博士論文（要約）

Structure-Function Studies on Enzymes from Intestinal Bacteria Involved in *C*-Deglycosylation of Medicinal Natural Products

(腸内細菌由来薬用天然物*C*-配糖体の脱グリコシル化に関わる酵素の構造機能解析)

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Introduction

Flavonoids mainly present as *O*- or *C*-glycosides in plants, which has gained more and more interest as result of their notable benefits to prevent modern diseases. Intestinal bacteria are able to cleave the *O*- or *C* glycosidic bond and the resulting metabolites exhibit various biological effects[1]. Previously, it has been reported that DgpA/DfgE (oxidoreductase), DgpB/DfgB (hypothetical protein), and DgpC (sugar phosphate isomerase) /DfgA (β-galactosidase) are involved in the C-C bond cleavage of *C*-glycoside flavonoids in intestinal bacteria strain PUE (92% similarity in 16S rRNA gene sequence with *Dorea longicatena*; Dgp cluster)[2, 3] and *Eubacterium cellulosolvens* (Dfg cluster)[3] (**Fig. 1**). However, their protein structures and detailed catalytic mechanism remain to be elucidated. In this study, we solved the protein structures with X-ray crystallographic and cryo-EM analyses, and characterized their biochemical properties.

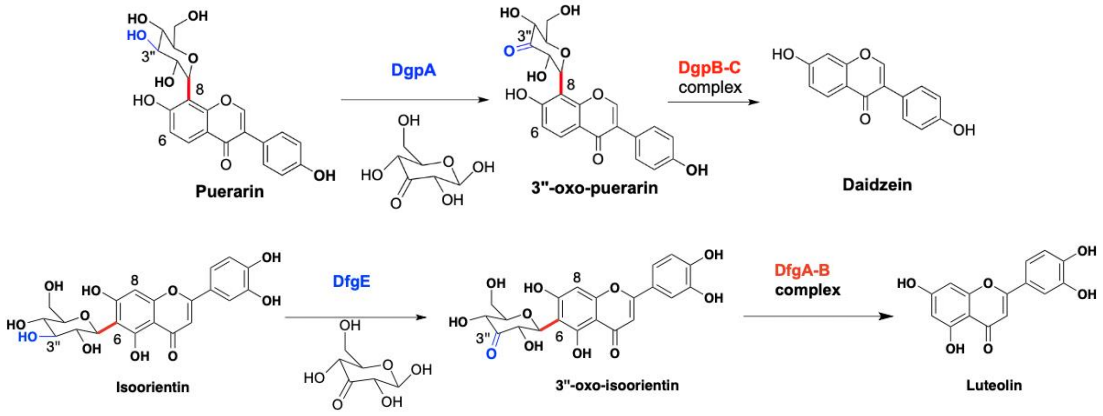


Fig.1 *C*-deglycosylation of puerarin by DgpA and DgpB-C, and isoorientin by DfgE and DfgA-B

1. Investigation of the Substrate Scope of DgpB-C and DfgA-B

Puerarin, orientin, isoorientin, vitexin, isovitexin and mangiferin were used to check the substrate specificity of Dgp B-C and DfgA-B. Dgp B-C complex can accept puerarin and orientin but not homoorientin or mangiferin as substrate with the combination of DgpA, which may indicate that Dgp B-C specifically catalyzes 8-C-glycoside. However, Dfg A-B complex can accept homoorientin and mangiferin but not puerarin or orientin as substrate with the combination of DfgE, which suggests that DfgA-B complex specifically catalyzes 6-C-glycosides. Therefore, DgpB-C and DfgA-B complex adopt different substrate specificity.

Furthermore, the biochemical properties of DgpB-C were investigated to reveal that 1) DgpB-C shows the highest activity at pH 6.0 and 60℃, and 2) DgpB-C loses 60% of its activity by EDTA treatment, but the activity can be restored after reconstitution with Mn^{2+} or Ni^{2+}.

