Purification and Properties of Amylases Extracellularly Produced by an Imperfect Fungus, *Fusidium* sp. BX-1 in a Glycerol Medium

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Amylases (I and II) extracellularly produced by an imperfect fungus, *Fusidium* sp. BX-1 in a medium containing glycerol as a carbon source, were purified as electrophoretically and isoelectrophoretically homogeneous proteins. The electrophoretical mobilities of amylase I and II on native and SDS-polyacrylamide gels exactly coincided with each other. Their molecular weights were estimated to be about 52,000. The sugar contents of amylase I and II were 3.4 and 4.7%, and the pI's were 8.70 and 8.55, respectively. The $K_0$'s of the enzymes for soluble starch were 0.053 and 0.044%. The actions of the enzymes on soluble starch, short chain amylases, and maltose were examined. Amylase I and II are identified as being to a $\alpha$-amylase and a glucoamylase, respectively.

In a previous paper, we reported the isolation of an imperfect fungus strain, BX-1, belonging to the genus *Fusidium*. Information on the physiological properties of *Fusidium* from the viewpoint of the uses of microorganisms is limited. Strain BX-1 was able to use a wide range of carbon sources at a low pH, particularly showed excellent yeast-like multiplication of cells in a liquid medium containing polysaccharides and polyols, and extracellularly produced several enzymes hydrolyzing polysaccharides. Furthermore, it was found that strain BX-1 produced a larger amount of these enzymes when incubated in a medium containing glycerol as a carbon source. Two types of amylases were detected in the glycerol medium. The productivity of amylases on glycerol was several times higher than that on starch. Generally production of amylases increases in the presence of glucosides and is repressed in the presence of glucose. It is known that glycerol also represses the enzyme production in some wild strains. However, there have been no reports on yeast-like fungi capable of producing larger amounts of several amylolytic enzymes in a glycerol medium than in a starch medium.

This paper deals with the purification and properties of amylases produced by *Fusidium* sp. BX-1 in a medium containing glycerol as a carbon source.

Materials and Methods

**Microorganism.** The microorganism used in this study was isolated from a pullulan-enrichment culture and identified as *Fusidium* sp. BX-1.

**Culture conditions.** Stock cultures were maintained on agar slants containing 1% pullulan as described previously. The medium for seed cultivation and enzyme production contained the following compounds (g per liter): glycerol, 20; yeast extract (Oriental Yeast), 5.0; KH$_2$PO$_4$, 2.0; MgSO$_4$, 7H$_2$O, 1.0; CaCl$_2$, 2H$_2$O, 0.1; and thiamine hydrochloride, 0.0001. The pH was adjusted to 5.0 with 1 N HCl, the medium was sterilized by autoclaving at 121°C for 15 min. A 100-ml Erlenmeyer flask containing 30 ml of the medium was inoculated with a loopful of cells taken from a stock slant and incubated at 30°C on a rotary shaker (180 rpm). After 48 hr of incubation, 5 ml of the culture was used to inoculate 200 ml of the medium in a 1-liter Erlenmeyer flask and incubated at 30°C with rotary shaking (180 rpm). The enzymes were produced extracellularly. After 68 hr of incubation, cells were removed by centrifugation. To the resulting supernatant, solid ammonium sulfate was added to give 80% saturation. The precipitate was collected by centrifugation and stored below 4°C until used.

**Purification of amylases.** The above 80% saturated ammonium sulfate precipitate was dissolved in a minimal amount of 10 mM sodium acetate buffer (pH 5.0) containing 5 mM CaCl$_2$, 2H$_2$O. The solution was dialyzed against the same buffer at 4°C overnight. The dialyzed solution was centrifuged again to remove insoluble substances and then put on a DEAE-cellulose D52 (Whatman) column ($\phi$ 2.6 x 50 cm) equilibrated with 10 mM acetate buffer (pH 5.0) containing 5 mM CaCl$_2$, 2H$_2$O and washed with the same buffer. Amylase activity was almost all collected in the fraction washed out with the buffer. This fraction was put on a CM-cellulose 52 (Whatman) column ($\phi$ 1.4 x 30 cm) equilibrated with the same buffer. The column was washed with 200 ml of the above buffer and eluted with a 600 ml linear gradient of 100 mM to 500 mM NaCl in the buffer at a flow rate of 25 ml/hr. Five ml fractions were collected. The active fractions were combined and centralized by a centrifugal freeze drier RC-11 (Yamotokagaku). The concentrated solution was dialyzed against 10 mM sodium acetate buffer (pH 5.0) containing 5 mM CaCl$_2$, 2H$_2$O overnight. The dialyze was put on to a Sephadex G-150 (Pharmacia) column ($\phi$ 1.6 x 90 cm) previously equilibrated with the above buffer. The column was eluted with the same buffer at a flow rate of 8 ml/hr and 2-ml fractions were collected. The fractions containing amylase activity were clearly separated into two groups (amylase I and II). Each group was combined and then rechromatographed on the same column. Fractions containing each enzyme activity were combined and concentrated by the centrifugal freeze drier. These preparations were stored as a purified enzyme at −20°C.

