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Characterization and crystal structure determination of β-1,2-mannobiose phosphorylase from *Listeria innocua*

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ABSTRACT

Glycoside hydrolase family 130 consists of phosphorylases and hydrolases for β-mannosides. Here, we characterized β-1,2-mannobiose phosphorylase from *Listeria innocua* (Lin0857) and determined its crystal structures complexed with β-1,2-linked mannooligosaccharides. β-1,2-Mannotriose was bound in a U-shape, interacting with a phosphate analog at both ends. Lin0857 has a unique dimer structure connected by a loop, and a significant open–close loop displacement was observed for substrate entry. A long loop, which is exclusively present in Lin0857, covers the active site to limit the pocket size. A structural basis for substrate recognition and phosphorolysis was provided.

1. Introduction

Glycoside phosphorylases (GPs) are useful tools for efficient oligosaccharide synthesis because of the reversible nature of their reactions [1–3]. Although increasing numbers of GPs have been reported recently, their utilization for the production of various oligosaccharides remains limited by the extent of known and characterized enzymes. In the Carbohydrate-Active enZyme database [4], anomer-inverting GPs are all categorized into glycoside hydrolase (GH) families: GH65, GH94, GH112, and GH130. Among them, GH130 is the most-recently established family, which mainly consists of GPs acting on β-mannosides. Approximately 800 members of GH130 were divided into at least two characterized subfamilies by phylogenetic analysis (Supplementary Fig. S1) [5]. GH130_1 contains two 4-*O*-β-D-mannosyl-D-glucose phosphorylases (EC 2.4.1.281) from *Bacteroides fragilis* (MGP, locus tag BF0772) [6], and *Ruminococcus albus* (RaMP1, Rumal_0852) [7], and GH130_2 consists of enzymes with relatively relaxed specificities: β-1,4-mannooligosaccharide phosphorylase (EC 2.4.1.319) from *R. albus* (RaMP2, Rumal_0099) [7], 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase (EC 2.4.1.320) from *Bacteroides thetaiotaomicron* (BT1033) [8], and β-D-mannopyranosyl-1,4-*N*,*N*'-diacetylchitobiose phosphorylase (EC 2.4.1.-) from the human gut bacterial metagenome (UhgbMP) [5]. These enzymes are involved in the bacterial utilization of mannans or *N*-linked glycans.

Recently, several GH130 enzymes with specificities for β-1,2-linked mannosidic linkages were found. From *Thermoanaerobacter* sp. X-514, two GPs were characterized: 1,2-β-oligomannan phosphorylase (Teth514_1788) and β-1,2-mannobiose phosphorylase (Teth514_1789) [9]. In the synthetic reaction, Teth514_1789 and Teth514_1788 produced β-1,2-mannooligosaccharides with degrees of polymerization up to 3 and 5, respectively. Moreover, two hydrolytic enzymes were very recently identified:

Abbreviations: GH, glycoside hydrolase; GP, glycoside phosphorylase; β-1,2-Man_2, β-1,2-mannobiose; β-1,2-Man_3, β-1,2-mannotriose; MGP, 4-*O*-β-D-mannosyl-D-glucose phosphorylase; α-Man1*P*, α-D-mannose 1-phosphate; P_i, inorganic phosphate

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β-1,2-mannosidases from *B. thetaiotaomicron* (BT3780) [10] and *Dyadobacter fermentans* (Dfer_3176) [11]. These two GHs have two conserved glutamic acid residues as candidates for catalytic bases at the position corresponding to the phosphate-binding site of GPs. These enzymes acting on β-1,2-linked mannosides might be involved in the bacterial utilization of glycans produced by *Candida albicans* and other human-associated microorganisms [9,10].

Currently, crystal structures of MGP [12], UhgbMP [13], and BT3780 [10] have been reported, and the coordinates of four uncharacterized proteins (TM1225, BT4094, BDI_3141, and BACOVA_03624) are available from structural genomics projects. However, the three-dimensional structure of β-1,2-linkage-specific phosphorylase has not yet been determined. In this study, we characterized a GH130 enzyme from *Listeria innocua* (Lin0857) as β-1,2-mannobiose phosphorylase and determined its crystal structures. Complex structures with D-mannose (Man), β-1,2-mannobiose (β-1,2-Man_2), and β-1,2-mannotriose (β-1,2-Man_3) revealed the detailed molecular mechanism of the substrate recognition.

2. Materials and methods

2.1. Protein preparation and enzyme assay

The gene encoding Lin0857 (GenBank ID: CAC96089) was amplified from the genomic DNA of *L. innocua* Clip11262 via a PCR, and the amplified gene was inserted into pET-24a(+) (Novagen, Madison, WI) to encode a C-terminally $(His)_6$-tagged protein. *Escherichia coli* harboring the expression plasmid was used to express the recombinant protein. The cells were harvested and sonicated, and the protein was purified to homogeneity by serial column chromatography. The activities of the synthetic and phosphorolytic reactions were measured as described previously [9,14]. Detailed procedures for the protein preparation, purification, enzyme characterization, and structure determination of the reaction products are described in the Supplementary Methods.

