

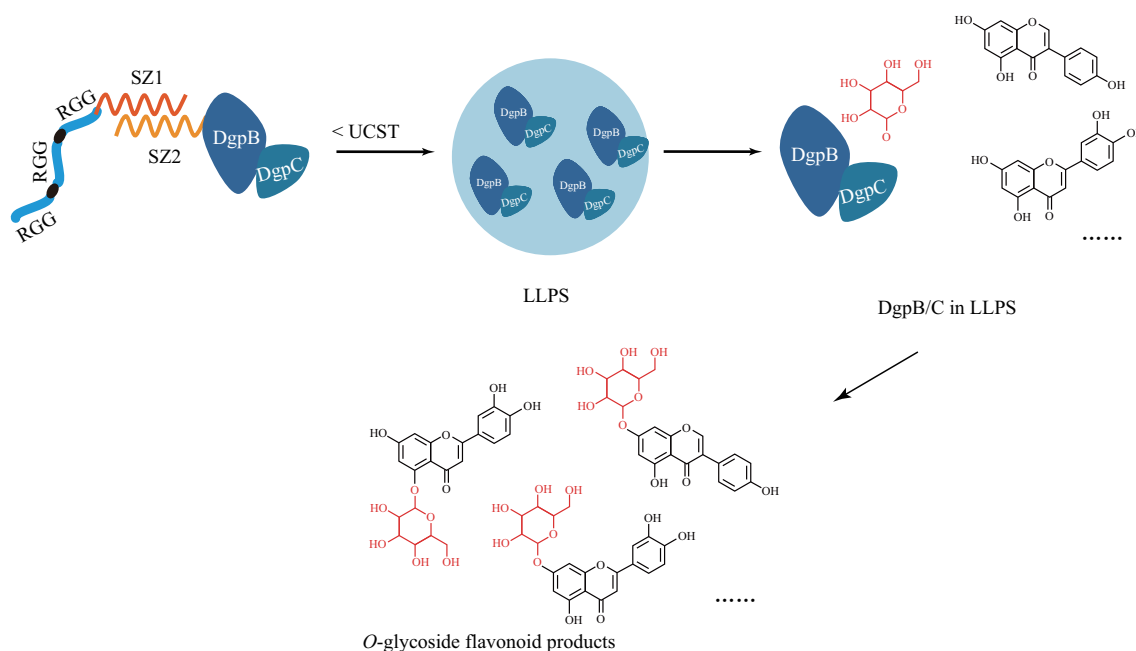


# Functional Remodeling of The DgpB/C Enzyme Into an *O*-Glycosyltransferase *via* Phase Transition\*

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## Graphical abstract



**Abstract Objective** Flavonoids are clinically significant natural products, yet their oxygen-glycosylation in aqueous environments relies heavily on expensive nucleotide-activated sugar donors such as UDP-glucose. Liquid-liquid phase separation (LLPS) creates specialized, membraneless physicochemical microenvironments capable of modulating enzymatic functions and overcoming mass transfer limitations. This study aims to investigate whether the gut microbiota-derived DgpB/C complex—a multienzyme system traditionally recognized for cleaving stable *C*-glycosidic bonds and facilitating isomerization—can undergo functional remodeling within phase-separated condensates. Our core objective is to elucidate the role of phase separation in expanding enzymatic catalytic plasticity and to provide a non-canonical, highly cost-effective biocatalytic mechanism for the direct utilization of free sugars in the synthesis of *O*-glycosylated natural products. **Methods** An artificial phase-separation platform was constructed utilizing the multivalent arginine-glycine-glycine motif (RGG)-repeat domain derived from the *Caenorhabditis elegans* LAF-1 protein. To ensure precise spatial compartmentalization, the DgpB/C complex was specifically recruited into the RGG condensates *via* a high-affinity SZ1/SZ2 heterodimerization tag system. Condensate formation and substrate partitioning were visualized using light and confocal fluorescence microscopy. The chemical structures and regioselectivity of the reaction products were rigorously characterized using high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry

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(LC-MS)/MS systems. Furthermore, molecular docking and 20-ns molecular dynamics (MD) simulations were performed *via* the Hermite platform and Uni-GBSA pipeline to elucidate the structural and thermodynamic basis underlying the phase-transition-induced functional shift. **Results** We observed that the condensates formed by RGG proteins selectively recruited and significantly enriched hydrophobic flavonoid substrates. Strikingly, within the LLPS microenvironment, the DgpB/C complex—which typically exhibits only degradative or isomerase activities—underwent a profound functional remodeling, transforming into an efficient *O*-glycosyltransferase. Diverging from canonical pathways that require high-energy donors, the sequestered DgpB/C complex directly utilized unactivated free glucose to catalyze the formation of new *O*-glycosidic bonds. This remodeled activity was found to be evolutionarily conserved across DgpB/C homologs from diverse gut microbiota strains, such as P581a and W974-1. LC-MS/MS analysis further revealed that the phase-separated environment reduced the regioselectivity constraints of the enzyme, enabling catalytic action on multiple hydroxyl positions of the flavonoid scaffold. MD simulations further indicated that the low-water-activity microenvironment of the condensates reconfigured the conformational dynamics of the catalytic pocket, favoring a spatial orientation highly conducive to dehydration condensation. **Conclusion** This study demonstrates that LLPS drives the functional remodeling of the gut microbiota enzyme DgpB/C through the reconfiguration of the physicochemical microenvironment. These findings challenge traditional perceptions regarding the functional boundaries of metabolic enzymes and reveal a novel biocatalytic pathway that bypasses the requirement for nucleotide-activated sugars. Consequently, this provides a highly promising artificial compartmentalization strategy for the green manufacturing of complex, high-value-added natural products.

**Key words** liquid-liquid phase separation (LLPS), DgpB/C complex, *O*-glycosyltransferase, functional remodeling, gut microbiota

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Flavonoids, a prominent class of natural products ubiquitous in the plant kingdom and human diet, predominantly occur in glycosylated form<sup>[1-2]</sup>. Based on the linkage between the carbohydrate moiety and the flavonoid aglycone, these compounds are often classified into *O*- or *C*-glycosylflavonoids<sup>[2-4]</sup>. In *O*-glycosides, the sugar is tethered *via* an oxygen atom, typically from a hydroxyl group, whereas in *C*-glycosides, it is directly coupled to a carbon atom of the flavonoid scaffold<sup>[5]</sup>. To date, over 4 000 natural *O*-glycosylflavonoids have been identified, significantly outnumbering the approximately 600 known *C*-glycosylflavonoids<sup>[6]</sup>. This structural disparity is attributed, in part, to the inherent stability of the *C*-glycosidic bond, which exhibits greater chemical and enzymatic resistance to both formation and cleavage compared to its *O*-glycosidic counterpart<sup>[7-9]</sup>.

