Purification of Cycloartenyl Ferulate, 24-Methylenecycloartanyl Ferulate, Campesteryl Ferulate and Sitosteryl Ferulate from Rice Bran and Their Effects on the NLRP3 Inflammasome

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INTRODUCTION

Much research has focused on the inflammasome-mediated immune responses in the past 10 years. Inflammasome is activated in response to pathogen associated danger signals and plays an essential role in innate and adaptive immune responses to clear pathogens. In addition, inflammasome is also involved in auto-immune diseases such as type 1 diabetes, inflammatory bowel disease, auto-immune encephalitis, and metabolic diseases including obesity and type 2 diabetes (Lamkanfi et al., 2011; Choi and Ryter, 2014). Among inflammasome pathways, nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inflammasome is a cytosolic multi-protein complex consisting of NLRP3, caspase-1, and ASC, which was the most well studied pathway (Wen et al., 2013). Assembly of NLRP3 inflammasome triggers the activation of caspase-1 leading to the maturation and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. It is well known that IL-1β plays an important role in innate immune responses. However, aberrant production of this cytokine often leads to various inflammatory disorders (Menu and Vince, 2011). Therefore, targeting NLRP3 inflammasome offers a considerable therapeutic promise for the treatment of inflammatory diseases.

γ-Oryzanol mixture is an important value-added co-product of rice bran processing. Therefore, many researches have been conducted to improve the recovery of γ-oryzanol in order to obtain enriched fractions in a mixture. The conventional chemical methods, degumming, dewaxing and alkali treatment of RBO removed 1.1, 5.9 and...
93.0 to 94.6% γ-oryzanol, respectively (Krishna et al., 2001). Hence, alkali treatment or neutralization results in a significant loss of γ-oryzanol. In addition, it gives rise to a change in the individual phytosterol composition. Actually, after bleaching, some isomers of 24-methylene cycloartanol were detected (Hoed et al., 2006). To obtain an abundant γ-oryzanol from RBO, it is very important to increase yields by minimizing a loss through purifying steps.

γ-Oryzanol is known to exert various biological effects such as anti-inflammatory, anti-cancer, anti-diabetic, antioxidant, and cholesterol lowering effects (Akhisa et al., 2000; Chandrashekar et al., 2014; Cheng et al., 2010; Kim et al., 2012; Klongpityapong et al., 2013; Kozuka et al., 2015; Wilson et al., 2007). However, it has not been studied yet whether γ-oryzanol components modulate the NLRP3 inflammasome. In this study, two-step crystallization of crude γ-oryzanol mixture was performed for effective isolation of individual γ-oryzanol components from rice bran of ‘Unkwang’ variety and then individual γ-oryzanol components were purified with an auto-purification preparative LC-MS system. Finally, a total of four main pure compounds were used to evaluate the inhibition of inflammasome.

MATERIALS AND METHODS

Plant materials

Rice bran of the representative variety, ‘Unkwang’ was obtained from the Korean National Institute of Crop Science (NICS) in 2012. Samples were used for purification of individual γ-oryzanol components and their mixture.

Instrumentation and reagents

A refrigerated multi-purpose centrifuge (Hanil Science Industrial Co. Ltd., Korea), an ultrasonic bath (Daian Scientific Co. Ltd., Korea) and a large scale of rotary vacuum evaporator (Eyela Co. Ltd., Japan) were used in sample pre-treatment. A standard γ-oryzanol mixture (Wako, Japan) was used as a comparative standard. Methanol (MeOH), methylene chloride (MeCl2), acetone, acetic acid (AcOH), acetonitrile (ACN), hexane, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), trichloroacetic acid (TCA), and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich Co. (St. Louis, MO, USA). CO2 incubator from Thermo Scientific (Logan, UT, USA) was used to culture bone marrow derived macrophages (BMDM). An electrophoresis system and reagents for Western blotting were purchased from Bio-Rad (Hercules, CA, USA). A POLARStar optima microplate reader (BMG Labtech, Ortenberg, Germany) and an Epoch microplate reader (BioTek, Winooski, VT, USA) were used to measure optical density. A micro-centrifuge and a swing rotor bench top centrifuge used were from Hanil Science (Incheon, Korea). A luminescent image analyzer LAS4000 system (GE Healthcare Bio-Science Co., Waukesha, WI, USA) was used. Antibodies were obtained from either Thermo Scientific or Young In Frontier Co.

