学位論文内容の要旨

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学位論文題名

Functional analysis of maltose phosphorylase MalE from *Bacillus* sp. AHU2001 and synthesis of oligosaccharides and sugar phosphates with the enzyme

(*Bacillus* sp. AHU 2001 由来マルトースホスホリラーゼ MalE の機能ならびに本酵素を用いたオリゴ糖および糖リン酸の合成に関する研究)

Maltose phosphorylase (MP, EC 2.4.1.8), belonging to glycoside hydrolase family (GH) 65, catalyzes the reversible phosphorolysis of maltose to β-D-glucose 1-phosphate (β-Glc1*P*) and D-glucose. Some bacteria such as *Bacillus subtilis* and *Lactobacillus acidophilus* utilize MP along with α-glucosidase and neopullulanase for the intracellular metabolism of maltodextrin and maltose. MP has a broad acceptor specificity in the reverse phosphorolysis, and synthesizes efficiently several non-natural α-(1→4)-glucosides through reverse phosphorolysis. Furthermore, MP is a useful enzyme for the production of β-Glc1*P*, because it uses maltose, which can be easily produced from abundant starch, as substrate. By coupling the reaction of MP and other α-glucoside phosphorylases, several oligosaccharides such as trehalose and nigerose are produced from maltose. Previously, the gene (*malE*), encoding GH 65 enzyme, was found in the downstream of the GH31 α-glucosidase gene (*BspAG31A*) of *Bacillus* sp. AHU2001. The deduced amino acid sequence of MalE shows 52–60% identity with that of characterized MPs. In this study, enzymatic characteristics of recombinant MalE produced in *Escherichia coli* were investigated, and acceptor specificity of this enzyme was described in detail to advance understanding of acceptor specificity of MP. Furthermore, the chemoenzymatic synthesis of oligosaccharides and sugar phosphates, including β-Glc1*P* and D-glucose 6-phosphate (Glc6*P*), using MalE was established.

1. Biochemical characterization of MalE

Recombinant MalE was produced in *E. coli* BL21 (DE3) transformant, and purified to homogeneity by nickel affinity column chromatography. From the cells obtained from 2 L of the culture broth, 13.6 mg of purified enzyme was obtained. The phosphorolytic activity of purified enzyme to 4 mM maltose in 10 mM sodium phosphate buffer (pH 8.0) was 15.9 U/mg. The molecular mass of MalE was 90 kDa as determined by SDS-PAGE and 193 kDa as determined by gel-filtration column chromatography, indicating that MalE exists as homodimer under nondenaturing conditions. MalE showed phosphorolytic activity toward maltose, but not toward other α-linked glucobioses and maltotriose. It showed the highest activity at pH 8.1 and at 45°C. It retained higher than 80% of original activity in a pH range of 4.5–10.4 after

the pH treatment at 4°C for 24 h and at ≤40°C after the heat treatment at pH 8.0 for 15 min. In the phosphorolysis of maltose, the reaction equation for a sequential bi bi mechanism fitted well to the reaction rates at various maltose and phosphate concentrations: k_{cat}, 30.9 ± 0.6 s^{-1}; K_{mA}, 0.295 ± 0.059 mM; K_{mB}, 0.835 ± 0.123 mM; and K_{iA}, 9.07 ± 1.74 mM (A, phosphate; B, maltose). In the reverse phosphorolysis, MalE utilized D-glucose, 1,5-anhydro-D-glucitol, methyl α-D-glucoside, 2-deoxy-D-glucose, D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, kojibiose, 3-deoxy-D-glucose, D-allose, 6-deoxy-D-glucose, D-xylose, D-lyxose, L-fucose, and L-sorbose as acceptors. In the kinetic analysis of reverse phosphorolysis in the presence of 10 mM β-Glc1*P*, the $k_{cat(app)}/K_{m(app)}$ of D-glucosamine was as high as that of D-glucose, although the $k_{cat(app)}/K_{m(app)}$ values for 2-deoxy-D-glucose and D-mannose were much lower than that for D-glucose, indicating that the equatorial 2-hydroxy group or amino group (hydrophilic chemical group) is required for good acceptors. MalE utilized also kojibiose as a sole disaccharide acceptor. Together with a modelled structure of MalE protein, the 2-OH group of the D-glucosyl residue in subsite +1 is possibly placed to be exposed to the solvent in the Michaelis complex structure of MalE. The $k_{cat(app)}/K_{m(app)}$ for 6-deoxy-D-glucose was comparable with that of D-glucose but that for D-xylose was lower, indicating that 6-C of D-glucose is involved in binding at subsite +1. Furthermore, MalE exhibited very low synthetic activity to all the tested 1- and 3-OH D-glucose derivatives, suggesting that 1- and 3-hydroxy groups of D-glucose are important for the substrate binding.

2. Chemoenzymatic synthesis of oligosaccharides and sugar phosphates by MalE

Oligosaccharides produced by the reverse phosphorolysis of MalE with 2-deoxy-D-glucose, D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, L-sorbose, D-allose, D-xylose and D-lyxose were prepared. MalE produced α-(1→3)-glucosides through the phosphorolysis with 2-deoxy-D-glucose and L-sorbose, whereas it produced α-(1→4)-glucosides in the reaction with the other acceptors. The digestion rate of synthesized oligosaccharides by rat intestinal acetone powder was 28.0–45.0% of that of maltose, indicating that the synthesized oligosaccharides might act as prebiotic oligosaccharides. Sugar phosphate β-Glc1*P* and Glc6*P* were produced through phosphorolysis of maltose by the action of MalE. The yield of β-Glc1*P* from 100 mM maltose and 400 mM sodium phosphate buffer (pH 7.0) reached 62.1% at 72 h in the presence of yeast, which consumed D-glucose generated by the phosphorolysis of maltose. By addition of β-phosphoglucomutase to the reaction, Glc6*P* was produced in a yield of 79.7% at 48 h.