— Research Letter —

Substrate Specificity of Mutanase of *Paenibacillus humicus* from Fermented Food

Hideaki Tsumori,[a] Atsunari Shimamura,[a] Yutaka Sakurai,[b] and Kazuo Yamakami*, [b]

[a]*Department of Chemistry and* [b]*Department of Preventive Medicine and Public Health, National Defense Medical College, Namiki, Tokorozawa 359–8513, Japan*

(Received August 27, 2010; Accepted November 9, 2010; Published online November 15, 2010)

Mutanase, α-1,3-glucanase, catalyzes the hydrolysis of α-1,3-glucans, and is expected for preventive medicine, since the enzyme has an ability for hydrolysis of insoluble glucans that are synthesized by cariogenic streptococci. We previously isolated the mutanase of *Paenibacillus humicus* from fermented soybeans. In the present study, *Paenibacillus* mutanase was characterized with respect to its hydrolysis efficiency of insoluble glucans, and mode of action on α-1,3-glucan oligosaccharides. Recombinant mutanase hydrolyzed insoluble glucans of cariogenic streptococci efficiently. Enzymatic reaction on hydrolysis of mutan, we assumed that the enzyme cleaved the substrate in an *endo*-catalytic manner. The hydrolysis of α-1,3-glucan oligosaccharides gave α-1,3-glucan tetrasaccharide as the primary final product but α-1,3-glucan pentasaccharide was the minimal size of substrate on which the enzyme catalyzed. Mutanase hydrolyzed borohydride-treated α-1,3-glucan hexasaccharide into the tetrasaccharide and the disaccharide-alditol. Thus, the enzyme cleaved the fourth α-1,3-glucosidic linkage from the non-reducing end of the oligosaccharides. Mutanase in fermented food should be capable of removing streptococcal insoluble glucans that can induce dental caries.

Key words —— mutanase, *Paenibacillus humicus*, fermented soybean, glucanase

*To whom correspondence should be addressed: Department of Preventive Medicine and Public Health, National Defense Medical College, Namiki, Tokorozawa 359–8513, Japan. Tel.: +81-4-2995-1563; Fax: +81-4-2996-5195; E-mail: yamakami@ndmc.ac.jp

INTRODUCTION

Mutan, α-1,3-glucan, synthesized from dietary sucrose by glucosyltransferases of cariogenic streptococci, is a major virulence factor for dental caries induction in humans.[1] Insoluble glucans, which mainly consist of α-1,3-glucosidic linkages, are compositions of biofilms from cariogenic streptococci.[2] Strategies to reduce the disease potential of biofilms have included the possible use of insoluble glucan degrading enzymes to disrupt the plaque. One of the glycosidases, termed mutanase, α-1,3-glucanase, has the possibility for preventive medicine.[3] However, mutanases used in caries prophylaxis have had little success due to difficulties with the enzyme in applications. In addition, mutanases from different sources have exhibited diverse catalytic preferences, one has *endo*-catalytic properties and another is an *exo*-catalytic enzyme.[3,4] For the prevention of dental caries, a candidate enzyme should efficiently suppress the formation of insoluble glucans and/or hydrolyze the glucans on tooth surfaces.[5]

A search for insoluble glucan-hydrolyzing activity from food sources should be advantageous from the perspective of convenience and safety in humans. Despite our interest in the glucolytic activity potential of foods, we found limited information on enzymes capable of hydrolyzing insoluble glucan in fermented food. Previously, we screened the fermented soybeans, and found a mutanase (α-1,3-glucanase; EC3.2.1.59) from *Paenibacillus humicus* (*P. humicus*) in the food.[6] The aim of the present study was to characterize mutanase activities with respect to removal of insoluble glucans from oral biofilms created by pathogenic microorganisms and to assess the potential of the enzyme for preventive medicine. In this article, we report on some of the catalytic properties of mutanase from *P. humicus* in fermented food. The efficient glucan-hydrolyzing activity in the fermented food provides support for the role of mutanase in preventive medicine.

