Purification, Characterization, Cloning, and Expression of a Novel Xyloglucan-specific Glycosidase, Oligoxyloglucan Reducing End-specific Celllobiohydrolase*

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A novel oligoxyloglucan-specific glycosidase, oligoxyloglucan reducing end-specific celllobiohydrolase (OXG-RCBH), with a molecular mass of 97 kDa and a pI of 6.1, was isolated from the fungus Geotrichum sp. M128. Analysis of substrate specificity using various xyloglucan oligosaccharide structures revealed that OXG-RCBH had exoglucanase activity. It recognized the reducing end of oligoxyloglucan and released two glucosyl residue segments from the main chain. The full-length cDNA encoding OXG-RCBH was cloned and sequenced, and it had a 2436-bp open reading frame encoding an 812-amino acid protein. The deduced protein showed 35% identity to members of glycoside hydrolase family 74. The cDNA encoding OXG-RCBH was then expressed in Escherichia coli. Although the recombinant protein was expressed as an inclusion body, renaturation was successful, and enzymatically active recombinant OXG-RCBH was obtained.

Xyloglucan is a major hemicellulose polysaccharide found in the primary cell wall of plants. It constitutes ∼20% of the total cell wall in dicot and non-graminaceous monocot plants and 5–10% of Gramineae cell walls. Xyloglucan associates with cellulose microfibrils by hydrogen bonding, forming a cellulose-xyloglucan network (1–3). Xyloglucan consists of a cellulose-like backbone chain of β-1,4-linked β-D-Glc residues that are frequently substituted at C-6 with side chains of α-D-Xylp-(1→6)-β-D-Galp or α-D-Xylp-(1→2)-α-D-Xylp-(1→6)-β-D-Galp. Each segment has a specific code letter depending on the side chains. The letters G, X, L, and F refer to an unbranched β-D-Glc residue, an α-D-Xylp-(1→6)-β-D-Glc segment, a β-D-Galp-(1→2)-α-D-Xylp-(1→6)-β-D-Glc segment, and an α-D-Fucp-(1→2)-β-D-Galp-(1→2)-α-D-Xylp-(1→6)-β-D-Glc segment, respectively (4). Structural studies suggest that most xyloglucan consists of XXXG or XXGG repeating units (5). In dicots, treatment of xyloglucan with endo-β-1,4-glucanase results in the cleavage of unbranched, 4-linked Glc residues in the backbone chain, generating mainly oligosaccharide subunits (XXXG, XLXG, XXLG, XLG, XXFG, XLFG, and so on) (6).

Disassembly of the cellulose-xyloglucan network is required for cell expansion and development. Xyloglucan metabolism is thought to have an important role in cell definition, cell expansion, and regulation of plant growth and development (7–11). Recently, it was reported that xyloglucan integration by xyloglucan endotransglycosylase in the cell wall suppresses cell elongation, whereas that of the xyloglucan oligosaccharide XXXG accelerates elongation (12). It was proposed that xyloglucan metabolism controls plant cell elongation. In the growing plant cell wall, xyloglucan oligosaccharides may provide positive or negative feedback control during cell elongation. Consequently, compositional analysis of oligosaccharide units in xyloglucan polymers is very important.

Glycosidases are useful tools for analyzing the fine structure of complex carbohydrates, and screening and characterizing novel glycosidases are important for ensuring progress in glycotechnology. In this study, we report the purification, characterization, cloning, and expression of a novel oligoxyloglan-specific β-glycosidase produced by Geotrichum sp. M128 fungus, which was isolated from soil in Tsukuba, Japan. The enzyme recognized the reducing end of the oligoxyloglucan heptasaccharide XXXG and produced the tetrasaccharide XXG and the trisaccharide XG. This enzymatic activity is very different from the activities of known glycosidases and constitutes a potentially powerful tool for the structural analysis of cell wall xyloglucan. It is also shown that the enzyme had minor activity for the reducing end of cello-oligosaccharides, releasing cellobiose. Based on these results, we propose oligoxyloglucan reducing end-specific celllobiohydrolase (OXG-RCBH) as the trivial name of this new enzyme.

