Purification and Characterization of a Novel α-Glucuronidase from Aspergillus niger Specific for O-α-d-Glucosyluronic Acid α-d-Glucosiduronic Acid

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A new α-glucuronidase that specifically hydrolyzed O-α-d-glucosyluronic acid α-d-glucosiduronic acid (trehalose dicarboxylate, TreDC) was purified from a commercial enzyme preparation from Aspergillus niger, and its properties were examined. The enzyme did not degrade O-α-d-glucosyluronic acid α-d-glucoside, O-α-d-glucosyluronic acid β-d-glucosiduronic acid, O-α-d-glucosyluronic acid-(1 → 2)-β-d-fructosiduronic acid, p-nitrophenyl-O-α-d-glucosiduronic acid, methyl-O-α-d-glucosiduronic acid, or O-α-(4-O-α-d-glucosyluronic acid)-d-glucosyl-β-cyclodextrine. Furthermore, it showed no activity on α-glucuronol linkage of 4-O-methyl-d-glucosyluronic acid-α-(1 → 2)-xylooligosaccharides, derived from xylan, a supposed substrate of α-glucuronidases.

The molecular mass of the enzyme was estimated to be 120 kDa by gel filtration and 58 kDa by SDS-PAGE suggesting, the enzyme is composed of two identical subunits. It was most active at pH 3.0–3.5 and at 40°C. It was stable in pH 2.0–4.5 and below 30°C. It hydrolyzed O-α-d-glucosyluronic acid α-d-glucosiduronic acid to produce α- and β-anomers of d-glucuronic acid in an equimolar ratio. This result suggests that inversion of the anomeric configuration of the substrate is involved in the hydrolysis mechanism.

Key words: α-glucuronidase; O-α-d-glucosyluronic acid α-d-glucosiduronic acid; Aspergillus niger; purification; hydrolysis

α-Glucuronidases (EC 3.2.1.139) that catalyze the hydrolysis of α-glucuronic linkages are distributed widely in fungi and bacteria.1–9) Most microbial α-glucuronidases degrade α-1,2 glucuronyl linkages to liberate 4-O-methyl D-glucuronic acids, which exist as side chains of xylan, although the enzymes do not hydrolyze p-nitrophenyl-O-α-d-glucosiduronic acid (PNP α-glucuronide).1–8) Among microbial α-glucuronidases, α-glucuronidase from Thermotoga maritima is the only enzyme that was known to hydrolyze PNP α-glucuronide.9) Substrate specificities of the α-glucuronidases, however, have not been examined in detail, due to the unavailability of various α-glucuronol oligosaccharides.

A new method using Pseudogluconobactor saccharoketogenes has been developed to oxidize sugars.10) Since the bacterial cells are able to oxidize not only the C-1 hydroxy group of glucopyranose residue but also the C-6 hydroxymethyl group, the method makes it possible to prepare various sugars containing glucuronol residues. In our present study, O-α-d-glucosyluronic acid α-d-glucosiduronic acid (trehalose dicarboxylate, TreDC) was converted from trehalose by this microbial oxidation method and used for the screening of α-glucuronidases. In consequence, an enzyme obtained from Aspergillus niger was found to hydrolyze TreDC.

It has been reported that α-glucuronidase from snail acetone powder hydrolyzed TreDC.11) The snail enzyme, however, hydrolyzed other glucuronides such as PNP α-glucuronide and 4-O-methyl-d-glucosyluronic acid-α-(1 → 2)-xylooligosaccharide (Me-GA-Xn) in addition. To our knowledge, there are no reports on α-glucuronidase extremely specific for TreDC. In this report, therefore, we tentatively call the TreDC specific enzyme trehalose dicarboxylate hydrolase (TreDCase) to distinguish it from other α-glucuronidases.
Materials and Methods

