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Cellouronate (β-1,4-linked polyglucuronate) lyase from *Brevundimonas* sp. SH203: Purification and characterization

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Abstract

Biodegradation of cellouronate (β-1,4-linked polyglucuronic acid sodium salt, β-1,4-linked glucuronan), which was prepared from regenerated cellulose by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) mediated oxidation, was investigated. A bacterial strain with the ability to degrade cellouronate was isolated from soil collected in a natural environment, and identified as *Brevundimonas* sp. SH203 by comparing the nucleotide sequences of its 16S rDNA with those registered in the GenBank database. Cellouronate lyase-I (CUL-I), being responsible for the depolymerization of cellouronate, was purified to homogeneity from cell-free extracts. CUL-I was a monomeric protein with the molecular mass of 39 kDa by SDS–PAGE and 37 KDa by size exclusion chromatography (SEC). The enzyme activity was optimum at pH 7.5 and was inhibited by some divalent metal ions such as Mg^{2+}, Fe^{2+} and Mn^{2+}. The enzymatic reaction products were analyzed by SEC, TLC and ^{13}C NMR. The results indicated that CUL-I catalyzed to depolymerize cellouronate endolytically to oligocellouronates and monomeric uronate.

Keywords: TEMPO; Cellulose; Cellouronate lyase; Biodegradation; *Brevundimonas* sp.

1. Introduction

Cellulose is the most abundant organic compound on the earth, and has been widely utilized due to its many favorable properties. Chemical modification of cellulose is one of the effective ways to provide additional functions. Many kinds of cellulose derivatives, such as cellulose esters and ethers, are manufactured industrially and used in various fields. Oxidation of cellulose is also expected as an interesting approach to make cellulose more valuable materials.

In 1980s, a new water-soluble oxidation catalyst, 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), became commercially available. The oxidation system using the combination of TEMPO, sodium hypochlorite and sodium bromide in aqueous media was applied to water-soluble polysaccharides, such as potato starch, amylodextrin and pullulan (de Nooy, Besemer, & Bekkum, 1995). This oxidation enabled selective and almost complete conversion of C6 primary hydroxyl groups in the polysaccharides to carboxyl groups, resulting in quantitative formation of corresponding polyuronates. Chang & Robyt (1996) applied the TEMPO-mediated oxidation to water-insoluble polysaccharides including amylose, cellulose and chitin, however, the oxidized products were partially soluble in water (approximately 10%) but complete water-solubility was not accomplished. Substantial work on the TEMPO-mediated oxidation was reported thereafter for various polysaccharides including starch (Kato, Matsuo, & Isogai, 2003), chitin (Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999), chitosan (Kato et al., 2004; Yoo, Lee, Park, Kim, Chang and Lee, 2005), galactomannan (Sierakowski, Milas, Desbrières, & Rinaudo, 2000) and maltodextrin (Thaburet, Merbouh, Ibert, Marsais, & Queguiner, 2001).

Isogai & Kato (1998) applied the TEMPO-mediated oxidation to cellulose. When native cellulose samples were subjected to the oxidation, only quite small amounts of carboxyl groups were introduced to the surface of the solid celluloses. On the other hand, when regenerated or mercerized celluloses was used, almost all C6 primary hydroxyl groups were selectively oxidized, and water-soluble β-1,4-linked polyglucuronic acid sodium salt (cellouronate) was obtained quantitatively. Cellouronate has a homogenous chemical structure and good water-solubility, and thus, is expected to be utilized as a novel modified cellulosic material with unique

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functions. To expand the potential application of cellouronate, more detailed characterization is necessary, and one of the important characteristics of cellouronate is its biodegrability. The biodegrability of cellouronate was previously studied and its higher biodegrability compared to carboxymethyl cellulose was reported (Kato, Habu, Yamaguchi, Kobayashi, Shibata and Isogai, 2002). Cellouronate was readily degraded by microorganisms in soil samples collected from a natural environment.

In the current study, a bacterial strain with the ability to degrade cellouronate was isolated from a natural environment in order to elucidate the degradation mechanism of cellouronate. Furthermore, an enzyme responsible for the depolymerization of cellouronate (cellouronate lyase) was purified, and characterized by some analytical methods.

2. Materials and methods

2.1. Samples

Bemlise, regenerated cellulose prepared from cotton linters using the cuprammonium system, was obtained from Asahi Chemicals Co., Japan, and used as a raw material for the preparation of cellouronate. TEMPO and a sodium hypochlorite solution (approximately 11%, w/w) were purchased from Wako Pure Chemicals, Co., Japan. Yeast extract and peptone were purchased from Oxoid Ltd, England. Other chemicals and solvents, unless otherwise mentioned, were of analytical grade (Kanto Chemicals Co., Japan or Wako Pure Chemicals Co., Japan) and used without further purification.

