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Coupled reaction of glycosyltransferase and sucrose synthase for high-yielding and cost-effective synthesis of rosin

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ABSTRACT

Rosin has wide applications in food and pharmaceutical fields. Herein, 96% yield of rosin was synthesized using *Arabidopsis thaliana* glucosyltransferase (UGT73C5) from 2 mM cinnamyl alcohol (CA) and excess UDP-glucose (UDPG). Furthermore, replacing expensive UDPG by uridine diphosphate (UDP), coupled reaction by *Arabidopsis thaliana* sucrose synthase (AtSUS1) and UGT73C5 led to 100% yield of rosin. In addition, crude UGT73C5 and AtSUS1 resulted in rosin yield of 95% (10 mM CA) and 94% (20 mM CA) without UDP addition. With elevated CA concentration, whole-cell catalysis led to even higher rosin yield than crude enzymes preparations with a final yield of 91% from 50 mM CA. Furthermore, in cascaded reaction with a newly introduced *Catharanthus roseus* glucosyltransferase (CaUGT3), rosavin B was synthesized with a yield of 62% from 50 mM CA. This developed coupled pathway provides a feasible and cost-effective approach for the production of rosin and its glucosides from CA.

1. Introduction

Rosin and its glycosylated derivatives, as the main active ingredients in *Rhodiola rosea* L., display multiple physiological and pharmacological actvitities such as antioxidation, improving depression, resisting altitude sickness, eliminating fatigue, and easing symptoms of asthenia subsequent to intense physical and psychological stress [1–5]. In consequence, they are widely applied in pharmaceutical [4–5] and food fields [6–7]. Rosin, the monoglycoside of cinnamyl alcohol (CA) via attaching a glucose unit at 6′-OH position, can be further modified to form rosarin and rosavin by adding an arabinofuranose or arabinopyranose unit to the glucose moiety, respectively (Fig. S1) [5,8]. Commonly, rosin, rosavin, and rosarin are collectively known as rosavins, and the content of rosavins is often used as an index substance to evaluate the quality of *R. rosea* extracts [5,9]. In addition, rosavin B (rosavin analogue) can be produced from rosin by attaching a glucose unit to the 6′-OH of glucose moiety (Fig. S1) [10]. Bioactivities of phenylpropanoid glycosides could be influenced by the introduction of the type of linked saccharide [11–13], thus the unnatural analogue rosavin B may lead to a new compound with extended application prospects in pharmaceutical and food fields [10].

Traditional production of rosavins mainly depends on directly extracting from *R. rosea* [14–15]. However, wild *R. rosea* has been listed as an endangered species in many countries due to its slow growth and overcutting [16]. To protect natural resources from excessive deforestation, many researchers have been devoted to the production of rosavins using organic synthesis and biosynthesis approaches. For instance, organic synthesis of rosin (11% yield) [17–18] is not environmental-friendly due to the usage of toxic organic solvent (e.g., DMF) and excess amount of alcohol. Biotransformation as an alternative way was performed for rosin production by feeding the precursor CA to the callus or the hairy root culture of *R. rosea* [19–22], and the yield of rosin was only about 0.7% [22]. To improve the productivity of rosin, metabolic engineering was performed for rosin synthesis from glucose, and the engineered *Escherichia coli* (integrated with the UGT73C5 gene from *Arabidopsis thaliana*) produced 0.26 g/L rosin (yield of 0.8%) by optimizing the phenylalanine metabolic pathway [23–24]. Similarly, rosavin B production could also be improved by metabolic engineering with a glycosyltransferase CaUGT3 [10,25], and the titer reached 4.7 g/L [10]. Although microbial synthesis of rosin and its glycosylated

Abbreviations: UGT73C5, uridine diphosphate-dependent glucosyltransferase from *Arabidopsis thaliana*; AtSUS1, sucrose synthase from *Arabidopsis thaliana*; CaUGT3, glucosyltransferase from Catharanthus roseus; CA, cinnamyl alcohol; UDP, uridine diphosphate; UDPG, UDP-glucose; PBS, phosphate buffer saline.

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derivatives has been extensively reported, the low yield and complex purification procedures limit its industrial production potential. Protein engineering as a promising strategy to improve enzyme properties was also explored to increase the production of rosavin E by generating the CaUGT3 variant (T145V/N375Q), and a titer of 0.78 g/L was finally achieved [26]. Until now, based on our knowledge, in vitro enzymatic bio-catalysis by glycosyltransferases as an alternative for the production of rosin and its derivatives have not been well studied.

Most glycosyltransferases used for glycosylation of natural products are Leloir (sugar nucleotide-dependent) enzymes, which depend on expensive uridine diphosphate-glucose (UDPG) as the glycosyl donor [27]. Sucrose synthase (SUS) has been recently reported to be able to regenerate UDPG from sucrose and UDP [28]. Therefore, it can be coupled with UDPG-dependent glycosyltransferase (UGT) for the synthesis of a number of natural products (e.g., polydatin [29], ginsenosides [30–31], orientin [32], vitexin [32], flavonoid glycosides [33], isoorientin [34], isovitexin [34], quercetin-3,4′-O-diglucoside [35], nothofagin [36–38]).

In this study, an in vitro enzyme-coupled catalytic strategy was established to produce rosin and rosavin B from CA. Initially, Rosin was produced by a coupled reaction catalyzed by recombinant glycosyltransferase from *Arabidopsis thaliana* (UGT73C5) and sucrose synthase from *Arabidopsis thaliana* (AtSUS1). Then, rosavin B was further synthesized by cascading with recombinant glycosyltransferase from *Catharanthus roseus* (CaUGT3). Under optimized reaction conditions, 91% rosin yield and 62% rosavin B yield were achieved at 50 mM CA without addition of UDPG and UDP. This study provides a promising in vitro enzyme-coupled strategy for cost-effective production of rosin and rosavin B.

2. Experimental

2.1. Chemicals, strains and plasmids

Plasmids pET-21a (+), pET-42b (+), pET-28a (+) and strain *E. coli* BL21 (DE3) were procured from Takara (Dalian, China). CA, rosin and rosavin B were purchased from Shanghai Yuanye Biological Co., Ltd. (Shanghai, China). UDP and UDPG were obtained from Guangzhou Angfei Co., Ltd. (Guangzhou, China). High performance liquid chromatography (HPLC) grade acetonitrile was procured from Titan Scientific Co., Ltd. (Shanghai, China). All other chemicals (reagent grade) used were obtained from local companies.

