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Modulation of acceptor specificity of *Ruminococcus albus* cellobiose phosphorylase through site-directed mutagenesis

Ken Hamura [†], Wataru Saburi [†], Hirokazu Matsui, Haruhide Mori [*]

Research Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

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ABSTRACT

Cellobiose phosphorylase (EC 2.4.1.20, CBP) catalyzes the reversible phosphorolysis of cellobiose to α-D-glucose 1-phosphate (Glc1*P*) and D-glucose. Cys485, Tyr648, and Glu653 of CBP from *Ruminococcus albus*, situated at the +1 subsite, were mutated to modulate acceptor specificity. C485A, Y648F, and Y648V were active enough for analysis. Their acceptor specificities were compared with the wild type based on the apparent kinetic parameters determined in the presence of 10 mM Glc1*P*. C485A showed higher preference for D-glucosamine than the wild type. Apparent k_{cat}/K_m values of Y648F for D-mannose and 2-deoxy-D-glucose were 8.2- and 4.0-fold higher than those of the wild type, respectively. Y648V had synthetic activity toward *N*-acetyl-D-glucosamine, while the other variants did not. The oligosaccharide production in the presence of the same concentrations of wild type and each mutant was compared. C485A produced 4-*O*-β-D-glucopyranosyl-D-glucosamine from 10 mM Glc1*P* and D-glucosamine at a rate similar to the wild type. Y648F and Y648V produced 4-*O*-β-D-glucopyranosyl-D-mannose and 4-*O*-β-D-glucopyranosyl-*N*-acetyl-D-glucosamine much more rapidly than the wild type when D-mannose and *N*-acetyl-D-glucosamine were used as acceptors, respectively. After a 4 h reaction, the amounts of 4-*O*-β-D-glucopyranosyl-D-mannose and 4-*O*-β-D-glucopyranosyl-*N*-acetyl-D-glucosamine produced by Y648F and Y648V were 5.9- and 12-fold higher than the wild type, respectively.

1. Introduction

Carbohydrate phosphorylases, catalyzing reversible phosphorolysis of glycosidic linkages at the non-reducing end of substrates, are useful for the efficient synthesis of sugars because generally, they have high substrate specificity and produce target carbohydrates specifically through the reverse reactions (synthetic reaction).[1] To increase the number of carbohydrates that can be synthesized by carbohydrate phosphorylases, identification of new enzymes is ongoing.[2–5]

Another strategy to expand application of carbohydrate phosphorylases for the synthesis of carbohydrates is to create mutant enzymes with specificities different from the parent enzymes. A mutant enzyme showing higher phosphorolytic activity toward lactose has been obtained through random mutation of cellobiose phosphorylase (EC 2.4.1.20, CBP).[6] Other mutant CBPs have been reported to be active toward various alkyl β-glucosides, methyl α-glucoside, and cellobiose in the synthetic reaction.[7] Recently, mutant trehalose phosphorylase was reported to produce lactotrehalose (α-D-glucosyl-(1,1)-α-D-galactoside) more efficiently than the wild type.[8]

On the basis of amino acid sequence similarities, CBP is categorized into glycoside hydrolase (GH) family 94 along with cellodextrin phosphorylase (EC 2.4.1.49), chitobiose phosphorylase (ChBP, EC 2.4.1.-), cyclic β-1,2-glucan synthase (CBGS, EC 2.4.1.-), and laminaribiose phosphorylase (LBP, EC 2.4.1.31).[9] In this family, only ChBP recognizes an *N*-acetyl-D-glucosamine moiety in the −1 and +1 subsites. It is evident from a comparison of the ChBP and CBP three dimensional structures that the amino acid residues forming the −1 subsite of ChBP are consistent with those of CBP, although the orientation of the Arg residue interacting with the chemical group at the C2 position is different.[10,11] In the +1 subsite of ChBP, Val forms a small pocket to accommodate an acetoamide group at the C2 position, while Tyr at the corresponding position of CBP forms a hydrogen bond to the 2-OH of D-glucose. This difference of amino acid residues suggests that it is possible to alter the acceptor specificities of the GH family 94 enzymes by site-directed mutagenesis.

