Characterization of Three β-Galactoside Phosphorylases from Clostridium phytofermentans

**DISCOVERY OF D-GALACTOSYL-β1→4-L-RHAMNOSE PHOSPHORYLASE**

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We characterized three D-galactosyl-β1→3-N-acetyl-d-hexosamine phosphorylase (EC 2.4.1.211) homologs from *Clostridium phytofermentans* (Cphy0577, Cphy1920, and Cphy3030 proteins). Cphy0577 and Cphy3030 proteins exhibited similar activity on galacto-N-biose (GNB; d-Galβ1→3-d-GalNAc) and lacto-N-biose I (LNB; d-Gal-β1→3-d-GlcNAc), thus indicating that they are D-galactosyl-β1→3-N-acetyl-d-hexosamine phosphorylases, subclassified as GNB/LNB phosphorylases. In contrast, Cphy1920 protein phosphorylated neither GNB nor LNB. It showed the highest activity with l-rhamnose as the acceptor in the reverse reaction using α-D-galactose 1-phosphate as the donor. The reaction product was D-galactosyl-β1→4-l-rhamnose. The enzyme also showed activity on l-mannose, l-lyxose, d-glucose, 2-deoxy-d-glucose, and D-galactose in this order. When d-glucose derivatives were used as acceptors, reaction products were β-1,3-galactosides. Kinetic parameters of phosphorolytic activity on D-galactosyl-β1→4-l-rhamnose were $k_{cat} = 45 \text{ s}^{-1}$ and $K_m = 7.9 \text{ mm}$, thus indicating that these values are common among other phosphorylases. We propose D-galactosyl-β1→4-l-rhamnose phosphorylase as the name for Cphy1920 protein.

Phosphorylases are a group of enzymes involved in formation and cleavage of glycoside linkage together with glycoside hydrolyses and glycosyl-nucleotide glycosyltransferases (syntheses). Phosphorylases, which reversibly phosphorolyze oligosaccharides to produce monosaccharide 1-phosphate, are generally intracellular enzymes showing strict substrate specificity. Physiologically, such strict substrate specificity is considered to be closely related to the environment containing bacteria possessing them. For example, D-galactosyl-β1→3-N-acetyl-d-hexosamine phosphorylase (GalHexNACp; EC 2.4.1.211) from *Bifidobacterium longum*, an intestinal bacterium, forms part of the pathway metabolizing galacto-N-biose (GNB; d-Galβ1→3-d-GalNAc) from mucin and lacto-N-biose I (LNB; d-Gal-β1→3-d-GlcNAc) from human milk oligosaccharides, both of which are present in the intestinal environment, with GNB- and LNB-releasing enzymes and GNB/LNB transporter (1–8). Another example is cellobiose phosphorylase from *Cellvibrio gilvus*, which is a cellulolytic bacterium. Cellobiose phosphorylase forms an important cellulose metabolic pathway with an extracellular cellulase system producing cellobiose (9, 10).

The reversible catalytic reaction of phosphorylases is one of the most remarkable features that make them suitable catalysts for practical syntheses of oligosaccharides. An oligosaccharide can be produced from inexpensive material by combining reactions of two phosphorylases, one for phosphorolyzing the material and the other for synthesizing the oligosaccharide, in one pot. Based on this idea, LNB is synthesized on a large (kg) scale using sucrose phosphorylase and GalHexNACp (11). Practical synthesis methods of trehalose and cellobiose have also been developed (12, 13). However, only 14 kinds of substrate specificities have been reported among phosphorylases (13), thus restricting their use. Therefore, it would be useful to find a phosphorylase with novel activity.

