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Studies on Screening and Isolation of α-Amylase Inhibitors of Soil Microorganisms(Ⅱ)

Isolation and Activities of the Inhibitor of *Streptomyces* Strain DMC-72

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토양균의 α-Amylase 저해제 검색 및 분리에 관한 연구(제 2 보)

스트렙토마이세스속 DMC-72 균주의 저해 성분의 분리 및 작용

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Abstract: Of 450 strains isolated from the soil microbes collected in various locations in Korea, a strain had a strong inhibitory activity against bacterial α-amylase and was named strain DMC-72 of the genus *Streptomyces*. The amylase inhibitory metabolite produced by this strain was purified by means of acetone precipitation, adsorption on Amberlite IRC-50 and SP-Sephadex C-25. The inhibitor was found to be a derivative of oligosaccharides by spectral and chemical data. The inhibitor was stable at the pH range of 1~13 and at 100°C for half an hour, also inhibited other amylases such as salivary α-amylase, pancreatic α-amylase, fungal α-amylase and glucoamylase. However, it showed no inhibitory activity against α-glucosidase, β-glucosidase, dextranase, and β-amylase. The kinetic studies of the inhibitor showed that its inhibitory effects on starch hydrolysis by α-amylase were noncompetitive.

Keywords: *Streptomyces* strain DMC-72, α-Amylase inhibitor, Fermentation, Enzyme kinetics, Oligosaccharide.

Recently several reports that were concerned with microbial amylase inhibitors appeared (Ueda *et al.*, 1973, 1978; Chyama *et al.*, 1977; Namix, 1982; Verbear, 1982). It was suggested that prevention and therapy of diabetes and obesity might be achieved by reducing the digestion of dietary starch with inhibitors of α-amylase (Murao 1975, 1977, 1979; Schindler, 1980; Niwa *et al.*, 1970; Udea *et al.*, 1976; Schmidt *et al.*, 1977). These inhibitors are also expected to decrease hyperglycemia and hyperinsulinemia (Namiki *et al.*, 1979; Kameda *et al.*, 1980). From these viewpoints, a number of α-amylase inhibitors have been isolated from the microbial cultures (Itoh *et al.*, 1981; Yokose *et al.*, 1983). In the course of our screening program for α-amylase inhibitors, an inhibitor was discovered in the culture filtrate of *Streptomyces* strain DMC-72. The inhibitor produced by strain DMC-72 had very effective inhibitory activity against amylase. It was purified by acetone precipitation, adsorption chromatography by XAD-2, column chromatography of Amberlite IRC-50, CG-50 and SP-Sephadex C-25. The inhibitor was stable

in a wide range of pH and temperature and had inhibitory activities against various amylases and related enzymes. This paper reports the production of the inhibitor by fermentation, purification, physico-chemical properties, its effects on various enzymes, and its kinetic studies.

Materials and Methods

Screening Method

1) Soil Sample

One gram of soil samples which were collected in various locations in Korea was used for screening procedure.

2) Medium

a) Oatmeal medium was used for isolation of colonies: oatmeal 20 g, yeast extract 1 g, agar 20 g and distilled water 1,000 ml.

b) Shake culture medium (oatmeal 20 g, yeast extract 1 g, distilled water 1,000 ml) was used for production of α-amylase inhibitory substances.

3) Cultivation

The colonies maintained in agar slants were inoculated into 10 ml of the shake culture media in a 50 ml flask and incubated for three days in rotary shaker at 27±1°C and 180 rpm.

4) Crude Inhibitor Solution

The culture broth was centrifugated at 3,000 rpm and the supernatant was used for the demonstration of amylase inhibitory activity.

The inhibitor solution was heated in a boiling water bath for three minutes before use in order to exclude interference by another enzyme.

5) Detection of α-Amylase Inhibitor in Culture Broth

a) Reagents

(1) α-Amylase sol'n (1.2 unit/ml)

Bacterial α-amylase (a commercial product from *Bacillus subtilis* by Sigma Chem. Co.) was dissolved in 200 mM phosphate buffer (pH 6.9).

