Food Chemistry 229 (2017) 75–83

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Glucosylation of flavonol and flavanones by *Bacillus* cyclodextrin glucosyltransferase to enhance their solubility and stability

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ARTICLE INFO

Article history:
Received 27 August 2016
Received in revised form 8 February 2017
Accepted 13 February 2017
Available online 16 February 2017

Keywords:
Flavonol
Flavanone
Glucosylation
Bacillus macerans
Cyclodextrin glucosyltransferase
Solubility

ABSTRACT

Enzymatically modified isoquercitrin (EMIQ), oligoglucosyl naringenin-7-(glucose [G]), and oligoglucosyl hesperetin (H)-7-G were produced via oligoglucosylation of quercetin-3-glucose, naringenin-7-G (prunin), and H-7-G, respectively, by cyclodextrin glucosyltransferase from *Bacillus macerans*. The aim was to explore the oligoglucosylation and the resulting changes in physicochemical properties. Water solubility of EMIQ, oligoglucosyl prunin, and oligoglucosyl H-7-G enormously increased in comparison with that of their aglycones. Glycosylation of an aglycone generally enhances its solubility. Resistance of the aglycones to oxidative degradation by the Cu^{2+} ion was strongly increased by the oligoglucosylation. This is probably because oligoglucosylation may protect sensitive parts of an aglycones molecule from the Cu^{2+} oxidation. Only EMIQ maintained its structure during thermal treatment much longer than quercetin did. Degradation of flavonoid aglycones by ultraviolet light C irradiation at 254 nm was not affected, and their antioxidant activities gradually decreased with the greater extent of oligoglucosylation.

1. Introduction

Bioflavonoids, commonly known as vitamin P, are some of the flavonoids with biological activities. They are found in many plant foods, such as fruits, vegetables, and cereal grains, particularly in citrus fruits, berries, green tea, and red wine. Some of the bioflavonoids include rutin, naringin, and hesperidin, which are used as food additives and antioxidants and have several beneficial properties, such as anticarcinogenic, anti-diabetic, anti-inflammatory, anti-bacterial, immuno-stimulating, anti-viral, bone-loss-preventive, estrogenic, free-radical-scavenging, and antioxidant effects (Bok et al., 1999; Liu, 2004; Ryu & Hur, 2005; Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007). However, they do not dissolve well in water and have a strong bitter taste, limiting their use in the food and pharmaceutical industries (Go, Kim, Lee, & Lee, 2007).

Enzymatic removal of terminal rhamnose from some flavonoids, such as rutin, naringin, and hesperidin, reportedly enhances the solubility and improves the properties (Chang et al., 2011; Lee, Huh, Nam, Kim, & Lee, 2013). Flavonoids are hydrolyzed by naringinases from *Penicillium decumbens* and from *Aspergillus sojae* and yield rhamnose-free flavonoid-intermediate: quercetin (Q)-3-G, naringenin (N)-7-(glucose [G]), and hesperetin (H)-7-G. This process helps to increase the potential bioavailability of the corresponding aglycones because of the higher water solubility (Chang et al., 2011; Lee et al., 2013). Conjugation of quercetin with glucose was reported to increase absorption of quercetin in the small intestine (Wach, Pyrzyńska, & Biesaga, 2007). H-7-G is better absorbed in the small intestine as compared to hesperidin owing to the removal of the rhamnose (Nielsen et al., 2006). It was also proven that bioavailability of hesperidin is increased when it is enzymatically transformed into H-7-G (Bredsdorff et al., 2010; Habauzit et al., 2009; Németh et al., 2003). Glycosylation is generally considered an effective method for conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and chemically stable ones (Shimoda & Hamada, 2010). In recent years, enzymatic modification has been applied to dietary flavonoids to improve their bioavailability (Nielsen et al., 2006; Yamada et al., 2006). Enzymatically modified isoquercitrin (EMIQ) is a mixture of isoquercetin and its α-oligoglucosides, which mainly comprise quercetin mono-glucoside and its α-glucosyl elongated derivatives with one to four additional linear glucose moieties (Akiyama, Washino, Yamada, Koda, & Maitani, 2000; Emura, Yokomizo, Toyoshi, & Moriwaki, 2007). EMIQ is absorbed more efficiently than quercetin according to a study on rodents (Makino et al., 2009) and on humans (Murota et al., 2010). It was also reported that transglucosylation of flavonoids, such as