GalHexNACp phosphorylizes GNB and LNB to produce α-D-galactose 1-phosphate (Gal 1-P) and the corresponding N-acetyl-d-hexosamine. To date, GalHexNACp is the only phosphorylase known to act on β-galactoside. This enzyme was first found in the cell-free extract of *Bifidobacterium bifidum* (14) and then in *B. longum* (1, 15), *Clostridium perfringens* (16), *Propionibacterium acetogenes* (17), and *Vibrio vulnificus* (18). These studies revealed that GalHexNACPs were classified into three subgroups based on substrate preference between GNB and LNB. These subgroups are as follows: 1) galacto-N-biose/lacto-N-biose I phosphorylase (GLNBP), showing similar activity on both GNB and LNB (*B. longum* and *B. bifidum*); 2) galacto-N-biose phosphorylase (GNBP), preferring GNB to LNB (*C. perfringens* and *P. acetogenes*); and 3) lacto-N-biose 1 phosphorylase (LNBP), preferring LNB to GNB (*V. vulnificus* (18)). The tertiary structure of GLNBP from *B. longum* (GLNBP$_b$) has been revealed recently (19). Based on the similarity in ternary structures between GLNBP$_b$ and β-galactosidase from *Thermus* β1→3-d-glucose; GalRhaP, D-galactosyl-β1→4-l-rhamnose phosphorylase; RG-I, rhamnogalacturonan I; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

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□

The abbreviations used are: GalHexNACP, D-galactosyl-β1→3-N-acetyl-d-hexosamine phosphorylase; GNB, galacto-N-biose; LNB, lacto-N-biose I; Gal 1-P, α-D-galactose 1-phosphate; GLNBP, galacto-N-biose/lacto-N-biose I phosphorylase; GNBP, galacto-N-biose phosphorylase; LNBP, lacto-N-biose I phosphorylase; GLNBP$_b$, galacto-N-biose/lacto-N-biose I phosphorylase from *B. longum*; GalRha, D-galactosyl-β1→4-l-rhamnose; L-Rha, l-rhamnose; l-Man, l-mannose; l-Lyx, l-lyxose; D-Glc2d, 2-deoxy-D-glucose; HPLC, high performance liquid chromatography; GalGlc, D-galactosyl-
Experimental Procedures

Cloning, Expression, and Purification—C. phytofermentans was purchased from the American Type Culture Collection (Manassas, VA). C. phytofermentans was cultured anaerobically using Anaeropack (Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan) at 30 °C for 4 days on a modified GAM agar plate (Nissui Pharmaceutical Co., Tokyo, Japan). Genomic DNA of C. phytofermentans was obtained from colonies on the plate, using InstaGene™ matrix (Bio-Rad). GalHexNACP homologous genes were amplified by PCR using KOD plus DNA polymerase (Toyobo, Osaka, Japan) and genomic DNA of C. phytofermentans as the template. Primer pairs for cphy0577, cphy1920, and cphy3030 genes were forward primer 5′-ggggtgaacctgagaaagatagctgt-3′ and reverse primer 5′-aacctgaacctgctgctct-acc-3′, forward primer 5′-agagagatcatagccacaaacagagag-3′ and reverse primer 5′-taagtctctgataggaattcagacg-3′, and forward primer 5′-gataacacaggtaagaaaacg-3′ and reverse primer 5′-gagttaatccgatctcctcataagg-3′, respectively. These forward primers contained Ncol or Ndel sites (underlined), and the reverse primers contained Xhol sites (underlined). Amplified cphy0577 and cphy3030 genes were inserted into the Ncol and Xhol sites of pET28a(+) (Novagen, Madison, WI), and the amplified cphy1920 gene was inserted into the Ndel and Xhol sites of pET30a(+) (Novagen, Madison, WI) to add His6 tags at the C termini. Escherichia coli BL21(DE3) was transformed by each constructed plasmid. Each transformant was grown in 150 ml of Luria-Bertani medium containing 30 μg/ml kanamycin at 37 °C to an absorbance of 0.8 at 660 nm in a 500-ml conical flask; the transformant was then treated with 0.1 mM isopropyl-β-D-thiogalactopyranoside. After induction, the transformant for the cphy0577 gene was cultured at 30 °C for 5 h, and the other two were cultured at 20 °C overnight with agitation at 160 rpm. Wet cells were collected by centrifugation at 5000 × g for 5 min, and then each sample was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl (buffer A). Each sample was disrupted by sonication and centrifuged at 15,000 × g for 15 min and was then loaded on an Ni2+-nitrilo-triacetic acid-agarose (7 ml) (Qiagen, Hilden, Germany) column equilibrated with buffer A containing 20 mM imidazole. The column was washed with buffer A containing 20 mM imidazole until almost all unbound components were removed, and each recombinant protein was eluted with a linear gradient of 20–250 mM imidazole at a flow rate of 1.8 ml/min. The buffer of each sample was then changed to 50 mM imidazole, and thus, the synthetic activity was determined (24). Synthetic activity was determined using Anaicon Ultra 10,000 molecular weight cut-off (Millipore, Billerica, MA). Finally, 22, 12, and 16 mg of recombinant Cphy0577, Cphy1920, and Cphy3030 proteins were obtained from 150 ml of culture medium, respectively. Single bands were detected at 80 kDa on SDS-PAGE, corresponding to the calculated molecular mass of recombinant proteins with His6 tag (84864, 84232, and 84937 Da, respectively).

