J. Microbiol. Biotechnol. (2007), **17**(8), 1291–1299

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization and Action Patterns of Two β-1,4-Glucanases Purified from *Cellulomonas uda* CS1-1

YOON, MIN-HO* AND WOO-YOUNG CHOI

Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea

Received: February 15, 2007 Accepted: March 28, 2007

Abstract Two β-1,4-glucanases (DI and DIII fractions) were purified to homogeneity from the culture filtrate of a cellulolytic bacteria, *Cellulomonas* sp. CS1-1, which was classified as a novel species belonging to *Cellulomonas uda* based on chemotaxanomic and phylogenetic analyses. The molecular mass was estimated as 50,000 Da and 52,000 Da for DI and DIII, respectively. Moreover, DIII was identified as a glycoprotein with a pI of 3.8, and DI was identified as a non-glycoprotein with a pI of 5.3. When comparing the ratio of the CMC-saccharifying activity and CMC-liquefying activity, DI exhibited a steep slope, characteristic of an endoglucanase, whereas DIII exhibited a low slope, characteristic of an exoglucanase. The substrate specificity of the purified enzymes revealed that DI efficiently hydrolyzed CMC as well as xylan, whereas DIII exhibited a high activity on microcrystalline celluloses, such as Sigmacells. A comparison of the hydrolysis patterns for pNP-glucosides (DP 2-5) using an HPLC analysis demonstrated that the halosidic bond 3 from the nonreducing end was the preferential cleavage site for DI, whereas bond 2, from which the cellobiose unit is split off, was the preferential cleavage site for DIII. The partial N-terminal amino acid sequences for the purified enzymes were ^{1}Ala-Gly-Ser-Thr-Leu-Gln-Ala-Ala-Ala-Ser-Glu-Ser-Gly-Arg-Tyr^{15}- for DI and ^{1}Ala-Asp-Ser-Asp-Phe-Asn-Leu-Tyr-Val-Ala-Glu-Asn-Ala-Met-Lys^{15}- for DIII. The apparent sequences exhibited high sequence similarities with other bacterial β-1,4-glucanases as well as β-1,4-xylanases.

Keywords: *Cellulomonas uda* CS1-1, β-1,4-glucanases, purification, hydrolysis patterns, substrate specificity

A number of studies on cellulolytic enzymes capable of degrading cellulose have already been carried out with various microorganisms. Cellulolytic enzymes from microorganisms are generally known to be induced as a multienzyme based on cellulose substrates used for cultivation, and the enzymatic hydrolysis of cellulose is exerted by the synergistic action of such enzymes as endo-1,4-β-glucanase (EDG), exo-1,4-β-glucanase (cellobiohydrolase; CBH), and β-glucosidase.

The efficient hydrolysis of cellulose is known to be performed by the simultaneous action of a nonprocessive EDG, which produces new ends in a random fashion within the polysaccharide chain, and a processive CBH, which splits off cellobiose from the free ends. The action mechanism of EDGs and CBHs toward various substrates has already been fairly well established with respect to fungal enzymes, such as *Trichoderma, Penicillium,* and *Talaromyces* species [4, 23, 37]. Although most cellulolytic enzymes have the same bond specificity, important functional differences are found in their mode of action toward cellulosic substrates. In particular, it is noteworthy that the attack of certain CBHs, unlike most CBHs, occurs from the nonreducing end of the glucose polymer as well as the reducing end, and the penultimate glycosidic linkage is not the exclusive site of hydrolysis for exo-type enzymes [31, 40]. These functional differences would seem to depend on the cellulases and substrates used, making it difficult to establish the action patterns of cellulolytic enzymes.

The genus *Cellulomonas* is representative of cellulolytic bacteria capable of producing cellulolytic as well as xylanolytic enzymes. Numerous *Cellulomonas* glucanases have already been purified and characterized with respect to studies on the substrate specificity and multifunctionality of these enzymes [8, 19, 26]. Furthermore, biochemical and molecular studies of the genes encoding glucanases have also been reported, including the structure of the catalytic and cellulose binding domain, and regulation of the gene [27, 38]. However, the previous reports on the mode of action of *Cellulomonas* glucanases are not extensive enough when compared which those on fungal enzymes, plus the reports on CBHs have been limited to those from *C. fimi* [19, 38].

Since Choi *et al.* [2] isolated a cotton wool-degrading bacterium, *Cellulomonas* sp. CS1-1, a series of work has

*Corresponding author
Phone: 82-42-821-6733; Fax: 82-42-823-9241;
E-mail: mhyoon@cnu.ac.kr

been conducted related to this organism, including the degradation of microcrystalline cellulose and sugar cane bagasse by Choudhury *et al.* [3] and mutant derivatives hyperproduced by Haggett *et al.* [11]. However, the extensive enzymatic studies and species-level identification for this strain are still inadequate, as no EDG, CBH, or β-glucosidase has yet been completely purified from the extracellular and cell-bound fractions. Accordingly, to elucidate the cellulolytic system of cellulases for this strain, this study attempted to separately purify extracellular β-1,4-glucanases with relatively endo-type and exo-type characteristics. Additionally, chemotaxanomic and phylogenetic experiments were performed to classify *Cellulomonas* sp. CS1-1. Therefore, this paper describes the identification of *Cellulomonas* sp. CS1-1, along with the characterization and action patterns of two extracellular β-1,4-glucanases purified from strain CS1-1.

MATERIALS AND METHODS

Organism and Culture Condition

The *Cellulomonas* sp. CS1-1 (KCTC 1371), isolated from the southern hemisphere, Australia, was grown on a Dubos'salt [7] solution containing 0.02% yeast extract and 1% Sigmacells (Sigma, St. Louis, MO, U.S.A.) as the carbon source. The cultivation was carried out in a Quickfit FV5L fermenter for 4 days at 30°C.