In biological systems, the formation of *O*- and *C*-glycosidic bonds is mediated by specialized enzymes termed *O*- and *C*-glycosyltransferases (GTs), respectively<sup>[10-11]</sup>. These enzymes utilize uridine diphosphate (UDP)-activated sugars as glycosyl donors. During catalysis, the glycosyltransferase facilitates the nucleophilic attack of the aglycone acceptor on the activated sugar moiety, resulting in the transfer of the carbohydrate and the concomitant establishment of the specific glycosidic linkage. While the deglycosylation of *O*-glycosides is efficiently catalyzed by glycoside hydrolases (GHs) *via* inverting or retaining mechanisms, the cleavage of *C*-glycosidic bonds is significantly more rare<sup>[8-9]</sup>. To

date, this activity has been primarily identified in specific human microbiota, such as *Eubacterium cellulosolvens* and PUE strains, which harbor the *dfg* or *dgp* gene clusters, respectively<sup>[12-14]</sup>. The encoded enzymes (DfgA-E or DgpA-C) are uniquely capable of cleaving the robust *C*-glycosidic bonds found in substrates such as puerarin (daidzein-8-*C*- $\beta$ -D-glucoside) and isovitexin (apigenin-8-*C*- $\beta$ -D-glucoside)<sup>[12-13]</sup>. In the enzymatic cleavage of *C*-glycosidic bonds by the DgpABC system, DgpA functions as a glucose oxidoreductase, while DgpB and DgpC constitute a Mn<sup>2+</sup>-dependent enzyme complex<sup>[7-8, 15]</sup>. The proposed catalytic pathway is initiated by the DgpA-mediated oxidation of the 3'-hydroxyl group of the glucose moiety<sup>[16]</sup>. This oxidative step activates the substrate for subsequent cleavage by the DgpB/C complex, which facilitates the removal of the glucosyl group<sup>[13]</sup>. Notably, DgpA appears to catalyze the final reduction of the oxidized glucose intermediate, suggesting a redox-neutral catalytic cycle. Beyond its canonical *C*-glycoside cleavage role, our recent findings demonstrated that this system catalyzes the isomerization of *O*-glycosides to their *C*-glycoside counterparts, representing a novel *in vitro* biosynthetic activity for *C*-glycosidic bond formation.

In recent years, liquid-liquid phase separation (LLPS) has emerged as an important theme in cell biology, particularly facilitating the assembly of membraneless organelles<sup>[17]</sup>. Also, these condensates sequester signaling molecules and enzymes, creating specialized microenvironments that enhance signal

transduction and catalytic efficiency beyond the levels achievable in the bulk cytosol<sup>[18-19]</sup>. For example, the catalytic activity of *Geobacillus thermocatenulatus* lipase 2 (BTL2) is approximately doubled within phase-separated condensates compared to bulk solution conditions<sup>[20]</sup>. Moreover, in multienzyme assemblies such as the purinosome, the recruitment of enzymes into these condensates significantly enhances the efficiency of purine biosynthesis<sup>[21]</sup>. However, while the role of LLPS in modulating catalytic potency is well-documented, its ability to reprogram enzymatic reaction specificity remains largely unexplored. Here, we demonstrated that natural product flavonoids preferentially partition into LLPS droplets formed by arginine-glycine-glycine (RGG) motif repeat fragments. Notably, we showed that within these condensates, the enzyme complex DgpB/C underwent a functional shift: while it acts as a C-glycoside-cleaving enzyme, it functions as an O-glycosyltransferase within the LLPS environment, utilizing free, unactivated glucose as a sugar donor. This finding underscored the capacity of LLPS to fundamentally reprogram enzymatic properties.

## 1 Materials and methods

### 1.1 Materials and reagents

The compounds used in this study were obtained from commercial sources. Daidzein (Cat. No. B20227), daidzin (Cat. No. B20266), puerarin (Cat. No. B20466), genistein (Cat. No. B21039), genistin (Cat. No. B21038), luteolin (Cat. No. B20888), cynaroside (Cat. No. B20887), and D(+)-glucose (Cat. No. B21882), all with a purity of  $\geq 98\%$  were obtained from Shanghai Yuanye BioTechnology Co. Ltd. (Shanghai, China). Anhydrous manganese chloride (Cat. No. S31112) with a purity of  $\geq 99\%$  was also obtained from Shanghai Yuanye.  $\beta$ -Nicotinamide-adenine dinucleotide (NAD<sup>+</sup>) (Cat. No. 0455), Isopropyl  $\beta$ -D-thiogalactoside (IPTG) (Cat. No. 0487) with a purity of  $\geq 99\%$  were from Beijing LABLEAD Biotechnology (Beijing, China). Acetonitrile (Cat. No. A998-4) and methanol (Cat. No. A452-4) both chromatographic grade with purity  $\geq 99\%$  were obtained from Thermo Fisher (Waltham, USA).

### 1.2 Recombinant plasmid construction

The DNA fragments encoding the RGG domains were synthesized by GENEWIZ (Suzhou, China) following codon optimization for expression in *E. coli*. The gene sequences for the DgpB/C variants were amplified *via* PCR from their respective genomic DNA templates: the DgpB/C complex was cloned from the PUE strain<sup>[12]</sup>, while the DgpB1/C1

and DgpB2/C2 variants were sourced from the W974-1 and P581a strains<sup>[22-23]</sup>, respectively. For the RGG domains, each PCR product was cloned into the linearized pET-24a vector containing a C-terminal 6 $\times$  His tag. For the Dgp proteins, the fragments were cloned into the linearized pET-28a vector containing N-terminal 6 $\times$ His and SUMO tags. All plasmid constructs were verified by Sanger sequencing (GENEWIZ, China).

### 1.3 Protein expression and purification

Purification of the RGG domain: the constructed plasmids for the RGG domain were transformed into *E. coli* BL21 (DE3) cells. The bacteria were cultured in Terrific Broth (TB) medium at 37°C until reaching an  $A_{600}$  of 0.6–0.8. The temperature was then lowered to 18°C, and protein expression was induced with 0.5 mmol/L IPTG, followed by 20 h expression while shaking at 180 r/min. The cells were harvested by centrifugation at 4 000g for 30 min. The pellet was resuspended in lysis buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication on ice and the lysate was clarified by centrifugation at 15 000g for 30 min. The supernatant was discarded, and the pellet was resuspended in urea buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl, 8 mol/L urea). After the second round of centrifugation, the supernatant was applied to a HiTrap His affinity column (Cytiva, USA). After washing with buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl, 8 mol/L urea) and buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl, 8 mol/L urea, 20 mmol/L imidazole), the bound proteins were eluted with buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl, 8 mol/L urea, 0.5 mol/L imidazole). Proteins were dialyzed overnight against PBS buffer (10 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 150 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) at 55°C, which exceeds their upper critical solution temperature (UCST).

