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<td>Author(s)</td>
<td>Kim, Young-Min; Kiso, Yoshiaki; Muraki, Tomoe; Kang, Min-Sun; Nakai, Hiroyuki; Saburi, Wataru; Lang, Weeranuch; Kang, Hee-Kwon; Okuyama, Masayuki; Mori, Haruhide; Suzuki, Ryuichiro; Funane, Kazumi; Suzuki, Nobuhiro; Momma, Mitsuru; Fujimoto, Zui; Oguma, Tetsuya; Kobayashi, Mikihiko; Kim, Doman; Kimura, Atsuo</td>
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<tr>
<td>Citation</td>
<td>Journal of Biological Chemistry, 287(24), 19927-19935</td>
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<tr>
<td>Issue Date</td>
<td>2012-06-08</td>
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<tr>
<td>Doc URL</td>
<td><a href="http://hdl.handle.net/2115/51765">http://hdl.handle.net/2115/51765</a></td>
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<td>Rights</td>
<td>This research was originally published in Journal of Biological Chemistry. Young-Min Kim; Yoshiaki Kiso; Tomoe Muraki; Min-Sun Kang; Hiroyuki Nakai; Wataru Saburi; Weeranuch Lang; Hee-Kwon Kang; Masayuki Okuyama; Haruhide Mori; Ryuichiro Suzuki; Kazumi Funane; Nobuhiro Suzuki; Mitsuru Momma; Zui Fujimoto; Tetsuya Oguma; Mikihiko Kobayashi; Doman Kim; Atsuo Kimura. Novel Dextranase Catalyzing Cycloisomaltooligosaccharide Formation and Identification of Catalytic Amino Acids and Their Functions Using Chemical Rescue Approach. Journal of Biological Chemistry. 2012; 287:19927-19935. © the American Society for Biochemistry and Molecular Biology.</td>
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<td>JBC287-24_19927-19935.pdf</td>
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Novel dextranase catalyzing cycloisomaltooligosaccharide-formation and identification of catalytic amino acids and their functions using chemical rescue approach* 

Young-Min Kim¹, Yoshiaki Kiso¹, Tomoe Muraki¹, Min-Sun Kang¹, Hiroyuki Nakai¹, Wataru Saburi¹, Weerauch Lang¹, Hee-Kwon Kang¹, Masayuki Okuyama¹, Haruhide Mori¹, Ryuichiro Suzuki², Kazumi Funane², Nobuhiro Suzuki³, Mitsuru Momma³, Zui Fujimoto³, Tetsuya Oguma⁴, Mikihiko Kobayashi⁵, Doman Kim⁶, and Atsuo Kimura¹

¹Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.
²National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba 305-8642, Japan.
³National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba 305-8602, Japan.
⁴Noda Institute for Scientific Research, 399 Noda, Noda 278-0037, Japan.
⁵Department of Food and Health Science, Jissenn Women's University, Hino 191-8510, Japan.
⁶School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea.

*Running title: Novel cyclic sugar-forming dextranase and its catalytic residues

To whom correspondence should be addressed: Atsuo Kimura, Research Faculty of Agriculture, Hokkaido University, Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan; Tel. and Fax: +81-11-706-2808; E-mail: kimura@abs.agr.hokudai.ac.jp

Keywords: dextranase; cycloisomaltooligosaccharide; glycoside hydrolase family 66; catalytic residues

SUMMARY

A novel endo-dextranase (PsDex) from Paenibacillus sp. was found to mainly produce isomaltotetraose and small amounts of cycloisomaltooligosaccharides (CIs) with a degree of polymerization=of 7–14 from dextran. The 1,696 amino-acid sequence belonging to the glycosyl hydrolase family (GH) 66 has a long insertion (632 residues; Thr451–Val1082), a portion of which shares identity (35% at Ala39–Ser1304 of PsDex) with Pro32–Ala755 of CI glucanotransferase (CI-Tase), a GH 66 enzyme that catalyzes the
formation of CIs from dextran. This homologous sequence (Val387–Met932 for PsDex and Tyr404–Tyr492 for CTTase), similar to carbohydrate-binding module 35, was not found in other endo-dextranases (Dexs) devoid of CTTase activity. These results support the classification of GH 66 enzymes into 3 types: (i) Dex showing only dextranolytic activity, (ii) Dex catalyzing hydrolysis with low cyclization activity, and (iii) CTTase showing CI-forming activity with low dextranolytic activity. The fact that a C-terminal truncated enzyme (having Ala39–Ser1304) has 50% wild-type PsDex activity indicates that the C-terminal 392 residues are not involved in hydrolysis. GH 66 enzymes possess 4 conserved acidic residues (Asp189, Asp340, Glu412, and Asp1254 of PsDex) of catalytic candidates. Their amide-mutants decreased activity (1/1500 to 1/40,000-time), and D1254N had 36% activity. A chemical rescue approach was applied to D189A, D340G, and E412Q using α-isomaltotetraosyl fluoride with NaN₃. D340G or E412Q formed a β- or α-isomaltotetraosyl azide, respectively, strongly implicating that Asp340 and Glu412 are nucleophile and acid/base-catalyst, respectively. Interestingly, D189A synthesized small-sized dextran from α-isomaltotetraosyl fluoride in the presence of NaN₃.

Endo-dextranases (EC 3.2.1.11; Dexs) randomly hydrolyze the α-1,6-linkage of dextran (1) and are classified into glycoside hydrolase families (GH) 49 and 66 based on amino-acid sequence similarity (2). Cycloisomaltooligosaccharide glucanotransferase (CTTase) is also classified into the same group of GH 66 and catalyzes the conversion of dextran to cycloisomaltooligosaccharide (CI) by intramolecular transglycosylation (3, 4). CI is an attractive cyclic sugar for the prevention of dental plaque formation due to its strong inhibition of mutansucrase-catalyzed insoluble glucan formation (5). CI with 10 glucose units (CI-10) exhibits stronger inclusion ability than do cyclodextrins (cyclic α-1,4-glucosidic sugars) (6).

GH 66 enzymes are composed of 5 regions from the N- to the C-terminus (7, 8) as follows: N-terminal variable region (N-VR), conserved region (CR), CTTase-specific region (CTT-SR), C-terminal conserved region (C-CR), and the C-terminal variable region (C-VR). All dextranases reported so far are devoid of CTT-SR. CTT-SR, which forms carbohydrate-binding module (CBM) 35, contributed to the CI-formation of CTTase (7). More recently, we resolved the three-dimensional (3D) structure of Dex from Streptococcus mutans (SmDex), which is truncated at N-VR and C-VR (9, 10). Our X-ray studies demonstrated that CR forms a catalytic (β/α)₈ barrel as has been observed in the GH 13, 27, and 31 proteins, indicating that they probably share a common evolutionary origin (11). Our studies also predicted that 3 acidic amino acids at CR of PsDex (Asp189, Asp340, and Glu412; PsDex, Dex from Paenibacillus sp.) are candidates for catalytic residues (9, 10, 12), although the functions of those candidates have yet to be elucidated.