Identification of *Cellulomonas* sp. CS1-1

The phenotypic and biochemical characteristics of strain CS1-1 were tested using API 20 NE, API ID 32 GN, and API ZYM test kits (bioMérieux). The chromosomal DNA for determining the G+C content was extracted from the cells and purified as previously described by Moore [25], and the DNA base composition determined using the HPLC method described by Mesbah *et al.* [24]. The cellular fatty acids of the organism grown on a TSA agar for two days were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). The isoprenoid quinones were analyzed using the method described by Shin *et al.* [33]. For the 16S rRNA gene sequencing and phylogenetic analysis, the DNA was extracted using a commercial genomic DNA extraction kit (Solgent Co., Korea), followed by PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product. The 16S rRNA gene full sequences were compiled using SeqMan software (DNASTAR, Madison, WI, U.S.A.). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database, and then the multiple alignments were performed using the Clustal_X program and the gaps edited using the BioEdit program [13]. The evolutionary distances were calculated using the Kimura two-parameter model [16], and the phylogenetic trees constructed using the neighbor-joining method [30] and maximum-parsimony method using the MEGA3 Program [17] with bootstrap values based on 1,000 replications. The DNA-DNA hybridization was performed fluorometrically using the method of Ezaki *et al.* [9], with photobiotin-labeled DNA probes and microdilution wells.

Enzyme Purification

The culture fluid (2.5 l) was harvested by centrifugation at 12,000 ×*g* for 30 min after cultivation for 4 days and concentrated using an ultrafiltration apparatus (Toyo UHP-25, Japan) to give a final volume of 200 ml. The pellet recovered from the concentrated solution by 20% to 90% ammonium sulfate (AS) precipitation was dialyzed with a 20 mM McIlvain buffer, pH 6.8, for 24 h, and the dialysate used as a crude enzyme solution for purification.

Step 1. Gel Filtration on Ultro-gel Ac54. An aliquot of the dialysed solution was separated on an Ultro-gel Ac54 column (2.6×100 cm; bed volume, 420 ml) equilibrated with a 20 mM McIlvain buffer, pH 6.8. The active fractions containing glucanases were pooled and concentrated by ultrafiltration using a PM10 membrane (Millipore, Beverly, MA, U.S.A.).

Step 2. Ion-Exchange Chromatography on DEAE-Sephadex A50. The active fraction obtained from the previous gel filtration step was loaded onto a DEAE-Sephadex A50 column (2.3×30 cm; bed volume, 130 ml) equilibrated with a 50 mM Tris buffer, pH 7.5, and then the elution was performed with a linear gradient of NaCl from 0 to 0.5 M in the same buffer. Two active fractions (DI and DIII fractions) were separated, and the DI fraction identified as homogeneous by SDS-PAGE. To remove the other contaminants, the DIII fractions were further purified using preparative isoelectric focusing (IEF).

Step 3. Preparative IEF. An aliquot (5 mg of protein) of the DIII fraction was separated on a Rotofor IEF Cell (Bio-Rad Lab.) using 1.5% ampholyte, pH 3–10. The electrofocusing was run for 5 h at 4°C with a constant power of 12 W using 0.1 M H_3PO_4 and 0.1 M NaOH as the anolyte and catholyte, respectively. The active fractions (IF-1, Nos. 4–6) were pooled and further purified by preparative HPLC.

Step 4. Preparative HPLC. Each 0.5 ml aliquot (2 mg of protein) of the DI and IE-1 fractions was subjected to a preparative HPLC column, and the DI and DIII enzymes finally purified by performing HPLC (510 pump system, Waters Association, U.S.A.) gel filtration on a GPC-100 column (Synchropack, 300×7.8 mm ID, Lafayette, Indiana, U.S.A.) using a 50 mM phosphate buffer, pH 6.8, containing 0.5 M NaCl as the eluent (Flow rate: 1.0 ml/min).

Assay of 1,4-β-Glucanase and Protein Content

Carboxymethyl Cellulose (CMC)-saccharifying Activity. The activity was determined using the method described by Choi *et al.* [2]. The reaction mixture consisted of 1.5 ml

of 1% CMC in a 0.05 M McIlvain buffer, pH 6.4, and 1 ml of the enzyme solution. After incubation for 20 min at 40°C, the amount of reducing sugar released was determined using the Somogyi method [34] with glucose as the standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of glucose per min under the assay conditions.

CMC-liquefying Activity. The activity was assayed according to the method described by Canevascini *et al.* [1]. The reaction mixture consisted of 9 ml of 0.3% CMC in a 50 mM McIlvain buffer, pH 6.8, and 1 ml of the enzyme solution. After adding the enzyme solution, 9 ml of the mixture was transferred to an Ostwald viscometer and the efflux time recorded at 2-min intervals during incubation for 10 min at 30°C. The activity unit was defined as the slope of the line obtained by plotting the ratios $\eta_{sp\cdot to}/\eta_{sp\cdot tx}$ ($\eta_{sp\cdot tx}$=specific viscosity after different incubation times) against the reaction time multiplied by 1,000.

Analysis of Protein and Carbohydrate

The protein content was determined using the method of Lowry *et al.* [22] with bovine serum albumin as the standard; the total carbohydrate was analyzed using the method of Dubois *et al.* [6] with glucose as the standard.

Estimation of Molecular Mass and Isoelectric Point (pI)

The enzyme proteins in each purification step were identified by native- or SDS-PAGE using a vertical slab gel (Model AE-6640, Atto Co., Japan) with a 10% acrylamide concentration. The molecular mass was determined by SDS-PAGE using the method of Laemmli [18], whereas the pI of the enzyme was analyzed by IEF using a 6% polyacrylamide gel containing 2% ampholyte with a pH range of 3 to 6 on a Mighty Small II system (Model SE250, Hoeffer Scientific Instruments, U.S.A.).

