



Title	Molecular basis for reactions of GH15 α -glucan 4(6)- α -glucosyltransferase
Author(s)	秦, 天翼
Degree Grantor	北海道大学
Degree Name	博士(農学)
Dissertation Number	甲第16773号
Issue Date	2026-03-25
DOI	https://doi.org/10.14943/doctoral.k16773
Doc URL	https://hdl.handle.net/2115/99618
Type	doctoral thesis
File Information	QIN_Tianyi.pdf, 全文



Molecular basis for reactions of
GH15 α -glucan 4(6)- α -glucosyltransferase
(GH15 α -グルカン 4(6)- α -グルコシル転移酵素
が触媒する反応の分子基盤)

Hokkaido University Graduate School of Agriculture

Frontiers in Biosciences

Applied Biochemistry Unit Doctor Course

Tianyi Qin

北海道大学 大学院農学院

応用生物化学ユニット 博士後期課程

秦 天翼

CONTENTS

1.	General introduction	1
1.1.	Isomaltosaccharides	1
1.2.	Synthesis of α -glucan by transglycosylation by α -glucosyltransferases	1
1.3.	Classification and reaction mechanism of GHs	2
1.4.	Carbohydrate synthesis via transglycosylation by retaining enzymes in GH families	3
1.5.	Functions, structures, and applications of GH15 anomer-inverting glucohydrolases	4
1.6.	Functions and structures of GH15 DDase and its homologues	5
1.7.	The purpose of this study	7
2.	Functional analysis of Td46GT and Pt46GT	11
2.1.	Introduction	11
2.2.	Materials and methods	11
2.2.1.	Materials	11
2.2.2.	Construction of expression plasmids for Td46GT and Pt46GT	11
2.2.3.	Production and purification of Td46GT and Pt46GT	12
2.2.4.	Blue native PAGE of Td46GT	12
2.2.5.	Time course of the reaction of Td46GT and Pt46GT with MOSs	13
2.2.6.	Preparation and structural analysis of 6II-O- α -isomaltosylmaltose	13
2.2.7.	Standard enzyme activity assay for Td46GT and Pt46GT	14
2.2.8.	Effects of pH and temperature on activity and stability	14
2.2.9.	Kinetic parameters in a single-substrate reaction	15
2.2.10.	Relative acceptor-substrate specificity constant in a two-substrate reaction	15
2.3.	Results	15
2.3.1.	Production and purification of recombinant Td46GT and Pt46GT	15
2.3.2.	Time course of Td46GT and Pt46GT reactions with MOS	16
2.3.3.	Effects of pH and temperature on activity and stability of Td46GT and Pt46GT	17
2.3.4.	Analysis of the initial reactions of Td46GT with MOSs, panose, and IMOs	18
2.3.5.	Analysis of the initial reactions of Pt46GT with MOSs and IMOs	18
2.3.6.	Evaluation of acceptor specificity via two-substrate reaction of Td46GT	19
2.3.7.	Prediction of product profile during the reaction with MOS and IMO based on specificity constants for donors and acceptors	20
2.4.	Discussion	21
3.	X-ray crystal structure analysis of Td46GT	48
3.1.	Introduction	48
3.2.	Materials and methods	48

3.2.1.	Construction of expression plasmids for Td46GT E666Q mutant	48
3.2.2.	Production and purification of Td46GT variants	48
3.2.3.	X-ray crystal structure analysis of Td46GT	49
3.2.4.	Effect of calcium ion on temperature activity and stability	50
3.2.5.	Inhibition experiment with acarbose	50
3.3.	Results	51
3.3.1.	Purification of Td46GT E666Q mutant	51
3.3.2.	Crystal structure analysis of Td46GT E666Q mutant	51
3.3.3.	Active center structure bound to acarbose	52
3.3.4.	Calcium ion binding-sites	53
3.3.5.	DSC analysis of wild-type Td46GT	53
3.3.6.	Effects of calcium ion on temperature activity and stability	53
3.4.	Discussion	54
4.	Molecular basis for donor and acceptor specificities of Td46GT and Pt46GT	76
4.1	Introduction	76
4.2.	Materials and methods	76
4.2.1.	Construction of expression plasmids for Td46GT variants	76
4.2.2.	Production and purification of Td46GT variants	77
4.2.3.	Kinetic parameters in a single-substrate reaction	77
4.2.4.	Evaluation of acceptor specificity via two-substrate reaction	77
4.3.	Results	78
4.3.1.	Preparation of Td46GT mutants	78
4.3.2.	Kinetic analysis of Td46GT mutants	78
4.3.3.	Kinetic analysis of Td46GT multiple mutants in single substrate reactions	79
4.4.	Discussion	79
5.	Conversion of reaction mechanism of Td46GT by rational mutagenesis	93
5.1.	Introduction	93
5.2.	Materials and methods	94
5.2.1.	Materials	94
5.2.2.	Construction of expression plasmids for Td46GT single mutants	94
5.2.3.	Construction of expression plasmids for Td46GT multiple mutants (MM)	94
5.2.4.	Production and purification of Td46GT variants	95
5.2.5.	Identification of reaction products from pNP-Glc and GF	95
5.2.6.	Measurement of reaction rates to pNP-Glc, GF, and G4	95
5.2.7.	Kinetic analysis pH profiles of Td46GT variants	96
5.2.8.	Analysis of the anomeric form of D-glucose produced from G4 using Td46GT MM8 mutant	96

5.3.	Results	97
5.3.1.	Functional analysis of the catalytic Glu residues in Td46GT	97
5.3.2.	Role of the hydrogen-bonding network involving Glu666	98
5.3.3.	Conversion of Td46GT to an inverting α -glucohydrolase through MM	98
5.4.	Discussion	99
6.	General discussion	112
	References	121

Abbreviations

B4: 6^{III}-*O*- α -glucosyl maltotriose

BSA: bovine serum albumin

CBM: carbohydrate binding module

CI: cycloisomaltooligosaccharide

CITase: cycloisomaltooligosaccharide glucanotransferase

DDase: dextran dextrinase

DP: degree of polymerization

DSC: differential scanning calorimetry

ESI-MS: electrospray ionization-mass spectrometry

GAase: glucoamylase

CAZy: Carbohydrate-Active enZyme database

GF: α -D-glucosyl fluoride

GH: glycoside hydrolase

Glc: D-glucose

Glc-Pan: 6^{II}-*O*-isomaltosylmaltose

GT: glucosyltransferase

G2: maltose

G3: maltotriose

G4: maltotetraose

G5: maltopentaose

GF: α -D-glucosyl fluoride

HMBC: heteronuclear multiple bond correlation

HPAEC-PAD: high performance anion exchange chromatography with pulsed amperometric detection

HSQC: heteronuclear single quantum coherence

IG2: isomaltose

IG3: isomaltotriose

IG4: isomaltotetraose

IG2F: α -isomaltosyl fluoride

IG3F: α -isomaltotriosyl fluoride

IGHase: isomaltose glucohydrolases

IMM: isomaltomegalosaccharide

IMO: isomaltooligosaccharides

LAB: lactic acid bacteria

MM: multiple mutation

MOS: maltooligosaccharide

NMR: nuclear magnetic resonance

pNP: *p*-nitrophenyl

pNP-Glc: *p*-nitrophenyl α -D-glucoside

RASC: relative acceptor-substrate specificity constant

Pt46GT: *Pseudoclostridium thermosuccinogenes* α -glucan 4(6)- α -glucosyltransferase

RMSD: root mean square deviation

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Td46GT: *Tepidibacillus decaturensis* α -glucan 4(6)- α -glucosyltransferase

TLC: thin layer chromatography

T_m : melting temperature

TtCITase: *Thermoanaerobacter thermocopriae* cycloisomaltooligosaccharide glucanotransferase

T-F-A: Three-Fragment-Assembly

α -Glucosyltransferase: α -GT

$\Delta 382C$: DDase lacking the C-terminal 382 residues.

ΔH_{cal} : calorimetric enthalpy for unfolding

1. General introduction

1.1. Isomaltosaccharides

Isomaltosaccharides, composed of α -(1 $\rightarrow$ 6)-D-glucosyl residues, have a variety of properties depending on degree of polymerization (DP). Isomaltooligosaccharides (IMOs) with DP 2–3 are widely utilized in food industry as texture modifiers and flavor improvers (Chen et al., 2001; Goffin et al., 2011; Lee et al., 2008). IMOs with DP 2–9 can stimulate growth of probiotic bacteria and inhibit growth of the pathogen bacteria, whereby acting as prebiotic oligosaccharides (Kohmoto et al., 1988; Rengarajan & Palanivel, 2025). IMOs (DP <10) can inhibit insoluble glucan formation to prevent dental caries (Nguyen et al., 2022).

Large-scale production of IMOs and its first commercialization (isomalto-500, Showa Sangyo, Japan) originated in Japan since 1985 (Kanno, 1990): starch was first liquified by amylase to produce maltose-enriched solutions, and transglucosylated using fungal α -glucosidase (Transglucosidase L ‘Amano’, Amano Enzymes, Japan; Okamoto et al., 2010) to produce IMOs primarily comprised of panose, isomaltose (IG2), and isomaltotriose (IG3). This classic strategy has been widely applied and continued for decades (Basu et al., 2016; Casa-Villegas et al., 2017; Xue et al., 2022).

An alternative strategy for IMOs synthesis involves the controlled hydrolysis of an α -(1 $\rightarrow$ 6)-D-glucosidic linked polysaccharide, dextran, catalyzed by dextranase (Goulas et al., 2004). This approach could avoid the D-glucose formation, even though it increases the production costs because dextran itself is a valuable and relatively expensive industrial material compared to starch hydrolysates.

Isomaltomegalosaccharides (IMMs) are isomaltosaccharides with DP 10–200 and show higher resistance than IMOs against digestive enzymes (Aizawa et al., 2021). IMMs could modulate immune response (Joe et al., 2016) and elicit a smaller blood D-glucose response (Aizawa et al., 2021), which is useful as slowly digestible carbohydrates especially for diabetic patients. Moreover, IMMs can also enhance the uptake of quercetin-3-*O*- β -D-glucoside by increasing its solubility (Shinoki et al., 2013).

1.2. Synthesis of α -glucan by transglycosylation by α -glucosyltransferases

Biosynthesis of α -glucan (e.g., glycogen, starch, and dextran) is commonly catalyzed by α -glucosyltransferases (α -GTs), which are classified as hexosyltransferases (EC 2.4.1.-). In general, α -GTs can be divided into Leloir and non-Leloir types according to the nature of their donor substrates (Lairson et al., 2008). Leloir α -GTs utilize activated nucleotide diphosphate sugars such as ADP -glucose (starch synthase;

EC 2.4.1.21) and UDP-glucose (glycogen synthase; EC 2.4.1.11), as glucosyl donors, whereas non-Leloir α -GTs employ glucans and other saccharides as donors. Many non-Leloir α -GTs have been identified in lactic acid bacteria (LAB) responsible for extracellular polysaccharides production (İspirli et al., 2023; Meng et al., 2016).

Dextran, an extracellular α -glucan of LAB and also an important biomedical material (Banerjee & Bandopadhyay, 2016; Petrovici et al., 2023), is biosynthesized by dextransucrase (EC 2.4.1.5), which is an α -GT secreted from *Leuconostoc mesenteroides* under sucrose-rich conditions (Naessens et al., 2005). In this extracellular process, sucrose serves as D-glucosyl donor for elongation of α -(1 $\rightarrow$ 6)-linked glucan catalyzed by extracellular dextransucrase, while the released D-fructose is taken up by the cells as an energy source for *L. mesenteroides* (Naessens et al., 2005). Dextran also contains minor amount of α -(1 $\rightarrow$ 2)-linked and α -(1 $\rightarrow$ 3)-linked D-glucosyl residues, which can be explained by the flexible and diverse binding model of glucan acceptors for dextransucrase (Leemhuis, Pijning, et al., 2013). Dextran can also be biosynthesized by dextran dextrinase (DDase; EC: 2.4.1.2), an α -GT found from ropy beer fermented by acetic acid bacteria consuming dextrin in beer to synthesize dextran, since from 1950s (Hehre, 1951; Kooi, 1958).

Mutan, another extracellular α -glucan of LAB, is water-insoluble and predominantly composed of α -(1 $\rightarrow$ 3)-linkages. Mutan could be biosynthesized by mutansucrase (EC 2.4.1.372) from some LAB such as *Streptococcus mutans* (Hanada & Kuramitsu, 1989). It is also a key factor contributing to dental plaque formation and dental caries development (Loesche, 1986).

Importantly, several non-Leloir α -GTs share similar catalytic mechanism and structure with retaining glycoside hydrolases (GHs), which reflects their evolutionary origin from common ancestors.

1.3. Classification and reaction mechanism of GHs

GHs catalyze the hydrolysis of glycosidic bonds in glycosides. GHs either invert or retain the anomeric configuration of the substrate in the product. The different outcomes of the anomeric configuration (inverting or retaining) has been explained by two different reaction mechanisms, based on Koshland's theory (Fig. 1-1A and B; Koshland, 1953). In most cases, two acidic amino acid residues act as the catalytic residues for the both types of the enzymes (Rye & Withers, 2000). Inverting reaction proceeds by a single-displacement mechanism: the aglycon is released upon proton donation from a general acid catalyst to the glycosidic oxygen, while a water molecule, activated by a general base catalyst, attacks the anomeric carbon from the

opposite side. Retaining enzymes utilize a double-displacement mechanism including two steps, in which the two catalytic acidic residues act as a general acid/base and a nucleophile, respectively. In the first step (glycosylation step), the general acid/base catalyst protonates the glycosidic oxygen and the nucleophile attacks the anomeric carbon from the opposite side, forming a covalent glycosyl-enzyme intermediate; In the second step (deglycosylation step), water and hydroxy group of sugars, deprotonated by general base catalyst, serves as acceptor to attack the anomeric carbon of the intermediate in hydrolysis or transglycosylation, respectively (Rye & Withers, 2000). Many non-Leloir α -GTs are anomer-retaining enzymes, and their catalysis is also proposed to follow the double-displacement mechanism, same to that of anomer-retaining GHs.

Based on amino acid sequence similarity, GHs and Leloir-GTs have been respectively classified into 194 and 139 of families in Carbohydrate-Active enZymes Database (CAZy) until 2025 October (Drula et al., 2022). However, as many non-Leloir α -GTs share the catalytic mechanism and sequence similarity with retaining GHs, they are always included in the same family. Retaining enzymes are not normally grouped within the same family as inverting enzymes (Fig.1-1C).

Notably, GH15, GH23 and GH97 families contain both inverting enzymes and retaining enzymes. GH23 and GH97 families conserve the residues acting as the proton donor (general acid and acid/base catalysts for inverting and retaining enzymes, respectively) in the primary state, but don't conserve the other catalytic residues acting as general base and nucleophile, respectively (Okuyama et al., 2009; Thunnissen et al., 1995). Interconversion of inverting and retaining mechanism by minimum mutation has been a hot topic for further understanding of catalytic mechanisms of GHs, but successful cases are seldomly reported. Conversion of an inverting GH24 viral lysozyme into a retaining GH was achieved by the single substitution of Thr26 in the active site, with a His (Brockerman et al., 2019; Kuroki et al., 1999). Conversion of an inverting GH181 sialic acid hydrolase into a retaining trans-sialidase was achieved through a double mutation, including a Gln→Tyr substitution that mimicked the nucleophile of GH33 trans-sialidase (Pichler et al., 2025). These studies have suggested a common structure basis for co-evolution of inverting and retaining GHs.

1.4. Carbohydrate synthesis via transglycosylation by retaining enzymes in GH families

Transglycosylation catalyzed by retaining enzymes has become an attractive and environmentally friendly approach for the synthesis of structurally diverse glycans in the pharmaceutical, cosmetic, and food

industries. In polysaccharide industry, in addition to previously described DDase used for dextran synthesis, GH68 inulosucrases are promising biocatalysts for large-scale production of inulin (Kido et al., 2023), a functional food ingredient whose hydrolysates exhibit well-documented prebiotic effects (Shoaib et al., 2016).

Not only polysaccharides, but diverse oligosaccharides have also been efficiently prepared by transglycosylation. Examples include IMOs as mentioned above, fructooligosaccharides and galactooligosaccharides. Fructooligosaccharides generated by GH68 β -fructofuranosidases and fructosyltransferases (Kralj et al., 2018; Maiorano et al., 2008; Sangeetha et al., 2005). This approach is more economically favorable than hydrolysis of inulin by inulinase (Nobre et al., 2015). β -Galactooligosaccharides, which function as effective prebiotics for bifidobacteria population promotion (Panesar et al., 2018), and can be efficiently synthesized by GH35 and GH2 β -galactosidases (Gao et al., 2019; Yang et al., 2025). In addition, glycoconjugates of diverse bioactive compounds, such as terpenoids (Chang et al., 2025; Kotik et al., 2023) and phenolic acids (de Boer et al., 2023) have been efficiently synthesized using GH1 and GH3 β -glycosidases. The glycosylation for these compounds typically exhibit enhanced solubility and bioavailability (Hofer, 2016; Zhao et al., 2019).

Transglycosylations catalyzed by retaining enzymes are typically accompanied by significant competing-hydrolysis. Because water molecule competes with the glycosyl acceptor in the deglycosylation step (Fig. 1-1B), transglycosylation/hydrolysis ratio is dependent on the glycosyl acceptor concentration (commonly 1 μ M - 1 M) relative to 56 M water molecule in aqueous condition, which is an important characteristic that varies among retaining GHs and ultimately determines their industrial efficiency. For instance, GH70 4,6- α -glucanotransferases catalyzed 56% of hydrolysis (D-glucose releasing) compared to transglucosylation, in reaction with 40 mM maltopentaose (G5) substrate (Leemhuis, Dijkman, et al., 2013). In clan-H of GH enzymes (clan means groups of families sharing a fold and catalytic mechanism), GH13 and GH77 4- α -glucanotransferases exhibited high transglycosylation specificity: maltotriose (G3) is a 5000-fold better acceptor than water (Kaper et al., 2007). Increasing the hydrophobicity at acceptor-binding sites, by site-directed mutagenesis to decrease water acceptor specificity, has successfully improved yields of the transglucosylation products such as cyclodextrin (Veen et al., 2001) and chitin (Zakariassen et al., 2011).

1.5. Functions, structures, and applications of GH15 anomer-inverting glucohydrolases

Most identified GH15 enzymes are α -glucohydrolases, including glucoamylases (GAase; EC: 3.2.1.3),

glucodextranase (GDase, EC: 3.2.1.70), α,α -trehalase (EC: 3.2.1.28), and isomaltose glucohydrolase (IGHase, EC: 3.2.1.205), which hydrolyze α -(1 $\rightarrow$ 4)-glucan, α -(1 $\rightarrow$ 6)-glucan, trehalose and IG2, respectively, to produce β -D-glucose from the nonreducing end of substrates.

Crystal structures of GH15 GAase and GDase in complex with acarbose (Aleshin et al., 2003; Coutinho & Reilly, 1994; Mizuno et al., 2004; Sauer et al., 2000) are compared. GH15 catalytic domain is commonly folded into an $(\alpha/\alpha)_6$ -barrel (Sauer et al., 2000). This fold is organized as a two-layered toroid, in which the inner layer consists of even-numbered helices and the outer layer contains odd-numbered helices (Fig. 1-2A; Aleshin et al., 2003). Two Glu residues, which catalytically function as general acid and base, position in $\alpha 5$ - $\alpha 6$ loop and $\alpha 11$ - $\alpha 12$ loop, respectively (Fig. 1-2C). Except this Glu-Glu catalytic dyad, several charged residues, including Arg and Asp on $\alpha 2$ helix, are highly conserved in subsite -1 (Fig. 1-3A). A completely conserved Trp in $\alpha 1$ - $\alpha 2$ loop formed CH- π interaction with the C6 atom of the valienamine unit at subsite -1, and the other completely conserved Tyr in $\alpha 9$ - $\alpha 10$ loop stacking interacted with the 6-deoxy-D-glucose moiety at subsite +1. Mutations of them occurred total loss of activity for a yeast GAase (Coutinho & Reilly, 1994; Itoh et al., 1989). The nucleophilic water at the β -face is coordinated by the general base Glu and 6-OH group of valienamine moiety, conserved in both GH15 GAase and GDase. This catalytic-water-mediated hydrogen bond is predicted to be essential for catalysis of GH15 glucohydrolases (Aleshin et al., 2003; Tagami et al., 2025).

GH15 GAases represent one of the oldest GHs, and are among the most extensively utilized industrial GH enzymes for starch saccharification (Ge et al., 2018; Liaw et al., 2000). D-Glucose produced from corn starch by GAase is widely used for high fructose corn syrup production (Kaneko et al., 2000). It is also utilized by *Saccharomyces cerevisiae* for continuous ethanol production (Li & Mitchinson, 2014), and promotes yeast fermentation to improve flavors in beverage industry (Durán et al., 2021) and bakery (Struyf et al., 2017).

1.6. Functions and structures of GH15 DDase and its homologues

As previously mentioned, DDase is a non-Leloir α -GT which uses dextrin or maltooligosaccharides (MOSs) as D-glucosyl donor to produce dextran via continuous α -(1 $\rightarrow$ 6)-transglucosylation (Hehre, 1951). DDase catalyzes three-types of reaction: first, α -(1 $\rightarrow$ 4)-transglucosylation, which transfers D-glucosyl residue from the nonreducing end of MOS donors to the nonreducing end of MOS acceptors to form α -(1 $\rightarrow$ 4)-linkage;

Second, α -(1 $\rightarrow$ 6)-transglucosylation from MOS donors to MOS acceptors to form α -(1 $\rightarrow$ 6)-linkage; Third, α -(1 $\rightarrow$ 6)-transglucosylation from IMO donors to IMO acceptors (Yamamoto et al., 1993). Based on sequence similarity, DDase has been classified into GH15 together with previously mentioned inverting α -glucohydrolases like GAase and GDase. Sequence similarity network analysis for 236 GH15 enzymes reveals further details for comparison of GH15 α -GTs and inverting GHs. It includes characterized fungal GAase (PDB entry: 1AGM), bacterial GAase (PDB entry: 1LF9), bacterial GDase (PDB entry: 1ULV), bacterial IGHase (PDB entry: 7C25) and DDase (PDB entry: 9JU0). Four groups are displayed (Fig. 1-2B), they are fungal GAases, bacterial/archaeal GAases and GDases, DDase-like enzymes and a single IGHase, respectively.

Sequence alignment of 21 DDase-like enzyme group and bacterial/archaeal glucohydrolases group showed that DDase-like enzymes share the same catalytic Glu-Glu dyad with GH15 inverting glucohydrolases (Fig. 1-2B and C). General acid catalyst Glu together with its N-terminal-adjacent Trp and C-terminal-adjacent Glu are completely conserved among all GH15 enzymes. However, residues adjacent to general base catalyst Glu in α 11- α 12 loop, are differently conserved in retaining and inverting GH15 enzymes.

Crystal structure of C-terminally truncated form of DDase- Δ 382C (Tagami et al., 2025), is organized as a dimer, and its catalytic domain is folded in an $(\alpha/\alpha)_6$ -barrel, similar to GH15 glucohydrolases. Its Glu671 and Glu858 are spatially aligned with the GH15 glucohydrolase general acid and base catalysts, respectively. Compared to GH15 glucohydrolases, Glu858 is approximately 1.2-1.6 Å closer to the acarbose equivalent anomeric carbon, facilitating its role as a nucleophile catalyst in the double-displacement mechanism (Fig. 1-3B). Spatial position of Glu858 is predicted to be exquisitely tuned by the α 11- α 12 loop and subunit interactions with domain N1 of the other protomer for its nucleophile catalyst (Tagami et al., 2025).

Another characterized GH15 α -GT is the C-terminal catalytic domain of a cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) from *Thermoanaerobacter thermocopriae* (TtCITase). It exhibited α -(1 $\rightarrow$ 6)-transglucosylation activity to MOSs, and produced IMOs up to DP 8 from G5 as initial substrate (Jeong et al., 2019). Recently, genome mining suggested that other possible retaining α -GT homologues of DDase and TtCITase are present in different domain organizations (Fig. 1-2D).

Tepidibacillus decaturensis, a rod-shaped Gram-negative bacterium, possesses U473_RS02300, which encodes a hypothetical extracellular protein GH15 Td46GT (NCBI Reference Sequence, WP_068722961.1) with a typical secretory signal sequence (Met1–Ala32). Td46GT is a 920-residue DDase-like protein,

possibly composed of domains N2 and A and a C-terminal stretch (Fig. 1-2D). Its GH15-like catalytic domain A exhibits sequence identity (29.8%) with that of DDase, and less sequence identity (11–17%) with GH15 glucohydrolases.

Pseudoclostridium thermosuccinogenes, a rod-shaped Gram-positive bacterium, possesses CDO33_RS17035, which encodes a hypothetical extracellular protein GH15 Pt46GT (NCBI Reference Sequence, WP_161496402.1) with a typical secretory signal sequence (Met1–Ala33) and shares high sequence similarity with domain A of Td46GT (46%). Pt46GT is predicted to possess an N-terminal carbohydrate binding module (CBM) 35 domain, which was not found in either Td46GT or DDase (Fig. 1-2D).

Both Td46GT and Pt46GT lack the domain N1 of DDase and the GH66 CITase domain of TtCITase. Therefore, the absence of these domains in Td46GT and Pt46GT raises questions regarding their enzymatic functions and substrate preference.

1.7. The purpose of this study

As previously described, GH15 DDase-like enzymes show a variety of domain organizations (Fig. 1-2), but most of them lack both structural and functional characterization. This study firstly biochemically characterized two 46GTs, Td46GT and Pt46GT. In chapter 2, the mechanism for IMO synthesis from MOS of Td46GT and Pt46GT was biochemically elucidated, by understanding the donor-substrate specificity and acceptor-substrate specificity. In chapter 3, the crystal structure of Td46GT was determined by X-ray crystallography. The calcium ion-dependent thermostability and the unique C-terminal domain sugar binding ability of Td46GT was also discussed. In chapter 4, the molecular basis important for donor and acceptor specificity of Td46GT, was analyzed by site-directed mutagenesis. Finally, the molecular basis that determine retaining/inverting reaction specificity between GH15 α -GTs and glucohydrolases was elucidated by a conversion of Td46GT to an anomer-inverting glucohydrolase through multiple mutagenesis.

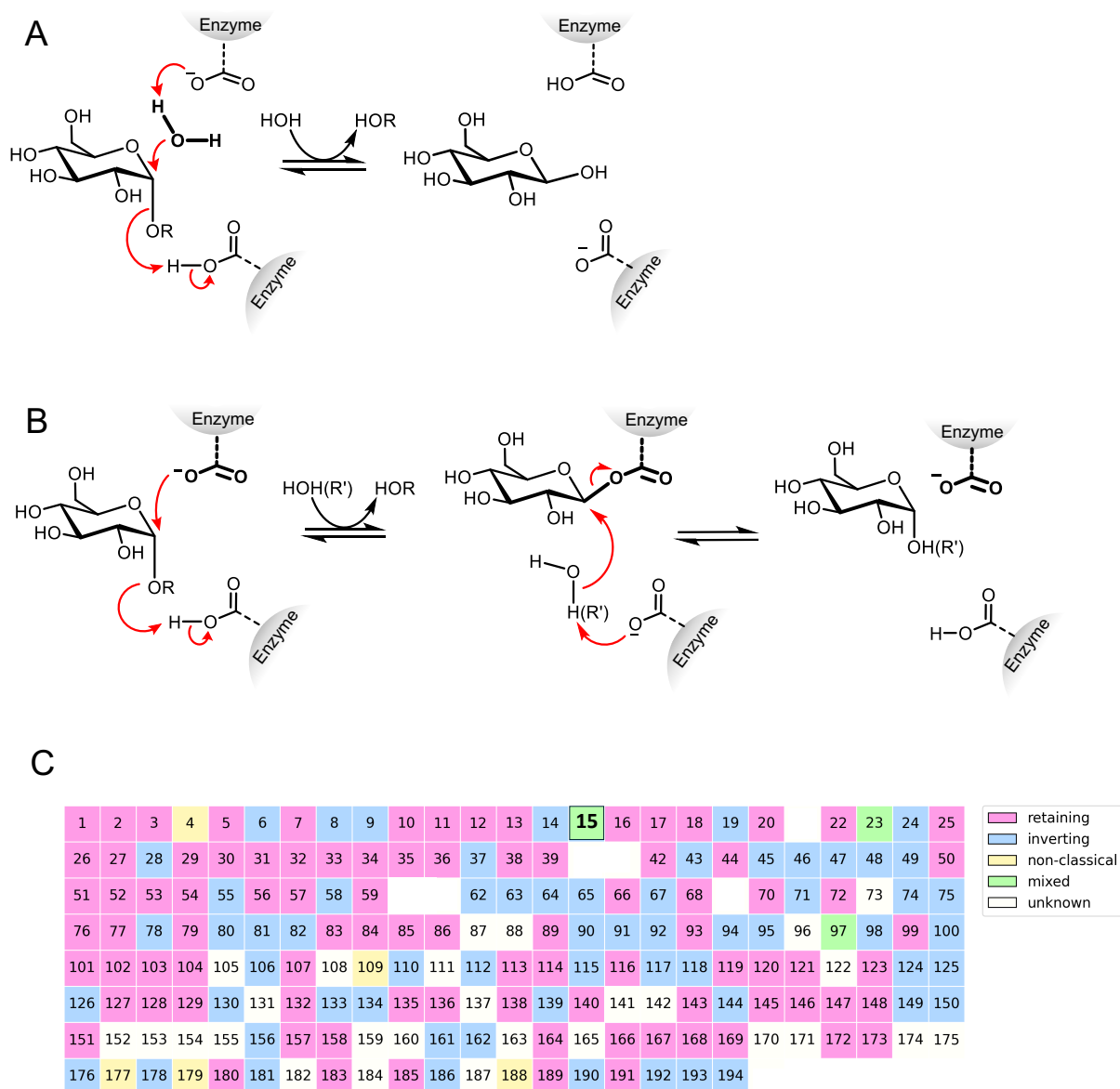


Fig. 1-1. Reaction mechanisms and classification of glycoside hydrolases

A, single-displacement mechanism of inverting glycoside hydrolase (GH). Nucleophile water facilitated by general base catalyst is shown in bold. B, double-displacement mechanism of retaining GH. Nucleophile catalytic residue is shown in bold. Red arrows represent the electron flow. The exo-acting α -glycoside hydrolases are used as example in A and B panels. C, sequence-based classification of GHs in Carbohydrate-Active enZyme database (CAZy). GH21, 40, 41, 60, 61 and 69 have been deleted from CAZy database. GH15 (in this study), GH23 and GH97 contain both retaining and inverting enzymes.

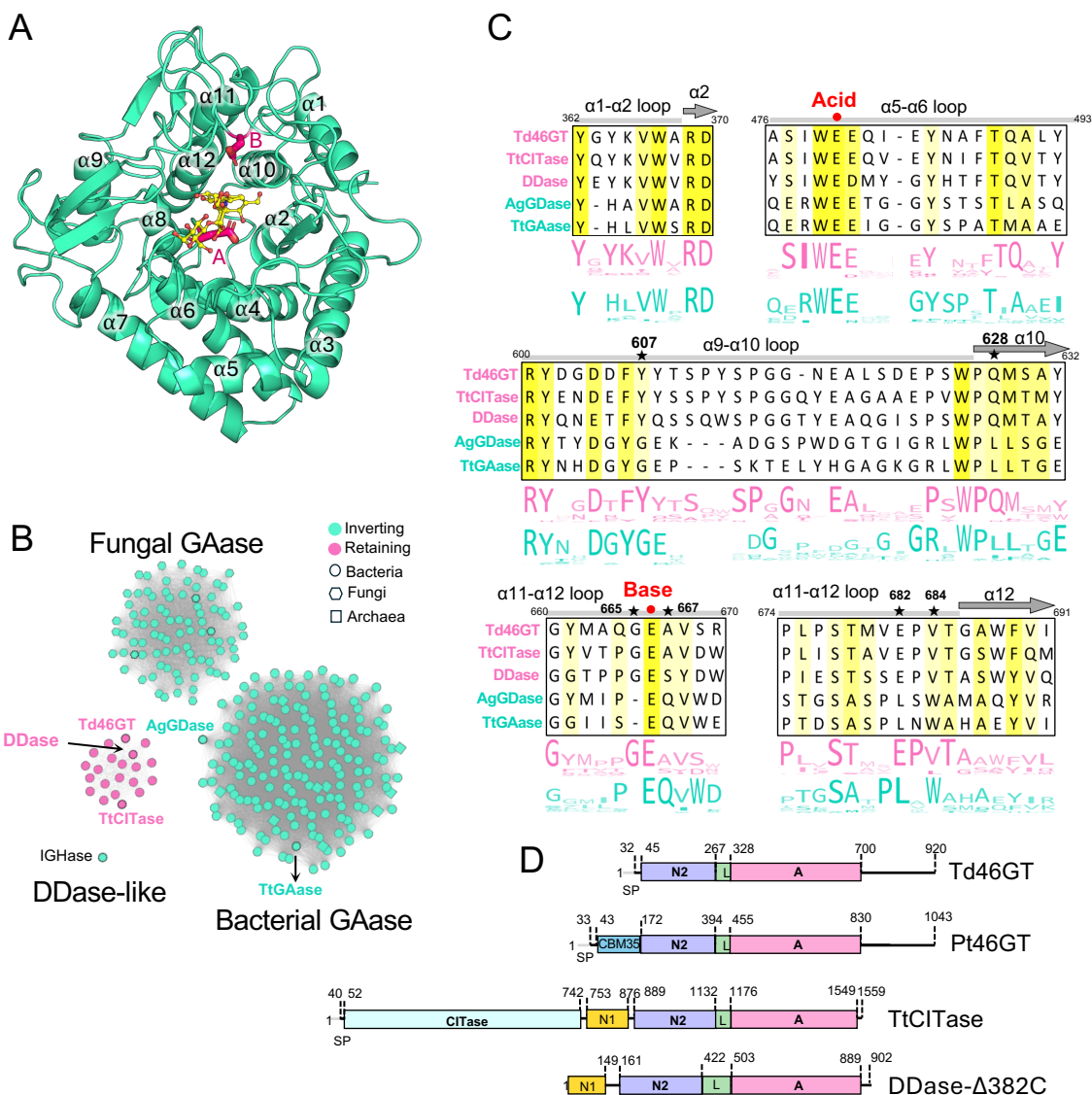


Fig. 1-2. Comparison of sequence similarity and domain organization of glycoside hydrolase family 15 (GH15) enzymes.

A, GH15 catalytic domain structure of *Thermoanaerobacterium thermosaccharolyticum* glucoamylase (TtGAase; PDB: 1LF9) in complex with acarbose (yellow). Symbol “A” in red represents general acid catalyst. Symbol “B” in red represents general base catalyst. B, sequence similarity network of 236 GH15 enzymes. Pink, DDase and its homologues. Green, inverting glucohydrolases. C, Sequence alignment of the GH15 catalytic domain and consensus logo of GH15 enzymes. D, domain organization comparison of GH15 glucosyltransferases. DDase, dextran dextrinase. Td46GT and Pt46GT, α-glucan 4(6)-α-glucosyltransferase from *Tepidibacillus decaturensis* and *Pseudoclostridium thermosuccinogenes* (in this study). TtCITase, *Thermoanaerobacter thermocopriae* JCM 7501 cycloisomaltooligosaccharide glucanotransferase (NCBI accession: WP_028992696.1). AgGDase, *Arthrobacter globiformis* I42 glucodextranase (PDB: 1ULV). IGHase, *Kribbella flavida* DSM 17836 isomaltose hydrolase (PDB: 7C25).

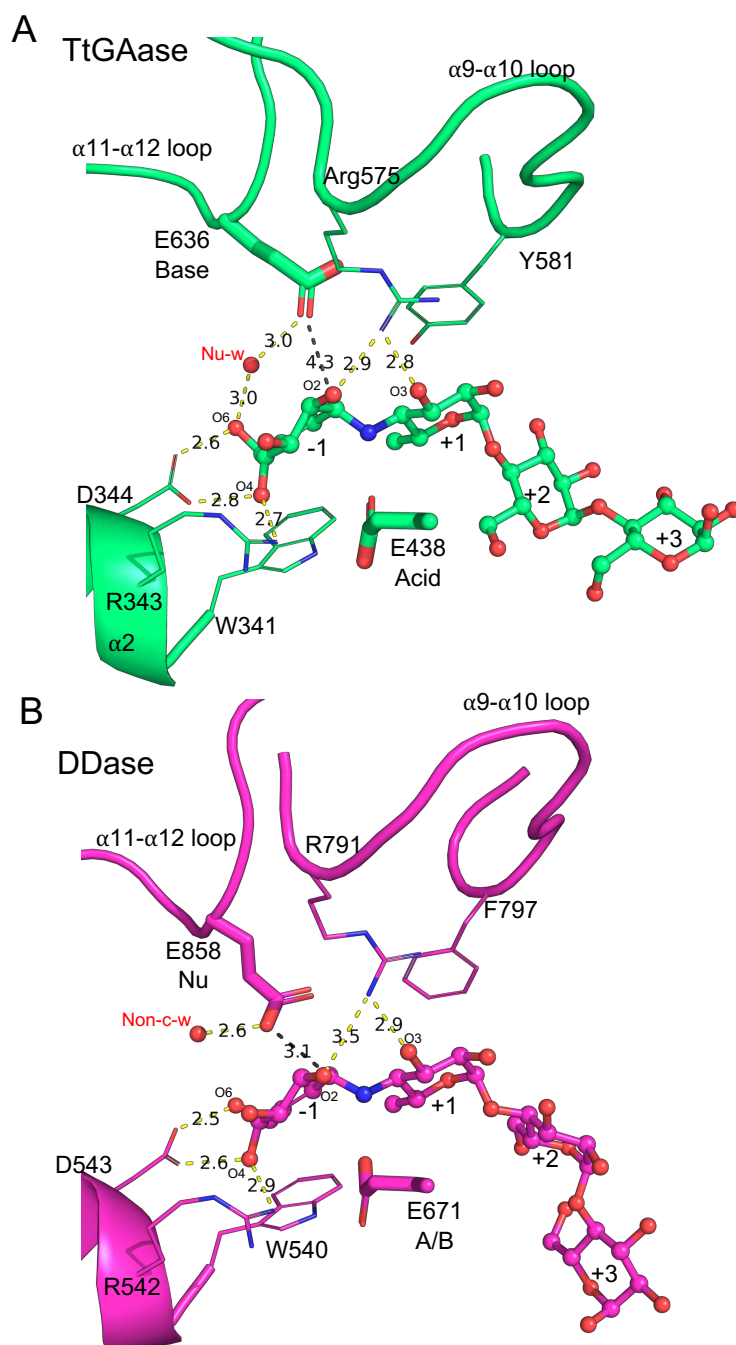


Fig. 1-3. Structural comparison of the active sites of GH15 enzymes.

A, *Thermoanaerobacterium thermosaccharolyticum* glucoamylase in complex with acarbose (TtGAase; PDB entry: 1LF9). B, DDase- $\Delta 382$ C in complex with acarbose (PDB entry: 9JU0). Predicted hydrogen bonds are shown in yellow dashed lines. Distance between general base/nucleophile (Nu) catalyst and anomeric carbon is shown in black (4.3 and 3.1 Å, respectively). A/B, acid/base catalyst. Nucleophilic water (Nu-w) for TtGAase and non-catalytic water (Non-c-w) observed at β -face in subsite -1 for DDase are shown in red sphere.

2. Functional analysis of Td46GT and Pt46GT

2.1. Introduction

As described above, two hypothesized proteins, Td46GT and Pt46GT, are predicted as DDase homologues, but have different domain organizations from DDase. In this chapter, the enzymatic function of Td46GT and Pt46GT were analyzed. They were identified as new α -GTs that catalyze successive transglucosylation to produce IMOs from MOSs.

2.2. Materials and methods

2.2.1. Materials

D-Glucose (Fujifilm Wako Pure Chemical, Osaka, Japan), maltose (G2; Nihon Shokuhin Kako, Tokyo, Japan), G3 (Nihon Shokuhin Kako), maltotetraose (G4; Nihon Shokuhin Kako), G5 (Nihon Shokuhin Kako), maltohexaose (G6; laboratory stock), panose (Pan; Nagase Viita, Okayama, Japan), IG2 (Tokyo Chemical Industry, Tokyo, Japan), IG3 (Tokyo Chemical Industry), isomalotetraose (IG4; Seikagaku, Tokyo, Japan), were used.

2.2.2. Construction of expression plasmids for Td46GT and Pt46GT

The Td46GT gene was amplified from genomic DNA of *T. decaturensis* DSM 103037 (DSMZ, Braunschweig, Germany) by PCR with primers (5' -ATGGATCGAATCATGAAAAA-3' and 5' -TTACTCTACATTACCATAAT-3') and KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). The DNA fragment encoding mature Td46GT without N-terminal signal peptide (Met1 – Ala32), predicted by SignalP 4.1 server (Nielsen, 2017), was amplified by PCR using the primers 5' -TTTAAGAAGGAGATATACATATGGATACATTACCCACTGA-3' and 5' -CAGTGGTGGTGGTGGTGGTGCTCTACATTACCATAATACC-3' (the Td46GT gene sequence underlined).

The Pt46GT gene was amplified from genomic DNA of *P. thermosuccinogenes* DSM 5807 (DSMZ) by PCR with primers (5' -ATGAATGGAGGTTTTGTTAT-3' and 5' -CTATTCAAAATTACCGTATA-3') and KOD FX Neo DNA polymerase. The DNA fragment encoding mature Pt46GT without N-terminal signal peptide (Met1–Ala33), predicted by SignalP 4.1 server (Nielsen, 2017), was amplified by PCR using the primers 5' -TTTAAGAAGGAGATATACATATGTTCAAYACCATATCTGC-3' and 5' -

CAGTGGTGGTGGTGGTGGTGGTTTCAAAATTACCGTATAACC-3' (the Pt46GT gene sequence underlined).

Amplified DNA fragments of Td46GT and Pt46GT were inserted into pET-23a (Novagene, Darmstadt, Germany) using an In-Fusion HD Cloning Kit (Takara Bio, Kusatsu, Japan). The inserted DNA and flanking regions were sequenced using an Applied Biosystems 3130 genetic analyzer (Life Technologies, Carlsbad, CA, USA).

2.2.3. Production and purification of Td46GT and Pt46GT

Transformants of *Escherichia coli* BL21(DE3) harboring the expression plasmid were grown in 1 L of LB medium containing 100 µg/mL ampicillin until $A_{600} = 0.5$. Recombinant protein production was induced at 18 °C for 22 h in the presence of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial cells, collected by centrifugation ($8,000 \times g$ at 4 °C for 5 min), were suspended in 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer A), and lysed by sonication on ice using Sonifier 250 (Branson, Danbury, CT, USA), under the conditions of duty cycle 50%, output control 3, and 1 min, three times. Cell free extract, obtained by centrifugation ($15,000 \times g$ at 4 °C for 10 min), was subjected to Ni²⁺-immobilized affinity column chromatography using Chelating Sepharose Fast Flow (2.6 cm I.D. $\times$ 3 cm; Cytiva, Tokyo, Japan). After washing the column with buffer A containing 30 mM imidazole, the adsorbed protein was eluted using a linear gradient of 30-500 mM imidazole in buffer A (elution volume, 160 mL). Purity of the fractions was judged using SDS-PAGE with 3 µg of Td46GT and 1 µg of Pt46GT. The pooled fractions were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and stored at 4 °C. Proteins concentrations of purified Td46GT and Pt46GT were determined by amino acid analysis using an amino acid analyzer L-8900 (Hitachi High-Technologies, Tokyo, Japan) after complete hydrolysis of the protein in 6 M HCl at 110 °C for 24 h. The specific coefficient at A_{280} was determined to be 2.47 for 1 mg/mL of Td46GT and 2.36 for 1 mg/mL of Pt46GT.

2.2.4. Blue native PAGE of Td46GT

An analytical sample of Td46GT (1 µg) was prepared using a Native PAGE Sample Prep Kit (Life Technologies). Electrophoresis was conducted using Native PAGE Novex 4–16% Bis-Tris Gels (Life Technologies) at 150 V for 60 min and 200 V for 60 min on ice. Molecular mass was determined using the

NativeMark Unstained Standard (Life Technologies).

