Full Paper

Functional analysis of α -1,3-glucanase domain structure from *Streptomyces thermodiastaticus* HF3-3

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α -1,3-Glucanase from *Streptomyces thermodiastaticus* HF3-3 (Agl-ST) has been classified in the glycoside hydrolase (GH) family 87. Agl-ST is a multi-modular domain consisting of an N-terminal β -sandwich domain (β -SW), a catalytic domain, an uncharacterized domain (UC), and a C-terminal discoidin domain (DS). Although Agl-ST did not hydrolyze α -1,4-glycosidic bonds, its amino acid sequence is more similar to GH87 mycodextranase than to α -1,3-glucanase. It might be categorized into a new subfamily of GH87. In this study, we investigated the function of the domains. Several fusion proteins of domains with green fluorescence protein (GFP) were constructed to clarify the function of each domain. The results showed that β -SW and DS domains played a role in binding α -1,3-glucan and enhancing the hydrolysis of α -1,3-glucan. The binding domains, β -SW and DS, also showed binding activity toward xylan, although it was lower than that for α -1,3-glucan. The combination of β -SW and DS domains demonstrated high binding and hydrolysis activities of Agl-ST toward α -1,3-glucan, whereas the catalytic domain showed only a catalytic function. The binding domains also achieved effective binding and hydrolysis of α -1,3-glucan in the cell wall complex of *Schizophyllum commune*.

Key Words: α -1,3-glucanase; α -1,3-glucan-binding activity; domain function; mycodextranase; *Streptomyces thermodiastaticus*

Introduction

α -1,3-Glucan, a water-insoluble linear α -1,3-linked homopolymer of glucose, is the main component of extracellular polysaccharides synthesized from sucrose from *Streptococcus mutans* and *S. sobrinus* by glycosyltransferases (GTFs) (GTF-I and GTF-SI) (Krzyściak et al., 2014; Marotta et al., 2002). GTF-I synthesizes mostly insoluble glucan with an α -1,3-glycosidic bond, and GTF-SI synthesizes a mixture of insoluble glucans with an α -1,3-glycosidic bond and an α -1,6-glycosidic bond. GTF-I and GTF-SI have been considered to be the most important GTFs, which are involved in the production of the major component of dental plaque in humans. Such insoluble glucans facilitate the accumulation of carcinogenic bacteria, and other bacteria and, consequently, enhance biofilm formation on dental surfaces, which causes dental caries, gingivitis, and periodontitis that, in turn, lead to cardiovascular disease, rheumatoid arthritis, and osteoporosis (Dervis, 2005; Griffin et al., 2009; Kinane et al., 2017; Pleszczyńska et al., 2015; Wiater et al., 2013).

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Fig. 1. Primary structure of Agl-ST from *S. thermodiastaticus* HF3-3 and its domain architecture.

The single underline represents the *N*-terminal amino acid sequence. The black triangle represents the signal peptide cleavage site, and the white triangle indicates the proteolytic cleavage point. The light gray region, black region, white region, and dark gray region represent β -SW (aa 58–195), catalytic domain (aa 209–426), uncharacterized region (aa 427–608), and discoidin domain (aa 609–735), respectively.

α -1,3-Glucan has been found in both ascomycetous and basidiomycetous fungi (Sipiczki et al., 2014). In *Aspergillus nidulans*, α -1,3-glucan was found during vegetative growth and shown to play an important role in sexual development as an endogenous carbon source (Yoshimi et al., 2017; Zonneveld, 1972). In *Schizosaccharomyces pombe*, α -1,3-glucan has roles in secondary septum formation and primary septum robustness during cell separation (Grün et al., 2005; Hochstenbach et al., 1998; Suyotha et al., 2016). Moreover, α -1,3-glucan is related to the virulent factors of pathogenic yeast, for example, in *Cryptococcus neoformans*, which is pathogenic in humans. In the fungus *Magnaporthe oryzae* (Bacon et al., 1968; Reese et al., 2007), a pathogen causing rice blast, α -1,3-glucan is used during invasion not only to protect the fungal cell wall from degradative enzymes secreted by the host but also to conceal chitin to delay the innate immune response in plants (Fujikawa et al., 2012).