2.2. Construction and expression of recombinant UGT73C5, CaUGT3 and AtSUS1

The recombinant plasmids and strains in this study (Table S1) were constructed as follows. The genes encoding glycosyltransferase UGT73C5 (GenBank: NP_181218.1), CaUGT3 (GenBank: BAH80312.1) and sucrose synthase AtSUS1 (GenBank: NP_001031915.1) were synthesized by Shanghai Exsyn-bio Biotechnology Co., Ltd (Shanghai, China) after codon-optimization. Subsequently, the synthesized gene *UGT73C5* was cloned into pET-28a(+) and pET-42b (+), and gene *AtSUS1* and *CaUGT3* were cloned into pET-28a (+), respectively, with restriction endonuclease *Bam* HI and *Xho* I (Fig. S2). Then, recombinant plasmids pET-28a(+)/UGT73C5, pET42b(+)/UGT73C5, pET28a (+)/CaUGT3 and pET28a(+)/AtSUS1 were transformed into *E. coli* BL21 (DE3), respectively.

The recombinant *E. coli* BL21 (DE3) strains harboring plasmids pET42b(+)/UGT73C5, pET28a(+)/CaUGT3 and pET28a(+)/AtSUS1 were inoculated to Luria-Bertani (LB) medium with 50 μg/mL kanamycin, and incubated at 37 °C with shaking at 220 rpm. When the optical density at 600 nm (OD_{600}) reached 0.6, expression was induced with a final concentration of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Then the culture was further incubated at 16 °C with shaking at 220 rpm for approximately 20 h. Subsequently, cells were harvested by centrifugation at 8000 × *g* and 4 °C for 5 min, then resuspended in phosphate buffer saline (PBS, 50 mM, pH 7.4). After disruption by high-press homogenization, the cell lysates were centrifugated at 8000 × *g* and 4 °C for 30 min and the supernatant was collected as the crude enzymes. Enzyme purification was performed by Ni-NTA agarose resin column (Qiagen, Chatsworth, CA, USA), and the purity was further determined by SDS-PAGE.

2.3. Construction and expression of UGT73C5/AtSUS1 co-expression strain

The *AtSUS1* gene was cloned into plasmid pET21a(+) between restriction endonuclease cutting sites of *Bam* HI and *Xho* I by ClonExpress II One Step Cloning Kit (Fig. S2). After verification by sequencing, the plasmid pET21a(+)/AtSUS1 was transferred into *E. coli* BL21 (DE3) together with pET42b(+)/UGT73C5. Then the recombinant *E. coli* BL21 (DE3)/UGT73C5-AtSUS1 strains were incubated on LB agar plates with 100 μg/mL ampicillin and 50 μg/mL kanamycin, and positive colonies were picked out. Then, the co-expression strain was incubated at 37 °C with shaking at 220 rpm in LB medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin, and induced with a final concentration of 0.2 mM IPTG. Finally, the UGT73C5-AtSUS1 co-expression cells were harvested and freeze-dried after 20 h incubation.

2.4. Enzyme activity

The activity of UGT73C5 was assayed by incubating at 40 °C for 10 min in a 300 μL reaction mixture containing 50 mM PBS (pH 7.5), 2 mM CA, 2 mM UDPG and 0.01 mg/mL UGT73C5. A double volume of methanol was added to terminate the reaction. Reaction products were analyzed by HPLC. One unit of UGT73C5 activity is defined as the amount of enzyme that produces 1 μmol rosin per minute.

The activity of CaUGT3 was assayed by incubating at 40 °C for 10 min in a 300 μL reaction mixture containing 50 mM PBS (pH 7.5), 10 mM rosin, 5 mM UDPG and 0.01 mg/mL CaUGT3. A double volume of methanol was added to terminate the reaction. Reaction products were analyzed by HPLC. One unit of CaUGT3 activity is defined as the amount of enzyme that produces 1 μmol rosavin B per minute.

The activity of AtSUS1 was assayed by incubating at 40 °C for 10 min in a 500 μL reaction mixture containing 50 mM PBS (pH 7.5), 500 mM sucrose, 10 mM UDP and 0.1 mg/mL AtSUS1. The resultant reducing sugar was determined by the addition of the DNS reagent (3, 5-dinitrosalicylic acid) and immediately boiled for 10 min. The reduced product (3-amino-5-nitrosalicylic acid) was detected by measuring the absorbance at 540 nm. One unit of AtSUS1 activity is defined as the amount of enzyme that produces 1 μmol fructose per minute.

Protein concentrations were determined by a Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China) with bovine serum albumin as standard.

2.5. Enzymatic characterization of UGT73C5

2.5.1. Effect of reaction pH and temperature on UGT73C5 glycosylation

All reactions were performed in the 2 mL Eppendorf tubule containing 500 μL mixture. Based on the initial reaction conditions (2 mM CA, 2 mM UDPG, 0.2 U/mL UGT73C5, incubation at 40 °C and pH 7.5), effect of reaction pH of UGT73C5 was investigated by conducting the reaction at 40 °C for 30 min in citric acid-disodium hydrogen phosphate buffer (50 mM, pH 5.0–8.0) and glycine-sodium hydroxide buffer (Gly-NaOH, 50 mM, pH 8.0–10.0). Effect of reaction temperature of UGT73C5 was investigated by conducting the reaction at the range of 20–50 °C for 30 min in the buffer of 50 mM Gly-NaOH buffer (pH 9.5).

2.5.2. Effect of the concentration of UDPG, UDP, sucrose or fructose on UGT73C5 glycosylation

The effect of UDPG concentration on UGT73C5 glycosylation

reaction was detected by conducting the reaction at 40 °C for 6 h in the mixture of 50 mM Gly-NaOH (pH 9.5), 2 mM CA, 0.2 U/mL UGT73C5 and various concentrations of UDPG (2–10 mM).

The effect of UDP concentration on UGT73C5 glycosylation reaction was detected by conducting the reaction at 40 °C for 6 h in the mixture of 50 mM Gly-NaOH (pH 9.5), 2 mM CA, 2 mM UDPG, 0.2 U/mL UGT73C5 and different concentrations of UDP (0–10 mM).

Various concentrations of sucrose (0–500 mM) or fructose (0–100 mM) were also added into the reaction mixture to investigated the effect of sucrose and fructose on the activity of UGT73C5.

The yield of rosin was calculated as the following equation:

$$\text{Yield} = \frac{\text{Concentration of produced rosin}}{\text{Concentration of initial CA}} \times 100\%$$

2.5.3. Kinetic analysis of UGT73C5 glycosylation

The kinetic analysis of UGT73C5 toward CA was performed as follows: 50 mM PBS (pH 7.5), excess UDPG (10 mM), 0.2 U/mL UGT73C5, and various concentrations of CA (1–10 mM). And the kinetic analysis of UGT73C5 toward UDPG was determined by the following mixture: 50 mM PBS (pH 7.5), excess CA (10 mM), 0.2 U/mL UGT73C5, and various concentrations of UDPG (1–10 mM). All reactions were conducted at 40 °C for 10 min. Data analysis were performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA).

