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A biocatalytic synthesis of diosgenyl-β-D-glucopyranoside by the use of four recombinant enzymes in one pot

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ABSTRACT

A system for the one-pot synthesis of diosgenyl-β-D-glucopyranoside (trillin) using multiple recombinant enzymes is developed. The enzymes maltodextrin phosphorylase (E1), glucose-1-phosphate thymidylyltransferase (E2), inorganic pyrophosphatase (E3), and solanidine glucosyltransferase (E4) involved in the work have been cloned and expressed in *Escherichia coli*. Under the optimized reaction conditions, the yield of trillin reached 28% (ca. 15.8 mg/l). The recovery yield of trillin after purification was 89%.

The majority of drugs used in therapeutics are inspired by, or derived from natural products, many of which are glycosylated by organisms in vivo.[1–3] A group of plant secondary metabolites which are glycosides is named steroidal saponins. Diosgenin, an example of a typical steroid, is an important industrial material for the synthesis of many steroidal saponins that have a broad range of activation including androgenic, contraceptive, anti-inflammatory, estrogenic and so on. Furthermore, these compounds are reported to possess cytotoxic, antitumor, antifungal, immunoregulatory, hypoglycemic, and cardiovascular properties.[4,5] Although it can be isolated from plants, this is extremely difficult and uneconomic, especially in large amounts, due to the lack of plant stuffs. Thus, glycosylations in vitro are important for the synthesis of steroidal saponins for the pharmaceutical industry. Chemical synthesis would provide a feasible route to obtain homogeneous saponins, however, the protection and deprotection of hydroxy groups are unavoidable and requires organic solvents.[6] A more economic and cleaner route is enzymatic synthesis.

The genes of maltodextrin phosphorylase (E1), glucose-1-phosphate thymidylyltransferase (E2), and inorganic pyrophosphatase (E3) were cloned from a genome of *Escherichia coli* K12 and expressed in *E. coli* BL21 (DE3). The solanidine glucosyltransferase (E4) gene was cloned from a genome of *Solanum tuberosum* and expressed in *E. coli* (DH5α). SDS–PAGE revealed that E2 and E3 were mainly expressed in soluble form, while E1 and E4 were expressed in insoluble form. The crude powders of the expressed enzymes were prepared and stored at 4 °C.

To increase the yield of trillin (Fig. 1), the reaction conditions were optimized. Due to the poor solubility of diosgenin and trillin in water, 10 cosolvents (10%, v/v) were tested to improve the solubility of substrate and the relative yield of product. Although the cosolvents improved the solubility of the substrate, the yield of product in the water-organic solvent reaction systems did not exceed that in the simple aqueous system. The enzymes were inactivated by the cosolvent, a visible flocculent precipitates appeared during the reaction on the addition of the cosolvent. Thus a phosphate buffer free of organic solvent was used to keep the enzymes active and stable in subsequent experiments. A study of the effect of pH revealed that the yield of trillin exhibited a maximum at pH 9. The optimum temperature for the enzymatic reaction was 40 °C. The maximum yield of trillin was observed when the reaction was run for 48 h. These data are displayed in Figures 2–5.

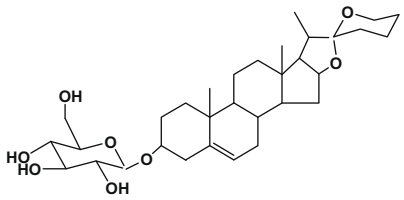

Figure 1. The chemical structure of diosgenyl-β-D-glucopyranoside (trillin).

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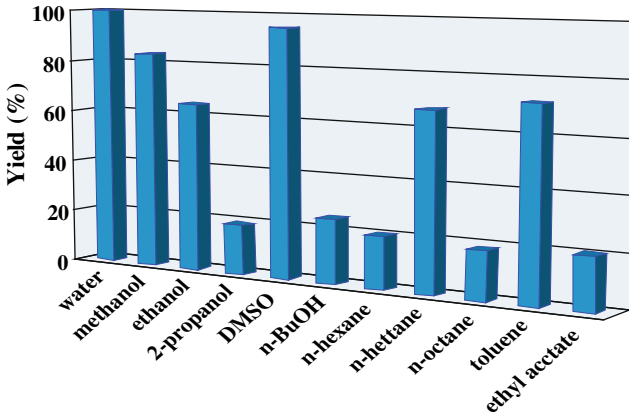

Figure 2. Effect of solvent on the reaction. The reaction mixture contained maltodextrin (5%, *m*/*v*), 5 mM UTP, 5 mM $MgCl_2$, 0.1 mM diosgenin, cosolvent (10%, v/v), 100 mM phosphate buffer (pH 7.5), and 6.58 mU E1, 125 mU E2, 7 U E3, 0.75 mU E4 for 8 h at 30 °C.

