Title: Kinetic and site-directed mutagenesis study on the catalytic mechanism of Flavobacterium \(\beta\)-glucosidase

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Abstract

A \(\beta\)-glucosidase from Flavobacterium meningosepticum has been purified and characterized. The enzyme exhibited a high specificity on the glycon portion of aryl-\(\beta\)-glucosides. NMR spectroscopy revealed the enzyme catalyzes hydrolysis of 4-nitrophenyl-\(\beta\)-glucoside with the retention of anomeric configuration, indicating that a double displacement mechanism was involved. The Bronsted relationship showed a concave-downward plot, which is consistent with the two-step mechanism. A series of site-directed mutagenesis experiments, which include 136E/Q, 137D/N, and 169H/A, have been performed to approach the essential groups of this enzyme. Kinetic studies showed the catalytic activity of 169H/A was at least 100-fold weaker than those of wild type enzyme. However, the Michaelis constant for the enzyme toward 4-nitrophenyl-\(\beta\)-glucoside were quite similar. The preliminary results revealed that H169 of the enzyme deeply involve in the catalytic activity.

Keywords: F. meningosepticum, \(\beta\)-glucosidase; mechanism; Bronsted relationship; site-directed mutagenesis
三、結果與討論

In order to study the catalytic mechanism of the cloned β−glucosidase at least 10 substrates with various leaving phenols (pKₐ 4 ~ 10) were synthesized according to literatures (1~3).

Substrate specificity

A variety of glycosides, including α-PNPG, β-PNPG, β-ONPG, β-PCPG, β-PG, β-CNPG, β-DNPG, β-PNPM, β-PNPAG, β-PNPA, were tested as substrates. Of all the substrates investigated only the aryl β-D-glucopyranosides were hydrolyzed effectively. The β-D-pyranoside configuration is essential for effective catalysis. PNPM and PNPAG are virtually inactive. Clearly, the C-2 hydroxy group on glucopyranoside is also important. Effect of hydroxyl group inversion at C-4 (being galactosides) weakens the catalytic efficiency by a factor of 160-fold (based on relative Vₘₐₓ/Kₘ value). Though it is highly specific with regard to the glycone moiety, the purified enzyme shows a broad tolerance for the aglycone portion. It can accommodate various substrates, such as 2,4-dinitrophenyl, 2,5-dinitrophenyl, 3,4-dinitrophenyl, 4-chloro-2-nitrophenyl, p-nitrophenyl, α-nitrophenyl, m-nitrophenyl, 4-cyanophenyl and phenyl-β-D-glucosides, with similar Michaelis constant (Kₘ).

However, the kₐₐ values can be different up to 2.6-order of magnitude.

Extended Bronsted Plot

The extended Bronsted plot has been shown to be a valuable tool to rationalize mechanistic actions of enzymes (4~6). Based on the kₐₐ values of arylglucosides (in Table 1), an extended Bronsted plot can be constructed by plotting the logarithmic form of the relative kₐₐ values (log₁₀kₐₐ, see Table 2) of aryl-β-D-glucosides against the pKₐs of leaving phenols. A biphasic characteristic with a concave-downward trend was clearly seen (Fig. 1). This provides an indication for a two-step mechanism: formation and breakdown of glucosy-enzyme intermediate or alternative carbonium-enzyme intermediate.

1H Nuclear magnetic resonance (1H-NMR) analysis of the stereoselectivity of the hydrolysis

1H-NMR spectroscopy has been used to investigate the stereoselectivity of various glycohydrolases such as cellulases and xylanase (7,8). Studies revealed that glycohydrolases often present a double-displacement mechanism, which involves the retention of the anomeric configuration. However, interestingly, a β-xylanosidase purified from Clostridium cellulolyticum was shown to act by inverting the β-anomeric configuration (9). To better understand the catalytic function of this purified β-glucosidase, a NMR study of stereoselectivity of the enzyme was carried out. Results were shown in Figure 2. In a glucose 1H-NMR study two doublets were found to be centered at δ = 4.48 ppm (J=7.8 Hz) and δ = 5.07 ppm (J=3.3 Hz), corresponding to the β and the α anomeric protons, respectively. The ratio of β/α is 64/36 when they are equilibrated. The C1 proton of PNPG was shown to be a doublet centered at 5.12 ppm (J=7.8 Hz). When F. meningosepticum β-glucosidase was added to PNPG, the β anomeric signal increased. At 18 minutes, a new doublet generated by mutarotation process and centered at δ = 5.07 ppm (J=3.3 Hz) emerged. The ratio of β-glucose to α-glucose was 90:10. At 35 minutes, the β and α anomeric signals constantly increased. The ratio of β to α was 81:19. The anomeric proton of PNPG was extinguished completely in 70 min (data not shown). This NMR study
clearly showed that *F. meningosepticum* β-glucosidase catalyzes the hydrolysis of PNPG with the retention of anomeric configuration.

