

Full Paper

Enzymatic and molecular characterization of  $\alpha$ -1,3-glucanase (AglST2) from *Streptomyces thermodiastaticus* HF3-3 and its relation with  $\alpha$ -1,3-glucanase HF65 (AglST1)

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Extracellular  $\alpha$ -1,3-glucanase HF90 (AglST2), with a sodium dodecyl sulfate (SDS)-PAGE-estimated molecular mass of approximately 91 kDa, was homogeneously purified from the culture filtrate of *Streptomyces thermodiastaticus* HF3-3. AglST2 showed a high homology with mycodextranase in an amino acid sequence and demonstrated specificity with an  $\alpha$ -1,3-glycosidic linkage of homo  $\alpha$ -1,3-glucan. It has been suggested that AglST2 may be a new type of  $\alpha$ -1,3-glucanase. The optimum pH and temperature of AglST2 were pH 5.5 and 60°C, respectively. AglST2 action was significantly stimulated in the presence of 5–20% (w/v) NaCl, and 1 mM metal ions Mn<sup>2+</sup> and Co<sup>2+</sup>. On the other hand, it was inhibited by 1 mM of Ag<sup>+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup> and Ni<sup>2+</sup>. Regarding the stability properties, AglST2 retained more than 80% of its maximum activity over a pH range of 5.0–7.0 at up to 60°C and in the presence of 0–20% (w/v) NaCl. Based on these results, the properties of AglST2 were comparable with those of AglST1, which had been previously purified and characterized from *S. thermodiastaticus* HF3-3 previously. The N-terminal amino acid sequence of AglST2 showed a good agreement with that of AglST1, suggesting that AglST1 was generated from AglST2 by proteolysis during cultivation. MALDI-TOF mass analysis suggested that AglST1 might be generated from

AglST2 by the proteolytic removal of C-terminus polypeptide (approximately 20 kDa). Our investigation thus revealed the properties of AglST2, such as tolerance against high temperature, salts, and surfactants, which have promising industrial applications.

**Key Words:**  $\alpha$ -1,3-glucan;  $\alpha$ -1,3-glucanase; characterization; mycodextranase; *Streptomyces thermodiastaticus* HF3-3

Introduction

$\alpha$ -1,3-glucan is a water-insoluble polysaccharide comprising a homopolymer of glucose with  $\alpha$ -1,3-glycosidic linkages. It is present in dental plaque and the fungal cell wall. To form dental plaque, the normal bacterial flora of the human oral cavity, principally *Streptococci* spp., including *Streptococcus mutans*, *S. sanguis*, and *S. salivarius*, produce various kinds of polysaccharides.  $\alpha$ -1,3-glucan, one of the main components of dental plaque, which accounts for approximately 20% of its total component, is synthesized by the catalysis of glucosyltransferase (GTF) using sucrose as the substrate (Ismail et al., 2006; Marotta et al., 2002).  $\alpha$ -1,3-glucan is involved in the development of cariogenic bacteria and other bacterial accumulation on tooth surfaces, which indicate the beginning of dental caries that consequently

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develops into severe periodontitis (Ismail et al., 2006).

Besides being the main component of dental plaque,  $\alpha$ -1,3-glucan plays physiological and biochemical roles in various fungi. The amount of  $\alpha$ -1,3-glucan in the cell wall varies with fungal strains.  $\alpha$ -1,3-glucan contributes to the overall strength of the cell wall structure and to the avoidance of host recognition as a part of the host immune systems (Choma et al., 2013; Reese et al., 2007; Synytsya et al., 2009). In some fungi,  $\alpha$ -1,3-glucan accumulates during vegetable growth as an endogenous carbon source and is involved in the formation of capsule in pathogenic fungi, with a function facilitating host cell invasion (Reese and Doering, 2003).

Previous reports have indicated that  $\alpha$ -1,3-glucanase (Agl; EC 3.2.1.59; or mutanase) is a promising enzyme for application in the decrease of dental plaque accumulation, control of pathogenic fungi, and preparation of fungal protoplast (Pleszczynska et al., 2015; Sanz et al., 2005; Yano et al., 2006). Agl hydrolyzes  $\alpha$ -1,3-glucan by recognizing the  $\alpha$ -1,3-glycosidic linkage. Agl was first reported in *Trichoderma harzianum* (Guggenheim and Haller, 1972) and then in other sources of both fungi and bacteria. Agl can be classified into two types: Glycoside Hydrolase family 71 (GH71) and Glycoside Hydrolase family 87 (GH87), as described in the Carbohydrate Active enzyme (CAZy) database based on their amino acid similarity (<http://www.cazy.org/>). GH71 Agl has been mainly detected in fungi, whereas GH87 Agl has been isolated only from bacteria. GH71 Agl from *T. harzianum* uses the inverting mechanism to hydrolyze the substrate (Grun et al., 2006; Suyotha et al., 2016), whereas the catalytic mechanism of GH 87 Agl remains unclear. With consideration of its application in commercial products, it is important to regulate Agl activity to counter its stability. Thermal stability is considered to be an important yardstick that helps to evaluate the enzyme stability. Therefore, to develop a thermostable enzyme, microorganisms have been screened for the production of Agl with a high thermal stability. In a previous study, *Streptomyces thermodiastaticus* HF3-3, which produces thermostable Agl, was isolated from native soil. Interestingly, this strain was found to produce two proteins with Agl activity but with different molecular masses. Agl with an apparent molecular mass of 65 kDa (designed as AglST1 in this study) has previously been purified and characterized by Suyotha et al. (2017).