Chemical rescue (ChR) is a reaction that is used to recover the activity of mutant enzyme by an exogenously added organic or inorganic compound that functions instead of the mutated residue. For example, the catalytic residue-mutated glycosylase displays its activity on a fluoride substrate by the addition of an anion (e.g., N₃⁻ or formate), which remains at the position of the altered residue. The glycosylase, mutated at its so-called nucleophile, catalyzes the formation of a product (glycosyl azide) that has an anomeric configuration opposite that of the substrate. The ChR of glycosylases, mutagenized at the acid/base catalyst, forms a product (glycosyl azide) with the same anomeric configuration as that observed in the intact enzyme-catalyzed hydrolytic reaction (13, 14). Therefore, the use of ChR is among the most convenient approaches to identifying catalytic residues functions.

We recently discovered PsDex from
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*Paenibacillus* sp. isolated from soil on the Hokkaido University campus. Interestingly, PsDex produced isomaltotetraose (IG-4) and small amounts of CLs [degree of polymerization (DP) = 7–14; CI-7 to CI-14]. The amino-acid sequence of PsDex harbors CBM 35 at its CTT-SR, permitting the classification of the GH 66 enzymes into 3 types: (i) pure Dex; (ii) Dex with low cyclization activity; and (iii) CTTase with low high cyclization activity and low hydrolytic activity. As mentioned before, the 3D structure analysis of SmDex (9, 10) implicated 3 acidic PsDex residues as candidates for catalytic residues (Asp189, Asp340, and Glu412). However, the function of those 3 residues remains unclear. To address this, we applied the ChR approach to their functional analysis. This study, for the first time, identifies the function of the catalytic residues of GH 66 enzymes.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and plasmids.** *Paenibacillus* sp. was isolated from soil on the Hokkaido University campus (Sapporo, Japan). This bacterium was aerobically incubated in 5 ml of pre-culture medium containing 0.1% dextran T2000 (Amersham Biosciences, Uppsala, Sweden), 1% Bacto peptone (Becton Dickinson and Company, Sparks, MD, USA), 0.01% yeast extract (Becton Dickinson and Company), and 0.05% NaCl (pH 7.0) at 30°C. For the production of PsDex, the pre-cultured *Paenibacillus* sp. was added to a fermenter containing 1,000 ml of medium [3 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 0.25 g of NaCl, 0.5 g of NH₄Cl, 0.5 mg of thiamine HCl, 0.5 mM MgSO₄, 0.05 mM of CaCl₂, 100 mM MES-NaOH buffer (pH 7.0), and 0.5% dextran T2000 per liter] and cultivated aerobically 3 times at 30°C for 20 h. *Escherichia coli* DH5α and *E. coli* BL21 (DE3)-CodonPlus-RIL (Stratagene, La Jolla, CA, USA) were used for the construction of expression plasmids and for the production of recombinant PsDex and its C-terminal-truncated PsDex (Ala39–Ser1304; PsDex-CT), respectively. Plasmids of pBluescript II SK (+) (Stratagene) and pET-23d (Novagen, Darmstadt, Germany) were used for the subcloning of DNA fragments amplified by polymerase chain reaction (PCR). *E. coli* was grown in LB medium [10 g of Bacto tryptone (Becton Dickinson and Company) 5 g of yeast extract (Becton, Dickinson), and 5 g of NaCl in 1 l of H₂O, pH 7.0] containing ampicillin (50 µg/ml).

**Purification of native PsDex.** *Paenibacillus* sp. cells were collected by centrifugation (8,000 x g for 10 min at 4°C), resuspended in 500 ml of 20 mM potassium phosphate buffer (pH 6.5; buffer-A), homogenized by an ice-chilled French press (Ohtake, Tokyo, Japan; 1,350 kg/cm², 3 times), and centrifuged (14,000 x g for 20 min at 4°C) to discard the insoluble components. Supernatants were pooled as crude extract containing 183 U of PsDex with 0.0178 U/mg. Solid ammonium sulfate was slowly added to the crude extract up to 30% saturation, and the turbid solution was maintained at 4°C overnight. The resulting precipitant was collected by centrifugation (12,000 x g for 20 min at 4°C), dissolved in 600 ml of buffer-A, dialyzed against buffer-A, and centrifuged (8,000 x g for 10 min at 4°C) to remove insoluble materials. The supernatant (865 ml, 178 U, 0.0548 U/mg) received a 0.02% final concentration of sodium azide. The solution was applied to a column of DEAE-TOYOPEARL 650M (Tosoh, Tokyo, Japan; 3.1 x 66 cm) equilibrated with buffer-A, followed by elution with a 0–1 M sodium chloride of linear gradient. The active fractions (65.2 U, 0.688 U/mg) were dialyzed against buffer-A containing 1.0 M ammonium sulfate (buffer-B), loaded onto a column of Butyl-TOYOPEARL 650M (Tosoh; 2.2 x 55 cm) equilibrated with buffer-B, and eluted with a linear gradient of 1.0–0 M ammonium sulfate. Active fractions (40.0 U, 8.51 U/mg) were dialyzed against buffer-A containing 0.05 M sodium chloride (buffer-C), concentrated to 3.5 ml, and added to a gel-filtration column using Sepharose 6B (Amersham Biosciences; 2 x 110 cm) equilibrated with buffer-C. The active fractions (17.9 U, 14.3 U/mg) were dialyzed against buffer-A. All purification steps were performed at 4°C.
Protein concentration of the crude extract and ammonium sulfate separation was measured using the Bradford method (15) with bovine serum albumin as a standard. Protein concentration at other purification steps was determined using 13.9 of absorbance at 280 nm obtained by determination of the amino acid contents of the hydrolysate (6 N HCl for 24 h at 110°C; 500 pmol sample) using an Amino Tac JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan). Dextranolytic activity was assayed using the copper bicinchoninate method (16) with glucose as the standard, by estimating the reducing power from 0.4% dextran T2000 in 20 mM sodium acetate (pH 5.5) at 35°C. One unit (U) of hydrolytic activity is defined as the amount of enzyme that released 1 μmol of reducing power per min under the assay conditions.

Electrophoresis. SDS-PAGE was performed using Laemmli’s method (17) with a Mini-Protein III cell apparatus (Bio-Rad, Hercules, CA, USA) and a 10% gel, followed by protein staining with Rapid CBB KANTO (Kanto Kagaku, Tokyo, Japan) or by sugar detection with a PAS kit (Nacalai Tesque, Kyoto, Japan) (18).

