## NOTE

# BIOSYNTHESIS OF MALTOTETRAOSE AS AN ANTIBIOTIC AGAINST *ERWINIA CAROTOVORA* by A STREPTOMYCES AMYLASE

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In our screening investigation, broth filtrates of 23 out of 159 *Streptomyces* strains were found to exhibit inhibitory zones by the paper disc-agar plate method and *Erwinia carotovora* as a test organism. No activity was found using other Gram-positive or negative bacteria. The active component was isolated from the broth filtrate of *Streptomyces* H 359 NSY 6, one of the above 23 strains, and its structure was proven to be maltotetraose[1]. In addition, antimicrobial activity of other malto-oligosaccharides against *E. carotovora* was also determined as described in a previous paper[1].

We have studied the biosynthesis of maltotetraose by *Streptomyces* H 359 NSY 6; the antibiotic was found to be produced from starch by the streptomyces amylase. Production, isolation and enzymatic properties of the streptomyces amylase and the analysis of the products of starch digestion by the enzyme will be described in this paper.

*Streptomyces* H 359 NSY 6 inoculum was prepared in shaking flasks (500 ml) containing 100 ml of 1.0% soluble starch-0.2% yeast extract medium (pH 7.0) incubated at 27°C for 24 hours. Two ml of the inoculum was inoculated into each of 20 shaking flasks (500ml) containing 100 ml of a medium composed of 1.0% potato starch, 1.0% glucose, 0.75% meat extract, 0.75% peptone, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ (pH 7.0) and cultured at 27°C for 96 hours on the shaker. The broth filtrate obtained (1,860 ml, pH 5.4) is saturated with ammonium sulfate (900 g) and kept at 5°C for 1 hour whereupon the precipitate is collected by centrifugation. A crude amylase (1,117 mg) is recovered from the precipitate by a 5-hour dialysis in a cellophane tube against 0.1 mM $CaCl_2$, followed by lyophilization of the dialyzate. The crude amylase, dissolved in 0.1 mM $CaCl_2$, is further purified on a Sephadex G-75 column (85 cm × 3.3 cm diam.) followed by an elution with 0.1mM $CaCl_2$. The eluate is collected in each 10 ml fractions and a partially purified amylase (14.1 mg) is obtained from the fractions 15~17 by lyophilization. (see Fig. 1) Thus, 18% of the activity is recovered from the crude amylase.

The amylase activity is determined by a cylinder-agar plate method on nutrient agar using *E. carotovora* as a test organism. Starch is hydrolyzed to malto-oligosaccharides by the amylase and the inhibitory zone against *E. carotovora* due to malto-oligosaccharides is measured. An inoculum of *E. carotovora* is prepared by incubation in nutrient broth at 27°C for 20 hours. The seed layer (5ml) containing 0.5% of the inoculum is placed on the basal layer (10 ml) and incubated at 27°C for 20 hours with a cylinder containing the test solution. The test solution consists of an incubated mixture of 0.5 ml of the test sample and 0.5ml of 6% soluble starch solution both dissolved in 1/10 M phosphate buffer (pH 6.5) after incubation at 37°C for 16 hours. Inactivation is done by heating at 100°C for 5 minutes. Test solutions containing 3,000 mcg/ml and 750 mcg/ml of maltotetraose show inhibitory zone of 21.0 mm and 16.5 mm by the above assay method.

The partially purified amylase is used to study enzymatic properties; amylase activity is expressed as maltotetraose activity (mg/ml) against *E. carotovora*.

Effect of incubation period on the enzyme activity is shown in Fig. 2; the activity becomes stationary after 16~20 hour incubation. Effect of pH on the activity is illustrated in Fig. 3; the optimum pH is around 7.0. The effect of temperature on the activity is shown in Fig. 4; the optimum temperature is around 40°C. The relationship between enzyme concentration and the activity is shown in Fig.

Fig. 1. Gel filtration on Sephadex G-75

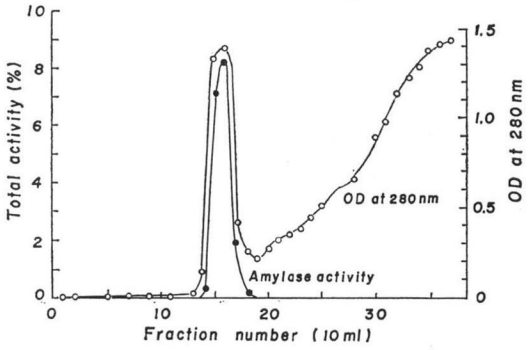



Fig. 2. Effect of incubation period on amylase activity

A mixture of 0.5 ml of the enzyme solution (600 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 6.5) and 0.5 ml of 6% starch solution in 1/10 M phosphate buffer (pH 6.5) was incubated at 37°C for various periods.

