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Prevention of oral biofilm formation and degradation of biofilm by recombinant α-1,3-glucanases from *Streptomyces thermodiastaticus* HF3-3

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The genes encoding α-1,3-glucanases (Agls; AglST1 and AglST2) from *Streptomyces thermodiastaticus* HF3-3 were cloned and were then expressed in *Escherichia coli* Rosetta-gami B (DE3). We purified the resultant histidine (His)-tagged α-1,3-glucanases (recombinant enzymes, rAglST1 and rAglST2). Both the recombinant enzymes were similar to the wild-type enzymes. We examined the effects of rAglST1 and rAglST2 on the formation and degradation of biofilms on glass plates with *Streptococcus mutans* NRBC 13955 by evaluating the biofilm content (%), release of reducing sugar (mM), release of *S. mutans* (log CFU/mL), and the biofilm structure using laser scanning microscopy (LSM). The results showed that after incubation for 16 h, rAglST1 and rAglST2 reduced the formation of biofilm to 52% and 49% of the control, respectively. The result may reflect the fact that the concentration of the reducing sugar and the number of *S. mutans* cells in the rAglATs-added medium were higher than in the control medium. After an 8-h treatment with rAglST1 and rAglST2, biofilms decreased to less than 60% of the control. The number of *S. mutans* cells in the reaction mixture gradually increased during the incubation period. The enzymes can degrade the biofilms that were pre-formed on the glass plate by more than 50% after a 30-min incubation in the presence of toothpaste ingredients (1% w/v of sodium fluoride, benzethonium chloride, and sodium dodecyl sulfate) at 50°C. Our study showed that rAglST1 and rAglST2 have advantageous properties for dental care applications.

Key Words: α-1,3-glucanase; biofilm; degradation; prevention; *Streptomyces thermodiastaticus*

Introduction

The first report that α-1,3-glucanase (Agl; EC 3.2.1.59), or mutanase that hydrolyzes α-1,3-glucan by recognizing the α-1,3-glycosidic linkage, was in the *Trichoderma harzianum* (Guggenheim and Haller, 1972), and later in other sources, such as fungi and bacteria. Agl is classified into two types: the glycoside hydrolase family 71 (GH71) and the glycoside hydrolase family 87 (GH87). These families are described in the Carbohydrate Active enzyme (CAZy) database on the basis of their amino acid similarity. GH71 Agl is primarily found in fungi. In *T. harzianum*, GH71 Agl uses an inverting mechanism to hydrolyze substrates. On the other hand, GH87 Agl is isolated only from bacteria, but its catalytic mechanism remains unclear (Grun et al., 2006; Suyotha et al., 2016).

Found in dental plaque and the fungal cell walls, α-1,3-glucan is a water-insoluble polysaccharide comprising a homopolymer of glucose with α-1,3-glycosidic linkages. Dental plaque is formed when the normal bacterial flora present in the human oral cavity, principally *Streptococci*

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spp., including *Streptococcus mutans*, *S. sanguis*, and *S. salivarius*, produce polysaccharides, also known as mutans. α-1,3-Glucan is synthesized by the catalysis of glucosyltransferase (GTF) from sucrose as a substrate (Ismail et al., 2006; Marotta et al., 2002). This polysaccharide is involved in the development of cariogenic bacteria and other bacterial accumulation on tooth surfaces. This accumulation indicates the beginning of dental caries, which eventually develops into severe periodontitis (Ismail et al., 2006).

Previous reports have suggested that α-1,3-glucanase could be used to decrease dental plaque accumulation, to control pathogenic fungi, and to prepare fungal protoplasts (Pleszczyńska et al., 2015; Sanz et al., 2005; Yano et al., 2006). Purified α-1,3-glucanase from *T. harzianum* F-470 effectively prevented mutan formation and removed mutan from the mixed streptococcal biofilm, particularly when combined with dextranase (Wiater et al., 2004). Likewise, the water-insoluble polysaccharide of the pooled dental plaque was completely solubilized after incubation with the unpurified mutanase from *T. harzianum* OMZ 779 at 40°C overnight (Guggenheim and Haller, 1972). Endo-α-1,3-glucanase from *Pseudomonas* sp. has been reported to remove almost all mutan on the glass surface at an enzyme concentration of only 0.4 mU/mL (Pleszczyńska et al., 2015).

Application of α-1,3-glucanase to animals and humans has been studied. The research in hamsters showed that α-1,3-glucanase, purified from *Pseudomonas* sp. and added to drinking fluid (2.5 U/ml), was able to decrease the total caries scores in an enzyme-treated group, compared with a non-treated group, significantly after 40 days, without any adverse effect on general health (Simonson et al., 1983). In humans, a mouthwash containing purified α-1,3-glucanase from *Pseudomonas* sp. was used with college students to investigate its effect on oral plaque. The results showed that, after 5 days, the mean plaque scores of the α-1,3-glucanase mouthwash-treated group was significantly lower than the placebo group (Inoue et al., 1990). Further study in the human oral system was performed by comparing the rinsing with a sucrose solution and the rinsing with a sucrose solution containing α-1,3-glucanase prepared from *A. nidulans*. The results showed that the proportion of *S. mutans* was lower after using the α-1,3-glucanase solution for 1 week than after using the sucrose solution only, with averages of 2.2% and 10.4%, respectively (Kelstrup et al., 1973).

In our previous studies, *Streptomyces thermodiastaticus* HF3-3, which was isolated from soil, produced two kinds of thermostable Agls. These Agls differed by molecular mass. The smaller protein and the larger one were designed as AglST1 and AglST2, respectively. AglST1 is generated by the proteolytic cleavage of C-terminal region of AglST2 during cultivation. Based on its amino acid sequence, AglST could be classified as a new group with the property of α-1,3-glucanase due to its high homology with mycodextranase (Cherdvorapong et al., 2019). However, preparation of the enzymes from original bacteria was a time-consuming, costly, and complicated purification procedure. It was challenging to control its expression, which can be easily affected by culture conditions. Moreover, there are some properties of AglSTs to be defined, such as domain structures, the functional properties of each domain, and the potential of this enzyme to be used in dental care treatment.

