The Condensation Reaction of Aspergillus niger Crude β-Xylosidase Using Xylose

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There have been several papers published reporting the synthesis of oligosaccharides using the condensation activity of a hydrolytic enzyme.1–5 Though it is very important to investigate condensation reactions for producing useful oligosaccharides, getting information on the yield of oligosaccharides, and elucidating the kinetics and substrate specificity of carbohydrases, no condensation reactions using pentoses catalyzed by a carbohydrase have yet been reported. In this study, we report the condensation reaction of a crude β-xylosidase of Aspergillus niger IFO-6662 using xylose as a substrate, and the identification of the main products.

A. niger IFO-6662 was grown on a reciprocal shaker at 30°C for 10 days in 500-ml Sakaguchi flask containing 100 ml of a medium of 2% insoluble xylan, 1% (NH₄)₂HPO₄, 0.4% KH₂PO₄, 1% peptone, and 0.3% corn steep liquor (pH 6.4). After filtration, the culture broth was made up to 80% saturation with solid ammonium sulfate, and left for 24 hr (pH 4.5). The precipitate was collected by centrifugation (8,000 × g, 15 min) and dissolved in 0.01 M acetate buffer (pH 4.5). After it was desalted and concentrated by ultrafiltration, the solution was designated the enzyme solution of A. niger β-xylosidase. β-Xylosidase activity was measured by the method of Shinozama et al.6 using phenyl-β-xyloside as a substrate.

For the condensation reaction, a reaction mixture consisting of 1 ml of enzyme solution and 1 g of xylose (about 60% (w/v) of xylose concentration) was incubated at 50°C, at pH 4.0. As a sample, 0.1 ml of the reaction mixture was taken at appropriate intervals and 1 ml of distilled water was added. The sample was then heated in boiling water for 5 min to inactivate the enzyme, mixed with resin (Amberlite IRA-68, OH⁻ form) to decolorize and neutralize the solution, and filtered with an ultrafiltration membrane (Ultrafree C3GC, Millipore Ltd.). Then, the products were analyzed by HPLC. HPLC analysis was done with a Water’s M-45 Solvent Delivery System and differential refractometer R-401 on a Tosoh TSX-GEL G-Oligo-PW column (7.8 × 100 mm) using double-distilled water as a eluent at a flow rate of 1.0 ml/min.

The yield of the condensation reaction product was defined as the percent ratio of the peak area of xylooligosaccharides against the total peak area of all detected sugars. When β-xylosidase in a solution was inactivated by heating, no product could be detected. The yield became almost 25% and the reaction was thought to reach equilibrium after 10 days when enzyme activity was 320 U/ml.

TLC was done for qualitative analysis on a silica gel 60 precoated plate (20 × 20 cm, 0.2 mm thickness, Merck), using acetone-chloroform–methanol–water (8:1:1:0.5, v/v) as a solvent system. After development by twice ascending, sugars were detected by spraying with 20% sulfuric acid in methanol and charring for 5 min at 130°C. After 24 hr of reaction, several products were detected including a product that had the same R₇ value as authentic β-1,4-xylodisaccharide (data not shown).

Products were separated from the reaction mixture by charcoal column chromatography. A reaction mixture consisting of 50 ml of crude enzyme solution (14 U/ml) and 40 g of xylose was incubated for 7 days. Then it was adsorbed on an activated charcoal (Wako Junyaku Co.) column (column volume 970 cm³) and eluted with distilled water, 5% (v/v) ethanol, and 35% (v/v) ethanol, stepwise. As a result of TLC analysis, the main products eluted with 5% (v/v) ethanol were five kinds of xylooligosaccharides, named P1, P2, P3, P4-1, and P4-2 as shown in Fig. 1. By silica gel column rechromatography, products were purified to give only one spot by TLC analysis.

Structural analysis of these five products were done by measurement of the degree of polymerization (DP) using the method of Timelli7 and HPLC analysis, by methylation analysis using the method of Cuccari et al.8 and by specific optical rotation measurement. These data are shown in Table 1. ²H-NMR was also done to P1 and P3. The ²H-NMR spectrum for a solution in deuterium oxide was recorded at 200 MHz with a super-conduction nuclear magnetic resonance instrument AC 200 (Nihon Bruker Co., Ltd.). From the above analytical data, P1 to P4-2 were identified as:

\[ \text{P1} : \beta,\beta-1,1-xylodisaccharide \]

Fig. 1. Charcoal Column Chromatogram of Condensation Reaction Products.

Fifty ml (700 U) of A. niger enzyme solution and 40 g of xylose were incubated at 50°C, pH 4.0. After 7 days, the reaction mixture was adsorbed on the activated charcoal column (column volume 970 cm³). The stepwise elutions were done with distilled water, and 5% (v/v) and 35% (v/v) ethanol. The figure shows a chromatogram of 5% ethanol elution. The flow rate of eluent was 1 ml/min. Concentration of total sugar was measured by the phenol-H₂SO₄ method.

Table 1. Methylation Analysis, Specific Optical Rotation, and DP of P1~P4-2

<table>
<thead>
<tr>
<th>Product</th>
<th>Methylation analysis*</th>
<th>[α]D² (degrees)</th>
<th>DP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,3,4</td>
<td>2,4</td>
<td>2,3,4/3,4</td>
</tr>
<tr>
<td>P1</td>
<td>+</td>
<td>−61.6</td>
<td>(c=0.58 H₂O)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
<td>+</td>
<td>−20.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(c=3.96 H₂O)</td>
</tr>
<tr>
<td>P3</td>
<td>+</td>
<td></td>
<td>+0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(c=0.36 H₂O)</td>
</tr>
<tr>
<td>P4-1</td>
<td>+</td>
<td></td>
<td>+91.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(c=0.06 H₂O)</td>
</tr>
<tr>
<td>P4-2</td>
<td>+</td>
<td></td>
<td>−11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(c=0.50 H₂O)</td>
</tr>
</tbody>
</table>

* 2,3,4=2,3,4-tri-O-methyl-d-xylopyranoside; 2,4=2,4-di-O-methyl-d-xylopyranoside; 2,3,4/3,4=2,3-di-O-methyl-d-xylopyranoside or 3,4-di-O-methyl-d-xylopyranoside.

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DP is degree of polymerization.
P2 : β-1,4-xylodisaccharide (xylobiose)
P3 : β-1,2-xylodisaccharide
P4-1: α-1,4-xylodisaccharide
P4-2: β-1,3-xylodisaccharide (rhodimenabiose).

So we found that all kinds of β-linked xylodisaccharides were synthesized by this reaction.

From the principle that an enzyme is able to hydrolyze a product of its condensation reaction,5 these four β-linked xylodisaccharides must be hydrolyzed by A. niger β-xylanase although there may be some differences in the hydrolysis rate. Then we thought that this result meant A. niger β-xylanase has a broad specificity for β-xyllosyl linkages. Yasui et al.10 reported that β,β-1,1-xylodisaccharide was one of the products of a transxyllosylation reaction with A. niger β-xylanase, and A. niger β-xylanase has a broad specificity for the hydroxy groups of an acceptor in transxyllosylation reaction.

α-1,4-Xylodisaccharide, which should not be synthesized in the condensation reaction catalyzed by β-xylanase, was thought to be synthesized by a contaminating α-xylanase that was isolated and purified from this fungus by Zong.11

Further investigations will be done on details of the condensation reaction using purified β-xylanase to elucidate the mechanisms of hydrolytic reactions of xylooligosaccharides.

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References