MUTASTEIN, A NEW INHIBITOR OF ADHESIVE-INSOLUBLE GLUCAN SYNTHESIS BY GLUCOSYLTRANSFERASES OF *STREPTOCOCCUS MUTANS*

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Mutastein, a new inhibitor of glucosyltransferases (GTase) of *Streptococcus mutans*, was isolated from cultural broth of *Aspergillus terreus* M3328. Mutastein was a heat-stable protein and was sensitive to pronase digestion. In the formation of glucans, that of adhesive water-insoluble glucans of *S. mutans* was specifically inhibited by mutastein.

Water-insoluble glucans (IG) synthesized by *Streptococcus mutans* form sucrose are known to be a major virulence factor for induction of dental caries in man and animals.[1~3] These glucans, which are synthesized by the action of glucosyltransferases (GTase) of *S. mutans*, firmly coat the tooth surfaces, forming a barrier which prevents the diffusion of acids produced by the bacteria.

Many chemical and enzymatic procedures for eliminating *S. mutans* from tooth surfaces have been explored.[3] Among these approaches, several were related to active compounds that specifically inhibit GTase, which include periodate-oxidized clinical dextran,[4] several amino sugars[5] and α-glucosidase inhibitors of microbial origin.[6] Recently, OKAMI *et al.*[7] isolated a specific GTase inhibitor, ribocitrin, from a culture filtrate of a strain of *Streptomyces* sp.

In the course of search for substances that inhibit GTase activity of *S. mutans*, we found a new inhibitor, named mutastein, in the culture filtrate of a strain of *Aspergillus terreus*. This report describes the isolation of mutastein and its effects on IG synthesis from sucrose by cell-free GTase of *S. mutans*.

Materials and Methods

Preparation of Cell-free GTase

S. mutans B13, serotype *d* (kindly supplied by K. FUKUSHIMA, Nihon University School of Dentistry, Matsudo, Chiba-ken) was grown at 37°C for 18 hours in brain heart infusion (BHI) broth (Nissui Medical Co., Tokyo). To produce cell-free GTase, the culture supernatant was brought to 60% saturation with $(NH_4)_2SO_4$. The precipitate formed was isolated by centrifugation, dissolved in 5 mM potassium phosphate buffer (KPB), pH 6.5, and dialyzed against the same buffer. The preparations were stored at −80°C until use.

Assay for Glucan Synthesis by Cell-free GTase

The reaction mixture consisted of sucrose (30 mg) and cell-free GTase (0~150 μg protein) in a total volume of 3 ml of 0.05 M KPB, pH 6.5 containing 0.02% NaN_3. The mixture was incubated at 37°C for 16 hours in a glass tube (13×110 mm) kept at an angle of 30° to the horizontal. After incubation, the adhesive-IG and nonadhesive-IG formed were harvested by decantation and centrifugation, dispersed by sonic oscillation, and their amounts were measured by optical density at 550 nm as described.[8] The water soluble glucans (SG) were determined as described by FUKUSHIMA *et al.*[9] SG was preci-

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pitated with 3 volumes of ethanol at 4°C. After the precipitate was washed with 75% ethanol, its sugar content was determined by phenol-sulfuric acid method.[10]

Unless otherwise described, 127 μg of cell-free GTase was used to determine glucan synthesis and one unit of mutastein was defined as the amount that gave 50% inhibition of adhesive-IG formation under these conditions, although as described later, inhibitory activity of mutastein varied with GTase concentration (Fig. 3).

Other Methods

Dextranase was assayed by incubating 0.2 ml of enzyme solution with 0.8 ml of 1.7% dextran T2,000 (Sigma) in 30 mM citrate buffer, pH 6.0, at 37°C for 4 hours, and the reducing sugars released were determined by the NELSON-SOMOGYI method.[11] The reaction mixture for assaying mutanase consisted of 0.2 ml of enzyme solution and 0.8 ml of 30 mM acetate buffer, pH 5.5 containing 1% carboxymethylated-IG from *S. mutans* OMZ176 (generous gift from T. NISHIZAWA, National Institute of Health, Tokyo), which was incubated at 37°C for 4 hours, followed by determining reducing sugars formed as described above. To detect dextranase and mutanase activity of mutastein preparations, one mg (in 0.2 ml) was assayed as described above.

