Enzymatic synthesis of β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside using *Escherichia coli* glycoside phosphorylase YcjT

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Short running head: Synthesis of Glc(α1-2)Glc(α1-2β)Fru*f* using YcjT

Abstract

YcjT is a kojibiose phosphorylase found in *Escherichia coli*. We found that sucrose was a good acceptor of YcjT in reverse phosphorolysis using β-D-glucose 1-phosphate as a donor. The product was identified as β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside. This sugar was also synthesized from sucrose and maltose using YcjT and maltose phosphorylase and promoted the growth of the probiotic *Bifidobacterium breve*.

Keywords: Glycoside phosphorylase, Kojibiose phosphorylase, Maltose phosphorylase, Non-reducing sugar, Prebiotic

Glycoside phosphorylases are enzymes that catalyze the phosphorolysis of oligosaccharides and polysaccharides to produce sugar 1-phosphate (Kitaoka 2015). These enzymes also catalyze the reverse reaction (reverse phosphorolysis), namely the synthesis of carbohydrates using sugar 1-phosphate as a donor, and can be applied to the production of oligosaccharides and polysaccharides *in vitro*. According to the classification by the CAZy database, glycoside phosphorylases belong to several distinct families of glycoside hydrolases and glycosyltransferases (Drula *et al.* 2022). Glycoside hydrolase family 65 (GH65) contains glycoside phosphorylases that cleave α-glucosidic bonds and produce β-D-glucose 1-phosphate (β-Glc1*P*) (Li *et al.* 2022). Kojibiose phosphorylase belongs to GH65 and catalyzes the reversible phosphorolysis of kojibiose (Glc(α1-2)Glc) into β-D-glucose 1-phosphate (β-Glc1*P*) and D-glucose. It has been reported to be present in several bacteria and archaea, including *Thermoanaerobacter brockii*, *Caldicellulosiruptor saccharolyticus*, *Halothermothrix orenii*, and *Pyrococcus* sp. (Chaen *et al.* 1999; Yamamoto *et al.* 2011; Jung *et al.* 2014; De Beul *et al.* 2021). In reverse phosphorolysis, many kojibiose phosphorylases can use kojibiose and larger kojioligosaccharides as acceptors instead of D-glucose. Furthermore, kojibiose phosphorylase from *T. brockii*, whose acceptor specificity has been well studied, has been shown to use various monosaccharides and oligosaccharides besides D-glucose and kojioligosaccharides, resulting in the synthesis of some rare oligosaccharides (Chaen *et al.* 2001a; Okada *et al.* 2003). Recently, Mukherjee *et al.* (2018) reported that the GH65 enzyme encoded by the *ycjT* gene of *Escherichia coli* exhibits kojibiose phosphorylase activity. However, no detailed information on the properties and potential applications of YcjT has been reported.

β-D-Fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside (Glc(α1-2)Glc(α1-2β)Fru*f*) is a kojibiose-related non-reducing trisaccharide. Glc(α1-2)Glc(α1-2β)Fru*f* and oligosaccharides with the "[Glc(α1-2)]$_n$Glc(α1-2β)Fru*f*" structure have been found in cyanobacterial cells (Fischer, Geyer and Loos 2006). These oligosaccharides accumulate in response to heat and salt stresses and are predicted to protect proteins from inactivation. However, there have been very few reports on the function of Glc(α1-2)Glc(α1-2β)Fru*f*. Developing a method for synthesizing Glc(α1-2)Glc(α1-2β)Fru*f* from inexpensive materials

would facilitate functional studies of this sugar and expand its potential in various future applications, such as food, pharmaceuticals, and cosmetics. Here we report that *E. coli* YcjT has suitable characteristics for synthesizing Glc(α1-2)Glc(α1-2β)Fru*f*.

The donor substrate (β-Glc1*P*) of YcjT was prepared by incubating a mixture (100 mL) containing 300 mM potassium phosphate buffer (pH 7.0), 300 mM maltose, and 10 μg/mL maltose phosphorylase (Oriental Yeast, Tokyo, Japan) at 37 °C for 72 h. The resultant β-Glc1*P* was purified as described by Van der Borght *et al.* (2010). Recombinant YcjT with a C-terminal histidine tag was overexpressed in *E. coli* and purified by nickel affinity chromatography as described in Supplementary Methods.

