大腸菌で発現させたClostridium thermocellum YM4株由来セロデキストリンホスホリラーゼ

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Cellodextrin Phosphorylase from *Clostridium thermocellum* YM4 Strain Expressed in *Escherichia coli*

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A cellodextrin phosphorylase (CDP) gene was cloned from *Clostridium thermocellum* YM4 strain by using a PCR cloning method. The nucleotide sequence of the gene contained an open reading frame of 2952 bp encoding a polypeptide of 984 amino acid residues. The deduced protein sequence was very close to that of CDP from *C. thermocellum* ATCC 27405 (92% identity) but not so similar to that of CDP from *C. stercorarium* (20%). The recombinant CDP phosphorolyzed cellooligosaccharides larger than cellotriose and utilized cellooligosaccharides larger than cellobiose as the acceptor molecule. The enzyme was stable up to 60°C and showed highest activity at pH 7.5.

Cellulose is the major component of plant cell walls and is consequently the most abundant biopolymer in the world. Many fungi and bacteria produce cellulase, which hydrolyzes cellulose, to utilize cellulose. It is important for utilization of the biomass to study enzymatic degradation of cellulose in the nature. The cellulase system comprises the most studied cellulolytic enzymes.[1]

It has been known that some bacteria have an intra-cellular phosphorolytic enzyme system to utilize cellooligosaccharides. The phosphorolytic cleavage of cellooligosaccharides was first demonstrated in *Clostridium thermocellum.*[2] Two enzymes are involved in the phosphorolytic system: cellobiose phosphorylase (CBP, cellobiose: orthophosphate α-D-glucosyltransferase, EC 2.4.1.20) and cellodextrin phosphorylase (CDP, 1,4-β-D-oligoglucan: orthophosphate α-D-glucosyltransferase, EC 2.4.1.49). CBP catalyzes a reversible phosphorolysis of cellobiose into α-glucose1-phosphate (G1-P) and glucose, but does not phosphorolyze cellooligosaccharides (CG_n: n, degree of polymerization) greater than $n=3$. Contrarily, CDP reversibly phosphorolyzes CG_n ($n \geqq 3$) (into glucose1-phosphate and CG_{n-1}), but does not phosphorolyze cellobiose.

CBP have been found in several bacteria such as *C. thermocellum,*[2,3] *Cellvibrio gilvus,*[4] *Cellulomonas fimi,*[5] *Cellulomonas* spec.,[6] *Ruminococcus flavefacience,*[7] and *C. sterocolarium.*[8] Alexander purified CBP from *C. thermocellum* strain 653.[9] CBP from *Cellvibrio gilvus* was purified into homogeneity[10] and its properties were investigated in detail.[11–13] On the other hand, CDP has been found only in Clostridia, *C. thermocellum*[14,15] and *C. stercorarium.*[8]

For the purpose of producing oligosaccharides, such phosphorolytic enzymes are considered to be very useful. Several reports have described syntheses of oligosaccharides by using CBP,[5,13,16–22] or CDP.[14,23]

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Abbreviations: CDP, cellodextrin phosphorylase; CBP, cellobiose phosphorylase; PCR, polymerase chain reaction; TAIL, thermal asymmetric interlaced; TLC, thin layer chromatography; CDP (YM4), cellodextrin phosphorylase from *Clostridium thermocellum* YM4; CDP (ATCC27405), cellodextrin phosphorylase from *Clostridium thermocellum* ATCC27405; CDP (643), cellodextrin phosphorylase from *Clostridium thermocellum* 643; CDP (ster), cellodextrin phosphorylase from *Clostridium stercorarium*; CG_n, cellooligosaccharides (n mer); G1-P, α-glucose1-phosphate; MOPS, (*N*-morpholino)propanesulfonic acid.

