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Gamma irradiation-assisted degradation of rosmarinic acid and evaluation of structures and anti-adipogenic properties

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ABSTRACT

Radiation is a promising technique for improving the safety and shelf-life of processed foods. In the present investigation, the degradation mechanism and bioactivity improvement of rosmarinic acid (RA) were studied in response to various gamma irradiation doses (10, 20, and 50 kGy). RA exposed to gamma irradiation at 50 kGy was completely degraded and showed an increased inhibitory effect against 3 T3-L1 preadipocyte compare to the parent compound. Structures of the newly generated compounds **2**–**4** from irradiated RA at 50 kGy were elucidated based on spectroscopic methods, including ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS). Interestingly, compounds **2** and **5** exhibited significantly enhanced anti-adipogenic properties in 3 T3-L1 cells compared to the original compound. These results provide evidence that structural changes in RA induced by gamma irradiation might enhance biological efficacy.

1. Introduction

Naturally occurring polyphenols, which contain at least two phenolic functional groups, are classified into several subclasses, including flavonoids, stilbenoids, lignans, and phenolic acids (Wang, Camp, & Ehlenfeldt, 2012). Polyphenols possess significantly potent antioxidant activity, which is closely correlated with various beneficial effects such as anti-aging, cancer prevention, and cardiovascular disorders (Chiara et al., 2017). Recent research has demonstrated that certain polyphenols are unstable, especially under oxidative and enzymatic conditions, and their readily oxidizable properties may be responsible for their efficient antioxidant activity (Atala, Fuentes, Wehrnhan, & Speisky, 2017; Shingai, Fujimoto, Nakamura, & Masuda, 2011). Oxidation reaction of polyphenols provides new compounds, and these newly generated products might accumulate in various foods as well as the human body (Masuda, 2010). Preservation of food products against degradation is a major goal of the food industry. Besides conventional thermal treatment of foods, gamma irradiation has been established as an advanced technology for food processing.

Gamma irradiation is known to play an important role in the destruction of pathogenic microorganisms since it can produce various reactive molecular species and free radicals, including methoxy ($CH_3O\cdot$), hydroxy alkyl ($\cdot CH_2OH$), hydrogen ($H\cdot$), superoxide anion ($O_2\cdot^-$), and peroxyl ($OOH\cdot$) radicals as well as hydroxyl ion (OH^-) (Butt & Qureshi, 2008). Although the efficacy of gamma irradiation for microbial inactivation has been demonstrated, its effects on the chemical compositions and biological efficacies of natural products have only been investigated a couple times (Khattak, 2012; Lacroix & Ouattara, 2000). These studies suggested that the biological functionalities of medicinal plants are dependent on the irradiation conditions, including the plant species, exposure time, irradiation dose, and presence of organic solvent. Recently, we reported that rutin and mangiferin were easily converted into new hydroxymethylated products by gamma irradiation as well as improved α-glucosidase and nitric oxide production inhibitory activities relative to the original compounds (Jeong & Kim, 2017; Jo, Yoon, Jang, & Kim, 2016).

Rosmarinic acid (RA), firstly isolated and characterized from rosemary (*Rosmarinus officinalis*), is a potent antioxidant polyphenol widely distributed in Lamiaceae herbs as well as the Boraginaceae, Rubiaceae, Apiaceae, and Araliaceae families. RA is one of the most abundant plant secondary metabolites in food additive and herb tea and has been revealed to possess a wide spectrum of substantial biological activities, including antioxidant, anti-inflammatory, anti-viral, antibacterial, and anti-allergenic properties (Razboršek, 2011). Until now,

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there is a lack of evidence regarding the degradation mechanisms of major food components induced by gamma irradiation. Changes in the chemical structures and functional properties of major polyphenols in foodstuffs induced by gamma irradiation exposure are closely linked to human health. Therefore, it is essential to evaluate levels of polyphenols under gamma irradiation treatment. In the current study, the main objective was to evaluate the effects of RA subjected to gamma irradiation at several doses on chemical degradation and the anti-adipogenic activity of 3T3-L1 preadipocytes related to obesity. In addition, a plausible mechanism behind the degradation pathways of RA is tentatively proposed.

2. Materials and methods

2.1. General information

Rosmarinic acid, acetonitrile, methanol, formic acid (HPLC grade), ethylacetate, and deuterated methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (KGaA, Darmstadt, Germany). All other reagents and chemicals purchased and used in this study were of analytical grade. UV (ultraviolet) spectra were obtained using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were measured on a Varian VNS600 instrument (Varian, Palo Alto, CA, USA) operated at 600 and 150 MHz, respectively. Chemical shifts are given in $_{\delta}$ (ppm) values relative to those of the solvent CD_3OD (δH 3.35; δC 49.0) on a tetramethylsilane (TMS) scale. Standard pulse sequences programmed into the instruments were used for each 2-dimentional measurement. The J_{CH} value was set at 8 Hz in the heteronuclear multiple bond connectivity (HMBC) spectra. Fast atom bombardment mass spectrometer (FABMS) was obtained on a Micro Mass Auto Spec OA-TOF spectrometer (Micromass, Manchester, UK). Column chromatography was performed using Toyopearl HW-40 (Tosoh Co., Tokyo, Japan) and YMC GEL ODS AQ 120-50S (YMC Co., Kyoto, Japan). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates (0.25-mm layer thickness, Merck, Darmstadt, Germany), and spots were detected by UV irradiation (254 and 365 nm) as well as spraying with 10% H_2SO_4 reagent.

