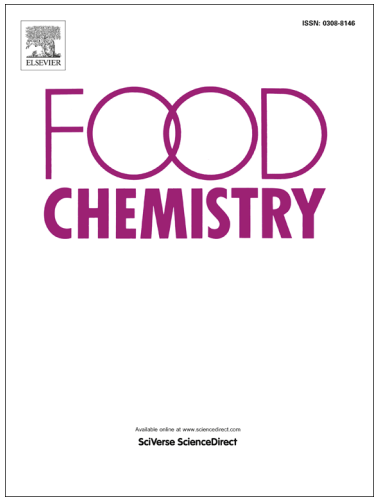

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Synthesis of rebaudioside D, using glycosyltransferase UGTSL2 and *in situ* UDP-glucose regeneration

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Abstract

Steviol glycosides from *Stevia rebaudiana* leaves are used in stevia-based sweeteners for their intense sweetness and low calories. Rebaudioside D is present in leaves in minute quantities (~ 0.4–0.5% w/w total dry weight), but it is ~350 times sweeter than sucrose, and sweeter than the more abundant rebaudioside A and stevioside. In the present study, pathways for rebaudioside D synthesis and UDP-glucose recycling were developed by coupling recombinant UDP-glucosyltransferase UGTSL2 from *Solanum lycopersicum* and sucrose synthase StSUS1 from *Solanum tuberosum*. Reaction parameters, including substrate ratio, sucrose concentration, temperature, crude extract concentration, and reaction time, were evaluated, and 17.4 g/l of rebaudioside D (yield = 74.6%) was obtained from 20 g/l of rebaudioside A after 20 h, using UDP or UDP-glucose in recombinant cell crude extracts. Extending the reaction time generated rebaudioside M2 from further glycosylation of rebaudioside D. K_m values for UGTSL2 indicated a higher affinity for rebaudioside D than for rebaudioside A.

Keywords

Stevia rebaudiana; UDP-glycosyltransferase; UGTSL2; Rebaudioside D; Rebaudioside M2

1. Introduction

Steviol glycosides (SGs) are plant-derived, natural zero-calorie (non-caloric) sweeteners that are attracting increasing interest due to increasing public awareness of adverse health impacts caused by excessive sugar consumption (Philippe, De Mey, Anderson, & Ajikumar, 2014). *Stevi*a *rebaudiana* Bertoni (family: *Asteraceae*) is a sweet herb native to Paraguay and Brazil, and leaf extracts contain more than 30 intensely sweet SGs (Chaturvedula, Rhea, Milanowski, Mocek, & Prakash, 2011; Wolwer-Rieck, 2012). Stevioside and rebaudioside A (Fig. 1) are the major SGs found in *stevia*, the latter of which has an additional glucose attached to the C-13 hydroxyl position of the common steviol aglycone cone, making it sweeter and more pleasant-tasting than stevioside (Brandle, Starratt, & Gijzen, 1998). As the number and type of sugars attached to the steviol C-13 hydroxyl and/or C-19 carboxylic acid functional group distinctly affect the taste, great attention has been paid to identifying novel SGs with more highly branched sugar chains at these positions to enhance sweetness (Chaturvedula & Prakash, 2011a, 2011b; Chaturvedula, Upreti, & Prakash, 2011). *Stevia* extracts contain minor components, such as rebaudioside D and rebaudioside M (Fig. 1), both of which have been isolated from the leaves of *S. rebaudiana* Morita that was developed by selective breeding from *S. rebaudiana* Bertoni, and reportedly characterised in 2009 (Ohta, et al., 2010). In 2013 and 2014, steviol glycoside preparations containing rebaudiosides D and M received Generally Recognized as Safe (GRAS) status from the United States Food and Drug Administration (USFDA). More isomers of rebaudioside D and M have since been identified, including rebaudioside I, D2, and M2 (Fig. 1) (Prakash, et al., 2014a; Prakash, et al., 2014b; Prakash, et al., 2014c). All steviol glycosides are likely metabolised to steviol, regardless of the

glucose combinations and β-1, β(1−2), β(1−3), or β(1−6) linkages in the glycosidic side chains (Purkayastha, et al., 2016). Therefore, safety data available for individual SGs, including rebaudiosides A, C, D, and M, can be used to support the safety of purified steviol glycosides in general.

