J Ind Microbiol Biotechnol (2017) 44:1343–1354
DOI 10.1007/s10295-017-1960-x

BIOCATALYSIS - ORIGINAL PAPER

# High production of genistein diglucoside derivative using cyclodextrin glycosyltransferase from *Paenibacillus macerans*

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Received: 21 February 2017 / Accepted: 9 June 2017 / Published online: 28 June 2017


**Abstract** Genistein has been regarded as one important soy isoflavone with multiple health benefits, whereas its applications are limited by the low hydrophilicity. To improve the water solubility, codon optimized cyclodextrin glycosyltransferase from *Paenibacillus macerans* was employed for genistein transglycosylation in this study. At least four transglycosylation products were produced and identified by HPLC and LC–MS: genistein monoglucoside, diglucoside, triglucoside, and tetraglucoside derivatives. Obviously, the yields of genistein monoglucoside and genistein diglucoside exhibited great superiority compared with other two products. To maximize the yield of genistein diglucoside, various reaction conditions such as genistein dissolvents, glycosyl donors, substrates concentrations and ratios, enzyme concentrations, reaction pH, temperature, and time were optimized. Finally, the yield of genistein diglucoside was enhanced by 1.5-fold under the optimum reaction system. Our study demonstrates that the production of genistein diglucoside could be specifically enhanced, which is one important genistein derivative with better water solubility and stability.

**Keywords** Genistein · Genistein diglucoside · Solubility · Co-α-CGTase · Optimization

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## Introduction

Soybeans and soybean-containing foods are usually of high nutritional and functional qualities due to their beneficial effects and therapeutic potential for various diseases [16, 34]. Isoflavones, as one main class of secondary metabolites in soybeans, are presumed to be involved in most of the health benefits of soybeans [32]. Isoflavones are often regarded as phytoestrogens due to their estrogenic activity through interaction with estrogen receptors in cells [2]. Among a variety of isoflavones, genistein (4′,5,7-trihydroxyisoflavone) has attracted considerable attentions due to its multiple pharmacological effects [16]. For instance, genistein was reported to possess anticancer activity and was capable of inhibiting the proliferation of various cancer cells, such as human ovarian cancer [3], prostate cancer [15], colon cancer [6], and so on. Genistein also exhibited antioxidant activity and could arrest the growth of malignant melanoma and inhibit ultraviolet light-induced oxidative DNA damage [29]. In addition, genistein also showed positive effects on bone-loss prevention [13], contractility inhibition of vascular smooth muscle [1], and intestinal [33]. Commonly, genistein predominantly presents as its glycoside form, genistin (genistein 7-*O*-β-D-glucoside) and sophoricoside (genistein 4′-*O*-β-D-glucoside), which can be converted to genistein by digestive enzymes to exert its biological effects.

However, one major disadvantage referring to genistein is their low water solubility, which seriously limits its applications in food and pharmaceuticals fields, as well as its bioavailability in aqueous systems in vivo. Therefore, improving the water solubility of genistein could expand its application fields and enhance their biological functions.

Transglycosylation is usually regarded as an effective modification reaction for a series of glucosides derivatives by transferring glycosyl groups to flavonoids compounds, which has successfully been used to increase the water solubility of many foods materials [17, 20]. For instance, *Thermus scotoductus* 4-α-glucanotransferase (TS4αGTase) was used for glycosylation of genistin to improve its water solubility [24]. Remarkably, the glycosylated products genistein diglucoside and genistein triglucoside showed dramatically increased water solubility by $3.7 \times 10^3$ and $4.4 \times 10^4$ folds, respectively. Subsequently, these soluble genistein glycosylated derivatives were proved to have similar antioxidant activity level as genistein [4], suggesting that they have great potentials as genistein substitutions for foods and pharmaceuticals applications.

Cyclodextrin glucanotransferase (CGTase, EC 3.2.1.19) is one multifunction glycosyltransferase capable of catalyzing four types of reactions such as cyclization, coupling, disproportionation, and hydrolysis [7]. Among them, disproportionation is commonly regarded as an important intermolecular transglycosylation performed by cleaving an α-glycosidic bond of linear maltooligosaccharide and transfer of one part to an acceptor [31]. CGTase has widely been used to improve the physiochemical properties of various raw materials in foods by transglycosylation, such as alleviating stevioside bitterness [14], improving L-ascorbic acid stability [35], and increasing isoflavones solubility [5, 24]. In our previous study, α-CGTase from *Paenibacillus macerans* was engineered by a series of site-saturation mutagenesis strategies [9–11] and chimeric modifications [8, 12] for the synthesis of 2-*O*-D-glucopyranosyl-L-ascorbic acid (AA-2G), which greatly improved the stability of L-ascorbic acid. In addition, to improve the heterologous expression of *P. macerans cgt* gene in *Escherichia coli*, systematic codon optimization strategy was employed in the previous work [27].

In the present study, we performed the transglycosylation of genistein using the α-CGTase from *P. macerans* after systematic codon optimization (co-α-CGTase). At least four genistein glycosylated derivatives were obtained and identified by LC–MS. We also optimized the reaction conditions to attain a high production of the major product genistein diglucoside derivative.

## Materials and methods

### Bacterial strains and chemicals

The α-*cgt* gene from *P. macerans* (CCTCC M203062) after systematic codon optimization (co-α-*cgt*, GenBank accession no. JX412224) was constructed previously [26]. In this study, the co-α-*cgt* gene was cloned into the plasmid pET-20b (+) (TakaRa, Dalian, China) after *pelB* signal peptide. In addition, the recombinant plasmid pET-20b (+)/co-α-*cgt* was transformed into *E. coli* BL21 (DE3) (TakaRa, Dalian, China) for enzyme production. Genistein, α-cyclodextrin (α-CD), and β-cyclodextrin (β-CD) were purchased from Sangon Biotech (Shanghai, China). HPLC grade acetonitrile and deionized water were purchased from Titan Scientific Co., Ltd. (Shanghai, China) and Watsons Group Ltd (Hong Kong, China), respectively. All other chemicals used were of reagent grade from China reagent companies.

