Enzymatic synthesis of chlorogenic acid glucoside using dextransucrase and its physical and functional properties

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ABSTRACT

Chlorogenic acid, a major polyphenol in edible plants, possesses strong antioxidant activity, anti-lipid peroxidation and anticancer effects. It used for industrial applications; however, this is limited by its instability to heat or light. In this study, we, for the first time synthesized chlorogenic acid glucoside (CHG) via transglycosylation using dextransucrase from *Leuconostoc mesenteroides* and sucrose. CHG was purified and its structure determined by nuclear magnetic resonance and matrix-associated laser desorption ionization–time-of-flight mass spectroscopy. The production yield of CHG was 44.0% or 141 mM, as determined by response surface methodology. CHG possessed a 65% increase in water solubility and a 2-fold browning resistance and it displayed stronger inhibition of lipid peroxidation and of colon cancer cell growth by MTT assay, compared to chlorogenic acid. Therefore, this study may expand the industrial applications of chlorogenic acid as water-soluble or browning resistant compound (CHG) through enzymatic glycosylation.

Keywords: Chlorogenic acid; dextransucrase; *Leuconostoc mesenteroides*

1. Introduction

Chlorogenic acid is one of the most abundant polyphenol in the human diet, and is consumed in coffee, fruits, and vegetables [1]. Chlorogenic acid is an ester -formed between quinic acid and certain trans-hydroxycinnamic acids, such as like caffeic, ferulic, or p-coumaric acids [2–3]. It has three different isomers: 3-*O*-caffeoylquinic acid (chlorogenic acid), 5-*O*-caffeoylquinic acid (neo-chlorogenic acid) and 4-*O*-caffeoylquinic acid (krypto-chlorogenic acid) [4–5]. Chlorogenic acid has antioxidant [2, 6], antimicrobial [7], and anxiolytic activity [8]. Recent reports indicate that this compound also exerts anti-proliferative effects against some types of cancer cell, such as those from liver, blood, or brain cancer [9–11].

Due to the various beneficial effects of chlorogenic acid, it has been continually demanded as a functional compound in the food and pharmaceutical areas [12]. Even though there are many usages of chlorogenic acid, it is readily oxidized and sensitive to heat and light, which restricts its application in industry [13]. There have been attempts to improve the bioactivity or stability of light and heat sensitive compounds using enzymatic modification, such as glucansucrase, for extending their industrial applications [14–18]. Dextranucrase produces dextran using sucrose and also transfers glucose units to other carbohydrates, or phenolic acceptors via glycosidic linkages [17]. In our previous studies, enzymatic transglycosylation using dextranucrase was applied to modify various bioactive compounds in order to improve their physical or functional properties such as acarbose to be used as anti-diabetic agents, salicin to increase blood coagulation, or hydroquinone to inhibit melanin synthesis [14, 15, 16]. Another bioactive compound, the glycosylated epigallocatechin gallate (EGCG) or glycosylated quercetin by dextranucrases were reported to be stable to UV radiation and were 100- or 12-fold more water soluble than the unstable EGCG or quercetin [17–18].

To our knowledge, there is no report of transglucosylation of chlorogenic acid using dextranucrase. In this study, chlorogenic acid-4-*O*- α -D-glucopyranoside (CHG) was synthesized using dextranucrase and sucrose. CHG was further purified by butanol extraction, silica gel column and preparative high-performance liquid chromatography (HPLC), and identified by nuclear magnetic resonance (NMR) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). CHG production was optimized using the response surface methodology. The purified CHG was characterized for physical stability as well as its antioxidant activity, inhibition of lipid peroxidation, and viability of HT-29 colon cancer cells.

2. Materials and methods

2.1. Materials

Chlorogenic acid, deuterium oxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH), α -tocopherol and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum, penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, USA). Silica gel beads (40–60 μ m) were purchased from Acros Organics (Geel, Belgium). Other chemical reagents were commercially available and of chemically pure grade.