The plasmids encoding DgpA/B/C were transformed into *E. coli* BL21 (DE3) cells. The bacteria were cultured in Luria Bertani (LB) medium at 37°C until reaching an  $A_{600}$  of 0.6–0.8. The temperature was then lowered to 16°C, and protein expression was induced with 0.2 mmol/L IPTG, followed by 16 h expression while shaking at 180 r/min. The cells were harvested by centrifugation at 4 000g for 30 min. The pellet was resuspended in lysis buffer (50 mmol/L Tris-HCl pH 8.0, 0.5 mol/L NaCl) containing 1 mmol/L PMSF. The cells were lysed by sonication on ice and the lysate clarified by centrifugation at 15 000g for 30 min. The supernatant

was applied to a HiTrap His affinity column. After washing with buffer (50 mmol/L Tris-HCl pH 8.0, 0.5 mol/L NaCl, 20 mmol/L imidazole) to remove impurities, bound proteins were eluted using a gradient of imidazole from 20 to 500 mmol/L in the buffer containing 50 mmol/L Tris-HCl pH 8.0, and 0.5 mol/L NaCl. The eluted proteins were dialyzed overnight into the buffer (50 mmol/L Tris-HCl pH 8.5, 0.1 mol/L NaCl) at 4°C. For proteins with a 6×His-Sumo tag, the tag was cleaved by ULP1 protease during dialysis. The dialyzed sample was applied to a Hitrap Q anion exchange column (Cytiva, USA) and proteins were eluted using a NaCl gradient from 50 to 500 mmol/L in the buffer (50 mmol/L Tris-HCl pH 8.5). All the purified proteins were flash frozen into liquid nitrogen and stored at -80°C in aliquots until use in activity assays.

#### 1.4 Imaging experiments

The RGG proteins were thawed at 55°C for 20 min (a temperature exceeding their UCST) to achieve complete dissolution, and subsequently mixed with DgpB/C in PBS buffer, yielding a final concentration of 5 µmol/L for all proteins. The capacity of the phase-separated droplets to enrich small molecules was evaluated by incorporating 0.01 mmol/L fluorescein isothiocyanate (FITC) or 0.2 mmol/L carminic acid into the mixtures. Phase separation was induced by rapidly cooling the thoroughly mixed samples from 55°C to 37°C, followed by a 20-min incubation at 37°C. All subsequent imaging was conducted at 37°C. Condensate enrichment of carminic acid and FITC was captured using a Nikon stereo microscope. High-resolution analysis of DgpB/C enrichment was performed utilizing an Olympus confocal microscope (100× oil immersion objective) with a 561 nm laser for RFP excitation.

#### 1.5 Sedimentation and enrichment assay

The 3×RGG protein was thawed at 55°C for 20 min to achieve complete dissolution, and subsequently mixed with daidzein at 55°C in PBS buffer, yielding a final daidzein concentration of 0.2 mmol/L. Phase separation was induced by incubating the thoroughly mixed samples at 37°C for 20 min, followed by centrifugation at 500g for 20 min to separate the condensate pellet from the soluble supernatant. The supernatant was carefully removed, and the pellet was gently washed with an equal volume of identical pre-chilled PBS. After the second centrifugation at 500g for 10 min, the wash supernatant was discarded. A parallel control experiment—subjecting daidzein alone to identical incubation, centrifugation, and washing procedures in

the absence of the 3×RGG protein—was conducted to rule out spontaneous small-molecule precipitation. Ultimately, the washed pellets from both groups were completely dissolved in a urea buffer (50 mmol/L Tris-HCl pH 8.0, 1 mol/L NaCl, 8 mol/L urea) to disrupt the condensates, and the extracted daidzein was analyzed *via* high-performance liquid chromatography (HPLC).

#### 1.6 HPLC analysis

The C-glycoside cleavage and O- to C-glycoside isomerization reactions were performed in a 500 µl 1× PBS (pH 7.4) system. The reaction mixture contained 0.1 mmol/L MnCl<sub>2</sub>, 0.1 mmol/L NAD<sup>+</sup>, 0.1 mmol/L substrate (daidzin or puerarin), and 1 µmol/L DgpA/B/C from the PUE strain. Reactions were incubated at 37°C, with aliquots collected for product analysis at 0, 2, 4, 8, 12, and 24 h. Each reaction was terminated by adding three volumes of methanol.

The O-glycoside synthesis reactions were conducted in a 500 µl 1×PBS (pH 7.4) system containing 0.1 mmol/L MnCl<sub>2</sub>, 0.4 mmol/L substrate (daidzein, genistein, or luteolin), and 1 mmol/L glucose. These reactions utilized 5 µmol/L SZ1-3× RGG protein alongside various enzyme combinations (DgpB/C from PUE strain, DgpB1/C1 from W974-1, DgpB2/C2 from W974-1, or DgpB1/C1 from P581a). The mixtures were incubated at 37 °C and the aliquots collected for product analysis at 0, 12, 24, 52, 72, and 96 h, after which the reactions were terminated by adding three volumes of methanol.

The reaction mixtures were centrifuged at 16 000 g for 20 min at 4°C to remove precipitated proteins. The resulting supernatant was filtered through a 0.22 µm PVDF syringe filter prior to analysis. Reverse-phase HPLC (RP-HPLC) was performed using a Shimadzu LC-20AT/ADXR system equipped with an SPD-M20A diode array detector and an Innoval C18 column (250 mm×4.6 mm, 5 µm) maintained at 30°C. The mobile phase consisted of acetonitrile (solvent A) and 0.1% glacial acetic acid in water (solvent B), delivered at a flow rate of 1 ml/min using the following gradient: 0–5 min, 5% A; 5–20 min, 5%–60% A; 20–25 min, 60%–100% A; 25–27 min, 100% A; 27–29 min, 100%–5% A; 29–31 min, 5% A.

An injection volume of 10 µl was used, with detection performed at 254 nm and full-spectrum scanning from 200–400 nm. Data acquisition and processing were managed *via* LabSolutions software. Products were identified by comparing retention times and UV spectra against reference standards and quantified using compound-specific calibration curves. All assays were executed as three independent experiments.

### 1.7 Liquid chromatography–mass spectrometry (LC–MS) /MS

The reaction was terminated by adding three volumes of methanol to 100  $\mu\text{L}$  of the sample. After centrifugation at 12 000g for 20 min, the supernatant was collected for analysis. LC-MS/MS analysis was carried out using a Waters Micromass Q TOF instrument (Waters Corporation, USA) equipped with a Shimadzu UPLC C18 column (2.1 mm $\times$ 100 mm, 2  $\mu\text{m}$  particle size). An injection volume of 5  $\mu\text{L}$  was used, and the elution procedure followed the above established protocol in 1.6. The structural identification of the target compounds *via* MS/MS was confirmed by comparing the results with reference standards analyzed under identical experimental conditions.