In the present study, the genes encoding AglST1 and AglST2 were cloned in *Escherichia coli* and produced as recombinant enzymes, designated as rAglST1 and rAglST2, respectively. The aim was to evaluate the effect of these enzymes on the *S. mutans* biofilm formation and degradation.

Materials and Methods

α-1,3-Glucan preparation. α-1,3-Glucan was prepared using glycosyltransferase II from *S. mutans* expressed in *E. coli* Rosetta Gami B (DE3) as described previously (Cherdvorapong et al., 2019).

Microorganism and culture conditions. *E. coli* JM 109 was used as a host to propagate the recombinant plasmid. It was incubated in Luria-Bertani (LB) broth (pH 7.0) containing 50 μg/ml ampicillin at 37°C for 18 h with shaking at 100 rpm. *E. coli* Rosetta Gami B (DE3) cells harboring the recombinant plasmid were used for α-1,3-glucanase production by incubating in 100 mL LB broth at pH 7.0 containing 50 μg/ml ampicillin, 20 μg/ml kanamycin, and 34 μg/ml chloramphenicol at 37°C with shaking at 100 rpm. *S. mutans* NBRC 13955 was incubated in 10-mL Heart Infusion medium (HI medium) for 18–24 h, then inoculated to 75 ml HI medium which contained 1% (w/v) sucrose for biofilm formation.

Cloning and expression of α-1,3-glucanases. Chromosomal DNA, which was isolated from *S. thermodiastaticus* HF3-3 by the general extraction method using phenol/chloroform, was used as a template DNA for PCR. We cloned two genes for α-1,3-glucanase 1 (AglST1) and α-1,3-glucanase 2 (AglST2) and designated as *aglst1* and *aglst2*, respectively. The primer AglST-F (5′-GGATCAGCATATGCACGGCACCACCAGAAC-3′) and the primer AglST1-R (5′-CGCAAGCTTTTCCGGGTTGCCCTGGCC-3′) were used to amplify *aglst1*, and the primers AglST-F and AglST2-R (5′-CGCAAGCTTGAAGGCCGTCGCGTACACCTC-3′) were used to amplify *aglst2*. The primers contained *Nde*I and *Hin*dIII restriction sites, indicated by underlining in the forward and reverse primers, respectively. PCR was performed in a reaction mixture of KOD-Plus-Neo kit (Toyobo, Japan). 50 μL of the PCR reaction contained 1.5 μL of each primer, 0.5 μL of *S. thermodiastaticus* HF3-3 DNA template, 5 μL of KOD buffer, 5 μL of dNTPs, 3 μL of $MgSO_4$ and 1 μL of KOD-Plus-Neo enzyme. Thermal cycling was one cycle of 94°C for 2 min, followed by 35 cycles at 98°C for 20 s and 72°C for 2 min. The PCR products (~2 kb) were digested with *Nde*I and *Hin*dIII, and then inserted into *Nde*I and *Hin*dIII sites of pET-21a(+) along with a His6 tag. The resultant plasmids were designed as pET21-aglST1 and pET21-aglST2, respectively. Each plasmid was introduced into *E. coli* Rosetta-gami B (DE3) cells. The transformants were cultured at 37°C under the conditions mentioned above. After the optical density at 600 nm reached 0.6–0.8, the cultures were cooled down, and isopropyl-β-D-

thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration 0.4 mM. The culture medium was further incubated at 15°C for 16–18 h.

Enzyme purification. After *E. coli* cells containing pET21-aglST1 or pET21-aglST2 were centrifuged at 12,000 rpm for 10 min at 4°C. The cell pellets were suspended with 10 mL of 50 mM Tris-HCl buffer (pH 7.4) containing 500 mM NaCl and 20 mM imidazole. The cell walls were disrupted by sonication, 15 s pulses at 45 s intervals, on ice. Cell debris was removed by centrifugation. The supernatants were applied to Ni Sepharose™ 6 Fast Flow column (GE Healthcare, Japan) equilibrated with the suspended buffer. The column was washed with 10 mL of washing buffer (50 mM Tris-HCl buffer, pH 7.4 containing 500 mM NaCl and 20 mM imidazole). Elution of the proteins was carried out with an elution buffer of 50 mM Tris-HCl buffer at pH 7.4 and containing 500 mM NaCl and 100 mM imidazole. Each fraction was collected separately, and then analyzed on a 10% SDS-PAGE. The active fractions were pooled, dialyzed against 10 mM citrate buffer (pH 5.5), and kept at –20°C until use. The purified enzymes were designated as rAglST1 and rAglST2, respectively.

Enzyme assay. The activities of rAglST1 and rAglST2 were determined by using α-1,3-glucan as a substrate. The reaction mixture contained 1% α-1,3-glucan, 50 mM citrate buffer (pH 5.5), and appropriate amounts of recombinant enzymes, and were incubated at 50°C for 30 min. The reaction was stopped by treating the reaction mixture at 100°C for 5 min. After centrifugation at 12,000 rpm for 2 min at 4°C to separate the undigested α-1,3-glucan, we used 250 μL of the resultant supernatant to determine the amount of reducing sugar by using the dinitrosalicylic (DNS) colorimetric method (Miller, 1959). One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (as glucose) per min.

