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# Biodegradation of (1→3)-β-polyglucuronate prepared by TEMPO-mediated oxidation

Erika Watanabe [a,b], Naoto Habu [a], Akira Isogai [b,*]

[a] Department of Applied Biological Chemistry, Utsunomiya University, Tochigi 321-8505, Japan
[b] Department of Biomaterial Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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ABSTRACT

2,2,6,6-Tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation was applied to curdlan to prepare water-soluble sodium (1→3)-β-polyglucuronate, and its biodegradation behavior was then investigated. A bacterial strain (EH621) having the ability to degrade (1→3)-β-polyglucuronate was isolated from soil, and identified as *Paenibacillus* sp. Strain EH621 cultured with (1→3)-β-polyglucuronate decreased the initial total carbon in the supernatant by approximately 60% within 3 days, showing that (1→3)-β-polyglucuronate can be readily degraded and metabolized by microorganisms present in the natural environment. Hydrolytic enzyme activity was detected in the cell-free extract of EH621, which was highly specific to (1→3)-β-polyglucuronate. Analyses of the enzymatic degradation products revealed that (1→3)-β-polyglucuronate was endolytically degraded to its monomeric unit, glucuronate, *via* some oligomers and dimers.



## 1. Introduction

Effective use of polysaccharides as natural resources has recently become required to establish a sound material-cycle society, and as a result, chemical modifications of polysaccharides have been attracting much attention. Among them, oxidation of the C6 primary hydroxyl groups of polysaccharides using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) as a catalyst under aqueous conditions has received a great deal of interest for its high reaction rate, regioselectivity, and environmental compatibility (Bragd, Besemer, & van Bekkum, 2001; de Nooy, Besemer, & van Bekkum, 1995). Conversion rates and ratios of primary hydroxyl groups to carboxyls depend on water-solubility and crystallinity of polysaccharides used as starting materials as well as oxidation conditions. This TEMPO-mediated oxidation has created opportunities to provide additional functionalities to polysaccharides. Various water-insoluble polysaccharides, such as regenerated cellulose, chitin, and β-cyclodextrin are made water-soluble by the TEMPO/NaBr/NaClO oxidation system (Fraschini & Vignon, 2000; Isogai & Kato, 1998; Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999). The water-soluble polyuronates thus obtained are expected to have new potential applications.

Curdlan, an extracellular bacterial polysaccharide, is a linear (1→3)-β-glucan. The repeating unit of curdlan is anhydroglucose, the same as those of cellulose, (1→4)-β-glucan, and amylose, (1→4)-α-glucan, which are the representative biomass resources (Harada, Misaki, & Saito, 1968; McIntosh, Stone, & Stanisich, 2005). Because curdlan has a triple-helical structure because of the (1→3)-β-linkage, many investigations focused on curdlan for its unique structure and peculiar functionality (Chuah, Sarko, Deslandes, & Marchessault, 1983; Deslandes, Marchessault, & Sarko, 1980; Shibakami, Sohma, & Hayashi, 2012).

When the TEMPO-mediated oxidation was applied to curdlan, water-soluble sodium (1→3)-β-polyglucuronates were quantitatively obtained (Tamura, Wada, & Isogai, 2009; Watanabe et al., 2013). The originally water-insoluble curdlan became soluble in the aqueous reaction medium as the oxidation of the primary hydroxyls proceeds. Partial depolymerization of curdlan was, however, inevitable during the oxidation reactions. Because water-soluble polysaccharides, which originally have triple-helical structures, are applicable as effective dispersants of carbon nanotubes and metal nanoparticles in water (Le et al., 2011; Li, Zhang, Xu, & Zhang, 2011), (1→3)-β-polyglucuronate is also expected to be suitable for this purpose. Thus, the biodegradability of (1→3)-β-polyglucuronate is an essential issue influencing its fundamental properties, applicability, and performance limits.

