J Incl Phenom Macrocycl Chem (2007) 57:53–59
DOI 10.1007/s10847-006-9162-6

ORIGINAL ARTICLE

A recombinant cyclodextrin glycosyltransferase cloned from *Paenibacillus* sp. strain RB01 showed improved catalytic activity in coupling reaction between cyclodextrins and disaccharides

Ratiya Charoensakdi · Masaru Iizuka · Kazuo Ito · Vichien Rimphanitchayakit · Tipaporn Limpaseni

Received: 5 May 2006 / Accepted: 20 October 2006 / Published online: 13 February 2007

Abstract Recombinant cyclodextrin glycosyltransferase (CGTase) was obtained by cloning the PCR gene fragment from thermotolerant *Paenibacillus* sp. strain RB01 screened from hot spring area in Thailand and cloned into the *Escherichia coli* expression vector. The nucleotide sequence was analyzed and aligned. Nucleotide sequence of the recombinant CGTase contained an open reading frame of 2139 bp encoding 713 amino acid residues. The recombinant required one-third of culture time and neutral pH to produce CGTase compared to wild type. CGTases from both wild type and transformant were purified in parallel by starch adsorption and DEAE cellulose column. Their biochemical properties such as molecular weight, optimum pH and temperature were quite similar. However, the recombinant enzyme showed improved catalytic activity in the coupling reaction between cyclodextrins (CDs) and some disaccharides. Among several sugars tested with excess βCD, cellobiose was the best substrate followed by leucrose. Very low activity was observed with trehalose, lactose and mellibiose. Sucrose and raffinose showed no activity. The K_m and other kinetic parameters of recombinant enzyme were determined for cellobiose and several cyclodextrin derivatives. Recombinant CGTase showed lower K_m for βCD and its derivatives, with improved activity compared to wild type enzyme.

Keywords Recombinant CGTase · Improved coupling activity

R. Charoensakdi · V. Rimphanitchayakit · T. Limpaseni (✉)
Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand
e-mail: tipaporn.l@chula.ac.th

M. Iizuka · K. Ito
Laboratory of Enzyme Chemistry, Faculty of Science, Osaka City University, 3-3138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

Introduction

Cyclodextrins (CDs) are cyclic α (1–4) linked oligosaccharides mainly consisting of 6, 7, or 8 glucose residues (α-, β- and γ-cyclodextrins) respectively. The glucose residues in the cyclodextrin rings are arranged in such manner that the secondary hydroxyl-groups are located on one edge of the ring and the primary hydroxyl-groups on the other edge, resulting in torus shaped molecules. The molecule with hydrophilic outside results in water solubility. The apolar cavity, which provides a hydrophobic matrix, enables cyclodextrins to form inclusion complexes with a wide variety of hydrophobic guest molecules. The formation of inclusion complexes leads to changes in the chemical and physical properties of the guest molecules. These altered characteristics of encapsulated compounds have led to various applications of cyclodextrins (or their derivatives) in analytical chemistry [1, 2], agriculture [3, 4], biotechnology [5], pharmacy [6–8], food [9] and cosmetics [5].

Many thermotolerant bacteria producing CGTase were screened from hot spring areas in Thailand for use in industrial production of products from CGTase such as cyclodextrins and oligosaccharides. *Paenibacillus* sp. RB01 was one of the isolated CGTase producing bacteria which showed potential for industrial

use. To improve CGTase production, its CGTase gene was cloned and expressed. The properties of wild-type and recombinant enzymes were compared.

Materials and methods

Materials

Cornstarch was obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Luria-Bertani (LB) media and Bacto agar for bacterial culture were acquired from Difco Laboratories (Detroit, MI, USA). Restriction enzymes were the products of (Amercham bioscience Co., USA and NEB Co., USA). All other chemicals were commercial products of analytical grade or molecular biological grade.

Bacterial strains

Paenibacillus sp. RB01 isolated from hot spring area in Ratchaburi province in Thailand, was characterized as thermotolerant bacterium producing CGTase by our research group. *E. coli* JM109 and the plasmid pGEM®-T Easy were the product of Qiagen.

