Characterization of Ruminococcus albus Cellodextrin Phosphorylase and Identification of a Key Phenylalanine Residue for Acceptor Specificity and Low Affinity to the Phosphate Group

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Abbreviations: CBP, cellobiose phosphorylase; CDP, cellodextrin phosphorylase; ChBP, \(N,N'\)-diacetylchitobiose phosphorylase; GH, glycoside hydrolase; Glc\(1P\), glucose 1-phosphate; RaCDP, Ruminococcus albus NE1 CDP;

Enzymes: \(\beta\)-glucosidase, EC 3.2.1.21; Cellobiose 2-epimerase, EC 5.1.3.11; Cellobiose phosphorylase, EC 2.4.1.20; Cellodextrin phosphorylase, EC 2.4.1.49; Cellulase, EC 3.2.1.4; \(N,N'\)-Diacetylchitobiose phosphorylase, EC 2.4.1.280; Cyclic \(\beta\)-1,2-glucan synthase, EC 2.4.1.-; Laminaribiose phosphorylase, EC 2.4.1.31; 4-\(O\)-\(\beta\)-D-Mannosyl-D-glucose phosphorylase, EC 2.4.1.281

Keywords: Cellodextrin phosphorylase; Glycoside hydrolase family 94; Substrate specificity; Acceptor binding site; Phosphate binding site
Abstract

*Ruminococcus albus* has the capability to intracellularly degrade cellooligosaccharides primarily via phosphorolysis. In this study, the enzymatic characteristics of *R. albus* cellodextrin phosphorylase (RaCDP), which is classified into glycoside hydrolase family 94, was investigated. RaCDP catalyzes the phosphorolysis of cellotriose through an ordered bi bi mechanism in which cellotriose binds to RaCDP before inorganic phosphate and then cellobiose and glucose 1-phosphate (Glc1P) are released in that order. Among the cellooligosaccharides tested, RaCDP had the highest phosphorolytic and synthetic activities towards cellohexaose and cellopentaose, respectively. RaCDP successively transferred glucosyl residues from Glc1P to the growing cellooligosaccharide chain, and insoluble cellooligosaccharides composed of an average of 8 residues were produced. Sophorose, laminaribiose, β-1,4-xylolbiose, β-1,4-mannobiose, and cellobiitol served as acceptors for RaCDP. RaCDP had very low affinity to phosphate groups in both the phosphorolysis and synthesis directions. A sequence comparison revealed that RaCDP has Gln646 at the position where His is normally conserved in the phosphate binding sites of related enzymes. Q646H showed approximately 2-fold lower apparent $K_m$ values for inorganic phosphate and Glc1P than the wild type. RaCDP has Phe633 at the position corresponding to Tyr and Val, conserved in the +1 subsites of cellobiose phosphorylase and $N,N'$-diacetylchitobiose phosphorylase, respectively. F633Y showed higher preference for cellobiose over β-1,4-mannobiose as an acceptor substrate in the synthetic reaction than the wild type. Furthermore, F633Y showed 75- and 1100-fold lower apparent $K_m$ values for inorganic phosphate and Glc1P, respectively, in the phosphorolysis and synthesis of cellotriose.

Introduction

*Ruminococcus albus* is an important ruminal bacterium involved in the digestion of dietary cellulose [1]. This bacterium has various cellulosytic enzymes including a cellulase (EC 3.2.1.4) [2], β-glucosidase (EC 3.2.1.21) [3], and cellobiose phosphorylase (CBP, EC 2.4.1.20) [4]. It was shown that within the cell, *R. albus* degrades cellooligosaccharides mainly through phosphorolysis.
catalyzed by CBP and cellobextrin phosphorylase (CDP, EC 2.4.1.49) rather than hydrolysis catalyzed by β-glucosidase [5]. Recently, R. albus CBP has been characterized [4], but the enzymatic properties of CDP from this bacterium remain unclear.

CDP, which was first identified in the cell-free extract of Clostridium thermocellum [6], catalyzes the reversible phosphorolysis of cellobiose to α-glucose 1-phosphate (Glc1P) and cellobiocarbohydrates with reduced chain-lengths. CDPs from C. thermocellum [7-9] and Clostridium stercorarium [10] have been characterized to date. They have the highest phosphorolytic activities towards cellotriose and cellotetraose, respectively.

According to the sequence based classification system of carbohydrate active enzymes [11], CDP is classified into the glycoside hydrolase (GH) family 94 together with CBP, N,N′-diacetylchitobiose phosphorylase (ChBP, EC 2.4.1.280), the C-terminal region of cyclic β-1,2-glucan synthase (EC 2.4.1.-) having phosphorolytic activity towards β-1,2-glucosaccharides [12], and laminaribiose phosphorylase (EC 2.4.1.31). The catalytic domains of GH family 94 enzymes are formed by an (α/α)6-barrel [13-15], which has a fold similar to the catalytic domain of clan GH-L enzymes including glucoamylase and maltose phosphorylase [16, 17]. Inorganic phosphate nucleophilically attacks the anomeric carbon of the glycosyl residue at the -1 subsite with the assistance of the conserved Asp, acting as the general acid catalyst to donate a proton to the glycosidic oxygen [18].

