— Research Letter —

Mutanase of *Paenibacillus humicus* from Fermented Food Has a Potential for Hydrolysis of Biofilms Synthesized by *Streptococcus mutans*

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Mutanase is an α-1,3-glucanase that cleaves the α-1,3-glucosidic linkages of glucan polysaccharides termed mutan. There has been interest in mutanase as it has the potential to be considered in preventive medicine. Previously, we identified the mutanase of *Paenibacillus humicus* from fermented soybeans and observed that the enzyme hydrolyzed insoluble glucans and mutan *in vitro*. Here we focused on the ability of mutanase in preventing dental caries. We proposed that the enzyme participates in the degradation of insoluble glucan biofilms in microplates, which were used as a model of biofilms on tooth surface. In this study, we investigated the potential of mutanase to hydrolyze insoluble glucan biofilms synthesized by a typical cariogenic streptococcus, *Streptococcus mutans* 10449, in 96-well microplates. The mutanase specifically hydrolyzed insoluble glucan biofilms composed of insoluble glucans synthesized by *S. mutans*. The mutanase hydrolyzed approximately 60% of *S. mutans* glucan biofilms in the wells and liberated oligosaccharides from the glucan films. We observed that recombinant mutanase could hydrolyze mutan biofilms, and the enzyme was characterized as an *endo*-α-1,3-glucanase because its hydrolysis of mutan biofilms, resulted in the predominant liberation of α-1,3-glucan tetrasaccharides. The present study suggests that *Paenibacillus* mutanase from fermented soybeans has potential application in preventive medicine because of its ability to degrade oral insoluble glucan biofilms.

Key words —— mutanase, *Paenibacillus humicus*, *Streptococcus mutans*, biofilm, glucan, mutan

INTRODUCTION

Mutan is an insoluble glucan consisting of α-1,3-glucosidic linkages. Mutanase (EC3.2.1.59) cleaves α-1,3-glucosidic linkages in mutan polysaccharides to oligosaccharides as the sole catalytic products.[1] If mutanase solubilizes glucan biofilms present in dental plaque, the enzyme has potential for preventive medicine. Dental plaque is formed by the adhesion of cariogenic streptococci such as *Streptococcus mutans* to tooth surface.[2] This adhesion is mediated by insoluble glucan biofilms, the components of which are synthesized by a series of glucosyltransferases from cariogenic streptococci.[3] Thus, formation of dental plaque leads to dental caries. The enzymatic glycolysis of mutan expects to liberate the biofilm from tooth surface and prevent dental caries.[3] Degradation of α-1,3-glucan polysaccharides can be catalyzed by mutanase, which is produced by fungi, *Trichoderma*, and bacterial genera *Bacillus* and *Paenibacillus*.[3–5] In addition, related studies indicated that mutanase activity should reduce the incidence of dental caries.[5] However, query of the safety of the mutanase producers as preventive agents have not yet been proven. Searching for mutanase producers among food should have an advantage of safety for human. Furthermore, few reports have characterized mutanase function in food in particular enzyme activity for oral hygiene.

Previously, we identified mutanase of *Paenibacillus humicus* from fermented soybeans.[6] The mutanase hydrolyzed insoluble glucans synthesized by cariogenic streptococci *in vitro*.[7] However, the ability of mutanase to hydrolyze insoluble glucan biofilms of cariogenic streptococci has not yet been proven. In the present study, mutanase from the fermented food has been investigated for its ability to hydrolyze insoluble glucan biofilm and its catalytic mechanism to assess the efficiency of the enzyme for preventive medicine.

