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An efficient chemoenzymatic production of small molecule glucosides with in situ UDP-glucose recycling

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Abstract A one-pot system for efficient enzymatic synthesis of curcumin glucosides is described. The method couples the activities of two recombinant enzymes, UDP-glucose: curcumin glucosyltransferase from *Catharanthus roseus* (CaUGT2) and sucrose synthase from *Arabidopsis thaliana* (AtSUS1). UDP, a product inhibitor of UDP-glucosyltransferase, was removed from the system and used for regeneration of UDP-glucose by the second enzyme, AtSUS1. The productivity was increased several-fold and UDP-glucose initially added to the reaction mixture could be reduced to one-tenth of the normal level. The concept of enhancing glucosylation efficiency by coupling a UDP-glucose regeneration system with glucosyltransferases should be applicable to enzymatic production of a wide range of glucosides.

Keywords: Chemoenzymatic synthesis; Glucosylation; Natural product; UDP-glucose recycling

1. Introduction

Glycosyl conjugation of lipophilic low molecular weight compounds is an efficient tool to enhance water solubility, to improve stability, and thereby to increase bioavailability and modify biological activity. Chemical synthesis of glycosides is usually difficult because it involves multiple blocking/deblocking steps before any product can be obtained. Enzymatic glycosylation offers several advantages over chemical methods, including (1) avoiding use of harsh conditions and toxic catalysts, (2) providing strict control of regio- and stereo-selectivity and (3) high efficiency [1].

One of the unique features of plant secondary metabolism is the capability to produce and accumulate structurally diverse glycosides. Application of plant cell cultures for glycosylation of exogenously supplied compounds has been extensively studied since Pilgrim [2] described glucosylation of xenobiotic phenolics in plant tissue cultures. In some cases, the glycosylation potential of cultured plant cells can be very high, as exemplified by efficient glucosylation of salicylic acid in *Mallotus japonica* suspension cultures [3]. However, plant cells usually accumulate glycosidic products in their vacuoles, which necessitates additional extraction and isolation steps in order to obtain pure products.

Family 1 glycosyltransferases (GTases) of higher plants transfer sugars to hydrophobic small molecules from activated sugar donors, typically UDP-sugars. GTases can recognize a wide range of acceptor substrates, including hormones, secondary metabolites and xenobiotics, and can therefore be used as general biocatalysts in glycoside synthesis [4]. Recombinant technologies have recently made it possible to exploit this capacity of plant GTases without the necessity of directly involving plant cells, and this increased interest means that a large number of GTase cDNAs have now been cloned and heterologously expressed [5]. Although enzymatic glycosylation using recombinant GTases has been shown to be efficient, the process still requires that activated sugar donors (in most cases UDP-sugars) be supplied in the reaction mix. These are usually expensive and difficult to obtain in large quantities. Furthermore, UDP, one of the products of the enzymatic glycosylation reaction, is a potent inhibitor of GTase activity. As a result, the glycosylation reaction rate gradually declines as UDP accumulates in the reaction mixture.

To alleviate these problems, in vivo systems using microbial cells expressing heterologous GTases have been explored as shown for the synthesis of quercetin glucosides in *Escherichia coli* [6] and human sugar chains in yeast [7]. However, to apply the whole-cell biocatalysis system the acceptor molecules should be permeable through cellular membranes and less toxic to the cells. Furthermore, extra steps to extract and separate the product from cellular metabolites will be generally required. Several methods have been reported for in vitro production of UDP-sugars [8], extending back to the first synthesis of UDP-glucose from UTP and glucose-1-phospahte with an enzyme preparation from yeast [9]. Enzymatic methods that allow in situ regeneration of UDP-sugars are quite attractive because this strategy not only reduces the cost of supplying the UDP-sugar substrate, but also avoids product inhibition by UDP [10]. However, combining such an in situ system for regeneration of UDP-sugar with glucosyl transfer by GTases has not been investigated for preparative in vitro glycosylation of lipophilic small molecules by plant GTases.

