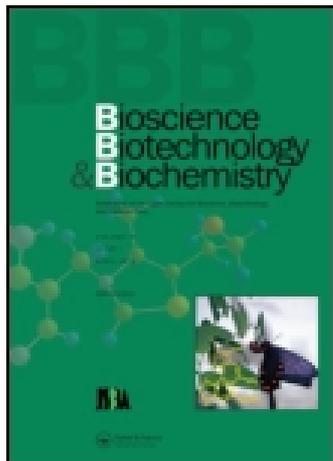


This article was downloaded by: [University of North Texas]

On: 21 November 2014, At: 19:57

Publisher: Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Bioscience, Biotechnology, and Biochemistry

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tbbb20>

Characterization of a Cellobiose Phosphorylase from a Hyperthermophilic Eubacterium, *Thermotoga maritima* MSB8

Eranna RAJASHEKHARA^a, Motomitsu KITAOKA^a, Yeon-Kye KIM^a & Kiyoshi HAYASHI^a

^a Enzyme Laboratory, National Food Research Institute 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Published online: 22 May 2014.

To cite this article: Eranna RAJASHEKHARA, Motomitsu KITAOKA, Yeon-Kye KIM & Kiyoshi HAYASHI (2002) Characterization of a Cellobiose Phosphorylase from a Hyperthermophilic Eubacterium, *Thermotoga maritima* MSB8, *Bioscience, Biotechnology, and Biochemistry*, 66:12, 2578-2586, DOI: [10.1271/bbb.66.2578](https://doi.org/10.1271/bbb.66.2578)

To link to this article: <http://dx.doi.org/10.1271/bbb.66.2578>

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

Characterization of a Cellobiose Phosphorylase from a Hyperthermophilic Eubacterium, *Thermotoga maritima* MSB8

Eranna RAJASHEKHARA, Motomitsu KITAOKA,[†] Yeon-Kye KIM, and Kiyoshi HAYASHI

Enzyme Laboratory, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Received May 27, 2002; Accepted July 25, 2002

The *cepA* putative gene encoding a cellobiose phosphorylase of *Thermotoga maritima* MSB8 was cloned, expressed in *Escherichia coli* BL21-codonplus-RIL and characterized in detail. The maximal enzyme activity was observed at pH 6.2 and 80°C. The energy of activation was 74 kJ/mol. The enzyme was stable for 30 min at 70°C in the pH range of 6–8. The enzyme phosphorylated cellobiose in an random-ordered bi bi mechanism with the random binding of cellobiose and phosphate followed by the ordered release of D-glucose and α -D-glucose-1-phosphate. The K_m for cellobiose and phosphate were 0.29 and 0.15 mM respectively, and the k_{cat} was 5.4 s⁻¹. In the synthetic reaction, D-glucose, D-mannose, 2-deoxy-D-glucose, D-glucosamine, D-xylose, and 6-deoxy-D-glucose were found to act as glucosyl acceptors. Methyl- β -D-glucoside also acted as a substrate for the enzyme and is reported here for the first time as a substrate for cellobiose phosphorylases. D-Xylose had the highest (40 s⁻¹) k_{cat} followed by 6-deoxy-D-glucose (17 s⁻¹) and 2-deoxy-D-glucose (16 s⁻¹). The natural substrate, D-glucose with the k_{cat} of 8.0 s⁻¹ had the highest (1.1×10^4 M⁻¹ s⁻¹) k_{cat}/K_m compared with other glucosyl acceptors. D-Glucose, a substrate of cellobiose phosphorylase, acted as a competitive inhibitor of the other substrate, α -D-glucose-1-phosphate, at higher concentrations.

Key words: hyperthermostable enzyme; cellobiose phosphorylase; *Thermotoga maritima*; phosphorolytic reaction; synthetic reaction

Cellobiose, a common intermediate in enzymatic cleavage of cellulose, can be degraded by hydrolysis or inorganic-phosphate-dependent phosphorolysis. Cellobiose phosphorylase (EC 2.4.1.20) catalyzes the reversible phosphorolysis of cellobiose into α -D-glucose 1-phosphate (G-1-P) and D-glucose with the inversion of the anomeric configuration, but does not phosphorylate cellobiose or higher cello-oligosaccharides. The presence of this enzyme was first demonstrated in *Clostridium thermocellum*¹⁾ and subsequently studied in several organisms.^{2–8)} The enzyme is absolutely specific for cleaving and synthesiz-

ing β -1,4 glycosidic bonds, but has a relaxed specificity with respect to the reducing sugars that function as glucosyl acceptors in the synthetic reaction. The absolute recognition of the β -anomeric hydroxyl group of the acceptor molecule was confirmed for the enzyme from *Cellvibrio gilvus* by using pseudo-glucoses.⁹⁾ However, β -glucosides are not accepted as the acceptor by any reported cellobiose phosphorylases. Syntheses of various disaccharides and branched trisaccharides using the synthetic reaction of cellobiose phosphorylase have been reported.^{10–14)} Direct synthesis of cellobiose from sucrose by the combined action of three enzymes including cellobiose phosphorylase was also reported,¹⁵⁾ and hence indicated the possible commercial importance of the enzyme. The enzyme was used in the selective colorimetric measurement of cellobiose in the presence of glucose and cellooligosaccharides,¹⁶⁾ a useful tool in investigation of biomass degradation.

The enzyme from *Cell. gilvus*¹⁷⁾ and *Cellulomonas uda*⁸⁾ catalyzed the phosphorolytic reaction in an ordered bi bi mechanism as follows: the enzyme at first bound cellobiose and then phosphate followed by the sequential release of glucose and G-1-P. Recently, we found that the enzyme from *Clostridium thermocellum* YM-4 also followed an ordered bi bi mechanism, but the order of the substrate binding was reverse.¹⁸⁾

The type strain of *Thermotoga maritima*, strain MSB8, is a hyperthermophilic fermentative bacterium that grows optimally at 80°C and its whole genome was sequenced for functional genomic studies.¹⁹⁾ Both *T. maritima* and *T. neapolitana* are able to use various carbohydrates for growth, and their cellulolytic enzymes are reported to be stable at extreme temperatures. Due to the extreme thermal resistance, there is an increasing interest for application of hyperthermostable enzymes in biotechnological processes.^{20–25)}

Recently, a cellobiose phosphorylase from a hyperthermophilic bacterium, *T. neapolitana*, was characterized,²⁶⁾ but not in detail. Because of the commercial importance of the enzyme and for fundamental understanding, a detailed functional analyses, phosphorolytic- and synthetic reaction of the recombinant

[†] To whom correspondence should be addressed. Fax: +81-298-38-7321; E-mail: mkitaoka@nfri.affrc.go.jp

cellobiose phosphorylase from another hyperthermophilic bacterium, *T. maritima*, was studied and reported in this paper.

