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Association of bi-functional activity in the N-terminal domain of glycogen debranching enzyme

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ABSTRACT

Glycogen debranching enzyme (GDE) in mammals and yeast exhibits α-1,4-transferase and α-1,6-glucosidase activities within a single polypeptide chain and facilitates the breakdown of glycogen by a bi-functional mechanism. Each enzymatic activity of GDE is suggested to be associated with distinct domains; α-1,4-glycosyltransferase activity with the N-terminal domain and α-1,6-glucosidase activity with the C-terminal domain. Here, we present the biochemical features of the GDE from *Saccharomyces cerevisiae* using the substrate glucose(n)-β-cyclodextrin (Gn-β-CD). The bacterially expressed and purified GDE N-terminal domain (aa 1–644) showed α-1,4-transferase activity on maltotetraose (G4) and G4-β-CD, yielding various lengths of $(G)_n$. Surprisingly, the N-terminal domain also exhibited α-1,6-glucosidase activity against G1-β-CD and G4-β-CD, producing G1 and β-CD. Mutational analysis showed that residues D535 and E564 in the N-terminal domain are essential for the transferase activity but not for the glucosidase activity. These results indicate that the N-terminal domain (1–644) alone has both α-1,4-transferase and the α-1,6-glucosidase activities and suggest that the bi-functional activity in the N-domain may occur via one active site, as observed in some archaeal debranching enzymes.

1. Introduction

Glycogen and starch are good sources of energy. Because glycogen acts as an emergency reserve when the human body needs an ample amount of energy, understanding the functional mechanism of glycogen degradation is important in maintaining the glycogen level and has wider implications in clinics and in the carbohydrate-processing industry [1–3]. For example, an imbalance of glycogen metabolism in the human body causes some glycogen process-related clinical disorders, such as glycogen storage diseases. Glycogen disease type III, known as Cori's disease, is an autosomal recessive metabolic disorder characterised by fasting hypoglycaemia, growth retardation and hepatomegaly in conjunction with liver cirrhosis, muscular weakness and cardiomyopathy [4,5]. Glycogen degradation is accomplished by the combined action of two enzymes, glycogen phosphorylase and glycogen debranching enzyme (GDE). Glycogen phosphorylase sequentially cleaves the α(1 → 4) glycosidic bonds between glucosyl residues at the non-reducing ends of the glycogen chains by simple phosphorolysis until four glucosyl units remain on each chain at a branch point [6]. Branch points are removed by GDE, which exhibits bi-functional enzymatic activities. The α-1,4-glycosyltransferase activity removes the outer three of the four glucosyl residues attached at the branch. The remaining single glucose residue attached by an α(1 → 6) linkage is then removed by the α-1,6-glucosidase activity [7]. In sequential action, glycogen phosphorylase and GDE can catalyse the phosphorolysis of α-1,4-linked glucose units and eliminate an α-1,6 branch point, releasing glucose 1-phosphate and glucose molecules. In the degradation of glycogen, two groups of debranching enzymes have been classified. One group is simple debranching enzymes such as isoamylase and pullulanase mainly found in bacteria, archaea and plants [8]. The other group is bi-functional GDEs mainly found in mammalian cells and yeast [8,9]. Most bacterial and plant debranching enzymes catalyse hydrolysis through a single α-1-6 glucosidase

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activity and produce only an α-(1 → 4)-glucan chain, whereas GDE shows both α-1-6-glucosidase activity and α-1-6-glucanotransferase activity in the same polypeptide chain in mammals and yeast [10,11]. GDE purified from rabbit [10,11] and yeast [6] showed both transferase and glucosidase activity; each activity occurred at two distinct catalytic sites [11–14]. Sequence alignment and biochemical studies have proposed that the C-terminal half of yeast GDE is associated with glucosidase activity, while the N-terminal half is associated with glycosyltransferase activity [15,16]. Several mutagenesis analyses have suggested candidate catalytic residues for each activity; for example, residues of Asp-535, Glu-564, and Asp-670 in the N-terminal domain were proposed to be the active site of the transferase activity, whereas the residues Asp-1086 and Asp-1147 located in the C-terminal domain may act as a general acid/base in catalysing the glucosidase reaction [13,15]. However, the structure of GDE has not been determined yet; therefore the understanding of its mechanism of action is limited. Several crystal structures of microbial or plant debranching enzymes that have smaller molecular weights with single glucosidase activity have been reported, including isoamylase, pullulanase and *Escherichia coli* GlgX [8,17], but none structure of a yeast or mammalian 1500–1600 aa GDE have been reported to date.