Curcumin (diferuloylmethane, **1**) is a water-insoluble yellow pigment of termeric (the dried rhizome of *Curcuma longa*). Curcumin has been used primarily as a food colorant, but is potentially also a neutraceutical because of its interesting pharmaceutical properties, including anti-oxidative, anti-inflammatory, anti-angiogenesis and anti-tumor activities [11,12]. We

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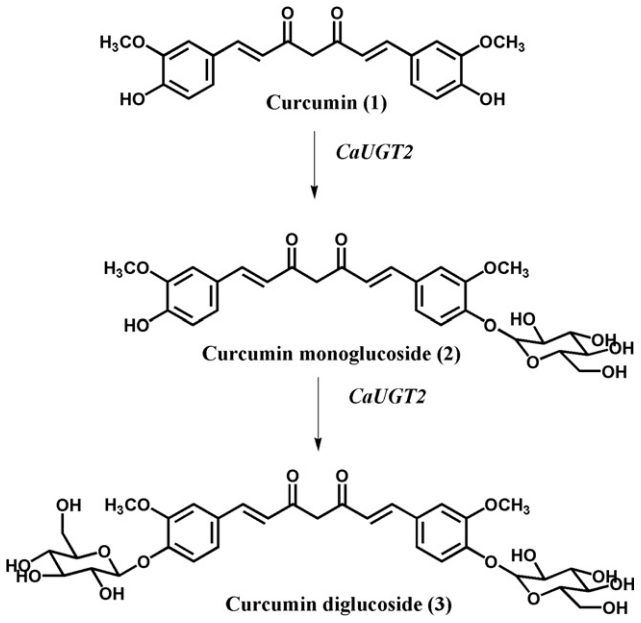

Fig. 1. A stepwise glucosylation of curcumin (**1**) to curcumin monoglucoside (**2**) and curcumin diglucoside (**3**) catalyzed by CaUGT2.

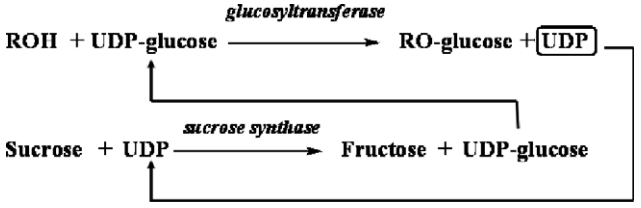

Fig. 2. General concept of the present one-pot two-enzyme system for efficient synthesis of glucosides from lipophilic small molecules.

earlier demonstrated that *Catharanthus roseus* cell suspension cultures actively convert curcumin to a series of glucosides, enhancing its water solubility by 2×10^7 [13]. Furthermore, we cloned a glucosyltrasferase (CaUGT2) catalyzing glucosylation of curcumin from *C. roses* cells [14]. The recombinant glucosyltrasferase converted curcumin to curcumin monoglucoside (**2**) and curcumin diglucoside (**3**), as shown in Fig. 1.

Here we describe a novel one-pot two-enzyme system for efficient production of curcumin glucosides in which glucosyl transfer from UDP-glucose to curcumin catalyzed by the recombinant CaUGT2 is coupled with removal of UDP and regeneration of UDP-glucose catalyzed by a recombinant *Arabidopsis* sucrose synthase (AtSUS1). We also show that this strategy is applicable to glucosylation of a wide array of acceptor molecules by various plant GTases utilizing UDP-glucose as a sugar donor. A diagram outlining this system is shown in Fig. 2.

2. Materials and methods

2.1. Glucosyltransferases

CaUGT2 cDNA previously isolated in our laboratory was subcloned into pQE30 (Qiagen) to create an N-terminal fusion protein with a His_6-tag. A cDNA clone of flavonoid 7-*O*-glucosyltransferase from *Scutellaria biacalensis* (F7GT) [15] was also subcloned into pQE30. The recombinant expression vectors were transformed into *E. coli* JM109.