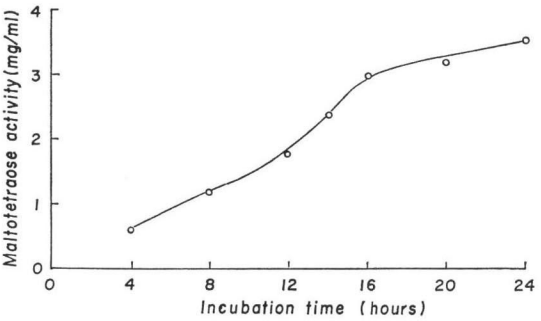



Fig. 3. Effect of pH on amylase activity

A mixture of 0.5 ml of the aqueous enzyme solution (600 mcg/ml) and 0.5 ml of 6% starch solution in 1/5 M phosphate buffer (pH 5.0~8.0) or 1/5 M glycine buffer (pH 3.0~5.0 and pH 8.0~11.0) was incubated at 37°C for 16 hours.

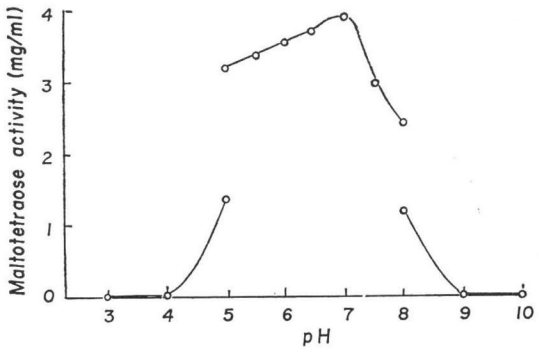



Fig. 4. Effect of temperature on amylase activity

A mixture of 0.5 ml of the enzyme solution (600 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 6.5) and 0.5 ml of 6% starch solution in 1/10 M phosphate buffer (pH 6.5) was incubated at the test temperature for 16 hours.

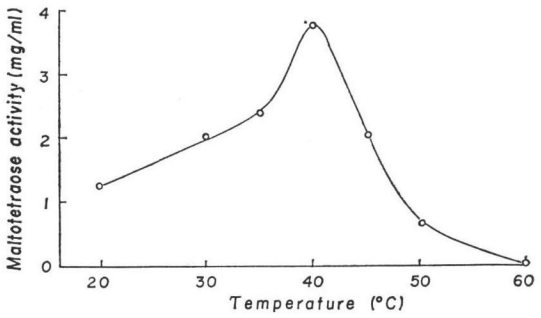



Fig. 5. Effect of enzyme concentration on amylase activity

A mixture of 0.5 ml of the enzyme solution (50~600 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 6.5) and 0.5 ml of 6% starch solution in 1/10 M phosphate buffer (pH 6.5) was incubated at 37°C for 16 hours.

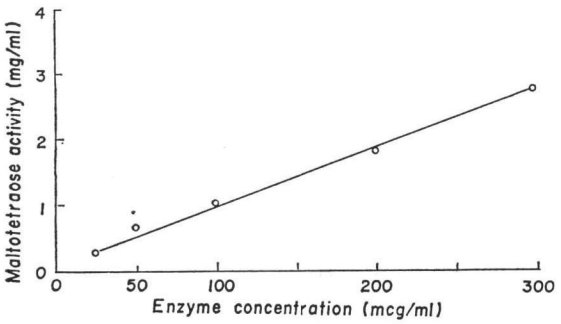
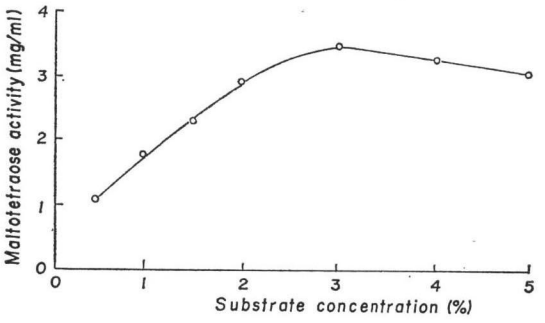



Fig. 6. Effect of substrate concentration on amylase activity

A mixture of 0.5 ml of the enzyme solution (600 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 6.5) and 0.5 ml of starch solution (1~10%) in 1/10 M phosphate buffer (pH 6.5) was incubated at 37°C for 16 hours.



5. Figure 6 shows the effect of substrate concentration on the activity; the optimum concentration is around 3%. The enzyme is most stable at pH 6.0 when kept at room temperature for 1 hour as may be seen in Fig. 7. The enzyme is stable at temperature below 45°C in a phosphate buffer (pH 6.5), but inactivated entirely at 60°C for 10 minutes as shown in Fig. 8. Various substrates, as shown in Table 1, are digested by the amylase. The resulting mixtures were deionized by treatment

Fig. 7. pH Stability

1/10 M Phosphate buffer (pH 5.0~8.0) or 1/5 M glycine buffer (pH 3.5 ~ 5.0 and pH 8.0 ~ 10.0) was used. A mixture of 0.05 ml of the aqueous enzyme solution (6,000 mcg/ml) and 0.25 ml of the buffer was kept at room temperature for 1 hour. After adjusting the pH value to 6.5 and the total volume to 0.5 ml, the mixture was incubated with 0.5 ml of 6% starch solution in 1/10 M phosphate buffer (pH 6.5) at 37°C for 16 hours.