Materials and Methods

Genomic DNA, plasmids and bacterial strains.

The genomic DNA of the type strain *T. maritima* MSB8 was kindly provided by Prof. Dr. K. O. Stetter (Lehrstuhl fuer Mikrobiologie, University of Regensburg, Germany). A plasmid pCR 2.1-TOPO vector (TOPO TA cloning kit, Invitrogen, USA) was used to insert the PCR-amplified product of the *cepA* gene described later. TOP 10 electrocompetant *Escherichia coli* cells F⁻ *merA*Δ (*mrr-hsdRMS-mcrBC*)φ80*lacZ*Δ*M15*Δ*lacX74**deoR**recA*Δ*araD139*Δ (*ara-leu*)7697*galUgalK*rpsL(*str*^R)-*endA1nupG*] were used as the host for the transformed by pCR2.1 plasmids harboring the cellobiose phosphorylase gene (*cepA*) of *T. maritima*. The plasmid vector pET 28a(+) from Novogen, USA, was used for the subcloning of the gene orienting the (His)₆ epitope tag at the gene's N-terminal end. The *E. coli* strain BL21-Codonplus-RIL competent cells [BF – *ompT* *hsdS*(rB – mB –)*dcm* + Tet^r *gal endA* Hte [*argU ileY leuW* Cam^r] were used for the heterologous expression of the *cepA* gene. All the recombinant DNA techniques followed were as described by Sambrook *et al.*²⁷⁾

Cloning, Sequencing of cepA, and Expression.

The ORF TM1848 (AE001822) which codes for the putative cellobiose phosphorylase of *T. maritima* MSB8 was amplified from the genomic DNA by a 25 cycle PCR (98°C, 30 s—55°C, 30 s—72°C, 150 s) using KOD-DASH DNA polymerase (Toyobo, Osaka, Japan), a forward primer, 5'CAT ATG GTG CGA TTC GGT TAT TTT GAT GAC GTC, and a reverse primer, 5'GAG CTC TCA GCC CAT CAC AAC TTC AAC CCT GTG with the incorporation of *NdeI* (N-terminal end) and *SacI* (C-terminal end) restriction sites (underlined) respectively. The amplicon was cloned in pCR 2.1-TOPO and transferred to electrocompetent *E. coli* TOP 10 cells. The nucleotide sequence of the cloned gene was confirmed using a Big dye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, CA) and GENETIX program (Software Development Co., Tokyo, Japan). The target gene, *cepA* from the pCR 2.1-TOPO-Tm*cepA* was digested with *NdeI* and *SacI* restriction enzymes and ligated to pET 28a(+) that had been previously digested with the above restriction enzymes. Ligation-High T4 DNA ligase (Toyobo) was used for gene-vector ligation during subcloning. The pET 28a(+) harboring *cepA* with (His)₆ residues at its N-terminal end was transferred to electrocompetent *E. coli* BL21-Codonplus-RIL cells. The transformed cells were cultured overnight at 37°C over a rotary

shaker in Luria-Bertani (LB) broth (100 ml) with 30 μg/ml of kanamycin to produce seed culture. This 100-ml seed culture was used as the inoculum for 900 ml of LB broth supplemented with 30 μg/ml of kanamycin. When the OD₆₀₀ of the broth reached approximately 0.6, the target gene was induced by the addition of isopropyl thio-galactopyranoside (IPTG) to a final concentration of 1 mM. Cultivation of the cells was continued for another 5 h at 30°C. Then the cells were harvested by centrifugation at 4,000 g for 20 min at 4°C.

Purification of recombinant enzyme. Approximately 2.7 g (wet weight) of cells were suspended in 20 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole (buffer A) and the cells were lysed by sonification. The sonified sample was centrifuged at 10,000 g for 30 min to remove the cell debris. The supernatant containing crude enzyme (71 ml) was mixed with 3 mL of fresh NiNTA resin (Qiagen, Germany) and kept on ice under gentle shaking for 15–20 min. The enzyme-bound resin was then packed into a column and eluted the enzyme with a linear gradient of 10–200 mM of imidazole in buffer A. The active fractions were pooled and dialyzed overnight at 4°C against 5 mM 2-[*N*-morpholino]ethane sulfonic acid (MES) buffer, pH 6.2. The dialyzed enzyme sample was further purified by an anion exchange column, Source 30 Q (Pharmacia, Sweden), of column volume 4 ml. The enzyme protein was eluted with a linear gradient of 0–100 mM of NaCl in 20 mM MES buffer, pH 6.2.

Protein analyses. Protein concentrations were measured using Coomassie Plus Protein assay reagent kit (Pierce, Rockford, USA) with bovine serum albumin as a standard. Sodium dodecyl sulfite/polyacrylamide gel electrophoresis (SDS-PAGE) of the enzyme samples was done and the protein bands were made visible by staining the gel with Coomassie brilliant blue.²⁸⁾ A 10-kDa ladder (Life technologies, USA) was used as a standard molecular mass protein marker. The purified enzyme (approximately 0.2 mg) from the Mono Q fraction was put on a gel filtration column of Superose 6 HR10/30 previously equilibrated with 50 mM MES buffer, pH 6.2, containing 0.1 M NaCl, at a flow rate of 0.1 ml per min to measure its native molecular mass with MW-Marker HPLC (Oriental Yeast Co., Osaka, Japan).