### Enzyme preparation and purification

The co-α-CGTase were prepared as the method reported previously [26] with slight modification. Briefly, Luria–Bertani (LB) medium (peptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L) was used as a seed culture, and modified Terrific Broth (TB) (peptone 12 g/L, yeast extract 24 g/L, glycerol 8 g/L, $KH_2PO_4$ 2.32 g/L, and $K_2HPO_4$ 16.43 g/L) medium as a fermentation culture. The recombinant *E. coli* BL21 (DE3)/pET-20b(+)/co-α-*cgt* was inoculated into 20 mL seed medium (in a 250 mL flask) with a final concentration of 100 μg/mL ampicillin, and incubated on a 200 rpm rotary shaker at 37 °C overnight. Then, the seed culture was inoculated (with 4% of ratio, v/v) into 50 mL fermentation medium (in a 500 mL flask) containing 100 μg/mL ampicillin. The initial culture condition was 30 °C, 200 rpm on a rotary shaker. Then, 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction when the optical density at 600 nm ($OD_{600}$) reached 0.6. After that, the culture temperature was changed into 25 °C for 40 h of incubation and then recovered to 30 °C for additional 50 h of incubation. The broth was centrifuged at 10,000×*g* and 4 °C for 5 min, and the supernatant was purified by 30% (w/v) ammonium sulfate salting precipitation, and then dialysis with 14 kDa dialysis membranes (Sangon Biotech, Shanghai, China). Subsequently, the dialysate was further purified by a Ni–NTA agarose column (Qiagen, Chatsworth, CA, USA) as described previously [25]. The purity of protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The activity of co-α-CGTase is represented by its cyclization activity [9]. Briefly, 0.1 mL of CGTase (appropriately diluted in 50 mM sodium phosphate buffer, pH 6.0) was mixed with 0.9 mL of 3% (w/v) soluble starch (dissolved in the similar buffer) and incubated at 40 °C for 10 min. Then, 1.0 mL of HCl (1.0 M) was added into the mixture for reaction termination, and 1.0 mL of 0.1 mM methyl orange in 50 mM phosphate buffer (pH 6.0) was added immediately. After standing for 20 min at 16 °C, the absorbance of mixture at 505 nm was measured. One

unit of co-α-CGTase activity was defined as the amount of enzyme capable of producing 1 μmol α-cyclodextrin per minute.

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

### Transglycosylation of genistein by co-α-CGTase

The initial reaction system (1 mL) included 300 μL of genistein (10 g/L) dissolved in dimethyl sulfoxide (DMSO), 500 μL of α-cyclodextrin (10 g/L) dissolved in PBS buffer (pH 6.5), and 200 μL of purified co-α-CGTase (150 U/mL) dissolved in PBS buffer (pH 6.5). The whole reaction solution was mixed in 2 mL inclosed tube and shaked for 24 h on a 120 rpm rotary shaker at 30 °C. Then, the reaction products were determined by HPLC.

Based on the initial transformation conditions, the influence factors in the transglycosylation reaction were investigated, such as genistein dissolvent, glycosyl donors, concentration and ratio of the glycosyl donor and acceptor, enzyme concentration, reaction temperature, pH, and time. The influence of glucoamylase on transglycosylation products was analyzed by adding different concentration of glucoamylase to reaction mixture and incubating at 65 °C for 6 h. The assay buffer for glucoamylase was 50 mM PBS buffer (pH 5.5). The tolerance of co-α-CGTase in DMSO was determined by incubating of the enzyme at different DMSO concentrations or different incubation time at 4 °C.

### HPLC analysis

An Agilent 1260 Infinity HPLC system equipped with a 250 × 4.6 mm Diamonisil C18 column and an Agilent 1260 WVD detector set at 260 nm were used to quantify the genistein and its glycosylated products. The mobile phase contained solution A (water/phosphoric acid, 100:0.1, v/v) and solution B (acetonitrile). For the gradient elution system, solution B increased gradually from 15 to 85% during a 15 min interval at a flow rate of 0.8 mL/min at 30 °C. 10 μL of sample was injected into the analytical HPLC with an automatic sampler.

### LC–MS analysis

The LC–MS analysis of transglycosylation products was performed using a MALDI SYNAPT Q-TOF Premier mass spectrometer (Waters, USA) with an electrospray ion source performed in the V-Optics negative mode. A BEH C18 column (2.1 × 100 mm) was used for liquid chromatogram on an ACQUITY UPLC (Waters, USA). Solution A (water/phosphoric acid, 100:0.1, v/v) and solution B (acetonitrile) were used as the mobile phase and the gradient elution conditions were as follows: solution B increased gradually from 10 to 60% during a 8 min and then increased to 100% during 4 min with a flow rate of 0.3 mL/min. The injection volume and column temperature were 0.2 μL and 45 °C, respectively. The mass spectrometer scanned from *m*/*z* 0–1500.

### Solubility determination of genistein

Solubility of genistein in different solvents was determined as described by the previous report [24]. Excess genistein were mixed with different solvents (DMSO, ethanol, methanol, ethyl acetate, and distilled water) and sonicated at room temperature for 1 h. After centrifugation (10,000 rpm, 5 min) at room temperature, supernatant of each sample was appropriately diluted and filtered through a 0.14 μm PVDF membrane for HPLC analysis. The standard curve was determined with different concentrations of genistein dissolved in DMSO.

## Results

### Expression and purification of co-α-CGTase

The recombinant *E. coli* BL21 (DE3) harboring plasmid pET-20b (+)/co-α-*cgt* was cultivated for co-α-CGTase expression. The extracellular expression level of crude co-α-CGTase was about 4.0 mg/mL, which was higher than that of the wild-type α-CGTase (approximate 2.6 mg/mL) (data not shown). The result showed that the yield of α-CGTase increased significantly after codon optimization. Furthermore, the cyclization activity of extracellular co-α-CGTase was about 29.3 U/mL, which was around twofold of that of WT α-CGTase (data not shown).

The crude co-α-CGTase was purified with 30% (w/v) ammonium sulfate precipitation and nickel affinity chromatography. As shown in Fig. 1, SDS-PAGE results revealed that there were still much impure proteins after ammonium sulfate precipitation. However, after nickel affinity chromatography, the purity of target protein reached more than 90% and the molecular mass of co-α-CGTase was approximate 75 kDa. In addition, the specific activity of purified co-α-CGTase was about 10 U/mg.

