

# Carboxy-Terminal Region of a Thermostable CITase from *Thermoanaerobacter thermocopriae* Has the Ability to Produce Long Isomaltooligosaccharides

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Received: October 13, 2019  
Revised: November 24, 2019  
Accepted: November 26, 2019

First published online:  
December 2, 2019

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pISSN 1017-7825, eISSN 1738-8872

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Isomaltooligosaccharides (IMOs) have good prebiotic effects, and long IMOs (LIMOs) with a degree of polymerization (DP) of 7 or above show improved effects. However, they are not yet commercially available, and require costly enzymes and processes for production. The N-terminal region of the thermostable *Thermoanaerobacter thermocopriae* cycloisomaltooligosaccharide glucanotransferase (TtCITase) shows cyclic isomaltooligosaccharide (CI)-producing activity owing to a catalytic domain of glycoside hydrolase (GH) family 66 and carbohydrate-binding module (CBM) 35. In the present study, we elucidated the activity of the C-terminal region of TtCITase (TtCITase-C; Met740–Phe1,559), including a CBM35-like region and the GH family 15 domain. The domain was successfully cloned, expressed, and purified as a single protein with a molecular mass of 115 kDa. TtCITase-C exhibited optimal activity at 40°C and pH 5.5, and retained 100% activity at pH 5.5 after 18-h incubation. TtCITase-C synthesized  $\alpha$ -1,6 glucosyl products with over seven degrees of polymerization (DP) by an  $\alpha$ -1,6 glucosyl transfer reaction from maltopentaose, isomaltopentaose, or commercialized maltodextrins as substrates. These results indicate that TtCITase-C could be used for the production of  $\alpha$ -1,6 glucosyl oligosaccharides with over DP7 (LIMOs) in a more cost-effective manner, without requiring cyclodextran.

**Keywords:** Cycloisomaltooligosaccharide glucanotransferase, cyclodextran, long isomaltooligosaccharides, *Thermoanaerobacter thermocopriae*

## Introduction

Prebiotics are non-digestible carbohydrates that are beneficial for the intestinal microbial balance in the human gut [1]. Among the various oligosaccharides, isomaltooligosaccharides (IMOs) comprising 40%  $\alpha$ -1,6-glucosidic linkages have shown promising prebiotic effects [2]. Commercialized IMOs with a degree of polarization (DP) ranging from 2 to 6 and lower numbers of  $\alpha$ -1,6-glucosidic linkages were produced from corn starch by  $\alpha$ -amylase and transglucosidase enzymes [3]. Dextranase has

also been used to synthesize pure IMOs via the hydrolysis of dextran with  $\alpha$ -1,6-glucan linkages, and longer IMOs with DP2–DP10 can be produced from sucrose-only substrates through the combined enzymatic action of dextranase and dextransucrase [4]. Furthermore, dextran dextrinase (DDase), belonging to the glycoside hydrolase (GH) family 15 isolated from *Gluconobacter oxydans*, was able to produce dextran with a high DP by the successive transfer of  $\alpha$ -1,6-glucosyl units from maltodextrins [5]. Long IMOs (LIMOs) with DP7 or higher are resistant to hydrolysis by human glycosidases, and are therefore

absorbed at much lower amounts and are retained in the colon for relatively longer periods [6, 7]. Moreover, LIMOs have been shown to promote quercetin-3-glucoside in the lumen [8, 9] and to improve the production of tumor necrosis factor- $\alpha$  in primary macrophages via Toll-like receptor 4 signaling with pro-inflammatory effects [9]. However, it has been a challenge to manufacture IMOs with DP2–DP4 for producing these potentially beneficial LIMOs above DP7.

Cycloisomaltooligosaccharide glucanotransferase (CITase, E.C. 2.4.1.248) is an enzyme belonging to GH family 66 that produces cyclic isomaltooligosaccharides (CIs). CIs are polymers of D-glucose units that are linked via  $\alpha$ -1,6 linkages with a DP of 7–17 through intra-molecular cyclization from dextran,  $\alpha$ -1,6-glucan [10–12]. CIs have been reported to exhibit encapsulating and anti-cariogenic activities [13], demonstrating their potential as alternatives for cyclomalto-dextrin with limitations in low water solubility. CITase was first reported in *Bacillus circulans* T-3040 (CITase-T3040) [12]. Since then, four kinds of CITases, CITase-598K from *Paenibacillus* sp. 598K [14], CITase-U155 from *Bacillus circulans* U-155 [15], PDCITase from *Paenibacillus daejeonensis* [16], and TtCITase from *Thermoanaerobacter thermocopriae* [17], have been investigated for their biochemical properties. Among them, CITase-598K [14] and PDCITase showed high activity at an alkaline pH, and TtCITase showed higher thermal stability than the others [17]. Recently, CIs from *B. circulans* T-3040 and *Paenibacillus* sp. 598K were produced from starch by co-catalysis with 6- $\alpha$ -glucosyltransferase (6-GT) and CITase [18, 19]. At present, CIs are typically produced from dextran by CITase alone or from an  $\alpha$ -1,4 glucan starch via cooperation between 6-GT and CITase [19].