There have not been so many molecular genetic studies on CBP and CDP. Only four CBP have been cloned: from *C. stercorarium* (U56424),[8] *Cellvibrio gilvus* (AB010707),[24] *C. thermocellum* ATCC 27405 (AB013109), and *Thermotoga neapolitana* (Z99777).[25] The number of CDP genes that have been cloned is only two: *C. stercorarium* (U60580)[8] and *C. thermocellum* ATCC27405 (AB 006822).[23]

C. thermocellum YM4 strain produces a cellulase system that has strong capability of hydrolyzing crystalline cellulose.[26,27] It has some physiological characteristics different from those reported for other *C. thermocellum* strains in spore formation, nutrient requirement and carbohydrate utilization.[28] We have been investigating the phosphorolytic enzymes of *C. thermocellum* YM4. The acetone-dried cells of *C. thermocellum* YM4 catalyzed the synthesis of cellotriose from cellobiose and G1-P, suggesting that the microorganism had intracellular CDP (unpublished result). In this paper, we report the cloning, sequencing, and expression of the *cdp* gene.

MATERIALS AND METHODS

Escherichia coli strains, vectors and media. TOPO TA Cloning Kit (including *E. coli* TOP10 and pCR2.1-TOPO) and TOPO XL PCR Cloning Kit (including *E. coli* TOP10 and pCR-XL-TOPO) (Invitrogen Corporation, USA) were used for cloning and sequencing. The recombinant protein was expressed in *E. coli* BL21-Gold(DE3) (Stratagene, USA) with a pET-28a(+) (Novagen, USA) vector. LB medium containing 50 μg/mL kanamycin was routinely used for cultivating *E. coli* harboring a plasmid. *E. coli* strains harboring a recombinant plasmid were selected by using LB medium containing 50 μg/mL kanamycin and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

DNA manipulation. Preparation of plasmid DNA, endonuclease digestion, ligation, and transformation were carried out using standard procedures.[29] *C. thermocellum* YM4 was cultivated as described previously[27] and its genomic DNA was extracted by using InstaGene matrix (Biorad, USA), according to its supplier's protocol.

Nucleotide sequence analysis. The nucleotide sequence was determined by the dideoxynucleotide chain terminating method using an automated DNA sequencer (Model 310A, Applied Biosystems, USA) with dRhodamine Terminator Kit (Perkin Elmer, USA). At least 3 independent clones of each PCR product were used sequenced. Sequence data was analyzed by using Genetyx Mac software Version 10.0 (Genetyx software Development Co., Ltd., Tokyo, Japan).

PCR cloning of the cdp gene. A pair of PCR primers for amplification of an internal sequence of *cdp* gene were designed based on the multiple alignment of amino acid sequences of cellodextrin phosphorylase and cellobiose phosphorylase: the sense primer, 5′-ATGATTACTAAAGTAACAGCGAGAAAT-3′ and the antisense primer, 5′-TCATTGTCTTTTACTTTCTTTGCAGC-3′. The first PCR was performed by using *C. thermocellum* YM4 genomic DNA as the template in the following conditions by using a thermocycler, Gene Amp system 9600 (Perkin-Elmer, USA): 98 °C for 1 min, 30 cycles at 98°C for 20 s, 58°C for 15 s and 68°C for 30 s, then 72°C for 10 min. The fragment amplified was then inserted in a pCR2.1-TOPO vector and sequenced.

The 5′ and 3′ regions of the *cdp* gene were amplified by the thermal asymmetric interlaced (TAIL) PCR method.[30] The specific antisense primers to amplify the 5′ region were CDP-278-REV: 5′-ATTCAACAACTCAACAGGTG-3′, CDP-475-REV: 5′-CCTTCTTCAACAAAACAAAC-3′, and CDP-749-REV: 5′-GGGAACGGAACGAACCTC-3′. Those specific sense primers to amplify the 3′ region were CDP-2637-FWD: 5′-GCATTACTTC-TTTGGTGACA-3′ and CDP-2531-FWD: 5′-TAG-CAATAATGGTGGATGTC-3′. A randomly degenerated primer was the same as described,[30] Therm-Hys: 5′-TCYKGGWAANGANTAT-3′ where K, N, W and Y mean G/T, A/C/G/T, A/T and C/T, respectively. The fragments amplified were inserted in a pCR2.1-TOPO vector and sequenced.

Finally the whole *cdp* gene was amplified by using the genomic DNA and two oligonucleotide primers on the basis of the nucleotide sequences of the 5′ and 3′ terminal sequence of the *cdp* gene. The amplified gene was inserted in a pCR-XL-TOPO

vector and subjected to nucleotide sequencing.

