Master's Thesis of Science in Agriculture

Synthesis and Characterization of Epigallocatechin Gallate Fructosides by using Levansucrase

Levansucrase를 이용한 epigallocatechin gallate (EGCG)의 수친화성 유도체 제조 및 기능성 연구

February 2018

Shina Jin

Department of International Agricultural Technology Graduate School of International Agriculture Technology

Seoul National University

Synthesis and characterization of epigallocatechin gallate fructosides by using levansucrase

A thesis
submitted in partial fulfillment of the requirements to the faculty
of Graduate School of International Agricultural Technology
for the Degree of Master of Science in Agriculture

By
Shina Jin

Supervised by
Prof. Doman Kim

Major of International Agricultural Technology
Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

February 2018

Approved as a qualified thesis
for the Degree of Master of Science in Agriculture
by the committee members

Chairman **Donghwa Chung, Ph.D.** _____

Member **Doman Kim, Ph.D.** _____

Member **Hyo Jin Kim, Ph.D.** _____

Abstract

Levansucrase is one of fructosyltransferases and synthesizes levan, c omposed of β-(2-6)-linked fructose residues, or inulin, composed of β-(2-1)-liked fructose residues. Levansucrase also catalyzes transfructosyla tion from sucrose to a variety of acceptors. In this study, I expressed of levansucrase from *Leuconostoc mesenteroides* 512 in *E. coli*. The o ptimum condition for levansucrase expression is 1 mM lactose, 25 μM ampicillin at 33°C. The levansucrase activity was 2.84 U/mg. The pro duction of levansucrase was used to catalyze transfructosylation using s ucrose as substrate and pigallocatechin gallate (EGCG), one of the mo st abundant catechin in green tea, as acceptors. The five novel epigallo catechin gallate fructosis were detected. When compare to EGCG, wate r solubility of 5'-EGCG-β-D-fructofuranosyl-β-2-6-fructofuranoside was i ncreased 350 mM, which is 64-fold water solubility of EGCG. Also a ntioxidant activities of EGCG-F2 (SC_{50}, 9.7 μM) are similar to EGCG (SC_{50}, 7.6 μM), and EGCG-F1 (IC50, 173.6 μM) showed higher inhi bitory activity against HMA than EGCG-F2 (IC50, 253 μM).

Key word: antioxidant activities, epigallocatechin gallate, fructosylation, levansucrase.

Student ID: 2016-22047

Contents

List of tables

List of figures

Introduction

Green tea is most widely consumed beverages in the world. It is distinguished by its remarkable content of polyphenols[1], Epigallocatechin gallate(EGCG) is a catechin polyphenol compound in the leaves of green tea, camellia sinensis[2]. It has various functional activities including antioxidant[3], anticancer activities[4] and antibacterial effects[5].Furthermore improvement of endothelial function and insulin sensitivity has been studied[6]. To utilize this positive biological characteristic there are various research is underway in cosmetic, pharmaceuticals, and food industries. Although EGCG possess various biological activities but EGCG has limited for application in function food, cosmetic because of its low solubility, stability, and browning effect[7, 8]. Therefore, the transfructosylation of EGCG is a useful method for the improvement of such weakness. Fructosyltransferase enzymes belong to glycoside hydrolase family 68 (GH68)[9], and composed of β-(2-6)-linked fructose residues, or inulin, composed of β-(2-1)-linked fructose residues[10]. Levansucrase is one of the fructosyltransferases and produced by various microorganisms[11]. Levansucrase expressed in *E. coli* BL21(DE3) was used as a host for the pRSET-M1FT[12]. Levansucrase catalyses trans

fructosylation from sucrose to a variety of acceptors. Three different re actions can be distinguished depending on the acceptor molecule:(1) H ydrolysis of sucrose when water is used as acceptor, (2) Transfer react ion when sucrose or gluco- and fructosaccharides are used as acceptor and (3)Polymerization when the growing fructan chain is used as acc eptor (levan synthesis)[11].

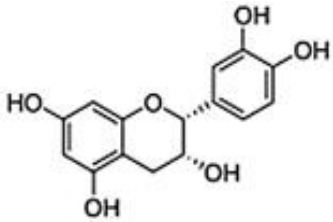
Through the previous study about acceptor reaction of EGCG found th at improving of solubility and stability and also manifested a higher d egree of browning resistance than was found in EGCG[13].

In this study, we reported the synthesis of EGCG fructosides by tra nsfructosylation using levansucrase. The EGCG-Fs were screened for bi ological activities, including antioxidant, human maltase intestinal malta se inhibition, browning effect, and EGCG-Fs were more solubility in water than EGCG.

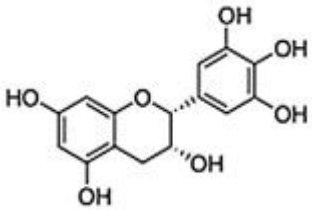
Literature Review

1. EGCG

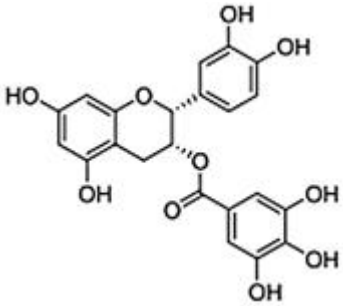
Tea is a worldwide popularity second only to that of water. Studies with animal models showed that green tea has preventive activity agai nst cancer of the oral cavity, stomach, intestine, colon, liver, lung, skin and other sites[9]. The compound (-)-epigallocatechin-3-gallate (EGCG) is the major catechin found in green tea. Several other polyphenolic compounds know as catechins are also found in lower abundance in g reen tea. These include (-)-epicatechin-3-gallate (ECG), (-)-epigallocatec hin (EGCG), (-)-epicatechin (EC) and (+)-catechin **(Figure 1).** Bioavail ability of catechins from tea is believed to be relatively poor. In huma ns, maximum catechin plasma concentrations of up to 1–2 μmol/L are achieved between 1 and 2 h after consumption followed by rapid clear ance restoring plasma concentrations to baseline levels within 24 h of i nitial consumption[14].



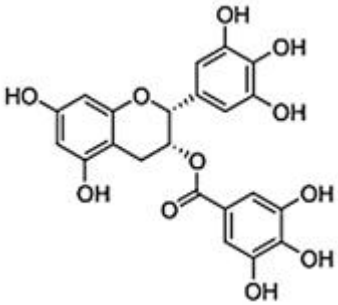
Epicatechin (E
C)



Epigallocatechin
(EGC)



Epicatechin-3-gallate
(ECg)



Epigallocatechin-3-gallate
(EGCg)

Figure 1. Structure of the four major catechins present in green tea.

2. Levansucrase

Glycosyltransferases are enzymes that catalyze the transfer of glycos yl residues from a donor molecule to a particular acceptor[15]. Among them, fructosyltranferases (FTFs) synthesize fructose polymers from su crose with release of glucose, in a similar way to glucosyltranferases (GTFs) that synthesize glucose polymers from sucrose with release of fructose[16]. Fructosyltransferases have a molecular weight ranging fro m 45 to 64 kDa, except for the enzymes from *Lactobacillus reuteri* an d *Streptococcus salivarius*, which are larger: 87 and 140 kDa respective ly[17]. Levan, a polymer of fructose linked by fructofuranosidic bond, is produced from sucrose by the transfructosylation reaction of levansu crase (β-2.6, fructan: D-glucose-fructosyl transferase). Levansucrase is f ound in various Gram-negative bacteria, such as *Zymomonas mobilis*[1 8], *Rahnella aquatilis*[19] and Gram-positive bacteria such as *Bacillus subtilis*[20]. The enzyme catalyses transfrucosylation from sucrose to fo ur acceptors: water, glucose, sucrose and levan. Levansucrase of *E. col i* is a transfructosylase which catalyses the following reaction: sucrose + acceptor⟶glucose + acceptor-fructose. The transfructosylation react ion occurs with the retention of the configuration of the fructose.