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http://dx.doi.org/10.1016/j.foodchem.2017.02.057
0308-8146/

naringin and neohesperidin, by alkalophilic *Bacillus* cyclodextrin glucanotransferase (CGTase) enhances their properties: a reduction in bitterness or higher water solubility (Kometani, Nishimura, Nakae, Takii, & Okada, 1996).

In this study, rhamnose-free flavonoid-intermediates, such as Q-3-G, N-7-G (prunin), and H-7-G (enzymatically converted from rutin, naringin, and hesperidin, respectively, by naringinases from *P. decumbens* and from *A. sojae*) were modified via subsequent transglucosylation by α-CGTase from *B. macerans* to generate their glucosides. The most transfer products were purified and confirmed by high-performance liquid chromatography (HPLC) and mass-assisted laser desorpion/ionization time-of-flight (MALDI-TOF) mass spectrometry. In addition, we evaluated the biological properties of the transglucosylated flavonoid-intermediates, including water solubility and resistance to oxidation by the Cu^{2+} ion, to thermal processing, and to ultraviolet light C (UV-C), as well as their anti-oxidant activity.

2. Materials and methods

2.1. Chemicals and enzymes

Rutin, quercetin, naringin, naringenin, hesperidin, hesperetin, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripyridyl-*S*-triazine (TPTZ), Trolox, naringinase from *P. decumbens,* amyloglucosidase from *A. niger*, and β-amylase from barley were purchased from Sigma Aldrich. α-Cyclodextrin (CD) was acquired from Tokyo Chemical Industry (T.C.I, Tokyo, Japan). All solvents were purchased from Fisher Scientific. All other chemicals were of analytical grade.

2.2. Preparation and analysis of naringinase from P. decumbens and A. sojae

Naringinase from *P. decumbens* was extensively dialyzed against 50 mM Tris-HCl buffer (pH 7.0) and then concentrated with an ultrafiltration membrane (0.45-μm pore size; Millipore Co., Bedford, MA, USA) for further analysis. Naringinase from the liquid culture of *A. sojae* was purified according to a previously published method, with some modifications (Chang et al., 2011). The crude enzyme in the culture medium was precipitated with 80% (w/v) $(NH_4)_2SO_4$ (centrifugation at 7000*g* for 30 min at 4 °C), dissolved in 50 mM sodium citrate buffer (pH 6.0), and dialyzed. The protein sample was purified by chromatography on a Q-Sepharose column in 50 mM Tris-HCl buffer (pH 7.0) at the flow rate of 1 mL/min. Active fractions that showed α-rhamnosidase activities were pooled, dialyzed, and concentrated by means of an ultrafiltration membrane for further analysis.

α-l-Rhamnosidase activity of the purified naringinase was spectrophotometrically assayed under standard conditions at 25 °C in 50 mM sodium citrate buffer (pH 6.0) using *p*-nitrophenol (NP)-α-rhamnoside as the substrate for the hydrolysis. One unit (1 IU) of the enzyme was defined as the amount of enzyme that releases 1 μmol of *p*-NP per min under standard conditions. The protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.3. Preparation and analysis of cyclodextrin glucosyltransferase from B. macerans

The Luria-Bertani medium (1% Bactotryptone, 1% NaCl, and 0.5% yeast extract) containing 100 μg/mL ampicillin was incubated with a colony of recombinant *Escherichia coli* BL21 and incubated at 37 °C overnight. Cyclodextrin glucosyltransferase (CGTase) from *B. macerans* was isolated according to a previously reported method (Han & Tao, 1999).

