Production of Flavonoid *O*-Glucoside Using Sucrose Synthase and Flavonoid *O*-Glucosyltransferase Fusion Protein

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Enzymatic glucosylation with glycosyltransferases can be used to regulate the water solubility of aglycone. The drawback of this process is the demand of UDP-glucose as a sugar donor. We made an in-frame fusion of the flavonoid *O*-glucosyltransferase *(OsUGT-3)* and sucrose synthase *(AtSUS)* genes. The resulting fusion protein, OsUGT3-AtSUS, was expressed in *E. coli* and purified. When sucrose and UDP were supplied, the fusion protein was able to convert quercetin into quercetin *O*-glucoside without the addition of UDP-glucose. In addition, UDP-glucose was recycled when sucrose was added to the reaction mixture. This fusion protein is useful for the enzymatic production of flavonoid *O*-glucosides.

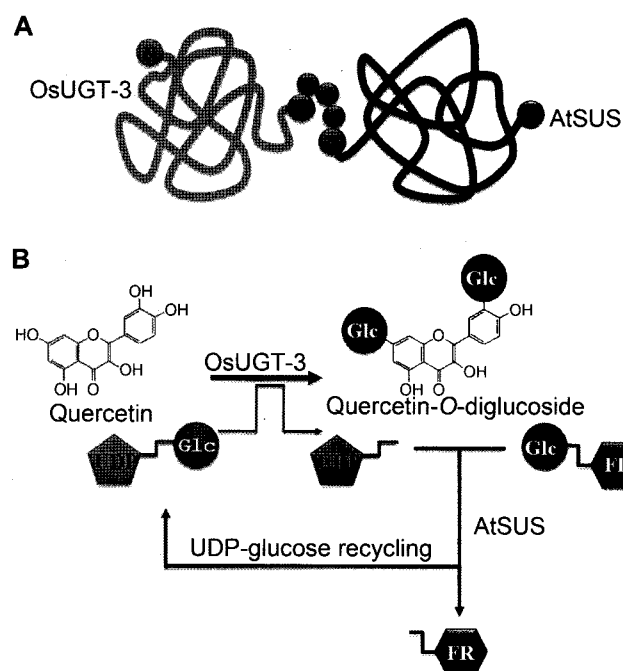**Keywords:** Flavonoid, glucosyltransferase, sucrose synthase, UDP-glucose

Flavonoids are typical plant secondary metabolites and are composed of a group of polyphenols. Over 10,000 natural flavonoids have been reported, and many of them are common in higher plants [14]. The structural diversity of secondary metabolites, including flavonoids, is due to modification reactions such as methylation, hydroxylation, acylation, glycosylation, and other reactions [15]. Among these modification reactions, glycosylation involves the attachment of a sugar unit to small compounds, which results in stabilization, enhancement of solubilization, and regulation of the biological activity of the resultant glycosides [7, 12]. Glycosyltransferases (GTs) transfer sugar to a wide range of acceptor molecules and have been classified into 78 families based on sequence similarity, according to CAZY (Carbohydrate active enzymes; http://afmb.cnrs-mrs.fr/CAZY/). Among these families, family 1 glycosyltransferases contain a UDP-glycosyltransferase (UGT) that is found in

plants, animals, fungi, and bacteria. UGTs transfer sugars from activated sugar donors, such as UDP-sugars, to hydrophobic small molecules including flavonoids, anthocyanins, terpenoids, plant hormones, alkaloids, and antibiotics [2, 3, 5].

The list of the biological impacts of flavonoids on human health is extensive [1, 6] and now flavonoids are considered to be a starting material in the development of new medicines or food additives [4]. Nevertheless, the low water solubility of flavonoids has been a drawback in their application as nutraceuticals. This problem could be resolved by attaching sugar molecules to hydroxyl groups using UGTs. However, UGTs require the expensive cofactor UDP-sugar from a sugar donor. In addition, the UDP released during enzymatic glycosylation usually acts as an inhibitor of UGTs [10]. An *E. coli* transformant expressing UGTs has been used as an alternative approach, because it does not require supplementation with UDP-sugars [8, 10, 12]. However, some flavonoids, such as quercetin, cause the lysis of *E. coli* cells, and the elimination of the cell lysate from the flavonoid glycones was problematic. The enzymatic synthesis of UDP-glucose has also been attempted using UDP-glucose pyrophosphorylase from UTP, and glucose 1-phosphate [13]. However, this method does not work with UGTs for the glycosylation of small compounds. Recently, sucrose synthase (SUS) has been suggested as a valuable tool for regenerating UDP-sugar [11]. SUS catalyzes the formation of UDP-glucose from sucrose and UDP. Thus, UDP generated by the reaction of UGTs can be converted into UDP-glucose in the presence of sucrose. Sucrose synthase from *Arabidopsis thaliana* (*AtSUS1*; GenBank Accession No. NM_001036838) was employed along with flavonoid glycosyltransferase (*OsUGT-3*; GenBank Accession No. CT830931) from *Oryza sativa* to construct a new artificial gene by the in-frame fusion of OsUGT-3 with AtSUS for efficient production of flavonoid glucoside. The *AtSUS1* open reading frame (ORF) was amplified with primers containing SmaI (5' end) and NotI (3' end) sites. The PCR product, after digestion with SmaI/NotI,
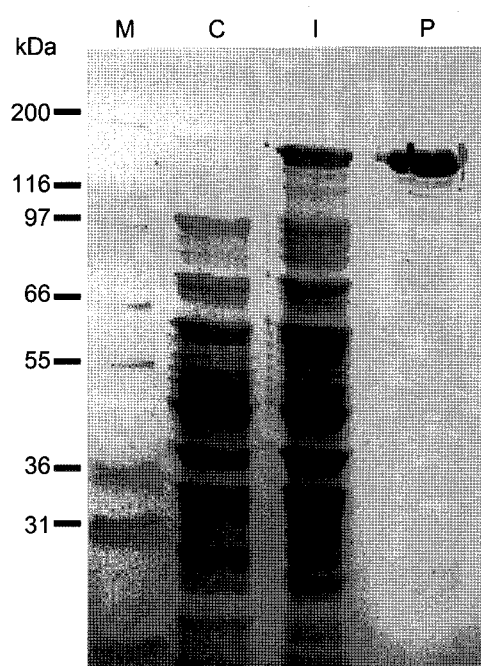
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A