MATERIALS AND METHODS

Recombinant Mutanase and Insoluble Glucan —— Recombinant mutanase was prepared according to the procedure described in our previous report.[6] Intrinsic glucosyltransferases for syn-

thesis of insoluble glucans were prepared as follows. The bacteria *Streptococcus mutans* Ingbritt, *Streptococcus sobrinus* 6715 and *S. sobrinus* B13 (American Type Culture Collection; Manassas, VA, U.S.A., and our culture collections), were grown in 1 liter of Todd-Hewitt broth (Invitrogen; Carlsbad, CA, U.S.A.), and after overnight incubation the cultured supernatant obtained by centrifugation at 7500 × *g* for 15 min was precipitated with solid ammonium sulfate at 60% saturation.[4] The sediment was dissolved in 20 ml of water and dialyzed against 10 mM sodium phosphate buffer (pH 6.5) for overnight. For the insoluble glucan synthesis, the enzyme solution (20 ml) was added to 0.1 M sodium phosphate buffer (pH 6.5) containing 10% sucrose (1 liter), and incubated at 37°C for 72 hr. Insoluble glucans were collected by centrifugation at 7500 × *g* for 15 min, then washed 2 times with water, and stored at 4°C until use.

Enzyme Assay —— For the assay of mutanase activity, mutan (α-1,3-glucan polysaccharide) was synthesized from sucrose by recombinant glucosyltransferase I of *Streptococcus downei* MFe28.[7] The standard assay mixture, contained 1.0 ml of 1.0% mutan or crude insoluble glucans in 20 mM ammonium acetate buffer (pH 5.7) and suitably diluted enzyme solutions (100 µl), and incubated at 37°C for 2 hr. After appropriate incubation periods, the reactions were terminated at 95°C for 10 min. The remaining substrates were removed at 20000 × *g* for 10 min. The amounts of the reducing sugars liberated from the substrates were quantified by the Somogyi-Nelson method and the amounts of the total sugars were determined by phenol-sulfuric acid method. One unit of activity was defined as the amounts of enzyme that released 1 µmol of glucose per min under standard conditions. Enzymatic assays were performed in triplicate, and the values reported here had standard deviations of less than 2% in all assays.

α-1,3-Glucan Oligosaccharides —— α-1,3-Glucan polysaccharides were synthesized as has shown in the above by recombinant glucosyltransferase I.[7] The preparation of defined α-1,3-glucan oligosaccharides was previously described.[8] In brief, α-1,3-glucans were partially hydrolyzed with 0.1 M sulfuric acid at 100°C for 2 hr with stirring under reflux. The mixture of partially hydrolyzed oligosaccharides was separated on a BioGel P2 (Bio-Rad; Hercules, CA, U.S.A.) column. The purities of individual saccharides were analyzed by TLC and assessed to be > 90%.[8] For labeling of the reducing ends, oligosaccharides were dissolved in 0.5 M ammonium hydroxide and reduced by adding excess borohydride at 20°C for 5 hr. The excess of borohydride was removed by adding 10% acetic acid. Boric acid was removed by co-evaporation from methanol under reduced pressure.

For the determination of cleavage sites by the enzyme, α-1,3-glucan pentasaccharide (G5) and hexasaccharide (G6) and their borohydride-treated saccharides (G5-ol and G6-ol) were used as the substrates. The enzyme was incubated with these substrates (1%) for 15 min and 4 hr in 50 mM ammonium acetate buffer (pH 5.7) at 37°C, and their hydrolyzates were assayed by TLC. The digests (3 µl) were spotted onto the silica gel plates (Wako, Osaka, Japan) and then developed with pyridine : butanol : water (3 : 15 : 4). TLC was performed using glucose (G1), nigerose (G2), and nigerose oligosaccharides (G3–G7) as the standards.[8] (G1, G2, G3, *etc*. correspond to the glucose and degree of polymerization of α-1,3-glucan oligosaccharides; *i.e*., glucose, nigerose, nigerotriose, and so on. Similarly, the suffix-ol indicates the alditol form of the corresponding oligosaccharides.)

