Additional Carbohydrate-Binding Modules Enhance the Insoluble Substrate-Hydrolytic Activity of \(\beta\)-1,3-Glucanase from Alkaliphilic Nocardiopsis sp. F96

Naoya Koizumi, Sumiko Masuda, Kiyoe Maeda, Yuya Isoda, Rie Yatsunami, Toshiaki Fukui, and Satoshi Nakamura

Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

Received December 2, 2008; Accepted December 24, 2008; Online Publication, May 7, 2009 [doi:10.1271/bbb.80846]

\(\beta\)-1,3-Glucanase (BglF) from Nocardiopsis sp. F96 is composed of only a catalytic domain. To improve the enzymatic properties of BglF, we attempted to construct chimeric enzymes consisting of BglF and some carbohydrate-binding modules, such as the C-terminal additional domain (CAD) and the N-terminal additional domain (NAD) of \(\beta\)-1,3-glucanase H from Bacillus circulans IAM1165 and the chitin-binding domain (ChBD) of chitinase from alkaliphilic Bacillus sp. 3813. CAD-fused BglF (BglF-CAD), NAD-fused BglF (NAD-BglF), and CAD-fused BglF (NAD-BglF-CAD) were constructed and characterized. The addition of CAD caused increases in binding abilities and hydrolytic activities toward insoluble \(\beta\)-1,3-glucans. As well as BglF-CAD, the binding ability and hydrolytic activity of BglF-ChBD toward pachymyan were also increased. The hydrolytic activity of BglF-CAD at pH 9.0-10.0 was higher than that of BglF. The relative activities of BglF-CAD and BglF-ChBD at around 50-70 °C were higher than that of BglF.

Key words: \(\beta\)-1,3-glucan; \(\beta\)-1,3-glucanase; glycoside hydrolase (GH) family 16; chimeric enzyme; carbohydrate-binding module (CBM)

\(\beta\)-1,3-Glucanase was first discovered by the lytic phenomenon of Aspergillus oryzae caused by accidentally contaminated Bacillus circulans.1) \(\beta\)-1,3-Glucanase hydrolyzes the \(\beta\)-1,3 bonds of \(\beta\)-1,3-glucan, a polymer of \(\beta\)-1,3-linked glucose. To date, \(\beta\)-1,3-glucanases have been isolated from bacteria,2-4) yeast,5) fungi,6,7) and plants.8,9) \(\beta\)-1,3-Glucanases play various physiological roles.10,11) The fungal cell wall is known to have a complex structure typically composed of \(\beta\)-1,3-glucan, \(\beta\)-1,6-glucan, chitin, mannan, and proteins.12,13) Although the wall composition frequently varies markedly among species of fungi, hydrolysis of \(\beta\)-1,3-glucan can weaken the cell wall structure. For example, \(\beta\)-1,3-glucanases from Paenibacillus sp. and Streptomyces siyaeensis had the ability to damage the cell wall structures of growing mycelia of the phytopathogenic fungi Pythium aphanidermatum and Rhizoctonia solani.14) These enzymes are classified as exo-\(\beta\)-1,3-glucanase (EC 3.2.1.58) and endo-\(\beta\)-1,3-glucanase (EC 3.2.1.6 and EC 3.2.1.39). Exo-\(\beta\)-1,3-glucanase sequentially releases glucose residues from the non-reducing terminus of a substrate, while endo-\(\beta\)-1,3-glucanase is capable of cleavage of internal \(\beta\)-1,3-linkages at random sites along the polysaccharide chain, releasing short oligosaccharides.

Alkaliphilic Nocardiopsis sp. F96 was isolated from a soil sample at Fuji in Shizuoka Prefecture, Japan, as a chitinase-producer.15) It has been shown that strain F96 also produces an endo-\(\beta\)-1,3-glucanase.16) The \(\beta\)-1,3-glucanase gene (bglF) was cloned from the chromosomal DNA of strain F96. It encoded a polypeptide comprising a signal peptide (25 amino acids) and mature enzyme (245 amino acids). The deduced amino acid sequence of mature BglF exhibited the highest homology to those of glycoside hydrolase (GH) family 16 \(\beta\)-1,3-glucanases, suggesting that the enzyme belongs to GH family 16 (http://www.cazy.org/index.html). Mature BglF was successfully expressed in Escherichia coli and characterized. The optimum pH and temperature of BglF were around pH 9.0 (at 37 °C) and 70 °C (at pH 6.0), respectively. This enzyme efficiently hydrolyzed insoluble \(\beta\)-1,3-glucans and showed the highest activity toward a \(\beta\)-1,3,1,4-glucan rather than \(\beta\)-1,3-glucans. Crystallization and structural studies have also been carried out on BglF.17,18) The overall structure of BglF exhibits a classical sandwich-like \(\beta\)-jelly roll motif. It is appeared that the N- and C-terminal ends are located at opposite sides of the catalytic cleft.

