Carbohydrate Polymers 77 (2009) 59–64

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Enzymatic degradation of amylouronate (α-(1 → 4)-linked glucuronan) by α-glucuronidase from *Paenibacillus* sp. TH501b

Natsuko Iihashi [a], Jun Nagayama [a], Naoto Habu [a,*], Naotake Konno [b], Akira Isogai [b]

[a] *Department of Bioproductive Science, Utsunomiya University, Tochigi 321-8505, Japan*
[b] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8567, Japan*

ARTICLE INFO

Article history:
Received 8 July 2008
Accepted 2 December 2008
Available online 11 December 2008

Keywords:
TEMPO
Starch
α-Glucuronidase
Amylouronate
Biodegradation
Paenibacillus sp.

ABSTRACT

Enzymatic degradation of amylouronate (α-(1 → 4)-linked polyglucuronic acid sodium salt, α-(1 → 4)-linked glucuronan), which was prepared from water-soluble starch by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation, was investigated. A bacterial strain TH501b capable of degrading amylouronate was isolated from soil samples collected in the natural environment. Molecular analysis of the 16S rRNA gene showed that TH501b belongs to the genus *Paenibacillus*. A hydrolytic enzyme responsible for the degradation of amylouronate, amylouronate hydrolase-I (AUH-I), was detected in the cell-free extract of TH501b. AUH-I was purified by four steps of column chromatography and some properties were characterized. The molecular mass of the native AUH-I was estimated to be approximately 115 kDa by size exclusion chromatography (SEC), whereas sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed two major bands at 80 kDa and 46 kDa, respectively. The enzyme was most active at pH 6.0–7.0 and 30 °C. The SEC analysis of reaction products revealed that AUH-I liberated glucuronate as a sole product from amylouronate, indicating that AUH-I hydrolyzed amylouronate exolytically, and thus, was classified as α-glucuronidase.

1. Introduction

Amylouronate is a homopolymer consisted of sodium glucuronate linked by α-(1 → 4)-glycoside bonds, which can be prepared from starch by selective oxidation of primary hydroxyl groups at C6 position to carboxyl groups (Fig. 1). TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical), a water-soluble and stable nitroxyl radical, is a suitable catalyst for such selective oxidation of polysaccharides. Various kinds of polysaccharides, such as starch (Kato, Matsuo, & Isogai, 2003), cellulose (Isogai & Kato, 1998), chitin (Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999), chitosan (Kato et al., 2004; Yoo et al., 2005) and galactomannan (Sierakowski, Milas, Desbrières, & Rinaudo, 2000), have been applied to TEMPO-mediated oxidation to prepare the corresponding polyuronates. These polyuroantes, thus obtained, are expected to acquire novel functions and potential applications in various industrial fields.

We have focused on the biodegradability of these polyuronates, especially amylouronate and cellouronate, which is one of the important functions for newly prepared polyuronates. In our previous work, biodegradation of cellouronate was investigated. Cellouronate was found to be readily degraded and metabolized by microorganisms in natural environment (Kato et al., 2002). Several bacterial strains were isolated as cellouronate-degraders. The degradation of cellouronate by these strains proceeded in common by intracellular lyases. One of the isolated strains, *Brevundimonas* sp. SH203, produced two different types of lyases as the enzymes responsible for the degradation of cellouronate. The combination of the two cellouronate lyases, which were purified and characterized as endo- and exo-type lyases, respectively, degraded cellouronate efficiently (Konno, Habu, Iihashi, & Isogai, 2008; Konno, Habu, Maeda, Azuma, & Isogai, 2006).

In this study, biodegradation of amylouronate was studied. We started screening and isolation of strains capable of degrading amylouronate, and then an enzyme responsible for the degradation of amylouronate was purified and characterized. In addition, a potential application of the enzyme for production of glucuronate was discussed.

2. Materials and methods

2.1. Materials

Amylouronate was prepared from water-soluble potato starch (Kanto Chemicals Co., Japan) by TEMPO-mediated oxidation (Kato et al., 2003). The chemical structure of the obtained amylouronate was confirmed by ^{13}C NMR (nuclear magnetic resonance), which gave the identical spectrum to that shown by Kato et al. (2003)

* Corresponding author. Tel.: +81 28 649 5458; fax: +81 28 649 5401.
E-mail address: habu@cc.utsunomiya-u.ac.jp (N. Habu).

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.12.004

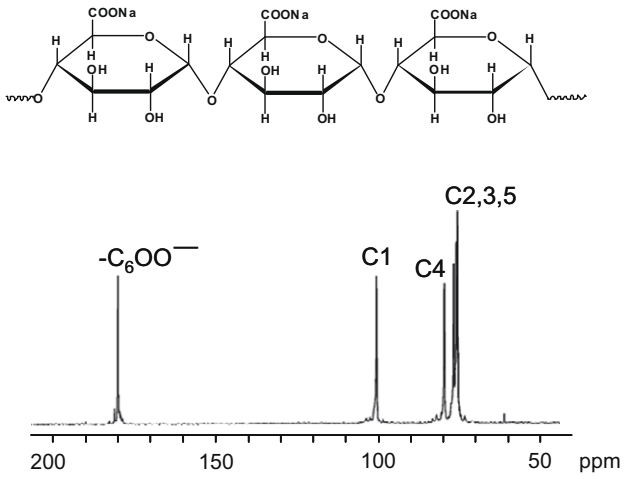

Fig. 1. Chemical structure and ^{13}C NMR spectrum of amylouronate, α-(1 → 4)-linked polyglucuronic acid sodium salt.

