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Biochemical Characterization of Alkaliphilic Cyclodextran Glucanotransferase from an Alkaliphilic Bacterium, *Paenibacillus daejeonensis*

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Cycloisomaltooligosaccharide glucanotransferase (CITase) was isolated from alkaliphilic *Paenibacillus daejeonensis* via an amino acid homology search for the reported CITase. The recombinant alkaliphilic CITase (PDCITase) from *P. daejeonensis* was expressed in an *Escherichia coli* expression system and purified as a single protein band of 111 kDa. PDCITase showed optimum activity at pH 8.0 and retained 100% of activity within a broad pH range (7.0–11.5) after 18 h, indicating alkaliphilic or alkalistable CITase properties. In addition, PDCITase produced CI-7 to CI-17, CI-18, and CI-19, which are relatively large cycloisomalto-oligosaccharides yet to be reported. Therefore, these large cycloisomaltooligosaccharides can be applied to the improvement of water solubility of pharmaceutical biomaterials.

Keywords: Cyclodextran, cycloisomaltooligosaccharide glucanotransferase, *Paenibacillus daejeonensis*, glycoside hydrolase family 66

Introduction

Cycloisomaltooligosaccharide (CI), also known as cyclodextran, is a cyclic oligosaccharide with a glucose moiety similar to that of cyclodextrin (CD), a cyclic α-1,4 oligosaccharide whereas CIs are linked by α-1,6-glucosidic bonds. CIs consist of 7–17 glucose units (CI-7 to CI-17) of α-1,6-linked glucose [1–3]. CIs are produced from dextran via intramolecular transglucosylation catalyzed by cycloisomaltooligosaccharide glucanotransferase (CITase; E.C. 2.4.1.248). CIs, especially small ones, CI-7 to CI-9, strongly inhibit glucansucrase activity in mutant streptococci [4], suggesting that CIs can be employed for the prevention of dental caries. Furthermore, CIs have 100-fold higher solubility in aqueous solutions than CDs do. Due to the structural nature of the α-1,6 linkage, it has a wider molecular diameter than that of the α-1,4 linkage, and a CI can encapsulate a guest molecule [2]. Therefore, CIs have attracted attention as potential solubilization reagents for functional materials that cannot be encapsulated by CDs. Among the reported CIs, relatively large cycloisomaltodecaose (CI-10) has a remarkable inclusion ability as compared with CDs and other CIs, and they have shown high stability according to Victoria blue B staining [2]. Thus, CIs may serve as a novel encapsulation agent for water-insoluble pharmaceutical biomaterials.

To date, three CI-producing bacteria, *Paenibacillus agaridevorans* T-3040 (formerly *Bacillus circulans* T-3040; CITase-T3040), *Paenibacillus* sp. 598K (CITase-598K), and *B. circulans* U-155 (CITase-U155), have been reported [1, 3, 5–7]. CITase catalyzes intramolecular transglucosylation of dextran [6]. Furthermore, the three reported enzymes have optimal pH around 6.0 and produce CI-7 to CI-17 [3]. Recently, *B. agaridevorans* T-3040 and *Paenibacillus* sp. 598K were shown to also produce CIs synergistically by means of 6-α-glucosyltransferase (6-GT) and CITase from starch

[5, 8, 9]. In particular, 6-GT catalyzes a reaction forming long isomaltooligosaccharides (α-1,6-glucosidic linkage from starch) and maltooligosaccharides (α-1,4-glucosidic linkage) with CITase and continually generates CIs from the produced isomaltooligosaccharides [9]. Thus, CIs are produced from various substrates such as dextran and α-1,6 glucan by CITase, and from starch and α-1,4 glucan via cocatalysis by 6-GT and CITase.

Although some CITases have been previously reported, various studies are needed to characterize and improve the function of these enzymes from bacterial sources. Recently, we discovered that an alkaliphilic bacterium, *Paenibacillus daejeonensis*, has a gene encoding a CITase-like enzyme according to the amino acid similarity with the CITase from *B. agaridevorans* T-3040 [9]. Besides, CIs were confirmed to be present in dextran-containing culture broth of *P. daejeonensis* [9].