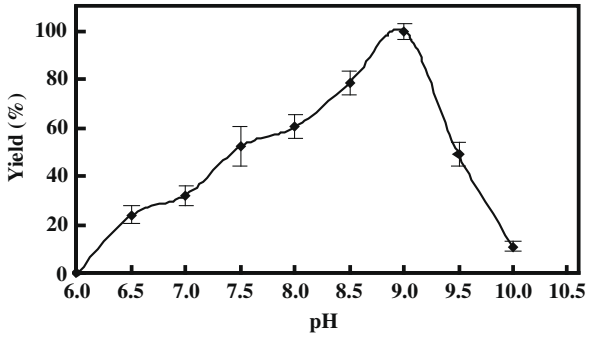

Figure 3. Effect of pH on the reaction. The pH was optimized at 30 °C in a reaction mixture that contained maltodextrin (5%, *m*/*v*), 5 mM UTP, 5 mM $MgCl_2$, 0.1 mM diosgenin, 100 mM phosphate buffer (pH 7.5), and 6.58 mU E1, 125 mU E2,7 U E3,0.75 mU E4 for 8 h at 30 °C.

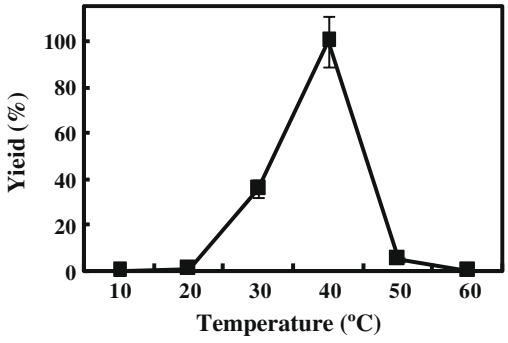

Figure 4. Effect of temperature on the yield of trillin. The temperature was optimized in a reaction mixture that contained maltodextrin (5%, *m*/*v*), 5 mM UTP, 5 mM $MgCl_2$, 0.1 mM diosgenin, 100 mM phosphate buffer (pH 9), and 6.58 mU E1, 125 mU E2, 7 U E3, 0.75 mU E4 for 8 h.

All the substrates in this system played critical roles in the synthesis of trillin. An orthogonal experiment (L3[4]) was designed to investigate the influence of substrate concentration on the trillin yield. Phosphate buffer (A), maltodextrin (B), UTP (uridine triphosphate) (C), and diosgenin (D) as four variables were studied and each variable was investigated at three levels (Table 1). The arrangements of each variable are displayed in Table 2. Under the optimum conditions, the reaction mixture containing 6.58 mU/ml E1, 125 mU/ml E2, 7 U/ml E3, 0.75 mU/ml E4, 5 mM $MgCl_2$, 100 mM phosphate buffer (pH 9), maltodextrin (5%, *m*/*v*), 10 mM UTP, 0.1 mM diosgenin was placed in a thermomixer at 1100 rpm at 40 °C for 48 h.

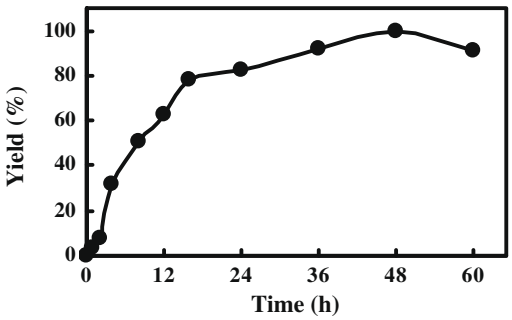

Figure 5. Time-course profiles for the synthesis of trillin. Conditions: 5% maltodextrin (*m*/*v*), 5 mM UTP, 5 mM $MgCl_2$, 0.1 mM diosgenin, 100 mM pH 9 phosphate buffer, and 6.58 mU E1, 125 mU E2, 7 U E3, 0.75 mU E4 at 40 °C.