**Enzyme assay.** Amylase activity was assayed in the reaction mixture containing 1 ml of 2% soluble starch solution, 2 ml of 100 mM acetate buffer (pH 5.0) and 1 ml of enzyme solution. After 20 min incubation at 30°C, the reducing sugar of the reaction mixture was measured by the 3,6-dinitrophenylhydrazide method. One unit (U) of the enzyme was defined as the amount of enzyme that liberated 1 µmol of glucose in the reaction mixture per min under these assay conditions.

**Protein assay.** Protein was measured by the method of Lowry et al. with bovine serum albumin as a standard protein. Protein from column effluents was monitored by measuring the absorbance at 280 nm.

**Electrophoresis.** Two procedures were used, one for native protein samples and the other for denatured protein samples. The native protein samples were directly analyzed with 7.5% polyacrylamide gel (pH 4.3) at 20 mA in 50 mM acetate–$\beta$-alanine buffer (pH 4.5). The denatured protein samples were prepared by heating them in 10 mM Tris–HCl buffer (pH
8.0) containing 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol for 3 min at 100°C. Electrophoresis of the heated samples was done in 40 mM Tris-acetate buffer (pH 7.4) containing 0.2% SDS on 12% gels at 20 mA by the method of Weber and Osborn.180

In both cases, protein was stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol-10% acetic acid solution.

Glycoprotein was stained with periodic acid-Schiff's reagent.200

Molecular weight estimation. The molecular weights of the denatured enzymes were estimated by SDS polyacrylamide gel electrophoresis as described above. An LMW Kit E (Pharmacia) was used as standard proteins.

Also, the molecular weights were estimated by gel filtration on a Bio-gel P-150 (Bio Rad) column (ø 1.6 x 70 cm) equilibrated with 10 mM acetate buffer (pH 5.0) containing 5 mM CaCl₂, 2H₂O. The column was eluted with the same buffer. Albumin (67,000), ovalbumin (45,000), and chymotrypsinogen (25,000) were used as standard proteins (Mann Research Laboratories).

Measurement of isoelectric point. Isoelectric focusing was done in a Pharmacia polyacrylamide gel (Pharmacia) with Ampholine ampholites for 3 hr at a constant voltage of 200 V at 4°C. Then, the gel was stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol-10% acetic acid solution.

Sugar content of the enzymes. Neutral sugars of the purified enzymes were measured by the phenol-sulfuric acid method using glucose as a standard.211

Substrate specificity. The reaction mixtures, containing 0.5—1.0 ml of 2% poly- and oligosaccharides, 1.0 ml of 100 mM acetate buffer (pH 5.0), and 0.5 ml of the purified enzyme solution (amyrase I, 5 U; II, 0.9 U) were incubated at 40°C. At intervals, samples of the reaction mixtures were taken out and inactivated by heating, and then 20 μl of each sample was chromatographed on paper.

Paper chromatography. Reaction products described above were assayed by paper chromatography. Each sample was spotted on a filter paper (No. 51C, Tomyoshir). The paper was developed by the multiple ascending method using the following solvent system: n-butanol-pyridine-water (6:4:3, v/v/v). Saccharides were detected by an aniline hydrogen phthalic acid salt solution.224

Identification of anomeric configuration. Anomeric configuration of the products formed by the action of the purified enzymes were identified by the method of Sawai and Niwa220 using a spectropolarimeter (JASCO J-20, Nihon Bunko Co.).

