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Protective effect of far-infrared treated rice hull extract on lipid peroxidation in phospholipid bilayer

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The protective effect of methanolic extract of far-infrared treated rice hull (FRH) on phospholipid peroxidation of liposome was compared with that of natural antioxidants (ascorbic acid, morin, quercetin and α-tocopherol). With induction of phospholipid peroxidation by 2,2'-azobis(2-amidinopropane), dihydrochloride (a water-soluble radical generator), 2,2'-azobis(2,4-dimethylvaleronitrile) (a lipid-soluble radical generator) and transition metal ions (Fe³⁺ and Cu²⁺), the phospholipid peroxidation was investigated for 6 h at 37 and 50°C. The added antioxidants prevent peroxidation of phospholipid in liposome, and FRH showed the strongest antioxidant activity in all cases. The results indicate that FRH showed significant antioxidant activity on lipid peroxidation in hydrophilic as well as hydrophobic conditions regardless of hydrophobicity of radical generators and transition metals.

Key words: Far-infrared treated rice hull, liposome, peroxidation, antioxidant activity.

INTRODUCTION

Lipid peroxidation in biological membranes has been considered as one of the major mechanisms of cell injury in aerobic organisms subjected to oxidation stress (Niki, 2009). Antioxidants have been widely used in pharmaceutical and food industry to prevent oxidation. Instead of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene, natural antioxidants have been received intensive attraction because they can remove free radicals without toxicity and carcinogenicity (Pokorny, 2007).

Currently, agricultural and industrial residues are attractive sources of natural antioxidants (Balasundram et al., 2006). Natural antioxidants such as flavonoids, tannins, coumarins, curcuminoinds, xanthons, phenolics, and terpenoids are found in various plant products such as fruits, leaves, seeds, and oils (Boskou, 2006), and some of these are as effective as synthetic antioxidants in model systems (Silberberg et al., 2006; Pajk et al., 2006). In our previous study (Lee et al., 2003a), antioxidant activity of methanolic extract of rice hull was greatly increased with increased amount of phenolic compounds by far-infrared treatment. The methanolic extracts from far-infrared treated rice hull (FRH) also showed antioxidant activity in irradiated turkey breast meat (Lee et al., 2003b) and cooked turkey meat (Nam et al., 2004) with reduction of thiobarbituric acid-reactive substances values and volatile aldehydes (hexanal, pentanal, and propanal).

Artificial phospholipid vesicles, so-called liposomes, have been frequently used as models of phospholipid bilayers constituting cellular and subcellular membranes. The unsaturated fatty acyl chains are vulnerable to oxidative degradation, that is, lipid peroxidation. Liposomes prepared from phospholipid containing polyunsaturated fatty acids could be used for investigating the antioxidative action of biological components occurring in phospholipid bilayers (Xu et al., 2009; Fagali and Catalá, 2009; Broniowska et al., 2007). In this study, FRH was incorporated into liposomes of soybean phosphatidylcholine (PC), and the antioxidant activity of FRH was investigated when lipid peroxidation was triggered by initiators such as lipid-, water-soluble azo compounds and transition metals.

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MATERIALS AND METHODS

L-Ascorbic acid, diethylene triamine pentaacetic acid (DTPA), 1,2-diacetyl-sn-glycero-3-phosphatidylcholine (PC) isolated from soybeans, morin, and quercetin dihydrate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). α-Tocopherol was purchased from Fluka Chemie AG (Buchs, Switzerland). 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) was purchased from Unichem Co. (Seoul, Korea), and 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) was from Sigma-Aldrich Chemical Co. All other chemicals and reagents were of analytical grade or purer.

Preparation of extract of far-infrared treated rice hull (FRH)

Rice hull was treated with far-infrared by the method described by Lee et al. (2003). Each batch of rice hull (5.0 g) was placed as a single layer in a Pyrex Petri dish (8.0 cm diameter) and treated by a far-infrared heater (35 × 10 cm, output 300 W, Hakko Electric Machine Works Co., Ltd., Nagano, Japan), which emitted radiation at the wavelength range from 2 to 14 μm in a far-infrared dryer (A-Sung Machinery, Kyungki-Do, Korea). Far-infrared treatment was carried out for 30 min at 100°C. Samples were turned 360° continuously during the irradiation process to achieve uniform irradiation, and the distance between the far-infrared heater and rice hull was 20 ± 1 cm. After treatment, the rice hull was allowed to cool to ambient temperature, then each 5 g of far-infrared treated rice hulls were extracted with 150 ml of methanol for 1 h of incubation with shaking at room temperature and filtered through a Whatman No. 1 filter paper. The filtrate was renamed as FRH and used for further experiments.