RESULTS AND DISCUSSION

Mutanase Hydrolyzed Insoluble Glucans, Which Were Synthesized by Cariogenic Streptococci

To investigate the ability of mutanase to hydrolyze insoluble glucans, we assayed the enzyme specificity for insoluble glucans, which were synthesized by glucosyltransferases from cariogenic streptococci (Fig. 1). The present mutanase showed specificity for hydrolysis of these insoluble glucans, and released saccharides ranging from 4.2 to 12.2 µmol/ml glucose equivalents. However, the details of compositions of insoluble glucans of these streptococci had not yet been identified completely.[9] The typical linkage structures of the insoluble glucans from these streptococci were composed of α-1,3 and α-1,6-glucosidic linkages with branched points of 1,3,6-linked glucose.[4] These glucans should contain approximately 60–90 mol% of α-1,3-linkages.[4,9] The variations in the glucolytic levels of these insoluble glucans may have depended on variations in the compositions of these glucans. It is likely that similar findings were observed wherein α-glucanase from *Trichoderma harzianum* cleaved α-1,3-linkages in some insolu-

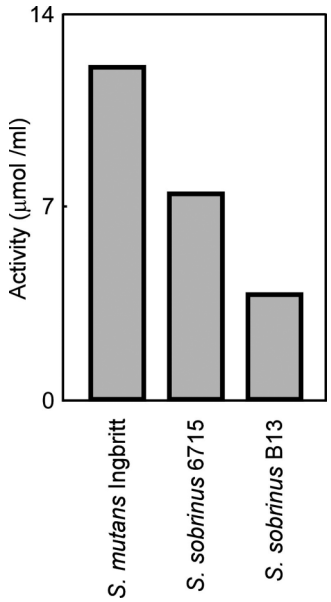

Fig. 1. Recombinant Mutanase Hydrolyzed Insoluble Glucans, Which Were Synthesized by Cariogenic Streptococci

Recombinant mutanase (16.8 munits) was incubated with 1.0% of insoluble glucans from *S. mutans* Ingbritt, *S. sobrinus* 6715 and *S. sobrinus* B13 in 50 mM ammonium acetate buffer (pH 5.7) at 37°C for 2 hr. Data indicates the amounts of reducing ends, which were released from the individual insoluble glucan. Activities are expressed as the glucose equivalents. Each bar is relative to the mean of triplicate assay, with a relative standard deviation of less than 2%.

ble glucans of streptococci.[10,11] Thus, the present mutanase from fermented food should be able to hydrolyze and solubilize glucans in biofilms formed by cariogenic streptococci *in vivo*.[12]

Recombinant Mutanase Hydrolyzed Mutan Efficiently

The hydrolysis potential of the recombinant mutanase was tested over a time course with mutan as the substrate. Enzymatic activity was assayed by determining the amounts of released oligosaccharides and the total amounts of glucose in the hydrolyzates. The profiles of both assay procedures showed that the hydrolysis levels increased linearly in the initial phase of process up to 0.5 hr and then became almost constant around 2 hr (Fig. 2). The combination of findings of high levels of glucose residues by the phenol-sulfuric acid method and low levels of the reducing ends by the Somogyi-Nelson method in the hydrolyzates indicate that the present enzyme hydrolyzed intrachain glucosidic linkages and released oligosaccharides from mutan. These characteristics of the enzyme activity suggest that the present mutanase exhibited *endo*-catalytic activity as also shown by the mutanase from *Paenibacillus* strain KSM-M86.[13]

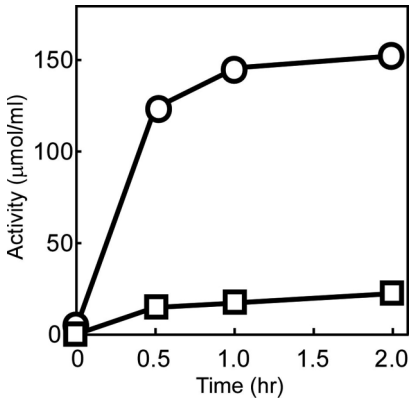

Fig. 2. Recombinant Mutanase Hydrolyzed Mutan Efficiently

The activity was performed in a reaction mixture consist of recombinant mutanase (16.8 munits), 1.0% of mutan, 20 mM ammonium acetate buffer (pH 5.7) at 37°C. Hydrolysis was conducted that the reducing sugars in the hydrolyzates were measured by the Somogyi-Nelson method (open square) and the total amounts of sugars were quantified with phenol-sulfuric acid (open circle). Activities are expressed as the glucose equivalents. Each point represents to the mean of triplicate assays.