Therefore, the purpose of this study was to describe the cloning and characterization of one of the putative CITase genes, *pdcit*, belonging to glycoside hydrolase family 66 from *P. daejeonensis*. The novel alkaliphilic CITase from *P. daejeonensis* was found to produce large CIs via high catalytic activity in a higher pH range relative to other CITases.

Materials and Methods

Materials

Standard CIs such as CI-7, CI-8, and CI-9 were kindly provided by Professor Funane Kazumi at Yamanashi University in Japan. *Escherichia coli* DH5α and *E. coli* Rosetta (DE3) cells were used for cloning and overexpression of the recombinant enzyme, respectively. They were aerobically grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates.

Cloning of Putative *cit* Genes from *P. daejeonensis*

The genomic DNA of *P. daejeonensis* (No. 3745, Korea Collection for Type Cultures, South Korea) was extracted using the genomic DNA extraction kit. A PDCITase-coding gene, *pdcit*, of *P. daejeonensis* was amplified by polymerase chain reaction by means of Pfu DNA polymerase (Enzynomics, South Korea) with genomic DNA and a primer pair (forward primer, 5′-AGCCATATG*GCTAGC* ATGGCAATCCAAAAGAAAGCC-3′ and reverse primer, 5′-TGC GGCCGC*AAGCTT*TCAATCTATTTCAATTTCAAAATCCG-3′) containing NheI and HindIII sites (italicized), respectively. The amplified gene was digested with NheI and HindIII and was inserted at the corresponding sites of pET28a(+) (Novagen, USA) encoding a C-terminal His-tag. DNA sequencing analysis was performed by Solgent Inc. (South Korea). Purified pET28a-*pdcit* was then transfected into *E. coli* Rosetta (DE3) for protein expression.

Expression and Purification of Recombinant CITase

E. coli Rosetta (DE3) carrying the constructed plasmid, pET28a-*pdcit*, was grown in LB broth containing 50 μg/ml kanamycin at 37°C to absorbance of 0.5 at 600 nm, and the recombinant PDCITase was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 21 h at 18°C. After the cells were collected by centrifugation at 10,000 ×*g*, they were resuspended in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl (buffer A). The resuspended cells were sonicated and centrifuged at 12,000 ×*g* for 30 min at 4°C, and the supernatant was loaded on a Ni^{2+}-chelating Sepharose column equilibrated with buffer A. The column was washed with buffer A containing 5 mM imidazole, and the sample protein was eluted with buffer A containing 200 mM imidazole. The protein was concentrated using an Amicon Ultra with molecular weight cutoff 5,000 (Millipore, Billerica, USA). Protein concentration was determined by absorbance at 280 nm during purification and was measured by the Bradford method [10].

An Enzyme Assay

An enzyme solution was added to 2% (w/v) dextran T2000 (molecular weight 200,000) in 40 mM Britton–Robinson buffer (pH 8.0). The reaction mixtures were incubated at 30°C for 2 h, and the reactions were stopped by boiling for 10 min. To each mixture, 100 mU of *Streptococcus mutans* dextran glucosidase (DexB) [11] and *Bacteroides thetaiotaomicron* α-glucosidase (SusB) [12] in 40 mM Na-acetate buffer (pH 6.0) were added to digest linear oligosaccharides to glucose at 37°C for 6 h. The reactions were stopped by boiling for 10 min, and each sample was mixed with an equal volume of acetonitrile. CITase activity was determined by measuring the amounts of CIs [the sum of the amounts of CI-7, CI-8, and CI-9 produced from 2% (w/v) dextran T2000] by HPLC on a TSK gel Amide-80 column (4.6 × 250 mm; Tosoh, Japan) [3]. The mobile phase was acetonitrile–water (55:45 (v/v)), and the flow rate was 1 ml/min. CIs were detected with a Refractive Index Detector (RID-20A, Shimadzu, Japan). One unit of CITase activity was defined as the amount of the enzyme producing 1 μmol of CIs (the sum of CI-7, CI-8, and CI-9) per minute [3].

