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

Isolation and Activities of the Inhibitor of *Streptomyces* Strain DMC-225

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Abstract □ To find amylase inhibitors produced by microorganisms from soil, a strain which had a strong inhibitory activity against bacterial α-amylase was isolated from the soil sample collected in Seoul. The morphological and physiological characteristics of this strain on several media and its utilization of carbon sources showed that it was one of *Streptomyces* species according to the International Streptomyces Project method. The amylase inhibitor of this strain was purified by means of acetone precipitation, adsorption on Amberlite XAD-2, and column chromatography on Amberlite CG-50 and SP-Sephadex C-25. The inhibitor was stable at the pH range of 1~10 and at 100°C for half an hour, and had inhibitory activities against other amylases such as salivary α-amylase, pancreatic α-amylase, fungal α-amylase and glucoamylase. The kinetic studies of the inhibitor showed that its inhibitory effect on starch hydrolysis by α-amylase was non-competitive.

Keywords □ *Streptomyces*, α-Amylase inhibitor, Enzyme Kinetics, Fermentation, Oligosaccharide.

Since certain diseases are associated with excessive or unregulated enzyme activities, it was reasoned that enzyme inhibitors of microorganisms might exhibit valuable pharmacological activities.

Recently it was suggested that diabetes and obesity might be prevented by reducing the digestion of dietary starch by using inhibitors on amylases.

Therefore, the agents inhibiting amylases and its related enzymes might be useful for persons who should consume only restricted quantities of carbohydrates to avoid hyperglycemia and increased synthesis of triglycerides in adipose tissue, liver and the wall of intestine. That is, patients suffering from carbohydrate-dependent diseases such as diabetes, type IV hyperlipoproteinaemia and obesity might utilize drugs of this type.

Naturally occurring amylase inhibitors were demonstrated in a variety of biological materials. Whereas the amylase inhibitors obtained from various plants such as wheat, rye, sorghums and resting potato tubers were mostly of macromolecular nature, the amylase inhibitors that were found in microbial culture broths were of small molecular nature.

Since Wojirimycin[1] as inhibitor of α- and β-glucosidases and some amylases was first isolated from the culture broth of certain species of *Streptomyces* in 1970 by T. Niwa *et al.*, about ten of amylase inhibitors[2~9] were isolated from the microbial culture broths.

In the course of our screening program for amylase inhibitors, a strain was isolated from the soil samples collected in Seoul and designated as strain DMC-225. This strain had remarkable inhibitory activities against various amylases and related enzymes.

This paper reports the results of taxonomical studies on the strain DMC-225, production by fermentation, isolation of the inhibitor produced

by this strain and its inhibitory activities.

EXPERIMENTAL METHODS

Isolation of Microorganisms

One gram of soil sample was diluted with 10 ml of sterilized water in sterilized cap tubes. After the suspension was agitated, one ml of this suspension was diluted in the same way to a final dilution of 1,000 times. One ml of this suspension was smeared on the oatmeal agar plate (oatmeal 20 g, yeast extract 1 g, distilled water 1,000 ml) with sterile glass rod and incubated for three days at 27±1°C. The colonies on the oatmeal agar plate were transferred aseptically into a new oatmeal agar plate and further incubated for three days at 27±1°C. Then, the colonies of *Actinomycets*[10] were selected and finally transferred into an oatmeal agar slant and screened for inhibitory activities against α-amylase.

Assay Systems for Amylase Inhibitory Activity

Amylase inhibitory activity was assayed by measuring the residual α-amylase activity after α-amylase was incubated with the inhibitor. α-Amylase activity was examined by the modified blue value method.

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 preincubated at 37 °C for 5 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 5 minutes on a vigorously boiling water bath, then the residual amylase activity was assayed by the modified blue value method. That is, 5 ml of 0.5 mM Lugol solution was added to 0.1 ml of each reaction mixture, 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 and the culture broth was incubated in parallel with the test sample. The percent inhibition of amylase activity was calculated from the following equation:

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

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 original activity.

