Domain Structure and Function of $\alpha$-$\beta$-Glucanase from Bacillus circulans KA-304, an Enzyme Essential for Degrading Basidiomycete Cell Walls

Wasana Suyotha,1 Shigekazu Yano,2 Kazuyoshi Takagi,3 Nopakarn Rattanakit-Chandet,4 Takashi Tachki,1 and Mamoru Wakayama1,†

1Department of Biotechnology, Faculty of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan
2Department of Biochemical Engineering, Graduate School of Sciences and Engineering, Yamagata University, Jonan, Yonezawa, Yamagata 992-8510, Japan
3Department of Applied Chemistry, Faculty of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan
4Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

Received November 26, 2012; Accepted December 20, 2012; Online Publication, March 7, 2013
[doi:10.1271/bbb.120900]

**Bacillus circulans** KA-304 $\alpha$-$\beta$-glucanase (Agl-KA) includes an N-terminal discoidin domain (DS1), a carbohydrate binding module family 6 (CB6), threonine and proline repeats (TPs), a second discoidin domain (DS2), an uncharacterized conserved domain (UCD), and a C-terminal catalytic domain. Domain deletion enzymes lacking DS1, CB6, and DS2 exhibited lower $\alpha$-$\beta$-glucan-hydrolyzing and -binding activities than the wild type, Agl-KA. An $\alpha$-$\beta$-glucan binding assay with fluorescent protein fusion proteins indicated that DS1, CB6, and DS2 bound to $\alpha$-$\beta$-glucan and fungal cell walls, and that binding efficiency was increased by their combined action. In contrast, UCD did not exhibit any $\alpha$-$\beta$-glucan-binding activity. A dramatic decrease in protoplast formation in the *Schizophyllum commune* mycelium was observed given only a DS1 deletion. An Agl-KA with deletion DS1, CB6, and DS2 produced no protoplasts. These results indicate that the combined actions of DS1, CB6, and DS2 contributed to increased cell-wall binding and were indispensable for efficient Agl-KA cell-wall degradation.

**Key words:** $\alpha$-$\beta$-glucanase; $\alpha$-$\beta$-glucan-binding activity; fungal cell-wall; mutan; protoplast

$\alpha$-$\beta$-Glucans are insoluble linear $\alpha$-$\beta$-linked homopolymers of glucose that are components of fungal cell walls1) and the extracellular polysaccharide (mutan) produced by oral *Streptococci* in dental plaques.2) $\alpha$-$\beta$-Glucanase (E.C.3.2.1.59), also called mutanase, hydrolyzes $\alpha$-$\beta$-glucans, and is widely distributed among fungi and bacteria. These enzymes are classified into two families, 71 and 87, of glycoside hydrolases based on their amino acid sequences, referred to in the Carbohydrate Active enZYmes (CaZy) Database. Type 71 $\alpha$-$\beta$-glucanases have been found in fungi, such as *Trichoderma*,3) *Penicillium*,4) *Aspergillus*,5) and *Schizosaccharomyces*,3) and type 87 enzymes have been found in *Bacillus*6) and *Paenibacillus*.7) These enzymes have been investigated not only to determine their physiological roles in these organisms,3,4) but also for application in degrading dental plaques,8,9) controlling pathogenic fungi,10) preparing fungal protoplasts,11) and for other uses.