(Fig. 1). The weight average degree of polymerization of the obtained amylouronate was approximately 250, which was determined by using SEC-MALLS (size exclusion chromatography equipped with a multi-angle laser light scattering detector) as described by Shibata, Yanagisawa, Saito, and Isogai (2006). Cellouronate was also prepared by TEMPO-mediated oxidation (Isogai & Kato, 1998; Konno et al., 2006) from regenerated cellulose (Bemliese, Asahi Chemicals Co., Japan). Alginate, pectin and carboxymethyl cellulose with degree of substitution of 0.5 were laboratory grades of commercial products (Wako Pure Chemicals Co., Japan), and used without further purification. Yeast extract was purchased from Oxoid Ltd., England, and other chemicals and solvents, unless otherwise mentioned, were from Wako Pure Chemicals Co., Japan and Kanto Chemicals Co., Japan.

2.2. Culture conditions

Microorganisms were aerobically cultured at 30 °C on a rotary shaker at 120 rpm in an amylouronate 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 amylouronate (pH 7.0). For a solid medium, 20 g/L agar was added to the above medium.

2.3. Isolation and identification of the bacterial strain

Bacterial strains growing on amylouronate as a carbon source were isolated from soil collected from natural environment in Tochigi Prefecture, Japan, according to the similar procedure described by Konno et al. (2006). The strains selected from single colonies on agar plates of the amylouronate medium were inoculated in the amylouronate liquid medium and cultured. The total organic carbon (TOC) in the culture supernatant was monitored by a TOC analyzer (TOC-V, Shimadzu Co., Japan). A bacterial strain TH501b with an ability to degrade amylouronate was thus isolated and used in this study.

The strain TH501b was identified by comparing its sequence of the gene encoding the 16S rRNA in databases. Primers of 10F (5′-GTT TGA TCC TGG CTC A-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′) were used to amplify the 16S rRNA gene according to a standard protocol (Schabereiter-Gurtner, Lubitz, & Rölleke, 2003). Polymerase chain reaction (PCR) was performed with 1 cycle of denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C each, annealing for 1 min at 55 °C and extension for 1 min at 72 °C, and a final cycle of extension for 7 min at 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 terminators v1.1 cycle sequencing kit. The obtained DNA sequence was registered in the DDBJ/GeneBank/EMBL database (Accession No. AB427095). 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. Enzyme purification

The strain TH501b was pre-cultured in the amylouronate medium of 100 mL for 3 days, and aliquots (1 mL each) were inoculated to 40 flasks containing 100 mL of the same medium, respectively. After cultivation for 2 days, cells collected by centrifugation at 10,000*g* and 4 °C for 10 min were washed three times with 50 mM sodium phosphate buffer, pH 7.0, and suspended in 100 mL of the same buffer. The suspension was divided into four portions (25 mL each), and ultrasonically disrupted (Ultrasonic Disrupter UD201, Tomy Seiko Co., Japan) on an ice-water bath at 20 kHz for 40 min. The obtained mixtures were combined and centrifuged at 11,000*g* and 4 °C for 30 min, and the resultant supernatant, i.e. cell-free extract, was used as the crude enzyme solution. The crude enzyme solution was concentrated and equilibrated with 25 mM sodium phosphate buffer, pH 7.5, using ultrafiltration membranes with 5000 molecular mass cut-off (Amicon model 8200 with a membrane PBCC, Millipore Co., USA). The crude enzyme solution thus obtained was fractionated by the following four steps of column chromatography. All procedures except for the following Step 4 were performed at 4 °C using an ÄKTAprime Plus (GE Healthcare UK Ltd., England).

2.4.1. Step 1

The crude enzyme solution was applied to a Toyopearl SuperQ-650M column (2.2 × 9.5 cm, Tosoh Co., Japan) previously equilibrated with 25 mM sodium phosphate buffer, pH 7.5. After elution with the same buffer (150 mL), proteins adsorbed to the resin were eluted with linear gradient of 0–0.66 M NaCl in the same buffer (660 mL) at a flow rate of 1.5 mL/min. Each fraction (9 mL) was collected and assayed for enzyme activity. As shown in Fig. 2, two different active peaks appeared separately, which were designated as amylouronate hydrolase-I (AUH-I) and AUH-II, respectively. The fractions corresponding to AUH-I were combined and concentrated in 50 mM sodium phosphate buffer, pH 7.0, containing 0.7 M ammonium sulfate by ultrafiltration.

