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Cloning of the *Trichoderma reesei* cDNA Encoding a Glucuronan Lyase Belonging to a Novel Polysaccharide Lyase Family▽†

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The filamentous fungus *Trichoderma reesei* produces glucuronan lyase (TrGL) when it is grown on β-(1→4)-polyglucuronate (cellouronate) as a sole carbon source. The cDNA encoding TrGL was cloned, and the recombinant enzyme was heterologously expressed in *Pichia pastoris*. The cDNA of TrGL includes a 777-bp open reading frame encoding a 20-amino-acid signal peptide and the 238-amino-acid mature protein. The amino acid sequence showed no similarity to the amino acid sequences of previously described functional proteins, indicating that the enzyme should be classified in a novel polysaccharide lyase (PL) family. Recombinant TrGL catalyzed depolymerization of cellouronate endolytically by β-elimination and was highly specific for cellouronate. The enzyme was most active at pH 6.5 and 50°C, and its activity and thermostability increased in the presence of Ca^{2+}, suggesting that its calcium dependence is similar to that of other PLs, such as pectate lyases.

In nature, polyuronates occur as components of cell wall and extracellular polysaccharides. Alginate, a heteropolysaccharide consisting of β-D-mannuronate and α-L-guluronate residues with exclusively (1→4)-glycosidic linkages, is produced by brown algae and certain bacteria. Pectin, which is partially methylated α-(1→4)-polygalacturonate, is present in the cell wall of higher plants. These anionic polysaccharides are degraded by lyases; i.e., alginate (34, 44), pectin (2, 37, 38, 40), glucuronan (6, 8, 9, 15), xanthan (12, 33), and hyaluronan (20, 21) are degraded by corresponding lyases. Currently, polysaccharide lyases (PLs) are classified into 19 families based on their amino acid sequence similarities in the carbohydrate-active enzyme (CAZy) server database (http://www.cazy.org/fam/acc_PL.html). PLs cleave their substrates by β-elimination at glycosidic bonds, resulting in the formation of a 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate in the newly formed nonreducing terminal unit (Fig. 1). In some cases, such as pectate lyases, Ca^{2+} plays an important role in the activity (2, 40).

In addition to the anionic polysaccharides mentioned above, natural polymers with β-(1→4)-glucuronan structures occur in bacteria (1, 16), fungi (10, 43), and algae (3, 31) as water-soluble polysaccharides. One of these polymers, a bacterial glucuronan from *Sinorhizobium meliloti* mutant strain M5N1CS, is partially acetylated at the C-2 and/or C-3 position (6). Recently, Isogai and Kato (19) prepared β-(1→4)-polyglucuronate (cellouronate) without any acetyl groups by oxidation of the primary hydroxyl groups at C-6 of regenerated cellulose to carboxylate groups using the 2,2,6,6-tetramethylpiperidine-1-oxyl radical, sodium hypochlorite, and sodium bromide. Cellouronate is a water-soluble homopolysaccharide and is readily depolymerized by microorganisms in the natural environment (23). We isolated a bacterial strain, *Brevundimonas* sp. strain SH203, that is able to degrade cellouronate (23, 28), and we purified two types of cellouronate lyase, cellouronate lyases I and II, which catalyze endo- and exodepolymerization of cellouronate, respectively. A combination of these two enzymes, moreover, resulted in synergistic degradation of cellouronate to its monomers (27, 28).

Recently, Delattre and coworkers isolated from compost a *Trichoderma* sp. that secreted a glucuronan lyase (GL) when it was cultured in the presence of bacterial glucuronan (9). These workers characterized this GL as an endo-type lyase that has a molecular mass of 27 kDa and reacts with deacetylated glucuronan rather than with the native glucuronan. Ascomycetes belonging to the genus *Trichoderma* have been widely studied as cellulolytic, pathogenic, or antipathogenic fungi (4, 26). The genome of the cellulolytic fungus *Trichoderma reesei* has recently been published (32), although no molecular biological information about the GL that it produces (TrGL) has been reported. In the present study, *T. reesei* was cultivated in medium containing cellouronate as a sole carbon source. The cDNA encoding TrGL was then cloned based on the genomic information for the fungus and heterologously expressed in the methylotrophic yeast *Pichia pastoris*. The recombinant enzyme was characterized.

