The Mode of Action of Endo \( \alpha,1,4 \) Polygalactosaminidase from *Pseudomonas* sp. 881 on Galactosaminooligosaccharides

Jun-ichi TAMURA, Toshiro ABE, Kaname HASEGAWA, and Kiyoshi KADOWAKI

Research Laboratory of Higeta Shoyu Co., Ltd., Chuo-cho 2-8, Choshi-shi, Chiba 288, Japan

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The mode of action of the endo \( \alpha,1,4 \) polygalactosaminidase from *Pseudomonas* sp. 881 on galactosaminooligosaccharides (GOSs) was studied. The enzyme could hydrolyze \( \alpha,1,4 \) polygalactosamine to GOSs by the endo-split manner. Tetraose and longer GOSs were hydrolyzed to galactosaminobiose and galactosaminotriose as the final products. Galactosaminomonomer (galactosamine) could not be produced as an enzymatic product. From the dependency of kinetic parameters on the chain lengths of the substrates, it was suggested that the enzyme has 8 subsites. A catalytic site of the enzyme is located between the third and the fourth sites from the non-reducing end, since the main product from GOSs was galactosaminotriose, and galactosaminotetraotril remained in the hydrolyzate of galactosaminohexaotril digestion. The enzyme showed transglycosylating activity on GOS4.

In our previous papers,\(^1\)\(^-\)\(^2\) we described the purification of an endo \( \alpha,1,4 \)-polygalactosaminidase from *Pseudomonas* sp. 881 and hydrolyzing products from polygalactosamine by the enzyme. It was unusual that galactosamine (GalN) could not be detected among the products even if the enzyme was believed to act in the endo-split manner. This paper deals with a mode of action by endo \( \alpha,1,4 \) polygalactosaminidase on GOSs and proposes a subsite model of the enzyme. The mode of action of many hydrolyzing enzymes (for example, amylases\(^3\)\(^-\)\(^6\)) on corresponding carbohydrate polymers has been investigated in detail, but ours is the first report on the mode of action of a purified endo \( \alpha,1,4 \) polygalactosaminidase.

**Materials and Methods**

**Materials.** CM-Sephadex C-25 was purchased from Pharmacia Fine Chemicals, Sweden. A Biofine IEC-CM packed column was purchased from Japan Spectroscopic Co., Ltd. All other reagents used were of guaranteed grade and purchased from Wako Pure Chemical Industries, Japan.

**Enzyme preparation.** Endo \( \alpha,1,4 \) polygalactosaminidase from *Pseudomonas* sp. 881 was prepared as described in our previous paper.\(^1\) High performance liquid chromatography of GOSs. GOSs were analyzed by a Biofine IEC-CM packed column with an HPLC system from Japan Spectroscopic Co., Ltd. Absorbed GOSs were eluted by a linear gradient of \((NH_4)_2SO_4\) from 0 to 1 M at room temperature. Optical rotation was monitored by DIP-181C (Japan Spectroscopic Co., Ltd.). Samples of 10–100 \( \mu \)g were injected into the column at a velocity of 0.7 ml/min. Each GOS was identified from the retention time.

**Enzyme reaction.** All the reaction was done in 20 mm sodium acetate buffer (pH 5.0) at 30°C. Enzyme activity was measured as described in our previous paper.\(^3\) The action of endo \( \alpha,1,4 \) polygalactosaminidase (molecular weight 31,000) on GOSs or alditois was studied using a reaction mixture of 0.5 or 2.5 mM substrates and 0.5 unit enzyme (about 0.25 \( \mu \)M). The mixtures (1 ml) were incubated for several intervals. The reaction was stopped by the addition of 1 M HCl (0.2 M). The hydrolyzates were evaporated to remove HCl at room temperature and were dissolved in water (200 \( \mu \)l). About 50 \( \mu \)g of GOS was put on the IEC-CM column to analyze the products. To estimate the frequency distribution of bond cleavage for individual GOSs or alditois, the products were separated by HPLC and the quantity of each isolated product was calculated from its peak area on HPLC using corresponding carboxylates as standards.

To measure the kinetic parameters of the enzyme, 1 ml of the reaction mixture (containing 20 mM sodium acetate buffer pH 5.0, 0.05 \( \mu \)M of enzyme and various concentrations of GOS) was incubated for 10 min and the net reducing power resulting from the enzyme reaction was measured by the Somogyi–Nelson method.\(^1\)

**Results**

**Products of endo \( \alpha,1,4 \) polygalactosaminidase on polysaccharide**

Typical HPLC patterns of hydrolyzates of polygalactosamine with endo \( \alpha,1,4 \) polygalactosaminidase incubated for 10 min and 20 hr and showed in Fig. 1. In the earlier stage, GOSs from dimer to decamer were obtained. Finally, the enzyme completely hydrolyzed GOSs upwards from galactosaminotetraose (GOS4) to galactosaminobiose (GOS2) and galactosaminotriose (GOS3) at the ratio of approximately 3:7:4.6: Galactosaminomonomer (GalN) was not produced by the enzyme reaction.

