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CHARACTERISTICS OF M-GTFI, A NEW INHIBITOR OF *STREPTOCOCCUS MUTANS* GLUCOSYLTRANSFERASE‡

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M-GTFI, originally screened as an inhibitor of *Streptococcus mutans* glucosyltransferase, strongly inhibited α-glucosidase, in a non-competitive manner especially when the synthetic substrate *p*-nitrophenyl-α-D-glucopyranoside was used. It also inhibited β-glucosidase, β-amylase and, to a lesser extent, β-glucuronidase.

The inhibitor was stable in neutral and alkaline pH ranges and dependency of the inhibition on pH and temperature was not observed. Some proteinases and polysaccharides-hydrolyzing enzymes as well as human saliva did not inactivate the inhibitor.

There was a correlation between the release of sulfate anions from the inhibitor molecule on incubation with HCl (0.2 N) at 100°C and loss of inhibitory properties of the molecule. It is suggested that the presence of sulfate ester linkages in the inhibitor molecule play an important role in the inhibition process.

KEY WORDS: Glucosyltransferase inhibitor, enzyme inhibitor, M-GTFI, *Streptococcus mutans*, dental caries.

INTRODUCTION

In the previous paper,[1] we reported that a strain (No. 731), identified as *Micromonospora narashinoensis* was found to produce a new inhibitor (designated as M-GTFI) of the glucosyltransferase from *S. mutans*, and a purification procedure and partial characterization of M-GTFI was described. From an examination of the effects of this inhibitor on representative strains of *S. mutans* other than K1-R, there was a suggestion of a similar selectivity for the water insoluble glucan-forming activity in other strains.

This paper reports the inhibitory spectrum of M-GTFI against enzymes other than glucosyltransferase, and some characteristics of this inhibitor.

MATERIALS AND METHODS

Enzymes

Subtilisin BPN′ from *B. subtilis*, α-amylase from *B. subtilis*, β-amylase from soybean, glucoamylase from *Rhizopus niveus*, and cellulase from *Aspergillus niger* were pur-

‡Studies on *S. mutans* glucosyltransferase inhibitor produced by *Micromonospora narashinoensis* strain No. 731. Part II. (See ref. 1 for Part I).

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chased from Nagase Biochemicals. Pronase from *Streptomyces griseus* was obtained from Kakenkagaku Co., and zymolyase from *Arthrobacter lutea* came from Seikagaku Kogyo Co. Trypsin from vovine pancrease, dextranase from *Penicillium* sp., α-glucosidase from bakers' yeast, α-galactosidase from green coffee bean, β-galactosidase from bovine liver and β-glucuronidase from *Escherichia coli* were obtained from Sigma Chemicals. β-Glucosidase from almond and β-fructosidase from yeast came from Boehringer Mannheim, Yamanouchi.

Substrates

Soluble starch, carboxymethylcellulose (Katayama Industries Co.), laminarin (Tokyo Kasei Kogyo Co.), dextran T70 (Pharmacia Fine Chemicals), *p*-nitrophenyl-α-D-glucopyranoside and phenolphthalein-β-D-glucuronide (Nakarai Chemicals) and salicin (Wako Pure Chemical Industries) were used.

Bacterial Strain and Growth Media

Human cariogenic *S. mutans* strain K1-R (serotype g) was routinely maintained in brain heart infusion agar strain stab cultures (BHIB, Nissui Seiyaku Co., Tokyo) at 4°C and transferred weekly. The strain was kindly supplied by Professor T. Morioka of the School of Dentistry, Kyushu University.