### 1.8 Enzymatic assays

The SZ1-3 $\times$ RGG protein was initially thawed at 55°C for 20 min to achieve complete dissolution. Subsequent serial dilutions were performed in 1 $\times$  PBS maintained at 55°C. Each 500  $\mu\text{L}$  reaction mixture comprised 1 $\times$ PBS (pH 7.4), 0.1 mmol/L  $\text{MnCl}_2$ , 0.4 mmol/L genistein, 1 mmol/L glucose, and a constant DgpBC concentration of 5  $\mu\text{mol/L}$ . The molar ratio of SZ1-3 $\times$ RGG to DgpBC was sequentially decreased from 1 : 1 to 1 : 2, 1 : 4, and 1 : 8, corresponding to final SZ1-3 $\times$ RGG concentrations of 5, 2.5, 1.25, and 0.625  $\mu\text{mol/L}$ , respectively. The resulting mixtures were incubated at 37°C, which induced the formation of condensates and initiated the catalytic process. Reaction aliquots were collected at specified time intervals (0, 12, 24, 52, 72, and 96 h), and the reactions were instantly terminated by the addition of three volumes of methanol. Quantification of the generated *O*-glycosides was subsequently performed *via* HPLC following the chromatographic protocols described above. All assays were executed as three independent experiments.

### 1.9 Computational modeling of substrate–enzyme complexes

The structure of the DgpB/C protein was preprocessed *via* the Hermite protein preparation module (<https://hermite.dp.tech/>) to refine the hydrogen-bonding networks and perform energy minimization with restrained heavy atoms. The preparation of the ligands, glucose and genistein, was conducted utilizing the Hermite Ligand preparation module (<https://hermite.dp.tech/>). Subsequently, the processed ligands were docked into the prepared protein receptor employing the LeDock software. For the evaluation of the generated binding

conformations, MM/GBSA binding free energies were computed using Uni-GBSA, which served as the criteria for pose ranking. The ultimate complex structures were determined by selecting conformations that exhibited optimal MM/GBSA scores, alongside ensuring the specific orientation of the glucose moiety toward the manganese ion. To verify the binding stability of each ligand within the protein pocket, three independent 20-ns molecular dynamics (MD) simulations were executed for each chosen conformation utilizing the automated MD pipeline of Uni-GBSA. The positional stability of the ligands was evaluated by computing the root-mean-square deviation (RMSD) across the simulation trajectories.

## 2 Results and discussion

### 2.1 Recruitment and partitioning of flavonoid into LLPS formed by RGG protein

The RGG domain of LAF-1 is a well-characterized driver of LLPS, facilitating the assembly of condensed membraneless organelles<sup>[24]</sup>. This phase transition behavior is governed by the domain's primary sequence, which features positively charged RGG repeats interspersed with aromatic hydrophobic residues, specifically tyrosine (Y) and phenylalanine (F). These constituents promote a distributed network of weak, multivalent interactions, including cation- $\pi$  and  $\pi$ - $\pi$  stacking, which stabilize the condensed phase<sup>[25]</sup>. The reported upper critical solution temperature (UCST) for the 3 $\times$ RGG system is approximately 50°C. This high UCST ensures that it is possible to keep in a phase-separated state at standard assay temperatures (*e. g.*, 37°C or room temperature), making it a suitable model for investigating how LLPS modulates enzymatic activity<sup>[24]</sup>. Then, we first investigated whether the distinct physicochemical environment of RGG-mediated condensates could selectively recruit amphiphilic, flavonoid-like molecules. Using purified recombinant 3 $\times$ RGG protein, LLPS was robustly observed at temperatures (Figure 1a,b)<sup>[24]</sup>. We initially assessed the partitioning of carminic acid—an anthraquinone derivative common in the food, cosmetic, and pharmaceutical industries—and observed significant enrichment within the RGG-mediated droplets (Figure 1c)<sup>[26]</sup>. To validate this recruitment, FITC was introduced; in alignment with the carminic acid results, FITC exhibited high levels of sequestration within the condensed phase (Figure 1c,d).

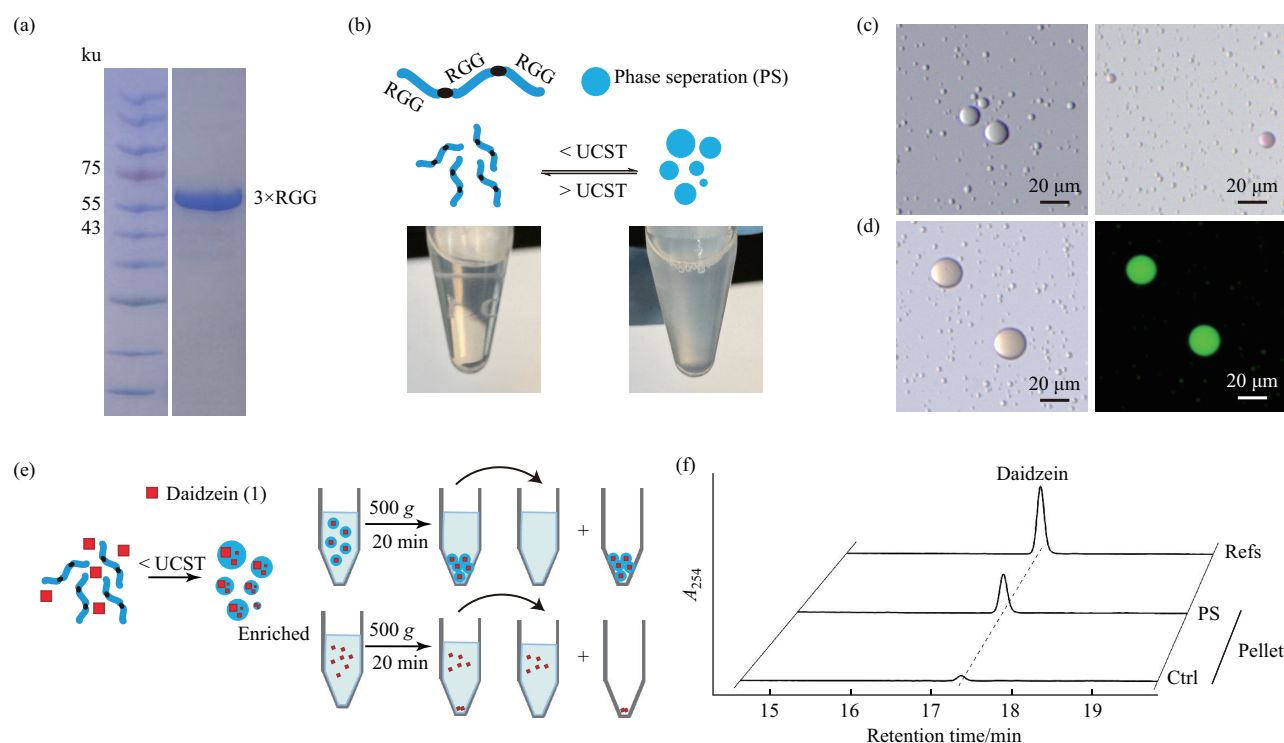


**Fig. 1 Recruitment and partitioning of hydrophobic natural compounds into LAF-1 RGG condensates**

(a) SDS-PAGE analysis of purified recombinant 3×RGG protein visualized by Coomassie Brilliant Blue staining. (b) The formation of LLPS droplets mediated by the 3×RGG protein. (c, d) Representative differential interference contrast (DIC) and fluorescence microscopy images demonstrating the selective recruitment of carminic acid (c) and FITC (d) into RGG-mediated condensates. (e, f) Quantitative analysis of daidzein partitioning into the condensed phase. (e) Schematic of the droplet isolation. (f) HPLC chromatograms showing the enrichment of daidzein in the LLPS fraction compared to the bulk aqueous phase.