Analyses of monosaccharide composition and sugar contents. Lyophilized native PsDex (50 μg) was dissolved in 100 μl of 2 M trifluoroacetic acid containing 0.1 M HCl, heated for 10 h at 100°C, neutralized with 1 M NaOH, and lyophilized. Monosaccharides were separated using high performance anion exchange chromatography equipped with pulsed an amperometric detector (Dionex, Sunnyvale, CA, USA) and CarboPac PA1 column (4 × 250 mm; Dionex), and eluted with 15 mM NaOH at a flow rate of 1.0 ml/min. Carbohydrate contents were measured by Robyt’s micro scale phenol-sulfuric acid method (19) with D-galactose as a standard.

Amino-acid sequence analysis. Purified native PsDex (50 pmol) was directly blotted on a polyvinylidene fluoride membrane using ProSorb (Applied Biosystems, Foster City, CA, USA), followed by sequencing using a model 477A protein sequencer (Applied Biosystems) with a model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems). For the analysis of the internal sequence, Lys-C peptidase (Wako, Tokyo, Japan)-digested peptides were prepared from 4-vinylpyridine-modified native PsDex (2 nmol), isolated using high-pressure liquid chromatography (HPLC) with a C8P-50 column (Asahikasei Asahipak Column, 4.6 × 150 mm; Osaka, Japan) using a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid, and subjected to the aforementioned sequence analysis.

Cloning of PsDex gene from Paenibacillus sp. All PCRs were performed using KOD DNA polymerase or KOD Dash DNA polymerase (Toyobo, Osaka, Japan), and DNA sequence analyses utilized an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The first PCR was performed using 2 degenerate primers: psdexAs (5'-115-GCIAATCAGGAGGAAGCGCA-3'); I, inosine; 115 or 134, counting nucleotide number from ATG of the initial Met; sense primer corresponding to N-terminal sequence of ANQEEKQ) and psdexBa (5'-783-GTGGTTCATTGGTAIGCCAT-761-3'; antisense primer corresponding to Lys-C peptidase-digested peptide of MAYQMN) with genomic DNA as a template, followed by successive PCR using psdexCa (5'-1976-CCIGGGAAAIATCGTTCICAT-1954 -3'; antisense primer corresponding to Lys-C peptidase-digested peptide of QGEAIFPG). Southern blot analysis was performed using a digoxigenin-labeled fragment (the first PCR-product of 669 bp amplified with psdexAs and psdexBa) using DIG-High Prime (Boehringer Mannheim Biochemical, Mannheim, Germany) on XbaI-digested chromosomal DNA, resulting in a positive signal with a 5 kb size. This XbaI-digested 7.7 kb-fragment (1 ng/μl) was subjected to self-ligation and used as a template for inverse PCR with psdexDa (5'-243-ACCGTCCAA TCTCTGGTGGCCCC-221-3', antisense primer) and psdexEs (5'-1837-GACGATTACATGACATTCCA TGTC-1860-3', sense primer), amplifying the 5.5 kb DNA fragment. For amplification of the 3’-region of

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the PsDex gene, a fragment (627 bp) isolated from the NdeI-SacI-digested inverse PCR product was used in the second southern blot analysis using XhoI-digested genomic DNA. A positive 7.5 kb XhoI-fragment was circulated by self-ligation and used as a template for inverse PCR with psdexFa (5'-3014-TACGTAAAGA TCGGCTTTGTTCTC-2990-3'; antisense primer) and psdexGs (5'-3636-CAATGATCCAACTGGAGA AATG-3658-3'; sense primer), amplifying a 6 kb fragment involving a PsDex gene at the 3'-end. Using psdexHs (5'-GGATCTACGGACTCGCTAGG-(-127)-3'; sense primer) and psdexIa (5'-3285-GGCATCCATAAGCGGTACAG-3266-3', antisense primer), the final PCR amplified a DNA fragment of 5.45 kb containing an open reading frame (ORF) of 5,091 bp with a 5'-flanking region of 126 bp and a 3'-flanking region of 230 bp. The 3 obtained clones were completely identical, confirming the absence of a PCR error.

A computer-based sequence analysis to find homologous regions was performed using the BLAST network service (http://www.isb-sib.ch) (20) with the Swiss-Prot/Trembl database. Prediction of a protein signal peptide was done using the Signal P server (http://www.cbs.dtu.dk/services/SignalP/) (21).

**Construction of expression vectors of PsDex and PsDex-CT.** After C761 was replaced by G to remove an inner NcoI site without amino acid substitution, NcoI and NotI sites were introduced to the 5'- and 3'-termini of the ORF, respectively, and the ORF was then introduced to the NcoI-NotI site of pET23d to produce a PsDex protein that lacked the original signal sequence (Met1–Ala38). PCR was performed using psdexRs (5'-TACCATGGAATACAGAAAGAAAGC-133-3'; where the NcoI site is underlined; sense primer) and psdexRa (5'-773-TGATAGCATTGCCCGTGAG CCG-750-3', where the bold-faced letter corresponds to C761-replacement by G antisense primer). The resultant DNA fragment was used as a primer for megaprimer PCR (22) with psdexRCA2 (5'-AAAGCGGCCGCTTGGATCAGATCCAAACAA -5071-3', where the NotI site is underlined, antisense primer). The PCR product and pET-23d were digested by NcoI and NotI (Takara, Kyoto, Japan), followed by connection with a DNA Ligation Kit ver. 2 (Takara) to construct an expression vector to produce PsDex (Ala39-Ala1696, an original Glu1696 replaced by Ala) with His6-tag at its C-terminus.

The DNA harboring PsDex-CT with the His6-tag at its C-terminus was constructed using PCR with psdexCTs (5'-2334-CATCTTCCCTAAA ACACCGGG-2354-3', sense primer) and psdexCTa (5'-3922-CAGCGGCGCCAGATCCATGCCGGGAAT AGC-3894-3', where the NotI site is underlined), replacing the original Pro1305 by Ala. The resultant PCR product and pET23d carrying the PsDex gene were digested by SacI and NotI (Takara) and connected using a DNA Ligation Kit ver. 2.

Eleven kinds of mutations at Asp189, Asp340, Glu412, Asp1254, and Cys1124 of PsDex-CT, having a His6-tag at the C-terminal, was performed by megaprimer PCR (22) using the appropriate primers. The amplified DNA fragment was digested by SacI and NotI, followed by introduction to the corresponding restriction sites of PsDex-CT gene-carrying pET-23d.