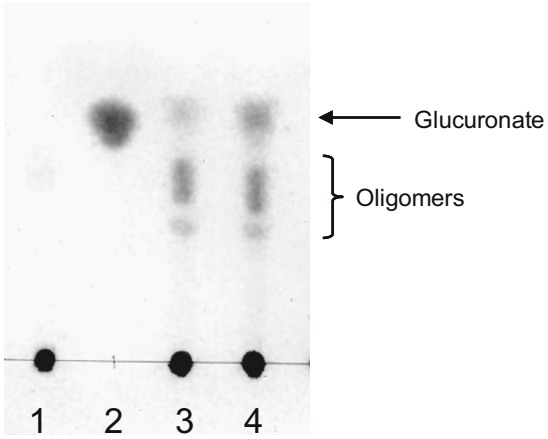

Fig. 2. TLC patterns of the degradation products by the crude enzyme. Amylouronate (10 mg) was incubated with approximately 0.04 U of crude enzyme in 1 mL of 25 mM sodium phosphate buffer at pH 7.5 and 25 °C. Lane 1: amylouronate, lane 2: authentic glucuronate, lanes 3 and 4: amylouronate incubated for 17 h and 24 h, respectively, by the crude enzyme.

2.4.2. Step 2

The AUH-I solution obtained by Step 1 was applied to a Toyopearl Phenyl-650M column (2.2 × 9.5 cm, Tosoh Co., Japan) previously equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.7 M ammonium sulfate. After elution with the same buffer (150 mL), proteins were eluted with linear gradient of 0.7–0.2 M ammonium sulfate in 225 mL of the same buffer at a flow rate of 1.5 mL/min, and each fraction (9 mL) was collected. The active fractions eluted with approximately 0.5 M ammonium sulfate were combined and concentrated in 25 mM sodium phosphate buffer, pH 7.5, by ultrafiltration.

2.4.3. Step 3

The enzyme solution obtained by Step 2 was applied to a packed column of Resource Q (0.64 × 3 cm, GE Healthcare UK Ltd., England) previously equilibrated with 25 mM sodium phosphate buffer, pH 7.5. After elution with the same buffer (150 mL), proteins were eluted with linear gradient of 0–0.5 M NaCl in the same buffer (300 mL) at a flow rate of 2.0 mL/min. Eluted fractions (5 mL each) were collected, and the active fractions were concentrated to 200 μL by ultrafiltration using Vivaspin 6 with 5000 molecular mass cut-off (Vivascience AG, Germany).

2.4.4. Step 4

The following procedure was carried out at room temperature. The enzyme solution obtained by Step 3 was subjected to size exclusion chromatography (SEC) by an HPLC system (LaChrom Elite, Hitachi High-Technologies Co., Japan) equipped with a packed column of TSKgel G3000SW$_{XL}$ (0.78 × 30 cm, Tosoh Co., Japan). After equilibrated with 25 mM sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl, proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. The fraction having enzyme activity, determined by the method described in the following section, was desalted and concentrated by ultrafiltration using Ultrafree-0.5 with 5000 molecular mass cut-off (Millipore Co., USA). The molecular mass of native AUH-I was estimated using a standard protein kit (MW-GF-200, Sigma, USA).

2.5. Standard assay of enzyme activity

The enzyme activity was assayed by determining the increased amount of reducing ends formed by the cleavage of glycoside bonds of amylouronate. A reaction mixture containing 0.3% (w/v) amylouronate and the enzyme 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 by Somogyi (1952) using a microplate reader (MTP-300, Corona Electric Co., Japan) with sodium glucuronate as a standard. One unit (U) of the activity was defined as the amount of enzyme necessary to liberate reducing ends equivalent to 1 μmol of sodium glucuronate per min.

2.6. Protein analyses

Protein concentration was determined according to the method by Bradford (1976) using bovine serum albumin as a standard or by measuring absorbance at 280 nm in a spectrophotometer cuvette of 1 cm light path, assuming that A_{280} = 1.0 mg/mL.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a slab gel (4% acrylamide for stacking gel and 8 % 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).

2.7. Characterization of enzymes

The effect of pH on the enzyme activity was examined using following buffers (50 mM); GTA (3,3-dimethylglutaric acid, tris(hydroxyethyl)aminomethane and 2-amino-2-methyl-1,3-propanediol)–HCl/NaOH buffer (pH 4.0–10.0), sodium phosphate buffer (pH 6.0–8.0) and Tris (tris(hydroxyethyl)aminomethane)–HCl buffer (pH 7.5–9.0). The pH stability was estimated by measuring the residual activity after incubation of the enzyme in above buffers at 4 °C for 24 h.

The effect of temperature on the enzyme activity was examined in 50 mM sodium phosphate buffer (pH 7.0) at various temperatures (10–50 °C). The thermal stability was estimated by measuring the residual activity after incubation of the enzyme in the same buffer at various temperatures (10–70 °C) for 10 min.

The substrate specificity of the enzyme was evaluated by using the following substrates in place of amylouronate; cellouronate, alginate, pectin, starch and carboxymethyl cellulose.

2.8. Analyses of the enzymatic reaction products

Amylouronate (40 mg) was incubated with 0.14 U of AUH-I in 2 mL of 20 mM sodium phosphate buffer at pH 7.5 and 25 °C with stirring. After desired time of incubation, aliquots of the mixture were withdrawn and immediately frozen until being subjected to analyses.