Materials. Oxidized saccharides, such as TreDC, O-α-D-glucosyluronic acid-(1→2)-β-D-fructosiduronic acid (SucDC), O-α-D-glucosyluronic acid β-D-glucosiduronic acid (α,β-TreDC), PNP α-glucuronide, methyl-O-α-D-glucosiduronic acid, and O-α-D-glucuronic acid α-D-glucoside (TreMC), were prepared from trehalose, sucrose, α,β-trehalose (Sigma Chemicals, St. Louis, MO), p-nitrophenyl-O-α-D-glucoside, and methyl-O-α-D-glucoside respectively by microbial oxidation as follows: Reaction mixtures consisting of 5 ml of 1% trehalose, sucrose, α,β-trehalose, p-nitrophenyl-O-α-D-glucoside, or methyl-O-α-D-glucoside were incubated with 0.29 g (wet weight) of P. saccharotonogenesIFO14483 cells prepared according to the method of Ishiguro et al.\(^\text{10}\). The reactions were carried out in the presence of 0.3% CaCO\(_3\) at 30 °C for 24 h (TreDC, SucDC, α,β-TreDC, PNP α-glucuronide, methyl-O-α-D-glucosiduronic acid), or 6.5 h (TreMC). After oxidation, the reaction mixtures were centrifuged at 5,000 g for 30 min to remove the cells, and put on an activated carbon column (3.0 × 20 cm). The passed solutions were evaporated, and applied onto a column (2.5 × 90 cm) of Bio-Gel P2 (Bio-Rad Laboratories, Hercules, CA) equilibrated with 10% ethanol. The eluates were collected, and the fractions corresponding to the oxidized products were combined, evaporated, and lyophilized. Their structures were confirmed by \(^1\)C-NMR: (1) TreDC NMR δ (D\(_2\)O): 73.2 (C-1), 73.9 (C-2), 74.2 (C-3), 74.8 (C-4), 74.9 (C-5), 74.7 (C-6), 97.1 (C-1′), 175.9 (C-2′). (2) SucDC NMR δ (D\(_2\)O): 64.2 (C-6), 73.8 (C-5), 74.8 (C-2), 75.3 (C-3), 75.5 (C-4), 78.5 (C-5′), 78.8 (C-3′), 82.4 (C-′), 94.5 (C-’), 107.2 (C-1), 179.4 (C-1′), 179.5 (C-6), glucuronyl residue (C-1 to C-6), furanuronyl residue (C-1′ to C-′), α,β-TreDC NMR δ (D\(_2\)O): 73.7 (C-4′), 73.8 (C-4′), 73.9 (C-2′), 74.0 (C-5′), 75.3 (C-3′), 75.3 (C-2′), 77.5 (C-5′), 77.6 (C-3′), 103.2 (C-1′), 105.9 (C-1), 174.8 (C-6′), 175.6 (C-6′). (3) PNP α-glucuronide NMR δ (D\(_2\)O): 73.5 (C-5), 74.5 (C-2), 75.4 (C-3), 75.7 (C-4), 99.4 (C-1′), 119.5 (C-2′,6′), 128.8 (C-3′), 145.1 (C-′), 164.1 (C-′), 175.2 (C-6), glucuronyl residue (C-1 to C-6), p-nitrophenyl residue (C-1′ to C-′), 6-Methyl-O-α-D-glucosiduronic acid NMR δ (D\(_2\)O): 57.9 (CH\(_3\)-O–), 73.8 (C-4), 74.6 (C-2), 74.7 (C-5), 75.6 (C-3), 102.1 (C-1′), 179.3 (C-2′,6′). (6) TreMC NMR δ (D\(_2\)O): 63.2 (C-6′), 72.4 (C-4′), 73.2 (C-3′), 73.6 (C-3), 73.8 (C-4), 74.9 (C-2′), 75.0 (C-5′), 75.2 (C-3′), 75.7 (C-3), 96.0 (C-1′), 96.0 (C-1), 175.6 (C-6), glucuronyl residue (C-1 to C-6), glucosyl residue (C-1′ to C-′), 6-O-α-(4-O-α-D-glucosyluronic acid)-β-D-glucosyl-β-cycloextrin (GUG-CD) was supplied by the Bio Research Corporation of Yokohama (Yokohama, Japan).\(^\text{11}\) Me-GA-X\(_6\)-OH (Aldouronic Acid Mixture) and 4-O-methyl-D-glucosyluronic acid-α-(1→2)-xylo-oligosaccharyl-α-(1→2)-xyitol (Reduced Aldo-uronic Acids, Me-GA-X\(_6\)-OH) were purchased from Megazyme (Dublin, Ireland). Me-GA-X\(_6\)-OH was composed of 4-