In this paper, we have reported the enzymatic properties of an 1,3-glucanase with apparent molecular masses of 91 kDa (designed as AglST2) and compared them with those of AglST1. In addition, we have described the primary structure of AglST2 obtained from the genomic analysis of *S. thermodiastaticus* HF3-3 and examined the relationship between AglST1 and AglST2.

## Materials and Methods

**$\alpha$ -1,3-glucan preparation.**  $\alpha$ -1,3-glucan was prepared using the GTF plasmid expressed in *Escherichia coli* Rosetta Gami B (DE3), as described previously (Suyotha et al., 2013). The recombinant cell was incubated in an auto induction medium broth (composition: 0.5% yeast extract, 1.0% hipolypeptone, 0.33%  $(\text{NH}_4)_2\text{SO}_4$ , 0.68%

$\text{KH}_2\text{PO}_4$ , 0.05% glucose, 0.20% lactose, 0.015%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.71%  $\text{Na}_2\text{HPO}_4$ ) at 30°C for 36 h. Cells were collected by centrifugation at 10,000 rpm for 10 min, followed by resuspension with 10 mM Tris-HCl buffer (pH 8.0) and the disruption of the pellet by sonication (15-s pulses at 45-s intervals) on ice. After centrifugation under the same conditions, the supernatant was dialyzed against the same buffer overnight. Next, 100 mL of the supernatant was mixed with 3 L of 20% (w/v) sucrose solution in 50 mM potassium-phosphate buffer (pH 7.0). This solution was incubated at 30°C for 3–4 d, and the subsequently synthesized  $\alpha$ -1,3-glucan was treated with 1.5 N NaOH. After adjusting to the neutral pH by adding 6 N HCl, the  $\alpha$ -1,3-glucan solution was precipitated with 95% ethanol at 4°C overnight. Finally,  $\alpha$ -1,3-glucan was collected and lyophilized. The resultant  $\alpha$ -1,3-glucan powder was stored at room temperature until further use (Suyotha et al., 2013).

**Microorganism and culture conditions.** *S. thermodiastaticus* HF3-3 previously isolated from the soil in the Shiga prefecture, Japan (Suyotha et al., 2017), was used for enzyme production. *S. thermodiastaticus* HF3-3 was incubated for pre-culturing in 10 mL LB broth (pH 7.0) for 16–18 h. Then, 500  $\mu\text{L}$  of the pre-culture was inoculated into 20 mL of  $\alpha$ -1,3-glucan supplement medium (1.0%  $\alpha$ -1,3-glucan, 0.05%  $\text{K}_2\text{HPO}_4$ , 0.05%  $\text{KH}_2\text{PO}_4$ , 0.01% yeast extract, 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0001%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.05% KCl, pH 5.0) and incubated on a rotary shaker at 100 rpm for 3 d at 50°C. The culture broth was collected by centrifugation at 10,000 rpm for 10 min at 4°C.

**Enzyme purification.** The culture broth was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and was applied on the DEAE-Cellufine column (7.5  $\times$  1.8 cm) equilibrated with the same buffer. After washing the column with the same buffer, it was developed with a series of the buffer containing 50, 100, 125 and 150 mM NaCl. The active fractions eluted with the buffer containing 100 mM NaCl were pooled, and dialyzed against the same buffer. The enzyme obtained was lyophilized and dissolved in 1 mL of 10 mM Tris-HCl buffer (pH 8.0), followed by application on the HiTrap Q HP-GE column (1 mL) equilibrated with the same buffer. This column was then developed with a linear gradient of 0–200 mM NaCl by ÄKTAprime plus (GE healthcare, UK). The active fractions were collected and dialyzed against 10 mM citrate buffer (pH 5.5), and the purified enzyme was used for protein analysis and the evaluation of the enzymatic properties.

**Agl activity assay.** Agl activity was determined by using  $\alpha$ -1,3-glucan as the substrate. The solutions of 1%  $\alpha$ -1,3-glucan, 50 mM citrate buffer (pH 5.5) and appropriate amounts of Agl were incubated at 50°C for 30 min. The reaction was stopped by incubating 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  $\alpha$ -1,3-glucan, 250  $\mu\text{L}$  of the resultant supernatant was used to determine the amount of reducing sugar as per the dinitrosalicylic colorimetric method (Miller, 1959). One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1  $\mu\text{mol}$  of reducing sugar (as glucose) per min.

**Table 1.** Purification of AglST2 from *S. thermodiastaticus* HF3-3.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	10.740	34.86	0.30	100.0	1.00
DEAD-Cellufine A500	5.044	0.78	6.47	47.0	21.00
HiTrap Q HP-GE	0.042	0.03	1.40	0.4	4.55

To determine the substrate specificity, Agl was incubated in 2% of various substrates including  $\alpha$ -1,3-glucan, cellulose, xylan, dextran, amylopectin, laminarin, amylose, and nigeran according to the activity assay as described above.