2. Structures of DgpB-C and DfgA-B Complex

Both of DgpB-C and DfgA-B complex structures were solved by cryo-EM and X-ray crystallography. DgpB-C structures consist of tetramer of heterodimers (**Fig. 2A** and **2B**). Hydrogen bonds and salt bridges are observed at the interface between DgpB and DgpC. A metal ion is coordinated by E141, D173, H263, E299 and two water molecules in the active site of DgpB-C (**Fig. 2E**). Also, DfgA-B consists of tetramer of heterodimers, and shares a similar heterodimer with that of DgpB-C(Fig. 2C and 2D) .

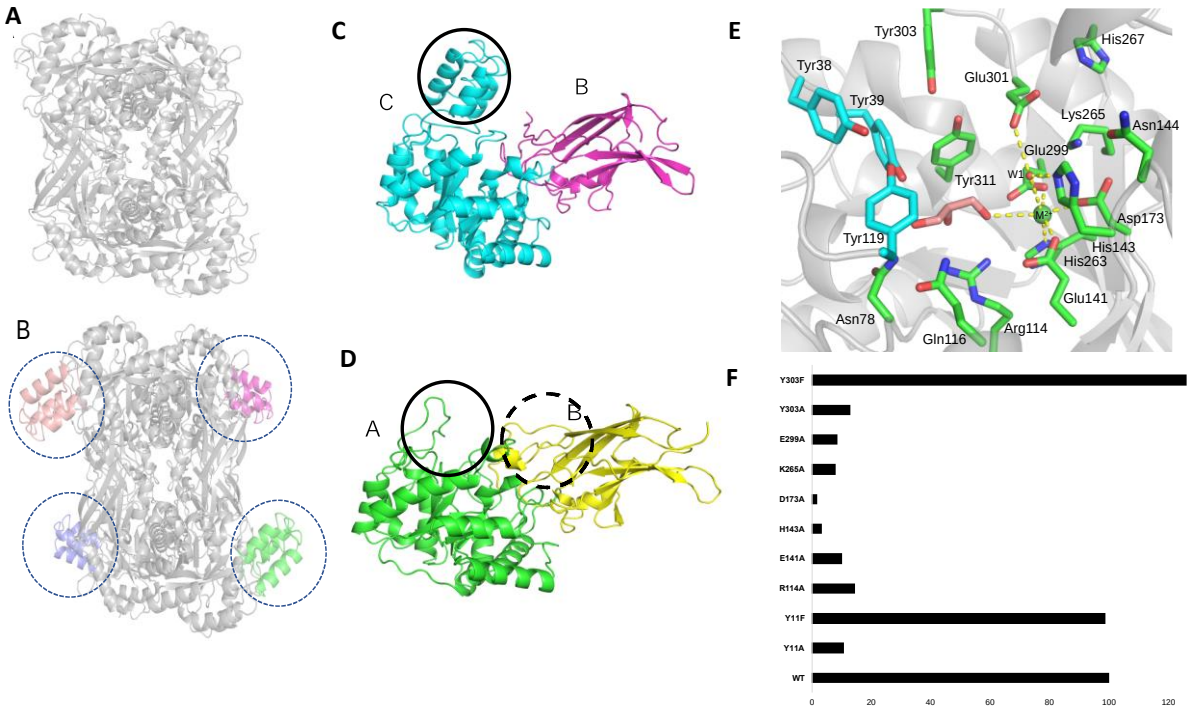


Fig. 2 overall structure of DgpB-C solved by **A**) cryo-EM and **B**) X-ray crystallography; Heterodimer structure of **C**) DgpB-C and **D**) DfgA-B; **E**) Active site of DgpB-C; **F**) Mutagenesis of DgpB-C