MATERIALS AND METHODS

Materials. Cellouronate and amylouronate [α-(1→4)-glucuronan] were prepared from regenerated cellulose (Bemliese; Asahi Chemicals Co., Tokyo, Japan) and water-soluble potato starch (Kanto Chemicals Co., Japan), respectively, by using 2,2,6,6-tetramethylpiperidine-1-oxyl radical-mediated oxidation as described previously (19, 24, 28). The weight-average degree of polymerization (DP) of cellouronate was approximately 50, which was determined using a size exclusion chromatograph (SEC) equipped with a multiangle laser light-scattering detector (39). Alginate, hyaluronate, pectin, polygalacturonic acid, carboxymethyl cellulose (degree of substitution, 0.5), and other chemicals and solvents

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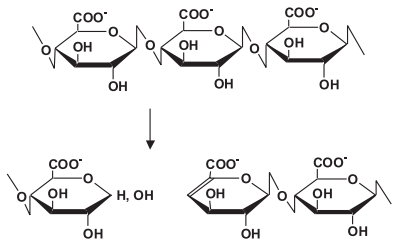

FIG. 1. Schematic diagram of the putative system for degradation of β-(1→4)-polyglucuronate by β-elimination.

were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) and used without further purification.

Cultivation of *T. reesei* on culture medium containing cellouronate. *T. reesei* was cultivated on a medium containing 2.6 g/liter $(NH_4)_2HPO_4$, 2.2 g/liter 2,2-dimethylsuccinic acid, 1.1 g/liter KH_2PO_4, 0.6 g/liter urea, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 10 mg/liter $FeSO_4 \cdot 7H_2O$, 74 mg/liter $CaCl_2 \cdot 2H_2O$, 6 mg/liter $ZnSO_4 \cdot 7H_2O$, 5 mg/liter $MnSO_4 \cdot 4H_2O$, 1 mg/liter $CoCl_2 \cdot 6H_2O$, and 0.1 mg/liter thiamine supplemented with 1 g/liter cellouronate or 20 g/liter microcrystalline cellulose (FUNACEL; Funakoshi Co., Ltd, Tokyo, Japan) as the carbon source (29). The initial pH of the culture was adjusted to 5.0, and 4.0×10^8 spores were inoculated into 400 ml medium in an Erlenmeyer flask (1 liter). The mixture was cultivated at 37°C with shaking at 150 rpm. After cultivation for 5 days, fungal mycelia were harvested, immediately frozen with liquid nitrogen, and stored at −80°C until RNA extraction was performed.

Identification of N-terminal amino acid sequence. A culture solution (1 ml) was concentrated by ultrafiltration with an Ultrafree-0.5 filter unit (Millipore Co., Billerica, MA) and was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% [wt/vol] polyacrylamide) (30). The protein with a molecular mass of 27 kDa was transferred to a polyvinylidene difluoride membrane (Millipore) with a Trans-Blot SD cell (Bio-Rad Laboratories, Hercules, CA), and the N-terminal amino acid sequence was determined with a protein sequencer (model 491 cLC; Applied Biosystems, Foster City, CA) as described previously (25). The amino acid sequence obtained was subjected to a BLAST alignment search using the *T. reesei* genome database (version 2.0) (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and the tblastn alignment program with the default settings except that the expect value was 1e-1 and the PAM30 scoring matrix was used.

Cloning of cDNA encoding TrGL. Total RNA was extracted from mycelial powder with an E.Z.N.A. fungal RNA kit (Omega Bio-tek, Doraville, GA), and mRNA was purified from the total RNA with an Oligotex-dT30<super> mRNA purification kit (Takara Bio, Shiga, Japan). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan), the 5′ random amplification of cDNA ends (RACE) cDNA synthesis primer of a SMART RACE cDNA amplification kit (Takara Bio), and the 3′ RACE adapter primer (Invitrogen, Carlsbad, CA) and was used as the template. Based on *T. reesei* genome information, a TrGL gene-specific forward primer (5′-ACCCGCAGCTTCTACAACGACG G-3′) was designed. PCR was performed with the forward primer and the universal adapter primer (Invitrogen) for 3′ RACE. The PCR product was inserted into a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen) and sequenced using a Thermo Sequenase primer cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) with an SQ5500E DNA sequencer (Hitachi Electronics Engineering, Tokyo, Japan).