In our previous work, the biodegradation behavior of cellouronate, (1→4)-β-polyglucuronate, and amylouronate, (1→4)-α-polyglucuronate, was investigated. These polyglucuronates were prepared from regenerated cellulose and water-soluble starch,

* Corresponding author at: 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Tel.: +81 3 5841 5538; fax: +81 3 5841 5269.
*E-mail address:* aisogai@mail.ecc.u-tokyo.ac.jp (A. Isogai).

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respectively, by TEMPO-mediated oxidation. Cellouronate was degraded with two types of intracellular lyases produced by *Brevundimonas* sp. SH203 isolated from the natural environment (Konno, Habu, Maeda, Azuma, & Isogai, 2006; Konno, Habu, Iihashi, & Isogai, 2008), while amylouronate was degraded with intracellular hydrolases produced by *Paenibacillus* sp. TH501b (Iihashi, Nagayama, Habu, & Isogai, 2009). These bacterial strains, however, were found to be unable to degrade (1→3)-β-polyglucuronate. In this study, therefore, bacterial strains with the ability to degrade (1→3)-β-polyglucuronate were isolated from the natural environment, and the biodegradation behavior of (1→3)-β-polyglucuronate by crude enzyme extracted from one stain was studied in detail.

## 2. Materials and methods

### 2.1. Materials

Commercially available curdlan (Wako Pure Chemicals, Co., Japan) was used as a starting material for the preparation of (1→3)-β-polyglucuronate. TEMPO, sodium bromide, 12% sodium hypochlorite solution, and other reagents and solvents were purchased from Wako Pure Chemicals Co., Japan and Kanto Chemicals Co., Japan and used without further purification. Yeast extract was purchased from Oxoid Ltd., UK. Curdlan (5 g) was suspended in water (500 mL) at pH 10 containing TEMPO (0.08 g, 0.5 mmol) and sodium bromide (0.5 g, 5 mmol), and the suspension was stirred at room temperature. The TEMPO-mediated oxidation was started by adding 12% NaClO solution (36 g, 15 mmol). The pH of the mixture was maintained at 10 by adding 0.5 M NaOH using a pH stat until no further NaOH consumption was observed. The reaction was quenched by adding excess ethanol (500 mL). The resulting precipitate was collected and washed three times with 80% aqueous ethanol and then acetone by centrifugation, and finally vacuum-dried (Tamura et al., 2009). The chemical structure of the obtained (1→3)-β-polyglucuronate was confirmed by $^{13}C$ nuclear magnetic resonance (NMR). The yield of the oxidized product was 92%.

### 2.2. Culture conditions

Microorganisms were aerobically cultured at 30 °C on a rotary shaker at 120 rpm in a (1→3)-β-polyglucuronate 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 $MgSO_4{\cdot}7H_2O$, 0.025 g/L $CaCl_2{\cdot}2H_2O$, 0.0023 g/L $FeCl_3{\cdot}6H_2O$, 0.1 g/L yeast extract, and 5.0 g/L (1→3)-β-polyglucuronate (pH 7.0). An L-Broth (LB) medium (pH 7.0) containing 10 g/L tryptone, 5.0 g/L yeast extract, and 5.0 g/L NaCl was also used as a nutrient medium. In the case of a solid medium, 20 g/L agar was added to the above media.

### 2.3. Isolation and identification of the bacterial strain

Several soil samples were collected from different sites in Tochigi and Shizuoka Prefectures, Japan, as sources of microorganisms occurring in the natural environment. Each soil sample (1 g) was suspended in distilled water (10 mL), and 0.5 mL of each suspension was added to 10 mL (1→3)-β-polyglucuronate medium in separated test tubes. The inoculated test tubes were shake-cultured (30 °C, 120 rpm) and repeatedly subcultured. The accumulated culture solutions were spread on (1→3)-β-polyglucuronate agar plates and incubated for 3 days. Four bacterial strains which made clear colonies on the (1→3)-β-polyglucuronate solid medium were isolated and designated as EH602a, EH602b, EH621 and EH622, respectively. These strains were then cultured in the (1→3)-β-polyglucuronate liquid medium and the total carbon (TC) in the supernatant of the culture solution was measured using a TOC analyzer (TOC-V, Shimadzu Co., Japan). Strain EH621, which showed the highest reduction in TC, was identified by comparing its sequence for the gene encoding the 16S rRNA with databases, using the same procedure described in our previous work (Iihashi et al., 2009). The obtained DNA sequence was registered in the DDBJ/GeneBank/EMBL database (Accession no. AB765931). The nucleotide sequence for the 16S rRNA was compared with the registered sequences in the GeneBank databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