Enzyme assays. The reaction of cellobiose phosphorylase was done at 60°C in 25 mM MES buffer, pH 6.2. In the phosphorylase reaction the amount of α-D-glucose 1-phosphate (G-1-P) or D-glucose formed was measured. G-1-P was estimated by the phosphoglucomutase-glucose-6-phosphate dehydrogenase system.²⁹⁾ D-Glucose was measured by the glucose oxidase-peroxidase method using a Glu

Table 1. Summary of Purification of *T. maritima* Cellobiose Phosphorylase

Purification step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Crude extract	92.3	208.7	0.44	1	100
NiNTA	54.4	22.6	2.44	5.4	59
Source 30 Q	19.7	7.4	2.66	6	21

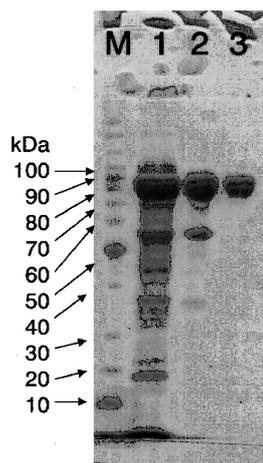
ICII kit (Wako Pure Chemicals, Osaka, Japan). In the synthetic reaction the product, phosphate was estimated by the method of Lowry and Lopez.³⁰ One unit of activity was defined as the amount of enzyme that produced one μmol of phosphate per minute from 10 mM G-1-P and 10 mM glucose at 60°C.

Optimum pH and temperature. To find the optimum pH, the phosphorolytic reaction was done at 60°C for 30 min with 10 mM cellobiose and 10 mM phosphate in 50 mM of various buffers prepared at 25°C such as sodium citrate (pH 2.2 to 4.1), sodium acetate (pH 3.7 to 5.7), MES (pH 5.1 to 7.2), 3-[*N*-morpholino]propane sulfonic acid (MOPS) (pH 6.4 to 8.8), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) (pH 6.5 to 8.6), Tris[hydroxymethyl]aminomethane hydrochloride (Tris/HCl) (pH 7.1 to 9.1), 2-[*N*-cyclohexylamino]ethane sulfonic acid (CHES) (pH 8.2 to 10.3), and 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS) (pH 9.6 to 11.5), and the amount of D-glucose formed was measured as mentioned earlier.

To find the optimum temperature, the phosphorolytic reaction was done at temperatures ranging from 25 to 95°C for 30 min in 25 mM MES buffer with 10 mM cellobiose and 10 mM phosphate. The amount of D-glucose formed after the reaction was measured and used for evaluating the average activity for 30 min.

pH and thermal stability. The purified enzyme (13.3 $\mu\text{g}/\text{ml}$) was incubated for 30 min at 70°C in various buffers as mentioned above and the residual enzyme activity was measured at 60°C for 30 min in MES buffer, pH 6.2 with 10 mM of cellobiose and 10 mM phosphate. To measure the thermal stability, the enzyme (13.3 $\mu\text{g}/\text{ml}$) was incubated for 30 min in 25 mM MES buffer (pH 6.2) at different temperatures ranging from 30 to 100°C and the residual enzyme activity was measured at 60°C for 30 min in MES buffer, pH 6.2 containing 10 mM of cellobiose and 10 mM phosphate.

Reaction mechanism. In the phosphorolytic direction, the reaction mechanism was measured using different combinations of cellobiose and phosphate concentrations from 0.25 to 2.5 mM. The enzyme ac-

**Fig. 1.** SDS-PAGE Analysis of Recombinant *T. maritima* Cellobiose Phosphorylase at Different Purification Steps.

lane M, molecular mass markers; lane 1, crude extract; lane 2, NiNTA fraction, lane 3, "Source 30 Q" fraction.

tivity at 60°C was estimated by measuring the D-glucose formed. The inhibition effects of the products, D-glucose (0–1 mM) and G-1-P (0–2 mM), against the substrates, cellobiose and phosphate, were also studied at several fixed concentrations of one substrate (2.5, 5, 20 mM) and varying the concentration of other substrate (0.25–2.5 mM) by measuring G-1-P and D-glucose, respectively, to find the orders of both substrates binding and products release.

Synthetic reaction. Various sugars and their derivatives mentioned in Table 2 were examined for the glucosyl acceptor of the cellobiose phosphorylase at 10 mM concentration with 10 mM G-1-P at 60°C in 25 mM MES (pH 6.2) buffer. The kinetic parameters were measured for those glucosyl acceptors that showed a rate higher than 1 $\mu\text{mole}/\text{min}/\text{mg}$.

Kinetic analyses. Kinetic parameters were calculated by regressing the experimental data to the appropriate rate formula based on the curve fit method by using a computer program, *Grafit-Ver.4* (Erithaus Software, UK).

Results

Purification and properties of the enzyme

The recombinant cellobiose phosphorylase was purified 6-fold to a final specific activity of 2.66 units/mg protein with an activity recovery of 21% through two purification steps (Table 1). Approximately 7.4 mg of pure enzyme was obtained from 1000 ml of culture. The purified cellobiose phosphorylase gave a single protein band on SDS-PAGE gel corresponding to a molecular mass (M_r) of approximately 85 kDa (Fig. 1), which agreed with the size calculated (94,322) on the basis of the prima-

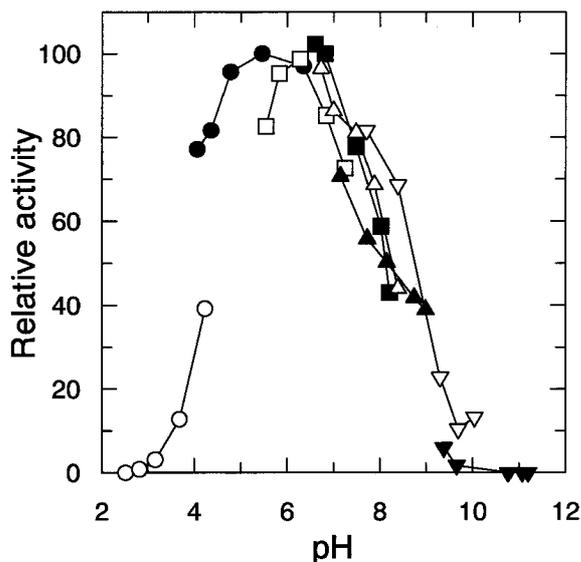


Fig. 2. Effects of pH on the Activity of the *T. maritima* Cellobiose Phosphorylase.

