Biocatalysis and Agricultural Biotechnology 10 (2017) 411–419

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Identification of the molecular species of acylglycerols containing hydroxy fatty acids in wild edible mushroom *Ganoderma lucidum*☆

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ARTICLE INFO

Keywords:
Philippine mushroom
Ganoderma lucidum
Hydroxy fatty acids
Odd carbon numbered fatty acids
Triacylglycerols
Mass spectrometry

ABSTRACT

Edible Philippine mushrooms including *Ganoderma lucidum* have many health benefits. Seventy-two molecular species of triacylglycerols and five molecular species of diacylglycerols containing hydroxy fatty acids (FA) in the lipid extract of this mushroom were identified by HPLC and MS. The mono-, di- and tri-hydroxy FA constituents of the molecular species of acylglycerols were identified by various degrees of dehydration of $[FA + Li]^+$. Many odd carbon numbered hydroxy FA constituents of acylglycerols were also identified. The contents of the 77 molecular species of acylglyerols and their constituent hydroxy FA in the lipid extract were estimated by the relative ion signal intensities of the molecular species of acylglycerols and the HPLC peak area % (ELSD). The contents (%) of the molecular species of acylglycerols containing hydroxy FA in the lipid extract were as: P-19:0-2OH18:2, 0.69%; 2OH18:2-2OH18:2-2OH18:2, 0.60%; P-OH19:1-OH19:1, 0.48%; S-OH19:1-OH19:1, 0.43%; 19:0-19:0-2OH18:2, 0.27% and the total of about 5.8%. "2OH18:2" stands for dihydroxy FA with 18 carbon atoms and two double bonds. The mushroom lipid extract was 1.67% of freeze-dried mycelium. The contents (%) of individual hydroxy FA in the mushroom lipid extract in decreasing order were: 2OH18:2, 1.4%; OH19:1, 1.1%; OH18:1, 0.38%; 2OH19:0, 0.19%; 2OH18:0, 0.13% and the total of about 3.5%. The odd carbon numbered hydroxy FA was about 1.44% of the mushroom lipid extract. The high contents of acylglycerols containing hydroxy FA, odd carbon numbered FA and odd carbon numbered hydroxy FA in edible mushroom might be related to one or more of its health benefits. As far as we are aware of, this mushroom contains the highest amounts of hydroxy FA and odd carbon numbered FA among the human foods.

1. Introduction

Mushrooms, Basidiomycetes of fungi, are rich in proteins, vitamins and minerals, and are low in fats (calories), and thus are considered as the healthy foods (Chang and Miles, 2004). The beneficial properties of mushrooms include antitumor, antiviral, antibacterial, antiparasitic and the preventing effects on hypertension, hypercholesterolemia, atherosclerosis and cancer (Wasser and Weis, 1999; Yilmaz et al., 2006). Some Philippine wild edible mushrooms have been studied for their nutritional and functional activities. Fruiting bodies of mushrooms like *Schizophyllum commune, Lentinus tigrinus, Lentinus sajor-caju, Ganoderma lucidum, Collybia reinakeana* and *Paneaolus antillarium* exhibited antidiabetic, antibacterial, anti-inflammatory, antioxidant, antihypertensive, and anti-coagulative properties (Reyes et al., 2004; Dulay et al., 2014, 2015; Eguchi et al., 2014). These valuable bioactivities of Philippine mushrooms are now being recognized for their health benefits and economic importance.

