学位論文内容の要旨

博士の専攻分野名称：博士（農学）　　氏　名：Yodai Taguchi

学位論文題名

Functions, structures, and applications of enzymes acting on trehalose and its derivatives

（トレハロースおよびその誘導体に作用する酵素の構造，機能ならびに応用に関する研究）

Trehalose (Glcα1-1αGlc) is a non-reducing disaccharide used as an energy source and a compatible solute in various organisms. In the universal pathway, trehalose is synthesized *via* trehalose 6-phosphate (Tre6*P*, Glcα1-1αGlc6P). Tre6*P* synthase (TPS) produces Tre6*P* from UDP-Glc and Glc6*P*, and Tre6*P* phosphatase (TPP) dephosphorylates Tre6*P* to produce trehalose. Trehalose is hydrolyzed to Glc by trehalase. In plants, the endogenous amount of Tre6*P* is very small, but Tre6*P* acts as an essential signal substance for controlling growth, flowering, and nutrition metabolism. Plants have multiple isozymes responsible for trehalose-synthesis. Since these enzymes are differently expressed in various organs, growth stages, and environments, plants strictly regulate the level of Tre6*P* using the multiple-enzyme system. In contrast to the trehalose synthesizing enzymes, plants possess a single trehalase responsible for the degradation of trehalose. Despite the physiological importance of trehalose related enzymes to regulate Tre6*P* signaling, enzyme functions and structures have been scarcely understood thus far. For precise functional and structural analysis of the enzymes, Tre6*P* and its derivatives are required abundantly. In this study, enzymatic synthesis of Tre6*P* and its derivative is established, and functions and structures of TPP isozymes and trehalase from *Arabidopsis thaliana* are investigated to provide a better understanding of the trehalose metabolism in plants.

1. Chemoenzymatic synthesis of Tre6*P* and its derivative using the reverse phosphorolysis catalyzed by Tre6*P* phosphorylase

Tre6*P* phosphorylase (TrePP) reversibly phosphorolyzes Tre6*P* into β-Glc1*P* and Glc6*P*. For Tre6*P* synthesis, the reverse phosphorolysis of Tre6*P* catalyzed by *Lactococcus lactis* TrePP was used. The TrePP produced 91.9 mM Tre6*P* from 100 mM β-Glc1*P* and 100 mM Glc6*P*. The one-pot reaction of TrePP and maltose phosphorylase enabled the production of 65 mM Tre6*P* from 100 mM maltose (Mal), 100 mM Glc6*P*, and 20 mM inorganic phosphate (Pi). By the addition of β-phosphoglucomutase, converting β-Glc1*P* to Glc6*P*, Glc6*P* was eliminated from starting materials. Besides, the addition of yeast, consuming Glc produced in the phosphorolysis of Mal, increased the yield of Tre6*P*. Finally, from 1 M Mal and 0.6 M Pi, 0.375 M Tre6*P* was synthesized in a 100 mL scale reaction, and 9.4 g (19 mmol) of highly purified Tre6*P* dipotassium salt was obtained by the purification procedures. Acceptor screening in the reverse phosphorolysis of TrePP showed that

Man6*P* also served as an acceptor substrate. From β-Glc1*P* and Man6*P*, a novel sugar phosphate, Glcα1-1αMan6*P*, was synthesized at 51% yield.

2. Comprehensive evaluation of enzymatic properties of TPP isozymes from *A. thaliana*

A. thaliana has 10 TPP isozymes, AtTPPA–AtTPPJ, with 45–82% identity in the amino acid sequences. For the investigation of the enzymatic functions of these TPP isozymes, recombinant AtTPPs were produced in *E. coli* and purified. All AtTPPs except for AtTPPI showed the dephosphorylation activity toward Tre6*P*. The k_{cat}/K_m of AtTPPs for Tre6*P* was diverse ranging from 0.113 $s^{-1}mM^{-1}$ to 23.0 $s^{-1}mM^{-1}$. AtTPPA and AtTPPB, whose gene expression level is high at the upper ground tissues and root, respectively, showed higher k_{cat}/K_m than the other isozymes. All active AtTPPs dephosphorylated Glcα1-1αMan6*P* as well as Tre6*P*. Since AtTPPs did not have the detectable activity to α-Glc1*P*, β-Glc1*P*, Glc6*P*, Fru6*P*, Gal6*P*, Man6*P*, α-Mal1*P*, and Suc6*P*, AtTPPs are considered to recognize the α1-1α glucosyl group of substrates strictly.

3. Molecular mechanism of substrate recognition of plant trehalase which changes substrate specificity depending on pH conditions

Trehalase from *A. thaliana* (AtTRE1) was characterized using truncated enzyme of the N-terminal transmembrane domain. AtTRE1 showed hydrolytic activity not only toward trehalose but also its analogs containing Tre6*P*. This substrate specificity was common in *Oryza sativa* trehalase. MS analysis of ^{18}O-labeled reaction products from Tre6*P* showed that Glc and Glc6*P* residues bind to subsite -1 and +1, respectively. AtTRE1 showed two peaks at pH 3.7 and pH 6.9 in the pH-k_{cat} curve of trehalose hydrolysis, indicating that AtTRE1 forms two types of ES complex, designated as ES_{acid} and $ES_{neutral}$, respectively, depending on pH conditions. This enzyme showed much higher activity to Tre6*P* at acidic pH than at neutral pH and showed a single peak at pH 3.9 in the pH-k_{cat} curve for the hydrolysis of Tre6*P*. Thus, AtTRE1 is considered to take a suitable structure for the hydrolysis of Tre6*P* at acidic pH. The structural analysis of inactive AtTRE1 mutant (D380A) in complex with trehalose revealed that α1→α2 loop and α11→α12 loop covered the substrate binding site, and the substrate binding site was closed (closed-form). The residues, Arg224 and Glu595, on these loops could form hydrogen bonds with 6-OH of Glc in subsite +1. By co-crystallization with Tre6*P*, the distorted structure of AtTRE1 was obtained. In this structure, the residues, located on α1→α2 and α11→α12 loops, flipped to open the substrate binding site (open-form). Since the structure of the closed-form AtTRE1 is not suitable for binding to Tre6*P* due to possible steric hindrance against the phosphoryl group, the open-form was considered to be suitable for the reaction with Tre6*P*. In the ES_{acid} and $ES_{neutral}$, AtTRE1 presumably takes the structures similar to the open- and closed-forms, respectively. Ser223 and Thr600 on α1→α2 and α11→α12 loops, respectively, could form hydrogen bonds differently in the closed- and open-forms. Since S223G mutation enhanced the preference for Tre6*P*, S223 was important for stabilizing the closed-form (open-form was more preferred in this mutant than in the wild type). On the other hand, T600D mutation to stabilize the closed-form reduced the preference for Tre6*P*. The mechanism for the interconversion of ES_{acid} and $ES_{neutral}$ through the structural change of the α1→α2 and α11→α12 loops is proposed.