### 2.4. Preparation of crude enzyme solution

The EH621 cells pre-cultured in the LB medium were cultured in 100 mL (1→3)-β-polyglucuronate medium. Ten mL of the culture was sampled daily and centrifuged at 10,000 × *g* and 4 °C for 10 min. The collected cells were washed three times with 50 mM sodium phosphate buffer (pH 7.0) and then suspended in 3 mL of the same buffer and treated using an ultrasonic disrupter (UD201, Tomy Seiko Co., Japan) at 20 kHz for 5 min over an ice-water bath. The mixture was centrifuged at 10,000 × *g* and 4 °C for 10 min, and the supernatant containing a cell-free extract was used as the crude enzyme solution. Selected conditions, such as pre-culture medium, culture time, and pH, were varied to obtain higher enzyme activities in the solution.

### 2.5. Enzyme assay

Enzyme activity was assayed by determining the increased amount of reducing ends formed by cleavage of the glycoside bonds of (1→3)-β-polyglucuronate. A reaction mixture containing the enzyme and 0.3% (w/v) (1→3)-β-polyglucuronate in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 30 °C for 0, 15, and 30 min. The amount of reducing ends was colorimetrically measured at 660 nm according to the method reported by Somogyi (1952) using a microplate reader (SH-1000 Lab, Corona Electric Co., Japan) with sodium glucuronate as a standard. One unit (U) of the enzyme activity was defined as an amount of enzyme necessary to liberate reducing ends equivalent to 1 μmol sodium glucuronate $min^{-1}$.

### 2.6. Analyses of the enzymatic degradation products

A reaction mixture of 1% (w/v) (1→3)-β-polyglucuronate and an amount of enzyme corresponding to 15 U was incubated at 26 °C with reciprocal shaking at 100 rpm. After the desired incubation time aliquots of the mixture were withdrawn and immediately frozen until subjected to further analyses. Thin-layer chromatography (TLC) was performed on a TLC plate of Silica 60 F254 (Merck Co., Germany) with a 3:2:2 1-butanol/acetic acid/water (v/v/v) developing solvent using glucuronate (50 ng) as a standard. The reaction products were visualized on the TLC plate by heating after dipping it in 10% (v/v) sulfuric acid in ethanol. The molecular mass distribution of the reaction products was evaluated by size-exclusion chromatography (SEC) using an SEC column for aqueous systems (SB-802.5 HQ, 8 mm $\phi$ × 30 cm, Shodex, Japan) and 0.1 M NaCl as the eluent. The samples were filtered using 0.2 μm polytetrafluoroethylene (PTFE) membranes (Millipore, USA) before injection. Elution was carried out at 40 °C with a flow rate of 0.5 mL/min, and the elution patterns were detected by refractive index (RI). The freeze-dried reaction mixture was dissolved in $D_2O$ and subjected to $^{13}C$ NMR analysis. The $^{13}C$ NMR spectra of the products were recorded on an ALPHA-500 (JEOL, Japan) using 3-trimethylsilyl-2,2,3,3-$d_4$-propionic acid sodium salt (Aldrich, USA) as an internal standard. The data accumulations for the $^{13}C$ NMR spectra were about 25,000 times.