In the genome of R. albus 7, two genes encoding GH family 94 proteins are found. The deduced amino acid sequence of Rumal_0187 is completely identical to that of CBP from R. albus NE1 [4]. On the other hand, it is difficult to predict the function of another gene, Rumal_2403, because its deduced amino acid sequence is not closely related to any known GH family 94 enzymes (Fig. 1). However, it was demonstrated that the cell-free extract of R. albus B199 grown on cellobextrin shows CDP activity [5], thus the CDP gene presumably encoded in the genome of R. albus. We considered Rumal_2403 to be a possible candidate gene encoding CDP, although the shared homology of the deduced amino acid sequence of Rumal_2403 to CDPs from C. stercorarium and C. thermocellum are low, 36% and 15%, respectively. In this study, the gene corresponding to the Rumal_2403 gene of R. albus 7 (the RaCDP gene) was cloned from R. albus NE1 [19]. The
recombinant protein produced in *Escherichia coli* was characterized in detail, and functions of some amino acid residues involved in the formation of acceptor- and phosphate binding sites are discussed based on the results of mutational analyses.

**Results and Discussion**

*Production, purification, and basic properties of recombinant RaCDP.*

The RaCDP gene of *R. albus* NE1 was obtained by PCR, in which the primers designed based on the Rumal_2403 gene of *R. albus* were used. The sequence of the amplified DNA fragment was completely identical to the Rumal_2403 gene of *R. albus*. The cell-free extract of the *E. coli* transformant overexpressing the RaCDP gene showed phosphorolytic activity towards cellotriose. The recombinant enzyme was purified to homogeneity by Ni-chelating column chromatography. From 0.5 L of culture medium, 25.1 mg of the purified enzyme (20.4 U/mg) was obtained. The molecular masses of the purified enzyme measured by SDS-PAGE and gel filtration column chromatography were 95 kDa and 177 kDa, respectively, indicating that RaCDP forms a homo-dimer in solution as observed for other GH family 94 enzymes [4, 18, 20, 21]. RaCDP showed the highest activity at pH 6.0 and 50°C, and was stable in the pH range of 5.8-8.3 and below 40°C.

**Kinetic mechanism of phosphorolysis of cellotriose by RaCDP.**

Phosphorolytic reaction velocities towards various concentrations of cellotriose and inorganic phosphate were measured (Fig. 2). The lines, obtained from double reciprocals plots for 1/v versus 1/[cellotriose] at various concentrations of inorganic phosphate, crossed at a certain point, indicating that the reaction occurred through a sequential bi bi mechanism. To determine the order of substrate binding and product release, product inhibition analysis of the phosphorolysis of cellotriose was carried out (Fig. 3). Competitive inhibition by Glc1P was observed in the reaction towards varying concentrations of cellotriose and a fixed concentration of inorganic phosphate. Mixed-type inhibition was observed under conditions of varying concentrations of cellotriose (the concentration of inorganic phosphate was fixed) versus cellobiose and varying concentrations of inorganic phosphate (the concentration of cellotriose was fixed) versus cellobiose or Glc1P. Thus in the phosphorolysis
of cellotriose, cellotriose and inorganic phosphate bind to RaCDP in that order, and Glc1P is
released after cellobiose. The kinetic parameters for the phosphorolysis of cellotriose were
determined as follows: $k_{cat} = 91.2 \pm 3.4$ s$^{-1}$, $K_{mA} = 5.41 \pm 0.88$ mM, $K_{mB} = 124 \pm 12$ mM, $K_{iA} = 12.6 \pm$
1.6 mM (A, cellotriose; B, inorganic phosphate). Generally, GH family 94 enzymes have low $K_m$
values for inorganic phosphate (0.14-1.5 mM) [4, 18, 20-24], but RaCDP had a significantly higher
$K_m$ value than the known enzymes (described below in detail). In the synthetic reaction, RaCDP also
had a high $K_{m \text{(app)}}$ value for Glc1P (166 ± 10 mM), which was determined by measuring the reaction
rates towards various concentrations of Glc1P and 40 mM cellobiose. RaCDP had a much higher $k_{cat}$
value than the characterized CDPs [10, 25], thus this enzyme may be functional in the degradation of
cellooligosaccharides in vivo.

Substrate specificity of RaCDP in the phosphorolytic and synthetic reactions.

The apparent kinetic parameters for the phosphorolysis of a series of cellooligosaccharides were
determined in the presence of 250 mM inorganic phosphate (Table 1). RaCDP had phosphorolytic
activity towards cellooligosaccharides longer than cellobiose. The $K_{m \text{(app)}}$ value decreased with an
increase of substrate chain-length, and the $k_{cat \text{(app)}}$ value of cellotetraose was the highest among the
cellooligosaccharides tested. Cellohexaose was the best substrate in terms of its $k_{cat \text{(app)}}/K_{m \text{(app)}}$. The
$k_{cat \text{(app)}}/K_{m \text{(app)}}$ towards this substrate was 4.2-fold higher than towards cellotriose. Cellotetraose and
cellotriose are the best substrates for the phosphorolytic reactions catalyzed by CDPs from \textit{C.}
\textit{stercorarium} and \textit{C. thermocellum}, respectively [10, 25], thus RaCDP has more preference for
long-chain substrate than the reported enzymes.

In synthetic reactions in the presence of 250 mM Glc1P, all cellooligosaccharides tested served as
acceptor substrates, although synthetic activity towards D-glucose was not observed, consistent with
the result that RaCDP did not phosphorolyze cellobiose. RaCDP had the highest $k_{cat \text{(app)}}/K_{m \text{(app)}}$
value toward cellopentaose. This result coincided with the fact that RaCDP had the highest
phosphorolytic activity toward cellohexaose. The $k_{cat \text{(app)}}/K_{m \text{(app)}}$ value towards cellohexaose was
approximately half of that towards cellopentaose. This indicates that the acceptor binding site, which
binds to the reducing-end part of the substrate from the scissile bonds, comprises 5 subsites with positive affinity. Sophorose, laminaribiose, β-1,4-xylobiose, β-1,4-mannobiose, and cellobiitol are also acceptor substrates of RaCDP. The $k_{\text{cat (app)}}/K_{\text{m (app)}}$ values toward β-1,4-mannobiose, β-1,4-xylobiose, and laminaribiose were 18%, 8.5%, and 7.9% of that towards cellobiose, respectively. Cellobiitol was a very poor acceptor for RaCDP, indicating that a closed glucose ring is recognized at the +2 subsite of the enzyme. Gentiobiose, lactose, $N,N'$-diacetylchitobiose, and α-linked glucobioses did not serve as acceptor substrates.