Scanning electron microscopic observation. Raw starch granules (3%, final concentration) incubated at 40°C with amyrase I (5 U) and II (0.9 U) for 20 hr were dried under reduced pressure. After gold coating, the granules were examined with a Hitachi S-808 scanning electron microscope operating at 10 kV.

Results

Purification of amylases

A typical purification of amylases from about 1.7 liters of the culture supernatant of Fusidium sp. BX-1 is summarized in Table I. The partially purified preparation obtained by CM-cellulose chromatography was homogeneous on non-denatured polyacrylamide gel electrophoresis and on SDS-polyacrylamide gel electrophoresis. However, this preparation was separated into two fractions (amyrase I and amyrase II) with amyrase activity with a Sephadex G-150 column (Fig. 1A). Finally, 5.32 mg of amyrase I and 5.70 mg of amyrase II were obtained as homogeneous preparation on the Sephadex G-150 column, respectively (Fig. 1B and C). The specific activities of amyrase I and II were 248 and 45.0 units/mg protein, respectively.

All preparations of amyrase I, II and I+II were seen as only one protein band with the same mobility by non-denatured and SDS–polyacrylamide gel electrophoresis (Fig. 2). As a final criterion of purity, these enzymes were tested by isoelectric focusing over the pH range of 3.50 to 9.30. Only a single protein band was detected with each

![Fig. 1. Affinity Chromatography of Amylases from Fusidium sp. BX-1 on Sephadex G-150.](image)

| Table I. Summary of Purification of Amylases Produced in Glycerol Medium by Fusidium sp. BX-1 |  |
|---|---|---|---|---|---|---|
| Purification procedure | Total volume (ml) | Protein (mg) | Total enzyme activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
| Culture supernatant | 1,670 | 743 | 12,900 | 17.3 | 100 | 1.00 |
| 80% sat. (NH₄)₂SO₄ | 17 | 76.5 | 6,120 | 80.0 | 47.4 | 4.62 |
| DEAE-cellulose DE52 | 150 | 24.8 | 4,520 | 182 | 35.0 | 10.5 |
| CM-cellulose CM52 | 50.0 | 23.2 | 4,370 | 188 | 33.9 | 10.9 |
| 1st Sephadex G-150 |  |  |  |  |  |  |
| Amylase I | 24.0 | 7.20 | 1,630 | 226 |  |  |
| II | 22.0 | 9.46 | 416 | 44.0 |  |  |
| 2nd Sephadex G-150 |  |  |  |  |  |  |
| Amylase I | 13.6 | 5.32 | 1,320 | 248 |  |  |
| II | 8.80 | 5.70 | 256 | 45.0 |  |  |

The enzymes were purified from 1.7 liters of the culture filtrate of the organism incubated in the medium containing glycerol as a carbon source at 30°C for 68 hr.
Fig. 2. SDS Polyacrylamide Gel Electrophoresis of Amylases.
Electrophoresis of denatured amylases and molecular markers was done on 12% SDS polyacrylamide gel. Lane 1, $M_r$ standards: rabbit muscle phosphorylase (97,400), bovine serum albumin (66,200), ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). Lane 2, amylase I. Lane 3, amylase II. Lane 4, amylase I + II.

Fig. 3. Isoelectric Focusing of Amylases on Pharmacia Gel.
Lane 1, amylase I. Lane 2, amylase II. Lane 3, $pI$ standards: amylglucosidase (3.50), soybean trypsin inhibitor (4.55), bovine carbonic anhydrase B (5.85) human carbonic anhydrase B (6.55), horse myoglobin (6.85), horse myoglobin (7.35), lentil lectin (8.15), lentil lectin (8.45), lentil lectin (8.65), trypsinogen (9.30).

enzyme (Fig. 3).

**Molecular weight of the enzymes**
The molecular weights of the amylases were estimated with SDS-polyacrylamide gel electrophoresis method. The molecular weights of amylase I and II were calculated to be the same value of about 52,000 (Fig. 4). Similar results (52,000—53,000) were obtained by gel filtration using a Bio-gel P-150 column.

**Isoelectric point of the enzymes**
The $pI$s of amylase I and II were 8.70 and 8.55, respectively (Fig. 3).

**Sugar content of the enzymes**
Bands of amylase I and II on PAGE were stained with Schiff's reagent. The bands stained with Coomassie brilliant blue and Schiff's reagent showed the same mobility on PAGE (data not shown). The sugar contents of purified preparation of amylase I and II were estimated to be about 3.4 and 4.7% by the phenol—sulfuric acid method.