2.2. Production and purification of chlorogenic acid glucoside

A dextranucrase mutant of *L. mesenteroides* B-1299 constitutive for dextranucrase (B-512FMCM) was isolated after ethyl methane sulfonate mutagenesis and was cultured on LM medium using 2% (w/v) glucose [18]. The activity of partially purified enzyme was determined by incubation for 6 h at 28°C with 100 mM sucrose in 20 mM sodium acetate, pH

5.2 [18–19]. One unit of dextranucrase activity was defined as the amount of enzyme required to generate 1 μ mol of fructose per minute at 28°C and pH 5.2 in 20 mM sodium acetate buffer [15].

The reaction mixture (1 L) in 20 mM Na-acetate (pH 5.2) consisting of 325 mM chlorogenic acid, 355 mM sucrose, and BF512 dextranucrase (650 mU/ml) was incubated at 28°C for 6 h, and boiled for 10 min to stop the reaction. Chlorogenic acid glucoside was confirmed by thin layer chromatography (TLC) analysis (Merck, Darmstadt, Germany) at room temperature according to previous study with some modifications [16]. The reaction digest (1 L) was partitioned with the same volume of n-butanol to obtain chlorogenic acid modified products from the n-butanol layer. These were further concentrated and applied to a 4.0 $\times$ 75 cm silica gel column and purified CHG was eluted with 85% (v/v) acetonitrile in water. The column was washed with distilled water to remove the remaining sugars (polymers, sucrose, and glucose). CHG purity was confirmed by HPLC under the following conditions: column TSK-GEL, amide-80, 5 μ m (Waters, Milford, MA, USA); mobile phase, acetonitrile: water = 80:20 (v/v); flow rate, 1.0 mL/ min; room temperature; detection, RID-10A RI detector (Shimadzu, Tokyo, Japan).

2.3. Structural identification

Purified CHG was assayed for its melting point (Stuart SMP10, Bibbly sterlin LTD, OSA, UK) and optical activity with $[\alpha]_D^{25} -10.3^\circ \pm 0.11$ c =1 in H₂O (Jasco P-2000 Polarimeter, Ishikawa-Cho, TOKYO, Japan). You do not report the melting point in the results section. Purified CHG (5 mg/ml) was diluted with deionized water and then mixed 1:1 (v/v) with 2,5-dihydroxybenzoic acid (1 mg/mL) dissolved in water. The mixed solution (1 μ L) was then spotted onto a stainless steel plate and slowly dried at room temperature. The mass spectrum was acquired using a Voyager DE-STR MALDI-TOF (Voyager DE-STR, Applied

Biosystems, Poster, CA, USA). The mass spectra were obtained in the positive linear mode with delayed extraction (average of 75 laser shots) with a 65 kV acceleration voltage. Approximately 10 mg of purified CHG were dissolved in DMSO-d₆ (250 µL) and placed into 5 mm NMR tubes. NMR spectra were obtained on Avance-500 (Bruker, Pleasanton, CA, USA) from the National Center for Inter-University Research Facilities (Seoul National University, Seoul) operating at 500 MHz for ¹H and 125 MHz for ¹³C at 25°C. The glucosidic linkage between chlorogenic acid and glucoside was determined via homo nuclear correlation spectroscopy (COSY), hetero nuclear single quantum coherence (HSQC), and hetero nuclear multiple bond correlation (HMBC) analyses [17].

2.4. Experimental design for optimized CHG production

The experimental response surface methodology (RSM) data were fitted via the response surface regression procedure using the following second-order polynomial equation [20]: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$. The Design-Expert 6.0.11 central composite design RSM software (State-Ease, Minneapolis, MN, USA) was also used for regression analysis and graphical analysis of the data obtained during whole experiments. Analysis of variance was used to estimate the statistical parameters. The significance of the model equation and model terms was evaluated by Fisher's test. The quality of fit for the polynomial model equation was expressed by the coefficient of determination (R^2) and adjusted R^2 . The fitted polynomial equation was expressed as three-dimensional surface plots to show the relationship between the responses and the experimental levels of each variables used in the design. The combination of optimized parameters producing the maximum response was determined to verify the validity of the model.

