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INHIBITION OF *STREPTOCOCCUS MUTANS* GLUCOSYLTRANSFERASE BY M-GTFI, A NEW INHIBITOR

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Two hundred strains of soil microorganisms were screened for the production of inhibitors of the glucosyltransferase activity of *Streptococcus mutans* strain, Kl-R. The strain producing the greatest amount of inhibitor was one recently isolated in our laboratory. It has now been identified as a strain of *Micromonospora narashinoensis* on the basis of morphological and physiological studies.

The inhibitor, M-GTFI, affects the glucosyltransferase that produces the water-insoluble glucan rather than that which produces the water-soluble glucan. Fuchsin-sulphite staining of the inhibitor after its purification by polyacrylamide gel electrophoresis indicates that it is probably an acidic substance. It had M_r 5700 as was determined by gel filtration.

From an examination of the effects of this inhibitor on representative strains of *S. mutans* other than Kl-R, there is a suggestion of a similar selectivity for the water-insoluble glucan-forming activity in other strains.

KEY WORDS: Glucosyltransferase inhibitor, inhibitor, M-GTFI, *Streptococcus mutans*, cariogenic glucan.

INTRODUCTION

Streptococcus mutans, a cariogenic bacterium, has several properties that make it especially virulent as a promoter of tooth decay. It contains glucosyltransferases that can form polymeric glucans from the glucose moiety of sucrose. This highly adhesive, extracellular polysaccharide enables *S. mutans* cells to cling to one another and also to accumulate on teeth, especially on smooth enamel surfaces.[1,2] In addition, *S. mutans* forms lactic acid readily and this, in turn, helps demineralize tooth enamel.[3] It readily forms colonies on tooth surfaces,[4] and it is a major component of plaque associated with dental caries.

The prospect of preventing dental caries had led to the search for inhibitors of the glucosyltransferase of *S. mutans*. Okami *et al.* isolated a specific glucosyltransferase inhibitor, ribocitrin, from a culture filtrate of a strain of *Streptomyces neyagawaensis*[5,6]; and Endo *et al.*[7] isolated an inhibitor, mutastain, from a culture filtrate of *Aspergillus terreus* M3328. Felgenhauer *et al.* reported the inhibition of the *S. mutans* glucosyltransferase by an α-glucosidase inhibitor.[8] The inhibitor reported here is different from these previously reported inhibitors (M. Uyeda, K. Suzuki, S. Yamane, Y. Sato and M. Shibata, Annu. Meeting Agric. Chem. Soc. Japan, 1982, Abstract 2M-5, p. 424). This paper reports the isolation and partial characterization of this new

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inhibitor and also the identification of the inhibitor-producing strain, based on its microbiological characteristics.

MATERIALS AND METHODS

Bacterial strains

Human cariogenic *Streptococcus mutans*, strain Kl-R (serotype g) was routinely maintained in brain heart infusion bouillon agar stab cultures (BHIB, Nissui Seiyaku Co., Tokyo) at 4°C and transferred weekly. This strain and the other strains used, HS-1 (serotype a), Ingbritt (serotype c), OMZ-176 (serotype d), and LM-7 (serotype f) were kindly supplied by Dr T. Morioka of the School of Dentistry, Kyushu University.

Growth media

BHIB supplemented with D-glucose (1.8%) was sterilized by autoclaving for 15 min at 121°C. *S. mutans* was routinely cultured anaerobically for 16 to 18 h at 37°C in a candle jar. Chemically defined medium was prepared according to Janda and Kuramitsu.[9] Tween 80 (1 mg/ml medium) was added to improve the bacterial growth.[10]

Purification of the glucosyltransferase

Purification of the glucosyltransferase (GTF) produced by *S. mutans* strain, Kl-R was carried out partly according to the method of Fukushima *et al.*[11] After 16 h of growth in the chemically defined medium at 37°C, the culture was filtered, and the filtrate was chilled to 0°C. An equal volume of cold (−25°C) absolute ethanol was slowly added, and the mixture was allowed to stand for 60 min at 0°C. The precipitate was collected by centrifugation for 10 min at 14 000 × g, and dissolved in triethanolamine (5 mM) and dialyzed against potassium phosphate buffer (0.02 M), pH 6.5.