N-Terminal Amino Acid Analysis

The purified enzymes were blotted from an SDS-PAGE minigel onto a polyvinylidene difluoride transfer membrane. The enzyme bands on the membrane were then excised for sequencing on a Porton PI 2090 protein sequencer. The sequencing was performed at the Department of Biochemistry, North Dakota State University, U.S.A., and the apparent protein sequences analyzed using the NCBI GenBank database.

Hydrolysis Products of p-Nitrophenyl-β-Glucosides ($pNPG_n$)

To estimate the hydrolysis patterns of the DI or DIII enzymes obtained during the degradation of $pNPG_n$ (DP 2–5, Seikagaku Kogyo Co., Japan) as substrates, the hydrolysis products released from $pNPG_n$ were separated by HPLC on a Develosil ODS-5 column (150×4.5 mm ID; Nomura Chemical Co., Japan). Two-hundred μl of reaction mixtures that contained 2 units of the enzyme and 0.2 mM of the substrates in a 50 mM McIlvain buffer, pH 6.5, was incubated at 40°C with the time interval ranging from 0.1 to 12 h, and then the reaction was stopped by placing the tube in a boiling-water bath for 15 min. Aliquots (10 μl) of the reaction mixture were applied to the column and the reaction products eluted using a linear gradient of 0.3% butanol in a 10 mM McIlvain buffer (A), pH 3.8, and 0.7% butanol in the same buffer (B) at 1.5 ml/min, and then monitored spectrophotometrically at 300 nm.

RESULTS

Identification of *Cellulomonas* sp. CS1-1

The DNA G+C content of strain CS1-1 was 73.2 mol%, a value that falls within the range reported for members of the genus *Cellulomonas*. Furthermore, the major fatty acids were anteiso-$C_{15:0}$ (61.3%) and anteiso-$C_{17:0}$ (15.9%), representing a similar fatty acid profile to those of other *Cellulomonas* species analyzed previously [15, 29]. Strain CS1-1 contained a predominant amount of tetrahydrogenated menaquinone with nine isoprene units MK-9 (H_4), which is the major lipoquinone found in members of the *Cellulomonadaceae* family (data not shown). Comparative 16S rRNA gene-sequence analyses of strain CS1-1 (1,460 bp, NCBI/EMBL/DDBJ Accession Number: AB259960)

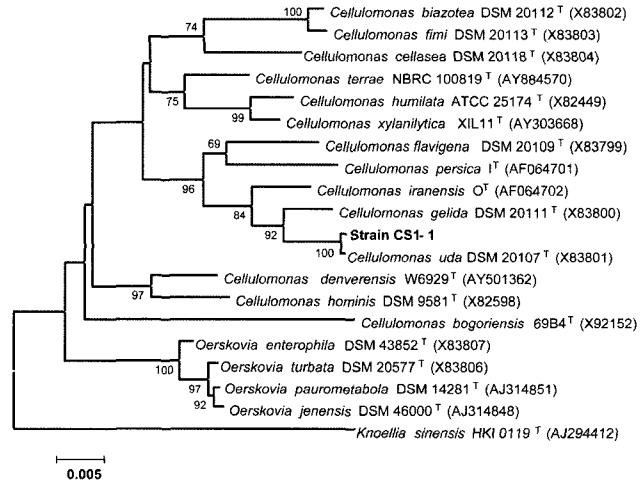

Fig. 1. Rooted phylogenetic tree based on 16S rRNA gene sequences of strain CS1-1 and related bacteria in the genus *Cellulomonas*.

This tree was constructed using the neighbor-joining method [30] with a Kimura [16] two-parameter distance matrix and pairwise deletion. The dots indicate generic branches that were recovered using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1,000 replications) greater than 70% are shown at the branch points. Bar, 0.005 substitutions per nucleotide position.

showed that the strain was phylogenetically affiliated to the *Cellulomonas* species, and a phylogenetic tree (Fig. 1) based on the neighbor-joining algorithm showed that strain CS1-1 appeared within the genus *Cellulomonas* and occupied the closest position to *C. uda* DSM 20107^T (99.7%). Therefore, the results obtained from the phenotypic and phylogenetic characterizations indicated that strain CS1-1 belonged to the genus *Cellulomonas*. Finally, the high DNA-DNA relatedness values between strain CS1-1 and *C. uda* DSM 20107^T (72%) revealed that strain CS1-1 belonged to *C. uda*, as recommended by Stackebrandt and Goebel [35].

Purification of Two 1,4-β-Glucanases

The two 1,4-β-glucanases from the culture filtrate of *C. uda* CS1-1 were purified to homogeneity by means of AS fractionation and subsequent column chromatographies, as summarized in Table 1. The fraction precipitated by AS at a 20–90% saturation was subjected to an Ultrogel Ac54 column, and two peaks, GFI (Nos. 17–25) and GFII (Nos. 33–47), were separated from the gel filtration (data not shown). The pooled GFII fraction with most of the glucanase activity was then loaded onto a DEAE-Sephadex A50 column and three fractions (DI, DII, and DIII) were separated by anion-exchange chromatography (Fig. 2). The DI fraction was eluted from the void volume without any interaction with the anion column, followed by the release of the DII and DIII fractions using a range of 0.3–0.5 M NaCl. The DI fraction, which showed a relatively high CMC liquefying activity, was finally purified by preparative HPLC gel filtration using a GPC-100 column. The purified DI fraction was detected as a single band on the SDS-PAGE and the specific activity and purification factor were 140 units/mg and a 13-fold higher CMC liquefying activity, respectively. The DIII fraction also appeared to have a high CMC-saccharifying