Biochemical Characterization of CITase

To determine the effect of pH, PDCITase was incubated at 30°C in 100 mM Britton–Robinson buffer (pH 3.0–11.0) [13] with 2% (w/v) dextran T2000. For determination of pH stability, the enzyme was kept at 4°C for 18 h in 100 mM Britton–Robinson buffer (pH 2.0–11.5), and the residual PDCITase activity was examined. To determine optimal temperature, the enzyme was incubated at 15–60°C for 1 h in 40 mM Britton-Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000. For evaluation of thermal stability, the enzyme was kept at 20–60°C for 18 h in 40 mM Britton-Robinson buffer (pH 8.0), and the residual enzymatic activity was examined at 30°C. For determining the effects of various cations on the reaction velocity of PDCITase, reactions were carried out in the presence of each cation (10 mM) at optimal pH and temperature. To determine kinetic constants, the initial

velocity (v) was measured by means of various concentrations (20, 32, 40, 60, 80, 160, 240, 320, and 400 μM) of dextran T2000 in 40 mM Britton-Robinson buffer (pH 8.0) at 30°C.

Product Analysis

Ethanol 66% (v/v) was added to the PDCITase reaction mixture to remove the dextran. After precipitation, the remaining dextran was hydrolyzed to isomaltooligosaccharides to form glucose by DexB [11] and SusB [12, 14]. The reaction mixture was next treated with 2 M NaOH at 100°C for 30 min to remove the reducing sugar. After the reaction products were passed through Amberlite MB3 (Merck, Germany) to remove ionic impurities, the reaction products were concentrated in a vacuum evaporator (EYELA, Japan) for further analysis. The molecular weight of products was analyzed by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) on an ABI 4800 Plus TOF-TOF mass spectrometer (Applied Biosystems, USA). A sample was dissolved in 2.5 μl of the sample solution [50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid], and 1 μl of the sample was dissolved in the matrix [0.06 g α-cyano-4-hydroxycinnamic acid, 50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid] [15].

Results and Discussion

Gene Cloning, Expression, and Purification of CITase (PDCITase) from *P. daejeonensis*

The gene was found to be 2,898 bp long and to encode a putative CITase from the alkaliphilic bacterium *P. daejeonensis* with the same sequence as that reported in NCBI (Accession No. WP_020619177) and was expressed in *E. coli*. The primary structure of PDCITase turned out to share high sequence identity, 65%, 69%, and 89%, with CITase-T3040, CITase-U155, and CITase-598K, respectively [1, 3, 5–7], all of which belong to the glycoside hydrolase family 66 [16]. The recombinant enzyme was purified, with a final purification yield of 9.4% and specific activity of 42 mU/mg. Overall, 6.4 mg of active protein was obtained from 1.5 L of culture broth. The final purified enzyme yielded a single band with a molecular mass of approximately 111 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1), in agreement with the calculated value of 107,809 Da based on the amino acid sequence plus six histidine residues at the carboxy terminus.

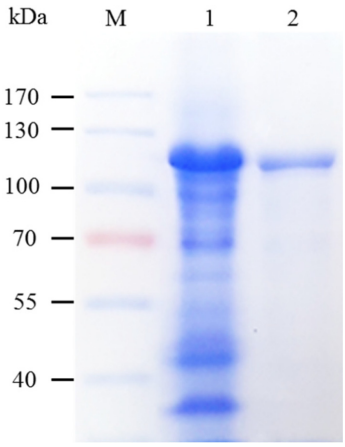

Fig. 1. Purification of recombinant PDCITase.
SDS-PAGE of purified proteins in an 8% (w/v) gel. Purified proteins (12 μg) were loaded onto a polyacrylamide gel, separated by electrophoresis, and visualized with Coomassie Brilliant blue staining. Lane M, size markers; lane 1, PDCITase cell-free extract; lane 2, purified PDCITase.

Effects of Temperature and pH on Activity and Stability

The purified PDCITase was examined for temperature and pH effects. PDCITase had optimum temperature at 45°C and retained 80% of activity at 40°C after 18 h. The observed thermal stability at 40°C was the same as or somewhat lower compared with CITase-T3040 at 40°C, CITase-598K at 50°C, or CITase-U155 at 50°C (Figs. 2A and 2B). The optimum pH for PDCITase activity was found to be 8.0, and the enzyme was stable across a broad pH range (7.0–11.5) after incubation at 4°C for 18 h (Fig. 2C). The optimum pH is comparable to that of CITase-598K (pH 5.5–8.0) and CITase-T3040 (pH 5.5–8.0). CITase-598K is 10°C more thermally stable (≤50°C) than PDCITase or

Table 1. A comparison of enzymatic properties of CITases from *B. circulans* U155, *P.* sp. 598K, *B. circulans* T-3040, and *P. daejeonensis*.