**Effects of pH on the enzymes**
Figure 5 shows the effects of pH on the activity and
stability of the two amylases. The optimum pHs of amylase I and II were about 4.5 and 5.0, respectively. Amylase I was stable in the wide pH range of 4 to 10, while amylase II was stable in the narrow pH range of 4 to 7.

**Effects of temperature on the enzymes**

The optimum temperatures of the amylase I and II were about 50 to 55°C under the standard assay conditions. Both enzymes were stable below 50°C, when they were heated for 1 hr at each given temperature (Fig. 6).

**Effects of metal ions on the enzyme activities**

Amylase I and II were purified in the presence of 10 mM acetic buffer (pH 5.0) containing 5 mM CaCl₂. The effects of metal ions on the enzyme activities were examined after the purified enzymes were dialyzed against the above buffer containing 1 mM EDTA and lacking CaCl₂ (Table II). Over 95% of the enzyme activities remained after the dialysis. No stimulation effects on the enzyme activities were observed with the metal ions examined. The activities were inhibited by Cu²⁺ and Hg²⁺. Particularly, amylase I was strongly inhibited by Hg²⁺.

**Substrate specificities of the enzymes and reaction products**

Figure 7 shows the courses of hydrolysis of soluble starch by amylase I and II. Amylase I produced some oligosaccharides (G₂ and G₃ as main products, and G₄ and G₅ as minor products) at the early stage of the reaction. A small amount of glucose was detected during 24 hr of incubation. On the other hand, amylase II produced only glucose at the early stage of the reaction but not oligosaccharides even after 24 hr of incubation. Similar results were obtained when these enzymes were incubated with waxy corn starch, amylose, dextrin, and glycogen. Amylase II was able to produce a small amount of glucose from pullulan, but amylase I was inert on pullulan.

The reactions of these amylases with various maltosaccharides (G₂ to G₆) were examined (Fig. 8). Amylase I hydrolyzed saccharides more than G₆ well and the reaction product of this enzyme was finally composed of the mixture of G₂ and G₃ with a small amount of glucose. However, the enzyme could hardly attack G₂. Amylase II produced glucose as the sole end reaction product from all of the oligosaccharides examined.

The optical rotations of the reaction products from soluble starch by these enzymes were measured before and after addition of anhydrous sodium carbonate to the reaction mixtures (Fig. 9). The products by amylase I and II showed downward and upward mutarotations, respectively. The anomic configurations of products from soluble starch by amylase I and II were confirmed to be α- and β-form.

From these results, it was seen that amylase I and II extracellularly produced by strain BX-1 in the glycerol medium were an α-amylase and a glucoamylase, respectively.

**Table II. Effects of Metal Ions on Activity of Amylases**

<table>
<thead>
<tr>
<th>Ion added (1 mm)</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylase I</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>106</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>102</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>101</td>
</tr>
<tr>
<td>Mn²⁺</td>
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<td>Ni²⁺</td>
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<tr>
<td>Cu²⁺</td>
<td>72</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>34</td>
</tr>
</tbody>
</table>

Fig. 7. Paper Chromatogram of Hydrolyzates Produced from Soluble Starch by Purified Amylases.

Soluble starch (20 mg) was incubated with about 5 U of amylase I (A) or 0.9 U of II (B) in 1.0 ml of 100 mM sodium acetate buffer (pH 5.0) at 40°C for times indicated in the figure, respectively. Standard saccharides: G₁, glucose; G₂, maltose; G₃, maltotriose; G₄, maltotetraose; G₅, maltpentaose.
Fig. 8. Paper Chromatogram of Hydrolyzates produced from Oligosaccharides by Purified Amylases.
Ten mg of each oligosaccharide (G₁, maltose; G₂, maltotriose; G₃, maltotetraose; G₄, maltopentaose, G₅, maltohexaose) were incubated with about 5 U of amylase I(A) or 0.9 U of II(B) in 1.0 ml of 100 mM sodium acetate buffer (pH 5.0) at 40°C for 5 hr, respectively.