Preparation of liposomes

Liposomes were prepared by the dehydration-rehydration method (Kirby and Gregoriadis, 1984; Lee et al., 2002) with some modifications. PC in chloroform/methanol (2:1, v/v) (60 mM final concentration) were added to a 100 ml round-bottomed flask. Lipophilic antioxidants such as α-tocopherol in chloroform or FRH, morin, and quercetin in methanol were also added to the flask when needed (0.1 g/ml final concentration). Solvent was evaporated on a rotary evaporator at 30°C to deposit a dry lipid film on the wall of the flask. The residual solvent was removed under a nitrogen stream for 15 min at 4°C. Then 5 ml of PBS (10 mM, pH 7.4) buffer containing DTPA (0.5 mM) and 0.5 g of glass beads were added to assist hydration of the lipids. The solution was then mixed on the rotary evaporator (without vacuum) to hydrate the lipids to form multilamellar vesicles (MLVs). The solution was centrifuged for 1 h 30 min at 80,000 × g and the pellet was washed and dispersed with 5 ml of PBS (10 mM, pH 7.4) buffer containing 0.5 mM of DTPA and hydrophilic antioxidant (ascorbic acid, 0.1 g/ml final concentration, when required).

Lipid peroxidation in liposomal suspension

Free radical generators were used to initiate lipid peroxidation of PC in liposomes. Lipid-soluble peroxide radical generator, AMVN in methanol was added to a final concentration of 0.1 M, prior to removal of solvent during liposome preparation. In case with water soluble radical initiator, 0.5 ml of 100 mM AAPH solution in PBS buffer (10 mM, pH 7.4, containing 0.5 mM of DTPA) was added to 2.0 ml of preincubated liposomal solution at room temperature for 10 min. When transition metals (Fe³⁺ and Cu²⁺) were used, liposome suspension was preincubated for 10 min at room temperature to saturate the oxygen fully, and the oxidation was started by the addition of 1 M FeCl₃ or 1 M CuCl₂ (0.5 ml) to the suspension (2.0 ml), respectively. In all experiments, 0.1 ml of liposome suspension was placed in glass vials and oxidation was carried out at 37 or 50°C for 6 h.

Measurement of phospholipid hydroperoxide (PCOOH)

PC hydroperoxide formed by peroxidation was measured by UV absorbance method (Klein, 1970). After incubation of liposome with natural antioxidants or FRH, 0.05 ml of liposome suspension mixed with 0.45 ml of 0.1 M NaOH, 0.5 ml of methanol, and 0.5 ml of chloroform. After vigorous mixing, the mixture was centrifuged for 5 min at 700 × g at 4°C. The lower layer was withdrawn and completely mixed again with 0.5 ml of methanol: chloroform: NaOH (1:1:1, v/v) solution. The mixture was centrifuged again at 700 × g at 4°C. This step was repeated for 4 times. The final 0.1 ml of aliquot of lower and 3 ml absolute ethanol was mixed. The absorbance at 233 nm was determined, and the oxidative index was calculated as the ratio of the absorbance values. The oxidation rate was calculated as follows:

\[
\text{Oxidation rate} = \left( \frac{[\text{PCOOH}]_{\text{t}}}{[\text{PCOOH}]_{\text{inh}}} \right) \div t
\]

Where [PCOOH]ᵢ is the concentration of produced PC hydroperoxide after incubation for 6 h, [PCOOHᵢnh] is the concentration of produced PC hydroperoxide after incubation for 1 h, and Tᵢ is the time of total incubation. PC hydroperoxide produced was calculated using a molar absorption coefficient of 28,000 at 233 nm (Sessa et al., 1977) and average molecular weight of soybean PC of 900 (Yamamoto et al., 1984).

Statistical analysis

All measurements were done in triplicate, and the mean value was represented.

RESULTS AND DISCUSSION

Inhibition of AAPH-derived lipid peroxidation in liposomes by FRH

AAPH is a water-soluble azo compound, and generates peroxyl radicals in the aqueous phase and thereby attacks phospholipids at the membrane surface. AAPH has been used extensively to study oxidations of liposomes and various cell membranes (Liang et al., 2009; Tang and Liu, 2007). The ability of FRH and some known natural antioxidants to prevent lipid peroxidation induced by AAPH was investigated at 37 and 50°C. Figure 1 shows the oxidation index of phospholipid in liposome induced by AAPH. There were little differences in oxidation index of PC after 1 h of incubation at 37°C in all liposomes (Figure 1A). However, after 6 h of incubation, PC of liposome without antioxidant showed the highest oxidation index (0.648), while liposome containing FRH represents the lowest oxidation index (0.124). The oxidation index of liposome with ascorbic acid (a water soluble antioxidant) showed slightly lower value than that with α-tocopherol (a lipid soluble
antioxidant) during incubation.