Cellouronate was prepared by TEMPO-mediated oxidation according to the procedure described by Isogai & Kato (1998) with slight modifications. Regenerated cellulose (5 g) was suspended in water (250 mL) containing 0.1 g TEMPO and 2.4 g sodium bromide. The oxidation was started by adding the sodium hypochlorite solution (35 mL) at 25 °C, and the pH of the reaction mixture was kept at 10.7 by adding a 0.4 M NaOH solution. After stirring the mixture for 12 min, the reaction was stopped by adding excess ethanol. Cellouronate precipitated was recovered by centrifugation at 570×*g* for 10 min, washed successively with ethanol/water (80/20, v/v), ethanol and acetone, and dried at 25 °C. Thus, obtained cellouronate was dissolved in water (2%, w/v) and the insoluble fraction was removed by centrifugation at 11,000×*g* for 20 min. The purified cellouronate was recovered from the supernatant by adding excess ethanol, washed thoroughly as described above, and dried in vacuo at 40 °C.

2.2. Media and cultural conditions

A cellouronate medium containing 0.82 g/L $(NH_4)_2SO_4$, 1.6 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 25 mg/L $CaCl_2 \cdot 2H_2O$, 2.3 mg/L $FeCl_3 \cdot 6H_2O$, 0.1 g/L yeast extract and 5 g/L cellouronate, at pH 7.0, was used for cultivation of microorganisms. L-broth, containing 10 g/L tryptone, 5.0 g/L yeast extract and 5.0 g/L NaCl, at pH 7.0, was also used to maintain the isolated strains. For a solid medium, 20 g/L agar was added to the above medium. Bacteria were aerobically cultured at 30 °C on a rotary shaker at 120 rpm.

2.3. Isolation and identification of the bacterial strain

As a source of microorganisms in nature, several soil samples were collected from different sites in Tochigi Prefecture, Japan. Each soil sample (1 g) was suspended in the 10 mL sterile water, and 1 mL of the suspension was added to 10 mL cellouronate medium in test tubes. The inoculated test tubes were shaken reciprocally at 120 rpm. Microorganisms that can grow on cellouronate as a carbon source were accumulated by subculturing, which was repeated more than ten times every 14 days. The accumulated culture solutions were spread on cellouronate agar plates and incubated for 3–4 days. Bacterial isolates were selected from single colonies on the cellouronate agar and re-streaked until pure cultures were obtained. Individual strains isolated were inoculated in the 100 mL cellouronate medium and cultured. Each 1 mL of the cultures was withdrawn, and the supernatant after centrifugation was subjected to total organic carbon (TOC) analysis (TOC-V, Shimadzu Co., Japan). From approximately 40 isolates tested, four bacterial strains that showed remarkable decrease in TOC were selected.

Bacterial 16S rDNA fragments were amplified using bacterial universal primers 341f (5′-CCT ACG GGA GGC AGC AG-3′) and 907r (5′-CCC CGT CAA TTC ATT TGA GTT T-3′) with LA *Taq* in GC buffer (Takara Co., Japan) according to a standard protocol (Schabereiter-Gurtner, Lubitz, & Rölleke, 2003). Polymerase chain reaction (PCR) was performed with 1 cycle of denaturation (5 min, 98 °C), followed by 35 cycles of denaturation (15 s, 98 °C), annealing (30 s, 45 °C) and extension (2 min, 72 °C), and a final cycle of extension (5 min, 72 °C). PCR products were sequenced by an automatic DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA) in combination with an Applied Biosystems BigDye terminator v3. cycle sequencing kit. Thus, obtained DNA sequences of the four strains were registered in the DDBJ/GeneBank/EMBL database (Acc. No. AB235160–AB235163). The nucleotide sequences for the 16S rDNA were compared with the registered sequences in the GenBank databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). One of the four bacterial strains, SH203, was used for further studies.

2.4. Preparation of crude enzyme solution

The strain SH203 was cultured in the 100 mL cellouronate medium. Each 6 mL of the cultures was sampled daily, and the cells were collected by centrifugation at 10,000×*g* and 4 °C for 10 min. The cells were washed twice with 50 mM sodium phosphate buffer (pH 7.0), suspended in 3 mL of the same buffer, and ultrasonically disrupted (Ultrasonic Disrupter UD201, Tomy Seiko Co., Japan) on an ice-water bath at 20 kHz for 4 min. The mixture was centrifuged at 10,000×*g* and 4 °C for 30 min and the resultant supernatant, the cell-free extracts, was used as the crude enzyme solution.

For the purification of cellouronate lyase, the strain SH203 was cultured in 3 L of the cellouronate medium (100 mL for each flask) for 4 days. The crude enzyme solution was prepared as described above except that the cells were suspended in a 50 mL buffer and the sonication time was prolonged to 20 min. The crude enzyme solution, thus obtained, was concentrated and equilibrated with 25 mM sodium phosphate buffer (pH 7.5) by ultrafiltration (Amicon model 8050 with a membrane PM10, Millipore Co., USA) to a final volume of 10 mL.