The activity was measured at 60°C for 30 min in 25 mM buffers of various pH levels. Values are shown as a percentage of the maximum activity, which is defined as 100%. Buffers used were sodium citrate (○), sodium acetate (●), MES (□), MOPS (■), HEPES (△), Tris/HCl (▲), CHES (▽), and CAPS (▼).

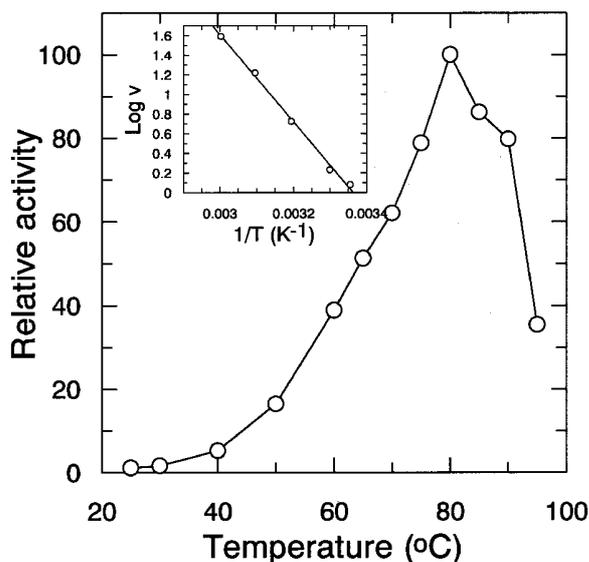


Fig. 3. Effects of Temperature on the Activity of the *T. maritima* Cellobiose Phosphorylase.

The activity was measured at various temperatures for 30 min in 25 mM MES buffer, pH 6.2. Values are shown as a percentage of the maximum activity, which is defined as 100%.

ry structure of the enzyme.¹⁹ The native molecular mass was estimated to be 150 kDa by gel filtration, indicating that the recombinant cellobiose phosphorylase existed in a dimer form. The enzyme phosphorylated cellobiose, but did not phosphorylate cellotriose or cellotetraose to form G-1-P even with a long incubation time.

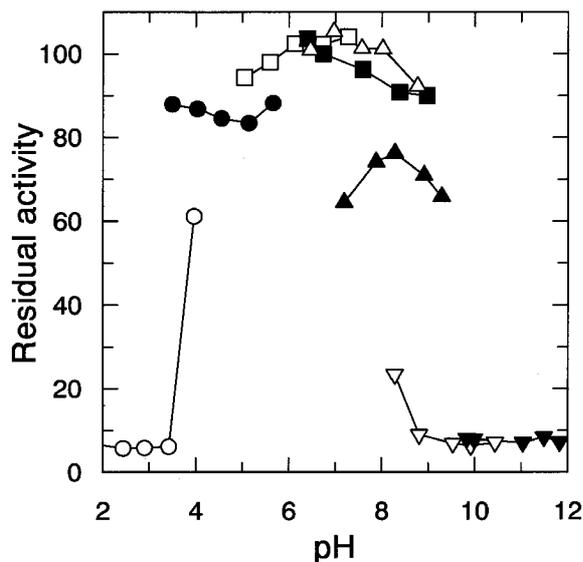


Fig. 4. Effects of pH on the Stability of the *T. maritima* Cellobiose Phosphorylase.

The stability of the enzyme was examined by treating the enzyme at 70°C for 30 min in buffers at various pH levels. The residual enzyme activity was measured at 60°C in 25 mM MES buffer of pH 6.2. Values are shown as percentages of the original activity (measured in 25 mM MES, pH 6.2, without incubation at 70°C) and this is defined as 100%. Buffers used were sodium citrate (○), sodium acetate (●), MES (□), MOPS (■), HEPES (△), Tris/HCl (▲), CHES (▽), and CAPS (▼).

The optimum pH for the maximal enzyme activity was found at 6.2 and, the enzyme showed more than 80% of the maximal activity between pH 4.5 to 7.5 (Fig. 2). The optimum temperature for the maximal enzyme reaction for 30 min was approximately 80°C (Fig. 3). From the linear part of the Arrhenius plot between 25 and 80°C, the activation energy for the phosphorylytic reaction was found to be 74 kJ/mol.

The enzyme retained more than 90% of its maximal activity in the pH range of 5 to 9 after 30 min of heating at 70°C (Fig. 4). The enzyme retained its original activity at temperatures lower than 70°C, but approximately 25% of the activity was lost upon incubating at 90°C and the activity was completely lost at 100°C after a 30-min incubation period (Fig. 5). It is notable that the reaction rate at 95°C for 30 min (35% of the maximum) was much lower than the value expected from the stability data (71% remaining after 30 min). The fact may suggest some reversible change in the enzyme structure to an inactive form at higher temperatures.

The course of the thermal inactivation of the enzyme at 60 and 80°C was studied in the absence of any substrate or stabilizer. At 60°C the enzyme was found to be stable for a period of 5 h, while at 80°C there was a 45% reduction in the enzyme activity after 60 min with the enzyme displaying a half-life of 70 min.

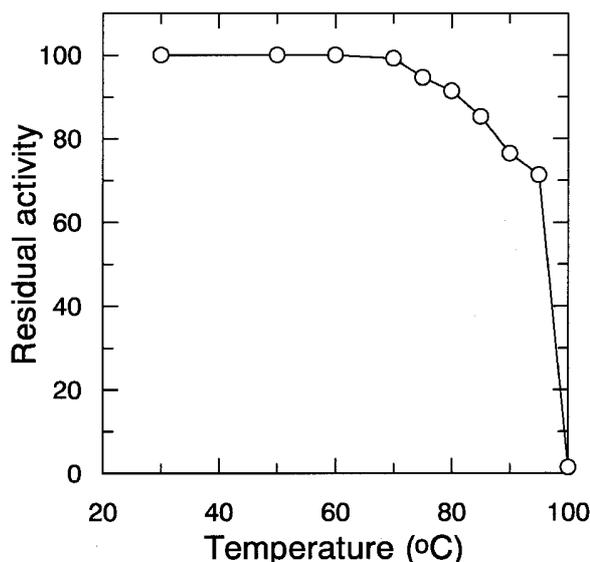


Fig. 5. Effect of Temperature on the Stability of the *T. maritima* Cellobiose Phosphorylase.