(2) Soluble starch sol'n (15 mg/ml)

Soluble starch was dissolved in 100 mM phosphate buffer (pH 6.9).

(3) Lugol's sol'n; 0.5 mM

b) Assay System for α-Amylase Inhibitory Activity

The reaction mixture was composed of 0.5 ml of enzyme solution (dissolved at the concentration of 1.2 unit/ml in 200 mM phosphate buffer, pH 6.9, containing 20 mM sodium chloride) and 0.5 ml of each culture broth. This reaction mixture was pre-incubated at 37°C for five minutes and 2 ml of soluble starch (dissolved at the concentration of 1.5 % in 100 mM phosphate buffer, pH 6.9) was added to this mixture. After incubation for 15 minutes at 37°C, the reaction was stopped by heating for five minutes on a vigorously boiling water bath. Then the residual amylase activity was assayed by the blue value method. That is, five ml of 0.5 mM Lugol's solution was added to 0.1 ml of each reaction mixture, and after this mixture was agitated, the optical density was measured at 620 nm.

As control, an assay mixture containing 0.5 ml of water in place of the culture broth was incubated in parallel with the test sample. As blank, an assay mixture containing 0.5 ml of 200 mM phosphate buffer (pH 6.9) and 0.5 ml of water in place of the enzyme solution and the culture broth was incubated in parallel with the test sample. The percent inhibition (=P.I.) of amylase activity was calculated from the following equation:

$$\text{P.I.} = \frac{T - C}{B - C} \times 100(\%)$$

where T, C and B are the optical densities of the test, control and blank.

One inhibition unit (I.U.) in this assay system was defined as the amount of inhibitor providing 50 % inhibition compared to the ori-

Table I. Screening method of amylase inhibitors.

Addition of	Test	Control	Blank
α-Amylase sol'n	0.5 ml	0.5 ml	Buffer
Culture broth	0.5 ml	Water	Water
Incubation for 5 min at 37°C in water bath.			
Soluble starch sol'n	2 ml	2 ml	2 ml
Mixing and incubation for 15min at 37°C in water bath. Reaction was stopped by heating.			
Addition of 0.1ml out of 3 reaction mixtures	0.1 ml	0.1 ml	0.1 ml
Addition of Lugol's sol'n	5 ml	5 ml	5 ml
Measurement of optical density at 620 nm.			

ginal activity.

The screening assay system for amylase inhibitors was shown in Table I.

Fermentation

1) Fermentation Procedure

A stock culture of strain DMC-72 was used to inoculate 100 ml of the seed culture medium in a 500 ml flask, and incubation was carried out at 27±1°C on a rotary shaker. A three-day culture (50 ml) was aseptically transferred into 500 ml of the production culture medium in a two-liter flask and shake culture was carried out for five days at 27±1°C.

The composition of the production medium was 2 % oatmeal medium.

2) Time Course of the Amylase Inhibitor Production

A stock of strain DMC-72 was inoculated into 100 ml of the oatmeal medium in a 500 ml flask and incubated at 27±1°C on a rotary shaker. A three-day culture (10 ml) was aseptically transferred into 100 ml of oatmeal medium in a 500 ml flask and incubated. To determine inhibitory activity and pH change of the culture broth, culture filtrate was obtained from one flask everyday for eight days. Inhibitory activity aganist α-amylase was determined by a modified blue value method and expressed by inhibition unit (I.U.) per ml.

Purification of the Amylase Inhibitor

The culture broth of the inhibitor from *Streptomyces* strain DMC-72 was centrifugated at 9,000 rpm for 20 minutes to remove mycelia (20 liters). The same volume of cold acetone (−20°C) was added to the supernatant. The inactive precipitate formed was removed by centrifugation at 10,000 rpm for 20 minutes and supernatnat was concentrated to dryness under reduced pressure at 50°C and dissolved in 1,000 ml of deionized water. The aqueous solution was applied to column of Amberlite XAD-8 (Rhom and Hass Co.), and the column was successively washed with deionized water. The active filtrate was evaporated to dryness. This dry material was dissolved in 1,000 ml of deionized water. This inhibitor solution was used for chromatography.