**Effects of pH, temperature and NaCl on the enzyme activity and stability.** The optimal pH for enzyme activity was determined by incubating the reaction mixture containing the enzyme, 1%  $\alpha$ -1,3-glucan, and 50 mM buffer of different pH values at 50°C for 30 min. To determine the pH stability, the enzyme was pre-incubated at 60°C for 1 h in 50 mM buffer of various pHs. After treatment, the residual activity was measured with 1%  $\alpha$ -1,3-glucan as the substrate. The buffers used for the reaction were citrate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 6.0–8.0), and This-HCl buffer (pH 8.0–9.5). To determine the optimal temperature, the activity was measured over a temperature range of 30–75°C for 1 h in 100 mM citrate buffer (pH 5.5). The thermal stability was determined after treatment of the enzyme across a temperatures range of 30–75°C for 1 h. The optimal NaCl concentration for the activity was measured by incubating the reaction mixtures with a range of NaCl concentrations (0–20% w/v) at 50°C for 30 min. The NaCl tolerance was determined after incubation of the enzyme in the buffer containing different concentrations of NaCl at 60°C for 1 h.

**Effects of metal ions, surfactants, and chemical reagents on the enzyme activity.** The effect of metals ions on the enzyme activity was examined by adding monovalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Li}^+$ , and  $\text{Ag}^+$ ), divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Ni}^{2+}$ ), and a trivalent cation ( $\text{Fe}^{3+}$ ) in the reaction mixture at 1 mM. 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). The influence of other chemical reagents, such as  $\text{NH}_4^+$  (at 1 mM), EDTA and DTT (at 1–50 mM), on the enzyme activity was also investigated.

**Analytical methods.** The protein concentration was measured by Lowry's method, using egg albumin as the standard (Lowry et al., 1951). In column chromatography, the protein concentration was measured by determining the absorbance at 280 nm. SDS-PAGE (10%) was performed by the method of Laemmli (1970) using the Pre-stained Protein Markers Broad Range (Nacalai Tesque, Japan) as marker. The N-terminal amino acid sequence of the enzyme was determined as described in a previous report (Suyotha et al., 2017). The molecular mass was determined using MALDI-TOF mass spectrometry (Autoflex Speed, Burkert Daltonics K.K., Kanagawa, Japan) with a matrix comprising 2,5-dihydroxybenzoic acid.

**Thin-layer chromatography assay.** The hydrolysis product of Agl 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. Next, 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 at 100°C for 10–15 min.

**Reagents.** Yeast extract and hipolypeptone were purchased from Nihon Seiyaku (Tokyo, Japan). The other pure grade chemical reagents were purchased from Nacalai tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Co. (Osaka, Japan).

## Results

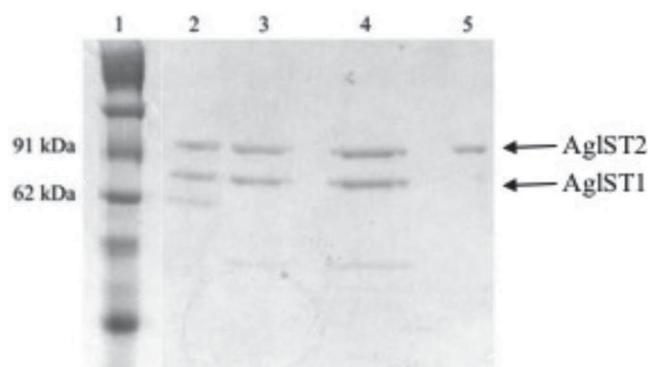
### Purification of Agl

AglST2 was separated from the other proteins by two-step column chromatography: DEAE-Cellufine A500, followed by HiTrap Q HP-GE. On the DEAE-cellufine A500 column, active fractions were eluted with 100 mM NaCl in 10 mM Tris-HCl buffer (pH 8.0). In this step, the activity fractions contained both AglST1 and AglST2. These proteins were further separated by the HiTrap Q HP-GE column to obtain the homogeneity of AglST2. From the HiTrap Q HP-GE, the fraction of AglST2 was collected, dialyzed against 10 mM citrate buffer (pH 5.5) and stored at –20°C until further use. The enzyme was purified 4.55-fold with a specific activity of 1.40 U/mg and a recovery of 0.4% (Table 1). SDS-PAGE confirmed that AglST2 was homogeneously purified and its molecular weight was slightly greater than 91 kDa (Fig. 1).

### Characterization of Agl

The specificity of AglST2 against various glycosidic linkage substrates including  $\alpha$ -1,3-glucan ( $\alpha$ -1,3-), cellulose ( $\beta$ -1,4-), xylan ( $\beta$ -1,4-), dextran ( $\alpha$ -1,6-), amylopectin ( $\alpha$ -1,4- and  $\alpha$ -1,6-), laminarin ( $\beta$ -1,3- and  $\beta$ -1,6-), amylose ( $\alpha$ -1,4-), and nigeran ( $\alpha$ -1,3- and  $\alpha$ -1,4-) was determined. AglST2 showed specificity toward  $\alpha$ -1,3-glucan and nigeran with relative activities of 100% and 10%, respectively. The other substrates were inert. AglST2 preferentially hydrolyzes on the  $\alpha$ -1,3-glycosidic linkage. Consequently, AglST2 should be categorized as Agl.