Some \(\beta\)-1,3-glucanases have one or several carbohydrate-binding modules (CBMs). The CBM generally enhances the hydrolytic activities of the catalytic domain toward insoluble substrates. For example, \(\beta\)-1,3-glucanase H (BglH) from Bacillus circulans IAM1165 is composed of a GH family 16 catalytic domain, an N-terminal additional domain (NAD), and a C-terminal additional domain (CAD).19) NAD and CAD of BglH have binding abilities toward insoluble substrates. NAD is comprised of 331 amino acids with three internal repetitive regions. The deduced amino acid sequence of NAD was highly homologous to the N-terminal region of BglF.

Abbreviations: GH, glycoside hydrolase; CBM, carbohydrate-binding module; ChBD, chitin-binding domain; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

1 To whom correspondence should be addressed. Fax: +81-45-924-5837; E-mail: nakamura.s.af@m.titech.ac.jp

Journal of Biosciences and Biotechnologies, 73 (S), 1078–1082, 2009
of $\beta$-1,3-glucanase (GlcA) from Bacillus circulans WL-12.20 On the other hand, the amino acid sequence of CAD, comprised of 193 residues, was highly homologous to the C-terminal region of a glycoside hydrodase from Geobacillus sp. Y412MC10 found in the NCBI database (EZJ039713).

In order to improve the enzymatic properties of BglF, we constructed various chimeric enzymes composed of BglF (catalytic domain) and CAD and/or NAD of BglH in this study. At the same time, a chitin-binding domain (ChBD) of chitinase (ChiJ) from alkaliphilic Bacillus sp. J813 was also used.21) The deduced amino acid sequence of ChBD of ChiJ exhibited the highest homology to those of CBM family 5 ChBDs (http://www.cazy.org/index.html).

Materials and Methods

Materials. Oligonucleotide primers were purchased from Operon Biotechnologies (Tokyo). Restriction enzymes, a DNA ligation kit, and DNA polymerase were from Toyobo (Osaka, Japan). Various polysaccharides, including laminarin from Laminaria digitata (Nacalai Tesque, Kyoto, Japan), curdlan from Alcaligenes faecalis (Wako, Osaka, Japan), lichenan from Cetraria islandica (Sigma-Aldrich, Tokyo), avicel (Merck, Darmstadt, Germany), and chitin (Funakoshi, Tokyo) were purchased, while pachyman was prepared from commercial fruiting bodies of Poria cocos (Suzu, Osaka, Japan).

Plasmids, strains, and medium. Recombinant plasmids pET-BglF bearing the bglf gene from strain F9620 and pET-B bearing the ChBD region of the chd gene from strain J81321 were used. Plasmid pBG200 contains the bglf gene from R. circulans IAM11657 Escherichia coli JM109 and E. coli BL21(DE3) were used as host strains for construction of plasmids and enzyme production, respectively. E. coli cells were grown in Luria-Bertani (LB) medium supplemented with 50 $\mu$g/ml of ampicillin.

Construction of chimeric genes. All DNA manipulations were performed using standard protocols. The DNA fragment encoding CAD of BglH was amplified by polymerase chain reaction (PCR) with pBG200 as the template. The primers used were as follows: a forward primer, 5'-GGATCCGGGGAAGGCCGCGGAGAATC-3', and a reverse primer, 5'-CGTCTGGATCTCATCCGTGTCC-TTGGGTAC-3'. The underlined sequence corresponds to a BamHI site that was introduced into each primer. The PCR products were digested with BamHI and ligated into the BamHI site of pET-BglF, and a noncoding region generated between the two DNA fragments was deleted by inverse PCR to yield pET-BglF-CAD. The DNA fragment encoding NAD of BglH was amplified by PCR with pET-B as the template. The primers used were as follows: a forward primer, 5'-ATAGAATCTCAAGCAGAAAATGCGGCG-3', and a reverse primer, 5'-GCCGGATCTACATTTTGTGC-3'. The underlined sequence is an EcoRI site. The DNA fragment encoding the ChBD region was prepared by EcoRI digestion of the PCR products and ligated into the EcoRI site of pET-BglF, and a noncoding region was deleted to construct pET-BglF-ChBD, as described above. DNA sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). If necessary, 10% (v/v) of dimethyl sulfoxide (DMSO) was added to the sequence reaction mixtures.