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MATERIALS AND METHODS

Degradation of Insoluble Glucans Adhered to Microplates by Intrinsic Mutanase —— The intrinsic mutanase of *P. humicus* NA1123 from fermented soybeans was prepared, and 60% ammonium sulfate fraction was used in this experiment.[6] For coating the insoluble glucan-biofilms on microplates, 96-well flat-bottom plates (Corning; Corning, NY, U.S.A.) were treated with 0.2 ml of 0.2% bovine serum albumin (Sigma; St. Louis, MO, U.S.A.) in phosphate buffered saline, pH 7.4 at 4°C for 16 hr. Cultures of cariogenic streptococci (*S. mutans* 10449; American Type Culture Collection; Manassas, VA, U.S.A.) in 0.2 ml of Todd Hewitt Broth (Invitrogen; Carlsbad, CA, U.S.A.) containing 1.0% sucrose were plated on to the microplates. Amounts of the streptococci were read at an absorbance of 600 nm to be 0.15. The cultures were incubated at 37°C for 6 hr under stationary conditions. Media were then removed, and the formed glucan biofilms adhered to the wells were washed with 20 mM sodium phosphate buffer (PB), pH 6.5. The intrinsic mutanase of *P. humicus* (16 munits/ml) in 20 mM PB, pH 6.5 (150 μl) were added to the wells, and the degradation of glucan biofilms was performed at 37°C for 4 hr in the presence of protease inhibitor cocktail (Sigma).[7] The released saccharides present in the supernatants in wells were recovered and quantified by Somogyi-Nelson method and by phenol-sulfuric acid method. The remaining insoluble glucans in each well were washed with 20 mM PB, pH 6.5 and were suspended in 0.2 ml of 1.0 M sodium hydroxide for 30 min at room temperature with gentle shaking. The alkaline-soluble saccharides in the wells were recovered, and the amounts of glucose were quantitated by the phenol-sulfuric acid method as the amounts of glucose in remaining biofilms.

Degradation of Mutan Biofilms Adhered to Microplates by Recombinant Mutanase —— In this experiment, recombinant mutanase of *P. humicus* was prepared according to our previous report, and was purified by metal affinity chromatography.[7] To prepare the mutan biofilms, streptavidin-coated 96-well microplates (Thermo Fisher Scientific, Roskilde, Denmark) were treated with biotin-labeled dextran (0.1 μg/well; Sigma) in phosphate buffered saline, pH 7.4. After washing the wells with the buffer to remove excess biotin-labeled dextran, mutan was formed on the dextran-coated microplates by recombinant glucosyltransferase I of *Streptococcus downei* MFe28 which synthesizes α-1,3-glucan at 37°C for 6 hr.[7] Then the wells were washed with 20 mM PB, pH 6.5. Recombinant mutanase (69 munits/ml) in 150 μl of 20 mM PB, pH 6.5 was added to the wells of microplates that were coated with mutan biofilms. Hydrolysis of mutan biofilm by the recombinant enzyme was performed at 37°C for up to 6 hr. The remaining mutan biofilms in the wells were washed with the buffer, and the amounts of saccharides in films were assayed by the phenol-sulfuric acid method. Supernatants in the well after 0 and 1 hr of hydrolysis of mutan biofilms were recovered to assay the saccharides by TLC.[7] Saccharides on the silica gel plates were developed with pyridine : butanol : water (3 : 15 : 4) for three times, and these were visualized with orcinol-sulfuric acid. TLC was performed using nigerooligosaccharides as the standards.

Statistics —— Assays to determine the amounts of hydrolysates by the enzyme were performed in triplicate. The values reported here were mean values with standard deviations being less than 5% in all cases.

RESULTS AND DISCUSSION

Mutanase of *P. humicus* from Fermented Food Hydrolyzed Insoluble Glucan Biofilms on Microplates

The experimental conditions for assaying mutanase activity were designed to the situation on tooth surface in the oral cavity. We examined the ability of intrinsic mutanase to degrade insoluble glucan biofilms in 96-well microplates. In this study, we prepared biofilms on plates that contained 1–4 μmol/ml glucose in each well. The finding shown in Fig. 1A demonstrated the hydrolytic action of mutanase of *P. humicus* towards the insoluble glucan biofilms that were synthesized by a typical cariogenic streptococcus, *S. mutans* 10449. The intrinsic mutanase degraded the insoluble glucan biofilms of *S. mutans* 10449 with 0.35 μmol of glucose equivalents after 4 hr of hydrolysis. Thus, the enzyme hydrolyzed approximately 60% of the insoluble glucans. This value reveals the composition of the glucans, synthesized by the *S. mutans*.[8] The streptococcal glucan biofilms may be composed of approximately 60–70 mol% α-1,3-glucosidic linkages.[2, 8, 9] The catalytic preference of our presented enzyme for hydrolysis of biofilms was similar to that of mutanase of *Trichoderma harzianum*.[9] The