2.5. Enzyme purification

All procedures were performed at 4 °C unless otherwise specified.

Step 1: The concentrated crude enzyme solution was applied to a Toyopearl DEAE-650M column (2.2×9.5 cm, Tosoh Co., Japan) which had been equilibrated with 25 mM sodium phosphate buffer (pH 7.5). The column was washed with the same buffer (150 mL), and then proteins adsorbed to the resin were eluted with a linear gradient of NaCl (0–0.9 M) in the same buffer (900 mL) at a flow rate of 1.5 mL/min. Eluted fractions collected every 9 mL were tested for the enzymatic activity. The active fractions were combined and concentrated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.8 M ammonium sulfate by ultrafiltration to a final volume of 12 mL.

Step 2: The concentrated enzyme solution from step 1 was applied to a Toyopearl Phenyl-650M column (2.2×9.5 cm, Tosoh Co., Japan) which had been equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.8 M ammonium sulfate. After the column was washed with the equilibration buffer (90 mL), proteins were eluted stepwise with the buffer containing 0.7, 0.2 and 0 M ammonium sulfate at a flow rate of 1.5 mL/min. The active fractions, which were eluted with the buffer containing 0.2 M ammonium sulfate, were combined, concentrated and equilibrated with 25 mM sodium phosphate buffer (pH 7.5) by ultrafiltration to a final volume of 10 mL.

Step 3: The enzyme solution from step 2 was applied to a Toyopearl DEAE-650S column (1.6×10 cm, Tosoh Co., Japan) which had been equilibrated with 25 mM sodium phosphate buffer (pH 7.5). The column was washed with the same buffer (150 mL), and proteins were eluted with a linear gradient of NaCl (0–0.4 M) in the same buffer (800 mL) at a flow rate of 1.0 mL/min. Eluted fractions were collected every 9 mL and the active fractions were concentrated to 400 μL by ultrafiltration.

Step 4: The following procedure was performed at room temperature. The concentrated fractions from step 3 were subjected to a size exclusion chromatography (SEC) using an HPLC system (L-7100, Hitachi High-Technologies Co., Japan) equipped with a TSK G3000SW column (0.8×30 cm, Tosoh Co., Japan). The column was previously equilibrated with 25 mM sodium phosphate buffer (pH 7.5) containing 1 M NaCl, and elution was done with the same buffer at a flow rate of 0.3 mL/min. Fractions were collected in 1 mL portions and the active fractions were used as a purified enzyme, cellouronate lyase-I (CUL-I).

2.6. Enzyme assay

The enzyme activity of cellouronate lyase was assayed by monitoring the increase in absorbance at 235 nm based on the formation of 4,5-unsaturated non-reducing terminals, using a spectrophotometer V-550 (JASCO Co., Japan) (Preiss & Ashwell, 1962; Yonemoto, Murata, Kimura, Yamaguchi, & Okayama, 1991). The enzymatic reaction was started by adding 100 μL of the enzyme solution to 900 μL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5% (w/v) cellouronate as a substrate at 25 °C. One unit (U) of the activity was defined as the amount of enzyme necessary to increase 0.01 in the absorbance at 235 nm/min.

2.7. Protein analyses

Protein concentration was determined according to the method by Bradford (1976) with bovine serum albumin as a standard or by measuring absorbance at 280 nm, assuming that $A_{280} = 1.0$ corresponds to 1 mg/mL.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a slab gel (4% acrylamide for stacking gel and 10% for resolving gel) using a Bio-Rad Mini-protean II system (Bio-Rad Laboratories, Inc., USA) according to the method by Laemmli (1970) with a standard protein mixture (Precision Plus Protein Standards, Bio-Rad). Protein was stained by Coomassie brilliant blue R-250 (Bio-Rad).

2.8. Analyses of the enzymatic reaction products

Cellouronate (300 mg) was incubated with 20 μg of purified CUL-I in 30 mL of 25 mM sodium phosphate buffer (pH 7.0) at 25 °C. After prescribed time of incubation, aliquots of the mixture were withdrawn and immediately frozen until being subjected to analyses.

Thin-layer chromatography (TLC) were done on TLC plates of Silica 60 F_{254} (Merk, Darmstadt, Germany) with a solvent system of 1-butanol/acetic acid/water (2/1/1, v/v/v) (Hashimoto, Miyake, Momma, Kawai, & Murata, 2000). The reaction products on the TLC plate were detected by heating after spraying 10% (v/v) sulfuric acid in ethanol. The unsaturated saccharides and α-keto acid on the plate were also detected using thiobarbituric acid (TBA) regent (Warren, 1960).

The molecular mass distribution of the enzymatic reaction products was evaluated by SEC using an HPLC system consisted of a Shodex OHpak SB-806M HQ column (0.8× 30 cm, Showa Denko K.K, Japan), a refractive index (RI) detector (RID-10A, Shimadzu Co., Japan) and a UV detector (Photodiode Array SPD-20A, Shimadzu). Elution was done with 0.1 M NaCl at 40 °C with a flow rate of 0.5 mL/min. The average degree of polymerization of the original cellouronate was estimated to be approximately 50 by SEC using pullulan standards.

