Note

Substrate Selectivity in *Aspergillus niger* KU-8 Acid Phosphatase II Using Phosphoryl Oligosaccharides

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The intracellular acid phosphatase II (ACPase II) produced by *Aspergillus niger* KU-8 preferentially dephosphorylates C-6 phosphate groups rather than C-3 phosphate groups of phosphoryl oligosaccharides. In this study, the kinetic parameters of ACPase II were measured. 3'-phosphoryl maltotriose and 6'-phosphoryl maltotriose, which differ only in the binding position of the phosphate group, were prepared and used as the substrates. The $K_m$ for both substrates were similar. However, the $k_{cat}$ value for the 6'-phosphoryl maltotriose was about three-fold of that for the 3'-phosphoryl maltotriose.

Key words: acid phosphatase; *Aspergillus niger*; phosphoryl oligosaccharides; kinetic; substrate selectivity

Acid phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.2) hydrolyze a wide variety of phosphate esters and usually exhibit pH optima below 6.0. The enzymes are widely distributed in nature and occur in a variety of organs and tissues. There have been many studies on the substrate specificity of acid phosphatases, including phytases (*myo*-inositol hexakisphosphate 3- and 6-phosphohydrolases; EC 3.1.3.8 and 3.1.3.26), toward the difference of binding position of phosphate groups of phytate. However, there have been few reports that have described the substrate selectivity of phosphatases toward C-3 phosphate groups of phosphoryl saccharides.

Potato starch is known to contain a small amount of covalently-bound phosphate groups in its component, one of 200 to 500 glucosyl residues on average being phosphorylated. Takeda and Hizukuri et al. have reported that approximately 70% of the phosphate groups were bound to the C-6 position of the glucosyl residues, with almost all the rest being bound to C-3, and a very small part possibly bound to C-2. Little is known of the properties of C-3 phosphate groups because of the difficulty in preparation of authentic samples of saccharides phosphorylated at C-3. Glucose 3-phosphate was detected by acid hydrolysis of potato starch. However, because of the inherent instability of the phosphoester linkage at C-3 compared to the C-6 position, acid hydrolysis results in the fatal loss of saccharides phosphorylated at C-3. Therefore, the preparation of glucose 3-phosphate by acid hydrolysis is essentially impossible.

Recently, we prepared phosphoryl oligosaccharides from potato starch hydrolysate using bacterial liquefying α-amylase (EC 3.2.1.1), glucoamylase (EC 3.2.1.3), and pullulanase (EC 3.2.1.4). The phosphoryl oligosaccharides were fractionated into two fractions, PO-1 and PO-2. The PO-1 fraction was composed of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides having one phosphate group in each molecule and the detailed structures of PO-1 fraction were analyzed.

In a previous paper, we reported a novel intracellular acid phosphatase, ACPase II, from *Aspergillus niger* KU-8. This enzyme preferentially dephosphorylates phosphate groups of 6-phosphoryl oligosaccharides rather than those of 3-phosphoryl oligosaccharides.

Here we compare the kinetic parameters of ACPase II for 3'-phosphoryl maltotriose and 6'-phosphoryl maltotriose.
phosphoryl maltotriose as authentic samples of 3-phosphoryl oligosaccharide and 6-phosphoryl oligosaccharide, respectively. These two oligosaccharides have the same maltotriose moiety and differ only in the binding position of the phosphate group.

ACPase II was purified from a cell-free extract of \textit{A. niger} KU-8 as described previously.\textsuperscript{14} PO-1 fraction (Fig. 1(a)) was prepared from a potato starch hydrolysate as described in our previous paper.\textsuperscript{15} Total saccharide contents were measured by the phenol-sulfuric acid method.\textsuperscript{16}

