Purification and Some Properties of a Novel $\alpha$-Amylase Produced by a Strain of Thermoactinomyces vulgaris

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An $\alpha$-amylase [$\alpha$-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1.], found in the culture filtrate of a strain of Thermoactinomyces vulgaris, was purified by ammonium sulfate fractionation, and DEAE-cellulose and CM-cellulose chromatographies. The purified enzyme showed a single band on disc gel electrophoresis. The optimum reaction pH and temperature were determined to be around pH 5.0 and 70°C. The isoelectric point was determined to be pH 5.2. The $\alpha$-amylase was stabilized by Ca++.

The $\alpha$-amylase was found to hydrolyze pullulan to panose. Therefore, the hydrolytic pattern of this enzyme is different from those of pullulanase and isopullulanase.

The authors have screened thermophilic actinomycetes strains for $\alpha$-amylase production and found that one actinomycetes strain isolated from a soil sample at 50°C produced a new type of $\alpha$-amylase. The $\alpha$-amylase had the ability to hydrolyze pullulan and showed different properties from the Thermoactinomyces $\alpha$-amylase reported by M. J. Kuo and P.A. Hartman. Since no $\alpha$-amylases have been reported to hydrolyze the part of pullulan consisting of maltotriose unit, this enzyme seems to be a new type of $\alpha$-amylase. The present report describes the procedure of purification, some properties, and the pattern of pullulan hydrolysis of this $\alpha$-amylase.

MATERIALS AND METHODS

Microorganism and culture. A thermophilic actinomycetes strain showing both $\alpha$-amylase and pullulan-hydrolyzing activities was isolated from soil at 50°C. The microorganism was identified as Thermoactinomyces vulgaris and was named strain R-47, which will be reported elsewhere. Flask culture of the isolated actinomycetes strain was carried out for 2 days at 50°C in 50 ml of medium in a 500 ml Sakaguchi flask on a reciprocal shaker. Inoculation was conducted by transferring spores and mycelia from a slant culture with a wire loop. The medium composition was as follows; 3% soluble starch (Junsei Chemical Co.), 0.5% Bacto-Tryptone (Difco), 0.5% yeast extract (Kyokuto Seiyaku), 0.05% CaCl$_2\cdot$2H$_2$O, 0.05% MnCl$_2\cdot$4H$_2$O, 0.05% MgCl$_2\cdot$7H$_2$O and 0.1% KH$_2$PO$_4$. The pH of the medium was adjusted to 6.0.

Materials. DEAE-cellulose (DE 32) and CM-cellulose (CM-23) were purchased from Whatman Ltd. Isomaltose, maltotriose, pullulan, and the pullulanase of Klebsiella aerogenes$^{3}$ were purchased from Hayashi-bara Biochemical Laboratories, Inc. Ampholine was purchased from LKB Produkter Co. Panose, isopanose, and the isopullulanase of Asp. niger$^{4}$ were kindly supplied by Dr. T. Kobayashi of Tokyo Noko University. Other chemicals were purchased from Iwai Kagaku Yakuhin Co.

Determination of $\alpha$-amylase activity.$^{5}$ One milliliter of suitably diluted enzyme solution was incubated with 10 ml of 0.5% soluble starch (Merck) dissolved in 0.03 M acetate buffer (pH 4.5) containing 0.03 M CaCl$_2$ at 60°C for 10 min. Then 1 ml of the reaction mixture was pipetted into a 100 ml volumetric flask containing 50 ml of 0.02 N HCl. After the addition of 3 ml of 0.05% iodine solution, the flask was filled up to 100 ml with distilled water. The optical density of the resulting blue color was measured at 620 nm. One unit of $\alpha$-amylase activity was the amount of enzyme catalyzing a 20% decrease in the optical density per min under the assay conditions as calculated by the following formula.

\[ \text{Unit of } \alpha\text{-amylase activity} = \frac{\text{Absorption units at 620 nm per min}}{0.02 \times 100} \]

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1 This paper was presented at the Annual Meeting of Agricultural Chemical Society of Japan, held in Yokohama on April 1~4, 1977.
Enzyme activity (units)  
\[
\text{Enzyme activity (units) = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} \times 100 \times \frac{1}{10} \times \text{(dilution factor)}}
\]

A linear relationship between the enzyme activity and the decrease in the optical density was found in the range of 10%~60% decrease in the optical density.