2.5. Water solubility and browning resistance

Excess chlorogenic acid or CHG was mixed with 200 μ l of water in an Eppendorf tube at room temperature. An ultrasonic cleaner was used to maximize solubility. After 1 h of sonication at room temperature, each sample was diluted and then filtered through a 0.45 μ m MFS membrane for HPLC analyses to determine the maximum concentrations dissolved in water [17]. Browning resistance of each chlorogenic acid or CHG after UV irradiation in an aqueous system was evaluated in water (1 mL) containing 1 mM chlorogenic acid or CHG. The sample solutions were exposed to UV irradiation at a distance of 10 cm from the 254 nm, 10 W UV source for 24 h at room temperature. Increases in absorbance at 460 nm were determined using a UV spectrophotometer [17].

2.6. Functional characterization

Chlorogenic acid and CHG were functionally studied with respect to their antioxidant, anti-lipid peroxidation, and HT29 cell growth inhibitory activities. The antioxidant activities of chlorogenic acid and CHG were assessed using DPPH radical scavenging assays [21]. IC₅₀ designated the concentration of the sample in which the levels of DPPH radicals were reduced by 50%.

Anti-lipid peroxidation activity was measured using a HP-CLA chemiluminescence-measuring device (Tohoku Electronic Industrial, Tokyo, Japan) and an ARAL kit (ABCD GmbH, Berlin, Germany). According to the thermo-initiated chemiluminescence (TIC) method, the antioxidant species in the sample was incubated with ample free radical-attached luminol to delay generation of photons until the antioxidant species were consumed. The lag time (s) was proportional to the amount of antioxidant species in each sample [22].

HT29 human colon cancer cells, from a colonic adenocarcinoma of a female Caucasian, were purchased from the American Tissue Culture Collection (HTB-38™, Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated

fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Inhibition of cell growth was assessed by MTT assay. Percent cell viability was calculated as follows: cell viability (%) = (absorbance of the sample tested/absorbance of the medium only) × 100. The concentration of chlorogenic acid or CHG compound at which 50% of the cells survived (IC₅₀) was also determined.

3. Results

3.1. Synthesis and purification

After the acceptor reaction of *L. mesenteroides* 512 FMCM dextransucrase with chlorogenic acid and sucrose, one reaction product, chlorogenic acid glucoside (CHG) was detected via TLC analysis (Fig. 1A). Butanol partitioning was used for the removal of unreacted or hydrolyzed carbohydrates and enzymes and the butanol layer was subjected to silica gel column chromatography and preparative HPLC purification (Fig. 1B). Chlorogenic acid and CHG in upper butanol layer were eluted by silica gel column chromatography using 85% (v/v) acetonitrile in water. Pure CHG with 89% purity was obtained as a single band by HPLC analysis. The yield of CHG purification was 19.2 g (38.4% of total CHG synthesized). The purified CHG was obtained as a brownish-white powder. The final optimum conditions for CHG synthesis were 320 mM chlorogenic acid, 365 mM sucrose, and 0.96 U or 963 mU/mL dextransucrase, which resulted in a yield of 141 mM (44.0% - v/v conversion of chlorogenic acid used). You can give the results of the melting point and optical activity here (see methods 2.3)

3.2. Structural determination

The number of glucose units attached to compounds was verified using MALDI-TOF MS. The molecular weight of compounds was increased by attaching one glucose. The NMR

data of CHG (¹H, ¹³C, COSY, HSQC, and HMBC) of chlorogenic acid and chlorogenic acid glucoside are shown in Table 1 and Fig. S1. The molecular ions of CHG were observed at m/z at 541 (M + Na)⁺. In Table 1, a doublet signal at 5.32 ppm (J=3.5 Hz) was assigned to the anomeric proton, showing that one glucosyl residue was α-linked to the chlorogenic acid (Fig. 2 and Table 1).