2.6. Optimization of UGT73C5-AtSUS1 coupled reaction

Original UGT73C5-AtSUS1 coupled reactions (containing 2 mM CA, 0.2 mM UDP, 500 mM sucrose, 50 mM PBS (pH 7.5), 0.2 U/mL UGT73C5 and 0.2 U/mL AtSUS1) were performed at 40 °C for 2 h in a 500 μL reaction system (in 2 mL Eppendorf tubule).

Based on the original coupled reaction conditions, influences of reaction temperature and pH were investigated at various temperatures (30, 35, 40, 45 and 50 °C) or pH (50 mM PBS buffer; pH 6.0, 6.5, 7.0, 7.5 and 8.0). Effects of different concentrations of UDP (0.05–1.0 mM) and sucrose (100–500 mM) on the coupled reactions were also investigated.

Based on the optimal reaction conditions, the ratio of UGT73C5/AtSUS1 was optimized, using various activities of UGT73C5 (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 U/mL) and a fixed activity of AtSUS1 (0.2 U/mL). Based on the optimal ratio, optimal activity of both UGT73C5 and AtSUS1 was investigated (0.2, 0.4, and 0.6 U/mL).

2.7. Rosin synthesis at elevated concentrations of CA by crude enzymes (cell extracts) or freeze-dried cells (UGT73C5/AtSUS1 co-expression strain)

Coupled reactions with elevated concentrations of CA (10–20 mM) were performed in 100 mL-Erlenmeyer flask containing 10 mL mixture, including 10 (or 20) mM CA, 500 mM sucrose, 50 mM PBS (pH 7.5), 1 U/mL UGT73C5 and 1 U/mL AtSUS1 (crude enzymes). All reactions were performed at 40 °C for 12 h.

In addition, to choose suitable co-solvent for the dissolution of elevated concentrations of CA (50 mM), the effect of different of co-solvents methanol, ethanol, DMSO, and hydroxypropyl β-cyclodextrin (HPCD) on coupled reaction was investigated.

Coupled reactions with elevated concentrations of CA (50 mM) were performed in 100 mL-Erlenmeyer flask containing 10 mL mixture, including 50 mM CA dissolved in 5% (w/v) HPCD, 500 mM sucrose, 50 mM PBS (pH 7.5), 2 U/mL UGT73C5 and 2 U/mL AtSUS1 (crude enzymes or freeze-dried cells). All reactions were performed at 40 °C for 12 h.

2.8. Rosavin B synthesis by cascade reaction

A 10 mL-cascade reaction mixture containing 50 mM PBS (pH 7.5), 50 mM CA dissolved in 5% (w/v) HPCD, 500 mM sucrose, 2 U/mL UGT73C5, 2 U/mL CaUGT3 and 4 U/mL AtSUS1 was performed at 40 °C for 12 h. Reaction products were determined by HPLC.

2.9. HPLC and NMR analysis of product rosin

Analysis of glycosylated products of CA (rosin and rosavin B) was performed using a HPLC 1260 system (Agilent Technologies, Santa Clara, CA, USA) with a 1260 VWD detector and a Diamonisil C18 column (250 mm × 4.6 mm × 5 μm). The mobile phases included solution A (H_2O) and solution B (acetonitrile). Analysis of CA and rosin was performed using 50% of solution A and 50% of solution B at 30 °C with a flow rate of 1 mL/min. Analysis of rosavin B was performed at 30 °C with a flow rate of 1 mL/min using the following gradient elution conditions: 0–2 min, 70% A and 30% B; 2–3 min, 70% A and 30% B to 30% A and 70% B (linear gradient); 3–6 min, 30% A and 70% B; 6–7 min, 30% A and 70% B to 70% A and 30% B (linear gradient); 7–12 min, 70% A and 30% B. All of these products were detected at wavelength of 254 nm with an injection of 10 μL.

NMR analysis of rosin was performed following the previous report [23]. Purification of product rosin was performed as follows: the reaction mixture was added to moderate ethyl acetate and stirred over 2 h for extraction [39]. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum. Crude products were purified by silica gel column chromatography with ethyl acetate-methanol (v/v = 12:1) as eluent. The purification process was monitored by TLC, using ethyl acetate-methanol (v/v = 6:1) as the mobile phase. Purified product was dissolved in 500 μL of CD_3OD, and analyzed by NMR on the Bruker Avance 400 (Karlsruhe, Germany). Chemical shifts are expressed in δ (ppm), and coupling constants (J) are given in hertz (Hz).

^{1}H NMR (400 MHz, CD_3OD) δ 7.43 (2H, m, H-3′, 5′), 7.32 (2H, m, H-2′, 6′), 7.24 (1H, dd, J = 5.0, 3.7 Hz, H-4′), 6.70 (1H, d, J = 16.0 Hz, H-3), 6.39 (1H, dt, J = 16.0, 6.3 Hz, H-2), 4.55 (1H, ddd, J = 12.8, 5.7, 1.6 Hz, H-1a), 4.39 (1H, d, J = 7.8, Hz, H-1″), 4.35 (1H, ddd, J = 12.8, 6.5, 1.4 Hz, H- 1b), 3.91 (1H, dd, J = 12.8, 2.4 Hz, H-6″a), 3.71 (1H, dd, J = 11.9, 5.5 Hz, H-6″b), 3.25–3.41 (4H, H −2″,−3″,−4″,−5″).

^{13}C NMR (100 MHz, CD 3 OD) δ 69.4 (C-1), 125.3 (C-2), 132.4 (C-3), 136.9 (C-1′), 126.1 (C-2′, 6′), 128.2 (C-3′, C-5′), 127.3 (C-4′), 101.9 (C-1″), 73.7 (C-2″), 76.6 (C-3″), 70.3 (C-4″),76.7 (C-5″),61.4 (C-6″).

3. Results

3.1. Expression of UGT73C5, CaUGT3, and AtSUS1 and enzymatic synthesis of Rosin

Recombinant plasmids pET-28a(+)/CaUGT3, pET-28a(+)/AtSUS1, pET-28a(+)/UGT73C5, pET-42b(+)/UGT73C5, and pET-21a(+)/AtSUS1 were successfully constructed (Fig. S2), and expressed in *E. coli* BL21(DE3). As shown in Fig. S3, most UGT73C5 was expressed as inclusion body in pET-28a(+) plasmid, but it was better expressed as soluble protein in pET-42b(+) plasmid. The molecular mass of recombinant UGT73C5 in pET-42b(+) was about 89 kDa, consisting of GST-tag (about 32 kDa) and UGT73C5 (about 57 kDa). CaUGT3 and AtSUS1 were expressed as soluble proteins in pET-28a(+), and their molecular mass were 56 and 93 kDa, respectively. After purification by Ni-NTA column, purified enzymes (> 90% purity) of UGT73C5, CaUGT3, and AtSUS1 were obtained (Fig. S3). Recombinant strain *E. coli* BL21(DE3)/AtSUS1-UGT73C5 was constructed for co-expression of UGT73C5 and AtSUS1. Only one significant band in SDS-PAGE was observed for the co-expression since recombinant UGT73C5 and AtSUS1 have similar molecular mass (about 90 kDa, Fig. S3).