2. Materials and methods

2.1. Cloning the GDE gene

Based on the genome sequence of *Saccharomyces cerevisiae* D349 (Accession No. AB018078.1) [18], two primers, GDE-NcoI (5′-CCG GCC ATG GAT ATG AAT AGA TCA TTA CTG-3′) and GDE-HindIII (5′-CCG GAA GCT TCT TCA GGA ATC ATC TTC GTA-3′), were synthesised to amplify the GDE gene. A 4.6-kb DNA fragment was PCR amplified from the chromosomal DNA of *S. cerevisiae* D349 using the two primers. After amplification, the PCR products were purified using the AccuPrep® gel purification kit (Bioneer). The DNA fragment was digested with NcoI and HindIII and ligated into pProExHta or pET30a vector at the appropriate sites such that the open reading frame of the gene was fused to six histidine residues in frame either at the N-terminus or C-terminus of the vector.

2.2. Expression and purification of the enzyme

Recombinant *E. coli* BL21-RIL carrying the gene for full-length or N-terminal domain *S. cerevisiae* GDE was grown in Luria-Bertani broth media supplemented with 30 μg/ml of antibiotics at 37 °C to an A600 = 0.6 before isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Induction of the expression of the enzyme was carried out overnight at 18 °C, and the cells were harvested by centrifugation at 4500 rpm for 30 min. The collected cell pellet was resuspended in lysis buffer [20 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM 2-mercaptoethanol, 20 mM imidazole]. The resuspended cells were lysed by sonication [amplitude-61%, pulse-1 s/1 s on/off, total time 90 s, three times] on ice. The cell lysate was centrifuged at 15,000 rpm for 50 min at 4 °C [19]. The His-tagged GDE protein was efficiently purified using a Ni–NTA column. The bound target protein was eluted with lysis buffer containing 250 mM imidazole. The eluted fraction was dialysed for 8 h with dialysis buffer containing 20 mM Tris–HCl pH 7.5, 50 mM NaCl and 5 mM 2-mercaptoethanol. After dialysis, the GDE protein was purified again by using anion exchange chromatography. The bound GDE protein was eluted by gradient in the dialysis buffer supplemented with 1 M NaCl. After the elution of GDE, the protein sample was treated by TEV protease at a ratio of 1:20. After cleavage of the His tag, the protein was applied to a Ni–NTA affinity column. Finally, the GDE protein was purified by gel filtration chromatography in a buffer of 20 mM Tris–HCl pH 7.5, 50 mM NaCl and 2 mM 2-mercaptoethanol.

2.3. Site-directed mutagenesis

Site-directed mutagenesis (Construction of mutants, D535N and E564Q) was performed using a Quick-Change kit (Stratagene, La Jolla, CA, USA). The DNA sequence encoding GDE 1-644 in pProExHta was used as template DNA for site-directed mutagenesis. The synthetic oligonucleotides used as primers for mutagenesis were as follows: D535N: 5′-GACGG TTCAGAATTAACTACTGC-CATTCTACTCCA-3′ and E564Q: 5′-AACCTATATGTCGTTGCACAGCT GTTTTCTGGTTCCGAA-3′. The amplified PCR products were purified using a QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) and transformed into DH10β and BL21(DE3-RIL). The sequence changes in the mutants were confirmed using the BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (Perkin-Elmer, Boston, MA, USA).

2.4. Trypsin digestion

The purified full-length GDE was digested with trypsin in 0.1 ml of ammonium bicarbonate. Trypsin was added to 10 μg of GDE at 2000:1, 1000:1, 100:1, 10:1 or 1:1 and incubated for 30 min at 25 °C. Digestion was terminated by the addition of 0.1 ml of 6 M GdmC1, 20 mM DTT and 50 mM Tris–HCl, pH 7.5. Tryptic peptides were separated by SDS–PAGE and transferred to a PVDF membrane. The sequence of each peptide was determined using N-terminal sequencing analysis at KBSI (Korea Basic Science Institute).