Thin-layer chromatography (TLC) was performed on TLC plate Silica 60 F_{254} (Merck Co., Germany) with a developing solvent of 3:2:2 1-butanol/acetic acid/water (v/v/v) using glucuronate as a standard. Organic compounds on TLC plate were visualized by heating after spraying 10% (v/v) sulfuric acid in ethanol (Konno et al., 2008).

The molecular mass distribution of the reaction products was evaluated by SEC using an HPLC (LaChrom Elite, Hitachi High-Technologies Co., Japan) system with a Shodex OHpak SB-802.5 HQ column (0.8 × 30 cm, Showa Denko K.K., Japan). Elution was done with 0.1 M NaCl at 40 °C with a flow rate of 0.5 mL/min, and elution patterns were detected by refractive index (RI).

3. Results and discussion

3.1. Isolation and identification of bacterial strains

Amylouronate, even though it is artificially prepared from water-soluble starch by TEMPO-mediated oxidation, was found to be biodegradable. However, the degradation rate was lower than that of cellouronate, when compared in a soil chamber containing microorganisms (Kato, Kaminaga, Matsuo, & Isogai, 2005). Thus, although both amylouronate and cellouronate are biodegradable, the former α-(1 → 4)-linked polyglucuronate is more resistant in biodegradation than the latter β-(1 → 4)-linked polyglucuronate. In our previous work, several bacterial strains degrading cellouronate were isolated and characterized (Konno et al., 2006, 2008). Because the cellouronate-degrading bacteria were unable to grow on amylouronate, new strains for amylouronate degradation were screened and isolated from the soil samples collected in the natural environment. During the screening process, the frequency of the colony appearance on agar plates containing amylouronate as a carbon source was much lower than that in the case of screening cellouronate-degraders, which also indicated higher resistance of amylouronate to biodegradation.

A bacterial strain TH501b degrading amylouronate was obtained as the most active isolate. TH501b was able to grow on not only amylouronate but also other polyuronates such as cellouronate and alginate. TH501b was identified by comparing its gene

sequence encoding the 16S rRNA (Accession No. AB427095) with the registered sequences in the data bases using BLAST search. The highest score, identity of 99% in the 713 bp fragments, was obtained against the sequence of *Paenibacillus* sp. (Accession No. EU442242). Thus, TH501b was recognized as *Paenibacillus* sp.

3.2. Degradation of amylouronate by TH501b

When *Paenibacillus* sp. TH501b was cultured in the amylouronate medium for 3 days, more than 55% TOC in the culture supernatant disappeared, and significant enzyme activity to produce reducing ends from amylouronate was detected in the crude enzyme solution. But no lyase activity to amylouroante, which was measured by monitoring changes in absorbance at 235 nm (Konno et al., 2006), was detected, indicating that the above enzyme activity resulted from the hydrolytic cleavage of glycoside bonds of amylouronate. On the other hand, the culture supernatant had no detectable activity of amylouronate degradation, although *Paenibacillus* sp. TH501b is a Gram-positive bacterium. These results show that amylouronate is degraded by some intracellular hydrolytic enzymes of *Paenibacillus* sp. TH501b. When amylouronate was incubated with the crude enzyme and the reaction products were analyzed by TLC, glucuronate, a monomer component of amylouronate, as well as some oligomeric products, was formed and accumulated with increasing incubation time as shown in Fig. 2.

3.3. Purification and characterization of AUH-I

The crude enzyme was fractionated by column chromatography to purify the enzyme responsible for the hydrolytic cleavage of amylouronate. As presented in Fig. 3, two different active peaks were observed by Step 1 using the anion exchange column (Toyopearl SuperQ). They were designated as amylouronate hydrolase-I (AUH-I) and AUH-II, respectively. AUH-I was eluted with approximately 0.2 M NaCl, while AUH-II was eluted without adsorbing to the SuperQ column. AUH-I was further purified by the successive column chromatographic series using the hydrophobic, anion exchange and size exclusion columns (Toyopearl Phenyl-650M, Resource Q and TSKgel G3000SW$_{XL}$, respectively). The results of purification process of AUH-I are summarized in Table 1. The specific activity of AUH-I was 2.8 U/mg with the purification fold and recovery of 41.5% and 2.1%, respectively.

The molecular mass of native AUH-I was estimated to be approximately 115 kDa by SEC, whereas SDS–PAGE showed two major bands at approximately 80 kDa and 46 kDa, respectively, with some weak bands of contaminants probably due to incomplete purification (Fig. 4). Although further purification of AUH-I was attempted by using an anion exchange column (TSKgel BioAssist Q (0.46 × 5 cm), Tosoh Co., Japan) and a hydroxyl apatite column (Seikagaku Co., Japan), complete homogeneity of AUH-I was not achieved. Thus, AUH-I obtained after the SEC (Step 4) was used in this study. The discrepancy between the molecular mass values obtained by SEC and SDS–PAGE can be explained by assuming that AUH-I consists of heterogeneous subunits with 80 kDa and 46 kDa.