We previously reported the characterization of a thermostable CITase from *T. thermocopriae*, and showed that the recombinant C-terminal region (Met740–Phe1,559) of TtCITase (TtCITase-C) could hydrolyze maltodextrin with  $\alpha$ -1,4- or  $\alpha$ -1,6-glucosidic linkages, and simultaneously transfer the glucosyl groups via  $\alpha$ -1,6-glucosidic linkage formation [17]. Based on these findings, we hypothesized that a LIMO with over DP7 could be produced from starch or commercially available maltodextrins by the enzymatic activity of TtCITase-C. This would be a highly economical approach for IMO production, as the typical substrates dextran and sucrose are more expensive than starch. Therefore, co-catalysis by the N-terminal region of TtCITase (from Met1 to Val876), comprising GH family 66 and carbohydrate-binding module (CBM) 35 with CITase activity, and the C-terminal region of TtCITase can be a

cost-effective approach to produce CIs from starch or maltodextrin without requiring any dextran.

To validate this possibility, in this study, we performed multiple sequence alignment analysis to predict the function of the C-terminal region of GH family 15, and determined the biochemical properties of TtCITase-C. Further, we analyzed the optimal pH, temperature, stability, and products formed from  $\alpha$ -1,4/ $\alpha$ -1,6-glucosidic-linked substrates.

## Materials and Methods

### Materials

*Escherichia coli* strains DH5 $\alpha$  and BL21 (DE3) were used for cloning and over-expression of TtCITase-C with the plasmid pGEX-6p-1 (GE Healthcare, USA). *E. coli* strains were cultured in Luria-Bertani (LB) medium (Difco, BD Biosciences, USA) supplemented with ampicillin (100  $\mu$ g/ml) at 37°C. Standards of maltooligosaccharides (MOs; maltose, M2; maltotriose, M3; maltotetraose, M4; maltopentaose, M5) and IMOs (isomaltose, IG2; isomaltotriose, IG3; panose, P; isomaltotetraose, IG4; isomaltopentaose, IG5) were purchased from Megazyme Co. (Copenhagen, Denmark), Carbosynth (Compton, UK), or TCI (Tokyo Chemical Industry, Japan). All other chemicals used in this study were of analytical reagent-grade and purchased from Sigma-Aldrich Chemical Co. (USA).

### Amino Acid Sequence Analysis

The TtCITase coding gene sequence (WP\_0289926961) with CITase activity was obtained from the National Center for Biotechnology Information (NCBI) database. The putative protein sequence (amino acids 1-1,559) was predicted using BLAST, an algorithm for comparing primary biological sequence information, including amino acid sequences, available online at the NCBI database (<http://blast.ncbi.nlm.nih.gov>) and Carbohydrate Active Enzyme information available at the CAZy database (<http://www.cazy.org>). Multiple sequence alignment of selected amino acids with reported enzyme activity from the GH family 66 and 15 domains, and the truncated C-terminal region of TtCITase (TtCITase-C) were carried out using CLUSTAL X 2.1.

### Cloning of the Partial CITase Gene from *T. thermocopriae*

The TtCITase-C coding gene was amplified by PCR from the genomic DNA of *T. thermocopriae* (RIKEN Bio-resource Center Gene Engineering Division, Japan). The gene was amplified with the following primer pair: forward primer, 5'-GGATCCCCG GAATTCATGATTTATGTAAAGCGTACGATAACCAC-3' and reverse primer, 5'-ATGCGGCCGCTCGAGTAAAAATCAGGT AATCGTAGATCAAACC-3' (underlines indicate the restriction enzyme sites EcoRI and XhoI, respectively). The amplified gene was cloned in *E. coli* via the pGEX-6p-1 vector with an N-terminal GST-tag, and confirmed by DNA sequencing analysis at Bionics Inc. (Korea).

### Protein Expression and Purification of Recombinant TtCITase-C

*E. coli* BL21 (DE3) harboring the TtCITase-C expression vector was grown in LB medium supplemented with 100 µg/ml ampicillin (Georgiachem, USA) until reaching an optical density of 0.5–0.8 at 600 nm. The *E. coli* cells were then induced by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (Sigma-Aldrich Chemical Co.) at 18°C for 18 h. The induced cells were harvested by centrifugation at 6,120 ×g for 10 min and resuspended in lysis buffer (20 mM sodium phosphate buffer pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM EDTA, or 1 mg/ml lysozyme). The cells were disrupted by sonication (SONICS, 10 s, 40% amplification) at 4°C and the cellular debris was removed by centrifugation at 16,200 ×g for 10 min. After centrifugation, the crude lysate was passed through a Glutathione Sepharose 4 Fast Flow affinity column (GE Healthcare). The column was washed with 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. Subsequently, the recombinant TtCITase-C was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM reduced glutathione. The protein concentration was determined according to the Bradford method with bovine serum albumin as the standard [20]. The final purified recombinant protein was resolved using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; SMOBIO Technology Inc., Taipei).