Measurement of Phosphorolytic and Synthetic Activity—Phosphorolytic activity was determined by quantifying the Gal phosphate in the reaction mixture.
containing 10 mM Gal 1-P and 10 mM acceptor in 100 mM MOPS buffer (pH 7.0), using the method of Lowry and Lopez (25) as described below. The substrate solution (142.5 μl; 10 mM Gal 1-P and 10 mM acceptor in 100 mM MOPS buffer (pH 7.0)) was preincubated at 37 °C for 10 min and then mixed with 7.5 μl of an enzyme solution to start the reaction. An aliquot (12.5 μl) was added to 100 μl of 0.2 M sodium acetate (pH 4.0) to stop the enzymatic reaction. Then 12.5 μl of 1% ammonium molybdate containing 25 mM sulfuric acid and 12.5 μl of 1% ascorbic acid with 0.05% potassium bisulfite were mixed with the samples. The mixtures were incubated at 37 °C for 1 h, and the concentration of phosphate formed was quantified by measuring absorbance at 700 nm.

Temperature and pH Profile—Thermostability was defined as residual activity after incubation of enzyme (2.2 mg/ml) at various temperatures in 20 mM MOPS buffer (pH 7.0) for 30 min. Residual activities were determined by measuring synthetic activities using D-GlcNAc as an acceptor for Cphy0577 and Cphy3030 proteins and L-rhamnose (L-Rha) as an acceptor for Cphy1920 protein. When pH activity was determined, synthetic activity was measured in various 100 mM buffers (NaOAc, pH 4.5–5.5; MES, pH 5.5–6.5; MOPS, pH 6.5–7.5; Tris-HCl, pH 7.5–9.0). The acceptors used were the same as those in thermostability experiments. Samples were taken every 0.7 min for 4.9 min to determine phosphate concentration. The time course increase in phosphate concentration was fitted with linear regression to determine the reaction velocity.

Thin Layer Chromatography—An assay was performed using 10 μl of substrate solution. The reaction mixture (1 μl) was spotted onto a TLC plate (Kieselgel 60 F254; Merck), and the sample was developed with a solution of acetonitrile/water (3:1, v/v). The TLC plates were briefly soaked in 5% sulfuric acid/methanol solution and heated in an oven until bands were sufficiently visualized.

Structural Analysis of the Products of Cphy1920 Protein—Each reaction mixture (1 ml) containing 50 mM Gal 1-P, 50 mM acceptor (L-Rha, L-mannose (L-Man), L-lyxose (L-Lyx), D-glucose (D-Glc), 2-deoxy-D-glucose (D-Glc2d), or D-Gal), and Cphy1920 protein (6.9 μg for L-Rha and 23 μg for other substrates) in 100 mM MOPS (pH 7.0) was incubated at 30 °C (overnight for L-Rha and 3 days for other substrates). The reaction mixtures were loaded onto a Toyopearl HW-40F column (2.6 cm × 90 cm; TOSOH, Tokyo, Japan) pre-equilibrated with distilled water, and the products were eluted by distilled water at a flow rate of 1.8 ml/min. Fractions containing a disaccharide were collected and desalted with Amberlite MB3 (Organo, Tokyo, Japan), followed by lyophilization. The amount of products obtained were 7.9, 9.1, 9.1, 10.1, 9.4, and 12.5 mg, respectively. The one-dimensional (1H and 13C) and two-dimensional (double-quantum-filtered COSY, heteronuclear single-quantum coherence, and heteronuclear multiple-bond correlation) NMR spectra of the products were taken in D2O using a Bruker Avance 500 spectrometer with 2-methyl-2-propanol as an internal standard. Proton signals were assigned based on double-quantum-filtered COSY spectra; 13C signals were assigned with heteronuclear single-quantum coherence spectra, based on assignment of proton signals. The position of linkage in each disaccharide was determined by detecting interring cross-peaks in each heteronuclear multiple-bond correlation spectrum.