Reverse phosphorolysis activities of YcjT were determined as follows: a reaction mixture containing 50 mM MES-NaOH buffer (pH 5.5), 10 mM acceptor substrate, 2 mM β-Glc1*P*, and 10 μg/mL YcjT was incubated at 37 °C for 10 min unless otherwise noted, and released inorganic phosphate was measured according to Saheki *et al.* (1985). The optimum pH and temperature of YcjT for reverse phosphorolysis activity using D-glucose as the acceptor were 5.2 and 37 °C, respectively (Figure S1). Mukherjee *et al.* (2018) conducted all assays for YcjT at pH 7.5 without determining the optimum pH. The enzyme exhibited only very low activity under such conditions. The substrate specificity in phosphorolysis was examined. A reaction mixture containing 100 mM MES-NaOH buffer (pH 5.5), 10 mM substrate, and 100 mM sodium phosphate buffer (pH 5.5) together with 0.2 mg/mL YcjT was incubated at 37 °C for 16 h and analyzed by TLC. YcjT degraded kojibiose in the presence of inorganic phosphate (Figure 1a). In contrast, this enzyme did not phosphorolyze the other disaccharides listed in Table S1. The substrate specificity in reverse phosphorolysis was also examined. A reaction mixture containing 100 mM MES-NaOH buffer (pH 5.5), 10 mM acceptor substrate, 10 mM β-Glc1*P*, and 0.2 mg/mL YcjT was incubated at 37 °C for 1 h and analyzed by TLC. Product spots were observed after the reaction with D-glucose, L-sorbose, and 1,5-anhydro-D-glucitol as acceptors (Figure 1b), which agrees with the previous report (Mukherjee *et al.* 2018). They reported that YcjT showed almost no enzymatic activity toward glucose–glucose disaccharide acceptors in contrast to kojibiose phosphorylase from *T. brockii*, *C. saccharolyticus*, and *H. orenii*; however, we observed an obvious product spot after the

reaction with nigerose. In addition, the reaction using sucrose as an acceptor resulted in a similar amount of product as the reaction using D-glucose. These findings indicate that some disaccharides also act as acceptors for YcjT. No products were observed in the reactions with the carbohydrates (monosaccharides, sugar alcohol, and disaccharides) listed in Table S1, other than D-glucose, L-sorbose, 1,5-anhydro-D-glucitol, nigerose, and sucrose. Apparent kinetic parameters for D-glucose were k_{cat} = 2.4 ± 0.1 s^{-1}, K_m = 20.4 ± 2.1 mM, k_{cat}/K_m = 0.12 $s^{-1}\cdot mM^{-1}$. Kinetic parameters for sucrose were k_{cat} = 5.3 ± 0.2 s^{-1}, K_m = 37.5 ± 3.1 mM, k_{cat}/K_m = 0.14 $s^{-1}\cdot mM^{-1}$. The k_{cat}/K_m values for D-glucose and sucrose were comparable, indicating that sucrose was a good acceptor substrate for YcjT. To date, there have been no reports of a kojibiose phosphorylase other than YcjT, which is as active for sucrose as for D-glucose in reverse phosphorolysis.

The structure of the reverse phosphorolysis product using sucrose as an acceptor was investigated. A mixture (15 mL) containing 50 mM MES-NaOH buffer (pH 5.5), 200 mM sucrose, 200 mM β-Glc1*P*, and 1.3 mg/mL YcjT was incubated at 37 °C for 40 h and heated at 100 °C for 5 min. After centrifugation, the supernatant was applied to a Dowex 1X8 column (acetate form, 2.5 cm id × 13 cm; Wako Pure Chemical, Osaka, Japan) using water as the mobile phase to remove inorganic phosphate and β-Glc1*P*. Fractions containing the product were collected, concentrated by evaporation, and applied to TOYOPEARL HW-40S columns (2.6 cm id × 100 cm × 3; Tosoh, Tokyo, Japan) using water as the mobile phase. Approximately 850 mg of the pure product was obtained (Figure 1c). NMR spectra of the product were essentially identical to those of β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside (Glc(α1-2)Glc(α1-2β)Fru*f*, CAS Registry Number [206553-36-8]) previously purified from a cyanobacterium, *Nostoc ellipsosporum* (Table S2) (Fischer, Geyer and Loos 2006). The molecular mass was determined using ESI-MS (*m/z* $[M + Na]^+$ 527), and was in agreement with the calculated molecular mass of Glc(α1-2)Glc(α1-2β)Fru*f*. Thus, we found that YcjT catalyzes the reaction to synthesize Glc(α1-2)Glc(α1-2β)Fru*f* from sucrose and β-Glc1*P* (Figure 2).