**Production and purification of PsDex and PsDex-CT.** PsDex, PsDex-CT, and the mutants of PsDex-CT at Asp189, Asp340, Glu412, Asp1254, and Cys1124 were produced in E. coli transformants carrying the respective expression plasmid. Each transformant was cultured in 1,000 ml of LB medium containing 50 µg/ml ampicillin at 37°C until the absorbance at 600 nm reached approximately 0.5. Protein production was induced with 0.2 mM isopropyl β-thiogalactoside, followed by further incubation with vigorous shaking at 18°C for 20 h. After E. coli cells were disrupted by sonication using a Sonifier 250 (Branson, Danbury, CT, USA), PsDex and PsDex-CT were purified to homogeneity by Ni-chelating chromatography using Chelating Sepharose Fast Flow (Amersham Biosciences). Active fractions were dialyzed against buffer-A and concentrated using a CentriPrep YM-50 apparatus (Millipore, Bedford, MA, USA). The concentration of purified protein was measured using...
12.2 (recombinant PsDex) and 5.54 (PsDex-CT and its 11 mutants) of $A_{\text{max}}^{\text{EOL}}$ at 280 nm determined by the aforementioned analysis of their amino acid compositions.

**Enzyme reaction.** The effects of pH on dextranolytic activity were examined by incubating each enzyme (26.0 nM PsDex and 30.0 nM PsDex-CT) with 0.4% dextran T2000 at 35°C in 16 mM modified Britton-Robinson buffer (pH 2.60-11.4; pH of mixture of acetate, phosphate and glycine was adjusted by NaOH; buffer-D). For pH-stability, PsDex (190 nM) and PsDex-CT (203 nM) were maintained at 4°C for 18 h in 36 mM buffer-D, followed by assay of residual enzyme activity under the aforementioned conditions. For thermal stability, PsDex (25.0 nM) and PsDex-CT (42.2 nM) were maintained at 25–60°C and 20 mM sodium acetate buffer (pH 5.5; Na-AB) for 15 min, and residual activity was then measured under the assay conditions.

PsDex- or PsDex-CT-catalyzing CI-production was performed by incubating 30 nM enzyme with 3% dextran T10 at 35°C and 20 mM Na-AB. The remaining dextran and long oligosaccharides were discarded by the addition of ethanol (2 volumes) to 25 ml of the reaction mixture, forming a precipitate at 4°C for 1 h. The supernatant was concentrated to 2 ml by a vacuum evaporator, reacted with buckwheat $\alpha$-glucosidase (7.6 U/ml) and *Aspergillus niger* $\alpha$-glucosidase (12 U/ml) at 37°C and 20 mM Na-AB for 15 h to digest short linear oligosaccharides, passed through a small column of Amberlite MB-4 (Organo, Tokyo, Japan), and applied to the Sep-PackC18 cartridge (Waters; Milford, MA, USA) followed by a first elution with milli-Q water (5 ml) for washing the remaining short linear oligosaccharides and by second elution with 20% ethanol (5 ml) to recover the CIs. The ethanol fraction was concentrated to 0.4 ml and analyzed by HPLC using a model D-2000 refractive index detector (Hitachi, Tokyo, Japan) using a Shodex RS Pak DC-613 column (6 x 150 mm; Showa Denko, Tokyo, Japan), followed by elution at 60°C with 61% acetonitrile. Molecular masses of the CIs were determined using model JMS-SX102A electrospray ionization-mass spectrometry (JEOL, Tokyo, Japan). $^{13}$C-NMR spectra were recorded using a Bruker AMX-500 Spectrometer at 125 MHz with an external standard of trimethylsilyl propionate.

Gram-level production of IG-4 was performed by reacting recombinant PsDex (1.0 mg) with 3% dextran T2000 (10 g) as a substrate at 20 mM Na-AB for 24 h. Resultant IG-n was concentrated, applied to carbon-Celite column chromatography (23), and eluted with 0–5% isopropanol linear gradient, resulting in about 4.5 g of pure IG-4. Purified IG-4 (100 mg) was converted to $\alpha$-isomaltotetraosyl fluoride (IG4F) (24) for the substrate used in the ChR reaction.

The ChR reaction was performed by the reaction of the mutated PsDex-CT at Asp189, Asp340, or Glu412 (see Table 2 for mutants used) with 6 mM IG4F and salt (0.2–2.4 M NaN$_3$, sodium formate or NaNO$_3$; Table 2) at 35°C and 200 mM Na-AB. Fluoride ions liberated from IG4F were measured by estimating the formation of a lanthanum complex (25). The ChR product was analyzed using thin-layer chromatography (TLC) with a silica-gel plate (60F$_254$; Merck, Darmstadt, Germany) and a solvent of nitromethane/1-propanol/water (4:10:3, v/v; 2-time development), followed by visualization at 110°C for 5 min after dipping of the TLC plate in 5% sulfuric acid in methanol containing 0.03% $\alpha$-naphthol. Purification of 2 ChR-products formed by D340A and E412Q was done using TLC. After development, each product was recovered from the TLC plate. Their structures were analyzed on a model JMS-SX102A fast atom bombardment (FAB)-MS and a model AMX-500 $^1$H-NMR at 500 MHz.

**RESULTS**

**Purification and characterization of PsDex.** A dextran-degrading bacterium was isolated from soil. DSMZ (Braunschweig, Germany) identified this strain as *Paenibacillus* sp. on the basis of the 16S rRNA sequence and cellular fatty acid composition analyses.
Dextranolytic activity at 24 h after cultivation reached 100 U per ml of medium, 3% and 97% of which were found in the supernatant and cell-homogenized fraction, respectively. The cell suspension, which reacted directly with 0.4% dextran T2000 at 35°C and 50 mM sodium acetate buffer (pH 5.5; Na-AB), displayed almost the same activity as that observed with the cell-homogenized fraction, implying that PsDex resided at the cell surface. Microscopic observation confirmed that the cells were not disrupted during incubation with dextran.

PsDex was isolated from the cell-disrupted fraction by 4 purification procedures; the final step entailed gel-filtration and purified a 200 kDa-protein with a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A; lane 1). This protein band was positive for periodic acid-Schiff staining (PAS) (Fig. 1A; lane 2). Both monosaccharide composition and sugar contents analyses confirmed that PsDex was a glycoprotein that had 5.1% D-galactose without glucosamine, galactosamine, or N-acetyl sugars thereof. PsDex was the most active at pH 5.5 and was stable at pH 5.0–9.0 and up to 40°C.

Table 1 summarizes the kinetic parameters of the hydrolytic reaction on IG-n (IG-3 to IG-7) and dextran T2000 with their cleavage patterns. PsDex had extremely low activity on IG-4, producing low amounts of glucose and IG-3 and no activity on IG-2 and IG-3. Isomaltooligosaccharides having a DP value > 5 became favorable substrates, and IG-5 was cleaved to glucose and IG-4. IG-5 was formed from IG-6, IG-7, and dextran T2000, indicating the preference of PsDex for the production of IG-5 at an initial stage of the reaction. As shown in Fig. 2B, PsDex formed IG-4 as a final product with a 53% yield, enabling the preparation of gram quantities of IG-4 from 10 g of dextran T2000.