Most carbon signals of CHG were significant with chlorogenic acid (Table 1), with chlorogenic acid exception of at 70.62 ppm to C-4, 68.40 ppm to C-5, 128.85 ppm to C-1', 116.84 ppm to C-5', and 116.43 ppm to C-8'. From the HMBC data, a correlation was observed between the proton signal H-1" of glucopyranoside and the C-4' of chlorogenic acid. The C-1" of the glucosyl residue was detected at 99.54 ppm from the HSQC data. According to these results, the structure of CHG was confirmed as chlorogenic acid-4'-O-α-D-glucopyranoside (Fig. 2).

3.3 Optimum CHG Synthesis

The effect of three variables (sucrose concentration, dextranase unit, and chlorogenic acid concentration) on the yield of CHG was determined. A total of 20 experiments were performed to investigate the interaction of these variables to CHG synthesis (Table S1 and S2). The response surface and their contour plots were described by the second-order polynomial equation (Fig. 3 and Table 2). As ascertained from the central points of the corresponding contour plots, the optimal conditions of the three variables were 365 mM sucrose, 963 mU/ml dextranase activity, and 320 mM chlorogenic acid. Values of “Prob > F” < 0.05 indicate that model terms, x_2 , x_3 , x_{21} , x_{22} , x_{23} , x_1x_2 , and x_1x_3 are significant (Table S2). The response, CHG synthesis by dextranase acceptor reaction, was expressed in terms of the following regression equation: $y = -178.7 + 0.29x_1 + 0.29x_2 + 0.74x_3 - 0.000001x_1^2 - 0.00002x_2^2 + 0.000005x_3^2 - 0.00037x_1x_2 - 0.00014x_1x_3 - 0.0011x_2x_3$, where x_1 is the sucrose

concentration (mM), x_2 is the dextransucrase units (mU/ml), and x_3 is the chlorogenic acid concentration (mM).

The regression equation obtained from ANOVA indicated a R^2 (multiple correlation coefficient) = 0.89, explaining 89% of the variation in response (Table S2). The “adequate precision value” of the present model was 7.78, suggesting that value above 4 are desirable for a good fitted model. Predicted response for CHG synthesis was 123.7 mM, and the observed experimental value was 124.8 ± 9.62 mM at 355 mM sucrose, 650 mU/mL dextransucrase, and 325 mM chlorogenic acid, suggesting near identical results between the predicted and actual CHG synthesis. The results indicated only 0.9% difference between experimental and predicted CG synthesis.

3.4. Solubility and stability of CHG

The water solubility of chlorogenic acid or CHG was tested using a method described previously [17, 23]. Chlorogenic acid and chlorogenic acid glucoside could be dissolved in water up to a concentration of 65.5 mM and 107.5 mM, respectively. The water solubility of chlorogenic acid was improved by 163% via glycosylation, indicating that attached glucosyl residues played a role in water solubility. These results agreed with those of our previous studies that reported higher solubility of EGCG or quercetin after glycosylation as compared to that of non-glycosylated compounds [17, 18].

Data on the browning resistance of chlorogenic acid or CHG in water is presented in (Fig. 4A). Irradiation-associated browning of the chlorogenic acid glucoside conferred stability against UV irradiation (48% of that of chlorogenic acid). Both chlorogenic acid and CHG were labile to UV exposure at 60-120 min incubation period with high absorbance value as presented in Fig. 4A.

3.5. Antioxidant or anti-lipid peroxidation activity

Progressive discoloration of DPPH in the presence of chlorogenic acid or CHG indicated that they were acting as antioxidant compounds. Chlorogenic acid and CHG, displayed different antioxidant activities depending on their structural configurations (Fig. 4B). The IC₅₀ value of CHG based on its DPPH radical-scavenging activity was 0.39 mM, which was lower than that of chlorogenic acid (IC₅₀ = 0.27 mM). As shown in Fig. 3B, the rate of scavenging activity increased with the increase of both concentrations. In addition, under the same chlorogenic acid concentration, CHG scavenged the DPPH radical more slowly than chlorogenic acid.