The enzyme was incubated in 25 mM final concentration of MES, pH 6.2 buffer at different temperatures for 30 min. Residual enzyme activities were measured at 60°C for 30 min. The residual activity are shown as percentages of the maximum activity (measured at 60°C for 30 min without a prior incubation step) taken as 100%.

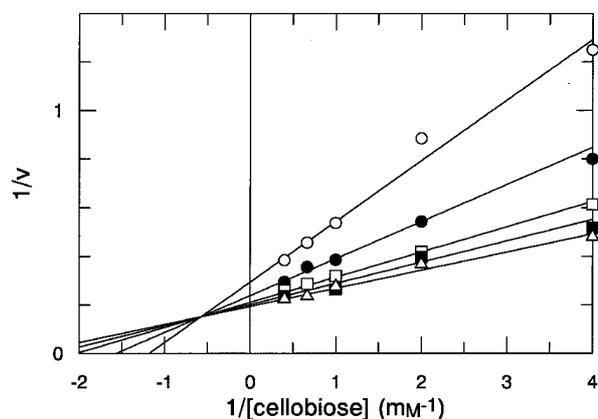


Fig. 6. Double Reciprocal Plot of the Phosphorolytic Reaction of Cellobiose.

The enzyme activity was measured as a function of the concentration of cellobiose at various concentrations of phosphate at 60°C for 30 min. Concentrations of phosphate were \circ , 0.25 mM; \bullet , 0.5 mM; \square , 1.0 mM; \blacksquare , 1.5 mM; \triangle , 2.5 mM.

Reaction mechanism

Kinetic analyses of the phosphorolytic reaction were undertaken to discover the reaction mechanism of the enzyme. When the concentration of cellobiose was varied, the $1/v - 1/[\text{cellobiose}]$ plot obtained with selected phosphate concentrations showed the lines crossing at a point in the upper left-hand quadrant (Fig. 6), indicating that the phosphorolytic reaction occurred in a sequential bi bi mechanism. The kinetic parameters in the rate equation for the se-

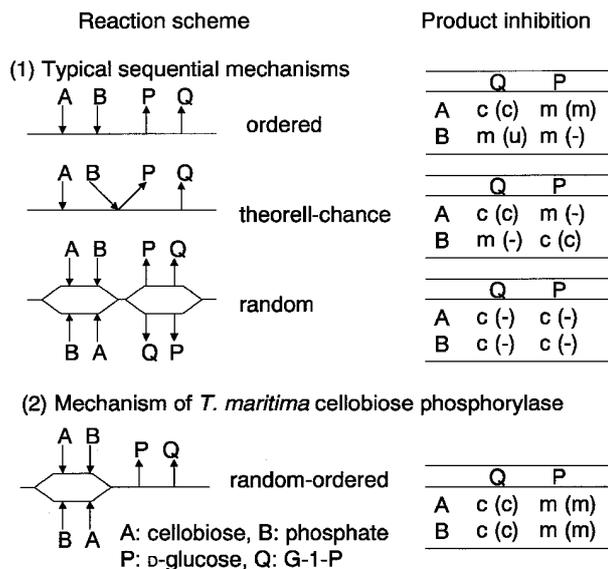


Fig. 7. Detailed Reaction Mechanisms in Sequential bi bi Mechanisms.

(1) The typical sequential bi bi mechanisms appeared in Cleland's report.³¹⁾ (2) Inhibition pattern and postulated mechanism of *T. maritima* cellobiose phosphorylase.

Left column, scheme of each mechanism; center column, name of each mechanism; right column, table of inhibition pattern of each product against each substrate. A and B are substrates and P and Q are products. In the table, c, m, and u mean competitive, mixed-type, and uncompetitive inhibitions, respectively. Inhibition patterns out of and in the parenthesis mean the inhibition pattern observed with unsaturated and saturated concentrations of the other substrate, respectively. The concentrations of the other substrate used in the table of (2) are 2.5, 5 mM (unsaturated), and 20 mM (saturated).

quential bi bi mechanism were calculated by regressing the experimental data with the following equation as shown below:

$$v = k_{\text{cat}}[E][A][B] / (K_{iA}K_{mB} + K_{mA}[B] + K_{mB}[A] + [A][B])$$

where [A] is the initial concentration of cellobiose, [B] is the initial concentration of phosphate, and [E] is the enzyme concentration.

$$k_{\text{cat}} = 5.4 \text{ s}^{-1}; K_{mA} = 0.29 \text{ mM}; K_{iA} = 1.7 \text{ mM}; K_{mB} = 0.15 \text{ mM}.$$

The inhibition patterns of the products, G-1-P and D-glucose, against the substrates, D-cellobiose and phosphate, are summarized in the table of Fig. 7(2). G-1-P acted as a competitive inhibitor and glucose as a mixed-type inhibitor against both cellobiose and phosphate at all of the three concentrations of the opposite substrates examined. This result did not match with any of three possible mechanisms in the sequential bi bi mechanism as shown in Fig. 7(1).³¹⁾ A possible explanation of the result is the "random-ordered bi bi mechanism" as described in Fig. 7(2), where the sequence of the substrate binding is ran-

Table 2. Substrate Acceptor Specificity of *T. maritima* Cellobiose Phosphorylase in the Synthetic Reaction

Substrate	Rate ($\mu\text{mol}/\text{min}/\text{mg}$)	Substrate	Rate ($\mu\text{mol}/\text{min}/\text{mg}$)
D-Glucose	8.7	D-Glucono- δ -lactone	—
D-Mannose	1.1	Methyl- β -D-glucoside	1.1
2-Deoxy-D-glucose	2.9	Methyl- α -D-glucoside	—
D-Glucosamine	2.9	D-Cellobiose	—
D-Mannosamine	—	D-Glucitol	—
N-Acetyl-D-glucosamine	—	D-Mannitol	—
N-Acetyl-D-mannosamine	—	D-Arabinose	—
D-Allose	—	L-Arabinose	—
D-Galactose	—	D-Ribose	—
D-Fucose	—	L-Sorbose	—
6-Deoxy-D-glucose	13.8	L-Glucose	—
D-Xylose	15.0	L-Fucose	—
D-Glucuronamide	—	L-Xylose	—
D-Fructose	—		

Values were measured using the concentrations of 10 mM G-1-P and 10 mM acceptor substrate at 60°C.