Expression of cellodextrin phosphorylase in E. coli. The coding region of CDP was amplified by PCR to create an *Nco*I site and an *Xho*I site at the 5′ end of the coding region and the 3′ end before the stop codon, respectively, and subcloned in pCR-XL-TOPO vector. Then the plasmid was hydrolyzed by *Nco*I and *Xho*I and the fragment containing the *cdp* gene was then inserted into pET28 a(+) at its *Nco*I-*Xho*I site, generating the plasmid pET28a-CDP, which codes CDP, added to the His 6 sequence at its C terminal. Then *E. coli* BL21-Gold(DE3) was transformed with pET28a-CDP by electroporation. The transformant was grown in a LB medium (1 L) containing 50 μg/mL kanamycin at 30°C until the A_{600} reached 0.6 and then CDP was induced by adding isopropyl-β-D-thiogalactopyranoside to the final concentration of 0.1 mM followed by the additional cultivation at 25°C for 20 h. The cells were harvested and CDP was extracted from the cell in 100 mL of 20 mM MOPS-Na buffer (pH 7.5) by using a sonifier (Model 250D, Branson, USA).

Purification of CDP. A portion of the cell free extract (45 mL) was first charged on a Ni-NTA agarose (Qiagen, Germany) column (2.4 mL) and the enzyme was eluted by a linear gradient of imidazole (10–250 mM, total 30 mL) in 20 mM MOPS-Na buffer (pH 7.5) containing 300 mM NaCl. The appropriate fractions were collected and ammonium sulfate was added to the fractions to make 1 M. The enzyme was adsorbed on a Butyl-Toyopearl 650M (Toso, Japan) column (5 mL), eluted with a linear gradient of ammonium sulfate (1–0 M, total 20 mL) in 20 mM MOPS buffer, to yield the purified enzyme. SDS PAGE was done by the method of Laemmli[31] and the protein bands were detected by staining with Coomassie Brilliant Blue R250.

Assay of the enzyme activity. The enzyme activity was routinely determined by measuring the increase in inorganic phosphate from 10 mM cellobiose and 10 mM G 1-P in 50 mM MOPS buffer (pH 7.5) at 37°C. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol of phosphate per minute under the above conditions. The amount of inorganic phosphate was determined by the method of Lowry and Lopez.[32] The phosphorolytic reaction of CDP was determined by measuring the increase in glucose 1-phosphate from cellooligosaccharide and inorganic phosphate in 50 mM MOPS buffer (pH 7.5). The amount of G1-P was quantified by using the phosphoglucomutase/glucose-6-phospate dehydrogenase method[33] after inactivating CDP by boiling the reaction mixture. Kinetic parameters were calculated by using a non-linear regression analysis program, Grafit Ver. 3 (Erithacus Softwear, London, U.K.).

RESULTS

Cloning of the cdp gene.

The results of the cloning are summarized in Fig. 1. The PCR amplification of genomic DNA by using a sense primer (CDP-MITK) and an antisense primer (CDP-AKKV) resulted an internal fragment of 2.6 kbp. Then, the TAIL-PCR strategy[30] was used to amplify each 5′ and 3′ region to obtain the complete *cdp* gene. By using TAIL-PCR, a 2.1 kbp fragment and a 1.4 kbp fragment were amplified from the 5′ and 3′ regions, respectively. Finally, a 3.3 kbp fragment containing the complete *cdp* gene was amplified.

Nucleotide and deduced amino acid sequence of cdp gene.

To determine the entire sequence of the *cdp* gene, three independent isolates of the pCRTOPOXL7 plasmid were sequenced. The nucleotide sequence of the *cdp* gene (accession number AB 061316) included an open reading frame of 2952 nucleotides encoding a protein with 984 amino acid residues having a molecular mass of 111,613.

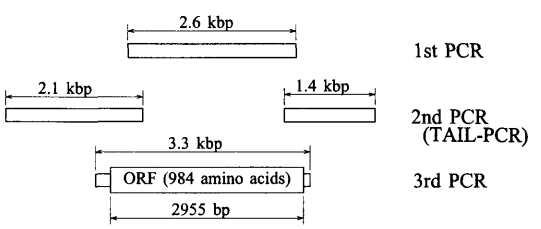

Fig. 1. PCR cloning of *C. thermocellum* YM4 CDP.