α -1,3-Glucanase is an enzyme that hydrolyzes the α -1,3-glycosidic bond of α -1,3-glucan, and has been stud-

ied for the removal of dental plaque and as a biological control agent of pathogenic fungi (Suyotha et al., 2016). This enzyme is classified into two types, fungal and bacterial, which are referred to as the glycoside hydrolase (GH) families 71 and 87, respectively.

In the previous studies, we have purified and characterized two types of α -1,3-glucanase (Agl-ST), Agl-ST (previously referred to as Agl-ST2) and the catalytic unit of Agl-ST (CatAgl-ST, previously referred to as Agl-ST1) from *Streptomyces thermodiastaticus* HF3-3, which are derived from the same gene. We clarified that Agl-ST could be categorized as a new group of α -1,3-glucanase (Cherdvorapong et al., 2019; Suyotha et al., 2017). Hakamada et al. (2008) and Suyotha et al. (2013) reported that GH 87 enzymes have a multi-domain structure containing α -1,3-glucan-binding modules. For example, α -1,3-glucanase from *Bacillus circulans* KA-304 (Agl-KA) consists of *N*-terminal discoidin domain I (DS1), carbohydrate-binding module 6 (CBM6), threonine and proline repeats, discoidin domain II (DS2), uncharacterized do-

Fig. 2. Schematic of the structure of domain GFP fusion proteins.

β -SW, β -sandwich; UC, uncharacterized domain; DS, discoidin domain; GFP, green fluorescence protein.

main (UC), and C-terminal catalytic domain. Meanwhile, Agl-ST consists of four domains, namely, N-terminal β -SW, a catalytic domain, UC, and C-terminal DS. Figure 1 shows the domain architecture of Agl-ST made on the basis of the amino acid sequence reported previously (Cherdvorapong et al., 2019). Comparison of Agl-ST with the related enzymes revealed a high similarity to mycodextranase, whereas it had a low identity with the known α -1,3-glucanases. However, Agl-ST is classified into α -1,3-glucanase based on its properties.

In this study, we constructed a fusion protein of putative domain with green fluorescence protein (GFP) described in Fig. 2 and evaluated the function of each domain of Agl-ST from *S. thermodiastaticus* HF3-3 by glucan binding and hydrolysis assays.

Materials and Methods

Microorganisms and culture conditions. *Escherichia coli* XL10-gold was used as a host for the construction of recombinant plasmids containing a DNA fragment of domain GFP-fusion proteins. It was cultivated in 5 ml of Luria Bertani (LB) broth (0.5% yeast extract, 1% hipolypepton, and 1% NaCl (pH 7.0)) containing 50 μ g/ml ampicillin at 37°C for 18 h. *E. coli* Rosetta-gami B (DE3), which was used for the expression of proteins, was cultivated at 37°C for 18 h in 5 ml of LB broth (pH 7.0) containing 50 μ g/ml ampicillin, 34 μ g/ml chloramphenicol, and 20 μ g/ml kanamycin. *S. commune* IFO 4928 was grown at 30°C for 4 days in 5 ml of Medium C containing 2% glucose, 1% hipolypepton, 0.3% yeast extract, 0.3% K₂HPO₄, and 5 mg/l thiamine, pH 7.0. For production on a large scale, the mycelial pellet was blended with a blender at high speed for 1 min (Waring no. 7009), after which it was transferred to 250 mL of Medium C and incubated with shaking at 100 rpm and 30°C for 4 days. The pellet was collected by centrifugation at 13,000 rpm for 20 min, after which it was washed with distilled water three times before lyophilization. The dried pellet was collected and ground as a powder, after which it was used as a substrate for analyzing α -1,3-glucanase activity.