### Enzyme Assay

The optimum pH and temperature of TtCITase-C activity were determined by measuring the release of para-nitrophenol (*p*NP) from 6 mM para-nitrophenyl-α-glucopyranoside (*p*NPG) as the substrate [21]. The optimum pH was determined by measuring the enzyme activity at 40°C in 120 mM Britton-Robinson buffer (B-R buffer, pH 2.0–9.0). The optimum temperature was determined in 80 mM B-R buffer (pH 5.5) at different temperatures (30–80°C). The temperature stability was analyzed by incubating 0.5 µg/ml of the purified enzyme for 1, 3, 6, 12, or 24 h. To determine the pH stability of TtCITase-C, the purified enzyme was kept in 100 mM B-R buffer (pH 2.0–9.0) at 4°C for 18 h, and the residual activity of enzymes from different test conditions at 40°C for 15 min in 120 mM B-R buffer with 6 mM *p*NPG was compared.

### High-Performance Anion-Exchange Chromatography-Amperometric Detection (HPAEC-PAD) Analysis

The reaction mixtures containing 5 mM maltopentaose (M5) and isomaltopentaose (IG5) in 120 mM B-R buffer (pH 5.0) with an equal volume of purified TtCITase-C were incubated at 40°C for 6 h and analyzed using an HPAEC system (ICS 5000 Dionex chromatography system, Thermo Fisher Scientific, USA) fitted with a CarboPac PA200 column (4 × 250 mm) and PA 200 pre-column (4 × 50 mm), and enabled with amperometric detection (HPAEC-PAD). The system was created using a PEEK tube (0.24 mm i.d.), gradient mixer (2 mm), ED amperometry cell with a 0.25-µl channel volume, pH-Ag/AgCl reference electrode, 0.002 in the gasket, and gold electrodes. NaOH (100 mM) and gradient sodium acetate (NaOAc) were used as the solvent for separating

and analyzing constituent saccharides. The NaOAc concentration was gradually increased from 0 to 150 mM during 10–40 min of the total running time (50 min). The column temperature was maintained at 40°C, the injection volume was 10 µl, and the flow rate was 0.5 µl/min. N<sub>2</sub> gas was used as the stabilizing gas to inhibit the carbonation of the solvent, and molecular-grade distilled water (Merck Millipore, Germany) was used for the analysis. To examine the reaction pattern of the enzyme, products formed from the substrate (5 mM isomaltotriose) during the time course of the reaction were determined. To obtain more accurate data of the α-1,6 glucanotransferase activity, the reaction products obtained using 10% (w/v) maltose and 10% (w/v) dextrin (D.E. 13.0–17.0) with TtCITase-C were compared during 16 h.

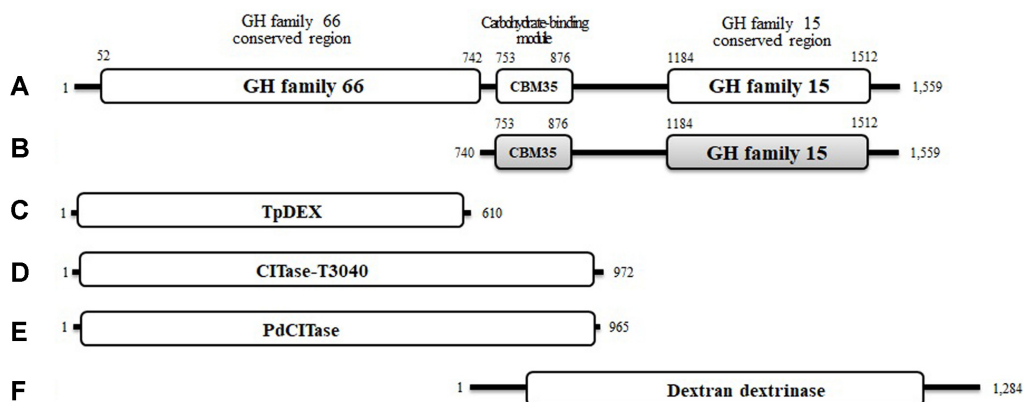
### Matrix-Assisted Laser Deionization-Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF-MS) Analysis

The molecular mass of the reaction products of TtCITase-C with M5 and IG5 was measured by MALDI/TOF-MS. The samples were dissolved in a 2,5-dihydroxybenzoic acid (DHB) matrix composed of a 7:3 (v/v) solution of DHB in 0.1% (v/v) trifluoroacetic acid/acetonitrile (1:1, v/v) and 10 mM NaCl. The sample was mixed with the matrix solution directly on the MALDI target and was vacuum-dried. MALD-TOF mass spectra were recorded and high-resolution MS data were obtained on a Bruker Ultra flexIII TOF/TOF-MS spectrometer. The data were analyzed with Flex Control 3.0 (Bruker Daltonics, USA). MS data were analyzed in positive reflection mode and spectra were acquired using a reflection mirror. The ion source voltage was 25.00 kV and the detectable *m/z* range was 100–7,000.