The presence of a signal peptide and N-glycosylation and O-glycosylation sites in the deduced amino sequence was predicted using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/), the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), and the NetOGlyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc/). Comparative analysis of homology with the enzymes registered in the GenBank databases was carried out using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST) with the default parameters. Protein sequences were aligned with MAFFT (version 6) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with the E-INS-I algorithm. A phylogenetic tree was inferred from the alignments using the minimum-linkage method and was drawn by using FigTree (version 1.1.2) (http://tree.bio.ed.ac.uk/software/figtree/).

Expression of recombinant TrGL in *P. pastoris*. Oligonucleotide primers 5′-TTTCTCGAGAAAAGAACCCGCAGCTTCTACAACGACGG-3′ and 5′-AA ATCTAGATTAAGCCTGGTCAGGGTCGACATCG-3′, which introduced XhoI and XbaI cleavage sites, respectively (underlined), were used to amplify the mature TrGL coding sequence. The fragment was subcloned into pCR4Blunt TOPO (Invitrogen) as described in the manufacturer's instructions. The vector pCR4Blunt-TOPO/*TrGL* was digested with XhoI and XbaI (TaKaRa Bio) and ligated into the expression vector pPICZα-A (Invitrogen) at the same restriction site. The vector pPICZα-A/*TrGL* was linearized with Bpu1102I (TaKaRa Bio) and transformed into *P. pastoris* strain KM71H by electroporation. The zeosin-resistant transformant was cultivated in 200 ml of growth medium (1% [wt/vol] yeast extract, 2% [wt/vol] polypeptone, 1% [wt/vol] glycerol) in a 500-ml Erlenmeyer flask for 1 day at 30°C with shaking at 150 rpm. The cells were harvested by centrifugation (5 min, 1,500 × *g*), suspended in 40 ml of induction medium (1% [wt/vol] yeast extract, 2% [wt/vol] polypeptone, 1% [wt/vol] methanol) containing 10 mM $CaCl_2$ and cultivated for another 3 days at 26.0°C (17, 18).

Purification of recombinant TrGL. All purification experiments were performed at 4°C unless otherwise specified. A culture of *P. pastoris* was centrifuged (30 min, 1,500 × *g*), and the supernatant was incubated with 5% (wt/vol) bentonite for 30 min at room temperature. The bentonite was removed by centrifugation (20 min, 10,000 × *g*), and proteins, including TrGL, were precipitated with ammonium sulfate (70% saturation). The resulting precipitates were then collected by centrifugation (20 min, 10,000 × *g*) and dissolved in 10 ml of 20 mM sodium phosphate buffer containing 1 M ammonium sulfate (pH 7.0). This solution was loaded on a Phenyl-Toyopearl 650S column (22 by 180 mm; Tosoh Co., Ltd., Tokyo, Japan) equilibrated with 20 mM sodium phosphate buffer containing 1 M ammonium sulfate (pH 7.0). The protein was eluted with a linear gradient of 1 to 0 M ammonium sulfate in 165 ml of the same buffer at a flow rate of 1.5 ml/min. The fractions containing cellouronate lyase activity were concentrated and applied to a Super Q-5PW column (7.5 by 75 mm; Tosoh) equilibrated with 20 mM Tris-HCl (pH 8.0) using a high-performance liquid chromatography system (LC-10AS, SPD-10AV, and SCL-10A; Shimadzu Co., Kyoto, Japan) at room temperature. The enzyme was eluted from the column with a linear gradient of 0 to 300 mM NaCl in 20 ml of the same buffer at a flow rate of 1.0 ml/min. The purity of the TrGL obtained was examined by SDS-PAGE (15% [wt/vol] polyacrylamide), and the protein concentration in the solution was estimated by the Bradford method using bovine serum albumin as a standard (5) or by measuring the absorbance at 280 nm (ε_{280} = 70,650 M^{-1} cm^{-1}).

Enzyme assay. The lyase activity was determined by spectrophotometry at 235 nm (absorbance due to unsaturated products formed from cellouronate). Unless otherwise specified, the standard assay mixture consisted of 50 mM sodium phosphate buffer (pH 6.5), 2 mM $CaCl_2$, and 0.5% (wt/vol) cellouronate. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of unsaturated product per min at 25°C. The molar extinction coefficient of unsaturated triglucuronate (DP, 3) was assumed to be 4,837 M^{-1} cm^{-1}.