This crude glucosyltransferase fraction was applied to a hydroxylapatite column (1.7 × 25 cm) equilibrated with potassium phosphate buffer (20 mM), pH 6.5. After the column was washed with this buffer, the sorbed enzyme was eluted with 200 ml of a linear gradient of potassium phosphate buffer (20–500 mM). The soluble GTF (S-GTF)-forming water soluble glucan (WSG) and the insoluble GTF (I-GTF)-forming water insoluble glucan (WIG) eluted in two peaks. Both enzymes were dialyzed against potassium phosphate buffer (0.01 M), pH 6.0, respectively and further purified (about two-fold based on protein) on hydroxyl apatite or on Sephadex G-100 and used as reference samples.

Thin layer chromatography[12] on silica gel was used to determine the sugars produced during enzyme assay. The only spots appearing were those due to fructose, glucan, and residual sucrose. The absence of glucose showed that there was no contamination of the samples by invertases or fructosyltransferases.

Assay for the glucosyltransferase inhibitor

The reaction mixture consisting of potassium phosphate buffer (1 ml, 0.2 M), pH 6.0, glucosyltransferase from *Streptococcus mutans* (1 ml), distilled water (1 ml) with or without inhibitor and sucrose (1 ml, 0.2 M) was incubated at 37°C for 30 min. After

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incubation, sodium hydroxide (1 ml, 0.2 M) was added to terminate the reaction. The reducing sugar (fructose) released was determined by the method of Somogyi–Nelson[13] and the resulting absorbance at λ 600 nm was used to measure the enzyme activity. The enzyme activity level was adjusted to give an absorbance of 0.4 to 0.5 (40 ~ 50 μg as reducing sugar) at λ 600 nm as determined by the Somogyi–Nelson method using 1 ml of the mixture. One inhibitor unit was defined as that amount which caused 50% inhibition by being extrapolated from one or more measurements.

Inhibition of the water insoluble and water soluble glucan-forming activities of the enzyme was determined turbidimetrically. An aliquot (1 ml) of the assay mixture, containing sodium azide (0.02%) as a preservative, was transferred to a test tube (1.2 × 10 cm) and incubated overnight at 37°C with the tube lying 30° from the horizontal. The water insoluble glucan that adhered to the glass wall and precipitated at the bottom of the tube was suspended with a glass rod, and quantitatively determined from the absorbance at λ 550 nm. The insoluble material was removed by centrifugation at 3000 × g for 10 min. Three volumes of ethanol (0°C) were added and the mixture kept at 4°C overnight. The absorbance at λ 550 nm was then used to measure the amount of the water soluble glucan formed.

Sucrose-agar plate method for the detection of the inhibition zone after polyacrylamide gel electrophoresis (PAGE)

A mixture of potassium phosphate buffer (30 ml, 0.2 M), pH 6.0, sucrose (30 ml, 0.5 M), water (40 ml) and agar (0.72 g) were mixed and autoclaved. After autoclaving, this liquid was kept in a water bath (50°C) and sodium azide solution (1.2 ml, 2%) was added. Aliquots (5 ml) were added to plates already containing the glucosyltransferase solution (1 ml). The polyacrylamide gel after electrophoresis was sliced longitudinally and half of the gel placed on the solidified agar, and the plate kept at 4°C for 30 min to allow some diffusion of the inhibitor into the agar gel. The plate was then incubated at 37°C for one day. The clear zone caused by the presence of the inhibitor was clearly seen against the turbid background caused by the formation of the water insoluble glucan.

PAGE of the inhibitor was carried out at pH 8.0 for 60 min with a constant current of 3 mA per tube. Fuchsin-sulphite staining of the gels was performed using the periodic acid-Schiff base technique of Zacharius *et al.*,[14] which was originally used for the detection of glycoprotein after gel electrophoresis.