Given that growth is potentially limited by agricultural sustainability, biotechnological production platforms for natural SGs can address supply scalability limitations associated with plant-based production. The development of microbial synthetic biology has facilitated *de novo* SG biosynthesis (Olsson, Carlsen, Semmler, Simon, Mikkelsen, & Moller, 2016; Wang, Li, Xiong, & Wang, 2016). Glycosyltransferases (GTs) are enzymes that have evolved naturally to catalyse glycosylation reactions, and they transfer sugar moieties from activated sugar donors to acceptor molecules with high efficiency and regiospecificity (De Bruyn, Maertens, Beauprez, Soetaert, & De Mey, 2015; Lim, 2005). GTs catalysing SG formation in *S. rebaudiana* belong to the Leloir GT family and use uridine diphosphate glucose (UDP-glucose) as the activated glycosyldonor (Madhav, Bhasker, & Chinnamma, 2013; Richman et al., 2005). Sucrose synthases (SuSys), that catalyse the transfer of a glucosyl moiety from sucrose to a nucleoside diphosphate (NDP) in a pH-dependent manner, have emerged as industrially attractive catalysts, and they pave the way for the application of Leloir GTs in cost-effective processes (Schmolzer, Gutmann, Diricks, Desmet, & Nidetzky, 2016). It is hoped that one-pot SuSy-GT cascade reactions for glycoside synthesis can be harnessed for novel SG sweetener production (De Bruyn, et al., 2015; Wang, et al., 2016).

The glycosyltransferase UGTSL2 from *Solanum lycopersicum* catalyses the

bioconversion of rebaudioside A to rebaudioside D, rebaudioside D2, and rebaudioside M2, with rebaudioside D as the major product (Prakash, et al., 2014a; Prakash, et al., 2014b). Rebaudioside D is only a minor constituent in natural SGs, but it is a natural sweetener with significantly less bitterness and comparable sweetness to rebaudioside A (Allen, Mcgeary, & Hayes, 2013; Nikiforov, Rihner, Eapen, & Thomas, 2013). In the present study, SuSy-GT cascade reactions for rebaudioside D synthesis and UDP-glucose recycling were developed by coupling the activities of recombinant UDP-glucosyltransferase UGTSL2 from *S. lycopersicum* and sucrose synthase StSUS1 from *Solanum tuberosum* (Sauerzapfe, Engels, & Elling, 2008). Initial UDP or UDP-glucose was provided by recombinant cell crude extracts. The generated products were analysed, and factors influencing product formation were investigated.

2. Materials and methods

2.1. Plasmid and strain construction

Coding regions derived from *S. lycopersicum* UGTSL2 (NCBI Reference Sequence: XP_004250485.1) and *S. tuberosum* sucrose synthase SUS1 (*St*SUS1; UniProtKB/Swiss-Prot: P10691), with codons optimised for *E. coli* heterologous expression, were synthesised and cloned into the *E. coli* expression vector pRSFDuet-1 (Novagen) by GenScript (Nanjing, China). The codon-optimised UGTSL2 gene, with a 6-histidine tag added at the C-terminus, was inserted between the *Nde*I and *Xho*I restriction endonuclease sites, and the modified *St*SUS1 gene was inserted between the *Nco*I and *Eco*RI restriction endonuclease sites, yielding plasmid pRSFDuet-SL2-SUS1 (Fig. 2). This plasmid was transformed into the *E. coli*

BL21(DE3) strain (TransGen Biotech, Beijing, China), resulting in the recombinant *E. coli* BL21 (pRSFDuet-SL2-SUS1) strain.

2.2. Expression of recombinant glucosyltransferase and sucrose synthase in E. coli

Individual colonies of *E. coli* BL21 (pRSFDuet-SL2-SUS1) were inoculated into 5 ml of Luria Bertani medium (10 g/l of tryptone, 5 g/l of yeast extract, and 5 g/l of NaCl) containing 50 μg/ml of kanamycin and incubated for 8 h at 37°C with shaking at 250 rpm. Next, 2 ml of the initial cultures were inoculated into 100 ml of fresh autoinduction medium (15 g/l of tryptone, 25 g/l of yeast extract, 10 g/l of NaCl, 2 g/l of glucose, and 0.5 g/l of lactose) in 500 ml flasks supplemented with antibiotics. Flasks were incubated at 30°C for 2 h, and when the culture turbidity (OD_{600}) reached approximately 0.2, cultures were kept at 25°C for a further 22 h with shaking at 200 rpm.