Taxonomical Studies of Strain DMC-225

Strain DMC-225 was grown in test tubes. The morphological and physiological characteristics on various media were noted and taxonomically classified according to International Streptomyces Project methods[11] and Bergey's Manual of Determinative Bacteriology[12]. Examination of carbon utilization was made according to the method of Pridham *et al*[13].

Fermentation

A stock culture of strain DMC-225 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 transferred into 500 ml of the production culture medium in a two-liter flask and shake culture was carried for five days at 27±1 °C. The composition of the seed and production medium was 2 % oatmeal medium.

Time Course of the Amylase Inhibitor Production

A stock culture of strain DMC-225 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 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 per day for seven days. Inhibitory activity against α-amylase was determined by modified blue value method and expressed by the inhibition unit (I.U.) per ml.

Assay System of Effect of the Amylase Inhibitor on Various Enzymes

A half ml of each solution was incubated with 0.5 ml of the amylase inhibitor at 37°C for 10 minutes and the residual enzyme activity was assayed. The activities of α-amylases were assayed by the modified blue value method. The activities of other amylases were assayed by measuring the amounts of reducing sugar released according to Somogyi-Nelson method. The substrates used for α-, β-amylase and glucoamylase were soluble starch, and the substrates for α-, β-glucosidase were maltose, and the substrate for dextranase was dextran. Each reaction was performed at the optimum pH of each enzyme.

Stability of the Amylase Inhibitor

A half ml of each enzyme solution was incubated with 0.5 ml of the amylase inhibitor at 37°C for 10 minutes and the residual enzyme activity was assayed. The substrates used were soluble starch.

Inhibitory ratings used were as follows:

+, 80~100% : −, 0% inhibition of enzyme activity.

Kinetic Studies of the Amylase Inhibitor

Kinetic studies have been conducted to observe effects of inhibitor on hydrolysis of soluble starch by α-amylase.

The concentration of the substrate (soluble starch) was changed from 0.25 % to 2 % and its effect on initial velocity (V_0) were determined at a fixed concentration of the inhibitor. Then a Lineweaver-Burk plot of $1/V_0$ vs. $1/[S]$ was prepared.

Purification of the Amylase Inhibitor

The culture broth (15 liters) of the strain DMC-225 was centrifugated at 10,000 rpm for 15 minutes to remove cells. 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 15 minutes and the supernatant was concentrated to dryness under reduced pressure at 50°C and dissolved in 500 ml of deionized water. The aqueous solution was applied to a column of Amberlite XAD-2 (Rhom and Hass Co.), and the column was successively washed with deionized water (X10) and then eluted with 60% MeOH. The active eluate was evaporated to dryness. This dry material was dissolved in 50 ml of distilled water. This inhibitor solution was used for a series of chromatography. A 50 ml of the inhibitor solution eluted from the Amberlite XAD-2 was adsorbed on a column of Amberlite CG-50 type II (H^+ form, 2.5×40 cm). The column was developed with about 2,000 ml of deionized water, and then eluted with 1,000 ml of 0.5N-NH_4OH. The eluates were obtained as a dark brown colored materials. This crude inhibitors were rechromatographed on a column of Amberlite CG-50(H^+), 2×50 cm. The column was washed with about 1,000 ml of deionized water, and then eluted with a linear gradient obtained with 1,000 ml of 0.01 N-NH_4OH and 1,000 ml of 0.5 N-NH_4OH. The elution was performed at a flow rate of 1 ml/min and collected into each fraction of 10 ml. The active fraction was obtained as a yellow colored materials.