The major difference between the crystal and cryo-EM structure of DgpB-C is the domain highlighted in dashed cycles in **Fig. 2B**. Those domains contain four helices and are not observed in the cryo-EM structure, indicating that they are flexible in solution state. Truncation of these domains in DgpB-C abolished its activity, suggesting their function as a closing cap when the substrate is bound in the active pocket. On the other hand, this additional domain (**Fig.2C and 2D,** highlighted in cycle, N179~D244) is not conserved in DfgA-B. Instead, a loop in DfgB covers the active site in the crystal structure. Furthermore, this loop is not observed in cryo-EM structure as in the case of DgpC(Fig.**2C** in dashed circle), indicating that the loop is also flexible in solution state and plays a similar role as a closing cap. In addition, when DgpB and DfgB were swapped in DgpB-C and DfgA-B, the expression of DgpC and DfgA was affected, even some deglycosylation activity of them was detected, which suggested that DgpB and DfgB have a chaperone function for forming the complex.

The mutation on metal coordinating residues (E141A, D173A, H263A and E299A) dramatically decreased the activity. Moreover, Y11A, Y303A variants also reduced the activity while Y11F, Y303F mutants still remained the comparable activity to wild type, suggesting that Y11 and Y303 are responsible for stabilizing and orienting substrate by π-π interactions (**Fig.2F**). H143A and K265A variants significantly decreased the activity, indicating their role as base to accept a proton in the reaction.

3. Proposed Reaction Mechansim

Based on the obtained protein strutures, we proposed a mechansim for DgpB-C catalyzing the 3''-oxo-peurarin to daidzein. 1) 3''-keto group of substrate binds to the metal ion in the active site;

2) Initially, 7- phenolic group is deprotonated by a basic residue, which facilitates enol-keto tautomerism of B ring; 3) Another basic residue abstracts 2''- proton which induces the following E1cb elimination to cleave the C-glycosidc bond and recover the aromaticity of B ring.

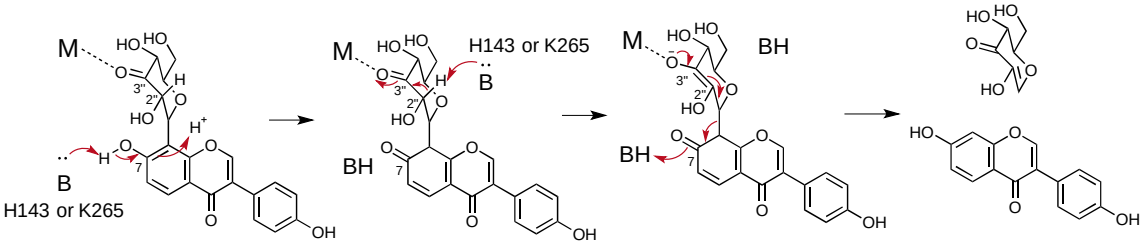
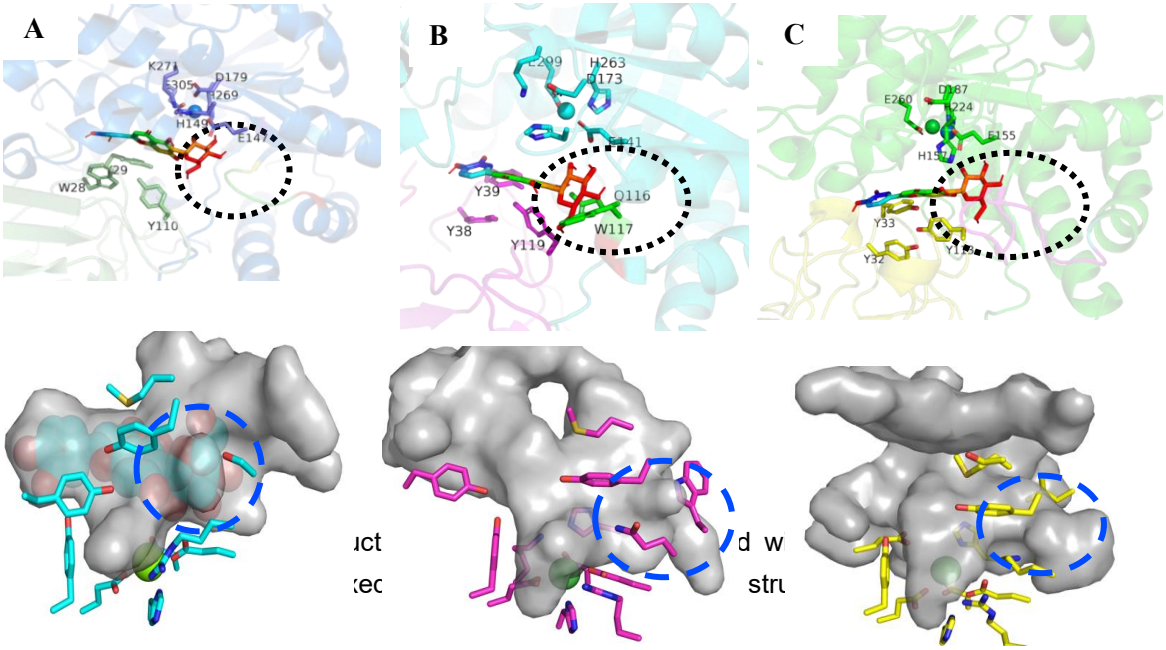