**Possible reaction mechanism**

NMR study suggested that the catalytic action of this β-glucosidase involve a double displacement mechanism. Bronsted plot also supported a two-step mechanism involved. A tentative mechanism (shown in Fig. 3) was proposed for the hydrolysis catalyzed by the purified β-glucosidase.

The observed retention of the configuration at C-1 can be explained either by a stereospecific hydration of the carbonium-enzyme intermediate (step 2a) or by a hydrolysis of the glucosyl-enzyme intermediate in an inversed displacement manner (step 2b). In spite of the uncertainty of the transition state structure of the reaction, the bottleneck step can be unequivocally predicted from the extended Bronsted relationship. Since substrates with good leaving phenols (such as 2',4'-dinitrophenol, *p*-nitrophenol) show no significant dependence of their reactivity on the leaving phenols' pKₐs, the reaction rate-limiting step (*r.l.s.*) of the good substrates is therefore the breakdown of the intermediate whereas the *r.l.s.* of the poor substrates such as PG is the formation of the intermediate. Similar result can be seen in β-glucosidase from sweet almond (10) and *A. faecalis* (11).

**Site-directed mutagenesis**

A series of site-directed mutagenesis experiments, which include 136E/Q, 137D/N, and 169H/A, have been performed to approach the essential groups of this enzyme. All mutants were sequenced to confirm the correct colony selection. Kinetics studies showed the catalytic activity of 169H/A was at least 100-fold weaker than those of wild type enzyme. However, the Michealis constant for the enzyme toward *p*-nitrophenyl-β-glucoside were quite similar.

Table 1. Km and relative kcat values

<table>
<thead>
<tr>
<th>Substrate phenol substituent</th>
<th>pKₐ</th>
<th>Km (mM)</th>
<th>kcat (M⁻¹ s⁻¹)</th>
<th>log ( \frac{k_{cat}}{K_m} )</th>
<th>log ( \frac{\text{Kcat}}{K_m} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dinitro</td>
<td>3.96</td>
<td>0.372</td>
<td>1.57E-6</td>
<td>-5.80</td>
<td>-2.38</td>
</tr>
<tr>
<td>2,5-dinitro</td>
<td>5.15</td>
<td>0.325</td>
<td>1.10E-6</td>
<td>-5.99</td>
<td>-2.51</td>
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<tr>
<td>3,4-dinitro</td>
<td>5.26</td>
<td>0.150</td>
<td>7.00E-7</td>
<td>-6.15</td>
<td>-2.33</td>
</tr>
<tr>
<td>4-chloro-2-nitro</td>
<td>6.45</td>
<td>0.788</td>
<td>1.42E-6</td>
<td>-5.85</td>
<td>-2.74</td>
</tr>
<tr>
<td>4-nitro</td>
<td>7.18</td>
<td>0.815</td>
<td>5.48E-7</td>
<td>-6.26</td>
<td>-3.17</td>
</tr>
<tr>
<td>2-nitro</td>
<td>7.22</td>
<td>1.470</td>
<td>5.63E-7</td>
<td>-6.35</td>
<td>-3.42</td>
</tr>
<tr>
<td>4-cyano</td>
<td>8.49</td>
<td>0.801</td>
<td>3.22E-8</td>
<td>-7.49</td>
<td>-4.39</td>
</tr>
<tr>
<td>H</td>
<td>9.99</td>
<td>1.100</td>
<td>4.19E-9</td>
<td>-8.38</td>
<td>-5.42</td>
</tr>
</tbody>
</table>

Fig. 1. Bronsted plot displays a concave-downward trend.
1. **Fig. 2** $^1$H NMR analysis of the stereoselectivity of the reaction catalyzed by β-glucosidase from *F. meningosepticum*. A suitable amount of enzyme was applied on 2.5 mM PNPG at pH = 7.1. The reaction was monitored about every 10 minutes after addition of the enzyme.

1. **Fig. 3** Proposed two-step reaction mechanism of β-glucosidase hydrolysis. The pathways A and B lead to the formation of carbonium-enzyme and glucosyl-enzyme intermediate, respectively. Step 1 is the formation of the intermediate while step 2 is the breakdown of the intermediate.


