Structural Analysis of Fungal *Endo*-β-1,2-Glucanase Reveals a Non-Canonical Reaction Mechanism

Endo-β-1,2-Glucanase from *Talaromyces funiculosus* specifically hydrolyzes β-1,2-glucan from the reducing end to sophorose (Glc-β-1,2-Glc) as a main product. Here, we determined the apo-structure of recombinant *Tf*SGL (*Tf*SGLr) using the SAD phasing and the Michaelis complex of inactive *Tf*SGLr with β-1,2-glucoheptose by soaking the crystal in β-1,2-glucan. The overall structure comprises an $(\alpha/\alpha)_6$ toroid fold and has a cleft. The ligand was observed in the large active pocket at the center of the cleft. Furthermore, structural analysis of the Michaelis complex revealed a novel catalytic mechanism catalyzed by non-canonical general acid (E262) and general base (D446).

Endo-β-1,2-Glucanase (SGL) is an enzyme that hydrolyzes linear and cyclic β-1,2-glucan comprising a β-1,2-linked glucosyl backbone. SGLs are basically classified into the glycoside hydrolase (GH) family based on amino acid sequence similarity [1]. Recently, identification and structural determination of a bacterial SGL from *Chitinophaga pinensis* (*Cp*SGL) have resulted in the discovery of a new GH family 144 [2]. However, fungal SGL was not identified. In our previous work [3], we identified a fungal SGL from *Talaromyces funiculosus* (*Tf*SGL) and there is no sequence similarity between *Cp*SGL and *Tf*SGL. In addition, analysis of the action patterns of *Tf*SGL indicates that *Tf*SGL preferably releases sophorose (Glc-β-1,2-Glc, Sop_2) as a main product from the reducing end of β-1,2-glucan and that *Tf*SGL is an inverting enzyme. Here, we report the structural information of *Tf*SGL [3]. The apo-structure of recombinant *Tf*SGL (*Tf*SGLr) was determined at 2.0 Å resolution using the iodide single-wavelength anomalous diffraction-phasing (SAD) method [**Fig. 1(A)**]. The overall structure folds into a single $(\alpha/\alpha)_6$ barrel domain. Although there is no sequence similarity between *Cp*SGL and *Tf*SGL, the overall structure of *Tf*SGLr is very similar to those of GH144 family members including *Cp*SGL. *Tf*SGLr has a cleft with a large pocket at its center. In the complex structure with Sop_2 and Glc obtained by soaking a TfSGLr crystal in Sop_2, the electron density of a Sop_2 molecule was clearly observed at the pocket [**Fig. 1(B)**]. Considering the action pattern of *Tf*SGLr, the Sop_2 molecule seems to be located at subsites +1 to +2. Substitution of E262 located at the substrate-binding pocket with a glutamine residue abolished the hydrolytic activity toward β-1,2-glucan. We succeeded in obtaining a Michaelis complex of the E262Q mutant with β-1,2-glucoheptose (Sop_7) by soaking a crystal of the mutant in β-1,2-glucan. In this complex, the fourth Glc moiety from the non-reducing end of the Sop_7 molecule forms a skew-boat conformation ($^1S_3$) (**Fig. 2**). This twisted conformation enables a nucleophile to be located where nucleophilic attack to an anomeric carbon is possible. Furthermore, a water molecule, which is a candidate for nucleophilic water, is located near the anomeric carbon of the Glc moiety with a $^1S_3$ conformation. These observations strongly suggest that the fourth Glc moiety is located at subsite –1. In the Michaelis complex of *Tf*SGLr, D446 interacts with the nucleophilic water via another water molecule and D177 and E262 interact with the anomeric oxygen at subsite –1 via the 3-hydroxy group of the Glc moiety of the substrate.

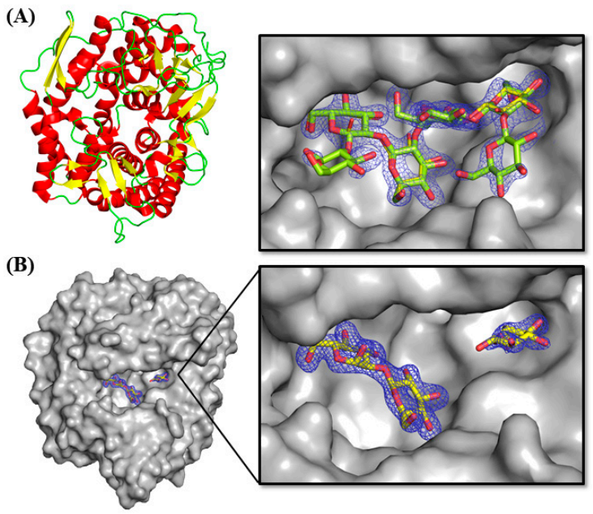

Figure 1: The overall apo-structure of *Tf*SGLr **(A)** and the complex structure with Sop_2 and Sop_7 **(B)**. The surface representation of the catalytic center with Sop_2 in *Tf*SGLr. The electron density of the Glc moieties of Sop_2 (bottom) and Sop_7 (top) is shown by a blue mesh.