The reaction of glycosylation by UGT73C5 was performed at 40 °C for 12 h with UDPG and CA as the glycosyl donor and acceptor, respectively. As shown in Fig. 1, compared with the control, the reaction sample displayed an additional peak at the retention time of 2.5 min, which is consistent with that of rosin standard. For further identification, the product was collected and purified for nuclear magnetic

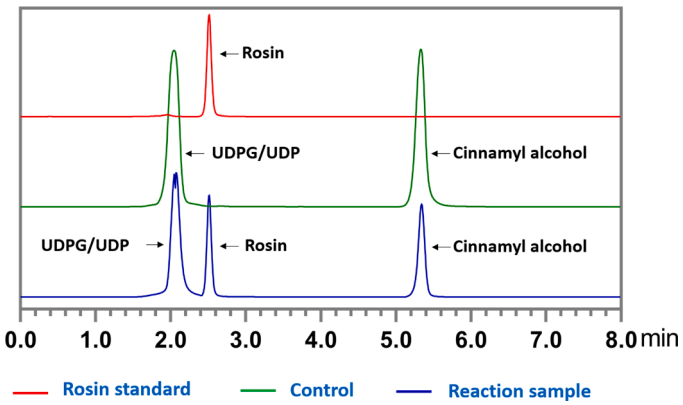

Fig. 1. HPLC of glycosylation by UGT73C5 with UDPG and CA as substrates. In the control group, methanol was added to stop the reaction at the beginning.

resonance (NMR). As shown in Fig. S4 (^{1}H NMR) and Fig. S5 (^{13}C NMR), the hydrogen and carbon spectrums are consistent with that of rosin [23], which further confirm the production of rosin.

3.2. Optimization of the glycosylation for rosin synthesis by UGT73C5

To optimize the reaction conditions of glycosylation catalyzed by UGT73C5, effects of pH and temperature on UGT73C5 activity were investigated. As shown in Fig. S6a, the activity of UGT73C5 kept increasing from pH 5.0 to 9.5, and dramatically decreased at pH 10.0. Therefore, the highest activity of UGT73C5 was obtained at pH 9.5. Under the optimum pH of 9.5, the effect of reaction temperature was also investigated (Fig. S6b). The activity of UGT73C5 gradually increased as from 20 to 45 °C, and dramatically dropped from 45 to 50 °C. Although UGT73C5 activity reached the highest at 45 °C, it still retained 98% relative activity at 40 °C. Considering that lower temperature is usually beneficial to enzymatic stability, 40 °C was selected as the reaction temperature for glycosylation by UGT73C5 in further investigation.

The effect of substrate ratio (UDPG/CA) on the yield of rosin by UGT73C5 was determined. As shown in Fig. S6c, the yield increased along with the increasing ratio of UDPG/CA. When the ratio of UDPG/CA reached 5:1, the final yield of rosin reached more than 95%. Therefore, excessive UDPG is crucial for the glycosylation of UGT73C5, which provides abundant energy and promotes the reaction equilibria for rosin synthesis.

Under optimal conditions, the kinetic parameters of UGT73C5 were determined (Fig. S7 and Table 1). The K_M value toward CA (3.99 mM) was over 16-fold of that of UDPG (0.28 mM), indicating that UGT73C5 has a significantly higher affinity toward UDPG than CA. Furthermore, the catalytic efficiency (k_{cat}/K_M) of UGT73C5 toward UDPG (2.75 $mM^{-1}{\cdot}s^{-1}$) was 10.6 times of that toward CA (0.26 $mM^{-1}{\cdot}\ s^{-1}$).

3.3. Coupled reaction of UGT73C5-AtSUS1 for rosin production

Although UDPG is an important glycosyl donor for rosin production by UGT73C5, the exorbitant price limits its industrial application as substrate for the production of rosin. In this study, AtSUS1 was introduced for UDPG regeneration. As shown in Fig. 2a, in the coupled reaction, UGT73C5 can catalyze the glycosylation of CA to produce rosin utilizing UDPG as glucose donor, meanwhile sucrose synthase can catalyze the conversion of sucrose to fructose along with the regeneration of UDPG from UDP.

In the UGT73C5-AtSUS1 coupled reaction, 10 mM UDP and 500 mM sucrose was added to replace 10 mM UDPG in the initial setting. As shown in Fig. 2b, the yield of rosin in coupled reaction was only half of the UGT73C5 catalyzed glycosylation reaction. To evaluate the effect of additional substrates (UDP and sucrose) and by-product (fructose) on the activity of UGT73C5, various concentrations of UDP, sucrose, and fructose were added. As shown in Fig. S8a and S8b, sucrose and fructose displayed no effect on the activity of UGT73C5. However, the activity of UGT73C5 gradually decreased with the increase of UDP concentration (Fig. 2c), indicating a significant inhibitory effect on UGT73C5 by high concentration of UDP. Therefore, the compromised yield of rosin in the UGT73C5-AtSUS1 coupled reaction (Fig. 2b) may be attributed to the inhibition of high concentration of UDP.

To enhance the yield of rosin in UGT73C5-AtSUS1 coupled reaction, reaction conditions were optimized as follows (Fig. 3). To avoid the inhibition of high concentration of UDP, 0.2 mM UDP was added initially in the reaction with excess sucrose (500 mM) and CA (2 mM). As shown in Fig. 3a, the optimum pH for the coupled reaction was 7.5. Then, under the optimum pH, the highest yield of rosin was obtained at both 40 °C and 45 °C (Fig. 3b). Considering the enzymatic thermostability, 40 °C was selected as the optimum temperature. The effect of UDP concentration on the coupled reaction was also investigated over the range of 0.05 to 1.0 mM. As shown in Fig. 3c, the yield of rosin significantly increased (58%–87%) when the concentration of UDP lifted from 0.05 to 0.2 mM, while slightly increased (87%–90%) as the UDP concentration increased from 0.2 to 1.0 mM. Thus 0.2 mM UDP was chosen as the final concentration considering the cost. Subsequently, the effect of sucrose was investigated, and the optimal sucrose concentration was determined to be 500 mM (Fig. 3d).