**Determination of pullulan-hydrolyzing activity.** A half milliliter of suitably diluted enzyme solution was incubated with 0.5 ml of 2% pullulan dissolved in 0.1 M acetate buffer (pH 4.5) containing 0.03 M CaCl₂ at 40°C for 1 hr. Then the reaction was stopped by heating the mixture in a boiling water bath for 5 min. The reducing sugar in the reaction mixture was determined by the Nelson-Somogyi method⁶) and calculated as glucose equivalents. One unit of pullulan-hydrolyzing activity is the amount of enzyme which produces reducing sugar equivalent to 1 µmole of glucose per min under the assay conditions.

**Determination of protein.** The protein content was determined according to the Folin-Lowry method.⁷) Bovine serum albumin was used as the standard.

**Hydrolyses of pullulan and starch.** Two percent pullulan or soluble starch was dissolved in a 0.05M acetate buffer (pH 4.5) containing 5 mM CaCl₂ and incubated with a given amount of T. vulgaris R-47 α-amylase at 60°C. The reducing sugar and total sugar in the hydrolyzed products were determined by the Nelson-Somogyi method⁸) and the phenol-sulfuric acid method,⁹) respectively. The degree of hydrolysis was expressed as the percent of the reducing sugar against the total sugar.

**Paper chromatography.** Samples were spotted on a Whatman No. 1 filter paper and this was developed with a solvent system of butanol:pyridine:water (6:4:3 v/v) by the descending method for 24 hr. After drying, the sugars were detected by the silver nitrate dip method.⁰) For the purification of the trisaccharide from pullulan hydrolyzed with T. vulgaris R-47 α-amylase, the hydrolyzed product (200 mg as dry substance) was linearly applied to a Whatman 3MM filter paper and the paper was developed with the same solvent system for 3 days. After drying, guide strips were cut off from both sides of the paper and the sugars were detected by the same method. Then the part of the paper corresponding to the position of the trisaccharide indicated by the guide strips was cut off. The trisaccharide was eluted from the paper with distilled water and concentrated at 60°C under a vacuum.

**Disc gel electrophoresis.** Electrophoresis of the enzyme was carried out using a pH 8.8~9.0 polyacrylamide gel, by the method of Davis.¹⁰) After the electrophoresis, the gel was stained for proteins with 1% Amino Black 10B in a 7% acetic acid solution and then destained electrophoretically with 7% acetic acid.

**Determination of isoelectric point.** Electrophoresis was carried out using an LKB Ampholine column at 300~700 volts for 70 hr under a linear gradient of glycerol from 0 to 60%. Ampholine of pH 3.5~10 was used. After the run, the Ampholine solution was drained from the column in 3 ml fractions. The pH and α-amylase and pullulan-hydrolyzing activities of each fraction were determined.

**RESULTS**

**Purification of T. vulgaris R-47 α-amylase**

**Step 1. Concentration by ammonium sulfate fractionation.** Solid ammonium sulfate was added to the culture filtrate until 90% ammonium sulfate saturation was reached. Then the precipitate was separated by centrifugation and dissolved in a minimal amount of 0.05 M acetate buffer (pH 5.5) containing 5 mM CaCl₂. The solution was dialyzed against the same buffer at 4°C overnight, changing the buffer twice.

**Step 2. First DEAE-cellulose column chromatography.** The dialyzed enzyme was applied to a DEAE-cellulose column (2.6 cmφ x 30 cm) equilibrated with 0.05 M acetate buffer (pH 5.5) containing 5 mM CaCl₂ and eluted with the same buffer.