Expression of chimeric enzymes. E. coli BL21(DE3) cells containing the expression plasmids were cultured at 37 $^\circ$C in 100 ml of LB broth containing 50 $\mu$g/ml of ampicillin. When the optical density at 660 nm of culture was reached at 0.6 (mid-log phase), 1 mM isopropyl-$\beta$-1-thiogalactopyranoside (IPTG) was added to induce chimeric gene expression. The cells were further incubated at 37 $^\circ$C for 2.5 h. They were harvested by centrifugation at 3,000 $\times$ g at 4 $^\circ$C for 10 min, suspended in 10 mM Tris-HCl buffer (pH 7.5), and then sonicated to prepare the cell extracts. The cell extracts were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels.22) After electrophoresis, the proteins in the gels were stained with Coomassie Brilliant Blue (CBB) R-250.

Enzyme assay and protein measurement. Unless otherwise stated, the standard assay for $\beta$-1,3-glucanase activity was carried out by measuring the amount of reducing sugars liberated from a $\beta$-1,3-glucan substrate (laminarin) by the 3,5-dinitrosalicylic acid (DNS) method. Briefly, 10 $\mu$l of the enzyme preparation was added to 40 $\mu$l of 1.5% laminarin suspension in 80 mM Britton-Robinson buffer (pH 7.0).24) The reaction was carried out at 37 $^\circ$C for 10 min. The enzyme was inactivated by adding 100 $\mu$l of the DNS reagent and boiled for 5 min. Absorbance at 545 nm was measured immediately after 600 $\mu$l of distilled water was added. One unit was defined as the amount of enzyme that produces reducing sugars equivalent to 1 $\mu$mol of glucose per min under the conditions described above. Hydrolytic activities toward several insoluble polysaccharides were performed by gently mixing 160 $\mu$l of each enzyme (0.08 U under the standard assay conditions) with 640 $\mu$l of 3 mg polysaccharide suspension in 80 mM Britton-Robinson buffer (pH 7.0) at 37 $^\circ$C. After various incubation times, the mixture was centrifuged at 20,400 $\times$ g at 4 $^\circ$C for 10 min. The liberated sugars in 50 $\mu$l of the supernatant were measured by the DNS method. In the case of analyzing pH profiles, Britton-Robinson buffers of various pHs were used. The protein concentrations were determined by the Lowry method using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard.

Polysaccharide-binding assay. The binding abilities of chimeric enzymes, as well as that of BglF, toward several insoluble polysaccharides were estimated by gently mixing 300 $\mu$l of enzymes (0.6 mg), polysaccharides (3 mg), and BSA (0.3 mg) in Britton-Robinson buffer (pH 7.0) at 4 $^\circ$C. After 1 h, the mixture was centrifuged at 20,400 $\times$ g at 4 $^\circ$C for 10 min. The enzyme remaining in the supernatant was measured by the standard assay. The amount of enzyme bound to each polysaccharide was estimated by subtraction of the activity recovered from the original activity.19)

Results

Preparation of chimeric enzymes

The schematic structures of the chimeric enzymes constructed in this study are shown in Fig. 1. The cell extracts of E. coli BL21(DE3) containing expression plasmids pET-BglF, pET-BglF-CAD (encoding BglF-CAD), pET-NAD-BglF (encoding NAD-BglF), pET-NAD-BglF-CAD (encoding NAD-BglF-CAD), and pET-BglF-ChBD (encoding BglF-ChBD) were applied to SDS-PAGE. All the chimeric enzymes were successfully expressed in E. coli, since the molecular masses of

![Fig. 1. Schematic Structures of BglF and Chimeric Enzymes.](image-url)
the recombinant enzymes estimated by SDS–PAGE were in agreement with those predicted from the amino acid sequences (Fig. 2). The amounts of chimeric enzymes produced were almost the same as that of BglF. Chimeric enzymes BglF-CAD and BglF-ChBD exhibited almost the same activity as BglF, although the NAD-fused enzymes, NAD-BglF and NAD-BglF-CAD, showed remarkably lower activities, approximately one tenth of that of BglF (data not shown).