2.2.5. Time course of the reaction of Td46GT and Pt46GT with MOSs

A reaction mixture (1200 μ L), containing 1.07 μ M Td46GT or 0.351 μ M Pt46GT (0.094 U/mL), 12 mM G5, 42 mM sodium acetate buffer (pH 4.5), 0.2 g/L bovine serum albumin (BSA) (Nacalai Tesque, Kyoto, Japan), was incubated at 37 °C. An aliquot (180 μ L) of the reaction mixture taken at the indicated time was heated at 100 °C for 3 min to stop the reaction. In the thin-layer chromatography (TLC) analysis of the reaction mixture, the sample (25 μ g of carbohydrate) was applied on silica gel 60 F₂₅₄ plate (Merck, Darmstadt, Germany) and developed twice using nitromethane/ethanol/water/1-propanol (2/3.5/4/5.5, v/v/v/v). GAase treatment was carried out by incubating the sample at 50 °C for 30 min in the presence of 42 U/mL *Rhizopus niveus* GAase (Fujifilm Wako Pure Chemical). For high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis, tenfold diluted samples were desalted with Amberlite MB-4 (Organo, Tokyo, Japan) and analyzed under the following conditions: injection volume, 10 μ L; column, Carbowax PA1 (4 mm I.D. $\times$ 250 mm; Thermo Fischer Scientific, Waltham, MA, USA); column temperature, 30 °C; elution, linear gradient of 0–200 mM sodium acetate in 200 mM NaOH (in 30 min); flow rate, 0.8 mL/min. As an internal standard, 100 μ M myo-inositol was used.

Time course of the reaction with G4 and IG2, as donor and acceptor substrates, respectively, was monitored at 37 °C for 1.5 h in 1 mL of a reaction mixture, containing 52 mM G4, 87 mM IG2, 440 nM Td46GT, 50 mM sodium acetate buffer (pH 4.5) and 0.05 g/L BSA. An aliquot (10 μ L) was taken at the indicated time, diluted tenfold by water, and heated at 100 °C for 3 min to terminate the reaction. Saccharide concentrations were measured using HPAEC-PAD as described above.

2.2.6. Preparation and structural analysis of 6^{II}-O- α -isomaltosylmaltose

A reaction mixture (10 mL), containing 472 mM G2, 218 nM Td46GT, 42 mM sodium acetate buffer (pH 4.5), and 0.2 g/L BSA, was incubated at 37 °C for 96 h and boiled to terminate the reaction. Bakery yeast-fermentable sugar in the sample was degraded by incubation with 20 g/L dry yeast (Nissin Food, Tokyo, Japan) at 37 °C for 3 h. The sample was boiled, desalted with Amberlite MB-4, and filtered through a 0.45 μ m cellulose acetate filter (Advantec Toyo, Tokyo, Japan). The reaction products were separated by gel filtration column chromatography using a Toyopearl HW-40S (5.0 cm i.d. $\times$ 100 cm; Tosoh, Tokyo, Japan)

with water as the mobile phase. 6^{II}-*O*- α -Isomaltosylmaltose-containing fractions were collected and lyophilized. The molecular mass of the product was measured using electrospray ionization-mass spectrometry (ESI-MS) by an Exactive Plus (Thermo Scientific, San Jose, CA, USA). The nuclear magnetic resonance (NMR) spectra were recorded in D₂O (Fujifilm Wako Pure Chemical) at 27 °C using Avance Neo (500 MHz; Bruker, Billerica, MA, USA). A series of one-dimensional (¹H and ¹³C) and two-dimensional homo- and heteronuclear correlated spectra (heteronuclear single quantum coherence (HSQC), HSQC total correlation spectroscopy, heteronuclear 2-bond correlation, and heteronuclear multiple bond correlation (HMBC)) were acquired to determine the chemical structure of the reaction product. Purity of 6^{II}-*O*- α -isomaltosylmaltose was evaluated by high performance liquid chromatography (HPLC) analysis under following conditions: sample injection volume, 10 μ L; column, tandem 2 HILIC Pak VG-50 4E (4.6 mm i.d. $\times$ 250 mm; Shodex, Tokyo, Japan); column temperature, 40 °C; eluent, 75% (v/v) acetonitrile, 25% (v/v) water; flow rate, 1 mL/min; detection, refractive index.

2.2.7. Standard enzyme activity assay for Td46GT and Pt46GT

The rate of D-glucose production from G2 was measured. A reaction mixture (50 μ L), containing an appropriate concentration of enzyme, 42 mM sodium acetate buffer (pH 4.5), 0.2 g/L BSA, and 20 mM G2, was incubated at 37 °C for 10 min. Reaction was terminated by incubating the samples at 80 °C for 3 min. After centrifugation (16,000 $\times$ g at 4 °C for 10 min) of the sample to remove insoluble material, D-glucose production was determined using the Glucose CII-test (Fujifilm Wako Pure Chemical).

2.2.8. Effects of pH and temperature on activity and stability

The optimum pH and temperature of Td46GT and Pt46GT were investigated in the same manner as in the standard assay, with a few modifications. To determine the optimum pH, 80 mM Britton Robinson buffer (the pH of a mixture of 80 mM acetic acid, phosphoric acid, and glycine was adjusted using 6 M NaOH in the range pH of 2.5–10) was used. For optimum temperature determination, the enzyme reaction was performed in the range of 30–80 °C. To evaluate stable ranges, the enzyme was subjected to pH and temperature treatments as follows: pH treatment, Td46GT or Pt46GT was incubated in 20 mM Britton Robinson buffer (pH 2.5–10) at 4 °C for 20 h; temperature treatment, the enzyme was incubated in 67 mM sodium acetate buffer (pH 4.5) at 30–80 °C for 15 min. Residual activity was measured using the standard

assay.

2.2.9. Kinetic parameters in a single-substrate reaction

The reaction rates on the following substrates were measured under the conditions of the standard enzyme assay: 5–120 mM G2, 1–24 mM G3, 0.5–50 mM G4, 0.6–18 mM G5, 10–120 mM IG2, 0.2–20 mM IG3, 0.2–20 mM IG4, and 4–20 mM panose. The velocity of D-glucose release was measured in reactions with G2 and IG2. Aglycone production was monitored using HPAEC-PAD in the presence of other substrates. The Michaelis-Menten equation was fitted to the initial reaction rates at various substrate concentrations by nonlinear regression using Grafit version 7.0.2 (Erithacus Software, East Grinstead, UK) to determine the kinetic parameters. Subsite affinities were calculated from k_{cat}/K_m values using Eq. 2-1 (Hiromi et al., 1973):

$$A_{+n} = RT \ln \{ (k_{\text{cat}}/K_m)_{\text{Gn}+1} / (k_{\text{cat}}/K_m)_{\text{Gn}} \} \quad (\text{Eq. 2-1})$$

in which R is the gas constant and T is the absolute temperature.

2.2.10. Relative acceptor-substrate specificity constant in a two-substrate reaction

The relative acceptor-substrate specificity constant (RASC), was calculated from the rates and concentration of each acceptor in the reactions, including the following sets of substrates, under standard assay conditions. The substrate concentrations were as follows: 10 mM G5 and 5–20 mM IG2; 10 mM G5 and 10–40 mM IG3; 10 mM G5 and 5–20 mM G2; 10 mM G5 and 50–250 mM Glc; 10 mM G4 and 20 mM IG2; and 7.5 mM G3 and 20 mM IG2. The RASC and affinities of the acceptor-binding subsites were calculated using the Eq. 2-2 and 2-3:

$$\text{RASC} = k_A/k_B = (v_A/[A]) / (v_B/[B]) \quad (\text{Eq. 2-2})$$

$$A_{+n} = RT \ln(k_{\text{Gn}}/k_{\text{Gn}-1}) \quad (\text{Eq. 2-3})$$

2.3. Results

2.3.1. Production and purification of recombinant Td46GT and Pt46GT

C-terminally His₆-tagged mature Td46GT (Asp33–Glu920) was heterologously produced in *E. coli* BL21(DE3) transformant and purified to homogeneity by Ni²⁺-immobilized affinity column chromatography (Fig. 2-1A). From 0.5 L of culture broth, 33 mg of Td46GT was obtained. The specific activity of D-glucose aglycone releasement from 20 mM G2 at 37 °C (pH 4.5) was 0.627 U/mg. Purified Td46GT showed a single

band of 106 kDa and 101 kDa in SDS-PAGE and blue native-PAGE, respectively (Fig. 2-1B and C). These molecular masses are close to the theoretical mass (100 kDa) and Td46GT was judged as monomer under nondenaturing conditions.

C-terminally His₆-tagged mature Pt46GT (Phe34–Glu1049) was heterologously produced in *E. coli* BL21(DE3) transformant and purified to homogeneity by Ni²⁺-immobilized affinity column chromatography (Fig. 2-2). From 1.0 L of culture broth, 9.6 mg of Pt46GT was obtained. The specific activity of D-glucose aglycone releasement from 20 mM G2 at 37 °C (pH 4.5) was 2.56 U/mg. Purified Pt46GT showed a single band of 110 kDa in SDS-PAGE (Fig. 2-2B). The molecular mass (110 kDa) is close to the theoretical mass (114 kDa).

2.3.2. Time course of Td46GT and Pt46GT reactions with MOS

Time-course of the reaction of Td46GT and Pt46GT with 12 mM G5 as substrate was monitored by TLC analysis and HPAEC-PAD (Figs. 2-3 and 2-4). For Td46GT, in the initial stage of the reaction within 3 min, longer and shorter products of various DP s than G5 were detected. The transglucosylation products longer than G5 were almost completely degraded by GAase and eluted at the same retention time as the MOSs standard in the HPAEC-PAD analysis. These results indicate that Td46GT predominantly catalyzed α -(1→4)-transglucosylation in the initial reaction with MOSs. GAase-tolerant α -(1→6)-transglucosylation products, derived from G2 and G3 as initial acceptors, increased at 20 min reaction. Short-chain MOSs such as G2 and G3 remained, while long-chain MOSs of DP ≥ 4 decreased. At 1.5–6 h reaction, D-glucose started to be observed, and trace amount of IMOs (IG2–IG4) was produced, while G3-derived α -(1→6)-oligosaccharides decreased. At 24–72 h reaction, D-glucose increased and IMOs were produced. Panose and its derived oligosaccharides gradually decreased with time-progress of the reaction. These observations suggest that Td46GT catalyzes α -(1 → 4)-transglucosylation to MOS acceptors longer than G3, and α -(1 → 6)-transglucosylation with G3, G2, D-glucose and the nonreducing terminal IMO moiety of the oligosaccharides, in an acceptor-dependent manner. Since no spot remained at the sample application position on the TLC plate in the reaction samples, polysaccharide formation was not considered to have occurred.

Time-course of the reaction of Pt46GT with 12 mM G5 as substrate was monitored by TLC and HPAEC-PAD (Fig. 2-4), the enzyme concentration used for reaction is 0.094 U/mL (20 mM G2, pH 4.5, 37°C) which was same with that of Td46GT (Fig.2-3). The product profile of Pt46GT highly resembled that of Td46GT,

but 6^{III}-*O*- α -glucosyl maltotriose (B4) and B4-derived α -(1 $\rightarrow$ 6)-oligosaccharides concentrations were lower in Pt46GT than Td46GT. Until 72 h reaction, production of IMOs up to DP 5 was observed. They were slightly shorter than Td46GT, which produced IG6 at the final stage of the reaction. These observations indicate that Pt46GT catalyzes similar reaction with Td46GT but holds possibly different specificity for substrate chain-length.

The reaction products obtained by using G2 as the starting substrate were also investigated (Fig. 2-5). Panose and panose-derived 6^{II}-*O*- α -isomaltosylmaltose were produced in the early reaction (1–4 h). IMOs with DP of 2–4 (IG2–IG4), which are products of α -(1 $\rightarrow$ 6)-transglucosylation to D-glucose and IMO acceptors, were produced in the later reaction (7–10 h). The tetrasaccharide derived from panose as acceptor (Glc-Pan) was separated from the reaction mixture by gel filtration column chromatography, and the chemical structure was analyzed by ESI-MS and NMR. ESI-MS detected m/z 689.23 ($[M + Na]^+$), confirming that Glc-Pan is a tetrasaccharide (Fig. 2-6). HPLC analysis of Glc-Pan identified its purity as 94% (Fig. 2-7). The D-glucosyl residues are numbered from the reducing end of Glc-Pan as Glc_I–Glc_{IV}. The chemical shifts of Glc_I–Glc_{III} matched well with those of the corresponding D-glucose units of panose (Best et al., 2002) except for C-6 of Glc_{III} (Table 2-1) of δ_c 66.3 ppm of Glc-Pan, which was higher than of the non-reducing end D-glucosyl residue of panose (δ_c 62.7 ppm) (Fig. 2-8). Correlation peaks between C-6 of Glc_{III} and H-1 of Glc_{IV} and between H-6 of Glc_{III} and C-1 of Glc_{IV} were observed in HMBC (Fig. 2-9). The $J_{1,2}$ of Glc_{IV} was 3.9 Hz, indicating an α -linkage between Glc_{III} and Glc_{IV}. The combined results indicated that the saccharide Glc-Pan was 6^{II}-*O*- α -isomaltosylmaltose.

2.3.3. Effects of pH and temperature on activity and stability of Td46GT and Pt46GT

The pH and temperature optima for D-glucose-release from G2 by Td46GT were pH 4.5 and 37°C, respectively (Fig. 2-10A and B). This enzyme retained $\geq 90\%$ of the original activity in a pH range of 3.6–8.5 (at 4°C for 20 h), and at $\leq 37^\circ\text{C}$ (at pH 4.5 for 15 min).

The pH and temperature optima for D-glucose-release from G2 by Pt46GT, were pH 4.5 and 50°C, respectively (Fig. 2-10C and D). This enzyme retained $\geq 90\%$ of the original activity in a pH range of 3.0–8.5 (at 4°C for 20 h), and at $\leq 50^\circ\text{C}$ (at pH 4.5 for 15 min).

2.3.4. Analysis of the initial reactions of Td46GT with MOSs, panose, and IMOs

Initial reaction products of Td46GT from MOSs, panose and IMOs were investigated by HPAEC-PAD (Fig. 2-11). G5 and G6 were detected as transglucosylation products in the reactions with G4 and G5, respectively. Td46GT mainly produced G4 from G3 but also slightly produced B4. Only α -(1 $\rightarrow$ 6)-transglucosylation products were detected in the reactions with G2, panose and IMOs. As D-glucose was not detected in reactions with substrates longer than disaccharides, Td46GT primarily catalyzed transglucosylation.

Aglycone releasing velocities at various substrate concentrations followed the Michaelis-Menten equation, and kinetic parameters, k_{cat} and K_{m} , were determined (Table 2-2). Td46GT exhibited high $k_{\text{cat}}/K_{\text{m}}$ for MOSs with DP ≥ 3 , and $k_{\text{cat}}/K_{\text{m}}$ for G4 ($76.3 \text{ s}^{-1} \text{ mM}^{-1}$) was highest of MOSs tested. The $k_{\text{cat}}/K_{\text{m}}$ values for G2 ($0.108 \text{ s}^{-1} \text{ mM}^{-1}$), panose ($1.41 \text{ s}^{-1} \text{ mM}^{-1}$), and IMOs ($0.0540\text{--}0.797 \text{ s}^{-1} \text{ mM}^{-1}$) were much lower than those for G3–G5. Td46GT showed very high preference for the α -(1 $\rightarrow$ 4)-linkage at the nonreducing end over α -(1 $\rightarrow$ 6)-linkage.

2.3.5. Analysis of the initial reactions of Pt46GT with MOSs and IMOs

Initial reaction products of Pt46GT from MOSs and IMOs were investigated by HPAEC-PAD (Fig. 2-12). G5 and G6 were detected as transglucosylation products in the reactions with G4 and G5, respectively. Pt46GT produced G4 from G3 but not B4, unlike Td46GT. Only α -(1 $\rightarrow$ 6)-transglucosylation products were detected in the reactions with G2 and IMOs.

Pt46GT exhibited high $k_{\text{cat}}/K_{\text{m}}$ for MOSs with $\geq \text{DP } 3$, and $k_{\text{cat}}/K_{\text{m}}$ for G4 ($75.2 \text{ s}^{-1} \text{ mM}^{-1}$) was highest among MOSs tested. This feature was similar to Td46GT. However, the $k_{\text{cat}}/K_{\text{m}}$ values for G2 ($0.464 \text{ s}^{-1} \text{ mM}^{-1}$) and G3 ($29.9 \text{ s}^{-1} \text{ mM}^{-1}$) were 4.3-fold and 3.8-fold higher than that of Td46GT, respectively. The $k_{\text{cat}}/K_{\text{m}}$ values for IG2 ($0.0219 \text{ s}^{-1} \text{ mM}^{-1}$) and IG4 ($1.98 \text{ s}^{-1} \text{ mM}^{-1}$) were 2.6-fold lower and 2.5-fold higher than that of Td46GT, respectively. The $k_{\text{cat}}/K_{\text{m}}$ value for IG3 ($0.536 \text{ s}^{-1} \text{ mM}^{-1}$) were comparable with Td46GT.

2.3.6. Evaluation of acceptor specificity via two-substrate reaction of Td46GT

The initial reaction in a two-substrate system of 10 mM G5 and 10 mM IG2 was analyzed by HPAEC-PAD (Fig. 2-13A). Increase in G4, G6 and IG3 was observed at rates of 68, 37 and 39 s^{-1} , respectively (Fig. 2-13C). In contrast, the D-glucose (aglycone of IG2 donor) release rate was below the detection limit ($< 0.1 \text{ s}^{-1}$). This indicated that G5 essentially serves as the sole D-glucosyl donor in this two-substrate reaction.

Transglucosylation products, G6 and IG3 were produced from G5 and IG2 as acceptors, at comparable rates: G6, 37 s⁻¹; IG3, 39 s⁻¹ (Fig. 2-13B). Notably, the IG3-production rate was 78-fold higher than in the single-substrate reaction with 10 mM IG2, whereas the rate for G6-production was 1.7-fold lower than with 10 mM G5 single-substrate reaction. The IG2 and G5 two-substrate reaction demonstrated that IG2 was as a good acceptor as G5 for Td46GT, although it was a much poorer donor substrate than G5. Reactions were performed with varying concentrations of IG2 (0–20 mM) and constant concentration (10 mM) of G5. The production rates for G6 formation (v_{G5}) decreased and that for IG3 formation (v_{IG2}) increased with the increasing IG2 concentration (Fig. 2-13C). The rate ratios for IG3 and G6 productions (v_{IG2}/v_{G5}) were positively proportional to the acceptor concentration-ratio [IG2]/[G5] (Fig. 2-14B). Based on the reaction scheme shown in Fig. 2-14A, v_{IG2}/v_{G5} is expressed as follows (Cornish-Bowden, 1984, 1993, 2013):

$$v_{IG2}/v_{G5} = (k'_{IG2}/k_{G5}) \cdot ([IG2]/[G5]) \text{ (Eq. 2-4)}$$

where k' and k represent the acceptor-substrate specificity constants for α -(1 $\rightarrow$ 6)- and α -(1 $\rightarrow$ 4)-transglucosylations, respectively, and k_{G5} and k'_{IG2} are defined by Eqs. 2-5 and 2-6:

$$k_{G5} = k_3k_4/(k_{-3} + k_4) \text{ (Eq. 2-5)}$$

$$k'_{IG2} = k_5k_6/(k_{-5} + k_6) \text{ (Eq. 2-6)}$$

The slope of 1.11 of the line shown in Fig. 2-14B, is regarded as the relative acceptor specificity constant (RASC) of IG2 and G5 (k'_{IG2}/k_{G5}). Similarly, linear correlation was obtained in reactions with G2, IG3, and D-glucose in the presence of G5 (Figs. 2-14, 2-15, 2-16 and 2-17). Both α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylation products were obtained in the two-substrate reactions with G2 and G5 (Fig. 2-17A), although only panose was detected as the initial reaction product in the single-substrate reaction with G2 (Fig. 2-11). The k'_{G2}/k_{G5} (2.47) was higher than k_{G2}/k_{G5} (1.31), indicating that G2 was an acceptor more preferable for α -(1 $\rightarrow$ 6)-transglucosylation than for α -(1 $\rightarrow$ 4)-transglucosylation (Fig. 2-14E). RASC for G3 (k_{G3}/k'_{IG2} , 5.58; and k'_{G3}/k'_{IG2} , 0.868) and G4 (k_{G4}/k'_{IG2} , 1.89) were determined from two-substrate reaction in the presence of IG2 (Figs. 2-18 and 2-19), because G3 and G4 served as the predominant D-glucosyl donor in these reaction systems as did G5.

RASCs of Td46GT were compared based on k_A/k'_{IG2} and k'_A/k'_{IG2} (Table 2-3). For α -(1 $\rightarrow$ 4)-transglucosylation, k_{G3}/k'_{IG2} was highest, indicating that G3 was the best acceptor, consistent with the fact that Td46GT displayed the highest k_{cat}/K_m to G4 in the single-substrate reaction. For α -(1 $\rightarrow$ 6)-transglucosylation, the k'_{G2}/k'_{IG2} (2.23) was highest, indicating G2 was a better acceptor for α -(1 $\rightarrow$ 6)-

transglucosylation than IMOs. RASC for D-glucose was lower than those of the other acceptors tested.

Time-course of the reaction of Td46GT with 200 mM G5 and 200 mM IG2 as substrate was monitored by TLC and HPAEC-PAD (Fig. 2-20). In the initial stage of the reaction within 5 and 20 min, G3, G4, G6 produced by α -(1→4)-transglucosylation to G5, and IG3 produced by α -(1→6)-transglucosylation to IG2 were detected. In 30 min reaction, IG4, produced from IG3, was detected. In 45 min reaction, IG5, panose, and B4 started to be detected. At 90–180 min reaction, the concentrations of IG3-IG5, panose and B4 were increased, by converse, the concentrations of MOSs significantly decreased. In 2d reaction, IMOs up to DP 7 were produced.

2.3.7. Prediction of product profile during the reaction with MOS and IMO based on specificity constants for donors and acceptors

In the reaction of Td46GT, the total reaction rate (V), the ratio of reaction amount (ξ) by time, can be obtained from the sum of reaction velocity for either donors or acceptors (Eq. 2-7).

$$d\xi/dt = V = \sum v_{donor,i} = \sum v_{acceptor,i} \text{ (Eq. 2-7)}$$

For a glucooligosaccharide with DP i (G_i), its concentration change follows Eq. 2-8.

$$d[G_i]/dt = v_{donor,G_{i+1}} + v_{acceptor,G_{i-1}} - v_{donor,G_i} - v_{acceptor,G_i} \text{ (Eq. 2-8)}$$

From Eq. 2-7 and Eq. 2-8, Eq. 2-9 is obtained.

$$d[G_i]/d\xi = (v_{donor,G_{i+1}} + v_{acceptor,G_{i-1}} - v_{donor,G_i} - v_{acceptor,G_i})/V \text{ (Eq. 2-9)}$$

The rate ratio for G_i as the donor and acceptor, relative to the total reaction rate was calculated using Eq. 2-10 and Eq. 2-11. These calculations used the $(k_{cat}/K_m)_{G_i}$ in the single G_i reaction for relative donor specificity constants and the $RASC_{G_i}$ values for acceptors.

$$v_{donor,G_i}/V = (k_{cat}/K_m)_{G_i}[G_i]/\sum\{(k_{cat}/K_m)_{G_i}[G_i]\} \text{ (Eq. 2-10)}$$

$$v_{acceptor,G_i}/V = RASC_{G_i}[G_i]/\sum\{RASC_{G_i}[G_i]\} \text{ (Eq. 2-11)}$$

Setting 52 mM G4 and 87 mM IG2 as initial conditions for *in silico* simulation of reaction product profile, every saccharide concentration against reaction amount ξ was simulated based on experimentally determined k_{cat}/K_m and RASC values using Python ODE solvers (Sundnes, 2024) (Fig. 2-21A). When ξ increases up to 200 mM, G4 and IG2 decreased to 2 and 42 mM, respectively. G5, α -(1→4)-transglucosylation product, accumulated until ξ reached 41 mM, and decreased at higher ξ concentrations. α -(1→6)-Transglucosylation products, IG3 and IG4, reaches 37 mM and 8 mM, respectively, at ξ of 200 mM.

The products profile in simulation was compared with the experimental values for a 90-min reaction (Fig. 2-21D). Using IG2 concentration aligned in the simulation and experiment, the relationship between ξ and reaction time was obtained as follows (Eq. 2-12; Fig. 2-21B):

$$\xi = -0.0078t^2 + 2.3t \quad (\text{Eq. 2-12})$$

The reaction at 90 min was equivalent to that at 144 mM ξ . From Eq. 2-12, change of total reaction rate ($d\xi/dt$) and the saccharide profiles over the reaction time were calculated (Fig. 2-21C and D). The $d\xi/dt$ linearly decreased during the reaction. The simulation accurately reflected the experimental data showing a decrease in G4 and an increase in G3, G5, IG3 and IG4 (Fig. 2-21D). The codes and data used for generation of Fig. 2-21 has been uploaded to <https://github.com/thank-u-my-mentor/Acceptor-Specificity-in-silico-simulation-program>.

2.4. Discussion

Isomaltosaccharides exhibit various beneficial functions depending on DP (Aizawa et al., 2021; Kohmoto et al., 1988; S. Kumar et al., 2023; Shinoki et al., 2013). Efficient production of high-DP IMOs from starch is required to expand their application in various industries. Previously, Jeong *et al.* demonstrated that the GH15 C-terminal domain of TtCITase produces IMOs with $DP \leq 8$ from MOSs via transglucosylation (Jeong et al., 2019). However, the underlying mechanism has remained unsolved.

In this chapter, the mechanisms for IMO production from MOS by Td46GT and Pt46GT were examined. They are homologues of TtCITase and DDase, but have different domain organizations (Fig. 1-2D). In the reaction with MOS as starting substrate, disproportionation of MOS by α -(1 $\rightarrow$ 4)-transglucosylation dominates initially, in both the reactions of Td46GT and Pt46GT, followed by the production of IMOs via isomaltooligosaccharyl maltose and isomaltooligosaccharyl maltotriose (Figs. 2-3 and 2-4). This change in saccharide profile in the reaction mixture is explained by the acceptor-dependent α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylation as clarified by using two-substrate reactions catalyzed by Td46GT (Table 2-3 and Figs. 2-14 to 2-20). Td46GT catalyzes α -(1 $\rightarrow$ 4)-transglucosylation toward MOS, and α -(1 $\rightarrow$ 6)-transglucosylation toward D-glucose, G2, G3 and IMOs in an acceptor-dependent manner. Therefore, the conversion of MOS into IMO proceeds via disproportionation of MOS by the α -(1 $\rightarrow$ 4)-glucosyl transfer reaction to generate G2 and G3, α -(1 $\rightarrow$ 6)-transglucosylation for G2 and G3 acceptor to accumulate isomaltooligosaccharyl maltose and isomaltooligosaccharyl maltotriose, and transferring D-glucosyl residues from the

isomaltooligosaccharyl maltose and isomaltooligosaccharyl maltotriose to D-glucose to generate IMO. Based on the similar reactions catalyzed by Td46GT and Pt46GT, they were proposed to be named by α -glucan 4(6)- α -GT.

The subsite map of the wild-type Td46GT indicated subsites +2^m and +3^m for MOS, +2ⁱ for IMOs, and +2^p for panose (G2 for α -(1 $\rightarrow$ 6)-transglucosylation) as high affinity subsites. Their affinities determined in the single-substrate reactions (see section 2.2.9 for method details) were 11.1, 5.9, 6.4, and 8.4 kJ mol⁻¹. These results correspond to affinities of +2^m and +3^m for MOS, +2ⁱ for IMOs, determined by two-substrate reaction as 8.2, 4.4, 9.3 kJ mol⁻¹, respectively (Fig. 2-22). These results suggest that subsite binding model is shared by both acceptor and donor substrates.

Although Pt46GT showed k_{cat}/K_m to G4 comparable with Td46GT. Pt46GT showed 3.8-4.3-fold higher k_{cat}/K_m to G2 and G3 than Td46GT. As subsite affinity of +3^m of Pt46GT was 2.4 kJ mol⁻¹, which was 2.5-fold lower than that of Td46GT. High k_{cat}/K_m for G2 and G3, observed in Pt46GT, might be attributed to high affinity at subsite +1 (Fig. 2-22).

Table 2-1. Chemical shifts of the 6^{II}-*O*-isomaltosylmaltose in the ¹H and ¹³C NMR spectra.

Residue	Number	δ _C (ppm)	δ _H (ppm)		<i>J</i> (Hz)
Glc _{IV}	1	98.5	4.96	d	3.9
	2	72.3	3.54	m	
	3	73.9	3.71	m	
	4	70.3	3.42	t	9.2
	5	72.6	3.73	m	
	6	61.3	3.84	m	
Glc _{III}			3.77	m	
	1	98.8	4.96	d	3.4
	2	72.3	3.58	m	
	3	74.1	3.73	m	
	4	70.3	3.50	m	
	5	71.0	3.90	m	
Glc _{II}	6	66.3	3.96	m	
			3.75	m	
	1	100.6	5.39	d	3.3
	2	72.1	3.59	m	
	3	73.9	3.70	m	
	4	70.3	3.46	m	
α-Glc _I	5	71.0	3.90	m	
	6	66.8	3.99	m	
			3.75	m	
	1	92.7	5.22	d	3.3
	2	72.0	3.59	m	
	3	74.0	3.97	m	
β-Glc _I	4	78.2	3.64	m	
	5	70.9	3.95	m	
	6	61.5	3.88	m	
			3.76	m	
	1	96.6	4.64	d	8.0
	2	74.8	3.27	dd	8.0, 9.5
	3	77.0	3.76	m	
	4	78.0	3.64	m	
	5	75.4	3.61	m	
	6	61.6	3.95	m	
			3.79	m	

d, doublet; dd, double doublet; t, triplet; m, multiplet.

Table 2-2. Kinetic parameters of Td46GT and Pt46GT wild type for single-substrate reaction.

Substrate	Td46GT					Pt46GT				
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	Relative $k_{\text{cat}}/K_{\text{m}}$ (-fold)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	Relative $k_{\text{cat}}/K_{\text{m}}$ (-fold)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	Relative $k_{\text{cat}}/K_{\text{m}}$ (-fold)
Maltose	3.12 ± 0.07	28.9 ± 1.7	0.108	1	9.22 ± 0.28	19.9 ± 1.7	0.463	1	0.463	1
Maltotriose	52.1 ± 3.0	6.63 ± 1.09	7.86	72.8	207 ± 2	6.92 ± 0.24	29.9	64.4	29.9	64.4
Maltotetraose	106 ± 2	1.39 ± 0.14	76.3	706	242 ± 4	3.22 ± 0.14	75.2	162	75.2	162
Maltopentaose	131 ± 3	3.03 ± 0.71	43.2	400	383 ± 23	17.1 ± 1.7	22.4	48.3	22.4	48.3
Isomaltose	9.40 ± 0.60	174 ± 16	0.0540	0.500	N.D.	N.D.	0.0219	0.0472	0.0219	0.0472
Isomaltotriose	N.D.	N.D.	0.640	5.93	N.D.	N.D.	0.536	1.16	0.536	1.16
Isomaltotetraose	N.D.	N.D.	0.797	7.38	N.D.	N.D.	1.98	4.27	1.98	4.27
Panose	N.D.	N.D.	1.41	13.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The values are mean ± standard deviation for triplicate measurements. N.D., not determined because a substrate saturation was not obtained under the analytical conditions (0.2–20 mM).

Table 2-3. Relative acceptor-substrate specificity constants of Td46GT.

Acceptor	k_A/k'_{IG2} (α -(1→4)-transglucosylation)	k'_A/k'_{IG2} (α -(1→6)-transglucosylation)
Maltopentaose	0.901 ± 0.023^a	N.D.
Maltotetraose	1.89 ± 0.07	N.D.
Maltotriose	5.58 ± 0.79	0.868 ± 0.041
Maltose	1.18 ± 0.08^b	2.23 ± 0.02^c
D-Glucose	N.D.	0.0535 ± 0.0080^d
Isomaltotriose	N.D.	0.505 ± 0.028^e

Subscript A indicates acceptor. k_A and k'_A , second-order constants of acceptor A for α -(1→4)-transglucosylation and α -(1→6)-transglucosylation, respectively. ^a, calculated from k'_{IG2}/k_{G5} . ^b, calculated from k_{G2}/k_{G5} and k'_{IG2}/k_{G5} . ^c, calculated from k'_{G2}/k_{G5} and k'_{IG2}/k_{G5} . ^d, calculated from k'_{G1c}/k_{G5} and k'_{IG2}/k_{G5} . ^e, calculated from k'_{IG3}/k_{G5} and k'_{IG2}/k_{G5} . N.D., not detected. The values are mean $\pm$ standard deviation for triplicate measurements.

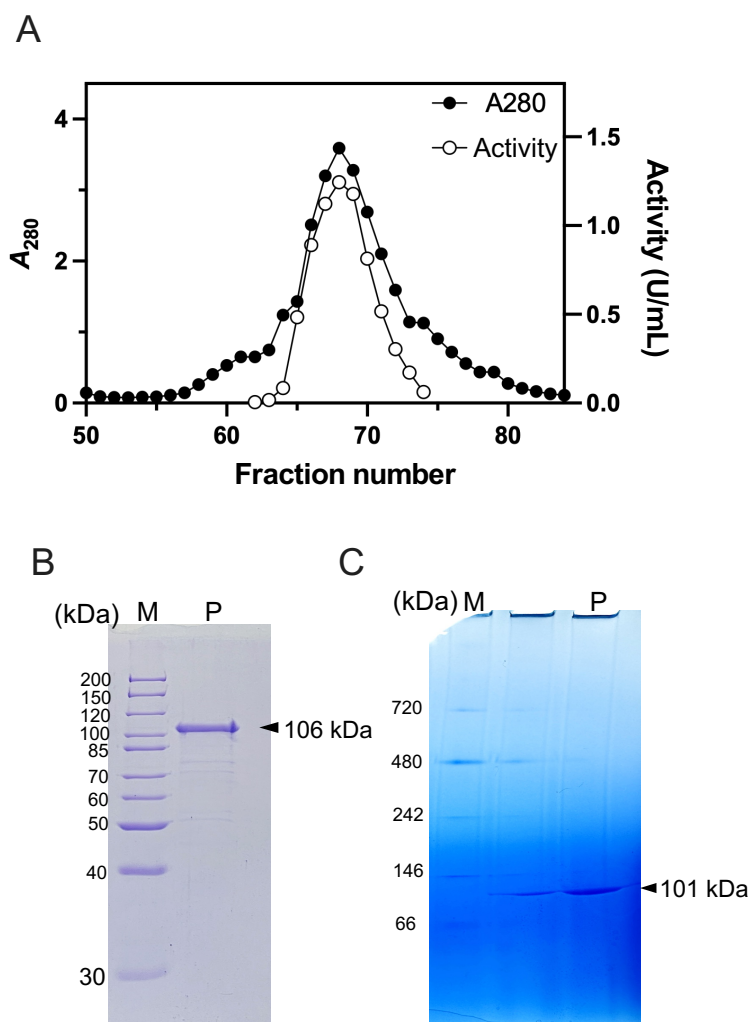


Fig. 2-1. Ni^{2+} -immobilized affinity column chromatography, SDS-PAGE and blue native PAGE analysis of purified Td46GT.

A, elution profile. Column, Ni^{2+} -immobilized Sepharose Fast Flow; equilibration and washing, 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl; elution, 0.03-0.5 M imidazole (elution volume, 160 mL); fraction volume, 5 mL; collected fractions, 65-70 (30 mL). Black circle, absorbance at 280 nm; white circle, activity (U/mL). B, SDS-PAGE. Lane P, 3 μg of purified Td46GT; Lane M, molecular mass standards (kDa). C, blue native-PAGE. Lane P, 1 μg of purified Td46GT; Lane M, molecular mass standards (kDa). Td46GT is indicated by an arrowhead. The theoretical mass of recombinant Td46GT from amino acid sequence is 99.9 kDa.

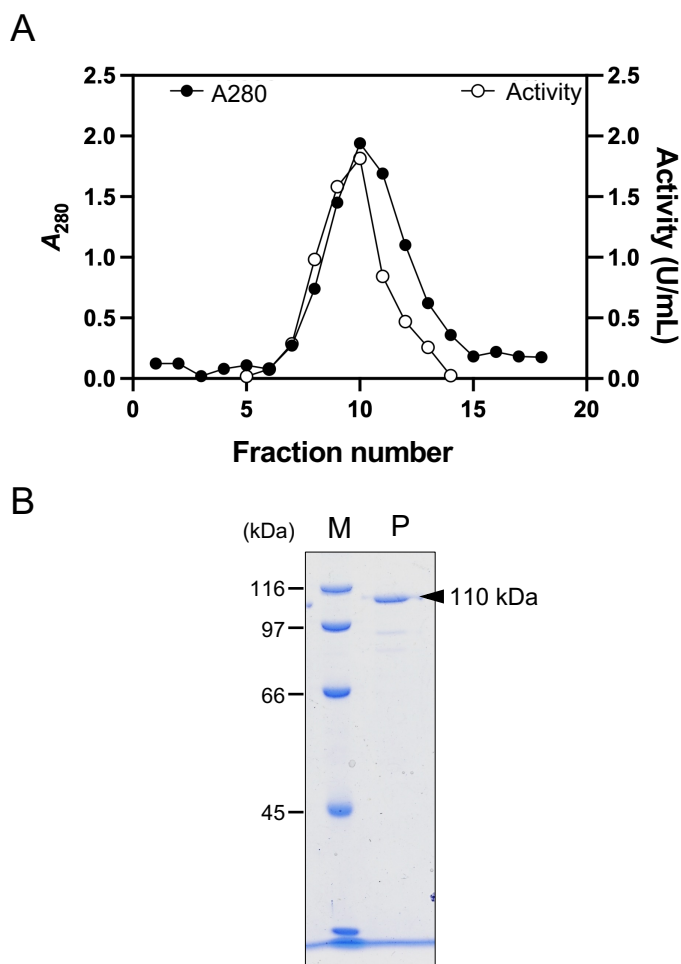


Fig. 2-2. Ni²⁺-immobilized affinity column chromatography and SDS-PAGE analysis of Pt46GT.

A, elution profile. Column, Ni²⁺-immobilized Sepharose Fast Flow; equilibration and washing, 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl; elution, 0.03-0.5 M imidazole (elution volume, 160 mL); fraction volume, 5 mL; collected fractions, 7-10 (20 mL). Black circle, absorbance at 280 nm; white circle, activity (U/mL). B, SDS-PAGE analysis of the recombinant Pt46GT. Lane M, molecular mass standards (kDa). Lane P, 1 µg of purified Pt46GT.

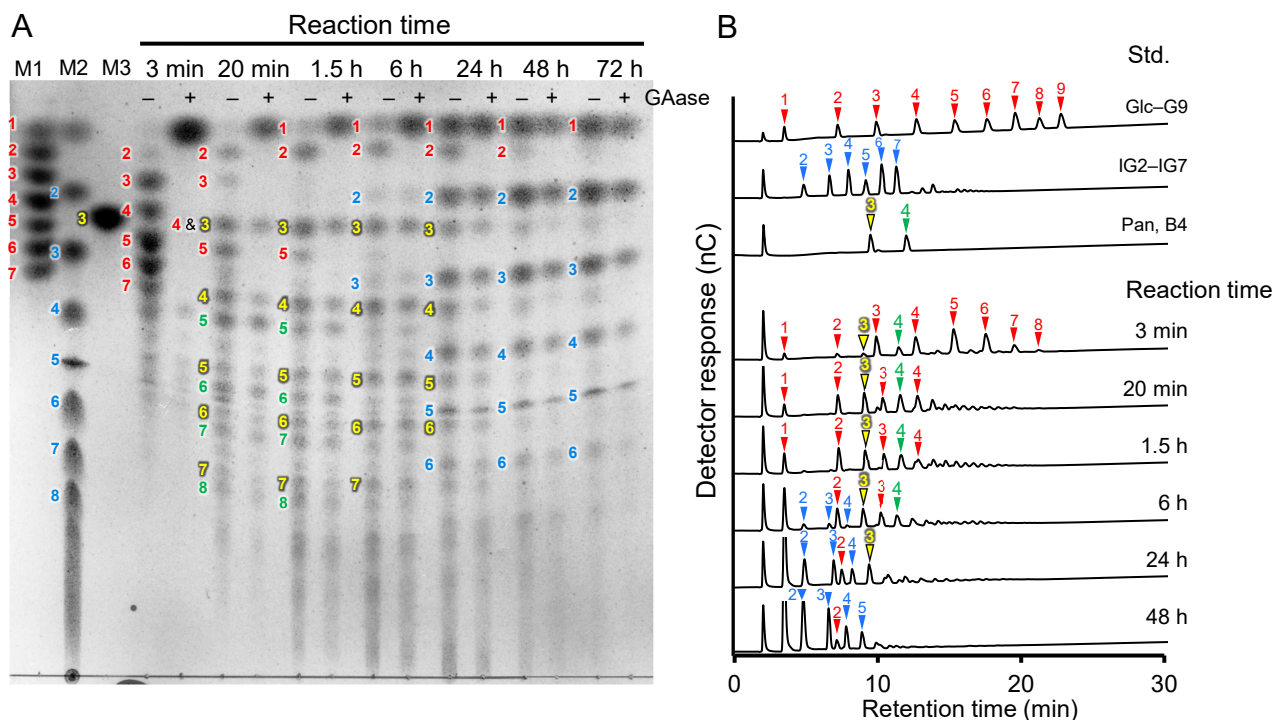


Fig. 2-3. Time course of Td46GT reaction with G5.

A, TLC analysis. M1, D-glucose (Glc) and MOS standards. M2, Glc and IMO standards. M3, panose (Pan). Plus and minus symbols indicate the samples with and without the GAase treatment, respectively. B, HPAEC-PAD analysis. Numbers in panels A and B indicate degree of polymerization of Glc and MOS in red, IMO in blue, panose and 6^{II}-O-isomaltooligosaccharyl maltose in yellow, and B4 and 6^{III}-O-isomaltooligosaccharyl maltotriose in green.

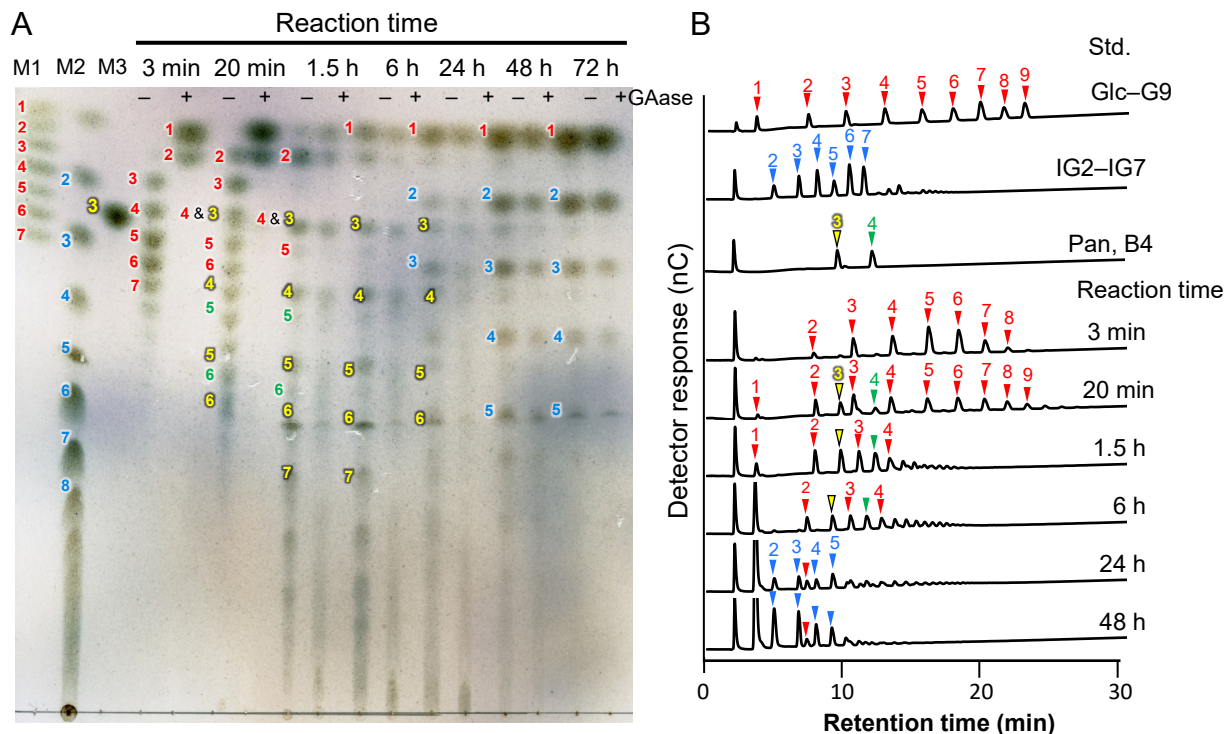


Fig. 2-4. Time course of Pt46GT reaction with G5.