## Results and Discussion

### Multiple Sequence Alignment of the *T. thermocopriae* CITase Gene

Multiple sequences of CITases were aligned and analyzed to predict the enzymatic function of the N- or C-terminal region of TtCITase using the protein BLAST (BLASTp) search. BLASTp analysis revealed that the N-terminal region (from Met1 to Val876) of TtCITase had high amino acid sequence homology (53.9–56.8%) with four previously reported CITases (CITase-T3040, CITase-598K, CITase-U155, and PDCITase). Moreover, TtCITase showed high amino acid sequence homology (36%) with thermostable dextranase (TpDex), a putative dextranase from *Thermoanaerobacter pseudethanolicus* belonging to GH family 66. This was an expected result given that these CITases originated from the same genus; moreover, CITase and endodextranase both belong to GH family 66. The CBM35 in the N-terminal region of TtCITase is important for CITase activity [22]. When compared with the other CITases, TtCITase can be used to produce relatively large CIs at



**Fig. 1.** Schematic diagram of the carbohydrate-binding protein catalytic domain of cyclodextrin glucanotransferases (CITases).

Aligned amino acid sequences were derived from (A) thermostable CITase of *Thermoanaerobacter thermocopriae* (TtCITase; GH family 66, CBM35, and GH family 15), (B) the carboxy-terminal of thermostable CITase from *T. thermocopriae* (TtCITase-C; CBM35 and GH family 15), (C) thermostable dextranase from *Thermoanaerobacter pseudethanolicus* (TpDEX, GH family 66), (D) CITase from *Bacillus circulans* T-3040 (CITase-T3040, GH family 66), (E) CITase from *Paenibacillus daejeonensis* (PDCITase, GH family 66), and (F) dextran dextrinase from *Gluconobacter oxydans* (GoDDase, GH family 15).

high temperatures [17]. Interestingly, the 819 amino acid C-terminal region (from Met740 to Phe1,559) of TtCITase without CBM35 (TtCITase-C) showed high amino acid sequence homology (31%) with the DDase from *G. oxydans* of GH family 15 (Fig. 1). These results suggested that TtCITase may have dual functions, with the N-terminal region acting like other members of GH family 66 and the C-terminal region acting like the DDase of GH family 15 linked by CBM35.

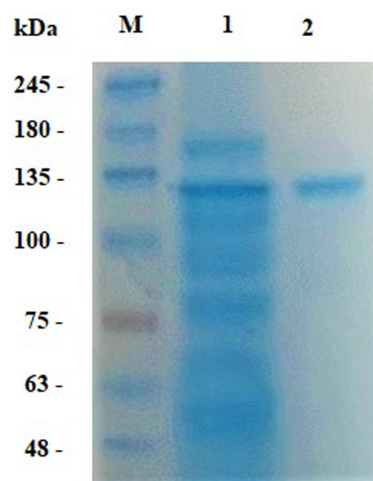
### Cloning and Expression of TtCITase-C

TtCITase is composed of CITase, a CBM35-like region, and a DDase-like region. We previously reported the functional properties of thermostable CITase [8]. However, when we cloned the C-terminal region alone, no enzymatic activity was detected (data not shown). Therefore, we cloned and stably expressed the partial gene sequence of TtCITase-C (Met740–Phe1,559 of TtCITase) encoding the C-terminal region that included the CBM 35-like region (Fig. 2). The purified TtCITase-C showed a high yield (24%) and specific activity (0.024 U/mg), and a molecular weight was 115 kDa as determined by SDS-PAGE analysis (Fig. 2).