Characterization of rAglST1 and rAglST2. The recombinant enzymes were incubated in a reaction mixture containing 1% α-1,3-glucan, 50 mM buffer of different pH values, and the enzyme at 50°C for 30 min, to determine the optimal pH. The enzyme was pre-incubated at 60°C for 1 h in 50 mM buffer of various pHs to determine the pH stability. After treatment, the residual activity was measured with 1% α-1,3-glucan as the substrate. The buffers used in the reaction were a citrate buffer (pH 4.0–6.0), a potassium phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 8.0–9.5). We measured the activity over a temperature range of 30–75°C for 30 min to determine the optimal temperature. The thermal stability was determined after the treatment of the enzyme across a temperature range of 30–75°C for 1 h in 100 mM citrate buffer (pH 5.5). The optimal concentration of NaCl for the enzyme activity was determined by incubating the reaction mixtures over a range of NaCl concentrations (0–20% w/v) at 50°C for 30 min. The NaCl tolerance was determined after incubating the enzyme in a buffer containing different concentrations of NaCl at 60°C for 1 h. The effect of metal ions on the enzyme activity was examined by adding monovalent cations (Na^+, K^+, Li^+, and Ag^+), divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}), and a trivalent cation (Fe^{3+}) in the reaction mixture at 1 mM in final concentration. To determine the effect of salts and surfactants on the enzyme activity, sodium fluoride (NaF), sodium dodecyl sulfate (SDS), and benzethonium chloride (BTC) were added to the reaction mixture at a final concentration of 0–1% (w/v) to determine the effect of salts and surfactants on the enzyme activity. The influence of other chemical reagents, such as NH_4^+ (at 1 mM), EDTA and DTT (at 1–50 mM) on the enzyme activity was also determined by incubating at 50°C for 30 min.

Thin layer chromatography assay. The hydrolysis product of rAglSTs for different reaction times (10 min, 30 min, 1 h, 12 h and 24 h) were spotted on the TLC Silica Gel 60 (Merck, Darmstadt, Germany) using 10% (w/v) of glucose, maltose, maltotriose, maltotetraose and maltopentaose as markers. The plate was developed in the chamber, which was saturated with a solution of distilled water: 1-butanol: acetic acid (1: 6: 8 v/v/v). TLC Silica Gel 60 was sprayed with a mixture of 5 mL sulfuric acid, 27 mL ethanol, 3 mL distilled water and 0.2 g orcinol, followed by heating on a hot plate at 100°C for 10–15 min.

Analytical methods. The protein concentration was measured by Lowry's method using egg albumin as the standard (Lowry et al., 1951). SDS-PAGE (10%) was performed by the protocol of Laemmli (1970) using the Pre-stained Protein Markers Broad Range (Nacalai Tesque, Japan) as markers.

Biofilm formation. Three 200 mL flasks with 75 mL of HI medium supplemented with 1% (w/v) sucrose were autoclaved at 121°C for 15 min. Afterward, we inoculated the flasks with *S. mutans* NBRC 13955. We added rAglST1 and rAglST2 to the culture medium at a final concentration 0.01 U/mL, respectively. We added 10 mM citrate buffer (pH 5.5) to the control instead of the enzyme. Five sets of three sterile glass plates were immersed into each flask, and were incubated at 37°C for 16 h under stationary conditions. Each set of glass plates was taken off of each flask after an incubation time of 0, 4, 8, 12, and 16 h. We immersed the plates in sterile, distilled water several times to remove excess cells attached to the glass plate. Biofilm conditions of the glass plate were examined using a Laser Scanning Microscope (LSM) (LEXT, Olympus, Japan). At the same time, we collected 1 mL of the culture from each flask to determine the number of *S. mutans* in HI agar with the drop plate method, and to estimate the released reducing sugar by the DNS method as previously described. Later, the biofilm on glass plates was dyed with alcian blue. After washing the alcian blue with 0.5 mL of 60% (v/v) acetic acid, the absorbance of the de-stained solution was measured at 340 nm (Wiater et al., 2004). Biofilm formation (%) was calculated by Eq. (1):

$$\text{Biofilm formation}\,(\%) = \frac{\text{absorbance at 340 nm of rAglST treated sample}}{\text{absorbance at 340 nm of rAglST non treated sample}} \times 100 \quad (1)$$

Biofilm degradation. We prepared a biofilm in advance on glass plates under the same conditions (without enzyme) as described in the biofilm formation assay. After a 16-h incubation, each set of glass plates was removed from the culture medium, immersed in sterile distilled water several times, and then transferred to the treatment solutions containing rAglST1 and rAglST2 (0.01 U/mL of enzyme at the final concentration and 50 mM citrate buffer, pH 5.5). All samples were incubated at 37°C for 16 h under stationary conditions. After incubation for 0, 4, 8, 12, and 16 h, each set of glass plates was removed, and the biofilm condition was examined through LSM. At the same time, 1 mL of the treatment solution was collected to determine the number of *S. mutans* in HI agar with the drop plate method, and to estimate the released reducing sugar. Later, each glass plate was dyed with alcian blue. After the plates were de-stained with 0.5 mL of 60% (v/v) acetic acid, the absorbance of the solution was measured with a spectrometer at 340 nm. Residual biofilm (%) was calculated using Eq. (1), as described above.

Biofilm degradation in the presence of toothpaste ingredients. The pre-formed biofilm was prepared as described above. Then, the set of glass plates was immersed in a treatment solution containing rAglST1 or rAglST2 (0.01 U/mL of enzyme at the final concentration), 1% (w/v) SDS, NaF and BTC in 50 mM citrate buffer (pH 5.5). Samples were incubated at 50°C for 30 min under stationary conditions. Each set of glass plates was removed, and the condition of the biofilm was exmined through LSM. The released reducing sugar in each solution was measured as a concentration of glucose equivalent. Each glass plate was dyed with alcian blue. After de-staining with 0.5 mL of 60% (v/v) acetic acid, the absorbance of the solution was measured with a spectrometer at 340 nm. Residual biofilm (%) was calculated with Eq. (1).

Reagents. Yeast extract and hipolypeptone were purchased from Nihon Seiyaku (Tokyo, Japan). HI medium was purchased from Nissui (Tokyo, Japan). The other pure grade chemical reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). The DNA primers and DNA sequence analysis were ordered in FASMAC, Co. Ltd. (Kanagawa, Japan).

Results

Purification of recombinant AglST

We purified rAglST1 and rAglST2 from *E. coli* Rosettagami B (DE3) with a Ni Sepharose™ 6 Fast Flow column. Each enzyme was completely eluted with 50 mM Tris-HCl buffer, pH 7.4 containing 500 mM NaCl and 20 mM imidazole. The active fractions of rAglST1 and rAglST2 were collected, dialyzed against 10 mM citrate buffer, pH 5.5, and stored at –20°C until further use. SDS-PAGE demonstrated that rAglST1 and rAglST2 were purified homogeneously (Figs. 1A and B). rAglST1 was purified 3.79-fold with a specific activity of 0.46 U/mg and a recovery of 62.5%. rAglST2 was purified 4.95-fold with a specific activity of 0.42 U/mg and a recovery of 44.7%.