Kinetic Analysis—In order to determine kinetic parameters, assays of phosphorolytic reactions were performed with various concentrations of LNB or GNB in the presence of 10 mM phosphate for Cphy0577 and Cphy3030 proteins and with various concentrations of GalRha and phosphate for Cphy1920 protein. Assays of synthetic reactions were performed with various concentrations of acceptors in the presence of 10 mM Gal 1-P. The kinetic parameters were calculated by curve fitting the experimental data with the theoretical equation, using Grafit version 4 (Erithacus Software, Middlesex, UK).

Anomeric Specificity of Cphy1920 Protein—The substrate solution was prepared by mixing 5 μl of 500 mM acceptors (L-Rha or D-Glc) and 25 μl of 100 mM Gal 1-P (adjusted pH at 7.2 with 1 N HCl) and keeping the mixture at room temperature (24 °C). The reaction was started by adding 20 μl of enzyme solution in 20 mM MOPS (pH 7.0, 0.48 mg/ml enzyme for L-Rha and 19 mg/ml enzyme for D-Glc), kept at room temperature, to the substrate solution (final concentration of acceptors and Gal 1-P was 50 mM). The anomeric forms of each substrate and product were quantified using high performance liquid chromatography (HPLC) under the following conditions. After 1- or 20-min incubation, 10 μl of the reaction mixture was injected onto a TSK amide-80 column (4.6-mm internal diameter × 25 cm; Tosoh, Tokyo, Japan) equilibrated with 75% acetonitrile. Samples were eluted with 75% acetonitrile at a flow rate of 1.5 ml/min. Saccharides were detected using a refractive index detector, RI model 504 (GL Science, Tokyo, Japan). Peaks of both anomers of the disaccharides were distinguished based on the existence ratio of the ρ-anomer over the β-anomer determined by NMR.

RESULTS

Sequence Analysis—Multiple alignment of GLNBP, GNBP, LNB, and three GalHexNAcP homologs from C. phytofermentans is shown in Fig. 2. Because catalytic and substrate recognition residues of GLNBP have already been identified from its ternary structure (19), we compared the corresponding residues of these proteins. The catalytic proton donor of GLNBP (Asp113) was conserved in Cphy0577, Cphy1920, and Cphy3030 proteins (Fig. 2). Recognition residues of galactose at subsite −1 (Asn166, Asp313, Tyr362, and Phe364), phosphate-binding residues (Arg32, Arg210, and Arg358), and residues that recognize the oxygen atom in the N-acetyl group of acceptors (Trp233) in GLNBP were also conserved in the other five proteins (Fig. 2). Val162 of GLNBP is a primary candidate for determining substrate preference between GNB and LNB. The residue is conserved in LNB from V. vulnificus but is substituted by threonine in GNBP from C. perfringens (19) (Fig. 2). Because Val162 of GLNBP is conserved in Cphy0577 and Cphy3030 proteins, they were predicted to be GLNBP or LNB. On the contrary, substrate specificity of Cphy1920 protein was unpredictable, because the corresponding residue of Cphy1920 protein was isoleucine, and Cphy1920 protein had low identity with the other proteins around the proton donor aspartate residue (Fig. 2).
Cphy0577, Cphy1920, and Cphy3030 proteins are predicted not to possess N-terminal signal peptides as well as known GalHexNAcPs (16–18) in accordance with the SignalP3.0 server (available on the World Wide Web) (26, 27). This suggests that these three proteins are cytosolic.

