Cycloartenyl Ferulate and β-Sitosteryl Ferulate - Steryl Ferulates of γ-Oryzanol - Suppress Intracellular Reactive Oxygen Species in Cell-based System

Shusuke Yasuda¹, a, Yoshihiro Sowa¹, a *, Hiroyuki Hashimoto², Takuya Nakagami², Takuo Tsuno², and Toshiyuki Sakai¹

¹ These authors contributed equally to this work.

¹ Department of Molecular-Targeting Cancer Prevention, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, JAPAN
² Tsuno Food Industrial Co., Ltd., Ito-Gun, Wakayama 649-7194, JAPAN

Abstract: γ-Oryzanol is a naturally occurring component of rice bran and consists of various steryl ferulates. The antioxidant activities of γ-oryzanol have mostly been demonstrated in cell-free systems. Therefore, we determined whether steryl ferulate of γ-oryzanol suppress spontaneous intracellular reactive oxygen species (ROS) in cell-based systems. We found that cycloartenyl ferulate and β-sitosteryl ferulate suppressed spontaneous intracellular ROS in a similar way to N-acetylcysteine and α-tocopherol.

Key words: γ-oryzanol, cycloartenyl ferulate, β-sitosteryl ferulate, intracellular reactive oxygen species

1 Introduction

Rice bran is a byproduct of rice milling process - i.e., the conversion of brown rice to white rice - and dietary rice bran is thought to have beneficial effects with regard to the prevention of several types of cancer³. γ-Oryzanol is a naturally occurring component of rice bran and comprises a mixture of steryl ferulates - i.e., ferulic acid esters of triterpene alcohols, mainly cycloartenol and 24-methylene-cycloartenol and phytosterols, mainly β-sitosterol and campesterol³ (Fig. 1). A particularly important property of γ-oryzanol is its antioxidant activity³, because oxidative stresses - e.g., the intracellular accumulation of reactive oxygen species (ROS) - induce nuclear and mitochondrial DNA damage, denaturation of intracellular proteins, and lipid peroxidation. However, the antioxidant activities of γ-oryzanol have mostly been demonstrated in cell-free systems. Therefore, we focused on the effect of each steryl ferulate of γ-oryzanol on spontaneous intracellular ROS in a cell-based system comprising HT-29 human colon cancer cells.
system. The system comprised an LC-8A pump, an SPD-M20A photodiode array detector, a manual recycle valve, and an FCV-20AH2 passage switching valve (all from Shimadzu, Kyoto, Japan), and was operated under the following conditions: column, Cadenza 5CD-C18 (28 x 250 mm, 5 μm; Imtakt, Kyoto, Japan); column temperature, 30°C; mobile phase, methanol-acetic acid (99:1, v/v); and flow rate, 18.6 mL/min. The detector was set to monitor at 320 nm. The sample was passed through the same columns repeatedly for better separation. Each peak was collected in test tubes using an FRC-10A fraction collector (Shimadzu).

2.2 Cell culture

We maintained the HT-29 human colon cancer cells in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceuticals, Tokyo, Japan) with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C, in a humid atmosphere comprising 5% CO₂.

2.3 Determination of the levels of intracellular ROS

We treated the cells with each steryl ferulate for 12 h, then treated them with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Molecular Probes, Carlsbad, CA, USA). N-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA) and α-tocopherol (Sigma-Aldrich) were used as antioxidant positive controls. After incubating the cells for 30 min with CM-H₂DCFDA, we measured the intensity of the fluorescence by flow cytometry, as described in the literature⁴. We calculated the geometric mean fluorescence intensity at 530 nm.

2.4 Statistical analysis

We carried out the statistical analysis using a two-tailed, paired Student’s t-test. Samples were considered significantly different at \( p < 0.05 \).

3 Results

3.1 Isolation of molecular species of γ-oryzanol in a recycling preparative HPLC

Figure 2 shows the chromatogram of γ-oryzanol in the recycling preparative HPLC. Peak 4 was fractionated in the second cycle, and the other peaks in the third cycle. The numbered peaks were identified as follows: cycloartenyl ferulate (peak 1); 24-methylenecycloartanyl ferulate (peak 2); campesteryl ferulate (peak 3); and β-sitosteryl ferulate (peak 4), in reference to the typical chromatogram of γ-oryzanol in reversed-phase HPLC⁵. The purification yield of each molecular species ranged from 75 to 99%.

3.2 Antioxidative effect of steryl ferulates on spontaneous intracellular oxidative stress in the cell-based system

To investigate the antioxidant effects on spontaneous oxidative stress of the four steryl ferulates of γ-oryzanol - i.e., cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate - we determined the levels of intracellular ROS using the fluorescent probe CM-H₂DCFDA. Since CM-H₂DCFDA reveals the amount of intracellular ROS in living cells, the suppression of CM-H₂DCFDA fluorescence reflected the antioxidant activity of each steryl ferulate. We used N-acetylcysteine and α-tocopherol as antioxidant positive controls.

Treatment with both cycloartenyl ferulate and β-sitosteryl ferulate reduced the level of intracellular ROS (Fig. 3). Both N-acetylcysteine and α-tocopherol, which are representative antioxidants, also reduced the level of intracellular ROS, but 24-methylenecycloartanyl ferulate and campesteryl ferulate had no such effect (Fig. 3).