2.2. Crystallography

Crystals of Lin0857 were obtained at 20 °C using the sitting drop vapor diffusion method by mixing 1.0 μl of solution containing 8 mg/ml protein with an equal volume of a reservoir solution, which contained 1.8 M $(NH_4)_2SO_4$, 10 mM $CoCl_2$, and 0.1 M MES-NaOH (pH 6.6). Crystals were cryoprotected using 20% (*v*/*v*) glycerol or 20% (*w*/*v*) Man (Man complex). Crystals complexed with β-1,2-Man_2 and β-1,2-Man_3 were prepared by soaking in the cryoprotectant solution supplemented with 50 mM β-1,2-Man_2 or 10 mM β-1,2-Man_3. The crystals were flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected at the Photon Factory of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan). The programs used for crystallography are described in the Supplementary Methods. The statistics for data collection and refinement are provided in Supplementary Table S1.

3. Results and discussion

3.1. Characterization of Lin0857

Because we noticed that Lin0857 is located between β-1,2- and β-1,4-specific enzymes in a phylogenetic tree of GH130 (Supplementary Fig. S1), we prepared and characterized its recombinant protein. The molecular masses of purified Lin0857 protein as deduced from the amino acid sequence and estimated by SDS–PAGE and calibrated gel filtration chromatography were 41, 40, and 88kDa, respectively, suggesting that it is dimeric in solution.

Table 1
The kinetic parameters for the synthetic reactions.

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Acceptor			
D-Mannose	31 ± 1	2.1 ± 0.4	15 ± 2
D-Fructose	3.9 ± 0.2	1.5 ± 0.2	2.7 ± 0.4
β-1,2-Man_2	12 ± 0.5	5.1 ± 0.7	2.3 ± 0.3
Donor			
α-Man1*P*	33 ± 3	3.1 ± 0.9	11 ± 3

The kinetic parameters were calculated by fitting the initial velocities to various concentrations of acceptor or donor substrates in the presence of 10 mM α-Man1*P* (donor) or 10 mM D-mannose (acceptor), using the Michaelis–Menten equation.

Table 2
The kinetic parameters for the phosphorolytic reactions.

	k_{cat} (s^{-1})	K_{mA} (mM)	K_{mB} (mM)	K_{iA} (mM)	k_{cat}/K_{mA} (s^{-1} mM^{-1})
β-1,2-Man_2	35 ± 2	0.44 ± 0.10	0.19 ± 0.03	1.1 ± 0.3	79 ± 15
β-1,2-Man_3	12 ± 1	4.1 ± 0.8	0.13 ± 0.05	10 ± 4	3.0 ± 0.4

The kinetic parameters were calculated by fitting the initial velocities to 0.3–10 mM substrate in the presence of 0.1–1.0 mM P_i using the following theoretical equation for a sequential Bi Bi mechanism: $v = k_{cat}[E]_0\,[A]\,[B]/(K_{iA}K_{mB} + K_{mA}\,[B] + K_{mB}\,[A] + [A]\,[B])$, where A is the substrate, and B is P_i.

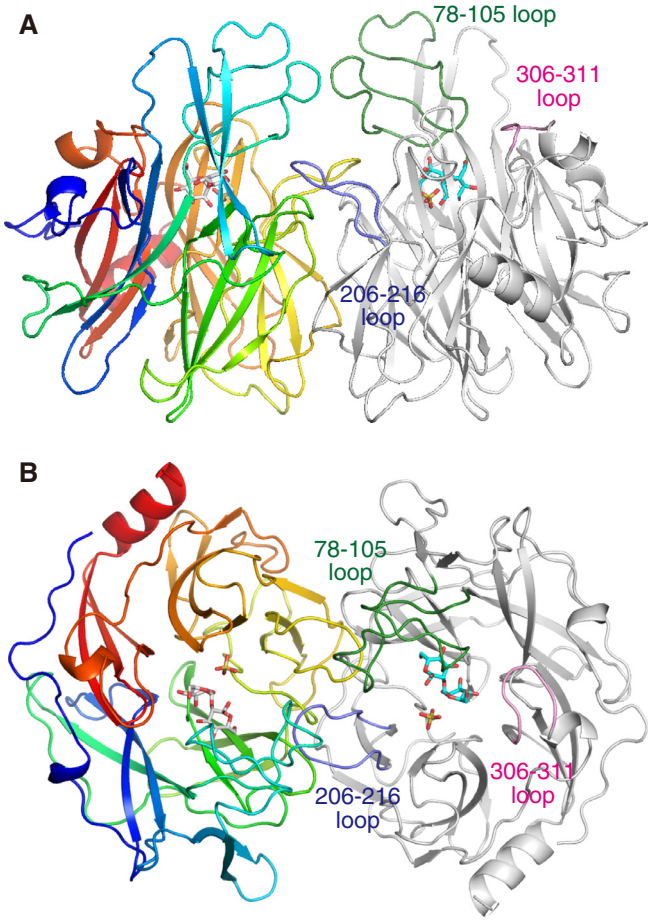

Fig. 1. Dimer structure of Lin0857 complexed with β-1,2-Man_2. One monomer is shown in rainbow color, and the other is shown mainly in gray with three loops highlighted. β-1,2-Man_2 and sulfate are shown as sticks. Two different views (90° rotation around a horizontal axis) are shown in (A) and (B).