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Enhanced production of β-glucosides by *in-situ* UDP-glucose regeneration

Fong-Chin Huang, Jens Hinkelmann, Alexandra Hermenau, Wilfried Schwab*

Technische Universität München, Biotechnology of Natural Products, Liesel-Beckmann-Str. 1, D-85354 Freising, Germany

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ABSTRACT

Glycosyltransferase (GT)-mediated methodology is recognized as one of the most practical approaches for large-scale production of glycosides. However, GT enzymes require a sugar nucleotide as donor substrate that must be generated *in situ* for preparative applications by recycling of the nucleotide moiety, e.g. by sucrose synthase (SUS). Three plant GT genes *CaUGT2*, *VvGT14a*, and *VvGT15c* and the fungal *SbUGTA1* were successfully co-expressed with *GmSUS* from soybean in *Escherichia coli* BL21 and W cells. *In vitro*, the crude protein extracts prepared from four *GT* genes and *GmSUS* co-expressing cells were able to convert several small molecules to the corresponding glucosides, when sucrose and UDP were supplied. In addition, GmSUS was able to enhance the glucosylation efficiency and reduced the amount of supplying UDP-glucose. In the biotransformation system, co-expression of *VvGT15c* with *GmSUS* also improved the glucosylation of geraniol and enhanced the resistance of the cells against the toxic terpenol. GT-EcW and GTSUS-EcW cells tolerated up to 2 mM geraniol and converted more than 99% of the substrate into the glucoside at production rates exceeding 40 $\mu g\,ml^{-1}\,h^{-1}$. The results confirm that co-expression of SUS allows *in situ* regeneration of UDP-sugars and avoids product inhibition by UDP.

1. Introduction

Glycosyl conjugation of low molecular weight compounds is an efficient tool to enhance water solubility, to improve stability, and thereby to increase bioavailability and modify their biological activity. Thus, glycosides are very important in various industrial applications. Glycosides derived from long chain alkanols possess good surfactant and emulsifying properties, and are therefore applied in detergents and cosmetics (de Roode et al., 2003). Terpene and phenolic glycosides are found to have antifungal and antimicrobial activity (Sahari and Asgari, 2013) and have attracted great attention in food industry (Mastelic et al., 2004). Glycosides of peptides and steroids are used in antitumor formulations and cardiac-related drugs, respectively (Ooi et al., 1985; de Roode et al., 2003). Aroma glycosides are an important class of nonvolatile precursors that are currently gaining increased interest and attention for their role in imparting unique aroma to food (Sarry and Günata, 2004; Sanchez Palomo et al., 2006; Schwab et al., 2015). Glycosides of flavors and fragrances are used as water soluble, storage-stable, and odorless pro-aroma molecules that can break down to the desired aroma compounds under controlled conditions (de Roode et al., 2001; Herrmann, 2007) and have been widely used in folk medicines (Mastelic et al., 2004).

Glycosides can be produced chemically or enzymatically. Currently, enzymatic preparation of glycosides is more favoured because consumers have a strong preference for natural food additives. The enzymatic glycosylation reactions are catalyzed by glycosyltransferases (GTs) (Lairson et al., 2008). GTs transfer sugars to a wide range of acceptors, from hormones and secondary metabolites to biotic and abiotic chemicals and toxins in the environment (Ross et al., 2001). Although *in vitro* glycosylation carried out by recombinant GTs has been shown to be efficient, the process requires activated sugar donors (in most cases UDP-sugars) that need to 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 GT activity. As a result, the glycosylation reaction rate gradually declines as UDP accumulates in the reaction mixture.

Sucrose synthase (SUS) is an important sucrose-cleaving enzyme in a variety of plant 'sink' tissues by virtue of its reversible conversion of sucrose and UDP into UDP-glucose and fructose (Thummler and Vermas, 1987; Sitthiwong et al., 2007). Enzymatic methods that allow *in situ* regeneration of UDP-sugars (Ichikawa et al., 1994; Chen et al., 2001) are quite attractive because this

* Corresponding author.
E-mail address: schwab@tum.de (W. Schwab).

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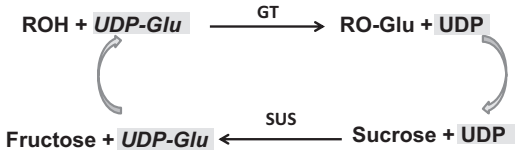

Fig. 1. *In situ* regeneration of UDP-glucose by coupling the activities of glucosyltransferase (GT) and sucrose synthase (SUS) for efficient synthesis of glucosides from lipophilic small molecules.

strategy not only reduces the cost of the required UDP-sugar substrate, but also avoids product inhibition by UDP (Masada et al., 2007; Fig. 1). Recently, an *in situ* system for regeneration of UDP-glucose with glucosyl transfer by GTs has been investigated. The method coupled the activities of two recombinant enzymes, UDP-glucose: curcumin glucosyltransferase from *Catharanthus roseus* (CaUGT2) and sucrose synthase from *Arabidopsis thaliana* (AtSUS1) (Masada et al., 2007). CaUGT2 has been shown to glycosylate curcumin, esculetin, scopoletin, *p*-nitrophenol, carvacrol and thymol (Kaminaga et al., 2004; Huang et al., 2015). SbUGTA1 from *Starmerella bombicola*, another catalytic protein used in the present study catalyzed the glycosylation of alkanols and hydroxyl fatty acids (Saerens et al., 2011; Huang et al., 2015) whereas VvGT14a and VvGT15c from *Vitis vinifera* have been shown to glycosylate monoterpenols (Bönisch et al., 2014).