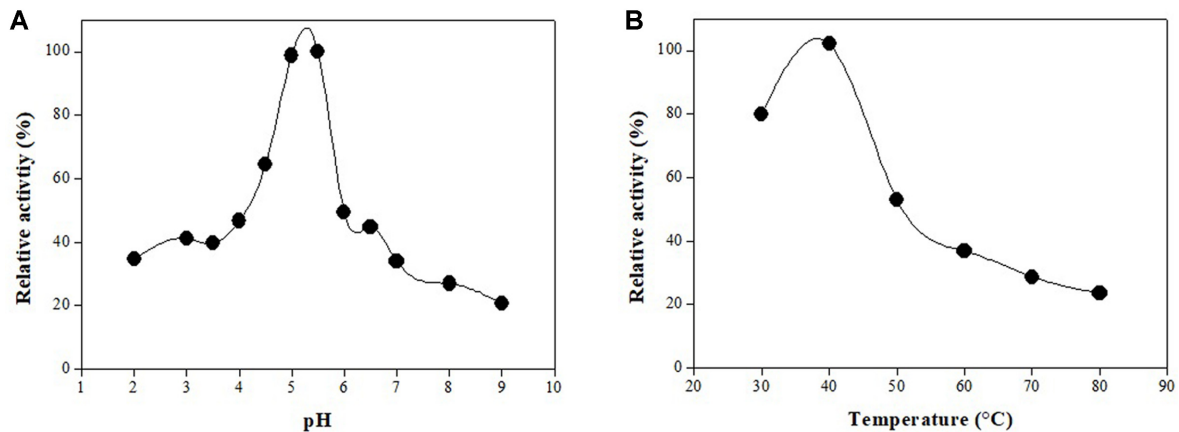
### Effects of pH and Temperature on TtCITase-C Activity and Stability

Next, we determined the effects of different pH and temperatures on the activity of purified TtCITase-C. TtCITase-C exhibited the highest activity at pH 5.5 (Fig. 3A),

and the enzyme was stable within only a narrow pH range (pH 5.0–5.5). The activity of TtCITase-C dropped to 74% at pH 7.0, 50% at pH 6.5, and was lower than 10% at pH 8.0 (Fig. 4A). Thus, the optimal pH for TtCITase-C activity differs from that reported for other CITases, including TtCITase (pH 6.0), CITase-T3040 (pH 5.5–8.0), and PDCITase (pH 8.0) [11, 13, 23]. The optimum temperature for TtCITase-C activity was found to be 40°C with 100% enzyme activity observed at 30°C and 40°C for 24 h; however, the activity



**Fig. 2.** SDS-PAGE gel of the expression of purified TtCITase-C. Lane M, protein molecular weight marker (48, 63, 75, 100, 135, 180, or 245 kDa); Lane 1, cell lysate supernatant of *E. coli* cells expressing TtCITase-C; Lane 2, purified TtCITase-C by a GST affinity column.



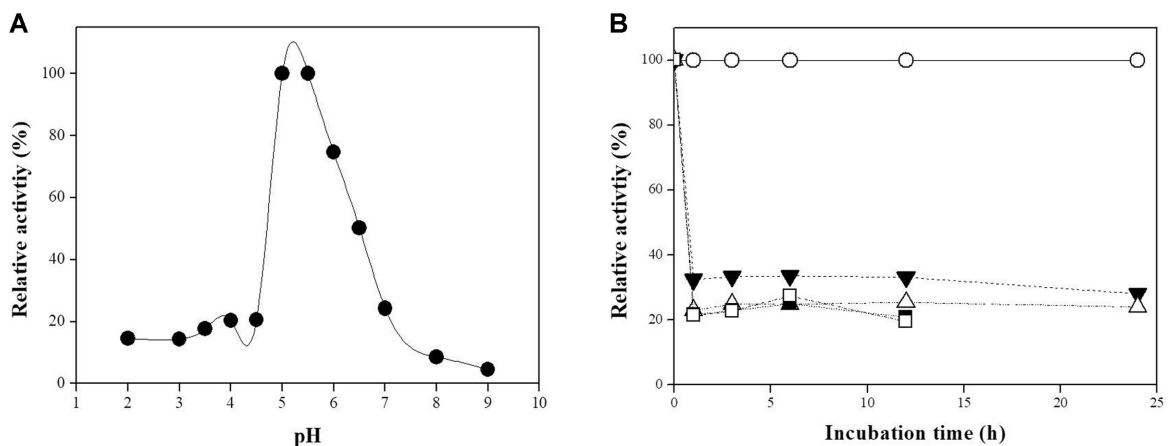
**Fig. 3.** Optimum pH (A) and temperature (B) for TtCITase-C activity.

sharply decreased over 50°C (Figs. 3B and 4B). These results were similar to those reported for TtCITase (60°C), CITase-T3040 (40°C), and PDCITase (40°C). The significant homology observed between the full sequence of TtCITase (53.9–56.8%) and these various other CITases could explain their similar optimum pH. Although TtCITase showed thermostable enzymatic properties, TtCITase-C showed inferior thermostability and reduced activity at higher temperatures. The thermophilic dextranase from *T. pseudethanolicus*, used for the structural comparison with other mesophilic enzymes, is truncated at the exposed loop region, thereby leaving many hydrophilic amino acid residues exposed at the surface [24]. Since charged residues can result in stable ion pairs that contribute to protein thermostability [23, 24], we presume that TtCITase-C was

less stable at 50°C and above due to the lack of the N-terminus that contributes to the thermal stability, such as TtDEX (Fig. 1).