^{13}C NMR spectra of the products were recorded on a Bruker AC-300 spectrometer. The freeze-dried reaction mixture was dissolved in D_2O, and subjected to ^{13}C NMR measurement. As

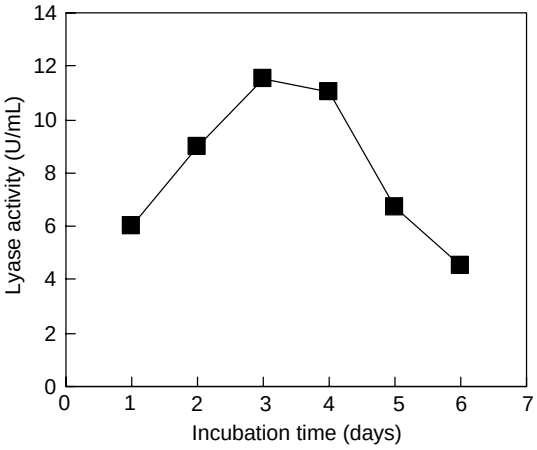

Fig. 1. Lyase activity for cellouronate by *Brevundimonas* sp. SH203.

an internal standard for 0 ppm, 3-trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (Aldrich, USA) was used.

3. Results

3.1. Isolation of bacterial strains

Approximately 40 bacterial isolates picked up from single colonies on cellouronate agar plates were cultured in liquid cellouronate medium, and the changes in TOC in the supernatant of the culture solutions were evaluated. Among them, a strain that showed notable reductions in TOC was chosen and designated as SH203. The strain SH203 cultured on cellouronate decreased more than 60% of the initial TOC within 4 days, indicating that cellouronate was mineralized or incorporated into cells.

In the BLAST search using the 16S rDNA sequence of the bacterial strain SH203 (Acc. No. AB235160), the highest score, identity of 99% in the 523 bp fragment, was obtained against the sequence of *Brevundimonas* sp. (Acc. No. AY169433). Thus, the strain SH203 was classified as *Brevundimonas* sp.

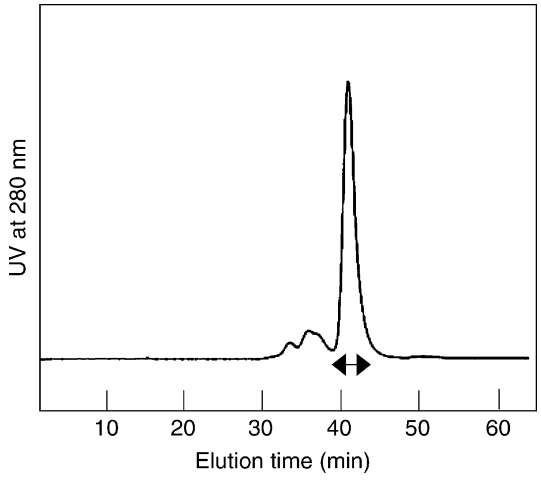

Fig. 2. Size exclusion chromatogram of cellouronate lyase-I on TSK G3000SW column. An arrow indicates fractions with the enzyme activity.

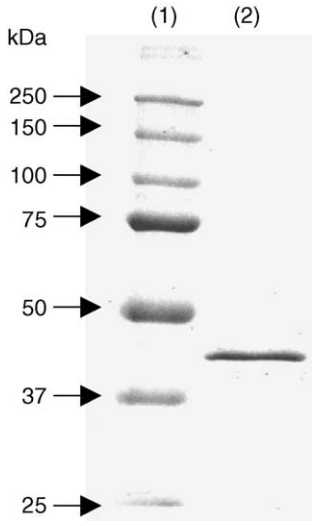

Fig. 3. SDS–PAGE of purified cellouronate lyase-I. Lane 1: molecular mass marker proteins; Lane 2: purified cellouronate lyase-I.

3.2. Enzyme purification

Brevundimonas sp. SH203 was cultured in the liquid cellouronate medium and the time-course of the enzyme production was investigated. As shown in Fig. 1, lyase activity was detected in the crude enzyme solution, and the highest activity was found after cultivation for 3–4 days. When *Brevundimonas* sp. SH203 was grown in L-broth, no lyase activities were detected, showing that the enzyme was induced by cellouronate.