To quantify these findings, the flavonoid aglycone daidzein (1) was introduced into the LLPS system. The resulting droplets were isolated from the bulk aqueous phase *via* centrifugation (500g for 20 min), and the concentration of daidzein within the condensed phase was determined using HPLC (Figure 1e). As shown in Figure 1f, daidzein was significantly enriched in the LLPS fraction compared to the control, consistent with the preferential partitioning observed for carminic acid and FITC (Figure 1c, d).

## 2.2 LLPS-induced O-glycosyltransferase activity of the DgpB/C complex

While recent studies established that DgpA/B/C enzymes from the PUE strain catalyze the cleavage of the C-glycosidic bond in daidzein-8-C-glucoside (puerarin (1b)) (Figure S1a), our previously published work demonstrated that these enzymes also possess the capacity to isomerize the O-glycoside daidzein-7-O-glucoside (1a) to puerarin as a novel type of C-glycoside isomerase (Figure S1b) [13, 16, 27]. This functional versatility suggests either broad substrate promiscuity or distinct catalytic mechanisms for

processing varying glycosidic linkages. To facilitate the recruitment of these enzymes into the LLPS environment, we utilized a high-affinity SZ1/SZ2 heterodimerization tag system. The DgpB/C complex was efficiently sequestered into RGG-mediated droplets *via* the SZ1 tag (Figure 2a, b) [24]. However, N-terminal SZ2-tagging of DgpA resulted in a complete loss of enzymatic activity, suggesting that either the tag itself or the physical environment of the condensate quenched the DgpA-catalyzed redox reactions (Figure S2a, b). To circumvent this, we alternatively introduced the SZ2 tag at the N-terminus of DgpB. Under these conditions, the efficiency of DgpA/B/C-catalyzed cleavage of the puerarin within the LLPS phase was comparable to that of the bulk solution control (Figure S2a, c). Furthermore, the O- to C-glycoside isomerization rate remained unaltered relative to the control for the SZ2 tag at N-terminus of DgpB, but not DgpA (Figure S2d-h). Collectively, these findings indicate that the DgpB/C complex maintains its catalytic competency when partitioned into the LLPS environment.

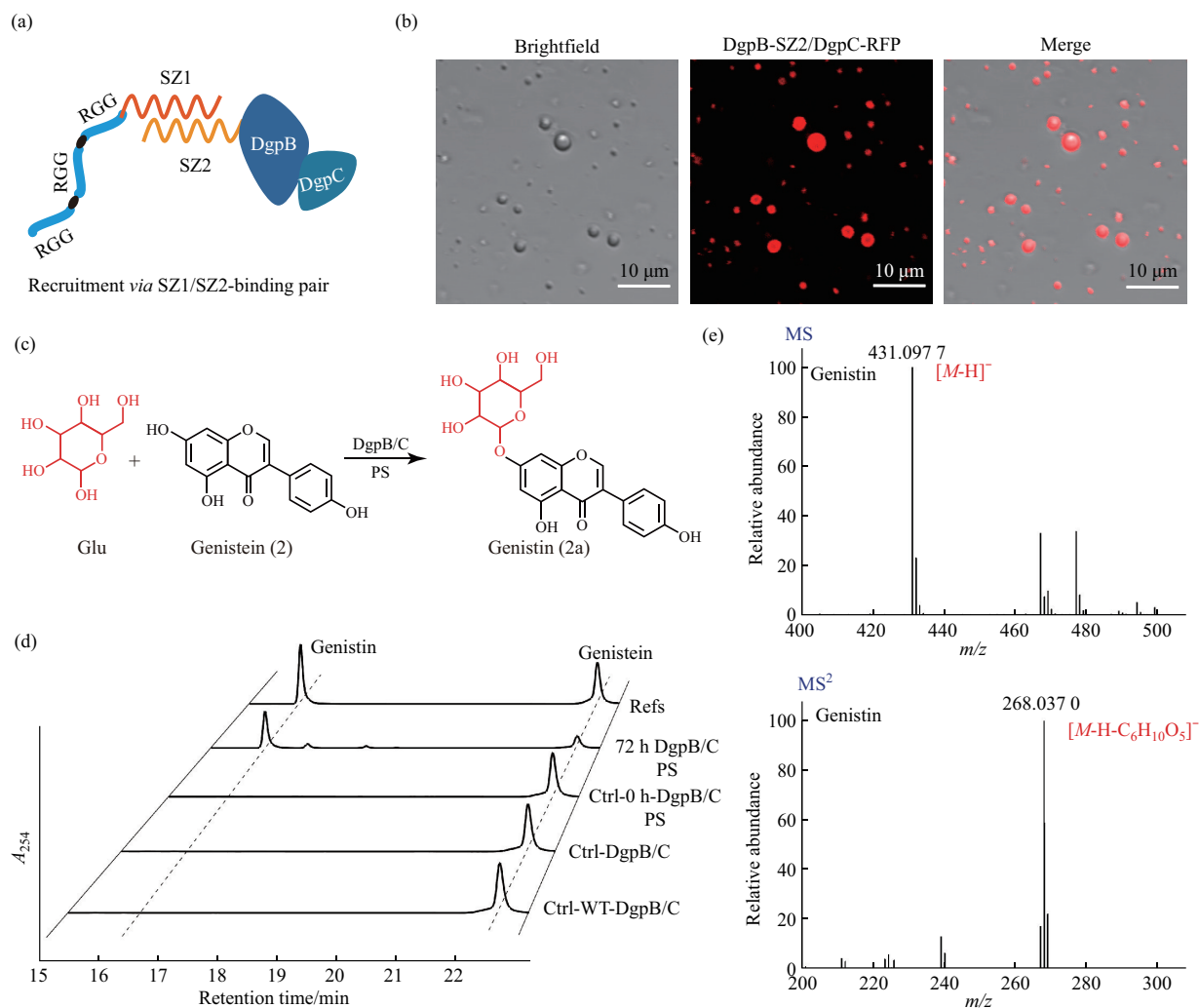


**Fig. 2 Unexpected *O*-glycosyltransferase activity of the DgpB/C complex within RGG-mediated condensates**

(a) Schematic representation of the DgpB/C complex recruitment into 3×RGG droplets utilizing the SZ1/SZ2 heterodimerization system. (b) Representative DIC and fluorescence microscopy images demonstrating the sequestration of the DgpB/C complex (DgpC-RFP) into LLPS droplets. (c) Reaction scheme showing the synthesis of *O*-glycoside genistin (2a) from glucose and genistein (2) catalyzed by the DgpB/C complex within the LLPS environment. (d) HPLC chromatograms monitoring the formation of *O*-glycoside genistin (2a). The analysis includes the wild-type DgpB/C (Ctrl-WT-DgpB/C), and the engineered DgpB/C system (Ctrl-DgpB/C) as controls, compared with the reaction profile in the phase separation (PS) system after 72 h. (e) Structural characterization of the reaction product *via* LC-MS/MS analysis, confirming the identity of the synthesized *O*-glycoside.