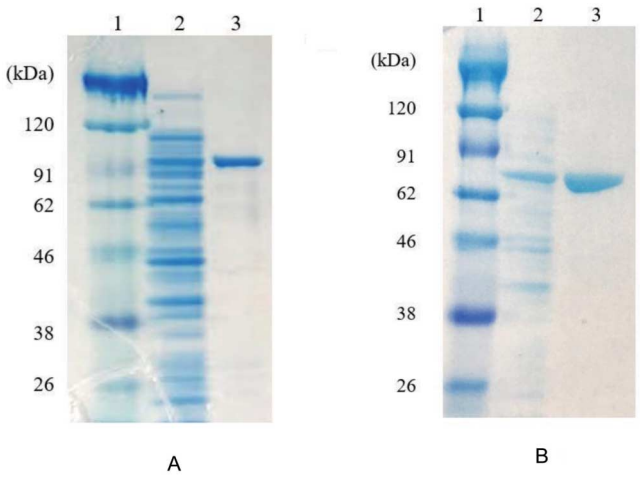

Fig. 1. SDS-PAGE of recombinant AglSTs.

SDS-PAGE (10%) was performed and the gel was stained with Coomassie Brilliant Blue R-250. A. rAglST2, B. rAglST1, Lane 1: marker, Lane 2: cell-free extract, Lane 3: Ni Sepharose™ 6 Fast Flow column.

Characterization of rAglST1 and rAglST2

The pH dependence of rAglST1 and rAglST2 were plotted as a bell-shaped histogram. Maximum activity was obtained at pH5.5, which is the same as that of wild-type Agls (Cherdvorapong et al., 2019; Suyotha et al., 2017). These results indicated that rAglST1 and rAglST2 retained more than 80% of the original activity over a pH range of 3.0–8.5 and 5.5–8.0, respectively. The effect of temperature was measured over a temperature range of 30–75°C. Maximum activity was observed at 65°C in the case of both recombinant enzymes. This agreed with previous research on AglST1, but was slightly higher in the case of AglST2 than in the previous reports (Cherdvorapong et al., 2019; Suyotha et al., 2017). Regarding thermal stability, the activities of both rAlgST1 and rAglST2 were retained at a greater than 80% activity after incubation at 65°C for 1 h. The activity of rAglST2 increased in the presence of NaCl at 5–10% (w/v), whereas the activity of rAglST1 slightly decreased when treated with 15–20% (w/v) NaCl and the activity was retained at greater than 80%.

As far as the effect of metal ions was concerned, the activity of rAglST1 and rAglST2 significantly decreased to less than 80% when compared to the original activity in the presence of Ag^{+}, Cu^{2+}, and Ni^{2+}. The activity of rAglST1 also decreased in the presence of K^{+}, but this did not affect the activity of rAglST2. The addition of Li^{+} and Mn^{2+} increased the relative activity in both recombinant enzymes, especially Mn^{2+}, which increased the enzyme activities by more than 200%. The presence of Ca^{2+} increased the activity of rAglST2, but did not affect the activity of rAglST1. When exposed to EDTA and DTT, the results revealed that a presence of 1 mM EDTA decreased the activities of both rAglST1 and rAglST2 to <80% and <50%, respectively. When exposed to 50 mM DTT, the activities of both enzymes slightly increased to almost 130%. The results of the general dental ingredients, NaF, SDS, and BTC, indicated that only BTC at 0.5–1.0% (w/v) decreased the activity of rAglST2 < 80%, whereas no inhibitory effect was observed in rAglST1. The other

Table 1. Characteristics of rAglST1 and rAglST2.

	rAglST1	rAglST2
Optimum pH	5.5	5.5
pH stability	3.0–8.5	5.5–8.0
Optimum temperature	65°C	65°C
Thermal stability	until 65°C	until 65°C
NaCl effect	Activated (5% w/v)	Activated (5–10% w/v)
Metal ions effect		
activators	Li^+, Mn^{2+}, DDT (50 mM)	Li^+, Ca^{2+}, Mn^{2+}, DDT (50 mM)
inhibitors	K^+, Ag^+, Cu^{2+}, Ni^{2+}, EDTA	Ag^+, Cu^{2+}, Ni^{2+}, EDTA
Toothpaste ingredients effect		
SDS	Activated	Activated (0.5% w/v)
NaF	Activated (0.25% w/v)	Activated (1% w/v)
BTC	Activated (0.25% w/v)	Inhibited (>0.5% w/v)
Cleavage type	endo-hydrolysis	endo-hydrolysis
Approximately molecular mass (kDa) by SDS-PAGE	higher than 65	higher than 91

chemicals, NaF and SDS, increased the activity of both enzymes to >120%.

The hydrolysis products of the rAglST1 and rAglST2 were observed by the TLC method and showed that rAglST2 and rAglST2 were endo-hydrolytic enzymes, equivalent to the wild-type enzymes. The summarized characteristics of rAglST1 and rAglST2 are shown in Table 1.

Effect of recombinant Agls on biofilm formation on a glass plate

To examine the effect of rAglSTs on biofilm formation, we added 0.01 U/mL of each recombinant enzyme to the batch culture medium attached to glass plates by the inoculation of *S. mutans* pre-culture. The result showed that the rAglSTs effectively inhibited biofilm formation. A lower amount of biofilm on glass plate than the control was clearly observed after a 12-h incubation, with 79% and 65% in the presence of rAglST1 and rAglST2, respectively. An almost 50% reduction of biofilm was observed when compared with the glass plate of the control after a 16-h incubation, without any difference between rAglsT1 and rAglST2 (Fig. 2A). After a 12-h incubation, the reducing sugar in the enzyme-treated samples was approximately 7 mM higher than the control, without any difference between rAglsT1 and rAglST2 (Fig. 2B). The number of *S. mutans* cells in the medium increased normally following an s-curve growth in both the non-treated and enzyme-treated samples. In addition, the number of *S. mutans* cells in enzyme-treated samples were higher than the control after incubation for 4 h. The same trends were seen until the end of the incubation (Fig. 2C).