Table 1
The variables and levels for orthogonal substrate concentration optimization

Variable	Level		
	1	2	3
A: Phosphate (pH 9)	50 mM	100 mM	200 mM
B: Maltodextrin	1%	5%	10%
C: UTP	1 mM	5 mM	10 mM
D: Diosgenin	0.1 mM	0.2 mM	0.5 mM

Table 2
Results of orthogonal experiments to optimize the substrate concentration in the one-pot enzyme reaction

Entry	Variable				(Trillin yield %)
	A	B	C	D	
1	1	1	1	1	12.8 (±0.043)
2	1	2	2	2	9.07 (±1.48)
3	1	3	3	3	2.21 (±0.16)
4	2	1	2	3	1.08 (±0.19)
5	2	2	3	1	20.6 (±4.17)
6	2	3	1	2	7.56 (±1.87)
7	3	1	3	2	3.86 (±1.17)
8	3	2	1	3	0.81 (±0.06)
9	3	3	2	1	14.3 (±1.57)

The milligram-scale synthesis of trillin was conducted in a mixture (100 ml) containing maltodextrin (5%, *m*/*v*), 10 mM UTP, 5 mM $MgCl_2$, 0.1 mM diosgenin, 100 mM phosphate buffer (pH 9), and 3 U E1, 20 U E2, 70 U E3, and 3 U E4 at 200 rpm and 40 °C for 48 h in shake flasks. The analytical yield of trillin relative to diosgenin in reaction was 28%, as analyzed by HPLC. The recovered yield of trillin after purification was 89%. The production of trillin was confirmed by high-resolution mass spectrum (*m*/*z*: 599.3540, $[M+Na]^+$). The chemical constitution of the product was verified by ^1H NMR and ^{13}C NMR spectroscopies.

In summary, the steroidal saponins which are glucosides with pharmacological properties are a topic of current interest. The presented system for the synthesis of diosgenin glucoside in vitro is successful, and benefits from enzyme-catalyzed reactions (mild conditions, high selectivity and so on) and reduces the cost of the enzymatic synthesis cost of trillin, due to the fact that the cheap maltodextrin was used to replace the expensive sugar donor UDP-Glc (uridine diphosphate glucose). This study significantly extends the technique for in vitro glycosylation of natural products. This one-pot bioconversion represents an efficient method for the synthesis of natural glycosides for the pharmaceutical industry.

Table 3
Endonucleases, vectors, and hosts for different genes

Gene	Endonucleases	Vector	Host
malpase	BamHI/NotI	pET24a (+)	*E. coli* BL21 (DE3)
g1pttase	BamHI/NdeI	pET11a (+)	*E. coli* BL21 (DE3)
ppase	BamHI/NdeI	pET11a (+)	*E. coli* BL21 (DE3)
sgt1.1	BamHI/NotI	PGEX-4T-1(+)	*E. coli* DH5α

Cloning, expression, and preparation of enzymes. The genes coding for *malpase*,[7–10] *g1pttase*[11–15], and *ppase*[16–20] were cloned from *E. coli* K12 genomic DNA by PCR (polymerase chain reaction). The gene of *sgt1.1*[21–25] was amplified from the total RNA of potato by RT-PCR (reverse transcription polymerase chain reaction). RNA extraction from the fresh buds of potato and RT-PCR were executed according to the instructions of the RNA extraction kit and the PrimeScript™ One-Step RT-PCR Kit (TaKaRa, Japan), respectively. Plasmid and strain construction are displayed in Table 3. All the experimental procedures are referred to in Molecular Cloning (3rd edition).[26] *E. coli* BL21 (DE3) or *E. coli* DH5α with exogenous recombinant plasmids was grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 37 °C, 200 rpm. When the OD_{600} of culture broth reached about 0.7, IPTG (isopropyl-β-D-thiogalactoside) was added and the cultivation was continued at a suitable temperature to induce the expression of the target enzyme protein. Cell disruption was carried out by sonication. After collecting the supernatant, the protein solution was finally lyophilized to give the crude enzyme powder.

Analytical procedures. The product (trillin) was extracted with *n*-BuOH (1:1, v/v) and analyzed using HPLC equipment (Shimadzu, 10AT, Japan) with a Hypersil BDS column (250 mm × 4.6 mm × 5 μm, Elitehplc, China). The mobile phase consisted of H_2O and methanol (1:9, v/v). The flow rate was 0.8 ml/min and UV detection was performed at 210 nm.

Milligram-scale synthesis, purification, and characterization of trillin. The product was isolated from the reaction system by extraction with *n*-BuOH (1:1, v/v). The *n*-BuOH phase was combined and concentrated by rotary evaporation. The powder was dissolved in methanol–H_2O (9:1, v/v) and the solution was centrifuged at 8000*g* for 10 min. After removing the precipitate, the supernatant was collected and concentrated by rotary evaporation. The concentrate was then separated by HPLC (250 mm × 4.6 mm × 5 μm, Hypersil BDS) to isolate the target product. The eluate containing the product was collected and dried by rotary evaporation. The product was analyzed using high-resolution MS (quadrupole time of flight) and NMR spectroscopy.

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Supplementary data

Supplementary data (spectrumoscopy (NMR and MS), HPLC and experimental details) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.01.077.

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