The effect of pH on the enzyme activity was examined using some buffers with various pHs. The optimum pH of AUH-I to amylouronate degradation was 6.0–7.0 (Fig. 5A). The pH stability of the enzyme estimated after incubation in buffers at various pHs from 4.0 to 10.0 and 4 °C for 24 h showed that AUH-I was the most stable at pH 7.0, and almost no enzyme activity appeared at pHs lower than 4.0 and higher than 9.0 (Fig. 5B).

The optimum temperature of AUH-I was 30 °C (Fig. 6A). The thermal stability of AUH-I was estimated by measuring the residual activity after incubation at various temperatures of 10–70 °C for 10 min. AUH-I was stable at 10–40 °C, but started to lose the activity above 40 °C (Fig. 6B).

The substrate specificity of AUH-I was estimated using starch, carboxymethyl cellulose and some polyuronates, such as cellouronate, alginate and pectin. AUH-I was highly specific to amylouro-

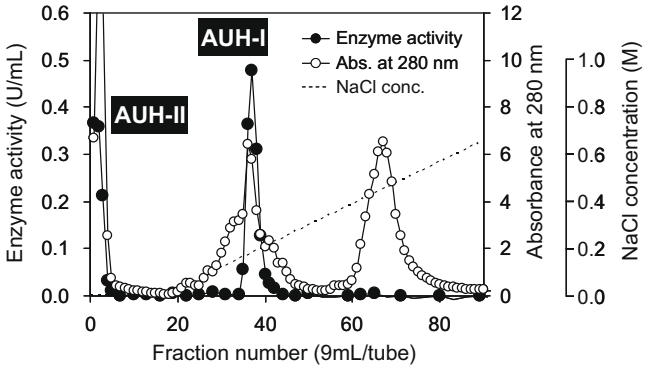

Fig. 3. Anion exchange chromatogram of the crude enzyme on a Toyopearl SuperQ column.

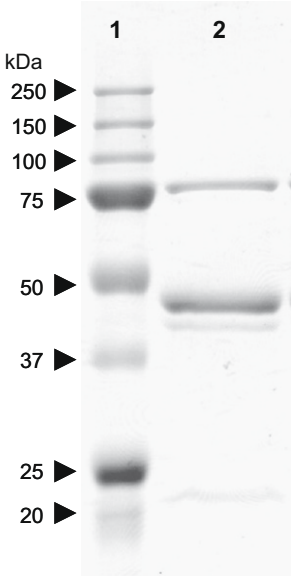

Fig. 4. SDS–PAGE of purified AUH-I. Lane 1: molecular mass standards, and lane 2: purified AUH-I.

Table 1
Purification of amylouronate hydrolase-I.

Purification step and the column used	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	1398	94.1	0.07	1.0	100
Step 1: Toyopeal SuperQ-650M	225	40.1	0.18	2.6	42.6
Step 2: Toyopeal Phenyl-650M	30.6	17.6	0.58	8.6	18.7
Step 3: Resource Q	2.60	8.6	3.31	49.1	9.1
Step 4: TSK G3000SW$_{XL}$	0.70	2.0	2.80	41.5	2.1

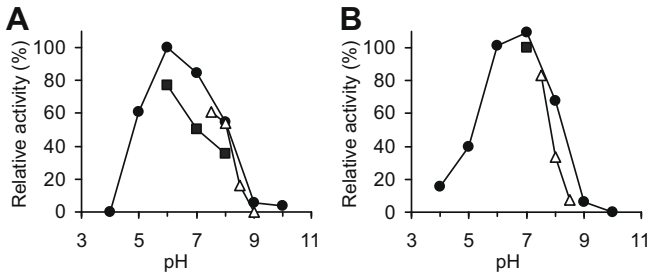

Fig. 5. Effects of pH on the activity (A) and stability (B) of AUH-I. Buffers used were GTA–HCl/NaOH (pH 4.0–10.0, ●), Na-phosphate (pH 6.0–8.0, ■) and Tris–HCl (pH 7.5–9.0, Δ).

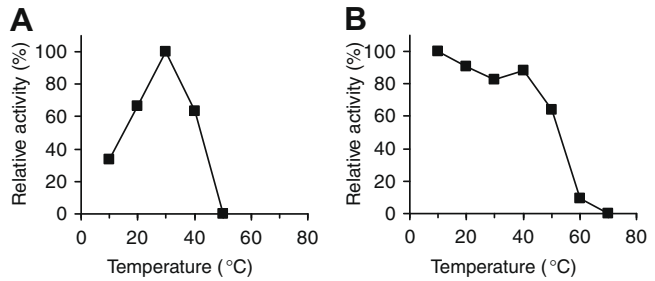

Fig. 6. Effects of temperature on the activity (A) and stability (B) of AUH-I.

nate, and inert to starch and carboxymethyl cellulose. Although AUH-I was slightly active to the polyuronates tested, the activities were less than 5% to that of amylouronate.