The enzyme which was responsible for depolymerization of cellouronate was purified and termed cellouronate lyase-I (CUL-I). The purification was achieved by four steps consisting of anionic exchange chromatography (Toyopearl DEAE-650M), hydrophobic chromatography (Toyopearl Phenyl-650M), anionic exchange chromatography (Toyopeal DEAE-650S) and size exclusion chromatography (TSK G3000SW). As shown in Fig. 2, the SEC elution profile, which was carried out at the last step of purification, had a sharp single peak with the enzyme activity, and the corresponding fractions were used as purified CUL-I. The homogeneity of purified CUL-I was confirmed by SDS–PAGE (Fig. 3). The results of each purification step are summarized in Table 1. The specific activity of purified CUL-I was 13.7 U/mg

Table 1
Purification of cellouronate lyase-I

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	187	56160	0.30	1.0	100
Toyopeal DEAE-650M	48.8	35814	0.73	2.5	63.8
Toyopeal Phenyl-650M	15.6	30636	1.96	6.6	54.6
Toyopeal DEAE-650S	0.50	3348	6.71	22.4	6.0
TSK G3000SW	0.17	2340	13.70	45.7	4.2

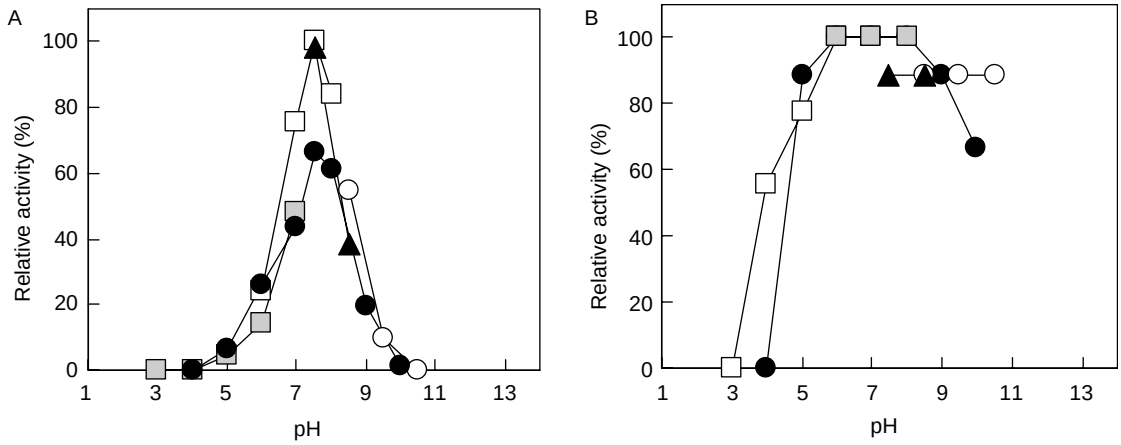

Fig. 4. Effects of pH on the activity (A) and stability (B) of cellouronate lyase-I. Buffers used were GTA–HCl/NaOH (pH 4.0–10.0, ●), citric acid–Na_2HPO_4 (pH 3.0–7.0, ▣), Na-phosphate (pH 6.0–8.0, □), Tris–HCl (pH 7.5–8.5, ▲), and glycine–NaOH (pH 8.5–10.5, ○).

with the purification fold of 45.7 and the recovery yield of 4.2%.

3.3. Characterization of cellouronate lyase

3.3.1. Molecular mass

The molecular mass of the native CUL-I estimated by SEC (TSK G3000SW) was 37 kDa. The denatured CUL-I gave a single band on SDS–PAGE at 39 kDa (Fig. 4). These results indicate that CUL-I is a monomeric protein.

3.3.2. Effects of pH and temperature

The effects of pH on the activity and the stability of CUL-I were examined by using following buffers (50 mM); GTA–HCl/NaOH buffer (pH 4.0–10.0), citric acid–sodium phosphate buffer (pH 3.0–7.0), sodium phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (pH 7.5–8.5) and glycine–NaOH buffer (pH 8.5–10.5). The optimum pH for the enzyme activity was 7.5, and the enzyme had the highest activity in sodium phosphate buffer (Fig. 4(A)). CUL-I was stable in a broad pH range from 5 to 9, when incubated at 25 °C for 24 h (Fig. 4(B)). Thermal stability of CUL-I was estimated by measuring the residual activities after incubation of the enzyme at various temperatures (10–70 °C) for 10 min. CUL-I was stable at 10–40 °C, and the activities decreased as the temperature increased above 40 °C.

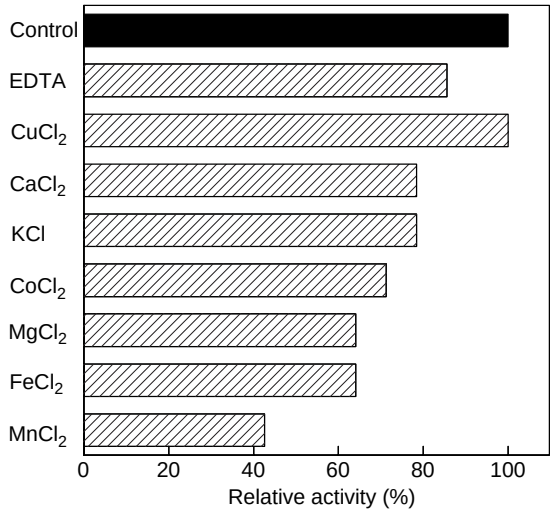

Fig. 5. Effect of metal ions on the activity of cellouronate lyase-I. The enzyme solution was treated with 2 mM metal compounds at pH 7.0 and 4 °C for 1 h.