In our previous study, $\alpha$-$\beta$-glucanase (designated Agl-KA) was isolated from culture filtrate of *Bacillus circulans* KA-304 grown on a cell-wall preparation (CWP) of *Schizophyllum commune* as inducer, and Agl-KA participated in *S. commune* protoplast formation.12) Agl-KA did not induce the formation of *S. commune* protoplasts by itself, but induced protoplast formation in cooperation with chitinase 1 of *B. circulans* KA-304, which is isolated from the same filtrate and is classified as a glycoside hydrolase family 19 type enzyme. We found that chitinase 1 too did not induce protoplast formation by itself, and both Agl-KA and chitinase 1 were a minimum requirement for protoplast formation.13) The Agl-KA gene has been cloned and expressed to examine the properties of Agl-KA in detail.14) The amino acid sequence of Agl-KA shows similarities to those of certain family 87 type enzymes, such as $\alpha$-$\beta$-glucanase (mutanase RM1) from *Paenibacillus* sp. strain KSM-M35, and $\alpha$-$\beta$-glucanase (MuC2) from *Paenibacillus* sp. strain KSM-126 with identities of 80%, 48.3%, and 46.7% respectively.15) Hakamada et al. compared the amino acid sequences of these $\alpha$-$\beta$-glucanases with those of other enzymes in databases, and reported that these enzymes included an N-terminal discoidin domain (DS1), a carbohydrate binding module family 6 (CB6), threonine and proline repeats (TPs), a second discoidin domain (DS2), an uncharacterized conserved domain (UCD), and a C-terminal catalytic domain.15) This indicates that Agl-KA can be classified as a type 87 enzyme that has a domain structure similar to that of *Paenibacillus* enzymes (Fig. 1A).

Shimotsuura et al.16) have reported that mutanase RM1 contains two major functional regions, an N-terminal region (277 residues) and a C-terminal region (937 residues), separated by TP. They also reported that the N-terminal region of mutanase RM1 exhibited $\alpha$-$\beta$-glucan-binding activity and that this region was indispensable in degrading the biofilms formed by *Strepto*...
**Materials and Methods**

Microorganisms and culture. *Bacillus circulans* KA-304 was grown at 30°C in medium B\(^{19}\) containing 0.5% polypeptone, 0.5% yeast extract, 0.1% K\(_2\)HPO\(_4\), 0.03% MgSO\(_4\)-7H\(_2\)O, 0.5% NaCl, and 0.5% CWP as inducer (pH 7). *Shizophyllum commune* IFO 4928 was grown at 30°C with shaking in medium C\(^{19}\) containing 2% glucose, 1% polypeptone, 0.5% yeast extract, 0.3% K\(_2\)HPO\(_4\), and 5 μg/L of thiamine (pH 7). *Escherichia coli* JM 109, used as host to construct various recombinant plasmids, was grown at 37°C with shaking (100 rpm) in LB medium containing 100 μg/mL of ampicillin. *E. coli* Rosetta-gami B (DE3), which harbored the recombinant plasmid, was grown at 30°C with shaking (100 rpm) in LB medium containing 100 μg/mL of ampicillin, 10 μg/mL of chloramphenicol, 25 μg/mL of kanamycin, and 15 μg/mL of tetracycline.

Preparation of \(\alpha\),\(\beta\)-Glucan. \(\alpha\),\(\beta\)-Glucan was prepared from sucrose using *Streptococcus mutans* ATCC700610 glucosyltransferase I (GTF-I) by the method described below. The GTF-I gene was amplified by PCR. An NdeI-linker sense primer (gtf-Nde) and an XhoI-linker antisense primer (gtf-Xho) were used for PCR: the sequences are given in Table 1. The PCR fragment was digested with Ndel and XhoI, the digested fragment was cloned into the Ndel and XhoI sites of pET-22b (+), and the recombinant plasmid (pET-gtf1) was introduced into...
**Expression of deletion enzyme genes.** DNA fragments encoding for deletion enzymes (Fig. 1B) were amplified from the genomic DNA of *B. circulans* KA-304 by PCR. All the primers are listed in Table 1. The primer pairs for amplifying AglΔDS1, AglΔDS1CB6, and AglΔDS1CB6DS2, and AglΔDCD-UCD were CBnNde/Cb6Nde, Ds1Nde/AglXho, Ds2Nde/AglXho, UcnNde/AglXho, and CtnNde/AglXho respectively. The PCR fragments were digested with NdeI and XhoI, and then the digested fragments were cloned into PET22b (+). The expression plasmids for recombinant proteins AglΔDS1, AglΔDS1CB6, and AglΔDCD-UCD were designated PET-aglΔDS1, PET-aglΔDS1CB6, and PET-aglΔDCD-UCD respectively. These expression plasmids were introduced into *E. coli* Rosetta-gami B (DE3) cells. The transformants were cultured at 30°C under the conditions described above until the optical density at 600 nm was about 0.6, and then IPTG was added to the culture medium at a final concentration of 0.4 mM. The cultures were incubated at 20°C for an additional 20 h.