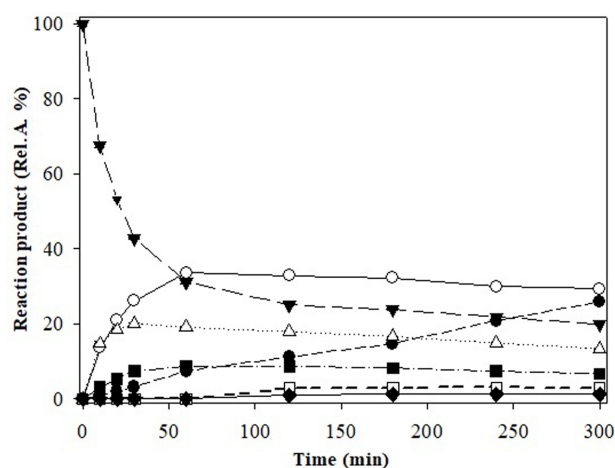
#### Analysis of Reaction Products

To determine the hydrolysis and glucosyl transfer activity of TtCITase-C, we performed a time-course analysis of the products formed from the substrates maltopentaose and isomaltopentaose. TtCITase-C produced G, IG2, IG4, and IG5 as the reaction products of transglucosylation from IG3 as the substrate in the early stage of the reaction (Fig. 5). Subsequently, the level of IG3 decreased rapidly over time, and the amount of IG2 (estimated by the relative area) exceeded that of IG3 after 1 h. IG2, generated from IG3, IG4, and IG5, was hydrolyzed and thus its level slightly



**Fig. 4.** The pH (A) and temperature (B) stability of TtCITase-C.

The purified TtCITase-C was incubated for various lengths of time and residual enzyme activity was measured at 40°C in Britton-Robinson buffer (pH 5.5). ●, 30°C; ○, 40°C; ▼, 50°C; △, 60°C; ■, 70°C; □, 80°C.



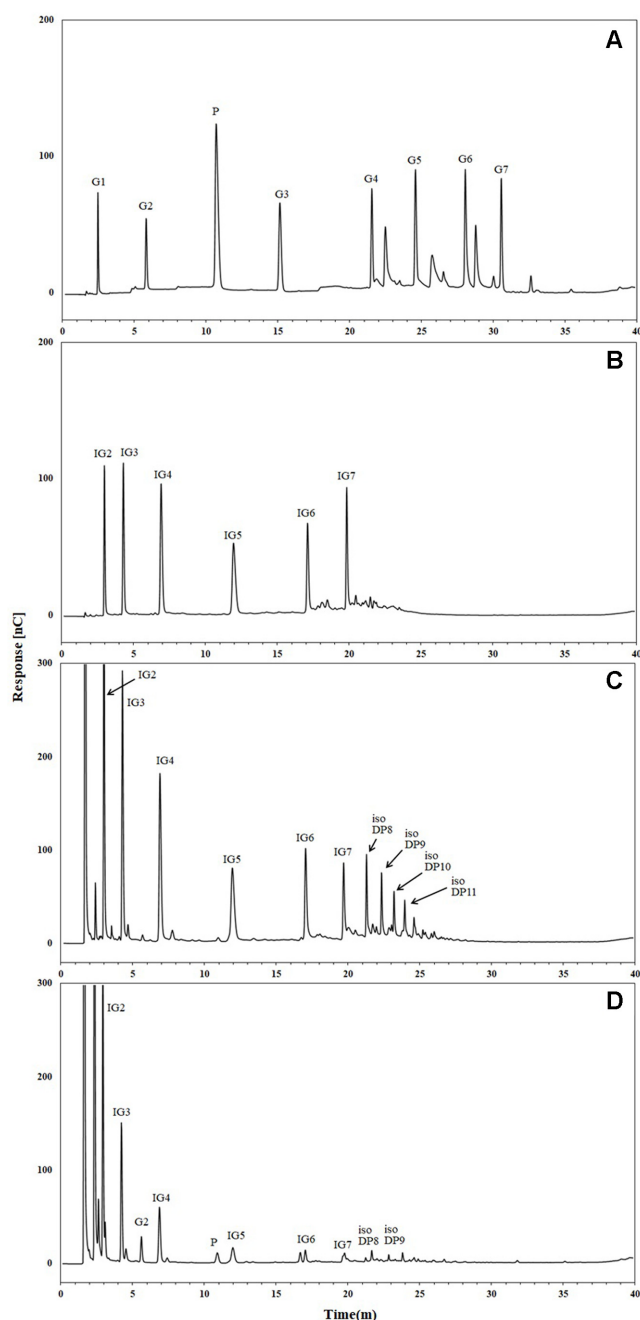
**Fig. 5.** Dynamics of reaction products during the reaction of TtCITase-C with 5 mM isomaltotriose at 40°C for 5 h.