DNA techniques

General molecular experiments involving DNA extraction, PCR reaction and transformation were performed in accordance with the methods described by Sambrook et al. 1989 [10] with some modification. Chromosomal DNA was prepared by dissolving 30 μl of cell pellet in 300 μl of SET (50 mM sucrose, 10 mM EDTA and 25 mM Tris pH 8.0), 200 μl of lysozyme (5 mg/ml in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and 10 μl of RNAse A (10 mg/ml in TE buffer) at 37°C for 1 h. The cell suspension was then incubated with 30 μl of 10% SDS and 3 μl of proteinase K (20 mg/ml in TE buffer) and incubate at 50°C for 4–5 h (or at 37°C overnight), after which 50 μl of 3 M sodium acetate was added and inverted gently for 2–3 times. An equal volume of TE-saturated phenol was added to the aqueous sample, vigorously mixed and centrifuged to get the phase separation. The upper aqueous layer was carefully transferred to a new tube, avoiding the phenol interface and was subjected to two ether extractions to remove residual phenol. An equal volume of water-saturated ether was added to the tube, mixed and centrifuged to allow phase separation. The upper ether layer was removed and discarded, including the phenol droplets at the interface. The extraction was repeated, the DNA was precipitated with 95% ethanol and resuspended in TE buffer and kept at 4°C.

Construction of expression plasmids

The genomic DNA was extracted and used as the template for preparation of CGTase gene by PCR technique. A pair of 24 nucleotide-length primers was used as PCR primers to amplify the chromosomal DNA of RB01. It was designed from the *N*- and *C*-terminal nucleotide sequence of CGTase gene from *Paenibacillus* sp. A11 [11], another mesophilic CGTase-producing bacteria studied in our laboratory. Amplification of DNA fragments was performed with 25 μl reaction mixture containing 100 ng of genomic DNA, 1× (Mg^{2+} free) Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotides (dNTPs), 10 ρmol of each primer and a total of 1.5 units of Taq DNA polymerase. The PCR reaction was started with 1 cycle of 94°C for 5 min followed by 30 cycles of the following program: 94°C for 1 min, 45°C for 2 min, 72°C for 3 min and finished with 1 cycle of 72°C for 5 min. Amplified PCR products were analyzed by 0.7% agarose gel electrophoresis in 1× TAE buffer (Tris–HCl 40 mM, Acetate 40 mM and Na_2EDTA 1 mM, pH 8.0) under the currency 100 V with the standard DNA marker, *Hin*d III. The PCR product was extracted and purified by the Geneclean III kit (Bio101 Inc., CA, USA). PCR product was ligated with pGEM®–T easy vector at 16°C for 18 h.

Cloning of CGTase gene

PCR ligated with pGEM®–T was transformed into *E. coli* JM109 using electroporation technique and grown in LB broth at 37°C for 18 h. Screening of bacteria producing CGTase on solid agar plates was usually performed on Horikoshi medium agar plate containing 1% soluble starch and stained with iodine solution [12] or phenolphthalein-methyl orange. The latter was more specific towards CDs since the dye staining was based on detection of colorless CD-Phenolphthalein complex formed around the positive colonies [13]. This technique was adapted for screening of CD producing activity in transformed *E. coli* which can not grew on Horikoshi plate by adding starch in LB medium. To identify the bacterial colonies contain ligated plasmid, the transformed *E. coli* was streaked on the replica plates of LB agar containing 1% soluble potato starch and 50 μg/μl of ampicillin, 0.002% X-gal and 0.0025% IPTG, incubated at 37°C overnight. The transformant containing the CGTase activity produced cyclodextrins

into the medium. CD forming activity was observed as the clear yellow zone around the positive colonies after staining with dye solutions, 0.2% I_2 in 0.2% KI and 0.03% phenolphthalein–0.01% methyl orange in 1% Na_2CO_3 which was specific test of β-CD.

Nucleotide sequencing

The plasmid DNA was isolated and purified using a Qiaprep Spin Miniprep Kit from Qiagen (Valencia, USA). PCR was performed with purified plasmid DNA as a template, M13 reverse and M13 forward (–20) primers, and Dye Terminator Cycle Sequencing Ready mixture (Perkin Elmer) according to the manufacturer's instructions. DNA sequences were determined by dye-terminator cycle sequencing with an automated DNA sequencer (Beckman Coulter, CEQ8000) and the results were analyzed using GENETYX-WIN software.