Phosphorolytic Activity of Cphy0577, Cphy1920, and Cphy3030 Proteins—The phosphorolytic activity of recombinant Cphy0577, Cphy1920, and Cphy3030 proteins on LNB and GNB was examined. Specific activities of Cphy0577 protein on LNB and GNB at 37 °C were 19 and 20 units/mg, respectively. Cphy3030 protein showed a specific activity of 21 and 14 units/mg on LNB and GNB, respectively, at 30 °C (Cphy3030 protein is not stable at 37 °C). Cphy0577 and Cphy3030 proteins did not exhibit phosphorolytic activity either on lacto-N-tetraose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) or galacto-N-tetraose (Galβ1→3GlcNAcβ1→4Galβ1→4Glc). These results indicate that both enzymes are GalHexNAcPs. However, Cphy1920 protein did not phosphorolysed either LNB or GNB, thus indicating that Cphy1920 protein is not GalHexNAcP.

Kinetic Parameters of Cphy0577 and Cphy3030 Proteins—Kinetic parameters for forward and reverse reactions of Cphy0577 and Cphy3030 proteins were examined. Cphy0577 protein exhibited similar kinetic parameters on LNB and GNB (Table 1). On the other hand, Cphy3030 protein showed similar $k_{cat}$ values on LNB and GNB (Table 1). The value of $K_m$ on LNB was ~4 times less than that on GNB, and the $k_{cat}/K_m$ value on LNB was ~4 times more than that on GNB (Table 1). Kinetic parameters for the reverse reaction of both enzymes showed a similar tendency for the forward reaction (Table 1). Because both enzymes showed significant activities on both LNB and GNB, we identified them as GLNBPs.

Comparison of Substrate Specificity among Cphy0577, Cphy1920, and Cphy3030 Proteins—We compared the specificities for the acceptor substrate of Cphy0577, Cphy1920, and Cphy3030 proteins in their reverse (synthetic) reactions. Although Cphy0577 and Cphy3030 proteins exhibited similar activity on d-GlcNAc and d-GalNAc in the presence of Gal-1-P, Cphy1920 proteins did not show activity on either of them (Table 2). Cphy1920 protein showed the highest activity on l-Rha and also exhibited activity on l-Man, l-Lyx, d-Glc, d-Glc2d, and d-Gal in this order (Table 2). However, Cphy0577 and Cphy3030 proteins did not show activity on either l-Rha, d-Glc, or d-Gal (Table 2). This result indicates that Cphy1920 protein is completely different from Cphy0577 and Cphy3030 proteins in substrate specificity.

We also investigated the specificities on the donor substrate of the three proteins. All of these proteins exhibited identical specificity (Table 2).

Basic Properties of Cphy0577, Cphy1920, and Cphy3030 Proteins—The phosphorolytic activity of recombinant Cphy0577, Cphy1920, and Cphy3030 proteins on LNB and GNB was examined. Specific activities of Cphy0577 protein on LNB and GNB at 37 °C were 19 and 20 units/mg, respectively. Cphy3030 protein showed a specific activity of 21 and 14 units/mg on LNB and GNB, respectively, at 30 °C (Cphy3030 protein is not stable at 37 °C). Cphy0577 and Cphy3030 proteins did not exhibit phosphorolytic activity either on lacto-N-tetraose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) or galacto-N-tetraose (Galβ1→3GlcNAcβ1→4Galβ1→4Glc). These results indicate that both enzymes are GalHexNAcPs. However, Cphy1920 protein did not phosphorolysed either LNB or GNB, thus indicating that Cphy1920 protein is not GalHexNAcP.

Identification of Products of the Synthetic Reaction of Cphy1920 Protein—After the synthetic reaction was performed in the presence of an acceptor (l-Rha, l-Man, l-Lyx, d-Glc,
**TABLE 2**  
Comparison of substrate specificity between Cphy0577, Cphy1920, and Cphy3030 proteins  
Values in parenthesis represent specific activity (units/mg).  