The protein having α-amylase activity was not adsorbed to the column. The α-amylase fractions were pooled and the α-amylase was concentrated by ammonium sulfate precipitation (90% saturation). The α-amylase solution was dialyzed against 0.05 M acetate buffer (pH 5.0) containing 5 mM CaCl₂.

**Step 3. CM-cellulose column chromatography.** The dialyzed enzyme solution was applied to a CM-cellulose column (1.6 cmφ x 20 cm) equilibrated with 0.05 M acetate buffer (pH 5.0) containing 5 mM CaCl₂. The α-amylase did not adsorb to the column and was eluted with the same buffer. The α-amylase was recovered by ammonium sulfate precipitation (90% saturation) and was then dissolved in 0.05 M Tris-HCl buffer (pH 7.8)
TABLE I. PURIFICATION OF R-47 α-AMYLASE

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>α-Amylase activity (αA)</th>
<th>Pullulan-hydrolyzing activity (PA)</th>
<th>PA/αA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total units</td>
<td>Specific activity (units/mg)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>1 Culture filtrate</td>
<td>3500</td>
<td>5500</td>
<td>1.6</td>
<td>100</td>
</tr>
<tr>
<td>2 (NH₄)₂SO₄ precipitate</td>
<td>580</td>
<td>5300</td>
<td>9.1</td>
<td>96.4</td>
</tr>
<tr>
<td>3 First DEAE-cellulose</td>
<td>28</td>
<td>4900</td>
<td>175</td>
<td>89.1</td>
</tr>
<tr>
<td>4 CM-Cellulose</td>
<td>6</td>
<td>2340</td>
<td>390</td>
<td>42.6</td>
</tr>
<tr>
<td>5 Second DEAE-cellulose</td>
<td>2</td>
<td>770</td>
<td>385</td>
<td>14.0</td>
</tr>
</tbody>
</table>

containing 5 mM CaCl₂. The enzyme solution was dialyzed against the same buffer.

Step 4. Second DEAE-cellulose column chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (1.6 cm × 20 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.8) containing 5 mM CaCl₂. The enzyme was eluted from the column by linearly increasing the NaCl concentration in the buffer from 0 to 0.5 M. The α-amylase fractions were collected, and the α-amylase was precipitated with ammonium sulfate (90% saturation), dissolved in 0.05 M CaCl₂ and dialyzed against the same buffer. A summary of this purification procedure is shown in Table I.

Disc gel electrophoresis

The disc gel electrophoresis pattern of the purified α-amylase is shown in Fig. 1. The purified enzyme protein (50 μg) was applied to polyacrylamide gel and electrophoresis was carried out at a constant current of 5 mA per tube for 1 hr. After the electrophoresis, the gel was stained for proteins with 1% Amido Black 10B in a 7% acetic acid solution and then destained electrophoretically with 7% acetic acid.

![Fig. 1. Disc Electrophoresis Pattern of Purified T. vulgaris R-47 α-Amylase.](image1)

The purified enzyme protein (50 μg) was subjected to polyacrylamide disc gel electrophoresis at pH 9.0. After electrophoresis, the gel was sliced into 2 mm-wide strips and each strip was separately eluted with 0.5 ml of 0.05 M acetate buffer (pH 5.0). Then, the α-amylase and pullulan-hydrolyzing activities of the eluted solution were determined.

○—○, α-amylase activity; ●—●, pullulan-hydrolyzing activity.

![Fig. 2. Pullulan-hydrolyzing and α-Amylase Activities on Acrylamide Gel.](image2)
purified enzyme showed a single band. The 
α-amylase and pullulan-hydrolyzing activities 
on the unstained gel were detected by slicing 
the gel and determining the enzymatic activi 
ties of each slice. The results are shown in 
Fig. 2. Both the α-amylase and pullulan-
hydrolyzing activities were seen to coincide with 
the protein band.