2.5. Thin layer chromatography

The detection and identification of transcosylation and hydrolysis products was performed using TLC analysis. Silica gel K5F TLC plates (Whatman, Maidstone, Kent, UK) were activated for 1 h at 110 °C. Samples were spotted onto plates using a pipette, and the plates were placed in a TLC chamber containing n-butanol:ethanol:water = 5:5:3 (v/v/v). Chromatography was conducted at room temperature. Reducing sugars were detected using the naphtol–sulphuric acid (H_2SO_4) method [20]. Each plate was thoroughly dried and developed by rapid dipping into a methanolic solution containing 3 g of N-(1-naphthyl)-ethylene-diamine and 50 ml of concentrated H_2SO_4 per litre. Each plate was dried and placed in an oven at 110 °C for 10 min; purple–black spots appeared on a white background.

2.6. High-performance anion exchange chromatography

The quantification of the transfer products and released glucose was performed using high-performance anion exchange chromatography (HPAEC). Reaction mixtures incubated with GDE were boiled for 10 min, centrifuged at 12,000*g* for 10 min, and filtered using a membrane filter kit (pore diameter 0.45 μm; Millipore, Billerica, MA). Products were applied to a CarboPacTM PA-1 column (0.4 × 25 cm; Dionex, Sunnyvale, CA) and analysed on an HPAEC platform fitted with a pulsed amperometric detector (ED40; Dionex). Sixty microlitres of sample was injected. Elution was achieved using a NaOAc gradient in 150 mM NaOH (increasing from 60 mM to 180 mM NaOAc over 0–10 min; from 180 mM to 240 mM over 10–16 min; from 240 mM to 300 mM over 16–27 min; from 300 mM to 360 mM over 27–44 min; and from 360 mM to 372 mM over 44–55 min). The flow rate was 1 ml per minute.

3. Results

3.1. Construction and purification of full length GDE and the N-terminal domain

Full length GDE was cloned into the pET30a vector with a six histidine tag at the C-terminus. Initially, a full-length protein with a histidine tag attached to the N-terminus was constructed and purified. However, this construct showed low binding affinity to the Ni column, yielding low purity and partial cleavage during purification at 4 °C. The full-length GDE with a C-terminal His tag yielded a high purity product adequate for further biochemical studies. Based on the observation of partial cleavage during purification, we subjected the full-length GDE protein to trypsin digestion in an attempt to cleave a flexible loop and define a putative stable domain. Two fragments were obtained, and one of them had a molecular weight of 70 kDa according to SDS–PAGE and remained stable for longer incubation times, as shown in Fig. 1. N-terminal sequencing of the band revealed that the peptide bond between Arg 644 and Cys 645 was cleaved by trypsin, indicating a putative boundary between domains that allowed for the construction of the N-terminal domain in this study. Among the various ranges of proteins constructed, two constructs of the N-terminal domain, corresponding to aa 1–644 and aa 140–529, produced soluble proteins. The N-terminal domain of aa 140–529 did not show any hydrolysis or transferase activity and therefore the N-terminal domain containing residues 1–644 was used for further biochemical study in addition to full-length GDE. Interestingly, GPC analysis showed an elution peak that corresponded to a tetrameric arrangement of both the full-length and N-terminal domain proteins, with molecular weights of 640 kDa and 273 kDa, respectively (data not shown).

3.2. Catalytic activity of full-length GDE

To investigate the bi-functional action of GDE, three types of substrates were used for activity assays, maltotetraose (G4); G1-βCD, a β-CD linked to a glucose G1 by α-1,6-glucosidic linkages; and G4-βCD, a β-CD linked to G4 by α-1,6-glucosidic linkages. The reaction products from each substrate were analysed by TLC and HPAEC. The results showed that the bacterially expressed full-length GDE exhibited bi-functional activity as expected (Fig. 2). For the G4 substrate, full-length GDE cleaved the α-1,4-glucosidic linkages and transferred the cleaved products to generate various lengths of oligosaccharides, exhibiting the α-1,4-transferase activity. For G1-βCD, GDE cleaved the α-1,6-glucosidic linkages and produced G1 and βCD, exhibiting the α-1,6-glucosidase activity. For the G4-βCD substrate, full-length GDE acted on the maltotetraose moiety with transferring activity and cleaved the α-1,6-glucosidic linkages to produce G1, βCD and various lengths of Gn-βCD as previously reported. Interestingly, no G1 product was detected from the G4 substrate, suggesting that GDE needs an acceptor, at least the glucose (G1) molecule, for its α-1,4-transferase activity.