OsUGT-3

AtSUS

B

OH
OH
HO
OH
OH O
Quercetin

OsUGT-3 →

Glc
Glc
OH
OH
OH O
Quercetin-O-diglucoside

Glc FR

UDP-glucose recycling   AtSUS

FR

Fig. 1. A. Construction of OsUGT3-AtSUS fusion protein linked by three proline residues. B. Glucosylation of quercetin using OsUGT3-AtSUS fusion protein with UDP-glucose recycling. Glc, glucose; FR, fructose.


Fig. 2. Expression and purification of the recombinant fusion protein OsUGT3-AtSUS.

was subcloned into the corresponding sites of the pGEX 5X-1 vector (Amersham, U.S.A.) and the resulting plasmid was named pAtSUS. The ORF of *OsUGT-3* was amplified with a forward primer containing an EcoRI site and a reverse primer (stop codon is deleted) containing CCACCACCA (Pro-Pro-Pro linker) and SmaI. The PCR products were digested with EcoRI and SmaI and subcloned into the corresponding sites of pAtSUS1 (Fig. 1). This construct was named pR3AtSUS1, and transformed into *E. coli* BL21 (DE3). The induction and the purification of the recombinant fusion protein were carried out as described in Ko *et al.* [9]. The expression of the fusion protein was verified using SDS–PAGE. The molecular mass of the fusion protein was approximately 172- kDa, which corresponds to the combined molecular mass of GST, OsUGT-3, and AtSUS (Fig. 2).

Sucrose synthase activity and flavonoid *O*-glycosyltransferase activity with the purified OsUGT3-AtSUS were measured to make sure both proteins were actively expressed. The reaction mixture for the sucrose synthase assay contained 10 μg of recombinant protein, 0.3 M sucrose, and 1 mM UDP in 100 mM Tris-HCl buffer (pH 7.5). After 1 h incubation at 37°C, the reaction was terminated by heating the reaction mixture in a boiling water bath for 2 min. The recombinant protein was precipitated by centrifugation for 15 min at 13,000 rpm. Analysis of the reaction mixture using high-performance liquid chromatography (HPLC) [11] revealed a new peak that had the same retention time as UDP-

glucose (data not shown). Flavonoid *O*-glucosyltransferase activity was tested with 10 μg of OsUGT3-AtSUS, 100 μM UDP-glucose, and 100 μM quercetin in 100 mM Tris-HCl buffer (pH 7.5). The production of quercetin *O*-mono- and diglucosides was observed by analyzing the reaction products resulting from the fusion protein combined with quercetin and UDP-glucose (data not shown). These results indicate

A

B
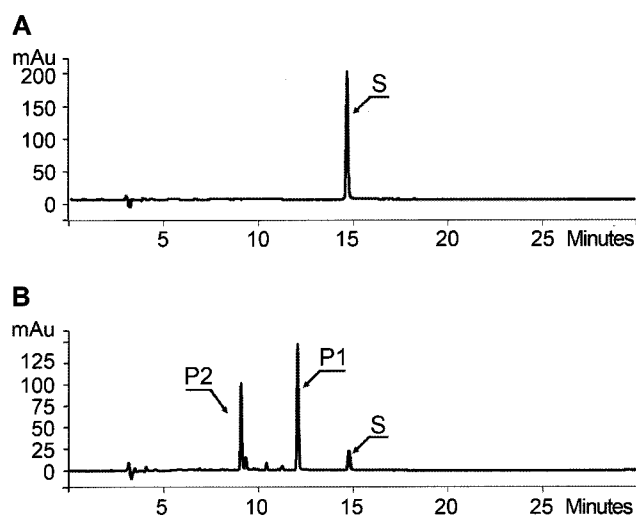
Fig. 3. Analysis of OsUGT3-AtSUS reaction product using HPLC.
The reaction mixture consisted of the purified OsUGT3-AtSUS, 100 μM quercetin, 1 mM UDP, and 0.3 M sucrose. Two reaction products (P1 and P2) were observed. S is the substrate (quercetin).

that both proteins of the fusion protein are functionally active.