2.3. Enzymatic synthesis of rebaudioside D using UGTSL2 and StSUS1

Induced cells were harvested by centrifugation at 7500 rpm (5289 ×*g*) at 4°C for 5 min, washed twice, and resuspended in 50 mM sodium phosphate buffer (pH 7.2). A lysate containing soluble proteins was obtained by sonicating the cell suspension in an ice bath, using an Ultrasonic Cell Disruptor JY92-IIN (SCIENTZ, Ningbo, China) and subsequent clarification by centrifugation at 6010 ×*g* and 4°C for 20 min. The resultant supernatant was used as the crude extract. Total protein concentrations were measured by the Bradford assay, using bovine serum albumin (BSA) as a standard (Bradford, 1976). For synthesis of rebaudioside D, reaction mixtures (20 ml) containing 20 g/l of rebaudioside A (a higher

concentration of rebaudioside A was heated to dissolve, then cooled to the reaction temperature), 60 g/l of sucrose, aliquots of the crude extract, 3 mM $MgCl_2$, and sodium phosphate buffer (50 mM, pH 7.2) were incubated in 50 ml flasks at 30°C for 24 h with shaking at 200 rpm. During incubation, samples were removed at defined time points, heated at 95°C for 10 min, clarified by centrifugation at 13523 ×*g* for 1 min, and the supernatant was immediately analysed by high-performance liquid chromatography (HPLC) or stored at 4°C.

2.4. Glucosyltransferase and sucrose synthase activity assays

UGTSL2 and *St*SUS1, expressed in *E. coli* (pRSFDuet-SL2-SUS1), were subjected to enzyme activity assays. The glucosyltransferase assay mix contained 1 mg of total protein from the crude extract, 1.2 mM rebaudioside A or rebaudioside D, 2 mM UDP-glucose, and 3 mM $MgCl_2$ in sodium phosphate buffer (50 mM, pH 7.2) in a total volume of 3 ml. Reactions were performed at 30°C for 30 min (in the linear range of product formation). Samples were removed and heated at 95°C for 10 min to terminate the reaction. The SG concentration was determined by HPLC (described below). One unit (U) of glucosyltransferase activity was defined as the amount of enzyme that produced 1 µmol of rebaudioside D from rebaudioside A, or rebaudioside M2 from rebaudioside D, per min under the described conditions.

The sucrose synthase assay mix contained 6 mg of total protein from the crude extract, 500 mM sucrose, and 10 mM UDP in 50 mM potassium phosphate buffer (pH 7.2) in a total volume of 3 ml. Reactions were incubated at 30°C, and samples were removed every hour and heated at 95°C for 10 min. Supernatants collected after centrifugation at 13523 ×*g* were measured, using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One unit (U) of

sucrose synthase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min under the given assay conditions. All reactions were performed in triplicate and corrected, using a reference of identical composition to the reaction mixture with crude extract containing only overexpressed glucosyltransferase UGTSL2.

2.5. Kinetic evaluation of glucosyltransferase UGTSL2

In vitro glycosylation reactions were carried out in 50 mM potassium phosphate buffer (pH 7.2) at 30°C in a total volume of 3 ml, using 1 mg of total protein from the crude extract, different concentrations of rebaudioside A or rebaudioside D (0.2–1.4 mM), 2 mM UDP-glucose, and 3 mM $MgCl_2$. After incubation for 40 min, samples were heated at 95°C for 10 min, and the supernatant was assayed by HPLC (described below). Glycosylation rates were calculated as μmol of glucosides formed per reaction time per amount of protein (μmol/min/mg). The Michaelis-Menten kinetic model was fitted to glycosylation rates versus substrate concentrations, and the Km of UGTSL2 for rebaudioside A or rebaudioside D was calculated according to the Lineweaver-Burk plot (Lineweaver & Burk, 1934).