Cultivation of the microorganisms used for screening

Microorganisms that had been isolated here from soils were inoculated into 200 ml Erlenmeyer flasks containing glycerin S media (50 ml), pH 7, consisting of glycerin (2%), potato starch (3%), peptone (0.5%), corn steep liquor (1%), soybean flour (1%), NaCl (0.3%) and $CaCO_3$ (0.3%). These cultures were grown aerobically at 28°C for several days on a rotary shaker (180 rpm). The broths were then filtered through Whatman No. 2 filter paper, the filtrates dialyzed against water and then assayed for inhibitory activity.

Taxonomic studies of strain No. 731, an inhibitor producer

Most procedures followed those in the International *Streptomyces* Project (ISP)[15] and Waksman's methods.[16] Morphological characteristics and physiological features of

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the strain on various media were observed, and the strain was classified according to Bergey's Manual of Determinative Bacteriology[17] and ISP descriptions.

A determination of carbon utilization was performed according to the method of Pridham[18] and the method of Luedemann.[19] Confirmation of cell wall type was carried out according to the method of Yamaguchi[20] and the method of Becker.[21]

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RESULTS AND DISCUSSION

Results of the screening

Four strains of actinomycetes producing glucosyltransferase inhibitors were obtained from about 200 actinomycetes studied. Strain No. 731, the best producer of the inhibitor, was chosen for further studies. The cultural and physiological characteristics of strain No. 731 are summarized in Table I. Comparison of these properties with those of known species[17] indicated that strain No. 731 resembled *Micromonospora narashinoensis*. The strain, therefore, was named as *Micromonospora narashinoensis* strain No. 731, and the glucosyltransferase inhibitor produced by this strain is now designated as M-GTFI.

Cultural conditions and time course of M-GTFI production by strain No. 731

The best medium for inhibitor production was found to be: glucose (5%), a mixture of peptone (0.5%), K_2HPO_4 (0.1%), NaCl (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), and the following trace salts (mg/l of medium): $Fe_2(SO_4)_3 \cdot 6H_2O$ (10), $CuSO_4 \cdot 5H_2O$ (1), $ZnSO_4 \cdot 7H_2O$ (1) and $MnSO_4 \cdot H_2O$ (1), maintained at 28°C and initially at pH 7.0.

The production of the inhibitor, along with the growth of the mycelium, the pH of the culture, and the consumption of glucose was followed over a 5 day period (Figure 1). The amount of inhibitor reached a maximum after 3 days, and this period was used for subsequent production of the inhibitor.

The absorbance at λ 483 nm was used to determine the amount of polysaccharide in a dialysate of the culture filtrate using the phenol-sulfuric acid method.[22] Inhibitor activity almost corresponded to the accumulation of the polysaccharides.

TABLE I
Cultural and physiological characteristics of strain No. 731

No true aerial mycelium.
Spores spherical or oval, either sessile or often on short sporophores, usually single. Spore wall ornamentation, smooth.
Colonies on yeast extract-malt extract agar producing abundant, folded growth of a deep orange color, later covered with a sparse dark brown spore layer. No soluble pigment produced.
Colonies on nutrient agar scant. Colonies on tyrosine agar (Shinobu's) scant, those on tyrosine-yeast extract agar abundant, some orange patches.
Colonies on Czapek's sucrose agar abundant, orange in color, later covered with black patches.
Milk liquefaction and coagulation, positive. Starch hydrolysis, positive. Nitrate reduction, negative.
Cell wall type, type II
Carbon utilization, positive on galactose, D-glucose, lactose, levulose, D-mannose, D-sucrose, L-rhamnose, starch, α-melibiose. Negative on L-arabinose, D-mannitol, inositol, raffinose, D-xylose, D-sorbitol, glycerol.