### HPLC analysis of transglycosylation products

The reaction mixture of transglycosylation by co-α-CGTase was analyzed using HPLC. As shown in Fig. 2a, the result showed that at least four novel peaks emerged in the chromatogram of reaction mixture compared with the genistein standard, indicating that at least four newly produced transglycosylation products might be formed after co-α-CGTase

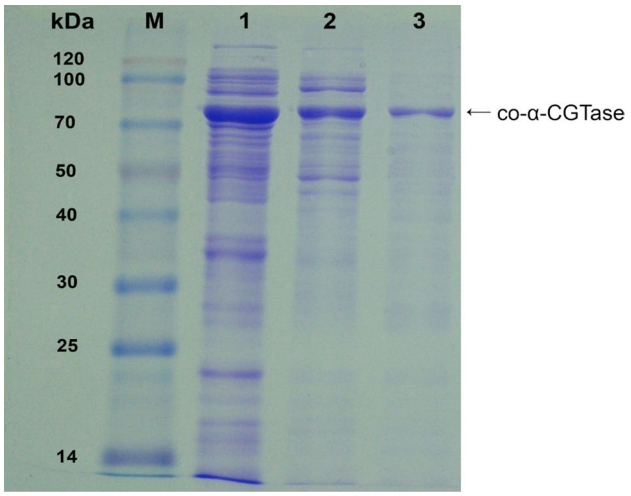



**Fig. 1** SDS-PAGE of the purification of co-α-CGTase. *M* protein molecular weight marker, *line 1* crude enzyme, *line 2* purified enzyme after ammonium sulfate precipitation, *line 3* purified enzyme after nickel affinity chromatography

catalysis. We also noticed that the newly produced peaks P1 and P2 were the major products, exhibiting significantly larger peak areas than those of P3 and P4. Whereas the peak Pu existed in both reaction mixture and genistein standard at the same retention time, suggesting that it might not be a transglycosylation product.

To further identify the transglycosylation products, two genistein monoglucoside derivatives, such as genistein 7-*O*-β-D-glucoside (genistin) and genistein 4′-*O*-β-D-glucoside (sophoricoside), were used as the standard sample for HPLC analysis. As shown in Fig. 2b, the retention time of P1 was corresponding to that of sophoricoside, suggesting that P1 should be genistein 4′-*O*-glucoside.

## LC–MS analysis of transglycosylation products

To analyze the molecular structure of above products, the reaction mixture was analyzed by LC–MS (Fig. 3). Five peaks successively appeared in the UPLC chromatogram at 1.90, 2.03, 2.13, 2.22, and 2.44 min (Fig. 3a), corresponding to the peaks P4, P3, Pu, P2, and P1 in the HPLC chromatogram, respectively (Fig. 2). As shown in Fig. 3b, the extracted ion chromatogram (EIC) showed the EIC peaks of possible transglycosylation products $(Glc)_n$-genistein ($n = 1, 2, 3, 4$) (corresponding to *m*/*z* values of 431, 593, 755 and 917, respectively), which was highly similar to the peaks shape in the UPLC chromatogram (Fig. 3a). The retention time of EIC peaks (Fig. 3b) was slightly delayed compared with the corresponding UPLC peaks (Fig. 3a). To identify the detailed molecular mass of each possible product, every EIC peak was analyzed by the MS. As shown in Fig. 4c (i), the EIC for *m*/*z* value of 431 assembled at 2.48 min (most) and 2.18 min (little). MS analysis of EIC peak at 2.48 min showed that the main *m*/*z* value was 431.1 (Fig. 4c-ii), suggesting that the possible product may be Glc-genistein (genistein monoglucoside) with molecular mass of 432. Figure 3d showed that the main *m*/*z* value of EIC peak at 2.27 min was 593.2, suggesting that it may be $(Glc)_2$-genistein (genistein diglucoside) with molecular mass of 594. The EIC peaks for *m*/*z* values of 755 and 917 mainly appeared at 2.08 min (Fig. 3e-i) and 1.93 min (Fig. 3f-i), which were corresponding to the detailed *m*/*z* values of 755.3 (Fig. 3e-ii) and 917.4 (Fig. 3f-ii), respectively. It suggested that the possible products of these two peaks are likely to be $(Glc)_3$-genistein (genistein triglucoside) with molecular mass of 756 and $(Glc)_4$-genistein (genistein tetraglucoside) with molecular mass of 918. Figure 3e (ii), f (ii) also exhibited many other *m*/*z* values, and possible reason may be that the EIC peaks at 2.08 and 1.93 min contained other impurities due to trace amount of genistein triglucoside and genistein tetraglucoside produced in the co-α-CGTase catalysis reaction. As for the EIC peak at 2.19 min, we could not detect any molecular mass of possible products. Therefore, this peak may not contain any genistein transglycosylation product, which further confirmed our conclusion about the peak Pu (Fig. 2a). In addition, we also noticed that there was one small peak before 1.90 (Fig. 3a), corresponding to the peak before P4 in Fig. 2a. However, it was too small to be detected in EIC chromatogram (Fig. 3b). Therefore, we could not determine its molecular weight.

On basis of the above analysis, we can conclude that the peaks P1, P2, P3, and P4 are corresponding to the products genistein monoglucoside, genistein diglucoside, genistein triglucoside, and genistein tetraglucoside derivatives, respectively. In addition, genistein monoglucoside and genistein diglucoside are the major products. Particularly, genistein diglucoside is of great interest to us due to its better water solubility than genistein monoglucoside [24].

## Reaction conditions optimization

To optimize the reaction conditions for the transglycosylation products especially for genistein diglucoside synthesis, various genistein dissolvents and different glycosyl donors were investigated in this study. Furthermore, we also studied the influences of different concentrations of enzyme, initial glycosyl donor and acceptor, and the substrates ratio (v/v) on transglycosylation products synthesis. At last, different reaction pH, temperature, and time were also examined.

Different solvents (DMSO, methanol, ethanol, water, and ethyl acetate) used as genistein dissolvents were investigated. As shown in Table 1, we found that genistein easily dissolved in DMSO, partly dissolved in ethanol, slightly

