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Production of Transglycosylated Rutin Using Novel Cyclodextrin Glucanotransferase

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Besides physical and chemical methods, the enzymatic method could be applied on the modification of rutin molecule. In this study, the transglycosylation of rutin using novel cyclodextrin glucanotransferase (CGTase) produced by *Bacillus* sp. SK13.002 has been investigated. The optimization of enzymatic reaction conditions using orthogonal experimental design has also been carried out based on temperature, pH, the amount of enzyme cyclodextrin glucanotransferase and the reaction time. The results indicated that among these enzymatic reaction conditions reaction time is the most important variable. Under the optimized enzymatic reaction conditions of pH 5.5, temperature 35 ºC, 20 U cyclodextrin glucanotransferase per mL reaction system and 24 h of reaction time, the transglycosylation conversation rate of rutin could reach as high as 65.7 %. Furthermore the change of the components of transglycosylated rutins during the rutin transglycosylation has also been investigated.

Key Words: Rutin, Transglycosylation, Transglycosylated rutin, Cyclodextrin glucanotransferase, Orthogonal experimental design.

INTRODUCTION

Rutin is one kind of bioflavonoids abundantly found and distributed in plants such as in buckwheat seed, fruits and fruit rinds, especially citrus fruits (orange, grapefruit, lemon) and presents important properties in human health such as significant scavenging properties on oxidizing species[1-6]. Furthermore, rutin has several pharmacological activities including antiallergic, antiinflammatory and vasoactive properties[3,7,8]. Rutin has been used as a nutritive element, pigment or an antioxidant in food, cosmetic and pharmaceutical industry. However, its application has been limited by its insolubility and instability in water. Great effort including physical, chemical in the last decade has been tried to overcome the poor water solubility of rutin. For example, a decrease of its particle size leads to rutin's increase in the saturation solubility, an enlarged surface and dissolution velocity[7,9]. Furthermore, enzymatic method has also been adopted in order to improve the physico-chemical characteristics of rutin.

Several studies showed that enzymatic transglycosylation improved rutin's water solubility[10,11]. Transglycosylation of rutin is an enzymatic reaction catalyzed mainly by cyclodextrin glucanotransferase (CGTase), which transfers glycosyl residues to rutin to form glycosyl-rutin. Cyclodextrin glucanotransferase can catalyze and transglycosylate various molecules, such as L-ascorbic[12], stevioside[13], rebaudioside A[14] and many other compounds in the field of biotransformation[15-18] as well as rutin. Furthermore, CGTases were mainly utilized to produce three kinds of cyclodextrin including α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin (CD_6, CD_7 and CD_8)[19-22].

We screened and obtained one microbial strain SK13.002 from one soil sample in starch factory using rapid screening method[23] combined with rutin transglycosylation enzymatic reaction. Based on cell morphological, biochem-physiological characteristics and 16S rDNA analyses, we found that strain SK13.002 was a novel strain in *Bacillus* family (*Bacillus* sp. SK13.002). The CGTase produced by *Bacillus* sp. SK13.002 transglycosylated rutin more efficiently compared to CGTases isolated from other strains reported.

EXPERIMENTAL

Rutin was purchased from Zelang Medical Technological Co., Ltd. (Nanjing, Jiangsu, China) Acetonitrile and methanol were purchased from Sigma Co., Ltd. (St Louis, MO, USA). Other chemicals and materials were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Microbial strain and culture conditions for CGTase production: *Bacillus* sp. SK13.002 was originally isolated from one soil sample in starch factory. The 16S rDNA gene

sequences for this strain have been deposited to the NCBI GenBank database under accession number GU570959.

The fermentation medium used for CGTase production contained (g/L): soluble starch 10, soy peptone 10, yeast extract 5, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2 and Na_2CO_3 8. A 3 % (v/v) culture inoculum of *Bacillus* sp. SK13.002 was transferred into a 250 mL conical flask containing 33 mL fermentation medium and incubated at 37 ºC for 96 h with continuous orbital shaking at 200 rpm. The cells and insoluble materials were removed by centrifugation at 5000 g for 15 min at 4 ºC and the supernatant was used as crude enzyme.