The optimum pH and temperature of AglST2 both appeared as a bell-shaped histogram. The maximum activity was obtained at pH 5.5 (Fig. 2A), which is the same as that of AglST1 (Suyotha et al., 2017). To evaluate the pH stability, the residual activities of AglST2 after pre-incubation with various pHs were determined at 50°C. The



**Fig. 1.** SDS-PAGE.

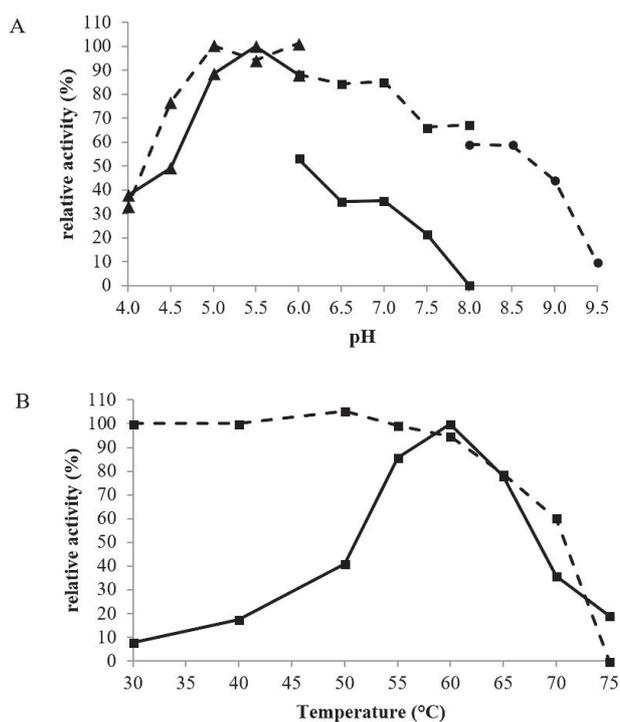
SDS-PAGE (10%) of AglST2 in each purification step was stained with Coomassie Brilliant Blue R-250. Lane 1: marker, Lane 2: culture filtrate, Lane 3: DEAD-Cellufine A500, Lane 4: Lyophilization, Lane 5: HiTrap Q HP-GE.

result indicated that AglST2 retained 80% of the original activity over a pH range of 5.0–7.0; however, the activity gradually decreased at a pH > 7.5 (Fig. 2A). The effect of temperature was estimated over a temperature range of 30–75°C. The maximum activity was observed at 60°C (Fig. 2B), which is slightly lower than that of AglST1 (Suyotha et al., 2017), which reached the highest activity at 65°C. For the thermal stability, the residual activity of AglST2 was >80% after incubation at 65°C for 1 h (Fig. 2B). The activity of AglST2 increased in the presence of NaCl with a maximum activity achieved at 15% (w/v) NaCl, with a relative activity >140%. In addition, AglST2 retained a full activity even when treated with 20% (w/v) NaCl for 1 h (data not shown).

Figure 3 shows the effect of metals ions. The activity of AglST2 significantly decreased to 57%, 44%, 55%, and 64% of the original activity, respectively in the presence of  $\text{Ag}^+$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Ni}^{2+}$ . On the other hand, the addition of  $\text{Mn}^{2+}$  and  $\text{Co}^{2+}$  increased the relative activity of AglST2 to >140%. In the case of other chemicals, such as EDTA and DTT, the results indicated that 1–50 mM EDTA decreased the relative activities by almost 50%, whereas DTT did not significantly affect AglST2 activity.

As mentioned earlier, the main application of Agl so far has been studied on dental plaque removal as well as on the lysis of the fungi cell wall. Therefore, the effect of general dental ingredients, including NaF, SDS, and BTC, was further examined. The results indicated no significant effect of benzethonium chloride and SDS on AglST2 activity, whereas NaF was found to promote the enzyme activity. These properties of AglST2 for oral care chemicals were comparable with those of AglST1 (data not shown).

The hydrolysis products of the AglST2 reaction were determined using  $\alpha$ -1,3-glucan as the substrate. Maltose and maltotriose were the major hydrolytic products of the AglST2 reaction, respectively (data not shown). These oligomers could be observed after 10 min of incubation, and they obviously appeared after 12 h of incubation. Similarly, the fade band of glucose also appeared after 12 h of incubation. These results indicate that AglST2 is an endo-hydrolytic enzyme. The properties of both AglST1 and AglST2 are summarized in Table 1.



**Fig. 2.** Effect of pH and temperature on AglST2 activity.

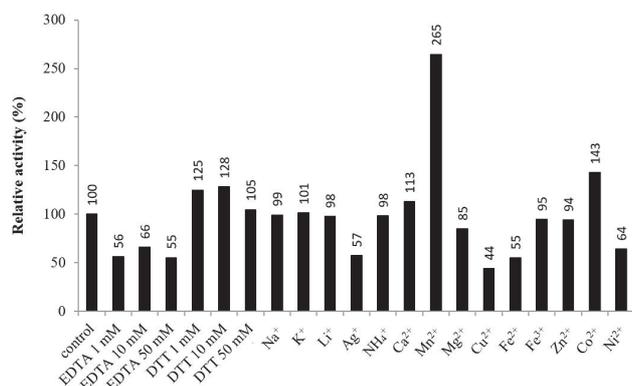
The effect of pH (solid line) and pH stability (dashed line) on AglST2 activity (A). Triangle, square and circle stand for a series of citrate buffer (pH 4.0–6.0), a series of potassium phosphate buffer (pH 6.0–8.0), and a series of Tris-HCl buffer (pH 8.0–9.5), respectively. The effect of temperature (solid line) and thermal stability (dashed line) on AglST2 activity (B).