Many fatty acids (FA) in many species of mushroom have been identified and quantified by gas chromatography-mass spectrometry (GC-MS), e.g., 31 FA in 15 species (Marekov et al., 2012), 30 FA in 12 species (Rebeiro et al., 2009) and 44 FA in 10 species (Pedneault et al., 2008). We have recently identified 16 molecular species of diacylglycerols (DAG) and 87 molecular species of triacylglycerols (TAG) in mushroom for the first time using LC-MS (Hou et al., 2016). All of the FA constituents of these acylglycerols (AG) and the previously reported FA in mushrooms were normal FA (long chain FA without functional group). We would like to report here the identification of the molecular species of AG containing hydroxy FA in mushroom. Hydroxy FA, e.g.,

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http://dx.doi.org/10.1016/j.bcab.2017.05.003
Received 14 March 2017; Received in revised form 8 May 2017; Accepted 8 May 2017
Available online 10 May 2017
1878-8181/

ricinoleate (R, $OH^{12}18:1^{9}$), has many industrial uses such as the manufacture of biodegradable and renewable plastics, nylon, plasticizers, lubricant, cosmetics and paints.

Castor oil contains about 90% of ricinoleate (Achaya et al., 1964) and is the only commercial source of ricinoleate. We have previously identified 13 molecular species of DAG, 65 TAG and 6 tetraacylglycerols mostly containing hydroxy FA in castor oil (Lin et al., 2006; Lin and Chen, 2010, 2011, 2012; Lin et al., 2009, 2013). Their constituent FA included mono-, di- and tri-hydroxy FA and normal FA (FA without functional group). Odd carbon numbered FA was very rare and only the TAG, RR-23:0, was identified (Lin and Chen, 2012). Odd carbon numbered hydroxy FA was not detected in castor oil. Sixteen AG in castor oil were quantified using HPLC with evaporative light scattering detector and the highest content was RRR at 71% (Lin et al., 2003).

Lesquerella oil contains 56.5% of lesquerolate (Ls, $OH^{14}20:1^{11}$) (Zhang et al., 2012). We have recently identified and quantified 10 molecular species of DAG, 74 TAG and 13 tetraacylglycerols mostly containing hydroxy FA in lesquerella oil (Lin and Chen, 2013a, 2013b, 2014a, 2014b; Lin et al. 2016). Their constituent FA included mono- and di-hydroxy FA and normal FA. Odd carbon numbered FA was also very rare and only the TAG, LsO-23:0, was identified and quantified as 0.13% (Lin and Chen, 2013a, 2014a). For abbreviations and structures of FA, see appendix of Table 1. Odd carbon numbered hydroxy FA was not detected in lesquerella oil. The content of ten DAG combined was about 1%, 74 TAG was about 98% and 13 tetraacylglycerols was about 1%. The highest content of the molecular species of AG in lesquerella oil was LsLsO at 31%.

Mushrooms are Basidiomycetes of fungi and are fast growing. The fungal oil of the sclerotia of the ergot fungus *Claviceps purpurea* contains 29.6% of ricinoleic acid (Zhang et al., 2012). The fungal oil of L. *lyrata* contains 41.1% of densipolic acid ($OH^{12}18:2^{9,15}$) and 8.2% of ricinoleic acid (Zhang et al., 2012).

In this study, we report the identification of seventy-two molecular species of triacylglycerols and five molecular species of diacylglycerols containing hydroxy fatty acids (FA) in the lipid extract of mushroom *Ganoderma lucidum*. Many odd carbon numbered hydroxy FA constituents of acylglycerols were also identified. The high contents of acylglycerols containing hydroxy FA, odd carbon numbered FA and odd carbon numbered hydroxy FA in edible mushroom might be related to one or more of its health benefits. This mushroom contains the highest amounts of hydroxy FA and odd carbon numbered FA among the human foods.

2. Material and methods

2.1. Source of mushroom strain

Pure culture of Philippine wild edible mushroom, *Ganoderma lucidum*, was obtained from the culture collections of the Center for Tropical Research and Development, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines.

2.2. Mass production and extraction of lipid fraction

Scale up production of *G. lucidum* mycelia was carried out by inoculating mycelia discs in a 2800 mL fernback flask containing 500 mL of SDB with the required pH 7 and incubated at 28 °C for 10 days as described in our previous report (Dulay et al., 2015). After the incubation, the mycelium was collected and freeze dried for 24 h.