Table 1. Identity of amino acid sequences of CDPs and CBPs.

		1	2	3	4	5	6	7
1	CDP (YM4)	100.0	92.1	21.8	22.5	19.8	20.8	20.6
2	CDP (ATCC27405)	92.1	100.0	22.5	21.7	23.3	22.5	19.8
3	CDP (stero)	21.8	22.5	100.0	41.0	41.1	41.1	38.1
4	CBP (ATCC27405)	22.5	21.7	41.0	100.0	71.6	70.9	62.8
5	CBP (stero)	19.8	23.3	41.1	71.6	100.0	68.5	61.7
6	CBP (TN)	20.8	22.5	41.1	70.9	68.5	100.0	59.0
7	CBP (CG)	20.6	19.8	38.1	62.8	61.7	59.0	100.0

Amino acid sequences of 3 CDPs and 4 CBPs were aligned by using Genentyx Mac Ver. 10, and the alignment scores (%) are given. 1, *C. thermocellum* YM4 CDP (AB061316); 2, *C. thermocellum* ATCC27405 CDP (AB006822); 3, *C. stercorarium* CDP (U60580); 4, *C. thermocellum* ATCC27405 CBP (AB013109); 5, *C. stercorarium* CDP (U56424); 6, *Thermotoga neapolitana* CBP (Z99777); 7, *Cellvibrio gilvus* CBP (AB010707).

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CDP(YM4)          (552)LYSDDSLWLL  (610)GDHGLPLLDLADWNDCLKIASNS  (889)SGTATWLN
CDP(ATCC27405)    (549)DCIPMTACLL  (607)GDHGLPLLDLADWNDCLKIDSNS  (885)PGTATWLN
CDP(stero)        (404)WYSDDHLWLP  (460)GPHALP-YSRADWNDTLNLDMGN  (690)TGTAAWNM
CBP(ATCC27405)    (412)NFNDDPLWLI  (469)GPHGLPLIGRADWNDCLNLNCFS  (718)TGTAAWNF
CBP(stero)        (412)GFNDDPLWLI  (469)GPHGLPLIGRADWNDCLNLNCFS  (718)TGTAAWNF
CBP(TN)           (427)GFNDDPLWLI  (486)GPHGLPLIGRADWNDCLNLNCFS  (744)TGTAAWSF
CBP(CG)           (419)GFNDDPLWLI  (476)GPHGLPLIGRADWNDCLNLNCFS  (731)TGTAAWNF
                             *          * * **     ***** *            *** *
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Fig. 2. Multiple alignment of amino acids from conserved regions of CDPs and CBPs.
*Completely conserved amino acid residue.

Table 2. Purification steps of the recombinant CDP from *C. thermocellum* YM4.

	Volume (mL)	Protein (mg)	Activity (IU)	Yield (%)	Specific activity (IU/mg-protein)
Sonic supernatant	45	2660	71.9	100	0.027
Ni-NTA agarose	11	11.0	59.2	82.3	5.4
Butyl-Toyopearl	10	7.69	30.4	42.3	5.5

Sequence comparison with CDP and CBP.

The amino acid sequence of CDP YM4 was compared with those of other cellodextrin phosphorylases and cellobiose phosphorylases cloned, as shown in Table 1. The CDP of *C. thermocellum* YM4 showed significant identity with CDPs of *C. thermocellum* ATCC 27405 (92%) and *C. stercorarium* (22%). It also has high similarity with CBPs of *C. thermocellum* ATCC 27405 (23%), *Clost. stercorarium* (20%), *Cellvibrio gilvus* (21%), and *T. neapolitana* (22%). No significant similarity was found to any other amino acid sequence known. The multiple alignment of amino acid residues of CBP and CDP, shows three blocks of high sequence conservation (Fig. 2).

Basic properties of the cloned CDP.