Production of cellooligosaccharides via the synthetic reaction of cellobiose and Glc1P.

Production of cellooligosaccharides from 250 mM Glc1P and 50 mM cellobiose was monitored (Fig. 4). At the initial stage of the reaction, cellotriose was produced rapidly, reaching a maximum at about 20 min. Cellotetraose, cellopentaose, and cellohexaose were also produced, indicating that RaCDP successively transferred a glucose moiety to the newly produced oligosaccharides. The highest concentrations of cellotriose, cellotetraose, cellopentaose, and cellohexaose obtained under the analytical conditions were 11.2 mM (40 min), 5.8 mM (60 min), 2.8 mM (80 min), and 1.3 mM (80 min), respectively. The yield of cellooligosaccharides calculated from the reacted cellobiose increased during the incubation time analyzed. After a reaction of 60 min, the yield was 52%, which is comparable to that for C. thermocellum ATCC27405 CDP. Yields of cellooligosaccharides from 200 mM cellobiose and Glc1P and from 20 mM cellobiose and 80 mM Glc1P were reported to be 48% and 54%, respectively [7, 9]. The cellobiose concentration decreased at a longer reaction time, and the concentration of cellobiose at 80 min was 13.3 mM, indicating that the yield of cellooligosaccharides was 73%. At 100 min, insoluble material was observed in the reaction mixture, similar to the reaction catalyzed by C. thermocellum CDP [25]. To analyze the chemical structure of the insoluble material, the synthetic reaction with a 5-fold increase of enzyme was carried out for 3 h. The products collected by centrifugation (7 mg) were dissolved in 4% NaOD, and the $^1$H-NMR was recorded. The spectrum of this product completely corresponded with that of the hydrolysate of cellulose [26], indicating that the insoluble product is a mixture of cellooligosaccharides. Calculated
from the relative peak areas of the 1-H of glucose at the reducing end and other glucose residues, the average degree of polymerization DP was estimated to be 8. From this length of cellobiooligosaccharides, the yield was estimated to be 11% based on the cellobiose used.

Comparison of amino acid sequences between RaCDP and other GH94 enzymes

The structure of the binding site of inorganic phosphate has been analyzed for several GH family 94 enzymes [13-15]. Highly conserved amino acid residues corresponding to Arg351, His666, Gln712, Thr731, Gly732, and Thr733 of Cellvibrio gilvus CBP are involved in the formation of this site. RaCDP has Gln646 in the position equivalent to His666 of C. gilvus CBP, although the other amino acid residues forming the inorganic phosphate binding site are well conserved (Fig. S1).

In ChBP from Vibrio proteolyticus, Val631 situated at the +1 subsite forms a small hydrophobic pocket to accommodate the N-acetyl group of N-acetyl-D-glucosamine. Most GH family 94 enzymes have a bulky residue, Tyr, at the corresponding position, thus Val631 of V. proteolyticus ChBP is thought to be a crucial residue for binding to the N-acetyl-D-glucosamine residue at the +1 subsite [13, 15]. In the case of CBP, the Tyr residue corresponding to Val631 of V. proteolyticus ChBP participates in a hydrogen binding interaction with the 2-OH group of the glucose residue at the +1 subsite [13]. RaCDP has Phe633 at the corresponding position (Fig. S1), implying that RaCDP has different acceptor specificity from other GH family 94 enzymes at the 2-OH position of a glycosyl residue bound to the +1 subsite. In fact, the $k_{\text{cat(app)}}/K_{\text{m(app)}}$ for β-1,4-mannobiose was 18% of that of cellobiose, making this a relatively good acceptor substrate for RaCDP, although interactions with the +2 subsite might contribute to the binding of β-1,4-mannobiose to the acceptor binding site. The synthetic activity of other known CDPs towards β-1,4-mannobiose have not been investigated. For CBPs, D-mannose is a poor acceptor substrate. The apparent $k_{\text{cat}}/K_{\text{m}}$ values of CBPs for the synthetic reaction of D-mannose are less than 1.2% of those for D-glucose [4, 22-24, 27]. Although the OH-group of the Tyr side chain is conserved in most GH family 94 enzymes other than ChBP, it might result in an unfavorable interaction with the axial OH-group at the C2-position of the glycosyl residue at the +1 subsite.
Functional analysis of the inorganic phosphate binding site of RaCDP

Gln646 of RaCDP, corresponding to the conserved His involved in binding to inorganic phosphate as described above, was replaced by His by site-directed mutagenesis. The mutant enzyme was produced and purified as for the wild-type enzyme. The apparent kinetic parameters for inorganic phosphate and Glc1P in the direction of phosphorolysis and synthesis, respectively, were determined. The $k_{\text{cat}}^{\text{(app)}}$ values of the mutant enzyme (Q646H) for inorganic phosphate and Glc1P were 9.5% and 3.9% of those of the wild type, respectively, while the $K_{\text{m}}^{\text{(app)}}$ values for inorganic phosphate and Glc1P were less than half of those of the wild type (Table 2), indicating that Gln646 is partly responsible for the high $K_{\text{m}}^{\text{(app)}}$ values for inorganic phosphate and Glc1P. Regardless, Q646H still has much lower affinity toward phosphate groups than the known GH family 94 enzymes. The substitution of Gln646 with His decreased the $k_{\text{cat}}^{\text{(app)}}$ of RaCDP for the synthetic reaction more than for the phosphorolysis. His666 of C. gilvus CBP is located at a position far from the acceptor binding site, thus Gln646 of RaCDP does not appear to have direct interactions with acceptor substrates. Introduction of His to position 646 of RaCDP might indirectly alter the interaction with the acceptor substrate, and decrease the synthetic activity.