In the synthesis of Glc(α1-2)Glc(α1-2β)Fru*f* as described above, β-Glc1*P* was used as a starting material. However, β-Glc1*P* is not easy to prepare and is expensive to obtain

commercially. Since the reaction of glycoside phosphorylases is reversible, using two glycoside phosphorylases simultaneously makes it possible to produce a variety of oligosaccharides and polysaccharides from inexpensive carbohydrates (Kitaoka 2015). We therefore investigated whether Glc(α1-2)Glc(α1-2β)Fru*f* could be produced by combining YcjT and maltose phosphorylase, using sucrose and maltose as starting materials. After a mixture (200 mL) containing 40 mM potassium phosphate buffer (pH 6.0), 200 mM sucrose, 200 mM maltose, 70 μg/mL YcjT, and 50 μg/mL maltose phosphorylase (Oriental Yeast, Tokyo, Japan) was incubated at 37 °C for 17 h and heated at 80 °C for 5 min, the production of Glc(α1-2)Glc(α1-2β)Fru*f* (approximately 90 mM) was observed by TLC and HPLC (Figure 1d, lane 4; Figure 1e). In this method, maltose phosphorylase produces β-Glc1*P* from maltose by phosphorolysis, and YcjT transfers a D-glucose residue to sucrose by reverse phosphorolysis using the resulting β-Glc1*P* as a donor substrate. Subsequently, residual substrates (sucrose and maltose) and D-glucose (produced by phosphorolysis of maltose) were removed by incubation with dry yeast (10 g; Nisshin Seifun Welna, Tokyo, Japan) at 30 °C for 23 h (Figure 1d, lane 5). In contrast, dry yeast did not consume Glc(α1-2)Glc(α1-2β)Fru*f*. The resulting sample (supernatant) contained a small amount of kojibiose, which was the reverse phosphorolysis product of YcjT from D-glucose and β-Glc1*P*. The by-product could be separated from Glc(α1-2)Glc(α1-2β)Fru*f* by size exclusion chromatography following anion-exchange chromatography as described above (Figure 1e). Thus, we found that Glc(α1-2)Glc(α1-2β)Fru*f* can be produced from inexpensive carbohydrates. Finally, 2.8 g (5.6 mmol) of Glc(α1-2)Glc(α1-2β)Fru*f* was obtained, and the yield was 14 % based on the amount of sugar substrates (40 mmol each). Optimization of production and purification conditions would improve the efficiency of the process yielding more of this oligosaccharide.

While sucrose is a good acceptor of YcjT in reverse phosphorolysis, kojibiose and maltose were unsuitable acceptors, as described above. Such relatively strict acceptor specificity has not been observed in the other kojibiose phosphorylases reported previously. For example, kojibiose phosphorylase from *T. brockii* can use sucrose, kojibiose or maltose as acceptors and produce Glc(α1-2)Glc(α1-2β)Fru*f*, kojitriose or α-D-glucopyranosyl-(1→2)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranose (Glc(α1-2)Glc(α1-4)Glc), respectively (Chaen *et*

al. 2001a, 2001b). The substrate specificity of YcjT is advantageous in producing Glc(α1-2)Glc(α1-2β)Fru*f* from low-cost maltose and sucrose in combination with maltose phosphorylase, with the benefit of low by-product formation because YcjT hardly uses maltose or kojibiose (synthesized from the product of maltose phosphorolysis) as acceptors. If kojibiose phosphorylase from *T. brockii* were used in the same way, then in addition to Glc(α1-2)Glc(α1-2β)Fru*f*, various oligosaccharides including kojitriose, larger kojioligosaccharides, and Glc(α1-2)Glc(α1-4)Glc would also be synthesized, and Glc(α1-2)Glc(α1-2β)Fru*f* would not be easy to purify.

Among the kojibiose phosphorylases, the most studied enzyme is that from *T. brockii*, whose acceptor specificity appears to differ from that of YcjT, as mentioned above (Chaen *et al.* 1999). Although the 3D structures of YcjT and the *T. brockii* enzyme have not been experimentally elucidated at present, the crystal structure of kojibiose phosphorylase from *C. saccharolyticus* complexed with kojibiose has been reported (Okada *et al.* 2014; PDB: 3WIQ). Superimposing AlphaFold models of YcjT and the *T. brockii* enzyme (Varadi *et al.* 2022) and the structure of *C. saccharolyticus* kojibiose phosphorylase showed that the structures of subsites −1 and +1 of these enzymes were very similar (Figure S2). On the other hand, the putative subsite +2 structure deduced based on the crystal structure of a GH65 α-1,2-glucosidase from *Flavobacterium johnsoniae* complexed with multiple D-glucose molecules (Nakamura *et al.* 2021; PDB ID: 7FE4) was considerably different between YcjT and kojibiose phosphorylase from *T. brockii*. Amino acid residues corresponding to Q555, L558, and R563 in YcjT were not found in the model of kojibiose phosphorylase from *T. brockii*. In contrast, amino acid residues corresponding to F424, D425, Y426, and L607 in kojibiose phosphorylase from *T. brockii* were not found around D-glucose at subsite +2 in the model structure of YcjT. These amino acid residues may be responsible for the differences in acceptor specificity of the two glycoside phosphorylases.