#### ***Analysis of the amino acid sequence of Agl from *S. thermodiastaticus* HF3-3***

The *N*-terminal amino acid sequence of AglST2 was determined to be AATAGADL, which was the same as that of AglST1 reported previously (Suyotha et al., 2017). Interestingly, only one gene encoding the protein of the *N*-terminal amino acid sequence, AATAGADL, was detected in the *S. thermodiastaticus* HF3-3 genomic sequence database (data not shown). This result indicates that AglST1 and AglST2 are derived from the same structural gene. However, the molecular weight of AglST1 was significantly lower than that of AglST2, suggesting that the C-terminal region of AglST1 may be truncated during cultivation. In the present study, the amino acid sequence of AglST2 (DDBJ Accession No. LC317049) was reported, and it comprised 741 amino acids with major compositions of 12.4% alanine, 11.1% threonine and 10.9% glycine (Fig. 4). The predicted molecular mass of AglST2 based on the amino acid sequence was 76.86 kDa. On the other hand, the molecular mass of AglST1 obtained from MALDI-TOF mass spectrometry analysis was 57.08 kDa (data not shown).

#### ***Comparison of amino acid sequences between AglST2 and the related enzymes***

The comparison between the amino acid sequences of AglST2 and those of mycodextranases registered in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) was performed using the Standard Protein BLAST program. The



**Fig. 3.** Effect of metal ions and chemicals on AglST2 activity.

Enzyme activity was determined in the presence of 1 mM final concentration of metal ions and final concentrations of 1, 10 and 50 mM of EDTA and DDT.

amino acid sequence of AglST2 showed a high similarity to mycodextranase from *Streptomyces* sp. NRRL F-5122 (WP\_059131821.1), *Streptomyces* sp. SAT1 (WP\_064536246.1), and *S. mirabilis* (WP\_037733756.1) with 83%, 81%, and 80% identities, respectively. However, the comparison with the mycodextranase characterized from *Streptomyces* sp. J-13-3 revealed only a 39% identity. Further comparisons with the mycodextranase from the other strains besides *Streptococcus* sp., including *Kutzneria albida* DSM 43870 (AHH97105.1), *Micromonospora lupini* str. Lupac 08 (CCH22333.1), *Stigmatella aurantiaca* DW4/3-1 (EAU64732.1), and *Catenulispora acidiphila* DSM 44928 (ACU73519.1), indicated 50%, 43%, 38% and 39% identities, respectively. Notably, no similarity of the AglST2 amino acid sequence to those of the known Agls was observed with BLAST searches. However, the homology search performed through the Conserved Domain Search Service from NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) demonstrated that AglST2 comprised two conserved modules that are frequently found among bacterial Agls. One module found in the *N*-terminal amino acid sequence (Ala<sup>1</sup>-Glu<sup>597</sup>) of AglST was similar to the catalytic domain at the *C*-terminus of Agls with approximately 20% identity. In contrast, the *C*-terminal amino acid sequence (Pro<sup>598</sup>-Ala<sup>741</sup>) of AglST was found to be similar to the discoidin domain II at the *N*-terminus of Agls with approximately a 30% identity. These results indicate a slight homology between AglST and the known Agls. Evolutionary relationships between mycodextranase including AglST and Agls are illustrated in Fig. 5.

## Discussion

The total Agl activity was the highest after *S. thermodiastaticus* HF3-3 was cultivated at 50°C for 3 d. The change of protein bands corresponding to AlgST1 and AglST2 was monitored using SDS-PAGE (data not shown). The density of the AglST1 protein band remained high until the fourth day of incubation, whereas that of the AglST2 protein band sharply decreased in the same time. Although, the altering mechanism between these Agls

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1 MHGTRTRPAA RRRLSAIGAA VALAAGMLVA LGAPTAQAAP AAQAAPAAQ A ATAGADLPPT
61 SVEAESATTT GTKIGPDYTO GTLASEASGR QAVRLDAGQR VEFTVPRAN ALTVAYSVPD
121 GQSGTLDDVYV NGTKLDRSLT VTSKYSYVDT GWIPGAKTHH FYDNTRLLGG RDVQAGDVTV
181 LQATNVQVTV DVADFEQVSA AAGQPAGSVS VTDKGDPTG QGDSTQAFRD AIAAAQGGVV
241 WIPPGDYRIT GPLSGVQVNT LQGAGSVYSV VSHSHPIDQT DSAGHVHLKD FAVIGEVTER
301 VDSSPDNFVN GSLGPGSSVS GMWQHVKVG LWLGTNDDL VVENNRILDT TADGLNLNGT
361 AKNVTVRDNF LRNQGDDALA MWSLYAPDTD CRFENNTITQ PNLANGIALY GGTDTITVKN
421 LISDTNALGS GIAISNOKFA EPPHPLAGTI TVDGNLTVRT GAINPNWNHP MGALRVDSYD
481 SAIEARVDIT DTTITDPSYS AFEFVSGGGQ GHAVKNVTVD GAAVKNTGIV VVQAEAPGEA
541 TFRNVTATGT GAAGIYNCFP PSGSGTFTVT DGGNSGWDT TWSDCSTWPO PQQGNPEPDP
601 NRNLAQGRPA TATGSQDVYT PGKAVDGDAT TWESANNAF PQSLTVDLGA PRAVRRLVLK
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721 VTANTGWFPAG QLSEVEVYAT A