Fig. 9. Mutarotation of Products from Soluble Starch by Purified Amylases.
Each reaction mixture was composed of 600 µl of 6% soluble starch solution in 100 mM sodium acetate buffer (pH 5.0) and 10 µl of amylase I (81 U, ●) or II (15.9 U, ○). Optical rotation was followed through a 2 mm cuvette for 5 min at 30°C. After addition of one drop of aqueous 30% Na₂CO₃ solution (△) to stop the reaction and to complete mutarotation, the size and the direction of the mutarotational curve was read.

Kinetic properties
The influence of substrate concentrations on the rate of reducing sugar formation from soluble starch, maltose, and maltooligosaccharides was examined. From these results, the $K_m$ of amylase I and II for soluble starch were calculated to be 0.053% and 0.044% from Lineweaver-Burk plots, respectively (Table III). Amylase II showed a ten-fold higher affinity for maltooligosaccharides than amylase I.

Hydrolyzing activity on raw starch
Raw starches of waxy corn starch, wheat, sweet potato, and potato in 3% concentration were incubated with the enzymes at 40°C for 20 hr. Amylase II could hydrolyze these raw starches except potato. On the other hand, amylase I produced no reducing sugar from any raw starch (Fig. 10). Amylase II bored holes in granules of wheat starch, but not potato (Fig. 11). The hydrolyzing activity for raw starch was not affected in the presence and absence of amylase I.

Table III. Summary of $K_m$ Values of Amylases
The assay mixtures containing about 2.98 U of amylase I and 0.54 U of amylase II were incubated at 30°C under standard conditions described in the text.

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Amylase I</th>
<th>Amylase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>ND*</td>
<td>1.4</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>ND*</td>
<td>0.39</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>8.3</td>
<td>0.30</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>2.7</td>
<td>0.20</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>1.9</td>
<td>0.13</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>0.053 (%)</td>
<td>0.044 (%)</td>
</tr>
</tbody>
</table>

* ND, not determined.
Discussion

It is taxonomically known that a large number of yeast strains can grow in a medium containing soluble starch as a sole carbon source,\textsuperscript{24} but information on extracellular amylase production and yeast amylase properties is limited.\textsuperscript{25,36} The extracellular amylolytic enzymes produced by species of Endomyces (Saccharomyces),\textsuperscript{25,26} Lipomyces,\textsuperscript{27} Schwanniomyces,\textsuperscript{32} and Filobasidium\textsuperscript{14} have been well characterized. *Fusidium* sp. BX-1 in this paper is different from the typical yeasts. However, when its culture was shaken in liquid media containing various carbon sources, the culture was kept in suspension containing uni-cells and short chain-cells like yeasts during incubation and its growth was excellent in these media.\textsuperscript{13}

Two kinds of amylases (I and II) were purified to homogeneity from the culture of *Fusidium* sp. BX-1 incubated in the glycerol medium. Amylase I and II were found to be 2-amylase and glucoamylase, respectively. Although it was impossible to distinguish these enzyme with polyacrylamide gel electrophoresis, they could be easily separated from each other by a Sephadex G-150 gel column and isoelectric focusing. The separation on Sephadex G-150 gel seems to be based on the difference of affinities of these enzymes of the gel. Color-intensity of the purified enzymes stained with the Schiff's reagent on polyacrylamide gels after electrophoresis was significantly between amylase I and II (data not shown). In contrast with this, there was a small difference between amylase I and II from the results of the sugar-content analysis of these enzymes. Further investigation is needed to define the role of sugars in these enzymes. Properties of amylase I and II with respect to pH, temperature and substrates seem to be in the ranges of those of many usual amylases. Activities of strain BX-1 amylases were not affected by Ca\textsuperscript{2+}. These results are similar to an amylase from *Bacillus* sp.\textsuperscript{37}

*Fusidium* sp. BX-1 was able to grow well at a wide range of initial pH (3 to 12) when incubated in a medium containing glucose or glycerol as a carbon source.\textsuperscript{13} However, the fungus showed no growth in the medium containing starch at an alkaline pH. The optimum pHs of the amylases produced by strain BX-1 were in the acidic range. Particularly these enzymes showed no activity over pH 8.0. The activity profiles of these enzymes to pH may be related to no growth on starch at the alkaline pH.

The mechanisms of amylase production in *Fusidium* sp. BX-1 incubated in the glycerol medium remain to be investigated. There have been no reports on fungi that simultaneously produce 2-amylase and glucoamylase with the same molecular weight in a glycerol-medium. The genus *Fusidium* strains seem to be an interesting microorganism.
as a genetic resource.

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References