**Purification of deletion enzymes.** All procedures were performed at 0–4°C, unless otherwise stated. *E. coli* cells harboring PET-aglΔDS1, PET-aglΔDS1CB6, PET-aglΔDS1CB6DS2, and PET-aglΔDCD-UCD were harvested by centrifugation (10,000 × g for 10 min). Cells harboring PET-aglΔDS1 were suspended in 50 mL of 10 mM Tris–HCl (pH 9.0), and those harboring PET-aglΔDS1CB6, PET-aglΔDS1CB6DS2, and PET-aglΔDCD-UCD were suspended in 50 mL of 10 mM Tris–HCl (pH 8.75). Each of the cell suspensions was sonicated (4°C, 10 min, 350–400 μA) on ice, after which cell debris was removed by centrifugation. The supernatant was dialyzed against the buffer used for the cell suspensions.

To purify AglΔDS1, the dialysate (cell-free extract) was applied to a DEAE-cellulose column (3 × 5 cm) equilibrated with 10 mM Tris–HCl (pH 9.0). After it was washed with the same buffer, the column was developed with a buffer containing 50 mM NaCl, which eluted the enzyme. The active fractions were dialyzed and collected against 10 mM Tris–HCl (pH 8.0), and then applied to a DEAE-cellulose column (3 × 5 cm) equilibrated with 10 mM Tris–HCl (pH 8.0). The column was washed with this buffer. After washing, the column was developed with a buffer containing 50 mM NaCl, which eluted the enzyme. The active fractions were dialyzed and collected against 10 mM Tris–HCl (pH 8.0). The column was washed with this buffer. After washing, the column was developed with a buffer containing 50 mM NaCl, which eluted the enzyme. The active fractions were dialyzed and collected against 10 mM Tris–HCl (pH 8.0). The column was washed with this buffer. After washing, the column was developed with a buffer containing 50 mM NaCl, which eluted the enzyme. The active fractions were dialyzed and collected against 10 mM Tris–HCl (pH 8.0).
of 10% to the dialyzed solution, and then the solution was applied to a Butyl-Toyopearl 650M column (2 × 3 cm) equilibrated with 10 mM Tris–HCl (pH 8.0) buffer containing 10% ammonium sulfate. After washing with a buffer containing 10% ammonium sulfate, the column was developed with a buffer containing 5% ammonium sulfate. GFP fusion proteins were found in the eluate with the buffer containing 5% ammonium sulfate.

Assay for α-1,3-glucanase activity. The reaction suspension contained 1% α-1,3-glucan, 50 mM potassium phosphate buffer (pH 6.5), and appropriate amounts of the enzyme. After incubation at 30 °C, the reaction was stopped by immersing the mixture in boiling water for 15 min. The suspension was centrifuged, and the precipitated α-1,3-glucan was removed. The reducing sugar formed in the supernatant was measured as glucose with dinitrosalicylic acid reagent by the method of Miller.20 One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per min.

Assay for insoluble substrate-binding activity. The mixture contained 2 mM/mL of protein, 1% substrate, and 50 mM potassium phosphate buffer (pH 6.5). After 1 h of incubation on ice with occasional stirring, the mixture was centrifuged, and the protein concentration in the supernatant was determined. The amount of bound protein was estimated by subtracting the amount of free protein in the supernatant from the initial amount of protein. The insoluble substrate-binding efficiency was calculated as a percentage of the initial amount of protein, and was used to express insoluble substrate-binding activity.