3.3. α-1,4-Transferase activity of the N-terminal domain

We carried out the same analysis for the activity of the GDE N-terminal domain, aa 1–644, using maltotetraose and G4-βCD as substrates. In Fig. 3, TLC analysis showed that various malto-oligosaccharides, ranging from maltose (G2) to maltoheptaose (G7), were produced from maltotetraose (G4) by the action of the N-terminal domain of GDE. The spot density of maltotetraose was significantly reduced over a 24 h reaction with GDE, and extended oligosaccharides as well as smaller molecules appeared in the reaction sample, demonstrating the α-1,4-transferase activity. HAPEC analysis of the products showed the same molecules in the elution profile, consistent with the TLC analysis. The G4-βCD substrate yielded compounds with higher molecular weights than G4-βCD, indicating that products were transferred from the maltotetraosyl moiety of the substrate, the same production pattern observed in full-length GDE. The intensity of the transferred products was significantly reduced compared to the intensity after treatment with full-length GDE, suggesting reduced activity of the N-terminal domain of GDE. These results indicated that the N-terminal domain has α-1,4-transferase functionality but with reduced activity compared to the full-length GDE.

3.4. α-1,6-Glucosidase activity of N-terminal domain

To investigate the α-1,6-glucosidase activity of GDE, we applied the N-terminal domain to the G1-βCD substrate in which the G1 glucose is linked to βCD by an α-1,6-glucosidic bond. As shown in Fig. 3, the N-terminal domain (aa 1–644) of GDE completely cleaved the α-1,6-glucosidic bond to produce only G1 and βCD. HAPEC analysis also showed that most G1-βCD was hydrolysed at 1 h and completely degraded at 5 h, showing α-1,6-glucosidase activity on the branched substrate comparable to that of full-length GDE. The activity was specific to the α-1,6-glucosidic bond because the βCD molecule linked by an α-1,4-glucosidic bond between glucose subunits was not degraded at all, demonstrating no α-1,4-glucosidase activity for either full-length GDE or its

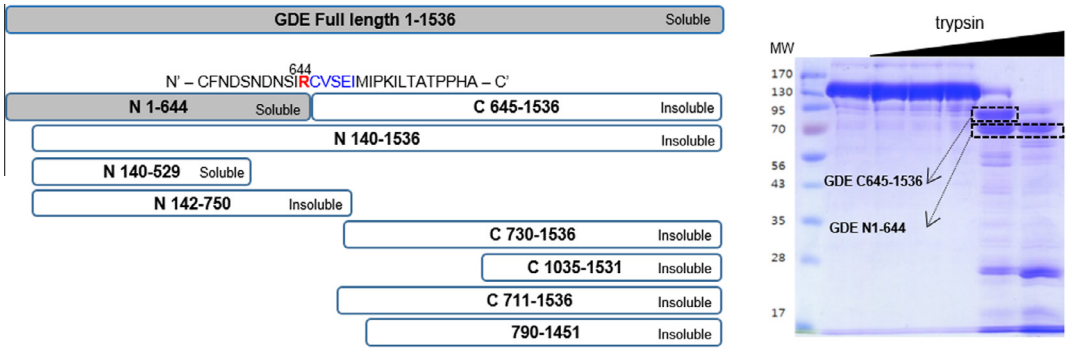

Fig. 1. Construction of various GDE proteins and the N-terminal domain construct. Various lengths of N- and C-terminal domain constructs were purified and tested for solubility (left). To define the N-terminal domain, a full-length GDE protein was treated with trypsin in a range of concentration and subjected to SDS–PAGE (right). Two significant bands (right, box) that remained after partial trypsin digestion at a 10:1 ratio were analysed by N-terminal sequencing to determine the cleavage site. The cleavage site (right, red) and its adjacent residues are shown in the amino acid sequence. The full length and the N-terminal domain used for biochemical assay in this study are highlighted in grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