Expression and purification of CGTases

Wild type *Paenibacillus* sp. RB01(RB01) and its *E. coli* transformant (pRB) were cultured in appropriate media and conditions by which optimum CGTase production were obtained. The *Paenibalillus* was grown in 1200 ml of Horikoshi medium pH 10.0 [14] containing 1% soluble corn starch for 72 h at 40°C while the recombinant *E. coli* was grown in 1200 ml of LB containing 1% soluble corn starch in the presence of 100 mg/ml ampicillin for 24 h at 37°C. The crude enzymes were harvested by centrifugation at 6,500 g for 15 min at 4°C and subjected to purification by starch adsorption and DEAE cellulose column chromatography. Starch adsorption was performed according to Kato and Horikoshi, 1984 [15] and modified by Kuttiarcheewa, 1994 [16]. Cornstarch was oven dried at 120°C for 30 min and cooled down to room temperature. It was then gradually sprinkled into stirring crude CGTase broth to make 5 g% concentration. After 3 h of continuous stirring, the starch cake was was collected by centrifucation at 8,000 g for 30 min and washed twice with 10 mM tris-HCl pH 8.5 containing 10 mM $CaCl_2$ (TB1). CGTase was eluted from the starch cake with 0.2 M maltose in TB1(2× 125 ml for starting broth of 1 liter) with stirring for 30 min. The eluate was collected by centrifugation at 10,000 g for 30 min. The solution was dialyzed against three changes of distilled water at 4°C.

DEAE-cellulose was packed in glass column (1.5 × 28 cm) and equilibrated with Tris-HCl pH8.0 (TB2). The dialyzed protein solution from starch adsorption step was applied, washed with TB2 and eluted with gradient of 0–0.3 M NaCl in TB2. Fractions of 4 ml were continuously collected. The elution profile was constructed by measuring absorbance at 280 nm and dextrinizing activity of the fractions. The fractions with enzyme activity were pooled, concentrated and kept for characterization. All purification steps, except enzyme dialysis, were performed at room temperature. The purified enzymes were kept at 4°C for characterization.

Electrophoresis and determination of protein concentrations

Non-denaturing polyacrylamide gel (ND-PAGE) was used to identify CGTase in each purification step by running the protein samples from each step on 7.5 % polyacrylamide gel with Tris-glycine buffer system. After electrophoresis, the non-denaturing gel was stained for dextrinizing activity or amylolytic activity by slightly modified technique of Kobayashi et al. 1978 [17] in 10 ml of substrate solution, containing 0.2% (w/v) potato starch in 0.2 M phosphate buffer, pH 6.0, at 40°C for 10 min. It was then quickly rinsed several times with distilled water and immersed in 10 ml I_2 staining reagent (0.2% I_2 in 2% KI) and left for color development at room temperature. The clear zone on the blue background represents starch degrading activity of the protein.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% polyacrylamide stacking and 7.5% polyacrylamide separating gels was carried out on a Bio-Rad Mini-Protean III gel apparatus (Bio-Rad Laboratories, Hercules, MA, USA) using the Laemmli buffer system (0.25 M Tris, 1.92 M Glycine, 1 % SDS, pH 8.3). The molecular weight markers used were phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (36.5 kDa), and carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The protein concentrations were measured by Coomassie blue G method according to Bradford (1976) [18] using bovine serum albumin as the standard.

CGTase activity assay

CGTase was monitored by two types of activity. Dextrinizing activity measured the starch hydrolytic activity of CGTase spectrophotometrically by measuring the decrease in absorbance of starch-iodine complex at 600 nm [12] with slight modification. Enzyme sample (10–1,000 μl) was incubated with 0.3 ml starch

substrate (0.2 g% soluble potato starch in 0.2 M phosphate buffer, pH 6.0) at 40°C for 10 min. The reaction was stopped with 4 ml of 0.2 N HCl and 0.5 ml of iodine reagent (0.02% I_2 in 0.2%KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and the absorbance at 600 nm was measured. For control tube, HCl was added before the enzyme sample. One unit of enzyme was defined as the amount of enzyme which produced 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition. Cyclization activity was determined by the phenolphthalein method of Goel and Nene, 1995 [19]. To 1.25 ml of 4.0% soluble starch, 0.25 ml purified CGTase was added. The reaction mixture was incubated for 30 min at 60°C. The reaction was stopped by boiling for 5 min and 1 ml of the reaction mixture was incubated with 4.0 ml of phenolphthalein solution. The decrease in phenolphathalein absorption at 550 nm reflected the amount of CD in the reaction which was quantitated from calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 mole of βCD per minute under the appropriate condition.