Preparation of GFP fusion proteins. The GFP gene was prepared from the pQBI-25fN1 plasmid as a template, as described previously (Suyotha et al., 2013). The recombinant plasmids were designed as pET- β -SW/Cat/UC/DS-GFP, pET- β -SW/Cat/UC-GFP, pET-Cat/UC/DS-

Table 1. Oligonucleotide primers use for construction of deletion enzymes fusion with GFP.

Primer name	Oligonucleotides
NdeI β -SW	5' GAGCATCATAT GCAGGCCGCGACCGCGGGC 3'
BamHI β -SW	5' CCTCCGGGATCCCTCGAAGTCGGCGAC 3'
NdeICat	5' GGCGCCCATATGTCGGTCTCCGTCACCGAC 3'
BamHCat	5' GCGCCGGGATCCCGCGTTGGTGTCCGA 3'
NdeIUC	5' GCGCCACATATGACCAACGCGCTGGGC 3'
BamHUC	5' GCGCCGGGATCCCGCGCCGCGCTGGGC 3'
NdeIDS	5' ACCGGTCATATGCGGCCGCGCCACCGCC 3'
BamHDS	5' GTATA GGGATCCCTCCACCTCGCTGAG 3'
GFPnBamH	5' AGGAAGCTTCGGGATCCCGGGGAGGCGGA 3'
GFPcXhoI	5' GAATAGGGC CCTCGAGTCTAGTTAGT 3'
T7 promoter	5' TAATACGACTACTA TAGG 3'
GFPBam1	5' TTAGCAGCCGGGATCCCGCTTGTACAGTT 3'
GFPBam2	5' ACCTCCGCCTCCATCTATGTTGTACAGTTCAT 3'
GFPBam3	5' TCCGGATCCCTCCACCTCCGCTCCATCTAT 3'

Restriction sites in the oligonucleotide sequence are underlined.

GFP, pET- β -SW-GFP, pET-Cat-GFP, pET-UC-GFP, and pET-DS-GFP (Fig. 1). The PCR fragments were amplified by using the primer pairs NdeI β -SW/BamDS, NdeI β -SW/BamUC, NdeICat/BamDS, NdeI β -SW/Bam β -SW, NdeICat/BamCat, NdeIUC/BamUC, and NdeIDS/BamDS, respectively (Table 1). PCR was performed in a reaction mixture of KOD-Plus-Neo kit (Toyobo, Japan) with the following condition for thermal cycling: 94°C for 2 min, followed by 35 cycles at 98°C for 20 s and 68°C for 2 min. Then, PCR products were purified using the Fast GeneTM Plasmid mini kit (Genetics, Japan). The fragments were digested with NdeI and BamHI, and then inserted into the NdeI and BamHI sites of the pET-22b (+) fusion plasmid containing the GFP gene, respectively. The plasmids were introduced into *E. coli* Rosetta-gami B (DE3) cells. The transformants were cultured at 37°C in 2 L of LB broth (pH 7.0) containing 50 μ g/ml ampicillin, 34 μ g/ml chloramphenicol, and 20 μ g/ml kanamycin. After the OD₆₀₀ reached about 0.4–0.6, the cultures were cooled down and supplemented with isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM, followed by incubation at 15°C for 20 h.

Purification of GFP fusion proteins. *E. coli* cells harboring pET- β -SW/Cat/UC/DS-GFP, pET- β -SW/Cat/UC-GFP, pET-Cat/UC/DS-GFP, pET- β -SW-GFP, pET-Cat-GFP, pET-UC-GFP, and pET-DS-GFP were centrifuged at 10,000 rpm for 10 min at 4°C, after which cell pellets were suspended in 10 mM Tris-HCl (pH 8.0), followed by disruption of the pellet by sonication (15 s pulses at 45 s intervals) on ice. After the removal of debris by centrifugation, the supernatant was dialyzed against 10 mM Tris-HCl (pH 8.0) overnight. The dialyzed solution was applied to a DEAE-Cellufine column (3 $\times$ 5 cm) that was equilibrated with 10 mM Tris-HCl (pH 8.0). The column was washed with the same buffer, after which each enzyme was eluted with the buffer containing 100 mM NaCl. Domain-GFP fusion proteins were detected by the absorbance at 474 nm. The fractions containing target proteins were pooled and dialyzed against the same buffer. Ammonium sulfate was added at a saturation of 10% to the dialyzed solution. The solution was applied to a Butyl-Toyopearl 650M column (3 $\times$ 5 cm) that was equilibrated

Fig. 3. 10% SDS-PAGE results of GFP fusion proteins.