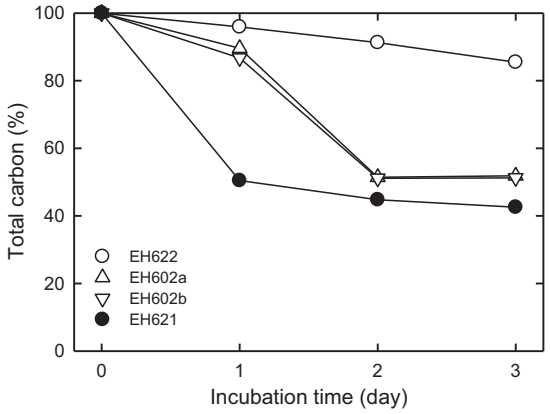


**Fig. 1.** Time-dependent change of total carbon in supernatant of the (1→3)-β-polyglucuronate medium incubated with isolated strains.

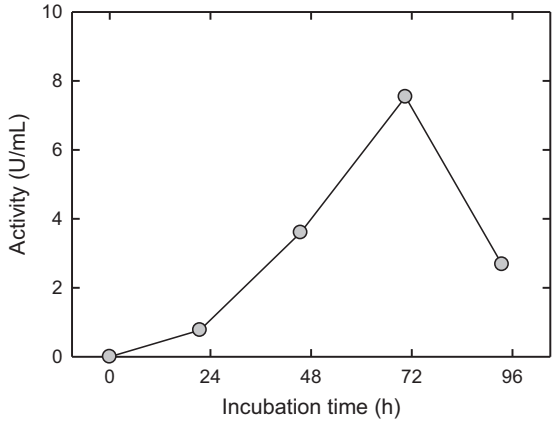


**Fig. 2.** Time-dependent change of intracellular hydrolase activity of the extracted crude enzyme.

## 3. Results and discussion

Curdlan was oxidized by TEMPO-mediated oxidation using NaClO and catalytic amounts of TEMPO and NaBr in water at pH 10 to prepare water-soluble TEMPO-oxidized curdlan. As will be described later, $^{13}C$ NMR analysis showed that almost all the C6 primary hydroxyl groups of curdlan were selectively oxidized to C6 carboxylate groups by the TEMPO-mediated oxidation. Thus, almost pure sodium (1→3)-β-polyglucuronate was successfully prepared from curdlan in high yield (Tamura et al., 2009), and this sample was used as the starting material in this biodegradation study.

### 3.1. Isolation and identification of the bacterial strain

In our previous work, several bacterial strains able to degrade polyglucuronates such as cellouronate with (1→4)-β-linkages (Konno et al., 2006, 2008) and amylouronate with (1→4)-α-linkages (Iihashi et al., 2009) were isolated and characterized. These strains, however, were found to be unable to grow in the presence of (1→3)-β-polyglucuronate. Thus, new strains for (1→3)-β-polyglucuronate degradation were screened and isolated from soil samples collected from various areas in a natural environment. Among 42 bacterial strains which colonized on (1→3)-β-polyglucuronate agar plates, 4 isolates, EH602a, EH602b, EH621 and EH622, showed remarkable growth. These were then picked up from single colonies and cultured in liquid (1→3)-β-polyglucuronate medium.

Time-dependent changes of the total carbon (TC) in the supernatant of the culture solutions were evaluated using a TOC analyzer and shown in Fig. 1. All the strains tested showed clear TC reduction in the culture solutions, although reduction rates varied between the strains. These observations suggest that microorganisms including bacterial strains, able to grow with (1→3)-β-polyglucuronate as a carbon source exist rather abundantly in the natural environment. Thus, (1→3)-β-polyglucuronate can be recognized as a biodegradable material, although it was artificially prepared from curdlan by the TEMPO-mediated oxidation in this study. Because EH621 exhibited the most rapid TC decrease during cultivation, further study was carried out using this strain. To identify the EH621 stain, its gene sequence encoding the 16S rRNA was compared with database-registered sequences using the BLAST search. The highest score, identifying 99.7% of 759 bp fragments, was obtained against the sequence of *Paenibacillus* sp. KSM-318 (Accession no. AB257600). Thus EH621 was recognized as *Paenibacillus* sp.