As seed culture, *S. mutans* was cultured anaerobically in the BHIB supplemented with D-glucose (1.8%) for 16–18 h at 37°C in a candle jar. Chemically defined medium was prepared according to Janda and Kuramitsu.[2] Tween 80 (10 mg/ml medium; ten times more concentrated than previously described) was added to improve the bacterial growth and I-GTF production.[3]

Purification of M-GTFI

Micromonospora narashinoensis strain No. 731, a producer of M-GTFI, was incubated into 200 ml Erlenmeyer flasks containing 50 ml of production medium,[1] and grown aerobically at 28°C for 3 days on a rotary shaker. M-GTFI used in this work was purified from the culture filrate by salting-out with ammonium sulfate, followed by dialysis, column chromatography on hydroxylapatite and gel filtration on Sephadex G-75. Its homogeneity was determined by polyacrylamide gel electrophoresis as previously described.[1]

Purification of the Glucosyltransferase

The insoluble GTF (I-GTF), which forms a water insoluble glucan from sucrose, was purified from *S. mutans* K1-R culture via column chromatography on hydroxylapatite by the method previously described.[1]

Assay for the Glucosyltransferase

The reaction mixture and conditions have been described previously.[1] Fructose liberated from sucrose by the glucosyltransferase reaction was determined by the method of Somogyi–Nelson.[4]

The standard curve for fructose by this method was linear up to 100 μg/ml which

gives an absorbance of 1.0 at λ600 nm. The enzyme activity level used in this report was adjusted to give an absorbance of 0.4 to 0.5 (40–50 μg as fructose) at λ600 nm.

Assay for Other Enzyme Reactions

The reducing power of the medium after reaction with the enzymes such as α-, β-amylase, glucoamylase, dextranase, cellulase, β-glucosidase, β-fructosidase, zymolyase and their substrates was determined by the method of Somogyi–Nelson.[4] *p*-Nitrophenol released by α-glucosidase reaction was measured colorimetrically at λ440 nm. The measurement of β-glucuronidase activity was based on the colorimetric determination (at λ550 nm) of phenolphthalein liberated from the substrate phenolphthalein-β-D-glucuronide.[5]

Digestion test of M-GTFI with human saliva was carried out using dialyzed and centrifuged saliva.

Inhibition Studies

The inhibitory activity of M-GTFI was determined from the degree of decrease in the absorbance caused by the addition of the inhibitor to each assay system as described in the above two sections. The activity of M-GTFI was usually expressed as ID_{50} which is the amount of the inhibitor required to give 50% inhibition. The concentration of M-GTFI used in other experiments was described in the text.

Determination of Sulfate Released by Acid Hydrolysis

After hydrolysis of M-GTFI with 0.2 N HCl at 100°C, determination of inorganic sulfate in the hydrolysate was performed using sodium rhodizonate[6] which formed a colored compound with barium ion. Sample (0.5 ml), ethanol (2.0 ml), $BaCl_2$ buffer (1.0 ml) and sodium rhodizonate solution (1.5 ml) were mixed in a test tube and the tube was shaken well. The tube was allowed to stand for 10 min in the dark after which the color intensity was measured spectrophotometrically at λ520 nm.

The absorbance value of water itself in the above assay method was usually 0.5 at λ520 nm, and the standard curve for sulfate (Na_2SO_4) by this method was linear up to 10 μg which causes a degree in absorbance of 0.4.

RESULTS AND DISCUSSION

Inhibitory Spectrum of M-GTFI

M-GTFI was originally screened as an inhibitor of *S. mutans* glucosyltransferase (GTF) and found to strongly inhibit GTFs from various strains of *S. mutans*, especially their water insoluble glucan-forming activity.[1] Felgenhauer *et al.* showed that α-glucosidase inhibitors such as 1-deoxynojirimycin and acarbose, also inhibit the GTF from *S. mutans*.[7] Therefore, the effect of our inhibitor, M-GTFI, on other enzymes was examined. As shown in Table I, M-GTFI strongly inhibited α-glucosidase especially when a synthetic substrate, *p*-nitrophenyl-α-D-glucopyranoside was used as substrate (ID_{50} = 0.01 μg).