To investigate the effect of the loading amount of two coupled enzymes on the yield of rosin, various ratios of UGT73C5/AtSUS1 were selected from 4:1 to 2:3 (Fig. 3e). At AtSUS1 loading of 0.2 U/mL, the highest rosin yield of 91% was obtained after 8 h reaction with 0.2 U/mL UGT73C5, thus the optimum UGT73C5/AtSUS1 ratio was 1:1. With UGT73C5 and AtSUS1 loadings of 0.4 U/mL (at 1:1), the rosin yield of 100% was achieved at 8 h (Fig. 3f), which is much higher than that (49%) at initial conditions without optimization (Fig. 2b).

3.4. Coupled reaction using cell-free extract or freeze-dried cells

To further promote the industrial potential, the coupled reaction using crude UGT73C5 and AtSUS1 without addition of UDP was investigated. When using 10 mM CA, purified enzymes (UGT73C5 and AtSUS1) led to 100% yield of rosin in the presence of 0.2 mM UDP, and crude enzymes resulted in 95% yield of rosin in the presence (0.2 mM) or absence UDP (Fig. 4a). Similarly, 94% yield of rosin was produced from 20 mM CA by crude enzymes (with or without UDP) compared with that (96% yield) by purified enzymes (Fig. 4b). Although a slightly compromised yield of rosin was observed for crude enzymes, the cost of substrates by the coupled reaction was greatly reduced due to no addition of UDP.

Under increased concentration of CA (e.g., 50 mM), a cloudy mixture was formed due to the low solubility of CA, and the reaction was hampered. Thus, different co-solvent (methannol, ethanol, DMSO and hydroxypropyl β-cyclodextrin (HPCD)) were investigated. As shown in Fig. 5a, effects of different co-solvents on the coupled reaction with 50 mM CA were investigated. Reaction with HPCD (5%, w/v) as the co-solvent achieved higher yield of rosin (58% yield) than those of other co-solvents (methannol, ethanol, and DMSO).

In a 10 mL-reaction mixtrue with 50 mM CA (in 5% HPCD), the final yield of rosin reached 84% by crude enzymes (Fig. 5b). Compared with crude enzymes, the coupled reaction using *E. coli* BL 21 (DE3)/UGT73C5-AtSUS1 freeze-dried cells reached 91% yield of rosin at 50 mM of CA, which were higher than that produced by in vitro enzymatic

Table 1
Kinetic parameters of UGT73C5 toward UDPG and CA.

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}{\cdot}\ s^{-1}$)
UDPG	0.28 ± 0.05	0.76 ± 0.03	2.75
CA	3.99 ± 0.43	1.02 ± 0.12	0.26

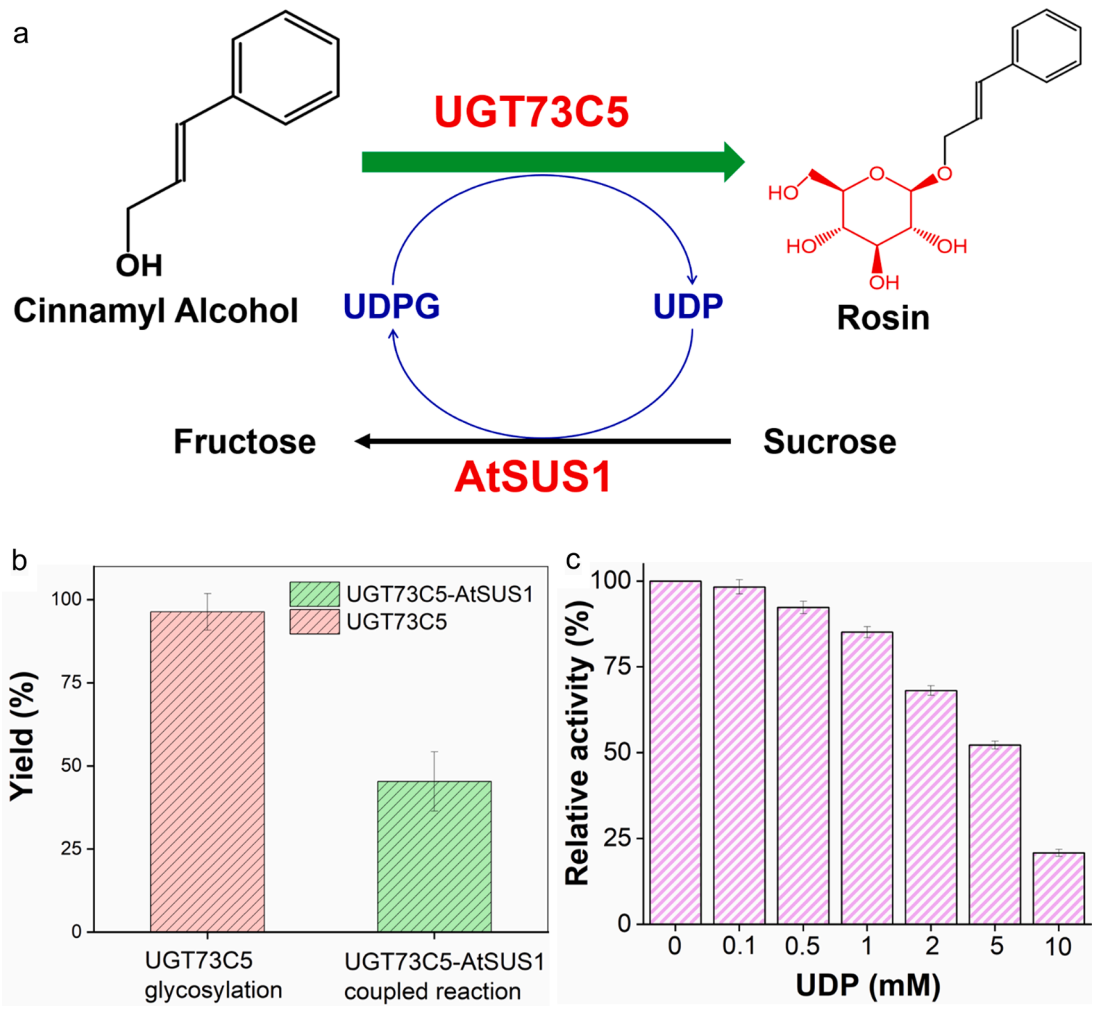

Fig. 2. Coupled reaction of UGT73C5-AtSUS1 (a-b) and effects of substrate and byproduct on UGT73C5 activity (e-f). (a) Schematic of UGT73C5-AtSUS1 reaction. (b) Comparison of UGT73C5 glycosylation and UGT73C5-AtSUS1 coupled reaction. (c) The influence of UDP on the activity of UGT73C5. (Coupled reaction conditions: 2 mM CA, 10 mM UDP, 10 mM sucrose, 0.2 U/mL UGT73C5, 0.2 U/mL AtSUS1, incubation at 40 °C and pH 7.5 for 6 h.).

catalysis (Fig. 5b). The detailed results of rosin production from various concentrations of CA in different reaction systems were summarized in Table 2.