To study the effect of pH on the activity, assays were performed using the following buffers: 50 mM sodium acetate at pH 4.0 to 6.0, 50 mM sodium phosphate at pH 6.0 to 8.5, and 50 mM Tris-HCl at pH 8.0 to 9.5. The pH stability of TrGL was estimated by measuring the residual activity after treatment in a buffer at 4°C for 24 h. The effect of temperature on the activity was investigated by conducting the standard assay at temperatures ranging from 10 to 70°C. To analyze thermostability, aliquots of the enzyme were treated in 50 mM sodium phosphate buffer (pH 6.5) at 10 to 70°C for 10 min. Then the TrGL activity was assayed at 25°C using the standard reaction mixture. To study the influence of metal ions on the activity, assays were performed by replacing 2 mM calcium chloride in the standard reaction mixture with 0.1 mM EDTA or 0.1 mM metal chloride. The following metal salts were used: $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CdCl_2$, $CuCl_2$, $NiCl_2$, $CoCl_2$, LiCl, and $MgCl_2$. The requirement of TrGL for Ca^{2+} was evaluated using $CaCl_2$ at concentrations ranging from 0.01 to 5 mM. The substrate specificity of TrGL was tested using 0.5% amylouronate, alginate, hyaluronate, pectin, polygalacturonic acid, or carboxymethyl cellulose in the standard reaction buffer.

SEC and ^{13}C NMR analysis. Cellouronate (1%, wt/vol) was incubated with 0.9 U of TrGL in 30 ml of 50 mM sodium phosphate buffer (pH 6.5) containing 2 mM $CaCl_2$ at 30°C, and the products were analyzed by SEC and ^{13}C nuclear magnetic resonance (NMR). SEC was performed with a high-performance liquid chromatography system consisting of a Shodex Ohpak SB-802.5 HQ column (0.8 by 30 cm; Showa Denko Co., Tokyo, Japan) as described previously (27, 39), and detection was carried out by determining both the refractive index (RID-10A; Shimadzu) and the UV absorption at 235 nm (SPD-M20A photodiode array; Shimadzu). ^{13}C NMR spectra of the products were collected with a Bruker AC-300 spectrometer as described previously (28).

Nucleotide sequence accession number. The nucleotide sequence encoding TrGL has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB440265.