PsDex gene and reduced primary structure. The PsDex gene was cloned from genomic DNA of *Paenibacillus* sp. by a series of polymerase chain reaction (PCR) and Southern blot procedures. The isolated gene had 2 putative promoters 5'-(−56)-TTAAA-(−52)-3' and 5'-(−29)-TAAAT-(−25)-3', together with a putative ribosome-binding site 5'-(−16)-AGGGAGGA-(−9)-3'. At the 3'-flanking region of 229 bp, no termination loop was found downstream of the stop codon (5'-TGAAT-3'). The deduced amino-acid sequence contained an N-terminal sequence of ANQEEKQSSQQAGLRA-LTVS and many internal sequences (data not shown) of Lys-C peptidase-digested peptides, indicating that a signal peptide was cleaved at the typical processing site between Ala38 and Ala39 (26) and that the isolated gene encoded PsDex protein. A BLAST homology search revealed PsDex to be a GH 66 enzyme family member without obvious similarity to GH 49 enzymes (27–29). Multiple alignments with GH 66 enzymes predicted that PsDex is composed of 5 regions together with signal sequence (Met1–Ala38) as follows: N-VR (Ala39–Ala53), CR (Leu54–Ala450), PsDex-specific region (PsDex-SR; Thr451–Val1082), C-CR (Gly1083–Val1287), and C-VR (Asp1288–Pro1696). PsDex-SR is composed of 3 portions: long insertion-1 (LI-1; Thr451–Glu812), CIT-SR (Ser813–Val946), and long insertion-2 (LI-2; Val947–Val1082). The similar sequences of LI-1 and LI-2 are not found in other GH 66 enzymes including CITase (4) and streptococcal Dexs (30–32). PsDex displays the highest identity with CITase (35%), while streptococcal Dexs display a range of only 25–26%.

Expression and characterization of recombinant PsDex and PsDex-CT. C-VR of PsDex contains a Pro/Ser-rich region (PSRR, Pro1305–Pro1329), followed by 3 sets of putative surface-layer homology domains (SLHDs; Tyr1518–Ala1685; refer to the Discussion section for details). Therefore, we constructed a PSRR/SLHD-truncated PsDex (PsDex-CT carrying Ala39–Ser1304) to elucidate the influence of those regions on catalytic function. Full-size PsDex or PsDex-CT was produced in *Escherichia coli* cells, and enzyme activities in the medium were increased to be 162 U/1,000 ml and 433 U/1,000 ml, which were larger than that of native
PsDex (100 U/1,000 ml) by 1.6-times and 4.3-times, respectively. Each enzyme was purified as a single band protein (Figs. 1A and 1B) with a specific activity of 14.0 U/mg for PsDex and 7.20 U/mg for PsDex-CT. The N-terminal sequence started at Ala37 in each protein, and electrospray ionization-mass spectrometry (ESI-MS) and SDS-PAGE analyses determined the molecular sizes of 186 kDa for PsDex and 143 kDa for PsDex-CT (Figs. 1B and 1C), indicating the absence of proteolytic digestion during production, which occurred in the production of SmDex (8). PsDex and PsDex-CT showed the same pH (stable at pH 5.2–10; optimum at pH 5.5) and temperature (stable at < 37°C) properties, together with the formation of mainly IG-4 from dextran T2000. All these properties were identical to those of native PsDex, allowing us to use those expressed enzymes for further experiments.

Investigation of CI-production. Since the primary structure of PsDex included CTF-SR, which is only found in CTase, we investigated the PsDex-CT-associated CI production from dextran T10 (Fig. 2C). Structures of 7 purified CIs were analyzed using ESI-MS and NMR. The mass signals of [M + Na]+ were 1157.29, 1319.57, 1481.67, 1644.11, 1806.28, 1968.21, 2130.62, and 2292.91, the estimated masses of which were multiples of 162.14, corresponding to a mass of 1 glucosyl unit, indicating that each carbohydrate was a non-reducing sugar with cyclic form. 13C-NMR (Fig. 2F) also supported the PsDex-CT-catalyzed CI formation of CI-7 to CI-14 (3, 6). Prolonged incubation with the enzyme resulted in CI degradation (Fig. 2D), since PsDex-CT had endo-wise dextranolytic activity, which could cleave the CIs with almost equal efficiency. Native and recombinant PsDexs without truncation also catalyzed the same CI production, which was followed by degradation.

Candidates for catalytic residues. Multiple alignments indicated that 4 acidic amino acids (Asp189, Asp340, Glu412 and Asp1254) of PsDex are highly conserved in GH 66 enzymes. Our 3D structure of SmDex (9, 10) indicated that 3 former residues are the most likely candidates for catalytic amino acids. We mutated those acidic amino acids of PsDex-CT, including Asp1254, and then measured their enzyme activities. D1254N displayed 4.3 U/mg (36% of PsDex-CT with 7.20 U/mg); D189N, 0.00481 U/mg; D340N, 0.000689 U/mg; and E412Q, 0.000178 U/mg. The latter mutants lost most of their dextranolytic activities.

ChR was applied to Asp189, Asp340, and Glu412 mutants (Fig. 3). Sodium azide or sodium formate enhanced the fluoride ion-releasing velocity from 6.0 mM α-isomaltotetraosyl fluoride (IG4F) by 1.5-times to 13-times (Table 2). Structures of products formed in the presence of the azide ion (Figs. 3A, 3B, and 4C) were analyzed by 3 approaches, namely, the reducing power test, FAB-MS, and 1H-NMR. D340G and E412Q synthesized the non-reducing sugars that had the same molecular mass (691 Da), while they exhibited different J 12 values of 8.5 Hz and 2.5 Hz in 1H-NMR, respectively, indicating that D340G formed β-isomaltotetraosyl azide and E412Q formed α-isomaltotetraosyl azide. On the other hand, D189A with 0.4 M NaN3 or 0.4 M sodium formate produced IG-4 or/and several IG-n from 6.0 mM IG4F, 20 mM IG-5 and 0.40% dextran T2000 (Figs. 3D and 3E), suggesting that a ChR of D189A catalyzed the hydrolytic reaction. The same products were also observed in the presence of 0.4 M NaN3 (lanes 11, 16, and 21 of Figs. 3D and 3E). Interestingly, the formation of IG-4 from IG4F and dextran T2000 appeared by D189A without any salt (lane 8 of Fig. 3D; lane 18 of Fig. 3E), and those IG-4 productions were markedly enhanced in the presence of salts (lanes 9–11 of Fig. 3D; lanes 19–21 of Fig. 3E).