O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-

β-(1→4)-D-xylose, 4-O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-β-(1→4)-D-xylosyl-β-(1→4)-D-xylose, and 4-O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-β-(1→4)-D-xylosyl-β-(1→4)-D-xylose and 4-O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-β-(1→4)-D-xylosyl-β-(1→4)-D-xylose and 4-O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-β-(1→4)-D-xylosyl-β-(1→4)-D-xylose and 4-O-methyl-D-glucosyluronic acid-α-(1→2)-D-xylosyl-β-(1→4)-D-xylosyl-β-(1→4)-D-xylose.

Enzymes. The enzyme preparations, Cellulorosine PE 60 and Orientase 20A, were donated by HBI Enzymes (Hyogo, Japan). TreDCase was purified from Cellulorosine PE 60 (HBI Enzymes). Porcine kidney trehalase was purchased from Sigma Chemical. A. niger α-glucuronidase that hydrolyzed Me-GA-X\(_6\)-OH\(^\text{2,12}\), was partially purified from Orientase 20A (HBI Enzymes) by DEAE-Toyopearl column chromatography, as follows: Enzyme preparation was put on a DEAE-Toyopearl column (3 x 20 cm, Tosoh, Tokyo, Japan). The enzyme was eluted by linear gradient from zero to 0.5 M NaCl in 10 mM acetate buffer (pH 5.0). The active fractions were collected and diaлизed against 10 mM acetate buffer (pH 5.0). Trehalase from A. niger\(^\text{13}\) was partially purified from Hemicellulase Amano 90 (Amano Enzyme, Nagoya, Japan) by SP-Toyopearl column chromatography, as follows: Crude enzyme solution was applied on a SP-Toyopearl column (3 x 20 cm, Tosoh). Elution was carried out by linear gradient from zero to 1.0 M NaCl in 50 mM acetate buffer (pH 5.0). The active fractions were combined and diaлизed against 10 mM acetate buffer (pH 5.0).

Enzyme activity assay.

(1) α-Glucuronidases. Standard assay method. A reaction mixture consisting of 180 μl of 10 mM TreDC in 100 mM sodium acetate–HCl buffer (pH 3.0) and 20 μl of enzyme solution was incubated at 40 °C for 15 min. The reaction was stopped by adding an equal volume (200 μl) of Somogyi solution. The reducing sugars were measured by the Somogyi–Nelson method using D-glucuronic acid (GlUCUA) as a standard.\(^\text{14,15}\) One unit of activity was defined as the amount of enzyme liberating 2 μmol of GlUCUA per min.

(2) Assay of hydrolysis activity against Me-GA-X\(_6\)-OH. The hydrolysis activity against Me-GA-X\(_6\)-OH was measured at 40 °C in a 15 min incubated reaction mixture consisting of 180 μl of 5.8 mg/ml Me-GA-X\(_6\)-OH in 100 mM sodium acetate–HCl buffer (pH 4.0) and 20 μl of enzyme solution. The reaction was terminated by boiling for 5 min. The amount of 4-O-methyl-D-glucuronidase was measured by high-performance anion-exchange chromatography with pulsed ampero-
metric detection (HPAEC-PAD), as described below, using GlcUA as a standard. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of 4-O-methyl-D-glucuronic acid per min.