The digestibility of Glc(α1-2)Glc(α1-2β)Fru*f* was evaluated using *in vitro* tests described by Yamamori et al. (2014), but with minor modifications, under the assumption that the sugar would be ingested by humans. Glc(α1-2)Glc(α1-2β)Fru*f* was not hydrolyzed by human salivary α-amylase, artificial gastric juice, or porcine pancreatic α-amylase. The

trisaccharide was slightly degraded by intestinal enzymes; however, its degradation rate was less than 3% that of maltose. Thus, these results suggested that the trisaccharide is almost unaffected by digestive juices. A further experiment was conducted to investigate the potential of Glc(α1-2)Glc(α1-2β)Fru*f* as a prebiotic. *Bifidobacterium bifidum* NBRC 100015, *Bifidobacterium breve* NBRC 115160, *Clostridium butyricum* NBRC 13949, and *Lacticaseibacillus casei* NBRC 15883 were purchased from NITE Biological Resource Center (Chiba, Japan) and maintained anaerobically in MRS medium. These strains were used as representative probiotic bacteria. In addition, *Collinsella aerofaciens* NBRC 114504, a major bacterium in the intestinal flora, was used. An anaerobic bacterial culture test was carried out as described by Hosaka et al. (2020) with minor modifications. A mixture (300 μL) containing MRS medium (without D-glucose), a bacterial cell suspension (OD_{655} of approximately 0.5) after washing with saline, and 30 mM Glc(α1-2)Glc(α1-2β)Fru*f* was added to each microplate well. After incubation at 37 °C for 24 or 72 h anaerobically, bacterial growth was evaluated by measuring the OD_{595} of the culture using a microplate reader. The pH of the culture was also measured. Control experiments confirmed that these strains did not grow anaerobically in a sugar-free medium. Among the strains, only *B. breve* grew in the medium containing Glc(α1-2)Glc(α1-2β)Fru*f*, and the pH of the medium dropped to 4.6 after 72-h incubation (Table 1). *B. breve* is known to improve gastrointestinal disorders such as constipation, and recent studies have shown that it can be a functional food ingredient to reduce body fat and has therapeutic potential for preventing cognitive impairment in Alzheimer's disease (Tabbers *et al.* 2011; Kobayashi *et al.* 2017; Minami *et al.* 2018). García-Cayuela et al. (2014) reported that kojibiose and its related oligosaccharide 4'-galactosyl-kojibiose promote the growth of *B. breve* but not *B. lactis* or *B. bifidum*. *B. breve* may have specific mechanisms for the uptake of kojibiose and related sugars. A simple experiment in this study showed that Glc(α1-2)Glc(α1-2β)Fru*f* may promote the growth of only certain strains of bacteria; however, experiments with a greater number of bacterial strains will be required to investigate the details.

An additional method to synthesize Glc(α1-2)Glc(α1-2β)Fru*f* was reported recently in which the reaction by glucosyltransferase was performed using UDP-α-D-glucose and sucrose

as substrates (Han *et al.* 2022). However, UDP-α-D-glucose is also expensive and unsuitable for the practical production of this sugar. In this study, we found that sucrose is a good acceptor for YcjT of *E. coli* in reverse phosphorolysis and developed a method of producing the non-reducing prebiotic oligosaccharide. The results of this study will contribute to further research on this sugar, including its roles in cyanobacteria. Moreover, these results will expand the sugar's potential in future applications.

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Supplementary material

Supplementary material is available at Bioscience, Biotechnology, and Biochemistry online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

N.I. conceived and designed the study and wrote the manuscript. N.I., S.Y., and K.Y. characterized YcjT. S.Y. and K.M prepared Glc(α1-2)Glc(α1-2β)Fru*f*. H.K. determined the structure of sugar. Y.M. characterized the sugar. All authors approved the final manuscript.

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Disclosure statement

No potential conflict of interest is reported by the authors.