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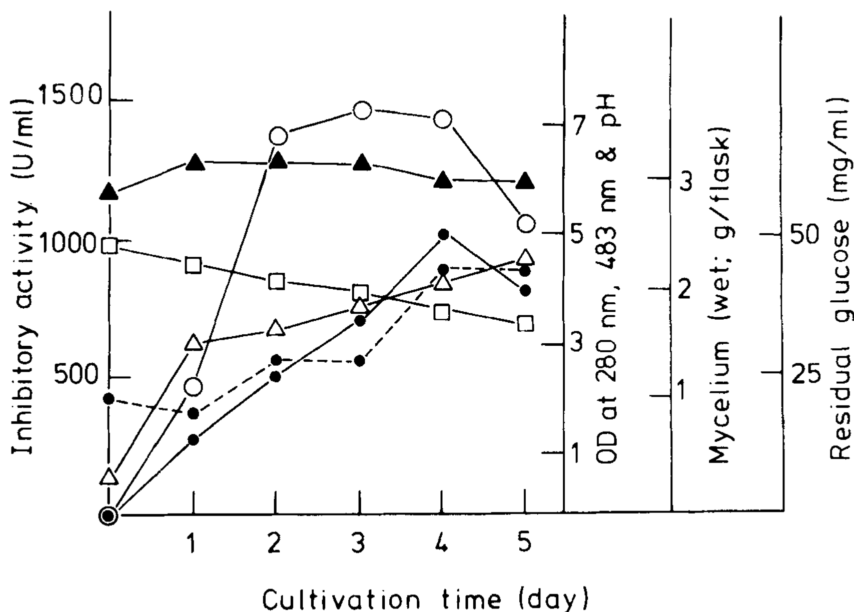

FIGURE 1 Time course of various criteria in the cultivation of *Micromonospora narashinoensis* strain No. 731. —o—, Inhibitory activity (U/ml); --●--, $A_{280\,nm}$; —△—, mycelium (wet; g/flask); —●—, $A_{483\,nm}$ (polysaccharide by the phenol-sulfuric acid method); —▲—, pH; —□—, residual glucose (mg/ml).

Partial purification procedure for M-GTFI

After cultivation, the culture was filtered through filter paper and to the clear filtrate was added solid ammonium sulphate (60% saturation). After standing overnight at 5°C, the precipitate was collected by centrifugation, dissolved in a minimal volume of potassium phosphate buffer (0.05 M), pH 5.5, and then dialyzed against the same buffer to remove ammonium sulphate. The dialysate was applied to a hydroxylapatite column (1.9 × 6.8 cm) equilibrated with the phosphate buffer. M-GTFI was eluted using a linear gradient from potassium phosphate buffer (0.05 M), pH 5.5, to dibasic ammonium phosphate (0.5 M), pH 8.2. The active fractions were combined and dialyzed against water to remove any salt, and then lyophilized. The preparation obtained was dissolved in a minimal volume of the dibasic ammonium phosphate (0.05 M) and the solution was applied to a Sephadex G-75 column previously equilibrated with this buffer and eluted with the same buffer. As shown in Figure 2, two peaks with inhibitory activity appeared, but the first peak eluted near the void volume of the column and contained impurities. The second peak was used for further purification. The fractions (30–36) were combined, and then subjected to gel filtration as described above. A typical elution pattern is shown in Figure 3. The active fractions (30–36) were combined, dialyzed against water to remove any salt, and lyophilized. M-GTFI thus obtained was subjected to PAGE at pH 8.0. After electrophoresis, the gel was placed on a sucrose-agar plate. As shown in Figure 4, the clear zone indicating the presence of the inhibitor was centered around a fuchsin-sulphite staining band that had migrated with bromophenol blue towards the anode. No protein could be detected in the gel using amidoblack 10B for staining. From these results, M-GTFI