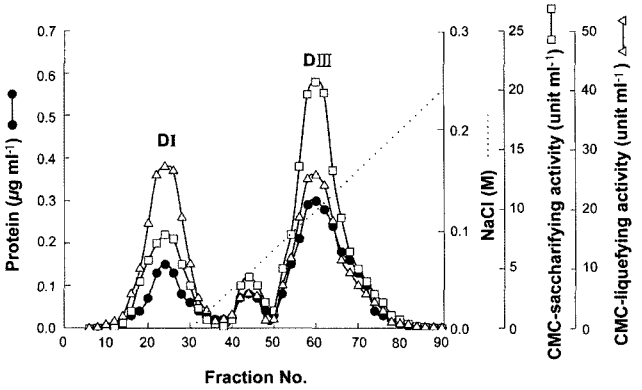

Fig. 2. Ion-exchange chromatography on DEAE-Sephadex A-50. Sample, 67 mg protein pooled fraction (GF-II) from gel filtration; Column dimensions, 2.3×30 cm; Elution, 50 mM Tris-HCl buffer (pH 7.3), with linear salt gradient 0–1 M NaCl; Flow rate, 20 ml/h; Fraction volume, 5 ml per tube.

activity and contained other isozymes, as well as the major protein. Further purification of the DIII fraction was performed using preparative IEF, and the protein patterns of the DIII fraction eluted at each pH range are presented on the SDS-PAGE in Fig. 3. The major protein was found in the fraction IE-1 (Nos. 4–6) between pH 3.7 and 3.9, and other proteins similar to those in the DII fraction were detected in fraction IF-2 (Nos. 8–11) between pH 4.2 and 6.0. As the IF-1 fraction possessed a high CMC-saccharifying activity and still appeared to include other proteins besides the major protein on the SDS-PAGE, the IF-1 fraction was subjected to preparative HPLC to separate the contaminants from the major protein. The major protein of the DIII fraction finally purified by preparative HPLC appeared to be free of other proteins on the SDS-PAGE, and the specific activity and purification factor were 5,387 units/

Table 1. Summary of purification steps for two 1,4-β-glucanases from *C. uda* CS1-1.

Purification step	CMC-saccharifying activity					CMC-liquefying activity			
	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purif'n factor (fold)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purif'n factor (fold)
Culture filtrate	360	524,160	1,456	100	1	4,070	11	100	1
AS ppt. (20–90%)	108	415,240	3,844	79	2.6	3,120	29	77	2.6
Ultro-gel Ac54									
GF-II (Fr. Nos. 33–47)	67	308,840	4,609	59	3.2	2,840	42	70	3.8
DEAE A-50									
DI (Fr. No. 20–30)	6	58,000	9,666	11	6.6	630	105	15	9.5
DIII (Fr. Nos. 55–70)	21	187,000	8,904	36	6.1	1,150	55	28	5
Prep. IEF on DIII									
IF-I (Fr. Nos. 4–6)	10	80,410	8,041	15	5.5	506	51	12	4.6
Prep. HPLC on GPC									
DI (RT: 9.94)	4	43,000	10,750	8.2	7.4	560	140	14	12.7
DIII (RT:9.00)	6	32,322	5,387	6.2	3.7	,135	23	3.3	2.1

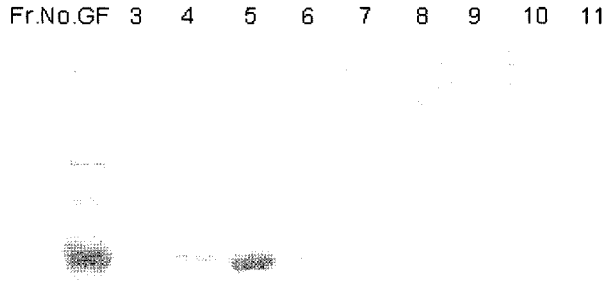

Fig. 3. SDS-PAGE of DIII fractions separated from preparative IEF.
The DIII fractions were separated on a Rotofor IEF Cell using 1.5% ampholyte, pH 3–10, and the protein profiles of each IEF fraction confirmed on a 10% SDS-PAGE. GF, GF fraction; lanes 4–6, IF-1 fraction (Nos. 4–6) between pH 3.7 and 3.9; lanes 8–11, IF-2 fraction (Nos. 8–11) between pH 4.2 and 6.0.

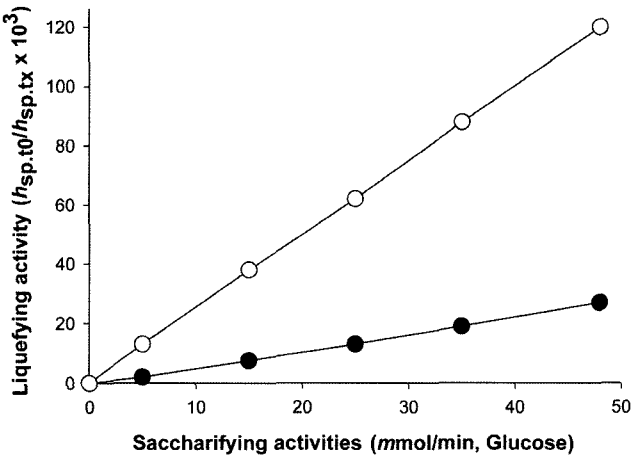

Fig. 5. Relationship between CMC-saccharifying and -liquefying activities of 1,4-β-glucanases purified from *C. uda* CS1-1.
○, DI enzyme; ●, DIII enzyme.

mg and a 3.7-fold higher CMC-saccharifying activity, respectively.