A, TLC analysis. M1, D-glucose (Glc) and MOS standards. M2, Glc and IMO standards. M3, panose. Plus and minus symbols indicate the samples with and without the GAase treatment, respectively. B, HPAEC-PAD analysis. Numbers in panels A and B indicate DP of Glc and MOS in red, IMO in blue, panose and 6^{II}-O-isomaltooligosaccharyl maltose in yellow, and B4 and 6^{III}-O-isomaltooligosaccharyl maltotriose in green.

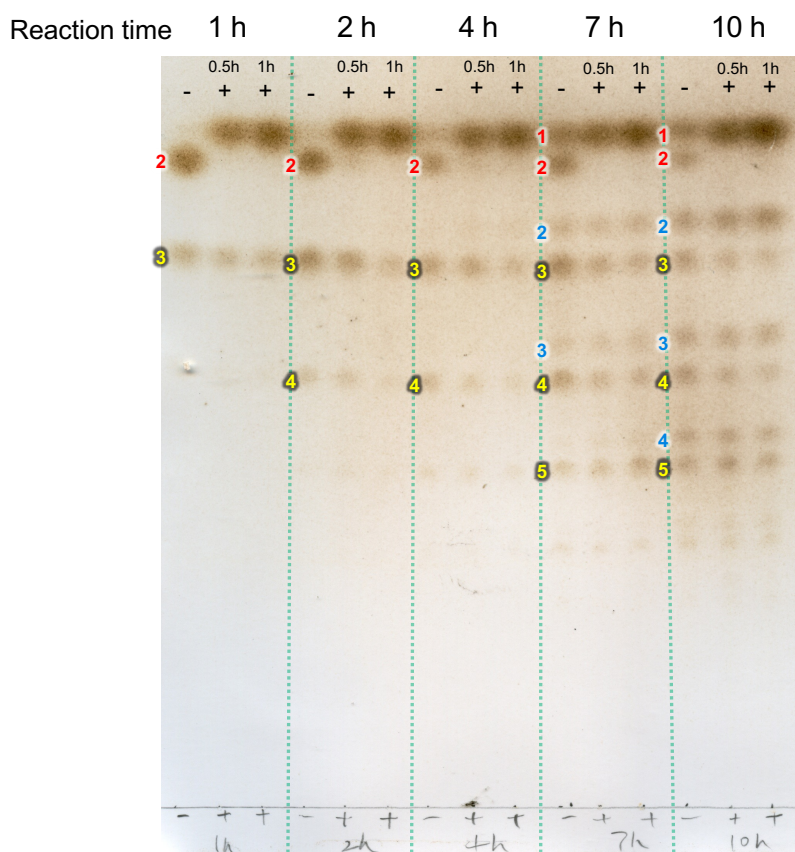


Fig. 2-5. Time course of Td46GT reaction with G2.

Td46GT (2.18 μ M) was incubated with 200 mM G2 in 42 mM sodium acetate buffer (pH 4.5) at 37°C. The reaction products were analyzed by TLC. Reaction samples were treated by GAase for 0.5 h or 1 h Plus and minus symbols indicate the samples with and without the GAase treatment, respectively. Numbers show degree of polymerization. D-Glucose (Glc) and maltose are shown in red; IMO, blue; panose and derived oligosaccharides, yellow. to digest MOS in the sample. G2 was completely digested by the GAase treatment under the analytical conditions, but panose, panose-derived product, and IMOs were not.

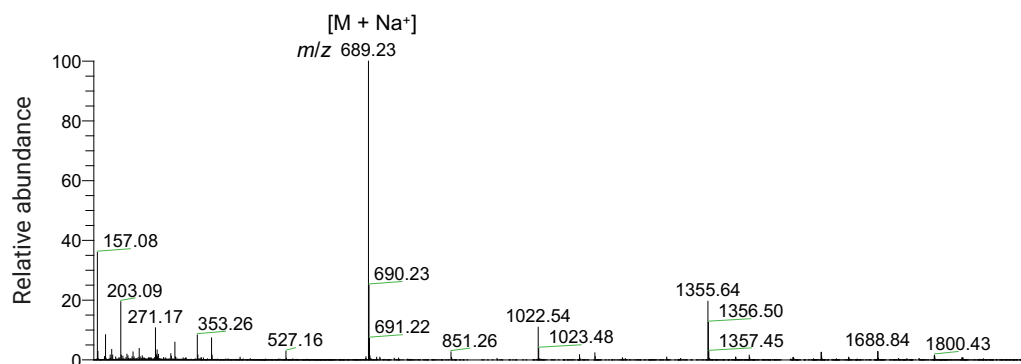


Fig. 2-6. Mass spectrum of 6^H-O- α -isomaltosylmaltose.

The tetrasaccharide produced by glucosylation of panose, separated from reaction mixture with G2 by gel-filtration chromatography.

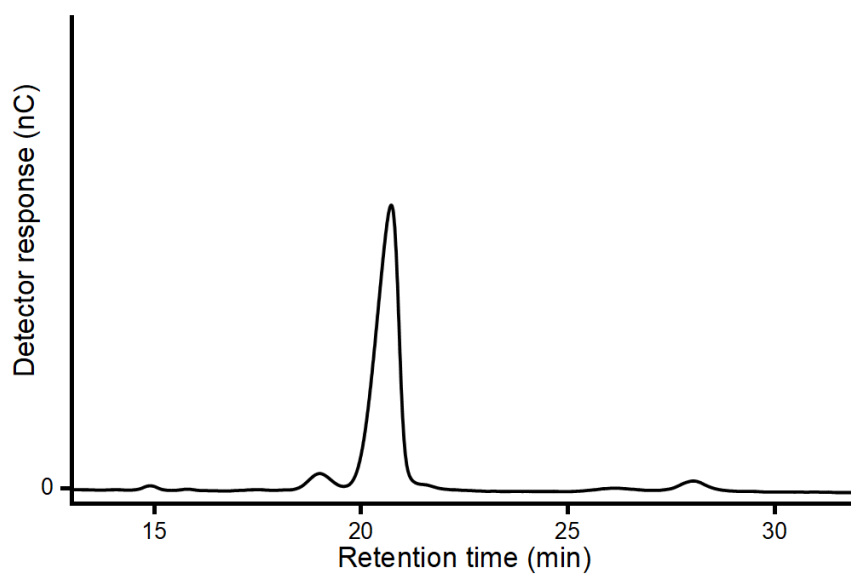


Fig. 2-7. HPLC analysis of purified 6^{II}-*O*- α -isomaltosylmaltose.

The tetrasaccharide produced by glucosylation of panose, separated by gel-filtration chromatography, was analyzed by HPLC analysis.

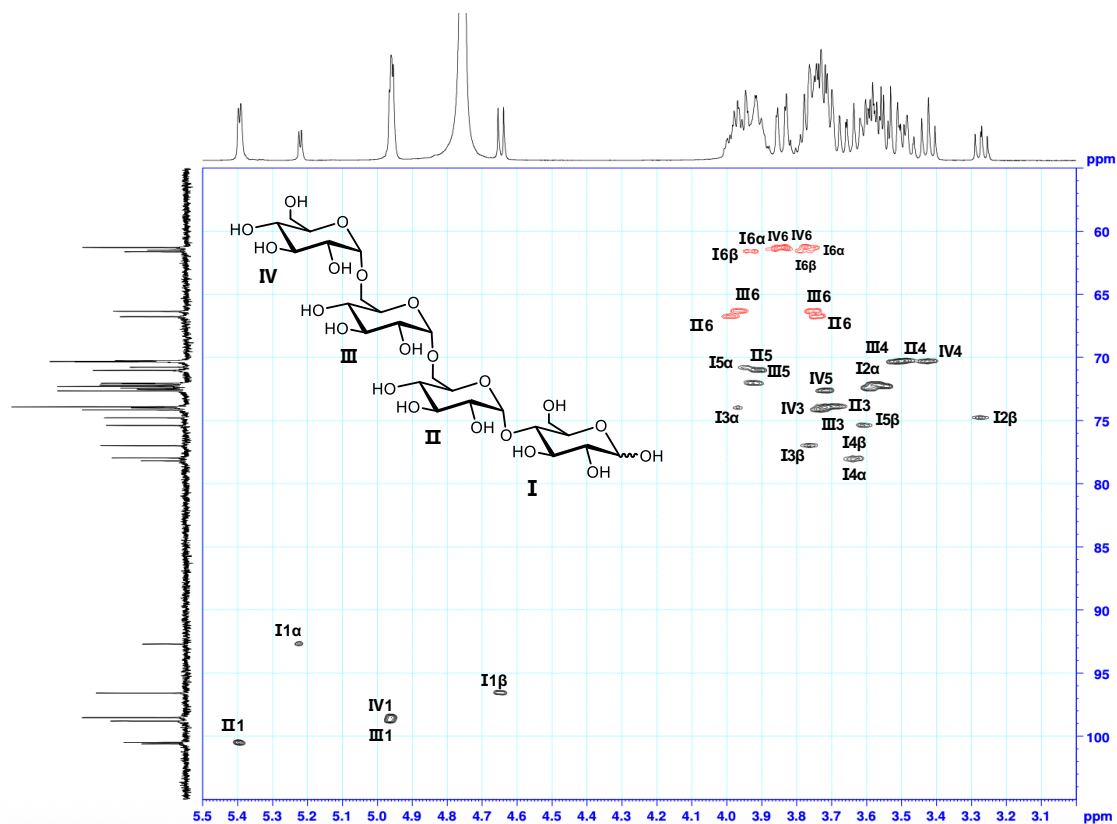


Fig. 2-8. The HSQC spectrum of 6^{II}-O- α -isomaltosylmaltose.

From the reducing end to nonreducing end, the D-glucose units are named as I, II, III, and IV, respectively. Each number shown after the roman numerical indicates the number of the carbon.

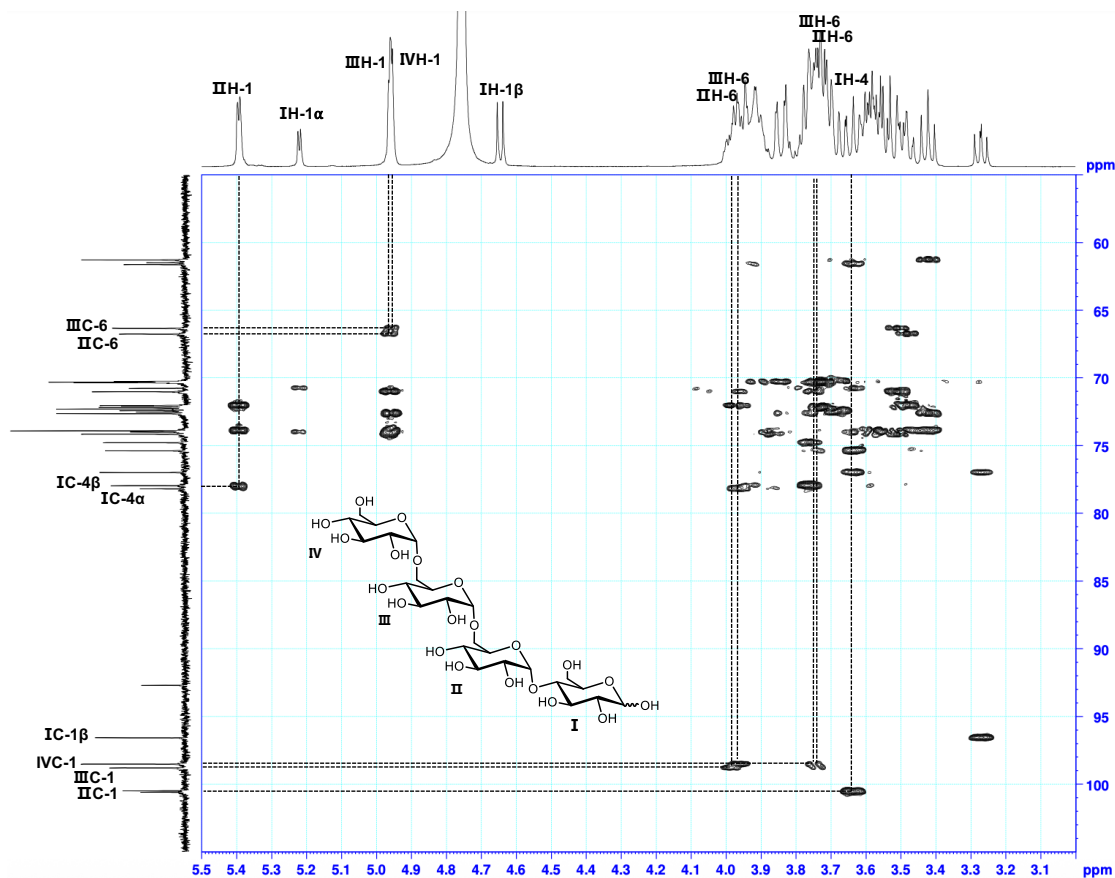


Fig. 2-9. The HMBC spectrum of 6''-O-α-isomaltosylmaltose.

From the reducing end to nonreducing end, the D-glucose units are named as I, II, III, and IV, respectively. Each number shown after the roman numerical indicates the number of the carbon.

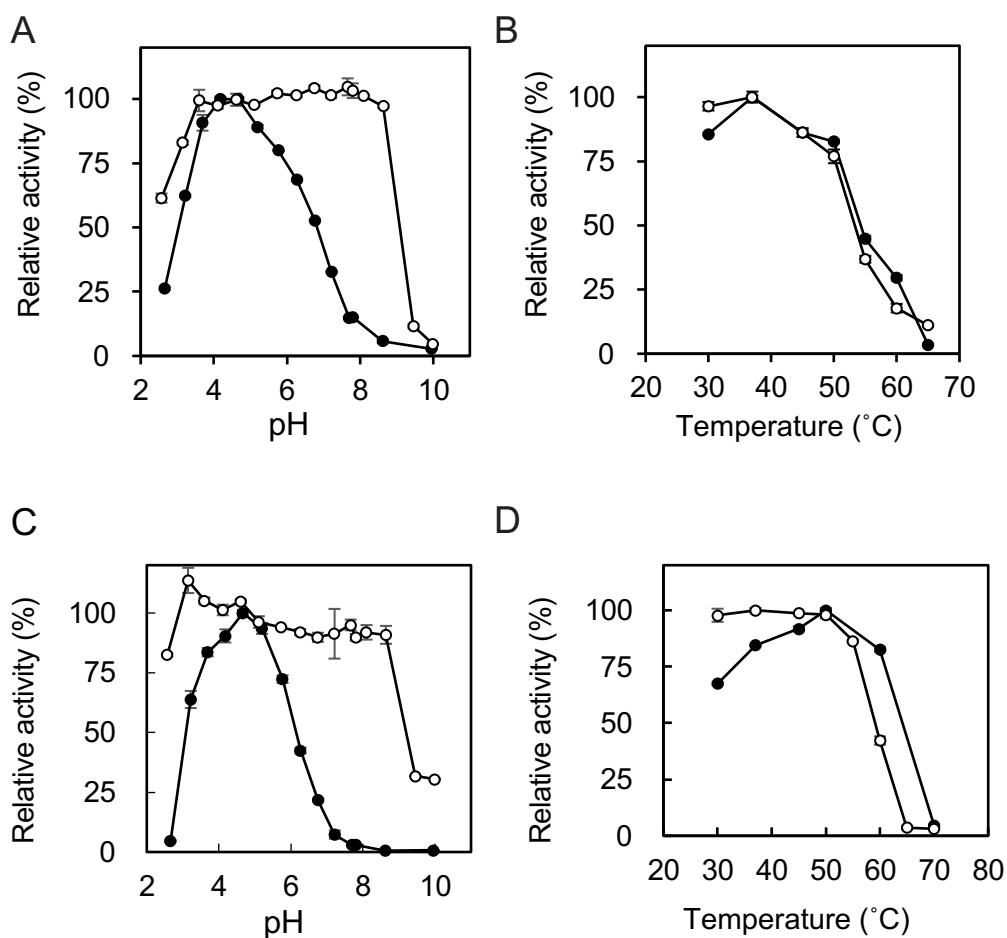


Fig. 2-10. Effects of pH and temperature on activity and stability of Td46GT and Pt46GT.

A, effect of pH for Td46GT. Black circle, relative activity; white circle, remaining activity after pH treatment at 4°C for 20 h. B, effect of temperature for Td46GT. Black circle, relative activity; white circle, remaining activity after heat treatment at pH 4.5 for 15 min. C, effect of pH for Pt46GT after pH treatment at 4°C for 20 h. D, effect of temperature for Pt46GT after heat treatment at pH 4.5 for 15 min.

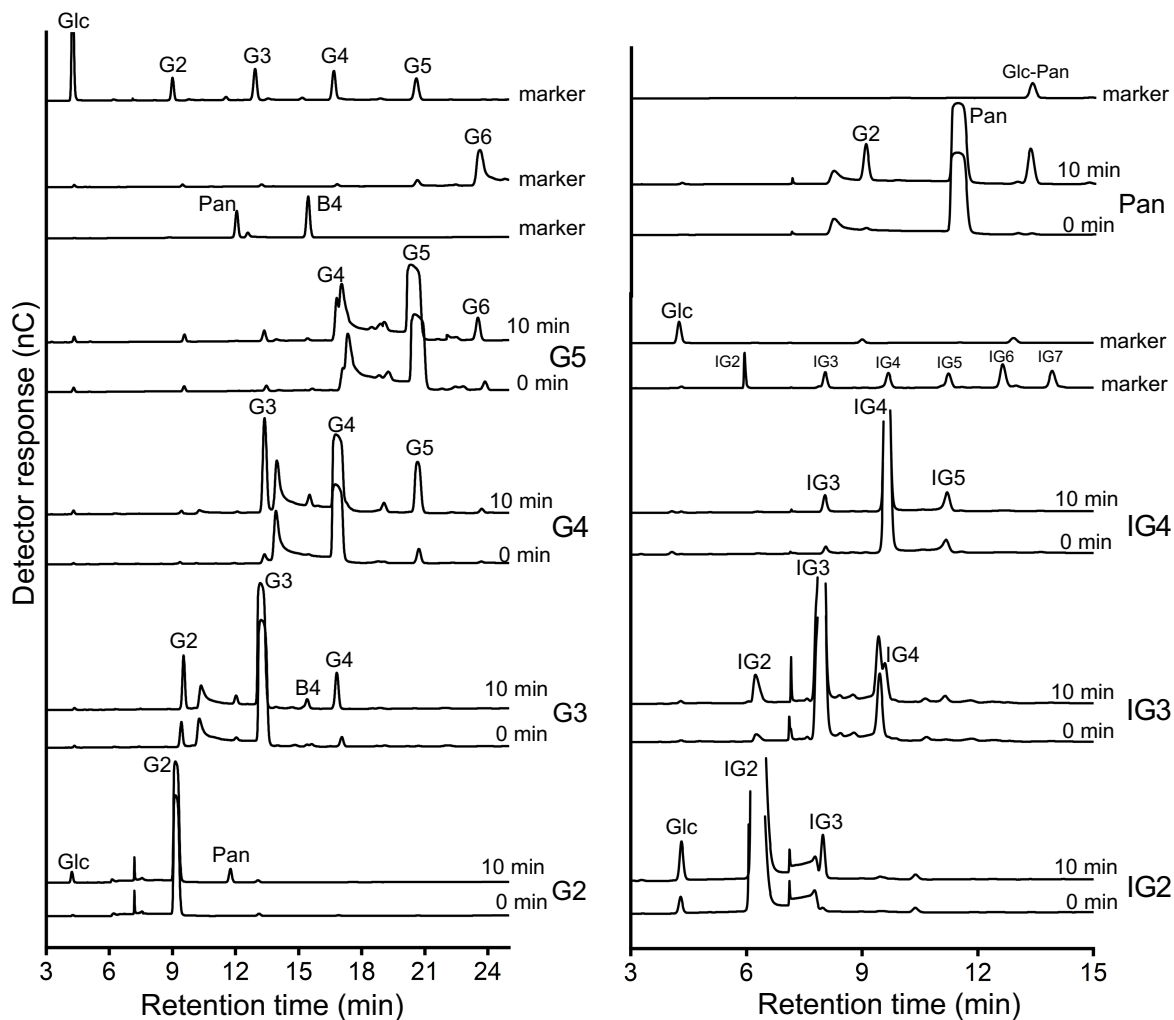


Fig. 2-11. HPAEC-PAD analysis of initial reaction products of Td46GT.

The initial reaction products of Td46GT from 20 mM MOSs, IMOs, and panose (Pan) were analyzed. Glc-Pan, 6^{II}-O- α -isomaltosylmaltose.

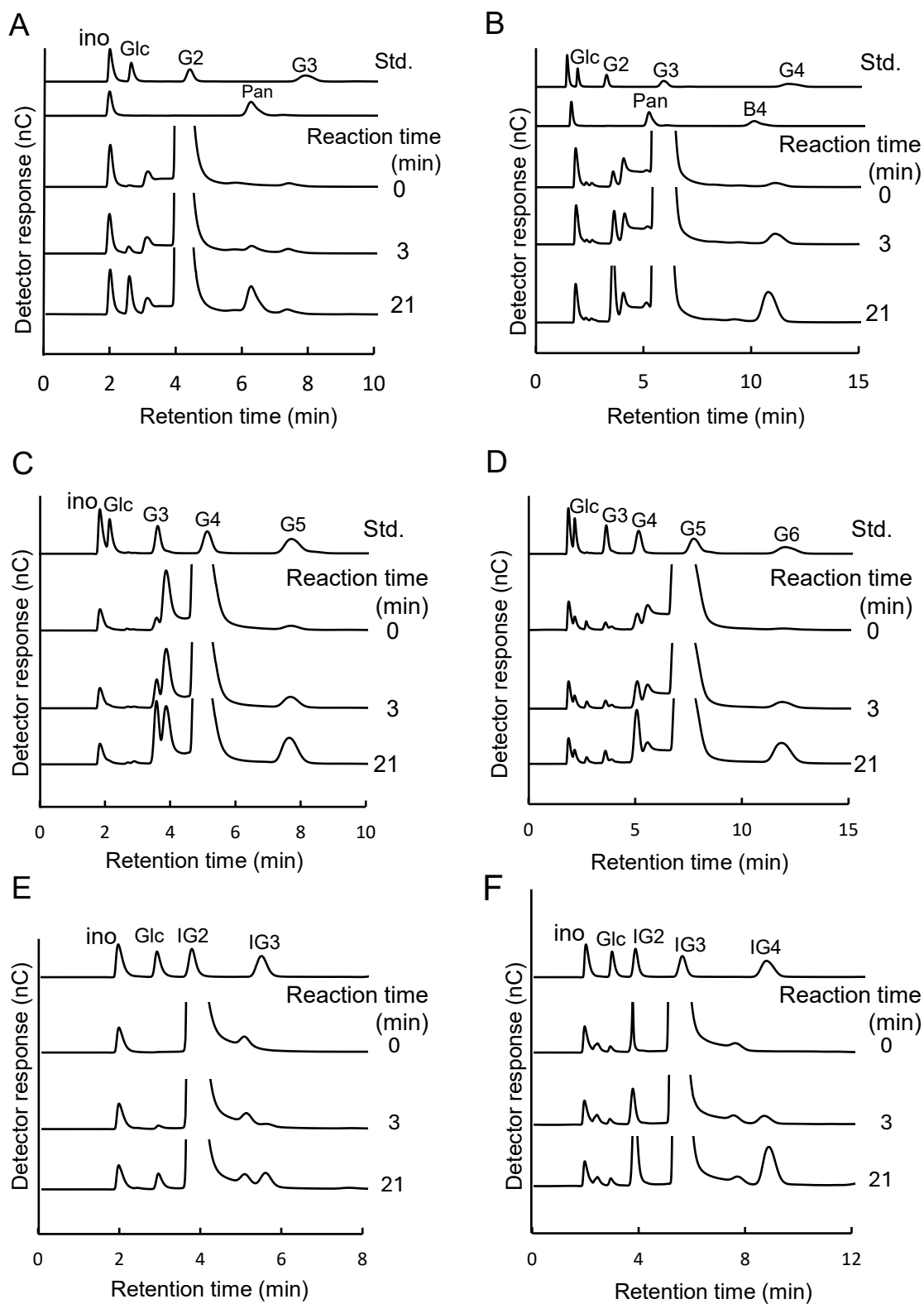


Fig. 2-12. HPAEC-PAD analysis of initial products on 12 mM MOSs and IMOs by Pt46GT.

Time course of Pt46GT reaction products from 12 mM G2 (A), G3 (B), G4 (C) and G5 (D), IG2 (E), IG3 (F), respectively. An internal standard, 100 μ M myo-inositol (ino) was used.

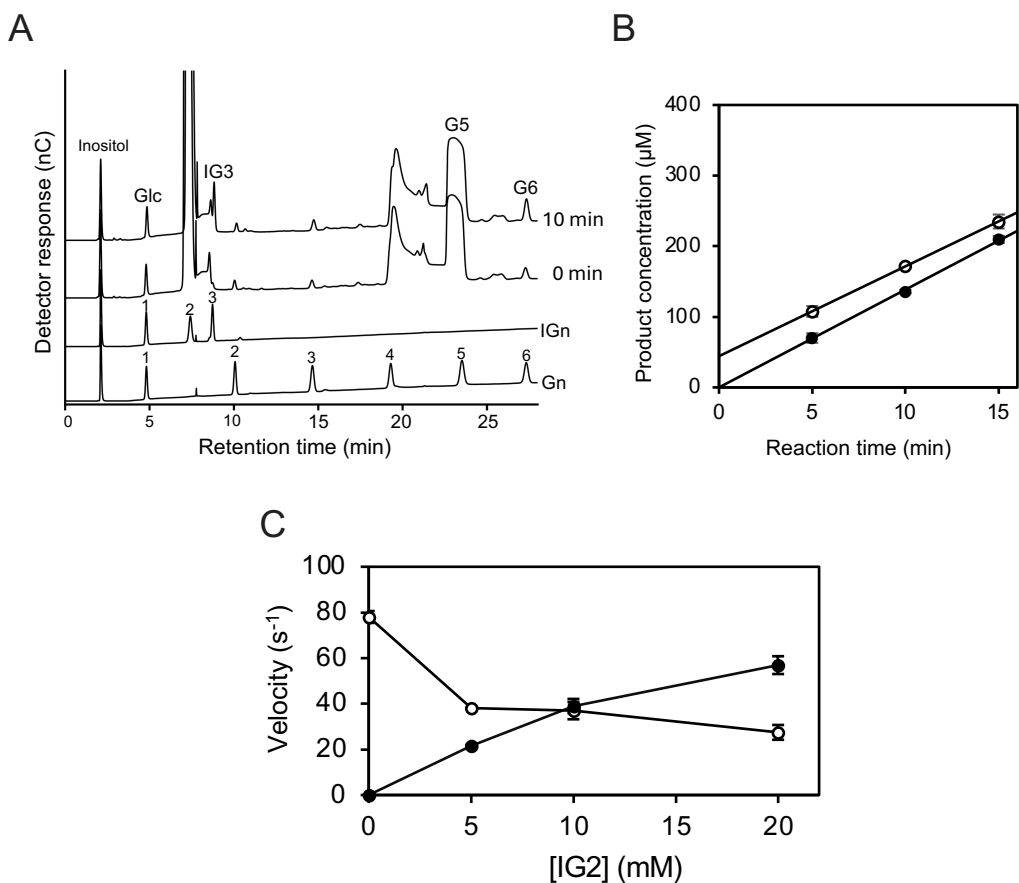


Fig. 2-13. Two-substrate reaction of Td46GT with G5 and IG2.

A, HPAEC-PAD analysis of initial reaction with 10 mM G5 and 10 mM IG2. B, time progress of the production of G6 and IG3 from 10 mM G5 and 10 mM IG2. C, initial velocities for G6- and IG3-productions at various concentrations of IG2 and 10 mM G5. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively. G6-production velocity, white circle. IG3-production velocity, black circle.

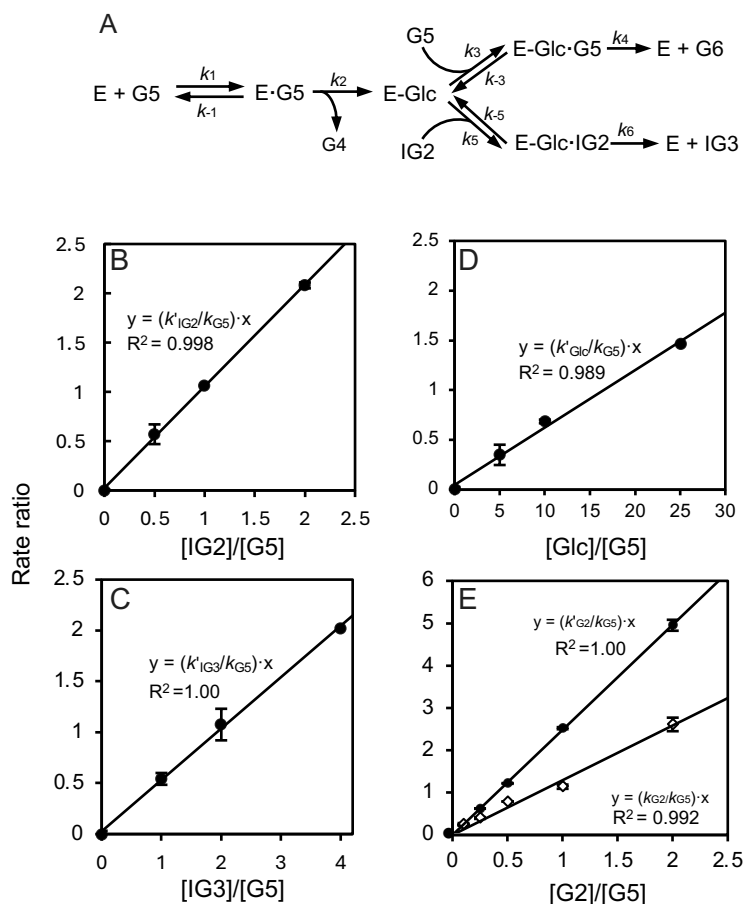


Fig. 2-14. Linear correlation between acceptor concentration ratio and transglucosylation rate ratio in a two-substrate reaction.

A, reaction scheme of the two-substrate reaction with G5 and IG2. B, reaction with IG2 and G5. C, reaction with IG3 and G5. D, reaction with Glc and G5. E, reaction with G2 and G5. In panel E, black circles indicate the reaction rate ratio for panose- and G6-production, and white diamonds indicate the reaction rate ratios for G3- and G6-production. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively.

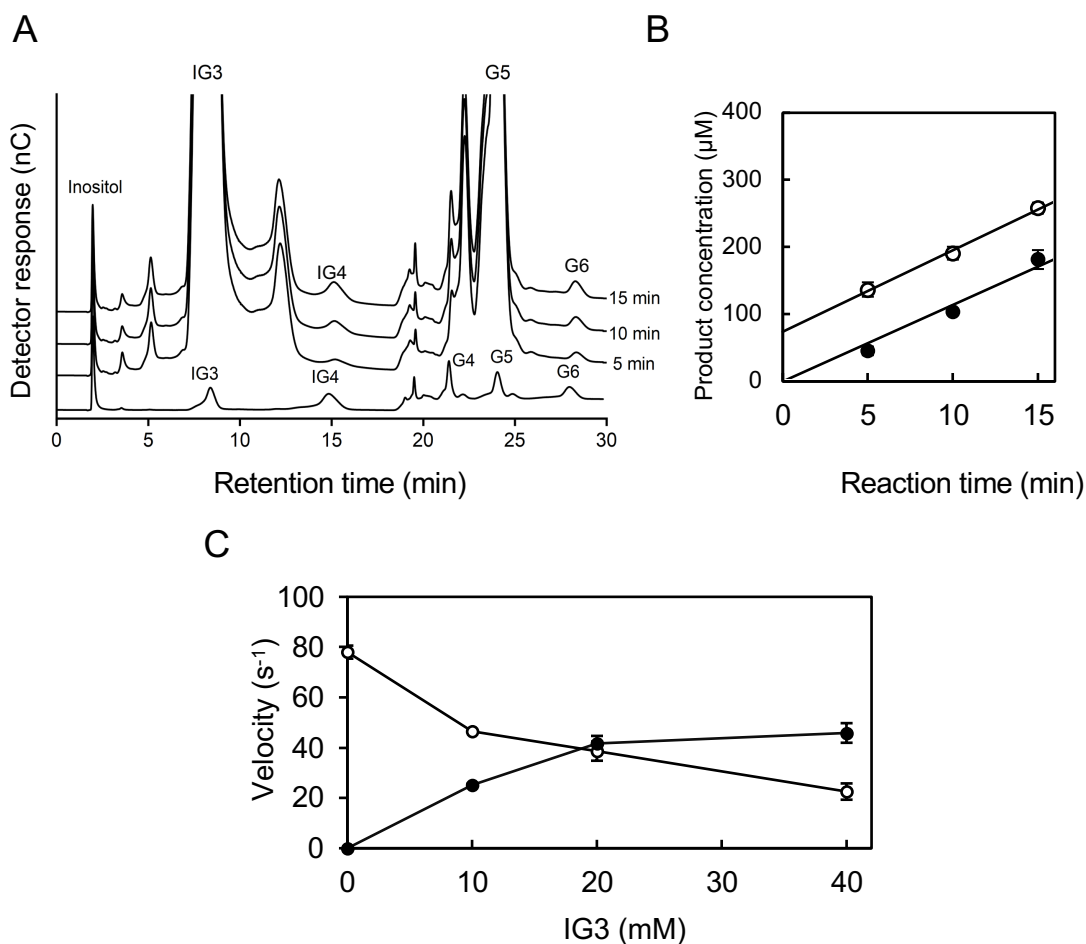


Fig. 2-15. Two-substrate reaction of Td46GT with G5 and IG3.

A, HPAEC-PAD analysis of initial reaction with 10 mM G5 and 20 mM IG3. B, time progress of the production of G6, and IG4 from 10 mM G5 and 20 mM IG3. C, initial velocities for G6-, and IG4-production at various concentrations of IG3 and 10 mM G5. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively. G6-production velocity, white circle. IG4-production velocity, black circle.

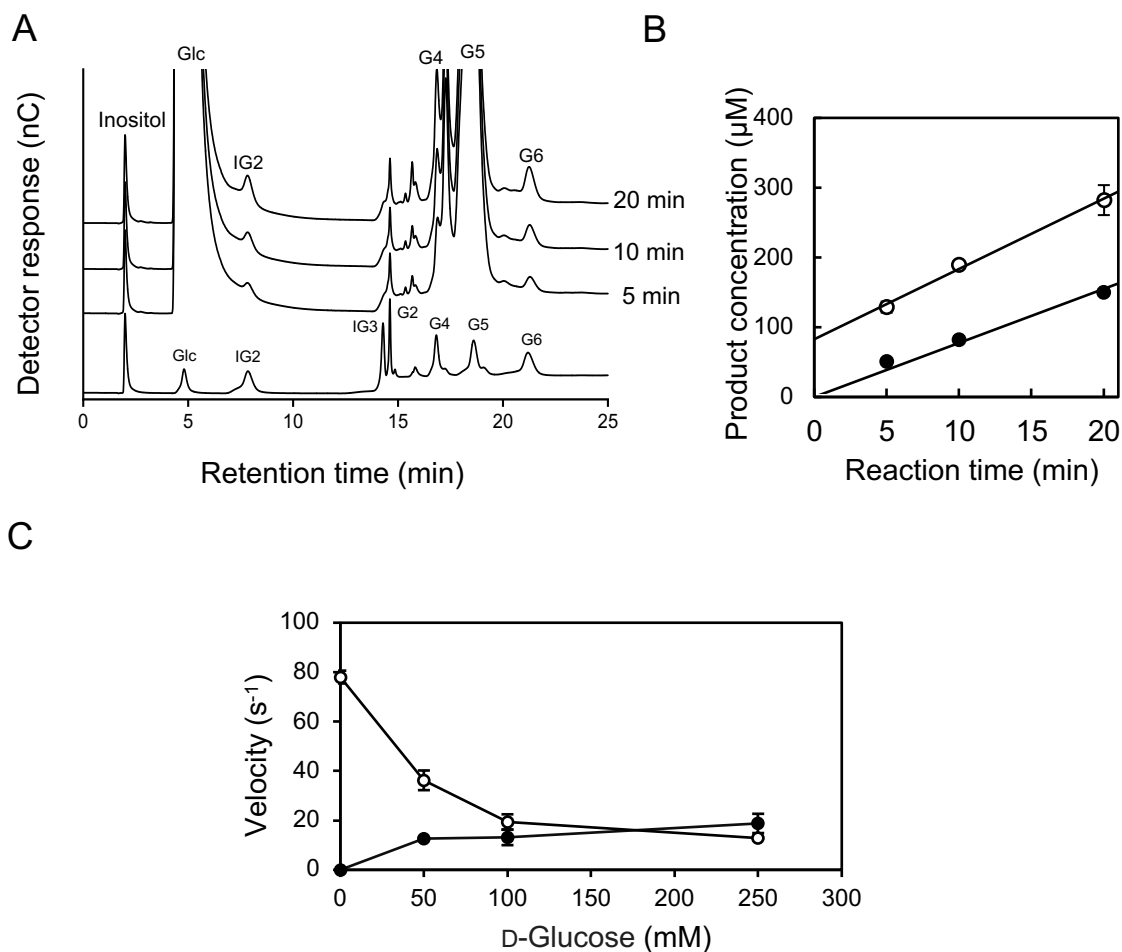


Fig. 2-16. Two-substrate reaction of Td46GT with G5 and D-glucose.

A, HPAEC-PAD analysis of initial reaction with 10 mM G5 and 100 mM D-glucose. B, time progress of the production of G6, and IG2 from 10 mM G5 and 100 mM D-glucose. C, initial velocities for G6- and IG2-production at various concentrations of D-glucose and 10 mM G5. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively. G6-production velocity, white circle. IG2-production velocity, black circle.

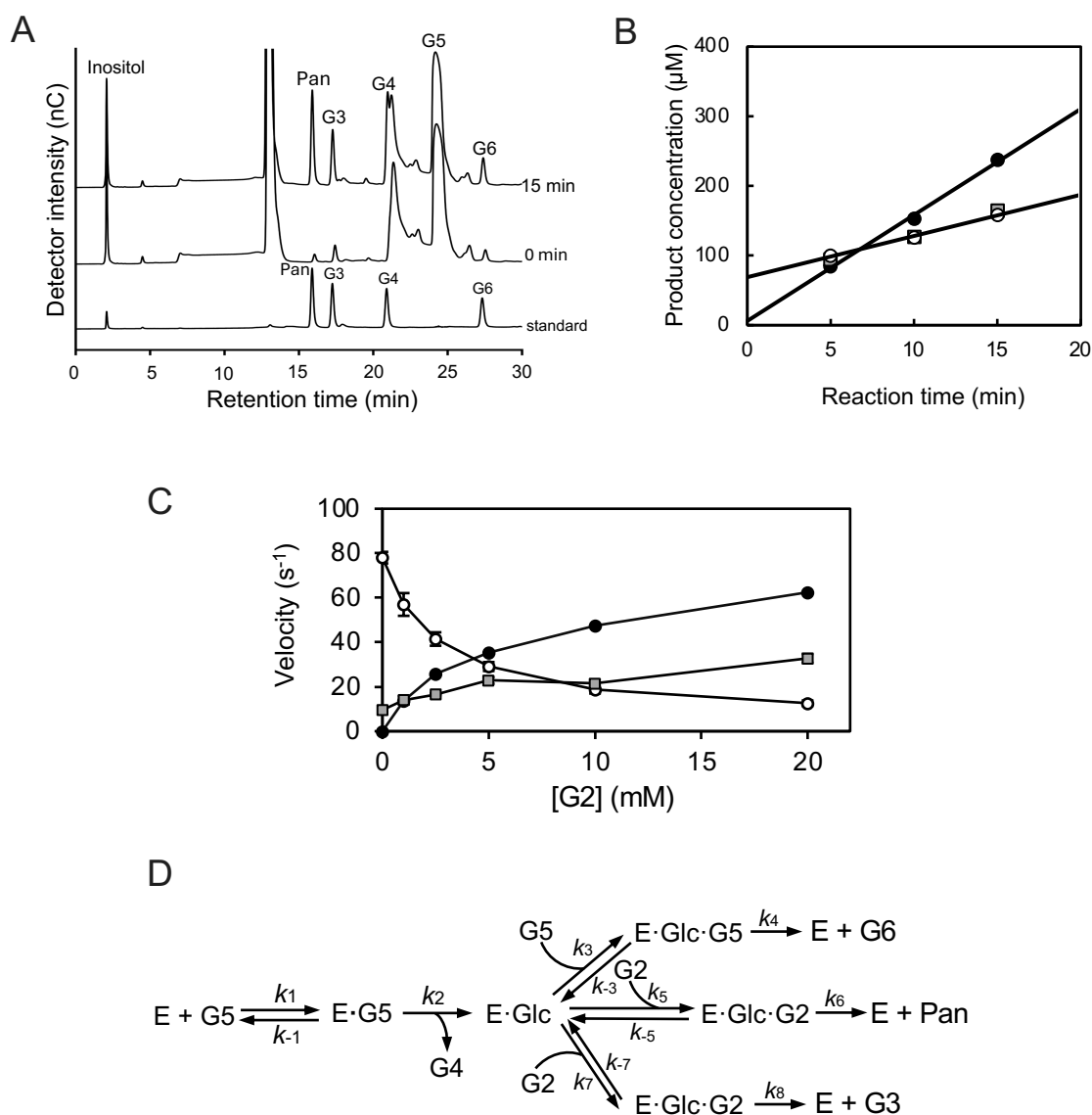


Fig. 2-17. Two-substrate reaction of Td46GT with G5 and G2.

A, HPAEC-PAD analysis of initial reaction with 10 mM G5 and 10 mM G2. B, time progress of the production of panose (Pan), G3, and G6 from 10 mM G5 and 10 mM G2. B, initial velocities for panose-, G3-, and G6-production at various concentrations of G2 and 10 mM G5. D, scheme of the reaction with G5 and G2. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively. G6-production velocity, white circle. Pan-production velocity, black circle. G3-production velocity, gray square.