3.4. Analyses of the reaction products by AUH-I

Amylouronate was incubated with AUH-I, and the reaction products were analyzed by TLC. Unlike the reaction products formed by the crude enzyme (Fig. 2), AUH-I gave glucuronate alone as the reaction product, and no other oligomeric intermediates were detected in the TLC plate (Fig. 7). The reaction products by AUH-I were further analyzed by SEC, and the profiles are shown in Fig. 8. In the SEC elution patterns, glucuronate and phosphate salts added as the buffer components were eluted together around 9.3 mL elution volume as an overlapping peak. However, it was clearly seen that the peak at 9.3 mL was increased with the incubation time, indicating the accumulation of glucuronate formed by the enzymatic reaction. On the other hand, the peak due to the original amylouronate around 6.4 mL elution volume was decreased with the incubation time as increased formation of the compound at 9.3 mL elution volume. Nearly no shifting to the lower molecular mass regions was observed even after the incubation for 24 h. These results show that AUH-I hydrolyzes amylouronate exolytically, and thus AUH-I is classified as α-glucuronidase (α-glucosiduronate glucuronohydrolase, EC 3.2.1.139). Various α-glucuronidases have been isolated from microorganisms and characterized (Heneghan, McLoughlin, & Tuohy, 2007; Kawabata, Nanri, Uchida, Kobayashi, & Kusakabe, 1994; Siika-aho, Tenkanen, Buchert, & Viikari, 1994; Uchida, Nanri, Kawabata, Kusakabe, & Murakami, 1992; Wood & Wilson, 1995). Considerable differences in the substrate specificities of the α-glucuronidases, depending on the source of the enzymes, have been reported. No enzymes were, however, reported so far to be active to the substrates having the same structure as amylouronate. In the SEC elution profiles for the degradation products by AUH-I, a small peak other than glucuronate was detected at 8.3 mL elution volume. The structure of this product is, however, unknown at present.

Paenibacillus sp. TH501b produced, in addition to AUH-I, other hydrolytic enzyme(s) for the degradation of amylouronate (AUH-II). As shown in Fig. 3, AUH-II was eluted without adsorbing to the anion exchange column, and was obtained as a somewhat turbid solution. AUH-II was then applied to a cation exchange (Toyopearl CM-650M) column, however, it was not adsorbed to the column either, indicating that AUH-II was not completely solubilized even by sonication treatment of the cells. The solubilization, purification and characterization of AUH-II are now under way. The preliminary result demonstrated that some oligomeric products including dimers and trimers were formed from amylouronate by AUH-II. Thus, *Paenibacillus* sp. KH501b is likely to degrade amylouronate efficiently by combination of multiple enzymes similarly

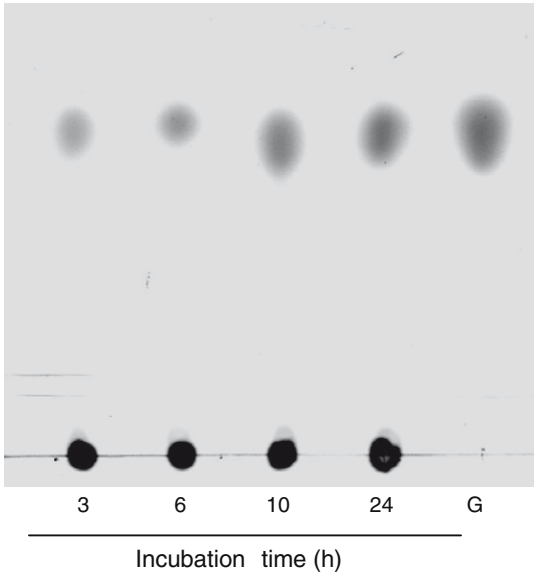

Fig. 7. TLC patterns of the degradation products by AUH-I. Amylouronate (40 mg) was incubated with 0.14 U of AUH-I in 2 mL of 20 mM sodium phosphate buffer at pH 7.5 and 25 °C for 3, 6, 10 and 24 h. G: Authentic glucuronate.

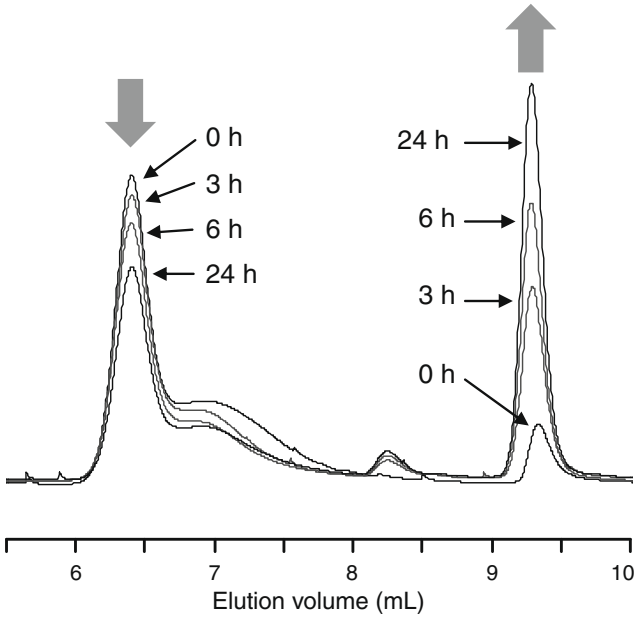

Fig. 8. SEC elution profiles of the degradation products of amylouronate by AUH-I, detected by refractive index. The reaction conditions were the same as those in Fig. 7.

for the degradation of other polyuronates such as alginate (Hashimoto, Miyake, Momma, Kawai, & Murata, 2000; Suzuki, Suzuki, Inoue, & Ojima, 2006), pectin (Shevchik, Condemine, Robert-Baudouy, & Hugouvieux-Cotte-Pattat, 1999; Shevchik et al., 1999), protuberic acid (Tsuchihashi, Yadomae, & Miyazaki, 1983, 1984a, 1984b) and cellouronate (Konno et al., 2008).