—, not detected (less than 0.6).

Table 3. Kinetic Parameters for Various Glucosyl Acceptors of *T. maritima* Cellobiose Phosphorylase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
D-Glucose ^a	0.69	8.0	1.1×10^4
D-Mannose ^b	67	4.4	6.5×10^1
D-Glucosamine ^c	5.7	5.2	9.1×10^2
2-Deoxy-D-glucose ^b	47	16	3.4×10^2
6-Deoxy-D-glucose ^c	4.1	17	4.3×10^3
D-Xylose ^d	14	40	2.8×10^3
Methyl- β -D-glucoside ^e	135	6.5	4.8×10^1

Concentrations used: a, 0.5–5 mM; b, 10–100 mM; c, 1–10 mM; d, 5–50 mM; e, 20–200 mM.

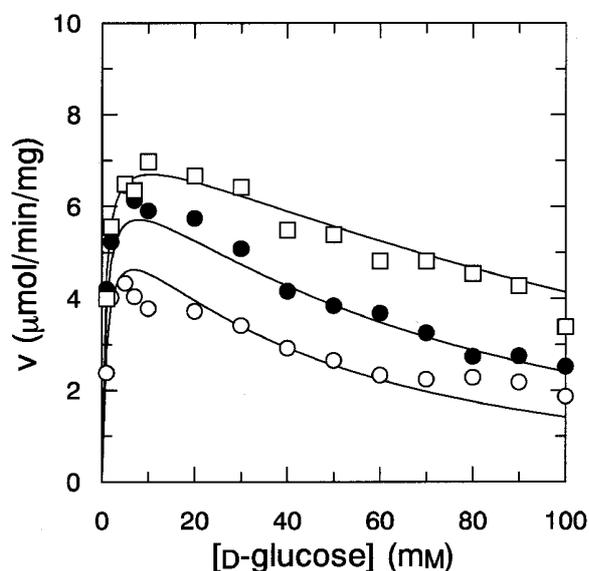
dom and the product release is ordered (glucose first and then G-1-P).

Substrate specificity for the synthetic reaction

Out of 27 compounds tested, seven were found to act as glucosyl acceptors for the cellobiose phosphorylase (Table 2). It is notable that the enzyme used β -methyl-D-glucoside as the acceptor. The product from G-1-P and β -methyl-D-glucoside was identified as β -methyl-cellobioside by NMR analysis (data not shown). Detailed kinetic studies were done by using these substrates, and their kinetic parameters are presented in Table 3. D-Xylose had the highest k_{cat} (40 s^{-1}) followed by 6-deoxy-D-glucose (17 s^{-1}) and 2-deoxy-D-glucose (16 s^{-1}). The natural substrate, D-glucose gave the k_{cat} of 8.0 s^{-1} , but it had the highest k_{cat}/K_m ($1.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) compared with other glucosyl acceptors.

Inhibition pattern of D-glucose

A substrate inhibition by D-glucose in the reverse synthetic reaction was observed previously for the cellobiose phosphorylases from *Cellv. gilvus*¹²⁾ and *Clostr. thermocellum*.^{18,32)} These inhibitions were explained as “competitive substrate inhibition”, in


Fig. 8. The v -[D-glucose] Plot of the Synthetic Reaction of the Cellobiose Phosphorylase at Fixed Concentrations of G-1-P.

Concentrations of G-1-P were \circ , 1 mM; \bullet , 2 mM; \square , 5 mM. The theoretical lines were obtained by regressing the experimental data to the formula of competitive substrate inhibition.¹²⁾

which one substrate, namely D-glucose, competitively inhibited the other substrate, G-1-P.¹²⁾ Therefore, the possibility of inhibition by D-glucose was studied at concentrations of D-glucose ranging from 1 mM to 100 mM with several fixed concentrations of G-1-P. As shown in Fig. 8, the [D-glucose]- v curves gave maxima around a D-glucose concentration of 10 mM, demonstrating that a similar inhibition also occurred in the synthetic reaction by the cellobiose phosphorylase from *T. maritima*. The theoretical lines obtained by regressing the experimental data with the formula explaining the competitive substrate inhibition,¹²⁾ as shown below, well explain the result (Fig. 8).

$$v = k_{\text{cat}}[E][Q][P] / \{ K_{iQ}K_{mP} + (K_{mQ} + K_{iQ}K_{mP}/K_{i1})[P] \}$$

$$\begin{aligned}
 &+ K_{mP}[Q] + [Q][P] \\
 &+ (K_{iQ}K_{mP}/K_{I1}K_{I2} + K_{mQ}/K_{I1})[P]^2 \\
 &+ (K_{mQ}/K_{I1}K_{I2})[P]^3
 \end{aligned}$$

where [Q] is the initial concentration of G-1-P, [P] is the initial concentration of D-glucose, and [E] is the enzyme concentration.

$$\begin{aligned}
 k_{cat} &= 13 \text{ s}^{-1}; K_{mQ} = 0.29 \text{ mM}; K_{iQ} = 1.7 \text{ mM}; \\
 K_{mP} &= 0.15 \text{ mM}; K_{I1} = 7.3 \text{ mM}; K_{I2} = 190 \text{ mM}.
 \end{aligned}$$

Discussion

The genome sequence results indicated the presence of a putative cellobiose phosphorylase in *T. maritima*.¹⁹⁾ According to the primary structure, cellobiose phosphorylase, cellodextrin phosphorylase, and chitinase phosphorylase are grouped in the family 36 of glycosyl transferases.³³⁾ (<http://afmb-mrs.fr/~pedro/CAZY/gtf.html>) The percent amino acid similarities among cellobiose phosphorylases found increasing from mesophilic (61% in *Cellv. gilvus*, AB010707) to thermophilic (73%, *Clost. thermocellum*, AB013109, and *Clost. stercorarium* U56424), and hyperthermophilic (92%, *T. neapolitana* AF039487 and Z99777) organisms. With the cellodextrin phosphorylase of *Clost. stercorarium* (U60580) the amino acid sequence similarity was found to be approximately 40%.