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Linkage of products by Cphy1920 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cphy0577</td>
<td>Cphy3030</td>
</tr>
<tr>
<td>Acceptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-GlcNAc</td>
<td>100 (26)</td>
<td>100 (25)</td>
</tr>
<tr>
<td>d-GalNAc</td>
<td>100 (26)</td>
<td>72 (18)</td>
</tr>
<tr>
<td>L-Rha</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-Man</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>L-Lyx</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>d-Man</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-Glc</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Donor  
Gal 1-P 100 (26) 100 (25) 100 (43)  
Glc 1-P ND ND ND  

* Specific activity of Cphy0577 and Cphy3030 proteins on d-GlcNAc and specific activity of Cphy1920 protein on L-Rha in the presence of Gal 1-P were defined as 100% relative activity.  
* Assay was performed at 30 °C.  
* ND, less than 0.5% of relative activity.  
* d-GlcNAc was used as an acceptor.  
* L-Rha was used as an acceptor.

**TABLE 3**  
Kinetic parameters of synthetic activity of Cphy1920 protein  
Values were obtained by regressing data with the following equation using Grafit Version 4.0.10:  

\[
\frac{v}{[\text{S}]} = \frac{k_{\text{cat}}[\text{A}][\text{B}]}{K_{\text{m}}[\text{A}] + K_{\text{m}}[\text{B}] + K_{\text{m}}[\text{A}] + [\text{A}][\text{B}]).
\]

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(K_{\text{m}}) (mM)</th>
<th>(k_{\text{cat}}/K_{\text{m}}) s(^{-1})mM(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Rha</td>
<td>74 ± 2</td>
<td>2.4 ± 0.2</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>L-Man</td>
<td>170 ± 2</td>
<td>78 ± 16</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>L-Lyx</td>
<td>120 ± 13</td>
<td>220 ± 40</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>d-Glc</td>
<td>85 ± 14</td>
<td>460 ± 90</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>d-Glc2d</td>
<td>28 ± 3</td>
<td>290 ± 50</td>
<td>0.095 ± 0.004</td>
</tr>
<tr>
<td>d-Gal</td>
<td>24 ± 6</td>
<td>610 ± 160</td>
<td>0.039 ± 0.002</td>
</tr>
</tbody>
</table>

* Up to 20 mM L-Rha was used.  
* Up to 100 mM acceptors were used.  
* Up to 150 mM acceptors were used.

Phosphorolytic Activity of Cphy1920 Protein—Double-reciprocal plots of initial velocities against various initial concentrations of GalRha and phosphate gave a series of lines intersecting at a point (Fig. 3). This indicates that the phosphorolytic reaction of Cphy1920 protein follows a sequential bi–bi mechanism, as do inverting phosphorylases (16, 18, 28–34). Kinetic parameters of the enzyme determined by regressing data are shown in the legend of Fig. 3. The values of \(k_{\text{cat}}\), \(K_{\text{m}}\), and \(K_{\text{m}}\) for each substrate were determined (Table S1). These results indicate that the disaccharides produced from the former three acceptors (L-Rha derivatives) differ from those produced from the latter three (d-Glc derivatives) in terms of the linkage position of the donor.

Kinetic Parameters of Synthetic Activity of Cphy1920 Protein—Kinetic parameters of Cphy1920 protein on six effective acceptors were determined. Only the \(K_{\text{m}}\) value on L-Rha was in the millimolar range and was 10–250 times smaller than those on the other acceptors (Table 3). On the contrary, the \(k_{\text{cat}}\) value on L-Rha was 0.4–3 times as large as those on the other acceptors (Table 3). This result indicates that \(k_{\text{cat}}/K_{\text{m}}\) values mainly depend on \(K_{\text{m}}\) values. The fact that the \(k_{\text{cat}}/K_{\text{m}}\) value on L-Rha was 15–800 times larger than those on the other acceptors indicates that L-Rha is the most effective acceptor for Cphy1920 protein (Table 3).
### DISCUSSION

**Classification of Cphy1920 Protein—**Cphy1920 protein shows activity that has not been reported elsewhere, whereas Cphy0577 and Cphy3030 proteins are GLNBPs (Fig. 5). This is consistent with the prediction of substrate specificity of these three enzymes, based on the ternary structure of GLNBPBl. Furthermore, we compared acceptors of these enzymes in a synthetic reaction and found that Cphy1920 protein and GLNBPs did not exhibit activity on each other’s acceptors (Table 2), thus indicating that β-1,4-galactosides are the main substrates for Cphy1920 protein. We also compared the orientation of substituting groups at corresponding positions of both L-Rha and D-Glc at linkage positions of a donor, and ring conformations of both acceptor moieties are stereochemically the same (Fig. 6A). We compared the orientation of substituting groups at corresponding positions of both L-Rha and D-Glc at linkage positions of a donor, and ring conformations of both acceptor moieties are stereochemically the same (Fig. 6A). Therefore, difference in the galactoside linkage position is rational in terms of ring conformations and orientations of substituting groups.