2.6. HPLC analysis

Sample analysis was conducted on an UltiMate 3000 HPLC system (Dionex China Limited, Beijing, China), using an Agilent TC-C18 column (250 mm × 4.6 mm; The Netherlands) maintained at 55°C with UV detection at 210 nm. Mobile Phase A was 0.1% HCOOH in acetonitrile (MeCN), and mobile Phase B was 0.1% HCOOH in water. The flow rate was 1.0 ml/min, the injection volume was 10 μl, and the gradient was as follows: 0.000 min (25% A, 75%

B), 15.000 min (47% A, 53% B), 20.000 min (100% A, 0% B), 20.010–25.000 min (100% A, 0% B), 25.010 min (25% A, 75% B), 25.010–30.000 min (25% A, 75% B) (Olsson, et al., 2016; Prakash, et al., 2014c). Rebaudioside D standard (97.6%) for HPLC was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (China). Quantification of rebaudioside M2 was performed using a rebaudioside M standard kindly provided by Xinghua GL Stevia Co., Ltd (China). The product yield was calculated as follows:

Rebaudioside D yield (%) = C(RD) / C(RA)

Rebaudioside M2 yield (%) = C(RM2) / C(RA)

where C(RA) represents the initial rebaudioside A molar concentration, and C(RD) and C(RM2) respectively represent the increased rebaudioside D or rebaudioside M2 molar concentration after reaction.

2.7. LC-MS analysis

LC-MS analysis was conducted on a Waters ACQUITY UPLC I-CLASS system coupled with negative electrospray ionisation (ESI) mass spectrometry (MS). Mass spectra in negative ion mode were acquired under the following conditions: capillary voltage = 3 kV, cone voltage = 40 V, desolvation gas flow = 800 l/h, cone gas flow = 50 l/h, LM resolution = 11.3, HM resolution = 14.0, ion energy = -0.3, m/z range =100–1500. An AXQUITY UPLC HSS T3 analytical column (2.1 × 100 mm, 1.8 μm) was used and maintained at 55°C, with UV detection at 210 nm. The flow rate was 1 ml/min, using 0.1% formic acid in water; acetonitrile (70:30, v:v)

as the mobile phase. The injection volume was 20 µl.

3. Results and discussion

3.1. Expression of recombinant UGTSL2 and StSUS1 in E. coli

The plasmid pRSFDuet-SL2-SUS1 was transformed into *E. coli* and expression carried out as described above. Presumably because translation occurs almost simultaneously with transcription, and because posttranslational modification systems are lacking in *E. coli*, many proteins, especially eukaryotic proteins, form undesirable aggregates (inclusion bodies) when heterologously expressed in this prokaryotic host (Georgiou & Valax, 1999), which restricts the production of active recombinant proteins. In the present study, we found that expression of plant-derived UDP-glucosyltransferase UGTSL2 and sucrose synthase StSUS1 in *E. coli* resulted mainly in insoluble inclusion bodies, with little soluble protein. However, the obtained recombinant UGTSL2 and StSUS1 were functional for rebaudioside D synthesis. Samples were removed after incubation of 19.2 mU/ml of UGTSL2 and 614.7 mU/ml of StSUS1 with crude extract, 10 g/l of rebaudioside A, 30 g/l of sucrose, and 3mM Mg^{2+} for 14 h, and 9.72 g/l of rebaudioside D was synthesised, representing a yield of 83.3%.

3.2. Effect of substrate ratio on rebaudioside D synthesis

With UDP-glucose as the direct sugar donor for transglycosylation, a higher UDP-glucose concentration in the reaction system was more conducive to the accumulation of product, but less recycling of UDP-glucose occurred at a higher initial concentration of UDP-glucose, probably due to inhibition of UDP-glucosyltransferase resulting from the high UDP

concentration (Wang, et al., 2016). Based on addition of only 0.006mM UDP, ~310 UDP-glucose regeneration cycles were achieved during the synthesis of rebaudioside A from stevioside, which is an overestimate because UDP and UDP-glucose in the cell lysate cannot be ignored (Schmolzer, et al., 2016; Wang, et al., 2016). Given that UDP-glucose is expensive, we used UDP-glucose and UDP in crude extracts for our transglycosylation reaction systems. Thus, neither UDP-glucose nor UDP were added, and only rebaudioside A and sucrose substrates were added to the SuSy-GT cascade reactions. When the substrate feed ratio was 1:1 (rebaudioside A: sucrose; w:w), the yield of rebaudioside D was only 34.8% (8.1 g/l), and 12.6 g/l of rebaudioside A remained in the reaction after 14 h (Fig.3(A)). Upon increasing the concentration of sucrose, the yield of rebaudioside D was effectively improved to 43.2% (10.1 g/l) and 55.6% (12.9 g/l) with substrate feed ratio of 1:3 and 1:5, respectively. These results suggest production of rebaudioside D was promoted by increasing the ratio of sucrose to rebaudioside A under our experimental conditions.