Solvent extraction was performed as follows: 7 g of the freeze-dried mycelium and 200 mL of ethyl acetate were mixed in a homogenizer for 1 min. The mixture was filtered with Whatman #1 filter paper to obtain the solvent extract. Extraction was done twice. The solvent was removed from the combined extracts using a rotary evaporator (Rotavapor R-215. BUCHI, Switzerland). The dried extracts were re-dissolved with 4 mL chloroform: methanol (2:1) to transfer into vials and concentrated to dryness under reduced pressure and temperature (45 °C). The weight of the extract was 0.117 g representing 1.67% of dried mycelium.

2.3. HPLC fractionation of the molecular species of acylglycerols in mushroom lipid extract

The mushroom lipid extract (1 mg in 50 μL) was fractioned on HPLC (Waters Associates, Milford, MA, USA), using a C_{18} analytical column (Gemini, 25 cm × 4.6 mm, 5 μm, C18, Phenomenex, Torrance, CA, USA) with a linear gradient from 100% methanol to 100% 2-propanol in 40 min, at 1 mL/min flow rate. UV detector at 205 nm was used for chromatogram in Fig. 1. Fractions were collected every 30 s and corresponding fractions were pooled from 8 HPLC runs. HPLC fractions were used for MS studies. The final methanol solutions of samples were prepared for direct infusion into the mass spectrometer by combining approximately one tenth of each HPLC fraction with 50 μL of methanol solution of 100 mM lithium acetate and diluting to a total volume of 250 μL.

2.4. Electrospray ionization mass spectrometry (ESI-MS)

An LCQ Advantage ion-trap mass spectrometer (MS 2.0) with Xcalibur 2.0 SR2 software (ThermoFisher Scientific, San Jose, CA, USA) was utilized for MS analysis of the various molecular species of acylglycerols. The infusion at a 2.5 μL/min flow rate from a syringe (250 μL) pump produced stable singly-charged lithiated parent ions which were subsequently fragmented for MS^2, MS^3, and MS^4 analysis.

2.5. Quantification of the molecular species of triacylglycerols using HPLC with ELSD and MS

The quantification method of the molecular species of AG was the same as that of our recently developed method (Lin and Chen, 2014a). The HPLC with evaporative light scattering detector (ELSD) was as our earlier report (Lin et al., 2003). The HPLC with ELSD provided the area % of the peak on the chromatogram (Fig. 2) and the area % of the peak represented the content % of total triacylglycerols in the peak. The ratio of the molecular species of triacylglycerols in a HPLC peak was assumed to be the same as the ratio of the total ion signal intensities of the individual molecular ions, $[M + Li]^+$, combined in the HPLC fractions of a HPLC peak. The contents of molecular species of AG were assumed to be proportional to their ion signal intensities.

3. Results and discussion

3.1. Identification of AG containing hydroxy FA using MS^2

Mushroom lipid extract was fractionated (0.5 min/fraction) by a C_{18} HPLC as shown in Fig. 1. Mass spectrometry of the HPLC fractions of the lipid extract of mushroom was used for the identification. HPLC elution characteristics of the molecular species of AG as well as the HPLC fraction numbers of the molecular species of AG previously identified were also used (Lin et al., 1997, 2009, 2013; Lin and Chen, 2012, 2013a). We started to figure out the identities of the molecular species of AG containing hydroxy FA from the least polar AG in the HPLC fraction #57 toward the earlier HPLC fractions with the more polar AG. Seventy-two molecular species of TAG (HPLC fractions #11-57) and five molecular species of DAG (HPLC fractions #17-22) containing hydroxy FA were identified by MS^2 as shown in Table 1. Table 1 lists the molecular species of AG identified in various HPLC fractions and the masses of their lithium adducts and the MS^2 fragment ions with the relative intensities. The underlined FA in the names of the molecular species of AG in Table 1 has the same mass. The following are the examples of the identifications of the molecular species of AG containing hydroxy FA in the mushroom lipid extract.