The visual examination by LSM showed a clear difference between the enzyme-treated samples and the non-treated sample under the glass surface condition after an 8-h incubation. A biofilm of the non-treated sample was formed on both the glass plates and the flask surface. In comparison, some biofilm in the enzyme-treated samples was loosened and detached from the glass plates and flask surface into the culture medium in which we could observe small particles (data not shown). From the observation with LSM, only *S. mutans* could be seen on the glass plates in the enzyme-treated samples after incubation for 8 h. After a 12-h incubation, an increasing amount of *S.*

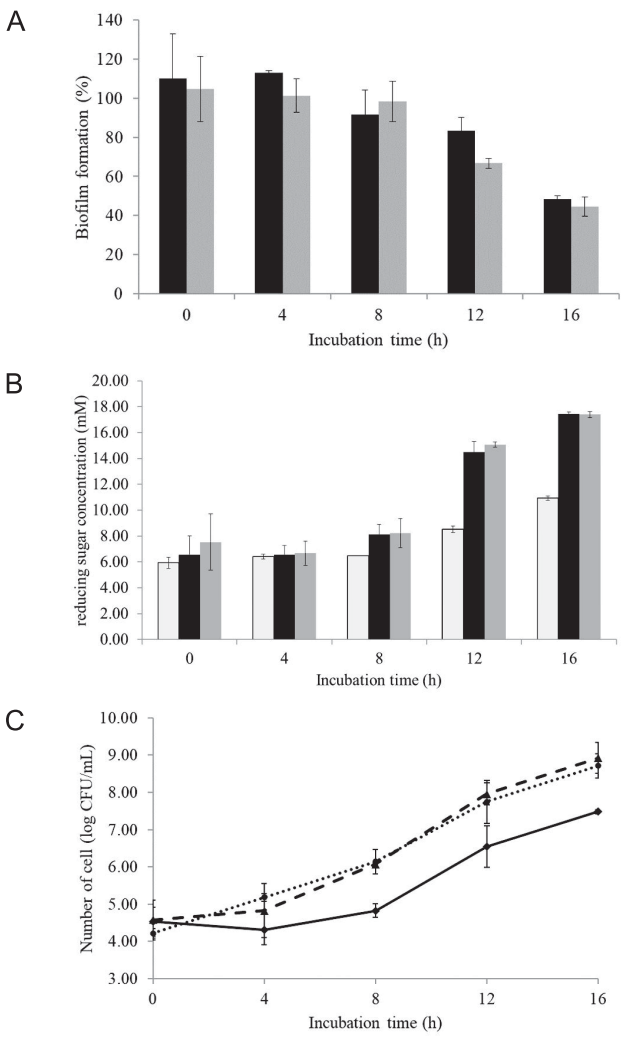

Fig. 2. Effect of recombinant AglSTs on biofilm formation.

A. Rate of biofilm formation in each incubation period. Black and gray bars represent rAglST1- and rAglST2-treated samples, respectively. Values are expressed relative to biofilm formation of the control (non-treated sample) as 100% at each incubation time. B. The releasing of reducing sugar in each incubation period. White, black, and grey bars represent the control (non-treated samples), rAglST1- and rAglST2-treated samples, respectively. Values are expressed as mM of glucose equivalent. C. The number of *S. mutans* cells in the medium in each incubation period. Solid line, dashed, and dotted lines represent the control (non-treated samples), rAglST1- and rAglST2-treated samples, respectively. Data are presented as the mean ± standard errors of the mean (SEM) of five independent experiments.

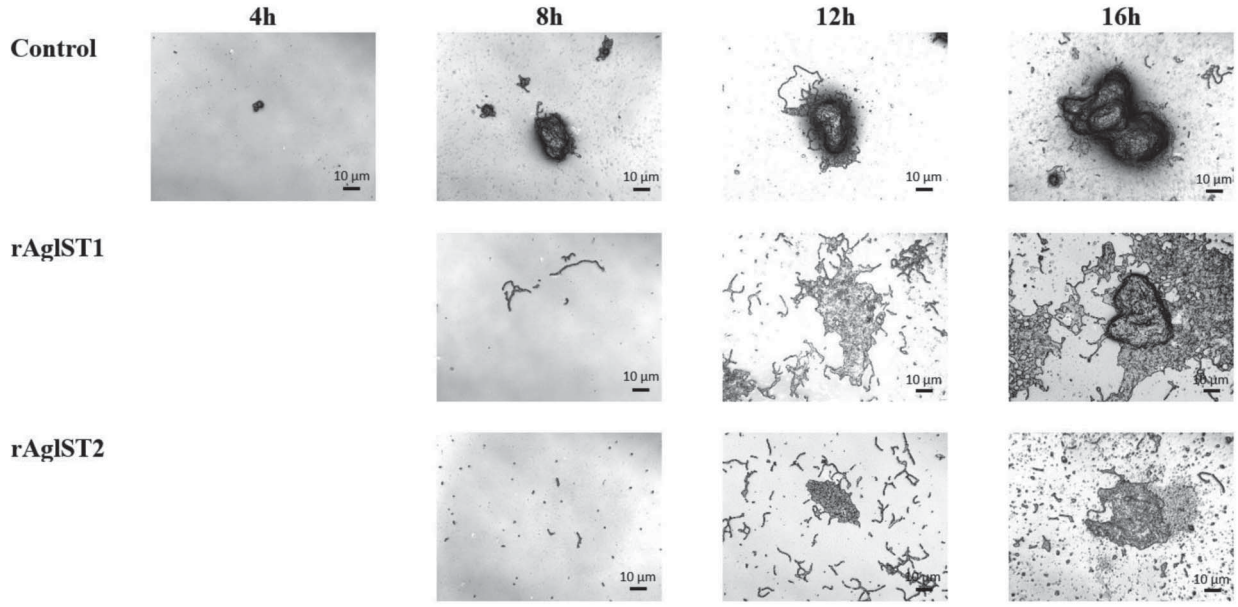

Fig. 3. Laser scanning microscopy of biofilm formed by *S. mutans*.
The control (non-treated sample), rAglST1- and rAglST2-treated samples were incubated for 4, 8, 12, and 16 h, respectively.

mutans cells formed a pellicle. The mature structure of the biofilm was clearly observed surrounding with pellicle and *S. mutans* cells after incubation for 16 h. On the other hand, the biofilm of the non-treated sample started to form after an 8-h incubation, and the size of the biofilm increased in proportion to the incubation time. In summary, even though biofilm could form on all glass plates after a 16-h incubation, the thickness of enzyme-treated biofilm was thinner than non-treated biofilm (Fig. 3).