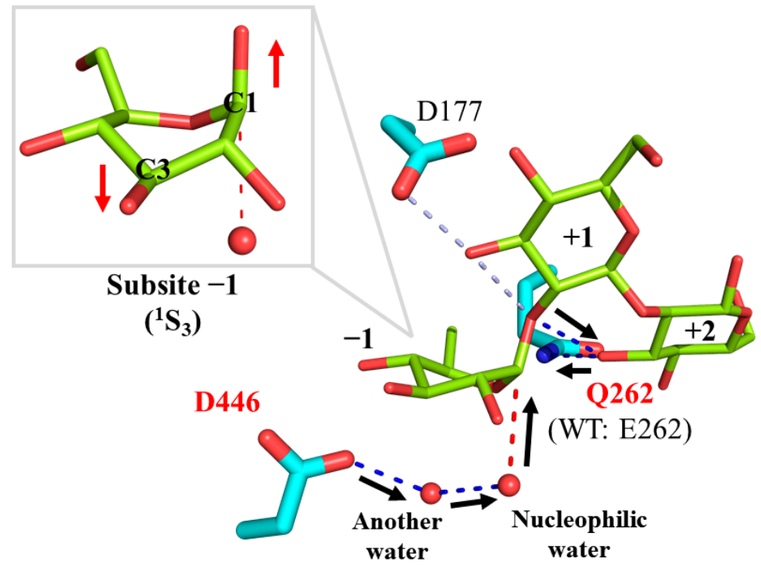

Figure 2: The non-canonical mechanism of *Tf*SGLr.

In general, an inverting GH enzyme hydrolyzes a glycosidic bond in the substrate through a single-displacement mechanism using two acidic residues. A general base activates a water molecule for nucleophilic attack on an anomeric carbon, whereas a general acid protonates a glycosidic bond oxygen atom directly [4].

In the case of *Tf*SGLr, however, no acidic residue directly interacts with the nucleophilic water molecule or the glycosidic bond oxygen atom (**Fig. 2**). To determine the general base and the general acid, mutational analysis of all candidates for catalytic residues was performed. The hydrolytic activity of D446N (the candidate for a general base), D177N and E262Q mutants (the candidates for a general acid) toward β-1,2-glucan was abolished. In addition, these candidate residues are highly conserved among *Tf*SGL homologs. These results suggest that D446 acts as a general base via two water molecules and D177 and/or E262 act as a general acid via hydroxy groups in the substrate. To determine the general acid, the action patterns for Sop_{5-6} deoxygenated at the hydroxy groups in the reaction pathways were investigated. The substrate with the 3-hydroxy group of the Glc moiety at subsite +2 deoxygenated was completely not hydrolyzed, suggesting that E262 indirectly protonates the glycosidic bond oxygen atom via the 3-hydroxy group of the Glc moiety at subsite +2.

Structural analysis of the Michaelis complex of *Tf*SGLr mutant revealed a unique reaction mechanism of *Tf*SGLr and led to the foundation of the new GH family 162.

The black and red arrows represent the pathway for proton transfer and the distorted positions (C1, C3) of the glucose moiety at subsite –1, respectively. The blue and red dotted lines represent hydrogen bonds and longer hydrogen bonds (over 3.5 Å) involved in catalysis, respectively. The gray dotted line represents hydrogen bonds.

REFERENCES

[1] B. Henrissat, *Biochem. J.* **280**, 309 (1991).
[2] K. Abe, M. Nakajima, T. Yamashita, H. Matsunaga, S. Kamisuki, T. Nihira, Y. Takahashi, N. Sugimoto, A. Miyanaga, H. Nakai, T. Arakawa, S. Fushinobu and H. Taguchi, *J. Biol. Chem.* **292**, 7487 (2017).
[3] N. Tanaka, M. Nakajima, M. Narukawa-Nara, H. Matsunaga, S. Kamisuki, H. Aramasa, Y. Takahashi, N. Sugimoto, K. Abe, T. Terada, A. Miyanaga, T. Yamashita, F. Sugawara, T. Kamakura, S. Komba, H. Nakai and H. Taguchi, *J Biol. Chem.* **294**, 7942 (2019).
[4] J. D. McCarter and G. S. Withers, *Curr. Opin. Struct. Biol.* **4**, 885 (1994).

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