3) Trehalases. A substrate solution consisting of 180 μl of 10 mM trehalose in 100 mM acetate buffer (pH 5.0) and 20 μl of enzyme solution was mixed and incubated at 40 °C for 15 min. The reaction mixture was boiled for 5 min to stop the reaction, and the amount of D-glucose was measured by glucose oxidase regent (Diacolor GC, Toyobo, Osaka, Japan). One unit of enzyme activity was defined as the amount of enzyme liberating 2 μmol of D-glucose per min.

Purification procedure for TreDCase.

Step 1. Sephadex G-25 column chromatography. Forty g of Cellulose PE60 was dissolved in 20 ml of 10 mM acetate buffer (pH 4.0) (buffer A). After centrifugation, the supernatant was applied onto a Sephadex G-25 column (4.0 × 40 cm, Tosoh) equilibrated with buffer A for desalting. The enzyme was eluted by the same buffer at a flow rate of 1 ml/min. The protein fractions were combined.

Step 2. Q-Sepharose column chromatography. The protein fraction was put on a column of Q-Sepharose (3.0 × 20 cm, Pharmacia, Uppsala, Sweden) equilibrated with buffer A. The enzyme was eluted by linear gradient from zero to 0.5 M NaCl in buffer A at 0.5 ml/min. The enzyme fractions were combined and dialyzed against buffer A.

Step 3. SP-Toyopearl column chromatography. The dialyzed solution was applied onto a column of SP-Toyoparl (1.5 × 20 cm, Tosoh) equilibrated with buffer A. The elution was done by linear gradient from zero to 1.0 M NaCl in the same buffer at 0.5 ml/min. The active fractions were collected.

Step 4. ω-Aminododecyl-agarose column chromatography. The active fraction was dialyzed against buffer A and put on a column of ω-aminododecyl-agarose (1.5 × 10 cm, Sigma Chemical) equilibrated with buffer A. The enzyme was eluted by linear gradient from zero to 0.1 M NaCl in the buffer A at 0.5 ml/min. The active fractions were collected and dialyzed against buffer A.

Step 5. ProtEX-DEAE column chromatography. ProtEX-DEAE was done under the following conditions: system, fast protein liquid chromatography (FPLC, Pharmacia) system; pump, P-500; controller, LCC-500; detection, absorbance at 280 nm; column, Superdex 200 (Pharmacia). The molecular mass standards used were alcohol dehydrogenase (150.0 kDa), albumin (66.0 kDa), and carbonic anhydrase (29.0 kDa) (Sigma Chemical).

Estimation of molecular mass by gel filtration. The molecular mass of TreDCase was estimated by gel filtration under the following conditions: system, FPLC system; pump, P-500; controller, LCC-500; detection, absorbance at 280 nm; column, Superdex 200 (Pharmacia). The molecular mass standards used were alcohol dehydrogenase (150.0 kDa), albumin (66.0 kDa), and carbonic anhydrase (29.0 kDa) (Sigma Chemical).

HPAEC-PAD. HPAEC-PAD was done under the following conditions: system, Dionex DX-500; detector, Model PAD II pulsed amperometric detector; column, Dionex Carboxpac PA-1 (4.0 × 250 mm); elution, linear gradient from 150 to 300 mM sodium acetate in 100 mM NaOH, flow rate, 1.0 ml/min; temperature, 35 °C.

Thin layer chromatography. Thin layer chromatography (TLC) was done with TLC plates (Kaisel Gel 60, Merck, Darmstadt, Germany) and a solvent system of ethyl acetate–acetic acid–water (3:1:1, v/v). Spots of sugars were detected by heating the plate to 150 °C after spraying with 50% (w/w) sulfonic acid in methanol.