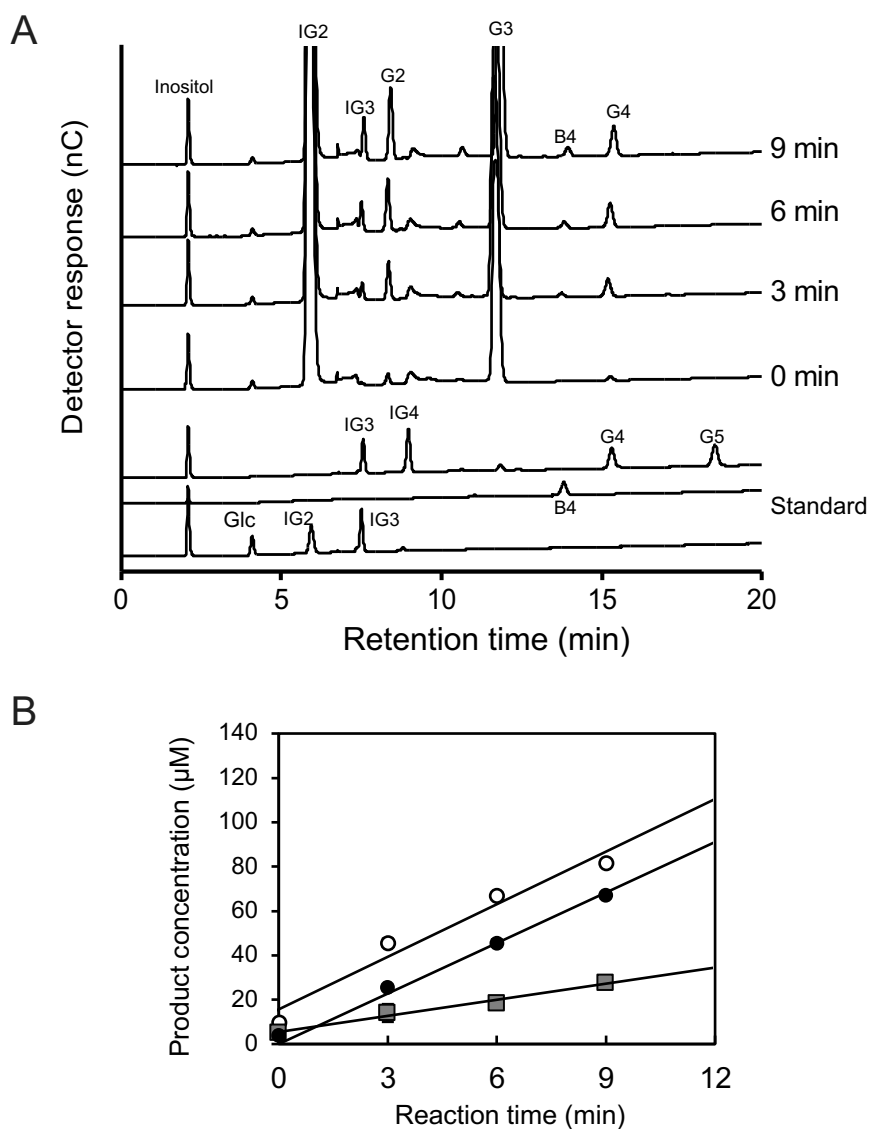


Fig. 2-18. Two-substrate reaction of Td46GT with G3 and IG2.

A, HPAEC-PAD analysis of initial reaction with 7.5 mM G3 and 20 mM IG2. B, Time progress of the production of G4, B4 (6^{III}-O- α -glucosylmaltotriose), and IG3. Values and error bars indicate the average and standard deviations of triplicate experiments (n=3), respectively. G4-production velocity, white circle. IG3-production velocity, black circle. B4-production velocity, gray square.

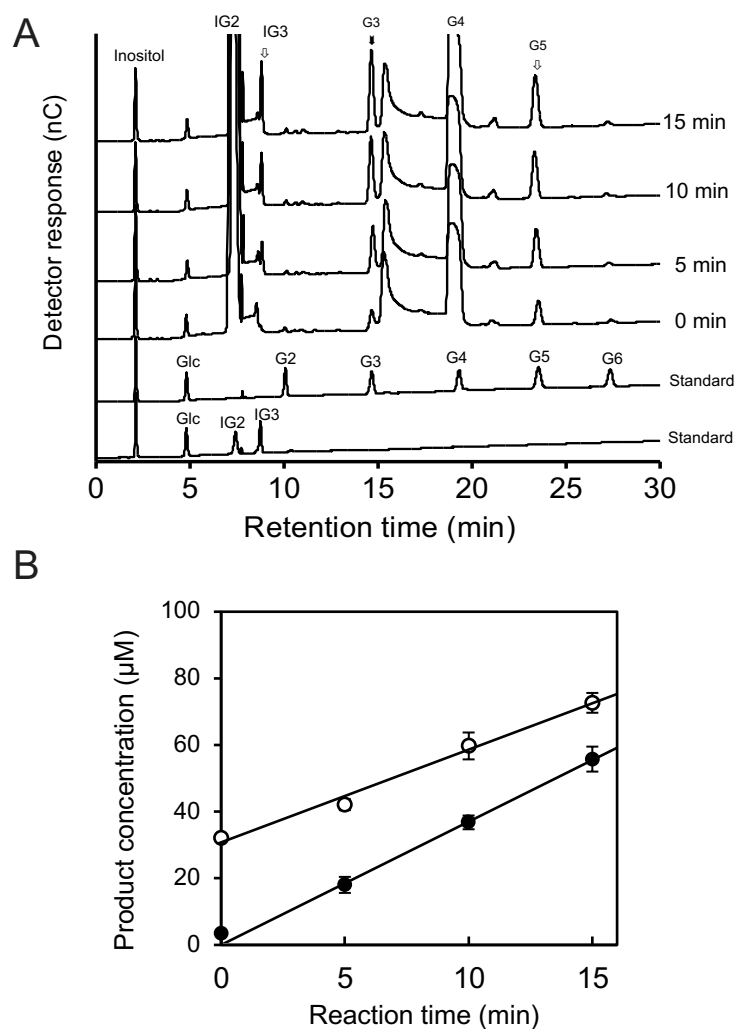


Fig. 2-19. Two-substrate reaction of Td46GT with G4 and IG2.

A, HPAEC-PAD analysis of initial reaction with 10 mM G4 and 20 mM IG2. B, Time progress of the production of G5 and IG3. Values and error bars indicate the average and standard deviations of triplicate experiments ($n=3$), respectively. G5-production velocity, white circle. IG3-production velocity, black circle.

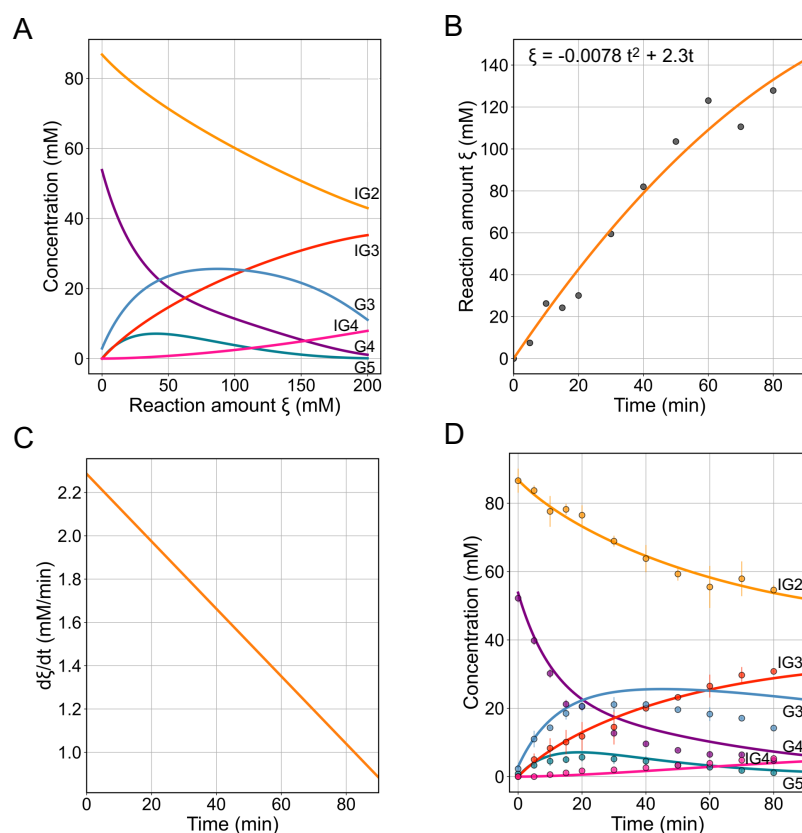


Fig. 2-21. Prediction of reaction product profile based on specificity constants for donor and acceptor substrates.

Prediction of reaction product profile based on specificity constants for donor and acceptor substrates. A, *in silico* simulation of the reaction amount (ξ) and product profile of the Td46GT reaction starting with 87 mM G4 and 52 mM IG2. B, relationship between ξ and time, determined using IG2 concentrations in simulation (panel A) and experiment (panel D). C, change in the total reaction rate over time. The rates were determined from the slope of the line shown in panel B. D, comparison of product profiles between *in silico* simulation and experimental data. Values and error bars indicate the average and standard deviations of triplicate experiments, respectively.

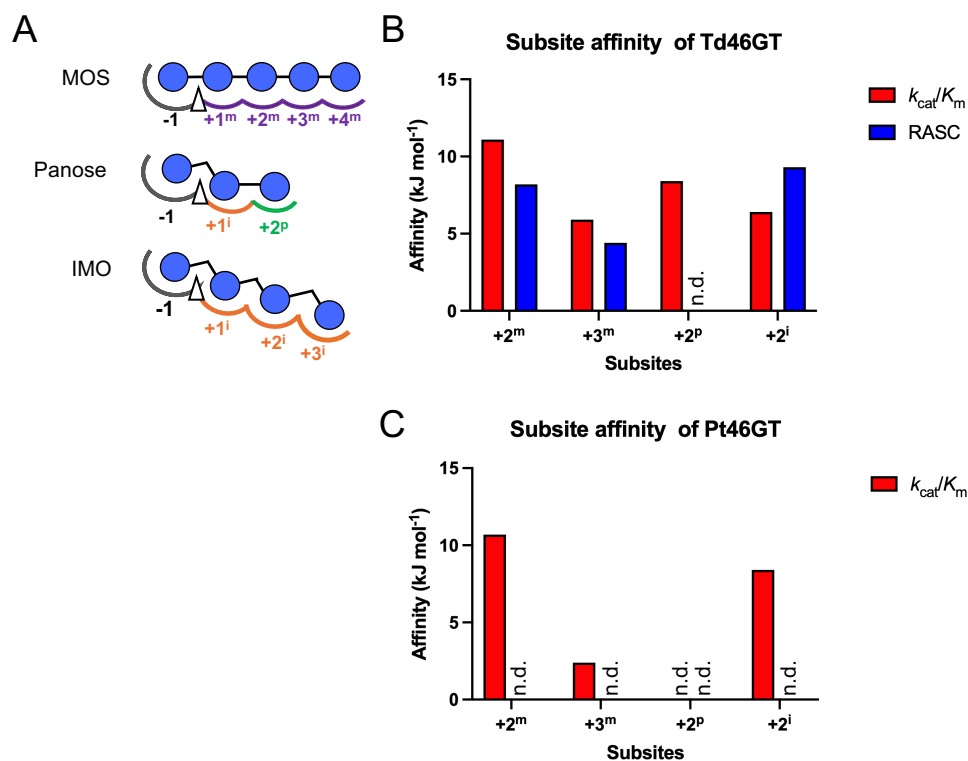


Fig. 2-22. Subsite affinity comparison of GH15 glucosyltransferases

A, Subsite definition of Td46GT for maltooligosaccharides (MOSs), isomaltooligosaccharides (IMOs), and panose. B, Subsite affinity of Td46GT. C, Subsite affinity of Pt46GT. Red: affinities calculated from k_{cat}/K_m values in single-substrate reaction. Blue: Affinities calculated using relative acceptor-substrate specificity constants in two-substrate reaction. n.d., not determined.

3. X-ray crystal structure analysis of Td46GT

3.1. Introduction

As described in chapter 1, GH15 had been regarded as a typical family of inverting α -glucohydrolases such as GAase and GDase (Aleshin et al., 2003; Mizuno et al., 2004), producing β -D-glucose from α -glucan. But two types of retaining α -GTs including DDase and 46GT were recently classified into this family. The structure of C-terminally truncated DDase mutant (DDase- Δ 382C; PDB entry: 9JU0) has been revealed as a dimer where the N1 domain of a protomer, essential for the enzyme activity, interacts with the α 11- α 12 loop of the catalytic domain of other protomer by hydrogen bonds (Tagami et al., 2025). However, in chapter 2, a DDase homologue, Td46GT has been shown to be a monomeric protein (Fig. 2-1) that lacks the regions equivalent to domains N1 of DDase (Fig. 1-2D). Product profile of Td46GT is also different from DDase, as it produces only short-chain IMOs up to DP 7 but not polysaccharides like dextran and IMMs (Fig. 2-3). To reveal the molecular basis for the diversity of GH15 retaining α -GTs, three-dimensional structure of Td46GT is indispensable. Furthermore, the structural comparison of Td46GT with GH15 inverting glucohydrolases could be greatly helpful to understand molecular determinants important for inverting and retaining catalytic mechanisms within GH15. In this chapter, the crystal structure of Td46GT is analyzed. Comparison with DDase and inverting GH15 α -glucohydrolases are described.

3.2. Materials and methods

3.2.1. Construction of expression plasmids for Td46GT E666Q mutant

The expression plasmid for the Td46GT E666Q mutant was prepared from the expression plasmid for the expression of Td46GT wild type as template (see section 2.2.2), using a PrimeStar Mutagenesis Basal Kit (Takara Bio). Primers used are 5' - CAGGGAGATGCCGTATCACGAATTTCA-3' and 5' - TACGGCATCTCCCTGGGCCATATAACC-3'.

3.2.2. Production and purification of Td46GT variants

E666Q was produced in transformants of *E. coli* BL21(DE3) and purified by two kinds of column chromatography. E666Q was firstly purified by Ni²⁺-immobilized affinity column chromatography using the same method for the wild type (see section 2.2.3). The pooled fractions were dialyzed against 10 mM HEPES-NaOH buffer (pH 7.0). Secondly, the collected sample was further purified by anion exchange column

chromatography using a Resource Q column (6 mL; Cytiva) equilibrated with 50 mM HEPES-NaOH buffer (pH 7.0). The adsorbed protein was eluted using a linear gradient of 0–0.5 M NaCl (total elution volume, 30 mL) at a flow rate of 2 mL/min. Purified sample was dialyzed against 10 mM HEPES-NaOH buffer (pH 7.0), and purity of the sample was judged using SDS-PAGE. Sodium azide was added up to 0.02% (w/v). The enzyme sample was stored at 4 °C.

Wild-type Td46GT used for acarbose inhibition kinetic analysis was prepared by methods described in section 2.2.3. Wild-type Td46GT used for thermostability, and differential scanning calorimetry (DSC) analysis, was produced from transformants of *E. coli* SoluBL21 (DE3) (Cosmo Bio, Tokyo, Japan). Wild-type Td46GT was purified by Ni²⁺-immobilized affinity column chromatography as described in section 2.2.3. The pooled fractions were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and stored at 4 °C.

Concentrations of the E666Q and wild-type Td46GT were determined using the specific coefficient at A_{280} of 2.47 for 1 mg/mL (section 2.2.3).

3.2.3. X-ray crystal structure analysis of Td46GT

The E666Q mutant of Td46GT purified as described in section 3.2.2 was dialyzed against 5 mM sodium acetate buffer (pH 4.5) and concentrated to 12.6 mg/mL via ultrafiltration (molecular weight cut off, 10 kDa; Merck, Darmstadt, Germany). Crystallization of Td46GT was achieved at 20 °C within one month via the sitting drop vapor diffusion method in the absence and presence of 2 mM acarbose (Carbosynth, Compton, Berkshire, UK). Protein drops were prepared by mixing equal volumes (0.75 μ L each) of protein and a crystallization buffer. Apo-form crystals were obtained from 0.1 M sodium acetate buffer (pH 4.2) containing 20 mM CaCl₂, and 31% (w/v) 2-methyl-2,4-pentandiol, while acarbose-complex crystals were grown using 0.1 M sodium acetate buffer (pH 4.3) containing 15 mM CaCl₂, and 29% (w/v) 2-methyl-2,4-pentandiol.

For X-ray diffraction, crystals were directly picked up from the crystallization solution and flash-cooled under a nitrogen stream at 100 K or in liquid nitrogen. Diffraction data were collected on beamline BL-45XU at SPring-8 (Hyogo, Japan). Data were processed (indexing, integration, scaling, and merging) using the XDS package (Kabsch, 2010). The asymmetric units of both the apo and acarbose-complex contained two molecules in the asymmetric unit. Matthews coefficients (Matthews, 1968) were 3.11 $\text{\AA}^3/\text{Da}$ and 3.12 $\text{\AA}^3/\text{Da}$, with estimated solvent contents of 60.5% and 60.6%, respectively. Data collection statistics are summarized in Table 3-1.

3.2.4. Effect of calcium ion on temperature activity and stability

The optimum temperature of the wild type was determined from the reaction rates under the standard assay conditions (section 2.2.7) but at 30–70 °C and in the presence of 0–40 mM CaCl₂. The stable temperature range of Td46GT wild type was determined from residual activity after incubation of 1.05 µM Td46GT in 40 mM sodium acetate buffer (pH 4.5) containing 0–10 mM CaCl₂ at 37–77 °C for 20 min. The range in which the enzyme retained ≥90% of the original activity was regarded as stable range. Three independent replications of each experiment were conducted.

3.2.5. DSC analysis of Td46GT

The buffer in the purified Td46GT wild type was exchanged with 50 mM sodium acetate buffer (pH 5.6) by repeated concentration via ultrafiltration and dilution. Sample (325 µL), containing 0.124 mg/mL of Td46GT and 25 mM sodium acetate buffer (pH 5.6) was analyzed by MicroCal PEAQ-DSC Automated Microcalorimeter (Malvern Panalytical, Malvern, Worcestershire, UK) under following conditions: temperature range, 30–85 °C; scan rate: 60 °C/h; pre-scan thermostat, 5 °C. Data of sample without the protein were subtracted and data were processed using the PEAQ-DSC software (Malvern Panalytical) to analyze a series of thermodynamic parameters using a non-two-state model. Samples with additive, 20 mM CaCl₂, 20 mM EDTA-2Na or 40 mM NaCl, were also analyzed in the same manner. Independent three analyses were performed for each additive.

3.2.5. Inhibition experiment with acarbose

In the reaction with *p*-nitrophenyl α-D-glucoside (pNP-Glc; Nacalai Tesque, Kyoto, Japan), liberation rate of *p*-nitrophenyl (pNP) was measured. A reaction mixture (50 µL), containing an appropriate concentration of enzyme, 42 mM sodium acetate buffer (pH 4.5), 4–31 mM pNP-Glc, and 0, 0.1 or 0.3 µM acarbose (Carbosynth), was incubated at 37 °C for 10 min. Reaction was terminated by incubating the samples at 80 °C for 3 min, and 75 µL of 1 M sodium carbonate buffer (pH 9.5) was added. After centrifugation (16,000 × *g* at 4 °C for 10 min) of the sample to remove insoluble material, pNP concentration was determined by absorbance at 400 nm. The competitive inhibition constant *K_i* of acarbose was determined by nonlinear regression using Grafit version 7.0.2.

3.3. Results

3.3.1. Purification of Td46GT E666Q mutant.

Td46GT E666Q mutant was produced in *E. coli* BL21(DE3) transformant and purified to homogeneity by Ni²⁺-immobilized affinity column chromatography and anion exchange column chromatography. The purified sample showed a single band in both SDS-PAGE and native PAGE (Fig. 3-1). The SDS-PAGE analysis showed the molecular mass of purified E666Q was 106 kDa, same as that of the wild type (see section 2.3.1).

3.3.2. Crystal structure analysis of Td46GT E666Q mutant.

Acarbose, a pseudo tetrasaccharide, is a strong inhibitor for α -glucohydrolases such as GAase. It served as a competitive inhibitor for wild-type Td46GT with a K_i value of 0.325 ± 0.017 μ M (Fig. 3-2). Although co-crystals of the wild-type Td46GT and acarbose were not obtained, the crystal structures of the apo and acarbose-bound forms of E666Q mutant were determined at 1.96 Å and 2.14 Å resolutions, respectively (Table 3-1). The asymmetric unit contains two molecules, although Td46GT exists as a monomer under nondenaturing conditions in solution. The region from Thr34 to Glu920 is modeled in both structures, which exhibits nearly identical conformations with 0.186 Å of overall C α root mean square deviation (RMSD). Molecule A (Mol A) is discussed in following contents (Fig. 3-3).

The Td46GT overall structure comprises three domains: N (Ser45–Ser287), catalytic (α/α)₆-barrel domain A (Gly329–Tyr696) and a sugar-binding domain C (Phe709–Glu920), domains N and A are linked by a helix-loop-helix (L) motif (Leu288–Asp328) (Fig. 3-3). Domain N of Td46GT corresponds to domain N2 of DDase (Ser161–Ala422; Tagami et al., 2025) and the N-terminal domains of some characterized bacterial α -glucohydrolases (Aleshin et al., 2003; Mizuno et al., 2004; Sakaguchi et al., 2015). Catalytic domain A of Td46GT that accommodates an acarbose ligand highly corresponds to the catalytic domain of DDase (Ala503–Thr889; RMSD, 1.05 Å for 349 C α atoms; Fig. 3-4), with similar secondary structures including two short β -hairpins (Table 3-2 and Fig. 3-5A). Domain N contacts with domain A by α 1 helix, α 1- α 2 loop, α 11 helix and α 11- α 12 loop. Domain C binds with one acarbose molecule, and is folded into a 12-stranded β -sandwich structure (Tables 3-3 and 3-4). One 3_{10} helix (Val723–Trp730) is inserted at β 1- β 2 loop (Table 3-3 and Fig. 3-5B). At the C-terminal loop (Gln913–Glu920), Asp710 on β 1 strand and Tyr735 on β 2 strand

form hydrogen bonds with $\alpha 8$ helix, $\alpha 9$ helix and $\alpha 8 \rightarrow \alpha 9$ loop of domain A (Fig. 3-6). The reducing-end trisaccharide portion of acarbose bound to domain C is stabilized through possible hydrogen bonds with Tyr806, Asp823, and His842 and hydrophobic interactions involving Trp821, Phe830, Phe839, and Trp895 (Fig. 3-7). This domain shows no significant structural similarity to any previously characterized functional domains in PDB database.

3.3.3. Active center structure bound to acarbose.

Electron density corresponding to acarbose in domain A was observed (Fig. 3-3C). Acarbose bound to the active site spanning subsites -1 to +3 (Fig. 3-8A). The valienamine unit occupies the bottom of the active pocket (subsite -1). The 6-deoxy-D-glucose unit, and two reducing-end side D-glucose units positioned at subsites +1, +2, and +3, respectively. The catalytic residues, Glu480 and Gln (Glu) 666, are suitably positioned to serve as general acid/base and nucleophile catalysts, respectively (Fig. 3-8B). The O ϵ 2 atom of Glu480 lies 2.7 Å from the amine nitrogen of acarbose, equivalent to the glucosidic oxygen in natural substrates, closely matching the configuration observed in other structurally characterized GH15 enzymes (Aleshin et al., 2003; Mizuno et al., 2004; Sakaguchi et al., 2015; Tagami et al., 2025). Gln (Glu) 666 is located on the β -face side of the valienamine ring, analogous to the catalytic nucleophile Glu858 in DDase (Fig. 3-8C; Tagami et al., 2025).

In subsite -1, each hydroxy group of the valienamine moiety of acarbose formed predicted hydrogen bonds with surrounding residues: O2 – Arg600; O3 – Ile478; O4 – Arg 369 and Asp370; O6 – Asp370, Gln (Glu) 666 and Glu682 (Fig. 3-8B). Trp367 participates in a CH- π interaction with the C6 atom of the valienamine unit. Asp370 and Trp367 are strictly conserved throughout GH15. Glu682 additionally forms a water-mediated hydrogen bond with Gln (Glu) 666, which highly resembles that of DDase. The water molecule in this hydrogen bond is almost overlapped in the two enzymes (Fig. 3-8C). Although this water molecule resides at the β -face of valienamine moiety, it is far away from the anomeric carbon by 5.8 Å (5.1 Å for DDase). Position of the OE1 atom of Gln (Glu) 666 resembles that of the nucleophilic water, in inverting GH15 α -glucohydrolases, and both are about 3.6 Å away from the anomeric carbon of acarbose ligand (Fig. 3-9).

In subsite +1 of Td46GT, Arg600 forms a hydrogen bond with the 6-deoxy-D-glucose unit. while Phe418 and Phe606 provide hydrophobic contacts from the α - and β -faces, respectively. In subsite +2, Tyr612 stacked

onto the D-glucosyl unit from the β -face. Mainchain of Phe418 forms a hydrogen bond with O1 of the D-glucosyl unit at subsite +3, which is not observed in DDase (Fig. 3-8C).

3.3.4. Calcium ion binding-sites

Five calcium ion-binding was observed and amino acid residues coordinating the calcium ions were identified (Fig. 3-10). In domain N, OE1 of Gln124 and Glu140 were responsible for Ca^{2+} ion binding. Asp220, Asp266 and Gln267 interact with the other calcium ion. In domain A, the mainchain oxygen atom of Asn590 on α 9 helix, OE1 and OE2 of Glu623 and OD1 of Asp602 on α 9- α 10 loop interact with a Ca^{2+} ion (Fig. 3-10C). In proximity of this calcium ion-binding site, Arg600 is predicted to form two hydrogen bonds with valienamine and 6-deoxy-D-glucose moieties at subsite -1 and +1, respectively (Fig. 3-8B). In domain C, two calcium ions are accommodated by residues in β 2- β 3 loop and β 12 strand (Figs. 3-5B and 3-10D).

3.3.5. DSC analysis of wild-type Td46GT

Unfolding behaviors of wild-type Td46GT in 25 mM sodium acetate buffer (pH 5.6) were monitored in the presence or absence of 20 mM CaCl_2 , 20 mM EDTA-2Na or 40 mM NaCl by DSC analysis (Fig. 3-11). The calorimetric enthalpy of unfolding (ΔH_{cal}) was not significantly different among control (2.62×10^3 kJ/mol), EDTA-2Na (2.94×10^3 kJ/mol), and NaCl (2.73×10^3 kJ/mol) conditions (Fig. 3-12A), but CaCl_2 enhanced ΔH_{cal} up to 4.67×10^3 kJ/mol, indicating a 1.8-fold higher energy required for Td46GT denaturation than the control (Fig. 3-12A). The T_m value of Td46GT in the presence of 20 mM CaCl_2 was 78 °C, whereas other three conditions both displayed a close value, 61 °C (Fig. 3-12B).

3.3.6. Effects of calcium ion on temperature activity and stability

Td46GT wild type retained $\geq 90\%$ of the original activity at 47 °C or lower temperature at pH 4.5 under the control condition (15 min incubation). In the presence of 1-10 mM CaCl_2 , Td46GT wild type retained $\geq 90\%$ of the original activity at ≤ 57 °C, and it also retained $\geq 75\%$ activity at 67 °C, in which the enzyme totally lost its activity in the absence of CaCl_2 (Fig. 3-13). Only 1 mM CaCl_2 was enough to increase the thermostability of Td46GT.

The activity profile of wild-type Td46GT was analyzed in the presence of various concentrations (0-40 mM) of CaCl_2 at 30–74 °C. At $\leq 67^\circ\text{C}$, 1-4 mM CaCl_2 additions enhanced activity compared to the control

condition, and did not change optimum temperature, 47 °C. The optimum activity in the presence of 4 mM CaCl₂ increased to 1.7-fold (1.51 U/mg) relative to the control (0.887 U/mg) at 47 °C. In the presence of 10-40 mM CaCl₂, the optimum temperature increased up to 57 °C and activity in the presence of 40 mM CaCl₂ was 1.68 U/mg (1.9-fold of activity in control condition; Fig. 3-14B).

3.4. Discussion

In this chapter, the crystal structure of Td46GT was determined, which possesses a simpler structure than DDase. Td46GT exists as a monomeric protein (Fig. 2-1) and lacks the domain corresponding to domain N1 of DDase (Figs. 1-2D and 3-4), which is required for dimerization and proper formation of the active site (Tagami et al., 2025). Domain N and GH15 catalytic domain including the substrate binding site (subsite -1 to +3) of Td46GT highly resembles with the corresponding structures of DDase. Especially their catalytic dyad was overlapped well (Fig. 3-8C).

Compared with crystal structures of inverting GH15 α -glucohydrolases (Aleshin et al., 2003; Coutinho & Reilly, 1994; Mizuno et al., 2004), most residues forming the subsites -1 and +1 are strictly conserved with retaining GH15 enzymes and they have similar hydrogen bond and hydrophobic/stacking interactions to the valienamine and 6-deoxy-D-glucose moieties (Fig. 3-9). Intriguingly, the catalytic nucleophile residue Gln(Glu)666 of Td46GT overlapped with Glu858 of DDase and specially positioned 1.2–1.6 Å closer to the anomeric carbon of the substrate in subsite -1 compared with inverting GH15 α -glucohydrolases (Fig. 3-9; Tagami et al., 2025). The OE1 atom of nucleophile catalytic residues of retaining GH15 α -GTs occupies the spatial position of the nucleophilic water in inverting GH15 glucohydrolases (Fig. 3-9). This structural evidence supports that nucleophile catalytic residue replaces the function of nucleophilic water—attack the anomeric carbon, for β -D-glucosyl-intermediate formation in the double displacement mechanism. The distance between nucleophile/base catalyst and anomeric carbon should be essential to determine inverting/retaining reaction mechanism among GH15 enzymes.

Subsites +2 and +3 are not conserved between retaining and inverting GH15 enzymes, which possibly explained their diverse linkage preferences of substrates (α -(1 $\rightarrow$ 4)- or α -(1 $\rightarrow$ 6)-glucosidic linkages or both). The most significant difference is on α 9- α 10 loop (Fig. 3-15A-C). For retaining enzymes, a semi-conserved aromatic residue (Y612 position of Td46GT) on α 9- α 10 loop, is protruded against the β -face of D-glucose moiety in subsite +2. This protrusion results from additional three residues insertion that is absent in inverting

enzymes (Fig. 1-2C and Fig. 3-15D), which instead have shallower pockets with no direct interaction toward the D-glucose unit at subsite +2.

Domain C of Td46GT has a unique 12-stranded β -sandwich fold that is distinguished from all other characterized GH15 enzymes. Its hydrogen bond interactions with $\alpha 8$ and $\alpha 9$ helices and $\alpha 8$ - $\alpha 9$ loop of GH15 catalytic domain A (Fig. 3-6), possible MOS-binding ability (Fig. 3-7), and two calcium ion-binding sites (Fig. 3-10) have been firstly revealed. Even though exhibiting low sequence identity (16.9%), foldseek search tool (<https://search.foldseek.com/>) implies an extremely thermostable C-terminal Ca^{2+} -binding CBM family 9 domain of xylanase from a hyper-thermophilic bacteria, *Thermotoga maritima* (PDB entry: 1I8U; Notenboom et al., 2001; Wassenberg et al., 1997) folded in similar 12-stranded β -sandwich with domain C of Td46GT (Fig. 3-16). But this domain was reported to bind with D-glucose and β -glucans including celooligosaccharides, distinct from domain C of Td46GT, which binds to a MOS mimic and might be classified in a new CBM family.

For further confirmation of functions of domain C of Td46GT, additional studies such like mutagenesis on residues interacted with acarbose ligand (Fig.3-7), and isothermal titration calorimetry analysis, might be required. Furthermore, it is reasonable to propose that domain C of Td46GT contributes to the calcium ion-dependent thermostability of the enzyme. Nine hydrogen bonds were identified at the interface between domains A and C (Fig. 3-6), suggesting that this interdomain interaction may play a role in maintaining the structural integrity of the catalytic domain A. Notably, the calcium ion-binding sites in domain C are in close proximity to this hydrogen-bonding region. Therefore, calcium ion-binding may indirectly stabilize the interface of domains A and C, resulting in enhancing thermostability by calcium ion.

In addition, another calcium ion-binding site in domain A is positioned near Arg600 (Figs. 3-5A, 3-8 and 3-10), a residue that forms two hydrogen bonds with the acarbose ligand at subsites -1 and +1. Accordingly, calcium ion-binding at domain A may also affect the catalytic activity of Td46GT, possibly by stabilizing substrate-binding.

Table 3-1. Summary of data collection and refinement statistics of X-ray crystallography of Td46GT.

		Acarbose complex	Apo-form
PDB entry		9XKN	9XKP
Data collection	Beamline	SPring-8 BL45XU	SPring-8 BL45XU
	Space group	$P4_3 2_1 2$	$P4_3 2_1 2$
	Unit cell parameters a, b, c, (Å)	112.9, 112.9, 391.4	112.8, 112.8, 390.4
	Unit cell parameters α, β, γ (°)	90, 90, 90	90, 90, 90
	Wavelength (Å)	1.00	1.00
	Resolution range (Å)	48.9–2.14 (2.27-2.14)	48.8–1.96 (2.08-1.96)
	Total number of reflections	5,684,016 (894,653)	7,415,599 (1,215,019)
	Number of unique reflections	267,317 (42,849)	346,245 (55,902)
	R_{meas} (%)*	29.3 (194)	36.9 (195)
	$\langle I/\sigma(I) \rangle$	16.0 (2.05)	16.3 (1.89)
Refinement	$CC_{1/2}$	99.9 (74.2)	99.8 (74.4)
	Completeness (%)	99.8 (99.1)	100 (99.8)
	Redundancy	21.3 (20.9)	21.4 (21.7)
	Number of reflections used	140,225	214,566
	Number of reflections for R_{free}	1,683	1,995
	$R_{\text{work}}/R_{\text{free}}$ (%)**	20.6/22.7	22.4/23.9
	Number of atoms		
	Macromolecules	14,032	14,032
	Ligand	179	52
	Water	698	1,059
	B-factors (Å ²)		
	Macromolecules	37.3	27.6
	Ligand	40.3	35.0
	Water	37.7	32.9
	RMSD from ideal		
	Bond lengths (Å)	0.002	0.006
	Bond angles (°)	0.47	0.84
	Ramachandran		
	Favored (%)	97.12	97.29
	Allowed (%)	2.82	2.60
	Outliers (%)	0.06	0.11

Values in parentheses are for the highest resolution shell.

* $R_{\text{meas}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

** $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, R_{free} was calculated for 5% randomly selected test sets that were not used in the refinement.

Table 3-2. Summary of secondary structure of (α/α)₆-barrel GH15 catalytic domain A of Td46GT.

Secondary structure	Residue number
α 1 helix	329–345
β 1 strand	365–367
α 2 helix	368–380
α 3 helix	384–397
β 2 strand	407–409
α 4 helix	424–440
α 5 helix	443–466
α 6 helix	487–507
α 7 helix	510–528
β 3 strand	548–550
β 4 strand	554–557
α 8 helix	562–570
α 9 helix	578–591
α 10 helix	627–639
α 11 helix	643–653
α 12 helix	683–696

Table 3-3. Summary of secondary structure of sugar binding C-terminal domain C of Td46GT.

Secondary structure	Residue number
β 1 strand	709–711
3_{10} helix	723–730
β 2 strand	735–737
β 3 strand	753–759
β 4 strand	763–770
β 5 strand	784–790
β 6 strand	816–821
β 7 strand	828–832
β 8 strand	836–842
β 9 strand	850–852
β 10 strand	856–862
β 11 strand	877–889
β 12 strand	893–907

Table 3-4. Summary of secondary structure of N-terminal domain N of Td46GT.

Secondary structure	Residue number
β 1 strand	45-49
β 2 strand	54-59
β 3 strand	74-80
β 4 strand	89-98
β 5 strand	103-105
β 6 strand	113-117
β 7 strand	123-127
β 8 strand	138-145
β 9 strand	153-160
β 10 strand	166-176
β 11 strand	188-193
β 12 strand	198-202
β 13 strand	210-215
β 14 strand	221-226
α 1 helix	239-246
β 15 strand	254-257
β 16 strand	260-270
β 17 strand	274-285

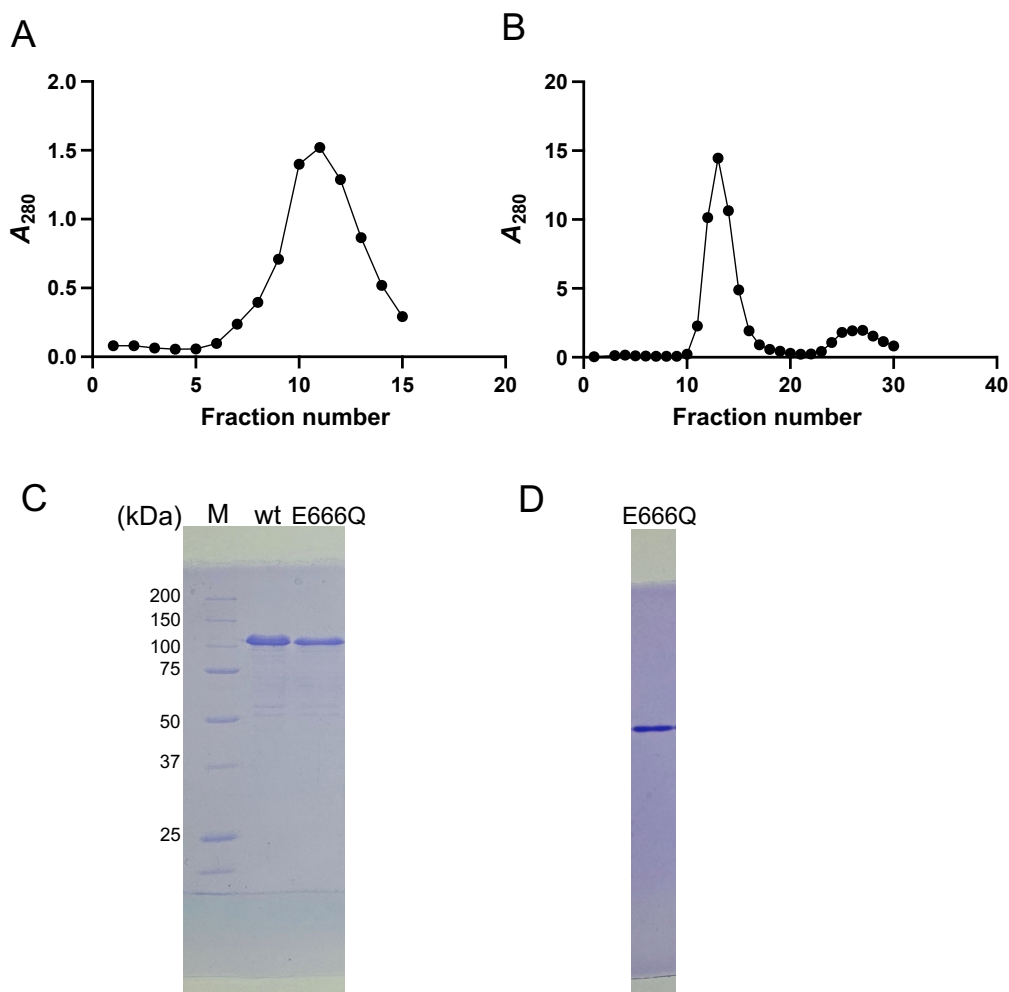


Fig. 3-1. Purification of Td46GT E666Q mutant.

A, elution profile. Column, Ni²⁺-immobilized Sepharose Fast Flow; equilibration, 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, elution, 0.03-0.5 M imidazole (elution volume, 160 mL), fraction volume, 5 mL. B, elution profile of anion-exchange column chromatography. Resource Q column (6 mL), ÄKTA purifier, 2 mL/min; equilibration, 10 mM HEPES-NaOH buffer (pH 7.0); elution, a linear gradient of 0-0.5 M NaCl (elution volume, 30 mL) in 10 mM HEPES-NaOH buffer (pH 7.0). C, SDS-PAGE analysis. Lane M, molecular mass standards (kDa), wt, wild-type Td46GT. D, native-PAGE analysis of E666Q, 1 µg of protein was applied.

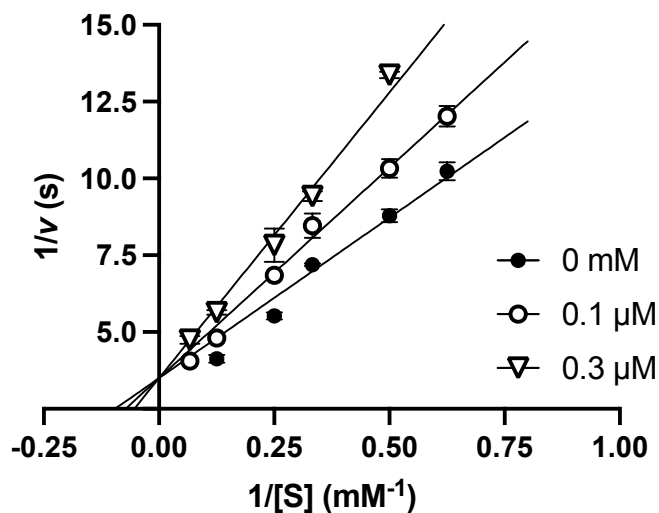


Fig. 3-2. Double reciprocal plots for reaction of wilt-type Td46GT with pNP-Glc in the presence of acarbose.

Black circles, white circles, and white triangles indicate acarbose concentrations of 0, 0.1, and 0.3 μM , respectively. The values shown are the average of independent triplicate experiments. Error bars show the standard deviation of the three values from triplicate experiments. $[S]$, the substrate concentration of pNP-Glc.

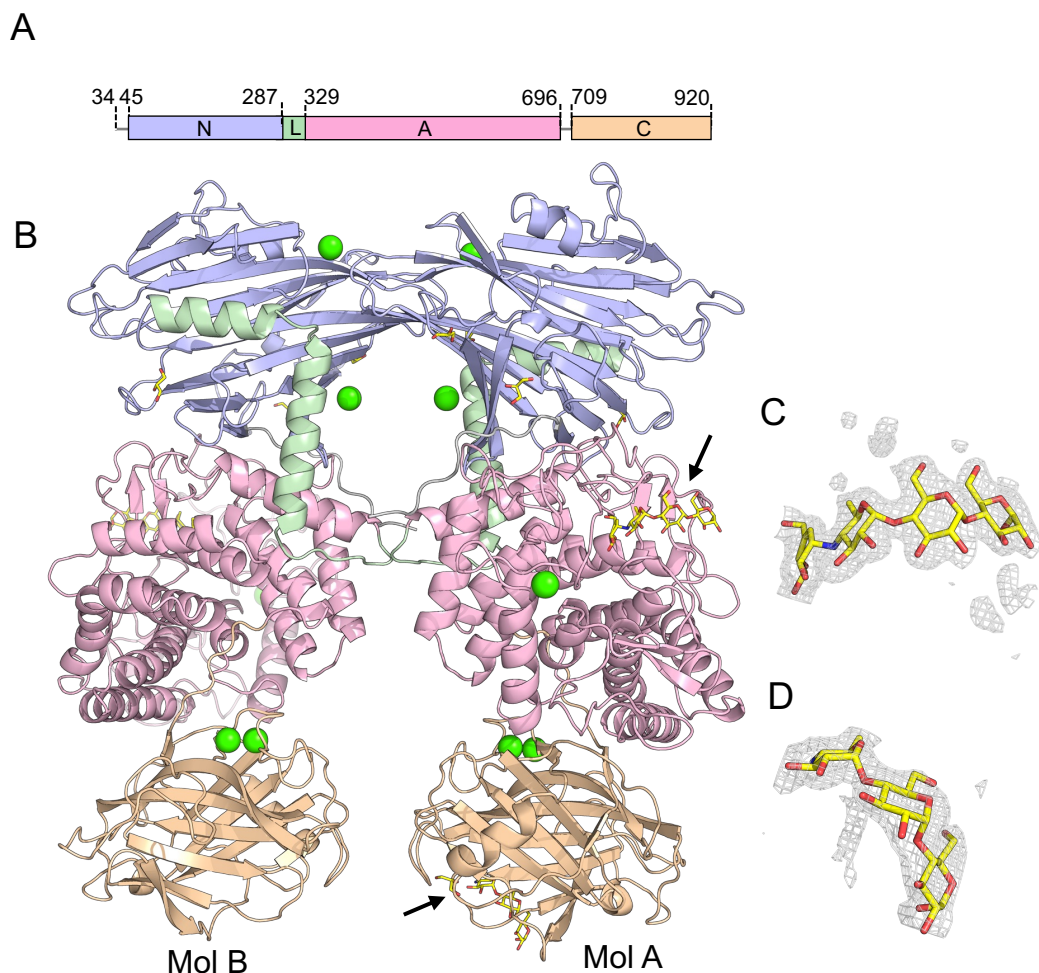


Fig. 3-3. Overall structure of Td46GT in complex with acarbose.

A, Domain organization of Td46GT (Thr34 to Glu920). B, ribbon diagram of overall structure of Td46GT (nucleophile mutant, E666Q) in complex with acarbose. The structure is shown in coloring from domain organization: blue, N-terminal domain N; pale green, a helix-loop-helix motif linker, L; pink, the GH15 (α/α)₆-barrel catalytic domain A; wheat orange, C-terminal domain C. Calcium ions are shown by green spheres. Acarbose and acarbose derived trisaccharides are shown by yellow sticks. C, D, the *Fo*–*Fc* OMIT map of acarbose and acarbose derived trisaccharide bound to domain A and domain C, respectively (contoured at 2.5 σ). Black arrows indicate acarbose-binding sites.