Materials and methods

2.1.1 Expression of levansucrase M1FT gene in *E. coli*

E. coli BL21(DE3) was used to propagate the plasmid pGEM-3Zf (-1) (Promega)[21] was grown in a 100 mL LB medium consisting of 0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 0.5 % (w/v) NaCl supplemented with 50 μg/mL ampicillin at 37℃ until the OD_{600} reached approximately 0.5. The cell was induced with 1 mM lactose with 15 h of shaking. The cell was collected by centrifugation (12, 600 X g for 20 min at 4℃).

2.1.2 Optimization of lactose concentration and temperature for induction

E. coli BL21(DE3) was selected to determine the effect of temperature for enzyme expression. It was induced with 0.5 mM~10 mM lactose or IPTG 1 mM at 33℃ for 15 h to determine the optimum lactose concentration. To access the effect of the temperature, the cells were grown in a 100 mL LB medium at 37℃ until OD_{600} reached approximately 0.5 and was induced with 1 mM lactose at different temperatur

e (20, 25, 28, 33, 37℃) with or 1mM IPTG at 37℃ for 15 h. and T he cells were centrifugation (12, 600 X g for 20 min at 4℃), resuspe nded in 4 mL of 20 mM phosphate buffer (pH 6.0), and disrupted by sonication (amplitude 30 for 30 S of sonication, 3 repeats on ice). T he supernatant obtained by centrifugation (12, 600 X g for 20 min, 4℃) was used as crude enzymes.

2.1.3 Levansucrase hydrolytic activity assay

Levansucrase activity was determined using 400 mM sucrose and en zyme in 50 mM phosphate buffer (pH 6.0). The reaction was conducte d at 37℃ for 30 min. The enzyme reacted samples were spotted on T LC silica gel 60 F_{254} plates (Merck Co. Germany) and were developed with three ascents of acetonitrile-water [85:15 (v/v)]. The fructose con centration liberated from sucrose was determined using integrated densi ty values (IDV) by employing the AlphaEaseFC 4.0 program (Alpha I notech, San Leandro, CA, USA). One unit of levansucrase activity was defined as the amount of enzyme that catalyzed the release of 1 μmo l glucose per min under the reaction condition.

2.1.4 Fermentation of levansucrase

Large-scale fermentation of levansucrase was performed in a 5000 L bioreactor that contained 2000 L medium consisting of 0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 0.5 % (w/v) NaCl supplemented with 50 μg/ml ampicillin at 37℃ until the OD_{600} reached 0.5 and induced with 1 mM lactose at 33℃ for 15 h.

2.2 EGCG acceptor reaction

800 M EGCG was prepared in DMSO (dimethyl sulfoxide) 100 mL of the acceptor reaction mixture contained 100 mM EGCG, 600 mM sucrose, and levansucrase 10 U/ml in 50 mM phosphate buffer (pH 6.0). It was incubated at 37℃ for 7 h, after which sucrose had been depleted. Then the reaction mixture was kept at 90 ℃ water bath for 5 min to halt the enzyme reaction. Analysis of acceptor reaction products using TLC silica gel 60 F_{254} plates (Merck Co.). 1 μL of a reaction mixture was spotted onto a lane in a silica gel plate, and the plate were developed with ethyl acetate-acetic acid-water (3:1:1, v/v/v) solvent, and was detected as described above.

2.3 Optimization of acceptor reaction using response surface methodology (RSM)

The experimental Response Surface Methodology (RSM) data was fit ted through the response surface regression procedure using the followi ng second order polynomial equation:

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} x_i x_j$$

Where Y_i is the predicted response, $x_i x_j$ are the independent variables, β_0 is the pffset term, β_i is the ith linear coefficient, β_{ii} is the ith quadrat ic coeffient, and β_{ij} is the ij^{th} interacyion coefficient. A central composit e (CCD) of RSM soft program (Design Expert 10.0.3, Stat-Ease, Minn eapolis, USA). A preliminary experiment indicated that three factors (s ucrose and EGCG concentration, enzyme unit) were considered in term s of obtaining the highest relative amount of EGCG converted to EGC G-Fs. The effect of different concentration of sucrose (400 mM ~ 1.2 M), enzyme unit (3 ~ 7 U/mL), EGCG (30 ~ 70 mM) were investigat ed. Twenty runs of the experiment were carried out with six replicatio ns at the central point, which were utilized in the fitting of a second-order response surface **(Table 2)**. The acceptor reaction mixture contain ed one of the condition at diverse concentrations and the other two co nditions fixed in 50 mM phosphate buffer (pH 6.0) at 37℃ for 16 h.

The amount of EGCG converted to products was calculated using the AlphaEaseFC 4.0 program (Alpha Inotech, SanLeandro, CA, USA). EG CG was dissolved in DMSO from 5 to 30 mM as the standard.

2.4 Purification EGCG and EGCG-Fs

The reaction mixture were separated using HP 20 column(2 cmX50 cm). after loading sample, sugars were removed with distilled water, and then successively eluted with 30% (v/v) ethanol. The eluent (whic h contained EGCG fructoside) was then concentrated at 35℃ with eva porator. After removed saccharides and polymers was prepared in water (A) and acetonitrile (B) in concentration of 200 mg/ml. Purification o f EGCG fructosides were performed using WATERS 2545 Binary Grad ient Module (Pump), 2767 sample manager (Injector), 2998 PDA detec tor HPLC system equipped with a Sunfire Prep OBD^{TM} C18 (19 mm X 100 mm, 5 μm) column and multiple wavelength detector.

Table 1. Purification condition of HPLC

System	WATER 2545 Binary Gradient Module (Pump), 2767 sample manger (Injector), 2998 PDA detector		
Column m)	Sunfire Prep OBD™ C18 (19 mm X 100 mm, 5 u		
Temperature	Room temperature		
Solvent	(A) Water (B) Acetonitrile		
Gradient	time	A (%)	B (%)
	1	93	7
	10	92	8
	20	92	8
	30	90	10
	40	85	15
	60	80	20
	65	0	100
Flow rate	17 mL/min		
Injection	341 uL		
Absorbance wave	254 nm		

2.5 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

The EGCG-fructosides (1 mg/mL) were diluted with deionized water. The mass spectrum was obtained using a Voyager DESTR MALDI-TOF mass spectrometer (Applied Biosystems, Foster, CA). The mass spectra were obtained in the positive reflector mode with delayed extraction (average of 300 laser shots) with a 25 kV acceleration voltage.

2.6 Nuclear magnetic resonance (NMR) analysis

10 mg of the purified EGCG-Fs were dissolved in 600 μL DMSO-*d*6 and were placed into 5 mm NMR tubes. The NMR spectra was recorded on the AVANCE Ⅲ 850 system (Bruker, Germany), operating at 850 MHz for ^{1}H and at 125 MHz for ^{13}C, at 25℃. Linkages between EGCG and fructose were determined with the homonuclear correlation spectroscopy (COSY), the heteronuclear single quantum coherence (HSQC), and the heteronuclear multiple bond correlation (HMBC).

2.7.1 Water solubility analysis

EGCG fructosides solubility in water was measured by HPLC-UV [22]. 2 mg of EGCG fructosides were mixed in 10 μL of water in an eppendorf tube, at room temperature. Then the mixture was diluted to c

heck the maximum concentration. Each sample was filtered through a 0.45 μm membrane (Agilent, Santa Clara, CA, USA) and analyzed High performance liquid chromatography. A Waters Associates liquid chromatography, model WATERS 2545, equipped with Binary Gradient Module (Pump), sample manager (Injector), 2998 PDA detector. The column system consisted of a C18 5 μM 4.6Χ100 mm column packed with SunFire packing. The mobile phase was acetonitrile and water. Operating conditions were flow rate 1 ml/min, absorbance 254 nm and injection volume was 20 μL. EGCG was prepared as a standard by placing 20 mg of EGCG in a 1 ml of water and diluted to 2 mg/ml.