Characteristics of Purified 1,4-β-Glucanases

The apparent molecular masses of the purified enzymes were estimated to be 50,000 Da for DI and 52,000 Da for DIII on the SDS-PAGE (Fig. 4A), and the pIs were pH 5.3 for DI and pH 3.8 for DIII (Fig. 4B). In the case of DIII, the pI of 3.8 matched well with the result obtained from the preparative IEF. DIII was identified as a glycoprotein containing 9.8% sugar, whereas DI was confirmed as a non-glycoprotein based on estimating the total sugar. When the relative ratios of the CMC-saccharifying and CMC-liquefying activities were compared with the purified enzymes, DI showed a steep slope, characteristic of an endoglucanase, whereas DIII showed a low slope, characteristic of an exoglucanase (Fig. 5). A Congo red plate assay also showed the same tendency, as DIII lacked the ability to produce a clear zone on a CMC plate, in contrast to DI (data not shown).

Substrate Specificity

The substrate specificity of the purified enzymes was investigated by assessing their ability to hydrolyze various cellulosic substrates (Table 2). Whereas DI exhibited a high specific activity on CMC, the hydrolysis of Sigmacells by

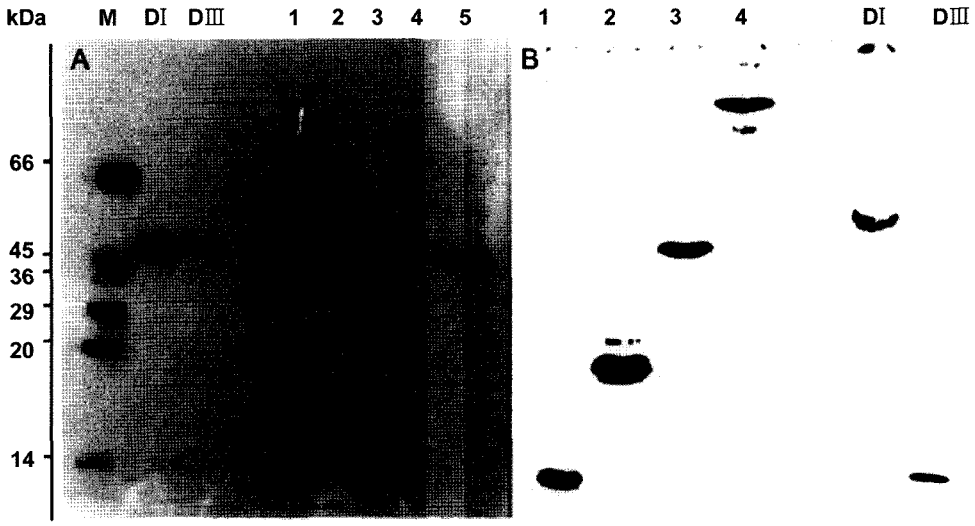

Fig. 4. SDS-PAGE (**A**) of fractions obtained during purification steps, and IEF (**B**) of DI and DIII purified from *C. uda* CS1-1.
A. M, protein marker; purified DI and DIII; lane 1, crude enzyme in culture filtrate; lane 2, GFII fraction from Ultro-gel Ac54; lane 3, DI fraction from DEAE A-50; lane 4, DII fraction from DEAE A-50; lane 5, DIII fraction from DEAE A-50. **B.** Lane 1, amyloglucosidase (pI 3.6); lane 2, trypsin inhibitor (pI 4.6); lane 3, β-lactoglobulin A (pI 5.1); lane 4, carbonic anhydrase III (pI 5.9).

Table 2. Substrate specificities of two 1,4-β-glucanases purified from *C. uda* CS1-1.

Substrates[a]	Activity (units mg^{-1})[b]	
	DI	DIII
CM-cellulose	59.8±1.3	24.0±1.8
Sigmacells	12.9±3.1	27.7±3.6
H_3PO_4-swollen CMC	49.3±4.5	13.4±5.3
Xylan	21.5±2.7	3.6±4.8
Barely glucan	2.7±3.2	1.4±4.5
pNPC	ND[c]	ND
pNPG	ND	ND
pNPX	ND	ND

[a]pNPC, p-nitrophenyl-β-cellobioside; pNPG, p-nitrophenyl-β-glucopyranose; pNPX, p-nitrophenyl-β-xylopyranoside. A reaction mixture consisting of 1.5 ml of a 1% substrate, pH 6.0, and 0.1 ml of the purified glucanases (50 μg) was incubated at 45°C for 2 h. The reducing sugar was measured using the assay conditions mentioned in Methods. The enzyme activities for pNPG, pNPC, and pNPX were determined as the amount of enzyme necessary to release 1 μmol of p-nitrophenol per min.
[b]The presented values are averages (±SD) of triplicate experiments.
[c]ND, not detectable.

DI was relatively very low. Furthermore, the activity of DI was fairly efficient against xylan, as well as glucan containing β-1,4- and β-1,3-linkages, such as barely glucan, demonstrating that DI simultaneously included both 1,4-glucanase and xylanase activity. However, DIII showed the highest activity on Sigmacells and a low activity on xylan and glucan, suggesting that DI and DIII acted separately as an EDG and CBH, respectively, since CMC and microcrystalline cellulose are known as a typical substrate for EDG and CBH, respectively.

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequences for DI and DIII were identified as [1]Ala-Gly-Ser-Thr-Leu-Gln-Ala-Ala-Ala-Ser-Glu-Ser-Gly-Arg-Tyr[15]- for DI and [1]Ala-Asp-Ser-Asp-Phe-Asn-Leu-Tyr-Val-Ala-Glu-Asn-Ala-Met-Lys[15]- for DIII. The apparent sequences were then used to compare the sequence similarities with other bacterial 1,4-β-glucanases based on the protein sequence database (Table 3). Although the specific activity of DI with CMC appeared to be three times higher than that with xylan, the protein sequence of DI shared a high degree of homology with the endo 1,4-β-xylanases from the *Streptomyces* species [5, 10, 32, 36] rather than an EDG. The sequence of DIII exhibited a partial high similarity with the EXG from *Cellulomonas fimi* [38], supporting that DIII could be a glucanase belonging to an EXG.

Table 3. Comparison of partial N-terminal sequences for two 1,4-β-glucanases purified from *C. uda* CS1-1.