Effect of chemicals and metal ions. Reaction mixtures (160 μl) consisting of TreDCase (0.01 U/ml) in 100 mM sodium acetate–HCl buffer (pH 3.0) were incubated with 20 μl of 20 mM CaCl₂, CuCl₂, MnCl₂, FeCl₃, MgSO₄, KCl, NaCl, AgNO₃, CoCl₂, NiCl₂, FeSO₄, HgCl₂, SnCl₂, SDS, or dithiothreitol at 40 °C for 30 min. After incubation, 20 μl of 100 mM TreDC was added and incubated at 40 °C for 15 min. The reaction was terminated by boiling for 5 min. The amount of GlcUA was measured by HPAEC-PAD, and the remaining activity was estimated.

Kinetic parameters toward TreDC. The Kₘ and Vₘₐₓ values toward TreDC were measured as follows: The enzyme (0.042 U/ml) in 100 mM sodium acetate–HCl buffer (pH 3.0) was incubated with TreDC in various concentrations (2.5, 5.0, 10, 20 mM) at 40 °C for 15 min. After incubation, the amount of GlcUA was measured by the standard assay method. The Kₘ and Vₘₐₓ values were calculated from a Hanes–Woolf plot. 18) The
amount of protein was estimated by the absorbance at 280 nm (light path, 1 cm), assuming that $E_{1%}^{1%}$ was 10.0.

**Substrate specificity.** Enzyme solution (0.1 U/ml, 180 μl) in 100 mM sodium acetate–HCl buffer (pH 3.0) was incubated with 20 μl of 100 mM TreDC, PNP α-α-glucuronic acid, SucDC, GUG-CD, trehalose, or 58 mg/ml Me-GA-Xn at 40°C. After 24 h of incubation, the amounts of GlcUA and 4-O-methyl-D-glucuronic acid were measured by HPAEC-PAD.

**Anomeric types of hydrolysis products from TreDC.** The anomeric configurations of the hydrolysis products were determined by the following method: A solution containing 2.0 U of the purified enzyme was frozen by liquid N2 and lyophilized. After lyophilization, the substrate solution consisting 0.5 ml of 50 mM TreDC in D2O at pH 3.0 was added (the pH was adjusted by mixing 50 mM sodium TreDC and 50 mM TreDC). The reaction mixture was incubated at 25°C for 8–50 min, and 1H-NMR spectra were recorded.

**Results**

**Screening and purification of TreDCase**

Commercial enzyme preparations were incubated with 1.0% TreDC at 40°C for 18 h and the product, GlcUA, was detected by TLC. Out of 120 different preparations, 6 exhibited TreDC hydrolysis activity. We purified the enzyme, which was involved in the hydrolysis of TreDC (TreDCase), from a commercial product from *A. niger*, Cellurosine PE60 (HBI Enzymes), which showed the strongest hydrolysis activity. The results of purification are summarized in Table 1. TreDCase was purified 586-fold. The yield was 0.76%. At all steps, a single active peak was observed. SP-Toyopearl, ω-Aminododecyl-agarose, and ProtEX-DEAE column chromatography were effective for increasing specific activities, although the recoveries in these steps were relatively low. Only one protein band was observed, when 10 μg of the purified TreDCase was analyzed with native PAGE followed by staining with a silver staining regent. The molecular mass of TreDCase was 120 kDa as estimated by gel filtration. On the other hand, one protein band was also observed by SDS–PAGE, and its molecular mass was estimated to be 58 kDa (Fig. 1). These results indicate that the enzyme is composed of two identical subunits. Fungal α-glucuronidases are monomers, and no dimeric enzymes from fungal origin have been reported.1,2,6–8) It is known that α-glucuronidases from bacteria and snail are composed of two subunits (Table 2).3–5,9,11)