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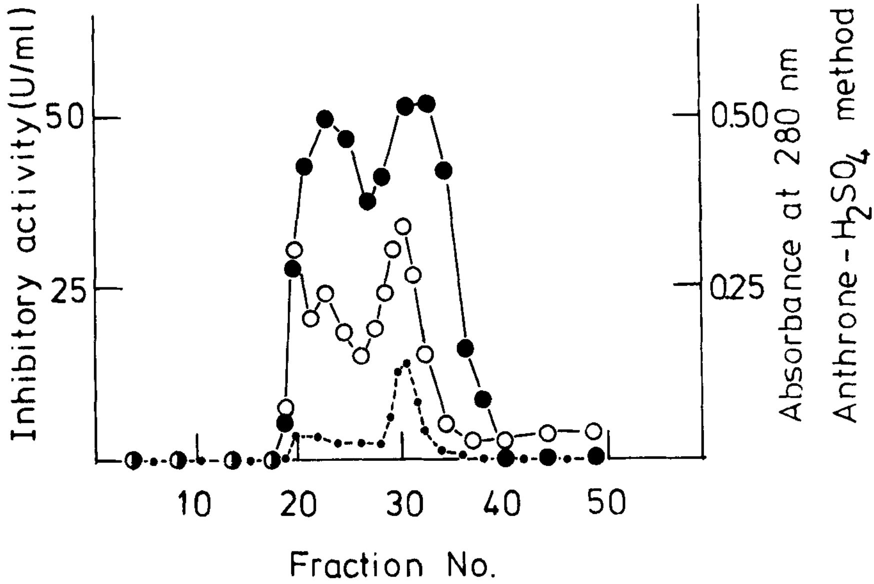

FIGURE 2 Column chromatography of active M-GTFI fraction on Sephadex G-75. Eluate, dibasic ammonium phosphate (0.05 M), pH 8.2; column size, 1.9 × 92 cm; fraction size, 5 ml; flow rate, 10 ml/h; —●—, inhibitory activity (U/ml); —○—, $A_{280\,nm}$; --●--, $A_{620\,nm}$ (anthrone-H_2SO_4 method).

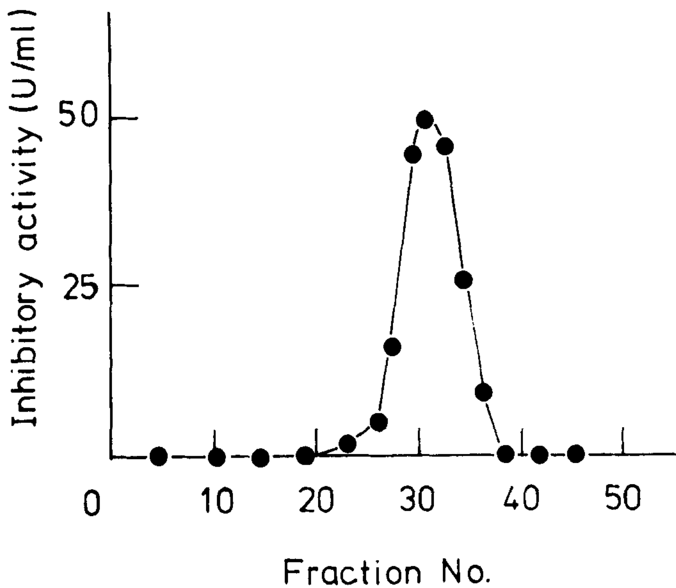

FIGURE 3 Refiltration of M-GTFI on Sephadex G-75.

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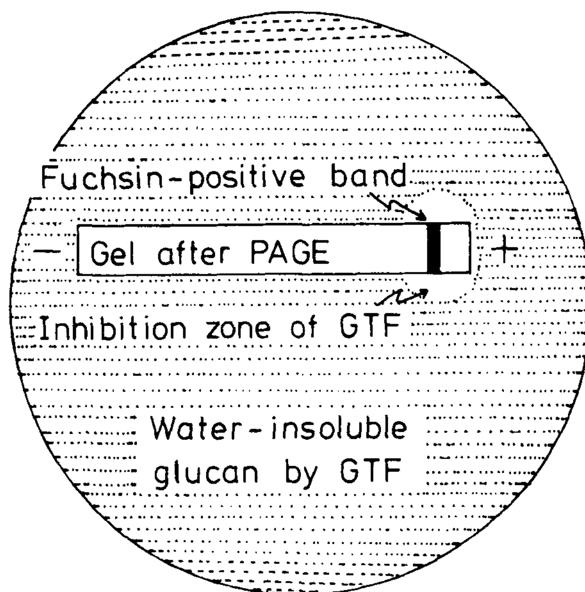

FIGURE 4 Inhibition zone of GTF on petri-dish and fuchsin-positive band on the gel after PAGE of M-GTFI at pH 8.0.

was considered to be an acidic substance. Further purification of the inhibitor is now in progress.