3.3.3. Effect of metal ions

The effects of metal ions on the enzyme activity were examined. The enzyme in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 4 °C for 1 h in the presence of metal compounds at a concentration of 2 mM, and then the residual activities were determined. The results were expressed as relative activities to the control (Fig. 5). Some divalent metal ions, such as Mg^{2+}, Fe^{2+} and Mn^{2+}, partially inhibited the activity, while other metal compounds and EDTA had no significant effect on the enzyme activity.

3.3.4. Substrate specificity

The substrate specificity of CUL-I was estimated by using some polyuronates, such as α-1,4-linked polyglucuronic acid sodium salt (amylouronate), alginate and pectin, and carboxymethyl cellulose sodium salt with the degree of substitution of 0.5. As shown in Table 2, CUL-I was highly specific to cellouronate, being slightly active toward alginate (5.7% relative to cellouronate) and amylouronate (2.8% relative to cellouronate).

Table 2
Substrate specificity of cellouronate lyase-I

	Relative activity (%)
Cellouronate	100.0
Alginate	5.7
Amylouronate	2.8
Pectin	ND
Carboxymethyl cellulose	ND

Each substrate (0.5%) in 50 mM sodium phosphate buffer (pH 7.0) was incubated with 5.7 μg of purified CUL-I and the enzyme activity was assayed by monitoring the increasing absorbance at 235 nm. ND, not detected.

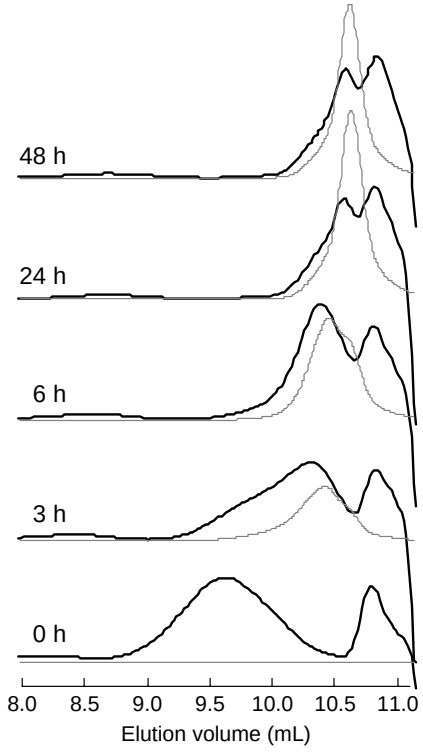

Fig. 6. Size exclusion chromatogram of the reaction products detected by refractive index (——) and absorption at 235 nm (—).

3.4. Analyses of the enzymatic reaction products

Cellouronate was incubated with CUL-I and the enzymatic reaction products were analyzed. The amount of unsaturated uronate residues monitored by the absorbance at 235 nm increased until incubation of 24 h, and leveled off thereafter (data not shown).

The SEC profiles of the reaction products are shown in Fig. 6. The original peak of cellouronate (incubated for 0 h) at the elution volume of 8.7–10.5 mL detected by RI was shifted to lower molecular mass regions after the enzymatic reaction. The shifted peak, unlike the original peak of cellouronate, had the absorption at 235 nm, which indicates that the depolymerization of cellouronate was accompanied by the formation of unsaturated uronate residues due to β-elimination. In the elution profile of the original cellouronate, another peak corresponding to phosphate salts as buffer components was detected by RI at the elution volume of 10.5–11 min. The area of this peak was increased as the enzymatic reaction proceeded, suggesting the accumulation of enzymatic reaction products having molecular mass values similar to those of phosphate salts.

The reaction products were analyzed by TLC (Fig. 7). The enzymatic reaction led to the formation of several reaction products corresponding to oligomeric cellouronates ((C) in Fig. 7), and finally to the accumulation of products (A) and (B). These reaction products were also detected when the TBA reagent was used for staining instead of sulfuric acid. According to the literatures (Shimokawa, Yoshida, Takeuchi, Murata, Kobayashi and Kusakabe, 1997), the accumulated products were tentatively identified as dimeric unsaturated uronic acid (B) and α-keto-glucuronic acid (A), respectively.

Fig. 8 shows ^{13}C NMR spectra of cellouronate before and after the enzymatic reaction. Two resonance peaks at approximately 110 and 147 ppm clearly appeared after the treatment. These peaks were assigned to C4 and C5, respectively, of unsaturated glucuronate residues formed by β-elimination. The signal at 42 or 47 ppm for the enzyme-treated product is probably due to methylene carbon at the β-position of α-keto acid formed from hexeneuronic acid monomer (Preiss & Ashwell, 1962; Shimokawa et al., 1997). Many signals at 85–108 ppm due to anomeric carbons indicate significant depolymerization of cellouronate by the enzymatic reaction. Details about signal assignment of the enzyme-treated product will be reported in the following paper after α-keto acid is isolated from the product.