Total carbohydrate was determined by phenol-sulfuric acid method.[10] Protein was determined by the method of LOWRY *et al.*[12]

Results

Identification of the Producing Fungus

The fungus strain M3328 employed in the production of mutastein was isolated from a soil sample collected at Musashino City, Tokyo, Japan. From the characteristics described below, the fungus was identified as *Aspergillus terreus* Thom, (in American J. Botany 5: 85, 1918).[13,14]

Colonies on Czapek-Dox agar grow rather rapidly, reaching a diameter of 3.0 to 4.5 cm in 10 days at 25°C, plane or marked by shallow radial furrows, velvety, with margins thin or irregular, light to medium sporulation, conidial heads in shades pale brown to reddish brown; exudate amber; no odor; reverse in dull yellow to brown shades. Conidial heads long, columnar, 400~500×30~50 μm; conidiophores more or less flexuous, smooth, colorless, 100~180×4.0~5.4 μm; vesicles hemispherical, domelike, 8~12 μm in diameter, merging imperceptibly into the supporting conidiophores; metulae 4.5~6.0×2.0 μm, no color; phialides 5.0~6.0×2.0 μm, no color; conidia globose, smooth, 1.9~2.4 μm, in diameter.

Colonies on malt-extract agar growing rather rapidly, plane, velvety, heavily sporulating throughout, conidial heads in reddish brown shade; exudate amber to yellowish brown; reverse in yellowish brown to reddish brown shades; no odor. Conidial heads as described on Czapek-Dox agar.

Isolation of Mutastein

A medium of the following composition was used for the fermentation of mutastein: 1% glucose, 2% potato starch, 2% soybean meal, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% Nissan Disform CB-442 (Nihon Yushi, Co., Tokyo). *A. terreus* M3328 was grown aerobically at 25°C for 7 days in a 30-liter fermentor containing 15 liters of the medium. The culture filtrate (10 liters) was adjusted to pH 3.5 by adding HCl and the resultant precipitate was obtained by centrifugation. The precipitate was dissolved in 2 liters of deionized water and the pH of the solution was adjusted to 10.0 with NaOH. The solution was brought to 30% saturation with $(NH_4)_2SO_4$. The precipitate formed was isolated by centrifugation, dissolved in 350 ml of water, and dialyzed against deionized water. The dialyzed solution was lyophilized, giving 10.2 g of dried powder.

Part of the powder (3.0 g) was dissolved in 300 ml of water and applied to a charcoal column (3×58 cm). The column was washed with water and the active fractions were pooled, which were then ap-

Table 1. Purification of mutastein from *Aspergillus terreus* M3328.

Purification step	Total protein (mg)	Total sugar (mg)	Total activity ($\times 10^3$ units)	Specific activity (units/mg protein)	Yield (%)
Lyophilized powder	3,000	210	385	125	100
Active charcoal	500	34	250	500	67
Hydroxyapatite	208	15	174	830	46
1st-gel filtration	78	—	78	1,000	21
2nd-gel filtration	22	1.5	36	1,670	10

plied to a hydroxyapatite column (4×4.5 cm) and the column was then developed successively with 0.005 M (150 ml), 0.025 M (250 ml), 0.05 M (300 ml), 0.2 M (350 ml) and 0.5 M (300 ml) KPB, pH 7.0. The fractions containing mutastein activity, which was found in the eluate of 0.2 M KPB, pH 7.0, were combined (300 ml) and concentrated to 21 ml using an ultrafiltration membrane UK-200 (Toyo Roshi Co., Tokyo). An aliquot of 3 ml of the concentrated solution was subjected to Toyoperl HW-75 column (3×95 cm) equilibrated with 25 mM KPB, pH 7.0 and the column was developed with the same buffer. The active fractions were combined, concentrated to 6 ml and then submitted to the second chromatography on Toyoperl HW-75 column under the same conditions (Fig. 1). Active fractions (Nos. 67~76) were combined and submitted to lyophilization. The lyophilized preparation was used in the experiments described below.

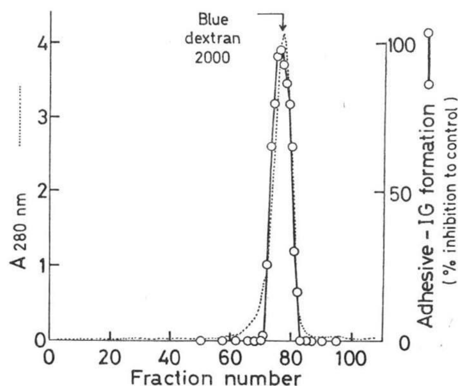
The purification procedure is summarized in Table 1. The overall purification was about 13 fold with a yield of 10%. The purity of the mutastein preparation obtained was 70~92% as judged from gel chromatography on Toyoperl HW-65 and from sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Properties of Mutastein

Mutastein was found to be a glycoprotein, since chemical analysis showed that it was composed of 85% protein and 6.5% carbohydrate. As shown in Fig. 1, mutastein was excluded from the column of Toyoperl HW-75 same as blue dextran 2,000, indicating that its molecular weight was 2,000,000 or more. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mutastein formed a broad protein band (data not shown). It was soluble in buffers at pHs higher than 4.0 but not soluble in buffers at pHs lower than 3.5. When mutastein was treated at 100°C for 10 minutes in buffers of pHs 4.5~11.0, no detectable changes in the inhibitory activity was observed. However, mutastein was sensitive to pronase digestion. Thus, its inhibitory activity was reduced by approximately 50% when 0.85 mg of mutastein was incubated with 0.1 mg of pronase

Fig. 1. A second Toyoperl HW-75 column chromatography.