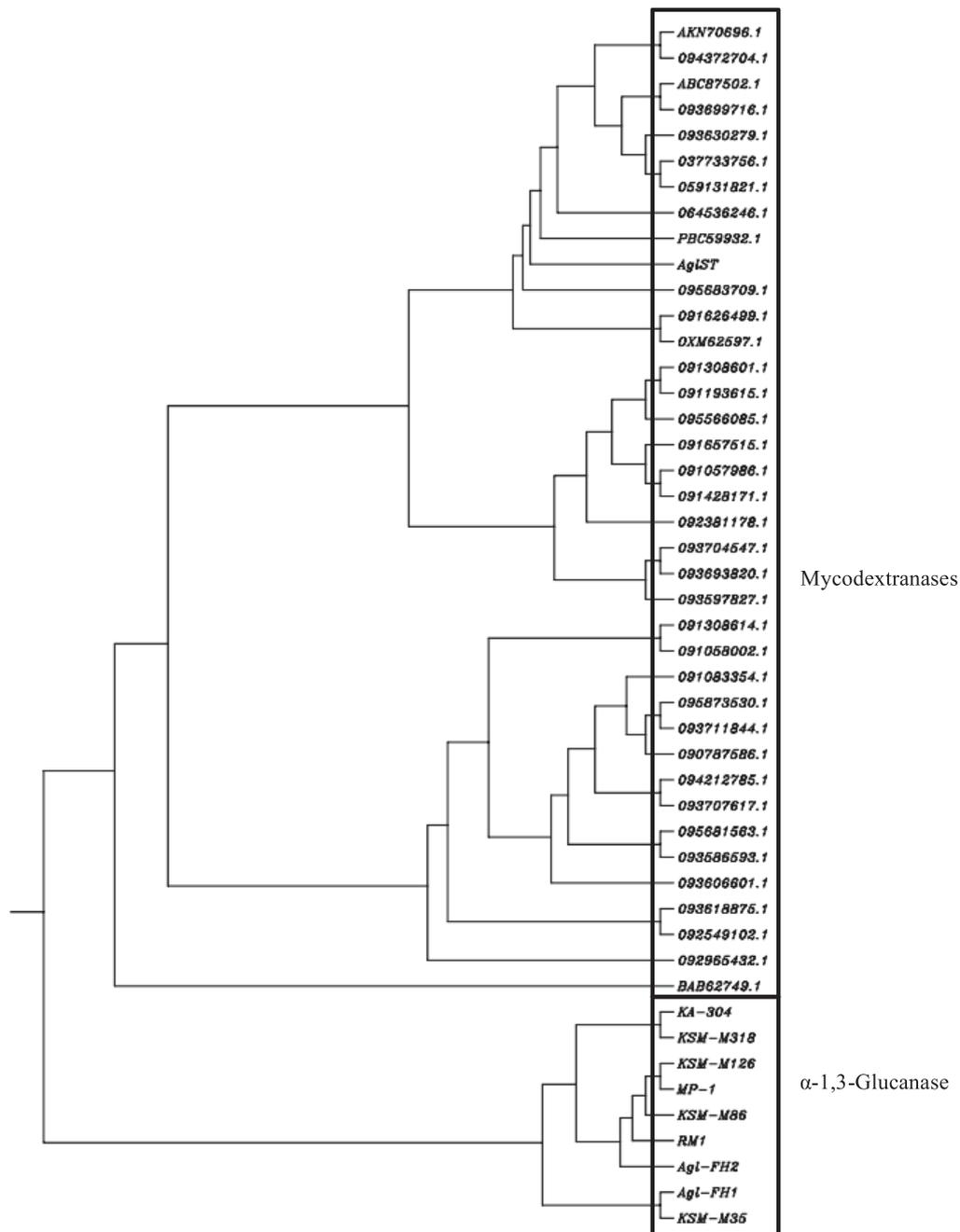
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**Fig. 4.** Amino acid sequence of AglST from *S. thermodiastaticus* HF3-3.

The amino acid sequences are indicated by single letters. The numbering of the amino acid sequence starts from the initial codon of the AglST gene (LC317049). The single underline represents the *N*-terminal amino acid sequence, the double underline reveals the internal amino acid sequence. The black triangle represents the signal peptide cleavage site, and the white triangle indicates the proteolytic cleave point.

is not well understood, a possible explanation is the proteolytic truncation from AglST2 to AglST1. A similar phenomenon has previously been reported in a study of mutanase from *Paenibacillus* sp. strain KSM-M86: the upstream region of the *N*-terminal sequence of the native enzyme was truncated by proteolysis, resulting in a decrease in the molecular mass from 116 kDa to 57 kDa (Sumitomo et al., 2007). The predicted molecular mass of AglST2 from the amino acid sequence was 76.86 kDa, whereas the molecular mass estimated using SDS-PAGE was slightly >91 kDa (Fig. 1). Furthermore, the molecular mass of AglST1 estimated using SDS-PAGE was also >62 kDa (Fig. 1), whereas that of AglST1, estimated using the MALDI-TOF mass spectrometer, was 57.08 kDa. The higher shift of molecular mass on SDS-PAGE can be related to the presence of positively-charged amino acids in the enzyme, which slow the migration during the run in SDS-PAGE, resulting in a higher molecular mass (Hames, 1998). The percentages of positively-charged amino acid residues in AglST1 and AglST2 were 6.1% and 5.7%, respectively. These values are greater than that of Agl (Agl-KA) from *Bacillus circulans* KA-304, the normally migrating protein in SDS-PAGE, which has 4.7% positively-charged amino acid residues (Suyotha et al., 2013).