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Table 1. Culture test of several human intestinal bacteria on media containing Glc(α1-2)Glc(α1-2β)Fru*f* [a].

Bacterial strain	ΔOD_{595} [b]		pH	
	24 h	72 h	24 h	72 h
Bifidobacterium bifidum NBRC 100015	NG	NG	6.4 ± 0.0	6.4 ± 0.1
Bifidobacterium breve NBRC 115160	0.35 ± 0.04	0.98 ± 0.04	5.4 ± 0.1	4.6 ± 0.1
Clostridium butyricum NBRC 13949	0.10 ± 0.02	0.08 ± 0.01	6.1 ± 0.0	6.1 ± 0.0
Collinsella aerofaciens NBRC 114504	NG	0.10 ± 0.21	6.7 ± 0.1	6.4 ± 0.2
Lacticaseibacillus casei NBRC 15883	0.02 ± 0.01	0.03 ± 0.03	6.3 ± 0.1	6.3 ± 0.0

[a] Data represents means ± SD of three replicates; NG, no growth.

[b] $\Delta OD_{595} = OD_{595}$ [+ Glc(α1-2)Glc(α1-2β)Fru*f*] − OD_{595} [without sugar].

Figure legends

Figure 1. TLC and HPLC analysis of YcjT products

(a) TLC analysis of phosphorolysis products from kojibiose. Glc, Koj, and G1P indicates D-glucose, kojibiose, and β-Glc1*P*, respectively. Plus (+) and minus (−) signs indicate the presence and absence of YcjT. The resulting samples were analyzed by TLC as described previously (Isono *et al.* 2022). (b) TLC analysis of reverse phosphorolysis products using various acceptor substrates (Glc, D-glucose; Sor, L-sorbose; AG, 1,5-anhydro-D-glucitol; Nig, nigerose; or Suc, sucrose). Arrows indicate products. (c) TLC analysis of purified Glc(α1-2)Glc(α1-2β)Fru*f*. Lane 1, purified Glc(α1-2)Glc(α1-2β)Fru*f*; lane 2, markers. (d) TLC analysis of products from sucrose and maltose using YcjT and maltose phosphorylase. Lane 3, a sample after 15 min of reaction; lane 4, a sample after 17 h of reaction; lane 5, the product after incubating with dry yeast. Mal indicates maltose. (e) HPLC analysis of products from sucrose and maltose using YcjT and maltose phosphorylase. The resulting Glc(α1-2)Glc(α1-2β)Fru*f* was purified using dry yeast, anion-exchange chromatography, and size-exclusion chromatography. A reaction mixture (1) before and (2) after purification was subjected to HPLC analysis using a COSMOSIL Sugar-D column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) and a refractive index detector. The mobile phase was acetonitrile/water (3:1) at a flow rate of 1.0 mL/min, and the column temperature was 30 °C.

Figure 2. Synthesis of Glc(α1-2)Glc(α1-2β)Fru*f* from sucrose and β-Glc1*P* by YcjT

Figure 1

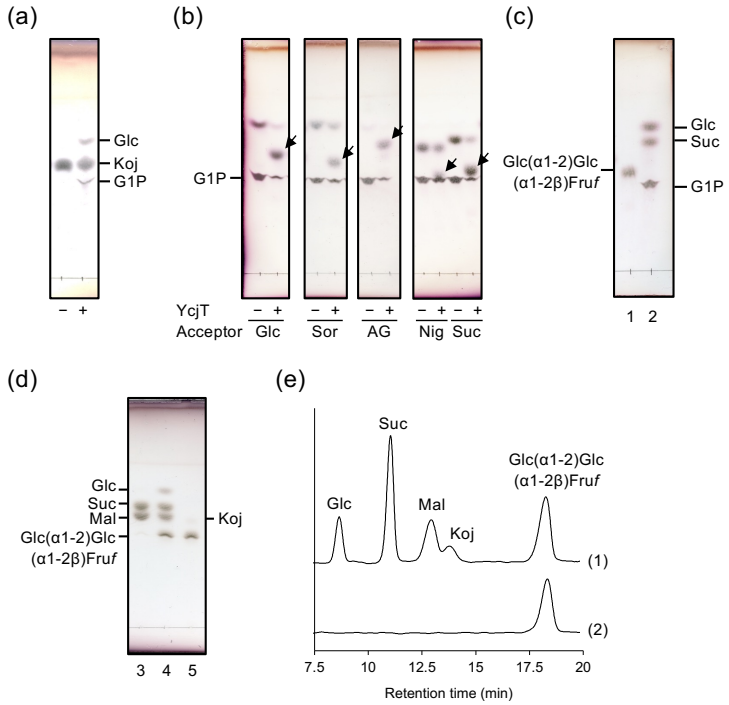
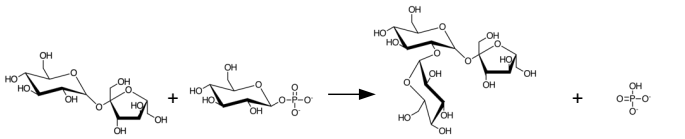