●, Glucose (G); ○, isomaltose (IG2); ▼, isomaltotriose (IG3); △, isomaltotetraose (IG4); ■, isomaltopentaose (IG5); □, isomaltohexaose (IG6); ◆, isomaltoheptaose (IG7).

decreased after 2 h, whereas IG6 and IG7 were produced in small amounts over the same time period. The amount of glucose, which is a by-product of the transglucosylation and hydrolysis of IMOs, was higher than the remaining amount of IG3 expected according to the relative area of the chromatogram peak.

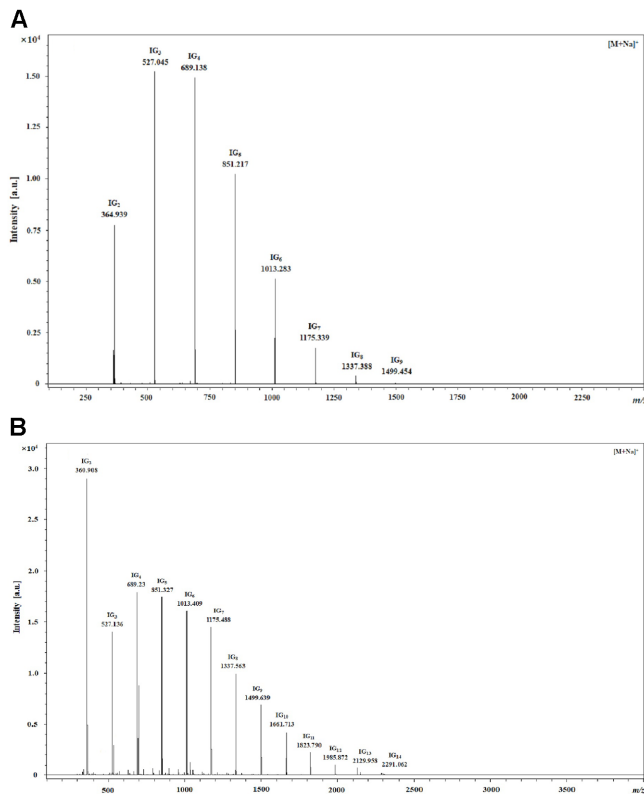
The production of M5 and IG5 was assessed via HPAEC analysis. TtCITase-C produced IMOs up to DP8 and DP14 from M5 and IG5, respectively. Because of the dual actions of TtCIT-C in the hydrolysis and transglucosylation with G5, the hydrolyzed glucose residue of the maltooligosyl  $\alpha$ -1,4-linkage (donor) would be transferred to the 6C-OH of the non-reducing end of another maltooligosaccharide (acceptor), thereby forming the  $\alpha$ -1,6 linkage (G-1,6-G5). The hydrolysis reaction was repeated three or four times, and G2 and G were produced as residual products. The panose-type materials mixed with  $\alpha$ -1,6/1,4 linkages were also produced as part of the intermediate reaction products of the transglucosylation to maltose. In the same manner, we expected that IG5 would be elongated by the glucosyltransferase activity of the glucose residue at the non-reducing end from the other IG5. Indeed, the reaction of IG5 showed a novel peak, which is presumed to be a LIMO (Fig. 6D).

MALDI-TOF MS identified the IMOs of DP8 to 14 produced by TtCITase-C as  $\alpha$ -1,6 glucosyl-transferring products. The molecular masses of  $\alpha$ -1,6-linked D-glucose products from M5 were confirmed to be 364.92 (DP2),



**Fig. 6.** HPAEC analysis of the product obtained from the reaction of TtCITase-C with maltopentaose and isomaltopentaose. (A) Maltooligosaccharides (G–G7) and panose (P) standards. (B) Isomaltohexaose (IG6) and isomaltoheptaose (IG7) standards. (C) Reaction products of TtCITase-C with maltopentaose. (D) Reaction products of TtCITase-C with isomaltopentaose.