Determination of kinetic parameters

The sugar most active towards βCD in coupling reaction was determined by incubation of 17 mM βCD with 20 mM of different sugars at 55°C in the presence of CGTase for 10 min and reaction stopped by boiling for 5 min. The most active sugar was used as sugar acceptor in subsequent study of kinetic parameters with several CDs and their derivatives as oligosaccharide donors. Kinetic parameters of the coupling reaction were determined by incubating various concentrations of α-, β- or γ-cyclodextrins and their derivatives (0.5–15 mM) with 10 mM cellobiose as glucosyl acceptor at 55°C, 50 mM acetate buffer, pH 6.0 was added to make the total volume of reaction mixture 0.25 ml. Cyclodextrin and cellobiose were preincubated for 5 min at 55°C. The reaction was started with 10 ml of 0.88 mg/ml of purified CGTase. After 10 min incubation, the reaction was stoped by boiling for 5 min. Subsequently, 0.2 units of *Aspergillus niger* glucoamylase (10 μl) was then added to convert linear oligosaccharides to glucose. The released reducing sugars was measured by the dinitrosalicylic acid method. K_m and V_{max} were determined from the Michealis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program.

Results and discussions

CGTase gene from *Paenibacillus* sp. RB01 was successfully isolated using the pair of primers previously used in our laboratory to clone CGTase gene from *Paenibacillus* sp. A_{11} [11]. DNA sequence was determined and amino acid sequence was deduced which revealed 2139 bp encoding 713 amino acids.

Optimum culture conditions for CGTase production were determined for both types of cells. The wild type produced maximum CGTase in Horikoshi medium at 40°C, pH10 at 60 h (Table 1). The transformed cells can not grow in Horikoshi medium but produce CGTase in LB medium containing 1% starch at 37°C, pH 7 at 24 h.

When CGTases from RB01 and pRB were purified in parallel by starch adsorption and DEAE-cellulose column, it was found that the transformant cells produced more enzyme with less contaminating proteins, resulting in 3 times higher specific activity (Table 2). Electrophoresis of both enzymes showed similar mobility on non denaturing gel and their dextrinizing activities were confirmed by activity stain of the gel. Their molecular weights were the same at 65 kDa on SDS-PAGE (Figs. 1 and 2), lower than the molecular weights calculated from the deduced amino acid sequences. Post-translational processes such as trimming, glycosylation or folding could result in the apparent lower molecular weight. Determination of optimum temperature and pH for dextrinizing and cyclization activities showed similar patterns for both enzymes (Table 2). The enzymes can work at high temperature up to 70°C and were quite stable up to 65°C at wide pH range from 6 to 10. Therefore, the recombinant CGTase possessed similar characteristics to wild type enzymes but the recombinant cells required only one-third culture time to produce maximum CGTase in

Table 1 Comparison of growth and CGTase production profiles between wild-type (RB01) and transformant(pRB)

	RB01	pRB
Growth	30–45°C, pH 10	37°C, pH 7
Maximal CGTase production	40°C, pH 10	37°C, pH 7
Time of cultivation	60 h	24 h
Optimum condition*		
Dextrinizing	60°C, pH 5–6	50–70°C, pH 5–9
Cyclization	60–70°C, pH 6.5	50–60°C, pH 6.5
Stability*		
Thermostability	40–65°C, pH 6.5	40–65°C, pH 6.5
pH	pH 6–10	pH 6–10

*Using purified enzymes, data were from 3 duplicated experiments

Table 2 Expression and purification of CGTases from wild-type (RB01) and transformant (pRB)

Step	Total dextrinizing activity (units)		Total protein (mg)		Specific activity (units/mg)		Purification fold		Yield (%)	
	RB01	pRB	RB01	PRB	RB01	pRB	RB01	pRB	RB01	pRB
Crude	59,500	21,0200	388.9	293.9	153	715	1	1	100	100
Starch adsorption	42,840	47,250	9.66	20.7	4,435	2,283	29	3	72	22
DEAE-cellulose	30,770	14,688	5.59	0.8	5,504	18,360	36	26	52	7