1) Amberlite IRC-50 Chromatography of Crude Inhibitor Solution

The inhibitor solution was adsorbed on a column of Amberlite IRC-50. Aqueous solution was applied on a column of Amberlite IRC-50 (H^+ form, 4.0×35 cm) equilibrated with water. The column was first washed with 1,000 ml of deionized water and then eluted with 2,000 ml of NH_4OH solution (0.1 M∼1.0 M NH_4OH gradient) and each 10-ml fraction was collected. Inhibitor-containing fractions were collected and concentrated by rotary vacuum evaporator.

2) Amberlite CG-50 Column Chromatography (H^+ form)

Inhibitors, re-dissolved in deionized water and adjusted to pH 7.0 with 1 N HCl, were applied on column of Amberlite CG-50 (H^+ form, 2.5

×60 cm) previously equilibrated with deionized water. The column was first eluted with 0.1 N ~1.0 N NH_4OH solution and then each 5 ml fraction was collected.

3) Gel Filtration on Sephadex G-25

Inhibitor solution was concentrated by rotary vacuum evaporator and applied on a column of Sephadex G-25 (2.5×70 cm) previously equilibrated with deionized water, and the inhibitor was eluted with deionized water at a flow rate of one ml per minute and each 5-ml fraction was collected.

4) SP-Sephadex C-25 (Na^+ form) Column Chromatography

The inhibitor solution was concentrated by rotary vacuum evaporator and applied on a column of SP-Sephadex C-25(2.0×60 cm) previously equilibrated with 0.05 M citrate buffer (pH 3.0), and was eluted with 0.05 M citrate buffer (pH 3~6 gradient) at a flow rate of one ml per minute and each 3-ml fraction was collected.

5) Gel Filtration on Sephadex G-15

The inhibitor solution was applied on a column of Sephadex G-15 and then eluted with deionized water to eliminate unnecessary salts.

Spectral Analysis

Physico-chemical properties of the purified α-amylase inhibitor of strain DMC-72 were examined by infrared spectroscopic methods.

Stability of the Amylase Inhibitor

One mg of purified inhibitor wad dissolved in various buffers and kept 100°C for 30 minutes and the residual inhibitory activity was assayed at pH 7.0.

Effects of the α-Amylase Inhibitor on Various Amylases

The enzymes used in the test were as follows;

α-Amylase : a commercial product produced from *Bacillus subtilis* by Sigma Chem. Co., was employed.

Salivary α-amylase : salivary α-amylase was prepared from human saliva.

Pancreatic α-amylase : pancreatic α-amylase was prepared from porcine pancreas.

Fungal α-amylase : fungal α-amylase was prepared from *Aspergillus oryzae*.

Glucoamylase : a commercial product produced from *Rhizopus* genus mold was prepared.

α-Glucosidase : brewers yeast.

β-Glucosidase : almonds.

Dextranase : *Penicillium* species.

β-Amylase : barley.

The Kinetic Studies of Inhibition

Kinetic studies were conducted to observe effects of the inhibitor on hydrolysis of soluble starch by α-amylase. The concentration of the soluble starch was changed from 0.25 % to 2.0 %, and its effects on initial velocity (Vo) were determined at a fixed concentration of the inhibitor. Then a Lineweaver-Burk plots of 1/Vo *vs.* 1/S was prepared.

The reaction system was as follows:

Each enzyme solution (0.5 ml, units) was pre-incubated with 0.5 ml of amylase inhibitor for three minutes at 37°C and then 2 ml of 1.5 % soluble starch (Commercial preparation for amylase test, Junsei Chemical Co., Ltd., dissolved in 100 mM phosphate buffer, pH 6.9) was added. After the mixture was incubated for 10 minutes at 37°C, the reducing sugar formed was determined by Somogyi method.

Results

Screening of the Strains for α-Amylase Inhibitor

Of 450 strains isolated from the soil samples

collected in various locations in Korea, a strain was isolated from the soil sample collected in Seoul and designated as strain DMC-72. This strain had a high inhibitory activity against α-amylase.