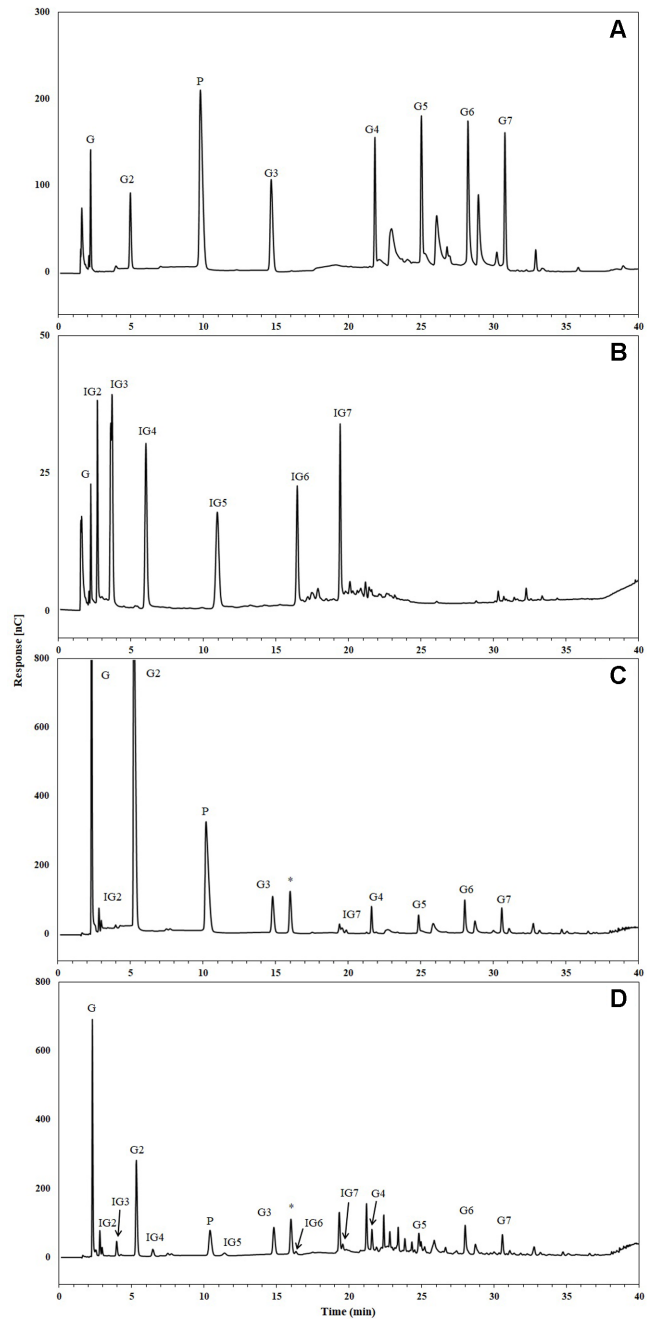
527.03 (DP3), 689.12 (DP4), 851.20 (DP5), 1,013.26 (DP6), 1,172.32 (DP7), 1,337.37 (DP8), and 1,499.42 (DP9) of mass value ( $m/z$ ) (Fig. 7A), whereas the reaction product from



**Fig. 7.** MALDI-TOF/TOF MS analysis of the reaction products of TtCITase-C with maltopentaose and isomaltopentaose. (A) Reaction products TtCITase-C with maltopentaose. (B) Reaction products of TtCITase-C with isomaltopentaose.

IG5 contained peaks up to 2,291.062 (DP14) (Fig. 7B). In addition to MOs with DP, panose with  $\alpha$ -1,6 glucosyl linkages was produced from M2 (Fig. 8C). Maltodextrin with a dextrose equivalent (D.E.) of 13.0–17.0 produced more complicated  $\alpha$ -1,6 glucosyl-transferring products with longer DPs (Fig. 8D).

The  $\alpha$ -glucosidase (transglucosidase) from *Aspergillus niger* was shown to rapidly transfer glucosyl residues to maltooligosaccharides; both the  $\alpha$ -1,4-glucosidic and  $\alpha$ -1,6-glucosidic linkages were gradually hydrolyzed at the non-reducing ends, and transformed into smaller molecules of mainly  $\alpha$ -1,6-glucosidic linkages [25]. TtCITase-C showed homology with DDase belonging to GH family 15 and showed strong  $\alpha$ -1,6 glucosyltransferase activity, similar to DDase from *G. oxydans* [26] and  $\alpha$ -glucosidase from *A. niger* [2]. DDase produced dextran as the main product and a small amount of IMOs, while *A. niger*  $\alpha$ -glucosidase produced IMOs with low DPs (2–6). However, in the present study, we did not detect the production of dextran or other high-DP products. Moreover, the  $\alpha$ -1,6 glucosyl-



**Fig. 8.** HPAEC analysis of the product obtained from the reaction of TtCITase-C with maltose and maltodextrin (D.E. 13.0–17.0).

(A) Maltooligosaccharides (G–G7) and panose (P) standards. (B) IG2–IG7 standards. (C) Reaction products of TtCITase-C with maltose. (D) Reaction products of TtCITase-C with maltodextrin (D.E. 13.0–17.0).

transferase enzyme activity of TtCITase-C was intermediate between the  $\alpha$ -glucosidase activity reported from *A. niger* and the DDase activity from *G. oxydans*. Further, TtCITase-

C produced IMOs (DP2–14) and LIMO-like products from substrates with  $\alpha$ -1,4-glucosidic and  $\alpha$ -1,6-glucosidic linkages.

In conclusion, this study confirmed that TtCITase-C has enzymatic properties suitable for producing LIMOs with various DPs. Further studies are required to elucidate the three-dimensional structure of TtCITase or TtCITase-C for more accurate classification of the observed enzymatic activity.

## Acknowledgements

This study was supported by the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01312601) Rural Development Administration, Republic of Korea.

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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