We prepared 3'-phosphoryl maltotriose as follows: 1.5 ml of 20% PO-1 solution (total 18.0 g) was chromatographed on an ODS preparative column (Daisopak SP-120-10-ODS-B column, 30 × 500 mm).\textsuperscript{17} The 3-phosphoryl oligosaccharides fraction (Fig. 1(b)) were collected and concentrated with a rotary evaporator. The phosphate buffer used for ODS chromatography was removed with activated charcoal chromatography, and 3-phosphoryl oligosaccharides were eluted by 50% EtOH. Then, the mixture of 3-phosphoryl oligosaccharides (4.55 g recovery) were treated with 1000 units of BSA in 0.2 M acetate buffer (pH 5.5) at 37°C for 16 h, and 3'-phosphoryl maltotriose was prepared. Measurement of BSA activity and its action patterns on 3-phosphoryl oligosaccharides were described previously.\textsuperscript{15,16} Neutral saccharides produced by BSA reaction were also removed with the 2nd activated charcoal chromatography. Finally, 0.45 g of 3'-phosphoryl maltotriose (Fig. 1(c)) was obtained.

The preparation step of 6'-phosphoryl maltotriose was almost the same as 3'-phosphoryl maltotriose. One-half ml of 20% PO-1 solution (total 6.0 g) was chromatographed on an ODS preparative column. After the 1st activated charcoal chromatography, the mixture of 6-phosphoryl oligosaccharides (4.53 g, Fig. 1(d)) was treated with 1000 units of neopullulanase\textsuperscript{17} in 0.2 M acetate buffer (pH 6.0) at 37°C for 16 h. Measurement of neopullulanase activity and its action patterns on 6-phosphoryl oligosaccharides were described previously.\textsuperscript{18,19} Phosphate buffer used at ODS chromatography was removed with activated charcoal chromatography, and 6-phosphoryl oligosaccharides were eluted by 50% EtOH. Neopullulanase produced 6'-phosphoryl maltotriose and a small amount of 6'-phosphoryl maltose; thus ODS chromatography was used again for the removal of 6'-phosphoryl maltose. Neutral saccharides produced by the neopullulanase reaction were removed with a 2nd activated charcoal chromatography. Finally, 2.70 g of 6'-phosphoryl maltotriose (Fig. 1(e)) was obtained. After the reaction, BSA and neopullulanase were removed by ultrafiltration.

The Michaelis constant (\(K_m\)) and molecular activity (\(k_m\)) of ACPase II for pNPP, Glc1P, Glc6P, PO-1, 3'-phosphoryl maltotriose, and 6'-phosphoryl maltotriose were obtained from Lineweaver-Burk plots (Fig. 2) and listed in the Table. ACPase II activity was measured as described previously.\textsuperscript{16} To ob-

![Fig. 1. High-performance Anion Exchange Chromatograms of Preparation Step of 3'-Phosphoryl Maltotriose and 6'-Phosphoryl Maltotriose.](a) PO-1 fraction prepared from potato starch. (b) 3'-phosphoryl oligosaccharides fractionated with ODS column chromatography. (c) 3'-phosphoryl maltotriose fraction obtained by BSA treatment. (d) 6-phosphoryl oligosaccharides fractionated with ODS column chromatography. (e) 6'-phosphoryl maltotriose fraction obtained by neopullulanase treatment. The HPAEC condition was reported in our previous paper.\textsuperscript{16} Peak J: 3'-phosphoryl maltotetraose, K: 3'-phosphoryl maltopentaose, L: 6'-phosphoryl maltotriose and 6'-phosphoryl maltotetraose, M: 6'-phosphoryl maltotetraose, N: 6'-phosphoryl maltopentaose, and P: 3'-phosphoryl maltotriose.
Fig. 2. Lineweaver-Burk Plots of ACPase II on 3'-Phosphoryl Maltotriose and 6'-Phosphoryl Maltotriose. Fifty microliters of the enzyme (fixed at 0.01 unit, measured by p-nitrophenol release from pNPP) was incubated with various concentrations of each substrate (125 µl) for 2, 5, 10, and 15 min at 37°C in 0.2 M glycine-HCl (pH 2.0) buffer (325 µl). Initial velocities were calculated by measuring the release of inorganic phosphate from each substrate. v, initial velocity for hydrolysis of substrate (nm product per min per mg protein); S, substrate concentration (mm). O, 3'-phosphoryl maltotriose; ●, 6'-phosphoryl maltotriose.