The ID_{50} when maltose was used as substrate for the enzyme was 0.7 μg, which was

TABLE I
Inhibitory spectrum of M-GTFI

Enzyme (E.C.)	Substrate (cleavage site)	Substrate (concn.*)	Inhibition (ID_{50}, μg)
α-Amylase	starch		> 200.0
(3.2.1.1)	(α-1,4)	(4.0)	
β-Amylase	starch		5.2
(3.2.1.2)	(α-1,4)	(4.0)	
Glucoamylase	starch		> 200.0
(3.2.1.3)	(α-1,4)	(4.0)	
Cellulase	cellulose		> 200.0
(3.2.1.4)	(β-1,4)	(1.0)	
Zymolyase	laminarin		> 100.0
(3.2.1.6)	(β-1,3)	(1.0)	
Dextranase	dextran		> 100.0
(3.2.1.11)	(α-1,6)	(4.0)	
α-Glucosidase	*p*-nitrophenyl-α-		0.01
(3.2.1.20)	D-glucopyranoside	(0.6)	
	maltose	(1.0)	0.7
β-Glucosidase	salicin	(2.0)	0.9
(3.2.1.21)			
β-Fructosidase	D-sucrose		> 100.0
(3.2.1.26)	(α-1, β-2)	(8.0)	
β-Glucuronidase	phenolphthalein-		30.5
(3.2.1.31)	β-D-glucuronide	(0.024)	
Glucosyltransferase	D-sucrose		0.4
(2.4.1.5)	(α-1, β-2)	(13.7)	

ID_{50}: Amount of M-GTFI required to give 50% inhibition (μg).
*Concentration was expressed as mg/ml-incubation mixture.

of the same order as that for inhibition of GTF. In addition to these enzymes, the inhibitor inhibited β-glucosidase, β-amylase and, to a lesser extent, β-glucuronidase.

K_i Value of M-GTFI in α-Glucosidase Reaction

In a previous paper,[1] 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 absence of M-GTFI, and the inhibition was shown to be non-competitive. The K_m value of I-GTF for sucrose was found to be 9.8×10^{-4} M, and the K_i value of M-GTFI was 1.3×10^{-7} M.

As described in the previous section, this inhibitor strongly inhibited α-glucosidase. The type of inhibition and K_i value was examined in the α-glucosidase reaction using *p*-nitrophenyl-α-D-glucopyranoside as substrate. The inhibition was non-competitive (Figure 1), as found for GTF, with a K_m value for α-glucosidase of 2.3×10^{-4} M, and a K_i value for M-GTFI of 9.4×10^{-10} M.

pH-Dependency of the Inhibition Reaction

GTF reactions were carried out in buffers of various pH values and the effect of pH on the inhibitory activity of M-GTFI was examined. As shown in Figure 2, GTF activity both in the presence and absence of M-GTFI was maximum at pH 6. The inhibitory activity of M-GTFI, calculated from these results, was independent of pH.

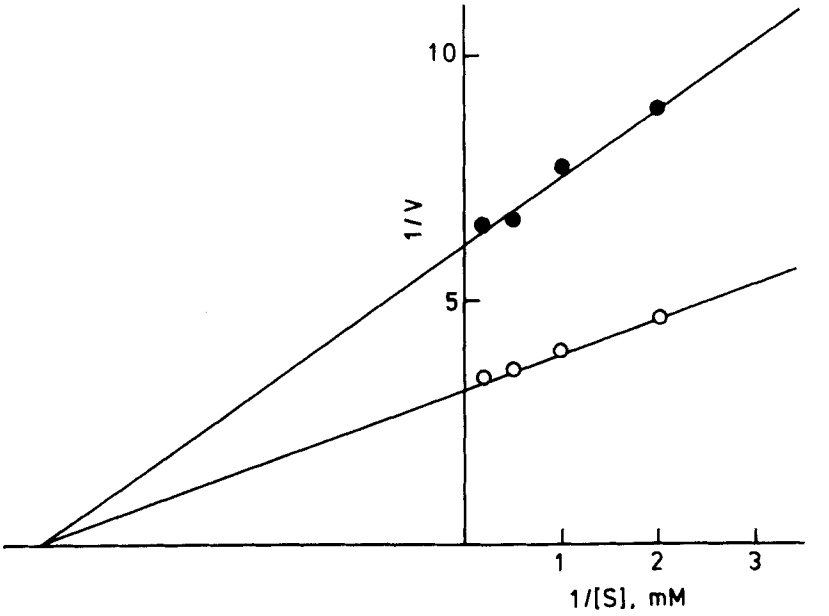

FIGURE 1 A Lineweaver-Burk plot of substrate (*p*-nitrophenyl-α-D-glucopyranoside) concentration against rate of hydrolysis by α-glucosidase with M-GTFI. –○–, Control (no inhibitor added); –●–, incubated with 0.01 μg of M-GTFI/incubation mixture.