Chlorogenic acid exhibited varying anti-lipid peroxidation effects, depending on the structural configuration. Chlorogenic acid and CHG exhibited different magnitudes of inhibition of lipid peroxidation (Fig. 4C). Use of α -tocopherol or Vit E (10-100 μ M) as a positive control, resulted in a dose dependent increase in chemiluminescent absorbance units ($y = 0.9671x + 2.8027$, $R^2 = 0.9889$). At 500 μ M, CHG showed 34% higher inhibition activity (27.2 U or 25.1 μ M Vit E eq.) than chlorogenic acid (20.2 U or 17.9 μ M Vit E eq.).

3.6. Inhibition of colon cancer cell growth

We evaluated inhibition of HT29 cell growth by chlorogenic acid and CHG (0.01–5 mM) after 48 h of incubation by the MTT assay (Fig. 4D). CHG exhibited 18.4% greater inhibition of cell growth (IC₅₀ 1.58 mM) than chlorogenic acid (IC₅₀ 1.87 mM) (Fig. 4D). Up to 0.25 mM, survival of HT29 cells by chlorogenic acid or CHG was not significantly affected. Treatment with 1.0 mM CHG or chlorogenic acid resulted in significantly decreased growth rate of HT29 cells by 46% or 52% survival rate of the control, respectively (Fig. 4D).

4. Discussion

So far, chlorogenic acid and its isomers in plant source have been increased using cost-effective process, physical or mechanical treatments. Chlorogenic acid and its isomers in

potatoes were 2-fold increased by treatment of exogenous amylolytic enzyme on wounded tuber surface [24–25]. Chlorogenic acid isomers in coffee bean were 1.9-fold increased by light brewing but 0.5–3 fold decreased by dark brewing [26]. Chlorogenic acid isomers are present in food materials and plants but information or reports about enzymatic transglucosylation is not available [5, 24–28].

CHG is synthesized via the acceptor reaction of dextranucrase with chlorogenic acid and sucrose. Chlorogenic acid was a suitable acceptor for the transglucosylation of dextranucrase with a high conversion yield (44.0%).

The water solubility and browning resistance of chlorogenic acid were improved after glycosylation. This result is consistent with other previous report that glycosylated caffeic acid showed 2–65-fold increase in solubility as compared to caffeic acid itself, depending on the binding position of glucose [28]. The water solubility of caffeoyl 4-O-glucoside was about 35-fold higher than that of caffeoyl 3-O-glucoside [28]. Glycosylated chlorogenic acid was 48% more stable to UV radiation as compared to the unglycosylated compound itself in this study (Fig. 4A). Similarly, glycosylated caffeic acid did not change color even after incubation for 4 d, while caffeic acid changed to a brown color after incubation for 1 d at 50°C [28]. Glycosylated EGCG or ampelisin by dextranucrase also exhibited a 17% or 14.5-fold improved browning-resistant quality than unglycosylated compounds [17, 23]. Interestingly, differing from water solubility, browning-resistant quality of compounds is conferred by glycosylation of the compounds, rather than the glycosylation positions and linkages of the resultant compounds [17, 23, 28].

Our data indicated that attachment of a glucose to chlorogenic acid decreased its antioxidant activity but increased its anti-lipid peroxidation effect (Fig. 4B and 4C). Esterification of caffeic acid by a sugar moiety or quinic acid decreased its DPPH radical-scavenging activity [29–30]. Glycosylated hydroquinone decreased its antioxidant activity

10-fold *in vitro* compared to that of hydroquinone [16]. Attachment of fructose and galactose to hydroquinone also resulted in six- to eight-fold decrease in antioxidant activity when compared to aglycon structures. However, a study reported that antioxidant activity can be determined by glucosidic linkage type (α or β) rather than attachment of sugar [20]. Most of the α -glucosidic linked hydroquinones displayed higher antioxidant activities than β -glucosidic linked hydroquinones, irrespective of the sugar group attached [16, 20, 29]. However, glycosylated caffeic acid exhibited five-fold greater prevention of lipid autoxidation of linoleic acid compared to caffeic acid [28]. Moreover, the hydroquinone fructoside exerted slightly greater inhibition of lipid peroxidation than hydroquinone [16].