2.2. Preparation of irradiated sample

Gamma irradiation was performed as previously reported at room temperature using a cobalt-60 experimental irradiator (point source AECL, IR-79, MDS Nordion International Co. Ltd, Ottawa, ON, Canada) located at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeongup, Korea) (Lee, Kim, Lee, Lee, & Chung, 2014). The source strength was around 320 kCi with a dose rate at the position of the sample of 10 kGy/h. Dosimeters were carried out using 5-mm diameter alanine dosimeters (BrukerInstruments, Rheinstetten, Germany) and calibrated against an International Standard Set by the International Atomic Energy Agency (Vienna, Austria). Sample solution of RA (1.0 g) in MeOH (1.0 L) was placed in 10 chapped glass test tubes and subjected to gamma irradiation at 10, 25, and 50 kGy (absorbed dose). The irradiated solution was concentrated immediately using a vacuum evaporator to remove methanol.

2.3. Determination of degraded compounds

High-performance liquid chromatography (HPLC) (Shimadzu, Tokyo, Japan) with a photodiode-array (PDA) detector was used for the chromatographic analysis of RA degradation products induced by gamma irradiation (Kim, Lee, Kim, & Jo, 2017). HPLC analysis was carried out using a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm; YMC Co., Kyoto, Japan), and the solvent system consisted of a gradient mode with an initial 1% HCOOH-H_2O increased to CH_3CN over 30 min (temperature: 40 °C; flow rate: 1.0 mL/min; UV detection; 280 nm). Successive column chromatography was conducted using YMC GEL ODS AQ 120-50S (YMC Co., Kyoto, Japan). Newly generated products from original RA were identified by comparing their retention times with that of pure RA. Quantification of the isolated compounds was carried out by HPLC analysis using the external standard method by constructing standard curves.

2.4. Isolation and structure elucidation of degraded products

RA in MeOH placed in glass test tubes was treated with gamma irradiation at 10, 25, and 50 kGy doses, and the newly formed products were immediately monitored by reverse-phase HPLC analysis. Among the dried reactants, sample solution containing RA treated with gamma irradiation at 50 kGy exhibited the most improved inhibitory activity (55.3%) against 3T-3L1 preadipocyte differentiation at a concentration of 10 μg/mL compared to that of original RA. Irradiated methanolic solution containing RA treated with gamma irradiation at 50 kGy was evaporated, directly subjected to column chromatography over a Toyopearl HW-40 column (coarse grade; 3.5 cm i.d. × 35 cm), and eluted in a gradient manner with H_2O-MeOH (0:100 to 100:0, then aqueous acetone) to produce five subfractions CR01-CR05. Subfraction CR01 was subjected to column chromatography over a YMC GEL ODS AQ column (1.1 cm i.d. × 41 cm) with aqueous MeOH to yield pure compound **4** (12.5 mg, t_R 8.3 min). Subfraction CR02 containing major degraded products was purified to passage over a YMC GEL ODS AQ column (1.1 cm i.d. × 42 cm) with aqueous MeOH using reverse-phase HPLC to yield pure compounds **2** (81.5 mg, t_R 14.3 min) and **5** (58.3 mg, t_R 9.1 min). Similarly, subfraction CR03 was purified using a YMC GEL ODS AQ 120-50S column (1.1 cm i.d. × 43 cm) with aqueous MeOH to yield pure compound **3** (14.8 mg, t_R 13.4 min). Chemical structures of these newly formed products were elucidated by interpretation of their spectroscopic data, including ^{1}H, ^{13}CNMR, 2D NMR, and MS. In addition, the purity of each individual compound isolated was also determined by HPLC with values more than 99%.

2.5. Cell culture

Cell culture media and supplements were obtained from Sigma-Aldrich (St. Louis, MO, USA) and cultured as described elsewhere (Park et al., 2012). In brief, mouse 3T3-L1 preadipocytes were grown in Dulbecco's modified eagle medium (DMEM) containing high glucose with 10% calf serum at 37 °C in a humidified atmosphere of 5% CO_2. Cells were subcultured after reaching a confluence of 80%. To induce adipogenesis, 3 T3-L1 cells were cultured until confluent. One day after reaching confluence (day 0), the culture medium was changed to differentiation/induction medium (MDI) consisting of 100 mM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin, and 0.25 μM dexamethasone in DMEM containing 10% fetal bovine serum. The differentiation/induction medium was changed every 2 days. 3-Isobutyl-1-methylxanthine, dexamethasone, indomethacin, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). RA and degraded compounds were added to the culture medium containing adipocytes on day 0. Cells were treated with 0, 5, or 10 μM RA and degraded compounds. After treatment with the tested compounds for 8 days, 3T3-L1 adipocytes were lysed for experimental analysis.

2.6. Cell viability assay

The effects of the newly generated products from irradiated RA on cell viability were evaluated by the 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Park et al., 2012). Cells were seeded at a density of 1×10^5 cells/well into 24-well plates. After 24 h, cells were treated with the degraded compounds for 8 days. MTT was added to each well, and the plates were incubated for 4 h at 37 °C. The liquid in the plate was removed, and dimethyl sulfoxide (DMSO) was added to dissolve the MTT-formazan complex. Optical density was measured at 540 nm.