Lane 1, Markers; 2, β -SW/Cat/UC/DS-GFP; 3, β -SW/Cat/UC-GFP; 4, Cat/UC/DS-GFP; 5, β -SW-GFP; 6, Cat-GFP; 7, UC-GFP; 8, DS-GFP.

Fig. 4. Change in reducing sugar contents with reaction time of α -1,3-glucan hydrolysis of GFP fusion proteins.

Black, dark gray, light gray, and white bars represent β -SW/Cat/UC/DS-GFP, β -SW/Cat/UC-GFP, Cat/UC/DS-GFP, and Cat-GFP, respectively. The mixture contained 0.15 nmol/mL domain GFP fusion proteins, 50 mM citrate buffer (pH 5.5), and 1% α -1,3-glucan. After each incubation time, the supernatants were collected by centrifugation and reducing sugar was determined as described in "Materials and Methods." Error bars represent reducing sugar the mean value and standard deviation from triplicate determinations.

with 10 mM Tris-HCl (pH 8.0) buffer containing 10% ammonium sulfate, after which each enzyme was eluted with the buffer containing 5% ammonium sulfate. The fractions were pooled and dialyzed against in 10 mM citrate buffer (pH 5.5) overnight. The dialyzed solution was concentrated using Amicon Ultra Centrifugal Filters MWCO 30 kDa (Merck, Darmstadt, Germany) and stored at -25°C . In the purification steps, each fraction was estimated by the absorbance at 280 nm. The total protein concentration was determined by Lowry's method with bovine serum albumin as a standard. The molecular weight of the protein was estimated by 10% SDS-PAGE using the Prestained Protein Marker Board Range (Nacalai Tesque, Japan) as a standard, comprising myosin (200 kDa), β -ga-

Fig. 5. Cell wall hydrolysis activity of GFP fusion proteins.

The mixture contained 0.15 nmol/mL domain GFP fusion proteins, 50 mM citrate buffer (pH 5.5), and 1% *S. commune* pellet. After 30 min of incubation, the supernatants were collected by centrifugation, and the reducing sugar amount was determined as described in "Materials and Methods." Values of reducing sugar are mean $\pm$ standard deviation from triplicate determinations.

lactosidase (116 kDa), phosphorylase B (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa).

Preparation of α -1,3-glucan. α -1,3-Glucan was prepared from sucrose using the recombinant GTF expressed in *E. coli* Rosetta-gami B (DE3) cells, as described previously (Suyotha et al., 2013).

Assay for α -1,3-glucanase hydrolysis activity. The reaction mixture including 0.15 nmol/mL of GFP fusion protein, 1% α -1,3-glucan, and 50 mM citrate buffer (pH 5.5) was incubated at 50°C for 0 min, 5 min, 15 min, 30 min, 1 h, 18 h, and 24 h. The reactions were stopped by boiling for 5 min. After the suspension had been centrifuged at 12,000 rpm for 2 min at 4°C , 250 μL of the supernatant was recovered, and the amount of reducing sugar was determined by the dinitrosalicylic colorimetric method of Miller (1959). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar (as glucose) per minute.

Assay for *S. commune* cell-wall hydrolysis activity. To determine *S. commune* cell-wall hydrolysis activity, the reaction mixtures containing 0.15 nmol/mL of GFP fusion proteins, 1% *S. commune* pellet, and 50 mM citrate buffer pH 5.5 were incubated at 50°C for 30 min. To determine hydrolysis activity, it was examined as described above.