Temperature-Dependency of the Inhibition Reaction

Similarly as in the above section, the effect of temperature on GTF activity with or without M-GTFI was examined. As shown in Figure 3, the inhibition was little affected by the reaction temperature.

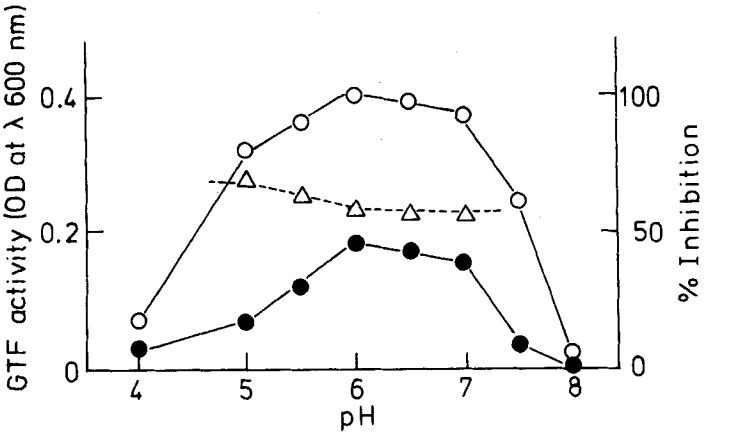

FIGURE 2 Effect of pH on GTF reaction with or without M-GTFI. –○–, pH-activity of GTF; –●–, pH-activity of GTF incubated with M-GTFI (1 unit); ---△---, Inhibitory activity (% inhibition) of M-GTFI derived from the two preceding curves. Buffer systems used were acetate (pH 4), phosphate (pH 5–7) and Tris-HCl (pH 8) in a final concentration of 0.05 M.

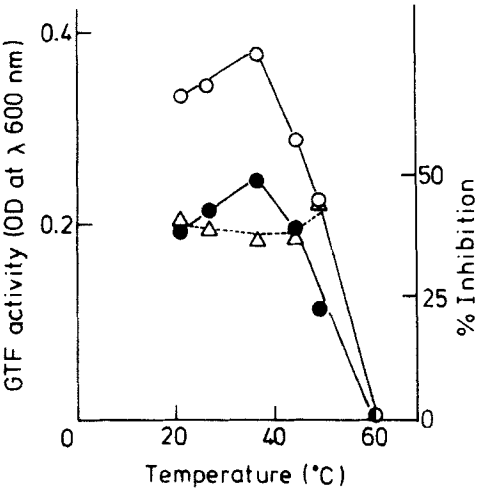

FIGURE 3 Effect of temperature on GTF reaction with or without M-GTFI. –○–, GTF activity (no inhibitor added); –●–, GTF activity incubated with M-GTFI; – – –△– – –, Inhibitory activity (% inhibition) of M-GTFI derived from the two preceding curves. 0.05 M potassium phosphate buffer (pH 6.0) was used in this experiment.

pH and Thermal Stability of M-GTFI

Stability of this inhibitor was examined by storage at 5°C for 3 days, 37°C for 24 h and 100°C for 10 min in buffers over the pH 1–13 range. Treatment even at 100°C for 10 min revealed that the inhibitor was stable except in the acidic pH 1–3 range (Figure 4).

