

Crystal Structure Unveiled the Reaction Mechanism of Novel C-C Bond Cleavage Proteins

C-glycosides are highly stable glycosides in which a carbon of the sugar moiety is bonded directly to a carbon of an aglycone, not bonded via a glycosidic bond. Unlike a general glycosidic bond, the sugar moiety of a C-glycoside is cleaved through a two-step reaction. We successfully identified two enzymes involved in the C-glycoside deglycosylation reaction in C-glycoside-catabolizing bacteria found in soil. Based on biochemical characterizations and bioinformatic analysis, we revealed bacterial C-glycoside metabolism in nature. Together with collaborators, we also reported the three-dimensional structures of these unique enzymes by X-ray crystallography and cryo-electron microscopy for the first time.

Enzymes are catalysts responsible for various reactions in living organisms. Peptide chains consisting of several hundred amino acids are folded to form an enzyme, and amino acid residues at the substrate-binding pocket are involved in the catalytic reaction.

In nature, there are still many metabolic reactions, of which catalytic enzymes have not been identified. For example, degradation pathways of various secondary metabolites produced by plants have never been identified in many cases, while such compounds are released into the environment as a result of defoliation etc., and should eventually be degraded by microorganisms.

This study focused on the degradation of compounds called C-glycosides. Glycosides are compounds, of which a sugar moiety is added to a non-sugar compound (aglycone) such as a flavonoid. The addition of a sugar improves water solubility and stability. Glycosides can be classified according to the mode of addition of the sugar. When the sugar is added to a hydroxyl group of an aglycone, it is called an O-glycoside; when it is added to an amino group, it is called an N-glycoside. When the anomeric carbon of the sugar is directly C-C bonded to the carbon atom of the aglycone, it is called a C-glycoside (Fig. 1). Compared to O-glycosides, C-glycosides are rare compounds but we have consumed them for many years. Puerarin involved

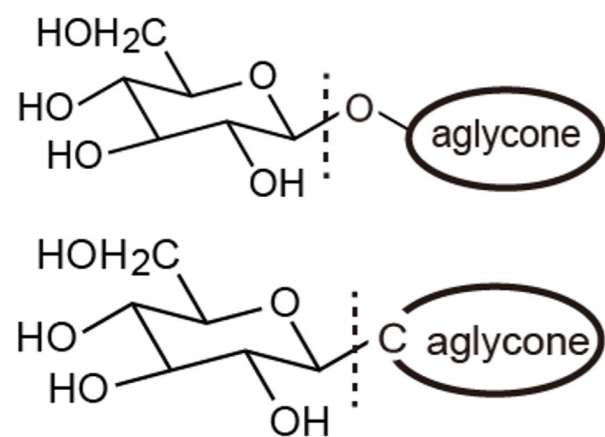


Figure 1: Structures of O-glycosides (upper) and C-glycoside (lower). The cleavage sites for deglycosylation are indicated by the dotted lines.

in Kakkon-to and carminic acid, an edible red dye used worldwide, are famous C-glycosides in our diet.

C-glycosides are not hydrolyzed like other glycosides, and a degradation pathway of them has not yet been elucidated in the enzymatic level at the beginning of this study. We succeeded in isolating the bacteria catabolizing carminic acid, a C-glycoside red compound synthesized by a scale insect and used worldwide as an edible pigment [1]. The C-glycoside degradation reaction we identified was a two-step reaction involving two enzymes. First, an FAD-dependent enzyme, named C-glycoside oxidase, oxidizes the 3-position of the sugar moiety of carminic acid. The resulting 3'-oxo-carminic acid is cleaved from the C-C bond between the sugar and the aglycon by a hitherto unknown enzyme we identified in this study and named C-glycoside deglycosylation enzyme (CGD) [2]. It shows little homology to known enzymes, as is described in the following.

When an enzyme that catalyzes a new reaction is found, X-ray crystallography is very useful in elucidating the reaction mechanism because it can reveal the binding mode of the substrate to the active site and the surrounding amino acids. We elucidated the 3D-structures of the two enzymes by X-ray crystallography and cryo-EM, and proposed their reaction mechanisms [1, 2]. The C-glycoside oxidase catalyzing the first oxidation reaction shows similarity to pyranose oxidases, and

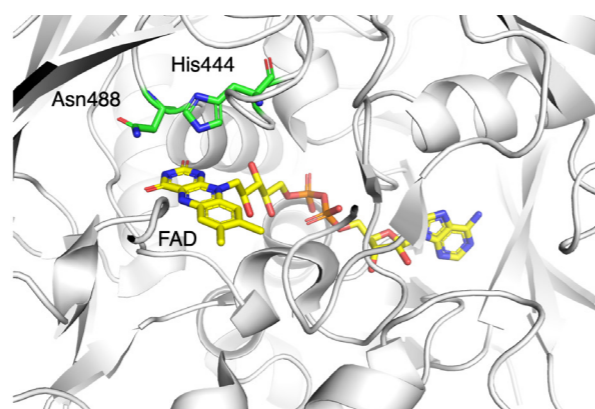


Figure 2: Superposition of the active site residues of C-glycoside oxidase. The conserved residues of histidine and asparagine are labeled.