2.7.2 Antioxidant activity

The antioxidant activities of EGCG and EGCG-Fs were evaluated by 2,2-dipheny-1-picrylhydrazyl (DPPH) radical scavenging [23]. Each of sample (1, 2.5, 5, 10, 15, 20, 25, 30, 40 μM) was mixed with 100 mM DPPH in ethanol solution. The mixture was incubated for 30 min at room temperature and the absorbance of each mixture was obtained at 517 nm on micro plate using the VERSA max (Molecular devices, USA). Water solution was used for negative control. The relative radical scavenging activity was obtained with the following equation:

DPPH radical scavenging activity (%) =[1-(B)/(A)] Χ100

A) represents absorbance of control without test samples, and *(B)* repre sents absorbance in presence of test sample. The results were expresse d with mean ± standard error of the mean. All analyses were carried out in triplicates. α-Tocopherol was used as a positive control. The SC_{50} value (the sample concentration necessary to decrease 50% of the a bsorbance of DPPH) was determined.

2.7.3 The human maltase intestinal maltase inhibition assay

The HMA gene was isolated from the pPICAαA vector. The HMA activity was measured by the amount of released glucose liberated fro m maltose[24]. Glucose was measured by glucose oxidase-peroxidase u sing Glucose-E kit (Asan Co, Korea). The enzymatic reaction was com posed of 10 μL of enzyme (1U/mL), 20 μL of maltose and 10 μL pot assium phosphate buffer (pH 6.5) at 37℃ for 15min. The reaction was stopped by adding 200 μL Tris-HCL buffer (pH 8.0) and 1ml of gluc ose-oxidase assay reagent. After 5min of reactions incubated at 37℃, a nd then 5min in the ice, the absorbance was measured at 505 nm usin g Visible spectrophotometer reader to determine the amount of glucose produced by HMA activity in the reaction. One unit (U) of activity

was amount of enzyme that hydrolyzes 1 μmol of maltose/min.

HMA inhibition activity (%) = [1 - B/A] X 100

A is the absorbance value of the control without compound, and B is the absorbance value of the containing EGCG and EGCG-Fs. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of HMA inhibitor necessary to reduce 50 % of HMA activity. Experiments were performed in triplicate.

2.7.4 Browning Effect of EGCG and EGCG-Fs

The browning effect of EGCG and EGCG-Fs were determined by dissolving in 1 mL of water containing 0.2% (w/v). The samples were then exposed to UV irradiation at a distance of 10 cm from the 254 nm, 10 w, G10T8-AN for 36 h at room temperature. The absorbance increments at 460 nm using SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA)[22].

Results and discussion

3.1 Levansucrase was expressed in *E. coli* using lactose induction

The effect of the induction temperatures on levansucrase expression i n different temperature of the induction (20, 25, 28, 33, 37℃) with 1 mM lactose or 1mM IPTG at 37℃ **(Figure 2).** The highest enzyme a ctivity was 63.3 U/mL at 33℃. In order to determine the optimum lac tose concentration for levansucrase expression, different lactose concent ration from 0.5 to 10 mM or 1 mM IPTG were used for levansucrase induction at 33℃. The highest levansucrase activity was 75 U/mL (**Fi gure 3**).

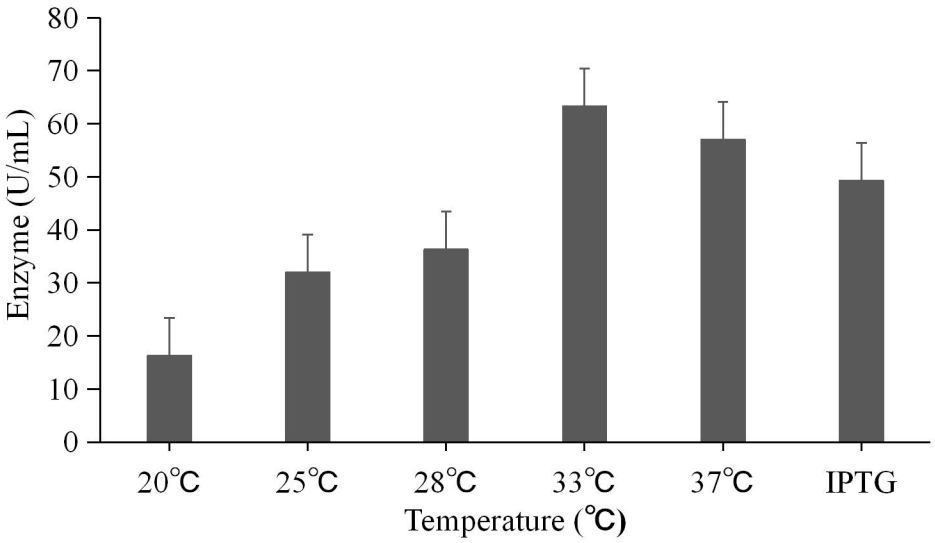


Figure 2. Effect of temperature for enzyme production.

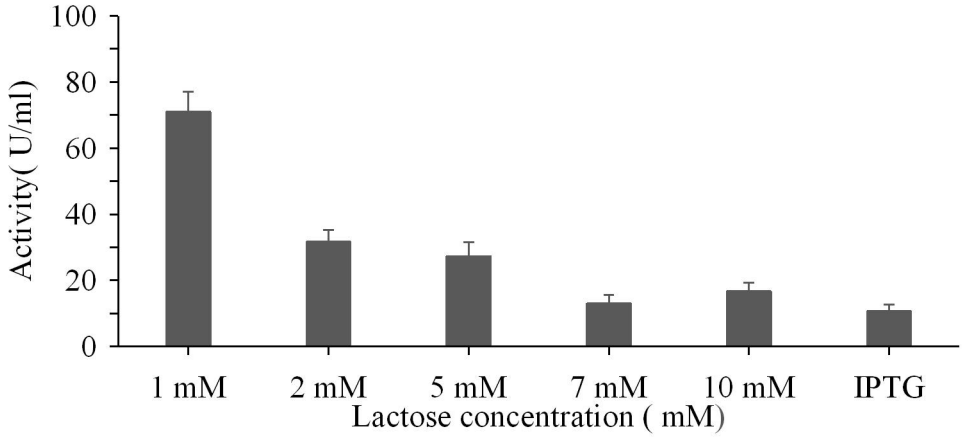


Figure 3. Effect of lactose concentration as an inducer for levansuc rase expression at 33℃.

3.2 Optimization of EGCG-Fs synthesis by RSM

In this study, RSM was used to optimize the conversion from EGCG to EGCG-Fs. The RSM experiments used to determine the effect of three factors [EGCG (x_1), sucrose (x_2), and enzyme (x_3)] are showed in Table 1. The 3D response surface and 2D contour plots of independent variables with respect to response are showed in figure and results of AVOVA (Analysis of variance) of EGCG conversion to EGCG-Fs indicated that the experimental data (**Table 3**). The model used to fit the response variables was found to be significant ($p<0.005$). the model had an F value of 22.13. the adjusted determination coefficient value (R^2_{adj}) for measuring the goodness of the fit of the regression equation was 0.9092. The amount of EGCG converted into EGCG-Fs was expressed with following regression equation:

$$Y=-84.48992+2.08490X_1+0.064799X_2+13.17232X_3+9.31453E\text{-}005X_1X_2-0.11167X_1X_3-4.99055E\text{-}003X_2X_3-0.015465X_1^2-2.00628E\text{-}005X_2^2-0.29863X_3^2$$

Where Y was EGCG conversion to product (%), X_1 was EGCG concentration (mM), X_2 was sucrose concentration (mM), and X_3 was enzyme concentration (U/mL). The regression equation obtained from ANOVA indicated a R^2 (multiple correlation coefficient) value of 0.9522. Based on the model, the predicted response for EGCG-Fs synthesis was

33.4%, and the observed experimental value was 5.3 U/ml levansucra se, 797.4 mM sucrose, 43.1 mM EGCG, representing near identical res ults from the predicted and actual EGCG-Fs synthesis.