Ruminococcus albus CBP (RaCBP) shows high synthetic activity toward D-glucose and 6-deoxy-D-glucose (apparent k_{cat}/K_m of these acceptors determined in the presence of 10 mM Glc1*P* were

Abbreviations: CBP, cellobiose phosphorylase; Glc1*P*, glucose 1-phosphate; GH, glycoside hydrolase; ChBP, chitobiose phosphorylase; CBGS, cyclic β-1,2-glucan synthase; LBP, laminaribiose phosphorylase; RaCBP, CBP from *Ruminococcus albus*; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

* Corresponding author. Tel./fax: +81 11 706 2497.
E-mail address: hmori@chem.agr.hokudai.ac.jp (H. Mori).

† These authors contributed equally to this work.

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63.2 s^{-1} mM^{-1} and 12.7 s^{-1} mM^{-1}, respectively).[12] 2-Deoxy D-glucose, D-mannose, and D-glucosamine are also acceptor substrates for RaCBP, but RaCBP shows low synthetic activities toward them (apparent k_{cat}/K_ms are 0.2–1.1% of that of D-glucose). *N*-Acetyl-D-glucosamine is inert as an acceptor. In this study, to enhance the synthetic activity toward C-2 derivatives of D-glucose, the +1 subsite of RaCBP was modulated through site-directed mutation.

2. Results and discussion

2.1. Selection of mutation sites of RaCBP

In the complex of *Celluvibrio gilvus* CBP and D-glucose, Cys491, Tyr653, and Glu659, corresponding to Cys485, Tyr648, and Glu654 of RaCBP, respectively, are located within 3 Å of the 2-OH group of D-glucose bound in the +1 subsite.[10] Glu residues, corresponding to Glu654 of RaCBP, are completely conserved in GH family 94 enzymes (Fig. 1). Most CBPs, CDPs, and ChBPs have Cys residues at the equivalent position of Cys485 of RaCBP, while CBGSs and LBPs have Gly residues at this position. ChBPs, acting on *N*-acetyl-D-glucosamine as an acceptor, have Val at the position of Tyr648 of RaCBP as described above. Other types of GH family 94 enzymes that bind to a D-glucose or D-glucosyl residue in the +1 subsite have a bulky amino acid residue at the corresponding positions: that is, CBPs, CDPs, and CBGS have Tyr, and LBPs have Phe. In this study, Cys485, Tyr648, and Glu653 of RaCBP were selected as the mutation sites to modulate the specificity of C-2 derivatives of D-glucose, and 6 mutant enzymes (C485A, Y648A, Y648V, Y648F, E654A, and E654D) were analyzed.

2.2. Production and physicochemical properties of the mutated RaCBPs

The mutated RaCBPs were produced in *Escherichia coli* as the wild type,[12] and all the enzymes were successfully purified to homogeneity by Ni-chelating column chromatography. Phosphorolytic activities of C485A, Y648F, and Y648V toward cellobiose were 43.0%, 23.0%, and 0.888% of the wild type, respectively (Table 1). Those of the other mutant enzymes were less than 0.1% of the wild type, and were not analyzed in the following experiments. In contrast to Y648F, Y648A, and Y648V had significantly lower activities than the wild type (0.0535% and 0.888% of that of the wild type, respectively), indicating that a bulky amino acid residue is required to maintain the enzymatic activity toward cellobiose. In *C. gilvus* CBP, Glu659, corresponding to Glu654 of RaCBP, interacts with the 3-OH of D-glucose at the +1 subsite in addition to the 2-OH group, suggesting that this residue is essential for catalytic activity.

C485A, Y648F, and Y648V showed the highest activity at pH 6.0 similar to the wild type. The C485A and Y648V mutants were stable over a pH range similar to the wilD-type (pH 5.5–8.8),[12] but Y648F was less stable at high pH than the wild type (pH 5.5–7.5). C485A was stable below 45 °C unlike the other mutants, which were stable below 40 °C similar to the wild type.