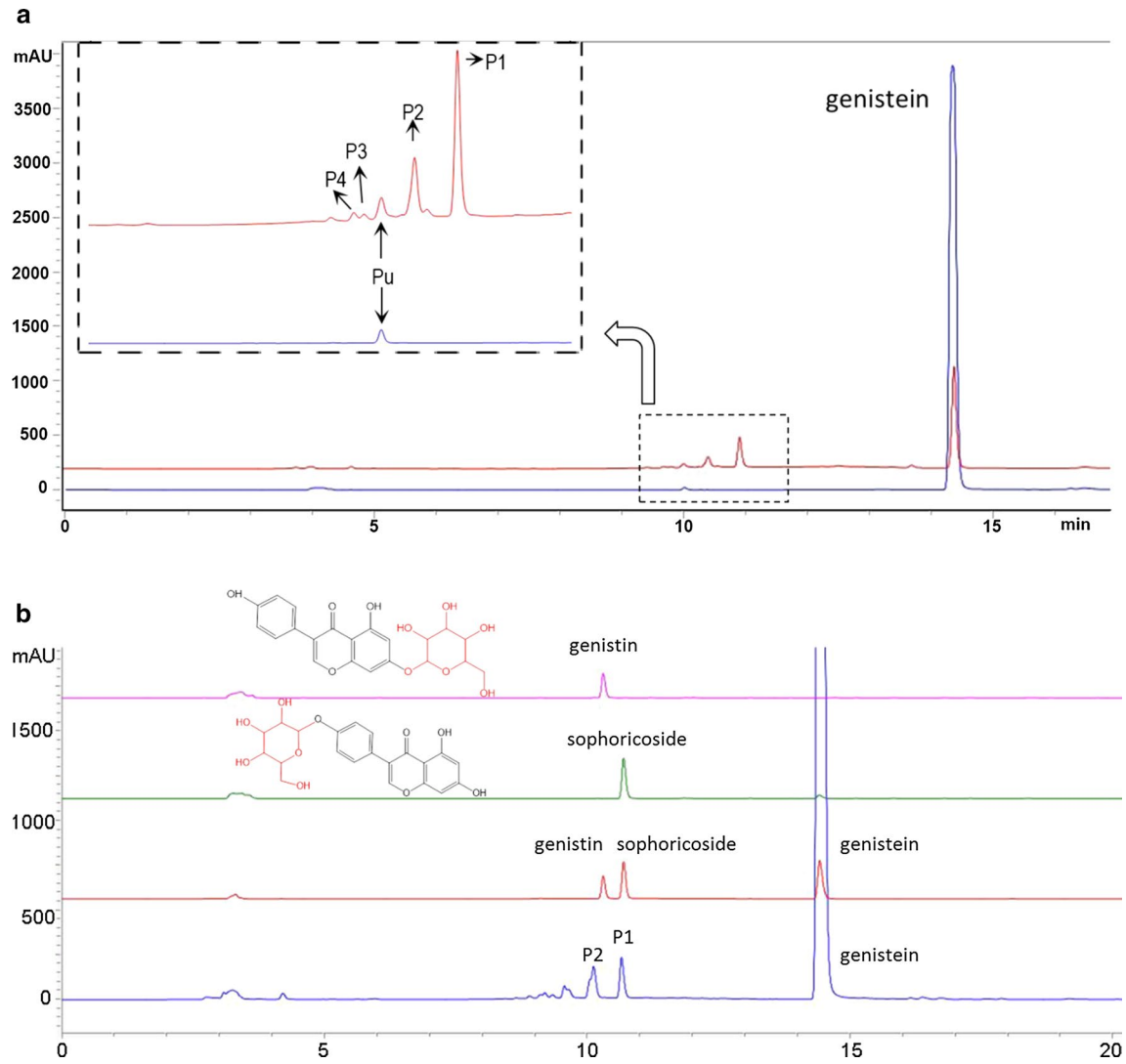
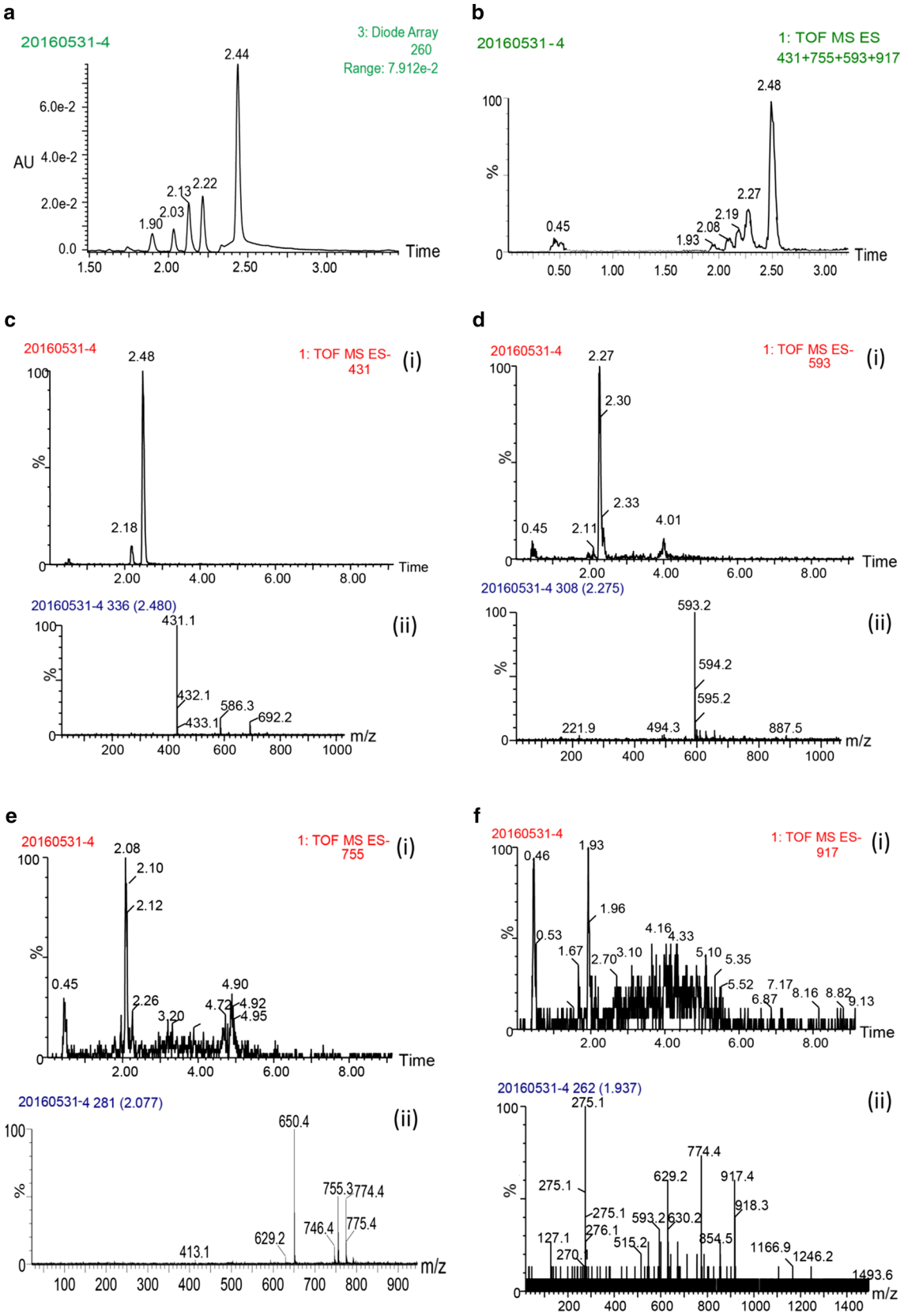