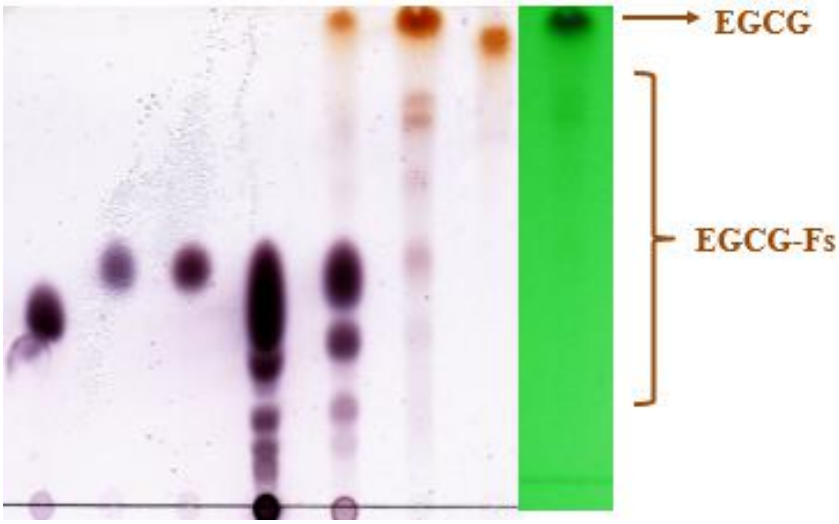


Figure 4. Thin-layer chromatogram analyses of EGCG acceptor rea ction. S, sucrose; F, fructose; G, glucose; 1 enzyme reaction with 10 U/ml and 400 mM sucrose; AR EGCG acceptor reaction before HP 20 column; 2 is EGCG acceptor reaction after purification; E, EGCG.

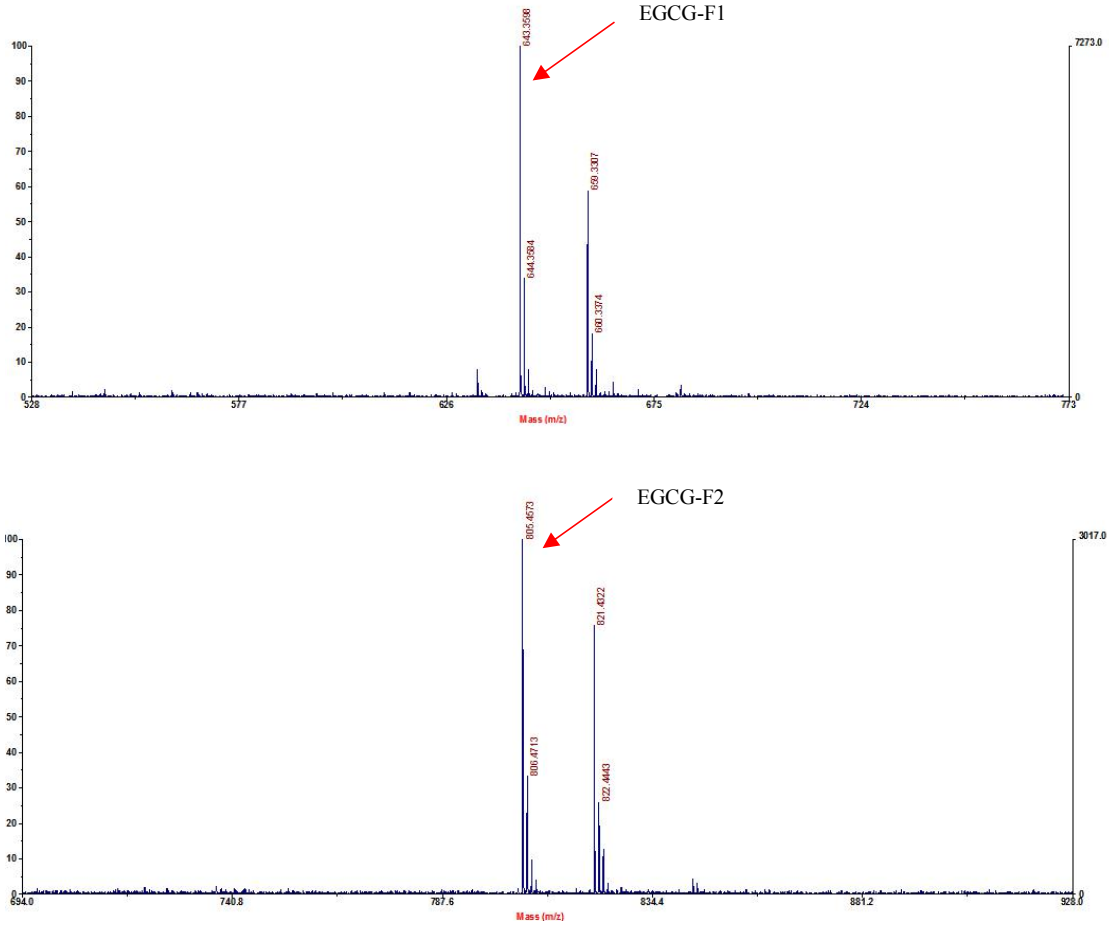


Figure 5. MALDI-TOF mass spectrum of the acceptor reaction pur ification productions. The number of the fructose units attached to E GCG were determined using MALDI-TOF MS analysis. EGCG-F1 (m/ z 643), EGCG-F2 (m/z 805).

Table 2. Running condition for EGCG acceptor reaction and EGC G conversion.

Run No.	Independent variables			EGCG conversion(%)	
	X1	X2	X3	Predicted	Actual
1	70	400	7	19.87	22.34
2	83.6	800	5	18.71	19.33
3	70	1200	3	37.78	38.27
4	50	800	5	34.33	35.27
5	30	400	3	8.756	13.27
6	50	1472	5	33.61	36.21
7	70	400	3	18.38	16.24
8	50	800	5	34.33	35.27
9	50	800	5	34.33	33.27
10	50	800	1.64	28.91	30.57
11	16.4	800	5	15.04	16.33
12	30	400	7	28.11	26.29
13	50	800	5	34.33	36
14	50	800	5	34.33	34.26
15	30	1200	7	28.56	29.36
16	30	1200	3	25.17	21.36
17	50	800	8.36	33.01	33.26
18	50	128	5	16.94	16.24
19	70	1200	7	23.3	15.96
20	50	800	5	34.3	31.6

X_1, the concentration of EGCG;

X_2, the concentration of sucrose;

X_3, the concentration of enzyme

Table 3. Results of two-way analysis of variance (ANOVA)

Source of Variation	Sum of Squares	Degrees of freedom	Mean Square	F Statistic	p-value Prob > F
Model	1313.73	9	145.97	22.13	< 0.0001
Residual	65.95	10	6.59		
Lack of Fit	52.77	5	10.55	4.01	0.077
Pure Error	13.17	5	2.63		
Cor Total	1379.68	19			