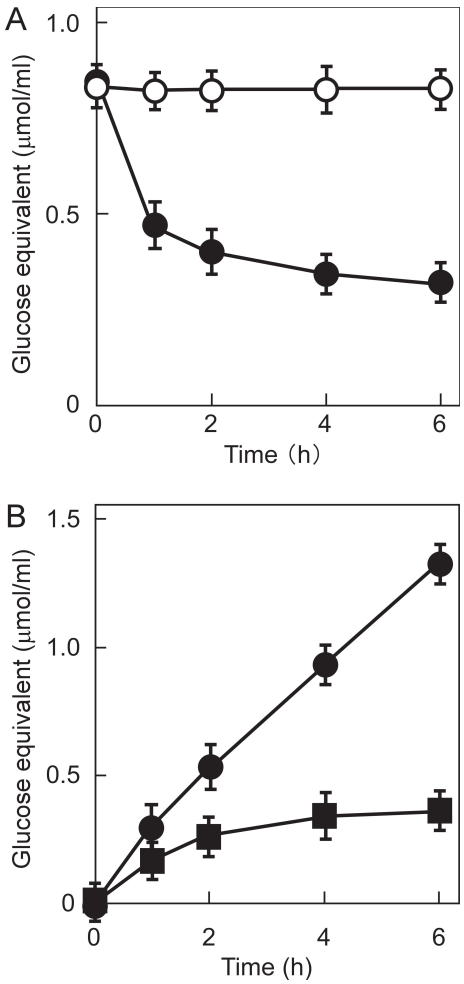

Fig. 1. Intrinsic Mutanase of *P. humicus* from Fermented Soybeans Hydrolyzed Biofilms of Insoluble Glucans Synthesized by the Glucosyltransferases of *S. mutans*

(A) Mutanase from fermented food hydrolyzed insoluble glucan biofilms synthesized by *S. mutans*. Insoluble glucan biofilms of *S. mutans* 10449 were preformed in 96-well microplates. Intrinsic mutanase in 20 mM sodium phosphate buffer, pH 6.5 was added to insoluble glucan-biofilms in wells and the plates were incubated at 37°C for up to 4 hr. The insoluble glucans in each well were washed with the buffer and suspended in 0.2 ml of 1.0 M sodium hydroxide. The alkaline-soluble saccharides in wells were assayed for the total amounts of glucose as the remaining glucan biofilms by the phenol-sulfuric acid method (closed circle). In the control experiment, buffer was added to the wells instead of enzyme (open circle). These were expressed as glucose equivalents. Data are presented as the means of assays that were performed in triplicate. (B) Mutanase released oligosaccharides from insoluble glucan biofilms. The released saccharides in the supernatants were recovered from each well and assayed by the Somogyi-Nelson method as the amounts of reducing sugars (closed square) and phenol-sulfuric acid method as the total amounts of sugars (closed circle). The values represent the mean of triplicate assay.

present mutanase from fermented food could hydrolyze insoluble glucan biofilms, indicating its potential of preventive medicine. The catalytic property of mutanase in the hydrolysis of insoluble glucan biofilms was assessed by assaying the level of liberated saccharides in the supernatants (Fig. 1B). The hydrolytic profiles of intrinsic mutanase indicate that the enzyme cleaved insoluble glucans in an *endo*-catalytic manner because of the relatively low levels of reducing end of saccharides by the Somogyi-Nelson method and the relatively high levels of glucose contents in hydrolysates detected by phenol-sulfuric acid method.

Recombinant Mutanase Hydrolyzed Mutan Biofilms on Microplates

To confirm the intrinsic mutanase activity on solubilization of insoluble glucans, we assayed the hydrolysis of α-1,3-glucan (mutan biofilm) by pure recombinant mutanase. Efficiency of recombinant mutanase was examined on the hydrolysis of mutan films on dextran-coated microplates. In this regard, we had previously observed that the presence of α-1,6-glucans (dextran) was required for the formation of mutan films to smooth surfaces.[10] The ability of mutanase to hydrolyze mutan biofilms was assessed as the levels of remaining glucose residues in biofilms (Fig. 2A). The hydrolytic profiles of recombinant mutanase revealed that the total amounts of glucose of remaining biofilms decreased linearly for up to 4 hr. The profile implies that the enzyme hydrolyzes mutan in biofilms *in vivo*. However, mutan biofilms were more resistant to enzymatic hydrolysis in this experiment than the suspension of mutan in reaction buffer used in our previous report.[7] The tendency of the present enzyme to catalyze the hydrolysis of biofilms was in agreement with the characteristics of mutanase of *T. harzianum*.[11] Thus, the ability of breakdown of mutan biofilms by mutanase from fermented food was demonstrated in this study. We analyzed the catalytic mode of recombinant enzyme in degrading mutan biofilms by TLC. The recombinant enzyme liberated primary α-1,3-glucan tetrasaccharides from mutan biofilms (Fig. 2B). The finding was supported by our previous study, that the enzyme liberated tetrasaccharides from non-reducing termini of α-1,3-glucan during the course of hydrolysis (for up to 4 hr incubation).[7] However, mutanase of *T. harzianum* F470 showed *exo*-catalytic property, that the enzyme liberated glucose monomer from insoluble glucans.[3] Thus, *Paenibacillus* mutanase hydrolyzed mutan biofilms in an *endo*-catalytic manner, resulting in the efficient breakdown of the structure of insoluble glucan biofilm.[12]