**Fig. 2** HPLC chromatogram of standard and reaction mixture. **a** HPLC chromatogram of genistein standard (*blue curve*) and reaction mixture (*reddish brown curve*); **b** HPLC chromatogram of genistin standard (*pink*), sophoricoside standard (*green*), mixture standards of genistein, genistin, and sophoricoside (*reddish brown*) and reaction mixture (*blue*) (color figure online)

dissolved in methanol and ethyl acetate, and hardly dissolved in water. As shown in Fig. 4a, significant yields of four transglycosylation products were obtained using DMSO, methanol, and ethanol as solvents, while little yields were produced with water and ethyl acetate as solvents. Compared with methanol and ethanol, DMSO could obviously increase the yields of genistein monoglucoside and genistein diglucoside. Whereas for the yields of genistein triglucoside and genistein tetraglucoside, there was a little change among the solvents of methanol, ethanol, and DMSO. Therefore, we selected DMSO as the genistein dissolvent.

The influences of different glycosyl donors (α-CD, β-CD, starch, maltodextrin, maltose, glucose, and sucrose) on the production of genistein glycosylated derivatives were also investigated. As shown in Fig. 4b, the highest yields of various transglycosylation products $(Glc)_n$-genistein ($n = 1, 2, 3, 4$) were obtained when using α-CD as the glycosyl donor. However, α-CD is not usually regarded as the best glycosyl donor due to its expensive price. When using glucose and sucrose as glycosyl donors, we hardly detected any transglycosylation product. Interestingly, the yields of two major products genistein monoglucoside and genistein diglucoside produced with starch were higher than those with β-CD, maltodextrin and maltose as glycosyl donors, although the yields of genistein triglucoside and genistein tetraglucoside were similar. Based on the cost and output, starch was selected as the optimum glycosyl donor for the transglycosylation by co-α-CGTase.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◂**Fig. 3** Extract ion flow and LC–MS chromatogram of reaction mixture. **a** UPLC chromatogram of reaction mixture; **b**: EIC chromatogram of multiple *m/z* (431 + 755 + 593 + 917); **c**: EIC chromatogram of *m/z* 431 (*i*) and its MS chromatogram (*ii*); **d** EIC chromatogram of *m/z* 593 (*i*) and its MS chromatogram (*ii*); **e** EIC chromatogram of *m/z* 755 (*i*) and its MS chromatogram (*ii*); **f** EIC chromatogram of *m/z* 917 (*i*) and its MS chromatogram (*ii*)

To investigate the influence of the initial concentrations of glycosyl donor on the products yields, different concentrations of starch (10, 20, 30, 40, and 50 g/L) were tested in the transglycosylation reaction. As shown in Fig. 4c, the yield of genistein monoglucoside was lower than that of genistein diglucoside with 10 g/L starch. Interestingly, when the starch concentration was more than 20 g/L, genistein monoglucoside began to exhibit higher yield than genistein diglucoside. Furthermore, the yields of all transglycosylation products produced with 40 g/L of starch were higher than those with other starch concentrations. Therefore, 40 g/L was regarded as the optimum initial concentration of starch.

The optimum initial concentration of the glycosyl acceptor was also investigated in this study. As shown in Fig. 4d, different concentrations of genistein (1.0, 2.5, 5.0, 7.5, and 10 g/L) were used for the transglycosylation by co-α-CGTase. The results showed that the yield of genistein diglucoside was lower than that of genistein monoglucoside with less than 5.0 g/L genistein concentration, while the yield of genistein diglucoside began to exhibit superiority when the genistein concentration was more than 5.0 g/L. Among all initial genistein concentrations, various transglycosylation products showed the highest yields with 7.5 g/L genistein. Thus, 7.5 g/L was selected as the optimum initial genistein concentration.

In the two-phase system, different ratios between organic and aqueous phases usually play a key role on the yields of products, and thus, we investigated different volume ratios of starch (dissolved in PBS aqueous solution) and genistein (dissolved in DMSO) in this study. As shown in Fig. 4e, we found that there was nearly no products synthesized when the substrates ratio (starch/genistein, v/v) was 2/6. Along with the ratio increasing, the yields of products (especially for genistein monoglucoside and genistein diglucoside) gradually increased. Moreover, all four products reached the highest yields at 6/2 of substrates ratio, while sharply decreased when the substrates ratio was further enhanced to 7/1. Thus, we selected 6/2 of substrate ratio for the latter study.

Enzyme concentration is also one important factor for a catalysis reaction. Here, we used different co-α-CGTase concentrations (20, 50, 100, 150, 200, 300, and 400 U/mL) for the transglycosylation. As shown in Fig. 4f, when the co-α-CGTase concentration was less than 150 U/mL, the yields of products gradually increased along with the increasing enzyme concentration. However, with more than 150 U/mL co-α-CGTase concentration, the yield of genistein monoglucoside gradually increased, while that of genistein diglucoside, genistein triglucoside, and genistein tetraglucoside gradually decreased along with the increasing CGTase concentration. To get more genistein diglucoside derivative, we selected 150 U/mL as the optimum enzyme concentration.

We also investigated the influences of reaction pH and temperature on the transglycosylation products synthesis. As shown in Fig. 5a, the yields of genistein triglucoside and genistein tetraglucoside showed no appreciable distinctions among different pHs. For the product of genistein monoglucoside, its yield reached the highest at pH 6.5, while the most genistein diglucoside was obtained at pH 7.0. We selected pH 7.0 as the optimum reaction pH for the highest yield of genistein diglucoside. The influence of different reaction temperatures on the transglycosylation products synthesis was shown in Fig. 5b. The yields of most products such as genistein monoglucoside, genistein diglucoside, and genistein triglucoside reached the highest levels at 40 °C. Hence, 40 °C was determined to be the optimum reaction temperature.

Based on the above optimum reaction conditions, we investigated the influence of reaction time on the yields of products. As shown in Fig. 5c, we found that the yields of genistein triglucoside and genistein tetraglucoside reached the maximum at 5 h, and then almost remained constant during the range from 5 to 25 h. At 15 h, the maximum yield of genistein diglucoside was obtained, which was approximate 2.5-fold of that under the initial conditions.