EXPERIMENTAL PROCEDURES

Organism—Geotrichum sp. M128 was isolated from soil in Tsukuba, Japan, and deposited in the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Ibaraki, Japan) under FERM P-16454.

Enzymatic Activity Assay of Geotrichum sp. M128 Culture Supernatant—Enzymatic activity was assayed by normal phase HPLC. The Geotrichum sp. M128 culture supernatant was incubated with 1% oligoxyloglucan heptasaccharide XXXG prepared using a previously described method (12). The reaction mixture (10 μl) was loaded onto a TSKgel Amide 80 column (Tosoh Co., Tokyo, Japan) and eluted with 58% acetonitrile (isocratic) at a flow rate of 0.6 ml/min to analyze the degradation products. Product structures were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and 13C NMR spectroscopy. MALDI-TOF mass spectra were recorded using a Compact MALDI IV instrument (Shimadzu Co., Kyoto, Japan) according to previously described methods (13). NMR spectra were obtained with a JNM-GX270 apparatus (Jeol Ltd., Tokyo)
at 68.5 MHz in the SGBCM mode, with complete proton decoupling at 24.5 °C, using D2O as a solvent in a 0.5-cm inner diameter tube. Chemical shifts were reported as δ (ppm) relative to the resonance of sodium 4,4-dimethyl-4-silopentano-1-sulfonate, which was used as an internal standard (δ = 0). The spectral width was 200 ppm.

**Purification of OXG-RCBH from Geotrichum sp. M128**—Geotrichum sp. M128 was cultivated in 4-liters cultures consisting of 0.8% xyloglucan from *Tamarindus indica* (Glyloid, Dainippon Pharmaceutical, Osaka, Japan), 0.8% Bactopeptone, 0.2% KH2PO4, 0.05% MgSO4, and 0.05% yeast extract (pH 6.0) at 30 °C for 6 days. Cells were removed by centrifugation, and the broth supernatant was concentrated by ultrafiltration. The concentrated supernatant was diluted with 25 mM imidazole HCl buffer (pH 7.4), loaded onto a chromatofocusing column containing Polybuffer Exchanger PBE media, PBE 94 (Amersham Biosciences), equilibrated with 25 mM imidazole HCl buffer (pH 7.4), and then eluted with a linear gradient of NaCl (0–0.5 M). Each separated fraction was assayed for XXXG hydrolytic activity by the method of Nelson (14) and Somogyi (15, 16). The active fractions were pooled and then applied to the same column with a pH gradient using Polybuffer 74 (Amersham Biosciences). The active fractions were gel-filtrated twice with an HW55F column (Tosoh Co.). The final active fraction was subjected to SDS-PAGE.

**Identification of Optimum pH, Temperature, and Stability**—McIlvain buffer solutions (pH 2.5–9.0), prepared from 0.2 M disodium hydrogen phosphate and 0.1 M citric acid, were used to identify the optimum pH and to determine pH stability. The optimum pH was examined by incubation with the oligoxyloglucan heptasaccharide XXXG at 45 °C for 30 min in Mcllvaine buffer solutions. The pH stability was assayed by incubating the enzyme, in the absence of substrate, at 45 °C for 30 min in buffer solutions. The buffer solutions were then adjusted to pH 4.0, and the remaining activity was assayed. The optimum temperature was determined by incubation in 50 mM sodium acetate (pH 4.0) at various temperatures. To analyze thermostability, the enzyme was incubated in the same buffer, without substrate, for 10 min at various temperatures. The remaining activity was then assayed at 45 °C.

**Substrate Specificity of OXG-RCBH**—The substrate specificity of purified OXG-RCBH was analyzed with various, well defined xyloglucan oligosaccharide structures prepared from tamarind seed using previously described methods (17). The resulting products were analyzed by normal phase HPLC.