Mutanase Cleaved α-1,3-Glucan Oligosaccharides in an *endo*-Catalytic Manner

To investigate the catalytic properties of the recombinant mutanase, the enzyme was incubated with oligosaccharides as the substrate (Fig. 3). TLC profiles of the pentasaccharide (G5, lane 3) hydrolyzates taken after 15 min incubation with the enzyme showed major spots corresponding to G4 and the remaining original G5 (lane 4). Increasing the incubation period to 4 hr showed the spots of G4 and G1 (lane 5). The hexasaccharide (G6, lane 9) hydrolyzates taken after 15 min reaction showed two major spots corresponding to G4 and G2 (lane 10). Increasing the reaction period to 4 hr did not show a change in the compositions anymore (lane 11). This finding demonstrates that G4 could not be a substrate since it was not cleaved over time, and that G5 was the minimum size substrate for the recombinant mutanase. We studied the action mode of mutanase on the hydrolysis of oligosaccharides, whose reducing end had been modified by borohydride reduction. On the hydrolysis of borohydride-treated G5 (G5-ol, lane 6), the mutanase did not show activity for G5-ol, at 15 min (lane 7) and 4 hr (lane 8) reaction periods. This finding indicates that G5-ol was proved a poor substrate for the enzyme. The enzyme cleaved G6-ol (lane 12) to give two major spots corresponding to G4 and G2-ol at 15 min (lane 13) and 4 hr (lane 14) reaction periods. The findings indicate that the mutanase recognizes a unit of intact form of α-1,3-glucan pentasaccharides in substrates and then cleaves the fourth α-

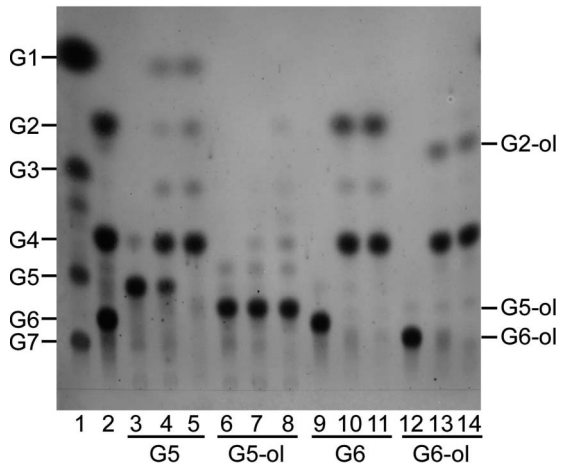

Fig. 3. Recombinant Mutanase Demonstrated *endo*-Catalytic Mode Against α-1,3-Glucan Oligosaccharides

α-1,3-Glucan pentasaccharide (G5, lane 3) and hexasaccharide (G6, lane 9), and their borohydride treated saccharides (G5-ol, lane 6 and G6-ol, lane 12) were used as the substrates. The enzyme (42 munits) was incubated with 1.0% of these substrates for 15 min (lanes 4, 7, 10 and 13) and 4 hr (lanes 5, 8, 11 and 14) in 10 mM ammonium acetate buffer (pH 5.7) at 37°C, and their hydrolyzates were analyzed by TLC. Each sample (3 µl) was placed onto the silica gel plates. Saccharides were developed with pyridine : butanol : water (3 : 15 : 4). The spots of hydrolyzates were visualized with orcinol-sulfuric acid. These profiles were one of two independent experiments. Saccharide standards were placed on lanes 1 and 2. The suffix-ol indicates the alditol form of saccharides.