Functional analysis of the acceptor binding site of RaCDP

Phe633 of RaCDP, corresponding to the conserved Tyr and Val of CBP and ChBP, respectively, was replaced by Tyr (F633Y). In the phosphorolysis of cellotriose, F633Y had 3.6- and 4.9-fold lower $k_{\text{cat}}^{\text{(app)}}$ and $K_{\text{m}}^{\text{(app)}}$ values for cellotriose, respectively, than the wild type, resulting in a 1.4-fold higher $k_{\text{cat}}^{\text{(app)}}/K_{\text{m}}^{\text{(app)}}$ (Table 3). Apparent kinetic parameters for the acceptor substrates, cellobiose and β-mannobiose, were measured in the presence of 250 mM Glc1P (Table 3). F633Y had 1.1- and 2.2-fold higher $K_{\text{m}}^{\text{(app)}}$ values for β-mannobiose and cellobiose than those of the wild type. The $k_{\text{cat}}^{\text{(app)}}$ values for β-mannobiose were more severely decreased than for cellobiose. The $k_{\text{cat}}^{\text{(app)}}$ values for cellobiose and β-mannobiose were 16.5- and 145-fold lower than those of wild type, resulting in a lower ratio of $k_{\text{cat}}^{\text{(app)}}/K_{\text{m}}^{\text{(app)}}$ values for β-mannobiose and cellobiose, indicating that F633Y has less preference for β-mannobiose than the wild type. The side chain OH-group of the
introduced Tyr633 might cause steric hindrance or unfavorable interactions at the +1 subsite when binding β-mannobiose as an acceptor.

Surprisingly, F633Y had significantly higher affinity to phosphate than the wild type. In the phosphorolysis of cellotriose, F633Y had 75-fold lower $K_m$(app) values for inorganic phosphate, $2.40 \pm 0.11$ mM, which is close to the values for known GH family 94 enzymes (Table 2). The $k_{cat}$(app) for inorganic phosphate was 15-fold lower, but the $k_{cat}$(app)/$K_m$(app) value for inorganic phosphate was 4.9-fold higher, because of the very low $K_m$(app) value. This mutant enzyme also had much higher affinity to Glc1P in the synthetic reaction than the wild type. The $K_m$(app) value for Glc1P was 1,100-fold lower than for the wild type. The $k_{cat}$(app)/$K_m$(app) value for Glc1P was 52-fold higher than the wild type, although the $k_{cat}$(app) for Glc1P was 20.5-fold lower than the wild type. Phe633 of RaCDP is predicted to be far from the phosphate binding site based on the three-dimensional structures of related enzymes, and this residue does not appear to have a direct interaction with inorganic phosphate and the phosphate group of Glc1P. In the complex of C. gilvus CBP, inorganic phosphate, d-glucose (+1 subsite), and glycerol (-1 subsite), the OH-group of the side chain of Tyr653 appears to participate in a hydrogen bonding interaction with Lys658 forming a hydrogen bond to Gln712, and thus is involved in the formation of the phosphate binding site. The orientation of Gln712, arranged through the interaction with Lys658, might be crucial for the observed high affinity to phosphate. In RaCDP, Lys638 and Gln692 are predicted to be located at positions equivalent to Lys658 and Gln712 of C. gilvus CBP, respectively. The side chain of Phe633 cannot form a hydrogen bond to Lys638, thus the orientation of Gln692 of RaCDP is thought to be unfavorable for binding to the phosphate group. Amino acid residues corresponding to Lys658 and Gln712 of C. gilvus CBP are conserved in most GH family 94 enzymes (Fig. S1), and the hydrogen bonding network, as discussed herein, likely plays an important role in the high affinity to the phosphate group. CDP from C. thermocellum has Arg and Ser at the positions equivalent to Lys658 and Gln712 of C. gilvus CBP, respectively, but in the synthetic direction, this enzyme has a $K_m$(app) value for Glc1P of 4.7 mM [6], which is similar in magnitude to the values of most GH family 94 enzymes. Two characterized laminaribiose phosphorylases have a Phe residue at the position.
corresponding to Tyr653 of C. gilvus CBP, but these enzymes have low $K_m$ values for inorganic phosphate (0.14-0.4 mM) for the phosphorolysis of laminaribiose unlike RaCDP [28, 29]. These enzymes show very low sequence identity to other GH family 94 enzymes including RaCDP, and they do not have an equivalent amino acid residue corresponding to Gln712 of C. gilvus CBP (Fig. S1). These structural differences suggest that different strategies for optimization of binding to the phosphate group might exist in these enzymes.

Possible functions of RaCDP in the metabolism of carbohydrates

RaCDP catalyzes synthetic reactions of both cellooligosaccharides and β-1,4-mannooligosaccharides, indicating that RaCDP also phosphorolyzes complex oligosaccharides in which cellooligosaccharides are linked to the non-reducing end of β-1,4-mannooligosaccharides (Fig. 5). These oligosaccharides may be produced by the hydrolysis of glucomannan having glucosyl residues in the main chain. RaCDP could produce a β-1,4-mannooligosaccharide through phosphorolysis of such complex oligosaccharides. The β-1,4-mannooligosaccharide produced is further metabolized by phosphorolysis and epimerization as demonstrated elsewhere [30].

