Note
Substrate Specificity of α-Glucuronidase Isolated from Snail Acetone Powder

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The substrate specificity of α-nitrophenyl α-D-glucopyranosyl-
uranic acid-hydrolyzing enzyme (PNP-GAase) isolated from snail acetone powder has been investigated with various substrates, such as α-nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA), 2-O-α-
D-glucopyranosyluronic acid-α-D-xylene (GA-2X), 2-O(4-O-methyl-
D-glucopyranosyluronic acid)-α-D-xylene (MeGA-2X), and 2-O-α-
D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid (GA-GA).

The \( K_m \) (mM) and \( V_{max} \) (μmol of glucuronic acid formed/minute) of enzyme protein/min toward these substrates were as follows: 0.13 and 3.21 for PNP-GA, 0.33 and 0.089 for GA-2X, 17.6 and 0.094 for MeGA-2X, and 0.36 and 0.015 for GA-GA, respectively. The results indicate that the PNP-GAase specifically hydrolyzed PNP-GA, however, the enzyme had broad substrate specificity.

Key words: α-glucuronidase; α-nitrophenyl α-D-glucopyranosyl-
uranic acid; substrate specificity of α-glucuronidase; snail acetone powder; Helix pomatia

α-Glucuronidase hydrolyzes the glycosidic linkages between α-D-glucopyranosyluronic acid and β-xylene, or 4-O-methyl-α-D-glucopyranosyluronic acid and α-xylene, and thereby liberates β-glucuronic acid or 4-O-methyl-β-glucuronic acid. There are some reports about α-glucuronidases (refer to the review by Puh3), but the enzyme has not yet been registered in the Enzyme Nomenclature.

Our earlier studies have shown that several species of basidiomycetes produce α-glucuronidases hydrolyzing GA-2X, but none of them produce PNP-GAase. Fontana et al. reported that an enzyme preparation from snail juice had both α-nitrophenyl α- and β-glucuronide-hydrolyzing enzyme activities. However, the enzymes have neither been purified nor characterized so far.33 Marsh and Levy have also reported that the enzyme preparation of abalone has these enzyme activities.34

In our previous paper, we described the purification and characterization of a new α-glucuronidase from snail acetone powder originating from the visceral hump of Helix pomatia.54 The enzyme was identified by using α-nitrophenyl α-D-glucopyranosyluronic acid as the substrate, and showed broad substrate specificity.

In this paper, we calculate the rate parameters of snail α-glucuronidase. Michaelis constants and maximum velocities, for hydrolysis of various α-glucuronides including aldobiouronic acid.

The snail α-glucuronidase was purified from snail acetone powder (Sigma) by the procedures described in our previous paper.54 The enzyme was homogeneous in polyacrylamide gel electrophoretic analysis. The apparent molecular weight of the enzyme was 180,000 as estimated by gel filtration with Superose 6 HR (10.30 (Pharmacia), and was 97,000 by SDS-polyacrylamide gel electrophoresis.

α-Nitrophenyl α-D-glucopyranosyluronic acid (PNP-GA), and Z-α-D-glucopyranosyluronic acid-Z-α-D-glucopyranosiduronic acid (GA-GA) were synthesized49 by catalytic oxidations of α-

Nitrophenyl α-D-glucopyranoside and Z-α-D-glucopyranoside (Nacarai Tesque Inc., Kyoto, Japan), as described by Marsh and Levy,33 and Goren and Jiang,38 respectively. 2-O-α-D-Glucopyranosyl-

uronic acid-α-D-xylene (GA-2X) and 2-O(4-O-methyl-α-D-glucopy-

ranosyluronic acid)-α-D-xylene (MeGA-2X) were prepared by the method of Matsuo et al.59

For PNP-GAase assay, 0.1 ml of enzyme solution was added to a mixture of 0.5 ml of 20 mM PNP-GA and 0.4 ml of 0.1 M NaOAc-HCl buffer (pH 3.0). The reaction was done at 50 C for 10 min, and stopped by adding 1.0 ml of 0.2 M Na2CO3. The amount of α-nitrophenol released was measured by the absorbance at 408 nm. One unit of PNP-GAase activity was defined as the amount releasing 1 μmol of α-nitrophenol per min.

For a kinetic study of PNP-GAase toward various α-glucuronides, reaction mixtures containing 50 μl of substrate solution of various concentrations, 150 μl of 0.1 M NaOAc-HCl buffer (pH 3.0), and 50 μl of enzyme solution were incubated at 50 C for various times. The amount of glucuronic acid or 4-

O-methyl-glucuronic acid released was measured by the Milner-Avigad method.109 The initial velocity, v, was expressed as μmol of glucuronic acid or 4-O-methyl-glucuronic acid liberated from the non-reducing end side of substrate mg of enzyme protein/min.

The courses of hydrolytic actions of PNP-GA, GA-GA, GA-2X, and MeGA-2X are shown in the Fig. PNP-GA was more rapidly hydrolyzed than other α-glucuronides by snail PNP-GAase. GA-GA, GA-2X, and MeGA-2X were slowly (20 times or more) hydrolyzed by the enzyme, and GA-2X was hydrolyzed faster than GA-GA and MeGA-2X. The rates of hydrolysis decreased in the following order: PNP-GA > GA-2X > GA-GA.