R^2 = 0.9522

Adj-R^2 = 0.9092

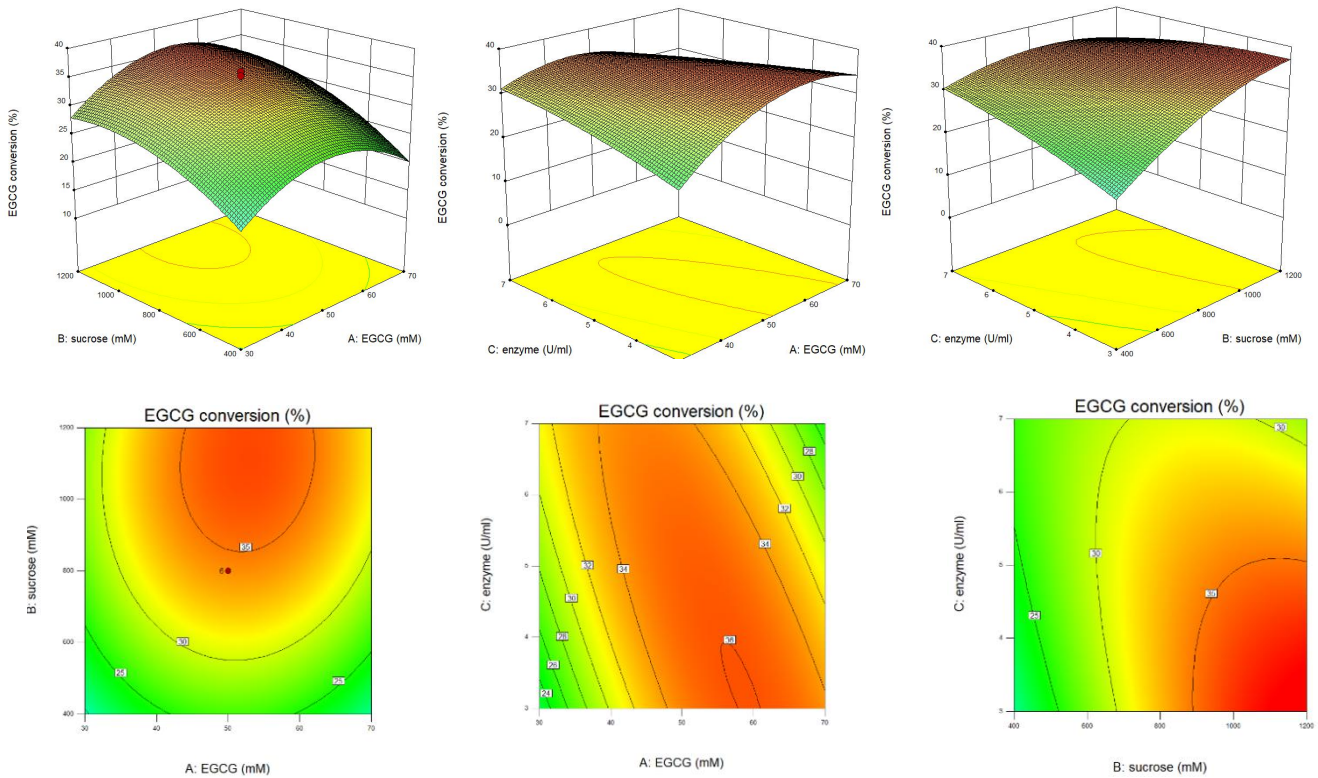


Figure 6. Response surface plot and contour plot. EGCG concentration vs. sucrose concentration (A), EGC G concentration vs. enzyme concentration (B), and sucrose concentration vs. enzyme concentration (C).

3.3 Two novel EGCG-Fs derivatives were identified by NMR

After removing the saccharides, the EGCG-Fs were purification usin g HPLC **(Figure 6)**. Two EGCG-Fs were purified. Through the MALD I-TOF MS analysis, the molecular weight is compound 1 is *m/z* 643 (M+ Na^+), indicating one fructose attachment to EGCG. Compound 2 were observed at *m/z* 805(M+ Na^+), two fructose unit attachment to E GCG. Two EGCG-Fs structures were identified using NMR (^1H, ^{13}C, HSQC, HMBC, and COSY).

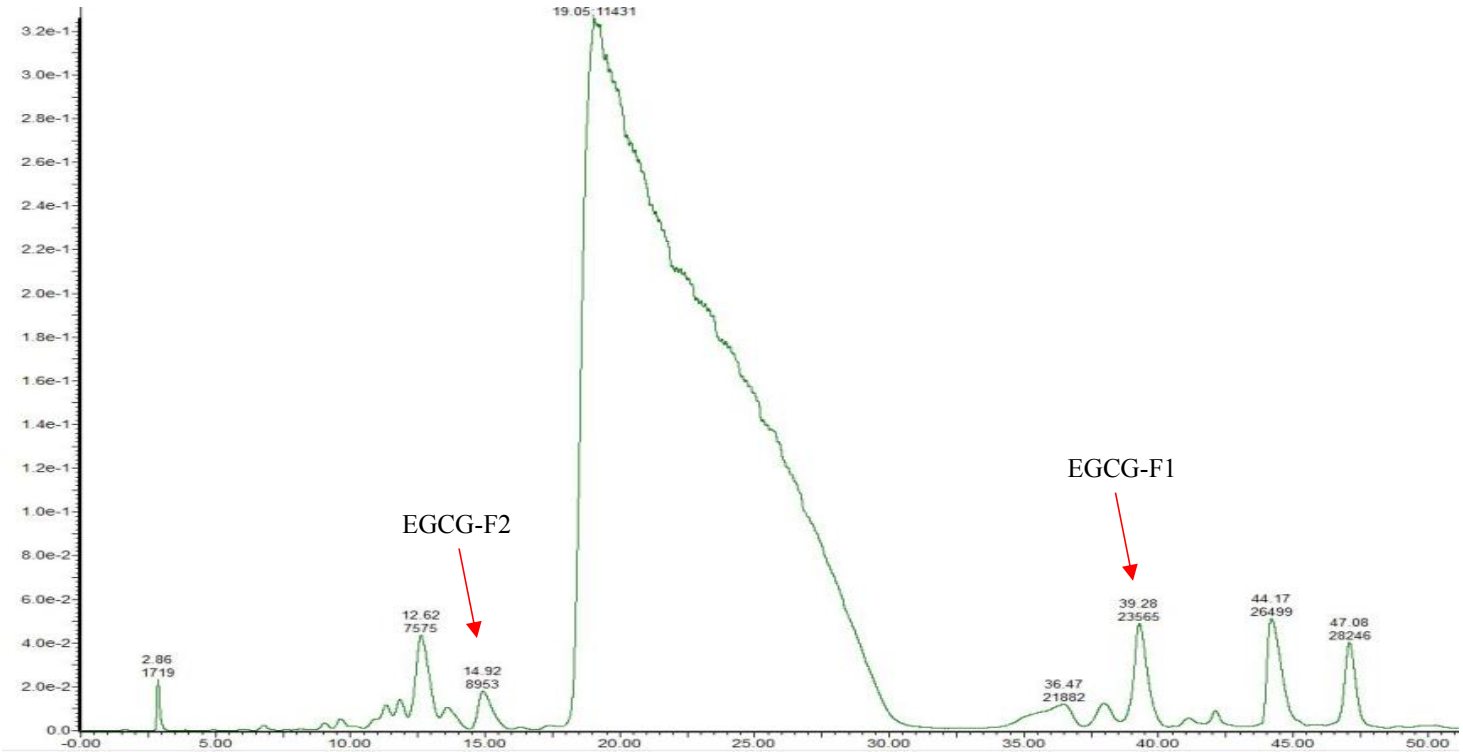


Figure 7. HPLC chromatogram of the EGCG acceptor reaction products using C_{18} prep column.

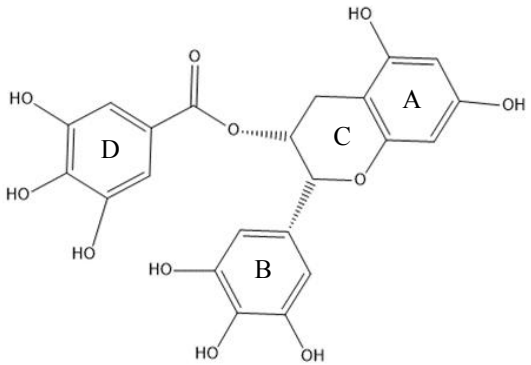
Compound 1(EGCG-F1): The levansucrase composed of β-(2-6)-linked fructose residues, so fructoside C-2 at 108.34 ppm than the other fru ctoside C. EGCG C-4'' at 138.7 ppm, but the EGCG-F1 C-4'' is 155.3 9 ppm, which higher than EGCG. Therefore, structure of the compoun d 1 was identified as 4''-EGCG-β-D-fructofuranoside (**Figure 8**).