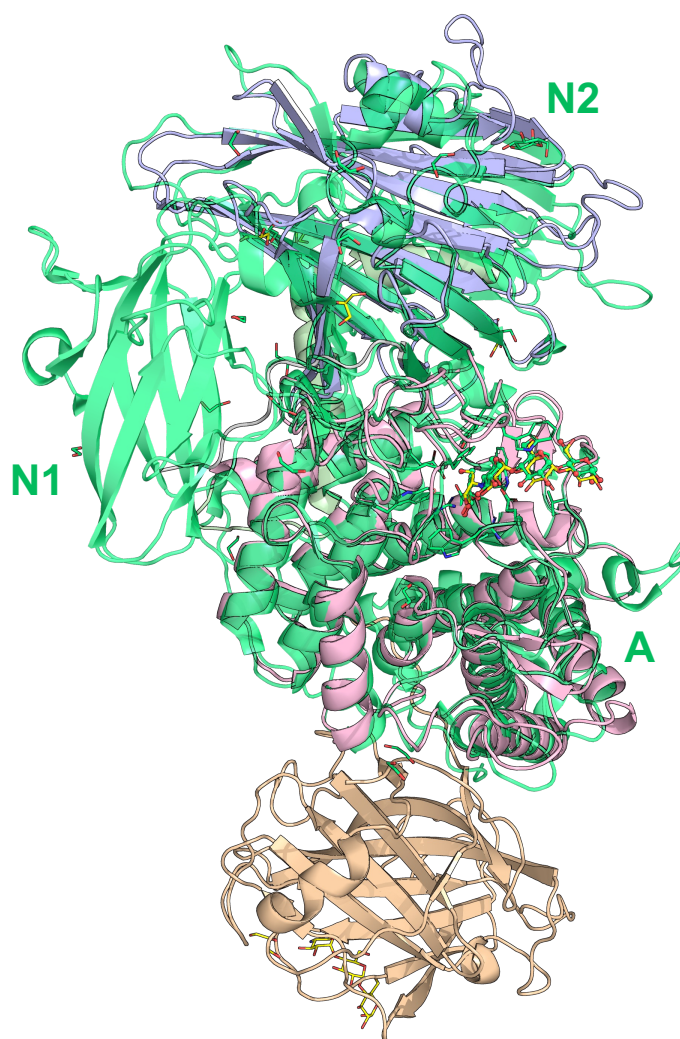


Fig. 3-4. Overall structural comparison of Td46GT and DDase.

Overlap of overall structure of Td46GT (same coloring as Fig. 3-3) and a protomer (chain B) of DDase-Δ382C dimer structure (green; PDB entry:9JU0).

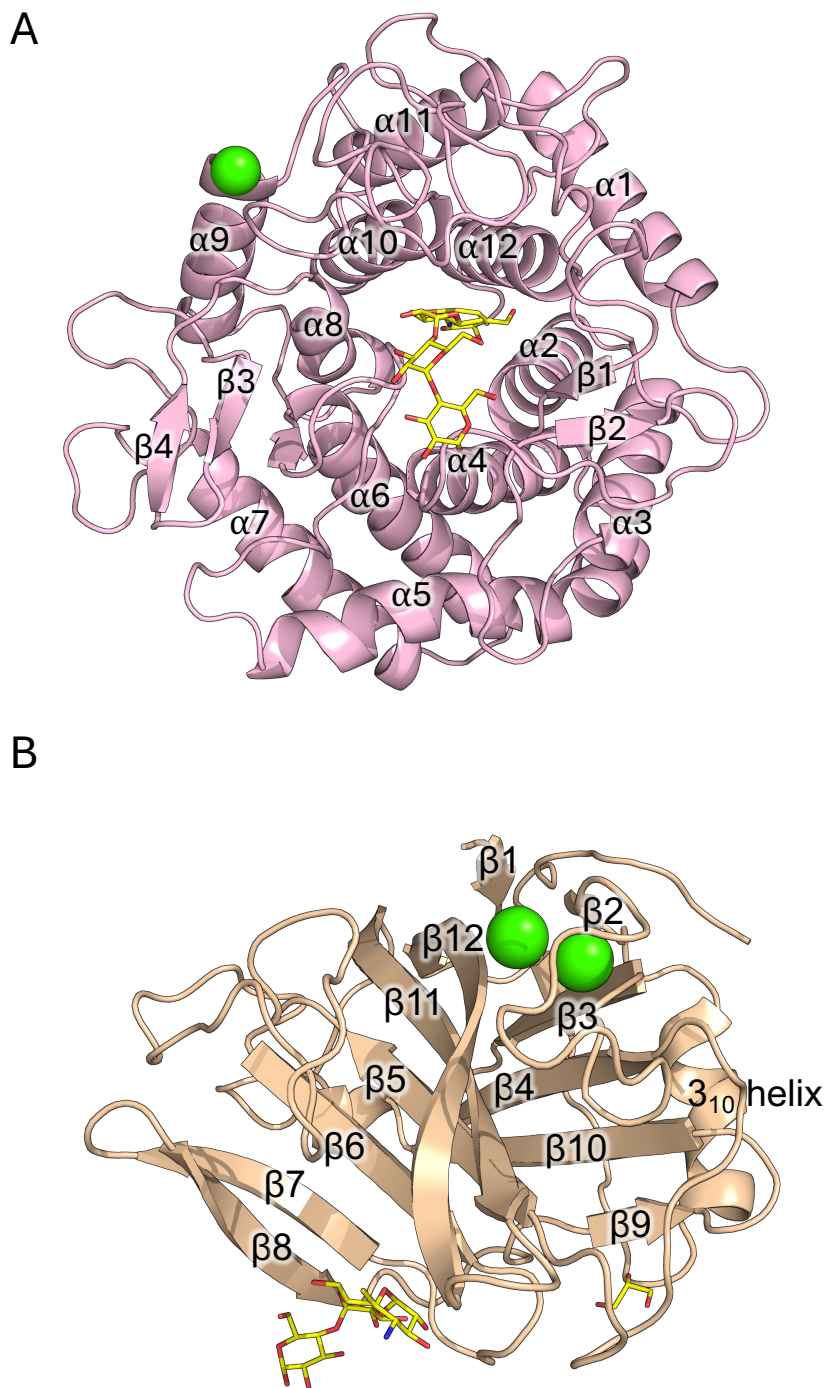


Fig. 3-5. Fold of domains A and C of Td46GT in complex with acarbose.

A, the $(\alpha/\alpha)_6$ -barrel fold of domain A of Td46GT. B, The 12-stranded β -sandwich fold of domain C of Td46GT, same coloring as Fig. 3-3.

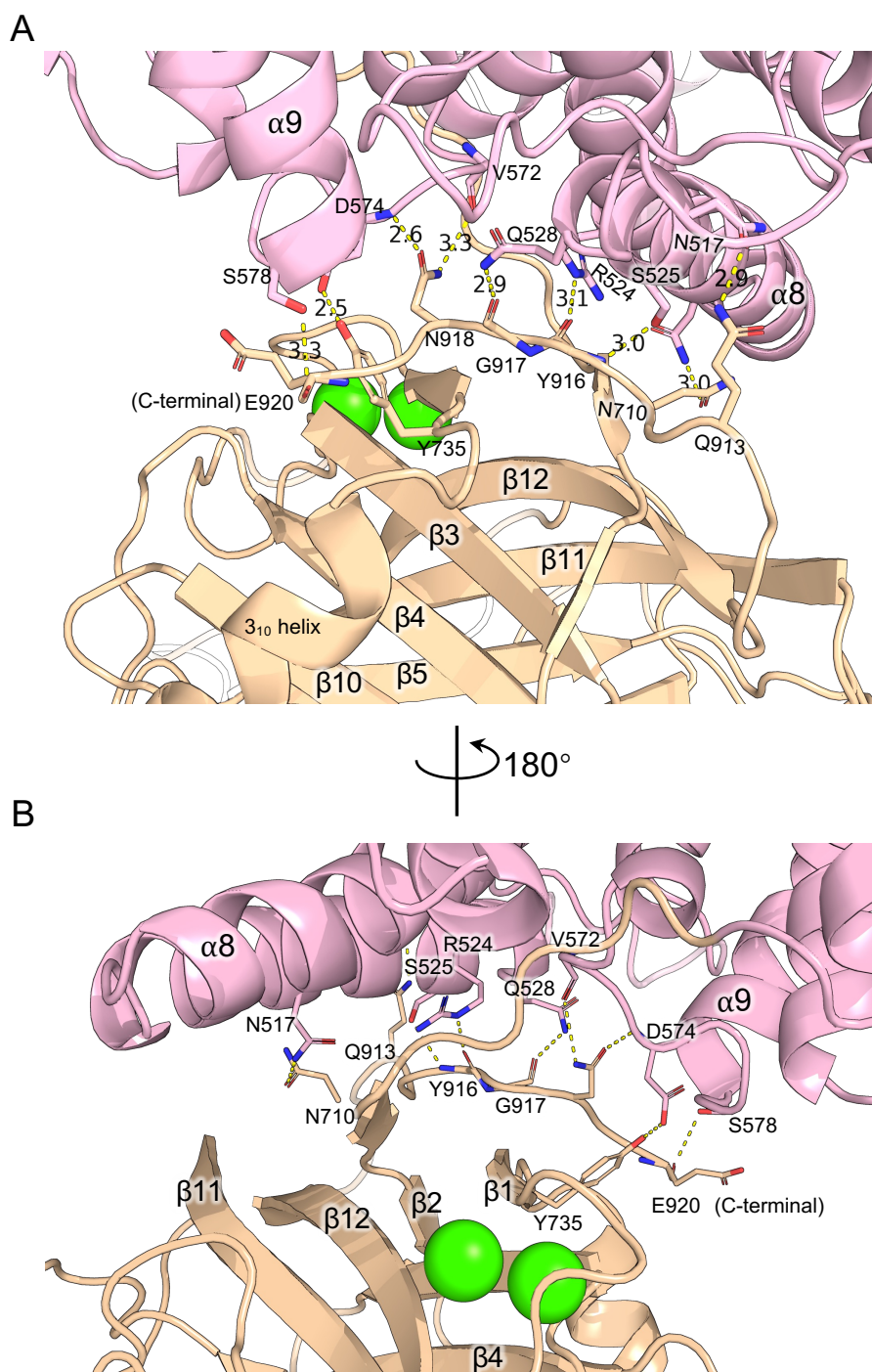


Fig. 3-6. Interactions between domains A and C of Td46GT.
 The structure in complex with acarbose (Mol A) is depicted, same coloring as Fig. 3-3. Predicted hydrogen bonds are shown by yellow dashed lines.

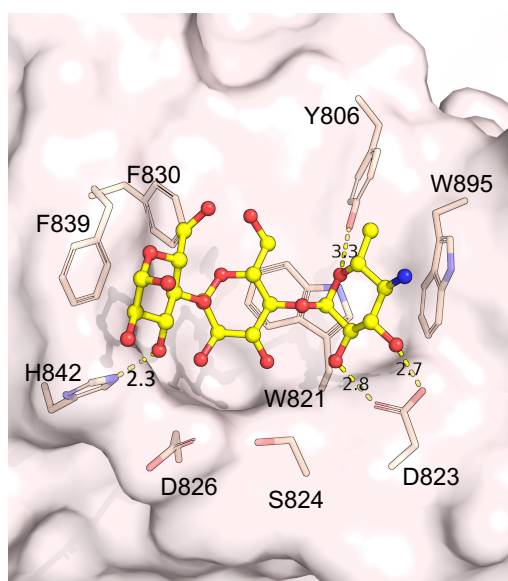


Fig. 3-7. Sugar-binding site in domain C of Td46GT.

Surface in pale pink. Residues around the acarbose reducing end trisaccharide unit are shown by yellow sticks. Predicted hydrogen bonds are shown in yellow dashed lines.

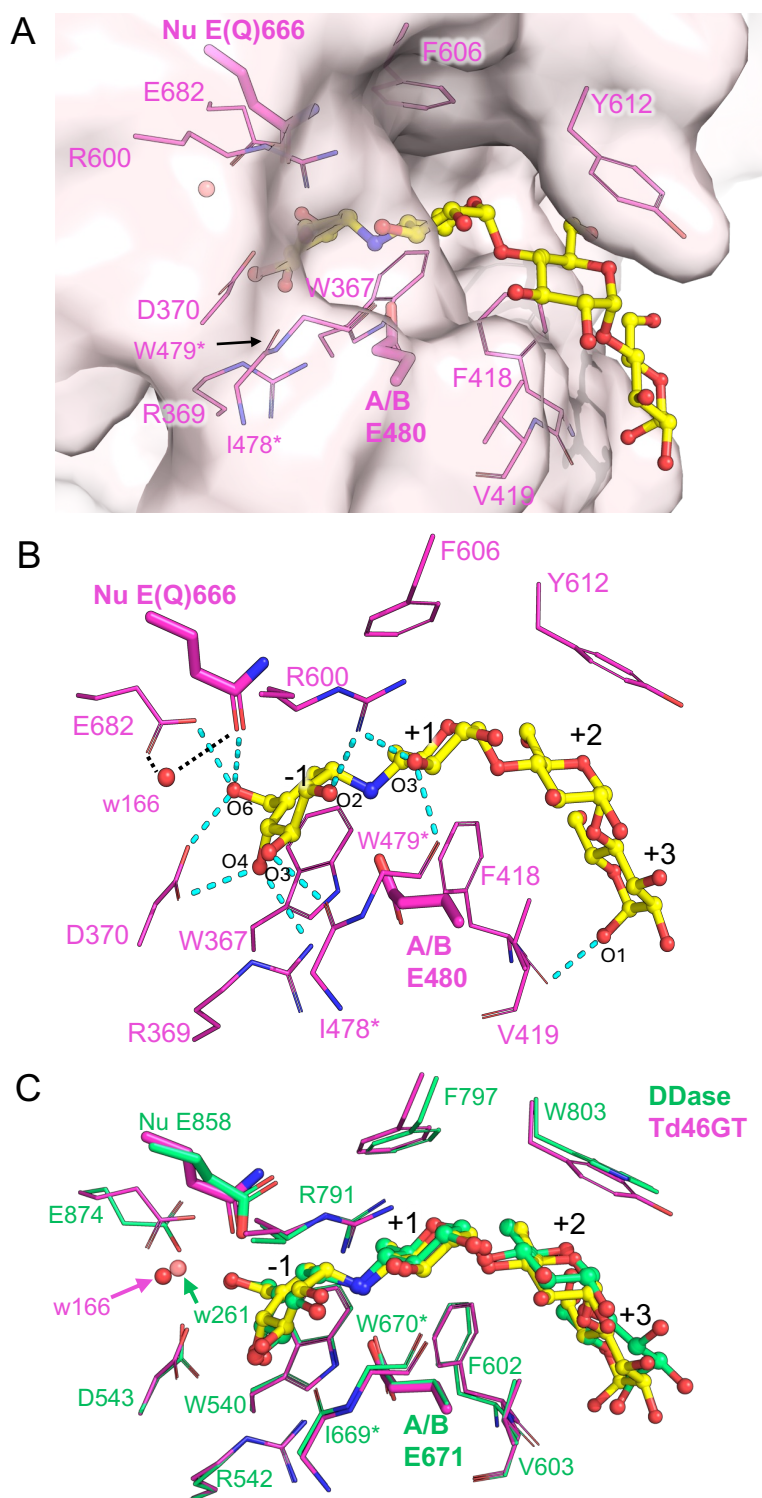


Fig. 3-8. Active pocket of domain A of Td46GT.

A, surface view in pale pink. Residues around the acarbose ligand are shown in magenta sticks. B, stick view. Predicted hydrogen bonds are shown by cyan dashed lines. The water-mediated hydrogen bond between Glu682 and Gln666 is shown by black dashed lines. C, overlap with DDase (green). Nu, nucleophile catalyst. A/B, acid/base catalyst. The arrows indicate water molecule observed in subsite -1 of Td46GT and DDase (semi-transparent), respectively. *, residue that only mainchain is shown.

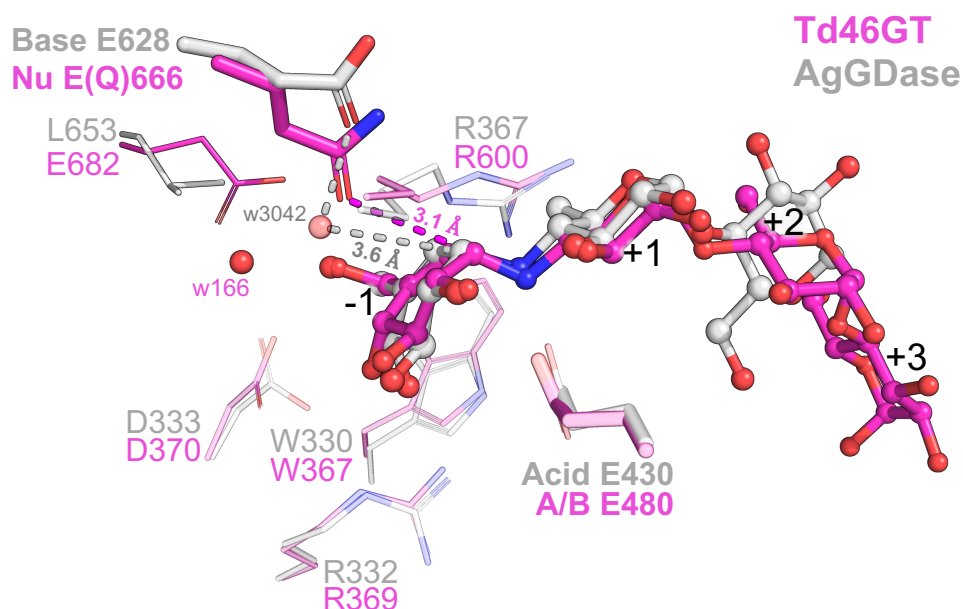
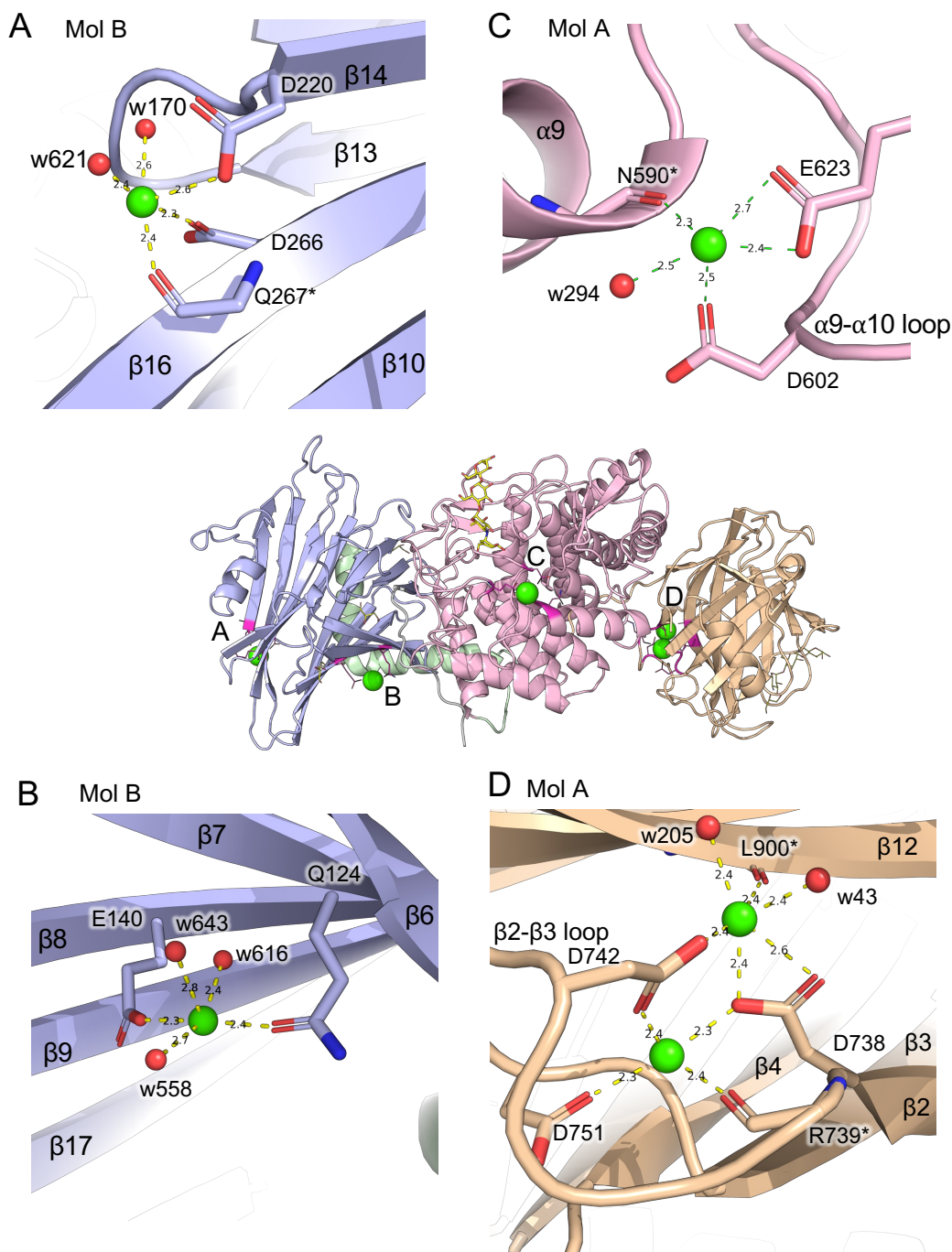


Fig. 3-9. Comparison of subsites -1 and +1 forming residues in GH15 enzymes.

Gray, *Arthrobacter globiformis* I42 glucodextranase (AgGDase; PDB: 1ULV). Magenta, Td46GT. Arrows indicate non-catalytic water molecule observed in subsite -1 of Td46GT and the nucleophilic water observed in AgGDase (semi-transparent). Catalytic residues are shown in bold sticks. Overlapped and conserved position are shown in transparency.



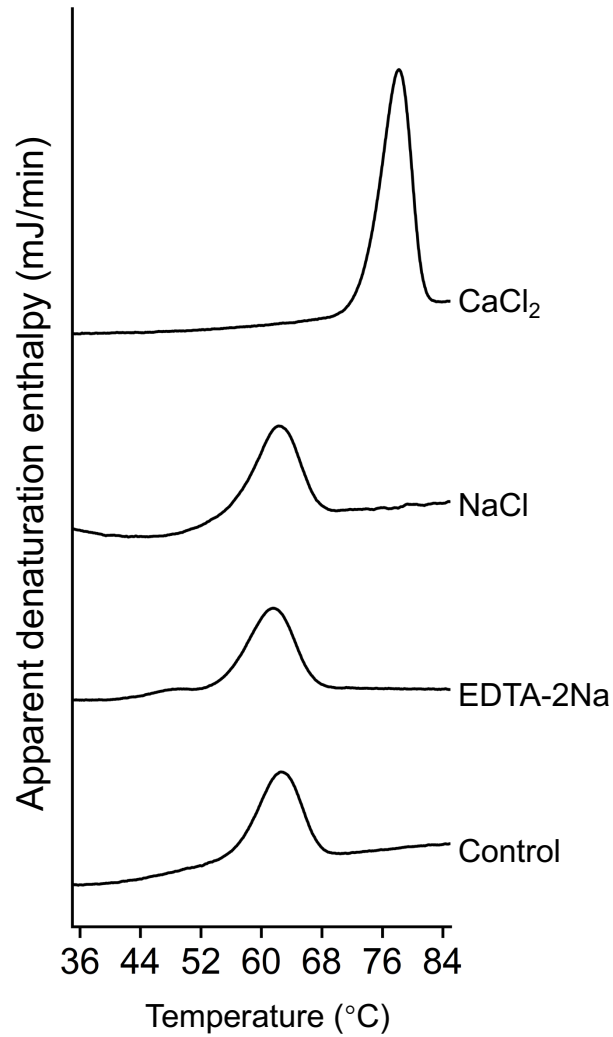


Fig. 3-11. DSC analysis profile for unfolding of wild-type Td46GT.

DSC analysis was performed using 1.24 μM wild-type Td46GT in 25 mM sodium acetate buffer (pH 5.6; control) containing 20 mM CaCl_2 or 40 mM NaCl or 20 mM EDTA-2Na .

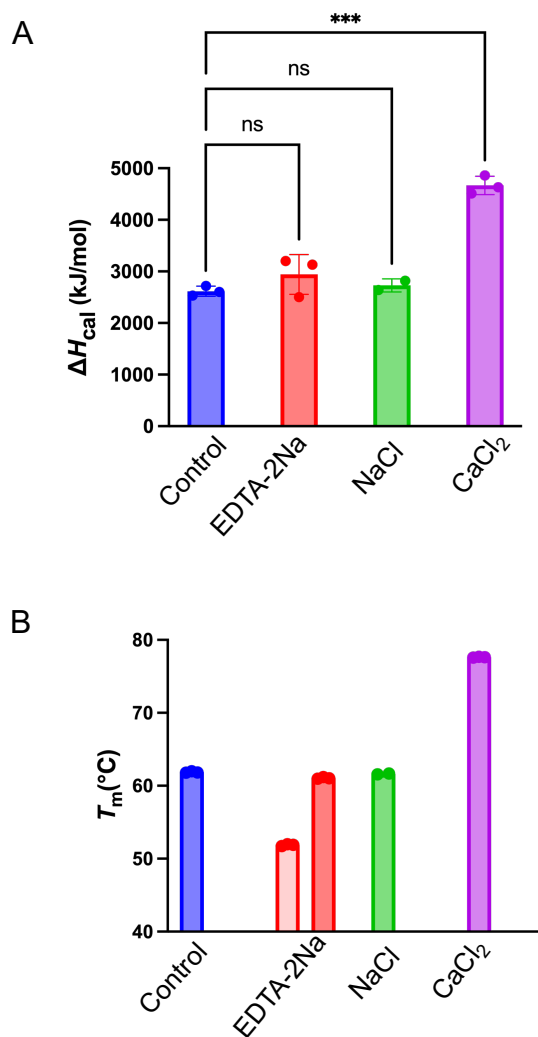


Fig. 3-12. T_m and enthalpy for unfolding of wild-type Td46GT.

A, calorimetric enthalpy for unfolding (ΔH_{cal}). A one-way ANOVA analysis was conducted (n=3; ns: $P > 0.12$; *** $P < 0.001$). B, melting temperature (T_m). The average values and standard deviations were calculated by three values from triplicate experiments.

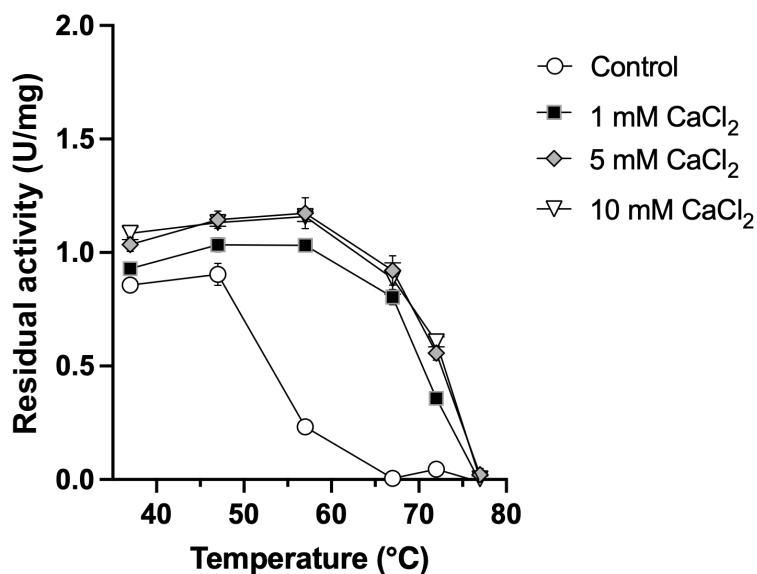


Fig. 3-13. Effect of calcium ion on thermostability of Td46GT.

Temperature-residual activity profile of Td46GT wild type. Enzymes were incubated in 37-77 °C for 15 min, then the residual activity was measured under the standard condition (pH4.5, 37 °C, section 2.2.7). Circle, control; square, 1 mM CaCl₂; diamond, 5 mM CaCl₂; triangle, 10 mM CaCl₂. Values and error bars show the average and standard deviation of the three values from triplicate experiments.

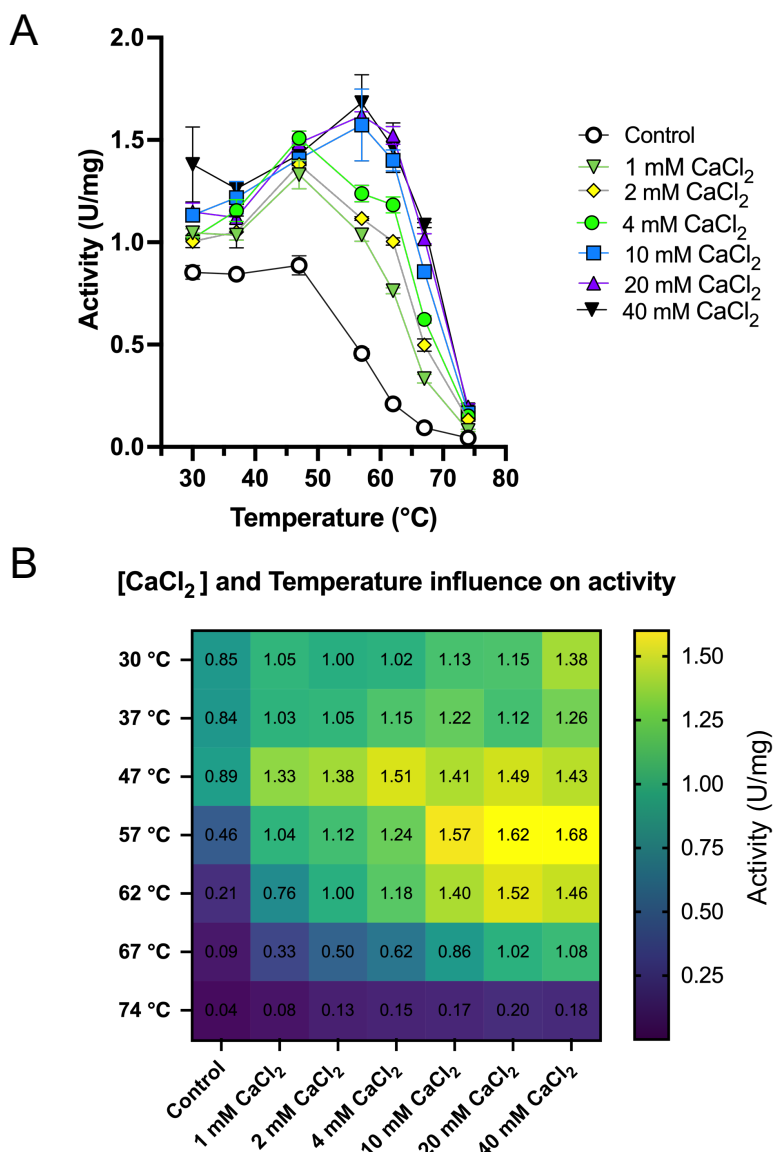


Fig. 3-14. Effect of calcium ion on activity and optimum temperature of wild-type Td46GT.

Effect of 0-40 mM of CaCl₂ on temperature-activity profile of wild-type Td46GT was evaluated, at 30-74 °C. The activity was measured under the standard condition (pH4.5, 37 °C, section 2.2.7). Values and error bars show the average and standard deviation of the three values from triplicate experiments. B, The heatmap representation of panel A data. Color linear gradient for activity is defined in range from 0 to 1.6 U/mg, while >1.6 U/mg, activity is colored by yellow.

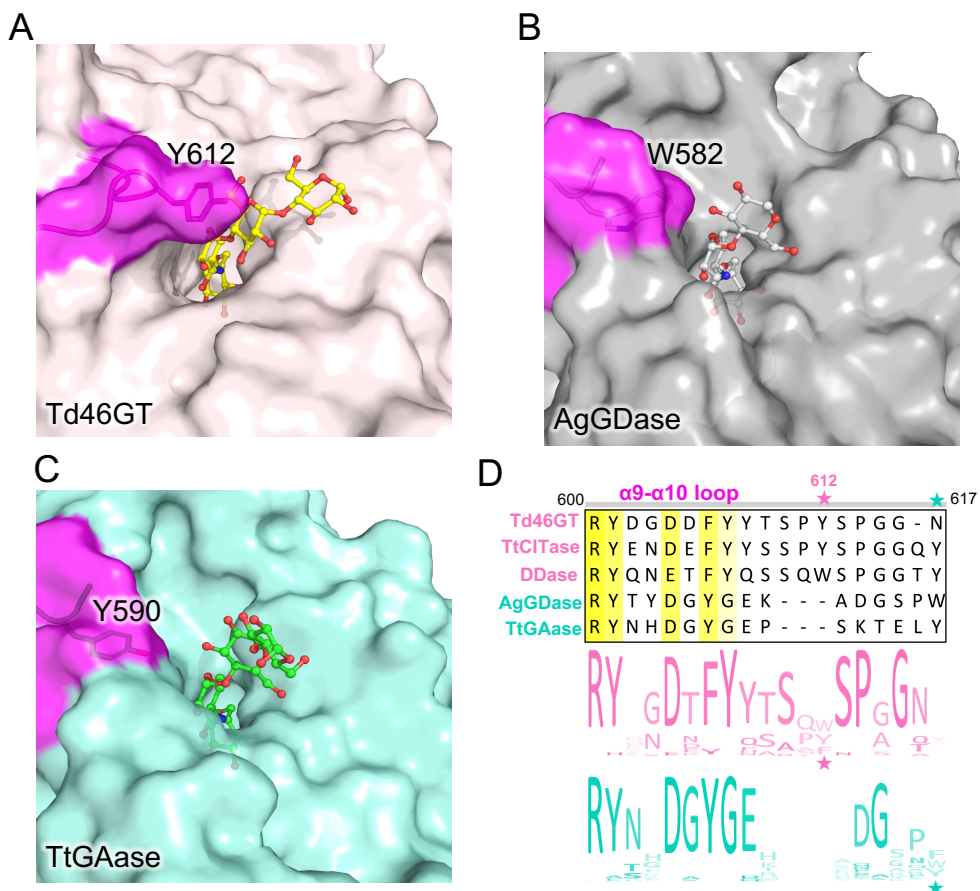


Fig. 3-15. Subsite +2 diversity of GH15 enzymes.

A-C, surface representations of the acarbose-bound active-site pockets of Td46GT (pink), *Arthrobacter globiformis* I42 glucodextranase (AgGDase, PDB entry: 1ULV, gray), *Thermoanaerobacterium thermosaccharolyticum* glucoamylase (TtGAase, PDB entry: 1LF9, green), respectively. The $\alpha 9$ - $\alpha 10$ loop close to subsite +2 is shown in magenta. D, Sequence alignment and sequence logo of GH15 enzymes, derived from Fig. 1-2. Red and green stars indicate residue in 612th position of Td46GT.

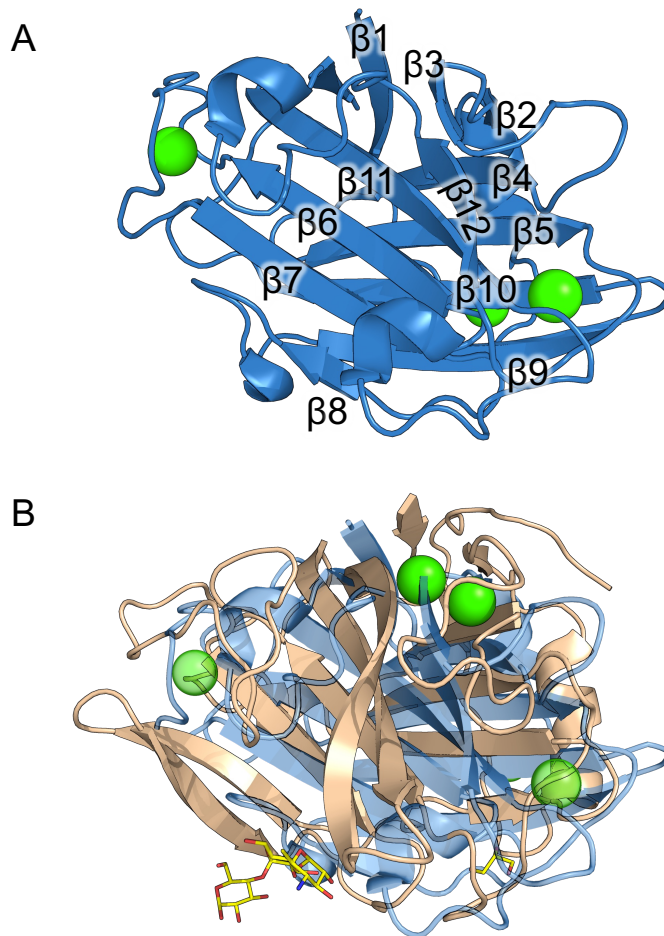


Fig. 3-16. Domain C of Td46GT exhibits weak similarity to an extremely thermostable, calcium-binding CBM9 domain.

A, ribbon diagram of an extremely thermostable C-terminal calcium ion-binding family 9 CBM domain of xylanase from a hyperthermophilic bacteria, *Thermotoga maritima* (PDB entry: 1I8U). B, overlap with domain C of Td46GT, in same view of Fig. 3-5B.

4. Molecular basis for donor and acceptor specificities of Td46GT and Pt46GT

4.1 Introduction

Td46GT catalyzes both α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylations in acceptor-substrate dependent manner as shown in chapter 2. In chapter 3, the crystal structure of Td46GT in complex with acarbose is revealed (Figs. 3-3 and 3-8), and the Phe418-Val419 dyad in the α 3- α 4 loop and Tyr612 in the α 9- α 10 loop of domain A of Td46GT are predicted to have hydrophobic interaction with the D-glucosyl residues of acarbose in subsites +2 (Fig. 3-8). These Phe-Val dyad and Tyr positions are completely conserved among GH15 α -GT homologues including DDase (Fig.1-2).

In this chapter, Phe418 and Val419 in the Phe-Val dyad and Tyr612 as key residues for the substrate-binding of Td46GT have been mutated and kinetically analyzed. Functions of these residues for donor/acceptor-substrate specificities and α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylation specificity were revealed.

Td46GT and Pt46GT showed different chain-length specificity for MOSs as shown in chapter 2. Based on structural comparison of these two enzymes, difference of α 5- α 6 loop structure was focused as structurally important for this difference of MOS specificity. Td46GT mutant harboring Pt46GT-like α 5- α 6 loop was analyzed.

4.2. Materials and methods

4.2.1. Construction of expression plasmids for Td46GT variants

The expression plasmids for the Td46GT mutants, F418A, F418W, V419A and Y612A, were prepared using a PrimeStar Mutagenesis Basal Kit using the plasmid for the expression of Td46GT wild type as template (see section 2.2.2). Primers used are as follows: for F418A, 5'-GTATCCGCAGTTGAGCCTGAACACGAT-3' and 5'-GTATCCTGCGTTGAGCCTGAACACGAT-3'; for F418W, 5'-GTATCCTGGGTTGAGCCTGAACACGAT-3' and 5'-CTCAACCCAGGATACGTAATTGTTAGT-3'; for V419A, 5'-TCCTTTGCAGAGCCTGAACACGATTCA-3' and 5'-AGGCTCTGCAAAGGATACGTAATTGTT-3'; for Y612A, 5'-TCTCCAGCAAGTCCCGGAGGGAATGAG-3' and 5'-GGGACTTGCTGGAGAGGTGTAATAGAA-3'.

The expression plasmid for the mutant Td46GT Q482I483→GDYS, was prepared by assembly of two Td46GT gene-derived DNA fragments and the linear pET-23a fragment. Preparation of DNA fragments includes PCR using KOD FX Neo DNA polymerase and primers 5'-GAGGGCGATTACTCGGAATATAACGCATTT-3' and 5'-CAGTGGTGGTGGTGGTGGTGCTCTACATTACCATAATACC-3', and the other PCR used primers 5'-CGAGTAATCGCCCTCTTCCCAGATGCT-3' and 5'-TTTAAGAAGGAGATATACATATGGATACATTACCCACTGA-3'. These two DNA fragments (1,376 bp, and 1,357 bp, respectively) were extracted after electrophoresis using 0.8% agarose gel and purified by Wizard SV Gel and PCR Clean-Up System. Assembly of the three DNA fragments was performed by using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA). Mutant Td46GT Q482I483→GDYS/A476K was prepared by Primestar Mutagenesis Basal Kit using Q482I483→GDYS as template, and primers 5'-GAAGATAAGAGCATCTGGGAAGAGGGC-3' and 5'-GATGCTCTTATCTTCCTCACCAAAACC-3'.

The mutant enzymes were produced in *E. coli* BL21(DE3) harboring the expression plasmids and purified by Ni²⁺-immobilized affinity column chromatography and anion exchange column chromatography as described in section 2.2.3.

Kinetic parameter and subsite affinities calculation methods were identical with that used for wild-type Td46GT, as described in section 2.2.9. However, 5–120 mM G2, 1–40 mM G3, 0.5–50 mM G4, 0.6–18 mM G5, 10–120 mM IG2, 0.2–40 mM IG3, 0.2–30 mM IG4 were used.

The method was same as that used for wild-type Td46GT, described in section 2.2.10, with modification of substrate concentrations. For F418W, 5 mM IG2 and 50 mM G5; 8 mM IG2 and 40 mM G4; 20 mM IG2 and G3; 20 mM IG3 and 10 mM G2; 10 mM IG3 and 25 mM G4; and 60 mM D-glucose and 10 mM G4 were used.

For F418A mutant, 5 mM IG2 and 50 mM G5; 10 mM IG2 and 50 mM G4; 20 mM IG2 and 20 mM G3; 10 mM IG3 and 15 mM G4; 20 mM IG3 and 10 mM G2; and 20 mM D-glucose and 15 mM G4 were used.

For V419A mutant, 7.5 mM IG2 and 25 mM G5; 12 mM IG2 and 20 mM G4; 60 mM D-glucose and 20 mM G5; 80 mM D-glucose and 15 mM G3; 12 mM IG3 and 20 mM G5; and 160 mM D-glucose and 30 mM G2 were used.

For Y612A mutant, 10 mM IG2 and 60 mM G5; 10 mM IG2 and 40 mM G4; 40 mM D-glucose and 20 mM G3; 20 mM G2 and 20 mM D-glucose; 20 mM IG3 and 20 mM G5; and 48 mM D-glucose and 40 mM G4 were used.

4.3. Results

4.3.1. Preparation of Td46GT mutants

Purified Td46GT mutants showed single band in both SDS-PAGE and native-PAGE analysis, indicating the homogeneity of the purified enzymes. For SDS-PAGE, mutants displayed identical molecular mass with that of wild-type, 106kDa, close to the theory mass, 100kDa (Figs. 4-1, 4-2 and 4-3). The activity of purified F418A, F418W, V419A, Y612A, Q482I483→GDYS, Q482I483→GDYS/A476K were 0.106, 0.571, 0.100, 1.71, 0.0667 and 0.644 U/mg, respectively. Activity of them were 16.9%, 91.1%, 15.9%, 273%, 10.6% and 103% compared to that of the wild type (0.627 U/mg).

4.3.2. Kinetic analysis of Td46GT mutants

In the single-substrate reactions with MOS, F418A and F418W showed considerable decreases in k_{cat}/K_m values (G3–G5, 1.5–13% compared to the wild type; Table 4-1 and Fig. 4-4). Although these mutant enzymes retained the highest preference toward G4 compared to the wild type, the relative k_{cat}/K_m values of F418A and F418W for G4 compared to those for G2, were 64.0 and 94.1, respectively (the wild type: 706). In the single-substrate reaction with IMOs (IG2–IG4), F418W retained the DP-dependent increase in k_{cat}/K_m observed in the wild type, but F418A displayed the highest k_{cat}/K_m for IG3. In two-substrate reactions, F418A/W exhibited much lower k_A/k'_{IG2} to MOS than the wild type (Fig. 4-5A), whereas they showed k'_A/k'_{IG2} to IMOs and MOSs comparable with the wild type (Fig. 4-5B). V419A exhibited decreased k_{cat}/K_m values for both MOS and IMO but retained chain-length specificity for MOS and

IMO (Table 4-1 and Fig. 4-4). Consistently, V419A retained more acceptor specificity in both α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylations than F418A (Table 4-2 and Fig. 4-5).