Sources	Protein sequences
DI component	[1]AGSTLEAAASESGRY[15]
S. thermoviolaceus xylanase [36]	[1]*E***G***AQ****[15]
S. roseiscleroticus xylanase [10]	[1]*E***G***QQ**Y*[15]
S. lividance xylanase [32]	[2]*E***G***AQ****[56]
DIII component	[1]ADSDFNLYVAENAMK[15]
C. fimi exoglucanase [38]	[33]***E***V*******[47]
T. saccarolyticum xylanase [20]	[387]TAKH**ML*******[401]
P. fluorescens cellulase [12]	[296]VRAE**QIT***I**[311]

*Asterisks mark the sequences identical with other bacterial glucanases.

Hydrolysis Patterns of pNP-Glucosides

The hydrolysis products of pNPGs with the DI or DIII enzymes were measured using HPLC analysis. The rate of formation of specific products obtained when degrading $pNPG_n$ (DP 3–5) with both enzymes was calculated based on the relative molar ratios of the reaction products (Table 4). $pNPG_1$ and $pNPG_2$ were not hydrolyzed by either DI or DIII, indicating that neither enzyme could attack the agluconic bond. There was no significant difference between the enzymes as regards the reaction products for $pNPG_3$,

Table 4. Rate of formation of specific products from pNP-glucosides with DI and DIII enzymes.

Substrates	DI component	DIII component
pNPβ-$(Glc)_3$	0.004	0.001
pNPβ-$(Glc)_4$	0.02 0.13 0.05	0.006 0.042 0.01
pNPβ-$(Glc)_5$	0.02 0.36 0.16	0.07 0.02 0.01

A reaction mixture (0.25 ml: 50 μl of 0.2 mM substrate+50 μl of 0.1 unit enzyme+150 μl of McIlvaine buffer, pH 6.5) was incubated at 40°C, while 50 μl aliquots were taken at certain time intervals and injected into the column (HPLC) to analyze the product amounts. The numbers are the relative molar ratio of the reaction products, where the product amounts are compared as mmol produced per min per mg of the enzyme protein. Symbols: □, β-(1→4)-glucopyranosyl; △, p-nitrophenyl.

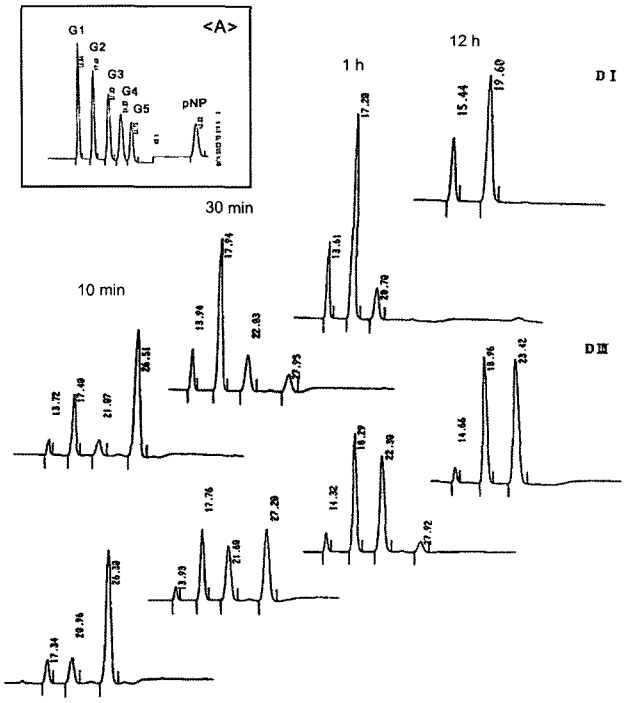

Fig. 6. HPLC analysis of reaction products from pNP β-cellopentaose with DI and DIII enzymes.

A. Standard curve for each 40 μM pNP β-(Glucoside)$_n$, n=1–5. The reaction mixture (0.25 ml: 50 μl of 0.2 mM substate+50 μl of 0.1 unit enzyme+150 μl of McIlvaine buffer, pH 6.5) was incubated at 40°C, while 50 μl aliquots were taken at certain time intervals and injected into the column (HPLC) to analyze the product amounts.

except the formation rate of $pNPG_1$ by DI as the major product was three times faster than that by DIII. In the case of $pNPG_4$, the relative formation of $pNPG_2$ by DIII, which was the dominant product of both enzymes, appeared to be somewhat faster than that by DI, yet the formation of $pNPG_1$ was 10 times faster with DI. The hydrolysis of $pNPG_5$ by DI gave $pNPG_2$ as the major product in the early stage of the reaction, but after 12 h, only $pNPG_1$ and $pNPG_2$ were detected as the final products. In contrast, although $pNPG_3$ was the major product, approximately equal molar amounts of $pNPG_2$ and $pNPG_3$ were produced as the hydrolysates of $pNPG_5$ catalyzed by DIII throughout the reaction (Fig. 6). Therefore, these results demonstrated that the halosidic bond 3 from the nonreducing end was the preferential cleavage site for DI, whereas bond 2, where the cellobiose unit is split off, was the preferential cleavage site for DIII.

DISCUSSION

The purification of an EDG and CBH from *Cellulomonas uda* CS1-1 was performed to elucidate the characteristics of a bacterial endo/exoglucanase and their action patterns. As an efficient assay method to compare the EDG and CBH activities, the relative ratio of the CMC-saccharifying and CMC-liquefying activity was used throughout the purification steps according to the definition of Wood *et al.* [39]. In addition, a zymogram test using the interaction between CMC and Congo red was also employed as an alternative. DI was purified 13-fold according to its CMC-liquefying activity after the final purification with preparative HPLC. However, it is worth noting that the final purification factor of DIII was decreased compared with that for the previous step, presumably due to a loss of activity during the purification step using preparative IEF.