3.3. Effect of reaction temperature on rebaudioside D synthesis

The reaction temperature had a great effect on rebaudioside D synthesis, as expected for most enzyme reactions. At 20°C, the enzyme activities were restricted, and the reaction rates were very slow; hence, 67.7% of rebaudioside A remained in the reaction after 14 h. Correspondingly, upon increasing the temperature, the yields of rebaudioside D were increased to 52.4.0% (12.2 g/l) and 58.3.0% (13.6 g/l) at 30°C and 40°C, respectively (Fig. 3(B)). The effect of temperature on the activities of glycosyltransferase UGTSL2 and sucrose synthase StSUS1 were investigated (**Table 1**). Both enzymes exhibited increased activity

when the temperature was increased from 20°C to 40°C. The highest temperature tested (40°C) was optimal for synthesis of rebaudioside D by the coupled activities of UGTSL2 and StSUS1.

Table 1 Specific activity of recombinant UGTSL2 at different temperatures

Temperature	Specific activity (mU/mg)	
(°C)	UGTSL2	StSUS1
20	2.43 ± 0.13	20.4 ± 2.26
30	4.24 ± 0.34	44.6 ± 2.75
40	6.12 ± 0.04	74.4 ± 3.24

3.4. Effect of crude extract concentration on rebaudioside D synthesis

As the crude extract contained, not only the two overexpressed recombinant enzymes, but also the cofactor UDP-glucose and UDP needed for biocatalysis, the effect of crude extract concentration on rebaudioside D synthesis was investigated. When the protein concentration in the reaction solution was 3 mg/ml following addition of crude enzyme, the yield of rebaudioside D was only 23.4% (5.5 g/l) after 14 h, which was the lowest value achieved. However, by increasing the amount of crude extract, the yields of rebaudioside D were effectively improved to 42.6% (10.0 g/l) and 62.0% (14.5 g/l) in with protein concentrations of 6 mg/ml and 9 mg/ ml, respectively (Fig. 3(C)).

3.5. Effect of reaction time on rebaudioside D synthesis

When the reaction was carried out under conditions involving a 1:3 substrate feed ratio and 6 mg/ml of crude extract at 30°C for 20 h, the yield of rebaudioside D was increased to 17.4 g/l (74.6%; Fig. 3(D)). However, when the reaction time was extended to 25 h, rebaudioside D did not accumulate any further. As shown in Fig. 3(E), this could be because any further conversion was catalysed by the recombinant UGTSL2. From the results of HPLC analysis, a new product, at 5.102 min, of larger molecular weight was found near the rebaudioside D peak (6.562 min). As demonstrated by LC-MS analysis of the product ion scan spectrum, the characteristic fragmentation of $[M+H]^+$ for the new steviol glycoside gave an *m/z* value of 1291.6 (Fig. 4). Therefore, this new product was predicted to be rebaudioside M2, presumably generated from rebaudioside D by glycosyltransferase UGTSL2 from *S. lycopersicum* (Prakash, et al., 2014b).

3.6. Substrate preference of glucosyltransferase UGTSL2

As glucosyltransferase UGTSL2 could catalyse, not only rebaudioside A, but also rebaudioside D, we investigated the substrate preference (**Table 2**). The specific activity of UGTSL2 for rebaudioside D was ~2.5-fold higher than that for rebaudioside A, suggesting that glucosyltransferase UGTSL2 catalyses rebaudioside D (to form rebaudioside M2) more readily than rebaudioside A (to produce rebaudioside D). The K_m values also indicated a higher affinity for rebaudioside D than for rebaudioside A. Thus, glycosyltransferase UGTSL2 catalyses the synthesis of rebaudioside D and M2 when rebaudioside A is added as substrate. When rebaudioside D is required as the major product, the catalytic reaction should be stopped at the appropriate time before further conversion to rebaudioside M2.

Table 2 Enzyme activity and Km of recombinant UGTSL2

Enzyme	Specific activity (mU/mg)	K_m (mM)
UGTSL2[a]	4.62 ± 0.15	0.89 ± 0.04
UGTSL2[b]	16.3 ± 0.09	0.29 ± 0.01

Note: All data were averaged from triplicate experiments.

[a] rebaudioside A was used as substrate,

[b] rebaudioside D was used as substrate.