**N-terminal and Internal Amino Acid Sequence Analyses**—The purified protein was separated by SDS-PAGE and transferred from the gel onto a polyvinylidene difluoride membrane filter (Millipore Corp.) in transfer buffer (10 mM CAPS (pH 11) and 10% methanol). The amino acid sequence was determined by automatic sequential Edman degradation using a Procise 494 HT protein sequencing system (Applied Biosystems). To determine internal amino acid sequences, the purified protein was digested with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Osaka). The resulting peptide fragments were fractionated by reverse phase HPLC on a TSKgel ODS-80Ts QA column (Tosoh Co.), and their N-terminal amino acid sequences were analyzed.

**cDNA Preparation**—Geotrichum sp. M128 total RNA was obtained from 3 ml of culture using a FastRNA RED kit (Bio 101, Inc.). The mRNA was prepared with an oligo(dT) column using a QuickPrep mRNA purification kit (Amersham Biosciences). The cDNA was synthesized from mRNA with an oligo(dT) primer using a TimeSaver cDNA synthesis kit (Amersham Biosciences).

**cDNA Cloning**—First, the cDNA fragment was amplified by PCR.
with degenerate primers designed from the N-terminal and internal (peptide 2) amino acid sequences: F1, 5′-GAAGACACCTTTATTGGAAGAAGG-3′; R1, 5′-TGCCGCAATTTTGTGGTTT-3′; F2, 5′-TATTTTTCACATAGCAGTGCGTGCCC-3′; and R2, 5′-GGACCCGCGGGCCGCAG-3′. The amplified fragment was sub- cloned into the T-overhang vector pGEM-T-Easy (Promega). Dideoxy double-stranded sequencing of the cDNA insert was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions, and the expected amplified fragment was obtained and confirmed. The complete nucleotide sequences were determined by 5′- and 3′-RACE using a SMART RACE cDNA amplification kit (Clontech). For 5′-RACE, the following gene-specific primers were designed: R2, 5′-CGTACAGCCAGTCTTGTCTTTGG-3′; and R3 (nested primer), 5′-ATATGATCCGGCCGCGGCAG-3′. For 3′-RACE, F2 (5′-GGACAGTTTCTGCTGTCGACGAC-3′) and F3 (nested primer, 5′-CCAACTGGACGGGACAAGGCT-3′) were used as the gene-specific primers.

Expression of OXG-RCBH in Escherichia coli Cells—The cDNA fragment encoding the mature region of OXG-RCBH was amplified by PCR with primers designed from 5′- and 3′-sequences corresponding to the N- and C-terminal regions of the mature protein: F4, 5′-TGCTCGAGCCG- CATATGGAACAGACTACTAGTCCGAAGAATG-3′; and R4, 5′-TGACGATCTTTAGCTTTCACATAGCAGTGCGTGCCC-3′. The NdeI and BglII sites (underlined) were added to the forward and reverse primers, respectively. The amplified DNA was digested with NdeI and BglII, subcloned into the pET-29a(+) expression vector (Novagen), digested with the same restriction enzymes, and transfected into E. coli BL21-CodonPlus(DE3) RP (Stratagene). The transfected cells were cultured, and expression was induced with 1 mM isopropyl-β-D-thiogalacto- pyranoside for 6 h at 37 °C. The recombinant protein was produced as an inclusion body that was extracted and partially purified with Bug-Buster protein extraction reagent (Novagen). The protein was solubilized at a concentration of 5 mg/ml in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 1 mM EDTA for 1 h at room temperature and renatured by removing the urea by dialysis against 25 mM imidazole HCl buffer (pH 7.4).

RESULTS AND DISCUSSION

Enzymatic Activity in Geotrichum sp. M128 Culture Supernatant—The oligoxyloglucan heptasaccharide XXXG was incubated with the Geotrichum sp. M128 culture supernatant, and the reaction products were separated by normal phase HPLC and analyzed by 5′- and 3′-RACE using a SMART RACE cDNA amplification kit (Clontech). For 5′-RACE, the following gene-specific primers were designed: R2, 5′-CGTACAGCCAGTCTTGTCTTTGG-3′; and R3 (nested primer), 5′-ATATGATCCGGCCGCGGCAG-3′. For 3′-RACE, F2 (5′-GGACAGTTTCTGCTGTCGACGAC-3′) and F3 (nested primer, 5′-CCAACTGGACGGGACAAGGCT-3′) were used as the gene-specific primers.