Effect of recombinant Agls on degrading biofilm on glass plate

The residual biofilm on the plate was measured to estimate the effect of rAglST1 and rAglST2 on the degradation of mature biofilm on glass plate. The decrease of biofilm was clearly observed after an incubation for 8 h to less than 60% of the original in the case of both rAglST1 and rAglST2. At the end of the incubation, rAglST1 and rAglST2 could decrease the biofilm to almost 40% of the control (Fig. 4A). Up to 4 h, the concentration of the reducing sugar produced from the degradation of the biofilm on the glass plate by rAglST2 increased rapidly. After an 8-h incubation, the concentration of reducing sugar of the sample treated by rAglST2 once decreased. The concentration of the reducing sugar of the sample treated by rAglST1 gradually increased and kept increasing for up to 16 h. On the other hand, the concentration of reducing sugar of the sample treated by rAglST2 apparently stopped increasing (Fig. 4B). The population of *S. mutans* in the sample solution treated by rAglST2 was rather higher than that of the sample solution treated by rAglST1 (Fig. 4C). It is thought that rAglST2 could degrade biofilm to the reducing sugars which *S. mutans* in the medium might use for growth more quickly than that formed from biofilm degradation by rAglST1. Therefore, *S. mutans* could grow better in the sample solution treated by rAglST2 than by rAglST1.

From LSM observations, the mature biofilms were gradually degraded after treatment with rAglST1 or rAglST2. The pellicle and the adhered *S. mutans* cells were detached from the glass plate. This resulted in a cleaner background on the glass plate after an 8-h incubation than the original background. Finally, only thinner biofilms remained after a 16-h incubation (Fig. 5).

Effect of rAglST1 and rAglST2 on biofilm degradation with the combination of toothpaste ingredients.

We wanted to evaluate the practical uses of these enzymes. The effect of α-1,3-glucanases on the degradation of a mature biofilm in the presence of mixed toothpaste ingredients (SDS, NaF, and BTC) was evaluated. Since rAglST1 and rAglST2 retained their activities after incubation at high temperatures as previously reported, rAglST1 and rAglST2 (0.01 U/ml of final concentration) were tested in a mixture of 1% (w/v) of SDS, NaF, and BTC, then incubated at 50°C for 30 min. After incubation, the result of alcian blue absorption showed rAglSTs could decrease the amount of biofilm by approximately 60% compared with non-treated samples, with no difference between rAglST1 and rAglST2 (Fig. 6A). After a 30-min incubation, a reducing sugar of 0.65 mM was generated in the enzyme-treated samples, whereas it was not observed in the non-treated sample (Fig. 6B). The difference in the biofilm quantity formed on the glass plate could be seen visually. In detail, the density of biofilm between non-treated and enzymes-treated samples was clearly different in the LSM spectroscopy observations (Fig. 7).

Discussion

The genes that encode α-1,3-glucanase from *S. thermodiastaticus* HF3-3 were successfully cloned and expressed in *E. coli* Rosetta-gami B (DE3). The purified recombinant α-1,3-glucanases, designated rAglST1 and

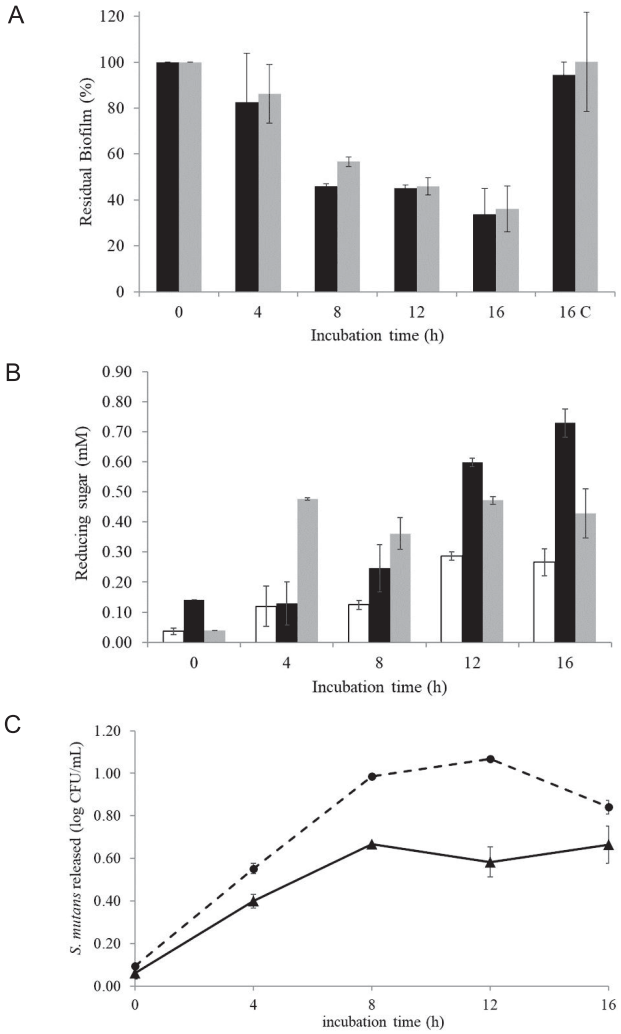

Fig. 4. Effect of recombinant AglSTs on biofilm degradation.