Due to their unique properties, the development of natural SG sweeteners is of great interest to the global food and beverage industries (Purkayastha, et al., 2016). Stevioside and rebaudioside A, as the main SG sweeteners in the market, are generally extracted and purified from the *Stevia* leaves with economic viability because of their relatively high content in *Stevia*. As for rebaudioside D, which is present in minute quantities in the selective breeding of *S. rebaudiana*, its enzymatic preparation from rebaudioside A or stevioside is probably an economically effective method for production of rebaudioside D on a commercial scale. In addition, glycosyltransferases, formerly believed to be rather selective towards acceptors and donors, can now be used in more flexible ways with structurally diverse analogues ((Křen & Thiem, 1998). Employing novel glycosyltransferases in order to alter the glycosylation patterns of SG products is an attractive route to improving their taste quality. Although rebaudioside M2 was the side-product in this transformation with glycosyltransferase UGTSL2, production of

rebaudioside M2 in a large amount is feasible under controlled experimental conditions. More work needs to done, in order to get enough pure rebaudioside M2 to test its sweetness and taste attributes.

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4. Conclusions

In the current work, we established a SuSy-GT cascade reaction system for rebaudioside D synthesis and UDP-glucose recycling by coupling the activities of recombinant UDP-glucosyltransferase UGTSL2 from *S. lycopersicum* and sucrose synthase StSUS1 from *Solanum tuberosum*. A higher ratio of sucrose and rebaudioside A, a higher reaction temperature, and a higher concentration of crude extract were favourable for conversion of rebaudioside A. Glucosyltransferase UGTSL2 not only catalyses the synthesis of rebaudioside D from rebaudioside A; it also converts rebaudioside D to rebaudioside M2.

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Author contributions

LC and YL conceived and designed the study. LC, PS and FZ performed the experiments and analysed the data. LC wrote the paper. KC, HJ, MY, DG and PO reviewed and edited the

manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interests

The authors declare that they have no competing interests.

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Figure legends

Fig. 1. Chemical structures of some steviol glycosides.

Fig. 2. Plasmid pRSFDuet-SL2-SUS1 used for expression of UDP-glucosyltransferase UGTSL2 from *Solanum lycopersicum* and sucrose synthase StSUS1 from *S. tuberosum*.

Fig. 3. The influencing factors for rebaudioside D production.

(A) Effect of the ratio of rebaudioside A to sucrose on rebaudioside D synthesis catalysed by the UGTSL2 and StSUS1 reaction system. UGTSL2 (16.4 mU/ml) and 235 mU/ml StSUS1 from the crude extract were incubated with 20 g/l of rebaudioside A, sucrose (20, 60, or 100 g/l), and 3 mM Mg^{2+} at 30°C and pH 7.2 for 14 h.

(B) Effect of reaction temperatures on rebaudioside D synthesis by the UGTSL2 and StSUS1 reaction system. UGTSL2 (16.4 mU/ml) and 235 mU/ml StSUS1 from the crude extract were incubated with 20 g/l of rebaudioside A, 60g/l of sucrose, and 3 mM Mg^{2+} at pH 7.2 for 14 h. The reaction temperature was 20, 30, or 40°C.

(C) Effect of crude extract concentration on rebaudioside D synthesis by the UGTSL2 and StSUS1 reaction system. UGTSL2 (8.2, 16.4, or 24.6 mU/ml) and StSUS1 (118, 235, or 353 mU/ml) from the crude extract were incubated with 20 g/l of rebaudioside A, 60 g/l of sucrose, and 3 mM Mg^{2+} at 30°C and pH 7.2 for 14 h.

(D) Effect of reaction time on rebaudioside D synthesis by the UGTSL2 and StSUS1 reaction system. UGTSL2 (28.3 mU/ml) and 361 mU/ml of StSUS1 from the crude extract were incubated with 20 g/l of rebaudioside A, 60 g/l of sucrose, and 3 mM Mg^{2+} at 30°C and pH 7.2 for 25 h.

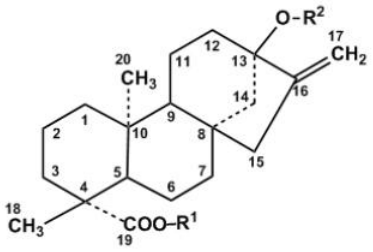
(E) HPLC analysis of the enzymatic synthesis of rebaudioside D from rebaudioside A using

crude enzymes. (a) 1.0 g/l of rebaudioside A and rebaudioside D standards; (b) Samples were removed immediately after reaction; (c and d) Samples were withdrawn after reaction for 12 and 24 h, respectively. UGTSL2 (25.5 mU/ml) and 268 mU/ml of StSUS1 from the crude extract were incubated with 20 g/l of rebaudioside A, 60 g/l of sucrose, and 3 mM Mg^{2+} at 30°C and pH 7.2.