Here, we describe the co-expression of a SUS gene from Soybean (*GmSUS*) and various GT genes (*VvGT14a*, *VvGT15c*, *SbUGTA1* and *CaUGT2*) in *Escherichia coli* cells for efficient production of small molecular glucosides *in vitro* and *in vivo*. The inhibitory effect of UDP on GT activity was determined. The results confirm that co-expression of SUS allows *in situ* regeneration of UDP-sugars and avoids product inhibition by UDP.

2. Materials and methods

2.1. Chemicals

Commercial chemicals were purchased in analytical grade from the following companies: 16-hydroxy palmitic acid, geraniol, eugenol, carvacrol (Sigma-Aldrich, Steinheim, Germany). Uridine 5′-diphosphate (UDP) and UDP-glucose (Sigma-Aldrich, Steinheim, Germany).

2.2. Cloning of sucrose synthase gene from Glycine max

Total RNAs were isolated from *Glycine max* seeds by CTAB extraction (Liao et al., 2004). The first-strand cDNAs were synthesized from 10 μg of total RNA using Superscript III RTase (Invitrogen, Karlsruhe, Germany) and a GeneRacer oligo-dT primer (5′-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG $T_{(18)}$-3′).

The coding region of sucrose synthase gene from *Glycine max* (*GmSUS*, accession number NM_001250596) was amplified by RT-PCR with the corresponding cDNA templates prepared as described above. The PCR primers used were 5′-CGC GGA TCC ATG GCC ACC GAT CGT TTG-3′ (forward) and 5′-ATT GCG GCC GCC TCA GCA GCA AGG GGC AC −3′ (reverse). The temperature program used was 5 min at 95 °C, 1 cycle; 45 s at 95 °C, 45 s at 55 °C, 2 min at 72 °C, 35 cycles; final extension at 72 °C for 10 min. The PCR product was digested and then ligated with the pET29a(+) vector (Novagen, Darmstadt, Germany), in frame with an C-terminal His-tag to yield *GmSUS*-pET29a. The recombinant genes were subjected to sequencing to confirm the sequence of the inserts.

2.3. Glycosyltransferase gene constructs

The full-length open reading frames of the *VvGT14a* (Bönisch et al., 2014), *VvGT15c* (Bönisch et al., 2014), *SbUGTA1* (Huang et al., 2014, 2015), and *CaUGT2* (Kaminaga et al., 2004; Huang et al., 2015) DNA sequences were cloned into the pGEX-4T-1 expression vector (Novagen, Darmstadt, Germany) to yield *VvGT14a*-pGEX-4T1, *VvGT15c*-pGEX-4T1, *SbUGTA1*-pGEX-4T1, and *CaUGT2*-pGEX-4T1.

2.4. Co-expression of glycosyltransferases and sucrose synthase *in* E. coli

Four GT constructs (in pGEX-4T1 vector) and the SUS construct (in pET29a vector) were singly transformed into the *E. coli* BL21(DE3) pLysS and W strains for expression of recombinant proteins and each GT co-transformed with SUS construct into BL21(DE3) pLysS and the W strains. A 4 ml overnight culture was used to inoculate a 400 ml culture in LB medium containing appropriate antibiotics. Cultures were grown at 37 °C until an OD_{600} of 0.6 was reached. Expression of the proteins was induced by the addition of 0.2 mM IPTG, and the cultures were grown at 18 °C for an additional 20 h. Cells were harvested by centrifugation (5000 g, 20 min, 4 °C) and re-suspended in 10 ml binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM Imidazole) which was suit for continued resin purification. Cells were lysed by sonication on ice with a MS 73 sonotrode (Bandelin electronic, Berlin Germany) 10 times for 30 s at 10% of maximal power. Cell debris was removed by centrifugation (12000 g, 30 min, 4 °C). Purification of SUS was performed using the IMAC Ni-charged resin (BIO-RAD, Munich, Germany) according to the manufacturer's instructions. Proteins were analysed by SDS-PAGE with 12% polyacrylamide gels and stained with Coomassie brilliant blue R-250. The protein concentration was determined by the Bradford assay (Bradford, 1976).

2.5. Enzyme assays

For determination of the GT activity, the standard assay (200 μl) contained 50 μl of crude protein extract, 100 mM Tris-HCl buffer (pH 7.5), 500 μM UDP-Glucose, and 600 μM substrate (dissolved in DMSO). The reaction was initiated by the addition of UDP-glucose, incubated at 30 °C with constant shaking for 60 min, and was stopped by the addition of 200 μl of ethyl acetate. The products were extracted with ethyl acetate, evaporated to dryness, re-suspended in methanol, and analysed by LC–MS.

For determination of SUS enzyme activity, SUS was co-incubated with a GT enzyme, sucrose, UDP, and substrate, and the formation of the glucoside was analysed by LC–MS. The reaction (200 μl) contained 50 μl of crude GT protein extract, 50 μl of crude SUS protein extract, 100 mM Tris-HCl buffer (pH 7.5), 500 μM UDP, 600 μM substrate (dissolved in DMSO), and 420 mM sucrose. The reaction was incubated at 30 °C with constant shaking for 60 min, and was stopped by the addition of 200 μl of ethyl acetate. The products were extracted with ethyl acetate, evaporated to dryness, re-suspended in methanol, and analysed by LC–MS. The SUS enzyme activity was indirectly determined following the formation of glucosides.

2.6. Analysis of the inhibition of GT activity by UDP

Different concentrations of UDP (varied from 500 to 50,000 μM) were first incubated with GT crude protein extract in 100 mM Tris buffer (pH7.5) for 10 min at room temperature, then substrate (600 μM) and UDP-glucose (500 μM) were added to start the reaction. After incubation for 60 min at 30 °C, the reaction was stopped by the addition of 200 μl of ethyl acetate. The products were extracted with ethyl acetate, evaporated to dryness, re-suspended