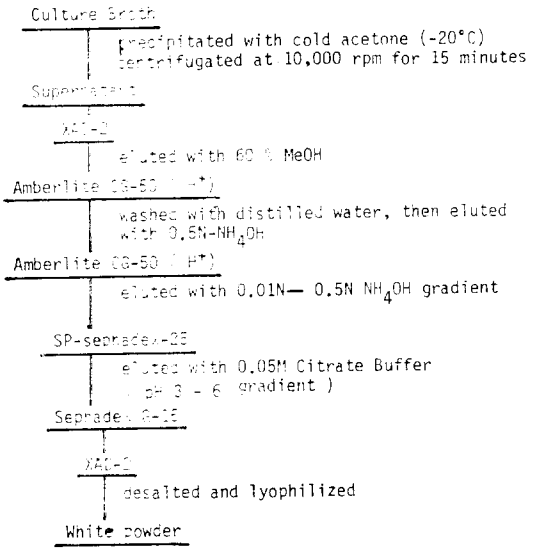

Fig. 1. Purification procedure of α-amylase inhibitor.

The eluted fraction from the column of Amberlite CG-50 were collected and concentrated to 3 ml, and then applied on the top of the SP-Sephadex C-25 column (3×60 cm) equilibrated with 0.05 M citrate buffer (pH 3).

The elution was carried out with 0.05 M citrate buffer(pH 3∼pH 6 gradient) at a flow rate of 0.5 ml/min and fractions of 10 ml were collected.

The fractions were applied to a column (2×60 cm) of Sephadex G-15 equilibrated with 0.01 M phosphate buffer (pH 6.8). The elution was carried out with starting buffer at a flow rate of 0.5 ml/min and collected into each fraction of 5 ml.

The active fraction from the Sephadex G-15 column was applied to a XAD-2 column and then washed with deionized water to eliminate salts. Adsorbed inhibitor was eluted with 60% MeOH and lyophilized to white powder. The schematic purification procedure of amylase inhibitor was outlined in Fig. 1.

Homogeneity of Amylase Inhibitor

The homogeneity of this amylase inhibitor preparation was examined by means of TLC using a Kiesel gel 60 plate (Merck Co) at room temperature in the following solvent system; A: n-butanol: pyridine: water=6 : 4 : 3, B: ethyl acetate: methanol: water=2 : 2 : 1, C: n-propanol: ethyl acetate: water=6 : 1 : 5.

Amylase inhibitor was detected by anisaldehyde-sulfuric acid and gave a single spot in all solvent systems, Rf values were 0.31(A), 0.12 (B) and 0.15(C). These results suggested that this amylase inhibitor was homogeneous.

Spectral Analysis

Physico-chemical properties of the purified α-amylase inhibitor of strain DMC-225 were examined by spectroscopic methods.

The conditions used for spectroscopy were as follows: UV spectrum; Hitachi Model ESP-35 (solvent: distilled water), IR spetrum; Beckman IR-20A (KBr disk), NMR spetrum; Varian FT-80A (solvent: D_2O).

RESULTS AND DISCUSSION

Taxonomical Studies of Strain DMC-225

Figures 2 and 3 showed that whole cell hydrolyzates of strain DMC-225 contained LL-DAP and no arabinose, galactose and xylose. This fact indicated that this strain was cell wall type 1 described in Bergey's Manual of Determinative Bacteriology and International Streptomyces Project method.[11] The cultural characteristic of strain DMC-225 on various media was shown in Table I. This strain grew very well on the glucose containing medium and ISP medium except yeast extract-malt extract agar (ISP No.2 medium) and produced abundant aerial mycelia. The color of spore was gray and the color of substrate mycelium was gray and slightly yellowish gray. Soluble pigment was not

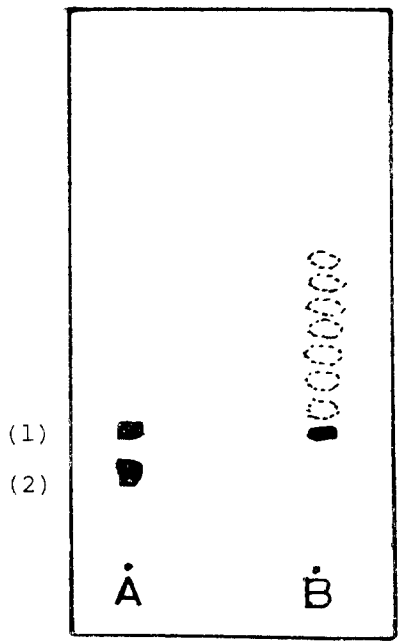

Fig. 2. Separation of DAP isomers by TLC.
(A) Standard DAP
(B) Hydrolyzates of strain DMC-225
(1) LL-DAP
(2) meso-DAP