Intriguingly, our investigation of DgpB/C activity within the LLPS environment revealed that the DgpB/C complex possesses the unexpected capacity to directly synthesize *O*-glycosidic bonds—a catalytic function observed exclusively within these condensates and not in the bulk solution control. Specifically, the complex utilizes glucose as a glycosyl donor and the aglycone genistein (2) as a glycosyl acceptor (Figure 2c–e). The *O*-glycosyltransferase activity within the LLPS environment was unequivocally confirmed *via* LC-MS/MS analysis. The detected mass ( $m/z$  431.0977) matches the theoretical  $m/z$  value of compound

genistin (2a), revealing a previously unrecognized catalytic plasticity of the DgpB/C complex under condensed-phase conditions.

### 2.3 The conservation of the *O*-glycosyltransferase activity of the DgpB/C complex in LLPS

While glycosyltransferase-catalyzed reactions typically depend on nucleotide-sugar donors, our findings demonstrated that the DgpB/C complex within the LLPS utilizes native sugars as substrates. Genomic analysis reveals that the *dgp* gene cluster is widely distributed across more than 400 bacterial strains. To determine whether this unconventional catalytic activity is conserved and to assess its

substrate breadth, we first evaluated the activity of PUE DgpB/C complex using daidzein as the sugar acceptor. The clear synthesis of daidzin (1a) confirmed that the DgpB/C complex facilitates *O*-glycosidic bond formation using aglycone daidzein (1) as the sugar receptor (Figure 3a, b). To further validate the ubiquity of this mechanism, we characterized DgpB/C orthologs from two additional human gut microbiota strains, P581a and W974-1 (Figure S3) [22-23]. The

DgpB/C enzyme from P581a strain successfully synthesized cynaroside (3a) from aglycone luteolin (3), while the W974-1 ortholog similarly exhibited robust *O*-glycosylation activity (Figure 3c, d). Collectively, these results provide strong evidence that DgpB/C-mediated *O*-glycosidic bond synthesis within LLPS is a conserved functional trait across diverse bacterial species and aglycone substrates.

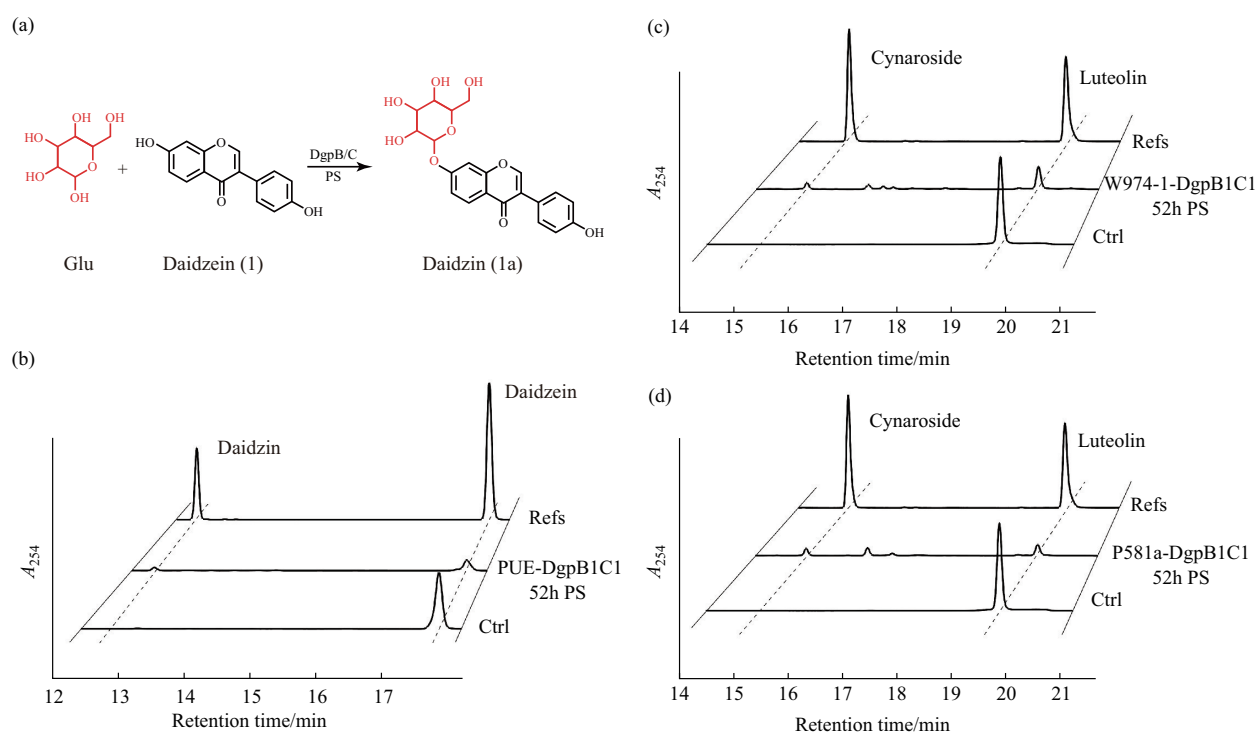


**Fig. 3 Conservation of DgpB/C-mediated *O*-glycoside synthesis within LLPS**

(a) Schematic representation of the DgpB/C complex catalyzing the synthesis of daidzin. (b) HPLC chromatograms showing the synthesis of daidzin mediated by the PUE DgpB/C complex. (c) HPLC analysis of cynaroside synthesis catalyzed by the DgpB/C homolog from strain W974-1. (d) HPLC analysis of cynaroside synthesis catalyzed by the DgpB/C homolog from strain P581a.

## 2.4 The *O*-glycosylation regioselectivity on flavonoids catalyzed by DgpB/C

To further confirm that the *O*-glycosidic bond synthesis catalyzed by the DgpB/C complex is dependent on RGG-mediated condensates, we systematically reduced the LLPS content within the reaction system. Our results demonstrated a corresponding decrease in the yield of genistin, indicating that the catalytic efficiency is directly correlated with the concentration of condensed phases (Figure 4a). These findings implied that DgpB/C-mediated *O*-glycoside synthesis is intrinsically coupled to the physiological environment provided by LLPS.