		*		* *
RaCBP	479:	RA**DWND**CINLSCY	645:	ISTYPGGYK**EN**AGIF
CgCBP	485:	RA**DWND**CLNLNCF	650:	VSTYPPGYK**EN**GGIF
CuCBP	485:	RA**DWND**CLNLNCF	650:	VSTYPPGYK**EN**GGIF
CtCBP	478:	RA**DWND**CLNLNCF	637:	ISTYPPGYK**EN**AGIF
TmCBP	479:	RA**DWND**CLNLNCF	639:	ISSYPPGYK**EN**AGIF
CsCDP	468:	RA**DWND**TLNLDMG	609:	MTTYPPGAK**EN**GGIF
CtCDP	619:	LA**DWND**CLKIASN	801:	TGHYFFGDR**EN**GAVF
AfCBGS	2523:	GG**DWND**GMNRVGV	2669:	IKGYPPGVR**EN**GGQY
AvCBGS	2525:	GG**DWND**GMNRVGV	2670:	IKSYPPGVR**EN**GGQY
PsLBP	521:	GA**DWND**GLDLAPE	723:	AFGFAFGHK**EN**GAMF
AlLBP	486:	NA**DWND**GLDMAKN	662:	AFGFSYNHK**EN**GAVF
VpChBP	487:	RA**DWND**CLNLGGG	628:	VTRVYQGVK**EN**GAIF
VfChBP	487:	RA**DWND**CLNLGGG	628:	VTRVYQGVK**EN**GAIF

Figure 1. Comparison of amino acid sequence of GH family 94 enzymes. Multiple-sequence alignment was constructed using Clustal W program (http://www.genome.jp/tools/clustalw/). Amino acid residues completely conserved are shown in boldface. Mutated sites are indicated by asterisks above the sequence. CgCBP, *C. gilvus* CBP (GenBank ID, BAA28631.1); CuCBP, *Cellulomonas uda* CBP (AAQ20920.1); CtCBP, *Clostridium thermocellum* YM4 CBP (AAL67138.1); TmCBP, *Thermotoga maritima* MSB8 CBP (AAD36910.1); CsCDP, *Clostridium stercorarium* CDP (AAC45511.1); CtCDP, *Clostridium thermocellum* YM4 CDP (BAB71818.1); AfCBGS, *Agrobacterium fabrum* C58 CBGS (AAK73356.1); AvBGS, *Agrobacterium vitis* F2/5 CBGS (AAQ08605.1); PsLBP, *Paenibacillus* sp. YM1 LBP (BAJ10826.1); AlLBP, *Acholeplasma laidlawii* PG-8A LBP (ABX81345.1); VpChBP, *Vibrio proteolyticus* ChBP (BAC87867.1); and VfChBP, *Vibrio furnissii* ChBP (AAG23740.1).

2.3. Phosphorolysis and synthesis of cellobiose by mutated RaCBPs

The apparent kinetic parameters of C485A, Y648F, and Y648V for cellobiose (phosphorolysis) and D-glucose (synthesis) were measured in the presence of 10 mM inorganic phosphate and Glc1*P*, respectively (Table 2). C485A and Y648F showed $k_{cat\ (app)}$ values for both substrates similar to those of the wild type, while their $K_{m\ (app)}$ values were 7.7- to 21-fold higher than those of the wild type. In the phosphorolytic reaction for Y648V, a saturation curve was not obtained under the analytical conditions, and only a $k_{cat\ (app)}/K_{m\ (app)}$ value was determined, 0.0681 s^{-1} mM^{-1}, which was 690-fold lower than the wild type. In the synthetic reaction, the $k_{cat\ (app)}$ and $K_{m\ (app)}$ of Y648V for D-glucose were 71-fold lower and 11-fold higher than the wild type, respectively. These results indicate that Cys485 and Tyr648 contribute to binding to D-glucose at the +1 subsite as predicted on the basis of comparison of the amino acid sequences between RaCBP and the structure-known *C. gilvus* CBP.