Influence of glucoamylase on transglycosylation products was also investigated in this study. As shown in Fig. 6, when treated by 5 U/mL of glucoamylase, the yields of genistein triglucoside and tetraglucoside gradually decreased, while genistein diglucoside and monoglucoside were slightly increased. As increasing of the glucoamylase concentration (5–20 U/mL), almost all genistein triglucoside and tetraglucoside were hydrolyzed along with the slightly decreasing yield of genistein diglucoside, while that of genistein monoglucoside greatly increased. When the glucoamylase concentration was more than 40 U/mL, genistein diglucoside and monoglucoside were gradually hydrolyzed into genistein. It suggested that genistein monoglucoside and diglucoside were more stable than genistein triglucoside and tetraglucoside.

## Discussion

There are three phenolic hydroxyl groups at 7-, 5-, and 4′-C position of genistein, respectively. Among them, the hydroxyl proton of 7-OH is easier for exchange than that

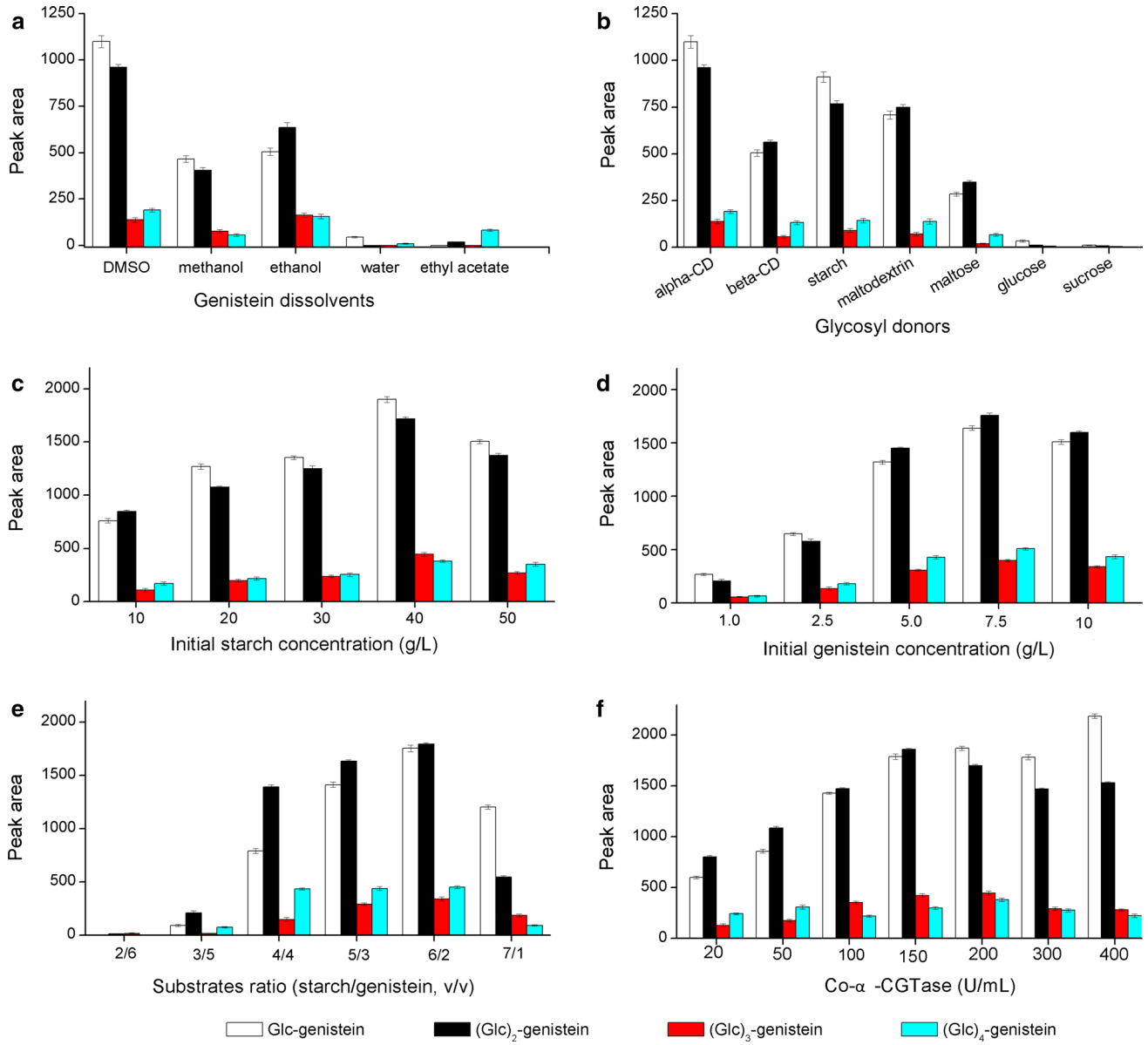


**Fig. 4** Influence of reaction system conditions on the transglycosylation products synthesis. **a** genistein dissolvents; **b** different glycosyl donors, **c** initial starch concentration, **d** initial genistein concentration, **e** substrates radio, and **f** enzyme concentration

**Table 1** Solubility of genistein in different solvents

| Solvent | Solubility (g/L, 25 °C) |
|---|---|
| DMSO | 357 ± 29.69 |
| Ethanol | 21.6 ± 3.52 |
| Methanol | 2.4 ± 0.21 |
| Ethyl acetate | 1.68 ± 0.24 |
| Water | 0.006 ± 0.0007 |

of 4′-OH, and the hydroxyl proton of 5-OH is most inactive [21]. To the best of our knowledge, many glycosyltransferases can glycosylate genistein resulting in the formation of genistein 7-*O*-glycoside [18, 19], but only one report reveals the glycosyltransferase which can specifically glycosylate genistein to genistein 4′-*O*-glucoside [28]. In our present study, transglycosylation catalyzed by co-α-CGTase could produce at least four genistein glucosides derivatives. We found that the retention time of genistein monoglucoside was identical to sophoricoside (Fig. 2b), suggesting that the co-α-CGTase could specifically glycosylate at 4′-OH position of genistein resulting in the formation of genistein 4′-*O*-glucoside. According to the results of transglycosylation reaction by CGTase in the previous reports [5, 24], we speculate that the glucosides may successively conjugate to the glucosyl group of genistein