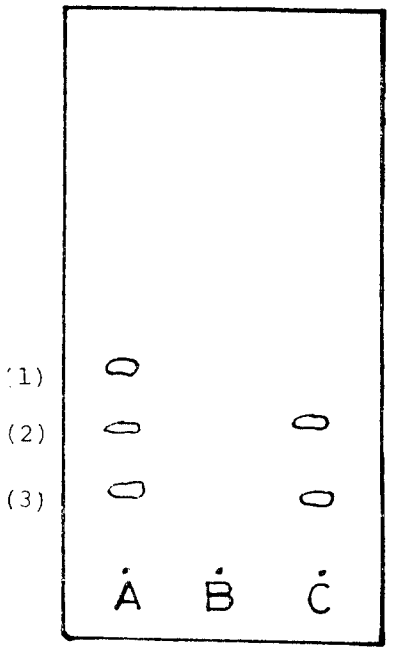

Fig. 3. Separation of monosaccharides in whole cell hydrolyzates.
(A) Standard mixture
(B) Strain DMC-225
(C) *Mycobacterium smegmatis*
(1) Xylose (2) Arabinose (3) Galactose

Table I. Cultural characteristics of strain DMC-225

Medium	Growth	Reverse phase	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No.2 midium)	no growth	none	none	none
Oatmeal agar (ISP No.3 medium)	good	gray	gray	none
Inorganic salts starch agar (ISP No.4 medium)	good	yellowish gray	yellowish gray	none
Glycerol-asparagine agar (ISP No.5 medium)	moderate	gray	gray	none
Glucose-asparagine agar	good	gray	gray	none
Glucose-peptone agar	good	whitish gray	whitish gray	none
Glucose nitrate agar	good	whitish gray	whitish gray	none

produced.

Figure 4 showed that aerial mycelium including the spore chain was spiral form. An electron micrograph of the spore showed oval with a smooth surface in Fig. 5.

The result of starch hydrolysis was positive and the melanin pigment was not formed. The results of the utilization of carbon sources were shown in Table II.

From these results, strain DMC-225 was ascertained to be one of *Streptomyces* species by ISP[11] method and Bergey's Manual of Determinative Bacteriology[12].

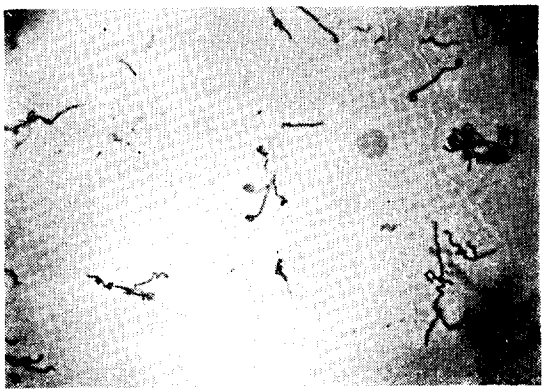

Fig. 4. Spore chains of strain DMC-225(X600)

Fig. 5. An electron micrograph of the spore of strain DMC-225.