In the present study, the biofilm solubilizing activity of mutanase from fermented soybeans

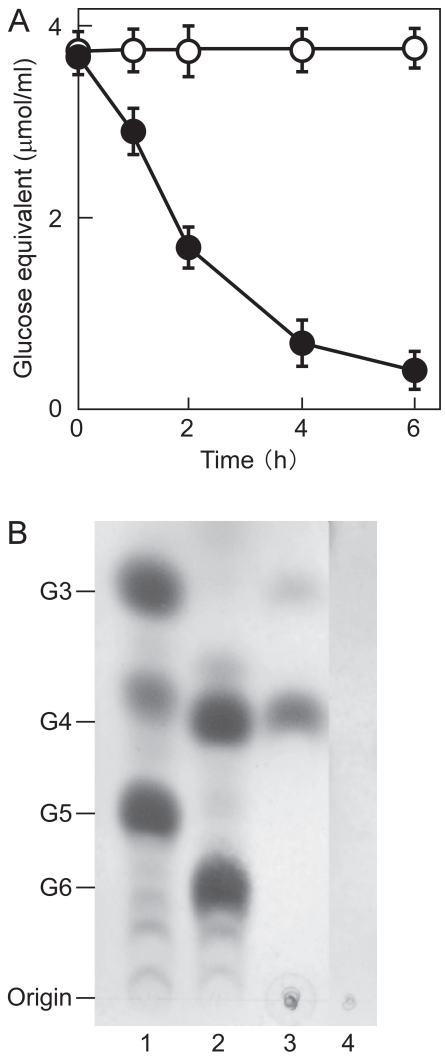

Fig. 2. Recombinant Mutanase of *P. humicus* Hydrolyzed Mutan Biofilms

(A) Recombinant mutanase hydrolyzed mutan biofilms in microplates. Mutan biofilms were preformed in dextran-coated microplates by recombinant glucosyltransferase I of *S. downei* MFe28. Recombinant mutanase in 20 mM sodium phosphate buffer, pH 6.5 (150 μl/well) was added to mutan biofilms on the wells, and the plates were incubated at 37°C. At regular intervals of enzymatic reactions, the remaining mutan biofilms in each well were washed with the buffer and were suspended in 0.2 ml of 1.0 M sodium hydroxide. The alkaline-soluble glucans in the wells were assayed by the phenol-sulfuric acid method for the amounts of glucose as the remaining mutan films (closed circle). The remaining mutan films were quantified as the glucose equivalents. For the control, buffer without the enzyme was added to the wells (open circle). Data are presented as the means of triplicate assay. (B) Recombinant mutanase hydrolyzed mutan biofilms into α-1,3-glucan tetrasaccharides. After the hydrolysis of mutan biofilms in 96-well microplates for 0 and 1 hr, the released saccharides in the portions of hydrolysates (3 μl) were analyzed by TLC. Saccharides on the silica gel plates were developed by pyridine : butanol : water (3 : 15 : 4) for three times, and these were visualized with orcinol-sulfuric acid. Standards, nigerooligosaccharides (G3, G4, G5 and G6 correspond to the degree of polymerization of α-1,3-glucan oligosaccharides), were placed on lanes 1 and 2. Hydrolysates after 1 hr and its control (0 hr) were placed lanes 3 and 4, respectively.

may have implications in dental caries prevention because of the breakdown of insoluble glucan biofilms. The mutanase offers an attractive approach to solubilize oral biofilm and addition to prevent from periodontal diseases. Further studies are required to delineate the usefulness of mutanase in promoting human health.

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