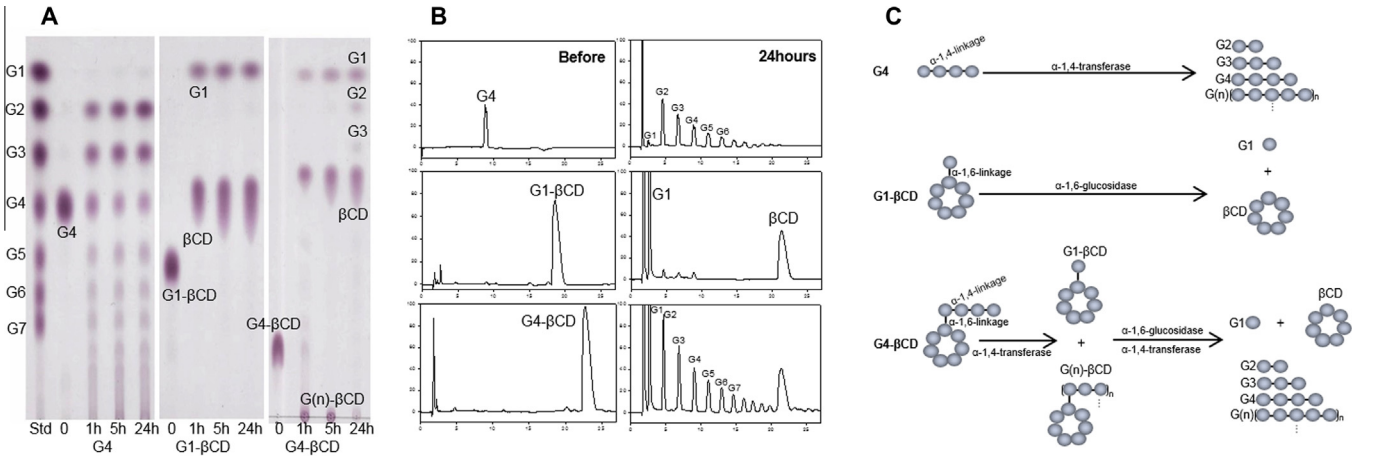

Fig. 2. TLC and HPAEC analyses of the reaction products generated by full-length GDE. (A) G4 (left), G1-βCD (middle) and G4-βCD (right) were individually incubated with GDE for 0, 1, 5 and 24 h before being subjected to TLC. The malto-oligosaccharide standard is shown in the left lane. (B) HPAEC analysis of products incubated with GDE for 24 h. The original substrate (left) is shown with the reacted sample (right). (C) Diagram depicting the mode of action of α-1,4-transferase and α-1,6-glucosidase activity for each substrate.