A 3-ml aliquot of the concentrated eluate of first Toyoperl HW-75 chromatography was applied to a Toyoperl HW-75 column (3×95 cm), and the column was developed with 25 mM KPB, pH 7.0. Fractions of 5 ml were collected. An aliquot of 15 μl of the fractions was submitted to the assay of inhibitory activity under the conditions described in Materials and Methods.



contained in KPB, pH 7.0 at 37°C for 2 hours.

Neither dextranase nor mutanase activity was detected in mutastein preparations under the conditions described in Materials and Methods. Mutastein showed no detectable effect on the growth of *S. mutans* in BHI broth at a concentration of 300 μg/ml.

Effect of Mutastein on the Glucan Formation by GTase

GTase of *S. mutans* B13 was known to produce mainly IG, highly adhesive to smooth-glass surfaces, from sucrose with a trace amount of SG[9].

As shown in Fig. 2, adhesive-IG formation by B13 GTase was strongly inhibited by mutastein. Complete inhibition of adhesive-IG formation was observed in the presence of 1 μg/ml of mutastein. On the other hand, the activity of SG formation was strongly activated by mutastein. Thus, SG synthesized was increased by approximately 10-fold at a concentration of 1 μg/ml. The formation of non-adhesive-IG was strongly activated by mutastein. The data indicate that mutastein specifically inhibits the production of IG from sucrose.

Fig. 3 shows the concentrations of mutastein required for giving 50% inhibition (I_{50}) of adhesive-IG formation at varying concentrations of GTase. Thus, the I_{50} values varied with GTase concentrations; I_{50} was 0.2 μg/ml in the presence of 5 μg/ml of GTase but 1 μg/ml of mutastein was required when 40 μg/ml of GTase was present.

Fig. 2. Effect of mutastein on the glucan synthesis by GTase.

Reaction mixture consisted of sucrose (30 mg), *S. mutans* B13 GTase (51 mg protein) and mutastein (0~3.2 μg protein) in 3 ml of KPB, pH 6.5 containing 0.02% NaN_3. After 16 hours of incubation at 37°C, the amount of adhesive-IG (○), nonadhesive-IG (△), and SG (●) synthesized was determined as described in Materials and Methods.

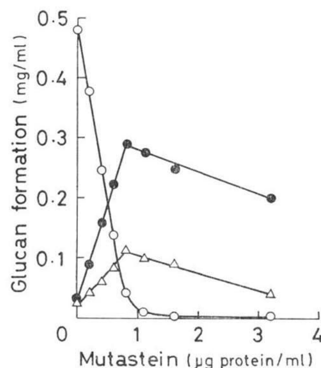
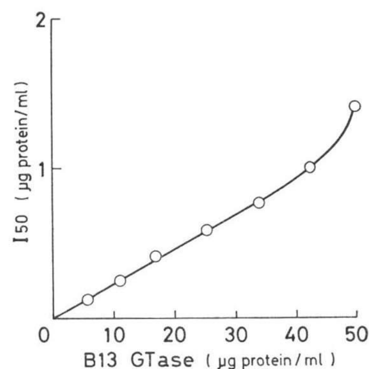


Fig. 3. Effect of mutastein on adhesive-IG formation by GTase.

Reaction mixtures containing various concentrations of mutastein and *S. mutans* B13 GTase as indicated were incubated at 37°C for 16 hours, and adhesive-IG formed was determined as described in Materials and Methods.



Discussion

A new inhibitor (mutastein) was found and partially purified from cultured broth of *A. terreus* M3328. It was a heat-stable glycoprotein with a molecular weight higher than 2,000,000. Preliminary experiments showed that treatments with Triton X-100 and NaSCN showed no significant changes in the molecular weight of mutastein. It was shown that mutastein specifically inhibited the synthesis of adhesive-IG from sucrose by GTase (Fig. 2).

GTase of *S. mutans* consists of at least two components.[15] One called GTase-S catalyzes the synthesis of SG, while the other (GTase-I) is essential to the formation of IG.[9]

Recently, FELGENHAUER and TRAUTNER[6] surveyed α-glucosidase inhibitors of microbial origin and found that 1-desoxynojirimycin, *N*-methyldesoxynojirimycin and acarbose inhibited extracellular GTase of *S. mutans*. OKAMI and his coworkers[7,16~18] isolated another type of GTase inhibitor, ribocitrin, from a culture broth of *Streptomyces neyagawaensis*, which consists of three D-ribose and one (+)-homocitric acid.[16] Mutastein is a heat-stable protein and distinct from these compounds.

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