**Effects of pH, temperature, and chemicals**

As shown in Fig. 2, the enzyme was stable in a pH range of 2.0–4.0 at 30°C, and the optimum pH was pH 3.0–3.5. TreDCase had the most acidic optimum pH among the fungal and bacterial α-glucuronidases previously reported (Table 2).2–9) The enzyme was stable up to 30°C after incubation for 24 h at pH 4.0, and activity was highest at 40°C. When the enzyme was treated with 2.0 mM of various chemicals and metal ions, Hg²⁺ (residual activity, 42%) and SDS (52%) inhibited activity (data not shown). K⁺, Na⁺, Ca²⁺, Cu²⁺, Mg²⁺, Ag⁺, and dithiothreitol slightly activated TreDCase (110–118%), and Mn²⁺, Fe₃⁺, and Fe²⁺ marginally inhibited it (80–87%). Co²⁺, Ni²⁺, and Sn²⁺ showed no effect (101–103%).

**Substrate specificity**

The kinetic parameters of TreDCase toward TreDC

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (A280)</th>
<th>Total activity (Unit)</th>
<th>Specific activity (Unit/A280)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>16,200</td>
<td>120</td>
<td>0.007</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>2,900</td>
<td>78.5</td>
<td>0.027</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>848</td>
<td>70.2</td>
<td>0.083</td>
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<td>59</td>
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<tr>
<td>SP-Toyopearl</td>
<td>39.0</td>
<td>21.1</td>
<td>0.54</td>
<td>77</td>
<td>18</td>
</tr>
<tr>
<td>ω-Aminododecyl-agarose</td>
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<td>5.70</td>
<td>1.6</td>
<td>229</td>
<td>4.8</td>
</tr>
<tr>
<td>ProtEX-DEAE</td>
<td>0.22</td>
<td>0.91</td>
<td>4.1</td>
<td>586</td>
<td>0.76</td>
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</table>

Table 1. Summary of Purification of TreDCase

![Fig. 1. SDS–PAGE of Purified Enzyme.](image)

SDS–PAGE was done by 8% polyacrylamide slab gel. The gel was stained for protein with silver staining regent. Lane 1, the purified TreDCase. Ten μg of purified enzyme was applied. Lane 2, SDS molecular mass standards including phospholipase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa).
were measured as described in Materials and Methods. The $K_m$ and $V_{max}$ values were calculated to be 4.4 mM and 50 μmol of TreDC hydrolyzed/min/mg protein respectively. Specificities of TreDCase on other substrates are shown in Table 3. TreDCase did not hydrolyze TreMC in which one of two glucosyl residues of trehalose was changed to a glucuronyl residue, /C11/C12-TreDC, which had the /C11/C12-1,1 glucuronyl linkage, and SucDC. Furthermore, other /C11-glucuronides, such as PNP/C11-glucuronide and methyl-O-/C11-D-glucosiduronic acid, and GUG-CD, which had an /C11-1,4 glucuronyl linkage, were not degraded at all.

The hydrolysis activities of TreDCase for Me-GA-X$_n$ and Me-GA-X$_n$-OH, which were known as substrates of usual /C11-glucuronidases (Table 3 and Fig. 3A) was examined. The Me-GA-X$_n$ hydrolyzing /C11-glucuronidase from A. niger was used as a control enzyme. TreDCase completely hydrolyzed TreDC below 200 mM of TreDC and produced a stoichiometric amount of GlcUA. Even at a higher concentration of TreDC (300 mM), about 80% of TreDC was degraded.

Figure 3B shows a comparison of the substrate specificity of TreDCase with those of trehalases. TreDCase did not hydrolyze trehalose. Trehalases from both porcine kidney and A. niger did not degrade TreDC. These results indicate that trehalases and TreDCase are entirely different enzymes.

**Time course of TreDC hydrolysis by TreDCase under high substrate concentration conditions**

In view of possible industrial applications of TreDCase, in, for instance, the production of GlcUA from TreDC, the effects of high concentrations of substrate on reaction rate were studied by incubating the enzyme with 100–300 mM of TreDC (Fig. 4). TreDCase completely hydrolyzed TreDC below 200 mM of TreDC and produced a stoichiometric amount of GlcUA. Even at a higher concentration of TreDC (300 mM), about 80% of TreDC was degraded.

