of molecular weights 56,000 and 65,000 indicating that the amylase had at least two subunits of different molecular weights.

The amylase from *N. asteroides* had an apparent $K_m$ value of 0.35 mg/ml for starch, which is similar to the $K_m$ value of amylase obtained from the *Clostridium* isolate (11). The $K_m$ value of amylase for maltose was 0.34 mg/ml, which is also comparable to the $K_m$ value of amylase from the *Clostridium* isolate. The physical properties of the purified *Nocardia* enzyme are comparable to those of the amylases obtained from other microorganisms.

The amylase of *N. asteroides* had an increased activity in the presence of CaCl$_2$. Similar results have been reported for the amylase from bean seeds, which required calcium for optimal enzyme activity (7). Enzyme activity was enhanced in the presence of MgCl$_2$ and inhibited by EDTA and metal ions like Fe, Co, Mo, Zn. Amylase was greatly induced by starch but not by glucose (data not shown).

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**REFERENCES**