1,3-glucosidic linkage from the non-reducing site of the unit. Thus, presented mutanase could be characterized as an *endo*-hydrolytic glucosidase. The mutanase in the *Paenibacillus* strain KSM-M86 obtained from a soil sample showed similar properties.[13)]

In this report, the mutanase showed an efficient hydrolytic potential for mutan solubilization in an *endo*-catalytic manner, and could potentially reduce the oral biofilm containing pathogenic microorganisms that induce dental caries. The mutanase from fermented food should be made available for preventive medicine.

REFERENCES

1) Davey, M. E. and Costerton, J. W. (2006) Molecular genetics analyses of biofilm formation in oral isolates. *Periodontol. 2000*, **42**, 13–26.
2) Tsumori, H. and Kuramitsu, H. (1997) The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. *Oral Microbiol. Immunol.*, **12**, 274–280.
3) Shimotsuura, I., Kigawa, H., Ohdera, M., Kuramitsu, H. K. and Nakashima, S. (2008) Biochemical and molecular characterization of a novel type of mutanase from *Paenibacillus* sp. strain RM1: Identification of its mutan-binding domain, essential for degradation of *Streptococcus mutans* biofilms. *Appl. Environ. Microbiol.*, **74**, 2759–2765.
4) Wiater, A., Szczodrak, J. and Pleszczyńska, M. (2008) Mutanase induction in *Trichoderma harzianum* by cell wall of *Laetiporus sulphureus* and its application for mutan removal from oral biofilms. *J. Microbiol. Biotechnol.*, **18**, 1335–1341.
5) Hayacibara, M. F., Koo, H., Vacca-Smith, A. M., Kopec, L. K., Scott-Anne. K., Cury, J. A. and Bowen, W. H. (2004) The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. *Carbohydr. Res.*, **339**, 2127–2137.
6) Tsumori, H., Kawauti, T., Shimamura, A., Hanada, N., Sakurai, Y. and Yamakami, K. (2010) Cloning and expression of the mutanase gene of *Paenibacillus humicus* from fermented food. *J. Health Sci.*, **56**, 456–461.
7) Monchois, V., Arguello-Morales, M. and Russell, R. R. B. (1999) Isolation of an active catalytic core of *Streptococcus downei* MFe28 GTF-I glucosyltransferase. *J. Bacteriol.*, **181**, 2290–2292.
8) Mukasa, H., Shimamura, A. and Tsumori, H. (2000) Nigerooligosaccharides acceptor reaction of *Streptococcus sobrinus* glucosyltransferase GTF-I. *Carbohydr. Res.*, **326**, 98–103.
9) Cerning, J. (1990) Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiol. Rev.*, **7**, 113–130.
10) Grün, C. H., Dekker, N., Nieuwland, A. A., Klis, F. M., Kamerling, J. P., Vliegenthart, J. F. and Hochstenbach, F. (2006) Mechanism of action of the *endo*-(1-3)-α-glucanase MutAp from the mycoparasitic fungus *Trichoderma harzianum*. *FEBS Lett.*, **580**, 3780–3786.
11) Wiater, A., Szczodrak, J. and Rogalski, J. (2004) Hydrolysis of mutan and prevention its formation in streptococcal films by fungal α-D-glucanases. *Process Biochem.*, **39**, 1481–1489.
12) Fuglsang, C. C., Berka, R. M., Wahleithner, J. A. Kauppinen, S., Shuster, J. R. Rasmussen, G., Halkier, T., Dalboge, H. and Henrissat, B. (2000) Biochemical analysis of recombinant fungal mutanases. A new family of α-1,3-glucanases with novel carbohydrate-binding domains. *J. Biol. Chem.*, **275**, 2009–2018.
13) Sumitomo, N., Saeki, K., Ozaki, K., Ito, S. and Kobayashi, T. (2007) Mutanase from a *Paenibacillus* isolate: Nucleotide sequence of the gene and properties of recombinant enzymes. *Biochim. Biophys. Acta*, **1770**, 716–724.