Table 2. Comparison of Properties of /C11-glucuronidases

<table>
<thead>
<tr>
<th>Properties</th>
<th>TreDCase</th>
<th>A. niger (Intracellular)</th>
<th>Snail acetone powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by SDS–PAGE (kDa)</td>
<td>58 (dimer)</td>
<td>130 (monomer)</td>
<td>97 (dimer)</td>
</tr>
<tr>
<td>by gel filtration (kDa)</td>
<td>120</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>3.0–3.5</td>
<td>4.8</td>
<td>3.0</td>
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<tr>
<td>pH stability</td>
<td>2.0–4.0</td>
<td>4.5–7.0</td>
<td>3.0–7.0</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>40</td>
<td>60</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Thermostability (°C)</td>
<td>&lt;30</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Reference</td>
<td>This study</td>
<td>1</td>
<td>11, 20</td>
</tr>
</tbody>
</table>
Anomeric type of hydrolysis products from TreDC

The anomeric configurations of the hydrolysis products of TreDC were determined by measuring the ratio of \( ^\text{1}H\)-NMR (Fig. 5). A doublet at 5.19 ppm \( (^3J = J, 3.9 \text{ Hz}) \) was assigned to H-1α and H-1β of TreDC, and doublets at 5.25 ppm \( (^3J = 3.9 \text{ Hz}) \) and 4.48 ppm \( (^3J = 7.9 \text{ Hz}) \) were assigned to H-1α and H-1β of GlcUA respectively. The intensity of a doublet from TreDC decreased and the intensity of doublets from GlcUA increased with the progress of hydrolysis. The intensity ratios of the products showed equimolar production of the two anomers during the early stage of reaction (8–50 min).

Since the rate of mutarotation from GlcUA to GlcUA was slow enough not to affect these results, GlcUA was probably formed by the inversion of anomeric configuration.

Fig. 3. Courses of Hydrolysis of TreDC, Me-GA-Xn-OH, and Trehalose by TreDCase, α-Glucuronidase, and Trehalases.

(A) Courses of hydrolysis of TreDC and Me-GA-Xn-OH by TreDCase and α-glucuronidase. Reaction mixtures (1.0 ml) containing 0.01 U of enzyme (TreDCase, \( \Delta \); partially purified A. niger α-glucuronidase, \( \Box \), and substrate (0.01 mmol of TreDC, —; 5.8 mg of Me-GA-Xn-OH, - - -) in 100 mM sodium acetate–HCl buffer (pH 3.0, TreDCase) or acetate buffer (pH 4.0, partially purified α-glucuronidase) were incubated at 40°C. After incubation, the amounts of GlcUA (—) and 4-O-methyl-D-gluronic acid (- - -) were measured by HPAEC-PAD. Note that no 4-O-methyl-D-gluronic acid was liberated by TreDCase from Me-GA-Xn-OH.

(B) Courses of hydrolysis of TreDC and trehalose by trehalases. Reaction mixtures (0.9 ml) consisting of 10 mM substrate (TreDC, —; trehalose, - - -) in 100 mM sodium acetate buffer (pH 3.0, TreDCase) or acetate buffer (pH 5.0, trehalases) were incubated with 0.1 ml of 0.01 U/ml enzyme (TreDCase, \( \Delta \); porcine kidney trehalase, \( \Box \); partially purified trehalase of A. niger, \( \Box \)) at 40°C. The amounts of GlcUA (—) and glucose (- - -) were measured by HPAEC-PAD and glucose oxidase reagent respectively.