Y612A mutation did not decrease k_{cat}/K_m change for G2 but severely decreased the values for G3–G5 (Table 4-1 and Fig. 4-4). Y612A showed the highest k_{cat}/K_m for G4, but the relative value to that of G2 was only 32.7. For IMOs, k_{cat}/K_m values of Y612A did not significantly increase with increasing substrate DP. This mutant showed much lower RASC values for both α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-transglucosylations using MOSs as acceptors (0.0405-0.268) than the wild type (0.868-5.58), while its RASC values for α -(1 $\rightarrow$ 6)-transglucosylations with D-glucose and IG3 acceptors were comparable with those of the wild type (Table 4-2 and Fig. 4-5).

4.3.3. Kinetic analysis of Td46GT multiple mutants in single substrate reactions

Compared with Td46GT, the α 5- α 6 loop of Pt46GT has two additional residues (Gly617-Asp618 dyad; Fig. 4-6). Intriguingly, this Gly-Asp dyad is highly conserved together with a Lys in 611 position of Pt46GT. In comparison, Td46GT replaces this Lys by Ala476 at the corresponding position, and this Ala is conserved together with the absence of Gly-Asp dyad. The Lys, only conserved in Pt46GT-like 46GT homologues, possibly makes the α 5- α 6 loop protruded, thereby the Asp618 of Pt46GT (lost in Td46GT) possibly forms hydrogen bond with MOS at subsite +3^m, according to the AlphaFold3 model of Pt46GT (Fig. 4-6, A and B).

In the single-substrate reactions with G2, G4 and G5 (Table 4-3), all k_{cat}/K_m values decreased in both mutants. Q482I483 $\rightarrow$ GDYS exhibited much more lower k_{cat}/K_m values (4.3-11% of the wild type) than Q482I483 $\rightarrow$ GDYS/A476K (25-63% of the wild type; Table 4-3). Q482I483 $\rightarrow$ GDYS/A476K showed threefold higher k_{cat}/K_m for G3, compared with the wild-type Td46GT. In the single-substrate reaction with IMOs (IG2–IG3), k_{cat}/K_m value of Q482I483 $\rightarrow$ GDYS/A476K decreased to similar degree with that for MOS (59-72% of the wild type). The k_{cat}/K_m value toward IG2 of Q482I483 $\rightarrow$ GDYS drastically declined to 0.19% of that of the wild type, and showed lower specificity to IG2 over G2 (0.221; Table 4-3).

4.4. Discussion

According to k_{cat}/K_m values for MOSs and crystal structure in complex with the G4 analogue, acarbose, in chapters 2 and 3, subsites +2^m and +3^m (“m” indicates subsites for MOS-binding) are estimated to have high affinity (Fig. 4-7) and probably have a critical role for high specificity to MOSs with $\geq$ DP 3. Crystal structure

comparison of Td46GT with DDase suggests that the Phe-Val dyad (Phe418 and Val419) in the $\alpha 3$ - $\alpha 4$ loop and an aromatic residue (Tyr612) in the $\alpha 9$ - $\alpha 10$ loop of Td46GT contribute to the formation of subsites +2^m and +3^m (Fig. 4-7). The affinity in subsite +2^m of the F418A, V419A, and Y612A mutants was estimated at 6.48, 11.9, and 2.54 kJ mol⁻¹, respectively (Fig. 4-7C, E, and F). This indicates that Phe418 and Tyr612 are pivotal residues in subsite +2^m. The loss of 8.46 kJ mol⁻¹ of binding affinity in subsite +2^m in the Y612A mutant aligns with energy contributions of CH- π stacking interactions (Wimmerová et al., 2012), consistent with findings on Td46GT crystal structure.

In single-substrate reaction with IMOs, Td46GT displayed the highest k_{cat}/K_m toward IG4 among tested IMOs, and the enzyme was estimated to have high affinity, 6.37 kJ mol⁻¹, in subsite +2ⁱ (“i” indicates subsites for IMOs; Fig. 4-7A and B). As this affinity is lower than of subsite +2^p 8.41 kJ mol⁻¹ (“p” indicates subsites for panose), calculated from k_{cat}/K_m values for panose and IG2, the α -(1→4)-linkage at the reducing end side of the trisaccharide is favorable than α -(1→6)-linkage. The role of the Phe-Val dyad and Tyr612 in forming subsite +2ⁱ was illustrated by the drastic decrease of affinity of F418A, V419A, and Y612A in subsite +2ⁱ which was estimated to be 1.83, 5.08, and 3.05 kJ mol⁻¹, respectively (Fig. 4-7C, E, and F), indicating that Phe418 and Tyr612 were important for the formation of subsite +2ⁱ as well as subsite +2^m. The F418W mutation had less effect on subsite +2ⁱ affinity (4.48 kJ mol⁻¹) than the F418A mutation and provided a positive affinity at subsite +3ⁱ (Fig. 4-7D) whereas this mutation retained great k'_A/k'_{IG2} for MOS acceptors. This implies that a bulky residue at this position is important for the formation of subsites +2ⁱ and +3ⁱ.

Comparison of Td46GT crystal structure with AlphaFold3 predicted model of Pt46GT demonstrated that C-terminal adjacent residues of Glu480 (acid/base catalyst) in $\alpha 5$ - $\alpha 6$ loop of Td46GT were different from those of Pt46GT (Fig.4-6). The Q482I483→GDYS/A476K mutation in Td46GT to mimic Pt46GT increased subsite affinity of +2^m by 4.0 kJ mol⁻¹ but decreased affinity of +3^m by 4.8 kJ mol⁻¹ (Table 4-4). These results suggested that the modified $\alpha 5$ - $\alpha 6$ loop might facilitate the MOS-binding in +2^m and cause steric hinderance in subsite +3^m. As Q482I483→GDYS mutation caused drastic loss of activity and did not change MOS chain-length preference, A476K mutation is required for proper function of the modified $\alpha 5$ - $\alpha 6$ loop in MOS binding. Pt46GT and its homologous 46GTs conserve the Lys residue at the 611 position of Pt46GT in addition to the Gly-Asp dyad. In contrast, Td46GT and its homologous 46GTs, lacking the Gly-Asp dyad, conserves Ala at the position corresponding to Lys611 of Pt46GT. Based on the structure of $\alpha 5$ - $\alpha 6$ loop of the catalytic domain, GH15 46GTs are classified into Td46GT type (high preference for G4) and

Pt46GT type (high preference for G2 and G3).

Table 4-1. Kinetic parameters of Td46GT mutants for single-substrate reactions.

Substrate	F418A			F418W			V419A			Y612A		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
G2	7.17 ± 0.43	84.4 ± 5.5	0.0850 (1)	0.263 ± 0.011	10.3 ± 1.0	0.0255 (1)	1.42 ± 0.09	139 ± 14	0.0102 (1)	24.4 ± 1.8	174 ± 22	0.140 (1)
G3	12.5 ± 1.8	12.2 ± 3.2	1.02 (12.0)	7.08 ± 0.49	17.4 ± 2.2	0.407 (16.0)	66.5 ± 8.6	63.6 ± 14.9	1.05 (103)	7.42 ± 0.42	19.9 ± 2.5	0.373 (2.66)
G4	35.5 ± 0.1	6.53 ± 0.40	5.44 (64.0)	6.59 ± 0.25	2.75 ± 0.32	2.40 (94.1)	80.4 ± 11.4	21.2 ± 4.7	3.79 (372)	44.2 ± 1.4	9.66 ± 0.89	4.58 (32.7)
G5	21.1 ± 1.7	21.5 ± 3.2	0.981 (11.5)	3.81 ± 0.20	5.95 ± 0.95	0.640 (25.1)	108 ± 16	51.4 ± 10.6	2.10 (206)	29.0 ± 1.2	25.5 ± 1.9	1.14 (8.14)
IG2	N.D.	N.D.	0.0416 (0.489)	N.D.	N.D.	0.0641 (2.51)	N.D.	N.D.	0.0142 (1.39)	N.D.	N.D.	0.101 (0.721)
IG3	N.D.	N.D.	0.0847 (0.996)	N.D.	N.D.	0.364 (14.3)	N.D.	N.D.	0.102 (10.0)	N.D.	N.D.	0.330 (2.36)
IG4	N.D.	N.D.	0.0391 (0.460)	N.D.	N.D.	0.447 (17.5)	N.D.	N.D.	0.311 (30.5)	N.D.	N.D.	0.228 (1.63)

The values are mean ± standard deviation for triplicate measurements. N.D., not determined because a saturation curve was not obtained under the analytical conditions. Values shown in parentheses are relative values to k_{cat}/K_m for G2.

Table 4-2. Relative acceptor specificity constants of Td46GT mutants.

Acceptor	F418A			F418W			V419A			Y612A		
	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}	k_A/k'_{IG2}
G5	0.0962 ± 0.0031	N.D.	N.D.	0.0575 ± 0.0019	N.D.	N.D.	0.192 ± 0.007	N.D.	0.0944 ± 0.0057	N.D.	N.D.	N.D.
G4	0.141 ± 0.009	N.D.	N.D.	0.181 ± 0.007	N.D.	N.D.	0.364 ± 0.010	N.D.	0.214 ± 0.012	N.D.	N.D.	N.D.
G3	0.414 ± 0.043	0.389 ± 0.050	0.172 ± 0.012	0.172 ± 0.012	1.09 ± 0.05	1.83 ± 0.02	0.837 ± 0.012	0.268 ± 0.008	0.114 ± 0.003	0.170 ± 0.014	0.0987 ± 0.063	0.441 ± 0.035
G2	0.166 ± 0.016	1.27 ± 0.04	0.133 ± 0.032	0.133 ± 0.032	2.82 ± 0.07	0.710 ± 0.071	1.40 ± 0.11	0.0405 ± 0.0030	N.D.	N.D.	N.D.	N.D.
Glc	N.D.	0.124 ± 0.001	N.D.	N.D.	0.0675 ± 0.0026	N.D.	0.0787 ± 0.028	N.D.	N.D.	N.D.	N.D.	N.D.
IG3	N.D.	0.166 ± 0.013	N.D.	N.D.	0.737 ± 0.037	N.D.	1.47 ± 0.05	N.D.	N.D.	N.D.	N.D.	N.D.

Subscript A indicates acceptor. Subscript A indicates acceptor. k_A and k'_A , second-order constants of acceptor A for α -(1 $\rightarrow$ 4)-transglucosylation and α -(1 $\rightarrow$ 6)-transglucosylation, respectively. k_A/k'_{IG2} , α -(1 $\rightarrow$ 4)-transglucosylation; k'_A/k'_{IG2} , α -(1 $\rightarrow$ 6)-transglucosylation.

Table 4-3. Kinetic parameters of Td46GT $\alpha 5$ - $\alpha 6$ loop mutants to mimic wild-type Pt46GT.

Substrate	Q482I483→GDYS/A476K				Q482I483→GDYS					
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	^a Relative to Td (-fold)	^b Relative to Pt (-fold)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	^a Relative to Td (-fold)	^b Relative to Pt (-fold)
G2	3.72 ± 0.07	54.8 ± 1.0	0.0677 (1)	0.627	0.146	N.D.	N.D.	0.00464 (1)	0.0430	0.0100
G3	154 ± 4	6.46 ± 0.34	23.8 (352)	3.03	0.795	N.D.	N.D.	0.595 (128)	0.0757	0.0199
G4	208 ± 25	5.71 ± 0.88	36.4 (538)	0.477	0.484	129 ± 7	14.9 ± 1.7	8.66 (1860)	0.113	0.115
G5	184 ± 3	17.0 ± 0.6	10.8 (160)	0.250	0.482	N.D.	N.D.	2.70 (582)	0.0625	0.121
IG2	N.D.	N.D.	0.0317 (0.468)	0.587	1.45	N.D.	N.D.	0.00103 (0.221)	0.0191	0.0470
IG3	N.D.	N.D.	0.459 (6.78)	0.717	0.856	N.A.	N.A.	N.A.	N.A.	N.A.

The values are mean ± standard deviation for triplicate measurements. N.D., not determined because a saturation curve was not obtained under the analytical conditions. N.A, not analyzed. Values shown in parentheses are relative values to k_{cat}/K_m for G2.

a: k_{cat}/K_m relative to wild-type Td46GT.

b: k_{cat}/K_m relative to wild-type Pt46GT.

Values in parentheses are ratio relative to k_{cat}/K_m for G2.

Table 4-4. Subsite affinity for MOS-binding in comparison of Td46GT variants and wild-type Pt46GT.

Enzyme	Subsite affinity (kJ mol ⁻¹)		
	+2 ^m	+3 ^m	+4 ^m
Td46GT wild type	11.1	5.9	-1.5
Q482I483→GDYS	12.5	6.9	-3.0
Q482I483→GDYS/A476K	15.1	1.1	-3.1
Pt46GT wild type	10.7	2.4	-3.1

Symbol “m” indicates subsites for MOS-binding, referred to Fig.4-7.

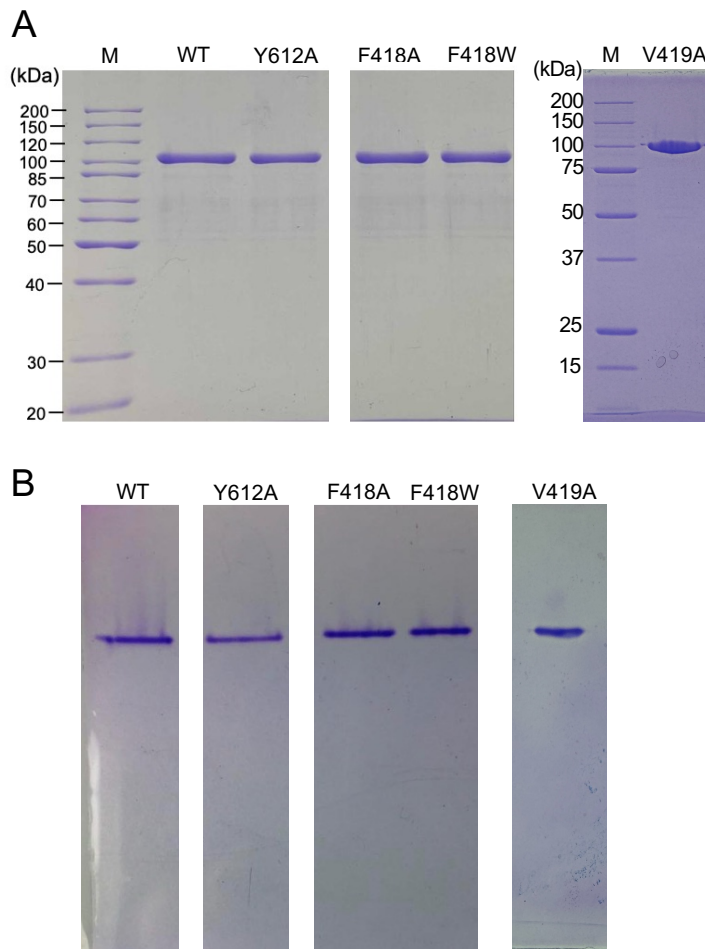


Fig. 4-1. SDS-PAGE and native PAGE of purified Td46GT mutants.
The wild type (WT) and mutants of Td46GT (1.0 μ g) were analyzed by SDS-PAGE (A) and native-PAGE (B). Lane M, molecular mass standards (kDa).

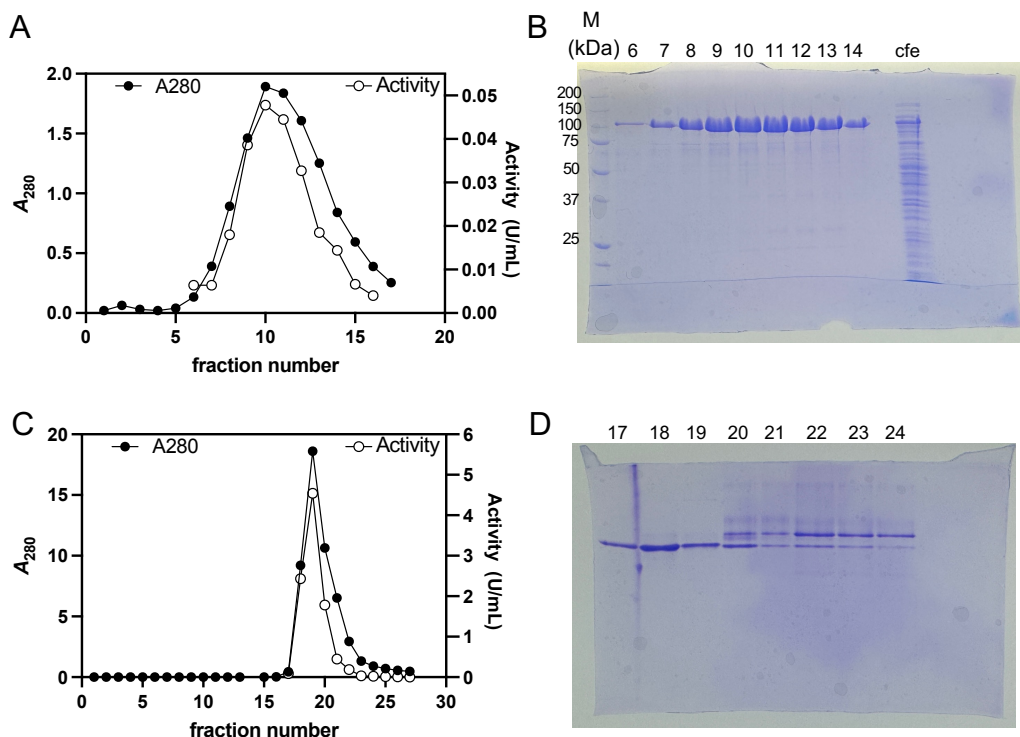


Fig. 4-2. Purification of Td46GT mutant Q482I483→GDYS/A476K.

A, elution profile. Column, Ni²⁺-immobilized Sepharose Fast Flow; equilibration and washing, 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl; elution, 0.03-0.5 M imidazole (elution volume, 160 mL); fraction volume, 5 mL. B, SDS-PAGE. C, elution profile of anion-exchange column chromatography. Resource Q column (6 mL), ÄKTA purifier, 2 mL/min; equilibration buffer, 10 mM HEPES-NaOH buffer (pH 7.0); elution, a linear gradient of 0-0.5 M NaCl (elution volume, 30 mL) in 10 mM HEPES-NaOH buffer (pH 7.0). D, native-PAGE analysis. Black circle, absorbance at 280 nm; white circle, activity (U/mL). Lane M, molecular mass standards (kDa), cfe, cell free extract.

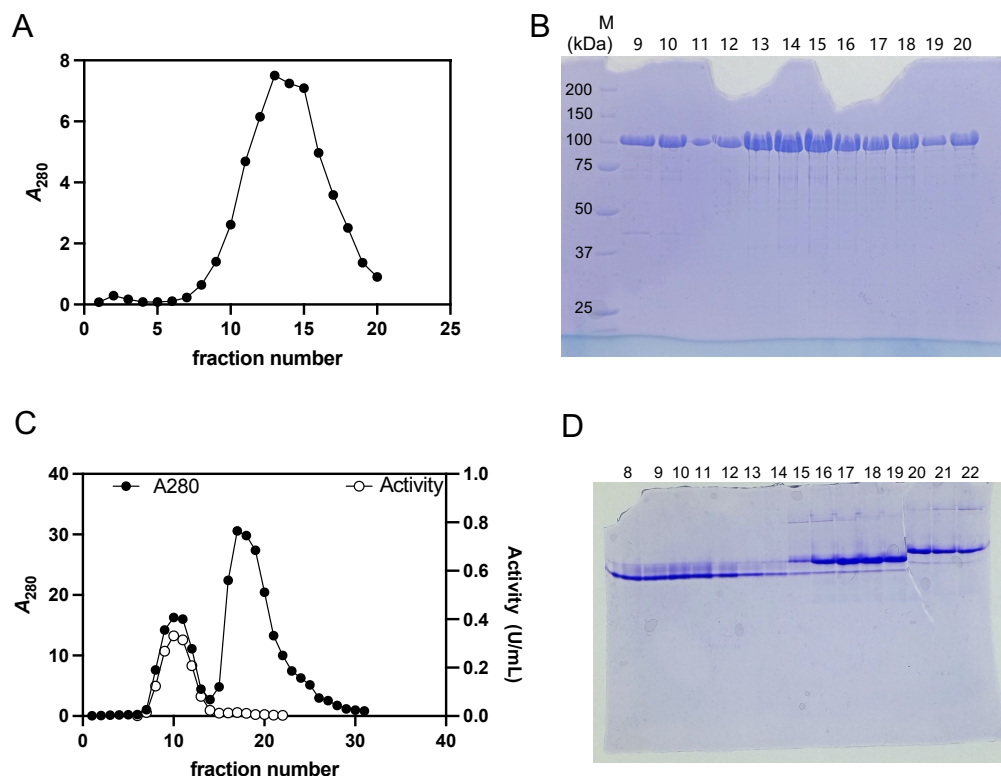


Fig. 4-3. Purification of Td46GT mutant Q482I483→GDYS.

A, elution profile. Column, Ni²⁺-immobilized Sepharose Fast Flow; equilibration and washing, 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl; elution, 0.03-0.5 M imidazole (elution volume, 160 mL); fraction volume, 5 mL. B, SDS-PAGE. C, elution profile of anion-exchange column chromatography. Resource Q column (6 mL), ÄKTA purifier, 2 mL/min; equilibration buffer, 10 mM HEPES-NaOH buffer (pH 7.0); elution, a linear gradient of 0-0.5 M NaCl (elution volume, 30 mL) in 10 mM HEPES-NaOH buffer (pH 7.0). D, native-PAGE analysis. Black circle, absorbance at 280 nm; white circle, activity (U/mL). Lane M, molecular mass standards (kDa).

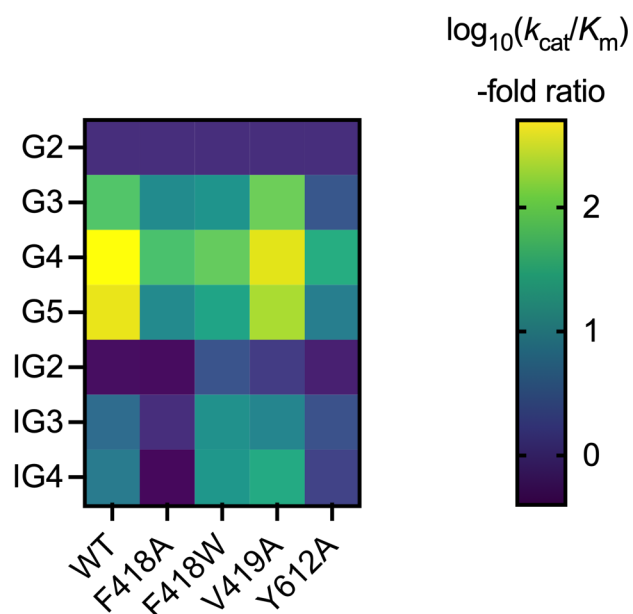


Fig. 4-4. Heatmap view of k_{cat}/K_m comparison of Td46GT variants. Kinetic parameter -fold ratios relative to G2, for Td46GT variants, analyzed in single-substrate reactions as listed in Tables 2-2 and 4-1.

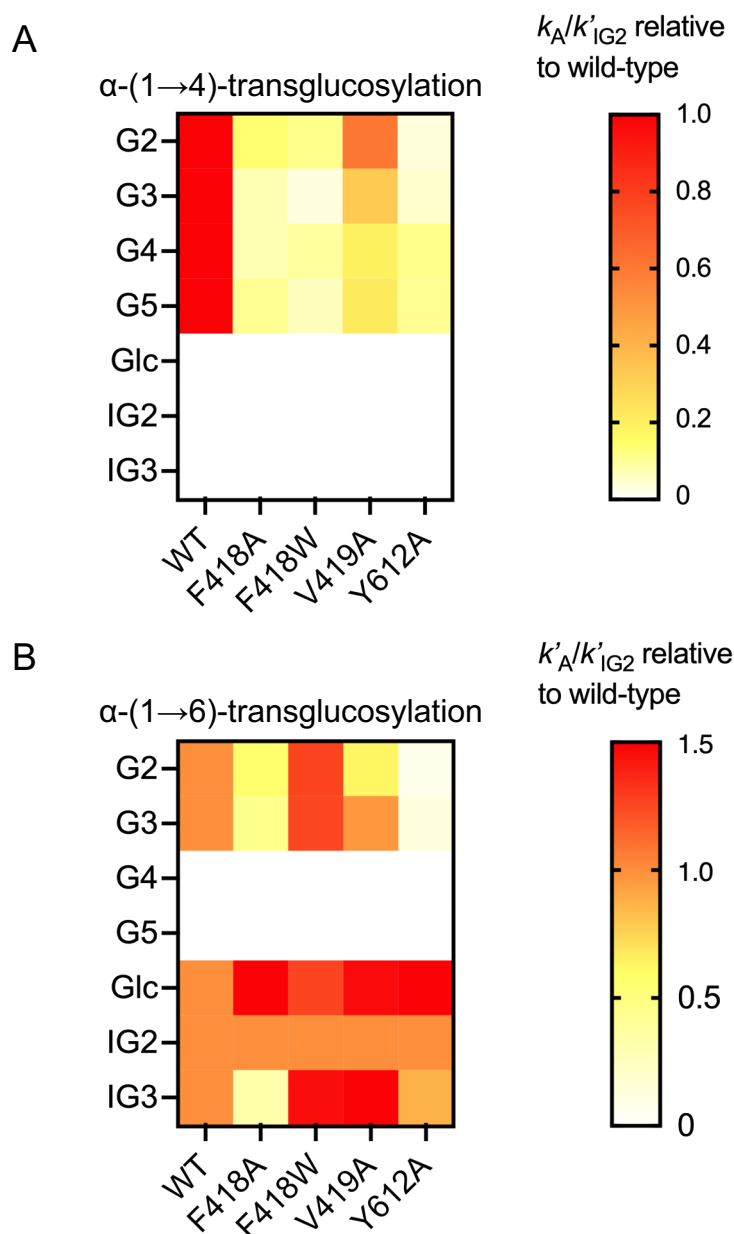


Fig. 4-5. Heatmap view of RASCs comparison of Td46GT variants.

Relative acceptor-substrate specificity constants (RASCs) of Td46GT mutants compared to wild type, as listed in Tables 2-3 and 4-2. A, α -(1→4)-transglucosylation acceptor specificity of different saccharides relative to the α -(1→6)-transglucosylation acceptor specificity of IG2. B, α -(1→6)-transglucosylation acceptor specificity of different saccharides relative to the α -(1→6)-transglucosylation acceptor specificity of IG2. Definitions of k_A/k'_{IG2} and k'_A/k'_{IG2} are described in section 2.3.6.

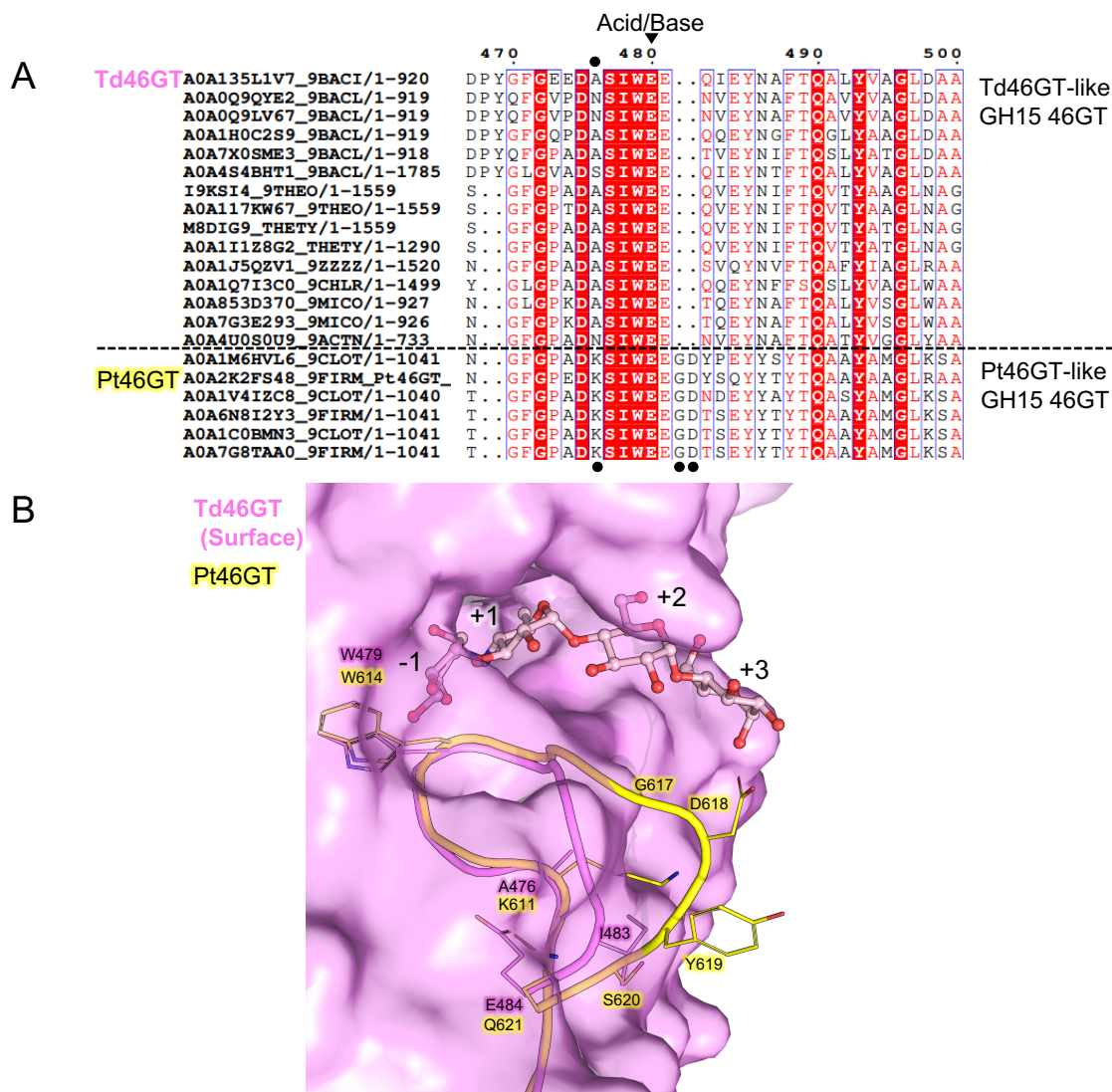


Fig. 4-6. Comparison of Td46GT and Pt46GT.

A, amino acid sequence alignment of Td46GT, Pt46GT and other 19 GH15 46GT homologues (Uniprot database IDs are shown). Comparison of $\alpha 5$ - $\alpha 6$ loop indicated two types of 46GT as either Td46GT-like or Pt46GT-like. Differently conserved positions annotated by black circle. B, structural comparison of $\alpha 5$ - $\alpha 6$ loop of Td46GT crystal structure (Mol A; chapter 3) and AlphaFold3 predicted model of Pt46GT.

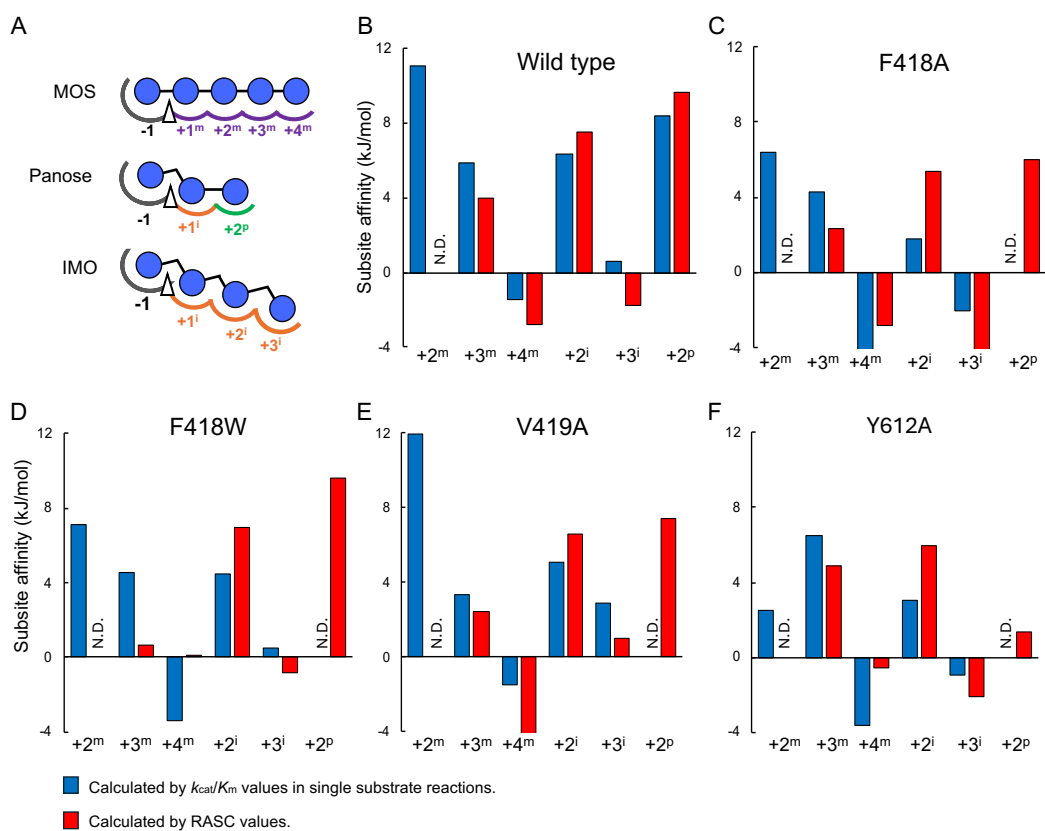


Fig. 4-7. Subsite affinities of Td46GT variants.

A, subsite definitions for MOSs, IMOs, and panose-derived saccharides. B–F, subsite affinity comparison of the Td46GT variants. Names of the Td46GT variants are indicated in each panel. Blue: affinities calculated from k_{cat}/K_m values. Red: Affinities calculated using relative acceptor-substrate specificity constants (RASCs). N.D., not determined.

5. Conversion of reaction mechanism of Td46GT by rational mutagenesis

5.1. Introduction

In inverting enzymes, the general base activates a water molecule for nucleophilic attack from the opposite face of anomeric carbon, which drives the reaction irreversibly into hydrolysis. Although an anomer-inverting transglycosylase capable of producing α -1,6-cyclized β -1,2-glucohexadecaose has recently been identified (Motouchi et al., 2024), such cases remain so exceptional. In contrast, retaining GHs provide much wider applications across various industrial sectors (see chapter 1), because their intrinsic transglycosylation activities are highly advantageous for the synthesis of diverse glycans.

To broaden the practical value of inverting GHs, identification of the molecular determinants that distinguish inverting/retaining mechanisms is necessary. It will give insights of possible structural targets for enzyme-engineering mutagenesis. Despite sustained interest for this topic, the current progress has focused on direct point mutation to place a catalytic residue to a proper position to alter the catalytic mechanism and only one case succeed: introduction of nucleophile catalyst residue Tyr to an inverting sialic acid hydrolase, gained a retaining trans-sialidase mutant (Pichler et al., 2025).

GH15, GH23 and GH97 are the only GH families comprise both inverting and retaining enzymes (Fig. 1-1C). GH23 retaining lytic transglycosylase (EC: 4.2.2.29) and inverting hydrolytic muramidase (EC: 3.2.1.17) only conserve the general acid or acid/base catalyst. Bacterial exolytic transglycosylases acting on peptidoglycan, employ the reaction mechanism of substrate-assisted catalysis with a favorable dipole provided by the *N*-acetyl carbonyl group from *N*-acetylmuramic acid substrate (Byun et al., 2018; V. Kumar et al., 2023; Vijayaraghavan et al., 2018), and they lost the counterpart acidic residue to general base catalyst of fungal inverting muramidase (PDB entry: 3GXR; Helland et al., 2009).

GH97 enzymes also conserve the general acid or acid/base catalyst, but structures around general base/nucleophile catalyst side are significantly different, according to studies of BtGH97a, an inverting α -glucoside hydrolase and BtGH97b, a retaining α -galactosidase both from *Bacteroides thetaiotaomicron* (Okuyama et al., 2009, 2014). BtGH97b lost a counterpart of general base Glu of BtGH97a that is positioned at the end of β 5 of the $(\beta/\alpha)_8$ barrel. Differently, BtGH97b has an Asp residue acting as the nucleophile catalyst positioned at the end of β 4, whereas this Asp is replaced by Gly in BtGH97a.

Instead of the direct catalytic residue substitutions observed in the inverting and retaining GHs of GH23 and GH97, non-catalytic residues are focused as the structural determinant to discriminate GH15 retaining

α -GTs and inverting α -glucohydrolases, as described in chapter 3. The difference of distance between nucleophile/general base catalyst and anomeric carbon, hydrogen-bonding networks involving the nucleophile/general base catalyst and water molecules at the β -face of D-glucosyl moiety of substrate in subsite -1 were observed in two-types of GH15 enzymes (Figs. 1-3, 3-8 and 3-9). However, their functions and importance to the catalytic mechanism are unclear. Therefore, in this chapter, a series of Td46GT variants carrying single- and multiple mutations were prepared and functionally characterized.

5.2. Materials and methods

5.2.1. Materials

α -D-Glucose and β -D-glucose were purchased from Sigma (St. Louis, MO, USA). pNP α -Isomaltotriose is a laboratory stock. α -D-Glucosyl fluoride (GF) was prepared from deacetylation of 2,3,4,6-tetra-*O*-acetyl- α -D-glucosyl fluoride, which was synthesized according to a previously described procedure (Hayashi et al., 1984).

5.2.2. Construction of expression plasmids for Td46GT single mutants

The expression plasmid of Td46GT wild type (chapter 2) was used as a template for constructing plasmids of Td46GT single mutants using a Primestar Mutagenesis Basal Kit. It includes E480D, E480Q, Y607G, Y607F, Q628L, E666D, E682L, E682Q, V684W, T685A mutations. Primers used for preparing these mutants, were listed in Table 5-1. This method will be abbreviated as “PriMax” in following texts.

5.2.3. Construction of expression plasmids for Td46GT multiple mutants (MM)

MM1 (Q628L, E682L, V684W and W688Q) was prepared from two PCRs in which plasmid of Q628L mutant was used as the template. Two sets of PCR were performed using primers EPV-LPW_688Q_s and Td_23a_a (5'-GCCATATGTATATCTCCTTCT-3'); and EPV-LPW_688Q_a and Td_23a_s (5'-TGCGGCCGCACTCGAGCACC-3'). KOD FX Neo DNA polymerase was used. Amplicons were extracted from agarose gel after electrophoresis using 0.8% agarose gel and purified by Wizard SV Gel and PCR Clean-Up System. These DNA fragments were inserted into pET-23a by using NEBuilder HiFi DNA Assembly Master Mix.

This process includes two sets of PCRs and DNA assembly using NEBuilder HiFi DNA Assembly,

would be abbreviated as T-F-A (Three-Fragments-Assembly) in following texts. By using either T-F-A or PriMax method, and a series of primers and templates (Table 5-2), plasmids of MM1-MM13 together with Y607G_A667Q were constructed.

5.2.4. Production and purification of Td46GT variants

All single mutants analyzed in this chapter were prepared with similar process as described in section 3.2.2. Mutant enzymes were produced in transformants of *E. coli* BL21(DE3) and purified by Ni²⁺-immobilized affinity column chromatography and anion exchange column chromatography using a Resource Q column. All MM mutants were purified only by Ni²⁺-immobilized affinity column chromatography as Td46GT wild type (chapter 2) with slight differences: MM8 was produced in BL21 Star (DE3) transformants (Thermo Fischer Scientific, Waltham, MA, USA); MM6 and MM7 was produced in SoluBL21(DE3) transformants. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of 2.47 for 1 mg/mL of the wild-type Td46GT.

5.2.5. Identification of reaction products from pNP-Glc and GF

For pNP-Glc reaction, a 20 μ L of mixture containing 15 mM pNP-Glc, 20 mM sodium acetate buffer (pH 6.0), and 218 nM wild-type Td46GT was incubated at 37 °C for 18 h. Two microliters of the reaction mixture were spotted on a silica gel 60 F₂₅₄ plate (Merck) at indicated time and developed twice using ethyl acetate/methanol/H₂O (7/2.5/1, v/v/v). Products were visualized using acetic acid/sulfuric acid/anisaldehyde (100:2:1, v/v/v) followed by heating or UV irradiation to detect *p*-nitrophenol group. For GF reactions, a 40 μ L mixture containing 100 mM GF, 120 mM sodium acetate buffer (pH 6.0), and 4.02 μ M wild-type Td46GT was incubated at 37 °C for 90 min. Products were analyzed as described above. Disaccharyl and trisaccharyl fluoride products were eluted with water from silica gel and analyzed by ESI-MS as described in section 2.2.6.

5.2.6. Measurement of reaction rates to pNP-Glc, GF, and G4

In pNP-Glc assay, pNP release was measured as described in section 3.2.5. In the reaction with GF, fluoride ion release from 20 mM GF was measured under the conditions described above. The reaction was terminated by addition of 200 μ L acetone. After centrifugation (16,000 $\times$ *g* at 4 °C for 10 min), 50 μ L Albusone reagent

(Dojindo Laboratories, Kumamoto, Japan) was added, and samples were incubated at room temperature for 60 min. Fluoride ion concentration was determined from absorbance at 620 nm. For G4 reactions, G3 production from 20 mM G4 was measured by HPAEC-PAD, as described in section 2.2.5.

5.2.7. Kinetic analysis pH profiles of Td46GT variants

Rates of pNP production from 0.6-12 mM pNP-Glc in 80 mM Britton–Robinson buffer (pH 3.0–9.0) were measured. The Michaelis-Menten equation was fitted to initial rates by nonlinear regression using Grafit version 7.0.2 to determine kinetic parameters. The pK_e and pK_{es} values of catalytic residues in the kinetic model for an enzyme with two catalytic ionizable groups were calculated using the following equations with the SciPy package in Python version 3.12.2:

$$\text{Equation 5-1: } k_{\text{cat}} = k_1 / (1 + 10^{pK_{es1}-\text{pH}} + 10^{\text{pH}-pK_{es2}})$$

$$\text{Equation 5-2: } k_{\text{cat}}/K_m = k_1/K_{s2} / (1 + 10^{pK_{e1}-\text{pH}} + 10^{\text{pH}-pK_{e2}})$$

The pK_e and pK_{es} values of catalytic residue in the kinetic model for an enzyme with single catalytic ionizable group (Figs. 5-3 and 5-6) were calculated using the following equations:

$$\text{Equation 5-3: } k_{\text{cat}} = k_1 / (1 + 10^{pK_{es1}-\text{pH}})$$

5.2.8. Analysis of the anomeric form of D-glucose produced from G4 using Td46GT MM8 mutant

A reaction mixture (143 μL), containing 60 mM G4, 68 μM Td46GT MM8 mutant, and 15 mM PIPES-NaOH buffer (pH 6.7), was incubated at 37°C for 18 min. At specified time points, aliquots were withdrawn and filtered through a 10 kDa cutoff centrifugal device (Merck) to remove the enzyme. Sample (10 μL) was immediately analyzed by HPLC under the following conditions: column, TSKgel Amide-80 (4.6 mm i.d. $\times$ 25 cm, 5 μm ; Tosoh, Tokyo, Japan); injection volume, 10 μL ; column temperature, 30 °C; elution, 80% acetonitrile; flow rate, 1 mL/min; detection, Corona Charge Aerosol Detector (ESA Biosciences, Chelmsford, MA, USA). Standard solution of equilibrated D-glucose, α -D-glucose, and β -D-glucose (1 mM each) were prepared. α - And β -D-glucose standards were obtained by dissolving 18 mg of crystalline material in 10 mL of water and diluting tenfold immediately prior to analysis. Ratios of α - and β -anomers in samples ($n = 3$) were calculated from peak areas and compared to standards using one-way ANOVA.

5.3. Results

5.3.1. Functional analysis of the catalytic Glu residues in Td46GT

Two α -D-glucosides with good leaving groups, pNP-Glc and GF, were tested as substrates for Td46GT. Td46GT wild type released the aglycone from pNP-Glc and GF at 0.349 and 29.0 s⁻¹, respectively, which are 209- and 2.52-fold lower than for maltotetraose (G4; 73.1 s⁻¹), the most favorable natural substrate (Table 5-3). The enzyme primarily catalyzed α -(1 $\rightarrow$ 6)-transglucosylation to pNP-Glc, producing pNP α -isomaltoside (pNP-IG2) and pNP α -isomaltotrioside (pNP-IG3; Fig. 5-1A). Transglucosylation products from GF were separated via TLC, and ESI-MS analysis revealed signals at m/z 367.10 ([M + Na]⁺) and m/z 529.15 ([M + Na]⁺), corresponding to disaccharyl- and trisaccharyl-fluoride, respectively (Figs. 5-1B and 5-2). Acid hydrolysis of these products yielded IG2 and IG3, indicating that Td46GT produced isomaltooligosaccharyl fluoride through α -(1 $\rightarrow$ 6)-transglucosylation with GF (Fig. 5-1C).

