ISOLATION OF MALTOTETRAOSE FROM *STREPTOMYCES* AS AN ANTIBIOTIC AGAINST *ERWINIA CAROTOVORA*

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Erwnia carotovora is known to cause bacterial stalk rot of plants. Culture filtrates of 159 strains of *Streptomyces* species freshly isolated in this laboratory have been screened for inhibitory activity against *E. carotovora* using the paper disc-agar plate method on nutrient agar. Twenty-six *Streptomyces* strains showed inhibitory zones against *E. carotovora*, but not against other gram-positive and negative bacteria. While 3 strains showed clear inhibitory zones against *E. carotovora* as well as against other gram-positive and negative bacteria. One of the above 26 *Streptomyces* strains, designated as *Streptomyces* H 359 NSY 6, was studied to isolate the antibiotic which was found to be maltotetraose. Until now, maltotetraose has not been reported as an antibiotic of *Streptomyces* origin. Cultivation, isolation and purification of the antibiotic and its identity with maltotetraose will be described in this paper.

Streptomyces H 359 NSY 6 was cultured in shaking flasks containing 100 ml of an inoculation medium composed of 1.0 % soluble starch and 0.2 % yeast extract (pH 7.0) at 27°C for 40 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). This inoculum was used to inoculate 16 shaking flasks (500 ml) each containing 150 ml of a production 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). The culture was grown at 27°C for 96 hours on the shaker. The mycelium was removed by filtration. Two liters of filtrate (pH 6.8) were obtained. The filtrate produced a turbid inhibitory zone of diameter 24 mm by the cylinder-agar plate method against *E. carotovora* (Table 1).

Table 1. Cultivation of *Streptomyces* H 359 NSY 6 and activity against *Erwinia carotovora*

Cultivation	2 days	3 days	4 days	5 days
pH	4.8	5.3	6.8	7.6
Inhibition diameter (mm)	0	0	24.0	0

The broth filtrate was treated with a small amount of activated carbon (10 g) for 30 minutes in order to adsorb chromogenic compounds. After filtration 80 % of the total antimicrobial activity remained in the filtrate. The crude antibiotic (3.2 g) was recovered from the filtrate by adsorption on activated carbon (30 g) for 60 minutes, followed by elution with MeOH as shown in Chart 1. The crude antibiotic (3.2 g) was purified on a carbon column,[1] eluted with a linear gradient concentration of aqueous EtOH to give 775 mg partially purified antibiotic with a total activity of 16.4 % (Chart 2). The partially

Chart 1. Isolation of the crude antibiotic

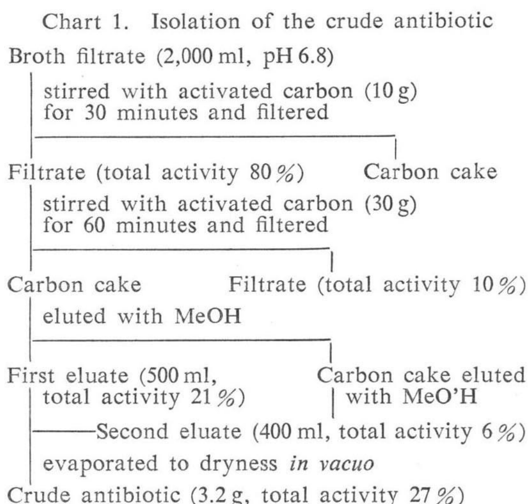

Chart 2. Partial purification of the crude antibiotic on a carbon column

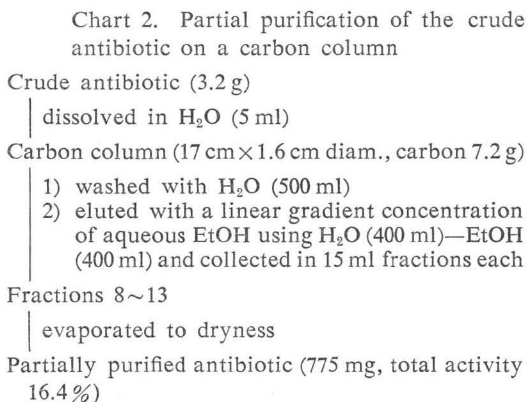

Chart. 3. Further purification of the antibiotic

Partially purified antibiotic (380 mg) dissolved in H_2O (2 ml)

| BuOH (20 ml) was added to make a homogenous solution

Cellulose powder column (74 cm×1.8 cm diam.)

