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Comparison of the activities of hydrophilic anthocyanins and lipophilic tocols in black rice bran against lipid oxidation

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ABSTRACT

The antioxidant capabilities of anthocyanin and tocol extracts from black rice bran were evaluated using an emulsion system containing either cholesterol (1.0 mg/ml) or fish oil (10 mg/ml). The cholesterol oxidation product, 7-ketocholesterol, increased to 180.1 μg/ml in the control emulsion after 168 h of oxidation, while it was only 15.4 and 39.0 μg/ml in the emulsions containing 1 μg/ml of the anthocyanin and tocol extracts, respectively; but below 1.2 μg/ml in the emulsion having 5 μg/ml of anthocyanins or tocols. In the fish oil emulsion, over 80% of C20:5 and C22:6 were oxidised after a 48 h incubation at 37 °C, while they were retained above 38% and 65% in the emulsions containing 10 μg/ml of anthocyanins and tocols, respectively, and above 85% in the emulsion containing 20 μg/ml of anthocyanins or tocols. Compared with the tocols extract, the capability of the anthocyanin extract was relatively greater in stabilising cholesterol but lower in inhibiting fatty acids oxidation.

1. Introduction

The lipophilic antioxidants in rice bran are different from other cereals and grains (Moreau & Lampi, 2012). Besides α- and γ-tocopherol, which are typically present in most grain and cereal oils, rice bran oil contains higher levels of α- and γ-tocotrienol. The tocotrienols in brown rice bran have been confirmed to possess a health promoting function of lowering serum cholesterol levels in animal and clinical studies (Godber & Juliano, 2004). Recently, black rice bran has also gained more attention for its potential application in functional foods (Jang & Xu, 2009; Kong et al., 2012). The black rice bran contains all the types of tocols present in brown rice bran, at a similar level, but also a high level of the hydrophilic antioxidants, anthocyanins (Jang & Xu, 2009). Anthocyanins are the natural pigments responsible for the red, purple, and black colour in fruits and vegetables. The capability of anthocyanins in berries and grapes in preventing or delaying various chronic diseases such as cardiovascular diseases, obesity, diabetes, and certain cancers has been extensively studied (Canter & Ernst, 2004; Prior & Joseph, 2004; Tsuda, Horio, Uchida, Aoki, & Osawa, 2003; Youdim, McDonald, Kalt, & Joseph, 2002).

Tissue inflammation has been considered to enact a pivotal role in many stages of the above mentioned chronic diseases (Gan, Man, Senthilselvan, & Sin, 2004; Handschin & Spiegelman, 2008). Inflammation can be caused by the accumulation of the oxidised lipid components from cholesterol and fatty acids in the tissue cell membrane and biological fluids (Bays, Mandarino, & DeFronzo, 2004; Paolisso et al., 1997). Cholesterol and/or fatty acids are oxidised through oxidative stress, initiated by free radicals or active oxygen. The leading cholesterol oxidation product, 7-ketocholerserol, and other cholesterol and fatty acid oxidation compounds, such as aldehydes and short chain fatty acid peroxides, can alter tissue cell membranes resulting in an inflammatory response by the cells. For example, cardiovascular diseases are strongly associated with higher low-density lipoprotein (LDL) cholesterol level and oxidised cholesterol (Cui et al., 2009). The toxic oxidation products damage the macrophage and endothelial cells in blood vessels and cause abnormal cholesterol metabolism, which eventually leads to formation of blood vessel plaques that block blood circulation (Hausenloy & Yellon, 2008). Elevated levels of fatty acids oxidation products have been shown to have strong correlations with cardiovascular disease (DeFronzo, 2009; Pilz et al., 2006). Thus, the benefits of anthocyanins and tocols in preventing those chronic diseases may be mainly associated with their antioxidant properties. However, information directly linking the antioxidant activity to the lipid oxidation of cholesterol and fatty acids is very limited, especially for cholesterol oxidation.

Many *in vitro* methods for the antioxidant activity of food components have been well documented and reviewed (Apak et al., 2007; Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012). Most of the antioxidant methods measure either hydrophilic or lipophilic

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antioxidant activity, depending on the reaction media system. These methods may not closely reflect the *in vivo* protection of cholesterol and fatty acids against oxidation in a biological fluid. Biological fluids are not as simple as an aqueous solution, but are more similar to an emulsion consisting of water, lipid micelles, and other components. In this study, cholesterol and fish oil emulsions were prepared as test media, which are miscible to both anthocyanins and tocols antioxidants. The results of these emulsion models could more closely predict the capability of antioxidants in preventing lipid oxidation in biological fluids and cells. The information could be very helpful in exploring the mechanism of health benefits of black rice bran.

2. Materials and methods

2.1. Chemicals and materials

HPLC grade hexane, methanol, and acetic acid were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Isopropanol was purchased from Mallinckrodt Co. (Paris, KY, USA). Ethyl acetate was purchased from EM Science (Gibbstown, NJ, USA). Tween 20, cholesterol, 7-ketocholesterol, menhaden fish oil, fatty acids (C20:5 and C22:6) standards, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), α-tocopherol and -tocotrienol, γ-tocopherol and -tocotrienol, and cyanidin chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rough black rice was obtained from a local store (Baton Rouge, LA, USA).