Transglycosylation of rutin: Briefly, a reaction mixture (*ca.* 10 mL), including crude enzyme (different amounts at 100, 150, 200, 250 U), 0.5 g maltodextrin (pre-dissolved in 5 mL buffer with different pHs (4.5, 5.5, 6.5, 7.5), 0.1 g rutin (pre-dissolved in 5 mL methanol) and 11.1 mg $CaCl_2$ are incubated at different temperatures (25, 30, 35, 40 ºC) for different time durations (8, 16, 24, 32 h) in the dark under gentle stirring. After the incubation, samples were boiled for 5 min to inactivate enzyme and then filtered through 0.45 μm membrane for carrying out high performance liquid chromatography assay.

Assay of transglycosylated rutin: We utilized HPLC assay to measure transglycosylated rutins (G-rutins) in the filtered samples[24]. HPLC assay of G-rutins was carried out using Agilent ZORBAX Eclipse XDB-C_{18} column (4.5 mm × 150 mm, 5 μm) and acetonitrile-water-formic acid (18: 81.9: 0.1, v/v/v) as mobile phase with a flow rate of 0.8 mL/min at 30 ºC. UV absorbance at 254 nm was recorded to calculate the concentration of different G-rutins. The transglycosylation conversion rate of rutin was defined as the ratio of the amount of G-rutins produced to the amount of remaining rutin plus G-rutins produced.

Identification of rutin transglycosylation enzymatic reaction: LC/MS (liquid chromatography/mass spectrometry) analysis of enzymatic reaction products was performed with a Waters platform ZMD 4000 system, which was composed of a Micromass ZMD mass spectrometer, a Waters 2690 HPLC and a Waters 996 photodiode array detector (Waters, Milford, USA).

Experimental design: Orthogonal experimental design was adopted and variables (temperature, the amount of enzyme, pH and reaction time) and levels are discussed.

RESULTS AND DISCUSSION

Optimization of enzymatic reaction conditions: The orthogonal experimental design chart with four variables (temperature, the amount of enzyme, pH and reaction time) at four levels and the corresponding values of the results of orthogonal experimental experiment are shown in Table-1. The sequences in which the experiments were carried out were randomized to avoid any kind of bias. All the results at each step of the design were expressed as the mean of three experiments. It is well known that orthogonal experimental design can be an efficient method in generating useful information on key parameters[25,26]. As seen from the results in Table-2, the variables that gave optimal levels were A3B2C4D4. In other words, optimal enzymatic reaction conditions for rutin transglycosylation were temperature 35 ºC, pH 5.5, the amount of CGTase 25 U per reaction system and 32 h of reaction time. At the meantime, we observed that almost the same transglycosylation conversion rate of rutin could be obtained when the amount of enzyme was 20 U per mL reaction system and reaction time was 24 h. Thus, we adjusted the final optimal reaction conditions as follows: temperature 35 ºC, pH 5.5, the amount of CGTase 20 U per mL reaction system and 24 h reaction time. Under this optimal reaction conditions, test experiment has been carried out and 65.7 % of the transglycosylation conversion rate of rutin could be obtained.

Furthermore, we investigated the analysis of variance of orthogonal experimental design and the results were provided in Table-3. As shown in Table-3, among those variables reaction time was proved the most important factor on rutin transglycosylation (significant).

TABLE-1
RESULTS OF ORTHOGONAL EXPERIMENTAL DESIGN

Experiment No.	A	B	C	D	E (blank)	Transglycosylation rate of rutin (%)
1	25	4.5	10	8	1	20.9
2	25	5.5	15	16	2	38.1
3	25	6.5	20	24	3	46.5
4	25	7.5	25	32	4	40.4
5	30	4.5	15	24	4	43.4
6	30	5.5	10	32	3	49.3
7	30	6.5	25	8	2	41.2
8	30	7.5	20	16	1	42.9
9	35	4.5	20	32	2	52.8
10	35	5.5	25	24	1	64.7
11	35	6.5	10	16	4	35.3
12	35	7.5	15	8	3	30.1
13	40	4.5	25	16	3	36.7
14	40	5.5	20	8	4	38.9
15	40	6.5	15	32	1	50.8
16	40	7.5	10	24	2	37.6
K_1	36.48	38.45	35.78	32.78	44.83	–
K_2	44.20	47.75	40.60	38.25	42.43	–
K_3	45.73	43.35	45.28	48.05	40.65	–
K_4	41.00	37.75	45.75	48.33	39.50	–
R	9.25	10.00	9.98	15.55	5.33	–