Molecular weight

The molecular weight of M-GTFI was estimated by the method of Andrews[23] using a Sephadex G-75 column, and plotting the ratio of elution volume to void volume against the logarithm of molecular weight for each reference polymer and M-GTFI (see Figure 5). An approximate molecular weight of 5700 was obtained for M-GTFI.

The effect of the inhibitor on the water insoluble and the water soluble glucan-forming activities

Glucosyltransferase has been reported to consist of a two enzyme system that forms either a water soluble–or a water insoluble glucan[23]. The inhibitor was tested with approximately equal amounts of the two enzymes that were obtained by hydroxyl-apatite chromatography. The inhibitor strongly repressed the formation of the water insoluble glucan but had little effect on the formation of the water soluble glucan (see Figure 6). (See M. Uyeda, S. Yamane, A. Yoshida, M. Imai, and M. Shibata, Annu. Meeting Agric. Chem. Soc. Japan, 1983, Abstract 2M-26, p. 213).

Type of inhibition

The type of inhibition was determined by a Lineweaver-Burk plot of substrate (sucrose) concentration against rate of hydrolysis by I-GTF in the presence and

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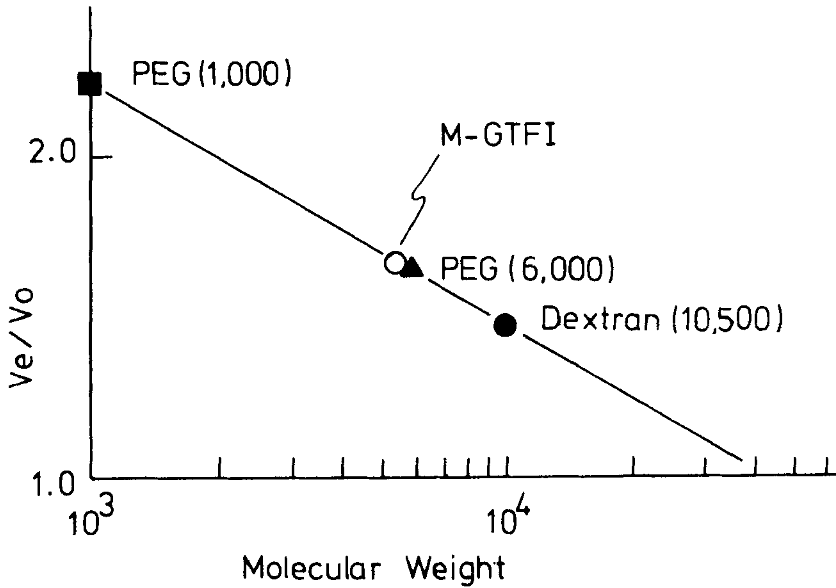

FIGURE 5 Plots of Ve/Vo, against log. of molecular weight for M-GTFI and authentic samples on Sephadex G-75. ■, Polyethylene glycol # 1000 (M_r, 1000); ▲, polyethylene glycol # 6000 (M_r, 6000); ●, dextran (M_r, 10,500); ○, M-GTFI.