Extraction of rice bran oil (RBO)

Fresh rice bran (200 g) of ‘Unkwang’ variety was placed in a 2 L-stopper glass vessel and extracted with 1.5 L of MeCl2:MeOH (2:1, v/v) for 30 min at 30°C in an ultrasonic bath. The supernatant solution was collected and the rice bran residue re-extracted twice using the same conditions. The combined extract was concentrated with a large scale of rotary vacuum evaporator and their concentrate (RBO) was stored at -20°C.

Dewaxing and degumming of RBO

Typically, crude RBO (50 g) was dissolved in acetone (300 ml) in a 500 ml-stopper glass vessel. The vessel was kept at 60°C for 1 h to obtain clear solution. After allowing the contents to cool to room temperature, the vessel was kept at 5°C for 24 h to crystallize waxes and phospholipids. The solid phase was separated by filtration. The filtrate was collected and again subjected to crystallization at -5°C for 24 h. The insoluble fraction was then separated by filtration. The filtrate was collected followed by solvent removal by rotary evaporation. The dewaxed and degummed RBO, the starting material used in this study for the isolation of γ-oryzanol is simply referred as DD-RBO. DD-RBO was stored at -5°C under nitrogen.

Two-step crystallization of crude γ-oryzanol mixtures from DD-RBO

Two-step crystallization of crude γ-oryzanol mixtures was performed by modification reference protocol (Lai et al., 2005). In the first crystallization, DD-RBO (10 g) was mixed with 400 ml MeOH: acetone (7:3, v/v) in a 500 ml-stopper glass vessel under magnetic stirring (700 rpm) for 1 h at ambient temperature. The solution was stored at -60 ± 1°C for 15 h. Liquid and solid phases were separated by filtration through filter paper (Advantec, Japan) under vacuum, followed by solvent removal from the liquid phase under rotary evaporation to obtain the γ-oryzanol rich product. The crystallized γ-oryzanol rich product was subjected to the second crystallization.

In the second crystallization, the γ-oryzanol rich product from the first crystallization was kept at room temperature for 72 h to allow the growth of γ-oryzanol crystal. Hexane (50 ml), as washing solvent was added to the γ-oryzanol rich lipid and white γ-oryzanol crystal was separated by filtration through a filter paper at room temperature. The white γ-oryzanol crystals obtained were stored at -20°C.
Two-step purification of major compounds from crystallized crude γ-oryzanol mixtures by autopurification preparative LC-DAD-ESI/MS system

The major individual compounds from crude γ-oryzanol mixtures obtained in the previous stage were purified with mass detector; Micromass ZQ (Waters Co., Milford, MA, USA), fractionation system and 2767 sample manager (Waters Co., Milford, MA, USA). In addition, a preparative YMC PACK ODS-AM reversed-phase column (20 × 250 nm I.D., 5 μm; YMC Co. Ltd, Japan) was used. The analysis was conducted at a flow rate of 18 ml/min using a representative wavelength of 325 nm. The mobile phases were MeOH: ACN: MeCl₂: AcOH (50: 44: 3: 3, v/v/v/v) with isocratic elution for 50 min. MS analysis was run in negative ionization mode using an electrospray ionization (ESI) source. The MS parameters were as follows: cone voltage, 90 V; source temperature, 100°C; desolvation temperature, 500°C; and desolvation N₂ gas flow and 480 L/h. The range of ions was m/z 100 to 800 in full scan mode. In addition, the fractionation parameters were as follows: maximum fractions and tubes per injection, 78; solvent front delay, 30 s; split/collector delay, 10 s; and maximum fraction width, 60 s. The crude γ-oryzanol mixture solution with concentration of 50,000 ppm to 1 ml per injection was used.