TABLE-2
VARIABLES AND LEVELS OF
ORTHOGONAL EXPERIMENTAL DESIGN

Level	Temp. (ºC) (A)	pH (B)	Amount of CGTase per mL reaction system (U) (C)	Reaction duration (h) (D)
1	25	4.5	10	8
2	30	5.5	15	16
3	35	6.5	20	24
4	40	7.5	25	32

Cyclodextrin glucanotransferase produced by *Bacillus* sp. SK13.002 has been proved suitable for rutin transglycosylation since the screening of CGTase-producing strain was based on rutin transglycosylation. Under the optimized enzymatic reaction conditions, *viz*., temperature 35 ºC, pH 5.5 and 20 U CGTase per mL reaction system, the transglycosylation conversion rate of rutin could reach as high as 65.7 % within 24 h reaction time. Compared with Suzuki's study on the optimal enzymatic reaction conditions, there were the same results such as pH (5.5), as well as different results such as temperature, reaction time and the amount of CGTase. In Suzuki's research work, the optimal temperature was 20 ºC, but it was 35 ºC in this study. Furthermore, in Suzuki's study, in order to get *ca.* 70 % of transglycosylation conversion rate of rutin, it should take 48-72 h reaction time; in this study, 65.7 % of transglycosylation conversion rate of rutin could be obtained within 24 h. The results indicated that CGTases with different sources had different enzymatic characterizations (including temperature, the amount of enzyme *etc.*)[27-32]. Go *et al.*[11] reported a similar conversion rate of rutin (70.8 %) and an even shorter reaction time (12 h). However, that shorter reaction time could be due to lower concentration of starting rutin in their reaction system (0.1 %), while the starting rutin concentration in our reaction system was 10 times higher (*ca.* 1 %).

Identification of rutin transglycosylation: We first examined whether high performance liquid chromatography analysis could effectively separate transglycosylated rutins from remained rutin (not transglycosylated). As shown in Fig. 1, HPLC successfully separated G1-rutin (glycol-rutin), G2-rutin (with two glucose units), G3-rutin, G4-rutin and G5-rutin from remained rutin, when the concentration of acetonitrile was 18 % (v/v). Furthermore, increase of acetonitrile percentage in the mobile phase decreased the retention time of each peak.

To confirm the released peaks were the right G-rutins, we performed liquid chromatography/mass spectrometry analysis for each released peak. The results revealed that the relative molecular masses of the two released peaks were 772 and 934, which were equal to the molecular weights of G1-rutin (glycosyl-rutin) and G2-rutin (with two glucose units). These

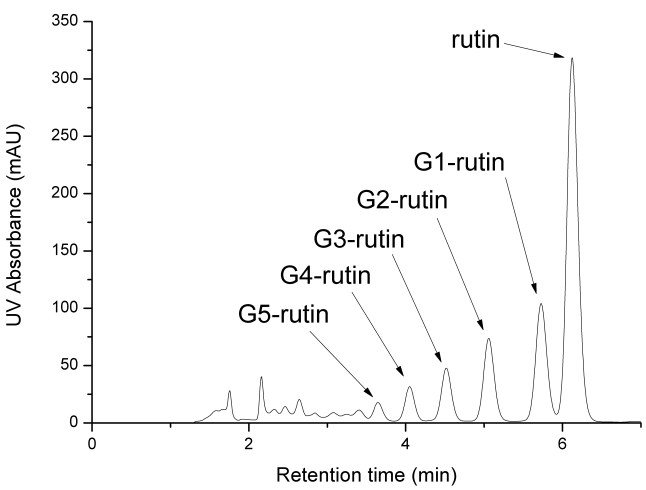

Fig. 1. HPLC chromatogram of tansglycosylated rutins

results demonstrated that HPLC analysis can be used to effectively separate transglycosylated rutins from rutin and the main products of rutin transglycosylation were G1-rutin (glycosyl-rutin) and G2-rutin(with two glucose units).

Suzuki and Suzuki[10] investigated the physical and chemical characteristics of glycol-rutin (G1-rutin in Fig. 1). However, other transglycosylated rutins with more than one glucose unit had not yet been investigated on their characteristics. Transglycosylated rutins with different glucose units could be separated and obtained through HPLC method. Comparing the physical-chemical characteristics of those transglycosylated rutins will be very important and interesting. Furthermore, it will also be very important and essential to investigate the mechanism of CGTase of transferring glucose units. Understanding the mechanism of CGTase on transferring glucose will undoubtedly help to farther optimize enzymatic reaction conditions and increase the transglycosylation conversion rate of rutin.