Properties of T. vulgaris R-47 α-amylase

1) Optimum pH. The effect of the reaction 
pH on the α-amylase and pullulan-
hydrolyzing activities is shown in Fig. 3. The 
optimum pH for the α-amylase and pullulan-
hydrolyzing activities was around pH 5 and 
both activities showed similar relationships to 
the pH.

Fig. 3. Effect of pH.
α-Amylase and pullulan-hydrolyzing activities were 
estimated at 60°C and various pH’s using a 0.05 M 
acetate buffer containing 0.03 M CaCl₂: ○—○, α-
amylase activity; ●—●, pullulan-hydrolyzing activity.

2) Optimum temperature. The effect of the reaction temperature on the α-amylase and pullulan-hydrolyzing activities is shown in Fig. 4. The optimum temperature for the α-amylase and pullulan-hydrolyzing activities was 70°C. The α-amylase activity showed higher relative activity than the pullulan-hydrolyzing activity at 80°C and 90°C. This was assumed to be caused by a difference between the starch and pullulan substrates in terms of the heat-protection they afford to the enzyme.

3) Heat stability. As shown in Fig. 5, the enzyme was fairly stable at 60°C and pH 5.0

Fig. 4. Effect of Temperature.
α-Amylase and pullulan-hydrolyzing activities were 
estimated at pH 4.5 and various temperatures: ○—○, α-
amylase activity; ●—●, pullulan-hydrolyzing activity.

Fig. 5. Heat-stability.
The purified enzyme was incubated at pH 5.0 and 60°C or 70°C in the presence of 10 mM Ca²⁺ or with no Ca²⁺. The residual α-amylase and pullulan-hydrolyzing activities were determined after 15, 30, 60 and 120 min: ○—○ α-amylase activity; ●—● pullulan-hydrolyzing activity.
1. 60°C, 10 mM Ca²⁺; 2. 70°C, 10 mM Ca²⁺; 3. 60°C, no Ca²⁺; 4. 70°C, no Ca²⁺.

4) Isoelectric point. The isoelectric point of both the α-amylase and pullulan-hydrolyzing activities was pH 5.20 ± 0.15 as shown in
Fig. 6. The elution patterns of protein, α-amylase and pullulan-hydrolyzing activities were consistent with each other. This shows the homogeneity of the enzyme.

Hydrolysis of pullulan. Pullulan (2%) was hydrolyzed with pullulanase, isopullulanase and the T. vulgaris R-47 α-amylase. The progress of these hydrolyses is shown in Fig. 7. The degrees of hydrolysis of the final products were 31.3% with pullulanase, 32.8% with isopullulanase and 33.1% with T. vulgaris R-47 α-amylase. The sugar composition of the hydrolyzates (4 hr reaction) was analyzed by paper chromatography. The paper chromatogram is shown in Fig. 8. The sugar composition of the hydrolyzate produced by the T. vulgaris R-47 α-amylase was similar to that in the isopullulanase hydrolyzate but different from that in the pullulanase hydrolyzate. Pullulan (2%) was completely hydrolyzed using the T. vulgaris R-47 α-amylase and the sugar composition of this hydrolyzate was determined by paper chromatography. The results are shown in Table II. The content of tri-saccharides reached 96.5%. This saccharide was thought to be panose or isopanose, or

Fig. 8. A Paper Chromatogram of the Products of Pullulan Hydrolyzed by Pullulanase, T. vulgaris R-47 α-Amylase and Isopullulanase.