**Binding abilities toward insoluble polysaccharides**

The binding abilities of BglF and chimeric enzymes to various insoluble polysaccharides were assayed by incubating the enzymes with each tested substrate at 4 °C at pH 7.0 for 1 h. The residual enzyme activity that remained in the supernatant was then determined. No glucose equivalent was released during the period of incubation at 4 °C for 1 h. As shown in Table 1, BglF slightly bound to chitin, although it did not bind to other polysaccharides. The binding abilities of BglF-CAD were moderately increased toward β-1,3-glucons (curdlan and pachyman), and slightly increased toward a β-1,3-1,4-glucon (lichenan) compared to BglF. The binding abilities of NAD-fused enzymes (NAD-BglF and NAD-BglF-CAD) were moderately increased toward curdlan and pachyman, and remarkably toward lichenan. On the other hand, the binding ability of BglF-ChBD remarkably increased toward chitin, and unexpectedly the binding abilities toward avicel, pachyman, and lichenan also increased. It is noteworthy that ChBD, a chitin-binding domain, binds to some species of β-1,3- and β-1,3-1,4-glucons.

**Hydrolytic activities toward insoluble polysaccharides**

Hydrolytic activities toward insoluble polysaccharides (curdlan, pachyman, and lichenan) were examined for BglF, BglF-CAD, and BglF-ChBD. The same amount of each enzyme equivalent to 0.08 U against laminarin, a soluble β-1,3-glucan, was used for the hydrolysis of insoluble polysaccharides. All the enzymes naturally showed the same activity toward laminarin (Fig. 3A). BglF-CAD showed higher activity toward curdlan than BglF, whereas the activity of BglF-ChBD was almost the same as that of BglF (Fig. 3B). The activities of both chimeric enzymes toward pachyman increased compared to that of BglF (Fig. 3C). On the other hand, the chimeric enzymes and BglF exhibited almost the same activity toward lichenan (Fig. 3D), although CAD and ChBD had distinct binding abilities to lichenan.

**Table 1. Binding Ability toward Insoluble Polysaccharides**

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Binding rate (%)</th>
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<tr>
<td></td>
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<tr>
<td></td>
<td>BglF</td>
</tr>
<tr>
<td>Curdlan (β-1,3-Glucan)</td>
<td>N.D.*</td>
</tr>
<tr>
<td>Pachyman (β-1,3-Glucan)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Lichenan (β-1,3-1,4-Glucan)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Avicel (β-1,4-Glucan)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Chitin (β-1,4-Glucan)</td>
<td>26</td>
</tr>
</tbody>
</table>

*N.D., not detected.*
Fig. 4. Effect of Reaction pH on the Activities of BglF, BglF-CAD, and BglF-ChBD.
Activity at pH 7.0 was regarded as 100%. Closed circles, BglF; open triangles, BglF-CAD; open squares, BglF-ChBD.

Fig. 5. Effect of Reaction Temperature on the Activities of BglF, BglF-CAD, and BglF-ChBD.
Activity at 37 °C was regarded as 100%. Closed circles, BglF; open triangles, BglF-CAD; open squares, BglF-ChBD.

Effects of reaction pH
The effects of reaction pH on the activities of BglF, BglF-CAD, and BglF-ChBD were determined using laminarin as a substrate. The activity was measured at 37 °C at various pHs according to the conditions of standard assay, and the activity of each enzyme at pH 7.0 was regarded as 100%. As shown in Fig. 4, the pH profiles of these enzymes were almost the same, and only BglF-CAD showed higher activity at pH 9–10 than BglF. It is unclear why the pH profiles have two-humped shapes.

Effects of reaction temperature
The effects of reaction temperature on the activities of BglF, BglF-CAD, and BglF-ChBD were determined using laminarin substrate. The activities were measured at various temperatures at pH 7.0 according to the conditions of standard assay, and the activity of each enzyme at 37 °C was regarded as 100%. As shown in Fig. 5, the optimum temperature of BglF, BglF-CAD, and BglF-ChBD was 60 °C. The activities of BglF-CAD and BglF-ChBD at around 50–70 °C were apparently higher than that of BglF.