Determination of crystallized γ-oryzanol mixtures and high-purity γ-oryzanol individual components by HPLC-DAD and LC-DAD-ESI/MS

The crystallized γ-oryzanol mixtures from rice bran of Unkwang variety as compared with commercial standard mixtures or the high-purity γ-oryzanol components were determined on a Micromass ZQ mass spectrometer and an Alliance e2695 HPLC system (Waters). The analysis was conducted at a flow rate of 1.4 ml/min, 30°C oven temperature and a representative wavelength of 325 nm. The mobile phases were MeOH: ACN: MeCl₂: AcOH (50: 44: 3: 3, v/v/v/v) with isocratic elution for 50 min. MS analysis was run with an electrospray ionization (ESI) source in negative ionization mode using a representative wavelength of 325 nm.

Animals

Six to eight weeks old C5BL6 mice were purchased from Samtako Bio Korea (Osan-si, Gyonggi-do, Korea) and housed at 24°C ± 1 under 50% humidity and 12 h light/dark schedule. Mice were given free access to food and water. All studies were performed in accordance with the guidance of Animal Care and Use Committee (ACUC) protocol and approved by the ACUC of Chonbuk National University (CBU 2014-00018).

Preparation of bone marrow-derived macrophages (BMDMs)

Mice were euthanized with CO₂ and BMDMs prepared as previously described. Briefly, BM cells were flushed from femurs and dispersed into single cell suspension by repeated pipetting, followed by red blood cell (RBC) lysis using ACK buffer (eBioscience, San Diego, CA, USA). After then, BM cells were resuspended in DMEM (Thermo Scientific, Logan, UT, USA) with 10% heat-inactivated FBS (Thermo Scientific), 1% antibiotics (penicillin-streptomycin solution, Thermo Scientific) and 20% M-CSF-containing L929 media. Cells were incubated in 95% air and 5% CO₂ atmosphere at 37°C for 5 to 7 days. Differentiated BMDM were harvested and used in the experiments.

Nitric oxide (NO) assay and cell viability assay

BMDMs (1.0 × 10⁴ cells/well) were plated in 96-well cell culture plate (SPL life Science Co.), incubated for overnight and then pretreated with rice bran extract or the vehicle solvent DMSO at appropriate concentrations. After 2 h, LPS was added into cell culture media at a final concentration of 200 ng/ml (Sigma-Aldrich) and incubated for 24 h at 24°C ± 1. Cell culture supernatant was collected and mixed with an equal volume of Griess reagent, followed by incubation for 10 min at room temperature. Absorbance at 570nm was read with a microplate reader (BioTek). The nitrite level was calculated from a sodium nitrite standard curve. For cell viability assay, cell culture medium was removed, followed by incubation of the cells in DMEM medium containing MTT (0.5 mg/ml) for 2 h at 37°C. After the medium was removed, DMSO was added into each well to dissolve formazan. The absorbance was quantified with a microplate reader at 540nm.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines such as IL-1β and TNF-α in the cell culture supernatants were measured with ELISA kits (eBioscience, Inc.) according to the manufacturers’ instructions. ELISA data were analyzed with the POLARstar optima-control software (BMG Labtech).

NLRP3 inflammasome induction

BMDMs (1.0 × 10⁶ cells/well) were plated in a 12-well cell culture plate (Corning Inc., NY, USA), and primed with 500 ng/ml of LPS. After 6 h, the medium was changed with...
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Table 1. The contents of oil and γ-oryzanol in bran of Korean rice variety, 'Unkwang'.