The acid/base mutant, E480Q, retained higher relative activity toward pNP-Glc and GF than toward G4, whereas the nucleophile mutant E666Q exhibited detectable reaction rate only toward G4 (0.0039 s⁻¹; 0.0053% of that of the wild type; Table 5-3). The reaction rates of E480Q toward G4, pNP-Glc, and GF were 0.0037%, 0.41%, and 0.25% of those of the wild type, respectively. The k_{cat}/K_m and k_{cat} values of E480Q for pNP-Glc were almost constant above pH 6 and fit a single-ionizable-group model, whereas those of the wild type followed a two-ionizable-group model (Fig. 5-3). The wild type exhibited nearly overlapping pH- k_{cat}/K_m and pH- k_{cat} curves with $pK_{e1} = 3.32 \pm 0.06$, $pK_{e2} = 7.07 \pm 0.07$, $pK_{es1} = 3.06 \pm 0.05$, and $pK_{es2} = 7.13 \pm 0.05$, indicating that substrate-binding did not substantially alter the ionization state of the catalytic residues. For E480Q, pK_{e1} and pK_{es1} were 3.66 ± 0.06 and 3.66 ± 0.12 , respectively, consistent with those of the wild type. These results confirm that Glu480 and Glu666 function as the general acid/base and nucleophile, respectively, in agreement with the structural data.

Effect of increasing the distance between the catalytic residues via substitution of the catalytic Glu with Asp was investigated (Table 5-3). E480D exhibited reaction rates for pNP-Glc and GF comparable to those of E480Q but displayed 25-fold higher activity toward G4 for E480Q. Similarly, E666D showed 9.8-fold greater activity toward G4 than E666Q and retained activity toward pNP-Glc and GF unlike E666Q. These results suggest that Asp substitutions partially restore catalytic functionality. HPAEC-PAD analysis revealed that E480D catalyzed both transglucosylation and hydrolysis, whereas E666D primarily catalyzed transglucosylation like the wild type (Fig. 5-4). The rate ratio of E480D for transglucosylation/hydrolysis

was approximately 75:25 in reaction with 20 mM G4. Anomer analysis of the hydrolytic product of E480D indicated exclusive formation of α -D-glucose, confirming an anomer-retaining hydrolytic mechanism.

5.3.2. Role of the hydrogen-bonding network involving Glu666

To assess the roles of Tyr607 and Glu682, which directly or indirectly interact with catalytic nucleophile Glu666. A three-water-molecules-mediated (waters 166, 290 and 326) hydrogen-bonding network involving Glu666 catalyst, Glu682 and Thr685 were observed (Fig. 5-5). Y607F, E682L/Q and T685A mutants were characterized. Reaction rates of Y607F, E682Q/L and T685A for 80 mM G4 (pH 6.7) were 0.409, 70.4, 1.57, and 68.3 s⁻¹, corresponding to 0.629%, 108%, 2.40% and 102% of rate of the wild type, respectively. Since E682L lacked detectable activity toward pNP-Glc, E682Q was used for pH-profile analysis using pNP-Glc (Fig. 5-6A). Y607F showed significantly elevated pK_{e1} and pK_{es1} values (5.53 ± 0.11 and 5.52 ± 0.09 , respectively) compared to that of wild type (Figs. 5-3C and 5-6A). No substantial shifts in pK_e or pK_{es} were observed for E682Q and T685A. The k_{cat}/K_m values at optimum pH of E682Q, T685A and Y607F decreased to 26.6%, 52.3% and 0.600% of that of the wild type, respectively. Product analysis revealed that all the mutant enzymes catalyzed transglucosylation to G4 (G5 produced) without detectable D-glucose production from hydrolysis (Fig. 5-6B).

5.3.3. Conversion of Td46GT to an inverting α -glucohydrolase through MM

To engineer Td46GT into an inverting α -glucohydrolase, MMs were introduced based on the structural comparison with GH15 α -glucohydrolases. Among MMs constructed, MM8–13, all of which contain Y607G, Q664/Gly665→Pro and V684W substitutions, predominantly catalyzed hydrolysis toward G4 (Figs. 5-7 and 5-8). The minimal mutant, MM8, hydrolyzed G4 with a rate of 0.00990 s⁻¹. The anomeric configuration of the hydrolysis product, D-glucose, was determined (Fig. 5-8B and C). The β -D-glucose to α -D-glucose ratios at reaction 6, 12, and 18 min were 3.39, 2.69, and 2.26, respectively, significantly exceeding that of equilibrated D-glucose (1.53) and closely approximating the β -D-glucose standard (2.67). These findings demonstrate that MM8 produced β -D-glucose from G4 via net inversion of the substrate's anomeric configuration.

5.4. Discussion

As discussed in chapter 3, structure comparison with GH15 inverting α -glucohydrolase indicates that the nucleophile catalyst of GH15 retaining α -GTs is positioned 1.2–1.6 Å closer position to the anomeric carbon of the substrate in subsite –1. This closer position of the catalytic nucleophile hinders binding of water molecule at the position of nucleophilic water in GH15 inverting glucohydrolases. These features are believed as critical determinants of inverting/retaining mechanisms within GH15 enzymes.

The single residue mutants E480Q and E666Q were prepared and analyzed, to biochemically validate the catalytic roles of Glu480 and Glu666 in Td46GT. The E480Q mutant retained higher reaction rate toward substrates with good leaving groups (pNP-Glc and GF) than toward those with poor leaving groups (G4) (Table 5-3). This property of E480Q likely reflects its diminished dependence on the general acid catalyst during aglycone departure in reactions with good leaving group substrates, a behavior characteristic of retaining glycosidase mutants lacking a general acid/base catalyst (Tagami et al., 2025; Williams & Withers, 2000). Furthermore, the pH dependence of both k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ for E480Q followed a single-ionizable-group model, in which an active enzyme contains a deprotonated catalytic group (Fig. 5-3B). E666Q displayed no detectable activity toward pNP-Glc and GF (Table 5-3), and significantly lost activity toward G4 substrate, which implied that Glu666 is essential for catalysis but in different manner of Glu480. These results establish Glu480 as the general acid/base catalyst and Glu666 as the nucleophile, consistent with the functional assignments in DDase.

The spatial distance between the two catalytic carboxy groups was modulated by substituting one of the catalytic Glu residues with Asp. Among the resulting variants (Fig. 5-4), E480D catalyzed the hydrolysis of G4 through an anomer-retaining mechanism (Fig. 5-4B). No mechanistic conversion was observed in E480D. The Asp at position 480 may be suboptimal for assisting nucleophilic attack by a sugar hydroxy group as a general base, and a water molecule likely occupies a favorable position in the E480D active site to attack the anomeric carbon of the D-glucosyl-enzyme intermediate to facilitate hydrolysis. E666D displayed only transglucosylation activity toward G4, with no detectable hydrolysis activity.

MM analysis demonstrated that Y607G, Q664/Gly665→Pro and V684W mutations are minimal set to alter 46GT to an inverting α -glucohydrolase. Adjacent to the N-terminal of Glu666, Gln664 and Gly665 were substituted with single Pro in inverting GH15 enzymes (Fig. 1-2C). The Gly insertion at the N-terminal side of the catalytic Glu in α 11- α 12 loop of Td46GT and DDase presumably brings the catalytic Glu residue

closer to the anomeric carbon of the substrate, and eliminating this Gly is necessary to bring the catalytic Glu to proper position to function as general base catalyst. The structural analysis of Td46GT suggested that Tyr607 in α 9- α 10 loop forms hydrogen bond with the catalytic nucleophile Glu666, and the hydrogen bonding interaction was confirmed by increase of pK_{el} and pK_{es1} values by Y607F mutation (Fig. 5-6). Hydrogen bonding interaction between the hydroxy group of Tyr607 and Glu666 maintains deprotonated form of Glu666 under acidic conditions, and is provably important for the proper positioning of the catalytic nucleophile Glu666. This residue may also cause steric hinderance upon binding of nucleophile water required for the anomer-inverting hydrolysis. Val684 is completely conserved in GH15 α -GTs, but substituted with Trp in inverting enzymes (Fig. 1-2C). The smaller Val residue in α -GTs may permit a three-water-molecules-mediated hydrogen-bonding network involving Glu682 and Thr685 (Fig. 5-5). The V684W mutation may disrupt the water-mediated hydrogen bonding network and obstruct the binding of the water molecule 166, coordinated by the Glu682 residue at the N-terminus of the 12th α -helix, thereby facilitating the proper accommodation of a nucleophilic water molecule activated by Glu666.

This chapter provides an unprecedented viewpoint on manipulation of non-catalytic residues for mechanism conversion between retaining GTs and inverting GHs, thereby advancing the understanding of catalysis of carbohydrate-active enzymes.

Table 5-1. Primers used for construction of expression plasmids for Td46GT variants.

Primer Name	Sequence(5'→3')
Td_23a_s*	TTGCGGCCGCACTCGAGCACC
Td_23a_a*	GCCATATGTATATCTCCTTCT
E480D_s	ATCTGGGATGAGCAAATTGAATATAAC
E480D_a	TTGCTCATCCCAGATGCTTGCATCTTC
E480Q_s	ATCTGGCAGGAGCAAATTGAATATAAC
E480Q_a	TTGCTCCTGCCAGATGCTTGCATCTTC
Y607G_s	GATTTTCGGTTACACCTCTCCATACAGT
Y607G_a	GGTGTAAACCGAAATCATCCCCGTCATA
Y607F_s	GATTTCTTCTACACCTCTCCATACAGT
Y607F_a	GGTGTAGAAGAAATCATCCCCGTCATA
Q628L_s	TGGCCACTTATGAGTGCTTATGCAGCT
Q628L_a	ACTCATAAGTGGCCAGGAAGGTTTCATC
E666D_s	CAGGGAGATGCCGTATCACGAATTTCA
E666D_a	TACGGCATCTCCCTGGGCCATATAACC
A667Q_s	GGAGAGCAAGTATCACGAATTTTCATTG
A667Q_a	TGATACTTGCTCTCCCTGGGCCATATA
E682Q_s	ATGGTACAGCCTGTAACAGGAGCATGG
E682Q_a	TACAGGCTGTACCATTGTACTGGGAAG
E682L_s	ATGGTATTACCTGTAACAGGAGCATGG
E682L_a	TACAGGTAATACCATTGTACTGGGAAG
V684W_s	GAGCCTTGACAGGAGCATGGTTTGTG
V684W_a	TCCTGTCCAAGGCTCTACCATTGTACT
T685A_s	CCTGTAGCAGGAGCATGGTTTGTGATT
T685A_a	TGCTCCTGCTACAGGCTCTACCATTGT
QG-P_A667Q_s	TGATACTTGCTCTGGGGCCATATAACC
QG-P_A667Q_a	CCAGAGCAAGTATCACGAATTTTCATTG
EPV-LPW_688Q_s	CTGTGCTCCTGTCCAAGGAAGTACCATTGT
EPV-LPW_688Q_a	CTTCCTTGGACAGGAGCACAGTTTGTGATT
V684_W688Q_s	GAGCCTTGGACAGGAGCACAATTTGTGATT
V684_W688Q_a	TTGTGCTCCTGTCCAAGGCTCTACCATTGT
Q664G_P_s	ATGGCCCCAGAGGCCGTATCACGAATT
Q664G_P_a	GGCCTCTGGGGCCATATAACCAACCGC
Y607_recovery_s	GATTTCTATTACACCTCTCCATACAGT
Y607_recovery_a	GGTGTAAATAGAAATCATCCCCGTCATA

*: The primers used in T-F-A, the Three-Fragments-Assembly process includes two turns of PCRs and DNA Assembly by NEBuilder HiFi DNA Assembly. Details in section 5.2.3.

Table 5-2. Summary of construction of expression plasmids for Td46GT multiple mutants (MM).

Plasmid	Method	Primers	Template
Y607G_A667Q	PriMax	A667Q_s and A667Q_a	Y607G
MM1	T-F-A	EPV-LPW_688Q_s and EPV-LPW_688Q_a	Q628L
MM2	PriMax	QG-P_A667Q_s and QG-P_A667Q_a	Y607G_A667Q
MM3	PriMax	Q628L_s and Q628L_a	MM2
MM4	PriMax	Y607_recovery_s and Y607_recovery_a	MM9
MM5	PriMax	Q664G_P_s and Q664G_P_a	Y607G
MM6	PriMax	Y607_recovery_s and Y607_recovery_a	MM8
MM7	PriMax	V684W_s and V684W_a	Y607G
MM8	PriMax	V684W_s and V684W_a	MM5
MM9	PriMax	Q664G_P_s and Q664G_P_a	MM10
MM10	PriMax	V684W_s and V684W_a	MM11
MM11	PriMax	V684_W688Q_s and V684_W688Q_a	MM13
MM12	T-F-A	EPV-LPW_688Q_s and EPV-LPW_688Q_a	MM2
MM13	PriMax	Q628L_s and Q628L_a	MM12

PriMax, Site-directed mutagenesis which uses Primestar Mutagenesis Basal Kit. T-F-A, the Three-Fragments-Assembly process, which includes two turns of PCRs and DNA assembly by NEBuilder HiFi DNA Assembly (section 5.2.3). Mutation sites of the mutants are shown in Fig. 5-7.

Table 5-3. Aglycone releasing rates of Td46GT wild type and the catalytic-residue mutants.

Variant	G4		pNP-Glc ^a		GF ^b	
	Rate (s ⁻¹)	Relative rate (%)	Rate (s ⁻¹)	Relative rate (%)	Rate (s ⁻¹)	Relative rate (%)
Wild type	73.1 ± 1.4	100	0.349 ± 0.012	100	29.0 ± 0.3	100
E480D	0.0668 ± 0.0009	9.10 × 10 ⁻²	(0.121 ± 0.012) × 10 ⁻²	0.35	0.111 ± 0.012	0.383
E480Q	(0.268 ± 0.019) × 10 ⁻²	0.367 × 10 ⁻²	(0.142 ± 0.006) × 10 ⁻²	0.41	0.0724 ± 0.0055	0.250
E666D	0.0410 ± 0.0015	5.61 × 10 ⁻²	(0.819 ± 0.021) × 10 ⁻⁴	0.023	(0.660 ± 0.126) × 10 ⁻²	0.023
E666Q	(0.388 ± 0.019) × 10 ⁻²	0.531 × 10 ⁻²	N. D.	N. D.	N. D.	N. D.

Values are mean ± standard deviation for three independent experiments. N.D., not detectable.

a: analyzed by pNP producing velocity quantification by reading A_{400} at pH 9.5.

b: analyzed by fluoride ion producing velocity quantification by reading A_{620} using Alfusone reagent.

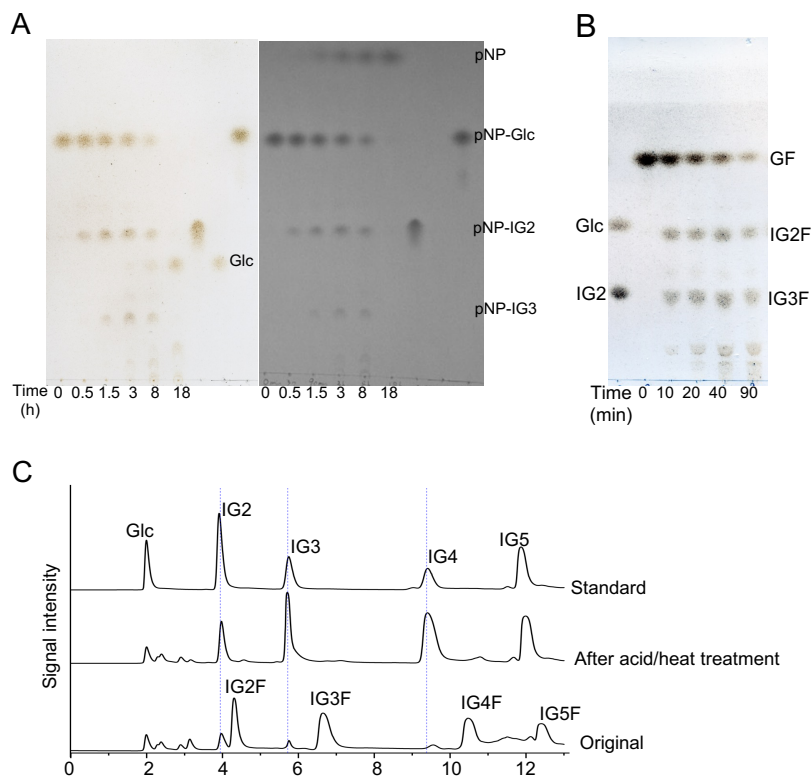
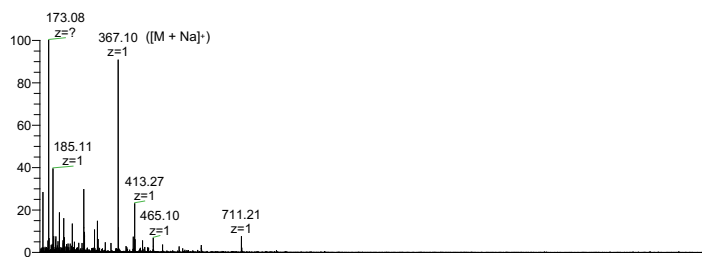


Fig. 5-1. Analysis of reaction products of Td46GT wild type from pNP-Glc and GF.

A, TLC analysis of reaction products from pNP-Glc. Reaction products were detected by the detection reagent (the left image) and UV (the right image). B, TLC analysis of the reaction products from GF. C, HPAEC-PAD analysis of the reaction products from GF before and after the acid and heat treatment.

A IG2F



B IG3F

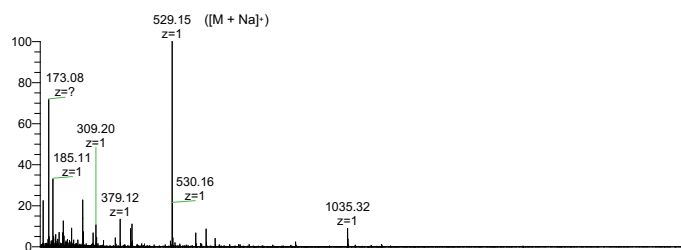


Fig. 5-2. ESI-MS analysis of transglucosylation products from GF.
A, IG2F, α -isomaltosyl fluoride. B, IG3F, α -isomaltotriosyl fluoride.

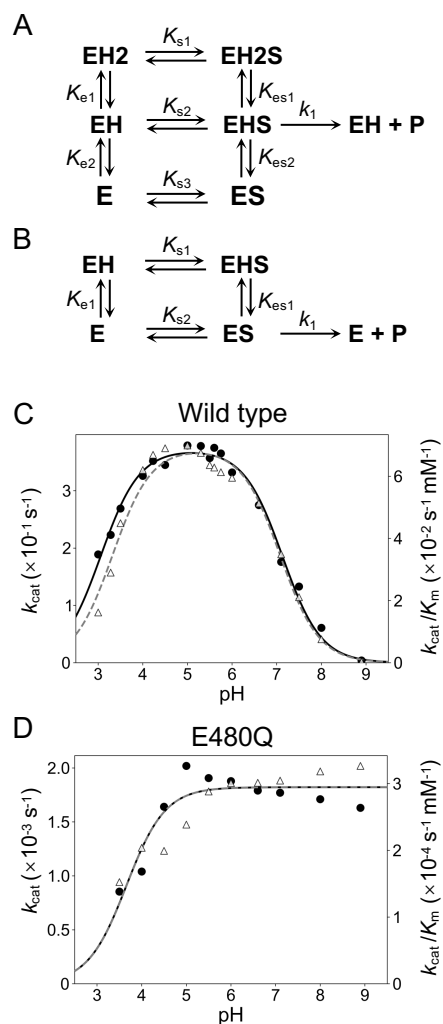


Fig. 5-3. Comparison of pH profiles between Td46GT wild type and E480Q mutant.

A, kinetic model for an enzyme with two catalytic ionizable groups. B, kinetic model for an enzyme with single catalytic ionizable group. C, pH- k_{cat}/K_m (triangle, dashed line) and pH- k_{cat} (circle, solid line) plots of wild-type Td46GT. D, pH- k_{cat}/K_m (triangle) and pH- k_{cat} (circle) plots of E480Q mutant.

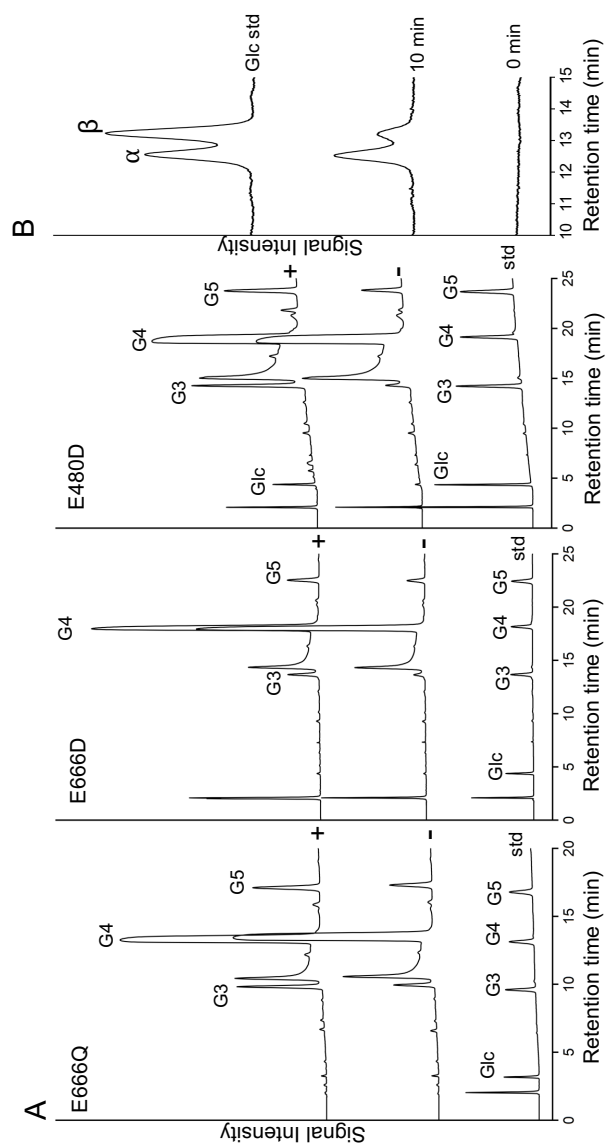


Fig. 5-4. Analysis of reaction products of E666Q, E666D and E480D Td46GT mutants from G4.

A, HPAEC-PAD analysis of the reaction products from G4 with E666Q, E666D and E480D. B, anomer analysis of the D-glucose (Glc) produced from G4 catalyzed by E480D.

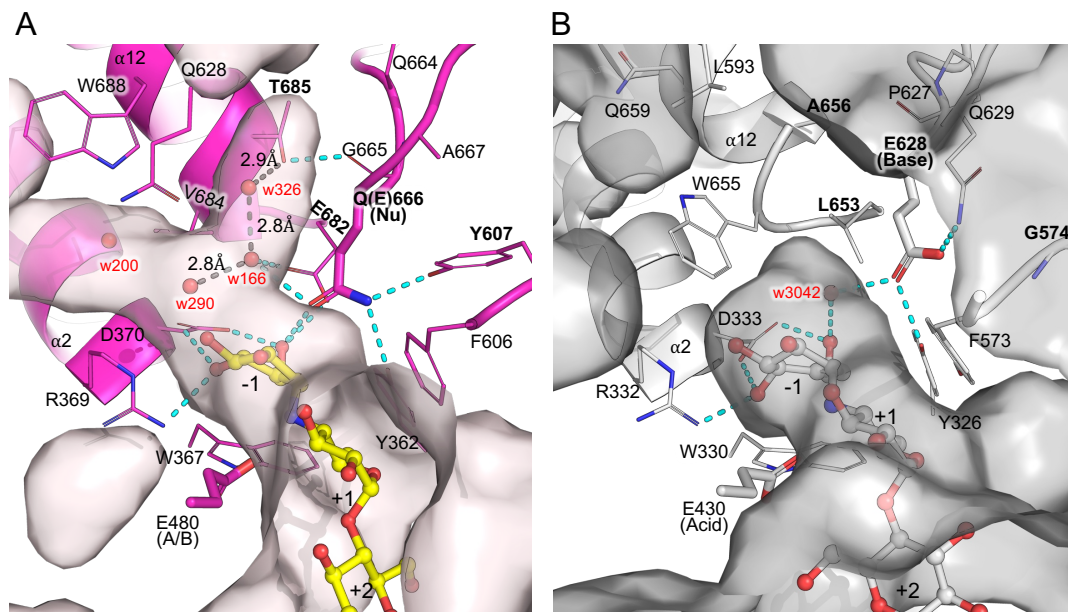


Fig. 5-5. Subsite -1 comparison with GH15 retaining glucosyltransferases and inverting glucohydrolases.

A, Surface look of the active pocket. Possible hydrogen bonds are shown in dotted lines. Red spheres indicate the water molecule (w) which mediate hydrogen-bonding networks involving the nucleophile catalyst (Nu), as shown in bold. Acid/Base catalyst, A/B. B, structure of catalytic site of *Arthrobacter globiformis* glucodextranase (AgGDase) in complex with acarbose (PDB entry, 1ULV), overlapped with Td46GT and counterpart residues (shown in same view, gray color). The predicted nucleophilic water of AgGDase is w3042.

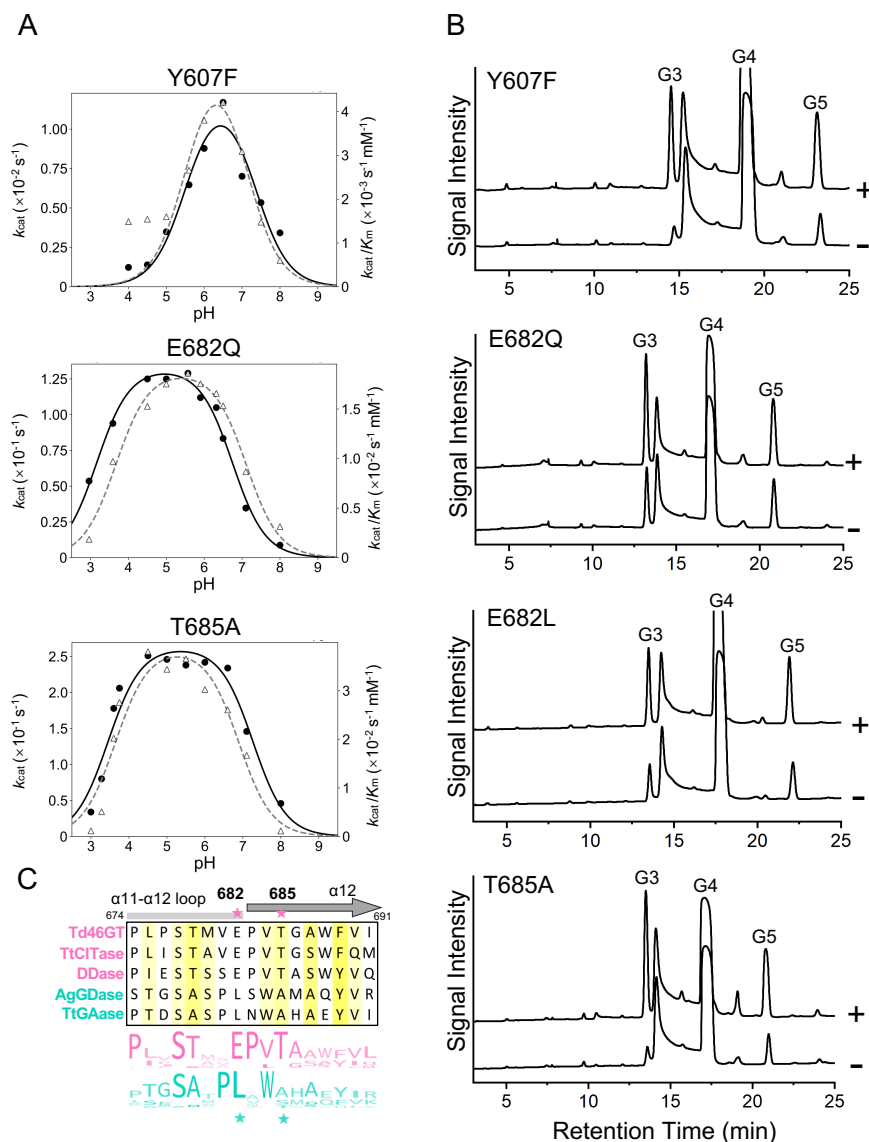


Fig. 5-6. Functional analysis of Td46GT Y607F and E682Q/L and T685A mutants.

A, pH- k_{cat}/K_m (triangle, dashed line) and pH- k_{cat} (circle, solid line) plots of Y607F, E682Q and T685A mutants. B, HPAEC-PAD analysis of the reaction products from G4 with Y607F, E682Q/L and T685A mutants. C, conserve-degree of Glu682 and Thr685 position at N-terminal of $\alpha 12$ helix, referred from Fig. 1-2, with same abbreviation for enzyme names. Pink, retaining GH15 enzymes. Green, inverting GH15 enzymes.

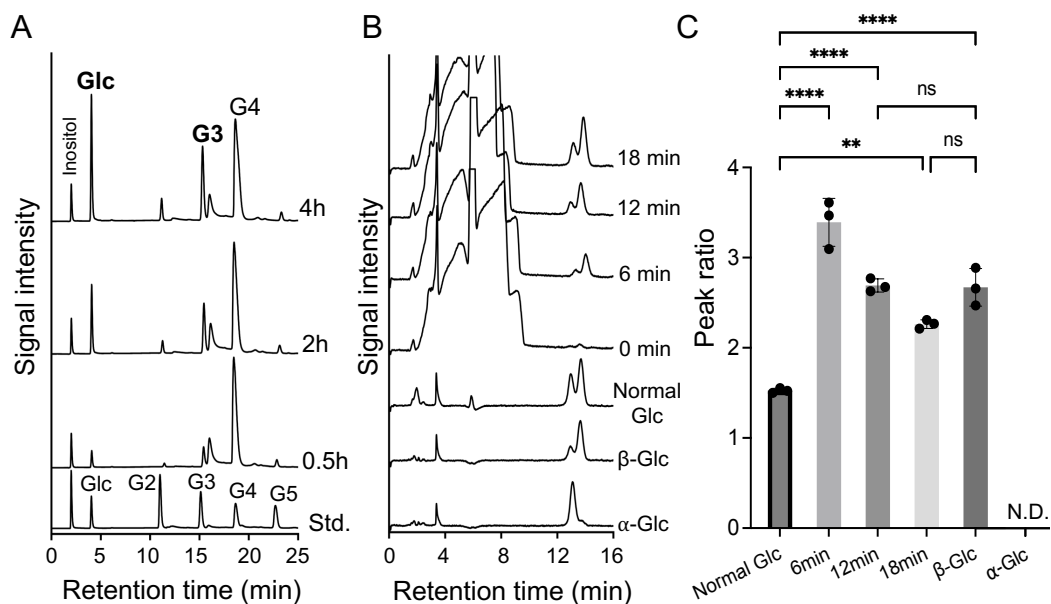


Fig. 5-8. Reaction of the MM8 mutant with G4 produces β-D-glucose.

A, HPAEC-PAD analysis of the reaction products from 80 mM G4 with MM8 mutant (pH 6.7, 37 °C). Products of D-glucose (Glc) and G3 were identified and shown in bold. B, Anomer analysis of Glc produced from G4 with MM8 mutant. C, peak ratio of α- and β-Glc produced from G4 with MM8 in different reaction times, and reference standards (one-way ANOVA, $n=3$). ns, $P > 0.12$; ** $P < 0.01$; **** $P < 0.0001$.

6. General discussion

Non-Leloir type GTs catalyze transglucosylation via the double-displacement mechanism as retaining GHs, and generally share structures of catalytic domains with retaining GHs. Some GH families of retaining enzymes such as GH13, GH31, and GH57 comprise both GTs and GHs. Within these families, GTs generally exhibit markedly higher transglucosylation specificity than their hydrolase homologues. For decades, this functional distinction has been predominantly attributed to differences in acceptor preference between saccharides and water molecules (Section 1.4; Bissaro et al., 2015). Recent structural study of DDase demonstrated that this α -GT shares pronounced structural similarity with inverting GH15 α -glucohydrolases, including a common $(\alpha/\alpha)_6$ -barrel catalytic domain and the conserved Glu-Glu catalytic dyad (Fig. 1-2; Tagami et al., 2025). In DDase, the catalytic nucleophile Glu occupies the spatial position of the water acting as nucleophile from β -face for anomer-inverting hydrolysis through single-displacement mechanism in inverting α -glucohydrolases (chapter 1). These observations raise a question for the structural determinants for anomer-retaining and inverting reaction mechanisms of GH15 enzymes. GH15 also contains TtCITase (the C-terminal catalytic domain), producing IMO from MOS, but its functional and structural analysis were only scarcely reported (Jeong et al., 2019). This study focuses on the DDase homologues Td46GT and Pt46GT, showing high sequence identity with TtCITase.

Td46GT and Pt46GT produced IMOs from MOSs through transglucosylation, but not dextran, indicating that these enzymes are α -GTs distinct from DDase. Kinetic analysis of two-substrate reaction (two-competing acceptor reaction), employed for characterization of Td46GT clearly demonstrated its acceptor-substrate dependent linkage formation: Td46GT forms α -(1 $\rightarrow$ 4)-linkage with MOS acceptor (DP $\geq$ 3) and α -(1 $\rightarrow$ 6)-linkage with MOS (DP 2–3), IMO, and D-glucose. Based on this acceptor specificity, production process of IMO from MOS was elucidated: (i) initial disproportionation of MOSs via α -(1 $\rightarrow$ 4)-D-glucosyl transfer reactions; (ii) α -(1 $\rightarrow$ 6)-transglucosylation toward G2 and G3 acceptors after consumption of long-chain MOSs, leading to accumulation of isomaltooligosaccharyl-maltose and isomaltooligosaccharyl-maltotriose; and (iii) α -(1 $\rightarrow$ 6)-transglucosylation toward D-glucose, produced as the aglycone from G2 donors, for continuous IMOs production. Judging from time progress of reaction products from MOS and initial reaction products from MOS and IMO, Pt46GT also produces IMO from MOS through similar processes to Td46GT. The two-substrate reaction kinetic analysis used in this study was shown to be applicable for quantitative evaluation of acceptor specificity. RASC, determined from this kinetic analysis,

was used for calculation of subsite affinity, and obtained affinity was in good agreement with that calculated by k_{cat}/K_m values in single-substrate reactions, supporting the interpretation that RASC and k_{cat}/K_m are specificity constants for acceptor and donor substrates, respectively. Furthermore, time-dependent change in MOS and IMO concentrations during the reaction is predictable based on RASC for acceptors and k_{cat}/K_m for donors (Fig. 2-21), offering a predictive framework for transglucosylation yield estimation. This simulation is helpful for efficient optimization for sugar synthesis using GTs.

The Phe418–Val419 dyad located in the $\alpha 3$ – $\alpha 4$ loop, is completely conserved among other GH15 α -GTs (Figs. 1-2C and 6-1B). This dyad has been biochemically confirmed to be essential for the formation of subsite +2 in Td46GT (Fig. 6-1A). Mutations at these residues severely impair the binding affinity for MOS-donors and MOS-acceptors for α -(1→4)-transglucosylation. The F418A mutation caused a larger decrease of activity toward MOS substrates than V419A (chapter 4). Essential role of Phe418 toward α -(1→4)-linkage selectivity of Td46GT highly resembles with function of its counterpart Trp, which is completely conserved in GH15 GAases (Figs. 1-2C and 6-1). They are both aromatic residues which hydrophobically interact with D-glucose unit at subsite +2, suggesting a common evolution of GH15 α -GTs and GAases. For GDase which significantly prefers α -(1→6)-linkage over α -(1→4)-linkage, this aromatic residue is substituted with a non-aromatic Gln (Fig. 6-1B). Therefore, the diversity on 418th position of Td46GT might be a structural factor influencing α -(1→4/6)-linkage specificity of GH15 enzymes.

Compared to bacterial GAases and GDases, 46GTs comprise of a long $\alpha 9$ – $\alpha 10$ loop, on which a unique aromatic residue (Tyr612 in Td46GT; Trp803 in DDase) stacks onto D-glucose unit at subsite +2 (Fig. 6-1B), which is lost in bacterial glucohydrolases. Y612A mutation caused significantly decrease of MOS-acceptors for both α -(1→4)-transglucosylation and α -(1→6)-transglucosylation specificity. This aromatic residue in $\alpha 9$ – $\alpha 10$ loop of GH15 46GTs is unique for MOS substrate binding.

A unique β -hairpin structure and long $\alpha 7$ – $\alpha 8$ loop between $\alpha 7$ and $\alpha 8$ helices is identified in bacterial GAases and GDases, but not in 46GTs (Fig. 6-2A and B). One Asn-Gly dyad in this structure is completely conserved in bacterial GH15 glucohydrolases (Fig. 6-2A) and the Asn residue possibly interacted with substrate at subsite +1 through hydrogen-bonding (Fig. 6-2C and D). In all, these structural differences might contribute to α -(1→4/6)-linkage selectivity in GH15 enzymes.

In chapter 2, Td46GT and Pt46GT have been identified to display poor transglucosylation specificity to long IMO acceptor. Different with DDase, they do not accumulate high-DP α -(1→6)-glucan products such

as IMM and dextran, during reaction with MOS, although these enzymes share similar substrate binding site, subsites -1–+3 with DDase. According to structural comparison, DDase possesses a unique extended $\alpha 3$ – $\alpha 4$ loop region (Asn573-Asn582) that is absent in Td46GT and Pt46GT (Fig. 6–3A). Molecular-docking analysis of DDase and isomaltoheptaose (IG7) suggests this loop possibly interacts with reducing end part of long-chain IMO substrates by hydrogen bond interactions with Asn576, Tyr579 and Asn582 (Fig. 6–3B). The $\alpha 3$ – $\alpha 4$ loop of DDase may contribute to enhanced binding affinity for long-chain saccharide substrates.

As discussed in Chapter 5, a Tyr (or Gly) residue on the $\alpha 9$ – $\alpha 10$ loop influences positioning of the nucleophile or general base catalyst, a hydrophobic Val (or Trp) residue located on the $\alpha 12$ helix, which presumably modulates β -face water molecules at subsite –1. These residues may play essential roles in determining the reaction mechanism of GH15 46GTs and glucohydrolases. To examine whether these residues represent a common and conserved mechanistic feature for GHs, their counterparts in other GH families evolutionarily related to GH15 were analyzed.

GH65, which belongs to the GH-L clan together with GH15, comprises inverting enzymes such as trehalase (EC 3.2.1.28) and kojibiose α -1,2-glucosidase (EC 3.2.1.216), whereas GH37 consists primarily of trehalase. Enzymes from GH15, GH37, and GH65 families share a common $(\alpha/\alpha)_6$ -barrel for catalytic domain. In these inverting hydrolases, the general acid catalyst on $\alpha 5$ – $\alpha 6$ loop, the general base catalyst on $\alpha 11$ – $\alpha 12$ loop, and an Asp residue located on the $\alpha 2$ helix are well conserved (Fig. 6-4A-C). However, the previously identified important residues, Tyr (or Gly) on $\alpha 9$ – $\alpha 10$ loop and Val (or Trp) on $\alpha 12$ helix for GH15 reaction mechanism determination, are not conserved among GH15, 37 and 65 families. GH15 bacterial glucohydrolases hold a much longer $\alpha 9$ – $\alpha 10$ loop than those of GH37 and GH65 hydrolases, Tyr (or Gly) residue in GH15 enzymes has been lost in GH65 kojibiose hydrolase (Fig. 6-4D), while the corresponding Gly residue in GH37 trehalase is structurally inconsistent with that in GH15 enzymes (Fig. 6-4D). The hydrophobic Val (or Trp) residue at the N-terminal of $\alpha 12$ helix, is substituted with a hydrophilic Thr, in GH65 kojibiose hydrolases (Fig. 6-4B and E).

These observations suggest that the distinct subsite –1 residue configurations observed between GH15 46GTs and glucohydrolases represent a family-specific adaptation, rather than a universally conserved mechanistic feature shared among other GH families.

Physiological functions of 46GT are discussed. The *Td46GT* gene is a part of a gene cluster encoding enzymes involved in cycloisomaltooligosaccharides (CIs) metabolism (Fig. 6-5). The adjacent upstream gene,

U473_RS02295, encodes a putative extracellular protein with 66% sequence identity to GH66 CITase from *Niallia cierculans* (Park et al., 2021), suggesting its cooperative function with Td46GT in CI biosynthesis from MOS: Td46GT generates α -(1→6)-isomaltooligosaccharyl glucans from MOSs, and U473_RS02295 uses this α -(1→6)-glucans to produce CIs. Unlike *T. thermocopriae*, which utilizes a multi-domain TtCITase, containing both CITase and 46GT catalytic domains, for CI production from MOS (Ichinose et al., 2017; Käll et al., 2007), *T. decaturensis* employs distinct enzymes. A similar cooperative strategy to produce CI from MOS is observed in *Penibacillus* sp. 598K, though its transglucosidase belongs to GH31, thus being structurally different from Td46GT (Mizushima et al., 2019). Based on TMHMM-2.0 prediction, U473_RS02295 likely functions at the cell membrane, enabling surface-level CI biosynthesis and subsequent import into the cytosol via an ATP-binding cassette transporter involving a solute-binding protein, U473_RS02270. Intracellular CI degradation into D-glucose is assumed to be facilitated by two predicted glycosidases, U473_RS02285 and U473_RS02265: U473_RS02285, a predicted membrane-associated protein with 28% of sequence identity to a GH66 CI-hydrolyzing dextranase from *Paenibacillus* sp. 598K (Ichinose et al., 2017; Mizushima et al., 2019); and U473_RS02265, a putative intracellular GH15 iGHase with 38% identity to *Kribbella flavida* IGHase (Tagami et al., 2022). This metabolic pathway of maltodextrin via α -(1→6)-glucan and CI is predicted to be shared by bacteria harboring Td46GT homologues, such as *T. thermocopriae*, because they possess a similar gene cluster to *T. decaturensis* (Fig. 6-5). This metabolic pathway potentially provides a competitive advantage under carbohydrate-limited conditions (Light et al., 2016).

Structural analysis confirmed the presence of calcium ion-binding sites and their contribution to the enhanced thermostability of Td46GT. These findings are consistent with adaptive features of *T. decaturensis*, which was originally isolated from deep groundwater of the Mt. Simon Formation in the Illinois Basin, an environment characterized by exceptionally high calcium concentrations (approximately 19,000 mg/L ;Dong et al., 2016; Ryan J. et al., 2018).