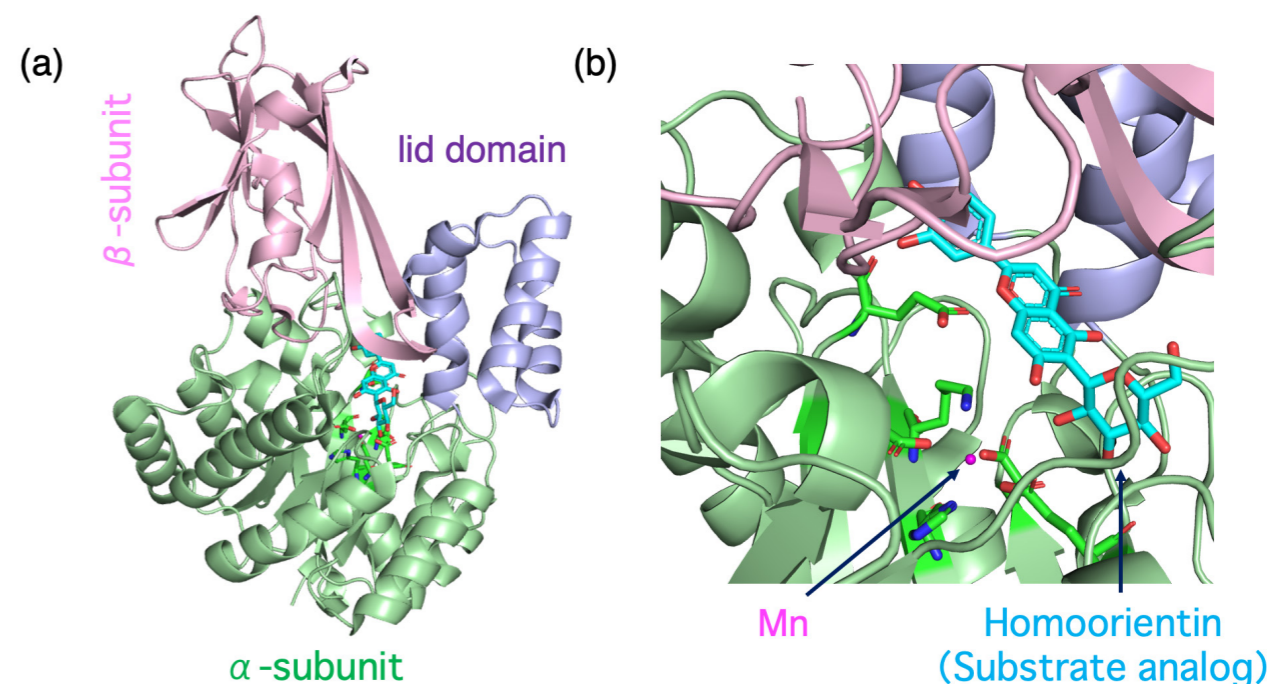


Figure 3: Crystal structures of C-glycoside deglycosylation enzymes. (a) Heterodimer structure. The α -subunit is shown in pale green and the β -subunit shown in light pink. The lid domain of α -subunit is shown in purple. (b) The active site of CGD.

the co-crystal structure with FAD revealed a similar active site to pyranose oxidases. The catalytic histidine residue and the asparagine residue located next to the histidine are conserved (Fig. 2). Since C-glycoside oxidase does not react with glucose, its substrate specificities were considered to be completely different from those of pyranose oxidases. Therefore, we named it as C-glycoside oxidase and proposed as a new enzyme.

The enzyme catalyzing the second reaction is a heterodimeric enzyme consisting of an α -subunit and a β -subunit [Fig. 3(a)]. The α -subunit shows only less than 20% homology with xylose isomerase and sugar phosphate isomerase/epimerase. The β -subunit showed no homology to known enzymes. Therefore, this second enzyme was considered to be a novel enzyme and named CGD as mentioned above.

The space formed at the dimer boundary in the co-crystal structure with the substrate analog is the active site [Fig. 3(b)]. It was also found that Mn^{2+} is bound in the active site of α -subunit side and the amino acids coordinated to Mn^{2+} are highly conserved among the homologous enzymes which we found by the database search. The metal was essential for enzyme activity because the activity was lost when the metal was removed by a chelating agent, and was restored when the metal was added again. The β -subunit side in the active site binds to the substrate at several sites, which may be important for active pocket formation and substrate recognition. Comparing the crystal structure containing a substrate analog with that of the apo-enzyme, it was found

that the binding of the substrate causes the movement of a domain named as a "lid domain," which consists of four α -helices. This observation suggests that conformational changes in the lid domain are important for the enzyme activity. The positional relationship between the substrate and the surrounding amino acids in the active site suggests that the reaction involves the metal, and amino acid residues of histidine and glutamate.

Although glycosides are abundant in nature, the C-glycoside has a relatively rare structure. We discovered microorganisms that catabolize C-glycosides through screening. And we found two novel enzymes from C-glycoside catabolizing microorganisms. It is expected that microorganisms still have many unknown enzymes for metabolizing unique compounds. Therefore, studies on the microbial metabolism of unique compounds are a frontier for the discovery of novel enzymes.

REFERENCES

- [1] T. Mori, T. Kumano, H. He, S. Watanabe, M. Senda, T. Moriya, N. Adachi, S. Hori, Y. Terashita, M. Kawasaki, Y. Hashimoto, T. Awakawa, T. Senda, I. Abe and M. Kobayashi, *Nat. Commun.* **12**, 6294 (2021).
- [2] T. Kumano, S. Hori, S. Watanabe, Y. Terashita, H. Y. Yu, Y. Hashimoto, T. Senda, M. Senda and M. Kobayashi, *Proc. Natl. Acad. Sci. USA* **118**, e2106580118 (2021).

BEAMLINES

BL-1A and BL-17A

T. Kumano and M. Kobayashi (Univ. of Tsukuba)