D189A displayed hydrolysis at its ChR, suggesting that transglycosylation might also occur. D189A was reacted with 30 mM IG4F at a high concentration in the presence of 0.4 M NaN3, and we found the product had a large molecular size, since this sugar stayed at its original position on the thin-layer chromatography (TLC) plate. The addition of ethanol to the reaction mixture resulted in the formation of
turbid material (Fig. 3F). This turbid material was identified as a dextran due to the production of IG-n by SmDex-treatment. Its size was analyzed by estimating DP using reducing power and total sugar, indicating an average DP of about 100. This size can be categorized as a small dextran.

**D189C/C1124Y activity restoration by KI-treatment.**

Asp189 of PsDex-CT was substituted by a Cys residue, followed by KI oxidation. Prior to this mutation, an original and sole Cys1124 of PsDex-CT was replaced with a Tyr residue to construct C1124Y, and the further mutation at Asp189 was then introduced to form D189C/C1124Y. C1124Y maintained the same dextranolytic activity (7.22 U/mg) as observed in PsDex-CT (7.20 U/mg), while D189C/C1124Y decreased its specific activity (0.0768 U/mg) by 1/94-times. An SH-group of D189C/C1124Y was probably converted to a sulfinate moiety by KI-treatment (26). No free Cys residue was confirmed using Ellman’s titration method (33). As shown in Fig. 2E, dextranolytic activity increased and reached its greatest value at 250 mM KI (0.522 U/mg; 6.8-fold higher than the original activity of D189C/C1124Y), followed by reduction by incubation with KI exceeding at more than 250 mM.

**DISCUSSION**

Relationship between structure and function of PsDex. In C-VR of PsDex, are 3 tandem 55 residue-repeated SLHDs (Tyr1518–Thr1560, Phe1578–Ala1620, and Tyr1642–Ala1685), that share great similarity to the N-terminal 3-repeat SLHDs (Phe34–Ala197) of the Bacillus anthracis surface array protein (Fig. 4A). This array protein remains at the cell surface-layer of Gram-positive bacteria by SLHD-associated non-covalent binding to a secondary cell wall carbohydrate in the bacterial cell wall (34).

SLHDs of PsDex seem to be active, since 97% dextranolytic activity of the native enzyme during cultivation was found on the cell surface of Paenibacillus sp. of Gram-positive bacteria. A possible linker of PSRR connects SLHR to C-CR. Both recombinant PsDex and PsDex-CT exhibited properties identical to those of the native enzyme, indicating that the C-terminal PSRR and 3 SLHRs of PsDex do not affect any catalytic functions.

PsDex-SR consists of 3 regions of LI-1 (Ile451–Glu812), CIT-SR (Ser813–Val946), and LI-2 (Val947–Val1082), and CIT-SR possesses a putative CBM 35 (PsDR1 in Fig. 4B). On the other hand, CITase carries 2 CBM 35-similar portions (BeCR1 and BeCR2 in Fig. 4B) (35, 36) at its CIT-SR and C-VR, respectively (7). The BeCR1-homologous region is found at CIT-SR of PsDex, although a region similar to BeCR2 is not found in PsDex. Even though the BeCR2-homologous sequence is missing at PsDex, a putative CBM 6 is observed at C-VR of PsDex [PsDR2 in Fig. 4C, having about 20% homology at β-agarase from Saccharophagus degradans strain 2-40 (EMBL ID: CP000282) (37) and α-agarase from Alteromonas agarilytica (PRF no.: 3319309A) (38)], indicating that PsDex selected CBM 6 during protein evolution. Interestingly, Funane et al. (7) predicted that CBM 35 at CIT-SR (BeCR1) catalytically contributes to CI production of CITase. This prediction prompted us to study CI production using PsDex and SmDex with and without CIT-SR, respectively. Obviously, both PsDex and PsDex-CT formed CI-7 to CI-14 from dextran (Fig. 2C), while SmDex did not display the production of any cyclic sugars, implying that PsDex-carrying CIT-SR might contribute to the production of CIs such as CITase (7). Since PsDR2-defective PsDex-CT produced CIs, the PsDR2 that was similar to CBM 6 did not contribute to cyclic sugar formation. These findings allow us to classify GH 66 members into 3 groups: (i) type 1-enzyme only catalyzing dextranolytic reaction (e.g., SmDex); (ii) type 2-enzyme exhibiting both hydrolytic and CI-forming activity (e.g., PsDex); and (iii) type 3-enzyme exhibiting CI-producing activity with low dextranolytic activity (e.g., CITase). Our attempts to find more type 2-enzymes among newly categorized GH 66 members are currently underway.

Interestingly, PsDex is a glycoprotein with
only galactose units. Many N-glycosylated and/or O-glycosylated proteins have been discovered among approximately 25 kinds of bacterial genera (39). To our knowledge, PsDex is the first glycoprotein in the genus *Paenibacillus* that contains only galactose. The O-glycosylation at the Tyr residue has been reported on an S-layer protein from *Paenibacillus alvei*, which has a polysaccharide composed of Glc, Gal, ManNAc, and Rha (40). The galactose residue in PsDex is not considered to be involved in dextranolytic activity, since the enzyme activity is available in the *E. coli*-expressing wild-type PsDex. This host lacks the glycosylation system.  

**Catalytic residues and their functions.** This study determined, for the first time, the catalytic residues of GH 66 enzyme and their functions. The 3 findings of (i) point mutation approaches at Asp189, Asp340, and Glu412 (Table 2), (ii) ChR reaction using mutated enzymes (Table 2, Fig. 3), and (iii) our 3D structure analysis (9, 10) are evidence that Asp340 and Glu412 are catalytic residues. Reaction products derived from ChR (Figs. 3A, 3B, and 3C) clearly revealed that Asp340 is a so-called catalytic nucleophile and Glu412 is an acid/base catalyst by virtue of the formation of β-isomaltotetraosyl azide and α-isomaltotetraosyl azide, respectively.