3.5. Potential application of AUH-I

Glucuronate and its intramolecular ester, glucuronolactone, are commercially manufactured and used as medicines and raw materials of medicines in the pharmaceutical and medical fields. The role of glucuronate in living bodies is involved in the catabolism and detoxification of hydrophobic substances such as endogenous hormones and xenobiotic toxins in the liver. The hydrophobic substances, generally after oxidized by cytochromes P-450, undergo conjugation reactions with glucuronide-derivatives, in a way that allows the enhanced hydrophilicity to be expelled through active transport mechanisms (Delaforge, Pruvost, Perrin, & Andre, 2005; Raftogianis, Creveling, Weinshilboum, & Weisz, 2000).

The manufacturing process for the production of glucuronate at industrial level generally consists of the following two stages: oxidation of natural glucans such as starch with, for example, dinitrogen tetraoxide, and hydrolysis of the glycoside bonds with strong acid such as sulfuric acid. However, because considerable amounts of by-products other than glucuronate are necessarily formed by rather low selectivity of these reactions, multiple procedures for isolation and purification of glucuronate from the reaction mixtures are inevitable, thus resulting in low production yields of glucuronate.

AUH-I has, therefore, the potential to be applied to a new process for the efficient production of glucuronate; the quantitative conversion of starch to amylouronate by TEMPO-mediated oxidation and the successive hydrolysis of amylouronate to glucuronate by AUH-I. The new process will provide an environment-friendly production of glucuronate as all processes can be achieved under mild conditions at ambient temperature and atmospheric pressure without using large amounts of strong or toxic agents. Furthermore, the reaction mixture after hydrolysis by AUH-I contains only glucuronate and residual amylouronate as major constituents, which will facilitate the isolation of glucuronate. To increase the process productivity in the next stage, the optimization of AUH-I production for scale-up and the detailed kinetic study on the hydrolysis of amylouronate by AUH-I are now under investigation.

4. Conclusions

A bacterial strain TH501b degrading amylouronate was isolated from the soil, and identified as *Panebacillus* sp. based on the analysis of its 16S rRNA gene. Amylouronate hydrolase-I (AUH-I) was purified from the cell-free extract as one of the enzymes responsible for the degradation of amylouronate, and some properties were studied. AUH-I was highly specific to amylouronate, and had the highest activity at pH 6.0–7.0 and 30 °C. AUH-I hydrolyzes the glycoside bonds of amylouronate exolytically and liberated glucuronate as a main product, thus was classified as α-glucuronidase. AUH-I has the potential to be applied to a new process for the efficient and environment-friendly production of glucuronate, which is significant in pharmaceutical fields.

Acknowledgement

This research has been partly supported by Grant-in-Aids for Scientific Research (Grant No. 19658067) from the Japan Society for the Promotion of Science (JSPS).

References

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Delaforge, M., Pruvost, A., Perrin, L., & Andre, F. (2005). Cytochrome P450-mediated oxidation of glucuronide derivatives: Example of estradiol-17β-glucuronide oxidation to 2-hydroxyestradiol-17b-glucuronide by CYP2C8. *Drug Metabolism and Disposition, 33*, 466–473.

Hashimoto, W., Miyake, O., Momma, K., Kawai, S., & Murata, K. (2000). Molecular identification of oligoalginate lyase of *Sphingomonas* sp. strain A1 as one of the enzymes required for complete depolymerization of alginate. *Journal of Bacteriology, 182*, 4572–4577.

Heneghan, M. N., McLoughlin, L., & Tuohy, M. G. (2007). Cloning, characterisation and expression analysis of α-glucuronidase from the thermophilic fungus *Talaromyces emersonii*. *Enzyme and Microbial Technology, 41*, 677–682.

Isogai, A., & Kato, Y. (1998). Preparation of polyuronic acid from cellulose by TEMPO-mediated oxidation. *Cellulose, 5*, 153–164.

Kato, Y., Habu, N., Yamaguchi, J., Kobayashi, Y., Shibata, I., Isogai, A., et al. (2002). Biodegradation of β-1,4-linked polyglucuronic acid (cellouronic acid). *Cellulose, 9*, 75–81.

Kato, Y., Kaminaga, J., Matsuo, R., & Isogai, A. (2004). TEMPO-mediated oxidation of chitin, regenerated chitin and N-acetylated chitosan. *Carbohydrate Polymers, 58*, 421–426.

Kato, Y., Kaminaga, J., Matsuo, R., & Isogai, A. (2005). Oxygen permeability and biodegradability of polyuronic acids prepared from polysaccharides by TEMPO-mediated oxidation. *Journal of Polymers and the Environment, 13*, 261–266.