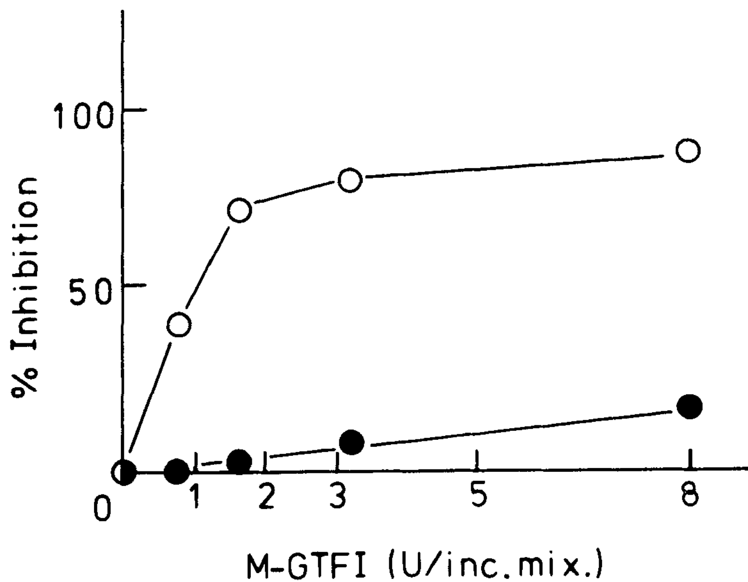

FIGURE 6 Effects of M-GTFI on S-GTF and I-GTF from *S. mutans* K1-R. —○—, I-GTF; —●—, S-GTF. Enzyme activity was adjusted to give 1.5 μg/min/ml of reducing sugar liberated at 37°C.

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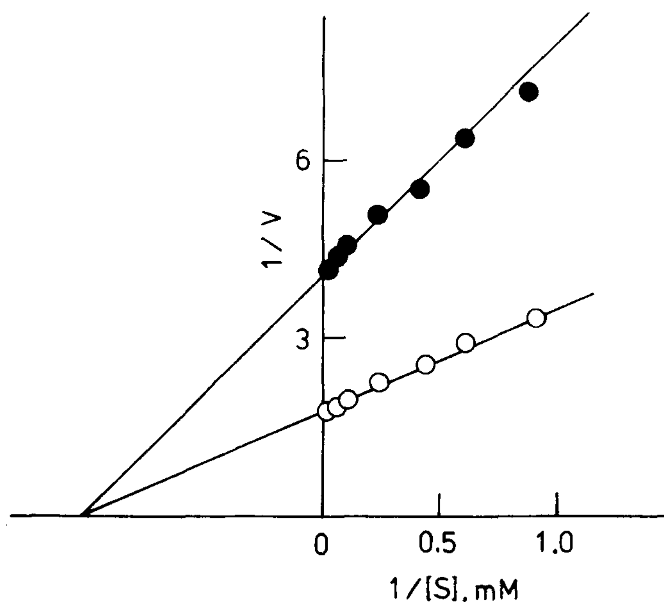

FIGURE 7 Lineweaver-Burk plots of substrate (sucrose) concentration against rate of hydrolysis by GTF with M-GTFI. —o—, Control (no inhibitor added); —●—, incubated with 5 μg of M-GTFI/incubation mixture.

absence of M-GTFI (Figure 7). The inhibition was shown to be non-competitive. The K_m value of I-GTF for sucrose was 9.8 ($\pm$ 0.20) $\times$ 10^{-4} M, and the K_i value of M-GTFI was found to be 1.3 ($\pm$ 0.34) $\times$ 10^{-7} M.

Effect of M-GTFI on the growing system of S. mutans K1-R

Growth of *S. mutans* was examined in the BHIB culture with Tween 80 (0.1%) in the presence of M-GTFI (0, 10, 100, and 1000 μg/ml). In these cultures, no complex of cells and adhesive glucan formed because of the absence of sucrose. To eliminate interference by the colour of the culture, the cells were collected by centrifugation, washed once with saline, and then resuspended in saline. The inhibitor did not affect the turbidity at λ 600 nm and so had no effect on the growth of *S. mutans* in the 15 h growth period used.

To examine the effect of M-GTFI on glucan formation in the growing system, *S. mutans* was grown at 37°C in the BHIB medium supplemented with sucrose (2%). After overnight incubation as described in MATERIALS AND METHODS, each culture tube was decanted. Highly adhesive glucan and cell aggregates remained attached to the glass walls while non-adhesive, flaky glucan and cells were decanted into another tube. The turbidity of each fraction was measured at λ 600 nm. The formation of the adhesive, water insoluble glucan was found to be strongly inhibited by 150 to 200 μg/ml of the inhibitor (Figure 8). This result suggests that the inhibitor may prevent *S. mutans* from adhering to the smooth surface of tooth enamel.