2.2. Cloning of AtSUS1 cDNA

The open reading frame of AtSUS1 was PCR-amplified with *KOD*-Plus DNA polymerase (Toyobo) using the first strand cDNAs prepared from young plants of *Arabidopsis thaliana* (ecotype Columbia) and gene-specific primers AtSUS1-FW, 5′-GGTACCATGGCAAACGCTGAACGTATG-3′, and AtSUS1-RV, 5′-CTGCAGTCAATCATCTTGTGCAAGAGGAA-3′. The primer sequences included appropriate restriction sites (underlined). The PCR product was cloned into pCR 2.1-TOPO vector (Invitrogen) and sequenced to ensure that no mutation had occurred during their construction. The coding region of AtSUS1 cDNA was subcloned into the expression vector pQE30 (Qiagen) to create an N-terminal fusion protein with a His_6-tag and transformed into *E. coli* JM109.

2.3. Expression and purification of the recombinant CaUGT2, F7GT and AtSUS1

The transformed bacteria were cultured at 30 °C in Luria-Bertani medium containing 50 μg/ml carbenicillin for a day and then harvested by centrifugation and stored at −80 °C until use.

Crude enzyme was obtained by sonicating the cell pellet in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole. The recombinant protein was affinity-purified with Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. The protein concentration was determined by the Bradford method with bovine serum albumin as a standard [16].

2.4. Assay of sucrose synthase activity

Sucrose synthase activity was measured as described by Uggla et al. [17]. Briefly, the recombinant AtSUS1 (0.07–0.42 μg) was incubated with 0.3 M sucrose and 10 mM UDP in 1 ml 100 mM Tris–HCl buffer (pH 7.5) at 30 °C, and the reaction was terminated by heating the reaction mixture in a boiling water bath for 1 min. The appropriate amount of the supernatant was then incubated with 10 mM NAD and 100 μg UDP-glucose dehydrogenase (Sigma) in a total volume of 800 μl 100 mM Tris–HCl buffer (pH 8.7) at 30 °C and NADH formation was monitored spectrophotometrically at 340 nm.

2.5. Assay of UDP-glucose:curcumin glucosyltransferase activity of CaUGT2

Curcumin glucosyltransferase activity was assayed with a reaction mixture containing 0.3 M sucrose, 0.5 mM curcumin (dissolved in DMSO), 5 mM UDP-glucose and appropriate amounts of the recombinant CaUGT2 in 100 μl 50 mM Tris–HCl buffer (pH 7.5) with or without appropriate amounts of the recombinant AtSUS1. The mixture was incubated at 30 °C and the reaction was terminated by adding 200 μl methanol. After centrifugation at $12000 \times g$ for 10 min, the supernatant was subjected to HPLC analysis for estimation of curcumin glucosides as described previously [13].

2.6. Assay of UDP-glucose: apigenin glucosyltransferase activity of F7GT

Apigenin glucosyltransferase activity was determined with a reaction mixture containing 0.3 M sucrose, 0.5 mM apigenin (Sigma), 5 mM UDP-glucose and 2.2 μg F7GT in 100 μl 50 mM Tris–HCl buffer (pH 7.5) with or without 3.9 μg AtSUS1. The mixture was incubated at 30 °C and the reaction was terminated by addition of 200 μl methanol. After centrifugation at $12000 \times g$ for 10 min, the reaction products were analyzed by HPLC. HPLC analysis was performed on a reversed phase column (COSMOSIL 5C18-ARII, Nacalai Tesque) and the eluates were monitored by a photodiode array detector. The solvent conditions were as follows (flow rate, 1 ml/min): 0–26 min, 15–52% acetonitrile; 26–29 min, 52–100% acetonitrile; 29–33 min, 100% acetonitrile. A standard sample of apigenin 7-*O*-glucoside was purchased from Funakoshi, Co.