During incubation at higher temperature (50°C), oxidation was enhanced than at lower temperature (37°C) (Figure 1B). Moreover, the differences between the antioxidants in liposomes were distinctly detected. The oxidation index of PC in liposome after incubation for 6 h without antioxidant and with FRH was 0.926 and 0.307, respectively, and that of ascorbic acid and α-tocopherol was 0.563 and 0.654, respectively.

The results indicated that FRH showed stronger antioxidant activity in AAPH (a hydrophilic radical generator)-induced phospholipid oxidation in liposome than any other antioxidants used in this study at same weight basis. FRH contains various compounds such as p-coumaric acid, 3-vinyl-1-oxybenzene, p-hydroxybenzaldehyde, vanillin, p-hydroxybenzoic acid, and 4,7-dihydroxyvanillic acid (Lee et al., 2003). Ramarathnam et al. (1979) identified isovitexin as a natural component in rice hull, which showed strong antioxidants inhibiting lipid peroxide. Asamarai et al. (1996) also identified a few phenolic compounds, such as anisole, vanilline and syringaldehyde, in wild rice hull extract. Although it is difficult to compare antioxidant activity of FRH extracts with pure antioxidant, the significant protection effects of FRH against oxidation of phospholipid induced by water-soluble radical generator could be coming from the synergistic effects of various compounds in FRH.

Inhibition of AMVN-derived lipid peroxidation in liposomes by FRH

The ability of FRH to protect PC against lipid peroxidation by AMVN-derived peroxyl radicals were evaluated (Figure 2). AMVN is a lipid-soluble radical
initiator, and the chain-initiating peroxy radicals are generated from AMVN within the liposomal membranes and chain-propagation reactions also proceed within the membranes (Niki, 1990). In the absence of any antioxidant, phospholipid hydroperoxides accumulated at a high rate. FRH showed significant protection effect on phospholipid peroxidation at 37°C (Figure 2A). After 1 h of incubation, oxidation index of PC with FRH was 0.125, and phospholipid hydroperoxides was not nearly accumulated during further incubation till 6 h. FRH might be an efficient antioxidant against radicals generated in the lipid phase. α-tocopherol, a lipidsoluble antioxidant, also very strongly inhibited oxidation of PC induced by lipid-soluble AMVN, however, ascorbic acid, a water-soluble antioxidant, could not suppress effectively the peroxidation efficiently. α-tocopherol is a major lipophilic antioxidant in cellular membranes and plasma lipoproteins, and its excellent antioxidant activity is well recognized (Laureaux et al., 1997).

The protective effect of FRH and natural antioxidants on AMVN-derived lipid peroxidation was also determined at 50°C. As shown in Figure 2B, though the oxidation index increased with increasing temperature, FRH showed superior inhibition activity against oxidation of phospholipid. FRH is a methanolic extract of rice hull, thus it might contain various hydrophobic antioxidant compounds (Lee et al., 2003). The well-known antioxidant compounds; ascorbic acid, morin, quercetin, and α-tocopherol also showed antioxidant activity. It is noticeable that morin exhibited relatively stronger antioxidant activity. Morin is a flavonol, which is widely present in plant such as sage orange and onion. Morin is an isomer form of quercetin with only one difference in the position of hydroxyl group in B ring.

Inhibition of metal ion-derived lipid peroxidation in liposomes by FRH

Transition metal ions such as Fe$^{3+}$ and Cu$^{2+}$ catalyze many of the reactions involved in lipid peroxidation of the cellular membranes (Benedet and Shibamoto, 2008; Zommara et al., 1995). They participate in production of a variety of potent oxidizing species which can initiate lipid peroxidation. The inhibition effect of FRH on lipid peroxidation in the presence of Fe$^{3+}$ and Cu$^{2+}$ was evaluated.

As shown in Figure 3, the lipid peroxidation in presence of Fe$^{3+}$ ion proceeds at 37 and 50°C for 6 h. In the absence of antioxidant, accumulation of PC hydroperoxide was rapidly increased, and the oxidation index was 0.905 after 6 h of incubation at 37°C (Figure 3A). On the other hand, the lipid peroxidation was significantly inhibited in the presence of antioxidants. The oxidation index being with ascorbic acid and quercetin after 6 h at 37°C was 0.270 and 0.328, respectively, while that with α-tocopherol was 0.602. Strong protection ability of FRH against lipid peroxidation in presence of Fe$^{3+}$ was also observed. After incubation for 6 h, oxidation index was 0.239, which was the lowest value in this study.