β-1,4-Mannooligosaccharides longer than β-1,4-mannobiose are phosphorolyzed to β-1,4-mannobiose by β-1,4-mannooligosaccharide phosphorylase, and β-1,4-mannobiose is epimerized to 4-O-β-D-mannosyl-D-glucose by cellobiose 2-epimerase (EC 5.1.3.11) for further phosphorolysis catalyzed by 4-O-β-D-mannosyl-D-glucose phosphorylase (EC 2.4.1.281). β-1,4-Mannooligosaccharide phosphorylase has a broad acceptor specificity in the synthetic direction similar to RaCDP, and cellobiose is a good acceptor substrate, indicating that complex oligosaccharides, in which a β-1,4-mannooligosaccharide is bound to the non-reducing end of a cellooligosaccharide, are substrates for phosphorolysis catalyzed by this enzyme.

β-1,4-Mannooligosaccharide phosphorylase could produce cellooligosaccharides from this type of complex-oligosaccharide for further phosphorolysis catalyzed by CDP and CBP.
Materials and Methods

Preparation of expression plasmids for wild type and mutant RaCDPs

The RaCDP gene was amplified by PCR, in which the genomic DNA of \textit{R. albus} NE1 as the template, a set of primers, 5′-CTTGTAAAAATGCGGATATATG-3′ and 5′-GGACTGCATATGACCAGATAG-3′, designed based on the Rumal_2403 gene, and PrimeStar HS DNA polymerase (Takara Bio, Otsu, Japan) were used. The amplified DNA fragment was cloned into the pBluescript II SK (+) vector (Stratagene, La Jolla, CA) via the EcoRV site. The DNA sequence of the amplified region was analyzed with an ABI Prism 310 Genetic Analyzer DNA sequencer (Applied Biosystems, Foster City, CA). This plasmid was used as the template for PCR to construct an expression plasmid of RaCDP. The DNA fragment, amplified with primers, 5′-GAGCATATGACTAGTATGGAATTT-3′ and 5′-GGAAGTAGGCCCATAACTACGGTGAT-3′, harboring the NdeI and XhoI sites, was cloned into the NdeI and XhoI sites of the pET-23a vector (Novagen, Darmstadt, Germany). The expression plasmids for Q646H and F633Y were prepared with a PrimeStar Mutagenesis Basal Kit (Takara Bio). The expression plasmid for the wild type was used as the template, and the sequences of primers were as follows: for Q646H, 5′-TTCAGCCACACACAGGGCTGGATAATC-3′ and 5′-CTGTGTGTGGCTGAATATACCGCCGTT-3′; F633Y, 5′-CACATATAACCCCGACACCAAGGAG-3′ and 5′-GGGGTTGTATATGTGCATCAGCGCACC-3′.

Production and purification of the wild-type and mutant RaCDPs

The transformant of \textit{E. coli} BL21 (DE3) harboring the expression plasmid for each RaCDP derivative was cultured in 500 mL of Luria-Bertani medium supplemented with 50 μg/mL ampicillin until the \(A_{600}\) reached 0.4. Production of the recombinant protein was induced by the addition of isopropyl \(\beta\)-D-thiogalactoside at the final concentration of 0.1 mM, and the incubation was continued with vigorous shaking at 18°C for 24 h. The bacterial cells harvested by centrifugation were
suspended in 75 mL of 20 mM MES-NaOH buffer (pH 6.0, buffer A) containing 0.5 M NaCl, and

disrupted by sonication using Sonifier 450 (Branson, Danbury, CT). Cell debris was removed by
centrifugation, and the cell-free extract obtained was applied to a Ni chelating Sepharose column
(1.6 x 5.0 cm) equilibrated with buffer A. After thorough washing with buffer A, adsorbed protein
was eluted with 0.5 M imidazole in buffer A. Highly purified fractions, confirmed by SDS-PAGE,
were collected, and dialyzed against 20 mM MES-NaOH buffer (pH 6.0). The purified sample was
frozen at -80°C until analysis.

Protein assay

The protein concentrations of the crude extract and fractions from the column chromatography
were measured by the Bradford method [31] and the UV method [32], respectively. Bovine serum
albumin (Nacalai Tesque, Kyoto, Japan) was used as the standard protein for the Bradford method.
The concentration of the purified enzyme was determined based on the concentration of each amino
acid after complete acid hydrolysis. The purified enzyme was hydrolyzed in 6 N HCl at 110°C for 24
h, and the resulting amino acids were measured by the ninhydrin colorimetric method with
JLC-500/V (Jeol, Tokyo, Japan) [33].

Standard enzyme assay

Twenty μL of a reaction mixture consisting of an appropriate concentration of enzyme, 50 mM
sodium phosphate buffer (pH 6.0), and 20 mM cellotriose (Seikagaku, Tokyo, Japan) was incubated
at 37°C for 10 min. The enzyme solution was diluted with 20 mM MES-NaOH buffer (pH 6.0)
containing 1 mg/mL of bovine serum albumin. The enzyme reaction was terminated by the addition
of 20 μL of 4 M Tris-HCl buffer (pH 7.0) and the Glc1P produced was measured by the
phosphoglucomutase-glucose-6-phosphate dehydrogenase method [34].