Kato, Y., Matsuo, R., & Isogai, A. (2003). Oxidation process of water-soluble starch in TEMPO-mediated system. *Carbohydrate Polymers, 51*, 69–75.

Kawabata, Y., Nanri, T., Uchida, H., Kobayashi, H., & Kusakabe, I. (1994). α-Glucuronidase-producing ability of Basidiomyces using 2-*O*-(α-D-glucopyranosyluronic acid)-D-xylose and *p*-nitrophenyl α-D-glucopyranosyluronic acid as substrates. *Mokuzai Gakkaishi, 40*, 336–339.

Konno, N., Habu, N., Iihashi., N., & Isogai, A. (2008). Purification and characterization of exo-type cellouronate lyase. *Cellulose, 15*, 453–463.

Konno, N., Habu, N., Maeda, I., Azuma, N., & Isogai, A. (2006). Cellouronate (β-1,4-linked polyglucuronate) lyase from *Brevundimonas* sp. SH203: Purification and characterization. *Carbohydrate Polymers, 64*, 589–596.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Muzzarelli, R. A. A., Muzzarelli, C., Cosani, A., & Terbojevich, M. (1999). 6-Oxychitins, novel hyaluronan-like regiospecifically carboxylated chitins. *Carbohydrate Polymers, 39*, 361–367.

Raftogianis, R., Creveling, C., Weinshilboum, R., & Weisz, J. (2000). Estrogen metabolism by conjugation. *Journal of the National Cancer Institute, 27*, 113–124.

Schabereiter-Gurtner, C., Lubitz, W., & Rölleke, S. (2003). Application of broad-range 16S rRNA PCR amplification and DGGE fingerprinting for detection of tick-infecting bacteria. *Journal of Microbiological Methods, 52*, 251–260.

Shevchik, V. E., Condemine, G., Robert-Baudouy, J., & Hugouvieux-Cotte-Pattat, N. (1999). The exopolygalacturonate lyase PelW and the oligogalacturonate lyase Ogl, two cytoplasmic enzymes of pectin catabolism in *Erwinia chrysanthemi* 3937. *Journal of Bacteriology, 181*, 3912–3919.

Shevchik, V. E., Kester, H. C. M., Benen, J. A. E., Visser, J., Robert-Baudouy, J., & Hugouvieux-Cotte-Pattat, N. (1999). Characterization of the exopolygalacturonate lyase PelX of *Erwinia chrysanthemi* 3937. *Journal of Bacteriology, 181*, 1652–1663.

Shibata, I., Yanagisawa, M., Saito, T., & Isogai, A. (2006). SEC-MALS analysis of cellouronic acid prepared from regenerated cellulose by TEMPO-mediated oxidation. *Cellulose, 13*, 73–80.

Sierakowski, M. R., Milas, M., Desbrières, J., & Rinaudo, M. (2000). Specific modifications of galactomannans. *Carbohydrate Polymers, 42*, 51–57.

Siika-aho, M., Tenkanen, M., Buchert, J., & Viikari, L. (1994). An α-glucuronidase from *Trichoderma reesei* RUT C-30. *Enzyme and Microbial Technology, 16*, 813–819.

Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry, 195*, 19–23.

Suzuki, H., Suzuki, K., Inoue, A., & Ojima, T. (2006). A novel oligoalginate lyase from abalone, *Haliotis discus hannai*, that releases disaccharide from alginate polymer in an exolytic manner. *Carbohydrate Research, 341*, 1809–1819.

Tsuchihashi, H., Yadomae, T., & Miyazaki, T. (1983). Protuberic acid-hydrolytic enzyme in *Kobayasia nipponica* and characterization of the hydrolytic products. *Journal of Biochemistry, 94*, 1037–1044.

Tsuchihashi, H., Yadomae, T., & Miyazaki, T. (1984a). Exo-β-D-glucuronidase from the fungus *Kobayasia nipponica*. *Journal of Biochemistry, 96*, 1789–1797.

Tsuchihashi, H., Yadomae, T., & Miyazaki, T. (1984b). Isolation and characterization of an endo-β-D-glucuronidase from the fungus *Kobayasia nipponica*. *Journal of Biochemistry, 96*, 1799–1805.

Uchida, H., Nanri, T., Kawabata, Y., Kusakabe, I., & Murakami, K. (1992). Purification and characterization of intracellular α-glucuronidase from *Asperigillus niger* 5–16. *Bioscience, Biotechnology, and Biochemistry, 56*, 1608–1615.

Wood, T. M., & Wilson, T. A. (1995). α-(4-O-Methyl)-D-glucuronidase activity produced by the rumen anaerobic fungus *Piromonas communis*: A study of selected properties. *Applied Microbiology and Biotechnology, 43*, 893–900.

Yoo, H.-Y., Lee, J.-S., Park, S. Y., Kim, Y.-S., Chang, P.-S., & Lee, H. G. (2005). Effects of selective oxidation of chitosan on physical and biological properties. *International Journal of Biological Macromolecules, 35*, 27–31.