2.4. Acceptor specificity of the mutated RaCBPs

Synthetic activities of C485A, Y648F, and Y648V toward various C2-derivatives of D-glucose were investigated (Table 3). C485A had 2- and 18-fold lower $k_{cat\ (app)}/K_{m\ (app)}$ toward D-mannose and 2-deoxy-D-glucose than the wild type. In contrast, its $k_{cat\ (app)}$ and $K_{m\ (app)}$ values for D-glucosamine were 3.7- and 4.4-fold higher than those of the wild type, respectively, while the $k_{cat\ (app)}/K_{m\ (app)}$ was similar to that of the wild type. D-Glucosamine did not serve as an acceptor substrate for Y648F and Y648V, suggesting that Tyr648 participates in interactions important for binding to D-glucosamine. The $k_{cat\ (app)}/K_{m\ (app)}$ values of Y648F for D-mannose and 2-deoxy-D-glucose were 8.2- and 4.0-fold higher than those of the wild type, respectively, while $K_{m\ (app)}$ values for these substrates were 2.7- and 1.4-fold higher. Replacement of Tyr648 with Phe enhanced the preference for D-mannose and 2-deoxy-D-glucose; thus the OH group of the Tyr might disturb the binding of these acceptors. LBPs have Phe at the corresponding position, but they possess low synthetic activity toward D-mannose.[13,14] The sequence identity of RaCBP to LBPs from *Paenibacillus* sp. YM-1 and *Acholeplasma laidlawii* PG-8A is very low, approximately

Table 1
Phosphorolytic activity of RaCBP variants toward cellobiose

Enzyme	v (s^{-1})	Relative v (%)
Wild type	76.9 ± 0.6	100
C485A	33.1 ± 0.3	43.0
Y648A	0.0412 ± 0.0013	0.0535
Y648F	17.7 ± 0.3	23.0
Y648V	0.683 ± 0.003	0.888
E654A	0.00155 ± 0.00016	0.00202
E654D	0.0275 ± 0.0011	0.0357

Reaction rates for phosphorolysis of 10 mM cellobiose were measured in the presence of 10 mM sodium phosphate buffer (pH 6.0). Data are mean ± standard deviation for three independent experiments.

Table 2
Apparent kinetic parameters of RaCBP variants for the phosphorolysis and synthesis of cellobiose

Enzyme	Phosphorolysis[a]			Synthesis[b]		
	$k_{cat\ (app)}$ (s^{-1})	$K_{m\ (app)}$ (mM)	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)	$k_{cat\ (app)}$ (s^{-1})	$K_{m\ (app)}$ (mM)	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)
Wild type	79.1 ± 5.2	1.68 ± 0.11	47.1	92.3 ± 12	1.46 ± 0.08	63.2
C485A	88.9 ± 1.4	16.6 ± 0.4	5.35	96.5 ± 0.6	11.3 ± 0.1	8.55
Y648F	73.9 ± 5.0	31.4 ± 3.2	2.36	64.2 ± 1.5	30.7 ± 0.9	2.09
Y648V	N. D.	N. D.	0.0681	1.30 ± 0.12	15.7 ± 2.0	0.0829

N. D. were not determined because a saturation curve was not obtained. Data are mean ± standard deviation for three independent experiments.
[a] Reaction rates for the phosphorolysis of 1–15 mM cellobiose were measured in the presence of 10 mM sodium phosphate buffer (pH 6.0).
[b] Reaction rates for the synthesis of cellobiose from 5–100 mM D-glucose and 10 mM Glc1*P* were measured.

Table 3
Apparent kinetic parameters of RaCBP variants for synthetic reactions toward C2 derivatives of D-glucose

Enzyme		D-Mannose	2-Deoxy-D-glucose	D-Glucosamine	*N*-Acetyl-D-glucosamine
Wild type	$k_{cat\ (app)}$ (s^{-1})	2.83 ± 0.05	21.5 ± 2.4	9.56 ± 0.60	N. D.
	$K_{m\ (app)}$ (mM)	23.5 ± 1.1	60.2 ± 10.4	13.3 ± 2.0	N. D.
	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)	0.112	0.357	0.719	N. D.
C485A	$k_{cat\ (app)}$ (s^{-1})	0.626 ± 0.007	N. D.	35.4 ± 0.2	N. D.
	$K_{m\ (app)}$ (mM)	11.8 ± 0.2	N. D.	58.1 ± 1.4	N. D.
	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)	0.0532	0.0198	0.6100	N. D.
Y648F	$k_{cat\ (app)}$ (s^{-1})	58.3 ± 1.8	119 ± 16	N. D.	N. D.
	$K_{m\ (app)}$ (mM)	62.6 ± 2.6	83.1 ± 15.5	N. D.	N. D.
	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)	0.931	1.430	N. D.	N. D.
Y648V	$k_{cat\ (app)}$ (s^{-1})	5.27 ± 0.34	7.83 ± 1.33	N. D.	6.54 ± 0.83
	$K_{m\ (app)}$ (mM)	200 ± 16	164 ± 35	N. D.	174 ± 28
	$k_{cat\ (app)}/K_{m\ (app)}$ ($s^{-1}\ mM^{-1}$)	0.0263	0.0477	N. D.	0.0376