Time Course of the Amylase Inhibitor Production

The results were outlined in Fig. 6. The variation of pH was not significant, and the

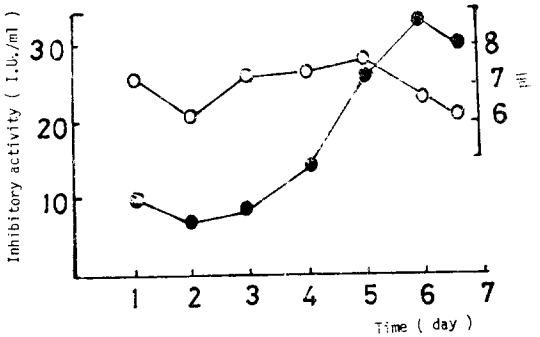

Fig. 6. Time course of the production of the α-amylase inhibitor by strain DMC-225.
●—● : Inhibitory activity
○—○ : pH

Table II. Utilization of carbon sources by strain DMC-225.

Carbon sources	Utilization
D-Glucose	+
D-Xylose	+
L-Arabinose	+
L-Rhamnose	−
D-Fructose	+
Cellulose	−
Raffinose	+
D-Mannitol	+
Inositol	+
Salicin	+
Sucrose	+

(+ : Utilized, − : Not utilized)

Table 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	Porcine pancreas	Starch	5.0	+
Glucoamylase	Rhizopus genus mold	Starch	5.5	+
α-Glucosidase	Brewers yeast	Maltose	6.0	−
β-Glucosidase	Almond	Maltose	6.0	−
Dextranase	Penicillium species	Dextran	6.0	−
β-Amylase	Barley	Starch	5.5	−

Inhibitory ratings used were as follows : +, 80~100% inhibition of enzyme activity
−, 0% inhibition of enzyme activity

inhibitory activity against α-amylase reached maximum at six days after the inoculation.

Effects of the Amylase Inhibitor on Various Enzymes

The results were given in Table III. The data showed that five enzymes including bacterial α-amylase, fungal α-amylase, pancreatic α-amylase, salivary α-amylase and glucoamylase were inhibited remarkably, and that α-, β-glucosidase, β-amylase and dextranase were not inhibited.

Stability of the Amylase Inhibitor

As shown in Figure 7, amylase inhibitor was stable at the pH range of 1~10 and at 100° C for half an hour, but it showed loss of its activity in strong alkaline condition.

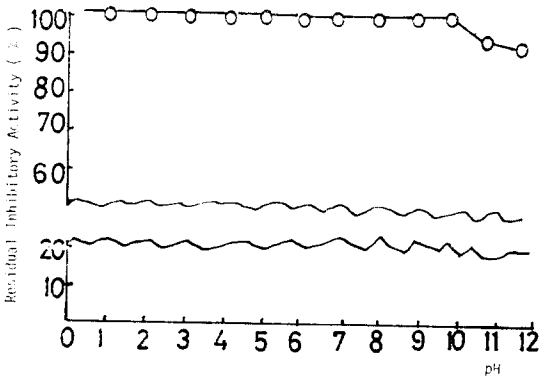

Fig. 7. Effects of pH and temperature on stability of the inhibitor.

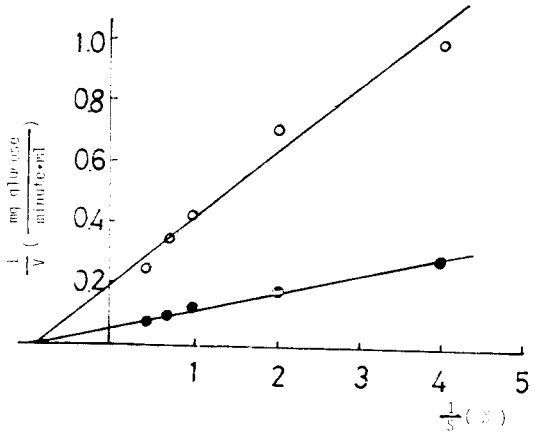

Fig. 8. Kinetic studies of the α-amylase inhibitor of strain DMC-225.
●—● : without inhibitor
○—○ : with inhibitor

Kinetic Studies of the Amylase Inhibitor

Typical result is shown as Lineweaver-Burk plots in Figure 8. The inhibition is the noncompetitvie type.