Furthermore, during the DgpB/C-catalyzed *O*-glycoside synthesis, we observed the formation of several minor peaks in addition to the primary product (Figure 4b, c). To characterize these secondary products, we performed LC-MS/MS analysis, which revealed an  $m/z$  of 447.093 7. This value is identical to that of cynaroside, suggesting that these minor peaks represent positional isomers of the main product. These results indicate that the *O*-glycosylation occurs at different hydroxyl groups of the luteolin aglycone, implying that the DgpB/C complex exhibits broad regioselectivity toward its aglycone substrates (Figure 4d-f).

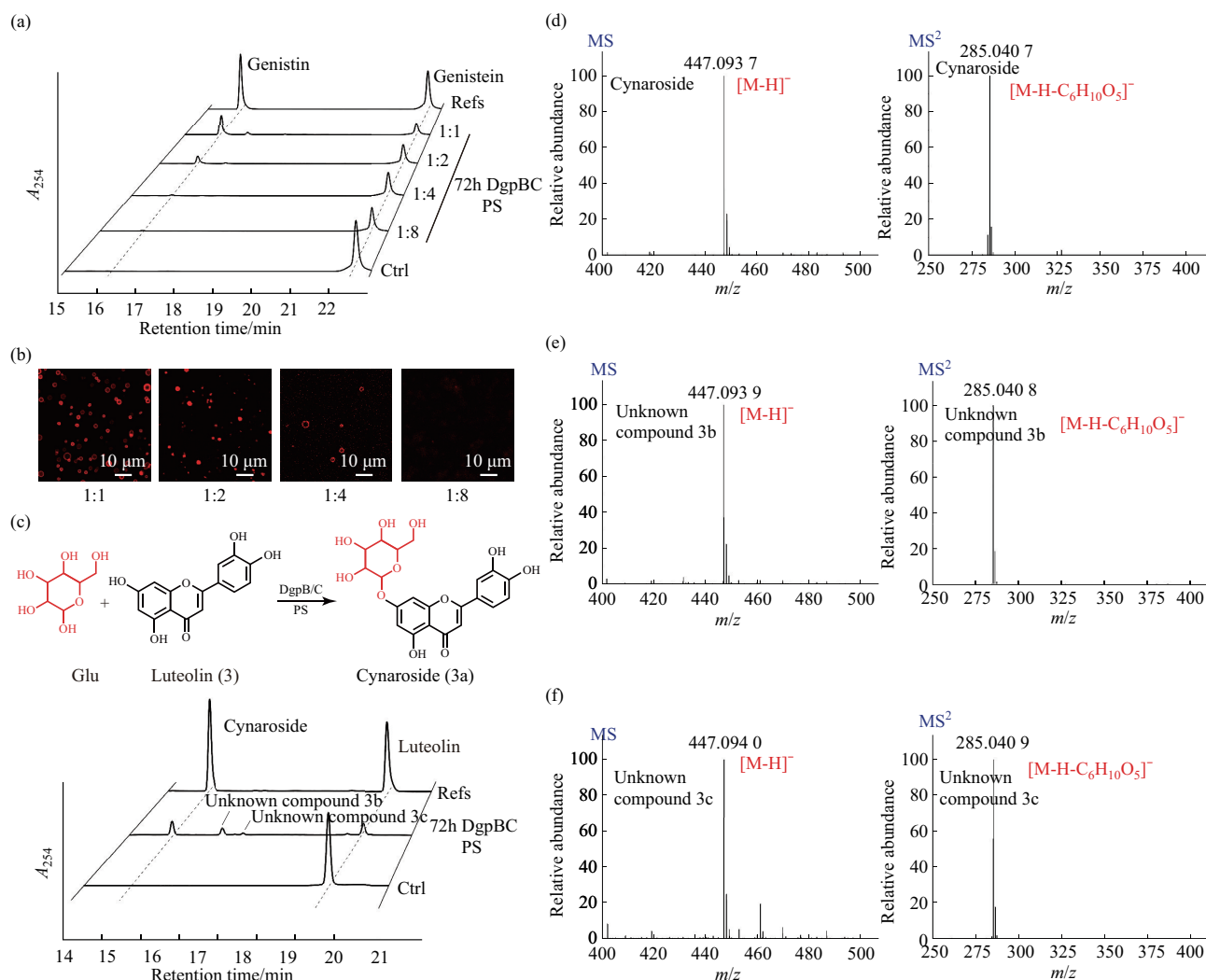


**Fig. 4** Flexible regioselective *O*-glycosylation by the DgpB/C enzyme toward aglycone substrates within LLPS condensates

(a) Quantitative analysis showing the decrease in *O*-glycoside genistin yield corresponding to the systematic reduction of LLPS components in the reaction medium. (b) Representative images of LLPS formation in (a). (c) Schematic representation of cynaroside synthesis catalyzed by the P581a DgpB/C complex and the HPLC chromatograms illustrating the formation of multiple products, including the primary product and secondary isomers. (d–f) Characterization of minor peaks via LC-MS/MS, confirming their identification as positional isomers of cynaroside.

## 2.5 The hypothesized mechanism model for DgpB/C-catalyzed *O*-glycosidic bond synthesis

The structure of the DgpB/C complex has been characterized by our group and others, revealing that DgpC functions as an isomerase-like domain, while DgpB adopts an IgG-like fold. The catalytic center resides within DgpC, whereas the substrate-recognition pocket is formed at the DgpB/C heterodimer interface<sup>[8, 27]</sup>. To elucidate the mechanism of DgpB/C-catalyzed *O*-glycosidic bond synthesis under LLPS conditions, we performed molecular docking of the DgpB/C complex with glucose and substrate genistein, followed by a 20 ns MD simulation (Figure 5a–c). Intriguingly, glucose

and substrate genistein occupied distinct positions within the binding pocket, resulting in a spatial orientation highly conducive to *O*-glycosylation at the hydroxyl groups of the B-ring of genistein.

These structural insights are consistent with our experimental observations, which showed that the sugar moiety could be linked to multiple hydroxyl positions on substrate genistein (Figure 4). Consequently, we hypothesized that the LLPS environment might reconfigure the activity of key catalytic residues, thereby enabling the non-canonical *O*-glycoside synthesis catalyzed by the DgpB/C complex (Figure 5d).