Table 3. Substrate Specificities of Various α-Glucuronidases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TreDC</td>
<td>+</td>
</tr>
<tr>
<td>Me-GA-Xn</td>
<td>—</td>
</tr>
<tr>
<td>PNP-α-GA</td>
<td>—</td>
</tr>
<tr>
<td>α,β-TreDC</td>
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</tr>
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<td>TreDC</td>
<td>—</td>
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<tr>
<td>Me-α-GA</td>
<td>—</td>
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<tr>
<td>SucDC</td>
<td>—</td>
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<tr>
<td>GUG-CD</td>
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Me-GA-Xn, 4-O-methyl glucosyluronic acid xylooligosaccharide; PNP-α-GA, PNP α-glucuronic acid; Me-α-GA, methyl α-α-glucosiduronic acid; N.D., not determined.

Fig. 4. Time Courses of TreDC Hydrolysis by TreDCase at High Substrate Concentration.

Reaction mixtures (1.0 ml) consisting TreDC (100 mM, \( \bigcirc \); 200 mM, \( \bigcirc \); 300 mM, \( \square \) and TreDCase (1.2 U/ml) in 100 mM sodium acetate–HCl buffer (pH 3.0) were incubated at 40°C for 0–72 h. The amounts of TreDC (\( \bigcirc \)) and GlcUA (—) were measured by HPAEC-PAD.
Discussion

We obtained a new type of α-glucuronidase which was highly specific for TreDC. The enzyme did not hydrolyze Me-GA-Xα or its derivatives, the common substrates for the reported α-glucuronidases. The enzyme described here hydrolyzed neither TreMC, αβ-TreDC, SucDC, GUG-CD, nor Me-GA-Xα (Table 3). This suggests that the enzyme is highly specific for TreDC, and that it recognized and required the two α,α-(1→1)-glucuronyl residues for the hydrolysis reaction. The strict substrate specificities were remarkable characteristics and differentiate TreDCase from other α-glucuronidases.

Various microbial α-glucuronidases have been reported and characterized. Most of them have been ranked as hydrolase, which degrade side chains of xylan and characterized. Most of them have been glucuronidases. Most of them have been

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Various microbial α-glucuronidases such as PNP α-glucuronidase, Me-GA-Xα, 4-O-methyl-d-glucosyluronic acid-α-(1→3)-xylose, and 4-O-methyl-d-glucosyluronic acid-α-(1→4)-xylose (Table 3). The V_{max} value toward TreDC was reported to be only a few percent of that toward PNP α-glucuronide, suggesting that TreDC is a poor substrate for the enzyme. The broad substrate specificity of the snail enzyme was antipodal against the strict substrate specificity of TreDCase.

TreDCase inverted the anomeric configuration of the substrate (Fig. 5). Biely et al. reported that extracellular A. tubingensis α-glucuronidase produced products with an inverted anomeric configuration. Since most α-glucuronidases have been classified into glycosyl hydrolase family 67 based on amino-acid sequence similarity, they proposed that the α-glucuronidases that belong to the family are inverting enzymes. Two mechanisms, inversion and retention, have been proposed for the reaction of glycosylases. In general, inverting enzymes catalyze hydrolysis by the single displacement mechanism, whereas retaining enzymes hydrolyze substrates by the double displacement mechanism. Some glycosidases, such as glucoamylase and trehalase, are known to be inverting enzymes. The catalytic properties of TreDCase, high substrate specificity for α,α-1,1 glycosidic linkage and inversion of the anomeric configuration of substrates, are similar to those of trehalases.

Since TreDC is composed of two glucuronyl residues, TreDCase is probably available for producing GlcUA. GlcUA has been used as a medicine, because it helps in detoxification, improvement of liver function, and recovery from fatigue. GlcUA is manufactured by chemical oxidation of starch followed by acid-catalyzed-hydrolysis. The yield of GlcUA chemical production, however, is still low (less than 20%), and many byproducts are produced. TreDCase completely hydrolyzed 200 mM of TreDC in 72 h to produce GlcUA in 100% yield (Fig. 4). TreDCase, therefore, might be applicable in the production of GlcUA.

References

5) Bronnenmeier, K., Meissner, H., Stocker, S., and