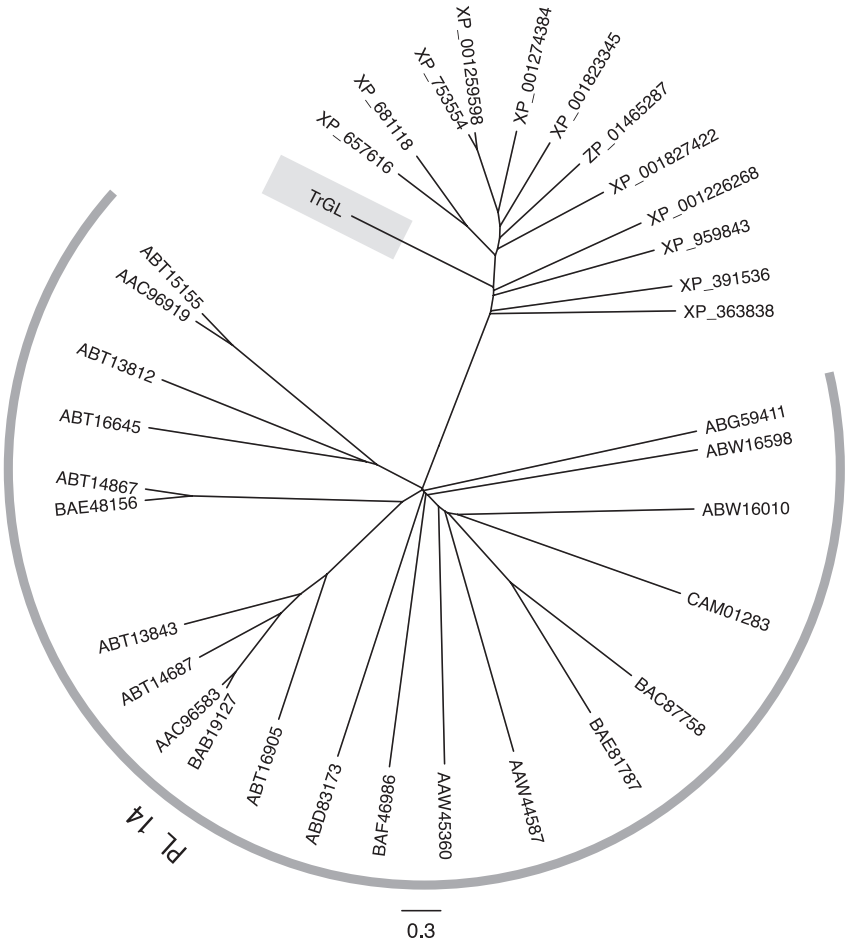

FIG. 2. Phylogenetic tree for sequences with significant homology with TrGL obtained by using BLASTP searches and PL family 14 protein data on the CAZy server. The sequences were aligned using MAFT, and the tree was drawn by using FigTree. XP_657616, hypothetical protein from *A. nidulans*; XP_681118, hypothetical protein from *A. nidulans*; XP_753554, hypothetical protein from *Aspergillus fumigatus*; XP_001259598, hypothetical protein from *Neosartorya fischeri*; XP_001274384, hypothetical protein from *Aspergillus clavatus*; XP_001823345, unnamed protein from *A. oryzae*; ZP_01465287, hypothetical protein from *Stigmatella aurantiaca*; XP_001827422, unnamed protein from *A. oryzae*; XP_001226268, hypothetical protein from *Chaetomium globosum*; XP_959843, hypothetical protein from *Neurospora crassa*; XP_391536, hypothetical protein from *Gibberella zeae*; XP_363838, hypothetical protein from *Magnaporthe grisea*; ABT15155, hypothetical protein from *Paramecium bursaria Chlorellavirus*; AAC96919, protein similar to DNA binding protein from *P. bursaria Chlorellavirus*; ABT13812, hypothetical protein from *P. bursaria Chlorellavirus*; ABT16645, hypothetical protein from *Acanthocystis turfacea Chlorellavirus*; ABT14867, hypothetical protein from *P. bursaria Chlorellavirus*; BAE48156, hypothetical methionyl-tRNA synthetase from *P. bursaria Chlorellavirus*; ABT13843, hypothetical protein from *P. bursaria Chlorellavirus*; ABT14687, hypothetical protein from *P. bursaria Chlorellavirus*; AAC96583, alginate lyase from *P. bursaria Chlorellavirus*; BAB19127, PL acting on glucuronic acid from *Chlorellavirus*; ABT16905, hypothetical protein from *Acanthocystis turfacea Chlorellavirus*; ABD83173, alginate lyase-like protein from *Saccharophagus degradans*; BAF46986, hypothetical protein from *Klebsiella pneumoniae*; AAW45360, expressed protein from *Cryptococcus neoformans*; AAW44587, hypothetical protein from *C. neoformans*; BAE81787, alginate lyase from *Haliotis discus hannai*; BAC87758, alginate lyase from *H. discus hannai*; CAM01283, candidate PL from *Saccharopolyspora erythraea*; ABW16010, hypothetical protein from *Frankia* sp.; ABW16598, hypothetical protein from *Frankia* sp.; ABG59411, candidate PL from *Cytophaga hutchinsonii*.

RESULTS

Isolation of cDNA encoding the 27-kDa protein in *T. reesei*. *T. reesei* grew in medium containing cellouronate as a carbon source, and a major protein with a molecular mass of 27 kDa was found in the culture solution. This protein was not produced when the medium contained cellulose (data not shown). The N-terminal amino acid sequence of the protein was determined to be Thr-Arg-Ser-Phe-Tyr-Asn-Asp-Gly-His-Leu-Asn. A search for this sequence using the *T. reesei* genome database yielded one matching region in the sequence, which was designated e_gw1.1.1968.1 (scaffold 1; positions 1478438 to 1479214).