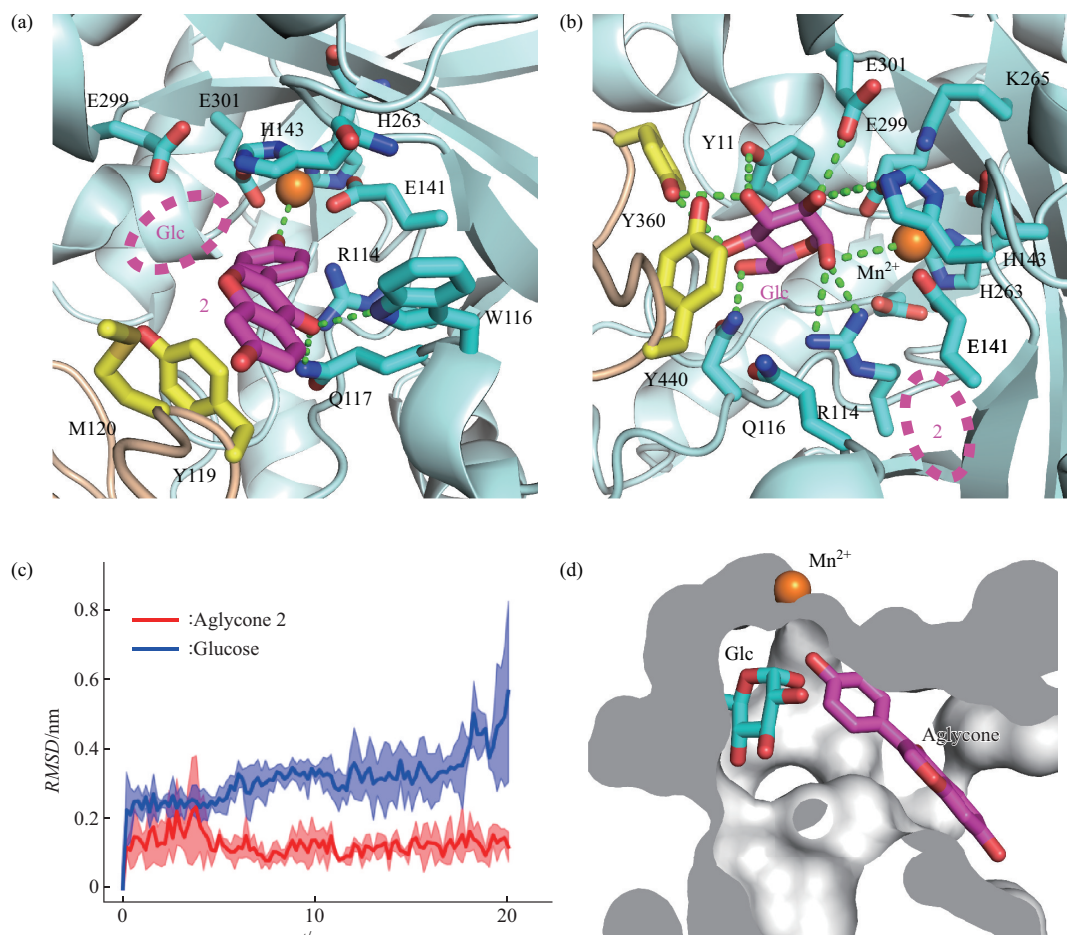


**Fig. 5 Proposed mechanism for LLPS-driven DgpB/C-mediated *O*-glycosidic bond synthesis based on molecular docking and dynamics simulations**

(a) Representative modeled structure of the DgpB/C complex (cartoon representation) in complex with glucose. (b) Modeled structure of the DgpB/C complex bound to flavonoid genistein. Key residues involved in substrate binding and catalysis are shown in stick representation: DgpC residues are colored cyan, DgpB residues are yellow, and flavonoid genistein is shown in purple. (c) Stability of the modeled complexes during 20 ns MD simulations. Data represent results from three independent replicates. (d) The proposed working model for LLPS-driven enzymatic remodeling, highlighting the possible *O*-glycosyltransferase activity of the DgpB/C.

### 3 Conclusion

In summary, this study revealed a remarkable functional shift in the DgpB/C complex driven by LLPS. We demonstrated that RGG-mediated condensates served as specialized microenvironments that selectively recruited and enriched hydrophobic flavonoid aglycones. Our results established that the sequestered DgpB/C complex functions as a non-canonical *O*-glycosyltransferase, utilizing free sugars rather than traditional nucleotide-sugar donors to catalyze *O*-glycosidic bond formation. This catalytic plasticity was intrinsically coupled to the phase-separated state; a reduction in LLPS components

leads to a proportional decrease in enzymatic yield. Furthermore, we confirmed that this mechanism is conserved across DgpB/C homologs from diverse human gut microbiota, including the P581a and W974-1 strains. Notably, DgpB/C in LLPS exhibits broad regioselectivity, facilitating *O*-glycosyl modification at multiple hydroxyl positions on the flavonoid scaffold. Finally, we proposed a structural model to elucidate the catalytic mechanism within these condensates. These findings underscored the critical role of the physical environment in modulating enzyme activity and offered a novel, biologically relevant pathway for the synthesis of *O*-glycosylated flavonoids.

**Supplementary** Available online (<http://www.pibb.ac.cn>, <http://www.cnki.net>):

PIBB\_20260054\_Figure\_S1.pdf

PIBB\_20260054\_Figure\_S2.pdf

PIBB\_20260054\_Figure\_S3.pdf

**Data Availability** The data related to this paper (DOI: 10.57760/sciencedb.33602/CSTR: 31253.11.sciencedb.33602) can be accessed from the Science Data Bank database (<https://www.scidb.cn/>).

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# DgpB/C通过相变实现O-糖基转移酶的功能重塑\*

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**摘要** **目的** 液液相分离创造了能够调控酶功能的特殊物理化学微环境。本研究旨在阐明相分离在拓展肠道微生物酶催化可塑性中的作用, 并为O-糖基化天然产物的合成提供新机制。**方法** 通过显微成像观察凝聚体形成及底物招募过程; 运用高效液相色谱法(HPLC)和液相色谱-质谱联用(LC-MS/MS)分析类黄酮富集、反应产物及区域选择性。**结果** 本研究表明, 富含精氨酸-甘氨酸-甘氨酸基序的相分离蛋白(RGG)介导的凝聚体能够选择性招募并富集疏水性类黄酮。更重要的是, 研究进一步发现, 通常以切割和异构化C-糖苷键著称的DgpB/C复合物, 在这些凝聚体中发生了功能重塑。与需要核苷酸糖供体的传统糖基转移酶不同, 被包裹的DgpB/C复合物在液相分离环境中利用天然糖直接催化O-糖苷键的合成。此外, 研究结果显示, 这种非常规的催化机制在不同的人类肠道微生物菌株(包括P581a和W974-1)中具有保守性, 并对多种类黄酮苷元表现出广泛的区域选择性。**结论** 这些发现凸显了相分离在扩展肠道微生物酶催化可塑性中的关键作用, 并为O-糖基化天然产物的合成提供了一种全新机制。

**关键词** 液液相分离, DgpB/C复合物, O-糖基转移酶, 功能重塑, 肠道菌群

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