The cloned CDP expressed in *E. coli* BL21Gold (DE3) was purified into homogeneity through two column chromatography steps (Table 2). The enzyme showed a single protein band at 105 kDa on SDS-PAGE where the molecular mass of the expressed protein, calculated based on the sequence (include the oligohistidine sequence), was 112,724 (Fig. 3). The specific activity of the purified enzyme was 5.5 IU/mg-protein and k_{cat} was 10.1 s^{-1} based on the calculated molar coefficiency of Abs_{280} of 108,240. The optimum pH of the enzyme was determined to be 7.5, and the enzyme was stable in the pH range of 5–8 at 37°C for 1 h (Fig. 4). The thermal stability (pH 7.5, 30 min) of the

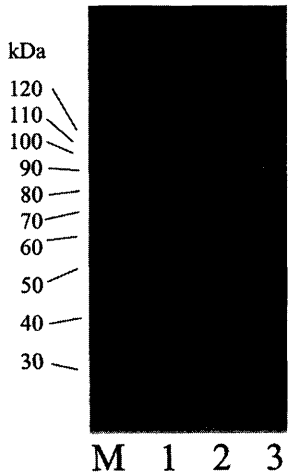

Fig. 3. SDS-PAGE of the recombinant CDP.

Lane M, molecular weight markers; Lane 1, sonic supernatant; Lane 2, after Ni-NTA agarose column chromatography; Lane 3, after Butyl-Toyopearl column chromatography.

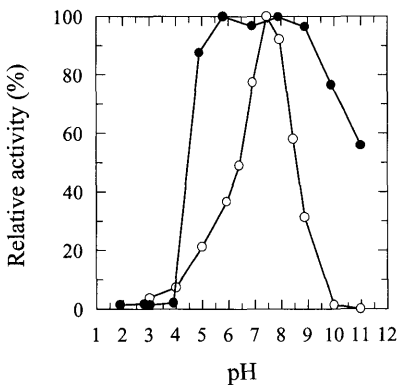

Fig. 4. Effect of pH on the activity and stability of the recombinant CDP.

○, activity; ●, stability. The activity was measured under the standard conditions while changing the pH in the reaction mixture by 200 mM buffers. The stability was determined by incubating the enzyme at 37°C for 1 h at each pH by using 200 mM buffers, followed by measuring the activity under the standard conditions. The following buffers were used; pH 2–3, glycine-HCl; pH 3–6, acetate-Na; pH 6–9, HEPES-Na; pH 9–11, CAPS-Na; pH 11–12, glycine-Na.

enzyme was lower than 60°C and the optimum temperature (reaction at pH 7.5 for 20 min) was 60°C (Fig. 5). The activation energy, E_a, of the reaction was determined to be 19.6 kcal/mol from the temperature-activity data between 20–60°C by the slope of the Arrhenius plot.

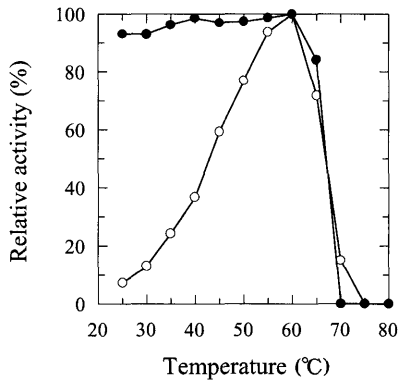

Fig. 5. Effect of temperature on the activity and stability of the recombinant CDP.

○, activity; ●, stability. The activity was measured for 20 min under the standard conditions while changing the temperature after pre-incubating the enzyme at the temperature for 5 min. The stability was determined by incubating the enzyme at each temperature for 30 min in 50 mM MOPS-Na buffer (pH 7.5) followed by the standard assay at 37°C.

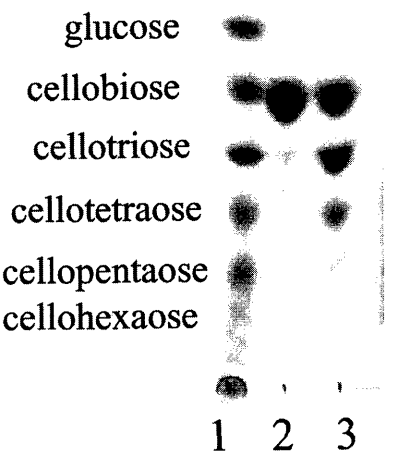

Fig. 6. TLC analysis of the reaction by the recombinant CDP.