Based on the molecular mass of AglST1 (57.08 kDa), the putative truncated point of AglST1 can be estimated using a calculation based on the amino acid sequence (Fig. 6). The cleavage site was between glutamic acid at position 597 and proline at position 598. The potential protease which is responsible for hydrolyzing the peptide linkage between Glu and Pro with a high possibility in the sequence of Gly(P3)-Asn(P3)-Pro(P2)-Glu(P1)-Pro(P'1)-Asp(P'2)-Pro(P'3) was predicted to be glutamyl endopeptidase II (GluII, EC 2.4.21.82). Glutamyl endopeptidases can be isolated from *Streptomyces* species, such as *S. griseus* (GluSGP) (Yoshida et al., 1988) and *S. fradiae* ATCC 14544 (GluSF) (Kitadokoro et al., 1993). The en-



**Fig. 5.** Phylogenetic tree of mycodextranases and  $\alpha$ -1,3-glucanases.

The amino acid sequences of mycodextranases and  $\alpha$ -1,3-glucanases were aligned using the CLASTALW software. The phylogenetic tree was constructed by using maximum likelihood analysis based on the alignment. Abbreviations and accession numbers for each sequence of mycodextranases are as follows: AKN70696.1, *Streptomyces* sp. PBH53; 094372704.1, *Streptomyces* sp. FBKL.4005 (WP\_094372704.1); ABC87502.1, *Streptomyces* sp. NRRL 30748; 093699716.1, *Streptomyces* sp. MnatMP-M27 (WP\_093699716.1); 093630279.1, *Streptomyces* sp. 3213 (WP\_093630279.1); PBC59932.1, *Streptomyces* sp. Tue6028; 095683709.1, *Streptomyces* sp. CLI2509 (WP\_095683709.1); 091626499.1, *Amycolatopsis saalfeldensis* (WP\_091626499.1); OXM62597.1, *Amycolatopsis* sp. H5; 037733756.1, *Streptomyces mirabilis* (WP\_037733756.1); 059131821.1, *Streptomyces* sp. NRRL F-5122 (WP\_059131821.1); 064536246.1, *Streptomyces* sp. SAT1 (WP\_064536246.1); 091308601.1, *Micromonospora chersina* (WP\_091308601.1); 091193615.1, *Micromonospora narathiwatensis* (WP\_091193615.1); 095566085.1, *Plantactinospira* sp. KBS50 (WP\_095566085.1); 091657515.1, *Micromonospora auratinigra* (WP\_091657515.1); 091057986.1, *Micromonospora humi* (WP\_091057986.1); 091428171.1, *Micromonospora tulbaghiaae* (WP\_091428171.1); 092381178.1, *Xiangella phaseoli* (WP\_092381178.1); 093704547.1, *Streptomyces* sp. 2131.1 (WP\_093704547.1); 093693820.1, *Streptomyces* sp. ScaeMP-e83 (WP\_093693820.1); 093597827.1, *Streptomyces jietaisiensis* (WP\_093597827.1); 091308614.1, *Micromonospora chersina* (WP\_091308614.1); 091058002.1, *Micromonospora humi* (WP\_091058002.1); 091083354.1, *Nonomuraea wenchangensis* (WP\_091083354.1); 095873530.1, *Streptomyces* sp. TLI\_235 (WP\_095873530.1); 093711844.1, *Streptomyces alni* (WP\_093711844.1); 090787586.1, *Asanoa ishikariensis* (WP\_090787586.1); 094212785.1, *Streptomyces* sp. 2R (WP\_094212785.1); 093707617.1, *Streptomyces* sp. 2131.1 (WP\_093707617.1); 095681563.1, *Streptomyces* sp. CLI2509 (WP\_095681563.1); 093586593.1, *Streptomyces* sp. LcepLS (WP\_093586593.1); 093606601.1, *Streptomyces indicus* (WP\_093606601.1); 093618875.1, *Actinoplanes philippinensis* (WP\_093618875.1); 092549102.1, *Actinoplanes derwentensis* (WP\_092549102.1); 092965432.1, *Agromyces* sp. CF514 (WP\_092965432.1); and BAB62749.1, *Streptomyces* sp. J-13-3. Abbreviations and accession numbers for each sequence of  $\alpha$ -1,3-glucanases are as follows: KA-304, *Bacillus circulans* KA-304 (AB248056); KSM-M318, *Paenibacillus* sp. KSM-M318 (MuE, AB292235); Agl-FH2, *Paenibacillus glycanilyticus* FH11 (AB898297); KSM-M126, *Paenibacillus* sp. KSM-M126 (MuC1, AB257603); RM1, *Bacillus* sp. RM1 (E16590); MP-1, *Paenibacillus curdianolyticus* MP-1 (HQ640944); KSM-M66, *Paenibacillus* sp. KSM-M66 (AB257602); KSM-M35, *Paenibacillus* sp. KSM-M35 (AB257601); and Agl-FH1, *Paenibacillus glycanilyticus* FH11 (AB898296). AglST represents  $\alpha$ -1,3-glucanase from *S. thermodiastaticus* HF3-3.