Compound 2(EGCG-F2): Two double signals at 3.78 ppm, 3.76 ppm, were assigned to the fructoside 1 C-2 108.1 at HMBC spectrum, so t wo fructosides is β-(2-6) linked. And second fructose C-2 is 104.14 p pm. EGCG C-5' at 145.3 ppm, which is the same with EGCG C-5'. T herefore, structure of the compound 2 was identified as 5'-EGCG-β-D-f ructofuranosyl-β-2-6-fructofuranoside (**Figure 9**).

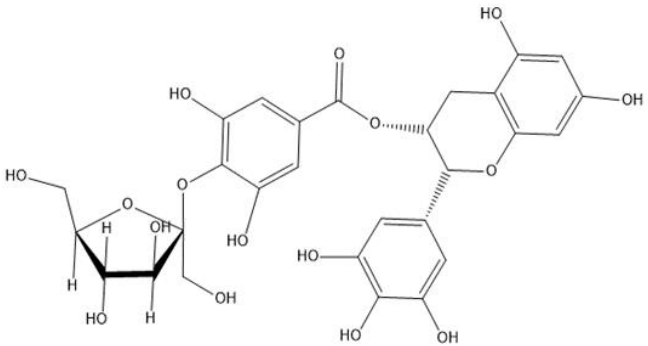
Table 4. ^{1}H, ^{13}C NMR of the EGCG-Fs.

	EGCG		EGCG-F1		EGCG-F2	
Carbon position	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**
2	76.6	4.95 (br s)	76.29	5.01 (br s)	76.25	5.01 (br s)
3	68.2	5.36 (br s)	67.68	5.39 (br s)	68.06	5.36 (br s)
4	25.9	2.92 (dd) (*J*=4.5, 17Hz)	25.71	2.92 (d) (*J*=17Hz)	25.66	2.92 (d) (*J*=17 Hz)
		2.65 (br d) (*J*=17Hz)		2.65 (br d)(*J*=16.85 Hz)		2.67 (br d) (*J*= 16.85Hz)
4a	97.6		97.32		97.34	
5	155.8		155.47		155.52	
6	94.5	5.82 (d) (*J*=2.5 Hz)	94.13	5.86 (d) (*J*=2.5 Hz)	94.3	5.85 (d) (*J*=2 Hz)
7	156.7		156.42		155.47	
8	95.7	5.93 (d) (*J*=2.5 Hz)	95.27	5.94 (d) (*J*=2.5 Hz)	95.6	5.93 (d) (*J*=2 Hz)
8a	156.7		156.36		156.43	
1'	128.8		127.61		128.19	
2'	105.7	6.4 (s)	105.27	6.44 (s)	109.44	6.79 (s)
3'/5'	145.6		150.93		145.43	
4'	132.5		135.24		136.61	
6'	105.7	6.4 (s)	105.13	6.41 (s)	111.9	6.79 (s)
1"	119.5		94.07		119.1	
2"/6"	108.8	6.81 (s)	108.41	6.8 (s)	108.57	6.81 (s)
3"/5"	145.8		145.37		145.43	

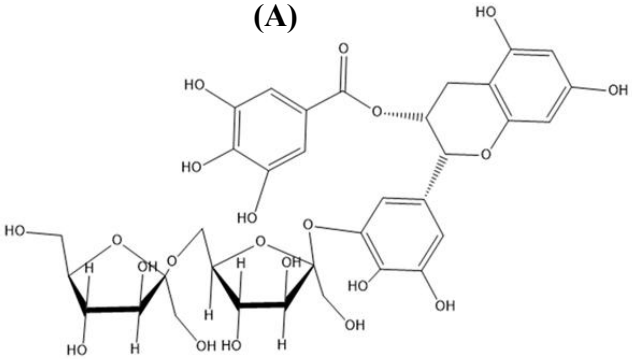
4"	138.7	155.39	136.61	
COO-	165.4	165.17	165.07	
Fructose (-F1)				
1'''		60.6	3.39/3.34	59.52	3.25/3.4
2'''		108.34	108.1	
3'''		76.09	4.0334	75.83	4.01
4'''		73.47	3.8642	75.16	3.95
5'''		82.17	3.5834	76.31	3.95
6'''		60.99	3.12/3.14	60.55	3.26/3.36
Fructose (-F2)				
1''''			63.11	3.69/3.68
2''''			104.14	
3''''			62.73	3.52
4''''			81.33	3.74
5''''			82.13	3.52
6''''			75.1	3.78/3.76



(A)



(B)



(C)

Figure 8. The structures of EGCG (A), EGCG-F1 (B), EGCG-F2 (C)

3.4 Assessment of the biological activities of EGCG-Fs.

3.4.1 Water solubility of EGCG and EGCG-Fs.

Solubility may play a major role in the therapeutic efficacy of catec hin. We carried out a comparison of the water solubility of the EGCG and EGCG-Fs. The solubility of EGCG-F1 and EGCG-F2 were 281.4 mM and 350.3 mM, whereas the solubility of EGCG was 5 mM. Th eir solubility was 52.1 and 64.9 times as high as that of EGCG. The EGCG-F2 which have two fructosyl units showed higher water solubili ty than EGCG-F1 which have one fructosyl unit, so EGCG have more fructosyl unit the water solubility will increase.

Table 5. Water solubility of EGCG-F and relative solubility

sample	Solubility in water (mM)	Relative solubility
EGCG	5.4	1
EGCG-F1(4"-EGCG-β-D-fructofuranoside)	281.4	52.1
EGCG-F2(5'-EGCG-β-D-fructofuranosyl-β-2-6-fructofuranoside)	350.3	64.9

3.4.2 Antioxidant activities (%) of EGCG and EGCG-Fs as their c oncentration.

Previous structure activity studies suggested that flavonoids with an *0*-dihydroxy or trihydroxy B ring are the most effective antioxidants[2 5]. So EGCG have the highest activity as antioxidants[26]. In this stud y, the antioxidant effects of EGCG and EGCG-Fs against DPPH were evaluated at concentration from 1μM to 40μM. The SC_{50} values of EG CG, EGCG-F1 and EGCG-F2 were 7.6±0.1, 14.1±0.1, 9.7±0.2 μM, res pectively (**Figure 9**). EGCG-F2 is similar antioxidant activity with EG CG but EGCG-F1 is lower than EGCG. Compared EGCG-F1 and EG CG-F2, EGCG-F2 is higher antioxidant activity than EGCG-F1. The re sults showed, the fructosylation at the 4''-OH group in the gallate ring have lower antioxidant activities than fructosyl units at the 5'-OH gro up[23]. And, the EGCG-F2 have fructosyl units at the 5'-OH in the B ring, it's have similar antioxidant activity with EGCG. The result imp lied that the 5'-OH in the B ring showed important influence on antio xidant activity.