The relationship between substrate concentrations and the enzyme activity was also investigated (data not shown). The Michaelis constant (K_m) and maximum velocity (V_max) were obtained by using Lineweaver-Burk plots. The K_m and V_max for PNP-GA, GA-GA, GA-2X, and MeGA-2X are listed in the Table.

The K_m for PNP-GA was 0.13 mM, the smallest among the glucuronides we tested. The V_max for PNP-GA was 3.21 μmol mg of enzyme min and the value was 35 times that for GA-2X and MeGA-2X and 200 times that for GA-GA.

Uchida et al. reported the K_m values of Aspergillus niger α-glucuronidases (CM-I and CM-II) toward 2-O-α-D-glucopy-

ranosyluronic acid-α-D-xylene (GA-2X), and 2-O(4-O-methyl-

α-D-glucopyranosyluronic acid)-α-D-xylene (MeGA-2X).61 The

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Abbreviations: PNP-GA; α-D-glucopyranosyluronic acid-α-D-glucopyranosiduronic acid; GA-2X, 2-O-α-D-glucopyranosyluronic acid-α-D-xylene; MeGA-2X, 2-O(4-O-methyl-α-D-glucopyranosyluronic acid)-α-D-xylene; GA-GA, 2-O-α-D-glucopyranosyluronic acid-α-D-glucopyranosyluronic acid-α-D-xylene; MeGA-2X, 2-O(4-O-methyl-α-D-glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid-α-D-xylene; PNP-GA, α-nitrophenyl α-D-glucopyranosyluronic acid; PNP-GAase, α-nitrophenyl α-D-glucopyranosyluronic acid-hydrolyzing enzyme.
**Substrate Specificity of α-Glucuronidase**

![Graph](image)

**Fig.** Progressive Hydrolytic Activity of Snail PNP-GAase on α-Glucuronides.

Reaction mixtures containing 50 μl of 5.0 mM substrate solution, 150 μl of 0.1 M NaOAc·HCl buffer (pH 3.0), and 50 μl of enzyme (2.0 x 10^{-3} mg) solution were incubated at 50°C. At the indicated times, the reaction was stopped by adding copper solution, and then the glucuronic acid or 4-O-methylglucuronic acid liberated was measured by the Milner-Avgad method.

- ● PNP-GA
- □ GA-2X
- ■ MeGA-2X
- ○ GA-GA

**Table**

Michaelis Constant ($K_m$) Values and Maximum Velocity ($V_{max}$) Values of Snail α-Glucuronidase toward Various α-Glucuronides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PNP-GA</th>
<th>GA-2X</th>
<th>MeGA-2X</th>
<th>GA-GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>0.13</td>
<td>0.33</td>
<td>17.6</td>
<td>0.36</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>3.21</td>
<td>0.089</td>
<td>0.094</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* μmol of glucuronic acid or 4-O-methylglucuronic acid mg of enzyme protein min.

$K_m$ values of CM-I toward GA-2X$_1$ and MeGA-2X$_1$ were 0.77 and 0.37, and those of CM-II were 0.82 and 0.47 mM, respectively. These results indicate that A. niger α-glucuronidase has a higher affinity for 4-O-methyl-α-glucuronic acid than unsubstituted α-glucuronic acid. However, in the case of snail α-glucuronidase, the $K_m$ values for GA-2X and MeGA-2X were 0.33 and 17.6 mM (Table). These results indicate that the O-methyl group at C-4 of glucuronic acid residue caused a great decrease in the affinity of the enzyme for the substrate. The $V_{max}$ values for GA-2X, MeGA-2X, and GA-GA were only a few percent of that for PNP-GA. Thus, it may be concluded that PNP-GAase specifically hydrolyzes PNP-GA, but the enzyme acts slowly on other α-glucuronides such as O-α-glucopyranosyluronic acid-α-xylene.

The substrate specificities of α-glucuronidases from A. niger and basidiomycetes have been reported. In these reports, we concluded that the α-glucuronidase of A. niger has a very strict substrate specificity as the enzyme hydrolyzes GA-2X and MeGA-2X only, while α-glucuronidase of basidiomycetes has a broad substrate specificity, hydrolyzing not only GA-2X and MeGA-2X but also 3-O- and 4-O-α-glucopyranosyluronic acid-α-xylases. But these α-glucuronidases do not hydrolyze PNP-GA. It may be concluded from this study that there are at least three types of α-glucuronidases:

- an α-glucuronidase that hydrolyzes only GA-2X, such as A. niger α-glucuronidase.
- an α-glucuronidase that hydrolyzes the regiosomers of O-α-glucopyranosyluronic acid-α-xylene but not PNP-GA, such as basidiomycete α-glucuronidases.
- an α-glucuronidase that specifically hydrolyzes PNP-GA and slowly hydrolyzes other α-glucuronides.

**References**