Kinetic analysis. Kinetic parameters for hydrolysis were determined by assays using 0.25–15 mg/mL of 1% α-1,3-glucan. A reaction mixture containing 0.15 mM/mL of enzyme and 50 mM potassium phosphate buffer (pH 6.5) was incubated at 30 °C. After 10 min, the reaction was stopped by boiling for 5 min. The amount of reducing sugar in the supernatant was determined.

Assay for protoplast-forming activity. Protoplast-forming activity was determined by previously described methods with Schizophyllum commune IFO 4928 as test strain.19

Cell-wall binding assay. S. commune mycelia treated with GFP-fusion proteins were visualized using an Olympus BX50 fluorescence microscope. Images were acquired and analyzed with a Retiga 2000R digital CCD camera (Qimaging, Surrey, BC) and Image-Pro software (Media Cybernetics, Bethesda, MD).

Analytical methods. During enzyme purification, protein concentrations were determined by Lowry’s method using egg albumin as standard.21 For the assays for mutan-hydrolyzing, insoluble substrate-binding, and protoplast-forming activities, the concentrations of Agl-KA and its derivatives were estimated by the absorbance at 280 nm using the following molar absorption coefficients: 178,142 M⁻¹ cm⁻¹ for Agl-KA, 147,202 M⁻¹ cm⁻¹ for AglΔDS1, 120,272 M⁻¹ cm⁻¹ for AglΔDS1CB6, 106,292 M⁻¹ cm⁻¹ for AglΔDS1CB6DS2, and 82,342 M⁻¹ cm⁻¹ for AglΔDCD-UCD. These were calculated based on their deduced amino acid compositions.22 The concentrations of GFP and GFP-fusion proteins used in the binding assays were estimated by the absorbance at 474 nm with the molar absorption coefficient of 26,200 M⁻¹ cm⁻¹ for GFP and Nterm-GFP.

SDS–PAGE was performed by Laemmli’s method.23 Myosin (MW 200,000), α-galactosidase (MW = 115,000), α-amylase (MW = 66,000), aldolase (MW = 42,000), carbonic anhydrase (MW = 30,000), and myoglobin (MW = 17,000) were used as molecular weight markers.

The effects of pH on the activity of Agl-KA and its derivatives were determined by incubation at 30 °C for 10 min with a mixture containing 1% α-1,3-glucan and 50 mM buffer of varying pH values. To determine the pH stability of the enzymes, a given enzyme was incubated at 45 °C for 10 min in 50 mM buffers of varying pHs. After treatment, α-1,3-glucanase activity was determined as described in the preceding section using 1% α-1,3-glucan as substrate. The unitary activity was measured as the reduction of 0.01 A₄₁₀ in 1 minute. 

To determine the optimum temperature, the activity was measured at temperatures in a range 20–70 °C for 10 min in potassium phosphate buffer (pH 6.5). Thermal stability was determined after treatment of the enzyme varius temperatures for 10 min.

Reagents. Chitinase I from B. circulans KA-304 was prepared from cell-free extract of E. coli harboring the pET22-chl plasmid by the previously described methods.23 GFP was purified from cell-free extract of E. coli harboring the pQBl-T7-GFP plasmid by DEAE-cellulose A-500 column chromatography and Butyl-Toyopearl 650M column chromatography. Corn starch was purchased from Wako Chemical (Tokyo), birchwood xylan from Sigma Chemical (Tokyo), and powdery crab shell chitin from Nacalai Tesque (Kyoto, Japan). Pachyman was purchased from Megazyme (Wicklow, Ireland) and lichenan from MP Biomedical (Illkirch, France). Microcrystalline cellulose was from Merck (Darmstadt, Germany). Chitosan 10B was from Funakoshi Chemical (Tokyo). The other reagents were chemically pure grades of commercial products.

