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Characterization of pH-tolerant and thermostable GH 134 β-1,4-mannanase SsGH134 possessing carbohydrate binding module 10 from *Streptomyces* sp. NRRL B-24484

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A GH 134 β-1,4-mannanase SsGH134 from *Streptomyces* sp. NRRL B-24484 possesses a carbohydrate binding module (CBM) 10 and a glycoside hydrolase 134 domain at the N- and C-terminal regions, respectively. Recombinant SsGH134 expressed in *Escherichia coli*. SsGH134 was maximally active within a pH range of 4.0–6.5 and retained >80% of this maximum after 90 min at 30°C within a pH range of 3.0–10.0. The β-1,4-mannanase activity of SsGH134 towards glucomannan was 30% of the maximal activity after an incubation at 100°C for 120 min, indicating that SsGH134 is pH-tolerant and thermostable β-1,4-mannanase. SsGH134, SsGH134-ΔCBM10 (CBM10-linker-truncated SsGH134) and SsGH134-G34W (substitution of Gly34 to Trp) bound to microcrystalline cellulose, β-mannan and chitin, regardless of the presence or absence of CBM10. These indicate that GH 134 domain strongly bind to the polysaccharides. Although deleting CBM10 increased the catalytic efficiency of the β-1,4-mannanase, its disruption decreased the pH, solvent and detergent stability of SsGH134. These findings indicate that CBM10 inhibits the β-1,4-mannanase activity of SsGH134, but it is involved in stabilizing its enzymatic activity within a neutral-to-alkaline pH range, and in the presence of various organic solvents and detergents. We believe that SsGH134 could be useful to a diverse range of industries.

[**Key words:** β-1,4-Mannanase; Carbohydrate binding module 10; Catalytic efficiency; Glycoside hydrolase 134; Hemicellulose]

The internal β-1,4-linkage of the mannan backbone is randomly hydrolyzed by β-1,4-mannanases that have been isolated from bacteria, fungi, plants and animals (1–4). Based on sequence similarity, β-1,4-mannanases have been classified into the glycoside hydrolase (GH) families 5 (GH 5), GH 26, GH 113 and GH 134 (1–5). The GH 26 β-1,4-mannanases were found predominantly in bacteria such as *Bacillus subtilis*, *Cellvibrio japonicus*, *Clostridium cellulovorans*, *Clostridium thermocellum*, *Klebsiella oxytoca* KUB-CW2-3, *Pantoea agglomerans* and *Reticulitermes speratus* (6–11). Some bacteria such as *Bacillus licheniformis*, *Cellulosimicrobium* sp. HY-13, *Streptomyces thermolilacinus*, *Thermotoga petrophila* and *Vibrio* sp. strain MA-138 reportedly produce GH5 β-1,4-mannanase (12–16). The primary structure of GH 5, GH 26 and GH 113 β-1,4-mannanases considerably differ, but their spatial arrangements are similar and they have a $(\beta/\alpha)_8$-barrel protein fold; thus they are grouped into the GH-A clan (2,4). The three-dimensional structure of the GH 134 β-1,4-mannanase SsGH134 from *Streptomyces* sp. NRRL B-24484 has recently been reported (17). A representative GH family 134 β-1,4-mannanase displays a fold closely related to that of hen egg-white lysozyme but acts with an inversion of stereochemistry (17). The structures of the Michaelis and product complexes suggest a Southern hemisphere $^1C_4 \rightarrow {}^3H_4^+ \rightarrow {}^3S_1$ conformational itinerary along the reaction coordinate, with the product relaxing to a Michaelis-mimicking $^1C_4$ conformation (possibly via a $^3H_4$ conformation) after the reaction is complete. *Streptomyces* sp. NRRL B-24484 has an operon encoding a series of proteins that are predicted to facilitate the deconstruction and metabolism of β-mannan, including a GH 2 β-mannosidase, two GH 5 β-mannanases, SsGH134, sugar ABC transporters, and a mannose-6-phosphate isomerase (17). In addition, SsGH134 possesses a carbohydrate binding module (CBM) 10 that is attached via a short Gly–Ser linker to its catalytic domain.

Glycoside hydrolases often display modular architectures comprising CBM (4,18). In general, CBM increases substrate concentrations around catalytic domains, which leads to increased catalytic efficiency (18). Carbohydrate binding modules have been classified into 81 families according to amino acid sequences, binding specificity and structure (CAZy database: www.cazy.org). Among them, CBM10 is mainly fused to cellulase, β-xylanase and β-1,4-mannanase from bacteria (19–22). CBM10 binds to insoluble microcrystalline cellulose and insoluble β-mannan (20,21).

The present study uncovered the enzymatic characteristics of SsGH134 and the role of the CBM10 domain at the N-terminal region. The enzymatic properties of SsGH134 differed from those of bacterial β-1,4-mannanases that belong to GH 5 and GH 26. We also found that CBM10 disruption increased catalytic efficiency and decreased pH, solvent and detergent stabilities.