Paper chromatography was carried out by the procedure described under MATERIALS AND METHODS:
1. glucose; 2, maltose; 3, maltotriose; 4, pullulanase hydrolyzate; 5, T. vulgaris R-47 α-amylase hydrolyzate; 6, isopullulanase hydrolyzate; 7, isopanose; 8, panose.
6) Identification of the trisaccharide. The trisaccharide was purified by Whatman 3 MM paper chromatography. Then the purified trisaccharide (2% solution) was incubated overnight with 10 units of isopullulanase per gram of substrate at 40°C and pH 4.5. The hydrolyzed products were analyzed by paper chromatography as shown in Fig. 9. This indicates that the trisaccharide was completely hydrolyzed to glucose and isomaltose. Therefore, the trisaccharide was panose, and was not contaminated with isopanose.

7) Hydrolysis of starch. Two percent soluble starch was hydrolyzed with 10 units of *T. vulgaris* R-47 α-amylase at 60°C and pH 4.5. The increase in the degree of hydrolysis and decrease in starch-iodine color reaction accompanying the hydrolysis are shown in Fig. 10. The blue color of the starch-iodine reaction disappeared when the hydrolysis

![Fig. 9. A Paper Chromatogram of the Products from the Trisaccharide Hydrolyzed by Isopullulanase.](image)

![Fig. 10. Hydrolysis of Soluble Starch.](image)

### TABLE II. COMPOSITION OF SACCHARIDES IN PULLULAN HYDROLYZATES

<table>
<thead>
<tr>
<th>Reaction time (hr)</th>
<th>Hydrolysis percentage</th>
<th>Saccharide composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>1</td>
<td>20.5</td>
<td>0.0</td>
</tr>
<tr>
<td>48</td>
<td>36.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a mixture of both saccharides based upon the Rf value in paper chromatography and the structure of pullulan.

### TABLE III. COMPOSITION OF SACCHARIDES IN STARCH HYDROLYZATES

<table>
<thead>
<tr>
<th>Reaction time (hr)</th>
<th>Hydrolysis percentage</th>
<th>Saccharide composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>1</td>
<td>38.7</td>
<td>2.0</td>
</tr>
<tr>
<td>48</td>
<td>52.9</td>
<td>11.8</td>
</tr>
</tbody>
</table>
Purification and Properties of *T. vulgaris* α-Amylase

Hydrolysis of starch and pullulan (2%) was carried out at 60°C and pH 4.5 using 20 α-amylase units of the *T. vulgaris* R-47 α-amylase per gram of substrate. Hydrolysis percentage of the hydrolyzates was determined by the Somogyi-Nelson method and the phenolsulfuric method: \(\text{starch; pullulan.}\) The mutarotation of the hydrolyzed product was determined according to the method described by H. Fuwa and Z. Nikuni. As a result, a decrease in the optical rotation was observed. Therefore, the hydrolyzed products should have an α-configuration. Figure 11 shows a comparison of the hydrolysis of soluble starch and pullulan when equal amounts of *T. vulgaris* R-47 α-amylase were employed. The sugar composition of the final hydrolyzate from the soluble starch was determined by paper chromatography and the results are shown in Table III. The main products were maltose (74.1%) and glucose (11.8%). These results indicate that this enzyme is a saccharifying α-amylase.

**DISCUSSION**

In 1967, P. A. Hartman et al. reported the purification of an α-amylase from *Thermoactinomycetes vulgaris*. The authors screened thermophilic actinomycetes strains for α-amylase production and found that two types of α-amylase were produced by thermophilic actinomycetes strains. One type was a neutral α-amylase similar to that reported by Hartman. The other type was an α-amylase with an acidic pH optimum, and this α-amylase had the ability to hydrolyze pullulan. The hydrolyzed product was found to be mainly panose.

Regarding pullulan-hydrolyzing enzymes, three types of enzymes have been known, as shown in Fig. 12. This α-amylase corresponds to a fourth type of enzyme, which has not hitherto been reported.

**Acknowledgements.** We wish to thank Dr. Y. Sakano and Dr. T. Kobayashi of Tokyo Noko University for their kind gifts of panose, isopanose and isopullulanase.

**REFERENCES**