Fig. 4. LC-MS analysis of the new steviol glycoside (peak #1) and rebaudioside D (peak #2) from the enzymatic catalysis of rebaudioside A.

Figures

Figure 1



	R^1	R^2
Stevioside	Glcβ1-	Glcβ1-2Glcβ1-
Rebaudioside A	Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
Rebaudioside D	Glcβ1-2Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
Rebaudioside I	Glcβ1-3Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
Rebaudioside D2	Glcβ1-6Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
Rebaudioside M	Glcβ1-2(Glcβ1-3)Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
Rebaudioside M2	Glcβ1-2(Glcβ1-6)Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-

Figure 2

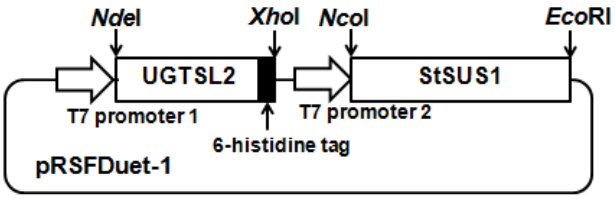
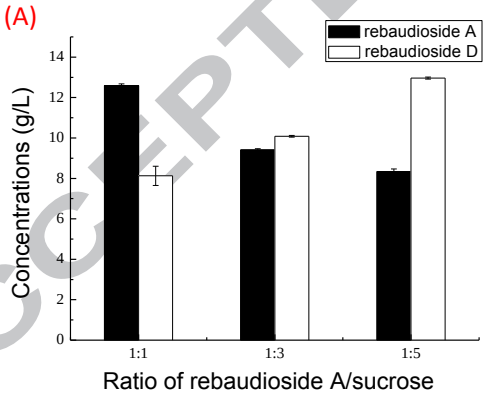


Figure 3



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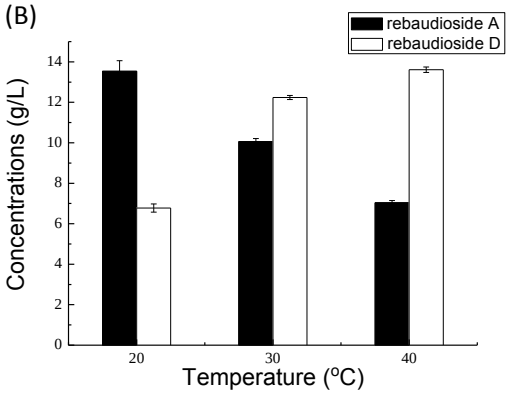

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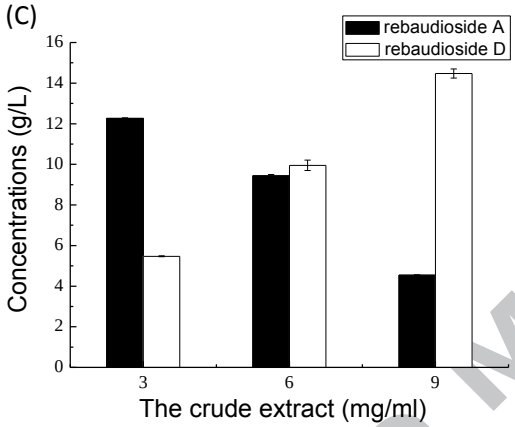

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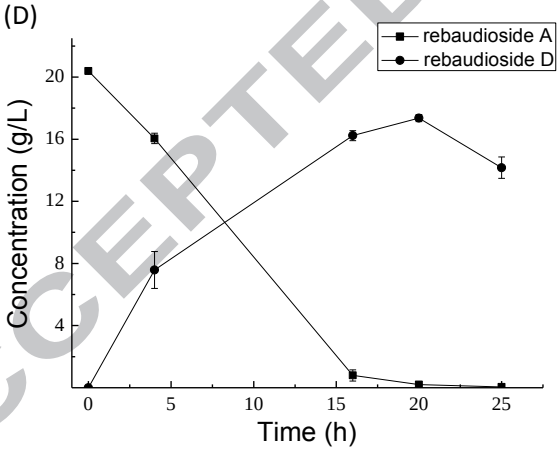