Fermentation

When strain DMC-72 was grown in the production medium by submerged culture, the mycelial growth showed its peak at the sixth day, but the maximum production of the amylase inhibitor was observed at the fourth day. At that time the fermentation broth had 3.2× 10 inhibition unit (I.U./ml), and the pH of the broth was 6.8 at this point. The results were shown in Fig. 1.

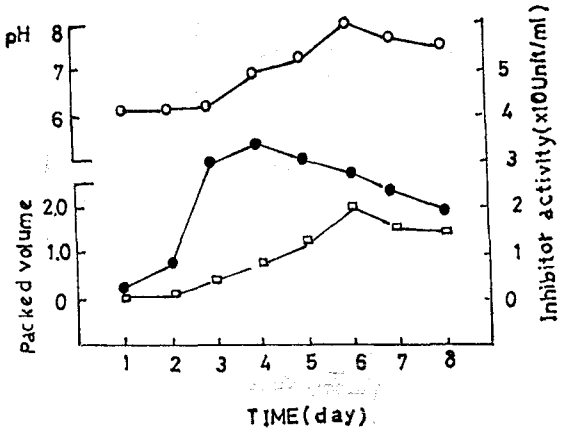

Fig. 1. Time course of the productions of the α-amylase inhibitor by strain DMC-72.
●—●: inhibitor activity
○—○: pH
□—□: packed volume of mycelia(ml)

Purification of the Amylase Inhibitor

Whole fermentation broth (20 liters, 3.2×10 I.U/ml, 6.2×10^5 I.U./total) was treated by acetone precipitation and then filtered. The filtrate was concentrated under reduced pressure to a volume of about 3 liters.

The concentrate (3 liters, 6.2×10^5 I.U./total) was passed through a column (3 liters) of amberlite XAD-8. The active fractions were combined and concentrated under reduced pressure to a volume of one liter of brownish color (6.0×10^5 I.U./total). This inhibitor solution was used for a series of chromatography as shown in Fig. 2.

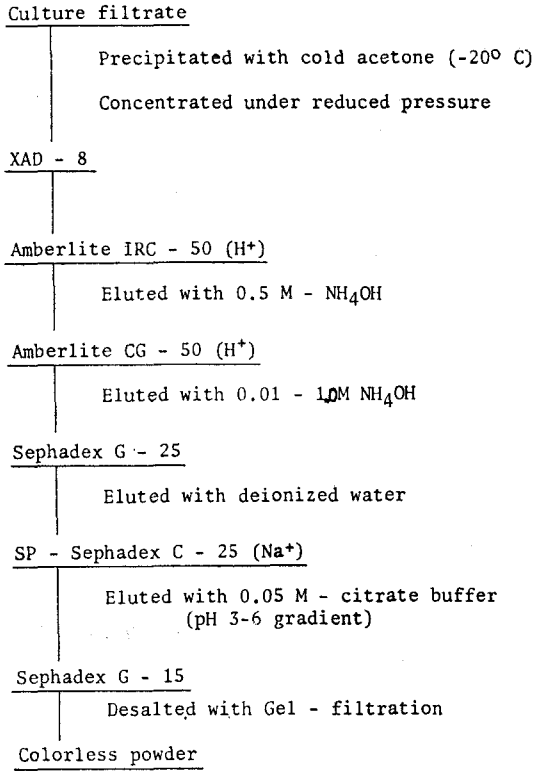

Fig. 2. Purification procedure.

1) Amberlite IRC-50 (H^+ form) Chromatography of the Amylase Inhibitor

The brown powder was dissolved in one liter of water and applied onto a column of Amberlite IRC-50.

The active eluate was combined and concentrated under reduced pressure and lyophilized to yield 10.5 g of yellow powder (5.4×10^5 I.U./total).