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FIG. 5. OXG-RCBH cDNA and deduced amino acid sequences. The putative N-terminal cleavable signal peptide is underlined. The consensus N-glycosylation sites are double-underlined. Partial amino acid sequences obtained from the purified protein are boxed.
stability was determined at 45 °C using Meltiave buffer solutions at various pH values. OXG-RCBH was stable between pH 4.0 and 8.0, where >90% of the activity was retained. Thermostability was analyzed at pH 4.0 by incubation at various temperatures for 10 min. More than 90% activity was retained.

**Substrate Specificity of OXG-RCBH**—Various oligosaccharide structures were used to analyze the substrate specificity of OXG-RCBH, and the results are shown in Table II. OXG-RCBH had very unique substrate specificity; the results indicated that it was an exoglucanase that recognized the structure of the reducing end of oligoxyloglucan and that released the two glucosyl main chain residues (GG, XG, or LG). When there were less than three Glc residues on the main chain, the activity decreased significantly. Based on these results, we propose the subsite image shown in Fig. 4.

**FIG. 6. Phylogenetic relationship between OXG-RCBH and the GH74 family.** The phylogenetic tree was constructed using ClustalW with the neighbor-joining method. The % identities to OXG-RCBH are indicated in parentheses. GenBank™/EBI accession numbers are as follows: Xanthomonas axonopodi pv. citri strain 306 ORF XAC1770, putative cellulase (CelA), AE011809; Xanthomonas campestris pv. campestris strain ATCC 33913 ORF XCC1752, putative cellulase, AE012276; Thermotoga maritima ORF TM3035, putative endoglucanase, AE001712; A. niger endoglucanase C (EglC), AY040839; A. aculeatus avicelase III, AB015511; A. bisporus d Biography 649 ORF cel, putative cellulase, AF292929; Clostridium acetobutylicum ATCC 824 ORF CAC0919, probably secreted sialidase (S7); Caldicellulosiruptor glycosyl hydrolase 5 (34%); C. acetobutylicum; putative secreted cellulase (G5); S. coelicolor putative secreted cellulase (G5%);

**FIG. 7. Recombinant OXG-RCBH expression in E. coli cells.** Recombinant OXG-RCBH was expressed in E. coli strain BL21-CodonPlus(DE3) RF and subjected to SDS-PAGE (7.5–15% gradient gel). The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lanes 2–4, E. coli cells transformed with pET-29a (+) alone (without OXG-RCBH cDNA); lanes 5–7, E. coli cells expressing recombinant OXG-RCBH; lane 8, purified recombinant OXG-RCBH with BugBuster. Lanes 2 and 5, whole cells; lanes 3 and 6, soluble fraction with BugBuster; lanes 4 and 7, insoluble fraction with BugBuster. The arrow indicates the position of recombinant OXG-RCBH.

**N-terminal and Internal Amino Acid Sequences**—Purified OXG-RCBH was analyzed to determine its N-terminal amino acid sequences. The following 22 amino acids were determined: KEHYEFKNVAIGGGGTTYTIVA. To analyze the internal amino acid sequences, OXG-RCBH was digested with lysyl endopeptidase, and the resulting peptide fragments were separated by reverse phase HPLC. Three peptide fragments were analyzed to obtain their N-terminal amino acid sequences. The amino acid sequences were as follows: peptide 1, DILLY—ARTDIGGA; peptide 2, ASAPSAVFIWGT; and peptide 3, VGVRVLYGT.