Based on the nucleotide sequence of e_gw1.1.1968.1, primers were designed, and cDNA encoding the 27-kDa protein was obtained by reverse transcription-PCR (see Fig. S1 in the supplemental material). This DNA contains a 777-bp open reading frame encoding 258 amino acid residues, without any intron. Based on the results of a SignalP analysis, the first 20 amino acid residues in the N-terminal region were considered to be a signal peptide, indicating that the mature protein consists of 238 amino acids. No putative N- and O-glycosylation sites were found in the sequence. The deduced amino acid sequence was analyzed using the BLASTP algorithm in the NCBI protein database, and a phylogenetic tree was drawn for

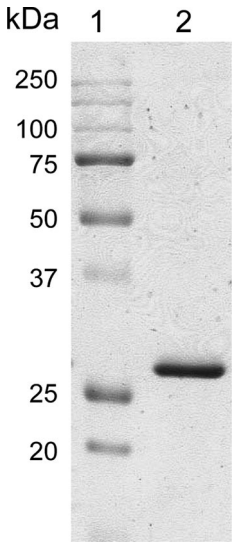

FIG. 3. SDS-PAGE of purified recombinant TrGL. Approximately 2 μg of a sample was separated on a 15% (wt/vol) polyacrylamide gel as described in Materials and Methods. Lane 1, molecular weight standards (kDa); lane 2, purified recombinant TrGL.

this protein and proteins belonging to PL family 14 on the CAZy server (Fig. 2). The BLAST search revealed no similar protein with a known function, and almost all the proteins similar to the 27-kDa protein were hypothetical proteins. Two hypothetical proteins of the fungus *Aspergillus nidulans* FGSC A4 (accession numbers XP_681118 and XP_657616) were clustered in the same clade as the protein, and the levels of similarity were 59 and 57%, respectively. When the sequence of the 27-kDa protein was analyzed using the BLASTP algorithm in NCBI, only one protein, a protein belonging to PL family 14 from *Cytophaga hutchinsonii* (ABG59411), showed a low level of similarity to the 27-kDa protein (less than 20%). Moreover, a PL acting on glucuronan from *Chlorellavirus* (BAB19127) is classified in PL family 14. However, as shown in Fig. 2, the 27-kDa protein does not belong to PL family 14.

Heterologous expression, purification, and characterization of recombinant TrGL. When the recombinant protein was heterologously expressed in *P. pastoris*, apparent cellouronate lyase activity was detected in the culture medium. The recombinant protein was purified by using two column chromatography steps, and the purified enzyme produced a single band at a molecular mass of 27 kDa on an SDS-PAGE gel (Fig. 3). Cellouronate was then treated with the purified protein, and the enzymatic reaction products were analyzed by ^{13}C NMR after 24 h (Fig. 4). Two resonance peaks, at approximately 110 and 147 ppm, were assigned to C-4 and C-5 of unsaturated glucuronate residues, respectively, which were putatively formed by β-elimination. To study the mode of action of the enzyme with cellouronate in more detail, aliquots obtained from the reaction mixture after 3, 12, and 24 h were analyzed by SEC (Fig. 5). During the reaction, the area of the peak corresponding to the original cellouronate at an elution volume of 6.0 to 6.8 ml (detected by using the refractive index) decreased, while products with adsorption at 235 nm appeared in the region corresponding to oligomeric compounds. Oligomers with DP of 2 to 4 accumulated. Because the formation of several oligomeric uronates was observed during the reaction, the 27-kDa protein is an endo-type GL (TrGL).

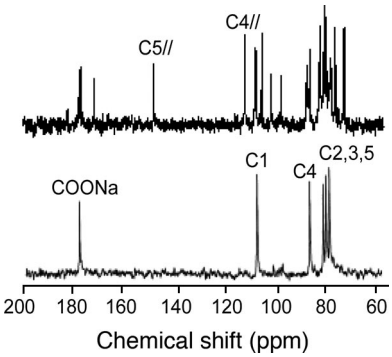

FIG. 4. ^{13}C NMR spectra of cellouronate before and after treatment with TrGL for 24 h. C4//and C5// indicate C-4 and C-5, respectively, of the unsaturated nonreducing terminus.

The enzyme was most active at pH 6.5 in 50 mM sodium phosphate buffer and at 50°C. TrGL was stable over a broad pH range (pH 5 to 9) when it was treated at 4°C for 24 h. Enzyme activity remained after incubation at 10 to 40°C for 10 min, but approximately 60% of the activity was lost at 50°C.