3.5. Extended application of coupled reaction for synthesis of rosavin B

Based on the coupled reaction of UGT73C5-AtSUS1, a cascade reaction route was constructed by introducing a 1, 6-glucosyltransferase from *Catharanthus roseus* (CaUGT3) for the synthesis of rosavin analogue (rosavin B) (Fig. 6a and Fig. S9). The cascade reactions catalyzed by UGT73C5, CaUGT3, and AtSUS1 were performed with 50 mM CA. As shown in Fig. 6b, within the first 2 h, the concentration of CA dramaticlly decreased while the production of rosin and rosavin B was significantly increased. After 2 h, the concentrations of both CA and rosin gradually decreased along with a continuous increase in rosavin concentration. The highest titer of rosavin B reached 31.2 mM with a yield of 62% (14.2 g/L) after 10 h.

4. Discussion

Rosin, a main component in *R. rosea.*, has a variety of physiological activities such as alleviating depression and psychological stress [1–5]. Here, we developed an efficient and cost-effective enzymatic route to synthesize rosin from CA. According to the heterologous expression of UGT73C5, recombinant plasmid pET-42b(+)/UGT73C5 showed better soluble expression than pET-28a(+)/UGT73C5 plasmid, which is attributed to the glutathione sulfhydryl transfer protein (GST-tag) in pET-42b(+) plasmid (Fig. S2), which is able to improve the soluble expression of recombinant protein [40]. According to the characterization of UGT73C5, the kinetic parameters showed 14 times lower K_m value toward UDPG than CA (Table 1), suggesting UGT73C5 has higher affinity toward UDPG. UDPG is an important glycosyl donor for the Leloir glycosyltransferase reactions, which can provide not only glucose unit to acceptors but also essential energy for promoting the reaction [27]. In the CA glycosylation by UGT73C5, although 96% yield of rosin was achieved from CA (2 mM) with UDPG/CA ratio of 5:1 (Fig. S6c), exorbitant cost of UDPG limits its practical application. To reduce the cost, herein AtSUS1 was introduced to couple with UGT73C5 for rosin production because it can sustainably regenerate UDPG from cheap UDP [41]. After optimization, 100% yield of rosin from 2 mM CA was obtained in UGT73C5-AtSUS1 coupled reaction (Fig. 3), which is higher than that of UGT73C5 catalyzed glycosylation reaction due to the sustainable UDPG regeneration and less UDP inhibition [42]. Since there are a certain amount of UDP and UDPG (around 20 μmol/g cell dry weight in total) existed in the cell extract [43], using curde enzymes in the coupled reaction could further eliminate UDP addition and reduce the cost of enzyme purification, despite a slight decrease (around 5%) in rosin yield (Fig. 4a). Therefore, enzymatic production of rosin by crude enzymes of UGT73C5 and AtSUS1 is a feasible and cost-effective approach since no enzyme purification and extra UDP addition are

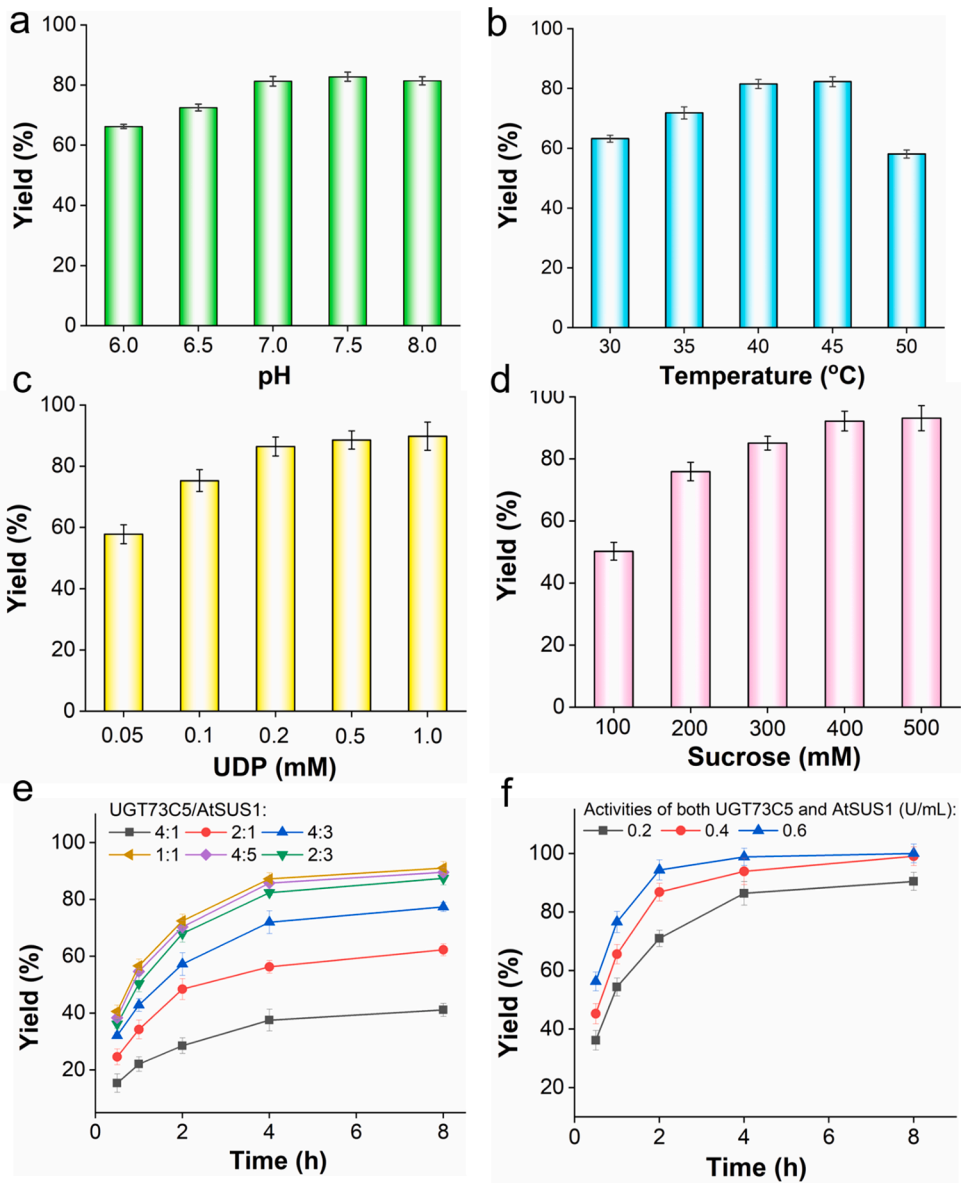

Fig. 3. Optimization of UGT73C5-AtSUS1 coupled reaction. (a) Effect of reaction pH; (b) Effect of reaction temperature; (c) Effect of UDP concentration; (d) Effect of sucrose concentration; (e) Effect of activity ratio of UGT73C5 and AtSUS1 (UGT73C5/AtSUS1); (f) Effect of activity of enzymes. Initial reaction conditions: a 500 μL-reaction mixture (in 2 mL Eppendorf tubule) contained 2 mM CA, 0.2 mM UDP, 500 mM sucrose, 50 mM PBS (pH 7.5), 0.2 U/mL UGT73C5 and 0.2 U/mL AtSUS1, incubation at 40 °C for 6 h.