(a)
Glc
Koj
G1P
− +
(b)
G1P
YcjT − + − + − + − + − +
Acceptor Glc Sor AG Nig Suc
(c)
Glc(α1-2)Glc
(α1-2β)Fruf
Glc
Suc
G1P
1 2
(d)
Glc
Suc
Mal
Glc(α1-2)Glc
(α1-2β)Fruf
Koj
3 4 5
(e)
Suc
Glc
Mal
Koj
Glc(α1-2)Glc
(α1-2β)Fruf
(1)
(2)
7.5 10 12.5 15 17.5 20
Retention time (min)

Figure 2



Supplementary materials for

Enzymatic synthesis of β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside using *Escherichia coli* glycoside phosphorylase YcjT

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Methods

Table S1 to S2

Figure S1 to S2

Methods

Preparation of recombinant YcjT

The expression vector pET-23a (Merck, Darmstadt, Germany) was linearized by inverse PCR using PrimeSTAR HS DNA polymerase (Takara Bio, Kusatsu, Japan) and the following set of primers: 5′-TATGTATATCTCCTTCTTAAAG-3′ and 5′-AGCTTGCGGCCGCACTCGAG-3′. The *ycjT* gene was amplified from the genomic DNA of *E. coli* K-12 DH5α using the following set of primers: 5′-AAGGA-GATATACATATGACCAGGCCAGTAACGTTATCAGAA-3′ and 5′-GTGCGGCCGC-AAGCTTTTCATCCTCCTGATGTTTGGTAGC-3′. The two amplified fragments were fused using an In-Fusion HD Cloning Kit (Takara Bio), and the resultant expression plasmid was sequenced.

Transformants of *E. coli* BL21(DE3) harboring the expression plasmid were cultured with shaking in 400 mL of LB medium (10 g/L tryptone (Nacalai Tesque, Kyoto, Japan), 5 g/L yeast extract (Nacalai Tesque), and 10 g/L sodium chloride) containing 100 μg/mL ampicillin at 25 °C. When the OD_{600} reached 0.6, IPTG was added to the medium to a final concentration of 0.4 mM. Incubation was continued for an additional 16 h. The cells were harvested by centrifugation, resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride, and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-NTA column (5 mL; FUJIFILM Wako Pure Chemical, Osaka, Japan) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM sodium chloride. The column was washed with the same buffer, and the adsorbed protein was eluted with a linear gradient of imidazole (0–250 mM, 100 mL) in the same buffer. The purified protein was concentrated using Amicon Ultra-15 centrifugal filter units (10 kDa NMWL; Merck) and desalted using a PD-10 column (Cytiva, Marlborough, MA, USA) equilibrated with 20 mM MOPS-NaOH buffer (pH 6.0).

Table S1. Carbohydrates used for substrate specificity analysis.

Carbohydrate	Manufacturer
D-Glucose	Kanto Chemical (Tokyo, Japan)
D-Mannose	Kanto Chemical
D-Galactose	FUJIFILM Wako Pure Chemical (Osaka, Japan)
D-Fructose	FUJIFILM Wako Pure Chemical
L-Sorbose	Tokyo Chemical Industry (Tokyo, Japan)
D-Xylose	FUJIFILM Wako Pure Chemical
D-Ribose	FUJIFILM Wako Pure Chemical
L-Arabinose	FUJIFILM Wako Pure Chemical
D-Glucitol	FUJIFILM Wako Pure Chemical
1,5-Anhydro-D-glucitol	FUJIFILM Wako Pure Chemical
Trehalose	FUJIFILM Wako Pure Chemical
Kojibiose	Hayashibara Biochemical Laboratories (Okayama, Japan)
Nigerose	Hayashibara Biochemical Laboratories
Maltose	Nacalai Tesque (Kyoto, Japan)
Isomaltose	Tokyo Chemical Industry
Sophorose	SERVA Electrophoresis (Heidelberg, Germany)
Laminaribiose	Seikagaku Biobusiness (Tokyo, Japan)
Cellobiose	Seikagaku Biobusiness
Gentiobiose	Kanto Chemical
Sucrose	Kanto Chemical
Lactose	FUJIFILM Wako Pure Chemical

Table S2. ^{13}C NMR chemical shifts of β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside [Glc(α1-2)Glc(α1-2β)Fru*f*] synthesized by YcjT.