Results

Expression and purification of Agl-KA derivatives

As described above, Agl-KA is a multi-domain enzyme, although the roles of the individual domains in α-1,3-glucan hydrolysis are not clear, except for the catalytic domain. To determine these roles, we constructed several deletion enzymes (Fig. 1B). Expression plasmids were introduced into E. coli Rosetta-gami B (DE3) cells and significant α-1,3-glucanase activities were detected in each of the cell-free extracts. All deletion enzymes were purified from the cell-free extracts by the procedures described above in “Materials and Methods.” As shown in Fig. 2A, the purified deletion enzymes were homogenous on SDS–PAGE. The predicted molecular masses of Agl-KA, AglΔDS1, AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD based on their amino acid sequences were approximately 135.5, 115.5, 97.5, 82.9, and 62.1 kDa respectively. Their molecular masses as estimated by SDS–PAGE were in agreement with these values.

Agl-KA was stable over a pH range of 6–10, and more than 70% of its activity was retained at pH 5 and 10. The optimum pH for Agl-KA activity was 8. Agl-KA exhibited maximum activity at approximately 50 °C and retained complete activity after 10 min of incubation at 50 °C. The properties of the deletion enzymes were similar to those of Agl-KA, which suggests that deleting DS1, CB6, DS2, and/or UCD of Agl-KA had little effect on the pH or temperature dependence of its reactivity and stability.

α-1,3-Glucan-hydrolyzing activities

Figure 3 shows the α-1,3-glucan-hydrolyzing activities for Agl-KA and the deletion enzymes. Agl-KA produced the largest amount of reducing sugars, followed by AglΔDS1, AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD in that order. A comparison of Agl-KA with the deletion enzymes, AglΔDS1, AglΔDS1CB6, and AglΔDS1CB6DS2, showed that an increased number of deletion domains caused gradual decreases in the amounts of reducing sugars, which suggests that DS1, CB6, and DS2 individually enhanced α-1,3-glucan hydrolyzing activity. In contrast, UCD appeared hardly to influence the enzymatic activity, since there was only a slight difference between the amounts of sugar formed by AglΔDCD-UCD and by AglΔDS1CB6DS2.
Fig. 2. SDS–PAGE Results. A, SDS–PAGE results for Agl-KA and deletion enzymes. 10% SDS–PAGE was used. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, Markers; 2, Agl-KA; 3, AglΔDS1; 4, AglΔDS1CB6; 5, AglΔDS1CB6DS2; 6, AglΔDCD-UCD. B, SDS–PAGE results for GFP fusion proteins. 12.5% SDS–PAGE was used. Lane 1, Markers; 2, GFP; 3, DS1-GFP; 4, CB6-GFP; 5, DS2-GFP; 6, UCD-GFP; 7, DS1CB6-GFP; 8, CB6DS2-GFP; 9, DS1CB6DS2-GFP.

Fig. 3. Time Course of α-1,3-Glucan Hydrolysis by Agl-KA and Its Derivatives. α-1,3-Glucan hydrolyzing activity was measured at 30°C in 50 mM potassium phosphate buffer using 1% substrate and 0.15 nmol/mL of the enzyme. Symbols: solid circles, Agl-KA; hollow circles, AglΔDS1; solid triangles, AglΔDS1CB6; hollow triangles, AglΔDS1CB6DS2; solid squares, AglΔDCD-UCD.

Table 2. Kinetic Parameters

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mg/mL)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mg$^{-1}$ mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agl-KA</td>
<td>5.6</td>
<td>91.3</td>
<td>16.3</td>
</tr>
<tr>
<td>AglΔDS1CB6</td>
<td>8.8</td>
<td>106.6</td>
<td>12.2</td>
</tr>
<tr>
<td>AglΔDS1</td>
<td>17.6</td>
<td>130.7</td>
<td>7.4</td>
</tr>
<tr>
<td>AglΔDS1CB6DS2</td>
<td>33.6</td>
<td>137.4</td>
<td>4.0</td>
</tr>
<tr>
<td>AglΔDCD-UCD</td>
<td>35.8</td>
<td>158.7</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Kinetic parameters were determined as described in “Materials and Methods” using 0.15 nmol/mL of enzyme and α-1,3-glucan as substrate. The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values were obtained from Lineweaver-Burk plots.