2) Amberlite CG-50 (H^+ form) Column Chromatography

The yellow powder was dissolved in 500 ml of water and applied onto a column of Amberlite

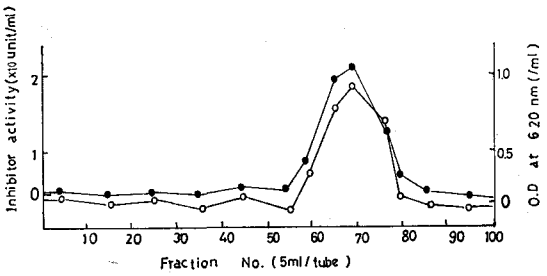

Fig. 3. Amberlite CG-50 column chromatography (H^+ form).
O—O : Anthrone test
●—● : Inhibitor activity

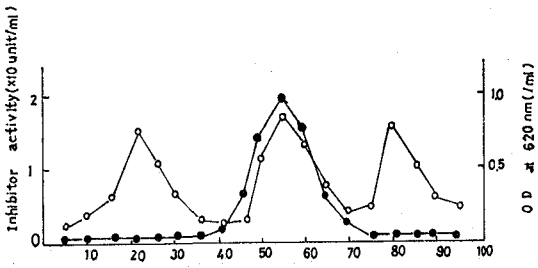

Fig. 4. Gel filtration on Sephadex G-25 column.
O—O : Anthrone test
●—● : Inhibitor activity

CG-50.

When the eluate was fractionated by column chromatography with Amberlite CG-50, the fractions between No. 65 and 75 had inhibitory activities and combined. They were positive to anthrone test. The results were shown in Fig. 3. The active eluate was combined and concentrated under reduced pressure and lyophilized to give 10 g of the inhibitor as pale yellow powder (5.2×10^5 I.U./total).

3) Gel Filtration on Sephadex G-25

The inhibitor (10 g, 5.2×10^5 I.U./total) obtained by Amberlite CG-50 was dissolved in 200 ml of water and applied onto a column of Sephadex G-25.

The water eluate fractions were monitored by the α-amylase inhibition assay and anthrone test. As shown in Fig. 4, there were 3 peaks which were positive to anthrone test. The fractions between No. 50 and 60 had inhibitory activities and were positive to anthrone test. The active fractions were collected and concentrated under reduced pressure and lyophilized to yield 8.5 g of the inhibitor as pale yellow powder (4.3×10^5 I.U./total).

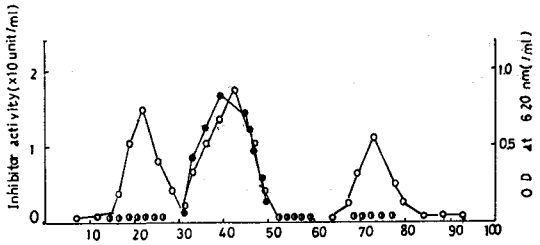

Fig. 5. SP-Sephadex C-25 (Na^+ form) column chromatography.
O—O : Anthrone test
●—● : Inhibitor activity
◑—◑ : Sample color

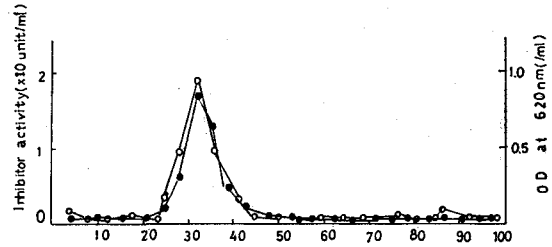

Fig. 6. Gel filtration on Sephadex G-15 column.
O—O : Anthrone test
●—● : Inhibitor activity

4) SP-Sephadex C-25 (Na^+ form) Column Chromatography

The inhibitor (4.3×10^5 I.U./total) obtained from Sephadex G-25 was applied onto a column of SP-Sephadex C-25. As shown in Fig. 5, There were 3 peaks which were positive to anthrone test. The fractions between No. 35 and 45 had inhibitory activities and were positive to anthrone test and were lyophilized to give colorless powder (2.8 g, 2.1×10^4 I.U/total).

5) Gel Filtration on Sephadex G-15

The inhibitor (2.8 g, 2.1×10^4 I.U./total), which was colorless powder was desalted by Sephadex G-15. The active eluate was collected

and concentrated under reduced pressure and lyophilized as colorless powder (0.5 g, 2.9×10^3 I.U./total). It was positive to anthrone test. The results were shown in Fig. 6.