Effects of pH and temperature

The optimum pH and temperature were investigated by measuring phosphorolytic activities at
various pH values and temperatures, respectively. For the analysis of optimum pH, a reaction mixture consisting of an appropriate concentration of the enzyme, 100 mM reaction buffer, 20 mM sodium phosphate buffer (pH 6.0), and 20 mM cellotriose was incubated at 37°C for 10 min, and liberated Glc1P was measured as described above. The reaction buffer was as follows: sodium acetate buffer for pHs 2.5-5.5, MES-NaOH buffer for pHs 5.2-6.7, and Tris-HCl buffer for pHs 6.5-9.5.

Stable ranges of pH and temperature were determined based on the residual activity after pH and heat treatments, respectively. For the pH treatment, the enzyme was incubated in 100 mM Briton-Robinson buffer (pH 2.0-12.0) at 4°C for 24 h. For the heat treatment, the enzyme was incubated in 62.5 mM sodium phosphate buffer (pH 6.0) for 15 min and then immediately cooled down on ice. The enzyme was considered to be stable in the ranges of pH and temperature over which the enzyme maintained more than 90% of its original activity.

Analysis of the kinetic mechanism of phosphorolysis of cellotriose

Phosphorolytic velocities at various concentrations of cellotriose (4-20 mM) and sodium phosphate buffer (pH 6.0, 80-400 mM) were measured as per the standard enzyme assay. The kinetic parameters were calculated by fitting the reaction rates to the following equation for a sequential bi bi mechanism [35] with Grafit version 7.0.2 (Erithacus Software, West Sussex, UK):

\[ v = \frac{k_{cat}[A][B]}{(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B])} \]

(A = cellotriose, B = inorganic phosphate)

Product inhibition analysis was carried out to determine the order of substrate binding and product release. The enzyme concentration was fixed at 100 nM. First, the phosphorolytic velocities towards 4-20 mM cellotriose and 50 mM sodium phosphate buffer (pH 6.0) in the presence of 0-20 mM Glc1P or cellobiose were measured. Then, the reaction rates towards 20 mM cellotriose and 20-100 mM sodium phosphate buffer (pH 6.0) in the presence of Glc1P or cellobiose were measured as described above. For the inhibition analysis by Glc1P, the cellobiose produced was degraded by CBP, and the resulting D-glucose was measured by the glucose oxidase-peroxidase method [36]. After
stopping the reaction by heating the reaction mixture at 80°C for 10 min, 20 μL of the reaction mixture was mixed with 100 μL of 60 mM sodium phosphate buffer (pH 6.0), 30 μL of 5.7 U/mL *R. albus* NE1 CBP [4], and 20 μL of Glucose CII-Test Wako (Wako Pure Chemical Industries, Osaka, Japan), and incubated at 37°C for 30 min. The absorbance at 505 nm was measured, and the cellobiose concentration was calculated based on the standard curve obtained with 0-0.5 mM cellobiose.

**Phosphorolysis of cellooligosaccharides**

The reaction rates for phosphorolysis of various concentrations of cellooligosaccharides and fixed concentration of inorganic phosphate were measured. Twenty μL of a reaction mixture consisting of enzyme, 250 mM sodium phosphate buffer (pH 6.0), and 1-20 mM of each cellooligosaccharide (cellobiose was purchased from Sigma, St. Louis, MO, and the other oligosaccharides were from Seikagaku) was incubated at 37°C for 10 min, and the liberated Glc1P was measured as described above. Concentrations of the wild type and F633Y were 20 nM and 610 nM, respectively. Apparent kinetic parameters, \(k_{\text{cat (app)}}\) and \(K_{\text{m (app)}}\), were calculated from the reaction rates obtained by fitting to the Michaelis-Menten equation.

**Acceptor specificity in the synthetic reaction**

The reaction velocities for the synthesis of various concentrations of oligosaccharides were measured in the presence of 250 mM Glc1P. Twenty μL of a reaction mixture consisting of the enzyme (wild type, 210 nM; F633Y, 490 nM for cellobiose and 3.1 μM for β-1,4-mannobiose), 250 mM Glc1P, 50 mM MES-NaOH buffer (pH 6.0), and 1-100 mM of each oligosaccharide (acceptor substrate) was incubated at 37°C for 10 min, and the liberated inorganic phosphate was measured by the method of Lowry and Lopez [37]. The apparent kinetic parameters were calculated as described above. A series of cellooligosaccharides, sophorose (Sigma), laminaribiose (Megazyme, Wicklow, Ireland), xylobiose (Wako Pure Chemical Industries), β-1,4-mannobiose (Megazyme), and cellobiitol (Sigma) were tested as acceptor substrates.
Apparent kinetic parameters of inorganic phosphate and Glc1P

The reaction rates for the phosphorolysis of cellotriose were measured at various concentrations of inorganic phosphate as described above to determine the apparent kinetic parameters for inorganic phosphate. Twenty µL of a reaction mixture consisted of enzyme, inorganic phosphate, 5 mM cellotriose, and 50 mM MES-NaOH buffer (pH 6.0). The concentrations of the wild type, Q646H, and F633Y were 63 nM, 7.7 µM, and 0.61 µM, respectively. The concentrations of inorganic phosphate were 25-250 mM for the wild type, 25-300 mM for Q646H, and 0.63-20 mM for F633Y.

To determine the apparent kinetic parameters for Glc1P, the reaction rates for the synthesis of cellotriose were measured as described above at a fixed concentration of cellobiose and various concentrations of Glc1P. Twenty µL of a reaction mixture consisted of enzyme, Glc1P, 40 mM cellobiose, and 50 mM MES-NaOH buffer (pH 6.0). Concentrations of the wild type, Q646H, and F633Y were 63 nM, 2.5 µM, and 0.61 µM, respectively. Concentrations of Glc1P were 40-400 mM for the wild type, 20-300 mM for Q646H, and 0.25-2.5 mM for F633Y.