| 1) washed with H_2O-saturated BuOH (400 ml)
| 2) developed with *n*-BuOH-MeOH-H_2O (2:1:2) and collected in 5-g fractions each

Fractions 12~17

| evaporated to dryness *in vacuo*

Antibiotic (104.5 mg)

Antibiotic (24 mg)

Paper chromatography (40 cm, wide×20 cm, high)

| 1) developed three times with *n*-BuOH-pyridine-H_2O (6:4:3)
| 2) eluted with H_2O and evaporated to dryness

Pure antibiotic (11 mg)

purified antibiotic (380 mg) was further purified by cellulose powder partition chromatography using *n*-BuOH-MeOH-H_2O (2:1:2) and 104 mg of the antibiotic was recovered. The antibiotic was shown to contain traces of impurities at 7.5 cm/15 cm and 4.1 cm/15 cm (mobility/developing distance) besides the antibiotic (5.8 cm/15 cm) by spraying with a silver nitrate-sodium hydroxide solution[2] on a filter paper, after developed three times with *n*-BuOH-pyridine-H_2O (6:4:3)[3]. Consequently, the impure antibiotic (24 mg) was separated on a filter paper (40 cm wide, 20 cm high) developed with the same solvent (three ascents). Elution with H_2O, followed by evaporation gave the pure white amorphous antibiotic showing only a single spot by paper chromatography (Chart 3). Decomposition at 169.5~173°C, $[\alpha]_D^{25}+196°$ (*c* 0.173, H_2O). Anal. calculated for $C_{24}H_{42}O_{21}$; C, 43.22; H, 6.35. Found, C, 43.18; H, 6.84.

The antibiotic shows only end absorption in UV spectrum in H_2O. The antibiotic is soluble in water and methanol, sparingly soluble in ethanol, iso-propanol, *n*-butanol and iso-butanol and insoluble in ether, chloroform, benzene, ethyl acetate and acetone. It gives positive aniline-phosphate[4] and silver nitrate-sodium hydroxide[2] reactions but a negative ninhydrin reaction.

The antibiotic shows inhibitory zones of 21.0 and 16.5 mm diameter respectively at concentrations of 3,000 mcg/ml and 750 mcg/ml against *E. carotovora* using the cylinder agar-plate method. Nevertheless, the antibiotic does not show growth inhibition at a concentration of 200 mcg/ml using the agar dilution method on glucose-nutrient agar against following microorganisms: *Staphylococcus aureus* FDA 209P, *Micrococcus flavus*, *Sarcina lutea*, *Bacillus subtilis*, *Corynebacterium bovis*, *Escherichia coli*, *Shigella sonnei*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella typhosa*, *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Candida albicans*, *Saccharomyces cerevisiae*, *Xanthomonas oryzae*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Erwinia carotovora*.

The antibiotic was thought to be a saccharide as is evidenced from physico-chemical properties. Two mg of the antibiotic was hydrolyzed with 0.5 N HCl at 80°C for 5 hours. The reaction mixture was examined periodically by silica gel thin-layer chromatography using *n*-BuOH-AcOH-H_2O (2:3:1) as a developing solvent as shown in Table 2. Three hydrolysis products were detected on the thin-layer plate at Rf 0.50, 0.61 and 0.67 by heating at 100°C for 10 minutes after spraying with a 2 N aniline-2N phosphate solution[4], while the original antibiotic was located at Rf 0.37. The three hydrolysis products were tentatively designated as Compounds I, II and III in order of increasing Rf values by the thin-layer chromatography. Compounds I and II were converted to Compound III during the hydrolysis, and Com-

Table 2. Silica gel thin-layer chromatography of the acid hydrolyzate of the antibiotic. Hydrolyzed at 80°C with 0.5 N HCl