4 Discussion

In the present study, we found that both cycloartenyl ferulate and β-sitosteryl ferulate reduced the level of spontaneous intracellular ROS in the cell-based system, but 24-methylenecycloartanyl ferulate and campesteryl ferulate did not.
Previously, Islam et al. reported that cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and \( \beta \)-sitosteryl ferulate exhibited radical scavenging and antioxidant activities in a cell-free system, and cycloartenyl ferulate also reduced H2O2-induced intracellular ROS levels in mouse fibroblasts\(^6\). In accordance with their findings, both cycloartenyl ferulate and \( \beta \)-sitosteryl ferulate reduced the level of spontaneous intracellular ROS in the cell-based system. However, 24-methylenecycloartanyl ferulate, which also exhibited radical scavenging and antioxidant activities in the cell-free system, did not reduce the level of spontaneous intracellular ROS in the cell-based system.

The activity of an antioxidant is primarily dependent on its structure\(^7\). Carbon-carbon double bonds in particular are considered to be one of the most important factors. However, all the four steryl ferulates investigated in the present study have one carbon-carbon double bond in the sterol moiety, and \( \beta \)-sitosteryl ferulate and campesteryl ferulate have a carbon-carbon double bond in the same position. Zhu et al. reported the correlation between the antioxidant activities of various steryl ferulates and their solvation energies\(^8\). However, they evaluated the antioxidant activities in a cell-free system, and there was no correlation between antioxidant activities in our cell-based system and the solvation energies. Because we evaluated the antioxidant activities in a cell-base system, we were able to focus on the hydrophobicity of the steryl ferulates from the perspective of cell membrane permeability. The LogP value of each compound reflects its hydrophobicity. Therefore, we compared the LogP values of the four steryl ferulates, which we obtained from the open chemical and spectral databases ChemSpider (http://www.chemspider.com). However, there was no correlation between the antioxidant activities and LogP values of cycloartenyl ferulate (LogP value: 12.85), \( \beta \)-sitosteryl ferulate (12.82), campesteryl ferulate (12.29), or 24-methylenecycloartanyl ferulate (13.20). The determinants of the intracellular antioxidant activities of the steryl ferulates are not clear, but interactions between the steryl ferulates and the cellular contents might be involved. So far, the difference between cell-free systems and cell-based systems for the evaluation of antioxidant activities is not clear. However, we consider that cell-based systems reflect the potency of each test compound more accurately than cell-free systems.

The proportions of cycloartenyl ferulate and \( \beta \)-sitosteryl ferulate in \( \gamma \)-oryzanol might influence its antioxidant activity with regard to spontaneous intracellular ROS.

**5 Conclusion**

We found that cycloartenyl ferulate and \( \beta \)-sitosteryl ferulate reduced the level of intracellular ROS in the cell-based system. These results suggest that the antioxidant activities of steryl ferulates are dependent on their sterol moieties, and the antioxidant activities of \( \gamma \)-oryzanol is dependent on the amount of steryl ferulates it contains. This raises the possibility that the intake of \( \gamma \)-oryzanol, which consists of large proportions of cycloartenyl ferulate and \( \beta \)-sitosteryl ferulate, may prevent the occurrence of chronic diseases through the suppression of intracellular ROS.
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References

1) Henderson, A.J.; Ollila, C.A.; Kumar, A.; Borresen, E.C.; Raina, K.; Agarwal, R.; Ryan, E.P. Chemopreventive properties of dietary rice bran: current status and future prospects. *Adv. Nutr.* 3, 643-653 (2012).

2) Kozuka, C.; Yabiku, K.; Takayama, C.; Matsushita, M.; Shimabukuro, M. Natural food science based novel approach toward prevention and treatment of obesity and type 2 diabetes: recent studies on brown rice and $\gamma$-oryzanol. *Obes. Res. Clin. Pract.* 7, e165-172 (2013).

3) Goufo, P.; Trindade, H. Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, $\gamma$-oryzanol, and phytic acid. *Food Sci. Nutr.* 2, 75-104 (2014).

4) Yogosawa, S.; Yamada, Y.; Yasuda, S.; Sun, Q.; Takizawa, K.; Sakai, T. Dehydrozingerone, a structural analogue of curcumin, induces cell-cycle arrest at the G2/M phase and accumulates intracellular ROS in HT-29 human colon cancer cells. *J. Nat. Prod.* 75, 2088-2093 (2012).

5) Cho, J.Y.; Lee, H.J.; Kim, G.A.; Kim, G.D.; Lee, Y.S.; Shin, S.C.; Park, K.H.; Moon, J.H. Quantitative analyses of individual $\gamma$-oryzanol (steryl ferulates) in conventional and organic brown rice (*Oryza sativa* L.). *J. Cereal Sci.* 55, 337-343 (2012).

6) Islam, M.S.; Yoshida, H.; Matsuki, N.; Ono, K.; Nagasaki, R.; Ushio, H.; Guo, Y.; Hiramatsu, T.; Hosoya, T.; Murata, T.; Hori, M.; Ozaki, H. Antioxidant, free radical-scavenging, and NF-$\kappa$B-inhibitory activities of phytosteryl ferulates: structure-activity studies. *J. Pharmacol. Sci.* 111, 328-337 (2009).

7) Young, A.J.; Lowe, G.M. Antioxidant and prooxidant properties of carotenoids. *Arch. Biochem. Biophys.* 385, 20-27 (2001).

8) Zhu, D.; Sánchez-Ferrer, A.; Nyström, L. Antioxidant activity of individual steryl ferulates from various cereal grain sources. *J. Nat. Prod.* 79, 308-316 (2016).