The molecular masses and pIs were 50,000 Da and pH 5.3 for DI, and 52,000 Da and pH 3.8 for DIII, respectively. The apparent molecular mass and pI for DI were comparable to 66,000 Da and pI 4.4 for the EDG purified from *C. uda* [26] and 45,000 Da for the EDG purified from *C. fimi* [19] produced on microcrystalline cellulose. Although the pI of 3.8 for DIII agreed well with the result of the preparative IEF on the Rotofor cell, this value is very low compared with those previously reported for other *Cellulomonas*.

A comparison of the relative ratio of the CMC-saccharifying and CMC-liquefying activity showed that DIII hydrolyzed CMC in a manner characteristic of a CBH, giving a relatively low slope when the specific fluidity was plotted against the release of reducing sugar. In contrast, the increase in the liquefying activity by DI was a steep slope (Fig. 6). In addition, the ability to hydrolyze various substrates revealed a high activity for DI on CMC and high activity for DIII on microcrystalline cellulose, such as Sigmacells.

The hydrolysis patterns of pNP-glucosides (DP 3–5) by both enzymes indicated that three contiguous sites occupied by glucosyl moieties are a prerequisite for hydrolysis, and the hydrolysis rate became faster in proportion to an increase in the glucosyl moiety. A comparison of the hydrolysis rate revealed that the halosidic bond 3 from the nonreducing end was the preferential cleavage site for DI, whereas bond 2 was the preferential cleavage site for DIII, suggesting that DI exhibited the behavior typical of an endoglucanase, which hydrolysis pNP-glucosides, as well as polysaccharides, in a random fashion. Although DIII exhibited the action of an exo-type enzyme similar to type II cellobiohydrolases, in that the bond cleavage frequencies for the halosidic bond 2 in $pNPG_n$ (DP 3–5) were high values like CBH II, the bond cleavage frequency patterns differed considerably from those for other exoglucanases [4, 31, 37].

Although parts of the amino acid sequences for the two enzymes differed from each other, the protein sequence data revealed that the isoforms had a significant homology with xylanase as well as cellulase. A particular sequence similarity with xylanase was found in DI, where the highest homology was with the xylanases from *S. thermoviolaceus*

[36], *S. roseiscleroticus* [10], and S. *lividans* [5]. The sequence for DIII showed the highest homology with a cloned EXG from *C. fimi* [38] and partial homology with the xylanases from *Thermoanaerobacterium saccharolyticum* [20] and *Pseudomonas fluorescens* [12]. There have been a few reports on cellulase with xylanase activity [21, 28], plus an homology between cellulase and xylanase sequences has also been observed in several bacterial cellulases [5, 14]. Previous studies on substrate specificity also found similar amino acid sequence results to the ability of DI to hydrolyze CMC as well as xylan. However, the specific activity of DI towards CMC was three times higher than that towards xylan, whereas the ability of DIII to hydrolyze xylan was remarkably low when compared with the ability of DI.

Although some details remain to be resolved, the present results indicate that DI can be classified as an endo-type β-1,4-glucanase, whereas DIII can be classified as an exo-type β-1,4-glucanase.

REFERENCES

1. Canevascini, G. and C. Gattlen. 1981. A comparative investigation of various cellulase assay procedures. *Biotechnol. Biochem. Biophys.* **25:** 377–379.
2. Choi, W. Y., K. D. Haggett, and N. W. Dunn. 1978. Isolation of a cotton wool degrading strain of *Cellulomonas* mutants with altered ability to degrade cotton wool. *Aust. J. Biol. Sci.* **31:** 553–564.
3. Choudhury, N., P. P. Gray, and N. W. Dunn. 1980. Reducing sugar accumulation from alkali pretreated sugar cane bagasse using *Cellulomonas*. *J. Appl. Microbiol. Biotechnol.* **11:** 50–54.
4. Claeyssens, M., H. Van Tilbeurgh, P. Tomme, T. M. Wood, and S. I. McCrae. 1989. Fungal cellulose systems; Comparisons of the specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei*. *Biochem. J.* **216:** 819–825.
5. Derewenda, U., L. Swenson, R. Green, Y. Wei, R. Morosli, F. Shareck, D. Kluepfel, and Z. S. Derewenda. 1994. Crystal structure, at 2.6-Å resolution, of the *Streptomyces lividans* xylanase A, a member of the F family of beta-1.4-β-D-glycanases. *J. Biol. Chem.* **269:** 20811–20814.
6. Dubois, M., K. A. Gilles, and F. Smith. 1956. Colorimetric method for determination of sugars and related substance. *Anal. Chem.* **28:** 350–356.
7. Dubos, R. J. 1928. The decomposition of cellulose by aerobic bacteria. *J. Bacteriol.* **15:** 223–234.
8. Emtiazi, G. and I. Nahvi. 2004. Production of thermostable-amylase and cellulose from *Cellulomonas* sp. *J. Microbiol. Biotechnol.* **14:** 1196–1199.
9. Ezaki T., Y. Hashimoto, and E. Yabuuchi. 1989. Fluorometric DNA-DNA hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Evol. Microbiol.* **39:** 224–229.
10. Grabski, A. C., I. T. Forrester, R. Patel, and T. W. Jeffries. 1993. Characterization and N-terminal amino acid sequences of beta-(1-4)endoxylanases from *Streptomyces roseiscleroticus*. *Protein Expr. Purif.* **4:** 120–129.
11. Haggett, K. D., P. P. Gray, and N. W. Dunn. 1979. Crystalline cellulose degradation by a strain of *Cellulomonas* and its mutant derivatives. *Eur. J. Appl. Microbiol. Biotechnol.* **8:** 183–190.
12. Hall, J., G. P. Hazlewood, N. S. Huskisson, A. J. Durrant, and H. J. Gilbert. 1989. Conserved serine-rich sequences in xylanase and cellulase from *Pseudomonas fluorescens* subspecies cellulosa. *Mol. Microbiol.* **3:** 1211–1219.
13. Hall, M. G. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* **41:** 95–98.
14. Heo, S. N., J. Y. Kwak, H. W. Oh, D. S. Park, K. S. Bae, D. H. Shin, and H. Y. Park. 2006. Characerization of an extracellular xylanase in *Paenibacillus* sp. HY-8 isolated from an herbivorous longicorn beetle. *J. Microbiol. Biotechnol.* **16:** 1753–1759.
15. Jones, B. E., W. D. Grant, A. W. Duckworth, P. Schumann, N. Weiss, and E. Stackebrandt. 2005. *Cellulomonas bogoriensis* sp. nov., an alkaliphilic cellulomonad. *Int. J. Syst. Evol. Microbiol.* **55:** 1711–1714.
16. Kimura, M. 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge.
17. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment briefings. *Bioinformatics* **5:** 150–163.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
19. Langsford, M. L., N. R. Gilkes, W. W. Wakarchuk, D. G. Kilburn, and R. C. Miller. 1984. The cellulase system of *Cellulomonas fimi*. *J. Gen. Microbiol.* **130:** 1367–1376.
20. Lee, Y. E., S. E. Lowe, and J. G. Zeikus. 1993. Gene cloning, sequence and biochemical characterization of endoxylanase from *Thermoanaerobacterium saccharolyticum* B6A-RI. *Appl. Environ. Microbiol.* **59:** 3134–3137.
21. Lowe, S. E., M. K. Theodorou, and A. J. Trinci. 1987. Cellulase and xylanase of an anaerobic rumen fungus grown on wheat straw holocellulose, cellulose and xylan. *Appl. Environ. Microbiol.* **53:** 1216–1223.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Medve, J., J. Karlsson, D. Lee, and F. Tjerneld. 1998. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*; Adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol. Bioeng.* **59:** 621–634.
24. Mesbah, M., U. Premachandran, and W. Whitman. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int. J. Syst. Evol. Microbiol.* **39:** 159–167.