### 3.2. Enzyme activity of Paenibacillus sp. EH621 to degrade (1→3)-β-polyglucuronate

When the *Paenibacillus* sp. EH621 was cultured with (1→3)-β-polyglucuronate as a main carbon source, rapid TC reduction, approximately 60% of the original TC within 3 days, was observed (Fig. 1), indicating that the microorganisms produced enzymes responsible for degradation of (1→3)-β-polyglucuronate. Thus, enzyme activities in both a cell-free extract (intracellular) and a culture supernatant (extracellular) were measured by determining the amount of reducing ends formed by cleavage of glycoside bonds. Significant enzyme activity producing reducing ends from (1→3)-β-polyglucuronate was detected in the cell-free extract. Absorbance at 235 nm, which is an indicator of lyase activity (Konno et al., 2006), was also measured during the enzymatic treatment, but no increase was observed. These results indicate that the above enzyme activity resulted from hydrolytic cleavage of glycoside bonds. The culture supernatant, however, had almost no detectable enzyme activity, indicating that *Paenibacillus* sp. EH621 degrades and metabolizes (1→3)-β-polyglucuronate mainly with intracellular hydrolytic enzymes under the culture conditions used in this study.

*Paenibacillus* sp. EH621 was cultivated under conditions with various factors such as pre-culture medium, culture time, and pH, and a crude enzyme solution (cell-free extract) was prepared to optimize enzyme production. The optimum culture pH for the enzyme production was found to be 7.6 (data not shown). The LB medium used as a pre-culture resulted in good growth of cells. After being pre-cultured in the LB medium for 1 day, the cells were collected by centrifugation, cultured in (1→3)-β-polyglucuronate medium for 1–4 days, and the crude enzyme solutions were prepared from them to measure their enzyme activities. Fig. 2 shows the time-dependent changes of the enzyme activity in the crude enzyme solution. The highest enzyme activity was obtained from cells cultured for 3 days, whereas prolonged culturing for 4 days led to a significant lowering of the enzyme activity.

The substrate specificity of the hydrolase activity in the crude enzyme solution was estimated using the following polyuronates; (1→4)-α-polyglucuronate (amylouronate), (1→4)-β-polyglucuronate (cellouronate), alginate, hyaluronate, pectin, starch and curdlan. As shown in Table 1, the hydrolase activity was highly specific to (1→3)-β-polyglucuronate. The enzyme solution was slightly active to pectin, although the relative activity was quite low, only 8.4% to (1→3)-β-polyglucuronate. The reason for this slight activity of the crude enzyme to pectin is unknown at present.

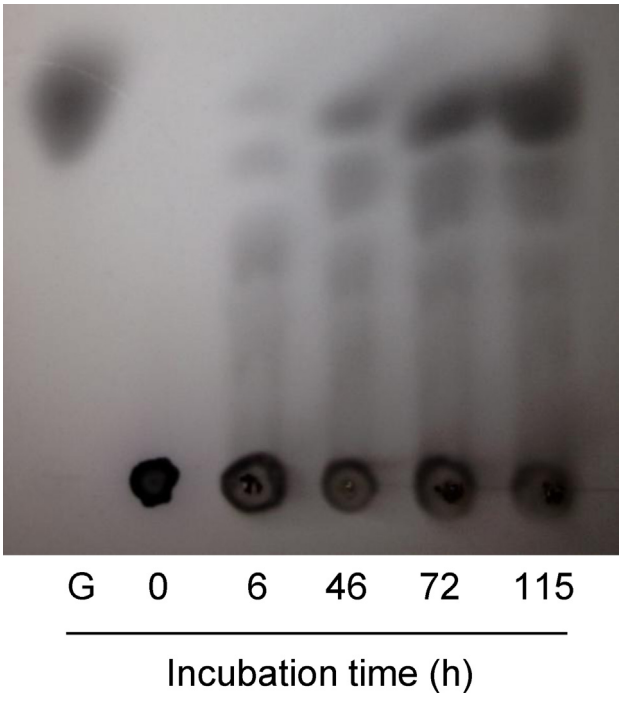


**Fig. 3.** TLC patterns of degradation products obtained from (1→3)-β-polyglucuronate after crude enzyme treatment for 0–115 h. G: authentic glucuronate.