Spectral Analysis

The physico-chemical properties of the inhibitor were summarized in Table II.

The inhibitor was soluble in water and acetonitrile but it was insoluble in other common orgnic solvents. The inhibitor was positive to phenol-sulfuric acid reaction, anthrone reaction and Somogyi-Nelson reaction.

From its IR spectrum, an absorption band for O-H stretching vibration was observed near 3300 cm^{-1}. The C-H and C-O stretching vibrations appeared in 2,900~2,950 cm^{-1} region and near 1,650 cm^{-1}, respectively. The band near 980~1,180 cm^{-1} was attributed to the C-H, C-O bending vibration. These absorption band showed the characteristics of oligosaccharidelike substances. Its infrared spectrum was shown in Fig. 7.

Table II. Some physico-chemical properties of the inhibitor.

Appearance	Colorless
UV Spectrum	End absorption
Color reactions	
Phenol-sulfuric acid	+
Anthrone reaction	+
Somogyi-Nelson reaction	+
Ninhydrin test	−

Soluble in pyridine, acetic acid, water and acetonitril.
Slightly soluble in acetone.
Insoluble in methanol, dimethyl sulfoxide and chloroform.

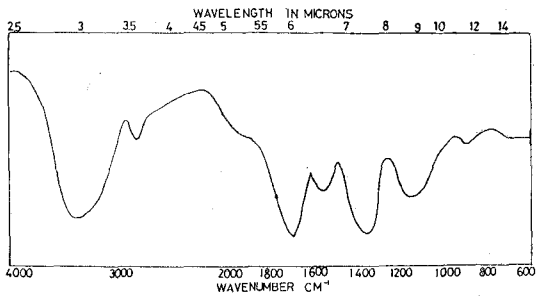

Fig. 7. IR spectrum of the inhibitor in KBr Disk.

Stability of the Amylase Inhibitor

The inhibitor was a very stable substance because it retained 100 % of its original inhibitory activity at 100°C for 30 minutes in the entire pH. range The results were shown in Fig. 8.

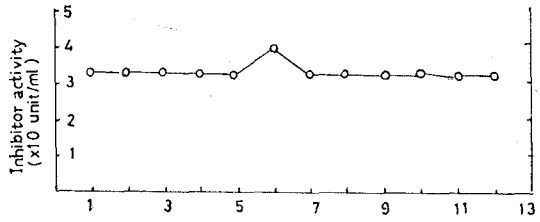

Fig. 8. Effects of pH and temperature on stability of the inhibitor.

Effects of the Amylase Inhibitor on Various Amylases

It was apparent from the data that five enzymes were inhibited remarkably, *e.g.* bacterial α-amylase, salivary α-amylase, pancreatic α-amylase, fungal α-amylase and glucoamylase. The resuts were shown in Table III.

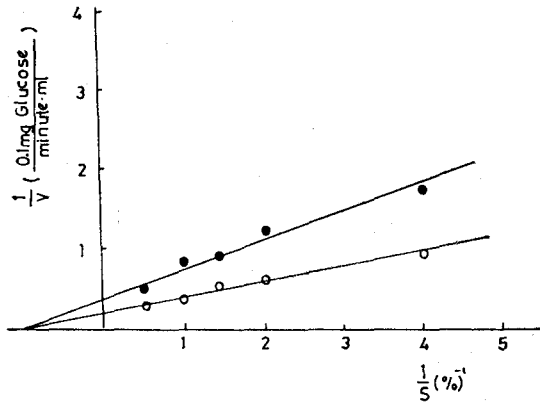

Fig. 9. Kinetic studies of inhibitor.
●—● : with inhibitor
○—○ : without inhibitor

Tabel III. Effects of the α-amylase inhibitor on various amylases.