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MATERIALS AND METHODS

Chemicals Low viscosity konjac glucomannan, carob galactomannan and insoluble β-mannan were purchased from Megazyme International (Bray, Ireland). Microcrystalline cellulose (MCC) and chitin were obtained from Funakoshi (Tokyo, Japan). Xylan from beech wood was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of recombinant proteins The codon-optimized form of a DNA fragment encoding *SsGH134* (NCBI accession no. WP_030268297.1) from *Streptomyces* sp. NRRL B-24484 was synthesized for expression in *Escherichia coli*. The *SsGH134* gene was inserted into pET28a (Novagen, Darmstadt, Germany). A CBM10-linker-truncated SsGH134 (SsGH134-ΔCBM10) was generated using QuikChange site-directed mutagenesis kits (Stratagene, San Diego, CA, USA) and the primer set: forward SsGH134-ΔCBM10: 5′-ACCTGTGGTTCTTATACGGTTGGCG-3′; reverse, SsGH134-ΔCBM10: 5′-CGGGGCGGTTTCGAATTCGGATCCG-3′. The Gly34 of SsGH134 replaced with Trp (SsGH134-G34W) was also constructed using QuikChange site-directed mutagenesis kits (Stratagene) and the primer set: forward SsGH134-G34W: 5′-TGGTATCCGTACTGCGCTAATGGTAGTG-3′; reverse SsGH134-G34W, 5′-GTTCGGGGCGGTTTCGAATTCGGAT-3′. The resulting plasmids were introduced into *E. coli* BL21 CodonPlus(DE3) (Novagen) and cultured in LB containing 50 μg/mL kanamycin sulfate for 16 h, and then 2-mL portions were agitated at 120 rpm in 200 mL of LB containing 50 μg/mL kanamycin sulfate at 37°C. When the optical density reached 1.0, isopropyl-thio-β-D-galactoside (0.2 mM) was added to the medium and the mixtures were further shaken at 120 rpm for 16 h at 25°C. The *E. coli* cells were harvested, suspended in 50 mL of 20 mM Tris−HCl buffer (pH 8.0) containing 150 mM NaCl and disrupted by sonication. Cell-free extracts were obtained from suspensions after centrifugation at 6000 ×*g* for 15 min, and then soluble fractions were further separated by centrifugation at 100,000 ×*g* for 30 min. These fractions were passed through columns (φ5 × 20 mm) containing nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) that were washed with 10 mL of 20 mM Tris−HCl buffer (pH 8.0) containing 50 mM imidazole. Proteins were eluted with the same buffer containing 300 mM imidazole. After dialysis with 20 mM Tris−HCl (pH 8.0), the protein solution was fractionated on 1-mL HiTrap Q columns (GE Healthcare, Waukesha, WI, USA) using a linear gradient of 0−0.5 M NaCl in 20 mM Tris−HCl (pH 8.0). The purified recombinant protein was finally dialyzed against 20 mM Tris−HCl (pH 8.0). All protein purification steps proceeded at 4°C. The protein concentrations were measured at 280 nm.

Binding assay of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W The reaction mixture containing 50 mM sodium acetate buffer (pH 5.0), SsGH134, SsGH134-ΔCBM10 or SsGH134-G34W, and 15 mg/mL of MCC, insoluble β-mannan, chitin or xylan was incubated at 37°C for 30 min and then separated by centrifugation at 15,000 ×*g*, for 10 min. The supernatant fraction was decanted and the precipitate was suspended in 50 mM sodium acetate buffer (pH 5.0). The wash and precipitate fractions were separated from the suspension by centrifugation and analyzed using SDS-PAGE.

Cellulose, insoluble β-mannan, chitin or xylan binding was quantified as described (20). Mixtures (100 μL) containing 0.5−10.0 μM of enzyme and 1.0 mg (w/v) MCC, insoluble β-mannan, chitin or xylan were incubated in 50 mM sodium acetate (pH 5.0), and then pelleted by centrifugation at 15,000 ×*g* for 10 min. The concentrations of the enzymes in the supernatant fractions were measured at 280 nm.

Enzyme assays The activity of β-1,4-mannanase was assayed in 0.5-mL reaction mixtures containing 0.2−5.0% (w/v) substrates and purified proteins that were incubated at 37°C. Enzymes were removed from reaction solutions using a Nanosep Centrifugal Device (Pall Corporation, Port Washington, NY, USA) as described by the manufacturers, and flow-through fractions were boiled at 100°C for 30 min. Reducing sugars produced by β-1,4-mannanase were measured using the DNS method. Standard curves were prepared based on solutions containing different concentrations of mannose.

Stability of pH and thermostability of purified SsGH134 and SsGH134-ΔCBM10 The pH stability of SsGH134 and SsGH134-ΔCBM10 was determined using glucomannan as a substrate. The β-1,4-mannanases were incubated in 50 mM sodium acetate (pH 3.0−5.0), 50 mM sodium phosphate (pH 5.0−7.0), and 50 mM Tris−HCl (pH 7.0−10.0) for 90 min, and then residual β-1,4-mannanase activity against 0.5% glucomannan was measured at the optimal pH of 5.0. The thermostability of SsGH134 and SsGH134-ΔCBM10 was determined using glucomannan as a substrate. Purified β-1,4-mannanases were incubated at temperatures ranging from 80°C to 100°C for 15, 30, 60 and 120 min. Residual β-1,4-mannanase activity was assayed at pH 5.0. The amount of reducing sugars produced by β-1,4-mannanase was measured using the DNS method.