Discussion
In this study, chimeric enzymes composed of BglF and several additional CBMs were prepared and characterized. BglF-CAD exhibited increased binding abilities and hydrolytic activities toward two insoluble β-1,3-glucans, curdlan and pachyman. On the other hand, BglF-ChBD also showed increased binding ability and hydrolytic activity toward pachyman, but binding ability and hydrolytic activity toward curdlan were almost the same as those of BglF. These results indicate that enzyme binding toward insoluble β-1,3-glucan by CBM contributes to increase the local concentration of enzyme around insoluble substrate and induces efficient hydrolysis. The difference in the binding rates of ChBD toward curdlan and pachyman might be caused by the conformational difference among these polysaccharides. Although BglF-CAD and BglF-ChBD bound to lichenan (β-1,3-1,4-glucan), the hydrolytic activities of the enzymes were not increased, suggesting that the CBMs and BglF bind to the same or neighboring regions of lichenan. The NAD-fused enzymes showed significantly higher binding abilities toward all the polysaccharides tested. The activities of these enzymes, however, were remarkably lower than that of BglF under the standard assay conditions, suggesting that fused NAD conceals the catalytic cleft of BglF. We are now trying to fuse NAD downstream of the C-terminal of BglF or, alternatively, to insert a linker sequence between NAD and BglF.

The hydrolytic activity of BglF-CAD in the high pH region was higher than that of BglF. The solubility of laminarin decreases in the higher pH region (pH 7.0) (data not shown). Thus, BglF-CAD might bind and hydrolyze laminarin more efficiently in the higher pH region. The optimum temperature of BglF-CAD and BglF-ChBD was similar to that of BglF. But the activities of both chimeric enzymes at around 50–70 °C were higher than that of BglF. This result does not contradict the fact that the solubility of laminarin slightly decreased at higher temperatures (data not shown).

CBMs have been grouped into three types based on the topology of binding sites, which reflects the macromolecular structure of the target ligands. Type A CBMs contain planar hydrophobic ligand binding surfaces that interact with crystalline polysaccharides. Type B CBMs contain clefts that accommodate a single polysaccharide chain, while the ligand binding sites in type C CBMs interact with mono-, di-, or trisaccharides. Family 5 CBMs, including ChBD of ChiJ, are classified into type A. A recent structural study revealed a unique feature of CBM family 5 ChBD of chitinase C from Streptomyces griseus HUT6037. In this ChBD, two exposed aromatic residues, Trp59 and Trp60, were found on the surface. The ligand molecule bound well on the surface-exposed tryptophans through two stacking interactions and two hydrogen bonds, and only Trp59 and Trp60 were involved in chitin binding. In this study, it was found that ChBD of ChiJ probably interacted with crystalline cellulose (avicel), β-1,3-glucan and β-1,3-1,4-glucan, as well as chitin. Two tryptophan residues of ChBD of ChiJ, corresponding to Trp59 and Trp60 of ChiC, might interact also with crystalline cellulose, β-1,3-glucan, and β-1,3-1,4-glucan. Binding of CBM family 5 ChBDs toward β-1,3-glucan and β-1,3-1,4-glucan has not been reported yet. On the other hand, CAD of BglH had binding abilities toward pachyman, curdlan, and lichenan, and did not bind to crystalline polysaccharides, such as chitin and avicel. These results suggest that CAD should be classified into type B.
To the best of our knowledge, CBM-fused β-1,3-glucanase has not been reported yet, whereas several chimeric enzymes based on other glycoside hydrolases have been investigated.\(^2\,1\,2\) The chimeric chitinases, for example, have been prepared by fusing a chBD of Nicotiana tabacum chitinase, ChiA, and a cellulose-binding domain of Trichoderma reesei cellobiohydrolase II to a chitinase (Chit42) of Trichoderma harzianum.\(^3\) Compared to the native Chit42, chimeric enzymes had similar hydrolytic activity toward soluble substrates and higher activity toward high-molecular-mass insoluble substrates such as ground crab-shell chitin and chitin-rich fungal cell walls. Chimeric enzymes constructed in this study showed increased binding abilities and hydrolytic activities toward insoluble polysaccharides, and might be useful as antifungal agents.

**Acknowledgments**

We would like to thank Dr. Mami Yamamoto of Toyo University for providing plasmid pBG200 and for helpful discussion. This study was partially supported by the Global COE Program from Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT), and a Grant-in-Aid for JSPS fellows from Japan Society for the Promotion of Science (JSPS).

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