**Actions of endo \( \alpha,1,4 \) polygalactosaminidase on GOS**

Table 1 shows the degree of hydrolysis and the product distribution of GOS3–GOS10. In the cases of GOS5 and longer GOSs, the dominant product was GOS3 and the sum of the degree of polymerization (DP) of GOS3 and
The Mode of Action of Galactosaminidase on Galactosaminooligosaccharides

Table I. Degree of Hydrolysis and Product Distribution

<table>
<thead>
<tr>
<th>Substrates</th>
<th>DH (%)</th>
<th>Product distribution (relative %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOS3</td>
<td>0</td>
<td>GOS1: 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>GOS4</td>
<td>87</td>
<td>GOS1: 44 35 13</td>
</tr>
<tr>
<td>GOS5</td>
<td>85</td>
<td>GOS1: 27 64 9</td>
</tr>
<tr>
<td>GOS6</td>
<td>83</td>
<td>GOS1: 50 25 9</td>
</tr>
<tr>
<td>GOS7</td>
<td>79</td>
<td>GOS1: 12 31 18 14 0 24</td>
</tr>
<tr>
<td>GOS8</td>
<td>85</td>
<td>GOS1: 10 42 11 21 7 0 9</td>
</tr>
<tr>
<td>GOS9</td>
<td>80</td>
<td>GOS1: 10 36 5 12 20 0 0 17</td>
</tr>
<tr>
<td>GOS10</td>
<td>70</td>
<td>GOS1: 5 25 9 10 7 19 0 0 24</td>
</tr>
</tbody>
</table>

The reaction mixture contained 0.5 mm substrate and 0.25 μm enzyme. The mixture was incubated for 30 min. Other experimental details are mentioned in Materials and Methods.

The boldface numbers are the second largest number of enzymatic products.

a) Product distribution was calculated from HPLC and expressed as % of isolated GOSs in total hydrolyzates.

b) Degree of hydrolysis (DH) was calculated from the amount of residual substrate.

Table II. Kinetic Parameters of the Enzyme

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ (mm)</th>
<th>$V_{max}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOS6</td>
<td>1.25</td>
<td>0.83</td>
</tr>
<tr>
<td>GOS7</td>
<td>0.71</td>
<td>0.52</td>
</tr>
<tr>
<td>GOS8</td>
<td>0.25</td>
<td>0.37</td>
</tr>
<tr>
<td>GOS9</td>
<td>0.24</td>
<td>0.42</td>
</tr>
<tr>
<td>GOS10</td>
<td>0.25</td>
<td>0.43</td>
</tr>
</tbody>
</table>

$K_m$ and $V_{max}$ were obtained from Lineweaver–Burk plots.

Molecular activity, $k_w$, is expressed as $V_{max}/c_e$, where $c_e$ is the molar concentration of the enzyme.

of the second most dominant product (bold number) was equal to the DP of the original substrate except in the case of GOS6 where GOS3 was the main product. These results indicate the enzyme has a tendency to cleave the glycosyl bond in each GOS3 unit. On the other hand, GalN was not produced from GOSs, even GOS4, by the enzyme reaction. In the case of GOS4, GOS2 and GOS3 were produced.

Fig. 1. HPLC Patterns of Enzymatic Products from Polygalactosamine.
Twenty-μl samples (containing about 50 μg GOSs) were analyzed by HPLC. The peaks of the chromatogram are as follows: 2—10 correspond to galactosaminobose (GOS2)—galactosamonomesoe (GOS10), respectively. Arrow: the elution position of GalN. Incubation time: 10 min (upper), 20 hr (lower).

Fig. 2. Dependence of Rate Parameters (RP) on the Degree of Polymerization of GOSs.

$K_m$ sec⁻¹; $K_w$ mm.

Fig. 3. HPLC Patterns of Digested GOS7 ad GOS7OH.
The experimental details are described in the text. The peaks: 2—7, GOS2—GOS7; GOS40H, GOS50H, GOS70H. Incubation time: 10 min (upper), 2 hr (middle), 24 hr (lower).

Kinetic parameters of the enzyme

The $K_m$ and $V_{max}$ were calculated from Lineweaver–Burk plots. These values of the enzyme on the various GOSs are listed in Table II. The $K_m$ decreased with the increase in chain length and $k_w$ became almost constant for GOSs more than GOS8. As shown in Fig. 2, the relationships between log[$k_w/K_m$], log[1/$K_m$], and DP of substrates were almost constant upward to GOS8.

Action of the enzyme on galactosaminohexose (GOS7) and galactosaminohexitol (GOS7OH)

Figure 3 shows HPLC elution patterns of the hydrolysate of GOS7 and GOS7OH by the enzyme. The enzyme was estimated to produce GOS2 and GOS5 or GOS3 and GOS4 from GOS7 at first, and GOS4 and GOS5 were digested to smaller products. Finally, the products were GOS2 and GOS3. On the other hand, the enzyme mainly produced GOS3, galactosaminotetraol (GOS40H), and a small amount of GOS2 from GOS7OH. GOS-alditols and corresponding GOSs were not separated clearly by HPLC column. But, as GOS40H can not be cleaved by the enzyme (Fig. 4), GOS40H remained in the hydrolyzate.