<table>
<thead>
<tr>
<th>Unkwang</th>
<th>Rice bran oil (g/100 g)</th>
<th>Total γ-oryzanol content (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hull rice</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>Rice bran</td>
<td>20.6 ± 1.5</td>
<td>34.6 ± 0.8*</td>
</tr>
<tr>
<td>Rice bran</td>
<td></td>
<td>242.2 ± 10.5*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD (n=3; n=5). The content of γ-oryzanol mixture crystallized was 91.4 mg from 100 g of rice bran.

DMEM without FBS. The cells were then treated with ATP for 1 h (5 mM) to induce NLRP3 inflammasome activation. At the end of incubation, cell culture supernatant and cell lysate were collected for further analysis. To determine the inhibitory effect of rice bran extracts on NLRP3 inflammasome activation, rice bran extract or DMSO (vehicle solvent) was added into the cell culture medium at indicated concentration and the mixture was sit for 30 min prior to ATP treatment. Cell culture supernatants were collected for ELISA and TCA protein precipitation, and cell lysates prepared with M-PER Reagent (Thermo Scientific) containing protease and phosphatase inhibitors for Western blot analysis.

Trichloroacetic acid (TCA) protein precipitation

For Western blot, cell culture supernatants were concentrated by TCA precipitation (Sigma-Aldrich Co.) precipitation. A quarter volume of TCA solution was added into supernatants and incubated for 10 min at 4°C, followed by centrifugation for 5 min at 14,000 rpm. The resulting precipitate was washed twice with 100% cold acetone and then centrifuged for 5 min at 13 K rpm. The pellets were air-dried and resuspended in 1 M Tri buffer (pH8.0). Sample buffer was added into each tube and boiled for 10 min.

Western blotting analysis

Samples from supernatants or cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10 or 15%) and blotted onto nitrocellulose membrane (Merck Millipore Ltd., Darmstadt, Germany) by electrophoretic transfer. The membrane was blocked with 5% nonfat milk (BioShop Canada Inc., Burlington, ON, Canada) in TBST and incubated overnight at 4°C with one of the following primary antibodies: anti-mouse IL-1β antibody (R and D system, Minneapolis, MN, USA), anti-caspase-1 p10 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-ASC antibody (Santa Cruz Biotechnology), anti-beta-Actin antibody (Young In Frontier co., Seoul, Korea). Protein bands were detected with HRP-conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibodies and visualized by West save gold (Young In Frontier) or West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Luminescent Image Analyzer LAS4000 System (GE Healthcare Bio-Science Co.).