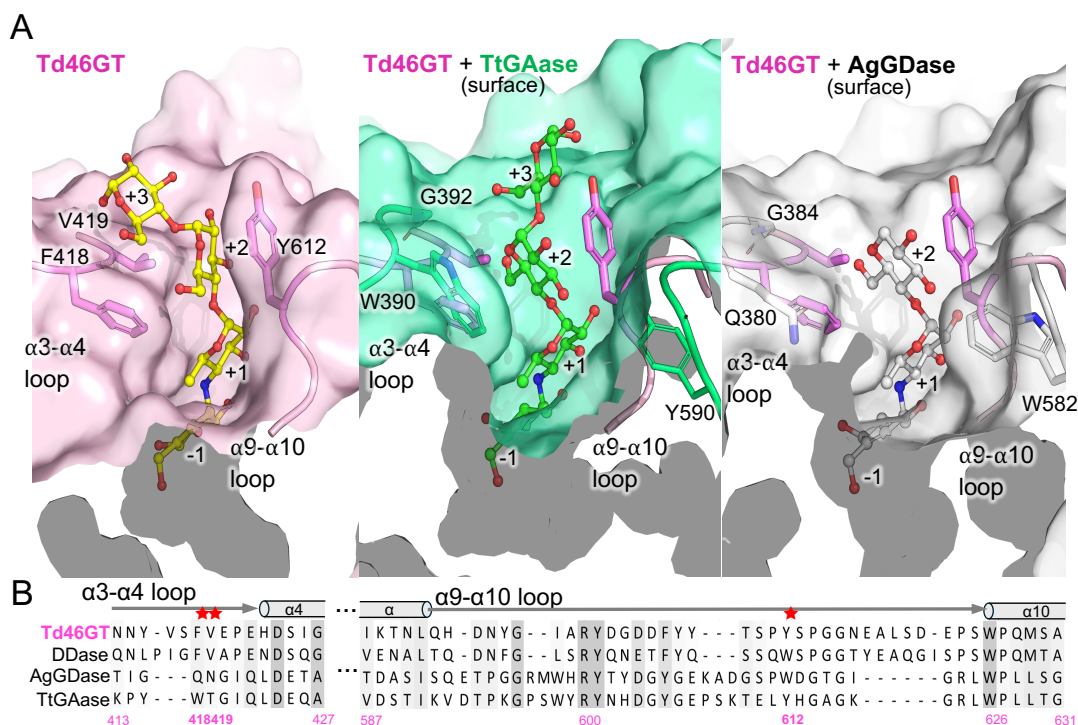


Fig. 6-1. Active center superimposition and sequence alignment of GH15 enzymes.

A, active center superimposition. Td46GT, pink; TtGAase (PDB: 1LF9), cyan; AgGDase (PDB: 1ULV), gray. B, sequence alignment of $\alpha 3$ - $\alpha 4$ and $\alpha 9$ - $\alpha 10$ loops. Red asterisks indicate residues forming subsite +2.

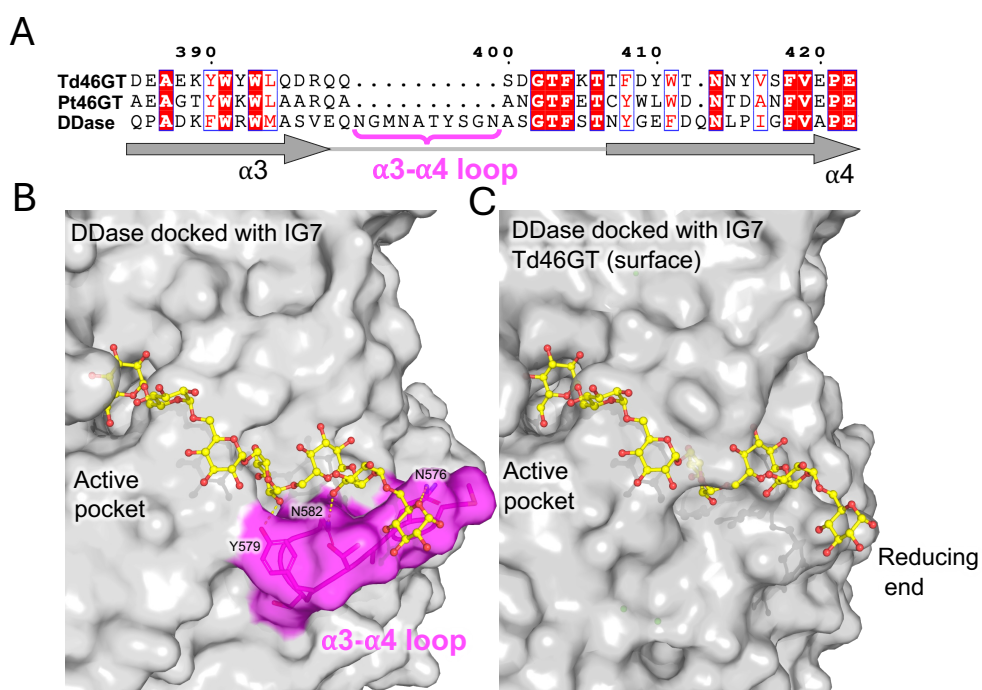


Fig. 6-3. Sequence alignment and structural comparison of GH15 46GTs and DDase. A, sequence alignment of $\alpha 3$ - $\alpha 4$ loop of Td46GT, Pt46GT and DDase. B, docking analysis of DDase (PDB entry: 9JU0) and isomaltoheptaose (IG7), the reducing end of IG7 ligand is located at the right side. Unique loop region as annotated in panel A is shown in magenta. C, docked IG7 ligand with DDase is overlapped with Td46GT crystal structure. The loop region corresponding to DDase is lost.

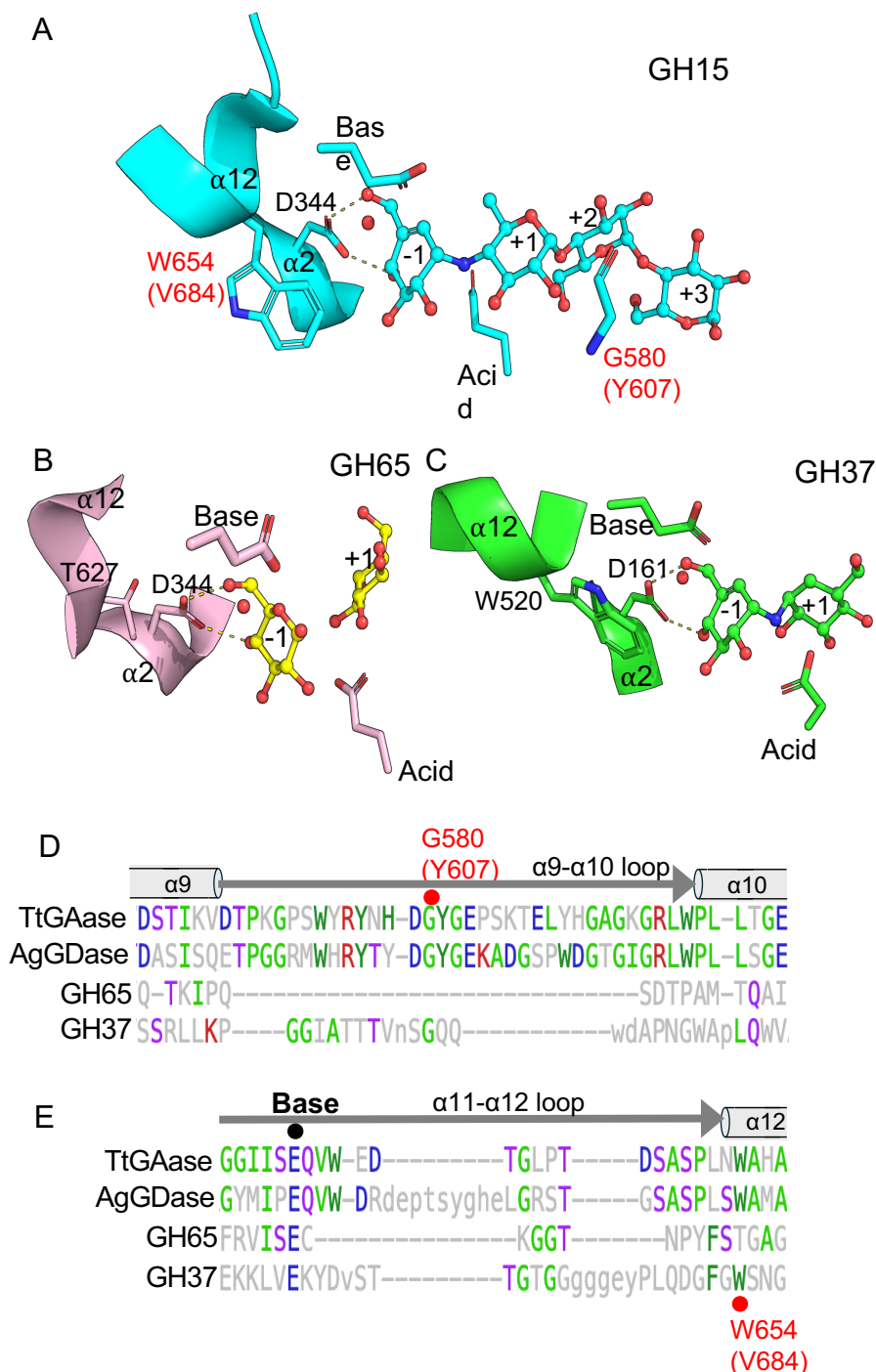


Fig. 6-4. Structural comparison of GH15, GH65 and GH37 enzymes.

A, TtGAase in complex with acarbose (PDB entry: 1LF9). Residue numbers of Td46GT identified to be important for GH15 reaction mechanism determination, including Tyr607 and Val684, are shown in brackets. B, kojibiose α -1,2-glucosidase in complex with D-glucose, from *Flavobacterium johnsonia* (PDB entry: 7FE3). C, trehalose α -1,1-glucosidases in complex with Validoxylamine A, from *Enterobacter asburiae* 2497 (PDB entry: 5Z66). Sequence alignments of GH15, GH65 and GH37 enzymes for $\alpha 9$ - $\alpha 10$ loop (D) and $\alpha 11$ - $\alpha 12$ loop (E) are shown.

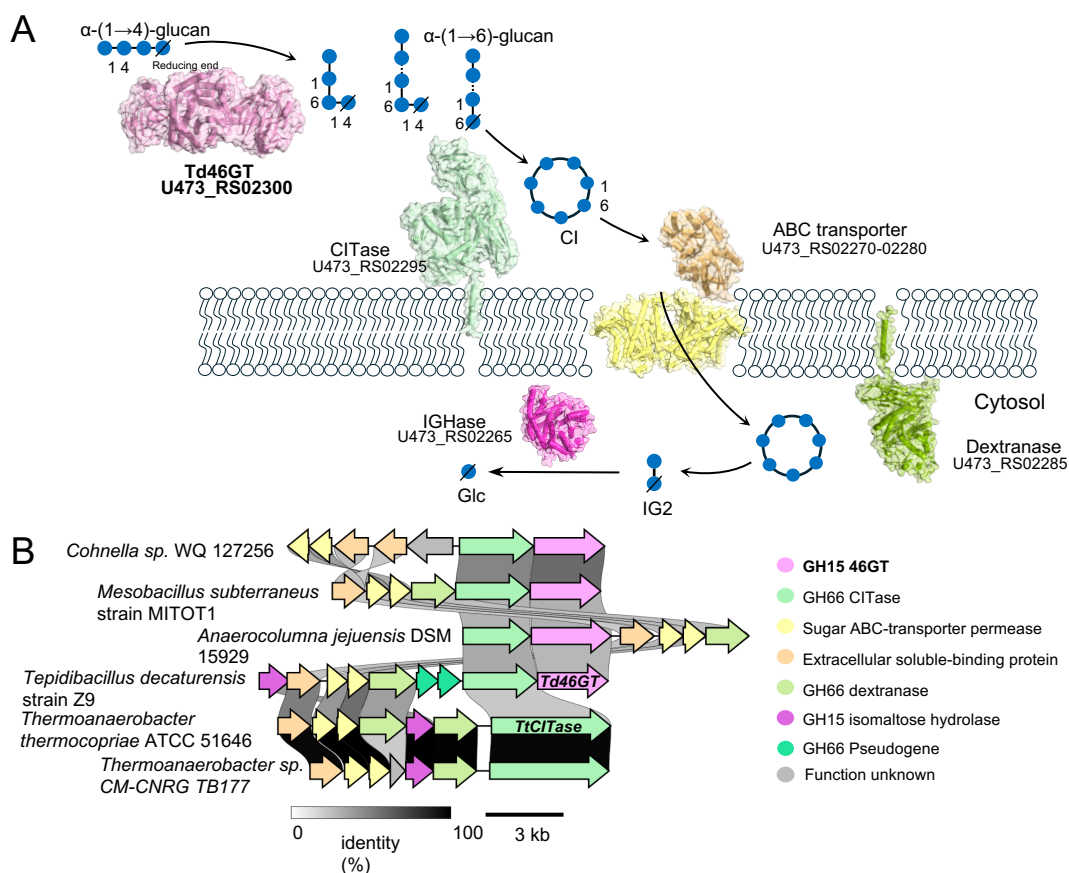


Fig. 6-5. Possible carbohydrate metabolic pathway involving Td46GT in *T. decaturensis*. A, possible metabolic pathway of α -(1→4)-glucans via α -(1→6)-linked glucans and CIs. Td46GT produces α -(1→6)-glucans from α -(1→4)-glucan through α -(1→6)-transglucosylation. Possible CITase (U473_RS02295) produces CIs from α -(1→6)-glucans, and CIs were predicted to be imported into cytosol by ABC transporter (U473_RS02275 and U473_RS02280) associated with a possible solute binding protein U473_RS02270. CI was predicted to be hydrolyzed by transmembrane dextranase (U473_RS02285) and isomaltose hydrolase (IGHase; U473_RS02265). B, comparison of the gene organization in *T. decaturensis* and bacteria with Td46GT homologous proteins. Figures were prepared using Clinker (<https://cagecat.bioinformatics.nl/tools/clinker>).

References:

- Aizawa, K., Takagi, H., Kokubo, E., & Takada, M. (2021). Preparation and analysis of α -1,6 glucan as a slowly digestible carbohydrate. *Journal of Applied Glycoscience*, 68(3), 53–61. https://doi.org/10.5458/jag.jag.JAG-2021_0001
- Aleshin, A. E., Feng, P.-H., Honzatko, R. B., & Reilly, P. J. (2003). Crystal structure and evolution of a prokaryotic glucoamylase. *Journal of Molecular Biology*, 327(1), 61–73. [https://doi.org/10.1016/S0022-2836\(03\)00084-6](https://doi.org/10.1016/S0022-2836(03)00084-6)
- Banerjee, A., & Bandopadhyay, R. (2016). Use of dextran nanoparticle: A paradigm shift in bacterial exopolysaccharide based biomedical applications. *International Journal of Biological Macromolecules*, 87, 295–301. <https://doi.org/10.1016/j.ijbiomac.2016.02.059>
- Basu, A., Mutturi, S., & Prapulla, S. G. (2016). Production of isomaltooligosaccharides (IMO) using simultaneous saccharification and transglucosylation from starch and sustainable sources. *Process Biochemistry*, 51(10), 1464–1471. <https://doi.org/10.1016/j.procbio.2016.06.008>
- Best, R. B., Jackson, G. E., & Naidoo, K. J. (2002). AN NMR INVESTIGATION INTO THE DYNAMICS OF PANOSE, AN $\alpha(1\rightarrow4)$ AND $\alpha(1\rightarrow6)$ -LINKED TRISACCHARIDE. *Spectroscopy Letters*, 35(5), 625–632. <https://doi.org/10.1081/SL-120014935>
- Bissaro, B., Monsan, P., Fauré, R., & O'Donohue, M. J. (2015). Glycosynthesis in a waterworld: New insight into the molecular basis of transglycosylation in retaining glycoside hydrolases. *Biochemical Journal*, 467(1), 17–35. <https://doi.org/10.1042/BJ20141412>
- Brockerman, J. A., Okon, M., Withers, S. G., & McIntosh, L. P. (2019). The pK a values of the catalytic residues in the retaining glycoside hydrolase T26H mutant of T4 lysozyme. *Protein Science : A Publication of the Protein Society*, 28(3), 620–632. <https://doi.org/10.1002/pro.3562>
- Byun, B., Mahasen, K. V., Dik, D. A., Marous, D. R., Sperti, E., Kumarasiri, M., Fisher, J. F., Hermoso, J. A., & Mobashery, S. (2018). Mechanism of the escherichia coli MltE lytic transglycosylase, the cell-wall-penetrating enzyme for type VI secretion system assembly. *Scientific Reports*, 8(1), 4110. <https://doi.org/10.1038/s41598-018-22527-y>
- Casa-Villegas, M., Marín-Navarro, J., & Polaina, J. (2017). Synthesis of isomaltooligosaccharides by saccharomyces cerevisiae cells expressing aspergillus niger α -glucosidase. *ACS Omega*, 2(11), 8062–8068. <https://doi.org/10.1021/acsomega.7b01189>
- Chang, T.-S., Wu, J.-Y., Ding, H.-Y., & Wang, T.-Y. (2025). Enzymatic glycosylation of ganoderma terpenoid via bacterial glycosyltransferases and glycoside hydrolases. *Biomolecules*, 15(5), 655. <https://doi.org/10.3390/biom15050655>
- Chen, H.-L., Lu, Y.-H., Lin, J.-J., & Ko, L.-Y. (2001). Effects of isomalto-oligosaccharides on bowel functions and indicators of nutritional status in constipated elderly men. *Journal of the American College of Nutrition*, 20(1), 44–49. <https://doi.org/10.1080/07315724.2001.10719013>
- Cornish-Bowden, A. (1984). Enzyme specificity: Its meaning in the general case. *Journal of Theoretical Biology*, 108(3), 451–457. [https://doi.org/10.1016/S0022-5193\(84\)80045-4](https://doi.org/10.1016/S0022-5193(84)80045-4)
- Cornish-Bowden, A. (1993). Enzyme specificity in reactions of more than one co-substrate. *Biochemical Journal*, 291(Pt 1), 323–324. <https://doi.org/10.1042/bj2910323b>
- Cornish-Bowden, A. (2013). *Fundamentals of enzyme kinetics*. John Wiley & Sons.
- Coutinho, P. M., & Reilly, P. J. (1994). Structure–function relationships in the catalytic and starch binding domains of glucoamylase. *Protein Engineering*, 7(3), 393–400. <https://doi.org/10.1093/protein/7.3.393>

- de Boer, R. M., Vaitkus, D., Enemark-Rasmussen, K., Maschmann, S., Teze, D., & Welner, D. H. (2023). Regioselective glycosylation of polyphenols by family 1 glycosyltransferases: Experiments and simulations. *ACS Omega*, 8(48), 46300–46308. <https://doi.org/10.1021/acsomega.3c08255>
- Dong, Y., Sanford, R. A., Boyanov, M. I., Kemner, K. M., Flynn, T. M., O'Loughlin, E. J., Locke, R. A., Weber, J. R., Egan, S. M., & Fouke, B. W. (2016). *Tepidibacillus decaturensis* sp. Nov., a microaerophilic, moderately thermophilic iron-reducing bacterium isolated from 1.7 km depth groundwater. *International Journal of Systematic and Evolutionary Microbiology*, 66(10), 3964–3971. <https://doi.org/10.1099/ijsem.0.001295>
- Drula, E., Garron, M.-L., Dogan, S., Lombard, V., Henrissat, B., & Terrapon, N. (2022). The carbohydrate-active enzyme database: Functions and literature. *Nucleic Acids Research*, 50(D1), D571–D577. <https://doi.org/10.1093/nar/gkab1045>
- Durán, A., Reyes-De-Corcuera, J., Garay, G., Valencia, P., & Urtubia, A. (2021). Development of an araucaria araucana beer-like beverage: Process and product. *Fermentation*, 7(3), 170. <https://doi.org/10.3390/fermentation7030170>
- Gao, X., Wu, J., & Wu, D. (2019). Rational design of the beta-galactosidase from *aspergillus oryzae* to improve galactooligosaccharide production. *Food Chemistry*, 286, 362–367. <https://doi.org/10.1016/j.foodchem.2019.01.212>
- Ge, J., Hua, L., Lee, S. H., Li, J., Shetty, J. K., Tang, Z., Zhang, B., & Zhong, K. (2018). *Trichoderma reesei* host cells expressing a glucoamylase from *aspergillus fumigatus* and methods of use thereof (European Union Patent No. EP2931872B1). <https://patents.google.com/patent/EP2931872B1/en?q=EP+2931872+B1>
- Goffin, D., Delzenne, N., Blecker, C., Hanon, E., Deroanne, C., & Paquot, M. (2011). Will isomaltoligosaccharides, a well-established functional food in Asia, break through the european and american market? The status of knowledge on these prebiotics. *Critical Reviews in Food Science and Nutrition*, 51(5), 394–409. <https://doi.org/10.1080/10408391003628955>
- Goulas, A. K., Fisher, D. A., Grimble, G. K., Grandison, A. S., & Rastall, R. A. (2004). Synthesis of isomaltoligosaccharides and oligodextrins by the combined use of dextranucrase and dextranase. *Enzyme and Microbial Technology*, 35(4), 327–338. <https://doi.org/10.1016/j.enzmitec.2004.05.008>
- Hanada, N., & Kuramitsu, H. K. (1989). Isolation and characterization of the streptococcus mutans gtfD gene, coding for primer-dependent soluble glucan synthesis. *Infection and Immunity*, 57(7), 2079–2085. <https://doi.org/10.1128/iai.57.7.2079-2085.1989>
- Hayashi, M., Hashimoto, S., & Noyori, R. (1984). Simple synthesis of glycosyl fluorides. *Chemistry Letters*, 13(10), 1747–1750. <https://doi.org/10.1246/cl.1984.1747>
- Hehre, E. J. (1951). The biological synthesis of dextran from dextrans. *Journal of Biological Chemistry*, 192(1), 161–174. [https://doi.org/10.1016/S0021-9258\(18\)55917-2](https://doi.org/10.1016/S0021-9258(18)55917-2)
- Helland, R., Larsen, R. L., Finstad, S., Kyomuhendo, P., & Larsen, A. N. (2009). Crystal structures of g-type lysozyme from atlantic cod shed new light on substrate binding and the catalytic mechanism. *Cellular and Molecular Life Sciences*, 66(15), 2585–2598. <https://doi.org/10.1007/s00018-009-0063-x>
- Hiromi, K., Nitta, Y., Numata, C., & Ono, S. (1973). Subsite affinities of glucoamylase: Examination of the validity of the subsite theory. *Biochimica Et Biophysica Acta (BBA) - Enzymology*, 302(2), 362–375. [https://doi.org/10.1016/0005-2744\(73\)90164-2](https://doi.org/10.1016/0005-2744(73)90164-2)
- Hofer, B. (2016). Recent developments in the enzymatic O-glycosylation of flavonoids. *Applied*

- Microbiology and Biotechnology*, 100(10), 4269–4281. <https://doi.org/10.1007/s00253-016-7465-0>
- Ichinose, H., Suzuki, R., Miyazaki, T., Kimura, K., Momma, M., Suzuki, N., Fujimoto, Z., Kimura, A., & Funane, K. (2017). Paenibacillus sp. 598K 6- α -glucosyltransferase is essential for cycloisomaltooligosaccharide synthesis from α -(1 $\rightarrow$ 4)-glucan. *Applied Microbiology and Biotechnology*, 101(10), 4115–4128. <https://doi.org/10.1007/s00253-017-8174-z>
- İspirli, H., Korkmaz, K., Arioglu-Tuncil, S., Bozkurt, F., Sağdıç, O., Tunçil, Y. E., Narbad, A., & Dertli, E. (2023). Utilisation of an active branching sucrase from *Lactobacillus kunkeei* AP-37 to produce techno-functional poly-oligosaccharides. *International Journal of Biological Macromolecules*, 236, 123967. <https://doi.org/10.1016/j.ijbiomac.2023.123967>
- Itoh, T., Sakata, Y., Akada, R., Nimi, O., & Yamashita, I. (1989). Construction and characterization of mutant glucoamylases from the yeast *Saccharomyces fibuligera*. *Agricultural and Biological Chemistry*, 53(12), 3159–3167. <https://doi.org/10.1080/00021369.1989.10869825>
- Jeong, W. S., Lee, Y.-R., Hong, S.-J., Choi, S.-J., Choi, J.-H., Park, S.-Y., Woo, E.-J., Kim, Y. M., & Park, B. R. (2019). Carboxy-terminal region of a thermostable CITase from *Thermoanaerobacter thermophilus* has the ability to produce long isomaltooligosaccharides. *Journal of Microbiology and Biotechnology*, 29(12), 1938–1946. <https://doi.org/10.4014/jmb.1910.10022>
- Joe, G.-H., Andoh, M., Shinoki, A., Lang, W., Kumagai, Y., Sadahiro, J., Okuyama, M., Kimura, A., Shimizu, H., Hara, H., & Ishizuka, S. (2016). Megalo-type α -1,6-glucosaccharides induce production of tumor necrosis factor α in primary macrophages via toll-like receptor 4 signaling. *Biomedical Research*, 37(3), 179–186. <https://doi.org/10.2220/biomedres.37.179>
- Kabsch, W. (2010). Xds. *Acta Crystallographica, Section D: Biological Crystallography*, 66(2), 125–132. <https://doi.org/10.1107/S0907444909047337>
- Käll, L., Krogh, A., & Sonnhammer, E. L. L. (2007). Advantages of combined transmembrane topology and signal peptide prediction—The phobius web server. *Nucleic Acids Research*, 35(suppl_2), W429–W432. <https://doi.org/10.1093/nar/gkm256>
- Kaneko, T., Takahashi, S., & Saito, K. (2000). Characterization of acid-stable glucose isomerase from *Streptomyces* sp., and development of single-step processes for high-fructose corn sweetener (HFCS) production. *Bioscience, Biotechnology, and Biochemistry*, 64(5), 940–947. <https://doi.org/10.1271/bbb.64.940>
- Kanno T. (1990). Some functional properties of so-called isomalto-oligosaccharides and their applications to food industry. *Journal of the Japanese Society of Starch Science*, 37(2), 87–97. <https://doi.org/10.5458/jag1972.37.87>
- Kaper, T., Leemhuis, H., Uitdehaag, J. C. M., van der Veen, B. A., Dijkstra, B. W., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2007). Identification of acceptor substrate binding subsites +2 and +3 in the amylomaltase from *Thermus thermophilus* HB8. *Biochemistry*, 46(17), 5261–5269. <https://doi.org/10.1021/bi602408j>
- Kido, Y., Saburi, W., Nagura, T., & Mori, H. (2023). Hydrolysis-transglycosylation of sucrose and production of β -(2 $\rightarrow$ 1)-fructan by inulosucrase from *Neobacillus drentensis* 57N. *Bioscience, Biotechnology, and Biochemistry*, 87(10), 1169–1182. <https://doi.org/10.1093/bbb/zbad100>
- Kohmoto, T., Fukui, F., Takaku, H., Machida, Y., Arai, M., & Mitsuoka, T. (1988). Effect of isomalto-oligosaccharides on human fecal flora. *Bifidobacteria and Microflora*, 7(2), 61–69. https://doi.org/10.12938/bifidus1982.7.2_61
- Kooi, E. R. (1958). *Production of dextran-dextrinase* (United States Patent No. US2833695A). <https://patents.google.com/patent/US2833695A/en>

- Koshland, Jr. D. E. (1953). Stereochemistry and the mechanism of enzymatic reactions. *Biological Reviews*, 28(4), 416–436. <https://doi.org/10.1111/j.1469-185X.1953.tb01386.x>
- Kotik, M., Kulik, N., & Valentová, K. (2023). Flavonoids as aglycones in retaining glycosidase-catalyzed reactions: Prospects for green chemistry. *Journal of Agricultural and Food Chemistry*, 71(41), 14890–14910. <https://doi.org/10.1021/acs.jafc.3c04389>
- Kralj, S., Leeftang, C., Sierra, E. I., Kemiński, B., Alkan, V., & Kolkman, M. (2018). Synthesis of fructooligosaccharides (FosA) and inulin (InuO) by GH68 fructosyltransferases from *Bacillus agaradhaerens* strain WDG185. *Carbohydrate Polymers*, 179, 350–359. <https://doi.org/10.1016/j.carbpol.2017.09.069>
- Kumar, S., Tissopi, T., & Mutturi, S. (2023). The successful synthesis of industrial isomaltooligosaccharides lies in the use of transglycosylating α -glucosidases: A review. *Carbohydrate Polymer Technologies and Applications*, 5, 100325. <https://doi.org/10.1016/j.carpta.2023.100325>
- Kumar, V., Boorman, J., Greenlee, W. J., Zeng, X., Lin, J., & van den Akker, F. (2023). Exploring the inhibition of the soluble lytic transglycosylase Cj0843c of *Campylobacter jejuni* via targeting different sites with different scaffolds. *Protein Science*, 32(7), e4683. <https://doi.org/10.1002/pro.4683>
- Kuroki, R., Weaver, L. H., & Matthews, B. W. (1999). Structural basis of the conversion of T4 lysozyme into a transglycosidase by reengineering the active site. *Proceedings of the National Academy of Sciences*, 96(16), 8949–8954. <https://doi.org/10.1073/pnas.96.16.8949>
- Lairson, L. L., Henrissat, B., Davies, G. J., & Withers, S. G. (2008). Glycosyltransferases: Structures, functions, and mechanisms. *Annual Review of Biochemistry*, 77(Volume 77, 2008), 521–555. <https://doi.org/10.1146/annurev.biochem.76.061005.092322>
- Lee, C.-C., Wang, H.-F., & Lin, S.-D. (2008). Effect of isomaltooligosaccharide syrup on quality characteristics of sponge cake. *Cereal Chemistry*, 85(4), 515–521. <https://doi.org/10.1094/CCHEM-85-4-0515>
- Leemhuis, H., Dijkman, W. P., Dobruchowska, J. M., Pijning, T., Grijpstra, P., Kralj, S., Kamerling, J. P., & Dijkhuizen, L. (2013). 4,6- α -glucanotransferase activity occurs more widespread in *Lactobacillus* strains and constitutes a separate GH70 subfamily. *Applied Microbiology and Biotechnology*, 97(1), 181–193. <https://doi.org/10.1007/s00253-012-3943-1>
- Leemhuis, H., Pijning, T., Dobruchowska, J. M., van Leeuwen, S. S., Kralj, S., Dijkstra, B. W., & Dijkhuizen, L. (2013). Glucansucrases: Three-dimensional structures, reactions, mechanism, α -glucan analysis and their implications in biotechnology and food applications. *Journal of Biotechnology*, 163(2), 250–272. <https://doi.org/10.1016/j.jbiotec.2012.06.037>
- Li, M., & Mitchinson, C. (2014). *Use of cellulase and glucoamylase to improve ethanol yields from fermentation* (European Union Patent No. EP2702161A1). <https://patents.google.com/patent/EP2702161A1/en>
- Liaw, G. C., Pedersen, S., Hendriksen, H. V., Svendsen, A., Nielsen, B. R., & Nielsen, R. I. (2000). *Enzymatic starch saccharification including a membrane separation step* (European Union Patent No. EP1034293A1). <https://patents.google.com/patent/EP1034293A1/en%C2%A0>
- Light, S. H., Cahoon, L. A., Halavaty, A. S., Freitag, N. E., & Anderson, W. F. (2016). Structure to function of an α -glucan metabolic pathway that promotes *Listeria monocytogenes* pathogenesis. *Nature Microbiology*, 2(2), 16202. <https://doi.org/10.1038/nmicrobiol.2016.202>
- Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews*, 50(4), 353–380. <https://doi.org/10.1128/mr.50.4.353-380.1986>

- Maiorano, A. E., Piccoli, R. M., da Silva, E. S., & de Andrade Rodrigues, M. F. (2008). Microbial production of fructosyltransferases for synthesis of pre-biotics. *Biotechnology Letters*, 30(11), 1867–1877. <https://doi.org/10.1007/s10529-008-9793-3>
- Matthews, B. W. (1968). Solvent content of protein crystals. *Journal of Molecular Biology*, 33(2), 491–497. [https://doi.org/10.1016/0022-2836\(68\)90205-2](https://doi.org/10.1016/0022-2836(68)90205-2)
- Meng, X., Ganggoiti, J., Bai, Y., Pijning, T., Van Leeuwen, S. S., & Dijkhuizen, L. (2016). Structure–function relationships of family GH70 glucansucrase and 4,6- α -glucanotransferase enzymes, and their evolutionary relationships with family GH13 enzymes. *Cellular and Molecular Life Sciences*, 73(14), 2681–2706. <https://doi.org/10.1007/s00018-016-2245-7>
- Mizuno, M., Tonozuka, T., Suzuki, S., Uotsu-Tomita, R., Kamitori, S., Nishikawa, A., & Sakano, Y. (2004). Structural insights into substrate specificity and function of glucodextranase*. *Journal of Biological Chemistry*, 279(11), 10575–10583. <https://doi.org/10.1074/jbc.M310771200>
- Mizushima, D., Miyazaki, T., Shiwa, Y., Kimura, K., Suzuki, S., Fujita, N., Yoshikawa, H., Kimura, A., Kitamura, S., Hara, H., & Funane, K. (2019). A novel intracellular dextranase derived from *paenibacillus* sp. 598K with an ability to degrade cycloisomaltooligosaccharides. *Applied Microbiology and Biotechnology*, 103(16), 6581–6592. <https://doi.org/10.1007/s00253-019-09965-y>
- Motouchi, S., Komba, S., Nakai, H., & Nakajima, M. (2024). Discovery of anomer-inverting transglycosylase: Cyclic glucohexadecaose-producing enzyme from *xanthomonas*, a phytopathogen. *Journal of the American Chemical Society*, 146(26), 17738–17746. <https://doi.org/10.1021/jacs.4c02579>
- Naessens, M., Cerdobbel, A., Soetaert, W., & Vandamme, E. J. (2005). *Leuconostoc* dextranase and dextran: Production, properties and applications. *Journal of Chemical Technology and Biotechnology*, 80(8), 845–860. <https://doi.org/10.1002/jctb.1322>
- Nguyen, T. T. H., Lee, D.-G., Apu, M. A. I., Jung, J.-H., Kim, M.-K., Lim, S., Chung, B., Pal, K., & Kim, D. (2022). The bifidogenic effects and dental plaque deformation of non-digestible isomaltooligosaccharides synthesized by dextranase and alternansucrase. *Enzyme and Microbial Technology*, 153, 109955. <https://doi.org/10.1016/j.enzmictec.2021.109955>
- Nielsen, H. (2017). Predicting secretory proteins with SignalP. *Methods in Molecular Biology (Clifton, N.J.)*, 1611, 59–73. https://doi.org/10.1007/978-1-4939-7015-5_6
- Nobre, C., Teixeira, J. A., & Rodrigues, L. R. (2015). New trends and technological challenges in the industrial production and purification of fructo-oligosaccharides. *Critical Reviews in Food Science and Nutrition*, 55(10), 1444–1455. <https://doi.org/10.1080/10408398.2012.697082>
- Notenboom, V., Boraston, A. B., Kilburn, D. G., & Rose, D. R. (2001). Crystal structures of the family 9 carbohydrate-binding module from *thermotoga maritima* xylanase 10A in native and ligand-bound forms. *Biochemistry*, 40(21), 6248–6256. <https://doi.org/10.1021/bi0101704>
- Okamoto, T., Wakabayashi, H., Nio, S., Shibata, M., & Numazawa, T. (2010). *Method and property improver for improving physical properties of starch-containing foods* (Patent No. JP4475276B2). <https://patents.google.com/patent/JP4475276B2/en>
- Okuyama, M., Kitamura, M., Hondoh, H., Kang, M.-S., Mori, H., Kimura, A., Tanaka, I., & Yao, M. (2009). Catalytic mechanism of retaining α -galactosidase belonging to glycoside hydrolase family 97. *Journal of Molecular Biology*, 392(5), 1232–1241. <https://doi.org/10.1016/j.jmb.2009.07.068>
- Okuyama, M., Yoshida, T., Hondoh, H., Mori, H., Yao, M., & Kimura, A. (2014). Catalytic role of the calcium ion in GH97 inverting glycoside hydrolase. *FEBS Letters*, 588(17), 3213–3217.

<https://doi.org/10.1016/j.febslet.2014.07.002>

- Panesar, P. S., Kaur, R., Singh, R. S., & Kennedy, J. F. (2018). Biocatalytic strategies in the production of galacto-oligosaccharides and its global status. *International Journal of Biological Macromolecules*, 111, 667–679. <https://doi.org/10.1016/j.ijbiomac.2018.01.062>
- Park, B.-R., Park, J. Y., Lee, S. H., Hong, S.-J., Jeong, J. H., Choi, J.-H., Park, S.-Y., Park, C. S., Lee, H.-N., & Kim, Y.-M. (2021). Synthesis of improved long-chain isomaltooligosaccharide, using a novel glucosyltransferase derived from *thermoanaerobacter thermocopriae*, with maltodextrin. *Enzyme and Microbial Technology*, 147, 109788. <https://doi.org/10.1016/j.enzmictec.2021.109788>
- Petrovici, A. R., Pinteala, M., & Simionescu, N. (2023). Dextran formulations as effective delivery systems of therapeutic agents. *Molecules*, 28(3), 1086. <https://doi.org/10.3390/molecules28031086>
- Pichler, M. J., Corbella, M., Meier, S., Haugaard Bohl Andersen, M., Nielsen, T. S., Banerjee, S., Morth, J. P., Rovira, C., & Abou Hachem, M. (2025). A Minimal Mutation Trajectory Separates Inverting and Retaining Mechanisms on the Same Sialidase Structural Scaffold. *ACS Catalysis*, 15(11), 9364–9378. <https://doi.org/10.1021/acscatal.5c01023>
- Rengarajan, S., & Palanivel, R. (2025). Evaluation of prebiotic potential and selective utilization of isomaltooligosaccharides by lactobacillus strains: An in vitro study. *Journal of Food Science and Technology*. <https://doi.org/10.1007/s13197-025-06305-z>
- Ryan J., K., David V., N., Edward N., S., & Charles D., G. (2018). *Nexus of water and CCS: Findings of the water working group (WWG) of the regional carbon sequestration partnerships plains CO2 reduction (PCOR) partnership phase III task 14 deliverable D107 - the PCOR partnership* (Research Repor No. No. DE-FC26-05NT42592; p. 12). Energy & Environmental Research Center (EERC), USA. <https://pcor.undeerc.org/products/product-search/nexus-of-water-and-ccs-findings-of-the-water-working-group-wwg-of-the-regional-carbon-sequestration-partnerships-plains-co2-reduction-pcor-partnership-phase-iii-task-14-deliverable-d107/>
- Rye, C. S., & Withers, S. G. (2000). Glycosidase mechanisms. *Current Opinion in Chemical Biology*, 4(5), 573–580. [https://doi.org/10.1016/S1367-5931\(00\)00135-6](https://doi.org/10.1016/S1367-5931(00)00135-6)
- Sakaguchi, M., Shimodaira, S., Ishida, S., Amemiya, M., Honda, S., Sugahara, Y., Oyama, F., & Kawakita, M. (2015). Identification of GH15 family thermophilic archaeal trehalases that function within a narrow acidic-pH range. *Applied and Environmental Microbiology*, 81(15), 4920–4931. <https://doi.org/10.1128/AEM.00956-15>
- Sangeetha, P. T., Ramesh, M. N., & Prapulla, S. G. (2005). Recent trends in the microbial production, analysis and application of fructooligosaccharides. *Trends in Food Science & Technology*, 16(10), 442–457. <https://doi.org/10.1016/j.tifs.2005.05.003>
- Sauer, J., Sigurskjold, B. W., Christensen, U., Frandsen, T. P., Mirgorodskaya, E., Harrison, M., Roepstorff, P., & Svensson, B. (2000). Glucoamylase: Structure/function relationships, and protein engineering. *Biochimica Et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1543(2), 275–293. [https://doi.org/10.1016/S0167-4838\(00\)00232-6](https://doi.org/10.1016/S0167-4838(00)00232-6)
- Shinoki, A., Lang, W., Thawornkuno, C., Kang, H.-K., Kumagai, Y., Okuyama, M., Mori, H., Kimura, A., Ishizuka, S., & Hara, H. (2013). A novel mechanism for the promotion of quercetin glycoside absorption by megalo α -1,6-glucosaccharide in the rat small intestine. *Food Chemistry*, 136(2), 293–296. <https://doi.org/10.1016/j.foodchem.2012.08.028>
- Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., Shakeel, A., Ansari, A., & Niazi, S. (2016). Inulin: Properties, health benefits and food applications. *Carbohydrate Polymers*, 147, 444–454. <https://doi.org/10.1016/j.carbpol.2016.04.020>

- Struyf, N., Verspreet, J., Verstrepen, K. J., & Courtin, C. M. (2017). Investigating the impact of α -amylase, α -glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. *Journal of Cereal Science*, 75, 35–44. <https://doi.org/10.1016/j.jcs.2017.03.013>
- Sundnes, J. (2024). *Solving ordinary differential equations in python* (Vol. 15). Springer Nature Switzerland. <https://doi.org/10.1007/978-3-031-46768-4>
- Tagami, T., Chen, M., Furunaga, Y., Kikuchi, A., Sadahiro, J., Lang, W., Okuyama, M., Tanaka, Y., Iwasaki, T., Yao, M., & Kimura, A. (2022). Structural insights reveal the second base catalyst of isomaltose glucohydrolase. *FEBS Journal*, 289(4), 1118–1134. <https://doi.org/10.1111/febs.16237>
- Tagami, T., Saburi, W., Sadahiro, J., Kumagai, Y., Lang, W., Matsugaki, N., Okuyama, M., Mori, H., & Kimura, A. (2025). Structural basis of transglucosylation in dextran dextrinase, a homolog of anomer-inverting GH15 glucoside hydrolases. *Journal of Biological Chemistry*, 301(9), 110541. <https://doi.org/10.1016/j.jbc.2025.110541>
- Thunnissen, A.-M. W. H., Rozeboom, H. J., Kalk, K. H., & Dijkstra, B. W. (1995). Structure of the 70-kDa soluble lytic transglycosylase complexed with bulgecin a. Implications for the enzymic mechanism. *Biochemistry*, 34(39), 12729–12737. <https://doi.org/10.1021/bi00039a032>
- Veen, B. A. van der, Leemhuis, H., Kralj, S., Uitdehaag, J. C. M., Dijkstra, B. W., & Dijkhuizen, L. (2001). Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of cyclodextrin-glycosyltransferase *. *Journal of Biological Chemistry*, 276(48), 44557–44562. <https://doi.org/10.1074/jbc.M107533200>
- Vijayaraghavan, J., Kumar, V., Krishnan, N. P., Kaufhold, R. T., Zeng, X., Lin, J., & Akker, F. van den. (2018). Structural studies and molecular dynamics simulations suggest a processive mechanism of exolytic lytic transglycosylase from campylobacter jejuni. *PLOS ONE*, 13(5), e0197136. <https://doi.org/10.1371/journal.pone.0197136>
- Wassenberg, D., Schurig, H., Jaenicke, R., & Liebl, W. (1997). Xylanase XynA from the hyperthermophilic bacterium thermotoga maritima: Structure and stability of the recombinant enzyme and its isolated cellulose-binding domain. *Protein Science*, 6(8), 1718–1726. <https://doi.org/10.1002/pro.5560060812>
- Williams, S. J., & Withers, S. G. (2000). Glycosyl fluorides in enzymatic reactions. *Carbohydrate Research*, 327(1), 27–46. [https://doi.org/10.1016/S0008-6215\(00\)00041-0](https://doi.org/10.1016/S0008-6215(00)00041-0)
- Wimmerová, M., Kozmon, S., Nečasová, I., Mishra, S. K., Komárek, J., & Koča, J. (2012). Stacking interactions between carbohydrate and protein quantified by combination of theoretical and experimental methods. *PLOS ONE*, 7(10), e46032. <https://doi.org/10.1371/journal.pone.0046032>
- Xue, N., Svensson, B., & Bai, Y. (2022). Structure, function and enzymatic synthesis of glucosaccharides assembled mainly by $\alpha 1 \rightarrow 6$ linkages – a review. *Carbohydrate Polymers*, 275, 118705. <https://doi.org/10.1016/j.carbpol.2021.118705>
- Yamamoto, K., Yoshikawa, K., & Okada, S. (1993). Detailed action mechanism of dextrin dextranase from acetobacter capsulatus ATCC 11894. *Bioscience, Biotechnology, and Biochemistry*, 57(1), 47–50. <https://doi.org/10.1271/bbb.57.47>
- Yang, X., Wu, X., Jiang, Z., Wang, K., Liu, Y., Qin, Z., Zhang, S., Liu, Y., & Cao, L. (2025). Fine-tuning the active-site microenvironment of β -galactosidase to enhance the synthesis ability of galactooligosaccharides while minimizing the impairment to transglycosylation activity. *Journal of Agricultural and Food Chemistry*, 73(19), 11866–11877. <https://doi.org/10.1021/acs.jafc.5c00679>
- Zakariassen, H., Hansen, M. C., Jøranli, M., Eijsink, V. G. H., & Sørle, M. (2011). Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating

mutant. *Biochemistry*, 50(25), 5693–5703. <https://doi.org/10.1021/bi2002532>

Zhao, J., Yang, J., & Xie, Y. (2019). Improvement strategies for the oral bioavailability of poorly water-soluble flavonoids: An overview. *International Journal of Pharmaceutics*, 570, 118642. <https://doi.org/10.1016/j.ijpharm.2019.118642>