Enzyme	Origin	Substrate	pH	Inhibition
α-Amylase	*Bacillus subtilis*	Starch	6.9	+
Salivary α-amylase	Human saliva	Starch	6.9	+
Pancreatic α-amylase	Porcine pancreas	Starch	6.9	+
Fungal α-amylase	*Aspergillus oryzae*	Starch	5.0	+
Glucoamylase	*Rhizopus* genus mold	Starch	5.5	+
α-Glucosidase	Brewer yeast	Maltose	6.0	−
β-Glucosidase	Almonds	Maltose	6.0	−
Dextranase	*Penicillium* species	Dextran	6.0	−
β-Amylase	Barley	Starch	5.5	−

Kinetic Studies

Kinetic studies were conducted on the effect of inhibitor on hydrolysis of soluble starch by α-amylase. The typical results were shown as a Lineweaver-Burk plots in Fig. 9. The inhibition of the inhibitor was a non-competitive type.

Discussion

In the course of our screening program for α-amylase inhibitors, strain DMC-72 was selected among 450 strains which were isolated from Korean soil samples. It had a very effective inhibitory activity against bacterial α-amylase. It was found that the strongest inhibitory activity of strain DMC-72 was shown by the four day culture broth.

The inhibitor was a strongly charged metabolite from the results of its behavior for ion-exchange resins and was adsorbed on the resins of cation ion-exchanger. To purify the inhibitor, acetone precipitation, adsorption on XAD-8, chromatography of Amberlite IRC-50, Amberlite CG-50 and SP-Sephadex C-25 were used. After it was purified by these methods, its colorless powder was obtained by lyophilization. The inhibitor was stable at various values of pH and temperature. The inhibitor had inhibitory activities on bacterial α-amylase, fungal α-amylase, salivary α-amylase, pancreatic α-amylase and glucoamylase, but did not inhibit β-amylase and β-glucosidase.

It appears that this amylase inhibitor showed inhibitory effects on amylase which could hydrolyze at α-1, 4-glucosidic linkages. The inhibitor showed an inhibition of the non-competitive type in the kinetic studies. The molecular weight of the inhibitor was estimated to be approximately 1,000 because it was eluted between the void volume of Sephadex G-10.

The IR spectrum of this compound showed strong absorption maxima at 3,200∼3,400 cm^{-1} and 980∼1,180 cm^{-1} attributable to its oligosaccharide nature.

From the results of spectra and anthrone test, the inhibitor of strain DMC-72 was found to be an oligosaccharide derivative.

Strain DMC-72 was postulated as a new *Streptomyces* species according to ISP method and Bergey's Manual of Determinative Bacteriology (Kim *et al.*, 1984). This strain also

produced a quinone derivative antibiotic, Soulomycin, whose structure elucidation is being studied.

And further studies are in progress to determine the complete chemical structure and physico-chemical characteristics of the inhibitor.

Conclusion

A metabolite which was produced by *Streptomyces* strain DMC-72 exhibited a potent inhibitory activity against α-amylase. It had strong inhibitory activities against bacterial α-amylase, fungal α-amylase, salivary α-amylase, pancreatic α-amylase and glucoamylase. The inhibitor was stable at relatively high temperature and at the range of pH 1~13. The kinetic studies of the inhibitor showed that the inhibitory effect on starch hydrolysis was non-competitive. This compound appears to be an oligosaccharide derivative on the basis of the results of spectral analysis and anthrone test.

Acknowledgments

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적 요

전국 각지 토양으로 부터 채취한 토양균을 screening 하여 α-amylase를 저해하는 물질을 생성하는 균주인 *Streptomyces* DMC-72를 선발하였다.

이 균주가 생성하는 저해제를 acetone 침전 및 이온교환 수지인 Amberlite IRC-50, Amberlite CG-50과 SP-Sephadex C-25를 이용하여 분리하였다. 이 물질은 α-amylase 뿐만아니라 다른 amylase와 기타 효소에 대해서도 억제작용을 나타내었다. 이 물질은 기질인 soluble starch의 가수분해에 대해서 non-competitive하게 저해작용을 보여주고 있으며 열 및 넓은 범위의 pH에서 안정한 물질이었다. 이 물질은 anthrone 시험과 IR spectrum에 의해서 oligosaccharide계 화합물임을 알 수 있었다.

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