**Table 2.** Comparison of characteristics between AglST1 and AglST2.

Characteristics	AglST1*	AglST2
Substrate specificity	$\alpha$ -1,3-glucan ( $\alpha$ -1,3-glycosidic linkage)	$\alpha$ -1,3-glucan ( $\alpha$ -1,3-glycosidic linkage)
Specific activity	2.32 U/mg	1.40 U/mg
Optimum pH	5.0–5.5	5.5
pH stability	4.5–6.5	5.0–7.0
Optimum temperature	65°C	60°C
Thermal stability	until 65°C	until 60°C
NaCl effect	activity was slightly increased at 5% (w/v)	activity was increased at 15% (w/v)
Metal ions effect		
activators	Co <sup>2+</sup> , Mn <sup>2+</sup> , Li <sup>+</sup> , Ca <sup>2+</sup>	Co <sup>2+</sup> , Mn <sup>2+</sup>
inhibitors	Ag <sup>+</sup> , Cu <sup>2+</sup>	Ag <sup>+</sup> , Ni <sup>+</sup> , Cu <sup>2+</sup> , Fe <sup>2+</sup>
Toothpaste ingredients effect	no effect	activity was increased at SDS 1.0% (w/v)
Cleavage type	endo-hydrolysis	endo-hydrolysis
Molecular mass	65 kDa (SDS-PAGE)	>91 kDa (SDS-PAGE)
	57.08 kDa (MALDI-TOF mass)	76.86 kDa (amino acid sequence)
N-terminal amino acid sequence	AATAGADL	AATAGADL

\*Data of AglST1 characteristics sourced from Suyotha et al. (2017).

zyme preferentially cleaves glutamic acid and proline at the P2 position. However, GluSGP activity decreases when proline or aspartic acid possesses the P'1 position (Stennicke and Breddam, 2013). According to this information, it can be considered that proline at position P2 contributes to the cleavage of glutamic acid at the P1 position, whereas proline at position P'1 decelerates the cleavage, consequently leading to the gradual increase in the activity of AglST1.

The optimum pH for AglST2 was also comparable with that reported in previous studies on Agl and mutanase from the other strains, which had an optimum pH in the acidic region (pH 4.5–7.0); for instance, *Trichoderma harzianum* CCM F-470, *T. asperellum* CECT 20539, and *Aspergillus nidulans*. However, the common remarkable property of AglST1 and AglST2 was the optimum temperature, which was in a comparatively high temperature range compared with that reported in previous researches (optimum temperature, between 40–55°C) (Pleszczynska et al., 2010, 2015). Because *S. thermodiastaticus* HF3-3 is classified as a thermophilic *Streptomyces*, it potentially produces AglST1 and AglST2 which exhibit optimum activity and stability at high temperatures (Haki and Rakshit, 2003; Kim et al., 1999).

Interestingly, the result of the effect of metal ions on AglST2 was in agreement with those of Agls and reported in previous studies, which are generally susceptible to the metal ions, particularly Cu<sup>2+</sup> and Fe<sup>2+</sup> (Sumitomo et al., 2007). The effect of EDTA on AglST2 was also different from that reported in a previous study on mutanase from *Paenibacillus* sp., where no effect of 10 mM EDTA was observed on mutanase (Sumitomo et al., 2007). Based on the observed DTT and EDTA properties, it can be concluded that AglST2 does not contain any disulfide bonds in the crucial parts that directly affect enzyme activity. Furthermore, some metal ions may be important in the stimulation of AglST2 activity resulting from the EDTA effect. NaF, SDS, and BTC are generally used in the dental industry for different purposes. NaF represents a fluoride source, which is the basic ingredient in dental care

products, due to its function in the prevention of tooth decay and enamel erosion. SDS is used as a detergent and surfactant, and BTC is normally used as an antimicrobial agent (<http://www.ada.org/en/member-center/oral-health-topics/toothpastes>). The pH of a healthy human oral system falls within the neutral range (5.6–7.9), within which the normal flora can function normally. In contrast, an acidic pH (pH < 5.5) facilitates the growth of acidic bacteria, which encourages enamel erosion, ultimately leading to severe dental problems (Majeed et al., 2011; Marsh et al., 2015). In addition, the average pH of toothpastes is neutral (pH 6.0–7.0) (Majeed et al., 2011). AglST2 is stable at this pH range and can thus be effectively used in the production of commercial dental care products aimed at the biofilm and dental plaque reduction.

Mycodextranase generally hydrolyzes on the  $\alpha$ -1,4-glycosidic linkage of glycosyl oligosaccharide containing both of  $\alpha$ -1,3- and  $\alpha$ -1,4-glycosidic linkage, suggesting that these linkages are required in the binding and cleavage processes (Tung et al., 1971). On the other hand, AglST2 demonstrated specificity with an  $\alpha$ -1,3-glycosidic linkage of  $\alpha$ -1,3-glucan proportion; this property is shared by Agl. Thus, AglST2 from *S. thermodiastaticus* HF3-3 can be classified in a new group of Agls.

In conclusion, the second Agl, AglST2, obtained from *S. thermodiastaticus* HF3-3 was purified and characterized. It was clarified to be the parental protein of AglST1 and was almost comparable with AglST1 regarding properties. Similarly to AglST1, AglST2 also possessed a high stability against temperature and chemical reagents. The characteristics of this enzyme are advantageous for an industrial application, for which a high stability is required. In addition, the amino acid sequence of AglST2 was found to be highly homologous with the amino acid sequences of the known mycodextranases, which specifically hydrolyze only  $\alpha$ -1,4-glycosidic linkages in polysaccharides comprising  $\alpha$ -D-glucose units alternatively linked (1 → 3) and (1 → 4). Overall, our results have indicated that Agls from *S. thermodiastaticus* HF3-3 are eligible for placement in a new group of Agls.

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