Table. Michaelis Constant ($K_m$) and Molecular Activity ($k_{cat}$/Km) of ACPase II

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ (mm)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}$/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPP</td>
<td>1.09</td>
<td>5.72</td>
<td>5.25</td>
</tr>
<tr>
<td>Glc1P</td>
<td>2.00</td>
<td>3.34</td>
<td>1.67</td>
</tr>
<tr>
<td>Glc6P</td>
<td>1.76</td>
<td>5.20</td>
<td>2.95</td>
</tr>
<tr>
<td>PO-1</td>
<td>21.2</td>
<td>8.10</td>
<td>0.38</td>
</tr>
<tr>
<td>3'-phosphoryl maltotriose</td>
<td>14.1</td>
<td>1.82</td>
<td>0.13</td>
</tr>
<tr>
<td>6'-phosphoryl maltotriose</td>
<td>14.5</td>
<td>4.85</td>
<td>0.34</td>
</tr>
</tbody>
</table>

tain the kinetic parameters of ACPase II, one unit of enzyme activity was defined as 1 µmol of inorganic phosphate release from pNPP or phosphorylated saccharides per min.10) Kinetic parameters for 3'-phosphoryl maltotriose, 6'-phosphoryl maltotriose, and PO-1 were calculated by using molecular weights of 3'-phosphoryl maltotriose and 6'-phosphoryl maltotriose (both 582.4, as free acid form), and PO-1 fraction (average 750). The $K_m$ of ACPase II for 3'-phosphoryl maltotriose was almost the same as that for 6'-phosphoryl maltotriose. However, the $k_{cat}$ for 3'-phosphoryl maltotriose was about one-third of that for 6'-phosphoryl maltotriose. Therefore, it is clear that the differences of ACPase II specificity toward phosphate groups of 3'-phosphoryl maltotriose and 6'-phosphoryl maltotriose were due to the difference of $k_{cat}$.

When reacted with the PO-1 fraction, ACPase II dephosphorylated all of the C-6 phosphate groups of 6-phosphoryl oligosaccharides irrespective of their DPs.10) Therefore, it would be reasonable that the dephosphorylation rates of ACPase II toward 6'-phosphoryl maltotriose and other 6-phosphoryl oligosaccharides are almost the same. In 3'-phosphoryl oligosaccharides, the activity of ACPase II toward 3'-phosphoryl maltotetrose was also almost the same for 3'-phosphoryl maltopentaose as the case of 6-phosphoryl oligosaccharides, although its dephosphorylation rate of C-3 phosphate groups was much slower than that of C-6.16) Therefore, the ACPase II activity toward 3-phosphoryl oligosaccharides (3'-phosphoryl maltotetrose and 3'-phosphoryl maltopentaose) would also be similar to 3'-phosphoryl maltotriose. From these results, described above, it seems reasonable to suppose that the differences of ACPase II specificity toward C-3 or C-6 phosphate groups of phosphoryl oligosaccharides were due to the difference of $K_m$.

With regard to other substrates, the $K_m$ of ACPase II for pNPP was higher than that of other acid phosphatases. For example, 0.27 mm with Aspergillus ficuum,5) 0.28 mm with yellow lupin,20) 0.27 mm (at pH 3.8) with human prostate,21) and 1.0 mm with A. niger.20) Glc6P was a slightly better substrate of ACPase II than Glc1P.

Further studies are now in progress to clone the encoding gene and to analyze the structure of ACPase II. The 3'-phosphoryl maltotriose and 6'-phosphoryl maltotriose will be useful substrates to analyze the catalytic center of ACPase II or other phosphatases including phytases.

References

5) Ullah, A. H. J. and Cummins, B. J., Purification, N-terminal amino acid sequence and characterization of pH 2.5 optimum acid phosphatase (E.C 3.1.3.2) from...