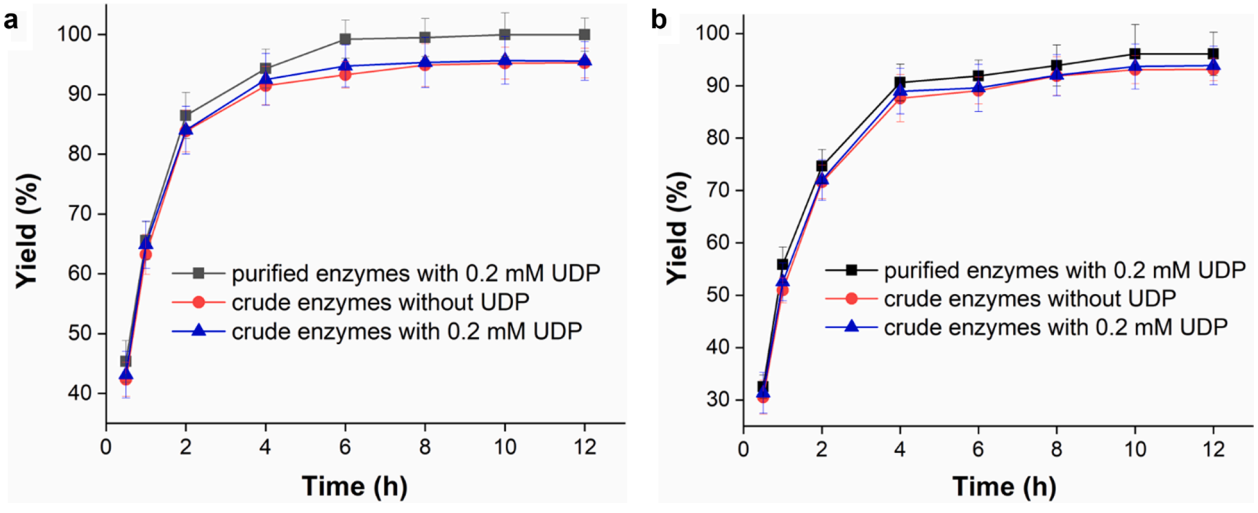

Fig. 4. Coupled reaction by purified/crude enzymes for rosin synthesis. (a) Coupled reaction by purified and crude enzymes at 10 mM CA; (b) Coupled reaction by purified and crude enzymes at 20 mM CA. .

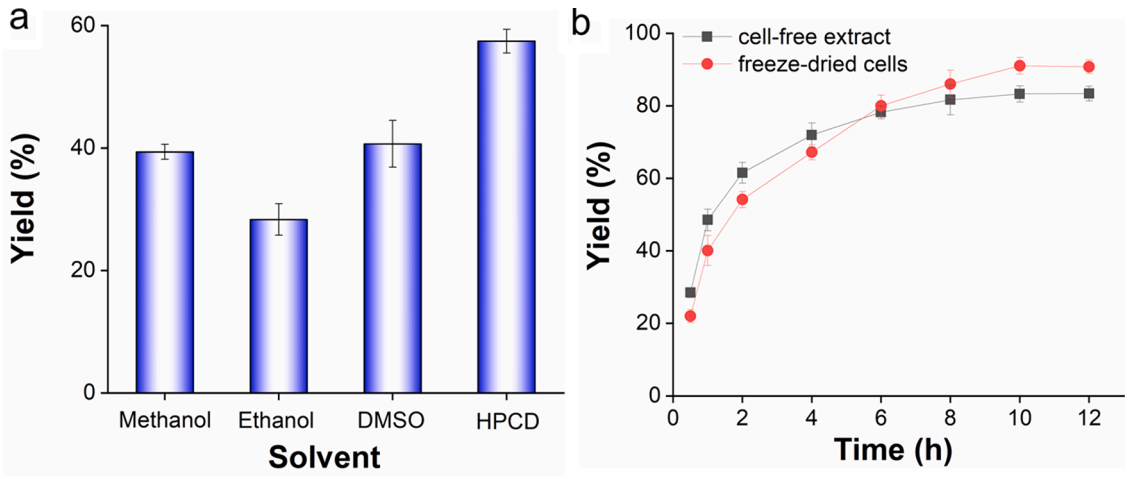

Fig. 5. Coupled reaction by crude enzymes and freeze-dried cells for rosin synthesis from 50 mM CA. (a) Effect of various co-solvents (5% methanol, 5% ethanol, 5% DMSO and 5% HPCD) on rosin synthesis from 50 mM CA (incubating at 40 °C for 2 h); (b) Coupled reaction by crude enzymes (cell extract) and freeze-dried cells with 50 mM CA (in 5% HPCD). .

Table 2
Rosin production from CA by different catalysts.

CA concentration (mM)	Yield (%)	Titer (gL^{-1})	UDPG (mM)	UDP (mM)	HPCD (w/v)	UGT73C5 (U/mL)	AtSUS1 (U/mL)	Biocatalysts status
2	96	0.56	10	–	–	0.2	–	purified enzyme
2	100	0.59	–	0.2		0.2	0.2	purified enzyme
10	100	2.96	–	0.2	–	1	1	purified enzyme
10	95	2.82	–	–	–	1	1	crude enzyme
10	95	2.82	–	0.2	–	1	1	crude enzyme
20	96	5.69	–	0.2	–	1	1	purified enzyme
20	94	5.51	–	–	–	1	1	crude enzyme
20	94	5.51	–	0.2	–	1	1	crude enzyme
50	84	12.4	–	–	5%	2	2	crude enzyme
50	91	13.5	–	–	5%	–	–	freeze-dried cells

(-: no addition)