Assay for insoluble substrate-binding activity. The mixture contained 2 nmol/mL of domain GFP fusion protein, 1% substrate, and 50 mM citrate buffer (pH 5.5). The reaction was performed on ice for 1 h with stirring and mixing by vortexing every 10 min, after which the supernatant was collected upon centrifugation at 12,000 rpm for 2 min. The amount of bound protein was estimated by subtracting the amount of free protein in the supernatant from the initial amount of protein. The enzyme-substrate binding activity was calculated as a percentage of the initial amount of protein.

Table 2. Insoluble-substrate binding activity of GFP fusion proteins.

Substrate	linkage	β -SW/Cat/UC/DS	β -SW/Cat/UC	Cat/UC/DS	β -SW	Cat	UC	DS
α -1,3-glucan	α -1,3-	78.16 ± 0.47	51.03 ± 1.77	43.77 ± 1.64	55.79 ± 7.88	n.d.	n.d.	45.35 ± 6.63
β -1,3-glucan	β -1,3-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Starch	α -1,4-	9.58 ± 2.36	n.d.	n.d.	4.49 ± 1.46	n.d.	n.d.	n.d.
Cellulose	β -1,4-	3.07 ± 0.72	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pachyman	β -1,3-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Xylan	β -1,4-	43.49 ± 1.18	27.98 ± 9.59	11.52 ± 1.70	33.33 ± 7.39	n.d.	n.d.	7.03 ± 2.31
Chitin	β -1,4-	10.15 ± 0.81	n.d.	n.d.	9.46 ± 0.33	n.d.	n.d.	n.d.

$\pm$ S.D. represent variation in the values of a variable from triplicate determinations; n.d. represent not determined.

The mixture contained 2 nmol/mL domain-GFP fusion proteins, 50 mM citrate buffer pH 5.5, and 1% substrate. The reaction was performed on ice for 1 h, then the supernatant was collected by centrifugation. The amount of bound protein was estimated by subtracting the amount of free protein in the supernatant from the initial amount of protein. The enzyme-substrate binding activity was calculated as a percentage of the initial amount of protein.

Fig. 6. Cell wall-binding activity of GFP fusion proteins.

S. commune mycelia were incubated with β -SW/Cat/UC/DS-GFP (A, B), β -SW/Cat/UC-GFP (C, D), Cat/UC/DS-GFP (E, F), β -SW-GFP (G, H), Cat-GFP (I, J), UC-GFP (K, L), and DS-GFP (M, N). A, C, E, G, I, K, and M are fluorescence images, and B, D, F, H, J, L, and N are light images.

β -1,3-Glucan was purchased from Sigma Chemical (Tokyo, Japan), corn starch from Wako Chemical (Tokyo, Japan), microcrystalline cellulose from Merck (Darmstadt, Germany), pachyman from Megazyme (Wicklow, Ireland),

birchwood xylan from Sigma Chemical (Tokyo, Japan), and powder crab shell chitin from Nacalai Tesque (Kyoto, Japan).

***S. commune* cell wall binding assay.** After *S. commune* had been cultured as mentioned above, the mycelial pellet was collected and washed with distilled water three times and then supplemented with 20 mL of distilled water and blended for 1 min with a blender. One hundred microliters of the mycelial suspension was pipetted and mixed with 2 nmol/mL GFP fusion proteins, and 50 mM citrate buffer (pH 5.5). The reaction was performed on ice for 1 h with shaking and mixing every 10 min. The fluorescence of domain-GFP fusion protein bound to mycelia was detected by using an Olympus BX53 fluorescence microscope (Olympus Tokyo, Japan). Images were analyzed with Olympus DP72 and cellSens standard software (Olympus, Tokyo, Japan).