To determine the contributions of DS1, CB6, DS2, and UCD to enzymatic activity, their kinetic parameters were obtained from Lineweaver–Burk plots. The affinities of these enzymes for α-1,3-glucan ($K_m$) and their turnover numbers ($k_{cat}$) and catalytic efficiencies ($k_{cat}/K_m$) are summarized in Table 2. The $K_m$ values for AglΔDS1, AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD were approximately 1.6-, 3.1-, 6.0-, and 6.4-fold higher than that of Agl-KA respectively. The $k_{cat}$ values for AglΔDS1, AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD were approximately 1.2-, 1.4-, 1.5-, and 1.7-fold higher than that of Agl-KA respectively. These results indicate that deleting DS1, CB6, and DS2 caused significantly increased $K_m$ values and slightly increased $k_{cat}$ values. Thus the $k_{cat}/K_m$ values of AglΔDS1, AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD were about 74%, 45%, 25%, and 27% of that of Agl-KA respectively. These kinetic parameters indicate that DS1, CB6, and DS2 increase the affinity of Agl-KA for the substrate, and that its catalytic efficiency is enhanced by these binding capabilities.

Deleting DS1, CB6, and DS2 of Agl-KA resulted in an increase in the turnover number. These domains might interfere with subsequent contact between the catalytic domain and a substrate by remaining in one place with strong binding affinity in the presence of excess substrates, since they act independently of the catalytic domain. Similar results have been reported by Okazaki et al. for xylanase from Alcaligenes sp. X3-1135 and by Santos et al. for cellulase from Bacillus subtilis 168. These enzymes contain carbohydrate binding and catalytic domains, and deleting those binding domains increased both the $K_m$ and the $k_{cat}$ value. DS1, CB6, and DS2 might behave like the carbohydrate binding domains of xylanase and cellulase in substrate hydrolysis. In contrast, the difference between the kinetic parameters of AglΔDS1CB6DS2 and AglΔDCD-UCD also suggest that UCD hardly influences the hydrolyzing activity.

α-1,3-Glucan-binding activity

The results shown in Fig. 4 confirm the α-1,3-glucan-binding activity of Agl-KA and the deletion enzymes as to α-1,3-glucan. They indicate that Agl-KA and AglΔDS1 bound to a substrate, while AglΔDS1CB6, AglΔDS1CB6DS2, and AglΔDCD-UCD did not. Deleting DS1 caused a decrease in binding activity that was nearly lost on deletion of DS1 and CB6, suggesting that at least DS1 and CB6 are necessary for the strong adsorption of this enzyme on α-1,3-glucan. Shimotsuura et al. reported that the binding activity of mutanase RM1 from Paenibacillus sp. RM1 remarkably decreased after deletion of the N-terminal region containing DS1 and CB6, which have high amino acid sequence similarities to DS1 and CB6 of Agl-KA. Their result is compatible with our results by α-1,3-glucan-binding assay with Agl-KA and deletion enzymes.
and 0

Fig. 5. Protoplast Forming Activity of Agl-KA and Deletion Enzymes.

Each of the enzyme (2 nmol/mL) was added to a reaction mixture for protoplast formation. The mixture contained 2 nmol/mL chitinase I, 50 mM potassium phosphate buffer (pH 6.5), 500 mM mononitol, and 0.1 mg of S. commune mycelia. The number of protoplasts released in 1 mL of the mixture was determined. Activity values for Agl-KA and the deletion enzymes are means ± standard deviations for three independent experiments.

Effects of deletion enzymes on protoplast formation

As described above, Agl-KA did not induce protoplast formation by S. commune mycelia by itself. Instead, it exhibited protoplast-forming activity when combined with chitinase I from B. circulans KA-304.

As shown in Fig. 5, the addition of Agl-KA to chitinase I resulted in protoplast-forming activity: approximately 3.6 × 10^6 protoplasts/mL were released after 13 h of incubation, while the addition of AglΔDS1 and of AglΔDS1CB6 released approximately 1.7 × 10^6 and 0.6 × 10^6 protoplasts/mL respectively. The decreased number of protoplasts can be attributed to decreased binding activity to α-1,3-glucan in the cell wall caused by deletion of DS1 and CB6. For AglΔDS1CB6DS2 and AglΔDCD-UCD, very few protoplasts were released during the incubation period. As the results shown in Fig. 5, DS1, CB6, and DS2 are necessary for efficient production of protoplasts.