The phosphorylolytic reaction by the cellobiose phosphorylase of *T. maritima* was found to proceed in a sequential mechanism. However, the product inhibition pattern did not match with the possible three sequential mechanisms presented by Cleland.³¹⁾ Judging from the inhibition pattern, a possible explanation of the reaction is a random-ordered bi bi mechanism. In this mechanism, the binding sequence of cellobiose and phosphate is random, and the release sequence of the glucose and G-1-P is ordered. The reaction mechanisms of *Cellv. gilvus*,¹⁷⁾ *Cellu. Uda*,⁸⁾ and *Clost. thermocellum*¹⁸⁾ cellobiose phosphorylases were reported to be ordered bi bi mechanisms. The first two enzymes bind cellobiose first and then phosphate followed by the sequential release of glucose and G-1-P. However, the *Clost. thermocellum* YM-4 enzyme binds phosphate before cellobiose and releases glucose and G-1-P with the same sequence of the other two enzymes.¹⁸⁾ The random-ordered mechanism of the *T. maritima* cellobiose phosphorylase seems intermediate between the above two types of ordered bi bi mechanisms because the difference in the mechanism is only in the sequence of the substrate binding.

In the synthetic reaction, out of 27 sugars or their derivatives tested, only D-glucose, D-xylose, D-mannose, D-glucosamine, 2-deoxy-D-glucose, 6-deoxy-D-glucose, and methyl-β-D-glucopyranoside served as

the glucosyl acceptors for the enzyme. The results indicated that the hydroxyl groups at positions 3 and 4 were essential for the recognition of acceptors. The kinetic parameters (Table 3) showed that the natural substrate of the enzyme, D-glucose, was found to have the lowest K_m (0.69 mM) followed by 6-deoxy-D-glucose (4.1 mM). The substitution or deletion of hydroxyl group at C1, C2 or C6 configurations of D-glucose resulted in significant increases in K_m , indicating that these hydroxyl groups were also recognized by the enzyme. D-Xylose acted as a good substrate with the maximum k_{cat} value of 40 s^{-1} as compared to D-glucose (8.0 s^{-1}). The cellobiose phosphorylases from different organisms that we studied strictly recognized the C1 configuration of β-D-glucopyranose^{8,9,12)} and it was believed that the enzymes did not recognize β-glucosides as the acceptor. However, the cellobiose phosphorylase of *T. maritima* was found to accept methyl-β-D-glucoside as a substrate in the synthetic reaction to produce methyl-(D-cellobioside. This unique specificity has not been reported for any other cellobiose phosphorylases. The substitution of a hydroxyl group at C1 of D-glucose with a methoxy group resulted in a 196-fold increase in the K_m values compared with that of D-glucose (0.69 mM) without significant difference in the k_{cat} values obtained.

Competitive inhibition by the natural substrate, D-glucose (Fig. 8), was again observed for the cellobiose phosphorylase from *T. maritima*. The phenomenon is supposed to be common in cellobiose phosphorylases.

In summary, the characterization of the cellobiose phosphorylase from *T. maritima* revealed a high degree of thermal stability and a distinct substrate specificity for reactions in the synthetic direction. The mechanism of the reaction was slightly different from other cellobiose phosphorylases reported. In the future, this hyperthermophilic cellobiose phosphorylase may find application in the syntheses of various oligosaccharides.

Acknowledgments

We thank Prof. K.O. Stetter of the Univ. of Regensburg, Germany for providing the genomic DNA of *T. maritima*. We also thank Dr. P. Selvakumar and Dr. Kshamata Goyal for their suggestions during this work. The STA fellowship of Japan awarded to the senior author is gratefully acknowledged. This work was supported in part by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences in Japan.

References

- 1) Sih, C. J., and McBee, R. H., A cellobiose phosphorylase in *Clostridium thermocellum*. *Proc. Mon-*