RESULTS AND DISCUSSION

Purification of crude γ-oryzanol mixture and their individual components

The most saponifiable lipids contained in RBO have been the main interferences for separation of crude γ-oryzanol mixture from RBO. In the removal process of saponifiable lipids, the previous alkali treatment (chemical) methods for dewaxing removed 93.0 to 94.6% γ-oryzanol from the original crude oil in the purification of γ-oryzanol (Hoed et al., 2006; Krishna et al., 2001; Xu and Godber, 2000). However, the more effective isolation of γ-oryzanol from crude RBO was achieved by a two-step crystallization process in a liquid-solid (physical) method (Zullaikah et al., 2009). In this study, crystallization of crude γ-oryzanol mixtures from the bran of the Korean rice variety 'Unkwang' was performed as a choice of selecting a gentle physical method rather than a harsh chemical method by modification of the published protocol (Zullaikah et al., 2009) and as choice being non-toxic to animals and humans. A large scale of rotary vacuum evaporation was used to obtain a large amount of crude γ-oryzanol mixtures for further purification of individual γ-oryzanol components. To measure recoveries of crude γ-oryzanol mixtures in the first crystallization step, actual γ-oryzanol content of rice bran was evaluated by analytical reverse-phase HPLC (Table 1). The content of crystallized crude γ-oryzanol mixtures was 91.4 mg from 100 g of rice bran and constituted approximately 40% of the total γ-oryzanol content (242.2 mg/100 g) in the rice bran, which was less than the value of 59% reported (Zullaikah et al., 2009). The most saponifiable and unsaponifiable lipids except γ-oryzanol could be entirely removed according to the reference protocol (Zullaikah et al., 2009). Crystallized γ-oryzanol mixtures from rice bran of 'Unkwang' was determined with a PDA detection and compared with the commercial standard mixture at a concentration of 1000 ppm. Finally, the mixture of the pure γ-oryzanol components with purity of 96% in a sum of total peak area was amorphous white powder and this was confirmed through UV spectra. In comparison of the γ-oryzanol extract of brown rice with the crystallized γ-oryzanol mixtures, the main impurities including glycerides (mono-, di-, tri- ) were removed from the γ-oryzanol extract (Figure 1). The process was, therefore, necessary to isolate...
individual γ-oryzanol components with high purity from prepared γ-oryzanol mixtures.
Until recently, the resulting crude γ-oryzanol products were purified by classical preparative HPLC in a laborious process. The autopurification preparative LC-MS system is commercially available and has been used for purification of early stage drug candidates in pharmaceutical companies (Guth et al., 2008; Fitz et al., 2009). It is a unique method that can collect exact compounds using a mass spectrometer to ‘signal’ fraction collection. However, as individual γ-oryzanol derivatives have similar molecular weights, it is often a challenge to isolate such analogues using the open tubular column chromatographic methods and therefore, a high resolution method is needed (Lai et al., 2005). Individual γ-oryzanol components could be purified by multiple steps along preparative column of small particle size. Figure 1A shows that individual γ-oryzanol analogues were isolated in the first autopurification step by retention times. The targeted components as major components of γ-oryzanol, cycoartenyl ferulate (p3), 24-methylene cycloartanyl ferulate (p4), campesteryl ferulate (p6) and sitosteryl ferulate (p8) were collected in Fractions f28, f31, f35 and f39, respectively. In the 1st step, purified cycloartenyl ferulate showed purity of 91.9% and further increased to 98.7% through the automated throwing away by personal programming in the 2nd step (Figure 1B). The other three major components were also purified through the 1st and 2nd steps, which included cycloartenyl ferulate with molecular weight of 602 in 98.7% purity and amorphous white powder. It was confirmed with the match of its UV absorbance spectrum with that of the cycloartenyl ferulate standard. The total amount of purified content was 437.9 mg from 200 g of rice bran. The purified 24-methylene cycloartanyl ferulate with molecular weight of 616 was in 94.8% purity and amorphous white powder and was confirmed through comparison of the UV absorbance spectrum with the standard. The total amount of purified content was 650.0 mg from 200 g of rice bran. Campesteryl ferulate with molecular weight of 576 was purified to purity of 97.0% amorphous white powder and was again confirmed through the UV spectrum (Figure 2). The total amount of purified content was 83.1 mg from 200 g of rice bran. The purified sitosteryl ferulate with molecular weight of 590 was 97.6% amorphous white powder confirmed through the UV spectral comparison. A total amount of purified content was 124.2 mg from 200 g of rice bran.

In this study, we purified a total of five compounds with an average of purity greater than 95%. High purity γ-oryzanol is valuable for testing their pharmaceutical effects such as anti-cancer, anti-inflammation and prevention of obesity and diabetes. Much research effort had been recently done to isolate γ-oryzanol components for evaluation of biological activities. The final purity, yield and the initial raw rice material used were not mentioned (Akihisa et al., 2000, 2001; Islam et al., 2009; Luo et al., 2005; Nagasaka et al., 2007; Oka et al., 2010).

**Inhibition of inflammasome by γ-oryzanol individual components**

Prior to investigating the physiological activities of the purified compounds, MTT assay was performed to examine the effect of γ-oryzanol compounds on cell viability of BMDDM. As shown in Figure 3A, CYF and MCF reduced cell viability to about 75% at 20 μM, while FA, CAF, and SF did...
Figure 2. HPLC-PDA chromatogram (including UV spectrum) of high-purified major compounds, cycloartenylferulate (p3), 24-methylene cycloartanyl ferulate (p4), campesterylferulate (p6), sitosterylferulate (p8) in autopurification 1st and 2nd steps.