4. Discussion

A bacterial strain that can degrade cellouronate was isolated from the soil in nature and the production of enzymes to depolymerize cellouronate was studied. Two types of enzymes, lyases and hydrorases, were considered to be responsible for the depolymerization of cellouronate, and these enzymes cleave glycoside bonds by β-elimination and hydrolysis, respectively. Depolymerization of cellouronate by lyases brings about the formation of double bonds between C4 and C5 in the uronate residues at the non-reducing ends, which can be measured by monitoring the increasing absorbance at 235 nm. The result in Fig. 1 clearly shows that *Brevundimonas* sp. SH203 acquired such lyase activities when cultured on cellouronate. To evaluate hydrorase activities of *Brevundimonas* sp. SH203, the profile of the increased amount of total reducing ends formed through cleavages of glycoside bonds of cellouronate by both lyases and hydrorases was monitored using the method reported by Somogyi (1952). The obtained time-course profile was similar to that for the lyase activities in Fig. 1 (data not shown). These results revealed that lyases

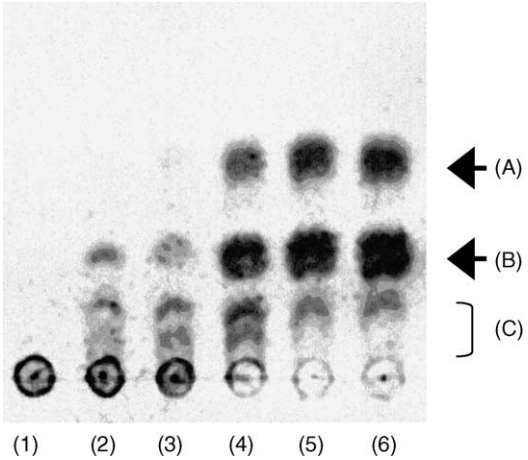

Fig. 7. TLC patterns of the reaction products. Cellouronate was treated by cellouronate lyase-I for 0, 3, 6, 24, 48 and 72 h on lanes 1, 2, 3, 4, 5 and 6, respectively.

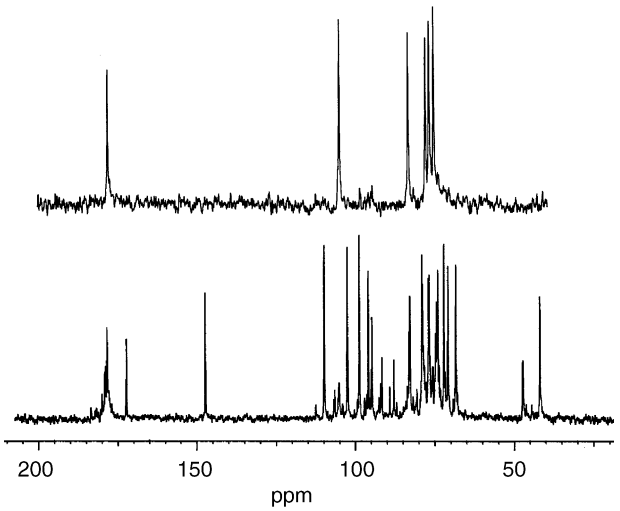

Fig. 8. ^{13}C-NMR spectra of cellouronate before (above) and after (below) the treatment with cellouronate lyase-I for 72 h.

primarily contribute to the depolymerization of cellouronate by *Brevundimonas* sp. SH203. Many of naturally occurring polyuronates are known to be depolymerized by lyases, such as alginate (Hashimoto et al., 2000; Preiss & Ashwell, 1962; Rehm, 1998; Yonemoto et al., 1991), pectin (Benen, Kester, Pařenicová, & Visser, 2000; Pissavin, Robert-Baudouy, & Hugouvieux-Cotte-Pattat, 1998; Singh, Plattner, & Diekmann, 1999), xanthan (Hashimoto, Miki, Tsuchiya, Nakai, & Murata, 1998, 2001) and hyaluronan (Jedrzejas, Mello, de Groot, & Li, 2002; Oettl, Hoechstetter, Asen, Bernhardt, & Buschauer, 2003; Ponnuraj & Jedrzejas, 2000).