There are two other differences that should be noted between the substrates of GalRhaP and GalHexNACPs. The difference can be explained by the ring conformations and orientations of substituting groups of L-Rha and D-Glc, because GalRhaP acts on β-1,3-galactosides as well as GalHexNACPs when D-Glc derivatives are acceptors. Although L-Rha has a 1C4 chair conformation (38), the stable ring conformation of D-Glc is 1C1. These ring conformations are conserved in the respective acceptor moieties of disaccharide products (Table S1). When hydroxyl groups of L-Rha and D-Glc at linkage positions of a donor are aligned, ring conformations of both acceptor moieties are stereochemically the same (Fig. 6A). We compared the orientation of substituting groups at corresponding positions of both L-Rha and D-Glc. The orientations of L-Rha 3-OH and C-6 methyl group are the same as those of D-Glc 2-OH and 4-OH, respectively (Fig. 6B). Moreover, both L-Rha 2-OH and the α-anomeric hydroxyl group of D-Glc are in an axial position (Fig. 6B). This orientation is very important, because GalRhaP did not exhibit activity on 1-glucose (C-2 epimer of L-Man) (Table 2) and was specific for the α-anomer of D-Glc (Fig. 4B). In addition, GalRhaP was not specific for the anomers of L-Rha (Fig. 4A). This may be because the anomeric carbon of L-Rha corresponds to O-5 atom of D-Glc (Fig. 6B). Therefore, difference in the galactoside linkage position is rational in terms of ring conformations and orientations of substituting groups.

Even after comparing acceptors with all of the phosphorylases that have been reported, no phosphorylase acting on L-Rha has been identified until now, although phosphorylases acting on D-Glc (35), glucooligosaccharides (30), D-Fru (36), D-GlcNAc (32), D-GalNAc (16, 18), or D-glucose 6-phosphate (37) have been found. Therefore, this study is the first report of a phosphorylase acting on L-Rha.

**Substrate Recognition of GalRhaP—**GalRhaP synthesized β-1,4-galactosides from L-Rha derivatives and β-1,3-galactosides from D-Glc derivatives as acceptors in the presence of Gal 1-P. Activity on L-Rha derivatives was higher than that on D-Glc derivatives (Table 2), thus indicating that β-1,4-galactosides are the main substrates for GalRhaP.

Linkage position of the donor is one of the most notable differences between the substrates of GalRhaP and GalHexNACPs. The difference can be explained by the ring conformations and orientations of substituting groups of L-Rha and D-Glc, because GalRhaP acts on β-1,3-galactosides as well as GalHexNACPs when D-Glc derivatives are acceptors. Although L-Rha has a 1C4 chair conformation (38), the stable ring conformation of D-Glc is 1C1. These ring conformations are conserved in the respective acceptor moieties of disaccharide products (Table S1). When hydroxyl groups of L-Rha and D-Glc at linkage positions of a donor are aligned, ring conformations of both acceptor moieties are stereochemically the same (Fig. 6A). We compared the orientation of substituting groups at corresponding positions of both L-Rha and D-Glc. The orientations of L-Rha 3-OH and C-6 methyl group are the same as those of D-Glc 2-OH and 4-OH, respectively (Fig. 6B). Moreover, both L-Rha 2-OH and the α-anomeric hydroxyl group of D-Glc are in an axial position (Fig. 6B). This orientation is very important, because GalRhaP did not exhibit activity on 1-glucose (C-2 epimer of L-Man) (Table 2) and was specific for the α-anomer of D-Glc (Fig. 4B). In addition, GalRhaP was not specific for the anomers of L-Rha (Fig. 4A). This may be because the anomeric carbon of L-Rha corresponds to O-5 atom of D-Glc (Fig. 6B). Therefore, difference in the galactoside linkage position is rational in terms of ring conformations and orientations of substituting groups.