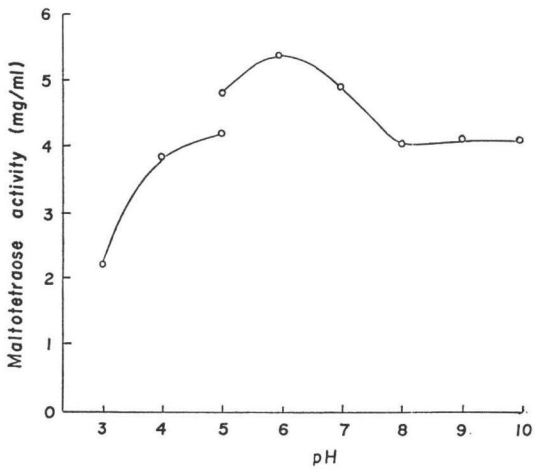



Fig. 8. Thermal stability

A half ml of the enzyme solution (600 mcg/ml) dissolved in 1/5 M phosphate buffer (pH 6.5) was heated at the test temperature for 10 minutes. After cooling, the resulting solution was incubated with 0.5 ml of 6% starch solution in 1/10 M phosphate buffer (pH 6.5) at 37°C for 16 hours.

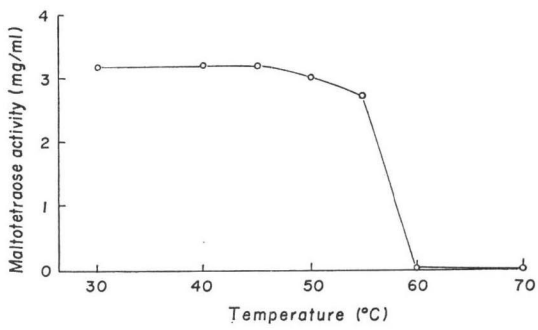



Table 1. Analysis of digested products by gas chromatography. I

| Substrates | Maltose (%) RT*: 6 min. at 230°C | Maltotriose (%) RT: 6 min. at 280°C | Maltotetraose (%) RT: 33 min. at 280°C |
|---|---|---|---|
| Amylase (M.W. 4,000) | 1.9 | 5.6 | 4.8 |
| Amylase (M.W. 21,000) | 3.8 | 4.7 | 4.7 |
| Corn starch | 0.2 | 1.2 | 0.9 |
| Amylopectin | 0.9 | 2.7 | 2.4 |

A mixture of 0.2 ml of the enzyme solution (1,500 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 7.5) and 0.8 ml of 2.5% starch solution in 1/10 M phosphate buffer (pH 7.5) was incubated at 37°C for 16 hours.

* RT: Retention time.

Table 2. Analysis of digested products by gas chromatography. II

| Substrate | Maltose (%) RT*: 6 min. at 230°C | Maltotriose (%) RT: 6 min. at 280°C | Maltotetraose (%) RT: 33 min. at 280°C |
|---|---|---|---|
| Amylase (M.W. 21,000) | 6.7 | 1.9 | 0.9 |

A mixture of 0.5 ml of the enzyme solution (1,800 mcg/ml) dissolved in 1/10 M phosphate buffer (pH 7.5) and 0.5 ml of 1% starch solution in 1/10 M phosphate buffer (pH 7.5) was incubated at 37°C for 16 hours.

* RT: Retention time.

with ion-exchange resins, IR 120 and IR 4B, to remove phosphates. The products were then analyzed by gas chromatography on a 1.5% OV-17 column (200 cm, under 1.6 kg/cm$^2$ of nitrogen gas) after being converted to the trimethylsilyl derivatives[2,3]. The results are shown in Table 1. Maltopentaose cannot be analyzed by gas chromatography; nevertheless presence of maltopentaose in the digested mixture is proven by paper chromatography, developed with BuOH-pyridine-$H_2O$ (6:4:3). The spot is detected by spraying with a silver nitrate-sodium hydroxide solution[4,5]. Low productivity of maltotetraose and maltotriose is observed when corn starch or amylopectin is digested with the amylase. This observation can be explained by the existence of the $\alpha$-1-6 glycosidic linkages due to side chains in both substrates. The quantity of maltose in the hydrolyzate is increased and that of maltotetraose and maltotriose is decreased when a lower concentration of the substrate, and a higher concentration of the amylase are employed as may be seen from Table 2.

Obviously, the antibiotic maltotetraose is produced from starch by the action of the amylase. The streptomyces amylase can be considered to be an $\alpha$-amylase, it hydrolyzes the $\alpha$-1-4 glycosidic linkage to yield maltooligosaccharides. The streptomyces amylase has a little higher optimum pH (pH 7.0) compared with that of *Bacillus subtilis* (pH 5.3~6.8, pH 5.6~6.0) or *Aspergillus oryzae* (pH 5.5~5.9, pH 5.2~5.6)[6].

## References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