Reaction rates for synthetic reaction to 5–100 mM acceptor and 10 mM Glc1*P* were measured. N. D. were not determined. In the reaction of C485A to 2-deoxy-D-glucose, $k_{cat\ (app)}/K_{m\ (app)}$ was determined from the slope of the *s*–*v* plot because the saturation curve was not obtained. Data are mean ± standard deviation for three independent experiments.

13%. Thus the acceptor binding mode of LBPs might be fundamentally different from CBPs. Y648V had a 760-fold lower $k_{cat\ (app)}/K_{m\ (app)}$ for D-glucose than the wild type, but it showed only 4.6- and 7.5-fold lower $k_{cat\ (app)}/K_{m\ (app)}$ values toward D-mannose and 2-deoxy-D-glucose, respectively. Only this mutated enzyme had synthetic activity toward *N*-acetyl-D-glucosamine. The introduced Val residue presumably forms a small pocket to accommodate the acetoamide group as observed for ChBP.[10,11]

2.5. Oligosaccharide synthesis using the mutated RaCBPs

Synthetic reactions of the mutant and wilD-type RaCBPs toward 10 mM Glc1*P* and the indicated acceptors were monitored (Fig. 2). Analyzed acceptors were as follows: for C485A, D-glucosamine; for Y648F, D-mannose; for Y648V, *N*-acetyl-D-glucosamine. The amount of oligosaccharides produced was estimated by the amount of inorganic phosphate liberated. At the same concentration of enzyme (1.0 μm), C485A and the wild type produced an oligosaccharide from 10 mM Glc1*P* and D-glucosamine at similar rates, and the concentrations of the oligosaccharide reached approximately equal concentrations, 6.2 mM and 5.9 mM (meaning 62% and 59% yield), respectively, after a 4 h reaction. The oligosaccharide produced by C485A was purified, and ^{13}C-nuclear magnetic resonance (NMR) was recorded. The chemical shifts of this oligosaccharide completely corresponded to those of 4-*O*-β-D-glucopyranosyl-D-glucosamine.[15]

Under conditions of 0.10 μm Y648F or wild type, Y648F much more rapidly liberated inorganic phosphate from 10 mM Glc1*P* and D-mannose than the wild type. After a 4 h reaction, concentrations of the oligosaccharide produced by Y648F and the wild type were estimated to be 5.6 mM and 0.95 mM (56% and 9.5% of yield), respectively. The ^{13}C NMR spectrum of the oligosaccharide produced by Y648F coincided with that of 4-*O*-β-D-glucopyranosyl-D-mannopyranose.[16] Y648V (0.90 μm) produced an oligosaccharide from 10 mM Glc1*P* and *N*-acetyl-D-glucosamine at a much higher rate than the same concentration of the wild type. Concentrations of the oligosaccharide produced were calculated to be 3.8 mM and 0.32 mM (38% and 3.2% yield), respectively, after a 4 h reaction. The chemical structure of the oligosaccharide produced by Y648V was confirmed to be 4-*O*-β-D-glucopyranosyl-*N*-acetyl-D-glucosamine[17] by ^{13}C NMR analysis.

Amino acid residues at the corresponding position of Tyr648 of RaCBP are considered to be an important structural element for determining acceptor specificity of CBP and ChBP.[10,11] In this study, we demonstrated that site-directed mutagenesis at Tyr648 of RaCBP modulated acceptor specificity, and the mutant enzymes Y648F and Y648V much more efficiently produced 4-*O*-β-D-glucopyranosyl-D-mannopyranose and 4-*O*-β-D-glucopyranosyl-*N*-acetyl-D-glucosamine, respectively, than the wild type.