Property	CITase-U155	CITase-598K	CITase-T3040	PDCITase
DP of CI	-	CI-7 to CI-17	CI-7 to CI-17	CI-7 to CI-19
Main product	CI-7	CI-7	CI-8	CI-7
Optimum pH	6.0	5.5–8.0	5.5–8.0	8.0
pH stability	-	5.0–9.0	5.0–9.0	7.0–11.5
# of amino acid residues	934	932	934	965
Reference	7	5	2	This study

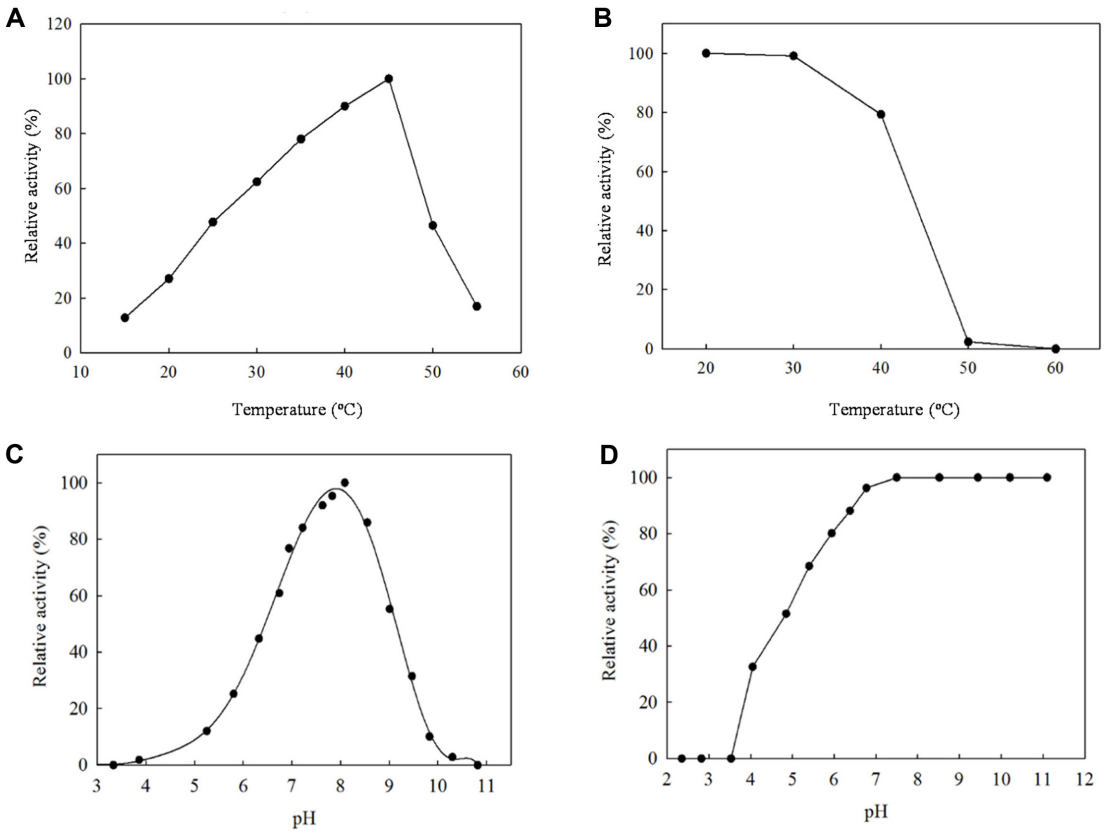

Fig. 2. Optimum temperature, **A**, thermal stability, **B**, optimum pH for activity, **C**, and pH stability, **D**, of PDCITase.
To examine the effect of temperature, PDCITase activity was measured at 15–60°C in 40 mM Britton-Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000. To evaluate the temperature stability of PDCITase, it was kept at 20–60°C for 18 h in 40 mM Britton–Robinson buffer (pH 8.0), and residual enzymatic activity was measured at 30°C in 40 mM Britton-Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000. To examine the effect of pH, enzymatic activity was measured at 30°C in 40 mM Britton-Robinson buffer (pH 3.0–11.0) with 2% (w/v) dextran T2000. To assess the pH stability of PDCITase, it was kept at 4°C for 18 h in 100 mM Britton-Robinson buffer (pH 2.0–11.5), and residual activity was measured at 30°C in 40 mM Britton-Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000. PDCITase was kept at 20–60°C for 18 h in 40 mM Britton-Robinson buffer (pH 8.0). The residual enzymatic activity was measured at 30°C in 40 mM Britton-Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000.