CGTase activity was assayed by determining the amount of reducing sugar. The reaction solution with 1% (w/v) soluble starch in 50 mM sodium acetate buffer (pH 6.0) as a substrate was incubated at 55 °C for 10 min according to the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of the enzyme (CU) was defined as the amount of enzyme increasing absorbance of the solution by 1 unit at 575 nm.

2.4. Enzymatic hydrolysis and transglucosylation of rutin, naringin, and hesperidin

Rutin (1.5 mM) in 50 mM sodium citrate buffer (pH 6.0) containing 20% of dimethylsulfoxide (DMSO) was incubated with naringinase (0.05–0.2 mg/mL) from *P. decumbens* at 37 °C for 12 to 24 h. A substrate solution of naringin (0.5–1.7 mM) in 50 mM sodium acetate buffer (pH 6.0) containing 10% of DMSO was incubated with naringinase from *A. sojae* at 37 °C for 12–24 h. Hesperidin (0.1–1.0 mM) in 50 mM sodium citrate buffer (pH 6.0) containing 20% of DMSO was incubated with naringinase (0.05–0.2 mg/mL) from *A. sojae* at 37 °C for 12 to 24 h (Lee et al., 2013). The reaction mixture was centrifuged at 12,000*g* for 15 min, and the resulting supernatant was then filtered through a membrane (0.22-μm pore size) for further experiments.

The hydrolyzed product solution containing 1% (w/v) α-CD was incubated with CGTase at 55 °C for 30–60 min. The reaction mixture was then filtered through a membrane (0.22-μm pore size). Each sample was analyzed by both thin-layer chromatography (TLC) and HPLC. TLC of the hydrolysates of rutin, naringin, and hesperidin involved the mixture isopropyl alcohol/ethyl acetate/water (3:1:1 or 3:1:0.3, v/v/v) as the solvent system. Each sample was also analyzed on a Waters Symmetry C18 column (4.6 × 250 mm; Waters Co., Milford, MA, USA) connected to a Waters model 510 system with a 996 photodiode array detector at 290 nm (Ribeiro & Ribeiro, 2008). The linear HPLC gradient was composed of two solvents: solvent A and solvent B, 10% and 90% (v/v) acetonitrile, respectively, in water. After a 20-μL injection of each sample, solvent B was increased from 10% to 30% in 20 min, and then increased to 100% in 5 min, held at 100% for 10 min, and finally reduced to 10% in 10 min. The analysis was performed at 25 °C (flow rate 1.0 mL/min). For quercetin (its hydrolysis and transfer products), a linear HPLC gradient was composed of two solvents: solvent A and solvent B, 10% and 90% acetonitrile, respectively, in water, with detection of absorbance at 254 nm. After a 20-μL injection of each sample, solvent B was increased from 10% to 30% in 20 min, increased to 100% in 5 min, held at 100% for 10 min, and then reduced to 10% for 10 min.

2.5. Purification of enzymatically modified isoquercitrin, oligoglucosyl naringenin-7-glucoside, and oligoglucosyl hesperetin-7-glucoside

The procedure was based on a previously published method (Chang et al., 2011; Lee et al., 2013). The reaction products were purified from the reaction mixture on a Prevail Carbohydrate ES column (300 × 20 mm; Alltech Associates, Inc., Deerfield, IL, USA) that was connected to a Waters Delta Prep 4000 preparative chromatography system with a Waters™ 486 absorbance detector at 254 nm for EMIQ and at 290 nm for oligoglucosyl prunin and oligoglucosyl H-7-G. A linear gradient in the HPLC was employed; it consisted of two solvents: 20% (v/v) acetonitrile in water (A) and 80% (v/v) acetonitrile in water (B) for 60 min at the flow rate of 4 mL/min at room temperature. Solvent B was increased from 10% to 30% in 25 min, then increased to 100% in 10 min, held at 100% for 10 min, and finally reduced to 10% in 10 min. The fractions of the reaction products according to their molecular weight