Cellouronate lyase-I (CUL-I) was purified as an enzyme responsible for the depolymerization of cellouronate. When cellouronate was incubated with CUL-I, the formation of reaction products having lower molecular masses and the absorption at 235 nm was observed in the SEC analysis. The results of TLC analysis also demonstrated the formation of several oligomeric uronates and α-keto-glucuronic acid. The formation of α-keto-glucuronic acid is explained by the non-enzymatic conversion from the unsaturated monomer due to its instability. Such conversion is also reported to occur in enzymatic degradation products from alginate (Preiss & Ashwell, 1962; Shimokawa et al., 1997). Based on these results, CUL-I is likely to depolymerize cellouronate endolytically by β-elimination to dimeric and monomeric uronates *via* oligo-cellouronates (Fig. 9). We confirmed in the purification process of CUL-I that lyase activities other than that of CUL-I were present in the crude enzyme solutions of *Brevundimonas* sp. SH203. The purification and the characterization of those lyases, as well as the relations with CUL-I, are actually under way.

Cellouronate is a semisynthesized polyuronate prepared by the oxidation of cellulose and is not recognized as an abundantly occurring natural polymer. However, a considerable number of apparently different microorganisms grew on cellouronate as a carbon source. Indeed, in addition to *Brevundimonas* sp.SH203 used in this study, three other strains with abilities to degrade cellouronate were also isolated. They were identified as *Brevundimonas* sp. KH403Y (Acc. No. AB235161), *Sphingomonas* sp. KH406 (Acc. No. AB235162) and *Sphingopyxis* sp. KYH-1 (Acc. No. AB235163), respectively. All these strains were confirmed to produce lyase activities when cultured on cellouronate. Moreover, the purified enzyme, CUL-I, was highly specific to cellouronate, being almost inert to other polyuronates.

Courtois, Seguin, Declomesnil, Heyraud, Colin-Morel and Dantas (1993) reported that a mutant strain *Sinorhizobium*

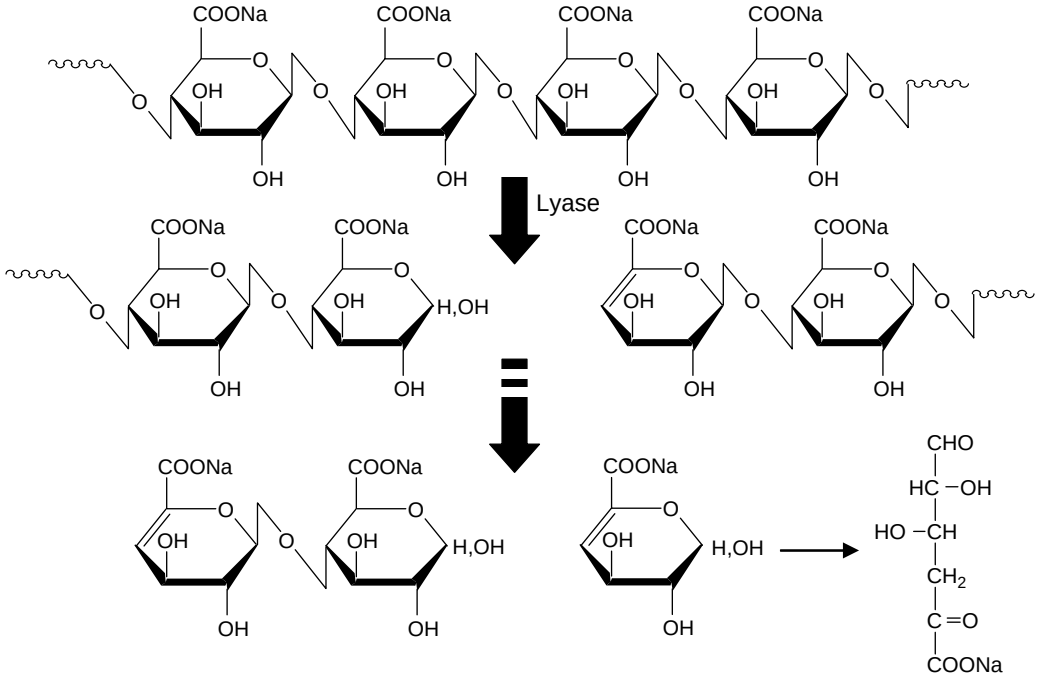

Fig. 9. Representative depolymerization scheme of cellouronate by cellouronate lyase-I.

meliloti M5N1CS (NVIMB 40472) produced partially acetylated β-1,4-linked glucuronan in the culture solutions. An endogenous lyase for that was purified from the same strain and characterized (Da Costa et al., 2001). The purified lyase was more active to deacetylated glucuronan, which has the same chemical structure as cellouronate, than the original acetylaed glucuronan. Very recently, it was reported that partially acetylated glucronan produced by the above strain of *S. meliloti* was depolymerized by lyase(s) present in the culture solutions from *Trichoderma* sp. GL2 (Delattre, Michaud, Lion, Courtois, & Courtois, 2005). Although detailed characteristics of the enzyme were not elucidated yet, the enzyme was more active to deaetylated glucuronan than to acetylated. This fungal strain was isolated from compost. Our results in the current study, as well as these reports, allow us to evoke the possibility that cellouronate, or similar polymers having β-1,4-linked polyglucuronate structures, may be present in nature more abundantly than we have recognized.

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