Spectral Analysis

To purify the inhibitor, acetone precipitation, adsorption on XAD-2 column, chromatography of Amberlite CG-50 and SP-Sephadex C-25 were used. After it was purified by these methods, a white powder was obtained by lyophilization.

UV, IR and NMR spectra of the α-amylase inhibitor of strain DMC-225 were shown in Figure 9, 10 and 11, respectively. The UV spectrum showed end absorption. From the IR spectrum, Absorption band for O—H stretching vibration was observed near 3300 cm^{-1}. The C—H and C—O stretching vibrations appeared in 2900~2950 cm^{-1} region and near 1650 cm^{-1}

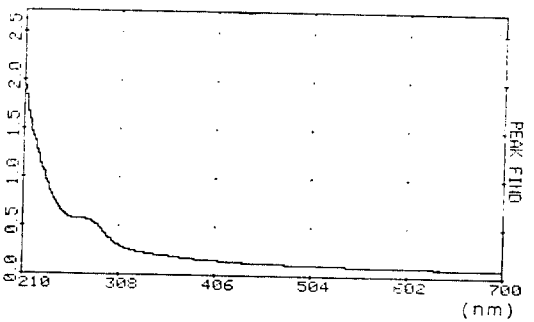

Fig. 9. UV absorption spectrum of amylase inhibitor of strain DMC-225 in H_2O.

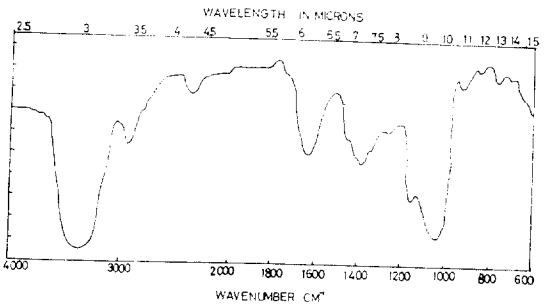

Fig. 10. IR absorption spectrum of amylase inhibitor of strain DMC-225 in KBr.

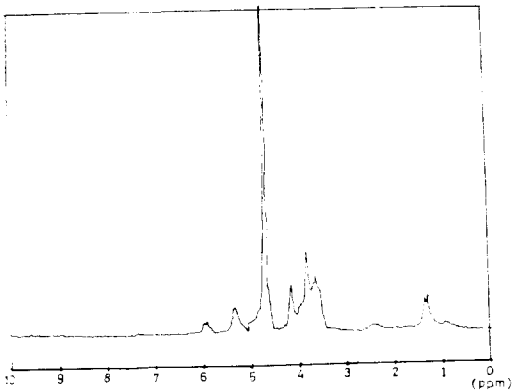

Fig. 11. NMR spectrum of amylase inhibitor of strain DMC-225.

respectively.

The band near 980~1180 cm^{-1} was attributed to the C—H, C—O bending vibration. These absorption bands showed the characteristic of oligosaccharide-like substances.

The NMR spectrum showed $CH_3—\underset{|}{CH}—$ signal at 1.38 ppm in D_2O from external TMS, $—\underset{|}{CH}—OH$ signals at around 3.6~4.2 ppm, $—O—\underset{|}{CH}—O—$ signals at around 5.2~5.4 ppm and olefinic protons at 5.9~6.0 ppm.

From the results of these spectra and anthrone test, the inhibitor of strain DMC-225 was found to be an oligosaccharide derivative.

CONCLUSION

A strain was found to produce an amylase inhibitor in its culture broth and named strain DMC-225. It was indentified as one of the genus *Streptomyces*.

The metabolite had inhibitory activities against bacterial α-amylase, fungal α-amylase, pancreatic α-amylase, salivary α-amylase and glucoamylase, and showed inhibition of the non-competitive type in the kinetic studies. This amylase inhibitor was considered to be an oligosaccharide derivative by the IR, NMR spectra and the anthrone test.

ACKNOWLEDGMENT

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