Results

Purification of GFP fusion proteins

The domain-GFP fusion proteins were produced in *E. coli* Rosetta gami-B (DE3) and were purified using DEAE-Cellufine and Butyl-Toyopearl 650M column, respectively. The molecular mass of purified GFP fusion proteins was determined by 10% SDS-PAGE, as shown in Fig. 3. The predicted molecular masses of β -SW/Cat/UC/DS-GFP, β -SW/Cat/UC-GFP, Cat/UC/DS-GFP, β -SW-GFP, Cat-GFP, UC-GFP, and DS-GFP were approximately 98, 88, 83, 44, 52, 49, and 42 kDa, respectively. Their molecular masses estimated by SDS-PAGE showed a good agreement with the values calculated based on the amino acid sequence.

α -1,3-Glucan-hydrolyzing activity

Among the domain-GFP fusion proteins, β -SW/Cat/UC/DS-GFP produced the highest reducing sugar, followed by β -SW/Cat/UC-GFP, Cat/UC/DS-GFP, and Cat-GFP, in this order (Fig. 4). β -SW-GFP, UC-GFP, and DS-GFP did not produce any reducing sugar, indicating their lack of catalytic function (data not shown).

Deletion of β -SW and DS domains caused a reduction of α -1,3-glucan-hydrolyzing activity. Suyotha et al. (2013) also reported that the DS domain of Agl-KA has a role in binding α -1,3-glucan and to enhancing α -1,3-glucan-hydrolyzing activity.

S. commune cell wall-hydrolyzing activity

We next checked the hydrolysis activities of the domain-GFP fusion proteins toward the cell of *S. commune*. As shown in Fig. 5, β -SW/Cat/UC/DS-GFP released the highest reducing sugar of approximately 0.3 mM after 30 min of incubation at 50°C, followed by β -SW/Cat/UC-GFP (0.2 mM), Cat/UC/DS-GFP (0.15 mM), and Cat-GFP (0.10 mM). The order of hydrolyzing strength of these fusion proteins toward the cell wall of *S. commune* was the same as that toward the synthetic α -1,3-glucan.

Binding activity of GFP fusion protein

Table 2 summarizes the water-insoluble substrate-binding activities of GFP fusion proteins. Among the insoluble substrates, none of the GFP fusion proteins showed binding activity to β -1,3-glucan and pachyman. Except for Cat-GFP and UC-GFP, all GFP fusion proteins can bind to β -1,3-glucan.

β -SW/Cat/UC/DS-GFP (78%) showed the highest binding activity to α -1,3-glucan, followed by β -SW-GFP (56%), β -SW/Cat/UC-GFP (51%), DS-GFP (45%), and Cat/UC/DS-GFP (44%). These results strongly suggested that β -SW and DS play important roles in α -1,3-glucan binding function and that the synergy of β -SW and DS promotes α -1,3-glucan binding activity and also enhances α -1,3-glucan hydrolysis activity.

β -SW and DS also could bind xylan, as shown in Table 2. β -SW/Cat/UC/DS-GFP (43%) showed the highest binding activity, followed by β -SW-GFP (33%), β -SW/Cat/UC-GFP (28%), Cat/UC/DS-GFP (12%), and DS-GFP (7%). These results indicated that Agl-ST could bind xylan but not hydrolyze it.

β -SW/Cat/UC/DS-GFP and β -SW-GFP could bind to starch and chitin with a lower affinity than α -1,3-glucan. β -SW/Cat/UC/DS-GFP showed a binding activity of starch and chitin of approximately at 9% and 10%, whereas for β -SW-GFP, the values were 5% and 9%, respectively. Meanwhile, β -SW/Cat/UC/DS-GFP could bind cellulose at a rate of approximately 3%.

Cell wall binding activity

To confirm the binding ability of each domain to α -1,3-glucan in the cell wall of *S. commune*, each GFP fusion protein was treated with *S. commune* mycelia and then observed using a fluorescence microscope. As shown in Fig. 6, β -SW/Cat/UC/DS-GFP, β -SW/Cat/UC-GFP, β -SW-GFP, β -SW/Cat/UC-GFP, Cat/UC/DS-GFP, and DS-GFP were bound to *S. commune* mycelia, whereas Cat-GFP and UC-GFP hardly bound to them at all.