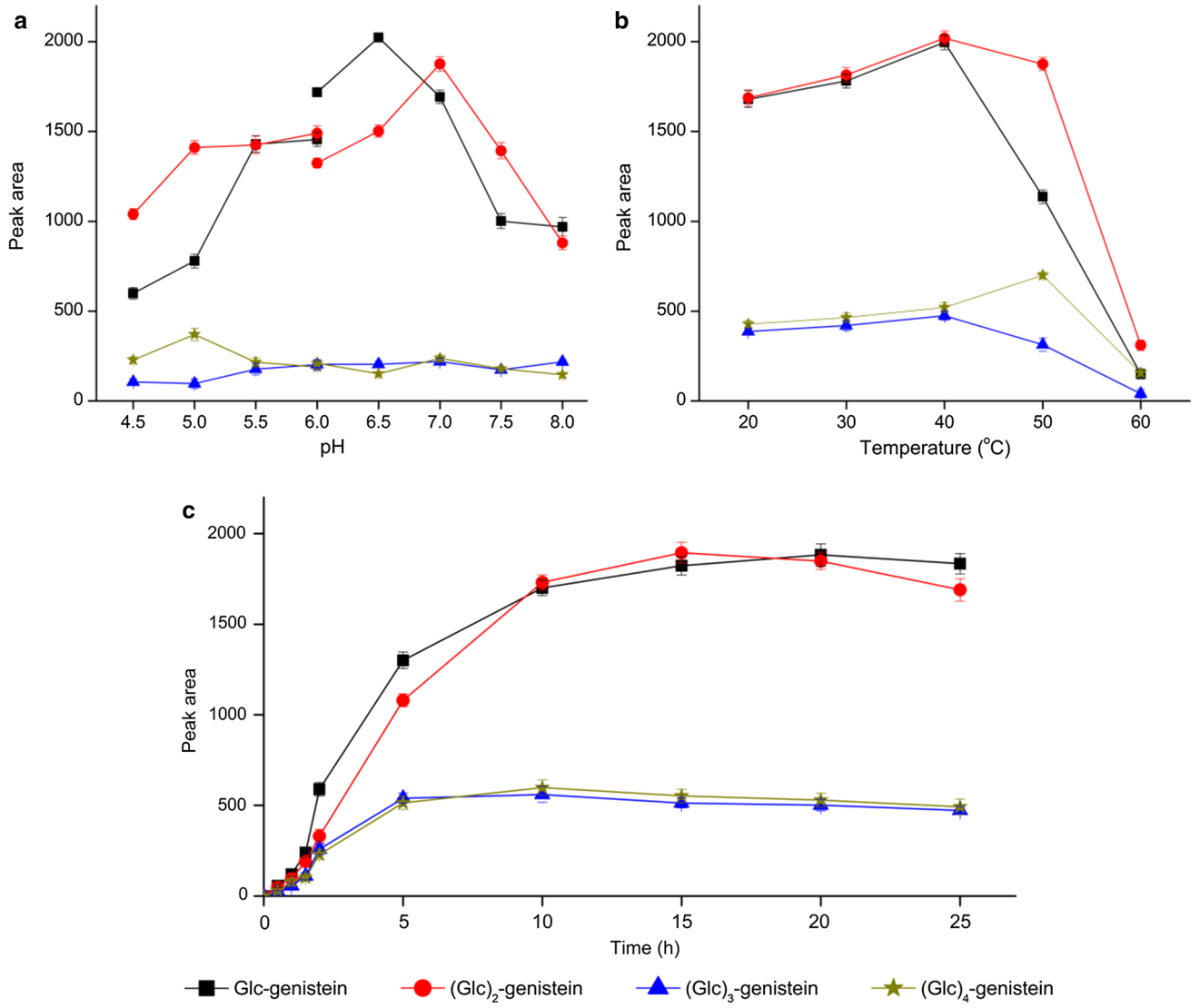


**Fig. 5** Influence of reaction pH (**a**), temperature (**b**), and time (**c**) on the transglycosylation products synthesis. pH 4.5–6.0 with acetic acid–sodium acetate buffer; pH 6.0–8.0 with phosphate buffer

4′-*O*-glucoside by α1-4 glycosidic bond to form genistein multiglucoside derivatives (such as genistein diglucoside, triglucoside, and tetraglucoside). This deduction is also in accordance with our results, as shown in Fig. 6, the glycosyl groups of genistein multiglucosides were gradually hydrolyzed by glucoamylase, resulting in the formation of genistein 4′-*O*-glucoside.

Transglycosylation has been often used for improving the water solubility of many isoflavones [22–24]. Different number of glucosides conjugate of isoflavones usually leads to different solubility. For instance, the solubility of genistein diglucoside and genistein triglucoside is 3700 and 44,000 times of that of genistin, respectively [24]. In addition, daidzein triglucoside showed 75,000 times improved solubility compared with daidzein [22]. It seems that the solubility increases along with the increasing number of glucosides. However, isoflavone multiglycosides may be unstable due to their easy hydrolysis property. For example, genistein triglucoside and tetraglucoside are more easily hydrolyzed by glucoamylase than genistein diglucoside and monoglucoside (Fig. 6). This observation is consistent with the previous report [24], in which the yields of glycosyl- and maltosyl-genistin increased along with the decreasing yields of multiple glycosyled genistin after β-amylase treatment. Therefore, genistein diglucoside, with better water solubility than genistein monoglucoside and better stability than genistein multiglucosides derivatives, may become an important genistein derivative for food and pharmaceuticals applications. There might be some oligosaccharides produced in the transglycosylation solution, since starch was