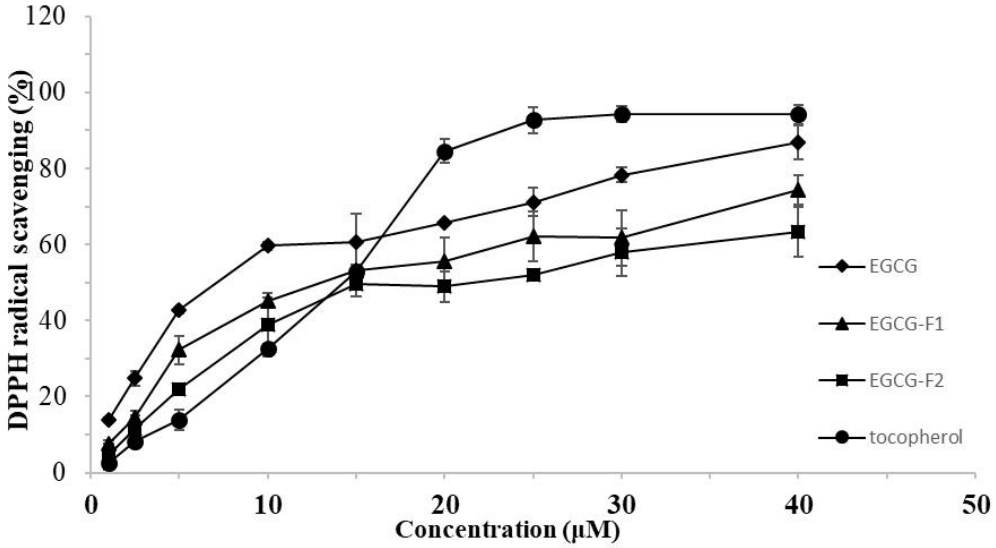


Figure 9. DPPH radical-scavenging activities of EGCG and the EG CG fructosides.

3.4.3 Inhibition activity of EGCG and EGCG-Fs against human int estinal maltase (HMA).

The α-glucosidase HMA has been an attractive treatment agent for t ype 2 diabetes and obesity[27]. α-glucosidase inhibition of its maltose-hydrolyzing activity can retard glucose production and decrease postpra ndial blood glucose level[28]. HMA is an N-terminal catalytic domain of human intestinal maltase-glucoamylase, which is responsible for α-gl ucosidase activity, hydrolyzes α-1, 4-linkage of maltose and results in t he production of glucose[29]. In this study, the HMA inhibition activiti es of EGCG, EGCG-F1, and EGCG-F2 were: 87.8 ± 5.8, 173.4 ± 11. 6 and 253 ± 16 μM (**Figure 10**). Compared EGCG-F1 and EGCG-F2, the EGCG-F1 have higher inhibitory activity against HMA than EGC G-F2. The results showed, fructosyl unit at the 4''-OH group showed higher inhibitory activity against HMA than 5'-OH group, and two fruc tosyl units have lower inhibitory activity against HMA than one fructo syl unit.

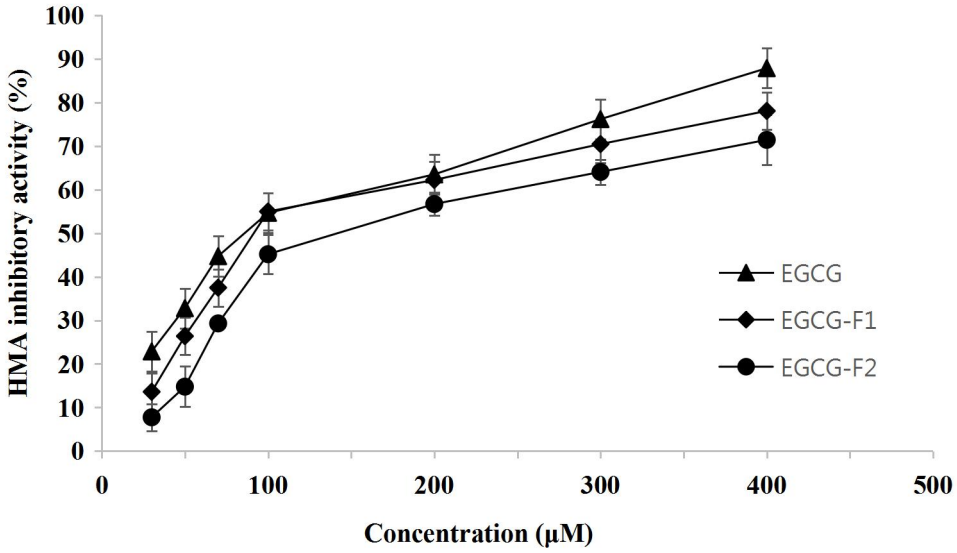


Figure 10. Inhibitory activity against human intestinal maltase of E GCG and the EGCG-fructosides.

3.4.4 The browning effect of EGCG and EGCG-Fs.

Catechin in water is susceptible to degradation and browning by UV irradiation[30]. (**Figure 11**) showed the browning effect of EGCG and EGCG-Fs after UV irradiation. The two compound showed higher bro wning effect than EGCG. And EGCG-F2 is higher browning effect tha n EGCG-F1. The result showed, fructosyl unit at the 5'-OH group is h igher browning effect than 4''-OH group, and two fructosyl units is hi gher than one fructosyl unit.

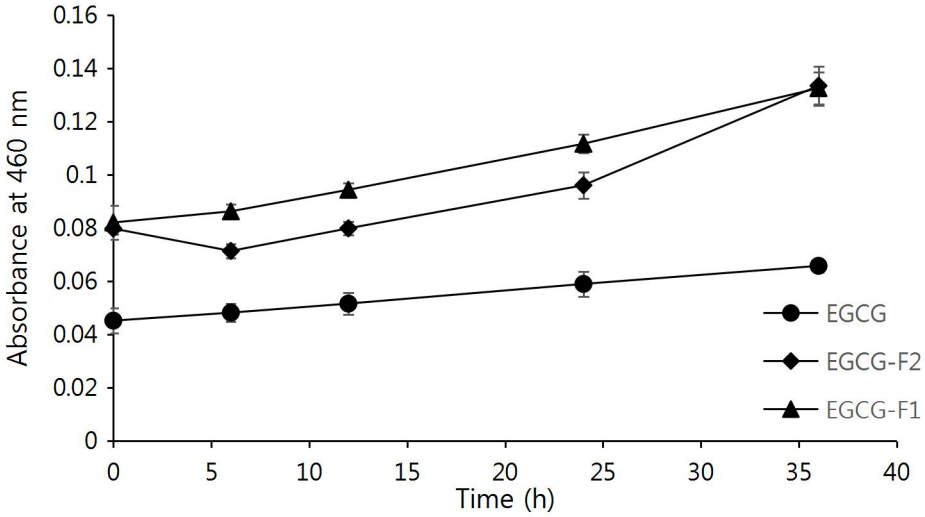


Figure 11. Browning effect to UV irradiation of EGCG and the E GCG fructosides.

Conclusion

In this study, conversion of EGCG into EGCG-Fs by levansucrase w as optimized through the Response Surface Method, the optimum condi tions for EGCG-Fs production were: 42.1 mM EGCG, 797.4 mM sucr ose, 5.3 U/mL levansucrase, and we purified two novel EGCG-Fs deri vatives, structure of two compounds were identified with MALDI-TOF MS and NMA data. The water solubility of EGCG-Fs had 60 times higher than EGCG. Antioxidant activities of EGCG-F2 is similar with EGCG. The EGCG-F1 have higher inhibitory activity against HMA tha n EGCG-F2. On the basis of the relationship between biological activit ies and structural configurations of transfructosylation EGCG. Compoun d with fructosyl unit at 4''-OH group in the gallate ring have lower a ntioxidant activities than fructosyl units at the 5'-OH group and 5'-OH group have similar antioxidant activity with EGCG. However, fructosy l unit at the 4''-OH group showed higher inhibitory activity against H MA than 5'-OH group.