Chlorogenic acid significantly reduces the proliferation of human colon and lung cancer cells in a concentration- and time-dependent manner [31]. The survival of HT29 cells treated with chlorogenic acid and CHG did not differ significantly after 24 h. However, chlorogenic acid and CHG significantly inhibited survival of HT29 cells following incubation for 48 h. The rate of inhibition of cell growth was ~50% at >1 mM chlorogenic acid and significant loss of viability was evident after 48 h of treatment [31].

In summary, we report here the synthesis of chlorogenic acid glucoside via transglycosylation using dextransucrase from *L. mesenteroides* and sucrose. CHG was purified and identified by NMR and MALDI-TOF MS. The production yield of CHG was determined using the response surface methodology. CHG showed improved physical stability, and greater inhibition of lipid peroxidation and growth of colon cancer cell, compared to chlorogenic acid. Thus, CHG could be applicable as a novel functional compound in the food and pharmaceutical industries.

Acknowledgements

327   This work was supported by Basic Science Research Program through the National
328   Research Foundation of Korea funded by the Ministry of Education (NRF-
329   2016R1D1A1B03936148), Republic of Korea. It was supported in part by the Basic
330   Science Research Program through the National Research Foundation of Korea funded by the
331   Ministry of Education (NRF-2015R1D1A1A01056929: D. Kim).

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Table and Figure captions

Fig. 1. Analysis of the transglucosylation product by TLC (A) and HPLC (B).

(A) Lane 1: sucrose; lane 2: fructose; lane 3: glucose; lane 4: enzyme; lane 5: standard chlorogenic acid; lane 6: dextransucrase reaction digest. (B) HPLC chromatogram of chlorogenic acid glucoside after butanol separation and silica gel column chromatography. Column, TSK-GEL, amide-80, 5 μ m (Waters, Milford, MA, USA); mobile phase, acetonitrile/water = 80:20 (v/v); flow rate, 1.0 ml/ min; room temperature; detection, RID-10A RI detector (Shimadzu).

Fig. 2. HMBC correlation of CHG between chlorogenic acid and glucosyl group

Table 1.

NMR analysis of chlorogenic acid and its glucoside, CHG

Table 2.

Central composite design matrix for the experimental design and predicted responses for CHG synthesis^a

Fig. 3. Response surface and contour plots of CHG production. Shown are the mutual interactions between dextransucrase and sucrose (A), chlorogenic acid and sucrose (B), and chlorogenic acid and dextransucrase (C). Synthesis of CHG was optimized by dextransucrase from *L. mesenteroides* (61–1238 mU/ml), sucrose concentration (10–700 mM), and chlorogenic acid concentration (30–619 mM).

458 **Fig. 4.** Browning resistance or functional study of chlorogenic acid and CHG with respect to
459 DPPH scavenging activity, lipid peroxidation inhibition, and growth inhibition of HT-29
460 human colon cancer cells. (A) After UV irradiation on at a distance of 10 cm from 254 nm,
461 10 W for 6 h at room temperature, browning resistance of chlorogenic acid or CHG (1 mM)
462 were determined by the increases in absorbance at 460 nm. (B) Each sample (30 μ L),
463 chlorogenic acid or CHG (0–2.0 mM), was mixed with 100 μ M 1,1-diphenyl-2-picrylhydrazyl
464 (270 μ L) in darkness at room temperature for 10 min, and then the absorbance at 517nm was
465 monitored. (C) Lipid peroxidation inhibition was measured using kits from ABCD GmbH
466 and an HP-CLA chemiluminescence detection instrument (Tohoku, Japan). α -Tocopherol
467 (10, 25, 50, and 100 μ M) was used as a control and chlorogenic acid or CHG at 500 μ M was
468 used to determine inhibition of lipid peroxidation. Different letters above the bar are
469 statistically different by Duncan's multiple range test ($P < 0.05$). (D) HT-29 cells (2×10^4 per
470 well) were treated with a series of chlorogenic acid or CHG concentrations (0–20 mM) for 48
471 h. Data are means $\pm$ SD of six independent experiments. * $P < 0.05$ indicate significant
472 difference from non- treated control.