Expression and purification of GFP fusion proteins

To determine the functions of DS1, CB6, and DS2 in detail, we constructed GFP fusion proteins of them (Fig. 1C) and examined their insoluble substrate- and cell wall-binding activities.

GFP fusion proteins were successfully expressed in E. coli Rosetta-gami B (DE3) cells, and all proteins were purified from the cell-free extracts by the procedure described above in “Materials and Methods.” As shown in Fig. 2B, the purified proteins were homogeneous on SDS–PAGE, and their molecular masses agreed with those predicated from their amino acid sequences.

Table 3 shows the binding activity of GFP fusions to insoluble polysaccharides, including α-1,3-glucan, starch, pachiman, chitin, chitosan, cellulose, xylan, and lichenan. For α-1,3-glucan as substrate, DS1-GFP, DS2-GFP, and CB6-GFP all bound to α-1,3-glucan, but UCD-GFP did not, which confirms that UCD was not involved in α-1,3-glucan binding. CB6-GFP showed a higher α-1,3-glucan-binding efficiency than DS1-GFP or DS2-GFP. Moreover, the binding efficiency of DS1CB6-GFP, CB6DS2-GFP, and DS1CB6DS2-GFP was much higher than that of CB6-GFP. These results indicate that the combined action of DS1, CB6, and DS2 enhance α-1,3-glucan-binding ability.

For xylan as substrate, all of the fusions were adsorbed, except for UCD-GFP. DS1-GFP, CB6-GFP, and DS2-GFP bound to xylan at approximately 17%, 21%, and 9% of the initial protein amounts respectively. DS1CB6-GFP, CB6DS2-GFP, and DS1CB6DS2-GFP fusions bound to xylan at approximately 49%, 29%, and 43% of the initial protein amounts respectively. This indicates that the combination of DS1, CB6, and DS2 promoted xylan-binding activity, as was the case for α-1,3-glucan-binding activity. The other substrates, DS1CB6-GFP, CB6DS2-GFP, and DS1CB6DS2-GFP bound weakly to starch and cellulose, and only 9.6% of DS1CB6DS2-GFP bound to lichenan.

Cell-wall binding activity

We used a cell-wall binding assay with GFP fusion to determine the roles of DS1, CB6, and DS2 in cell-wall binding. As shown in Fig. 6, DS1CB6-GFP, CB6DS2-
GFP, and DS1CB6DS2-GFP certainly bound to the cell wall. In contrast, DS1-GFP, CB6-GFP, and DS2-GFP hardly bound to the *S. commune* mycelia, and UCD-GFP and GFP alone were not found on the cell wall (data not shown). These results support the findings, shown in Fig. 5, that deleting DS1, CB6, and DS2 from Agl-KA resulted in reduced protoplast-forming activity.

### Discussion

GH 87 type α,1-3-glucanases have been proposed to have DS1, CB6, DS2, and UCD at the N-terminal, and the catalytic domain at the C-terminal. A biochemical study of mutanase RM indicated that the N-terminal region, corresponding to DS1 and CB6, exhibited /C11-1,3-glucan binding activity and enhanced degrading activity as to biofilms from *Streptococcus mutans*, but no functions of individual domains, DS1 and CB6, have been investigated in it. In this study, we determined in detail the role of each N-terminal domain, containing DS1, CB6, DS2, and UCD, of Alg-KA by characterizing the domain deletion enzymes, and confirmed their relevance to cell-wall lysis. We found that a combination of these domains was indispensable for efficient substrate-binding and cell-wall degradation.