Effects of organic solvents and detergents on β-1,4-mannanase activity We determined the effects of 20% solvents, methanol, ethanol, isopropanol and acetone, 10% detergents, SDS, Triton X-100 and Tween-20 on the enzymatic activity of β-1,4-mannanase by measuring residual activity against 0.5% glucomannan after a 60-min incubation at 30°C.

Sequence analysis Amino acid sequences of CBM10 in GH 134 β-1,4-mannanase from *Streptomyces* sp. NRRL B-24484 (SsGH134, WP_030268297.1) were aligned with the SsGH134 homologs, KIQ61562 from *Kitasatospora griseola*, WP_062059199 from *Cellvibrio* sp. OA-2007, AIF91528 from *Alteromonadaceae bacterium* Bs12 and AIF91558 from *A. bacterium* Bs02, and GH 5 β-1,4-mannanase from *C. japonicus* (CjMan5A, WP_012488914.1), GH 5 β-1,4-mannanase from *C. japonicus* (CjMan5B, WP_012488072.1), GH 10 β-xylanase from *Pseudomonas fluorescens* (PfXyn10A, WP_012488068.1), and GH 5 cellulase from *Lyrodus pedicellatus* (Cel5AB, ABS72374.1) using ClustalW (http://www.clustal.org).

RESULTS

Alignment of CBM10 from various glycoside hydrolases We aligned CBM10 amino acid sequences of GH 134 β-1,4-mannanase from *Streptomyces* sp. (SsGH134, WP_030268297.1) as described above. Ponyi et al. (20) found using site-directed mutagenesis that aromatic amino acid residues in CBM10 sequences from PfXyn10A (GH 10 β-xylanase from *P. fluorescens*) are highly conserved, and that W497, Y498, W512 and W514 (previously represented as W7, Y8, W22 and W24) of CBM10 play roles in binding to cellulose and β-mannan. Four of the CBM10 amino acid residues that interact with polysaccharides (boxed in Fig. 1) were highly conserved. In contrast, the essential residue W497 of CBM10 in PfXyn10A sequence was not conserved in SsGH134 (Fig. 1). SsGH134 homologs from bacteria are distributed in some actinobacteria and proteobacteria (5). Among them, SsGH134 from *Streptomyces* sp. NRRL B-24484, NCBI accession no. KIQ61562 from *K. griseola*, NCBI accession no. WP_062059199 from *Cellvibrio* sp. OA-2007, NCBI accession no. AIF91528 from *Alteromonadaceae bacterium* Bs12 and NCBI accession no. AIF91558 from *A. bacterium* Bs02 were fused with CBM10. The first Trp of CBM10 was highly conserved in WP_062059199, AIF91528 and AIF91558 from a proteobacterium, while the substitution of the first Trp with Gly was presented in SsGH134 and KIQ61562 from an actinobacterium. These indicate

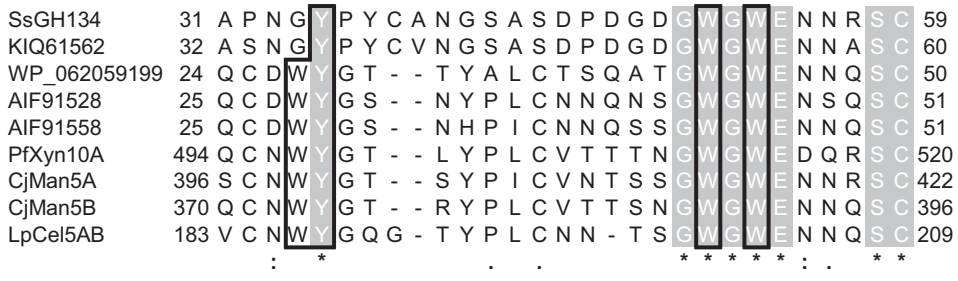

FIG. 1. Amino acid alignment of CBM10. Amino acid sequences of CBM10 were aligned using ClustalW. Amino acids that might interact with cellulose are boxed, and identical amino acids are indicated in gray. Dots and colons indicate conserved amino acids with substitutions. CBM10 of SsGH134 (WP_030268297.1) from *Streptomyces* sp., CBM10 of KIQ61562 from *Kitasatospora griseola*, CBM10 of WP_062059199 from *Cellvibrio* sp. OA-2007, CBM10 of AIF91528 from *Alteromonadaceae bacterium* Bs12, CBM10 of AIF91558 from *A. bacterium* Bs02, CBM10 of CjMan5A (WP_012488914.1) from *Cellvibrio japonicus*, CBM10 of CjMan5B (WP_012488072.1) from *Cellvibrio japonicus*, CBM10 of PfXyn10A (WP_012488068.1) from *Pseudomonas fluorescens*, CBM10 of Cel5AB (ABS72374.1) from *Lyrodus pedicellatus*.