CITase-T3040 (≤40°C). Nonetheless, the pH stability of PDCITase (pH 7.0–11.5) has a broader range than that of CITase-598K (pH 5.0–9.0) or CITase-T3040 (pH 5.0–9.0) [5]. This feature is likely due to the characteristics of the alkaliphilic parent strains whose growth is stimulated in an alkaline environment from pH 8.0 to 12.0 [17]. Therefore, PDCITase had the higher optimum pH area than other CITases. This stability in a broad pH range needs to be further elucidated on the basis of the three-dimensional structure of PDCITase. Additionally, the broad pH stability of PDCITase and other CITases may be useful in industrial processes involving alkaline conditions.

Effects of Metal Ions on Enzymatic Activity

CITase-T3040 is activated and stabilized by the presence of Ca^{2+} owing to a metal-binding site [18, 19]. Enzymatic activity of PDCITase increased by 107%, 117%, and 123% in the presence of Mg^{2+}, K^{+}, and Na^{+}, respectively, but there was 74% and 54% of residual activity in the presence of Mn^{2+} and Co^{2+}, respectively. Nonetheless, the activity of the enzyme was strongly inhibited in the presence of such metal ions as Cu^{2+} (data not shown). PDCITase did not manifest activation by Ca^{2+} ion addition (activity ~98%) compared to the 130% increase in the activity of CITase-T3040 by Ca^{2+} [18]. In the three-dimensional structure of CITase-T3040, there are two Ca^{2+}-binding sites in the CBM35 structure. PDCITase contains the same amino acids (*e.g.*, Glu418, Glu420, Thr437, Gly440, and Asp543) as CITase-T3040 does in the highly conserved first Ca^{2+}-binding site [19]. Nevertheless, the second Ca^{2+}-binding

Table 2. Kinetic parameters of CITases toward dextran.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	Reference
CITase-T3040	0.18	3.2	17.8	5
CITase-598K	0.18	5.8	32.2	5
PDCITase	0.02	1.3	67.4	This study

site was found to be not strictly conserved between CITase-T3040 and PDCITase. The lesser effect of Ca^{2+} on PDCITase suggests that Ca^{2+} is more tightly bound to CITase-T3040 as compared to PDCITase or CITase-598K [5, 19]. This loose binding of PDCITase or CITase-598K to Ca^{2+} ions may influence the patterns of production of CIs.

Enzymatic Kinetics of PDCITase

Kinetic parameters of PDCITase toward dextran T2000 were determined (Table 2 and Fig. 3), and v denotes the rate of production of CIs (the sum of CI-7, CI-8, and CI-9). The enzymes turned out to have similar k_{cat} values toward dextran (3.2 for CITase-T3040, 5.8 for CITase-598K, and 1.3 for PDCITase), but K_m of PDCITase is 9-fold lower than those of CITase-T3040 and CITase-598K [5]. Therefore, the second-order rate constant, k_{cat}/K_m of PDCITase (67.4 $s^{-1}\cdot mM^{-1}$) is approximately 2.1- and 3.7-fold higher than those of CITase-598K (32.2 $s^{-1}\cdot mM^{-1}$) or CITase-T3030 (17.8 $s^{-1}\cdot mM^{-1}$), respectively [5].