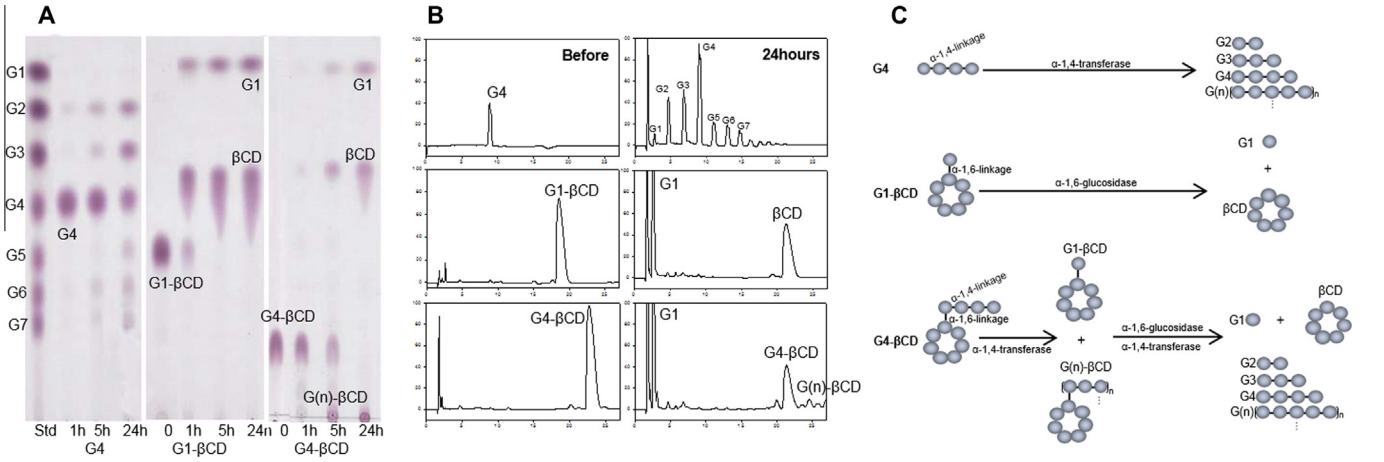

Fig. 3. TLC and HPAEC analysis of the reaction products from the GDE N-terminal domain. (A) (left), G1-βCD (middle) and G4-βCD (right) were individually incubated with GDE for 0, 1, 5 and 24 h before being subjected to TLC. The malto-oligosaccharide standard is shown in the left lane. (B) HPAEC analysis of products incubated with the GDE N-terminal domain for 24 h. The original substrate (left) is shown with the reacted sample (right). (C) Diagram depicting the mode of action of α-1,4-transferase and α-1,6-glucosidase activity for each substrate.

N-terminal domain. The N-terminal 1–644 aa generated mainly G1 and βCD molecules from G4-βCD in addition to higher molecular weight products of G4-βCD as a result of transferase activity. Because neither the full-length or N-terminal domain GDE produced a G1 molecule from the G4 substrate, the presence of G1 in the reaction sample of G4-βCD was likely the result of the hydrolysis of glucose from G1-βCD by the α-1,6-glucosidase activity after three glucose molecules at the non-reducing end from G4-βCD substrate were transferred away by the α-1,4-transferase activity.

3.5. Essential residues for α-1,4-transferase activity

Four conserved motifs of α-amylase that are essential for enzymatic activity were reported to be in the N-terminal region. Asp-224, Asp-535, Glu-564 and Asp-670 were suggested to be crucial for α-1,4-transferase activity [15,16]. The N-terminal 1–644 aa used in this study do not contain Asp-670 in the conserved motif IV. To investigate the functional role of these conserved motifs, we mutated Asp-535 in motif II and Glu-564 in motif III to asparagine and glutamine, respectively, and examined the activities of the mutants. As shown in Fig. 4, both the Asp-535 and Glu-564 mutants in the N-terminal domain were not able to cleave the maltotetraose substrate at all, revealing the complete abolishment of the α-1,4-transfer activity by mutation at these sites. However, both the Asp-535 and Glu-564 mutants cleaved the G1-βCD substrate completely, revealing that these residues are not essential for the α-1,6-glucosidase activity (Fig. 4). For the G4-βCD substrate, neither mutant cleaved the substrate despite its α-1,6-glucosidase activity, suggesting that the use of the α-1,4-transfer activity to transfer residues from the non-reducing end of the branched point is required before GDE cleaves the residual glucose unit by the α-1,6-glucosidase activity, in agreement with the previously observed functional pattern of GDE.

4. Discussion

GDE is a key enzyme involved in glycogen metabolism and is of special interest because it exhibits two enzymatic activities in one polypeptide chain [10,11]. Previous studies suggested that each activity occurs at distinct sites, the α-1,4-transferase activity in the N-terminal half and the α-1,6-glucosidase activity in the C-terminal half [12,15]. However, we showed in this study that