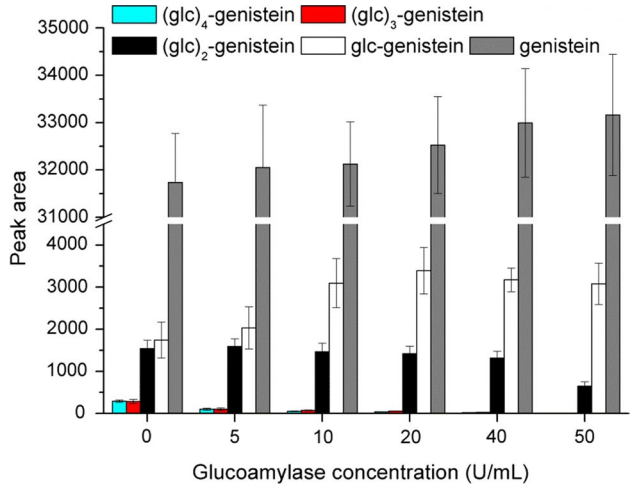


**Fig. 6** Influence of glucoamylase on transglycosylation products

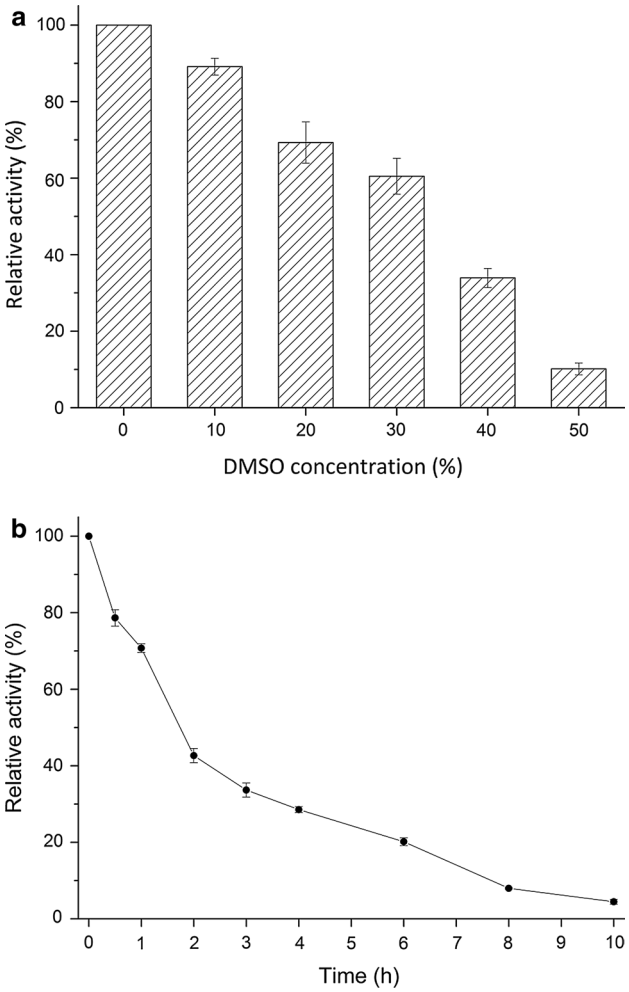


**Fig. 7** Assessment of DMSO solvent tolerance of co-α-CGTase. **a** Relative residual activity of co-α-CGTase in the presence of different concentrations of DMSO for 1 h. **b** Relative residual activity of co-α-CGTase in the presence of 20% of DMSO for different times

used as the substrate. Sep-Pak Plus C18 cartridge can be used to absorb genistein glycosides and to remove the by-product oligosaccharides and salts in our further product purification.

CGTase is commonly regarded as one multifunctional enzyme capable of catalyzing not only transglycosylation but also hydrolysis reaction [7, 30]. In the reaction optimization, obvious distinctions between genistein monoglucoside and genistein diglucoside were observed among different concentrations of starch and co-α-CGTase. As a comparison between Fig. 4c and d, the yield of genistein monoglucoside was lower than that of genistein diglucoside under low starch concentration (10 g/L) and high genistein concentrations (5.0, 7.5, and 10 g/L), while higher yield of genistein monoglucoside than genistein diglucoside was attained with high starch concentrations (20, 30, 40, and 50 g/L) and low genistein concentrations (1.0 and 2.5 g/L). It is speculated that more genistein monoglucoside would be formed along with the increasing ratio between glycosyl donor and acceptor, which is also corresponding to the result of Fig. 4e. In addition, we found that the ratio of genistein monoglucoside and genistein diglucoside increased along with the increasing concentration of co-α-CGTase (Fig. 4f), suggesting that high concentration of CGTase may hydrolyze multiglucosides genistein into genistein monoglucoside. Therefore, with less than 150 U/mL co-α-CGTase, both yields of genistein monoglucoside and genistein diglucoside enhanced with the increasing enzyme concentration. Whereas when the co-α-CGTase concentration was more than 150 U/mL, genistein monoglucoside yield continued to increase, while genistein diglucoside yield gradually decreased, possibly because more genistein diglucoside derivatives were hydrolyzed into genistein monoglucoside by co-α-CGTase. In addition, considering the long production period and high consumption of CGTase, the co-α-CGTase used in this study is much beneficial for economic and industrial application than wild-type CGTase due to its significantly enhanced expression level.

In the present study, we also noticed that the optimum reaction temperature and pH of co-α-CGTase for genistein diglucoside synthesis were 40 °C and pH 7.0, respectively. However, the optimum reaction temperature and pH of the same enzyme for 2-*O*-D-glucopyranosyl-L-ascorbic acid synthesis were 36 °C and pH 6.5 [9] and for α-CD synthesis were 45 °C and pH 5.5 [26]. Therefore, we can conclude that different reactions for different products synthesis catalyzed by the same enzyme may have different optimum reaction conditions.

One main bottleneck for the transglycosylation reaction may be the low tolerance of co-α-CGTase to organic solvents. In Fig. 7a, more than 50% activity of co-α-CGTase

was lost when DMSO concentration in the reaction system was higher than 30%. Even at 20% DMSO concentration, the residual activity of co-α-CGTase was lower than 50% after 2 h of treatment, and only 10% activity remained after 8 h. Therefore, improving the organic solvent tolerance of CGTase is one primary approach to enhance the yield of transglycosylation products in our future work.

In conclusion, co-α-CGTase was used for transglycosylation to produce genistein glycosylated derivatives in this study, and at least four products $(Glc)_n$-genistein ($n = 1, 2, 3,$ and 4) were obtained and identified by LC–MS. After optimization of reaction conditions, the major product genistein diglucoside yield was enhanced by 1.5 times compared with that at the initial condition. However, low conversion is the primary problem for this reaction due to the low tolerance of co-α-CGTase to organic solvent. Therefore, the organic solvent tolerance of this enzyme will be improved by protein engineering approaches in our future study.

**Acknowledgements** We are grateful to the National Natural Science Foundation of China (31401634), Natural Science Foundation of Jiangsu Province (BK20150003 and BK20140135), Six talent peaks project of Jiangsu Province (2015-SWYY-008), Research cooperation project of Jiangsu Province (BY2015019-19), the Program of Introducing Talents of Discipline to Universities (111-2-06), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions for the financial support of this research.

**Author contributions** Ruizhi Han participated in the design of the study, performed research, analyzed data, and drafted the paper. Binbin Ge performed the HPLC and LC–MS experiments and analyzed the data. Mingyang Jiang performed the conditions optimization experiments and analyzed the data. Guochao Xu helped to design the experiments and revise the manuscript. Jinjun Dong helped to design and revised the experiment approaches. Ye Ni conceived the study, designed and coordinated the experiments, and critically revised the manuscript. All authors read and approved the final manuscript.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical statement** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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