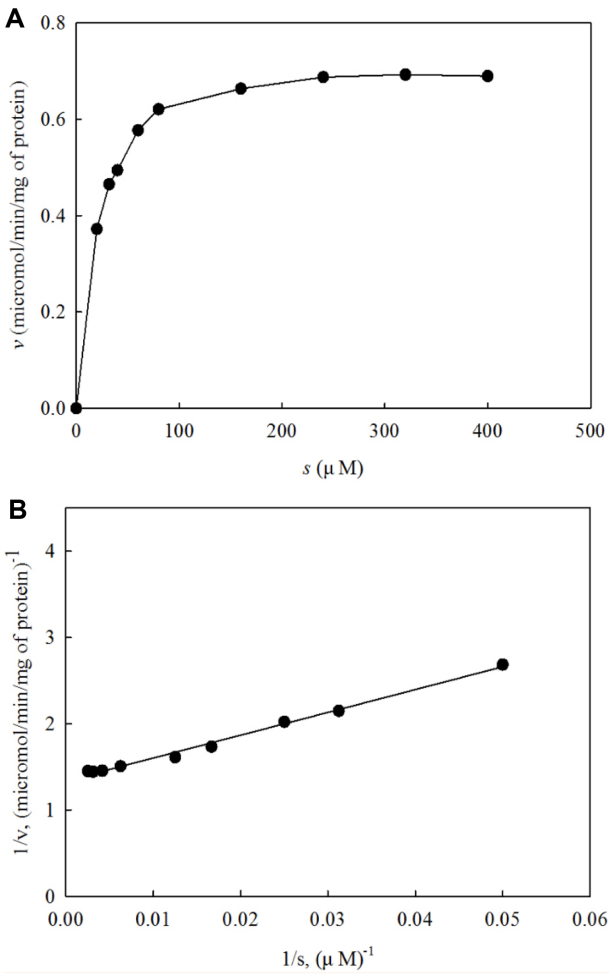

Fig. 3. A Michaelis–Menten plot, **A**, and a Lineweaver–Burk plot, **B**, of the PDCITase reaction with dextran T2000.

CI Production Patterns and Amino Acid Sequence Similarity

CI-7 to CI-19 were produced by PDCITase, with CI-7 to CI-9 being the main products (Fig. 4), and MALDI-TOF/TOF MS analysis revealed that PDCITase produces CI-7 to CI-19 (Fig. 5). These CIs are likely to be novel large CIs. This finding points to the necessity of further research on the production and utilization of the high polymerization degree of CI. PDCITase was found to share high primary-sequence identity, namely, 66% with CITase-T3040, 71% with CITase-U155 (71%), and 89% with CITase-598K.

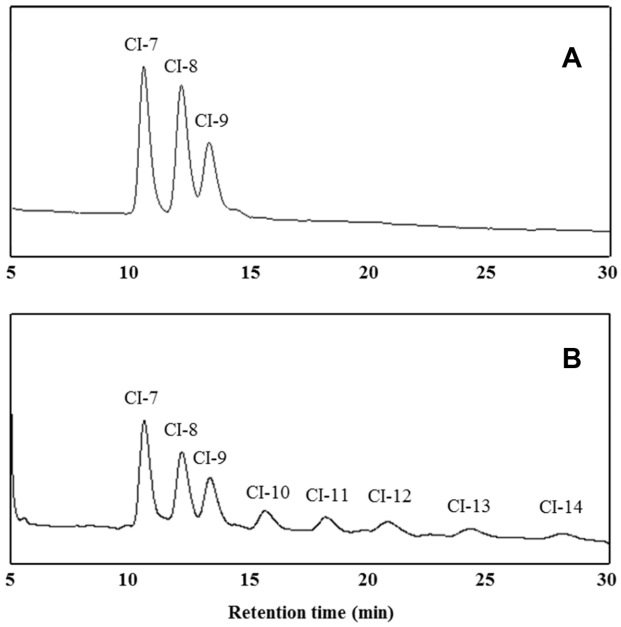

Fig. 4. HPLC analysis of CIs produced by PDCITase.
PDCITase was incubated in 40 mM Britton–Robinson buffer (pH 8.0) with 2% (w/v) dextran T2000 at 30°C for 2 h, and the enzymatic reaction was stopped by boiling for 10 min. The reaction mixture was treated with DexB and SusB to digest linear oligosaccharides and the remaining dextran to glucose. The produced CIs were quantified on a TSKgel Amide-80 column by a previously described method [3]. **A**, CI standards; **B**, PDCITase with dextran T2000. Standard CIs (CI-7, CI-8, or CI-9) were kindly provided by Professor Funane Kazumi.