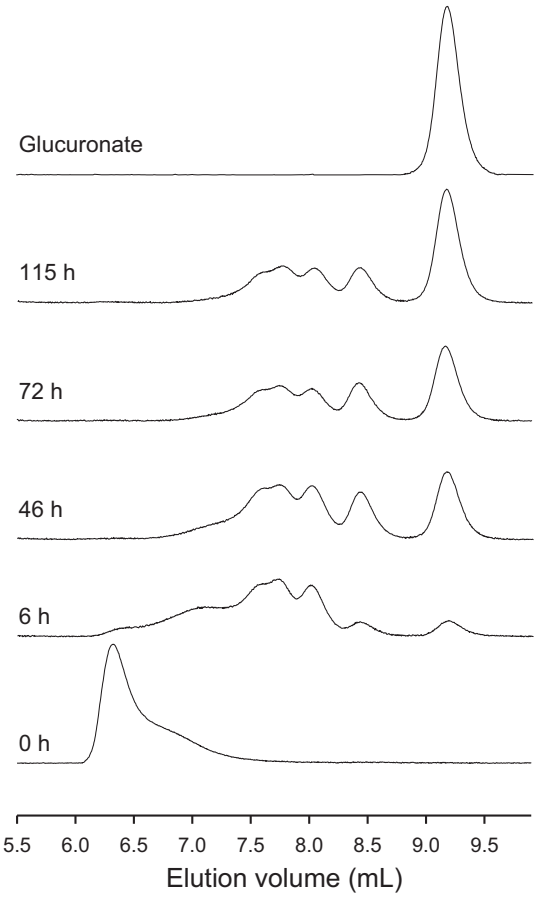


**Fig. 4.** SEC elution profiles of degradation products obtained from (1→3)-β-polyglucuronate after crude enzyme treatment for 0–115 h, detected by refractive index.

**Table 1**
Substrate specificity of the crude enzyme to (1→3)-β-polyglucuronate.

| Substrate | Relative activity (%) |
|---|---|
| (1→3)-β-Polyglucuronate | 100.0 |
| Amylouronate | ND |
| Cellouronate | ND |
| Alginate | ND |
| Hyaluronate | ND |
| Pectin | 8.4 |
| Starch | ND |
| Curdlan | ND |

ND, not detected.

### 3.3. Analyses of degradation products by crude enzyme

(1→3)-β-Polyglucuronate was incubated with the crude enzyme and the resulting enzymatic degradation products were analyzed by TLC, SEC, and $^{13}$C NMR. Fig. 3 shows TLC patterns of the degradation products. The enzymatic degradation led to formation of glucuronate, a monomer component of (1→3)-β-polyglucuronate, and several products corresponding to dimer and oligomers. The amount of glucuronate formed during the reaction increased with incubation time.

The SEC elution profiles of the original (1→3)-β-polyglucuronate, its degradation products by the crude enzyme, and glucuronate standard are shown in Fig. 4. The peak of the original (1→3)-β-polyglucuronate (incubated for 0 h) at an elution volume of 6.1–7.5 mL was shifted to higher elution volumes or lower molecular mass regions with increasing enzyme-treatment time. Along with the peak shift, the peak due to glucuronate was detected at an elution volume of 9.0–9.6 mL, and increased with the treatment time, indicating the accumulation of glucuronate by the enzymatic degradation. The TLC and SEC results revealed that some