Production of cellooligosaccharides by the synthetic reaction

The synthetic reaction toward cellobiose and Glc1P was monitored. One hundred µL of a reaction mixture consisting of 69 nM RaCDP, 50 mM cellobiose, 250 mM Glc1P, and 100 mM MES-NaOH buffer (pH 6.0) was incubated at 37°C for 0-60 min. The enzyme reaction was stopped by heating the reaction mixture at 80°C for 10 min. Cellooligosaccharides produced were measured by HPLC under the following conditions: injection volume, 10 µL; column, Asahipak NH2P-50 4E (4.6 x 250 mm; Shodex, Tokyo, Japan); column temperature, 40°C; elution, descending linear gradient of 60-50% acetonitrile over 10 min; flow rate, 0.8 ml/min; detection, pulsed amperometry.

The insoluble product was prepared to analyze the structure. One mL of a reaction mixture consisting of 343 nM RaCDP and the other components the same as described above was incubated at 37°C for 3 h. After heating the mixture at 80°C for 10 min, the insoluble material was collected by centrifugation. The insoluble product dried in vacuo was dissolved in 1 mL of 4% NaOD-D2O, and the 1H-NMR was recorded using a Bruker AMX-500 (Bruker Daltonics, Billerica, MA).
Acknowledgements

We thank Dr. Eri Fukushi of the GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University and Mr. Tomohiro Hirose of the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution, Hokkaido University for NMR analysis and amino acid analysis, respectively.

References


1 225-232.


**Supplementary information**

Fig. S1. Multiple sequence alignment of catalytic domain of GH94 enzymes.
Figure legends

Fig. 1. Phylogenetic tree of the characterized GH family 94 enzymes. Multiple-sequence alignment was constructed with a ClustalW program (http://www.genome.jp/tools/clustalw/), and a phylogenetic tree was visualized with the Mega 5 program (http://www.megasoftware.net/). RaCDP, Ruminococcus albus NE1 cellodextrin phosphorylase (CDP); CsCDP, Clostridium stercorarium CDP (AAC45511.1); CtCDP A, Clostridium thermocellum YM4 CDP (BAB71818.1); CgCBP, Cellvibrio gilvus cellobiose phosphorylase (CBP) (GenBank ID, BAA28631.1); RaCBP, R. albus NE1 CBP; CtCBP, C. thermocellum YM4 CBP (AAD36910.1); CuCBP, Cellulomonas uda CBP (AAQ20920.1); AfCBGS, the C-terminal part of Agrobacterium fabrum C58 cyclic β-1,2-glucan synthetase (compared region was 1400-2832; AAK73356.1); AvCBGS, the C-terminal part of Agrobacterium vitis F2/5 cyclic β-1,2-glucan synthetase (1401-2831; AAQ08605.1); PsLBP, Paenibacillus sp. YM1 laminaribiose phosphorylase (BAJ10826.1); AILBP, Acholeplasma laidlawii PG-8A laminaribiose phosphorylase (ABX81345.1); VpChBP, Vibrio proteolyticus N,N’-diacetylchitobiose phosphorylase (ChBP) (BAC87867.1); VfChBP, Vibrio furnissii ChBP (AAG23740.1)

Fig. 2. Double-reciprocal plots of the phosphorolysis of cellotriose in the presence of various concentrations of inorganic phosphate. Phosphorolytic velocities at 4-20 mM cellotriose and a fixed concentration of sodium phosphate buffer (pH 6.0) were measured. Concentrations of inorganic phosphate are 80 mM (black circle), 160 mM phosphate (white circle), 240 mM (black triangle), and 400 mM (white triangle). Error bar shows mean ± standard deviation for three independent experiments.

Fig. 3. Product inhibition analysis of the phosphorolysis of cellotriose. Inhibition of phosphorolysis of cellotriose by Glc1P and cellobiose was analyzed. Fixed concentrations of inorganic phosphate and cellotriose are 50 mM (A, C) and 20 mM (B, D),
respectively. A, inhibition of Glc1P against cellotriose; B, inhibition of Glc1P against inorganic phosphate; C, inhibition of cellobiose against cellotriose; D, inhibition of cellobiose against inorganic phosphate. Circle, no inhibitor; black triangle, 10 mM Glc1P; black square, 20 mM Glc1P; white triangle, 10 mM cellobiose; white square, 20 mM cellobiose. Error bar shows the mean ± standard deviation for three independent experiments. In panel A, data were fitted to the equation for competitive inhibition, and in other panels, data were fitted to the equation for mixed inhibition.

Fig. 4. Time course of the synthesis of cellooligosaccharides by RaCDP.
Synthesis of cellooligosaccharides from 50 mM cellobiose and 250 mM Glc1P was monitored. Black circle, white circle, black triangle, and white triangle show cellotriose, cellotetraose, cellopentaose, and cellohexaose, respectively. The black square indicates the yield of cellooligosaccharides calculated from the reacted cellobiose. Error bar shows mean ± standard deviation for three independent experiments.

Fig. 5. Possible degradation mechanism of complex-oligosaccharides derived from glucomannan.
Left pathway: cellooligosaccharide part linked at the non-reducing end of an oligosaccharide is phosphorolyzed by CDP. The resulting β-1,4-mannooligosaccharide longer than β-1,4-mannobiose is further phosphorolyzed by β-1,4-mannooligosaccharide phosphorylase (MP2). β-1,4-Mannobiose is epimerized by cellobiose 2-epimerase (CE), and phosphorolyzed by 4-O-β-D-mannosyl-D-glucose phosphorylase (MP1). Right pathway: the β-1,4-mannooligosaccharide part linked at the non-reducing end of an oligosaccharide is phosphorolyzed by MP2. The cellooligosaccharide liberated is degraded by CDP and CBP.
Fig. 1, Sawano et al.