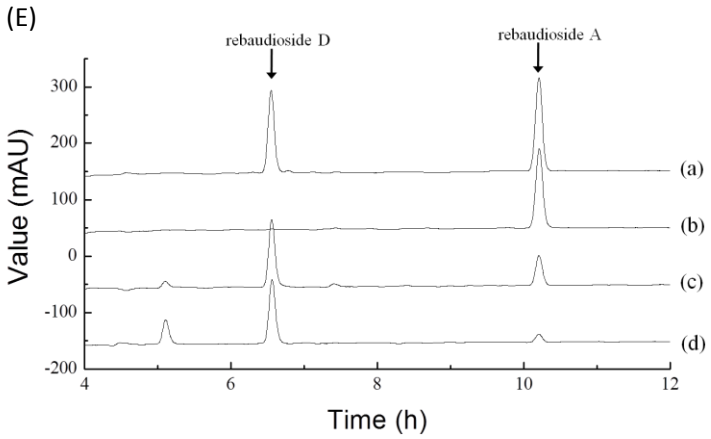
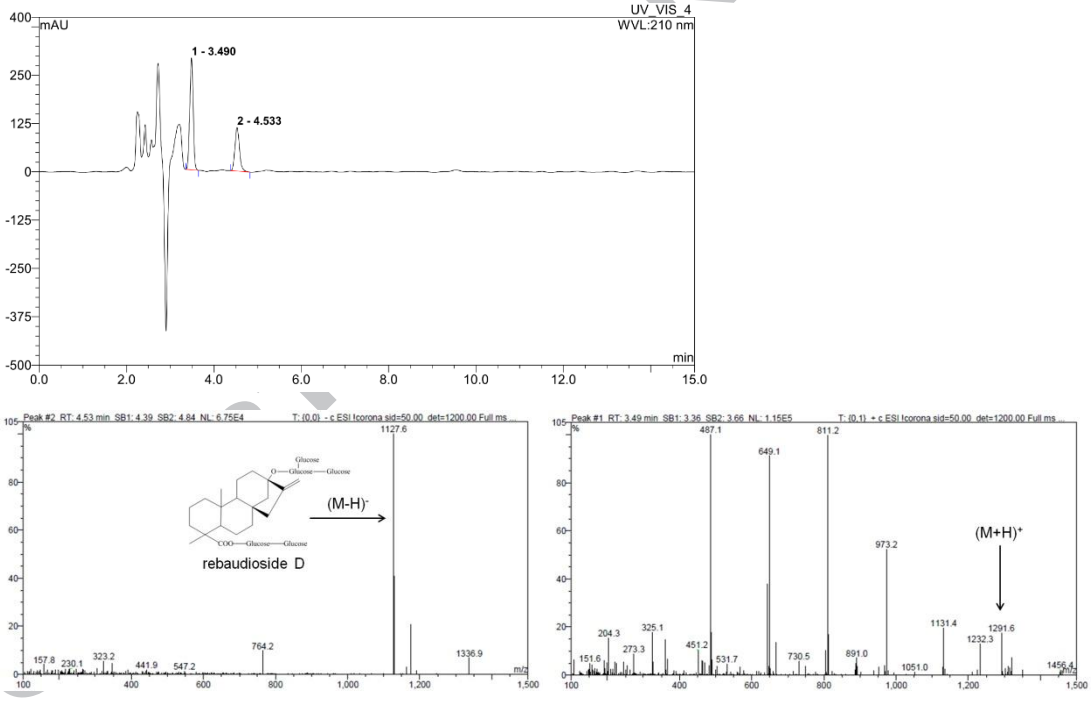

(E)
rebaudioside D
rebaudioside A
Value (mAU)
Time (h)
(a)
(b)
(c)
(d)

Figure 4


UV_VIS_4
WVL:210 nm
mAU
min
1 - 3.490
2 - 4.533
rebaudioside D
(M-H)-
(M+H)+

Highlights:

1. A SuSy-GT cascade reaction system is established for rebaudioside D synthesis.

2. The yield of rebaudioside D by enzymatic synthesis is up to 74.6% (17.4 g/L).

3. UGTSL2 not only catalyses the synthesis of rebaudioside D but also rebaudioside M2.

4. The K_m values of UGTSL2 indicate a higher affinity for rebaudioside D than for rebaudioside A.