3. Experimental

3.1. Preparation of mutated RaCBPs

The expression plasmids for the mutant RaCBPs were prepared using a Primestar Mutagenesis Basal Kit (Takara Bio, Otsu, Japan). The expression plasmid of the wild type[12] was used as the template. Primers used in the PCR are summarized in Table 4. The mutant enzymes were produced in the transformant of *Escherichia coli* harboring the expression plasmid of each derivative of RaCBP, and purified to homogeneity as described elsewhere.[12] To improve protein production, induction culture was carried out at 18 °C for 24 h in the presence of 0.1 mM isopropyl β-D-thiogalactopyranoside. Enzyme concentrations of purified RaCBP variants were determined by amino acid analysis after complete hydrolysis.[12]

3.2. Enzyme assay

3.2.1. Phosphorolysis of cellobiose

The reaction rate for phosphorolysis of 10 mM cellobiose (Sigma, St. Louis, MO) was measured in the presence of 10 mM

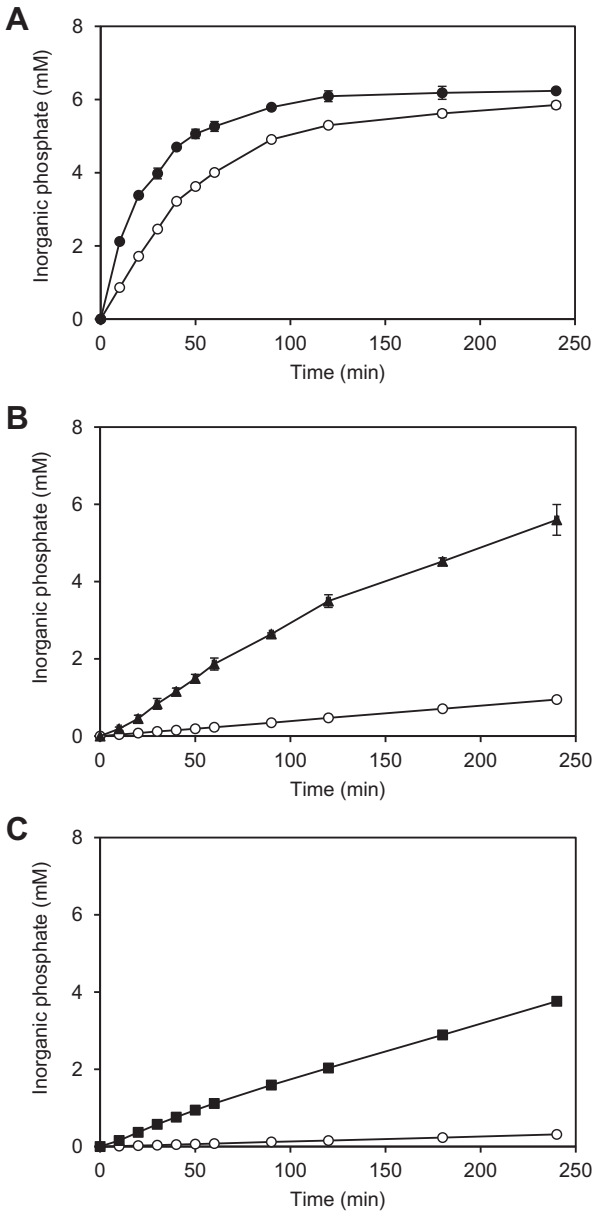

Figure 2. Time course of synthetic reactions carried out by mutated RaCBPs. Synthetic reactions toward 10 mM Glc1*P* and 10 mM acceptor substrates were monitored. Equimolar inorganic phosphate is liberated during the oligosaccharide synthesis. Enzyme concentrations of wild type and the mutant enzymes were 1.0, 0.10, and 0.90 μm for the reactions with D-glucosamine (A), D-mannose (B), and *N*-acetyl-D-glucosamine (C), respectively. Open circle, filled circle, triangle, and square indicate the wild type, C485A, Y648F, and Y648V, respectively.

sodium phosphate buffer (pH 6.0) as described previously.[12] Apparent kinetic parameters for the phosphorolysis of cellobiose were determined from phosphorolytic velocities at various concentrations of cellobiose and 10 mM sodium phosphate buffer (pH 6.0) as described previously.[12]