Hydrolysis period (min.)	Rf values			
0	0.37			
15	0.37 (m)	0.50	0.61 (t)	0.67 (t)
30	0.37 (m)	0.50	0.61	0.67
60	0.37	0.50	0.61	0.67
120	0.37	0.50	0.61 (m)	0.67 (m)
180			0.61	0.67 (m)
240			0.61 (t)	0.67 (m)
300				0.67 (m)

Developing solvent: *n*-BuOH-AcOH-H_2O (2:3:1)
(m): Main component. (t): Trace component.

pound III was obtained as the only hydrolysis product by the hydrolysis for 300 minutes. Compound III was confirmed to be glucose by paper and thin-layer chromatography[5]. Identification of Compound III as glucose was also proven by its gas chromatogram (retention time, 24 and 39.8 minutes) on 1.5 % SE-30 column (200 cm) at 150°C after conversion to its trimethylsilyl derivative[6~8]. Compound II was identified to be maltose by AcONa-treated silica gel thin-layer chromatography developed with $CHCl_3$-MeOH (6:4) or AcOEt-iso-PrOH-H_2O (32:12:6)[5] and

Table 3. Paper chromatography of the antibiotic in comparision with maltotetraose[3]

Solvent	Mobility (cm)/Developing distance (cm)			
	I	II	III	IV
Antibiotic	5.8/15.0	2.4/16.8	5.6/17.3	0/16.0
Maltotetraose	5.8/15.0	2.4/16.8	5.6/17.3	0/16.0

Solvent I: *n*-BuOH-Pyridine-H_2O (6:4:3) (three ascents)
Solvent II: *n*-BuOH-EtOH-H_2O (5:3:2) (three ascents)
Solvent III: *n*-BuOH-EtOH-H_2O (2:1:1) (three ascents)
Solvent IV: H_2O-saturated *n*-BuOH

Table 4. Silica gel thin-layer chromatography of the antibiotic in comparision with maltotetraose

Solvent	Rf values		
	I	II	III
Antibiotic	0.21	0.47	0.37
Maltotetraose	0.21	0.47	0.37

Solvent I: *n*-BuOH-EtOH-H_2O (5:3:2)
Solvent II: *n*-PrOH-AcOEt-H_2O (6:1:3)
Solvent III: *n*-BuOH-AcOH-H_2O (2:3:1)

the spot was detected by heating at 100°C for 10 minutes after spraying 2 N aniline-2 N phosphate solution[4]. This suggested that Compound I and the antibiotic were thought to be maltotriose and maltotetraose respectively. Indeed, the structure of the antibiotic was proven to be maltotetraose by the paper and silica gel thin-layer chromatography as listed in Tables 3 and 4. The gas chromatogram of the trimethylsilylated antibiotic had the same retention time (24 min.) with that of trimethylsilylated maltotetraose[9] on 1.5 % OV-17 column (200 cm) at 280°C under 1.8 kg/cm^2 of nitrogen gas.

Table 5. Antimicrobial activity of malto-oligosaccharides against *Erwinia carotovora* by the cylinder-agar plate method

Malto-oligosaccharides	Relative activity (%)
Maltose	8
Maltotriose	105
Maltotetraose	100
Maltopentaose	75
Maltohexaose	40
(Glucose 30 mg/ml)	0

The malto-oligosaccharides were tested at concentrations of 3,000 mcg/ml and 750 mcg/ml except for maltose (10,000 mcg/ml and 2,500 mcg/ml).

Small quantities (10^{-5} M) of glucose, arabinose, galactose, fructose and lactose are known to enhance growth of *E. carotovora*, but higher concentrations ($10^{-3}\sim10^{-2}$ M) are inhibitory for this microorganism[10].

The antimicrobial activity of various malto-oligosaccharides against *E. carotovora* was determined by the cylinder-agar plate method on nutrient agar to study structure and activity relationship. The relative antimicrobial activities are listed in Table 5. Maltotriose and maltotetraose had almost equal activities, weaker activity was observed with maltopentaose and maltohexaose, and almost none with maltose.

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