A. Survival rate of biofilm in each incubation period. Black and gray bars represent rAglST1- and rAglST2-treated samples, respectively. Values are expressed relative to biofilm degradation of the control (16C; 16-h incubated samples without any enzymes) as 100%. B. The releasing of reducing sugar in each incubation period. White, black, and grey bars represent the control (non-treated samples), rAglST1- and rAglST2-treated samples, respectively. Values are expressed as mM of glucose equivalent. C. The number of *S. mutans* cells comparing rAglST1-treated with rAglST2-treated in each incubation period. Black line and dashed-line represent rAglST1- and rAglST2-treated samples, respectively. Data are presented as the mean ± standard errors of the mean (SEM) of five independent experiments.

rAglST2, were obtained in a one-step purification using a Ni Sepharose^{TM} 6 Fast Flow column according to the C-terminal His-tagged. As we reported in a previous study, AglST1 is generated from AglST2 by proteolytic truncation of the C-terminus region. It was interesting to identify the function of this truncated region (~400 aa). Both rAglST1 and rAglST2 were characterized, and compared for their effectiveness on biofilm formation and degradation.

Comparing the characteristic of rAglST1 and rAglST2, both enzymes showed similarities in properties such as optimum pH, optimum temperature, thermostability, the effect of NaCl, and endo-type hydrolysis. However, they differed slightly in some properties, including pH stability, the effect of metals as activators and inhibitors, and the effect of benzethonium chloride (BTC). rAglST1 retained more than 80% of its activity in a pH range of 3.0–8.5, whereas rAglST2 activity spanned a range of 5.5–8.0. Moreover, it was found that Ca^{2+} increased the activity of rAglST2, whereas it did not affect the activity of rAglST1. K^{+} decreased the activity of rAglST1, but did not have any effect on rAglST2. On the contrary, the presence of 0.5–1.0% of BTC decreased the activity of rAglST2 without any effect on rAglST1. These differences might be derived from the truncated C-terminus region. Similar information of the slightly different characteristics between two kinds of α-1,3-glucanases, or mutanases, from the same species has been reported. However, those reports do not explain about any structural information (Guggenheim and Haller, 1972; Tsunoda et al., 1977). We have been undertaking X-ray crystallographic analysis of AglFH1, one of the isozymes of α-1,3-glucanases from *Paenibacillus glycanilyticus* FH11, and obtained same results, including a part of three-dimensional structure of AglFH1 (unpublished data). The 3D structure obtained showed that the region located adjacent to catalytic domain contained one calcium ion. In the case of AglFH1, Glu-X-Glu, Ser-X-X-Ser, and Asp in the region have been shown to involve Ca^{2+} binding. In the case of AglFH2, the isozyme of AglFH1, Asp-X-Asp, Ser-X-X-Lys, and Asp are found in the corresponding region. In the C-terminal region of AglST2, Asp^{626}-Gly^{627}-Asp^{628}, Ser^{683}-Ser^{684}-Phe^{685}-Ser^{686}, and Asp^{711} are present in the sequence (DDBJ Accession No. LC317049). These results suggest that these amino acid residues might be involved in the formation of the Ca^{2+} binding site. At present, X-ray crystallographic analysis of AglST2 is underway.

We have investigated the effects of both rAglST1 and rAglST2 on the formation and degradation of *S. mutans* biofilm. As shown in Fig. 3, both rAglST1 and rAglST2 especially suppressed biofilm formation during incubation for 12 h even though the concentration of the enzymes was low (0.01 U/mL). *S. mutans* uses sucrose to produce a biofilm of α-1,3-glucan and releases fructose in the medium as a byproduct (Colby and Russell, 1997). Accordingly, when rAglST1 or rAglST2 was added to the medium in the course of biofilm formation, glucose and α-1,3-glucosyloligosaccharides were released into the medium by hydrolytic action of the enzyme on the biofilm under formation, and they exhibited a reducing ability as with fructose. In addition to an increase of reducing sugar in the medium with reaction time, the number of *S. mutans* cells was also increased in the medium by suppressing the habitation of *S. mutans* in the biofilm when it was treated with rAglST1 or rAglST2 (Fig. 2).

Considering previous reports, the study on α-1,3-glucanase in the application of biofilm removal were basically in a combination of commercial dextranase. Oral biofilm containing pathogenic microorganisms was effectively removed after a 3-h incubation with an enzymatic mixture of 0.25 U/mL mutanase from *T. harzianum* F-340 and 1 U/mL commercial dextranase, and was completely removed after a 6-h incubation with the same mixture (Wiater et al., 2008). A favorable result also reported in the study of Shimotsuura et al. (2008) that 1.4 U/mL of

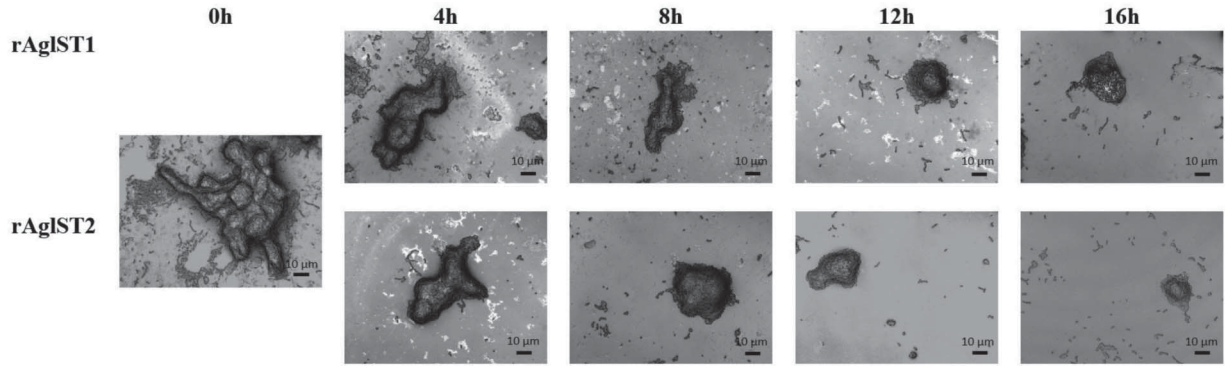

Fig. 5. Laser scanning microscopy of *S. mutans* biofilm degradation.
The biofilm formed was incubated in the solution of rAglST1 and rAglST2 for 0, 4, 8, 12, and 16 h, respectively.