Diagram showing the relationships between different CBPs (CuCBP, RaCBP, TmCBP, CICBP, CsCDP, VChBP, VpChBP, RaCDP, AfCBGS, AVCBGS, CICDP, CiCDP, PsLB, AllBP) with a scale of 0.2.
Fig. 2, Sawano et al.
Fig. 3, Sawano et al.
Fig. 4, Sawano et al
Fig. 5. Sawano et al.
Table 1. Apparent Kinetic Parameters of Phospholytic and Synthetic Reactions of RaCDP. Initial reaction velocities for phosphorolysis and synthesis were measured at varying concentrations of cellooligosaccharides and acceptor substrates in the presence of 250 mM inorganic phosphate and Glc1P, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphorolysis</th>
<th></th>
<th></th>
<th>Synthesis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (app)</td>
<td>$K_m$ (app)</td>
<td>$k_{cat}/K_m$ (app)</td>
<td>$k_{cat}$ (app)</td>
<td>$K_m$ (app)</td>
<td>$k_{cat}/K_m$ (app)</td>
</tr>
<tr>
<td></td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
<td>(s$^{-1}$mM$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
<td>(s$^{-1}$mM$^{-1}$)</td>
</tr>
<tr>
<td>Cellobiosine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>47.1 ± 2.6</td>
<td>13.2 ± 1.1</td>
<td>3.56</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>76.2 ± 0.8</td>
<td>6.04 ± 0.22</td>
<td>12.6</td>
<td>43.7 ± 1.0</td>
<td>5.01 ± 0.26</td>
<td>8.71</td>
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<tr>
<td>Cellotetraose</td>
<td>92.8 ± 2.6</td>
<td>4.16 ± 0.25</td>
<td>22.3</td>
<td>37.8 ± 0.8</td>
<td>3.97 ± 0.27</td>
<td>9.50</td>
</tr>
<tr>
<td>Cellopentaose</td>
<td>83.8 ± 1.4</td>
<td>2.41 ± 0.12</td>
<td>34.8</td>
<td>28.9 ± 0.7</td>
<td>2.73 ± 0.16</td>
<td>10.6</td>
</tr>
<tr>
<td>Cellohexaose</td>
<td>55.8 ± 0.6</td>
<td>1.04 ± 0.11</td>
<td>53.4</td>
<td>18.2 ± 0.5</td>
<td>3.22 ± 0.26</td>
<td>5.63</td>
</tr>
<tr>
<td>Sophorose</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>14.2 ± 0.7</td>
<td>343 ± 18</td>
<td>0.0414</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>33.3 ± 3.5</td>
<td>119 ± 19</td>
<td>0.281</td>
</tr>
<tr>
<td>Xylobiose</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>15.4 ± 0.6</td>
<td>50.9 ± 3.8</td>
<td>0.302</td>
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<tr>
<td>Mannobiose</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>41.3 ± 1.7</td>
<td>65.0 ± 3.7</td>
<td>0.635</td>
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<tr>
<td>Cellobiitol</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>3.26 ± 0.12</td>
<td>73.7 ± 7.7</td>
<td>0.0443</td>
</tr>
</tbody>
</table>

N.D., not detected at 40-fold higher enzyme concentration than for other substrates. N.T., not tested.
Table 2. Comparison of Apparent Kinetic Parameters of Inorganic Phosphate and Glc1P between the Wild Type and Mutated Enzymes. a, Phosphorolytic velocities towards various concentrations of inorganic phosphate were measured in the presence of 5 mM cellotriose. b, Synthetic velocities towards various concentrations of Glc1P and 40 mM cellobiose were measured.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inorganic phosphate&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th>Glc1P&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{\text{cat (app)}} )</td>
<td>( K_m (\text{app}) )</td>
<td>( k_{\text{cat (app)}}/K_m (\text{app}) )</td>
<td>( k_{\text{cat (app)}} )</td>
<td>( K_m (\text{app}) )</td>
<td>( k_{\text{cat (app)}}/K_m (\text{app}) )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>11.8 ± 0.1</td>
<td>181 ± 5</td>
<td>0.0652</td>
<td>39.6 ± 1.5</td>
<td>166 ± 10</td>
<td>0.239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F633Y</td>
<td>0.769 ± 0.011</td>
<td>2.40 ± 0.11</td>
<td>0.320</td>
<td>1.93 ± 0.04</td>
<td>0.155 ± 0.015</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q646H</td>
<td>1.12 ± 0.027</td>
<td>80.2 ± 5.8</td>
<td>0.0140</td>
<td>1.53 ± 0.07</td>
<td>75.1 ± 3.4</td>
<td>0.0204</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Comparison of Apparent Kinetic Parameters between the Wild Type and F633Y. Apparent kinetic parameters for phosphorolysis of cellotriose, which were determined in the presence of 20 mM inorganic phosphate, are shown. Apparent kinetic parameters for synthetic reactions towards cellobiose and β-1,4-mannobiose are the values determined from the reaction rates towards various concentrations of the acceptor substrates and 250 mM Glc1P.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>F633Y</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat (app)}}$ (s$^{-1}$)</td>
<td>$K_{m (app)}$ (mM)</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>0.821 ± 0.019</td>
<td>0.855 ± 0.075</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>2.86 ± 0.20</td>
<td>28.9 ± 3.0</td>
</tr>
<tr>
<td>β-1,4-Mannobiose</td>
<td>0.285 ± 0.025</td>
<td>72.4 ± 11.0</td>
</tr>
</tbody>
</table>