Fig. 3 Proposed reaction mechanism of *C*-deglycosylation reaction

4. Structural basis of different substrate specificity between DgpB-C and DfgA-B

Prof. Kobayashi Michihiko (Tsukuba University) solved a binary crystal structure of DgpB-C homologous enzyme H3 complexed with isoorientin (Fig. 4A). Differently, this homologous enzyme H3 is derived from soil bacteria (*Arthrobacter globiformis* NBRC12137), which can catalyze C-deglycosylation reaction of orientin. Based on this structure, I performed a structural modeling for DgpB-C and DfgA-B complexed with isoorietin (Fig. **4B** and **4C**). DgpB-C, DfgA-B and homologous H3 share similar heterodimer structures, and most of residues in the deep cavity are conserved. However, the sugar moiety of isoorietin is steric clashed by a short loop in H3 and helix structure in DgpB-C, but it is a long and flexible loop structure there in DfgA-B (**Fig.4** in dashed circle). In addition, the homologous enzyme H3 and DgpB-C possess more spacious active site which can accept substrate with wide size like 8-C-glycoside, but DfgA-B has a narrow active site to accept compounds with linear size like 6-C-glycoside. Therefore, the substrate specificity of DgpB-C and DfgA-B depends on the difference of substrate size (wide or linear) and shape of active site (spacious or narrow).



5. Summary

Cytochrome P450 (CYP450) enzymes in human body are necessary for the detoxification of foreign chemicals and the metabolism of drugs. Similar to cytochrome P450, the enzymes from

gut bacteria can accept broad C-glycosylated natural products and catalyze C-deglycosylation reaction, which thereby affects their health-promoting effects in the human host. To the best of my knowledge, this is the first report of structure-function study on enzymes derived from intestinal bacteria involved in *C*-deglycosylation reaction. Both of DgpB-C and DfgA-B are unique two-enzyme complex, which is not seen in reported sugar isomerase or β- galactosidase. DgpB-C homologous are widely distributed in not only intestinal bacteria but also other source bacteria, and they may share the similar function and catalytic mechanism to metabolize various glycosylated natural products.

Reference: [1] Braune, A., Blaut. M., *Gut Microbes*, **2016**, *7*, 216-234. [2] Nakamura K, *et al*., *Appl Environ Microbiol*., **2020** *in press*. DOI: 10.1128/AEM.00607-20. [3] Braune, A., *et al., Environ Microbiol*., **2016**, *18*, 2117-2129.