Figure 3. Effect of γ-oryzanol and purified compounds on cell viability, and the production of NO and pro-inflammatory cytokines. BMDM cells were pretreated with γ-oryzanol or each compound at various concentration as indicated in graphs. DMSO was also included as vehicle control. After 2 h, cells were stimulated with LPS (200 ng/ml) for additional 24 h. (A) Cell viability was measured by MTT assay and (B) the level of NO was evaluated using Griess reagent; (C and D) TNF-α and IL-1β in cell culture supernatants were measured by ELISA. Data are expressed as mean ± SEM of triplicated samples and the representative result of at least three independent experiments is shown. (FA: ferulic acid, ORY: oryzanol, CYF: cycloartenylferulate, MCF: 24-methylene cycloartanyl ferulate, CAF: campesterylferulate, SF: sitosterylferulate).
not alter cell viability at the concentrations up to 20 μM.

When macrophages are stimulated with LPS, various inflammatory molecules including NO, TNF-α and IL-1β are released. To explore the anti-inflammatory effects of γ-orizanol and purified compounds, BMDMs were pretreated with γ-orizanol or purified compounds at varying concentrations for 2 h and cells were then stimulated with LPS. All compounds including FA tested did not affect NO production induced by LPS (Figure 3B). This result was not consistent with the previous findings that FA suppressed NO production in microglia cells (Huang et al., 2011). It could be due to different cell types. Similarly, when the level of inflammatory cytokines, TNF-α and IL-1β, was measured by ELISA, there were no significant differences among groups (Figure 3C and D).

Recent studies well demonstrated that inflammasome plays a central role not only in innate immune responses against microbial infection but also in inflammatory disorders (Menu and Vince, 2011). Among inflammasomes, NLRP3 inflammasome is the most comprehensively studied pathway. This inflammasome is activated by ATP, nigericin or bacteria including Listeria monocytogenes and Salmonella typhimurium (Amer et al., 2006; Kim et al., 2010; Locovei et al., 2007; Mariathasan et al., 2006; Meixenberger et al., 2010; Miao et al., 2008). In the present study, NLRP3 inflammasome was induced by ATP in LPS primed BMDM cells and it was determined whether γ-orizanol or purified compounds modulated the activation of NLRP3 by measuring IL-1β secretion. As depicted in Figures 3A and 4B, FA, ORY, CYF, MCF and CAF did not affect NLRP3 inflammasome activation in a significant level. However, SF suppressed IL-1β secretion
in a dose dependent manner and reduced IL-1β secretion by more than 60% at 20 μM (Figure 4B and C). To further confirm this result, the effect of SF on caspase-1 activation and IL-1β maturation through NLRP3 inflammasome was studied. In addition to efficient suppression of IL-1β secretion by SF (Figure 4C), SF suppressed IL-1β maturation (Figure 4D). These results were well correlated with the reduced level of cleaved and activated caspase-1 in cell culture supernatants, implying that SF inhibits IL-1β production through suppression of caspase-1 activation. To our knowledge, this is the first report that demonstrated the inhibitory effect of one of the purified compound from γ-oryzanol on NLRP3 inflammasome.

Conclusion

Major components of γ-oryzanol, cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, campesteryl ferulate and sitosteryl ferulate were purified in high purity and a novel function of sitosteryl ferulate in inflammasome activation was identified. Among them, sitosteryl ferulate inhibited IL-1β secretion through NLRP3 inflammasome activation, putatively due to its suppression of caspase-1. This will be useful to define the molecular mechanisms of NLRP3 inflammasome and its biological activity in inflammatory disorders such as type 1 diabetes using mice model.

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