- tana Acad. Sci.*, **15**, 21–26 (1955).
- 2) Ayres, W., Phosphorylation of cellobiose and glucose by *Ruminococcus flavifaciens*. *J. Bacteriol.*, **76**, 515–517 (1958).
 - 3) Hulcher, F. H., and King, K. W., Metabolic basis for disaccharide preference in *Cellvibrio*. *J. Bacteriol.*, **76**, 571–577 (1958).
 - 4) Hutterman, A., and Volger, C., Cellobiose phosphorylase in *Fomes annosus*. *Nature New Biol.*, **245**, 64 (1973).
 - 5) Matheron, C. A., Delrot, M., Gaudet, G., and Forano, E., *In vivo* ¹³CMR study of glucose and cellobiose metabolism by four cellulolytic strains of the genus, *Fibrobacter*. *Biodegradation*, **9**, 451–461 (1998).
 - 6) Schimz, K.-L., Broll, B., and John, B., Cellobiose phosphorylase of *Cellulomonas*: Occurrence, induction and its role in cellobiose metabolism. *Arch. Microbiol.*, **135**, 241–249 (1983).
 - 7) Sasaki, T., Tanaka, T., Nakagawa, S., and Kainuma, K., Purification and properties of *Cellvibrio gilvus* cellobiose phosphorylase. *Biochem. J.*, **209**, 803–807 (1983).
 - 8) Nidetzky, B., Eis, C., and Albert, M., Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*. *Biochem. J.*, **351**, 649–659 (2000).
 - 9) Kitaoka, M., Ogawa, S., and Taniguchi, H., A cellobiose phosphorylase from *Cellvibrio gilvus* recognizes only the β -D-form of 5a-carba-glucopyranose. *Carbohydr. Res.*, **247**, 355–359 (1993).
 - 10) Alexander, J. K., Synthesis of 4-O- β -D-glucopyranosyl-D-xylose, 4-O- β -D-glucopyranosyl-D-arabinose, 4-O- β -glucopyranosyl-2-deoxy-D-glucose, 4-O- β -glucopyranosyl-D-mannose and 4-O- β -D-glucopyranosyl-D-mannoseamine by cellobiose phosphorylase from *Clostridium thermocellum*. *Arch. Biochem. Biophys.*, **123**, 240–246 (1968).
 - 11) Kitaoka, M., Taniguchi, H., and Sasaki, T., Production of glucosyl-xylose using *Cellvibrio gilvus* cells and its properties. *Appl. Microbiol. Biotechnol.*, **34**, 178–182 (1990).
 - 12) Kitaoka, M., Sasaki, T., and Taniguchi, H., Synthetic reaction of *Cellvibrio gilvus* cellobiose phosphorylase. *J. Biochem. (Tokyo)*, **112**, 40–44 (1992).
 - 13) Percy, A., Ono, H., and Hayashi, K., Acceptor specificity of Cellobiose phosphorylase from *Cellvibrio gilvus*: synthesis of three branched trisaccharides. *Carbo. Res.*, **308**, 423–429 (1998).
 - 14) Tariq, M. A., and Hayashi, K., Synthesis of three heterodisaccharides, 4-O- β -D-glucopyranosyl-6-deoxy-D-glucose, 4-O- β -glucopyranosyl-D-mannoseamine, 4-O- β -D-glucopyranosyl-D-mannose and confirmation of their structures by C¹³ NMR and MS. *Biochem. Biophys. Res. Comm.*, **214**, 568–575 (1995).
 - 15) Kitaoka, M., Sasaki, T., and Taniguchi, H., Conversion of sucrose into cellobiose using sucrose phosphorylase, xylose isomerase and cellobiose phosphorylase. *Denpun Kagaku*, **39**, 281–283 (1992).
 - 16) Kitaoka, M., Aoyagi, C., and Hayashi, K., Colorimetric quantification of cellobiose employing cellobiose phosphorylase. *Anal. Biochem.*, **292**, 163–166 (2001).
 - 17) Kitaoka, M., Sasaki, T., and Taniguchi, H., Phosphorolytic reaction of *Cellvibrio gilvus* cellobiose phosphorylase. *Biosci. Biotechnol. Biochem.*, **56**, 652–655 (1992).
 - 18) Kim, Y.-K., Kitaoka, M., Krishnareddy, M., Mori, Y., and Hayashi, K., Kinetic studies of a recombinant cellobiose phosphorylase (CBP) of *Clostridium thermocellum* YM4 strain expressed in *Escherichia coli*. *J. Biochem. (Tokyo)*, **132**, 197–203 (2002).
 - 19) Nelson, K. E., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M., Stewart, A. M., Cotton, M. D., Pratt, M. S., Phillips, C. A., Richardson, D., Heiderberg, J., Sutton, G. G., Fleischmann, G. G., Eisen, J. A., White, Salzberg, S. L., Smith, H. O., Venter, J. C., and Fraser, C. M., Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature*, **399**, 323–329 (1999).
 - 20) Bronnenmeir, K., Kern, A., Liebl, W., and Staudenbauer, W. T., Purification of *Thermotoga maritima* enzymes for degradation of cellulosic materials. *Appl. Environ. Microbiol.*, **61**, 1399–1407 (1995).
 - 21) Evans, R. B., Gliman, A. K., Cordray, K., and Woodward, J., Mechanism of substrate hydrolysis by a endoglucanase from *Thermotoga maritima*. *Biotechnol. Lett.*, **22**, 735–740 (2000).
 - 22) Goyal, K., Selvakumar, P., and Hayashi, K., Characterization of a thermostable β -glucosidase (BglB) from *Thermotoga maritima* showing transglycosylation activity. *J. Mol. Catalysis. B. Enzymatic.*, **15**, 45–53 (2001).
 - 23) Huber, H., and Stetter, K. O., Hyperthermophiles and their possible potential in biotechnology. *J. Biotechnol.*, **64**, 39–52 (1998).
 - 24) Liebl, W., Cellulolytic enzymes from *Thermotoga* spp. In “Methods in Enzymology” vol. 330, eds. Adams, M. W. W., and Kelly, R. M., Academic Press, San Diego, pp. 290–300 (2001).
 - 25) Liebl, W., Brem, D., and Gotschlich, A., Analysis of the gene for β -fructosidase (invertase, inulinase) of the hyperthermophilic bacterium *Thermotoga maritima* and characterization of the enzyme expressed in *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **50**, 55–64 (1998).
 - 26) Yernool, D. A., McCarthy, J. K., Eveleigh, D. E., and Bok, J.-D., Cloning and characterization of the glucooligosaccharide catabolic pathway β -glucan glucohydrolase and cellobiose phosphorylase in the marine hyperthermophilic *Thermotoga maritima*. *J. Bacteriol.*, **182**, 5172–5179 (2000).
 - 27) Sambrook, J., Fritsch, E. F., and Maniatis, T., In “Molecular cloning: A laboratory manual, 2nd ed.”, Cold Spring Harbor Laboratory, New York (1989).
 - 28) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
 - 29) Michael, G., In “Methods in Enzymatic Analysis, 2nd ed.” vol. 4, ed. Bergmeyer, H. U., Academic Press, San Diego, pp. 185–191 (1974).

- 30) Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421–428 (1946).
- 31) Cleland, W. W., The kinetics of enzyme-catalyzed reactions with two or more substrates or products. *Biochim. Biophys. Acta*, **67**, 104–137 (1963).
- 32) Tanaka, K., Kawaguchi, T., Imada, Y., Ooi, T., and Arai, M., Purification and properties of cellobiose phosphorylase from *Clostridium thermocellum*. *J. Ferment. Bioeng.*, **79**, 212–216 (1995).
- 33) Campbell, J. A., Davies, G. J., Bulone, V., and Henrissat, B., A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.*, **326**, 929–939 (1997).