25. Moore, D. D. 1995. Preparation and analysis of DNA, pp. 2–11. *In* F. W. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.), *Current Protocols in Molecular Biology.* Wiley, New York, U.S.A.
26. Nakamura, K. and K. Kitamura. 1983. Purification and some properties of a cellulase active on crystalline cellulose from *Cellulomonas uda*. *J. Ferment. Technol.* **61:** 379–382.
27. O'Neil, G., S. H. Goh, R. A. J. Warren, D. G. Kilburn, and R. C. Miller Jr. 1986. Structure of the gene encoding the exoglucanase of *Cellulomonas fimi*. *Gene* **44:** 325–330.
28. Pason, P., G. H. Chon, K. Ratanakhanokchai, K. L. Kyu, O. H. Jhee, J. Kang, W. H. Kim, K. M. Choi, G. S. Park, J. S. Lee, H. Park, M. S. Roh, and Y. S. Lee. 2006. Selection of multienzyme complex-producing bacteria under aerobic cultivation. *J. Microbiol. Biotechnol.* **16:** 1269–1275.
29. Rivas, R., M. F. Trujillo, P. F. Mateos, E. Martínez-Molina, and E. Velázquez. 2004. *Cellulomonas xylanilytica* sp. nov., a cellulolytic and xylanolytic bacterium isolated from a decayed elm tree. *Int. J. Syst. Evol. Microbiol.* **54:** 533–536.
30. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
31. Sanchez, M. M., F. I. Javier Pastor, and P. Diaz. 2003. Exo-mode of action of cellobiohydrolase Cel48C from *Paenibacillus* sp. BP-23. *Eur. J. Biochem.* **270:** 2913–2919.
32. Shareck, F., C. Roy, M. Yaguchi, R. Morosoli, and D. Kluepfel. 1991. Sequences of three genes specifying xylanases in *Streptomyces lividans*. *Gene* **107:** 75–82.
33. Shin, Y. K., J. S. Lee, C. O. Chun, H. J. Kim, and Y. H. Park. 1996. Isoprenoid quinone profiles of the *Leclercia adecarboxylata* KTCT 1036^T. *J. Microbiol. Biotechnol.* **6:** 68–69.
34. Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* **195:** 19–23.
35. Stackebrandt, E. and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44:** 846–849.
36. Tsujibo, H., K. Miyamoto, T. Kuda, K. Minami, T. Sakamoto, T. Hasegawa, and Y. Inamori. 1992. Purification, properties, and thermostable xylanases from *Streptomyces thermoviolaceus* OPC-520. *Appl. Environ. Microbiol.* **58:** 371–375.
37. Tuohy, M. G., D. J. Walsh, P. G. Murray, M. Claeyssens, M. M. Cuffe, A. G. Savage, and M. P. Coughlan. 2002. Kinetic parameters and mode of action of the cellobiohydrolases produced by *Talaromyces emersonii*. *Biochim. Biophys. Acta* **1596:** 366–380.
38. White, A., S. G. Withers, N. R. Gilkes, and D. R. Rose. 1994. Crystal structure of the catalytic domain of the beta-1,4-glycanase cex from *Cellulomonas fimi*. *Biochemistry* **33:** 12546–12552.
39. Wood, T. M., S. I. Mcrae, and K. M. Bhat. 1989. The mechanism of fungal cellulase action. *Biochem. J.* **260:** 37–43.
40. Zverlov, V. V., G. A. Velikodvorskaya, and W. H. Schwarz. 2002. A newly described cellulosomal cellobiohydrolase, CelO, from *Clostidium thermocellum*: Investigation of the exo-mode of hydrolysis, and binding capacity to crystalline cellulose. *Microbiology* **148:** 247–255.