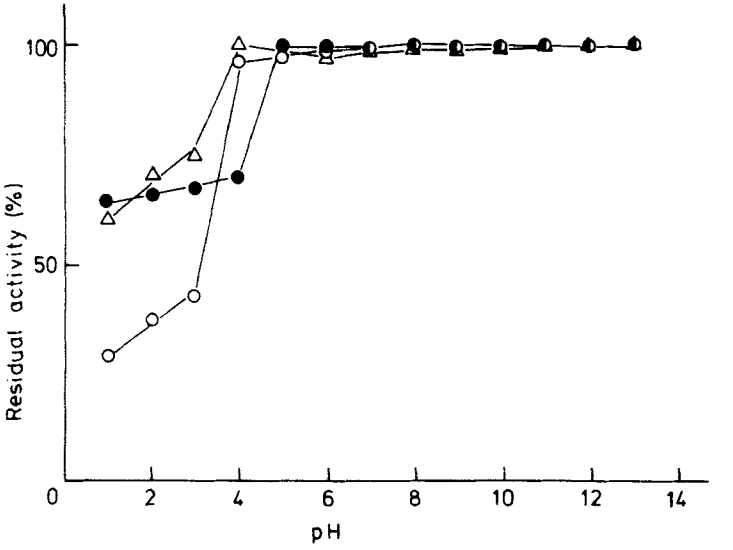

FIGURE 4 pH and thermal stability of M-GTFI. –○–, 100°C, 10 min; –△–, 37°C, 24 h; –●–, 5°C, 72 h. Buffer systems used were HCl (pH 1), KCl-HCl (pH 2), Gly-HCl (pH 3), acetate (pH 4), phosphate (pH 5–7), Tris-HCl (PH 8–9), Gly-NaOH (pH 10–12) and NaOH (pH 13) in a final concentration of 0.05 M.

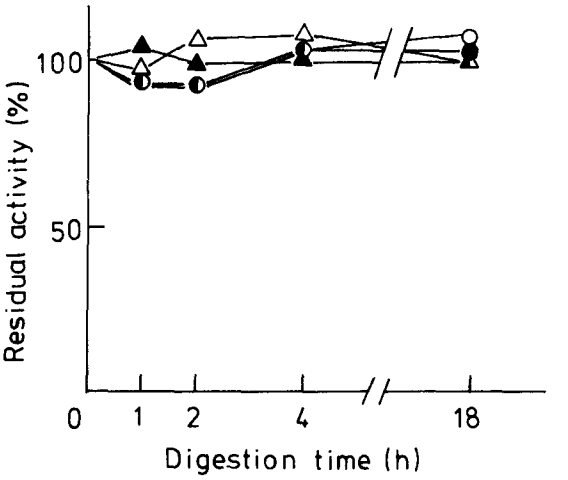

FIGURE 5 Digestion test of M-GTFI with human saliva. Saliva was provided by 4 students. The reaction mixture consisting of 1 mg/ml solution of M-GTFI (1 ml), saliva without dilution (0.3 ml), 0.2 M phosphate buffer at pH 6.0 (0.5 ml) and 0.2% NaN_3 solution (0.2 ml) was incubated at 37°C. After incubation for the indicated time, the reaction mixture was heated for 10 min at 100°C, and then the residual inhibitory activity was measured by the usual assay method.

Digestion Test of M-GTFI with Human Saliva

As described in the previous paper,[1] the inhibitor had no effect on the growth of *S. mutans* itself (i.e. the inhibitor had no antimicrobial activity against Gram-positive, and -negative bacteria, fungi and yeasts examined; data not shown); however, it prevented *S. mutans* from adhering to the smooth surface of glass tubes. Therefore, it was suggested that it could be a useful agent for the prevention of human dental caries. Consequently it was important to establish the stability of the inhibitor in the mouth.

TABLE II
Digestion test of M-GTFI with various enzymes

Enzyme	Enzyme amount[a]	pH[b] (buffer)	Digestion time[c] (h)	Residual activity[d] (%)
No digestion	0			100
Subtilisin BPN′	50	9.0(Tris)	0.5	101
Pronase E	50	7.4(Tris)	0.5	100
Trypsin	50	8.0 (Ph)	0.5	95
α-Amylase	50	6.0 (Ph)	0.5	96
β-Amylase	100	5.0 (Ac)	0.5	107
Cellulase	100	5.0 (Ac)	4.0	100
Zymolyase	100	7.0 (Ph)	2.0	100
Dextranase	50	6.0 (Ph)	4.0	95
β-Fructosidase	100	6.0 (Ph)	18.0	100
α-Galactosidase	25	6.0 (Ph)	4.0	97
β-Galactosidase	50	7.3 (Ph)	18.0	96

[a], Amount (μg) of enzyme to 1 mg of M-GTFI.
[b], 0.2 M buffer used: Tris = Tris-HCl, Ph = phosphate, and Ac = acetate.
[c], Digestion was carried out at 37°C.
[d], Each value was obtained from duplicate experiments.