Through α,1-3-glucan hydrolysis and binding assays (Figs. 3 and 4), we confirmed that deleting DS1 and CB6 from Agl-KA resulted in reduced hydrolyzing and binding activities, as was the case for deletion of the N-terminal domain of mutanase RM1. In addition, we determined in detail the role of each N-terminal domain, containing DS1, CB6, DS2, and UCD, of Alg-KA by characterizing the domain deletion enzymes, and confirmed their relevance to cell-wall lysis. We found that a combination of these domains was indispensable for efficient substrate-binding and cell-wall degradation.
found that deleting DS1 alone caused reduced hydrolyzing and binding activities, although the reduced activity due to deletion of DS1 was lower than that due to deletion of both DS1 and CB6. Moreover, deleting DS1, CB6, and DS2 potentiated the reduced hydrolyzing activity. These results suggest that DS1, CB6, and DS2 independently formed domain structures and that each of them had a particular role in substrate binding. DS1, CB6, and DS2 bound mainly to α-1,3-glucan, and UCD had no binding activity (Table 3). Surprisingly, DS1, CB6, and DS2 also bound weakly to xylan, a component of the plant cell wall. We found that a combination of them enhanced binding efficiency to α-1,3-glucan and other substrates. The amino acid residues considered to be involved in substrate binding are conserved in DS1, CB6, and DS2 (data not shown), but these domains do not have high sequence similarities to known carbohydrate binding domains, suggesting that substrate recognition by DS1, CB6, and DS2 of Agl-KA is different from those domains.

As for cell-wall binding, we confirmed that DS1, CB6, and DS2 bound to the cell wall independently, while UCD did not, and that binding efficiency to the cell wall was also increased by their combined action. The cell wall of basidiomycetes consists of a water-soluble β-glucan in the outer layer, an alkaline-soluble α-1,3-glucan (S-glucan) in the middle layer, and an alkaline-insoluble highly branched β-1,3,1,6-glucan (R-glucan) and chitin in the inner layer. In addition, the heterogeneity of the cell walls of multicellular basidiomycetes should be taken into consideration. The cell-wall of hyphal tips consists chitin and a small amount of water-soluble β-glucan. In the lateral wall, glucan and chitin are cross-linked to form a chitin-glucan complex, and this is overlaid by other glucans necessary for strong binding to cell wall. On the other hand, even though water-soluble β-glucan covers α-1,3-glucan in the middle layer of the cell wall, GFP fusion proteins, except for UCD-GFP, bound to the cell wall. These fusion proteins contacted α-1,3-glucan, because water-soluble β-glucan is loosely bound to the outside of the wall and the layer formed was very thin, and DS1, CB6, and DS2 did not bind to β-glucan (Table 3).

In this study, we determined that the DS1, CB6, and DS2 domains of Agl-KA are novel α-1,3-glucan-binding domains, and are indispensable to the efficient hydrolysis of α-1,3-glucan in fungal cell walls. In contrast, deletion of UCD hardly influenced the catalytic reaction of Agl-KA, and UCD did not bind to insoluble substrates or S. commute mycelia. It is possible that UCD is a linker separating the binding domains from the catalytic domain in Agl-KA that provides flexibility to both. In general, TP in polysaccharide-degrading enzymes is described as a linker, the so-called TP linkers. To understand UCD and TP functions in Agl-KA further, detailed investigation is necessary, including deletion of them from Agl-KA.

We also found that GFP fusion proteins, particularly DS1CB6DS2-GFP, bound to the cell walls. For the cell structure analysis, fluorescent probes with moderately intense fluorescence are required to determine the localization of a target. DS1CB6DS2-GFP might be used as a moderate probe for α-1,3-glucans in the cell wall to determine the cell-wall structures of fungi. To clarify the molecular mechanism of binding to α-1,3-glucan and other polysaccharides, the 3D structures of DS1, CB6, and DS2 of Agl-KA by X-ray crystallographic analysis should performed. Our studies aim to elucidate the details of the cell wall structure of basidiomycetes and the mechanism of cell-wall lysis by α-1,3-glucanase.

Acknowledgment

This work was supported in part by the Japan Society in Japan, and in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Grant 24780085).

References


