学位論文内容の要旨

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学位論文題名

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)

Glucans play important roles in *in vivo* metabolism and various industries. Glycosyltransferases (GTs) and glycoside hydrolases (GHs) are the principal enzymes responsible for glucan synthesis and hydrolysis, respectively. GHs either retain or invert the anomeric configuration of the substrate in the product, through different catalytic mechanisms. Anomer-retaining GHs can catalyze both hydrolysis and transglycosylation, and some of them share sequence similarity with GTs, which catalyze transglucosylation exclusively. GTs are promising biocatalysts for the industrial synthesis of functional carbohydrates.

GH family 15 (GH15) mainly comprises inverting α-GHs, including glucoamylases that release β-glucose from α-(1→4)-linked maltooligosaccharides (MOSs) and starch. Recently, however, several retaining GTs including dextran dextrinase (DDase) and α-glucan 4,6-α-glucosyltransferase (46GT), have also been assigned to GH15 based on sequence similarity. 46GTs use MOSs as substrates to efficiently produce valuable α-(1→6)-linked isomaltooligosaccharides (IMOs), which are prebiotics with diverse physiological benefits.

GH15 46GTs are potential enzymatic tools for IMO industry but require detailed kinetic and mechanism evaluation. GH15 46GTs are also valuable targets for understanding anomer-retaining and anomer-inverting catalytic mechanism distinction, but devoid of structural solution. This study focuses on these issues from three parts.

1. Mechanism for synthesis of IMOs from MOSs by GH15 46GTs

Enzymatic reactions of GH15 46GTs from *Tepidibacillus decaturensis* (Td46GT) and *Pseudoclostridium thermosuccinogenes* (Pt46GT) were analyzed. Both enzymes catalyzed α-(1→4)-transglucosylation to MOS, and α-(1→6)-transglucosylation to maltose (G2) and IMOs. Comparison of k_{cat}/K_m values in single-substrate reactions revealed that Td46GT and Pt46GT prefer α-(1→4)-linked substrates to α-(1→6)-linked ones. Compared with Pt46GT, Td46GT displayed lower k_{cat}/K_m values to G2 and maltotriose (G3), but higher k_{cat}/K_m value to isomaltose (IG2). Kinetic analyses of the two-substrate reactions of Td46GT revealed that the acceptors determined linkages of transglucosylation products. Relative acceptor-substrate specificity constants (RASCs) indicated that G3 and G2 were the best acceptors for α-(1→4)- and α-(1→6)-transglucosylations, respectively. The subsite affinities calculated from the RASCs were consistent with those obtained from k_{cat}/K_m values for single-substrate reactions. These results indicate that Td46GT possesses distinct MOS- and IMO-binding subsites in a single active pocket, which are shared by both acceptor and donor substrates, and that the binding manner of the acceptor determines the product specificity of transglucosylation. The

RASCs and k_{cat}/K_m values enabled *in silico* simulation of time-dependent quantitative changes of MOS and IMO-derived saccharides, in reaction catalyzed by Td46GT. These results mathematically demonstrate that the kinetic parameters govern both the initial reaction state under fixed substrate concentrations and the long-term reaction phase, during which substrate and product concentrations continuously change.

2. Structure of Td46GT and functions of substrate-binding residues

Crystal structure of Td46GT inactive mutant E666Q (the nucleophile catalyst mutant) in complex with a pseudo-G4 inhibitor, acarbose, is determined. Td46GT is monomer and comprises three domains N, A, and C. Domains N and A correspond to N-terminal and catalytic $(\alpha/\alpha)_6$-barrel domains of DDase. The acarbose-binding manner in domain A highly resembles that of DDase, indicating a strictly conserved active pocket for retaining GH15 GTs. Hydrogen bonds formed with acarbose at subsites -1 and +1 are also similar with those in inverting GH15 α-GHs.

Differently, in subsite -1, the catalytic Glu666 of Td46GT locates in closer proximity to the anomeric carbon and forms additional hydrogen bonds with Tyr607 and Glu682 (mediated by a water), compared to inverting GHs. In subsite +2, Tyr612 stacks onto glucose unit from its β-face and Phe418 has hydrophobic contact with this glucose unit from α-face. Importance of Tyr612 and Phe418 were identified by large reduction of affinity in subsite +2 for both MOS and IMO-binding in F418A and Y612A mutants. Domain C of Td46GT is a unique 12-stranded β-sandwich absent in characterized protein database, and binds to a second acarbose. Five calcium ion binding sites were determined. Biochemical assays revealed that addition of calcium ions significantly enhanced thermostability and activity of Td46GT.

3. Conversion of reaction mechanism of Td46GT by rational mutagenesis

Glu480 and Glu666 located in α5-α6 and α11-α12 loops of Td46GT were biochemically verified as general acid/base and nucleophile catalysts. Sequence alignment revealed that eight residues in subsite -1 were differently conserved in inverting GHs and retaining GTs within GH15. The multiple mutant targeting all these residues converted Td46GT to an inverting GH, with largely lost activity, but produced β-glucose in the reaction with a MOS, maltotetraose.

Further dissection of this multiple mutant set identified that only four-residues-mutation was essential to convert Td46GT toward inverting GH. As two of the four residues, Gln664 and Gly665, lie adjacent to the nucleophile Glu666, and their substitution with a single Pro could change the position of Glu666, together with Y607G mutation which diminished a direct hydrogen bond with Glu666. In addition, V684W possibly caused steric hinderance to water molecules at β-face of valienamine unit of acarbose in subsite -1.

This study provides an unprecedent 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.