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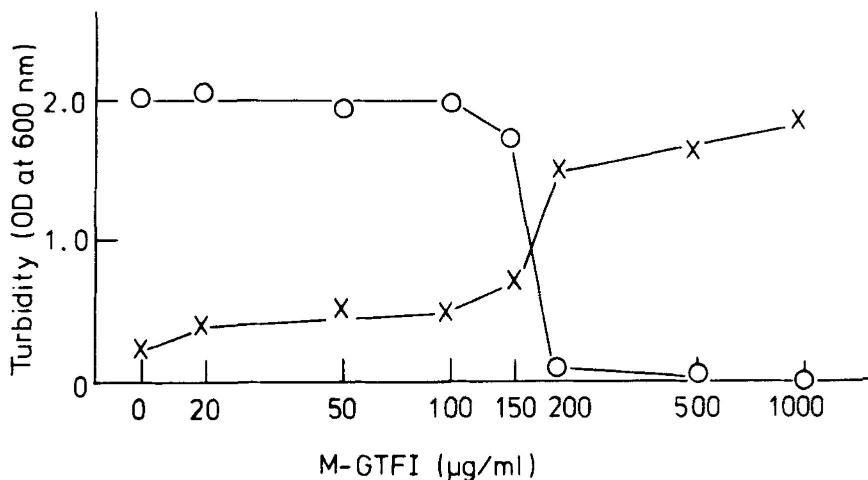

FIGURE 8 Effect of M-GTFI on insoluble glucan formation in a growing system. The effect of inhibitor on the culture of *S. mutans* Kl-R was examined in the presence of sucrose. —O—, insoluble glucan formed ($A_{600 nm}$); —X—, turbidity in the supernatant ($A_{600 nm}$).

The effect of the inhibitor on the glucan-forming activities of various S. mutans strains

Table II shows the effects of the inhibitor on the glucosyltransferases of strains HS-1 (a), Ingbritt (c), OMZ-176 (d) and LM-7 (f) in addition to Kl-R (g). The transferases forming the water insoluble glucan (WIG) and water soluble glucan (WSG) were purified on hydroxylapatite as described in MATERIALS AND METHODS. For all of the strains tested, both WIG and WSG synthesis were inhibited although the former was more sensitive to inhibition than the latter. The results suggest that I-GTF may be more sensitive to the inhibitor than S-GTF.

In general, WIG was synthesized from sucrose by the coupled reaction of I-GTF and S-GTF, although WSG was synthesized exclusively by S-GTF. M-GTFI is considered to inhibit the formation of WIG by initially inhibiting I-GTF activity.

From the present study, M-GTFI differs from other previously described in-

TABLE II
Effect of M-GTFI on WIG- and WSG-forming activities from some representative strains of *S. mutans*

Strain (serotype)	Amount of M-GTFI required to give 50% inhibition (μg) WIG-forming activity	WSG-forming activity
Kl-R (g)	1.0	25.0
HS-1 (a)	0.7	4.7
Ingbritt (c)	0.6	7.6
OMZ-176 (d)	0.6	9.6
LM-7 (f)	0.4	16.7

WIG; water insoluble glucan.
WSG; water soluble glucan.

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hibitors. Ribocitrin[5,6] is a small molecule (M_r 602), consisting of 3 ribose molecules and a homocitric acid and is produced by *Streptomyces neyagawaensis* MF-980-CFI. Another inhibitor, mutastain, is a glycoprotein ($M_r > 2 \times 10^6$) containing 85% protein and 6.5% carbohydrate, and is produced by *Aspergillus terreus* M3328. The glucosyltransferase from *S. mutans* is also inhibited by α-glucosidase inhibitors such as l-desoxynojirimycin and acarbose.[8] The inhibitor M-GTFI, whose isolation is reported here, clearly differs from the above inhibitors in that it is an acidic substance with a molecular weight of approximately 5700 and is derived from *Micromonospora narashinoensis* strain 731.

As pointed out by Tanzer,[25] the virulence of dental caries can be ascribed to the production of an alkaline-soluble α-1,3-glucan from sucrose by *S. mutans*. Mutants that are defective in the synthesis of glucan[26] have a diminished virulence that is probably due to the lack of the α-1,3-rich glucan. The inhibitor that we have isolated shows no direct antibiotic activity toward *S. mutans* but represses the formation of the water insoluble glucan and therefore has potential in decreasing production of the cariogenic α-1,3-rich glucan.

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