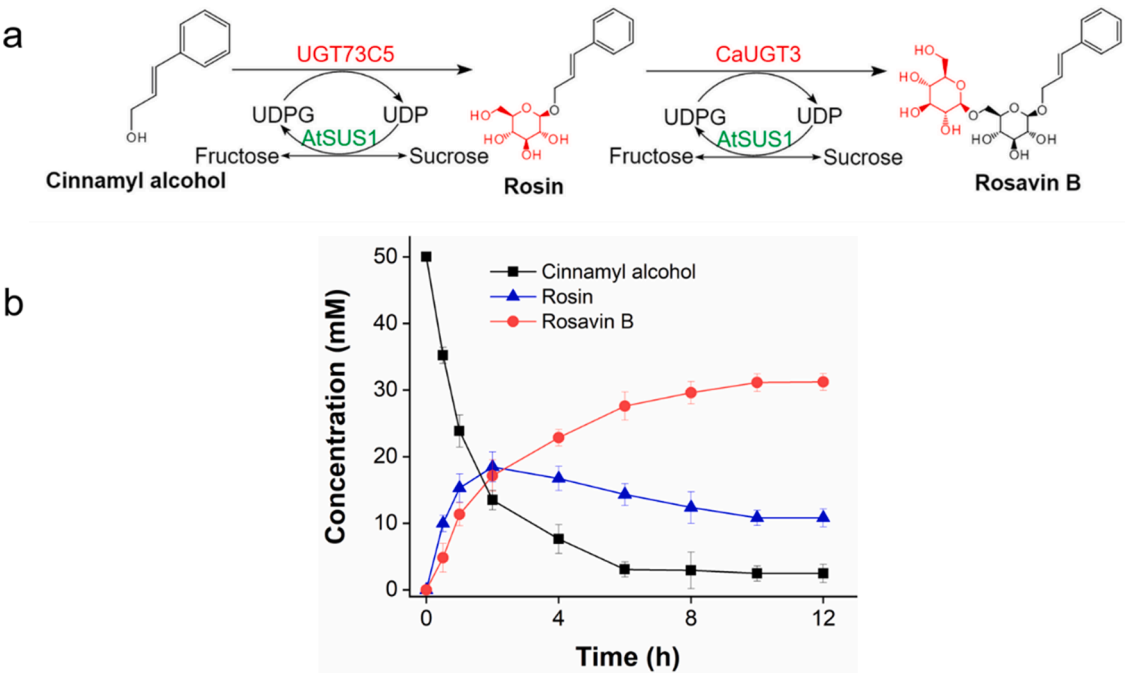

Fig. 6. Cascade enzymatic reaction for rosavin B synthesis. (a) Schematic of cascade enzymatic reaction; (b) Synthesis of rosavin B in cascade reaction. The reaction was performed under the condition of 50 mM PBS (pH 7.5), 50 mM CA dissolved in 5% (w/v) HPCD, 500 mM sucrose, 2 U/mL UGT73C5, 2 U/mL CaUGT3 and 4 U/mL AtSUS1 and conducted at 40 °C for 12 h.

required.

UGT73C5 has an optimum pH of 9.5 (Fig. S6b), which is beneficial to the production of rosin from CA. However, AtSUS1 showed better performance at pH 5.5 and 7.5, and lower pH favors high yield produciton of UDPG from sucrose [28,44-45]. In the coupled reaction, the compatibility of activity and stability between AtSUS1 and glycosyltransferase is important for the production of glucoside products [36–37], which could lead to the optimum pH of 7.5 (Fig. 3a) for the UGT73C5-AtSUS1 coupled reaction. Elevated concentration of CA usually leads to insolubility and mass transfer issues [46–47]. Herein, CA was easily dissolved in HPCD (Fig. 5a), which also shown great solubilizing potential for phloretin [36]. Addition of co-solvents could promote the solubility of CA, whereas also inhibit the enzymatic activity. As shown in Table 2, at elevated concentration of CA (50 mM), *E. coli* BL 21 (DE3)/UGT73C5-AtSUS1 freeze-dried cells reaction displayed higher yield (91%) of rosin than crude enzymes (84%), which may be attributed to better resistance to HPCD of freeze-dried cells than crude enzymes. To our best knowledge, this is highest yield among all reported synthesis routes of rosin, which is much higher than that via chemical synthesis route (11% yield) [17,18], biotransformation by plant callus (0.7% yield) [22], and fermentation of recombinant *E. coli* (0.8% yield) [23].

As a glycosylated derivative of rosin, rosavin has higher physiological activities and more broad application prospects [5,8]. However, based on our knowledge, no suitable glycosyltransferase has been identified for direct production of rosavin. CaUGT3 was reported to catalyze the glycosylation of rosin for the synthesis of one of rosavin analogues (rosavin B) [10], which may lead to a new extended application prospects because different linked saccharide of phenylpropanoid usually lead to different bioactivities [11–13]. Herein, 62% yield of rosavin B (14.2 g/L) was achieved from 50 mM CA in the enzymatic cascade reaction by UGT73C5, CaUGT3, and AtSUS1, which is 3.0-fold of that produced by fermentation of engineered *E. coli* (4.7 g/L) [10]. However, 20% of rosin is still not completely transformed into rosavin B in this cascade reaction (Fig. 6b), suggesting that engineering of CaUGT3, efficient enzyme formulation [48], and cascade process optimization should be further investigated in future work.

5. Conclusion

In summary, glycosylation catalyzed by UGT73C5 was performed for the synthesis of rosin from CA, and rosin yield of 96% was achieved with UDPG/CA ratio of 5:1. To avoid usage of expensive UDPG, a UGT73C5-AtSUS1 coupled reaction was performed with addition of merely 0.2 mM UDP, and a finial yield of 100% was obtained for rosin from 2 mM CA. Also coupled reaction with crude enzymes was attempted to further reduced the costs (no addition of UDP), giving a slightly decreased rosin yield compared with purified enzymes. To investigate rosin production from elevated concentrations of CA (50 mM) using HPCD (5%, w/v) as a co-solvent, freeze-dried cells biocatalysis was conducted and higher rosin yield of 91% was achieved. Furthermore, the synthesis of rosavin B was explored in cascade reaction catalyzed by UGT73C5, CaUGT3, and AtSUS1, and a yield of 62% was obtained from 50 mM CA. This study provides a high-yielding and cost-effective approach for the synthesis of rosin.

CRediT authorship contribution statement

Ruizhi Han: Supervision, Project administration, Writing – original draft, Writing – review & editing. **Honghui Fang:** Investigation, Methodology, Writing – original draft. **Zhaoyue Fan:** Investigation, Methodology. **Yu Ji:** Methodology, Writing – review & editing. **Ulrich Schwaneberg:** Methodology, Writing – review & editing. **Ye Ni:** Supervision, Project administration, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors have declared that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mcat.2023.113035.

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