Asp189 exhibited the interesting ChR phenomena involving catalysis of the hydrolytic reaction (Figs. 3D and 3E), meaning that Asp189 mutants display the ordinal ChR reaction simply by occupying the Asp189 position with an external anion of $\text{N}_3^-$, formate, or $\text{NO}_3^-$. We thought that an Asp189-mutated enzyme (D189A) would catalyze the transglycosylation on IG4F, since D189A becomes a simple hydrolase in the presence of an anion. Surprisingly, a dextran-type polysaccharide was formed (Fig. 3F), indicating that D189A catalyzed a “sequential ChR reaction”; this, to our knowledge, is a novel discovery in the endo-lytic hydrolase reaction reacting on α-glucan. The reaction mechanism of sequential ChR still cannot be explained clearly, but the produced short IG-n was used for further D189A-mediated ChR. A negative charge is essential for the position of Asp189 since D189C/C1124Y enhanced its dextranolytic activity by KI-treatment (Fig. 2E), which oxidizes the SH-group of C1124 to form a possible anionic sulfinate (26).
REFERENCES
Novel cyclic sugar-forming dextranase and its catalytic residues


FOOTNOTES

*This work was partially supported by the Grants-in-aids from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN). The nucleotide sequence reported in this paper has been submitted to the GenBank under accession number JN935295.
**To whom correspondence should be addressed: Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan. Tel: 81-11-706-2808; Fax: 81-11-706-2808; E-mail: kimura@abs.agr.hokudai.ac.jp.
Abbreviations used: CBM, carbohydrate-binding module; C-CR, C-terminal conserved region; CI, cycloisomaltooligosaccharide with DP (degree of polymerization) = 7–14 (CI-7 to CI-14); CITase, cycloisomaltooligosaccharide glucanotransferase; CIT-SR, CITase-specific region; ChR, chemical rescue.
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CR, conserved region; C-VR, C-terminal variable region; Dex, endo-dextranase; DP, degree of polymerization; GH, glycosyl hydrolase family; HPLC, high-pressure liquid chromatography; IG-n, isomaltooligosaccharide having DP of n; IG4F, α-isomaltotetraosyl fluoride; LI-1 or LI-2, long insertion-1 or 2, respectively; Na-AB, sodium acetate buffer (pH 5.5); N-VR, N-terminal variable region; ORF, open reading frame; PAS, periodic acid-Schiff staining; PCR, polymerase chain reaction; PSRR, Pro/Ser-rich region; PsDex, endo-dextranase from *Paenibacillus* sp.; PsDex-CT, C-terminal truncated PsDex; PsDex-SR, PsDex-specific region; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLHD, surface-layer homology domain; SmDex, Dex from *Streptococcus mutans*; TLC, thin-layer chromatography; 3D, three-dimensional.

Table 1. Kinetic parameters for IG-n and dextran T2000 by PsDex and products with their levels.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_0$ (sec$^{-1}$)</th>
<th>$k_0/K_m$ (μM/sec)</th>
<th>Products with levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IG-2, IG-3, IG-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No or weak activity</td>
</tr>
<tr>
<td>IG-5</td>
<td>0.414</td>
<td>4.36</td>
<td>10.5</td>
<td>+++*, Glc/IG-4</td>
</tr>
<tr>
<td>IG-6</td>
<td>0.386</td>
<td>7.53</td>
<td>19.5</td>
<td>+++*, Glc/IG-5; ++, IG-2/IG-4</td>
</tr>
<tr>
<td>IG-7</td>
<td>0.288</td>
<td>7.93</td>
<td>27.5</td>
<td>+++*, IG-2/IG-5; ++, IG-3/IG-4; +, Glc/IG-6</td>
</tr>
<tr>
<td>Dextran T2000</td>
<td>0.000183</td>
<td>44.3</td>
<td>242000</td>
<td>+++*, IG-5/IG-4; +, &gt;IG-6/&lt;IG-3</td>
</tr>
</tbody>
</table>

* +++*, ++, and + indicate strong, moderate, and weak production levels, respectively, tested by TLC.

Table 2. Chemical rescue of mutants constructed from PsDex-CT*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Salt added</th>
<th>$\nu$ (μmol/min/mg)</th>
<th>Ratio (-fold of $\nu$ without salts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D189G</td>
<td>0</td>
<td>0.050</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.2 M NaN$_3$</td>
<td>0.16</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2.4 M Sodium formate</td>
<td>0.55</td>
<td>11</td>
</tr>
<tr>
<td>D189A</td>
<td>0</td>
<td>0.17</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.4 M NaN$_3$</td>
<td>1.54</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>2.4 M Sodium formate</td>
<td>1.49</td>
<td>8.2</td>
</tr>
<tr>
<td>D189N</td>
<td>0</td>
<td>0.12</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Novel cyclic sugar-forming dextranase and its catalytic residues

<table>
<thead>
<tr>
<th></th>
<th>0.2 M NaN₃</th>
<th>2.4 M Sodium formate</th>
<th>0.4 M NaN₃</th>
<th>2.4 M Sodium formate</th>
<th>0.4 M NaN₃</th>
<th>2.4 M Sodium formate</th>
<th>0.4 M NaN₃</th>
<th>2.4 M Sodium formate</th>
<th>0.4 M NaN₃</th>
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</tr>
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<tbody>
<tr>
<td>D340G</td>
<td>0.19</td>
<td>1.6</td>
<td>0.18</td>
<td>1.5</td>
<td>0.00016</td>
<td>1.0</td>
<td>0.0027</td>
<td>1.7</td>
<td>0.0065</td>
<td>4.0</td>
</tr>
<tr>
<td>D340A</td>
<td>0.18</td>
<td>1.5</td>
<td>0.0030</td>
<td>1.0</td>
<td>0.0019</td>
<td>6.3</td>
<td>0.0038</td>
<td>12.6</td>
<td>0.0032</td>
<td>4.4</td>
</tr>
<tr>
<td>E412G</td>
<td>0.00072</td>
<td>1.0</td>
<td>0.0038</td>
<td>5.3</td>
<td>0.0038</td>
<td>5.3</td>
<td>0.0032</td>
<td>4.4</td>
<td>0.0032</td>
<td>5.1</td>
</tr>
<tr>
<td>E412A</td>
<td>0.00052</td>
<td>6.3</td>
<td>0.0032</td>
<td>5.1</td>
<td>0.0042</td>
<td>5.1</td>
<td>0.00082</td>
<td>1.0</td>
<td>0.0065</td>
<td>1.0</td>
</tr>
<tr>
<td>E412Q</td>
<td>0.00072</td>
<td>11</td>
<td>0.0032</td>
<td>5.1</td>
<td>0.0072</td>
<td>11</td>
<td>0.0065</td>
<td>9.8</td>
<td>0.0064</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*ChR was evaluated by the determination of F' from 6.0 mM IG4F, where the v for PsDex-CT was 4.63 μmol/min/mg.

FIGURE LEGENDS

FIGURE 1.  SDS-PAGE of purified native PsDex (A), recombinant PsDex (B), and PsDex-CT (C). Lane M, size markers; lanes 1, 3, 4, protein staining; lane 2, detection of glycoprotein using PAS.