Time-course for the transglycosylation enzymatic reaction of rutin: We also investigated the time-course of rutin transglycosylation using CGTase produced by *Bacillus* sp. SK13.002. It could be observed in Fig. 2 that in order to obtain *ca.* 65 % of the conversion rate of rutin (the largest quantity of transglycosylated rutin), the reaction time should be lasted as long as 24 h or more[33]. However, in cyclodextrins (CDs) production using CGTase, it usually took only several hours to finish enzymatic reaction and get the largest quantity of cyclodextrins products. Compared with other enzymatic reactions, there was nearly half part of volume of methanol in the enzymatic reaction system of rutin transglycosylation. It is well known that except several kinds of enzymes[34], organic solvents usually have exhibitive effects on enzymatic reactions.

TABLE-3
ANALYSIS OF VARIANCE OF ORTHOGONAL EXPERIMENT

Source	Sum of squares	df	Mean square	F value	*p*-Value ($\alpha = 0.05$)	Significant
Temperature	200.605	3	92.486	3.107	9.280	–
pH	262.960	3	95.151	4.072	9.280	–
Enzyme amount	261.635	3	54.641	4.052	9.280	–
Time	702.725	3	295.029	10.882	9.280	*
Error	64.575	3		1.000	9.280	–

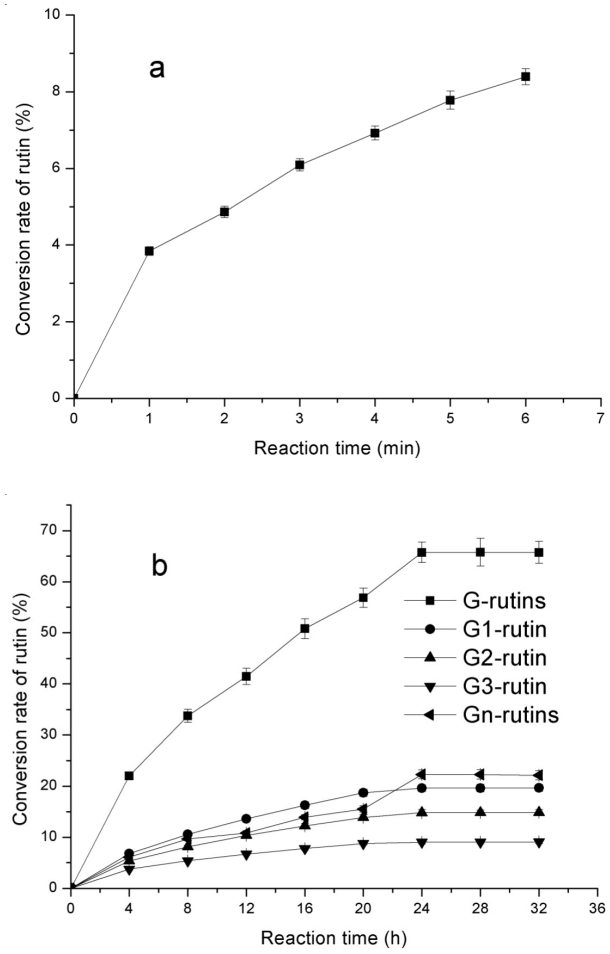

Fig. 2. Time-courses of the transglycosylation of rutin and components change

Thus we deduced that methanol in the reaction system of rutin transglycosylation could have negative effects on transglycosylation enzymatic reaction, because organic solvents could induce protein denaturation and inactivation of enzyme. How to sustain the activity of CGTase during rutin transglycosylation is a very important and interesting topic in rutin transglycosylation, because it could solve the problem of very long enzymatic reaction time (24 h in this study and 48-72 h in Suzuki's study).

Conclusion

The CGTase produced by *Bacillus* sp. SK13.002 showed high transglycosylation activity of rutin and could transglycosylate rutin more efficiently comparing with other CGTases. The optimized enzymatic reaction conditions have been obtained based on orthogonal experimental design as follows: pH 5.5, temperature 35 ºC, 20 U CGTase per mL reaction system and 24 h of reaction time.

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