At 50°C, the lipid oxidation was more rapidly increased in the presence of Fe$^{3+}$ (Figure 3B). Though most of phospholipid was oxidized in the presence of Fe$^{3+}$ after 6 h of incubation at 50°C (oxidation index = 0.964), FRH showed marvelous antioxidant activity at same condition (oxidation index = 0.350). It was worth of notice that antioxidant activity of α-tocopherol and ascorbic acid declined noticeably at 50°C, maybe
caused by heat unstability of them.

Copper-induced oxidative damage on lipid peroxidation has been generally attributed to the formation of highly reactive ·OH by a mechanism analogous to the metal-driven Haber-Weiss reaction, or the Fenton reaction for Fe$^{2+}$ and hydrogen peroxide (Alexandrova et al., 2007). Similar oxidation pattern was observed in the presence of Cu$^{2+}$ (Figure 4). Oxidation index was increased with increasing incubation time and temperature. At both studied temperatures (37 and 50°C), FRH showed superior antioxidant activity than other natural antioxidants such as ascorbic acid, morin, quercetin, and α-tocopherol.

Transition metals are strongly implicated in the generation of free radicals by decomposition of H$_2$O$_2$ or lipid hydroperoxide (LOOH) to give hydroxyl radicals or alkoxyl radicals, respectively (Vidrio et al., 2008). Therefore, we examined whether the endogenous LOOH in PC liposomes are directly involved in the onset of the stimulatory effect of Fe$^{3+}$ and Cu$^{2+}$, respectively. In the present study, natural antioxidants and FRH efficiently scavenged transition metal ion-induced radicals either 37 or 50°C. In particular, FRH significantly limited the formation of PC hydroperoxide regardless of temperature and incubation time. Therefore, FRH effectively protected on phospholipid peroxidation by metal ion-induced radicals.

### Comparison of the rates of oxidation on phospholipid peroxidation by radical generator-induced radicals

The rates of PC hydroperoxide production by radical initiators or transition metal ions in the presence of natural antioxidants and FRH were determined (Table 1). The PC hydroperoxides were more rapidly accumulated at 50 than at 37°C in all situations. For example, in absence of any antioxidant, the oxidation rate of AAPH-derived lipid peroxidation at 37 and 50°C was 9.59 ± 0.25 and 81.25 ± 0.29 μM/h, respectively. A higher temperature not only activate reacting molecules but also enhance decomposition of hydroperoxides and may shorten the induction period prior to the start of rapid lipid peroxidation.

FRH showed the lowest oxidation rates regardless of hydrophobicity of radical initiators and transition metals.

Table 1. Comparison of the rates of oxidation by the reaction of soybean PC with peroxyl radicals generated via various radical generators in liposomes in the presence of variety of natural antioxidants and FRH extracts of rice hull at 37 and 50°C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>37°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAPH</td>
<td>AMVN</td>
</tr>
<tr>
<td>Control</td>
<td>9.59 ± 0.25</td>
<td>12.42 ± 0.12</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>1.43 ± 0.14</td>
<td>8.39 ± 0.19</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>1.91 ± 0.19</td>
<td>1.05 ± 0.25</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.67 ± 0.21</td>
<td>2.75 ± 0.21</td>
</tr>
<tr>
<td>Morin</td>
<td>1.46 ± 0.16</td>
<td>5.32 ± 0.19</td>
</tr>
<tr>
<td>FRH</td>
<td>0.16 ± 0.13</td>
<td>0.20 ± 0.17</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation of triplicate measurements.
Figure 5. Effect of different concentration of FRH extracts of rice hull on the formation of PCOOH in soybean PC liposomes after 6 h of incubation at 37°C in the presence of FeCl₃ (▲), AAPH (●), CuCl₂ (◆), and AMVN (■). The amount of PCOOH formed in the presence of FRH extracts of rice hull is expressed as a percentage of that formed in the control, with FRH extracts of rice hull.

This phenomenon might be resulted that FRH is a mixture of various antioxidant compounds (Lee et al., 2003). It is impressive that hydrophilic antioxidant (ascorbic acid) showed stronger antioxidant activity than hydrophobic antioxidant (α-tocopherol) in the presence of hydrophilic radical initiator (AAPH), and vice versa in the case of hydrophobic radical initiator (AMVN).

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