Residue		δ (ppm)	
		This work[a]	Literature[b]
αGlc	C-1	97.1	97.1
	C-2	72.0	71.9
	C-3	73.6	73.5
	C-4	70.0	70.0
	C-5	72.8	72.6
	C-6	60.9	60.9
αGlc	C-1	90.3	90.3
	C-2	76.3	76.3
	C-3	71.9	71.8
	C-4	70.0	70.0
	C-5	72.5	72.6
	C-6	62.8	62.7
βFru*f*	C-1	62.5	62.4
	C-2	104.9	104.9
	C-3	76.9	76.9
	C-4	74.4	74.3
	C-5	81.8	81.8
	C-6	61.0	60.9

[a] NMR spectrum was recorded in D_2O using a JNM-ECZ500R NMR spectrometer (JEOL, Tokyo, Japan).

[b] Chemical shifts of Glc(α1-2)Glc(α1-2β)Fru*f* purified from *Nostoc ellipsosporum* (Fischer, Geyer and Loos 2006).

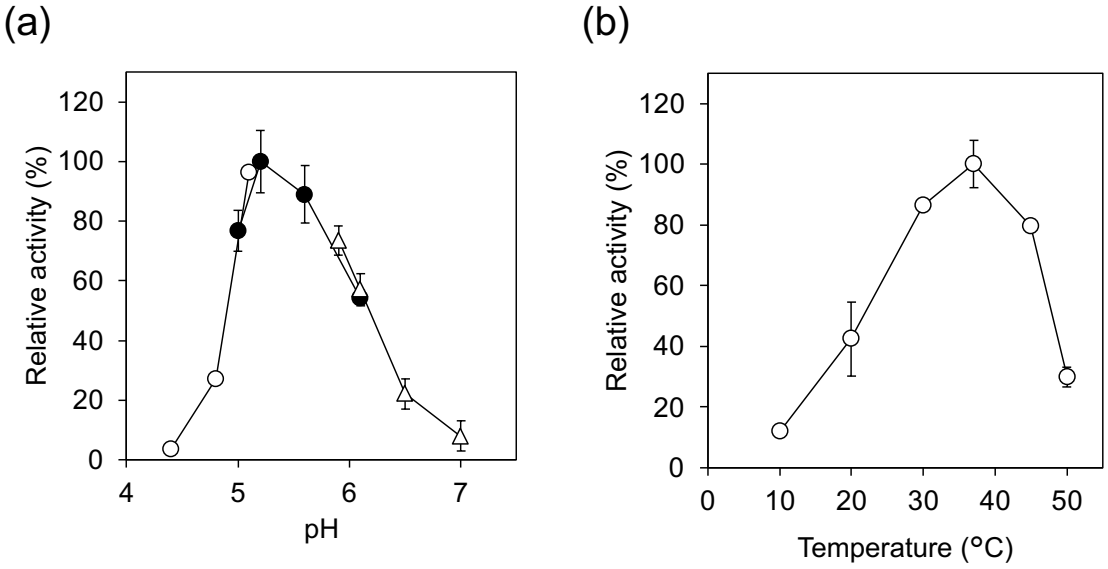

Figure S1. Optimum pH and temperature of YcjT

(a) Optimum pH. Reverse phosphorolysis activity using D-glucose as the acceptor substrate was assayed at different pH values. Sodium acetate buffer (open circles), MES-NaOH buffer (closed circles), and MOPS-NaOH buffer (open triangles) were used as the reaction buffers. (b) Optimum temperature. Reverse phosphorolysis activity using D-glucose as the acceptor substrate was assayed at different temperatures.