References

1. Graham, H.N., *Green tea composition, consumption, and polyphenol c hemistry.* Preventive medicine, 1992. **21**(3): p. 334-350.
2. Singh, B.N., S. Shankar, and R.K. Srivastava, *Green tea catechin, epigall ocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical appli cations.* Biochemical pharmacology, 2011. **82**(12): p. 1807-1821.
3. Elbling, L., et al., *Green tea extract and (–)-epigallocatechin-3-gallate, t he major tea catechin, exert oxidant but lack antioxidant activities.* Th e FASEB journal, 2005. **19**(7): p. 807-809.
4. Wang, Y.-C. and U. Bachrach, *The specific anti-cancer activity of green tea (−)-epigallocatechin-3-gallate (EGCG).* Amino acids, 2002. **22**(2): p. 131-143.
5. Zhao, W.-H., et al., *Inhibition of penicillinase by epigallocatechin gallat e resulting in restoration of antibacterial activity of penicillin against p enicillinase-producing Staphylococcus aureus.* Antimicrobial agents and chemotherapy, 2002. **46**(7): p. 2266-2268.
6. Potenza, M.A., et al., *EGCG, a green tea polyphenol, improves endothe lial function and insulin sensitivity, reduces blood pressure, and protec ts against myocardial I/R injury in SHR.* American Journal of Physiolog y-Endocrinology and Metabolism, 2007. **292**(5): p. E1378-E1387.
7. Kitao, S., et al., *Enzymatic syntheses of two stable (-)-epigallocatechin gallate-glucosides by sucrose phosphorylase.* Bioscience, biotechnology, and biochemistry, 1995. **59**(11): p. 2167-2169.
8. Zimeri, J. and C. Tong, *Degradation Kinetics of (−)-Epigallocatechin Gal late as a Function of pH and Dissolved Oxygen in a Liquid Model Sy stem.* Journal of Food Science, 1999. **64**(5): p. 753-758.
9. Meng, G. and K. Fütterer, *Structural framework of fructosyl transfer in Bacillus subtilis levansucrase.* Nature Structural & Molecular Biology, 2 003. **10**(11): p. 935-941.

10. Henrissat, B., *A classification of glycosyl hydrolases based on amino a cid sequence similarities.* Biochemical Journal, 1991. **280**(2): p. 309-316.
11. van Hijum, S.A., et al., *Purification of a novel fructosyltransferase from Lactobacillus reuteri strain 121 and characterization of the levan prod uced.* FEMS microbiology letters, 2001. **205**(2): p. 323-328.
12. Kang, H.K., et al., *Cloning and expression of levansucrase from Leucon ostoc mesenteroides B-512 FMC in Escherichia coli.* Biochimica et Biop hysica Acta (BBA)-Gene Structure and Expression, 2005. **1727**(1): p. 5-15.
13. Moon, Y.-H., et al., *Enzymatic synthesis and characterization of novel e pigallocatechin gallate glucosides.* Journal of Molecular Catalysis B: En zymatic, 2006. **40**(1): p. 1-7.
14. Chow, H.S., et al., *Pharmacokinetics and safety of green tea polyphen ols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals.* Clinical Cancer Research, 2003. **9** (9): p. 3312-3319.
15. Monsan, P. and F. Paul, *Enzymatic synthesis of oligosaccharides.* FEMS Microbiology Reviews, 1995. **16**(2-3): p. 187-192.
16. Funane, K., et al., *An active-site peptide containing the second essenti al carboxyl group of dextransucrase from Leuconostoc mesenteroides by chemical modifications.* Biochemistry, 1993. **32**(49): p. 13696-13702.
17. Jacques, N.A., *The fructosyltransferase of Streptococcus salivarius.* New phytologist, 1993. **123**(3): p. 429-435.
18. Lyness, E. and H. Doelle, *Levansucrase from Zymomonas mobilis.* Biote chnology letters, 1983. **5**(5): p. 345-350.
19. Ohtsuka, K., et al., *Characterization of levansucrase from Rahnella aqu atilis JCM-1683.* Bioscience, biotechnology, and biochemistry, 1992. **56** (9): p. 1373-1377.
20. Chambert, R., G. Treboul, and R. Dedonder, *Kinetic studies of levansuc rase of Bacillus subtilis.* The FEBS Journal, 1974. **41**(2): p. 285-300.
21. Xu, W., et al., *Enzymatic Production of Melibiose from Raffinose by th*

e Levansucrase from Leuconostoc mesenteroides B-512 FMC. Journal o f Agricultural and Food Chemistry, 2017.

22. Moon, Y.-H., et al., *Synthesis, structure analyses, and characterization o f novel epigallocatechin gallate (EGCG) glycosides using the glucansuc rase from Leuconostoc mesenteroides B-1299CB.* Journal of agricultural and food chemistry, 2006. **54**(4): p. 1230-1237.
23. Kim, J., et al., *Functional properties of novel epigallocatechin gallate gl ucosides synthesized by using dextransucrase from Leuconostoc mese nteroides B-1299CB4.* Journal of agricultural and food chemistry, 2016. **64**(48): p. 9203-9213.
24. Ryu, H.-J., et al., *Expression, purification, and characterization of huma n intestinal maltase secreted from Pichia pastoris.* Food Science and B iotechnology, 2011. **20**(2): p. 561-565.
25. Pietta, P.-G., *Flavonoids as antioxidants.* Journal of natural products, 20 00. **63**(7): p. 1035-1042.
26. Salah, N., et al., *Polyphenolic flavanols as scavengers of aqueous phas e radicals and as chain-breaking antioxidants.* Archives of biochemistry and biophysics, 1995. **322**(2): p. 339-346.
27. Nguyen, T.T.H., et al., *Inhibitory effects of epigallocatechin gallate and its glucoside on the human intestinal maltase inhibition.* Biotechnolog y and bioprocess engineering, 2012. **17**(5): p. 966-971.
28. Park, H., et al., *Discovery of novel α-glucosidase inhibitors based on t he virtual screening with the homology-modeled protein structure.* Bio organic & medicinal chemistry, 2008. **16**(1): p. 284-292.
29. Rossi, E.J., et al., *Inhibition of recombinant human maltase glucoamyla se by salacinol and derivatives.* The FEBS journal, 2006. **273**(12): p. 26 73-2683.
30. Nakano, H., et al., *Transglycosylation of Hydroquinone and Epicatechin by β-Fructofuranosidase from Arthrobacter sp.* Journal of Applied Gly coscience, 2002. **49**(2): p. 115-121.

Abstract in korean

과당전이효소의 일종인 러반수크레이즈는 β-2, 6 혹은 β-2, 1 결합으로 과당을 연결하는 특성을 가지고 있다. *Leuconosto mesenteroids* 유래의 러반수크레이즈를 *E. coli* BL21(DE3)에서 발현하였으며 러반수크레이즈의 발현 최적화 조건은 최종 농도 1 mM 유당, 25 μM 암피실린을 이용하여 33℃에서 배양을 하였고 2.84 U/mg의 활성을 나타내었다. 본 연구에서는 러반수크레이즈를 이용하여 에피갈로카테킨 갈레이트 (EGCG) 배당체를 합성하였다. 5개의 새로운 에피갈로카테킨 갈레이트(EGCG) 프락토사이드가 합성되었으며, 고분자와 단당류는 각각 90% 에탄올 침전법과 HP-20 컬럼을 이용하여 제거되었으며, 최종적으로 C18컬럼을 장착한 고성능 액체 크로마토그래피를 이용하여 2개 산물을 정제하였다. 또한 NMR 분석을 통해 그 구조를 밝혔다. 정제한 산물을 이용하여 기능성을 평가하였다. 그 결과, 5'-EGCG-β-D-fructofuranosyl-β-2-6-fructofuranoside은 EGCG에 보다 수용성이 64배 증가하였고 EGCG와 비슷한 수준의 항산화능(SC50, 9.7 μM)을 나타내었다. 그리고 EGCG-F1(IC50, 173 μM)은 EGCG-F2(IC50, 253 μM)보다 항당뇨 효과도 뛰어 나는것으로 나타났다.

주요어: 에피갈로카테킨 갈레이트, 러반수크레이즈, 항산화능

학 번: 2016-22047