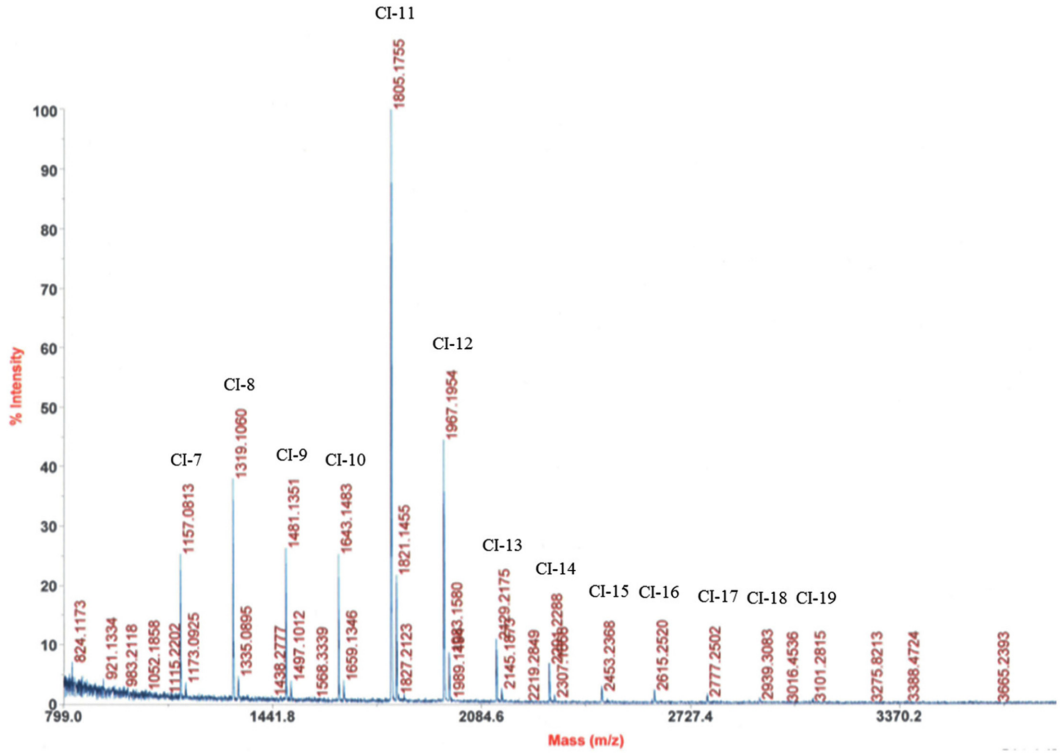

Fig. 5. A MALDI-TOF/TOF MS spectrum of CIs produced by PDCITase.

CITase-T3040 mainly produces CI-8 [18], whereas CITase-598K and CITase-U155 mainly produce CI-7 [5]. The amino acid sequence domains of CITase-T3040 were identified as the N-terminal Conserved Region (Ser1–Gly403), CITase-Specific Insertion (R1, Tyr404–Tyr492), C-terminal Conserved Region (R2, Glu493–Ser596), C-terminal Conserved Region (R3, Gly597–Met700), and C-terminal Variable Region (R4, Lys701–Ser934) [18]. It has been reported that the presence of R1 contributes to the preference for CI-8 production [5]. It is believed that this product pattern is related to the amino acid similarity of the R1 region. In a comparison of the R1 sequence similarities, PDCITase showed 59% similarity with CITase-T3040. By contrast, PDCITase is 89% similar to CITase-derived CITase-598K. These results indicated that PDCITase shares greater similarities with CITase-598K, including CI production patterns and a high amino acid similarity (over 80%). Nonetheless, PDCITase has high optimum pH and pH stability under alkaline conditions. The amino acid sequence differences between PDCITase and CITase-598K involve the R1 region, which contributes to the production of large CIs (from CI-18 to CI-19) under alkaline conditions.

In conclusion, the previously uncharacterized PDCITase from *P. daejeonensis* was expressed in an *E. coli* system and its biochemical properties were characterized. CITase exerts CITase activity toward dextran T2000 in a broad pH range and produces relatively large CIs: from CI-7 to CI-19. Regarding CIs, this study is the first to report CI-18 and CI-19. The high activity at relatively high pH and broad ranged pH stability suggest that this enzyme may be useful in the industrial production of CIs at high pH area without contaminating bacteria. Furthermore, directed evolution experiments aimed at increasing the thermostability of PDCITase are currently underway with the goal of maximizing the utility of the enzyme for CI production.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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