2.2. Preparation of hydrophilic and lipophilic rice bran extracts

The rough rice sample (500 g) was milled using a McGill No. 2 mill (McGill, Brookshire, TX, USA). Rice bran collected from milling was mixed well and stored at −20 °C before use. Hydrophilic anthocyanins and lipophilic tocols extracts were prepared by the method described by Jang and Xu (2009). Fifteen gram of each bran sample was extracted twice using hexane (50 ml) at 60 °C. After extraction and isolation, the hexane solution was evaporated using a vacuum centrifuge evaporator (CentriVap Mobile System; Labconco, Kansas City, MO, USA). The dried defatted bran was mixed twice with methanol (50 ml) to perform hydrophilic extraction. Both of the anthocyanins and tocols extracts were stored at −20 °C before use.

2.3. Determination of tocopherols, tocotrienols, and anthocyanins using HPLC

The tocopherols and tocotrienols content in the tocols extract and anthocyanins were determined using normal phase and reversed phase HPLC methods, respectively. The operational conditions of the methods described by Jang and Xu (2009) were used. Tocopherol and tocotrienol concentrations were calculated based on standard curves. The total tocols content was calculated by summing α- and γ-tocopherol and α- and γ-tocotrienol concentrations and converting to μg/g of extract or bran. The concentration of each anthocyanin was calculated based on its standard curve of cyanidin chloride. The total anthocyanins content was calculated by summing each anthocyanin and converting to μg/g of extract or bran.

2.4. Preparation of cholesterol and fish oil emulsion and oxidation reaction

Cholesterol emulsion (1000 ml) consisted of 1000 mg cholesterol, 100 mg AAPH, 10 ml Tween 20, and phosphate buffer (pH 7.0). Fish oil emulsion (1000 ml) was prepared by homogenising 10.0 g menhaden fish oil, 10 ml Tween 20 and phosphate buffer (pH 7.0). Total anthocyanins (100 μg/ml) in methanol and total tocols (100 μg/ml) in hexane were prepared using anthocyanins and tocols extracts, respectively. An aliquot of 0.2, 1.0, 2.0, or 4.0 ml of the prepared extraction solutions was added in a 40-ml vial with a Teflon seal cap. After the solvent in the vial was totally evaporated, 20 ml of each emulsion was immediately added and homogenised with the dried extract using a sonication method. The emulsion without the extract was used as a control sample. Then, the vials were incubated in a water bath at 37 °C. The emulsions were continuously agitated by a multiple magnetic stirrer (Multistirrer, VELP Company, Italy). At day 0, 1, 2, 3, 5, and 7, 1.0 ml of each cholesterol emulsion was taken to determine 7-ketocholesterol level. At day 0, 1, and 2, 1.0 ml of each fish oil emulsion was taken to measure the retained concentration of C20:5 and C22:6.

2.5. Determination of 7-ketocholesterol using HPLC and C20:5 and C22:6 fatty acids using GC

The sample taken from the cholesterol emulsion was mixed with 2.0 ml of hexane and vortexed for 1 min. Then, it was centrifuged at 5000*g* for 10 min to separate the hexane and aqueous layers. The hexane layer was added to a test tube containing 0.1 g of Na_2SO_4 to remove any possible moisture before it was transferred to an HPLC injection vial. The oxidation product, 7-ketocholesterol, was analysed using the HPLC method according to Tian, Wang, Abdallah, Prinyawiwatkul, and Xu (2011). The concentration was calculated using a calibration curve of 7-ketocholesterol standard. The C20:5 and C22:6 fatty acids in the fish oil emulsion were determined using the method of Yue et al. (2008). The sample was mixed with 2 ml of hexane containing the internal standard heptadecanoic acid (C17:0) (0.1 mg/ml) to extract the fish oil. The hexane supernatant was separated by centrifugation and evaporated to obtain dried fish oil. The dried oil was reacted with 2 ml BCl_3-methanol and 1 ml 2,2′-dimethoxypropane to perform the derivatisation of fatty acid methyl esters. A GC, with a FID detector, was used to determine the fatty acid concentrations. The column was a Supelco SP2380 (30 m × 0.25 mm) (Bellefonte, PA, USA). The concentrations of C20:5 and C22:6 were calculated using the C17:0 internal standard as a reference. The percentage of retained C20:5 or C22:6 in the fish oil emulsion was obtained by comparing its concentration in the incubated sample to its initial concentration in the emulsion at day 0.

2.6. Statistical analysis

Determination of tocols and anthocyanins concentrations were duplicated for each extract. The experiment for the cholesterol or fish oil emulsion mixed with each level of anthocyanins or tocols from the extract and its control group was performed three times. Means and standard deviations were calculated and all data were analysed using Microsoft Excel statistical program (Redmond, WA, USA) to evaluate the significant difference at $P < 0.05$.

3. Results and discussion

3.1. Anthocyanins and tocols in the black rice bran extracts

The chemical structures of anthocyanin and tocol consist of at least one hydroxyl group linked with a benzene ring which enables them to savage free radicals and singlet oxygen and reduce oxidation stress on vulnerable compounds. Generally, most fruits, vegetables, and grains are only rich in either water or fat soluble antioxidants. However, black or purple rice bran contains both types of the antioxidants at a significant level. Jang and Xu