**cDNA Cloning of OXG-RCBH**—PCR was carried out using cDNA from Geotrichum sp. M128 as a template and degenerate primers F1 and F2, designed from the N-terminal and internal (peptide 2) amino acid sequences. The resulting DNA fragment of ~2 kilobase pairs was subcloned and sequenced. The complete cDNA nucleotide sequences were determined by 5’ and 3’-RACE, and the full-length cDNA encoding OXG-RCBH was successfully cloned. The cDNA sequence contained a 2436-bp open reading frame encoding a putative 812-amino acid protein (Fig. 5). The criteria for a Kozak consensus translational initiation site, QATATGG (19, 20), were observed. The deduced amino acid sequence perfectly matched the partial amino acid sequences obtained from the purified protein. There were nine potential N-glycosylation sites (NX/S/T). The N-terminal amino acid sequence of the mature protein started at the 24th amino acid residue, indicating that 23 amino acids were cleaved from the N terminus of the mature form. These 23 amino acids seem to be a signal sequence. N-terminal proteolytic cleavage resulted in an 85-kDa protein containing 789 amino acid residues. Because the native enzyme isolated from the Geotrichum sp. M128 culture had a molecular mass of 97 kDa, the difference in molecular mass between these two proteins may result from the presence of a carbohydrate or other post-translational modification.

The deduced amino acid shows ~31–37% identity to several proteins in the glycos hydrolase 74 (GH74) family (Fig. 6) (21–26), indicating that OXG-RCBH can be classified in this family. The functions of most of the enzymes in this family are unknown. Aspergillus aculeatus avicelase III (GenBank™/EBI accession number AB015511) was proposed as an exoglucanase, but functional analysis has not yet been conducted. Aspergillus niger EglC has been analyzed, and it was reported that it exhibits endoglucanase activity for azo-carboxymethyl cellulose, azurein-dyed and cross-linked-cellulose, carboxymethyl cellulose, β-glucan, and xyloglucan (26). On the other
hand, OXG-RCBH had exoglucanase activity, recognized the reducing end of oligoxyloglucan, and produced two glucosyl residue segments, as described above. This action is similar to that of Trichoderma reesei cellulohydrolase I (27), which cleaves cellobiose at the reducing end to produce cellobiose. However, no sequence similarity was observed between these proteins.

Expression of OXG-RCBH in E. coli Cells—To demonstrate that the protein encoded by the cloned cDNA had enzymatic activity, cDNA was expressed in E. coli strain BL21-CodonPlus(DE3) RP. This strain has extra copies of the genes argU and ploL, which encode tRNAs that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively. Many of them, particularly CCC, are found in OXG-RCBH cDNA. Although we tried expressing OXG-RCBH in another host strain (BL21(DE3)), the expression level was lower than in BL21-CodonPlus(DE3) RP (data not shown). A protein of ~85 kDa was expressed in the cells (Fig. 7, lane 5). Under the conditions we tested, recombinant OXG-RCBH was expressed as an insoluble inclusion body (Fig. 7, lane 7), like many other enzymes. Even when induction conditions were changed (e.g. induction temperature of 20°C and isopropyl-β-D-thiogalactopyranoside concentration of 0.1 mM or a different host cell strain), no OXG-RCBH activity was observed in the soluble fraction (data not shown). We tried to renature recombinant OXG-RCBH. Purified inclusion body (Fig. 7, lane 8) was solubilized in 8 M urea and renatured by dialysis. Using this procedure, renaturation was successful, and enzymatically active recombinant OXG-RCBH was obtained (11.60 units/mg of protein). The yield of refolded recombinant OXG-RCBH was >100 mg from 1 liter of culture. Testing the effects of pH and temperature on recombinant OXG-RCBH revealed that they were very close to those on native OXG-RCBH. Therefore, the recombinant OXG-RCBH found in this study will be useful for further analyses such as studies of molecular mechanisms of action and structural analysis.

Conclusion—In this study, we described a novel oligoxyloglucan-specific β-glucosidase, OXG-RCBH, from Geotrichum sp. M128 that was purified, characterized, cloned, and expressed in E. coli cells. OXG-RCBH recognized the reducing end of oligoxyloglucan and produced two glucosyl residue segments. Although several β-1,4-glucanases with specific activity for xyloglucan have been reported (26, 28, 29), OXG-RCBH activity was very different from known glycosidase activities. OXG-RCBH should prove to be a powerful tool for identifying the fine structure of oligoxyloglucan.

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