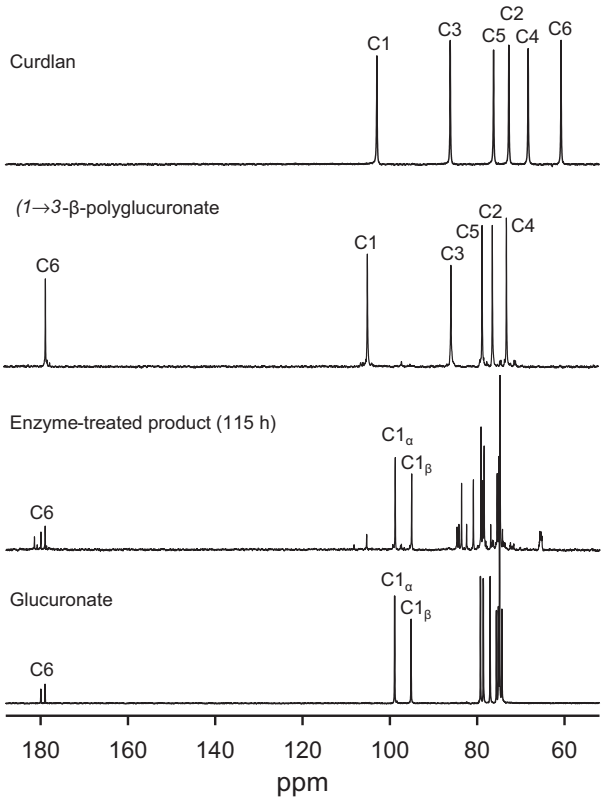


**Fig. 5.** $^{13}$C NMR spectra of the original curdlan dissolved in DMSO-$d_6$, and (1→3)-β-polyglucuronate, its degradation product by the crude enzyme treatment for 115 h, and glucuronate dissolved in $D_2O$.