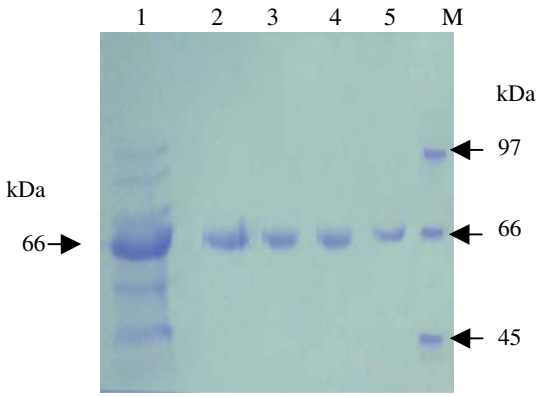

Fig. 1 SDS-PAGE of purified CGTase from pRB. Samples were run on 7.5% gel and stained with Coomassie brilliant blue. Sample loadings in each lanes are as follows: (lane 1–3, from pRB) lane 1, crude; lane 2, starch adsorbed; lane 3, DEAE-cellulose chromatography; lane 4, starch adsorbed from wild type RB; lane 5, BSA; M, molecular weight marker

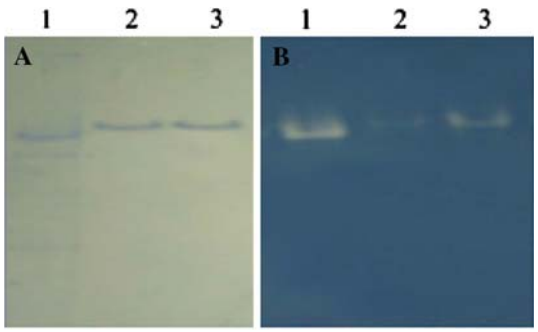

Fig. 2 Native-PAGE of CGTase from pRB. Samples were run on 7.5% gel stained with Coomassie brilliant blue staining (**A**), dextrinizing activity staining (**B**). Lanes in A and B were as follows: lane 1, crude enzyme; lane 2, starch adsorbed; lane 3, DEAE-cellulose column chromatography

neutral pH with higher enzyme purity (higher specific activity).

In addition to the study on cyclodextrin production by CGTase, the coupling reaction which can produce different oligosaccharides from cyclodextrins and some sugars were also investigated. Suitable sugar for coupling activity was selected by determining the coupling activity with βCD as substrate and various sugars as oligosaccharide acceptor. It was found that cellobiose was the best substrate (Table 3). Other sugars produced very low activity except luecrose, a disaccharide synthesized by dextran sucrase [20], which gave moderate activity. Kinetic analysis of the coupling activity of the purified enzymes using various cyclodextrins and cellobiose showed that both enzymes can catalyze the reactions with many cyclodextrins especially the natural occuring CDs. (Fig. 3 and Table 4). In general, K_m for all CDs tested were lower in pRB except α-CD.

The transformant CGTase preferred β-CD and its derivatives as substrates. Since the nucleotide and deduced amino acid sequences of both enzymes were almost 98% similar, the difference in catalytic activity observed might be the results of the difference in post translational modifications and folding of the enzyme molecule caused by the different cell compositions and environment including pH and composition of the culture medium. New oligosaccharides were observed on analysis of the reaction mixture by TLC and HPLC (unpublished data) and the structures of the products will be further analyzed. The distinct preference of pRB enzyme for β-CD and its derivatives in the coupling reaction may provide new synthetic reactions for new oligosaccharide products. In conclusion, the recombinant cells produced more purified CGTase at a shorter time and also showed high potential as an enzyme for production of new oligosaccharides from β-CD.

Table 3 Relative coupling activity of CGTases from RB01 and pRB towards different sugars

Sugar acceptor	Relative activity (%)[a]	
	RB01	pRB
Cellobiose	73.6	100[b]
Leucrose	35.6	48.3
Mellibiose	9.7	10.3
Trehalose	5.6	6.7
Lactose	1.1	2.8
Sucrose	0	0

[a] 10 units of each enzyme was used in the reaction with 17 mM βCD with 20 mM of each sugars at 37°C for 30 min

[b] The highest activity observed at the condition in a) was taken as 100%. Data was average of 3 duplicated experiments