When Ca^{2+} was present in the reaction mixture, the activity of TrGL increased substantially (Fig. 6). No significant effect was observed with other metals, while approximately 50% inhibition of TrGL was observed when 0.1 mM EDTA was present compared with the reference. The activity of TrGL with Ca^{2+} was about 8 times that of the reference. An increase in the calcium concentration led to a significant increase in the activity of the enzyme, and the maximum activity was observed with 2 mM Ca^{2+} (data not shown). The effect of Ca^{2+} on TrGL

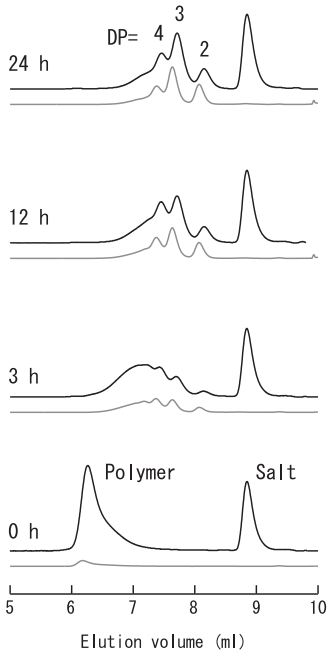

FIG. 5. SEC elution patterns of the enzyme-treated products. Detection was carried out by measuring the refractive index (black line) and the UV absorbance at 235 nm (gray line).

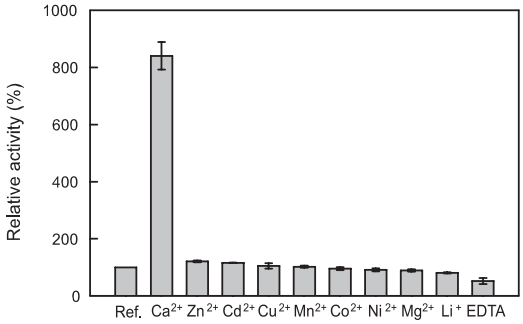

FIG. 6. Effect of metal ions or EDTA on the activity of recombinant TrGL. The activity was assayed in the presence of 0.1 mM metal chloride or EDTA. Ref, reference. The error bars indicate one standard error.

stability was tested by measuring the thermal inactivation at 50°C (Fig. 7). Without Ca^{2+}, a >85% decrease in TrGL activity was observed after 30 min, although the enzyme was stable after incubation for 30 min at 50°C in the presence of 2 mM Ca^{2+}. This result indicates that the thermostability of TrGL increased in the presence of Ca^{2+}.

The substrate specificity of recombinant TrGL was examined using various polyuronates, including alginate, hyaluronate, pectin, polygalacturonic acid, amylouronate, and carboxymethyl cellulose (sodium salt) (Table 1). The results indicate that there was high substrate specificity of the enzyme for cellouronate.

DISCUSSION

Species of *Trichoderma* have been intensively studied to determine their cellulolytic, pathogenic, or antipathogenic activities. However, there has been little study of the PLs of *Trichoderma* spp., and no PL family gene from *Trichoderma* spp. is listed on the CAZy web server. The GL produced by a *Trichoderma* strain was purified and characterized by Delattre et al. (9). The cellulolytic ascomycete *T. reesei* is one of the best-studied cellulose-degrading fungi, and its plant cell wall degradation systems have been intensively investigated. The

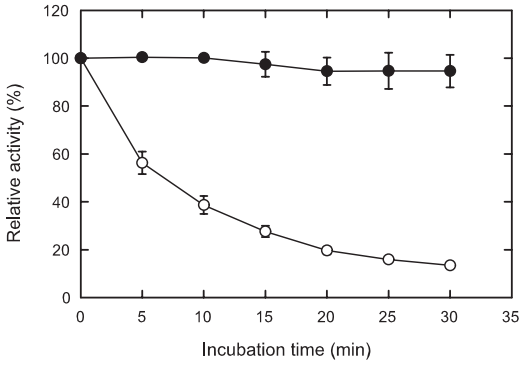

FIG. 7. Effect of Ca^{2+} on the thermostability of recombinant TrGL. An enzyme solution was incubated in the presence or absence of $CaCl_2$ (2 mM) at 50°C. After various incubation times, the residual activity was assayed using the standard assay described in Materials and Methods. The error bars indicate one standard error.