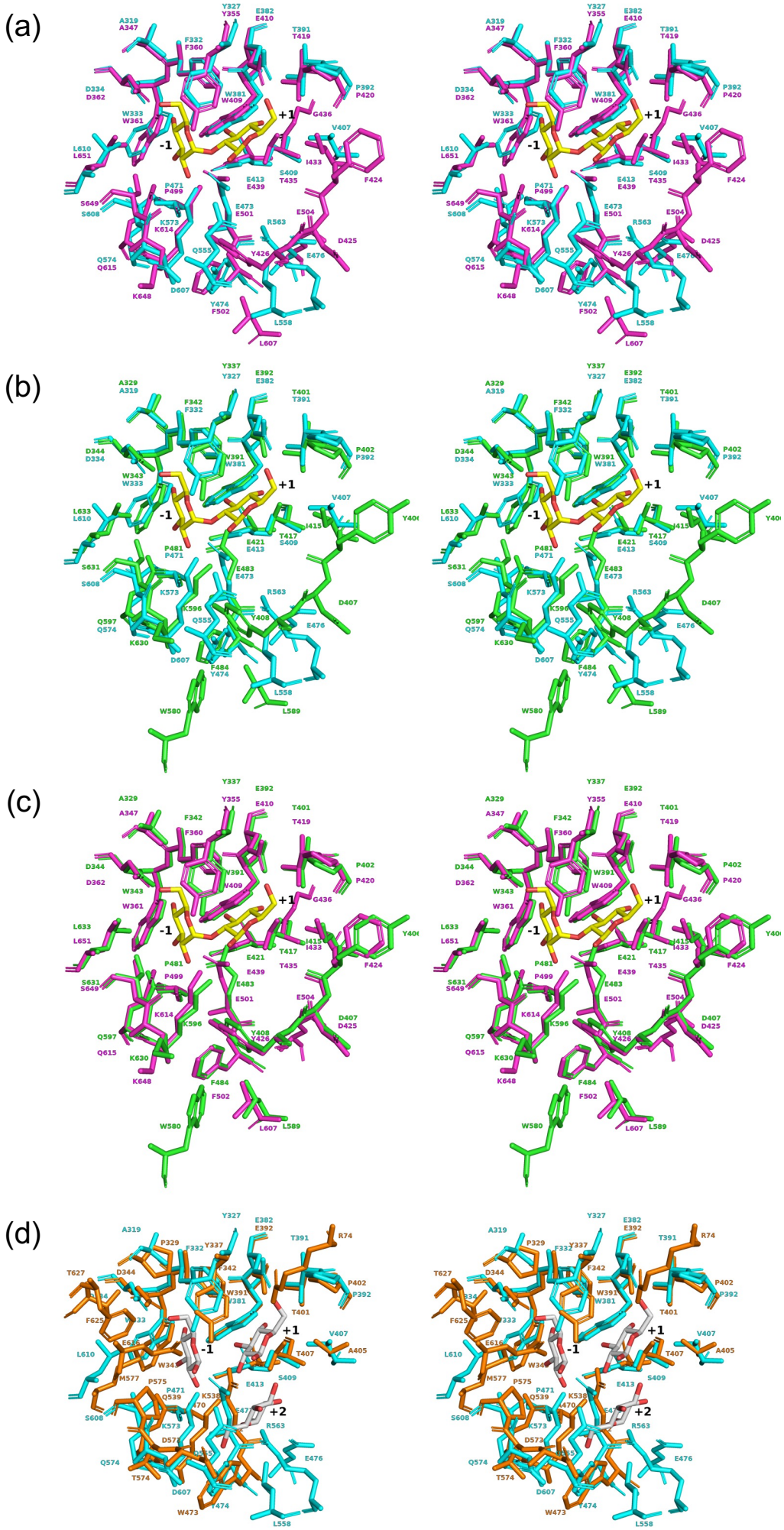

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Figure S2. Structural comparison of YcjT, GH65 kojibiose phosphorylases from *T. brockii* and *C. saccharolyticus*, and GH65 α-1,2-glucosidase from *F. johnsoniae*.

The model structures of YcjT (cyan) and kojibiose phosphorylase from *T. brockii* (magenta) were obtained from the AlphaFold Protein Structure Database. The structure of kojibiose phosphorylase (green) complexed with kojibiose (yellow) was retrieved from RCSB Protein Data Bank (PDB: 3WIQ). The structure of GH65 α-1,2-glucosidase from *F. johnsoniae* (orange) complexed with D-glucose (white) was retrieved from RCSB Protein Data Bank (PDB: 7FE4). Figures were prepared using PyMOL (Schrödinger, New York, USA). Residues around the active site were shown, including subsites −1 and +1 and putative subsite +2. (a) YcjT and kojibiose phosphorylases from *T. brockii.* Kojibiose (yellow) bound to kojibiose phosphorylase from *C. saccharolyticus* is superimposed on the models. (b) YcjT and kojibiose phosphorylase from *C. saccharolyticus.* (c) Kojibiose phosphorylases from *T. brockii* and *C. saccharolyticus.* (d) YcjT and α-1,2-glucosidase from *F. johnsoniae.*