3.2.2. Synthetic reaction to various monosaccharides

Apparent kinetic parameters for the synthetic reaction to D-glucose (Nacalai Tesque, Kyoto, Japan), 2-deoxy-D-glucose (Tokyo Chemical Industry, Tokyo), D-mannose (Wako Pure Chemical Industries, Osaka, Japan), D-glucosamine (Tokyo Chemical Industry), and *N*-acetyl-D-glucosamine (Nacalai Tesque) were determined from reaction rates at various concentrations of monosaccharides and 10 mM Glc1*P* (Sigma) as described previously.[12]

Table 4
Primer sequences used in this study

Name	Sequence (5′ to 3′)	Direction
C485A sense	AACGACGCAATCAACCTGTCATGCTAC	Forward
C485A antisense	GTTGATTGCGTCGTTCCAGTCAGCACG	Reverse
Y648A sense	TCCACAGCTCCCGGCGGATACAACGAG	Forward
Y648A antisense	GCCGGGAGCTGTGGAGATCTCACCGTA	Reverse
Y648F sense	TCCACATTTCCCGGCGGATACAACGAG	Forward
Y648F antisense	GCCGGGAAATGTGGAGATCTCACCGTA	Reverse
Y648V sense	TCCACAGTTCCCGGCGGATACAAGGAG	Forward
Y648V antisense	GCCGGGAACTGTGGAGATCTCACCGTA	Reverse
E654A sense	TACAAGGCGAACGCAGGTATCTTCACA	Forward
E654A antisense	TGCGTTCGCCTTGTATCCGAAGGGATA	Reverse
E654D sense	TACAAGGACAACGCAGGTATCTTCACA	Forward
E654D antisense	TGCGTTATCCTTGTATCCGAAGGGATA	Reverse

Primer names include names of the mutant enzymes prepared.

3.2.3. Heat and pH stabilities and optimum pH of mutated RaCBPs

The optimal pH, pH and heat stabilities of mutated RaCBPs were investigated as described previously.[12]

3.3. Time course of oligosaccharide production by mutant RaCBPs

A reaction mixture (600 μL) consisting of enzyme, 20 mM MES–NaOH buffer (pH 6.0), 10 mM Glc1*P*, and 10 mM acceptor substrate was incubated at 37 °C. Fifty microliters of the reaction mixture was taken at the indicated time, and mixed with 50 μL of 4 M Tris–HCl buffer (pH 7.0) to stop the reaction. Inorganic phosphate liberated was measured following Lowry and Lopez.[18] In the reactions with D-mannose, D-glucosamine, and *N*-acetyl-D-glucosamine, enzyme concentrations were 0.10, 1.0, and 0.90 μM, respectively.

3.4. Preparation and structural analysis of oligosaccharides produced by mutant RaCBPs

One milliliter of a reaction mixture consisting of enzyme, 10 mM MES–NaOH buffer (pH 6.0), 100 mM Glc1*P*, and 100 mM of each acceptor substrate was incubated at 37 °C for 5 h, and heated at 70 °C for 10 min to stop the reaction. For the reactions with D-mannose, D-glucosamine, and *N*-acetyl-D-glucosamine, 4.7 μM C485A, 3.1 μM Y648F, and 2.8 μM Y648V were used, respectively. The reaction mixture containing D-mannose or *N*-acetyl-D-glucosamine was desalted using Amberlite MB4 (Rohm and Haas, Philadelphia, PA) to remove any remaining Glc1*P*, and subjected to gel filtration column chromatography using a Bio-Gel P2 extra fine column (1.8 × 100 cm). Column chromatography was carried out at 3 mL/h using water as the eluent. The product from the reaction with D-glucosamine was removed by treatment with Amberlite MB4; thus this was purified by thin layer chromatography (TLC). The reaction mixture of D-glucosamine was concentrated to approximately 200 μL, and was subjected to TLC in which 1-butanol/acetic acid/water = 8:3:2 (v/v/v) was used as the developing solvent. Silica gel containing the product was scraped off, and the oligosaccharide was extracted with 5 mL of water.

Chemical structures of the purified oligosaccharides were analyzed by ^{13}C NMR. ^{13}C NMR was recorded at 300 K in D_2O using ECP-400 (100 MHz, JEOL, Tokyo). 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt was used as the standard. The chemical shifts of the samples were compared with known oligosaccharides.[15–17]

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