473 (A)

474

475 (B)

476

477

478 **Fig. 1. Nam et al.**

479

480

481 **Fig. 2. Nam et al.**

482

483

Table 1.
NMR analysis of chlorogenic acid and its glucoside.

Carbon position	Chlorogenic acid (δ)		Chlorogenic acid glucoside (δ_1)		
	δ_C	δ_H	δ_{1C}	($\delta_1 - \delta$)	δ_H
Chlorogenic acid					
1	73.50				
2	36.45	5.07 (1H, m)	36.56	(0.11)	1.93, 2.02 (2H, m)
3		5.11 (1H, m)	71.14		5.09
4	68.40	3.63 (1H, m)	70.62	(2.22)	3.56
5	70.44	3.94 (1H, m)	68.40	(-2.04)	3.92
6	37.18	5.07 (1H, m)	37.27	(0.09)	1.77, 2.07 (2H, m)
7	174.85		175.15	(0.3)	
1'	125.60		128.85	(3.25)	
2'	115.74	7.04 (1H, d, $J=1.17$ Hz)	115.00	(-0.73)	7.15 (1H, d, $J=2$ Hz)
3'	145.53	9.15 (1H, s)	147.32	(1.79)	8.91 (1H, s)
4'	148.30	9.57 (1H, s)	147.12	(-1.17)	
5'	114.73	6.77 (1H, d, $J=8.20$ Hz)	116.84	(2.11)	7.21 (1H, d, $J=8.5$ Hz)
6'	121.31	6.99 (1H dd $J=1.17$ 8.20 Hz)	120.76	(-0.55)	7.09 (1H, dd, $J=2, 8.5$ Hz)
7'	144.89	7.43 (1H, d, $J=16.11$ Hz)	144.31	(-0.58)	7.47 (1H, d, $J=16$ Hz)
8'	114.31	6.16 (1H, d, $J=16.11$ Hz)	116.43	(2.12)	6.32 (1H, d, $J=16$ Hz)
9'	165.71		165.61	(-0.1)	
Glucose					
1"			99.53		5.32 (1H, d, $J=3.5$ Hz)
2"			71.97		3.34 (1H, m)
3"			73.08		3.69 (1H, m)
4"			69.90		3.18 (1H, m)
5"			73.87		3.45 (1H, m)
6"			60.67		3.49, 3.60 (2H, m)

Table 2.

Central composite design matrix for the experimental design and predicted responses for
CHG synthesis^a

Run no.	Coded levels			CHG synthesis (mM)	
	X ₁	X ₂	X ₃	Actual	Predicted
1	150	300	150	35.2	16.7
2	560	300	150	29.4	24.9
3	150	1000	150	111.2	87.5
4	560	1000	150	108.7	95.2
5	150	300	500	26.0	14.2
6	560	300	500	20.7	19.1
7	150	1000	500	107.2	86.4
8	560	1000	500	97.6	90.8
9	10.2	650	325	41.3	73.6
10	699.8	650	325	80.8	84.3
11	355	61.4	325	3.3	12.7
12	355	1238.6	325	106.2	132.5
13	355	650	30.7	2.7	26.3
14	355	650	619.3	8.4	20.5
15	355	650	325	126.4	123.7
16	355	650	325	112.8	123.7
17	355	650	325	125.4	123.7
18	355	650	325	138.6	123.7
19	355	650	325	115.1	123.7
20	355	650	325	130.3	123.7

$$^a y = -178.7 + 0.29x_1 + 0.29x_2 + 0.74x_3 - 0.000001x_1^2 - 0.00002x_2^2 + 0.000005x_3^2 - 0.00037x_1x_2 - 0.00014x_1x_3 - 0.0011x_2x_3$$

Fig. 3. Nam et al.

(A)

(B)

(C)

(D)

Fig. 4. Nam et al.