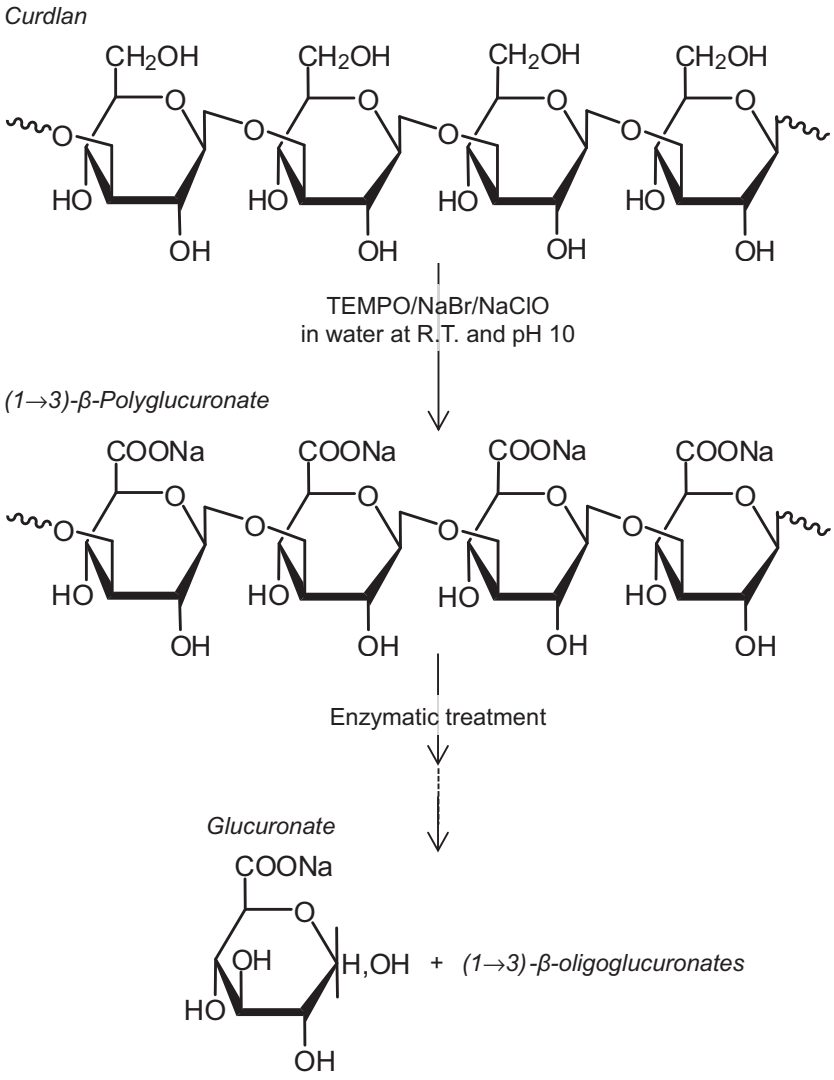


**Fig. 6.** Preparation of (1→3)-β-polyglucuronate from curdlan by TEMPO-mediated oxidation, and formation of glucuronate from (1→3)-β-polyglucuronate by crude enzyme treatment.

hydrolytic enzymes that depolymerize (1→3)-β-polyglucuronate endolytically (and probably exolytically as well) were present in the crude enzyme solution.

Fig. 5 shows $^{13}$C NMR spectra of the original curdlan, (1→3)-β-polyglucuronate prepared from curdlan by the TEMPO-mediated oxidation, its degradation product by the crude enzyme for 115 h, and glucuronate. The signal due to C6 primary hydroxyl groups of the original curdlan at ~60 ppm disappeared in the spectrum of the TEMPO-oxidized curdlan. Conversely, the signal due to the C6 carboxylate groups at 178 ppm appeared for the oxidized curdlan, indicating that almost all the C6 primary hydroxyl groups of curdlan were selectively oxidized to C6 carboxylate groups by the TEMPO-mediated oxidation to form sodium (1→3)-β-polyglucuronate. Signals due to the C1, C3, and C6 carbons of (1→3)-β-polyglucuronate at 105, 86, and 178 ppm, respectively, were obviously decreased by enzymatic treatment for 115 h. Correspondingly, peaks due to glucuronate were detected after the enzymatic treatment. Especially, two pairs of peaks at 95/99 and 178/179 ppm, assigned to anomeric C1 carbons and C6 carbons influenced by the anomeric C1 carbons, respectively (Guard, Coleman, & Ross, 1992), were clearly observed. Thus, the $^{13}$C NMR analysis revealed that the monomer formed and present in the enzymatic degradation products was glucuronate.

Based on the obtained results, it was concluded that *Paenibacillus* sp. EH621 produces a hydrolytic enzyme system that depolymerizes (1→3)-β-polyglucuronate in an endolytic manner. Glucuronate, a repeating unit of (1→3)-β-polyglucuronate, formed one of the primary degradation products in the enzymatic degradation products (Fig. 6). The results in Figs. 4 and 5 also suggest the presence of enzymes that exolytically hydrolyzed the endolytically degraded products to efficiently form glucuronate. Similar combinations of endo- and exo-type multiple enzymes in effective enzymatic degradation have been reported for some other polyuronates such as alginate (Suzuki, Suzuki, Inoue, & Ojima, 2006), pectin (Shevchik, Condemine, Robert-Baudouy, & Hugouvieux-Cotte-Pattat, 1999), and cellouronate (Konno et al., 2008).

## 4. Conclusions

A bacterial strain, EH621, with the ability to degrade (1→3)-β-polyglucuronate was isolated from the natural environment, and identified as *Paenibacillus* sp. When EH621 was cultured with (1→3)-β-polyglucuronate as a carbon source, approximately 60% of the original total carbon in the culture solution disappeared within 3 days, showing that (1→3)-β-polyglucuronate can be recognized

as a biodegradable material. The enzyme activity responsible for the degradation of (1→3)-β-polyglucuronate was detected in the cell-free extract of EH621, and the culture conditions for the enzyme production were optimized. Analyses of the enzymatic degradation products revealed that the enzyme system produced by EH621 depolymerizes (1→3)-β-polyglucuronate hydrolytically to form glucuronate, a monomer component, in an endolytic manner. The hydrolase activity of the crude enzyme was highly specific to (1→3)-β-polyglucuronate.

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