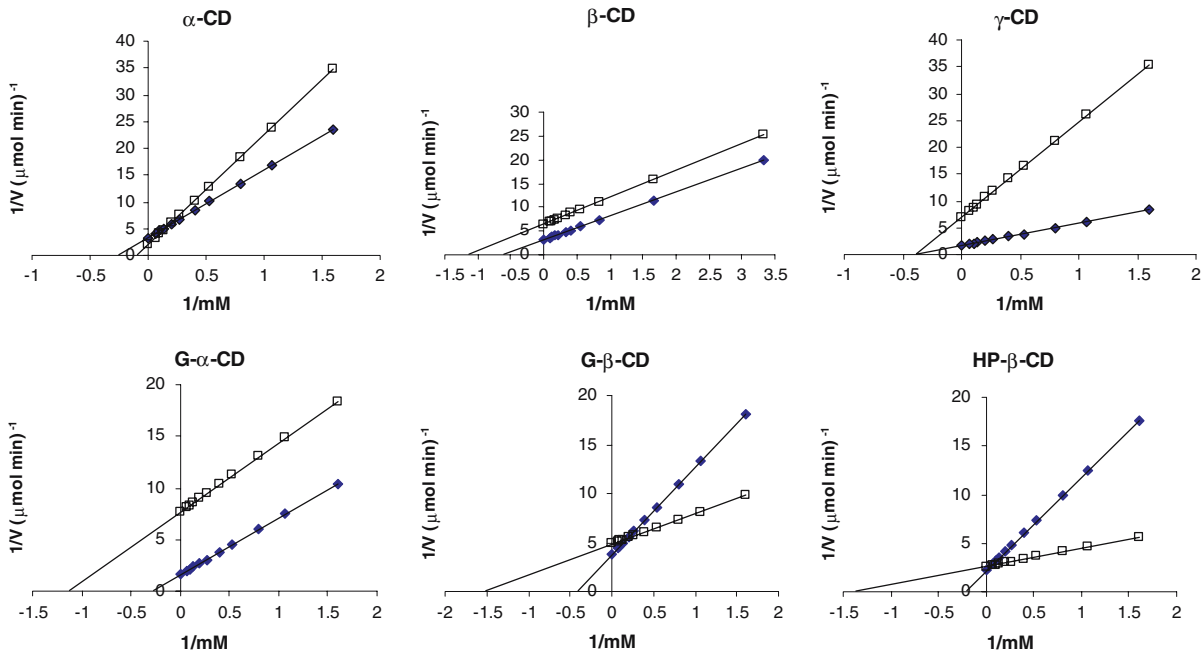

Fig. 3 Lineweaver-Burk plot of CGTase coupling activity with various cyclodextrins and cellobiose. CGTase was incubated with 10 mM cellobiose and various concentrations of each cyclodextrins in 50 mM acetate buffer, pH 6.0 at 40°C for 5 min. 0.2 unit of *Aspergillus niger* glucoamylase was added to convert linearized oligsaccharides to glucose. The amount of CD degraded was monitored by the dinitrosalicylic acid method. (◆) RB01 (□) pRB

Table 4 Kinetic parameters of coupling activity of CGTases from RB01 and pRB with cellobiose and various cyclodextrins as substrates

Substrate	K_m (mM)		V_{max} (mM min^{-1} mg^{-1})		k_{cat} (sec^{-1})		k_{cat}/K_m (mM^{-1} sec^{-1})	
	RB01	pRB	RB01	pRB	RB01	pRB	RB01	pRB
α-Cyclodextrin	3.777 ± 0.196	10.443 ± 2.457	0.300	0.509	0.022	0.037	0.006	0.004
β-Cyclodextrin	1.620 ± 0.134	0.887 ± 0.135	0.322	0.156	0.024	0.012	0.015	0.013
γ-Cyclodextrin	2.574 ± 0.660	2.527 ± 0.160	0.608	0.142	0.004	0.011	0.002	0.004
Glucosyl-α-Cyclodextrin	3.423 ± 0.401	0.876 ± 0.220	0.624	0.131	0.046	0.009	0.013	0.011
Glucosyl-β-Cyclodextrin	2.385 ± 0.231	0.634 ± 0.050	0.265	0.206	0.002	0.015	0.001	0.024
Hydroxypropyl-β-Cyclodextrin	4.445 ± 0.298	0.718 ± 0.044	0.462	0.383	0.034	0.028	0.008	0.039

*Data were from 4 duplicated experiments

Acknowledgements This work was under the financial supports of Ratchadapisek Research Fund granted to Starch and Cyclodextrin Research Unit, Graduate School of Chulalongkorn University and the JSPS-NRCT Core University Program.

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