FIGURE 2.  Linear sugar products (B) or cyclic sugar products (D, F) from dextran, and activity restoration of D189C/C1124Y by KI-treatment (E). A and B: Dextran T2000 (1%, w/v) was incubated with PsDex (14 μg/100 ml of reaction mixture) at 20 mM Na-AB and 35°C for 24 h. Products were analyzed by high-pressure liquid chromatography (HPLC) using a Shodex RS pack DC-16 column with elution of 55% acetonitrile at 60°C. Panel-A shows standards of glucose (peak 1) and IG-2 to IG-7 (peaks 2–7, respectively). C, D, and F: HPLC profiles at reaction times of 6 h (C) and 24 h (D). ¹³C-NMR recorded signals (F) of C1 to C6 of glucosyl units of C1 formed by PsDex-CT (signals from a produced CI-8). E: D189C/C1124Y (10 μg/0.1 ml) was treated with various concentrations of KI at 35°C and 100 mM sodium phosphate buffer (pH 7.0) for 24 h, followed by measurement of dextranolytic activity under assay conditions.
**FIGURE 3.** ChR catalyzed by mutated PsDex-CT. A, D340A-catalyzed ChR: lanes 1 and 2, reaction time of 0 h and 24 h, respectively. B, Purified product observed in lane 2: Std, standard sugars of glucose and IG-2 to IG-7; lane 3, product purified by HPLC. C, ChR catalyzed by Glu412-mutants: lanes 4–6, ChRs by E412G/Q/A, respectively. D and E, D189A-catalyzed ChR: IG4F, IG-5 and dextran T2000 were used for substrates in lanes 7–11, 12–16, and 17–21, respectively; substrate with NaN3 was spotted to lanes 7, 12, and 17; substrate with D189A was spotted to lanes 8, 13, and 18; substrate with D189A and NaN3 was spotted to lanes 9, 14, and 19; substrate with D189A and sodium formate was spotted to lane 10, 15, and 20; substrate with D189A and NaNO3 was spotted to lanes 11, 16, and 21. F, Polysaccharide-formation by D189A-catalyzed ChR: Cont, without D189A; R, with D189A.

**FIGURE 4.** Multiple alignments of SLHDs of PsDex and *Bacillus anthracis* surface array protein (A), CBM 35 (B), and CBM 6 (C). A, SLHD: Upper and lower 3 amino-acid sequences, C-VR of PsDex and N-terminal of *Bacillus anthracis* surface array protein, respectively; white letters with black background, identical residues at more than 5 sequences; letters with gray background, identical residues at more than 2 sequences of PsDex or surface array protein; region with square, LTRAE-motif of SLHD. B, CBM 35: PsDR1, this study; BcCR1 and BcCR2, 2 sequences of CITase from *B. circulans* T-3040 (GenBank ID: AB073929) (7); BgCts, 6-glucosyltransferase CBM 35 from *Bacillus globisporus* (AB073929) (35); AoGac, exo-β-D-glucosaminidase from *Amycolatopsis orientalis* (AY962188) (36); white letters with black background, identical residues at 3 sequences. C, CBM 6: PsDR2, this study; SdAga, β-agarase from *Saccharophagus degradans* strain 2-40 (EMBL ID: CP000282) (37); AaAga, α-agarase from *Alteromonas agarilytica* (PRF no.: 3319309A) (38); white letters with black background, identical residues at all sequences; letters with gray background, identical residues at 2 sequences, including PsDR2.
Novel cyclic sugar-forming dextranase and its catalytic residues

Fig. 1.
Novel cyclic sugar-forming dextranase and its catalytic residues

Fig. 2.

Retention time (min)

Specific activity (U/mg)

Retention time (min)

ppm
Novel cyclic sugar-forming dextranase and its catalytic residues

Fig. 3.

A

B

C

D

E

F

Product
IG4F
IG-4

1 2

Stn 3

4 5 6

Stn 7 8 9 10 11

Glc
IG2
IG3
IG4
IG5
IG6
IG7

Cont R

Glc
IG2
IG3
IG4
IG5
IG6
IG7

Product
IG4F
Novel cyclic sugar-forming dextranase and its catalytic residues

Fig. 4.

A

1518: YAVGTP-THAYEAIASSLSSKYVTGCMGNDKELQGQT ATRAO QFT :1560
1578: FTDIPA-NAGYAKEVAAYAYEVGLVQKSDGKEDENGS VTRQE MM:1620
1642: YAFAISIAADAEAVMQASASYQCRRGNANGSFAEVLIL TSKAB LA:1685

34: FPQVPA-DHNGIDSINYLVEKCAVGKNDKGMFEFGKE LTRAB AA:76
95: FADSQ--GGTTTFAAIAEVAKGVKGT-NGEBNGK LTRVS MM:135
156: FKELETL-NQGKEKANILVEISVGTG-DQWIEKKT VTKAB LA:197

B

PsDR1: 837 VTTGVPVFVSAYASDFNNSDRIEVEHVQA--LTAGSYDGVWIKRNKAPGAT
BcCR1: 404 YTGS-----GFVQFQASTGQKVSAINA--PEAGKXSVREVYGNNT-GAN
BcCR2: 736 YTGS-----GFVQGFSSTNQSVSFVVK---TASDEYALKRLRYANG--GSD
BgCts: 883 YTGT-----GFVQGLG---NSAGVTFYPKVKTGDYNVSRYANA---SGT
AoGac: 736 YTGT-----GFYK-YN-DVAGSSVEWTVTVPSAGYDVVVRYANG--TTT

PsDR1: TVTRSVYY-GQDGIPVSSLAPTANSAGAEI----LQGVBEFLCCGNOITIKM 931
BcCR1: STLNLVYQGVFQK-LYFF--NQSSNGTSDKDAWYQVPLCQGHTVELRY 492
BcCR2: ATRD-YVVDEKLQG-TVSEK--STGWSTSTSYGE-ITARLEGHHTIVLVQ 817
BgCts: AKSVSIIFVNGKRVK-STSLA---NLANWTTG-TQSETLPLTACVNVTVKY 970
AoGac: SRPLDFSVNCISASVGAE---STGTRNT Pt-TRTVTIAACVNKIKAVA 970

C

PsDR2: 1344 ENGQKVTIALNTEITNV-RIPLNAGELCTNDVLNFE---LSLRW
SdAga: 59 VNQATNAYNVN-ADYTDYQINVAT--HCYNNVQYAICTSVASGAI
AaAga: 56 VNQGQNFINVFAGADYDVNYNLG---GEYDIEYFQGTGVSNGPI

PsDR2: TAAAL-KEELNKTGDAGAYLDDLARSTKDPQLLSKLPGLMLIQNYL 1434
SdAga: ELLQV-NGSSNESQGOTNVPHWDSFOPLNASHEV1LPACKTVEHNLVYG 150
AaAga: EVLVDVNGT-WSQGSGVAVPYSWDDFQLPTSHTVLPQSTVRLIA 146