Lane 1, cellooligosaccharides (each 5 mg/mL); Lane 2, before the reaction; Lane 3, after the reaction for 1 h. The reaction conditions were as follows; 50 mM cellobiose, 50 mM G1-P, and 5 IU/mL CDP in 200 mM MOPS-Na buffer (pH 7.5) at 30°C for 1 h. The reaction mixture was deionized with Amberlite MB3 before spotting on the TLC plate. The plate was irrigated 2 times with acetonitrile/water (85/15 by volume) after spotting 1 μL of each sample. The compounds were detected on the plate by baking after dipping the plate in 5% sulfuric acid in methanol.

Table 3. Kinetic parameters for cellooligosaccharides.

	Phosphorysis		Synthesis	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Glucose	—	—	nd	nd
Cellobiose	nd	nd	0.89	10.1
Cellotriose	0.81	4.0	1.75	13.9
Cellotetraose	0.82	3.2	2.65	16.2

nd, not detected; —, not tested.

Reaction of the enzyme.

When the enzyme was incubated with an equimolar solution of cellobiose and G1-P, a series of celloologosaccharides were formed in the reaction mixture (Fig. 6). However no reaction was found when the enzyme was incubated with glucose and G1-P. The kinetic parameters of the enzyme for various cellooligosaccharides in both the phosphorolytic and synthetic reactions are summarized in Table 3. This enzyme did not phosphorolyze cellobiose and did not use glucose as an acceptor. The kinetic parameters of the higher cellooligosaccharides were close to each other.

DISCUSSION

The CDP from *C. thermocellum* YM4 (CDP (YM4)) is the third *cdp* gene cloned. As shown in Table 1, the amino acid sequence of CDP(YM4) was close to that of *C. thermocellum* ATCC 27405 CDP (CDP(ATCC27405)) but not so similar to *C. stercorarium* CDP (CDP(ster)), which showed higher alignment scores with CBPs than *C. thermocellum* CDPs. The difference in the amino acid sequence may represent the difference in species.

Only three CDPs were characterized before this report. Sheth and Alexander[14] separated CDP with CBP from *C. thermocellum* strain 651 (CDP(651)) and found its optimum pH at 7.5. The second one is CDP(ATCC 27405) and its optimum pH was reported at 9.0.[15] The third one is CDP(ster) showing optimum pH between 6–7.[8] CDP(YM4) showed optimum pH at 7.5, similar to CDP(651) and CDP(ster). It is interesting that the optimum pH of CDP(YM4) is considerably different from that of CDP(ATCC27405) whereas both the enzymes are very similar in their amino-acid sequence (92% identity).

Sheth and Alexander described that CDP(651) require a thiol compound.[14] Arai *et al.* also reported that CDP(ATCC27405) was activated 14 fold by 10 mM dithiothreitol.[15] We found that dithiothreitol did not affect the reaction of CDP (YM4), quite unlike the other *C. thermocellum* CDPs. However, it is uncertain whether the recombinant CDP(YM4) shows identical characteristics to the native CDP(YM4), since the native enzyme has not been characterized.

Comparing the amino-acid-sequences of CBPs and CDPs, only the small regions are conserved as shown in Fig. 2. Though the 3D structure of any CBP and CDP has not been made available yet, these regions are probably involved in the catalytic center of the enzymatic activity, considering that both the enzymes catalyze the phosphorolytic cleavage of β-1,4-glucosidic linkages.

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大腸菌で発現させた *Clostridium thermocellum* YM4 株由来セロデキストリンホスホリラーゼ

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PCR 法を用いて, *Clostridium thermocellum* YM4 株由来セロデキストリンホスホリラーゼ遺伝子のクローニングを行った. セロデキストリンホスホリラーゼ遺伝子は, 984 アミノ酸残基のポリペプチド鎖をコードする 2952 bp のオープンリーディングフレームを含んでいた. 得られたアミノ酸配列は, *C. thermocellum* ATCC27405 株由来セロデキストリンホスホリラーゼと高い相同性 (92%) を示したが, *C. stercorarium* 由来のセロデキストリンホスホリラーゼとは相同性が低かった (20%). 組替セロデキストリンホスホリラーゼはセロトリオース以上のセロオリゴ糖を加リン酸分解し, セロビオース以上のセロオリゴ糖をアクセプターとして認識した. 温度安定性は 60°C 以下であり, 至適 pH は 7.5 であった.