TABLE 1. Substrate specificity of recombinant TrGL[a]

Substrate	Glycosidic linkage	Activity (U/mg)
Cellouronate	β-(1→4)	81.3 ± 3.3
Amylouronate	α-(1→4)	ND[b]
Alginate	β-(1→4), α-(1→4)	ND
Hyaluronate	β-(1→4), β-(1→3)	ND
Pectin	α-(1→4)	ND
Polygalacturonic acid	α-(1→4)	ND
Carboxymethyl cellulose	β-(1→4)	ND

[a] Assays were performed using a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5), 2 mM $CaCl_2$, and 0.5% (wt/vol) substrate.
[b] ND, not detectable.

genome sequence of this fungus is publicly available on the web server of the U.S. Department of Energy's Joint Genome Institute (http://genome.jgi-psf.org/Trire2/Trire2.home.html), and an initial analysis of the sequences has been described (32). In the present study, we determined the sequence of a cDNA encoding a GL in *T. reesei* and we also characterized the recombinant enzyme.

In the CAZy classification system, PLs are classified into 19 families on the basis of amino acid similarity. However, PL family 14 contains only one PL which exhibits activity with glucuronan, and the gene encoding it was cloned from *Chlorellavirus* CVK2 and designated vAL-1. This gene encodes a 349-amino-acid protein which degrades β- or α-(1→4)-linked polyglucuronate in the cell wall of *Chlorella* strains (41, 42). However, the TrGL obtained in the present study has no similarity to this enzyme. Moreover, amino acid sequence analysis of TrGL revealed no significant homology with any previously described functional protein when a BLAST search was performed, indicating that this enzyme should be classified in a new PL family. This conclusion is supported by the results of a hydrophobic cluster analysis, and the sequences of TrGL and related proteins have been placed in PL family 20 (B. Henrissat, personal communication). The nucleotide sequence encoding TrGL showed 91 and 69% similarity with regions of scaffold 2 (positions 773189 to 773966) in the *Trichoderma virens* genome database (http://genome.jgi-psf.org/Trive1/Trive1.home.html) and scaffold 11 (positions 1405722 to 1405892) in the *Trichoderma atroviride* genome database (http://genome.jgi-psf.org/Triat1/Triat1.home.html), respectively. The amino acid sequence of TrGL also showed high levels of similarity to some hypothetical proteins from filamentous fungi belonging to *Aspergillus* spp., reflecting the apparent growth of *Aspergillus oryzae* in the medium containing cellouronate as a sole carbon source. Moreover, we have isolated at least four bacterial strains with the ability to degrade cellouronate, indicating that the degradation of β-(1→4)-polyglucuronate may be a common phenomenon in nature, although this polysaccharide is a minor polysaccharide compared with other polyuronates. β-(1→4)-GLs might play an important role in the degradation of cell walls with complex structures.

Recombinant TrGL has a molecular mass of 27 kDa and catalyzes endo-type depolymerization of β-(1→4)-polyglucuronate. Previously, an endo-GL acting on bacterial O-acetylated β-(1→4)-glucuronan was purified from *Trichoderma* sp. as a 27-kDa protein (7, 9), and this protein may be a homologue of the GL from *T. reesei* described here. The activity of TrGL in

the presence of 2 mM Ca^{2+} was about 80-fold higher than that in the absence of Ca^{2+}, suggesting that Ca^{2+} is necessary for the catalytic activity of TrGL (2, 40). PLs catalyze β-elimination, resulting in the formation of 4,5-unsaturated products, and the mechanism has been thought to consist of three steps: (i) neutralization of the carboxyl group on the substrate to lower the pK_a of the H-5 proton, (ii) general base-catalyzed abstraction of this proton, and (iii) β-elimination of the 4-O-glycosidic bond with concomitant formation of the C-4—C-5 double bond within the uronic acid moiety at the nonreducing end (45). Some PLs, especially pectate lyases, require Ca^{2+} for activity, and structural studies of such enzymes have shown that Ca^{2+} has a role in the first step of the β-elimination reaction (11, 38). The Ca^{2+} is coordinated by acidic residues on the protein and/or by the carboxyl groups on the polyuronate in the active site (13, 35). Jenkins et al. (22) argued that the role of Ca^{2+} is to withdraw electrons from the C-6 carboxylate of the substrate. This effect acidifies the C-5 proton, making it more susceptible to attack by a base (14, 36). Ca^{2+} in TrGL may play a similar role in substrate binding and catalysis. We are currently analyzing the three-dimensional structure of TrGL to shed light on the degradation mechanism.

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