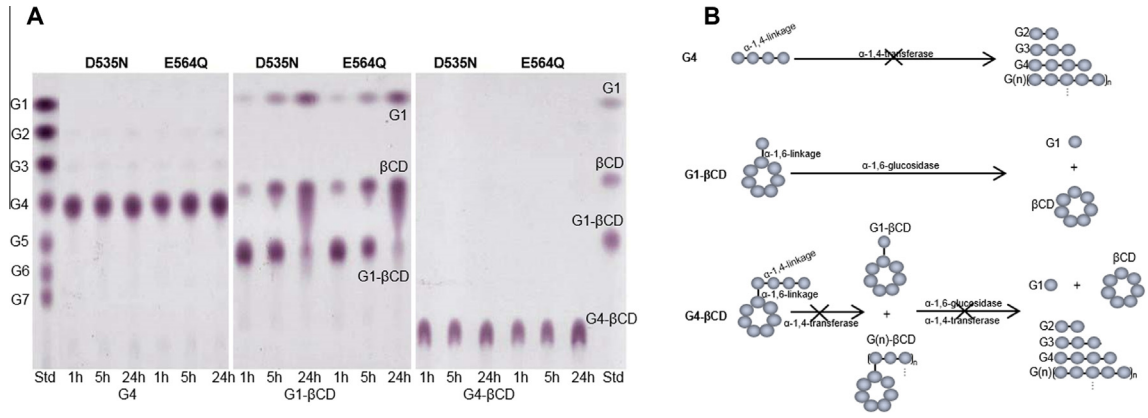

Fig. 4. TLC and HPAEC analysis of the reaction products from the GDE N-terminal mutants. (A) TLC analysis of the reaction products from the GDE N-terminal domain with mutations of Asp 535 or Glu 564. G4 (left), G1-βCD (middle) and G4-βCD (right) were individually incubated with GDE for 1, 5 and 24 h before subjected to TLC. The malto-oligosaccharide standard is shown in the left lane. (B) Diagram depicting the lack of α-1,4-transferase and α-1,6-glucosidase activity for each substrate.

the N-terminal domain of aa 1–644, the boundary of which was defined by protease digestion, exhibited a novel α-1,6-glucosidase activity as well as the known α-1,4-transferase activity. The finding of two activities in the N-terminal domain of GDE is reminiscent of the function of the archaeal debranching enzyme TreX. TreX is the GH13 family enzyme from *Sulfolobus sulfotaricus* that removes the side chain of glycogen in maltodextrin [21,22]. Despite high sequence similarity to plant and microbial debranching enzymes (74% homology to *Sulfolobus acidocaldarium* isoamylase and 42% homology to the *E. coli* debranching enzyme GlgX), TreX exhibits both α-1,4-transferase and α-1,6-glucosidase activities. The three-dimensional structure revealed that both activities are associated with the same active site in which the dimer subunit has only α-1,6-glucosidase activity, but an additional α-1,4-transferase activity results from the lid architecture generated by tetrameric oligomerisation of dimers to reshape the active site, conferring an additional function to the same catalytic residues [8,23,24]. Intriguingly, the N-terminal domain of GDE, which has a molecular weight of 70 kDa MW, similar to TreX, appears to form tetramers in solution [8]. In fact, sequence analysis showed that the N-terminal domain of GDE has high similarity to GH13 family proteins, suggesting that they might share the same overall structure.

It is conceivable that the novel activity of α-1,6-glucosidase observed in the N-terminal 1–644 aa was an artefact of the alteration of the activity after the truncation of the C-terminal domain. The removal of the C-terminal domain, which shapes the substrate-binding site for the α-1,4-transferase activity may have altered the substrate-binding geometry and resulted in α-1,6-glucosidase activity as an artefact. However, despite its α-1,6-glucosidase activity, both the Asp-535 and Glu-564 mutants of the N-terminal domain could not cleave the G4-βCD substrate and cleave only G1-βCD substrate. The requirement of transferring residues from the non-reducing end of the branched point before α-1,6-glucosidase activity is in good agreement with the unique functional feature of GDE in vivo, indicating that both the α-1,4-transferase and α-1,6-glucosidase activities associated with the N-terminal domain are the genuine functions of GDE. Further biochemical studies and structural information will help to clarify the functional role of the C-terminal domain.

In conclusion, we observed that the N-terminal region containing residues 1–644 exhibited both α-1,4-transferase and α-1,6-glucosidase activities. Based on a sequential and functional comparison to the archaeal debranching enzyme TreX, we suggest that the active sites for these activities may be arranged in one location in three-dimensional geometry. This result would provide a new insight into the functional mechanism of glycogen degradation by GDE and guide the development of an activity modulator for clinical and industrial applications.

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