A study of the digestion of the inhibitor was made using saliva provided by 4 students. The results are shown in Figure 5. The inhibitor was found to be very stable in the presence of saliva but its usefulness as an anticaries agent will depend on how long its action persists in the mouth which remains to be determined.

Digestion Test of M-GTFI with Various Enzymes

In some cases, enzymatic digestion gives infomation on the type of the structural linkage present in a compound. For this reason, a digestion test of M-GTFI was carried out with various enzymes. As tabulated in Table II, some proteinases and some polysaccharides-hydrolyzing enzymes did not digest M-GTFI which indicates that there were no linkages in the molecule such as a peptide linkage, sugar α-1,4-, β-1,4-, β-1,3-, α-1,6-, and α-1, β-2-linkages, *etc.* although substrate specificity should be taken into consideration.

Identification of Sulfate Esters in the Inhibitor Molecule

Complete structural elucidation of this inhibitor is now in progress and will be reported elsewhere. For the confirmation of the presence of a sulfate ester the infrared spectrum (KBr) of the inhibitor was measured. The IR spectrum showed a strong absorption peak at 1226 cm^{-1} and this, taken together with the strongly acidic nature of the inhibitor, indicated that it may be sulfate ester ($-O\cdot SO_3^-$). After sulfate ion release by acid hydrolysis (see later), this strong peak disappeared from the spectrum indicating the presence of sulfate esters in the inhibitor molecule.

Correlation of the Release of Sulfate Anions from the Inhibitor Molecule with Inactivation of the Inhibitor

As mentioned in the previous section, the inhibitor is very stable over a wide pH

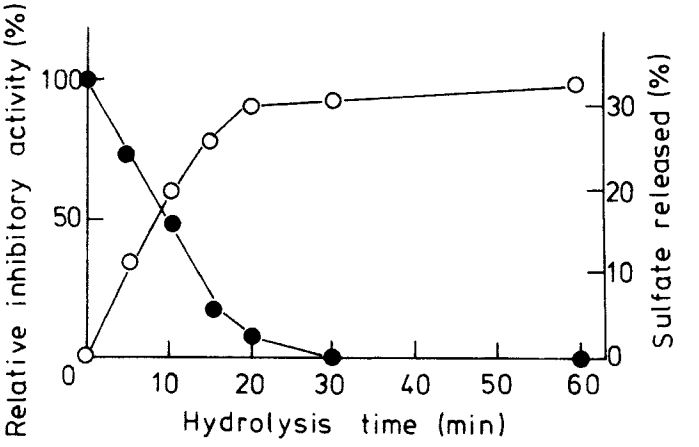

FIGURE 6 Time course of acid hydrolysis of M-GTFI. –●–, Inhibitory activity after incubation with 0.2 N HCl at 100 C; –○–, Sulfate released (%). The concentration of M-GTFI used for the hydrolysis was 2 mg in 1 ml of 0.2 N HCl. After incubation for the indicated time at 100°C, the solution was diluted by water, and then the residual inhibitory activity and the amount of inorganic sulfate in the hydrolysate were measured.

range. After incubation of this compound in 0.2 N HCl at 100°C for 0.5 h, the inhibitor was found to be inactivated. As shown in Figure 6, as the hydrolysis time increased, sulfate anions were released from the molecule, and this was associated with a decrease in inhibitory activity.

The correlation of the release of sulfate anions from the inhibitor molecule with inactivation of the inhibitor suggests that ionised sulfate ester groups play an important role in the enzyme inhibition.

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