There are two other differences that should be noted between the substrates of GalRhaP and GalHexNACPs in substituting groups. First, GalRhaP acts on D-Glc but not on D-GlcNAc, although GalHexNACPs act on D-GlcNAc, thus indicating that GalRhaP cannot accept the N-acetyl group. Despite this fact, Trp 233 of GLNBPBl, which recognizes the oxygen atom in the N-acetyl group of acceptors, is conserved in GalRhaP (Fig. 2). In GalRhaP, two residues (Ser-Asn) are inserted around the Trp residue (Fig. 2), corresponding to the loop region of GLNBPBl (19). Therefore, such an insertion might cause the corresponding Trp residue of GalRhaP to change its position and hinder acceptance of the N-acetyl group sterically. We also compared kinetic parameters of D-Glc and D-Glc2d to evaluate the contribution of D-Glc 2-OH to substrate recognition of GalRhaP. The value of kcat/Km on D-Glc2d was approximately half
of that on D-Glc (Table 3), thus suggesting that D-Glc 2-OH is not important for D-Glc recognition. This result may be related to the fact that there is no hydrogen bond with the nitrogen atom in the N-acetyl group of acceptors in GLNBPβ₁ (19). Second, the C-6 methyl group of α-Rha corresponds to the 4-OH of D-GlcNac (Fig. 6B). Because GalRhaP showed a much smaller $K_m$ value on α-Rha than that on α-Man and α-Lyx, where only the C-6 methyl group of α-Rha is substituted, the methyl group must be important for substrate recognition of GalRhaP (Table 3). Such a difference in substituting groups between α-Rha and D-GlcNac may exist because the residue of GalRhaP corresponding to Val¹⁶² of GLNBPβ₁ is not valine (GLNBP or LNB type) but threonine (GNBP type) but isoleucine (Fig. 2).

Physiological Role of GalRhaP and GLNBPs from C. phytofermentans—Phosphorylases usually have strict substrate specificities that are closely related to the growth environment of bacteria possessing them. Because C. phytofermentans can utilize various kinds of plant polysaccharides for growth (23), it is speculated that GalRhaP and GLNBPs are involved in the metabolism of some plant polysaccharides. GNBs from plant polysaccharides are little known, and LNB is known to exist in biosynthetic intermediates of N-linked glycoproteins during the processing of plant N-glycans in the Golgi apparatus, which is quite a minor component in plants (39). Moreover, there are no endo-α-N-acetylglactosaminidase (7, 40, 41) and lacto-N-biosidase (5) (GNB- and LNB-releasing enzyme, respectively) homologs in the genome of C. phytofermentans. Physiological functions of GLNBPs are still uncertain.

GalRha is found in rhamnogalacturonan I (RG-I) contained in pectin as a structure with one galactose residue that distinguishes RG-I from RG-II. The residue of GalRhaP corre-sponding to Val¹⁶² of GLNBPβ₁ is not valine (GLNBP or LNB type) but isoleucine (Fig. 2). Sec-ondary structure containing GalRha was found in the Glycoscience.de data base (available on the World Wide Web). Because C. phytofermentans has some RG-I-metabolizing gene ho-mologs (cphy0343 gene, rhamnogalacturonan lyase (polysaccharide lyase family 11) (43)), GalRhaP is probably involved in the metabolism of RG-I.

GalRha structures are also found in some bacterial exopolysaccharides (44, 45) aside from plant polysaccharides. GalRhaP may be involved in salvage of exopolysaccharides produced by C. phytofermentans.

Conclusions—Phosphorylases are more suitable for synthesis of oligosaccharides than glycoside hydrolases in terms of reversibility of the reaction. Several large scale preparation methods of oligosaccharides taking advantage of this feature have been developed (11–13). However, the narrow variation of phosphorylases restricts their utility. In this study, we found a unique β-galactoside phosphorylase. Discovery of the enzyme will further expand utilization of phosphorylases.
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Characterization of Three β-Galactoside Phosphorylases from Clostridium phytofermentans: DISCOVERY OF d-GALACTOSYL-β1→4-l-RHAMNOSE PHOSPHORYLASE

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