Frequency distribution of bond cleavage of GOS-alditols

The degree of hydrolysis of GOS-alditols by the enzyme at an early stage is shown in Fig. 4. The dominant cleaved glycosyl bond, which could be estimated from enzymatic products, was indicated by arrows. In cases of GOS-alditols
Fig. 4. Distribution of Cleavage on GOS-Alditols.
The experimental details are described in the text. Symbols: ○, GaIN unit; A, GaIN-alditol. Products: 2, GOS2; 3, GOS3; 4, GOS40H; 5, GOS4OH; 6, GOS6OH; 7, GOS7OH. Incubation time: 10 min.

Fig. 5. HPLC Patterns of Digested GOS4.
The reaction mixture contained 2.5 mM GOS4 and 0.05 μM enzyme. About 50-μg samples were applied. The peaks of chromatogram are: 2, GOS2; 3, GOS3; 4, GOS4; 6, GOS6. Incubation time: 10 min (upper), 2 hr (middle), 24 hr (lower).

upwards to GOS7OH, the enzyme mainly cleaved the bond between the third and the fourth from the non-reducing end. But on GOS6OH, the enzyme only cleaved the bond between the second and third because of the products (GOS2 and GOS40H). On the other hand, on GOS5OH, the enzymatic products were GOS2, GOS3, and GOS40H. GaIN was not produced in any case like GOSs digestion.

Transglycosylating activity of endo α-1,4 polygalactosaminidase
To analyze the unusual phenomena of the digestions of GOS4 and GOS5OH, the digesting-profile of GOS4 was studied at higher substrate concentration. The results are shown in Fig. 5. By the enzyme reaction, GOS6 was made from GOS4 as an intermediate, and GOS3 was thought to be from the GOS6.

Discussion
Under the described conditions, purified endo α-1,4 polygalactosaminidase from Pseudomonas sp. 881 did not produce galactosaminomonomer from polygalactosamine (Fig. 1) and this is the characteristic distinguishing the enzyme from many other endo-type glycanases that produce monomers from corresponding polymers.

The product distribution analysis provided detailed information on the enzyme reaction mode with GOSs. The enzyme adhered to produce GOS3 from GOSs (DP = 4—10) except GOS4 (Table 1). On GOS5, the enzyme divided into GOS2 and GOS3 or GOS3 and GOS2 from the non-reducing end. On the other hand, on GOS4, not only did the enzyme produce GOS2, but it produced GOS3 without producing GaIN.

The subsite size of endoglycanase can be directly estimated from the dependency of the $k_a$ on the chain length of the substrate. The $k_a$ was reduced according to the decrease of DP, when DP of the substrate were under 8 (Table II). On the other hand, $K_{m}$, log($k_0/K_{m}$) and log($k_0/K_{m}$) (Fig. 2) were almost constant upwards to DP 8. These results strongly suggested that the enzyme had an 8 galactosamine-unit subsite size.

From the enzyme reaction on GOS7 (Fig. 3) it was found that the enzyme reaction to produce GOS3 was the main reaction, but the sub-reaction to produce GOS2 was also detected. On the other hand, GOS40H produced from GOS7OH was not divided any more, and so the amount of GOS2 produced was very small. These results strongly suggested the catalytic site of the endo α-1,4 polygalactosaminidase was between the third and the fourth site from the non-reducing end.

The enzyme mainly cleaved the glycosyl bond between the third and the fourth from the non-reducing and of GOS-alditols upwards to GOS7OH (Fig. 4). On the other hand, on GOS6OH, the enzyme only produced GOS2 and GOS40H. This result suggests that the enzyme cannot recognize the structure of the GaIN-alditol residue as that of GaIN, and that GOS5 was divided into GOS2 and GOS3, not into GOS3 and GOS2 from the non-reducing end. On GOS5OH, the transglycosylating activity was believed to occur from the final products (GOS2, GOS3 and GOS40H).

The best evidence of transglycosylation is given in Fig. 5. As an intermediate, GOS6 was made from GOS4 by the enzyme reaction. The production of GOS6 from GOS4 was explained as the result of translating activity. By the translating activity, the enzyme could transfer a GOS2 unit from a substrate to another substrate, and the intermediate was further divided to two GOS3 molecules, but the intermediate was not observed at lower substrate concentrations (see Table I). The velocity of the digestion of the intermediate might be too fast to detect it by the methods used here.

In conclusion, the subsite structure of α-1,4 polygalactosaminidase from Pseudomonas sp. 881 is proposed in Fig. 6. The enzyme has 8 subsites and the catalytic site is between the third and the fourth sites from the non-reducing end. Upwards from GOS6, the substrates can bind one after another from site number one. On GOS5,
the substrate does not bind to subsite 1 but binds subsites 2 to 6, probably because of a strong affinity to site 6. GOS4 binds subsites 2 to 5 and is cleaved by the transglycosylation. By model, we can explained how the enzyme does not produce galactosamine monomer from polygalactosamine.

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References