Discussion

As shown in Fig. 1 in Section “Introduction”, Agl-ST, which belongs to bacterial type GH87 family, has a domain architecture different from those of the known GH87 family enzymes. Furthermore, the previous report indicated that Agl-ST showed a high similarity to mycodextranase that specifically hydrolyzes α -1,4-glycosidic linkages in polysaccharide comprising α -D-glucose units alternatively linked (1 $\rightarrow$ 3) and (1 $\rightarrow$ 4) (Cherdvorapong et al., 2019). Given these results, to investigate the function of each domain of Agl-ST and their synergetic effect, and to compare them with those of the other α -1,3-glucanases and mycodextranases, is of great interest from the viewpoint of their evolution and their future applications.

We prepared GFP fusion proteins to clarify the function of each domain of Agl-ST. For α -1,3-glucan and cell wall hydrolysis, β -SW/Cat/UC/DS-GFP showed the highest activity, followed by β -SW/Cat/UC-GFP, Cat/UC/DS-GFP, and Cat-GFP. Conversely, β -SW-GFP, UC-GFP, and DS-GFP showed no hydrolysis activity, indicating that only the catalytic domain is important for the hydrolysis of α -1,3-glucan.

The result of insoluble substrate binding activity demonstrated that β -SW and DS play important roles in substrate binding, and the combination of β -SW and DS showed a synergistic effect on the binding activity and enhanced hydrolysis efficiency. The comparison of Agl-

ST with Agl-KA in terms of the activity of binding to α -1,3-glucan revealed that β -SW (55.8%) and the DS domain (45.4%) of Agl-ST exhibited almost the same binding ability as the corresponding domains CBM6 (61.5%), DS1 (42.6%), and DS2 domains (32.4%) of Agl-KA (Suyotha et al., 2013). For xylan binding, Agl-ST demonstrated a binding activity of β -SW domain (33.3%) higher than that of CBM6 domain (20.9%), whereas the DS domain (7%) revealed an ability similar to that of the DS2 domain (8.9%) of Agl-KA.

The amino acid sequences of each substrate-binding domain were compared between Agl-ST and Agl-KA was analyzed using the Standard Protein BLAST program. The amino acid sequences of the β -SW and DS domains of Agl-ST showed a similarity to Agl-KA with CBM6 (28.5%) and DS2 (50.8%) identities, respectively. The comparison between known α -1,3-glucanases and mycodextranases in the phylogenetic tree was conducted using CLUSTAL Omega software. The β -SW domain revealed a high similarity to mycodextranase, whereas the DS domain showed a similarity to α -1,3-glucanase, as well as to mycodextranase. These results suggested that the β -SW domain might be evolutionarily conserved in the relationship between mycodextranases and α -1,3-glucanases. Unfortunately, there are currently no reports concerning the substrate binding properties in mycodextranases.

Cell wall binding of GFP fusion proteins has clarified the binding ability of each domain to the cell wall visually. Similar to the binding of α -1,3-glucan, it has been suggested that β -SW and DS domains might synergistically function in the binding to the cell wall of *S. commune*.

The catalytic domain and UC hardly bound to any insoluble substrates. This result indicated that the catalytic domain is specific to hydrolyzing substrates. As for UC, Suyotha et al. (2013) described that it might be a linker connecting the binding domain with the catalytic domain in Agl-KA. However, the comparison of amino acid sequences of UC between Agl-ST and Agl-KA showed only a 22% identity. Therefore, UC of Agl-ST might have a different function other than a linker; for example, supporting the stability of the catalytic domain. A three-dimensional structure analysis of Agl-ST should provide further information on its function.

In conclusion, we have attempted to clarify the function of each domain function of α -1,3-glucanase (Agl-ST) from *S. thermodiastaticus* HF3-3 by constructing GFP fusion proteins. The results demonstrated their functions for binding and hydrolysis toward α -1,3-glucan and the cell wall of *S. commune*. However, we still lack the structural details that are essential to describe their functions regarding substrate recognition and catalytic mechanism. X-ray crystal analysis of the 3-D structure is currently being pursued.

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