Enzymatic synthesis of flavonoid *O*-glucoside without the addition of UDP-glucose was performed using OsUGT3-AtSUS. Initially, the reaction mixture included purified OsUGT3-AtSUS, 100 μM quercetin, 1 mM UDP, and 0.3 M sucrose. Analysis of the reaction product using HPLC revealed two new peaks. MS analysis of the reaction products showed that the molecular mass of the compound corresponding to P1 (Fig. 3A) was increased by 162- Da and that corresponding to P2 was increased by 324- Da, relative to that of quercetin. This indicates that P1 was quercetin *O*-mono- and P2 was *O*-diglucoside. The quercetin *O*-diglucoside was observed because OsUGT-3 is able to convert quercetin *O*-monoglucoside into *O*-diglucoside (unpublished results). These results indicate that AtSUS successfully converted sucrose into fructose and UDP-

glucose. Then, OsUGT-3 utilized UDP-glucose and quercetin to produce quercetin *O*-glucosides. However, when sucrose was omitted from the reaction mixture, no reaction product was observed. The reaction product was obtained when other flavonoids, kaempferol or luteolin, were used as the glucose donor (data not shown).

In order to test the recycling of UDP-glucose in this system, the same concentrations of quercetin and UDP-glucose were added without sucrose. The reaction product, quercetin *O*-monoglucoside, was produced until the UDP-glucose was used up, and lengthening the incubation time did not result in quercetin *O*-diglucoside production (Fig. 4A). However, when sucrose was added to this reaction mixture, quercetin was converted into quercetin *O*-monoglucoside, which was eventually converted into quercetin *O*-diglucoside (Fig. 4B). This result also indicates that the UDP produced by the reaction of OsUGT-3 was recycled by AtSUS in the presence of sucrose.

The price of UDP-glucose is a major obstacle for the enzymatic glucosylation of small molecules. However, this approach allows us to circumvent this obstacle. By varying the UGT that is fused to the SUS, we can extend the range of small molecules that can be glycosylated.

Glycosylation of small molecules with separately expressed UGT and SUS has been attempted [11] and was successful for the glucosylation of curcumin and apigenin. However, this approach involved the expression and purification of both proteins separately. Our approach utilizes a fusion of the two proteins and is therefore more convenient because it only requires one purification step.
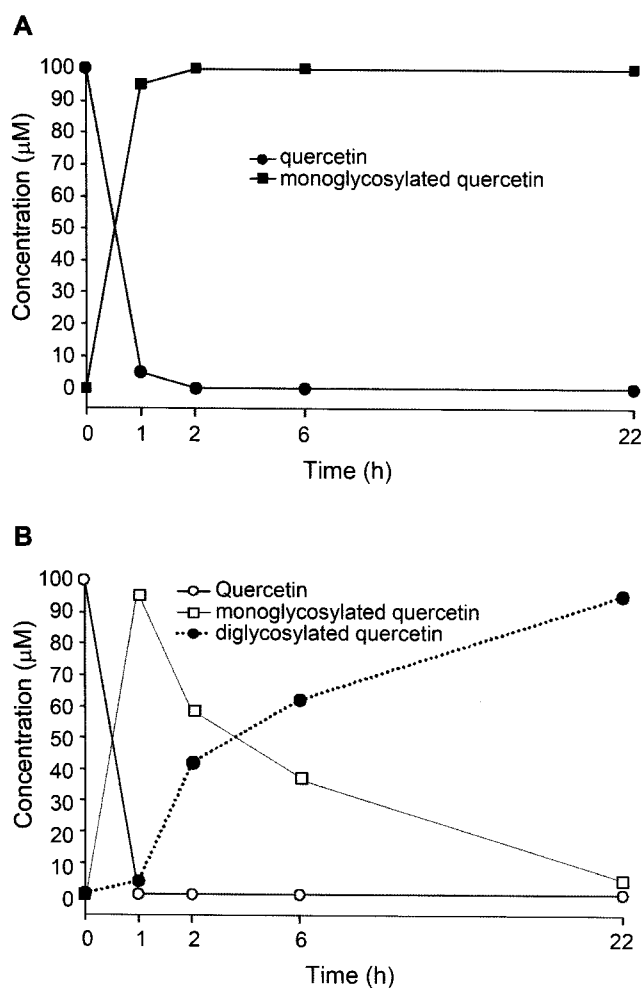
Fig. 4. Glucosylation of quercetin catalyzed by OsUGT3-AtSUS with (**B**) or without (**A**) sucrose.
The assay was performed at 37°C for 1, 2, 6, and 22 h in 100 mM Tris-HCl buffer (pH 7.5) containing 100 μM quercetin, 100 μM UDP-glucose, and purified OsUGT3-AtSUS with (**B**) or without (**A**) 0.3 M sucrose.

Acknowledgments

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