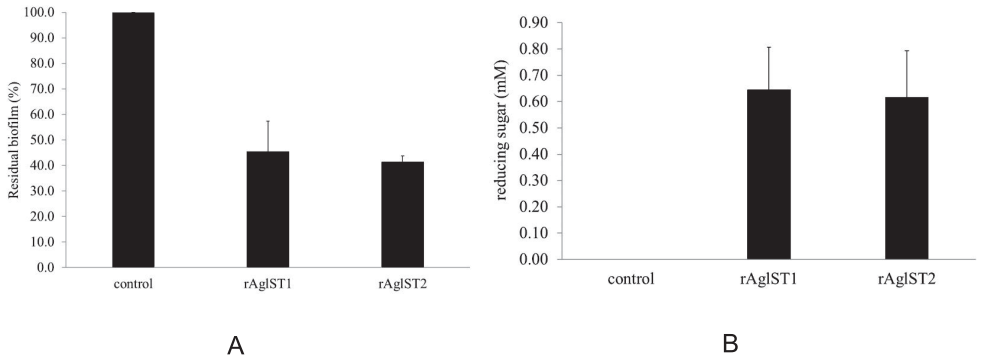

Fig. 6. Effect of recombinant AglSTs on biofilm degradation in the presence of toothpaste ingredients.
Survival rate of biofilm (A) and the concentration of released reducing sugar (B) after 30-min incubation at 50°C in the presence of AglSTs and 1% (w/v) of SDS, NaF, and BTC. The reactions were performed according to the procedures described in Materials and Methods. Values are expressed as mM of glucose equivalent. Data are presented as the mean ± standard errors of the mean (SEM) of five independent experiments. Control: distilled water (without AglSTs)

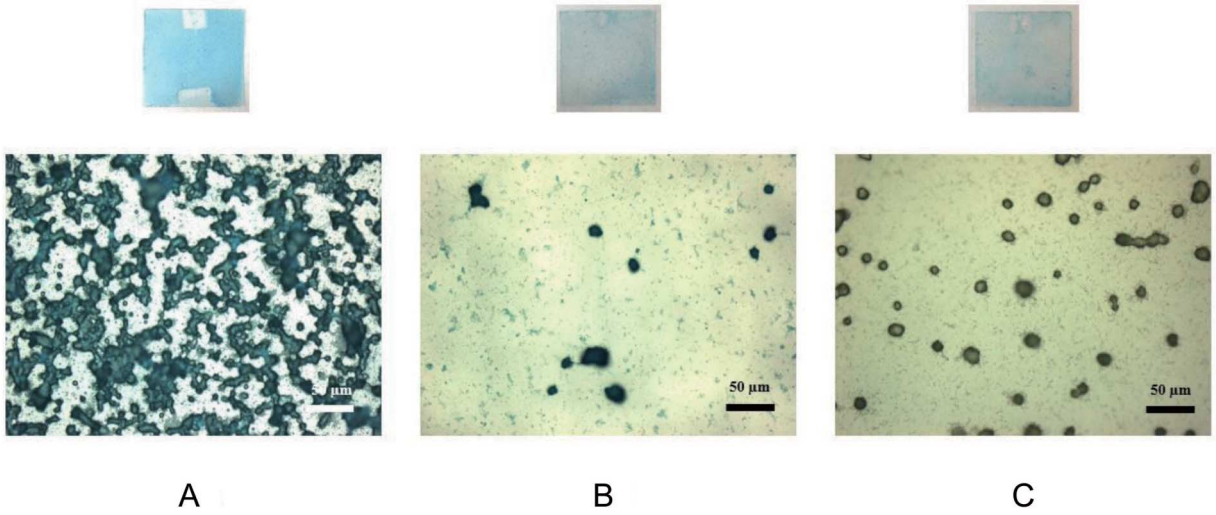

Fig. 7. Laser scanning microscopy of *S. mutans* biofilm degradation on the glass plates.
S. mutans biofilm on the glass plate was incubated in distilled water (A), rAglST1 treatment with 1% (w/v) of SDS, NaF and BTC (B), rAglST2 treatment with 1% (w/v) of SDS, NaF and BTC (C) 30 min at 50°C.

recombinant mutanase from *Paenibacillus* sp. showed biofilm degrading activity comparable to that of wild-type enzyme after incubation for 6 h. Another study on the effect of mutanase from *T. harzianum* F-470 and commercial dextranase (Sigma-Aldrich, USA) both individually and in combination were reported. The most effective prevention was shown with the combination of 0.3 U/mL mutanase and 5 U/mL dextranase, which decreased the accumulation of streptococcal film to about 99.5% in biofilm formation, and 95% in biofilm degradation (Wiater et al., 2004). To our knowledge, there are few reports on the biofilm degradation in short-time experiments (Pleszczyńska et al., 2010; Shimotsuura et al., 2008). There are no reports on the effect of the combination of α-1,3-glucanase and toothpaste ingredients on biofilm degradation. The synergistic result of rAglSTs and a present of 1% (w/v) SDS, NaF and BTC combining at a high temperature (50°C) indicated that the decrease of biofilm was greater than 50%, compared with the control, after an incubation of only 30 min. The higher temperature and some toothpaste ingredients could increase the activity of rAglSTs, which is characteristic of these enzymes. These ingredients reduced the strength of attachment of the biofilm on glass plates which may improve the hydrolysis efficiency of rAglSTs. Our demonstration revealed that rAglSTs can be applied in toothpaste and possibly adopted in related products such as mouthwash. Further, it should be noted that the concentration of both rAglST1 and rAglST2 in this experiment was only 0.01 U/mL. A presence of dextranase would provide a synergistic effect due to its cleavage of a minor α-1,6-glycosidic bond in *Streptococcus biofilm*, which leads to an increase in the decomposition efficiency in dental plaque.

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